Pharmaceutical Intervention in Apoptotic Pathways

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Pharmaceutical Intervention in Apoptotic Pathways

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Preface

'Apoptosis' or 'programmed cell death (PCD)' recently emerged as a new scientific field. With thousands of publications, two journals ('Cell Death and Differentiation' and 'Apoptosis') and a series of international meetings it is now one of the hottest areas in biomedical research; among the ten most cited papers published and cited in 1995, four were about apoptosis/PCD (1).

The concept of 'cell suicide' is interesting not only for specialists working in different areas like oncology, immunology, embryology, developmental biology, pathology, pharmacology, toxicology, and virology, but has been drawing the attention of a broad public: it happens to be an eye-catching item in numerous newspapers and popular science magazines (2) and even was the subject of two documentary films ('Death by Design' and 'Death Wish') that have been broadcasted by many networks. This massive interest is driven both by scientific curiosity and by the awareness that aberrant regulation of apoptosis probably contributes to some of the worlds most frightening diseases; too much apoptosis has been shown (or is likely) to be involved in AIDS, neurodegeneration, osteoporosis, multiple sclerosis, chronic neutropenia, liver failure, heart infarcts, type I diabetes mellitus, ulcerative colitis, Wilson disease and anaplastic anemia. On the other hand, too little apoptosis results in lymphoma, leukemia, solid tumors, and various autoimmune diseases.The prospect that modulation of apoptosis might provide new opportunities for the treatment of these diseases is gradually affecting research funding systems and especially pharmaceutical and biotechnology industries are making impressive investments. In addition, many companies have been formed whose principal scientific and commercial activities are aimed at apoptosis/PCD.

Because of the interdisciplinary character of the field of cell death and because of its explosive growth during the past few years, there is clearly a great need for informal meetings in which the significance of apoptosis for health and disease and its opportunities for pharmaceutical intervention can be discussed with leading experts. Since no such meeting had been held in the Netherlands before, we, ie: a biochemist working in the field of toxicology (J. Fred Nagelkerke), a cell biologist working in the field of oncology (Jan Hein van Dierendonck), and a molecular biologist working in the field of virology and oncology (Mathieu H. M. Noteborn), decided to bring the subject to the attention of the Royal Netherlands Academy of Arts and Sciences. This resulted in a very successful colloquium of 50 Dutch and foreign scientists, held in the residence and under the auspices of the Royal Netherlands

Academy of Arts and Sciences at the 17th century 'Trippenhuis' in Amsterdam, from July 7 till 9, 1997.

The present Proceedings of the Colloquium in this volume contain three parts, respectively representing session I: The morphology, genes and detection of apoptosis; session II: Deregulated control of signal integration: consequences for the nerve system, immunity, and cancer development; and session III: Apoptosis-based therapies and drug development.

We owe special gratitude to Mrs Manita Giribaldie-Kooy from the Royal Netherlands Academy of Arts and Sciences for expert support in organising the scientific meeting and the staff of the Editorial Department of the Royal Netherlands Academy of Arts and Sciences for their support in the process of preparing this volume.

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Jan Hein van Dierendonck, Mathieu H.M. Noteborn and J. Fred Nagelkerke

Pharmaceutical Intervention in Apoptotic Pathways

In this introductory chapter, we attempt to briefly outline the development of the field of apoptosis research in general, the definition of the concepts, the present state of the art of molecular mechanisms involved, and the prospects for treatment of various diseases, incorporating some of the findings and ideas that were presented during the meeting in Amsterdam.

Part I: Morphology, Genes and Detection of Apoptosis

The first day of the meeting, John Cohen, Wilfried Bursch and Michael Hengartner summarized from different perspectives the development of cell death research (p. 17, 31, 41 respectively). The problem with the cell death concept is that it happens to be far more difficult to define than a phenomenon like cell division; as a result, the terminology used is not very straightforward and even rather confusing. In part this could stem from the fact that during the past 150 years various aspects of cell death have been rediscovered over and over again, most often within entirely different fields of research.

In their search for old literature on cell death research, Peter and Stephany Clarke discovered that cell death was already reported in 1842 in Carl Vogt's writings on the metamorphosis of midwife toads. In their review they listed no less than 24 names of scientists who during the second half of the nineteenth century reported (mostly in German) cell death in a wide variety of animals and tissues in different stages of development (Clarke & Clarke, 1996). For instance, Walter Flemming, who had experimented a lot with fixatives and stains and was the creator of words like 'mitosis' and 'chromatin', noticed that in regressing ovarian follicles in rabbits there were many cells with fragmented nuclei; he named the observed changes in staining patterns associated with dying cells 'chromatolysis' (Flemming, 1885).

In 1914, Ludwig Gräper stated in his 'A new point of view regarding elimination of cells' that in all tissues, Flemming's chromatolysis was a counterbalance to mitosis and responsible for various forms of tissue shrinking and that phagocytosis by neighbouring cells was a crucial factor in the removal of cellular debris (Gräper, 1914).

After the World Wars, Glücksmann wrote an extensive review of the many observations on cell death in the field of embryology that had been reported during the preceding decennia. He carefully described the shrinkage, chromatolysis, the falling

apart of nuclei and the striking feature that there were rarely any changes in the mitochondria, which seemed to be in sharp contrast to what happened after exposure of cells to very toxic agents (Glücksmann, 1951). Few years later, the French hematologist Marcel Bessis made time-lapse movies of leukocytes dying under coverslips, being probably the first cinematographic recording of what he called 'death by fragmentation' as opposed to 'accidental cell death' (Bessis, 1955).

The term 'programmed cell death' was first identified by Lockshin and Williams to describe the death of metamorphosing larval cells, recognizing that predictable developmental events should always have some genetic background (Lockshin and Williams, 1964). After the discovery of the lysosomes by the Duve, who had proposed that cells might be killed from within by an explosion of these 'suicide bags', many researchers gained renewed interest in the phenomenon of cell death. The pathologist John Kerr, who had carefully studied the appearance of lysosomes in dying hepatocytes, noticed that many cells did not die by lysosomal activities, but showed a morphology he called 'shrinkage necrosis' (Kerr, 1971). Building on this observation, he and his colleagues Wyllie and Currie established that most physiological forms of cell death, observed both in normal and pathological conditions, in fact follow a common morphological pattern. The powerful insight that this was a 'basic biological phenomenon with wide-ranging implications for tissue kinetics' justified a special name: 'apoptosis' (Kerr et al., 1972). Independently, Farber and coworkers were aware of the occurrence of 'active' or 'suicidal' cell death in various organs after treatment with anticancer drugs (Farber et al., 1971) and it became clear that this drug-induced cell death was indistinguishable from 'physiological' apoptosis (Searle et al., 1974).

But these (re)discoveries did not readily lead to wide recognition of the wide-ranging implications. The answer generally given to the question why it took another 15 years before the attention of the scientific community became focussed on these extremely interesting and important observations and ideas is that apoptosis needed the recent progess in the fields of molecular biology and genetics.

Only very recently 'ap-o-pto-sis' has entered the Medical Dictionary, under the rather cryptic definition of 'single deletion of scattered cells by fragmentation into membrane-bound particles which are phagocytosed by other cells; believed to be due to programmed cell death. [greek: Falling or dropping off]' (Stedman, 1995). But it is clear that the typical morphology of this deletion of scattered cells, the importance of the phenomenon for sculpting structures and for deletion of unneeded structures, and the concept that cells may compete for survival factors as a solution to the problem of matching of cell numbers and proper interactions between different cell types were established before the invention of terms like PCD and apoptosis.

It had been observed in the early seventies that irradiation of cells can induce fragmentation of chromatin in a very regular pattern, implicating the occurence of DNA breaks between nucleosomes. Wyllie linked these internucleosomal DNA breaks with apoptosis, contrasting the total DNA degradation that was observed in samples from cells treated in a way leading to blunt necrosis (Wyllie, 1980). The psychological

importance of his observation was that it vizualized, on a biochemical level, that apoptosis should be under tight control and that it provided a badly needed biochemical marker. The belief that internucleosomal DNA cleavage was crucial to apoptotic morphology led to the application of enzymatic methods to mark DNA breaks with labeled nucleotides, methods that could be used to detect apoptotic cell fractions by flow cytometry or to help identifying sporadic apoptotic cells *in situ* in tissue sections; these techniques, known as Terminal deoxynucleotidyl tranferase-mediated dUTP-biotin Nick End Labeling (TUNEL) (Gavrieli et al., 1992) and In Situ End-Labeling (Wijsman et al., 1993), were quickly commercialized and are still widely used.

However, the relevance of apoptosis-related DNA fragmentation is still a very controversial subject. It has been described that although specific DNA breaks (leading to 50,000-300,000 basepair fragments) are prerequisite for chromatin condensation and nuclear fragmentation (Cohen et al, 1994, Oberhammer et al., 1993), the occurence of massive internucleosomal cleavage are not (Schultze-Osthoff et al., 1994). Moreover, although it may very rapidly occur in specific cell types, such as leukocytes, in other cell types it may occur rather late in the apoptotic process (Collins et al., 1997). Internucleosomal cleavage may even occur in cells dying without apoptotic morphology, including cells dying as a result of severe ischemia and/or membrane damage. There is increasing evidence, however, that a nuclease exists that is exclusively activated in apoptosis; this nuclease is expressed in many types of cells, but is usually located in the cytoplasm in an inactive, latent form as a result of binding to an inhibiting partner molecule. Destruction of the latter leads to nuclear translocation of the active endonuclease (Enari et al., 1998).

A very striking feature of apoptosis is that, unless it occurs so massively that there is an overload of the 'phagocytotic machinery', the dying cells generally disappear very quickly from the tissues or from the bloodstream, without generating significant inflammation; apoptotic cells are visible only a few hours, and the majority of this time is being spent within the cells that have eaten them. Conditions causing apoptotic cell death are even associated with active suppression of inflammation and cell-mediated immunity; for instance, the presence of apoptotic cells during monocyte activation increases their secretion of the anti-inflammatory and immune-regulatory cytokine interleukin 10 and decreases secretion of the pro-inflammatory cytokines tumour necrosis factor, interleukin 1, and interleukin 12 (Voll et al., 1997).

An interesting concept proposed by Chris Reutelingsperger (p. 53) and Stephan van den Eijnde (p. 63) is that tolerance of the existence of the individual cell in multicellular organisms is mediated by the distribution of the various phospholipid species across the bilayer of the plasma membrane. *In vitro* studies have established that cell-surface exposed phosphatidylserine (PS) on ageing erythrocytes and apoptotic leukocytes triggers elimination of these cells by phagocytosis, but that blood cells are inert in this respect when this aminophospholipid is predominantly residing in the plasma membrane leaflet facing the cytoplasm. The hypothesis is that all cells spend energy to generate and maintain a state in which the plasma membrane leaflet facing the environments contains a very low level of PS, but that activation of apoptotic pathways leads to rapid changes in PS topography. As demonstrated in

chapters 5 and 6, the finding that one member of the family of Annexin proteins, i.e. Annexin V, exhibits a high affinity for PS led to the development of a promising marker for apoptotic cells, applicable on living cells *in vitro*, or even *in vivo* (Van den Eijnde et al., 1997).

Is signalling of the process of dying to the environment, ie, the 'eat me' signal, the stimulation of heterophagic elimination by other cells, a general feature exclusively associated with the typical morphology related to apoptosis? It is clear that this feature is clearly no universal hallmark of PCD; for instance, PCD is an extremely important phenomenon during plant growth and development, whereas phagocytosis is simply no issue here (Beers, 1997).

There is litlle doubt, however, that in animals and human beings phagocytosis by neighbour cells is a key phenomenon in the process of apoptotic cell death. The typical structural changes associated with this type of cell death relate to efficient recognition, uptake and digestion. John Cohen (p. 17) made us clear why: as in an adult body about 25 million cells divide each second, this should be counterbalanced by a similar amount of cell deaths; if his calculations are correct, each day about 1.5 kg cells are made available for recycling.

PS externalization in the plasma membrane is probably one of the earliest indicators of apoptotic cell death and is associated with a decrease in lipid polar-head group packing; these features seem to occur independently of alterations in plasma membrane permeability, which induce a very rapid loss of fluid. Visually, this rapid cell shrinkage is the most dramatic feature of apoptosis and there is increasing evidence that this phenomenon results from Na^+ or K^+ efflux by active pumping via Na^+, K^+-ATPase pumps or $Ca2^+$-dependent K^+ channels. It has been proposed that one reason for the dependence of apoptosis on functional mitochondria is that these processes demand a considerable amount of energy (ie ATP) (McCarthy and Cotter, 1997). An interesting question is whether the decrease in ionic strength plays a necessary and perhaps pivotal role in the execution of the cell death program (Bortner et al., 1997).

The contribution of Wilhelm Bursch (p. 31) added another level of complexity to the discussion on the nature of apoptosis. He prefers to speak of 'active cell death' (ACD) as a general term for all forms of cell death that depend on sufficient energy supply. This ACD can have a variety of different forms, depending on the organism, cell type involved or even the type of cell death induction. Whereas a lot of attention is being paid to one of these forms, apoptosis, other forms, such as the typical 'autophagic' or 'lysosomal' cell death which most often occurs in cells with a relatively large cytoplasm, certainly deserve more intense investigation.

But both apoptotic and non-apoptotic ACD is clearly quite different from cell death occuring after severe trauma or ischemia: in mammalian tissues, latter cells tend to swell, the plama membrane ruptures, cells loose internal materials and some of these compounds may induce strong inflammatory reactions. This type of cell death is generally known as 'necrosis', although Majno and Joris recently pointed out that this terminology is badly chosen: 'necrosis' or 'necrobiosis' has always been used to describe drastic tissue changes and very dead cells and to say 'cell death by necrosis'

would be like saying that clinical death occurs by postmortem autolysis. They proposed the term 'oncosis' (oncos=swelling) to oppose 'apoptosis', implying that features of true 'necrosis' are the inevitable fate of apoptotic cells not phagocytosed by other cells (Majno and Joris, 1995). But then the question remains whether all existing forms of eukaryotic cell death in which intracellular energy levels and mitochondrial function are rapidly compromised show all the characteristics of oncosis.

It is clear that the term apoptosis should not be confined to situations that involve (reduction of) immune responses, because some basic features of apoptotic cell death are phylogenetically older than immune systems. Investigators like Horvitz and Hengartner (p. 45), who performed pioneering research in the primitive nematode *Caenorabditis elegans*, have not readily adopted the term apoptosis, but there is little doubt that apoptosis owes its fame in large part to the fact that this little worm happened to be an excellent system for identifying genes that function in PCD.

The success story is well known: whereas over 15 genes have now been identified that function in PCD (most of them being somehow involved in efficient recognition and phagocytosis by neighbour cells), two (*ced*-3 and *ced*-4) are required for the execution of this process during development and a third gene (*ced*-9) is required to protect *C. elegans* cells that should survive from inappropiately activating the death program. The finding that *ced*-9, *ced*-3 (and as was announced during the meeting, but was published shortly thereafter: *ced*-4) are similar in sequence and function to genes that function in mammalian apoptosis suggested that primitive worms and mammals share similar conserved mechanisms to get rid of cells. CED-9 resembled the proto-oncogene product Bcl-2, and CED-3 was similar to interleukin 1-converting enzyme (ICE), but it became soon clear that the ritual of cell death in higher organisms shows much higher levels of complexity than in the worms: Bcl-2 became the founding member of an increasing family of proteins whose members are either involved in inhibition (Bcl-2, Bcl-Xl, Bcl-W, Mcl-1, A1) or in the induction (Bax, Bak, Bcl-Xs, Bad, Bik, Bid) of apoptosis, whereas ICE appeared to be one member of a family of cysteine proteases that specifically cleave proteins after Asp residues (caspases). At present, this family comprises eleven members that are involved in the activation of proinflammatory cytokines, in the induction phase of apoptosis, or in the execution process of apoptosis (see also p. 179). Caspases cleave a number of cellular proteins, usually only at one site and resulting in activation or inactivation, but never in degradation of their substrate. Substrates may include other caspase zymogens, suggesting a proteolytic network resembling the blood coagulation cascade. These caspase activities lead to activation of proteases and endonucleases responsible for the induction of apoptotic morphology; if the activity of certain caspases is inhibited, cell death will be delayed and result in a morphology more resembling necrosis.

Thus, caspase activation is a key event in both the initiation and execution of apoptotic pathways, and the regulation of the caspases by pro- or anti-apoptotic molecules constitutes the major basis of precise control of the whole process (Villa et al., 1997). In this context, it is interesting that certain anti-apoptotic viral proteins may directly

inhibit activated caspases. A very effective response of animal cells to stop the propagation of a virus infection is the induction of the apoptotic machinery; therefore, to replicate, a virus has to block this machinery either by interfering with the signals that induce the caspase cascade and/or interfering with the execution program. The discovery of inhibitor of apoptosis proteins (IAPs) in baculovirus (Clem and Miller, 1994) led to the identification of similar proteins in mammals, some of which were recently found to be direct inhibitors of specific caspases (Roy et al., 1997)

A large variety of stimuli can trigger the caspase cascade, suggesting that many of these stimuli somehow trigger a central event. In that context, much attention is being focussed nowadays on cytochrome c (cyt c). This protein is required for oxidative phosphorylation in mitochondria, where it assists with production of life-sustaining ATP by participating in electron transport. A remarkable recent discovery is that following a variety of apoptotic stimuli, cyt c is rapidly released from the mitochondria into the cytosol, where it induces the activation of the caspase cascade (Liu et al., 1996; Kluck et al., 1997). The emerging picture is that cyt c acts as a co-factor for the recently discovered Apaf-1 protein. The latter protein contains three distinct domains: a so called caspase recruitment domain (CARD), a domain homologues to CED-4, and a part believed to mediate protein-protein interactions (Zou et al., 1997). In the presence of cyt c and dATP (or much higher concentrations ATP), the CARD of Apaf-1 can bind to CARD-containing procaspases, such as caspase 9 precursor, immediately resulting in activation of the latter (Li et al., 1997). Active caspase 9 then cleaves and activates procaspase-3, which has a variety of targets, including the inactive precursor of the above mentioned DNase (Liu et al., 1997).

Of course, one consequence of massive release of cyt c would be prevention of oxidative phosphorylation, promoting free-radical production and depletion of ATP; if caspase activity is inhibited selectively, cells will then inevitably die by oncosis. But perhaps the escape of a limited amout of cyt c to the cytosol will suffice to trigger a caspase cascade, ensuring adequate production of ATP (Reed, 1997).

It is possible that any mutation in cyt c that compromises its caspase-inducing capacity may also affect its functioning in electron-chain transport, and therefore the clonogenic survival of the cell. An intriguing question is therefore whether cyt c release from the intermembrane space of mitochondria is a universal marker of apoptosis. In this context it is remarkable that although CED-4, CED-3 (and CED-9) can form a complex at mitochondrial sites ('apoptosomes'), CED-4 does not seem to depend on cyt c for processing CED-3 *in vitro* (Chinnaiyan et al., 1997; see also p. 45).

The fact is that cyt c release is strongly inhibited by proteins like Bcl-2 and Bcl-Xl. Therefore, it is tempting to conclude that at least in mammalian cells this specific action is in part responsible for aborting the apoptotic response (Yang et al., 1997; Kim et al., 1997). Co-immunoprecipitation studies have shown that Bcl-Xl associates with cyt c, whereas a pro-apoptotic splicing variant of BCL-X (BCL-Xs) does not (Kharbanda et al., 1997). Microinjection of cyt c, however, seems to result in apoptosis that cannot be inhibited by Bcl-Xl expression and it is becoming evident that Bcl-2 family proteins operate upstream of the activation of Apaf-1 (Vander Heiden, 1997).

Some evidence exists that pro-apoptotic members like Bax can directly induce cyt c release and that Bcl-2, Bcl-Xl, and Bax not only resemble the poreforming domains of certain bacterial toxins, but indeed can form ion channels in synthetic lipid membranes. It has been proposed that the anti-apoptotic Bcl-2 acts on mitochondria to stabilize the membrane integrity and to prevent opening of megachannels. Interaction of these outer and inner mitochondrial membrane proteins may regulate accesibility of both intermembrane space and the matrix. It is intriguing that Bcl-2 seems to localize preferably at putative contact sites of the inner and outer mitochondrial membranes (de Jong et al., 1994).

However, Bcl-2 family members are not only localized in mitochondria, but also in nuclear and endoplasmic membranes and possibly at other sites. Moreover, Bcl-2 can interact with a large variety of different proteins, a property which inspired John Reed to compare this protein with a multi-functional Swiss army knife. So, although it is clear that the dogma of preservation of mitochondrial morphology as a hallmark of apoptosis seems to be violated by recent findings, there is at present no consensus about whether mitochondria are indeed the central executioners in all forms of PCD.

Part II: Deregulated Control of Signal Integration: Consequences for the Nerve System, Immunity and Cancer Development

Is there a Grand Unifying Theory of PCD, a basic feature being fundamental to all phenomena hitherto observed? Would it be possible, eventually, to define PCD by the activity of a limited number of key genes? We can only answer these questions when all the different forms of PCD have been more fully characterized and when we have a clear picture of how these complex mechanisms evolved in the first place.

Jean-Claude Ameisen focussed in his presentation on the evolutionary origin of PCD. He reasoned that although it has always been assumed that the 'altruistic' form of cell survival regulation arose with multicellularity, and that this would have been rapidly counterselected in unicellular organisms, it is quite evident that a similar process of socially advantageous regulation of cell survival operates in unicellular eukaryotes too: in primitive mitochondrial eukaryotes, such as certain species of the protozoan parasites *Trypanosoma*, environmental stress (such as starving) and extracellular signals can lead to a form of PCD that shares many characteristics with apoptosis. Ameisen argued that in colonies of unicellular organisms, PCD would allow constant selection of the fittest cells, optimal adaptation of cell numbers to the environment, and tight regulation of the cell cycle and cell differentiation. Primitive forms of 'PCD' even occur in prokaryotes; competition between bacteria from different species could have led to a selection of killer genes encoding toxins used for offence in evolutionary arms races, and concommitant selection of genes encoding toxin antidotes for defense purposes. In this context, it is interesting that bacteria can easily become 'addicted' to plasmids encoding both toxin and antidote: provided that the antidote is less stable than the toxin, losing the plasmid would eventually result in a surplus of toxin and therefore inevitably to the death of the cell (Yarmolinsky, 1995).

Kroemer proposed that a similar mechanism could be involved in the generation of endosymbiosis: the pro-mitochondrion invading the ancestral eukaryote might have developed one or several 'addiction molecules' to stabilize the host/parasite micro-ecosystem. Another possibility is that these bacteria contained pre-formed host-specific toxins; these could have been compartmentalized (e.g. between the inner and outer membrane of the pro-mitochondrion) and only released in particular circumstances, ie, after damage leading to rupture of their outer membrane. If ancestral eukaryotes had developed bactericidal enzymes (e.g. specific proteases) to digest intruding or phagocytozed bacteria, they now needed to develop strategies to keep these enzymes inactive, either by maintaining them as immature precursors or by sequestering them in subcellular compartments (e.g. lysosomes) well-separated from the intruding bacterium. The coevolution of nucleus and endosymbiont subsequently might have led to a condition in which large parts of the bacterial genome were gradually incorporated into the nuclear genome, including genes encoding proteins now known to be crucial for cell death regulation, such as cyt c and Bcl-2 lookalikes (Kroemer, 1997).

Although Ameisen agreed that a multistep process in het emergence of apoptotic machineries is plausible, he also pointed to another possibility: because many genes controlling the cell cycle and cell differentiation also participate in the control of PCD, could it be that the requirement for coupling cell survival to the prevention of self-destruction is as old as the origin of the cell? The inability of cells to avoid random genetic mutation has led to the selection of both DNA proofreading and repair mechanisms and the amplification of DNA diversity by genetic reassortment: could it be possible that originally the inability to avoid self-destruction was an inherent consequence of progression through the cell cycle, and that natural selection forced the regulation of this machinery to evolve in such a way that this self-destruction was effectively repressed (Ameisen, 1996)?

It has been proposed repeatedly that apoptosis is a form of aberrant mitosis (Shi et al., 1994), but there is no hard evidence that the mitotic process by itself is apoptogenic. Gerald Evan, the next speaker in this session, had been among the first to demonstrate that genes like the proto-oncogene *c-myc*, that drive certain cells into the cell cycling mode, also force these cells to undergo apoptotic cell death (Evan et al., 1992) unless the expression of intracellular factors (such as Bcl-2) or certain extracellular signals (such as insulin-like growth factor, IGF-1) prevent the cells from doing so. More recently, his group found evidence that the cell survival signalling pathway from the IGF-1 receptor runs through Ras, but that Ras also activates a pro-apoptotic pathway and that this signal is dominant unless mitigated by interactions with other signalling pathways (Kauffmann-Zeh et al., 1997). Thus, key components of signalling pathways that are often mutated in cancer, such as *myc* and *ras*, have innate 'booby traps' that trigger the death of a cell in which they are activated in the wrong context.

Another intriguing phenomenon in this context is that Bcl-2 family members may affect the level of certain proteins involved in cell cycle regulation. As was demonstrated by Hugh Brady (p. 75), Bax overexpression can facilitate the entry of

T cells into S phase, whereas Bcl-2 delays this entry via modulation of the level of p27/Kip1 protein. Latter protein is a member of cyclin-dependent kinase (cdk) inhibitors; it is implicated in mediating G1 arrest in response to a variety of growth inhibitory signals and its overexpression induces apoptosis (Wang et al., 1997).

This would suggest indeed that part of the cell cycle machinery is used during apoptosis. But these cell cycle factors are likely to function upstream the machinery involved in the actual execution process.

One may speculate that perhaps the genetic modules allowing regulated 'altruistuic' PCD may originally have become selected and have spread for their 'selfish' property of being addictive, but that in a context of multicellularity a variety of pathways evolved to regulate the ancient mechanisms for many purposes. Perhaps even cell death signalling pathways evolved that bypassed the requirement for mitochondria for activating caspase enzymes.

A major breakthrough in apoptosis research is related to the discovery of receptors that can specifically trigger apoptosis: activation by cognate ligand or agonistic antibodies can lead to cell death. These receptors belong to the tumor necrosis factor (TNF) (or nerve growth factor) receptor superfamily of transmembrane proteins, characterized by exracellular cysteine-rich domains. Intracellularly, a number of these receptors share an area of weak homology (Death Domains) required to couple them to specialized adaptor molecules that in turn recruite the apoptosis-inducing machinery. The best studied DD containing receptors are Fas (CD95/Apo-1) and TNFR1 and more recently DR3, DR4 and DR5 have been added to this series.
Krammer focussed in this meeting on Fas (CD95/APO-1) and its ligand, important for apoptosis of peripheral T cells, for down-regulation of an immune response and most likely, at least in part also for peripheral T cell tolerance. In AIDS, apoptosis mediated by this system might contribute to the depletion of T helper lymphocytes, and also in certain liver diseases the Fas system is believed to play a major role.

Most tumor cells have lost Fas surface expression or are resistant to Fas ligand (Fas L) induced death signals. Interestingly, a family of specific inhibitors of this signalling system exists (designated FLIPs), that were first identified in certain viruses (Thome et al., 1997); high levels of a human FLIP have recently been detected in colon carcinoma and malignant melanoma tumors (Irmler et al, 1997).

Fas can activate different sets of adaptor molecules that in turn may activate different caspase enzymes or activate different signalling pathways. Some of these pathways can be inhibited by FLIP, others by Bcl-2; some herpesviruses have both Bcl-2 and FLIP analogues, possibly to block all Fas-transduced apoptotic pathways in infected cells. Recently, Strasser concluded from his experiments that some Fas signalling routes bypass the Bcl-2 system, and probably the mitochondria, but that both routes converge upon activation of certain caspases responsible for induction of the cellular collapse (Strasser et al., 1997).

Krammer showed us that various DNA damaging drugs can upregulate both Fas and Fas-L, probably by their activation of the transcription factor p53 (the promotor of Fas contains several p53 binding sites). Could this phenomenon contribute to the efficacy of certain anticancer agents (Krammer, 1997)?

Fas-L exists in two forms, the insoluble membrane-bound mFas-L and a soluble form, sFas-L, that is cleaved (or shed) from mFas-L by a metalloproteinase. Efficient recruitment of adaptor molecules requires crosslinking of Fas molecules, and mFas-L is much more potent in crosslinking Fas than sFas-L (Strasser & O'Connor, 1998).

Another recent discovery is that the ligand for DR4 and DR5, designated TRAIL, is cytotoxic for a number of tumor cell lines and yet relatively non-toxic for normal cell lines. The reason for this is that TRAIL can bind to decoy receptors that have a cytoplasmic domain, but lack DD and are preferably expressed in normal human tissues, but not in most cancer cell lines (Pan et al., 1997).

Although Fas and TNF-R are widely expressed in many different tissue types and cells, Fas-L and TNF can induce apoptosis only in a limited number of cell types and require the presence of the protein synthesis inhibitor cycloheximide to induce apoptosis in others (Yuan, 1997). In case of TNF, resistance to cell death induction may result from the ability of TNF to activate NF-κB-mediated transcription (Liu et al., 1996).

Thus, multiple intracellular signals, pro-apoptotic and antiapoptotic, are concurrently activated when a cell is stimulated; the prevailing signal(s) will simply determine the fate of the cell (Beg et al., 1996).

The effect of neurotrophins to block neural cell death has been appreciated since the classic work of Levi-Montalcini and Hamburger (Levi-Montalcini, 1987). During prenatal development much more nerve cells are initially formed than necessary and the excess of cells is eliminated by PCD. This has probably evolved during evolution to allow exact match of nerve and target cells (Jacobson et al., 1997).

Neurotrophins are produced which can bind to two distinct groups of receptors: the p75NTR receptor, previously known as nerve growth factor (NGF) receptor, which is a member of the TNF receptor family, and Trk receptor tyrosine kinases (Bredesen & Rabizadeh, 1997; Dechant & Barde, 1997). Yves-Alain Barde (p. 87) described that for all neurotrophins, interaction with TrkA receptor is necessary for prevention of PCD. However, interaction with of NGF with p75NTR promotes PCD.

Interestingly, as with binding of Fas-L to Fas and by binding of TNFα to TNFR1, binding of NGF leads to generation of ceramide (Cassaccia-Bonnefil et al., 1996), which is considered to be a key component of intracellular stess response pathways (Hannun, 1996).

In the sphingomyelin cycle, a number of extracellular agents and insults (including certain chemotherapeutic agents, heat, certain cytokines, TNF, Fas-L and NGF) cause activation of specific sphingomyelinases, which act on the plasma membrane phospholipid sphingomyelin and release the metabolite ceramide. Ceramide acts as a second messenger and has emerged as an important regulator of various stress responses in mammalian cells. Jannie Borst discussed recent data supporting the idea that ceramide acts in conjunction with the caspase cascade in Fas-induced apoptosis. On p. 97-112, she and her co-workers discuss the role of Fas in apoptosis induced in T cells as a result of recognition by the T cell antigen receptor of antigenic peptides. They conclude that within this context both Fas-dependent and -independent mechanisms exist.

Cells can 'learn' to protect themselves against induction of stress-induced apoptosis. One mechanism how this is achieved was presented by Bob van de Water. Cellular exposure to environmental stresses such as ischemia/reperfusion, drugs, heat shock, environmental pollutants or toxic chemicals results in upregulation of stress proteins like c-Myc, c-Jun, and c-Fos, and members of the heat shock family, including heat shock proteins HSP70, HSP90, and HSP110 and the glucose-regulated proteins GRP78 and GRP94. These proteins function as molecular chaperones and bind to partially folded newly synthesized transmembrane and secretory proteins, including integrins, viral proteins, and MHC class I and II proteins.
Besides the chaperone function most, if not all, of the chaperones of the endoplasmic reticulum (ER) are also calcium-binding proteins, but it is not entirely clear whether the calcium-binding capacity is necessary for the chaperoning function of these proteins. The relevance of induction of ER stress proteins in apoptosis/PCD is evaluated on p. 113-126.

Session III: Apoptosis-based Therapies and Drug Development

Whereas the first days of the colloquium were mainly devoted to the definition, morphology, genetics and relevance of apoptosis/PCD, the last session attempted to translate some of the overwhelming amount of knowledge obtained during the past few years into prospects for treatment of various diseases, especially cancer. As was stressed by John Hickman (p. 127), the concept of apoptosis has led to a paradigm-shift in thinking about the origin, progression and treatment of cancer. Chemical and physical damage to mammalian cells induces complex cellular and molecular responses; the ability of a cell to indure and survive such insults may be influenced by factors other than the extent of cellular damage sustained.
Earlier studies identified p53 as an important regulator of apoptosis and demonstrated that mutations could promote oncogenic tranformation, tumor progression, and resistance to cytotoxic agents by reducing a cell's apoptotic potential. Studying the effects of anticancer agents on colonic crypt epithelia in normal mice and mice lacking p53, Hickman's group concluded that 'toxicity' is a composite of p53-dependent apoptosis, a prolonged p53-dependent cytostasis and an inhibition of mitosis. The observed cell deaths, however, did not always show the characteristics of apoptosis: many cells simply lose their proliferative capacity and eventually die, probably without induction of the caspase cascade.

Also Scott Lowe's presentation (p. 143) was focused in part on the role of p53 in oncogenic transformation and chemosensitivity. How do p53 and other cell death regulators affect caspase activation and which apoptotic pathways are p53-independent? What are the prospects of introducing a wildtype p53 gene (e.g. with the aid of viral vectors) into tumors cells that have lost their p53 function?

Prostate and breast cancers most often depend on a specific hormonal environment for their growth; hormones and growth factors may not only act mitogenic, but can also function as survival factors, or, as Gert-Jan van Steenbrugge (p. 151)

showed, even as apoptogenic factors: in the human prostate cancer cell line LNCap, depending on the concentration administered, cell proliferation can be stimulated and apoptotic cell death induced. Development of androgen-resistance towards these effects was found to be associated with expression of Bcl-2.

In androgen-dependent cells, Bcl-2 levels can be modulated by androgens and, as Henk-Jan van Slooten demonstrated (p. 165), a similar relationship has been found between Bcl-2 and estrogens in human breast cancer cells. An important question then is whether treatments based on endocrine manipulation act in part by modulation of Bcl-2 and whether a combination of different treatment modalities, such as treatment with anti-estrogens and cytotoxic drugs respectively, should be given in a such a way that timing and choice of drugs guarantee that advantage is taken from down-regulation of anti-apoptotic Bcl-2 family members.

A similar strategy has been proposed for drug combinations which include compounds that affect formation of mitotic spindle figures (such as taxol): latter compounds tend to trigger a pathway that leads to phosphorylation, and thereby inactivation of Bcl-2.

Other strategies to down-regulate or inactivate Bcl-2 are treatment with Bcl-2 antisense messenger RNAs, as has recently been pursued a series of 9 patients with B-cell lymphoma, and the development of small molecular and highly specific inhibitors. In fact, many pro-apoptotic Bcl-2 family members act by binding to a hydrophobic pocket on the surface of Bcl-2, thereby abrogating its anti-cell death activity.

Apart from modulating tumor cell sensitivities to apoptosis-induction it is clear that knowledge on factors involved in the regulation of apoptosis could be of value in predicting the response to various treatment modalities. As was stated by Van Slooten: if one gives chemotherapy that works through the apoptotic pathway, and show that the tumor is not apoptosis-competent for that particular treatment, it is obvious that one is just poisoning the patient: paying attention to apoptosis-competency in tumors, diagnostically and prognostically, may eventually provide a way to tailor therapy. In the previous session, Gajja Salomons had shown interesting data indicating that the ratio between Bcl-2 and Bax is an important indicator for the survival of drug-induced apoptosis in leukemic cell lines and that information of these gene products could be relevant for childhood acute lymphatic leukemia (p. 179).

Although it takes 15 years on average for an experimental drug to become approved and registrated, there exists little doubt that the recent developments in apoptosis research have provided many new opportunities. The last three presentations of the colloquium involved drug development, but from entirely different perspectives.

Donald Nicholson (p. 187) focussed entirely on the human caspase family, their roles in homeostatic and repair processes, as well as in apoptosis-related proteolysis, their X-ray crystal structure and the determination of precise substrate specificities. The elucidation of the caspase structure, mechanisms of activation and place within apoptotic pathways has been helped tremendously by a number of selective inhibitors. Nicholson envisioned an important role for such inhibitors in diseases caused by too much apoptosis, including Huntington's disease and Alzheimers's disease.

This type of drug design is based on profound knowledge of structure and function of specific molecular targets and systematic search, but in fact the development of most of the drugs used today was based on trial and error and unexpected discoveries.

Danen-van Oorschot (p. 197) described the apoptosis-inducing protein Apoptin, which is derived from an avian virus. Apoptin induces apoptosis in a p53-independent way, is stimulated by overexpression of Bcl-2, and is insensitive to BCR-ABL. Preliminary data suggest that caspases do not play a key role in the induction pathway. Surprisingly, Apoptin induces apoptosis in a large variety of human tumorigenic/transformed cells, but not in normal diploid cells. This differential behavior is likely to be related to the observation that Apoptin is present in the nucleus of tumor cells, whereas in normal diploid cells it is localized in peri-nuclear structures. Danen-van Oorschot concluded that the unique features of Apoptin makes it a potent anti-tumor agent, and that elucidation of the mechanism of Apoptin-induced apoptosis could possibly result in novel molecular targets for drug treatment.

Finally, David Tomei told us how the observation that dogs tested to a certain

that soy flour was responsible for the effect; in an *in vitro* screen it was found that an extract of this soy flour was particularly effective at inhibiting apoptosis. The active ingredient appeared to be a mixture of protein-bound lysophopholipids. An optimized formulation of these lysophospholipids blocked apoptosis in several culture and whole-organ systems and applications for reduction of chemotherapy-induced death of intestinal crypt cells and organ preservation in transplantation look promising.

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J. John Cohen

Apoptosis and its Significance in Health and Disease

Abstract

Apoptosis is the method by which normal cells die normal deaths. It is essential throughout life; some cells live just a few hours. But apoptosis can also be triggered abnormally, as in degenerative diseases. Conversely, we want to induce apoptosis in dangerous cells. So we need to know how apoptosis is regulated, and how we can influence the process.

Necrosis and Apoptosis

The invention of tissue staining over a hundred years ago was perhaps the most significant advance in pathology. It made it possible to move from gross to microanatomic insights into disease processes. Cell death was one of the first phenomena described, and necrosis was the term applied to its appearance. The early pathologists looked most at tissues where injury was extreme and sudden: ischemia following occlusion of a major artery, physical or chemical trauma, overwhelming infection. In necrosis, regardless of the inciting injury, the cellular response is rather stereotyped. The target organelle is the mitochondrion, which begins to swell within the cytoplasm. At the stage called 'high-amplitude swelling' crystals, probably of calcium phosphate, form in the mitochondrial lumen; at this point the mitochondrion can no longer maintain its ionic gradients or oxidative phosphorylation, and the cell runs out of energy. Without enough ATP, plasma membrane ion pumps fail, water rushes in, and the cell swells and bursts. Lysis releases the cell's intracellular contents into the extracellular milieu, where they have no business being; their internal lipids, proteases, and small molecules are intensely proinflammatory. They attract white cells, primarily macrophages, from around the body. Overall this is desirable, since necrosis always involves many cells at the same time in one area, and the local facilities for dealing with damage can be overwhelmed. The net effect of the inflammatory process is the eventual removal of debris, resolution of damage, and, sometimes, scar formation.

In 1972, Kerr, Wyllie, and Currie (Kerr, Wyllie, and Currie, 1972) described a different morphology of cell death. It was seen in cells for which death was a normal stage of their life style, that is, cells scheduled to live a shorter time than the body as

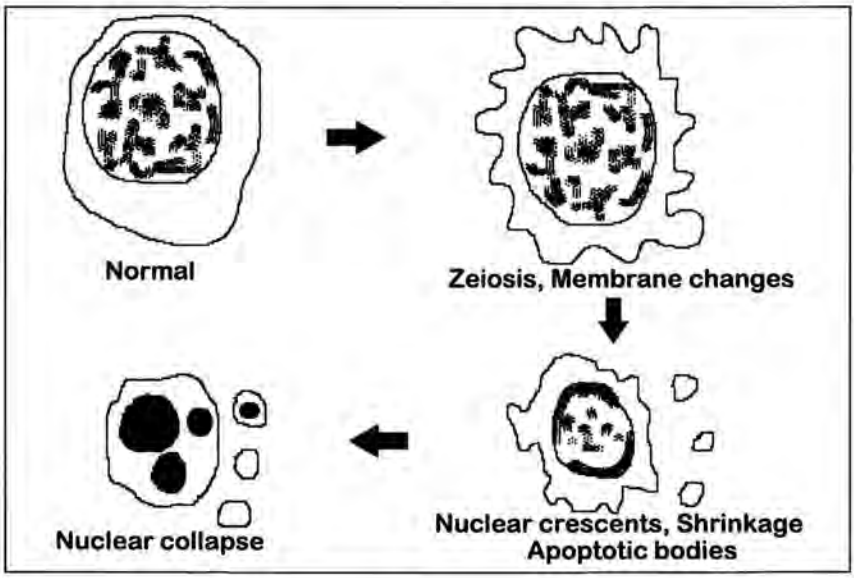

Fig. 1. The morphological progression of apoptosis. Zeiosis, from the Greek word for yeast, refers to the bubbling appearance of the plasma membrane.

a whole. It was also seen in cells dying of relatively minor injury compared to that which led to necrosis, for example, around the periphery of ischemic lesions. Because this death seemed to be more physiological than necrosis it was named apoptosis, from a word in the Hippocratic corpus meaning the loss of leaves from trees in the autumn. It was a nice choice, as it invokes a loss that is expected, and it implies renewal.

Characteristics of Apoptosis

The main morphological feature of apoptosis is a collapse of the nucleus; chromatin, which is normally composed of mixed condensed and open regions, becomes super-condensed, appearing as crescents around the nuclear envelope and, eventually, spherical, featureless beads (Figure 1). When stained with fluorescent DNA dyes these beads are extremely bright. The biochemical correlate of this morphological change is the fragmentation of DNA, initially into quite large pieces of about 50 to 70 kilobase pairs, and then, in some but not all cells, into units of one or several nucleosomes in length. (A nucleosome consists of a core of histone proteins wrapped by about 200 base pairs of DNA, and is the first stage of compaction of DNA so that a meter of it can fit in the nucleus of a cell.) This nucleosomal degradation reflects the action of an endonuclease, whose identity has not yet been clearly established, on the DNA in the linkers between nucleosomes; this stretch of DNA is not very well

protected by histones (Stratling and Klingholz, 1981). Because a cell can only repair about 7 simultaneous double-stranded breaks in its DNA, the extensive DNA damage in apoptosis (up to 300,000 breaks/chromosome) means that even if there were no other changes, the cell would certainly never divide again.

But other changes do take place. Early in apoptosis cells shrink remarkably, losing about a third of their volume in a few minutes. The mechanism for cell shrinkage is not yet understood, but it must involve shifts in ions as well as water (Bortner and Cidlowski, 1996), perhaps through the unusual activation of plasma membrane-associated ion channels or pumps. This shrinkage is quite apparent in cell culture, and also in vivo, where apoptotic cells in tissue sections often call attention to themselves by the clear halo which surrounds them. As might be expected there are cytoskeletal changes that accompany shrinkage, and the result is a peculiar, vigorous 'boiling' action of the plasma membrane, which has been called zeiosis (Costero and Pomerat, 1951; Godman et al., 1975). By this action the apoptotic cell usually tears itself apart into apoptotic bodies, some of which contain chromatin. It is not known how, or even if, these changes lead to cell death. This is because early in apoptosis, while the cell is still fully 'viable', that is, able to exclude vital dyes like trypan blue, it is recognized by another cell and phagocytosed; it dies within the phagocyte. So the real goal of all the morphological changes may be to ensure that the cell gets taken up by another, before it has had a chance to spill its contents.

Recognition of Apoptotic Cells

Apoptosis is also accompanied by changes in the plasma membrane, the most interesting of which involves the phospholipid phosphatidylserine (PS). All the PS in a normal plasma membrane is confined to the inner leaflet of the lipid bilayer; in fact, a special enzyme, aminophospholipid transferase, exists just to ensure that any PS molecule that strays to the outer leaflet is quickly returned (Connor and Schroit, 1991). Soon after apoptosis begins, the distribution of PS becomes equal on both sides of the membrane, by a 'scrambling' mechanism not yet clearly defined (Fadok et al., 1992). This means that PS is now exposed on the cell's exterior surface. Phagocytic cells have receptors for PS, and recognize, bind to, and ingest cells that have committed to the apoptotic pathway, consuming them while they are still alive. In this way the apoptotic cell never has a chance to lyse and release inflammation-causing molecules to the extracellular space. Furthermore, a macrophage that recognizes a cell as apoptotic does not become activated (Meagher et al., 1992). So the removal of apoptotic cells is physiological and silent, as would be appropriate for an event that occurs constantly in the normal human body. Thanks to a clever recent observation it is possible to detect externalized PS quite simply, taking advantage of the fact that this phospholipid has high affinity for annexin V; tagging annexin V with a fluorescent label makes it easy to see its binding to apoptotic cells (Koopman et al., 1994; Homburg et al., 1995; Vermes et al., 1995). The removal of apoptotic cells is so vital that there are multiple mechanisms for their recognition, in addition to the PS system (Savill et al., 1993; Hall et al., 1994; Stern, Savill, and Haslett, 1996).

It is interesting to speculate that there might be abnormalities and pathological versions of this important function. For example, a parasite that 'learned' to get inside a macrophage by binding to its receptors for apoptotic cells might not activate the macrophage's killing systems. Such a parasite would find itself in an enviable position, protected from the immune system like the warriors in the Trojan Horse. It seems also probable that its antigens would not be presented to the T cell surveillance system.

Induction of Apoptosis

The model system we have used for many years to study apoptosis is the rodent thymocyte exposed to glucocorticoids. We had found, to our surprise, that thymocytes die when exposed in culture to a concentration of glucocorticoid equal to that achieved each day at the peak of the circadian cycle. But it soon became known that over 95% of cells that are generated in the thymus will die there, and fewer than 5% will mature to competent T cells; something must contribute to all this cell death. We suspected that thymocyte death-by-steroid was a physiological phenomenon. Although we were not able to demonstrate it at the time, Ashwell's group has now clearly shown the essential role that steroids play in thymus cell maturation (Vacchio, Papadopoulos, and Ashwell, 1994; Ashwell, King, and Vacchio, 1996). Wyllie made it plain in 1980 that glucocorticoids killed thymus cells by apoptosis (Wyllie, 1980). Steroids, we knew, work almost exclusively by inducing changes in gene expression, usually by transcriptional activation. But could transcriptional activation lead to death? It seemed impossible, but in our first experiments we treated thymocytes with a lethal concentration of the synthetic glucocorticoid dexamethasone, and found that if we incorporated blockers of transcription in the medium, the cells did not die (Cohen and Duke, 1984). The steroid was not killing the cells; rather, it was inducing them to kill themselves. When we prevented them from expressing what we came to call 'death genes' at either the transcriptional (mRNA) or translational (protein) levels, they could not die.

The important point that these experiments made was this: if one cell type has 'death genes', then all cells in the body do, since they share the same genome. So any cell in the body could be made to undergo apoptosis if we could understand how to get it to turn on these genes, and any cell that intended to die might be prevented from doing so by a reverse strategy. It also suggested that in addition to programmed cell death, there was a cell death program.

Biochemistry of Cell Death

What are the steps that constitute the cell death program? They are not yet all clear, but one consistent pattern is emerging: the involvement of proteases as signals for the apoptotic events that follow, and possibly as important players in those events themselves (Figure 2). In the nematode worm *Caenorrhabditis elegans*, two genes

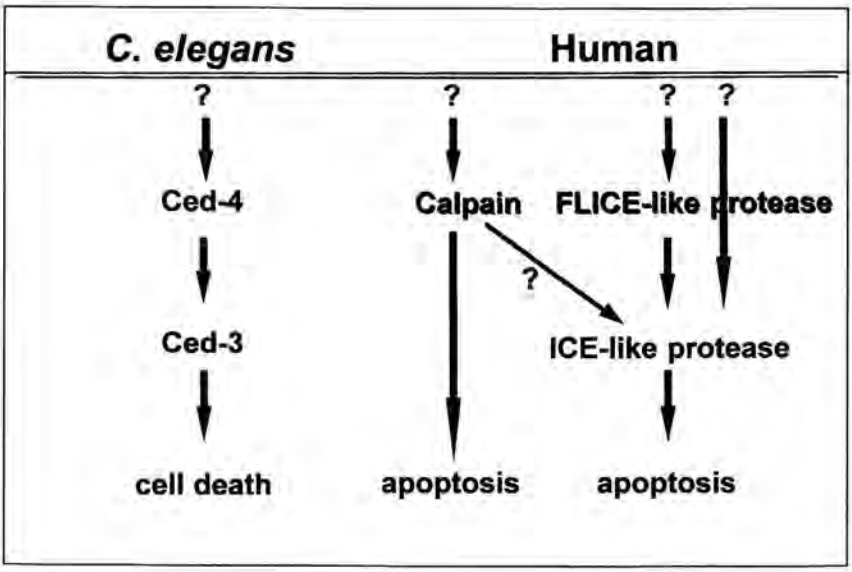

Fig. 2. Protease signals in programmed cell death. Ced-3, the members of the caspase family (FLICE-like and ICE-like proteases), and calpain are all cysteine proteases. The two pathways in the human may be interconnected, because proteases can activate other proteases.

are known to be essential for programmed cell death of some 131 cells that develop but are lost upon maturation. These are called *ced-3* and *ced-4* (Yuan and Horvitz, 1990; Xue, Shaham, and Horvitz, 1996; Yuan et al., 1993). The function of Ced-4 is not yet known; it acts upstream of Ced-3, and probably binds to it when Ced-3 is active. Ced-3 is a cysteine protease. There is also a death-inhibiting gene, *ced-9*, whose protein product competes with Ced-3 for binding to Ced-4; if more Ced-4 is bound to Ced-9 than to Ced-3, the Ced-3 is inactive and the cell does not die. In humans, there are a number of cysteine proteases with homology to Ced-3; they are related to the interleukin-1ß converting enzyme, ICE. Recently it has been agreed to call these enzymes caspases, for cysteine proteases that cleave at an aspartic acid residue. One of them, caspase 3 (also called CPP32,) seems to be the most important apoptosis-associated enzyme of the group. It is activated in many, but not all, models of apoptosis studied so far. It has a number of substrates but the ones that are critical for apoptosis are not yet identified. Caspase 3 can be activated when it is cleaved by an upstream caspase, FLICE (caspase 8). FLICE itself is activated when it interacts with a complex of plasma membrane proteins including CD95, or Fas; thus external signals are transduced into death signals within the cell.

The caspases are not the only proteases that have been implicated in apoptosis. Calpain, a calcium-dependent cysteine protease unrelated to the caspases, seems to be essential for apoptosis in a number of normal cell systems, including thymocytes and neutrophils (Squier and Cohen, 1997; Squier et al., 1994). Interestingly, calpain

inhibition reduces the extent of lesions in the brain of rats undergoing experimental stroke (Bartus et al., 1994); this reminds one that apoptosis is a significant part of lesions once thought to be purely necrotic (Kerr, Wyllie, and Currie, 1972). It seems reasonable to suggest that proteases are involved as apoptosis signals because, unlike the other well-known signaling pathways such as G proteins and membrane receptor kinases, proteolytic cleavage in the cell is irreversible, and so moves the cell inexorably towards its destruction.

The Ubiquity of Programmed Cell Death

A few examples serve to illustrate how widespread programmed cell death is, and how important to the organism. In 1986, Duke showed (Duke and Cohen, 1986) that growth factor deprivation causes death by apoptosis. During the response to antigen, helper T cells produce cytokines to which other cells, such as cytotoxic (killer) T cells respond by proliferation and differentiation. The cytotoxic cells become dependent on the cytokine interleukin-2 for their survival, and die by apoptosis when it is removed. Thus many of what we commonly refer to as growth factors may be also, or even solely, survival factors. It has been argued that all cells in the body depend for their continued existence at all times on input, diffusible or contact, from their neighbors (Raff, 1992). Local conditions could determine whether a cell will live or die. Very recently it has been shown that even cell shape, as influenced by the local tissue geometry, affects whether a cell will survive or die (Chen et al., 1997).

A very important phenomenon during development called morphogenetic death determines the final shape of body parts and organs. In limbs, the death by apoptosis of cells between the digits gives the final form to fingers and toes (Saunders, 1966; Hurle et al., 1996). Even in the nervous system, many more cells than the organism needs develop; if these form the correct contacts at the correct time, they are bathed in survival factors by the target they have innervated, and if not, they are dispensable (Oppenheim, 1991). Indeed, the formation of as precise a structure as the brain depends on a Darwinian-style selection of cells that have chanced to make the best connections. In the thymus of the young rodent, 95-99% of the lymphocytes that develop there fail to be selected, and die by apoptosis; the entire organ is replaced every 3 days (Shortman and Jackson, 1974; Matsuyama, Wiadrowski, and Metcalf, 1966).

As mentioned earlier, apoptosis is estimated to occur 25 million times a second in a human adult. This is based on a basic assumption: In a steady-state system like the adult human, whenever a cell in a particular compartment divides, another must die, or else the size of the compartment will change. It is not easy to estimate the rate of apoptosis in a solid tissue, because the efficiency of removal of live but apoptotic cells is superb, and counting the number of classically apoptotic cells in a section will certainly underestimate the true figure (for example, we know that a third of all lymphocytes in the young mouse thymus die every day, but it is difficult to find even one on a standard section.) But using the 1 mitosis = 1 apoptosis assumption, we can estimate apoptosis as matching the rate of mitosis, which is a more tractable number to

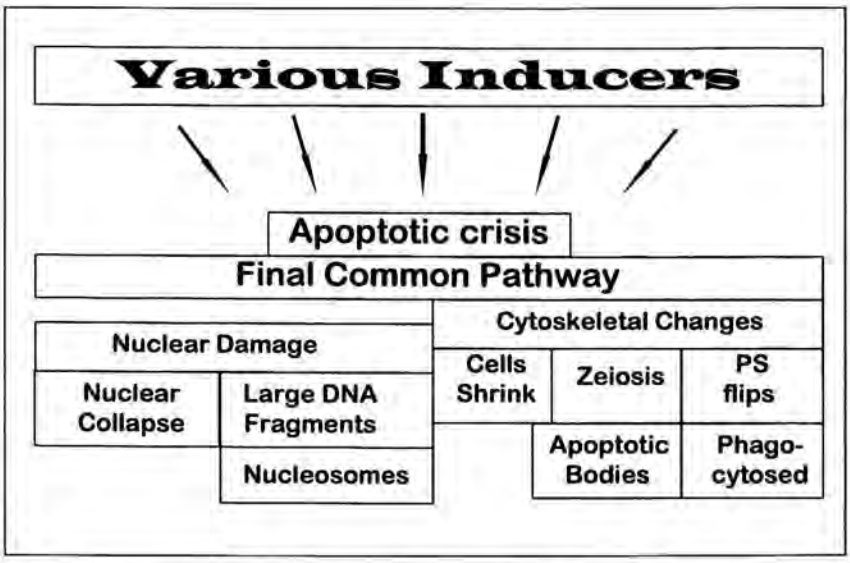

Fig. 3. Different inducers can activate cell-specific pathways that eventually funnel into a single crisis point that triggers stereotyped apoptotic events.

measure (David Prescott, personal communication.) Using this estimate, we come up with some surprising numbers: 2.2×10^{12} cells die (and are born) per day, a weight of at least 2.2 kilograms! For a 70 kg man who needs 2,500 kCal/day to maintain basal functions, this suggests that about 75% of the apoptotic cell mass is recycled, and 25% used to provide the necessary metabolic energy. No wonder so many apoptotic processes and mediators (the nucleases and proteases) are designed for efficient dismantling and recycling of building blocks.

Whenever a cell undergoes apoptosis, regardless of the cell type and for whatever reason, the morphology tends to be stereotyped, suggesting that the underlying mechanism is the same. This means that there must be multiple pathways leading to a final common pathway of apoptosis (Figure 3). Clearly, for some purposes we might want to attack the common pathway, but in most cases therapeutic interventions will be directed toward influencing a particular cell-specific signal. For example, it might be valuable to be able to prevent apoptosis in cardiomyocytes after a coronary occlusion, or in neurons early after a stroke.

Throughout adult life, cells continue to die and to be replaced. Life expectancies in the human body range from half a day or so for neutrophils to a century or more for neurons. Again, the decision is made partly by the cell itself, and partly by its environment in the form of survival signals. The importance of the environment cannot be estimated yet, but the following scenario is interesting in this context. It has been estimated that in the average adult, about one cell per hour sustains a mutation of the type that could lead to malignant transformation. We obviously do not get tumors at that rate; what limits the success of the mutated cell? If we suppose that the

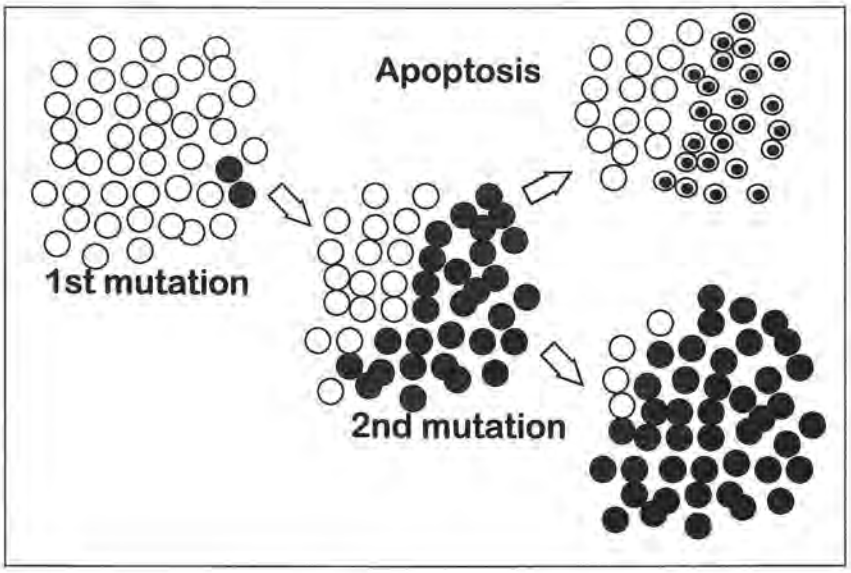

Fig. 4. A first mutation that stimulates cell growth will produce a clone of abnormal cells that will outgrow the local supply of survival factors, and undergo apoptosis. But if there is a second mutation that produces a new phenotype, more resistant to apoptosis, growth may continue.

mutation confers a growth advantage, a clone will develop from the original cell. At some point the ability of the local environment to sustain this unanticipated growth will be exceeded, and then the cells, deprived of growth/survival factors, will die by apoptosis (Figure 4). This must happen virtually all the time. But if during this critical period a second mutation (or adaptation) takes place, such that the cells are now more resistant to apoptosis, the clone may survive. There will be subsequent crises, and a new adaptation will be required each time; most estimates are that it takes about 7 mutations for a cell to become fully, clinically, malignant. But this simple model stresses a key point: for cancer progression, mutations that inhibit death may be just as important as those that stimulate growth. We are learning a lot about anti-apoptotic genes and their protein products, for example the Bcl-2 family (Boise et al., 1995; Reed, 1995; Yang and Korsmeyer, 1996).

Cellular Responses to Damage

Lymphocytes are more sensitive to radiation than any other cell; it has been reported that as little as 5 rad (0.05 Gy) will kill a lymphocyte (Anderson and Warner, 1976), while it takes thousands of rads to kill fibroblasts or macrophages. This death is clearly by apoptosis (Sellins and Cohen, 1987). Why are lymphocytes so sensitive? It may be because they are so dangerous. A very minor change in the environment — the

binding of antigen to the cell's receptor — can drive a lymphocyte into rapid cycle, so that the one cell can become 64,000 cells within 4 days. If such a cell were to be damaged, perhaps mutated, the error would rapidly be locked into a substantial clone. This poses a risk of autoimmunity, or even lymphoma. So it seems reasonable that a damaged lymphocyte would respond not by repair, but by committing suicide. This is biologically sound, since the sole function of the body is to preserve and perpetuate gametes, and any single somatic cell may be sacrificed, if it presents a possible risk to the community of cells. We call this the 'better dead than wrong' rule. Cells that are less risky, like fibroblasts, have the leisure to repair much more severe damage. So there is a continuum of response to injury: first, repair; if repair is impossible or unwise, apoptosis; if the damage is overwhelming, necrosis. For different cell types, the crossover to the next response will occur at different levels of damage. This could explain, for example, why certain toxins and chemicals are more harmful to specific tissues or cell types. At a more subtle level, if a population of cells were relatively resistant to apoptosis they might under some circumstances be more susceptible to malignant transformation, as they would survive better — possibly harboring mutations — after exposure to carcinogens. This observation might underlie the resistance of small intestinal crypt cells to cancer, and the much greater sensitivity of similar cells in the large intestine (Merritt et al., 1995).

The tendency of lymphocytes to undergo apoptosis after mild injury is well illustrated by their response to confusing signals. T cells require simultaneous signals at both the receptor for antigen (TCR) and accessory molecules, notably (for helper T cells) CD4. If both signals are delivered together, as can be done experimentally using antibodies to the TCR and to CD4 to cross-link these molecules, the T cell, fooled into thinking it is recognizing antigen, becomes activated, produces lymphokines, and prepares to divide. But if the CD4 signal is delivered alone, and the TCR signal delivered a half hour later, the cell undergoes apoptosis. Receiving the correct signals in the wrong order is enough to make the T cell commit suicide (Newell et al., 1990). In people who are HIV seropositive, circulating gp120 shed from virus-infected cells binds to CD4 on uninfected cells, and the antibody to gp120 which seropositive people make will cross-link it, and therefore CD4, too. These cells can be thought of as storing the first signal; if they now have their TCR cross-linked, they will undergo apoptosis rather than respond to the antigen (Banda et al., 1992). It is possible that this mechanism is responsible for considerable cell loss in AIDS, especially of cells that are not actually infected by the virus (Ameisen et al., 1995).

Apoptosis Transduction

Cytotoxic T cells are responsible for surveillance of the surfaces of all body cells. Nucleated cells display the proteins they are making on their plasma membranes, in the form of peptides transported there bound to Class I major histocompatibility complex proteins. If a cytotoxic T cells recognizes this combination, the target cell undergoes apoptosis. Although there are several models for the mechanism of this process, the most clearly established involves the cytotoxic cell upregulating expression of a

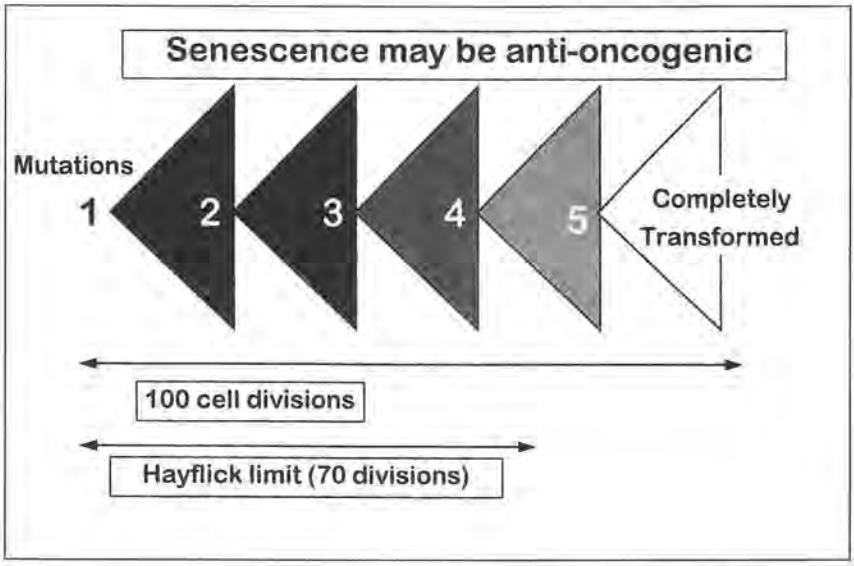

Fig. 5. It takes a series of mutations or similar changes (estimates range from 5 to 8) for an 'initiated' cell to become completely transformed, that is, to develop the full malignant phenotype. If about 20 doublings are required before the next change is locked in, then it might take up to 100 doublings for the malignant state to be reached. But if normal cells reach their Hayflick limit of division before that, then these senescent cells can no longer be transformed. The cancers seen in old people were initiated in their youth.

surface molecule called Fas (or CD95) ligand, which then engages and cross-links a corresponding molecule on the abnormal cell's surface, Fas or CD95. CD95 then transduces a signal into the cell's interior, which activates a cascade that includes the caspases FLICE and CPP32 (Fernandes-Alnemri et al., 1996). Mice deficient in the CD95/CD95-ligand pathway develop autoimmunity and huge overgrowth of lymphoid tissues with cells that are abnormal although not malignant in the usual sense. Recently, children with the same problems have been reported (Sneller et al., 1997). Their enormous lymphadenopathy suggests lymphoma or Hodgkin's disease, although the pathogenesis is a failure of cells to die rather than an uncontrolled proliferation. Again it is worth considering that in every cell compartment of the adult at steady state, a cell must die for each one that divides. If proliferation exceeds death, the compartment grows; and this can happen because cells are dividing too fast, or not dying fast enough. This is a novel way of looking at malignancy, and no doubt both processes are often of equal importance.

Apoptosis and Aging

It is reasonable to suppose that death from old age is a physiological or even a 'programmed' event, one that will occur at about the same time in everyone if 'accidental'

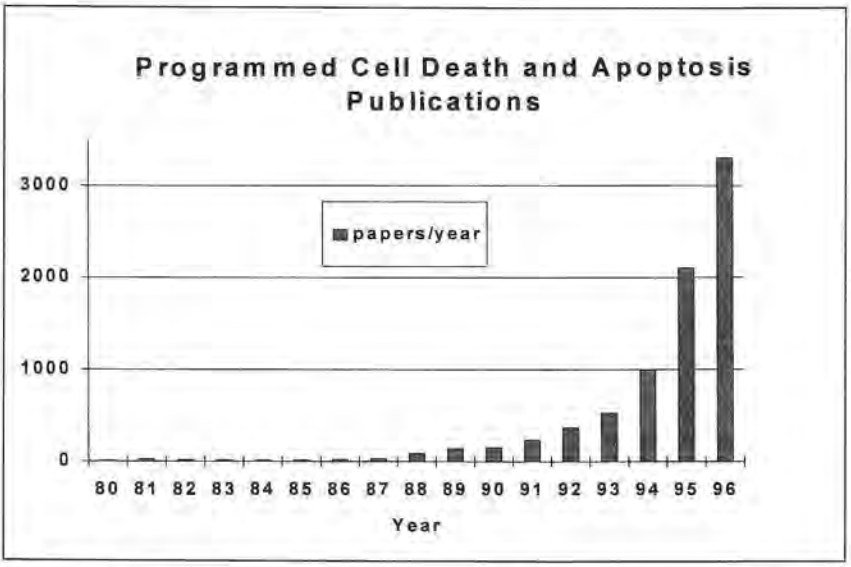

Fig. 6. Publications having to do with apoptosis in the world's literature since, 1980.

death does not supersede it. If we define 'senescence' as the decreases in function that characterize the aging process, then it is also reasonable to think that because senescence leads to death, in fact death is the purpose of senescence. This is maybe just a truism, but it is also possible that senescence has benefits for the organism that are not directly connected with death as an outcome. Because if senescence is the process by which we die of old age, why does it take so long — most of our life span — instead of coming on explosively as it does in the Pacific salmon, as soon as reproductive function is over?

Given that mice and humans are made of cells of about the same size, complexity, and metabolic diversity, with the same operating temperature and the same cell cycle times, and about the same biochemical requirements, it is amazing that a mouse is old and gray at 2.5 years and dead by 3, while humans take 80 to 100 years or more to reach the same state. So another issue that has not been resolved is the locus of the changes that lead to senescence and death. Are these organismal or systemic? Cellular or molecular? Environmental or genetic? The safe answer here is: probably some of each. As someone who studies the death of cells, but only thinks about the death of organisms, what I will discuss here seems interesting from my perspective, but may strike as naive the sophisticated student of senescence.

Is senescence in any way linked with apoptosis? It is tempting to think that the processes that lead to cellular and organismal 'programmed' death might be connected. However, at present there is not much direct evidence to support this idea. It is possible that with age there is a relative increase in apoptosis over mitosis, so that the mass and therefore function of critical cell systems may decline, leading to an overall decrease in efficiency of the organism. Clearly, in the steady state, mitosis

and apoptosis should be exactly balanced. Loss of mass would follow either a decreased rate of mitosis or an increased rate of apoptosis. As we discover the genetic regulation of both these complicated processes we will be able to see to what extent they are affected by aging.

Human fibroblasts that senesce in culture after 60 or 70 population doublings go out of cycle but do not usually die, and exhibit few if any of the hallmarks of apoptosis. It is easy to see how this process, if happening generally in the body, could cause its parts to wear out through lack of repair. It is intriguing to speculate on the reasons for senescence as compared to apoptosis. Apoptosis seems to exist as a process for the removal of redundant or effete cells. Senescence may be a way of taking cells out of cycle to prevent their undergoing malignant transformation (Figure 5). As age progresses, the mutations that can lead eventually to transformation accumulate in individual cells. Since transformation requires multiplying cells, and since senescent growth cessation is dominant over proliferation, it may be that we senesce in order to keep from getting more and more cancer (Sager, 1991). Dying of old age, then, could simply be an unavoidable side effect of an otherwise excellent design.

Conclusions

There is hardly another area in cell biology that has exploded so rapidly in recent years. Although the first publication to specifically mention apoptosis appeared in 1972 (Kerr, Wyllie, and Currie, 1972), it was not until 1980 that a biochemical approach became available with Wyllie's description of DNA fragmentation (Wyllie, 1980). Even then, interest was desultory until the late 1980s, when apoptosis research entered the log-growth phase in which we still find it (Figure 6). There is still much to be learned about this fascinating and important process, which pervades all aspects of life from gametes to centenarians, skin to bones, worms to humans.

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R. Schulte-Hermann

Active Cell Death (ACD): Concepts, Subtypes and Quantitative Detection in vivo

Abstract

The occurrence of cell death as a physiologic event in multicellular organisms has been known since more than 150 years. In 1972 the term apoptosis was introduced on morphological grounds. Accumulating evidence suggests that cells seem to use different pathways for active self-destruction as reflected by different morphology: condensation prominent, type I or apoptosis; autophagy prominent, type II; etc. Morphologically different types of ACD were found to be associated with different biochemical and molecular events eventually leading to cell collapse. Thus, induction of transglutaminase and cytoskeleton breakdown appears to occur during type I (e.g. hepatocellular apoptosis; HT29/HI1) but not during type II ACD (e.g. autophagic, lysosomal ACD of mammary tumor cells (MCF-7)). Furthermore, discrimination between active cell death and necrosis may not always be unequivocal. Thus, DNA fragmentation as detected by TUNEL assay and DNA ladder has also been found in necrotic cells. Currently no simple biochemical or molecular marker for detection of cell death subtypes is available. Therefore, for unequivocal identification of the various types of cell death morphological (electron- and light-microscopy), biochemical and functional criteria should be used in combination. During tumor development in various organs of animals and humans not only rates of cell proliferation (α), but also rates of cell death (β) may increase with increasing malignancy. Morphological and functional criteria (anti-promotion, withdrawal of survival factors, net growth rate α-β) indicate that ACD is a major determinant for the development and growth of tumors.

Concepts on Cell Death

The occurrence of cell death under a variety of physiological and pathological conditions in multicellular organisms has been documented manyfold during the past 150 years (Vogt 1842 for review: Majno and Joris, 1995, Clarke and Clarke 1995). Virchow (1871) described the diversity of cell death as 'necrosis' and 'necrobiosis'. Subsequently, cell death was reported to occur during the development of mammals

and metamorphosis of invertebrates and lower vertebrates, in hormone-dependent tissues such as prostate and mammary gland after removal of trophic hormones by gonadectomy, after treatment with glucocorticoids in lymphocytes and leukemic cells ('cytolysis'), and after various kinds of damage by chemicals, injury, radiation, hypoxia etc. In developmental biology cell death early was recognized as a 'programmed' event (Glücksmann 1951, Saunders 1966, Lockshin and Williams 1964, 1965). On the other hand, in toxicology and pathology cell death traditionally was considered as a passive, degenerative phenomenon occuring after severe damage of tissues. It was not before the early 1970ies when Farber et al. — based upon a characteristic morphology of cell death and its requirement for protein synthesis — suggested the occurrence of an 'active' or 'suicide' type of cell death in liver, intestine, and other organs after treatment with cytotoxic anti-cancer drugs (Farber et al. 1972). Nevertheless, the widespread occurrence and biological relevance of active cell death was only recognized by Kerr, Wyllie and Currie and in 1972 they proposed a classification of cell death into two broad categories. According to this proposal the term 'apoptosis' was coined to describe a category of cell death 'which appears to play a complementary but opposite role to mitosis in the regulation of animal cell populations. Its morphological features suggest that it is an active, inherently programmed phenomenon which can be initiated or inhibited by a variety of environmental stimuli, both physiological and pathological' (Kerr et al. 1972). The term 'necrosis' which usually was used for all types of cell death was re-defined and restricted to events caused by environmental perturbation, 'which must be violent and lead to rapid incapacitation of major functions and to collaps of internal homeostasis' (Kerr et al. 1972). This proposal initiated a controversial discussion on cell death in the scientific community but eventually helped to elucidate that active cell death is as important as cell proliferation for normal development and regulation of cell homeostasis in organisms.

In the last decade, a significant progress has been achieved in understanding the control of apoptosis by survival and death factors as well as the intracellular events eventually associated with cell suicide (for review: Tenniswood et al. 1992, Dexter et al. 1994, Schulte-Hermann et al. 1995, Peter et al. 1996, Cohen 1996, Kroemer et al. 1997, Lockshin 1997). Interest in cancer research was triggered when it was recognized that tumors may exhibit high rates of active cell death (Kerr et al. 1972, Gullino 1980, Sarraf and Bowen 1986) and that tumor promoters may selectively increase survival of preneoplastic cells (Bursch et al. 1984). Subsequently, blockade of apoptotic elimination of B-cells consequently to overexpression of the oncogene bcl-2 was found to be involved in the pathogenesis of Burkitt lymphoma, showing that prevention of apoptosis is not a phenomenon specifically for liver tumor promotion (Vaux et al. 1988). Unfortunately, the knowledge about apoptosis to date still is scarce and sometimes misconceptions are apparent which may obscure the clear recognition of phenomena and access to underlying causes. Thus, apoptosis often is equated with 'programmed' or 'active' cell death (ACD). However, accumulating evidence suggests that ACD is not confined to apoptosis as originally defined (see below; Schweichel and Merker 1973, Clarke 1990, Tenniswood et al. 1992, Schwartz et al. 1993, Zakeri et al. 1995, Bursch et al. 1996).

Types of Active Cell Death

Apoptosis originally was defined on the basis of a specific pattern of morphological changes in the dying cell (Kerr et al. 1972): condensation of cytoplasm, in solid tissues separation from neighbouring cells, condensation of chromatin at the nuclear membrane to sharply delineated masses and cell fragmentation into apoptotic bodies. In highly condensed dead cells or cell fragments organelles are still intact as shown by electron microscopy; cellular membranes are well preserved and consequently, cell contents are not liberated. In vivo, apoptotic bodies are rapidly phagocytosed and degraded by neighbouring cells. An important feature of apoptosis is that lysosomes (autophagy) do not play a distinct role in early stages but are involved later in the heterophagic degradation of apoptotic bodies (Kerr et al. 1972, Wyllie et al. 1980, Bursch et al. 1985). However, Schweichel and Merker (1973) and Clarke (1990) noted that active cell death may exhibit morphological features different from apoptosis. These authors described 3 morphologically distinct types of cell death in the developing embryo: type I is most likely identical to apoptosis. Type II is characterized by a prominent formation of autophagic vacuoles ('autophagic cell death'). In vivo, cell residues undergoing type II cell death, like those of apoptosis (type I) are finally phagocytosed by neighbouring cells (Schweichel and Merker, 1973). Type III is described as occurring through disintegration of cells into fragments without involvement of the lysosomal system and without marked condensation (Clarke 1990).

We have studied the occurrence of different types of cell death in human mammary carcinoma cells (MCF-7) and in the liver in vivo as well as in vitro. As to mammary carcinoma cells, previous studies on MCF-7 cells revealed an inhibitory effect of antiestrogens on DNA synthesis and enhanced cell death (Bardon et al. 1987, Jordan and Murphy 1990, Wärri et al. 1993). We have confirmed and extended these results using tamoxifen, 4-hydroxy-tamoxifen and ICI 164.384 (Bursch et al. 1996). Tamoxifen at high doses (10^{-5} M) causes lysis (necrosis) of almost all cells within 24 hours, which cannot be prevented by estradiol. The cytotoxic action of tamoxifen may result from perturbations in membrane fluidity (Wiseman, 1994), formation of reactive oxygen species (Tuner et al. 1991), DNA damage by DNA adducts or chromosomal aberrations, which have been found to occur in kidney and liver (Han and Liehr, 1992, Sargent et al. 1994). On the other hand, lower concentrations of tamoxifen (10^{-6} M and below) or ICI 164.384 induce a gradual appearance of cell death starting to occur 3 days after treatment. This type of cell death is considered to be a receptor-mediated, active cell suicide because of its inhibition by estradiol ('mitogen rescue') even six days after anti-estrogen treatment. In view of the screening for death factors such as antiestrogens it is worth to note that our studies with MCF-7 cells suggest that the anti-survival and anti-proliferative activity of antiestrogens can be regarded, at least to some extent, as independent, distinct pharmacological properties: 1. the anti-survival effect of tamoxifen and ICI 164.384 becomes manifest several days later than their anti-proliferative effect; 2. the lowest tamoxifen and ICI 164.384 concentration tested (10^{-8} M) exerts an anti-proliferative, but no anti-survival effect; 3. estradiol reversed the anti-survival effect of ICI 164.384, but not its

anti-proliferative effect; 4. tamoxifen and ICI 164.384 differed about 10-fold in anti-proliferative potency, but their anti-survival effect was equal.

Tamoxifen Induced ACD of MCF-7 Cells: Nuclear Changes

Electron microscopical studies revealed that the nuclei of MCF-7 cells undergoing ACD showed one of two distinct changes: a) apoptosis-like condensation and fragmentation of chromatin to crescent masses abutting to the nuclear envelope. b) condensation of the chromatin to a single, pyknotic mass in the center of the nucleus, detached from the nuclear envelope. Light microscopic evaluation revealed the predominance of the pyknotic type of cell death. Biochemically, activation of a non-lysosomal endonuclease that degrades DNA into (oligo)nucleosomes yielding a characteristic 'DNA-ladder' after gel electrophoresis is often regarded as halmark of apoptosis (for review: Arends et al. 1990, Bursch et al. 1992a, b, Schulte-Hermann 1995). However, apoptosis may not always be associated with DNA fragmentation into (oligo)nucleosomes, as exemplified by TGF-ß1-induced apoptosis in primary hepatocyte cultures (Oberhammer et al. 1993). Recent concepts suggest a sequential DNA degradation into high and low molecular weight fragments, the degree of which seems to depend on cell type and stimulus under study (Walker et al. 1995). As outlined above, most of the dying MCF-7 cells after Tamoxifen morphologically exhibit nuclear pyknosis which clearly differs from 'classical' apoptotic nuclei. However, by pulsed field and conventional gel electropheresis DNA fragmentation into large fragments (50 Kb) and further into oligonucleosomal fragments could be demonstrated (Bursch et al. 1996). Apparently apoptotic and pyknotic types of chromatin condensation may share DNA fragmentation into high molecular fragments. Studies on the biochemistry of DNA degradation underlying the different morphological features of nuclear changes are in progress.

Tamoxifen Induced ACD of MCF-7 Cells: Cytoplasmic Changes

By electron microscopy we noted early formation of autophagic vacuoles (AV) and a gradual loss of cytoplasmic organisation during active cell death. The number of cells with a normal looking nucleus but signs of extensive autophagy exceeded the number of those cells exhibiting a pyknotic or apoptotic nucleus, i.e. irreversible signs of cell death. The importance of autophagic vacuoles in the preparation of cells for death is supported by the inhibition of both types of nuclear alterations by 3-methyladenine (3-MA; Bursch et al. 1996). 3-MA has previously been characterized as a specific inhibitor of formation of autophagic vacuoles in liver cells (Seglen et al. 1982). In view of functional criteria for the differentiation between subtypes of ACD it is of interest to mention preliminary data suggesting that 3-MA does not inhibit TGF-ß1 induced 'classical' apoptosis of hepatocytes (W. Parzefall, personal communication).

Interestingly, in Tamoxifen treated MCF-7 cells structures required for protein synthesis such as polyribosomes, ER, and Golgi disappear completely, whereas a few clusters of intact mitochondria persist in close vicinity with AVs and the nuclear envelope. Remarkably, most of the dead cells remain attached to the substrate until 8-10 days after treatment (Bursch et al. 1996). In these cells the integrity of cytokeratin,

F- and G-actin appear well preserved. This was demonstrated in individual cells by parallel staining with Hoechst fluorescent dye (H 33258), which exhibits the nuclear structure, and with antibodies directed against G-actin and cytokeratin; F-actin was detected by FITC-phalloidin. Cytokeratin of cells detached from the substrate, however, shows signs of degradation into smaller fragments as detected by Western blot analysis. The protein cross-linking enzyme transglutaminase, which is activated in apoptotic hepatocytes (Bursch et al. 1992), apparently is not involved in tamoxifen induced ACD of MCF-7 cells. Currently, we investigate the mechanisms of cytoplasmic degradation in MCF-7 cells in more detail.

In conclusion, the features of antiestrogen-induced ACD in MCF-7 cultures are clearly different from those of TGF-ß1 induced apoptosis (electron microscopically proven) in the liver in vivo and in primary hepatocytes cultures previously described elsewhere (Bursch et al. 1985, Oberhammer et al, 1991; for review: Schulte-Hermann et al 1995). Rather, antiestrogen induced death of MCF-7 cells resembles that of type II or autophagic cell death (Schweichel and Merker, 1973, Zakeri et al. 1995). The most prominent morphological features of both types of cell death are schematically depicted in figure 1. Type II cell death has been described to occur in vivo under a variety of biological conditions (Schweichel and Merker 1973, Clarke 1990, Zakeri et al. 1995). It therefore appears that the autophagic mode of cell death is not peculiar to MCF-7 cells but is an important mechanism in tissue homeostasis of wide-spread importance.

No specific biochemical pattern has so far been attributed to type III cell death. Schwartz et al. have identified a type of cell death in insect tissues characterized by strong expression of the polyubiquitin gene and of the multicatalytic proteinase (proteasome) (Schwartz et al. 1993, Jones et al. 1995). Ubiquitin binds to cellular proteins to label them for proteolytic degradation by the proteasome protease. So far, enhanced expression of ubiquitin was not observed in models of apoptosis (Schwartz et al. 1993). Likewise, in our studies MCF-7 cells treated with tamoxifen showed no induction of ubiquitin expression above controls.

In conclusion, the different morphology and biochemistry of dying cells most probably reflects differences in cellular signalling and mechanisms. However, as recently pointed out by Zakeri et al. (1995) type I cell death (apoptosis) and type II may not be strictly separated or mutually exclusive phenomena. As shown in our MCF-7 cell culture studies, a subfraction of dying cells shows autophagic cell death with an apoptotic nuclear morphology. Therefore, in a given cell type several pathways leading to active death may coexist.

Active Cell Death versus Necrosis

An apparent misconception on apoptosis and ACD is that its discrimination from necrosis is unequivocal and, in principle, always possible. First, toxic injury may cause necrosis or active cell death depending on the extent of damage to the membrane, cytoskeleton or vital functions such as ATP-synthesis etc. As exemplified by the effect of different doses of tamoxifen on MCF 7 cells (see above) there may exist a threshold of injury beyond which necrosis occurs in a given cell. Single hepatotoxic

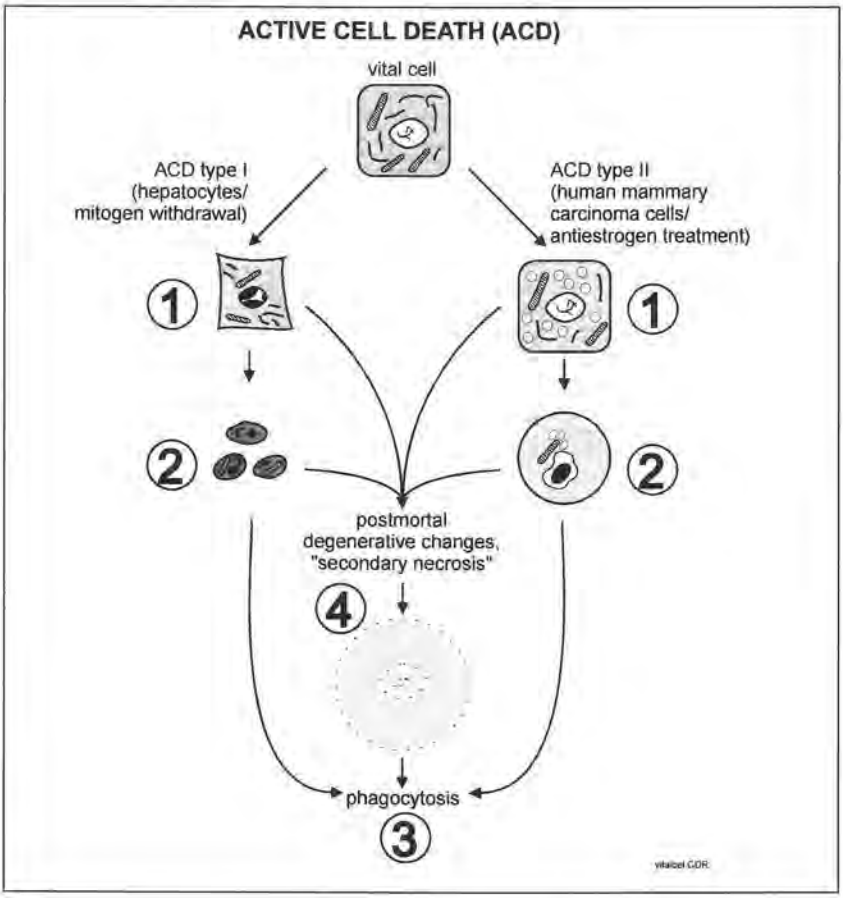

Fig. 1. Active cell death (ACD): Morphological sequence of type I (apoptosis) and type II (autophagic/lysosomal) ACD.

ACD Type I (apoptosis): 1. condensation of cytoplasm and of chromatin at the nuclear membrane to sharply delineated masses (often like crescents). 2. cell fragmentation into apoptotic bodies. 3. Phagocytosis (in vivo) and heterophagic degradation.
Note: autophagy/lysosomes do not play a distinct role early in apoptosis.

ACD Type II (autophagic/lysosomal cell death): 1. Autophagy: formation of autophagic vacuoles (AVs; open circles) and degradation of cytoplasmic constituents; 2. Pyknosis, single pyknotic mass in the center of the nucleus, nuclear envelope still intact, cytoplasm amorphous with few clusters of AVs and mitochondria. 3. Phagocytosis (in vivo) and final degradation.
4. Note: a cell may enter a given ACD pathway which, however, may not be completed and secondary necrosis ensues (see text for details).

doses of thioacetamide or dimethylnitrosamine first induced apoptosis and then necrosis in rat liver in vivo (Pritchard and Butler 1989, Ledda-Columbano et al. 1991). Secondly, *in vivo* massive physiological signals triggering ACD may overload the degradative machinery (autophagy, phagocytosis and heterophagic digestion of

cell fragments) which may result in lytic changes resembling necrosis (figure 1). Thus, in hepatocellular carcinoma produced in rats by longterm treatment with the non-genotoxic carcinogen nafenopin, a dramatic increase of cell death (initially mainly apoptosis) and tumor regression was triggered by withdrawal of nafenopin. With increasing time of withdrawal cancers developed large areas of hydropic and fatty degeneration and lytic cell death which was also reflected by a concomitant increase of glutamate dehydrogenase in serum. Such lesions were not found in tumors *during* nafenopin treatment indicating that the formation of lytic cell death was a secondary phenomenon, probably due to insufficiency of phagocytotic mechanisms in rapidly regressing tumors (Schulte-Hermann et al. 1995, Grasl-Kraupp et al. 1997). Likewise, in *cell cultures* dead cells — in particular upon detachment from the substrate — may undergo postmortal degenerative changes ('secondary necrosis' figure 1; Wyllie et al. 1980). Unfortunately, upon a given stimulus many cell types enter cell death asynchronously. Consequently, at a given time point all stages of active cell death as well as secondary necrosis may be present and render biochemical and molecular studies on *cell homogenates* very difficult or even impossible (Cejna et al. 1994).

Furthermore, in our experiments with MCF-7 cells 50 Kb DNA fragments as well as oligo-nucleosomes were also detected during non-active, *lytic* cell death. This may suggest that activation of the enzymes producing the regular pattern of DNA fragmentation is not restricted to ACD, although DNA degradation during lytic cell death is much more rapid than in the active one (24 hours vs. 3-5 days, resp.). Most probably membrane destruction results in an increase in cytoplasmic and nuclear Ca^{++} and Mg^{++} ions thereby activating DNAses (Walker at al. 1993, Cain et al. 1994). Likewise, necrosis of neuronal cells has been found to be associated with transient formation of oligonucleosomes (Collins et al. 1992). These observations emphasize current notions that certain patterns of DNA fragmentation (e.g. DNA ladders) should not be considered specific for the apoptotic mode of ACD.

Recently, techniques have been developed which allow detection of DNA fragments in situ on histological sections using DNA polymerase or terminal transferase to label DNA ends ('TUNEL' assay). Pyknotic nuclei of MCF-7 cells undergoing ACD exhibited DNA strand breaks as demonstrated histochemically in individual cells using the TUNEL technique (Sikorska, Walker, Bursch, unpublished observation). However, a positive result not necessarily is a reliable and specific indicator of active cell death or of apoptosis as exemplified by in vivo studies on rat liver. A positive response after applying the TUNEL technique was also obtained in nuclei of necrotic liver cells after intoxication with CCl_4 and even as a result of tissue autolysis (Grasl-Kraupp et al. 1995).

In conclusion, to date there is no single biochemical or molecular marker for detection of active cell death subtypes available. Therefore, morphological, biochemical and functional criteria applicable to the specific conditions under study (in vivo, in vitro, cell suspension or not, etc.) should be used in combination (table 1). Morphological methods are still required to identify cells undergoing active cell death, and electron-microscopy may be necessary for unequivocal identification. Detection of mRNAs or proteins if specific for active cell death in a given tissue, is an additional possibility but candidate genes or proteins generally applicable have not yet been found. As to the

Table 1. Morphological, Biochemical and Functional Criteria Helpful for Identification and Quantification of Active Cell Death:

Cytoplasmic and nuclear changes		
	Morphologically:	Light microscopy (H&E, Hoechst 33258 stain in vitro)
		Electron microscopy
	FACS:	Suspendable cells, isolated nuclei
DNA degradation		
	DNA ladders (not always positive, not specific)	
	TUNEL (not specific)	
Cytoplasmic degradation		
	Proteasome/ubiquitin (non-apoptotic types of ACD)	
	Autophagic/lysosomal activity (-//-)	
Functional		
	Induction by mitogen withdrawal	
	Cell rescue by survival factors	
	Receptor mediated	
Rates of cell death (in vivo)		
	Apoptotic index:	Determination requires information about duration of visible stages of ACD, circadiane rhythm, number of cell fragments ('apoptotic bodies') deriving from *one* cell upon its fragmentation. Technical requirements: standardized, equal thickness of sections, intensity of stain; control of histological counts for inter- and intra-individual deviations caused by investigator(s)

discrimination between ACD and necrosis, functional characteristics such as inhibition of ACD (i.e. rescue) by cell/tissue specific mitogens and survival factors may be helpful and have been widely used, both in vivo and in vitro (for review: Schulte-Hermann et al. 1995).

Quantification of Active Cell Death in vivo and Implications for Carcinogenesis

Quantitative determination of the incidence of cell death in vivo requires counting of histological tissue sections and good quality sections stained with HE are essential (Goldsworthy et al. 1996, Schulte-Hermann et al. 1995). However, the incidence of histological signs of cell death is not sufficient to assess the quantitative role of cell death in the kinetics of growth and involution of a given tissue. Rather, this approach requires determination of the frequency of cell death per unit time, i.e. death rate. Therefore we have estimated the duration of the histologically visible stages of apoptosis of individual hepatocytes in vivo (both in normal cells as in preneoplastic tissue) which was found to be about 3 hours (Bursch et al.1990). This short duration explains why apoptosis is relatively rarely observed in histological sections even in

states of considerable cell loss. Moreover, the incidence of apoptosis in the liver and probably in other tissue exhibits a circadiane rhythm. Thus, the feeding/fasting state of the animal profoundly modifies the incidence of apoptosis, yielding high rates at the end of the daily light (fasting) period, and low rates in the dark (feeding) phase (Schulte-Hermann et al. 1988, Grasl Kraupp et al. 1994,). Thus, if rodents are sacrificed in the morning according to laboratory routine, apoptotic rates in the liver may be minimal and any increase is due to a specific state or treatment of animals might be easily missed.

As to the implications of apoptosis for carcinogenesis, we have long been interested in the mechanisms of tumor promotion using rat liver as the major experimental model. In the liver, initiated cells can be detected as single cells or small foci of cells by immunocytochemical and histochemical means (Schulte-Hermann et al. 1990, 1995). In the early 80ies we discovered that these foci exhibit higher rates of cell proliferation than normal liver which, however, may be counterbalanced by apoptosis. These foci can be stimulated to grow by tumor promoters such as phenobarbital and many others (Schulte-Hermann et al. 1990, 1995). Closer analysis showed that tumor promoters have little effect on cell proliferation but their predominant effect is inhibition of apoptosis in the foci (i.e. survival factor activity), thereby allowing rapid accummulation of preneoplastic cells (Schulte-Hermann et al. 1982, 1990, Bursch et al. 1984). These findings showed that tumor promoters can act as survival factors for preneoplastic cells in vivo and provided a new approach to understand the mechanism of action of this class of risk factors. Even malignant cells in the liver were found to still depend on the presence of survival factors as exemplified by a dramatic increase in cell death and rapid regression of liver tumors upon withdrawal of nafenopin (see above). The occurrence of apoptosis in liver foci was subsequently confirmed and extended by other groups (Columbano et al. 1984; Garcea et al. 1989). In vitro studies by Kanter et al. (1984) showed that tumor promoters such as TPA (12-0-tetradecanoyl-13-0-phorbol acetate) and epidermal growth factor protect fibroblasts from cell death upon serum withdrawal.

More recently we have carefully determined rates of cell birth and death in various stages of cancer in the liver. It was found that both birth (α) and death ($ß$) rates gradually increased from normal liver to putative preneoplastic foci to hepatocellular adenoma and to hepatocellular carcinoma, the net growth rate of tissue is determined by $\alpha - ß$ (Grasl-Kraupp et al. 1997). (Pre)neoplastic cell populations (pn, tu) in general exhibit higher net growth rates than the tissue of origin (n*(ormal)*), namely $(\alpha_{pn,tu} - ß_{pn,tu}) > (\alpha_n - ß_n)$. As in rats, in human liver cancer birth and death rates were increased manyfold, indicating a species-independent phenomenon (Grasl-Kraupp et al. 1997). Likewise, in human pre-malignant and malignant colonic tumors rates of cell proliferation and active cell death were much higher than in normal tissue (although cell death rates in carcinoma were somewhat lower than in adenoma; Partik et al. 1997). The gradual increase of cell turnover during the sequential stages of carcinogenesis has important implications. Thus, the more cell turnover is accelerated, the greater should be the impact even of *small* imbalances between cell birth and death on cell number. Experimental evidence for this prediction has been provided: a 90 days period of food restriction results in an increase of apoptosis in preneoplastic liver foci

and in selective disappearance of the majority of these lesions (Grasl-Kraupp et al. 1994). Likewise, injection of TGF-ß1, one of the physiological death signals for normal hepatocytes, strongly enhanced apoptosis also in preneoplastic cells resulting in their selective elimination (Müllauer et al. 1996). A further important implication of increasing death rates has first been described by Moolgavkar and Luebeck on mathematical grounds, namely that cell clones may become extinct (Luebeck et al 1995). The probability of extinction of a clone is expressed by ß: α. Thus, if food restriction would cause an eradication of preneoplastic lesions (in other words: reduce the number of 'promotable' lesions), fewer tumors eventually should develop as compared to animals feed ad libitum during their life. We have experimentally provided prove for this prediction by showing that the food restriction indeed partially protects against carcinogenesis by subsequent treatment with the tumor promoter nafenopin (Grasl-Kraupp et al. 1994).

In conclusion, these findings clearly indicate that active cell death may well occur in malignant tumors. The current notion by some authors that cancers develop because of an inability of cells to undergo active cell death does not apply to the cancers studied here and probably not to others. Rather, it appears that preneoplastic and neoplastic cells are *more* susceptible to signals inducing proliferation or death than normal cells. Therefore, tumor promoters will induce selective (preferential) growth of (pre)neoplastic lesions. Conversely, anti-promotion (food restriction, TGF ß1, lack of survival factors due to promoter withdrawal) may cause selective regression by preferential increase of active cell death. These findings may be important for risk assessment of non-genotoxic carcinogens. Furthermore, more detailed knowledge of survival factors and death factors controlling birth and death rates of cells in tissue may provide new possibilities for selective induction of active cell death and thereby, for cancer prevention and therapy.

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Michael O. Hengartner

The Apoptosome: Molecular Control of Programmed Cell Death in the Nematode *C. elegans*

Abstract

The proapoptotic genes *ced-3* and *ced-4* are essential for programmed cell death in the nematode *C. elegans*. The activity of these killer genes in antagonized by the survival gene *ced-9*. All three genes have mammalian homologs that also function in the apoptotic pathway. Recent work suggests that CED-3, CED-4, and CED-9 form a multiprotein complex — the apoptosome — that regulates apoptosis in *C. elegans*. A similar complex might exist in mammalian cells.

Introduction

Programmed cell death (apoptosis) is an important component of animal development and homeostasis. This process, which removes cells that are not needed or are potentially dangerous, can be observed in a wide variety of tissues in both vertebrates and invertebrates (Ellis et al., 1991; Glücksmann, 1951; Saunders, 1966). Proper control of programmed cell death is very important: breakdown in the regulation of this process appears to be associated with the etiology or pathology of many types of cancer, certain autoimmune diseases, myocardial heart infarct, stroke, and possibly neurodegenerative diseases (Thompson, 1995).

Programmed Cell Death during *C. elegans* Development

The small nematode *Caenorhabditis elegans* has been used with great success as a model organism for the genetic analysis of programmed cell death. Of the 1090 cells generated during *C. elegans* hermaphrodite development, 131 undergo programmed cell death (Kimble and Hirsh, 1979; Sulston and Horvitz, 1977; Sulston et al., 1983). As with most of *C. elegans* development, these deaths are highly reproducible: The identity of the dying cells and the time in development at which these cells die are essentially invariant among individuals. Cells dying by programmed cell death in *C. elegans* undergo a series of morphological changes that, at both the light and electron microscopy levels, show many features that are characteristic of apoptotic deaths

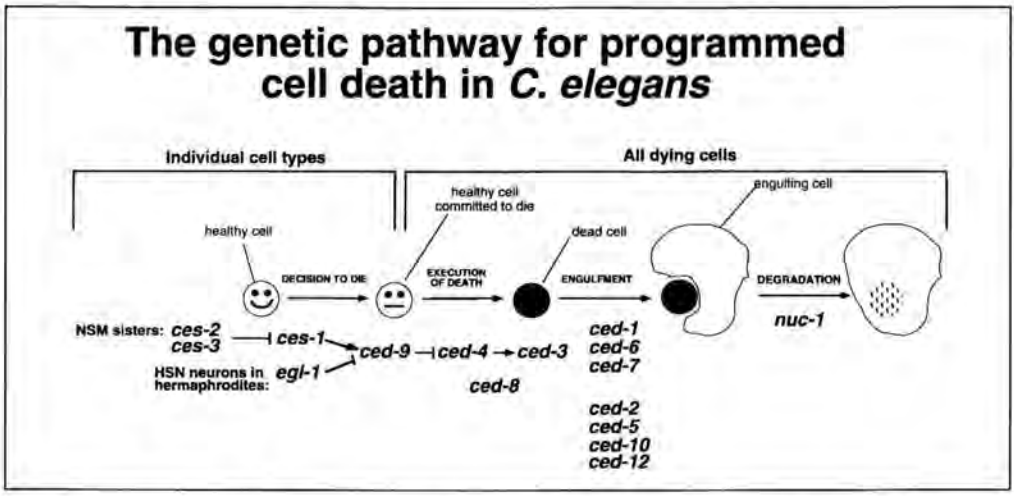

Fig. 1. The genetic pathway for programmed cell death in *C. elegans*. Mutations in 14 genes affect programmed cell deaths. These mutations divide the process of programmed cell death into four steps; genes that act in the last three steps are common to all programmed cell deaths, while mutations in genes that act in the first step affect only a few cells. Regulatory interactions deduced from genetic studies are shown. ⟶, positive regulation; ⊣, negative regulation. Adapted from Ellis et al., 1991a).

in mammals, suggesting that the subcellular events that are occurring with the dying mammalian and nematode cells are similar (Robertson and Thomson, 1982; Sulston and Horvitz, 1977; Wyllie et al., 1980).

Genetic dissection of programmed cell death in *C. elegans* has led to the identification of a large number of mutations that affect this process (reviewed by Driscoll, 1992; Hengartner and Horvitz, 1994). These mutations identify 14 genes that function in programmed cell death and that can be placed into a genetic pathway (Figure 1). Mutations in 11 genes affect all 131 programmed cell deaths. These genes divide the death process into three distinct steps: execution of the death sentence, engulfment of the dying cells by neighboring cells, and degradation of the engulfed cell. Three additional genes, *ces-1*, *ces-2*, and *egl-1*, have been identified that act upstream of the general cell death pathway: These genes affect the decisions of a very small number of cells whether to live or die. One attractive hypothesis is that these genes are involved in the cell type-specific control of the activation of the death program (reviewed by Driscoll, 1992; Hengartner, 1997; Hengartner and Horvitz, 1994).

Three genes act in the execution step of the cell death pathway (Figure 1). The activities of two of these three genes, *ced-3* and *ced-4* (**ce**ll **d**eath abnormal), are necessary for programmed cell deaths to occur: Mutations that inactivate either *ced-3* or *ced-4* result in the survival of all 131 cells that normally die during hermaphrodite development (Ellis and Horvitz, 1986). Both genes act cell-autonomously, indicating that the dying cell plays a central role in bringing forth its own demise and suggesting that programmed cell deaths in *C. elegans* might not be 'murders' but rather 'suicides' (Yuan and Horvitz, 1990). The third gene acting in this step, *ced-9*, is required

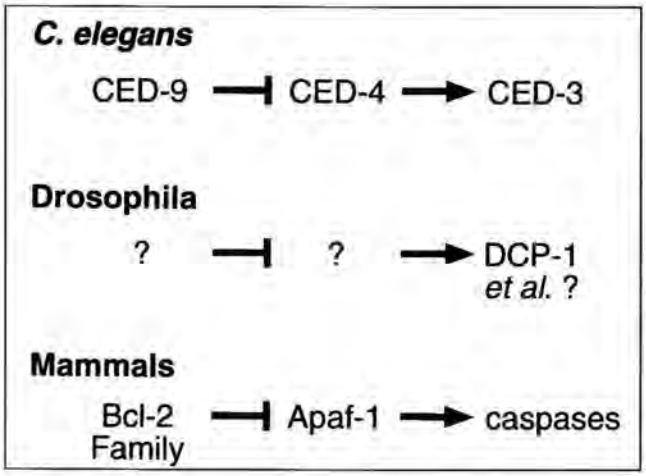

Fig. 2. Conservation of the genetic pathway for apoptosis between *C. elegans*, Drosophila, and mammals. All three of the key cell death regulators identified in *C. elegans* have homologs in mammals that perform similar genetic functions in apoptosis. While CED-9/Bcl-2 and CED-4/Apaf-1 homologs have not yet been identified in Drosophila, the requirement for caspases for programmed cell death in this species suggest that a similar pathway for apoptotic death also exists in insect.

to prevent activation of the cell death program in cells that should live: Either a gain-of-function mutation in the *ced-9* gene or overexpression of wild-type *ced-9* results in the survival of cells that normally die; by contrast, mutations that reduce *ced-9* function cause many cells that normally survive to undergo programmed cell death.

Mutations in seven genes (*ced-1, 2, 5, 6, 7, 8, 10*) affect the engulfment of dying cells in *C. elegans* (Ellis et al., 1991; Hedgecock et al., 1983b). In these mutants, many dying cells fail to be engulfed and persist for many hours or even days. Absence of any one of these genes does not completely block the engulfment process: While some cell corpses accumulate, most dying cells are still engulfed properly, suggesting an element of redundancy among the various genes. Indeed, analysis of all double mutant combinations led to the division of these seven genes into two subgroups, which are proposed to be involved in two distinct but partially redundant processes that act in the engulfment of cell corpses (Ellis et al., 1991b).

The gene *nuc-1* (nuclease abnormal) is involved in the last step of the cell death pathway (Figure 1): *nuc-1* mutants lack a nuclease activity that is required to degrade the DNA of the dead cell. In these animals, cells die and are engulfed normally (Hedgecock et al., 1983), suggesting this nuclease is not required for killing cells, but rather is involved in the subsequent 'cleaning up'.

Conservation of the Genetic Pathway for Programmed Cell Death between Nematodes and Mammals

The molecular characterization of the *C. elegans* cell death genes revealed that *ced-3*, *ced-4*, and *ced-9*, the three key genes involved in the control and execution of the death sentence, are similar in sequence to mammalian cell death genes, suggesting the cell death program found in *C. elegans* also functions in mammals (Figure 2; reviewed by Hengartner, 1997; Hengartner and Horvitz, 1994).

For example, we have found that the CED-9 protein shows significant similarity to the Bcl-2 family of cell death regulators. Like CED-9 *in C. elegans*, several members of this family, including Bcl-2, Bcl-Xl, and Bcl-W, protect cells from apoptotic death (Reed, 1997). Similarly, CED-3 shows significant sequence similarity to the caspase family of cysteine proteinases (Yuan et al., 1993). Mammalian caspases, like CED-3 in *C. elegans*, are essential positive mediators of apoptosis. Finally, Wang and colleagues recently reported the isolation of a mammalian homolog of CED-4, called Apaf-1 (Zou et al., 1997). Like CED-4 in *C. elegans* (Shaham and Horvitz, 1996; Yuan and Horvitz, 1992), Apaf-1 is a pro-apoptotic protein, and acts upstream of caspases.

The involvement of conserved functional homologs in the process of apoptosis in both *C. elegans* and humans strongly suggest that nematodes and mammals share a common molecular pathway for programmed cell death. If so, then it seems likely that not only CED-3, CED-4, and CED-9, but also the rest of the cell death pathway that has been characterized in *C. elegans* will be conserved through evolution. This common genetic program for cell death presumably predates the evolutionary separation of nematodes and vertebrates and thus seems likely to be of ancient origin. Consistent with this hypothesis, caspases homologs also have been identified in Drosophila (Fraser and Evan, 1997; Song et al., 1997). As is the case in worms and mammals, at least some of these fly caspases are essential for apoptosis, as overexpression of the caspase antagonist p35 prevents cell death during eye development (Hay et al., 1994).

Dissecting the Cell Death Machinery: the Apoptosome

How do CED-3, CED-4, and CED-9 act to regulate cell death in C. elegans? Early genetic studies have suggested that the genetic order of function of the three genes is *ced-9* > *ced-4* > *ced-3*, but gave no hints as to the molecular interpretation of this genetic ordering. However, recent work from a number of groups, including our own, have led to the emergence of a simple model for the control of apoptosis in *C. elegans*.

The first series of experiments, published nearly simultaneously by several groups, revealed that the cell death suppressor CED-9 binds tightly and specifically to the killer protein CED-4 (Chinnaiyan et al., 1997; James et al., 1997; Spector et al., 1997; Wu et al., 1997). This interaction, which can be detected in vitro, in yeast (*both S. cerevisiae* and *S. pombe*), and in mammalian cells, appears to be fairly stable, as co-expression of CED-4 with CED-9 leads to recruitment of the CED-4 protein to the subcellular localization observed for CED-9. CED-9 mutants defective in their ability to prevent apoptosis are also impaired in their ability to interact with CED-4, suggesting that the binding of CED-9 to CED-4 is biologically relevant (James et al., 1997; Spector et al., 1997; Wu et al., 1997).

How does CED-4, once 'freed' from CED-9's clutches, promote apoptosis? Recent work by three groups has demonstrated that CED-4 efficiently promotes the processing of the inactive proCED-3 proenzyme into the active enzyme (Chinnaiyan et al., 1997; Seshagiri and Miller, 1997; Wu et al., 1997). CED-4 exerts this activating effect by directly binding to proCED-3, suggesting that CED-4 might act as a

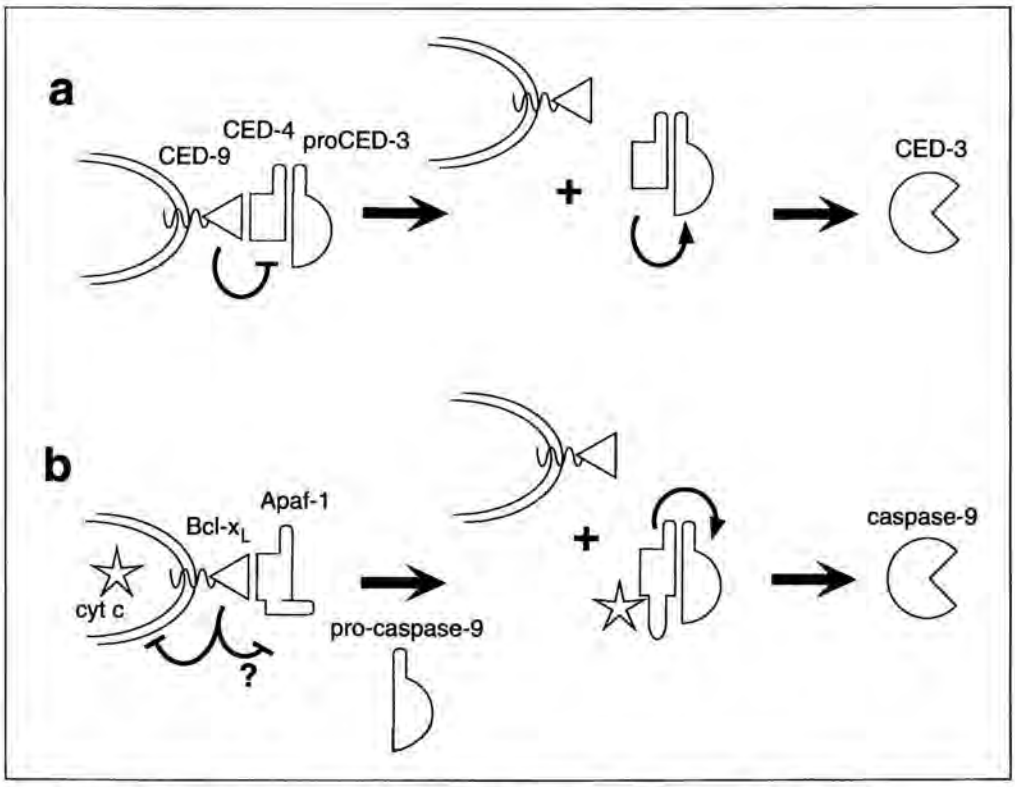

Fig. 3. The *C. elegans* apoptosome: a model for the mechanism of action of the cell death machinery. The cell death regulators CED-3, CED-4, and CED-9 are predicted to be stably associated in a multiprotein complex localized, by analogy with Bcl-2 family members in mammals, to the outer surface of mitochondria. This complex would be expected to be present in all cells, but is inactive. In cells fated to die, a proapoptotic stimulus modifies the complex, possibly resulting in the dissociation of CED-3/4 from CED-9. Once freed, CED-4 allows the CED-3 proenzyme to autoactivate. The active protease then cleaves the relevant apoptotic substrates, bringing on the death of the cell. Adapted from Hengartner (1997).

chaperonin or co-factor in the activation of CED-3. As for the CED-9/CED-4 interaction, the binding of CED-4 to CED-3 is crucial for its ability to promote CED-3 activation: point mutations that inactivate the proapoptotic activity of CED-4 also abolish both interaction with proCED-3 and stimulation of CED-3 activation.

As might be expected from the available genetic data, binding of CED-9 to CED-4 abolishes its ability to promote CED-3 activation (Chinnaiyan et al., 1997; Seshagiri and Miller, 1997; Wu et al., 1997). However, CED-9-bound can still interact with CED-3. Thus, in normal *C. elegans* cells, all three key cell death proteins are likely to be associated together in a multiprotein complex, which has been termed the 'apoptosome', that controls cell death (Figure 3). In cells that are fated to die, a proapoptotic stimulus would be expected to somehow modify one or several components of the apoptosome either through post-translational modification, protein-protein interaction, or modification in protein levels), thereby abolishing CED-9's negative influence on

CED-4. One simple way in which this could occur is by promoting a physical dissociation of CED-4/3 from CED-9 (as shown in Figure 3). However, a simple conformational change might also be sufficient.

Is there a Mammalian Apoptosome?

Since all three components of the *C. elegans* apoptosome have mammalian homologs, it is worth asking whether a similar death complex might exist in mammalian cells. So far, the evidence is rather limited. A direct interaction between Bcl-2 family member Bcl-Xl and CED-4 has been reported, presumably mimicking an interaction with an endogenous CED-4 family member. The nature of this interactor has, however, not yet been determined.

The most obvious similarity is at the level of the CED-4/CED-3 interaction. In a very elegant series of papers, Wang and his colleagues have purified Apaf-1, a mammalian homolog of CED-4, based on its activity to promote the activation of caspase-9 (Apaf-3), a mammalian homolog of CED-3 (Zou et al., 1997). Indeed, Apaf-1 and caspase-9 can interact directly, as do CED-4 and CED-3. However, unlike the situation in *C. elegans*, this interaction requires the presence of another protein, cytochrome c (Apaf-2). While this twist does not invalidate the concept that interaction between CED-4 family members and caspases promotes the autocatalytic activation of the latter, it clearly indicates that we do not yet have the full picture in view, and many more embellishments to the basic model are to be expected.

Conclusion

Genetic studies in *C. elegans* have been very successful in identifying key regulators of the apoptotic machinery. Because this machinery is conserved, we predict that not only *ced-3*, *ced-4*, and *ced-9*, but also all the other cell death genes that have been identified in *C. elegans* will have mammalian homologs that perform similar functions in the control of apoptosis.

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Chris P.M. Reutelingsperger, Istvan Vermes, Clemens Haanen
and Waander L. van Heerde

Phosphatidylserine, the Remarkable Feature of the Face of Death

Abstract

The aminophospholipid phosphatidylserine (PS) locates in viable cells predominantly in membrane leaflets facing the cytosol. Cells spend energy to generate and maintain a steady state in which the plasma membrane (PM) leaflet facing the environment contains very low levels of PS. Activation of apoptotic pathways changes this asymmetry by targeting on PM molecular machineries responsible for PS topography. Consequently, PS becomes exposed persistently at the outer PM leaflet where it signals the process of dying to the environment. Annexin V, a member of the Annexin family and exhibiting a high affinity for PS, binds to apoptotic cells with surface exposed PS. Using Annexin V as a tool has shown that cell surface exposure of PS is a ubiquitous and conserved feature of apoptosis.

Phosphatidylserine, its Localisation and its (Patho)physiological Significance

The aminophospholipid phosphatidylserine (PS) carries a phosphoserine headgroup, which gives this phospholipid a negative charge at pH 7.4. As was firstly shown for erythrocytes and platelets PS localizes predominantly in those membrane leaflets facing the cytosol. Now it is generally accepted that this PS asymmetry is ubiquitous and characterizes plasma membranes (PM) of anucleated as well as nucleated cell types. After its generation PS asymmetry of the PM requires maintainance. In 1984 Seignereut and Devaux demonstrated that the cell spends energy to generate and maintain the phospholipid asymmetry of its membranes (Seignereut and Devaux, 1989). To date a model exists in which various membrane associated proteins are directly responsible for the distribution of the phospholipid species between the two leaflets of the membrane (Diaz and Schroit, 1996). These proteins, which have not been identified sofar, transport PS from the outer to the inner leaflet (aminophospholipid translocase) or vice versa (floppase) or bi-directionally (scramblase) (Zwaal and Schroit, 1997). The aminophospholipid translocase transports selectively aminophospholipids whereas the floppase and scramblase exhibit less specificity and translocate also cholinephospholipids. Under viable and non-perturbing conditions the aminophospholipid translocase activity dominates by creating a situation in which PS is

exclusively localized to the leaflets facing the cytosol. Blood platelets were the first cells for which it was demonstrated that a pathophysiological change of PS asymmetry is invoked by the action of agonists like thrombin and collagen (Bevers et al., 1982 and 1983).

Stimulation of these cells results in a rise of cytosolic Ca^{2+}, which causes at the one hand inhibition of the aminophospholipid translocase and on the other hand activation of the scramblase (Williamson et al., 1995). Within minutes the architecture of the PM is changed such that the cell exposes significant amounts of PS at its surface. Comparable mechanisms operate in erythrocyte membranes and plasma membranes of nucleated cells (Zwaal and Schroit, 1997). Erythrocytes have Ca^{2+} controlled regulation of phospholipid asymmetry and show an age/density dependent accumulation of PS in the outer leaflet of the plasma membrane (Connor et al., 1994). As was firstly demonstrated for the lymphocyte, nucleated cell types expose PS at their surface during apoptosis, a well-organized process of cell-suicide (Fadok et al., 1992). It was demonstrated that during the execution of apoptosis by lymphocytes the aminophospholipid translocase is inhibited while concomittantly a scramblase gets activated (Verhoven et al., 1995). Obviously the cell spends energy to maintain its surface devoid of PS and as soon as termination of existence is preluded the cell transports PS to the outer leaflet of the plasma membrane with a speed, which is orders of magnitude faster then the passive diffusion rate of phospholipids between the membrane leaflets. Hence, the PS topology seems to be of major physiological importance under viable as well as dying conditions.

Cell surface exposure of PS has been connected to scavenging mechanisms. Ageing of erythrocytes is associated with an accumulation of PS at the cell surface (Connor et al., 1994). The reticulo-endothelial system recognises PS and removes cells with surface exposed PS from the circulation by virtue of an as yet unidentified PS receptor (Schroit et al., 1985). A similar scavenging mechanism for removal of unwanted cells is operative in the tissues where tissue macrophages recognise and engulf PS exposing cells through receptor mediated processes. Valerie Fadok and co-workers recognized that cell surface exposure of PS is entangled with apoptosis entailing recognition and engulfment of the dying cell by phagocytes (Fadok et al, 1992). Amongst other plasma membrane structures cell surface exposed PS appears to signal the termination of viability of the cell to the environment. This signal is picked up by phagocytes likely through receptor-ligand type of mechanisms eliciting phagocytosis (Savill, 1995). The functionality of this recognition based removal of cells resides within the physiological need to prevent the dying cell from spilling its pro-inflammatory content into the environment. Studies employing Annexin V as a tool have revealed that a cell in apoptosis exposes PS at its surface well before plasma membrane integrity becomes compromised by inhibiting the aminophospholipid translocase and activating the scramblase and possibly the floppase (see figure 1 and the section below).

Hence, cell surface exposed PS is employed by physiology to entombe the dying cell before it desintegrates thereby preventing undesirable inflammatory responses.

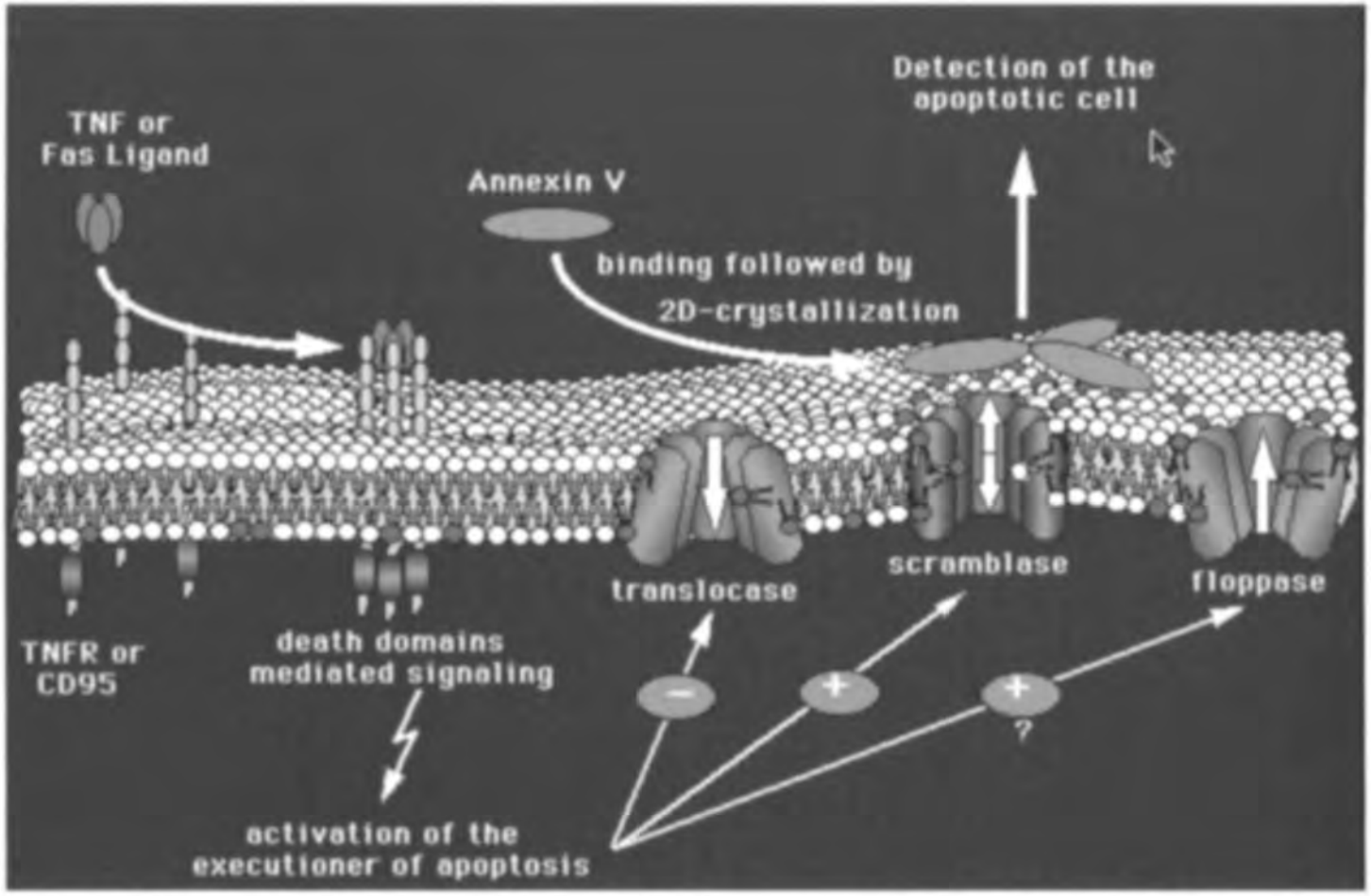

Fig. 1. Schematic presentation of the regulation of PS asymmetry of the plasma membrane during life and death and the role for Annexin V if PS (closed symbol) is exposed at the cell surface by activation of apoptotic pathways. For a detailed explanation see text.

Annexin V, its Binding to Phosphatidylserine

Annexin V was originally isolated from the human umbilical cord artery by virtue of its anticoagulant activity (Reutelingsperger et al. 1985), which in retrospect could be explained by its binding to and shielding of negatively charged phospholipids (Andree et al., 1990; Tait et al., 1989). In model systems Annexin V binds in the presence of calcium ions to most phospholipid species (Andree et al, 1990; Blackwood and Ernst, 1990; Meers et al., 1991; Tait et al., 1989 and 1992). Whereas Annexin V hardly associates with phosphatidylcholine (PC) and sphingomyelin (at < 5 mM Ca^{2+}) (Andree et al., 1990), it binds with high affinity to negatively charged phospholipids like PS likely because Annexin V bears a binding pocket specific for the phosphoserine headgroup (Swairjo et al., 1995). Binding to a PC model membrane containing PS occurs with a Kd of less then 0.1 nM (Andree et al., 1990; Tait et al., 1989). Its interaction with the membrane is one of cooperativity and depends on both the phospholipid composition and the Ca^{2+} concentration (Schlaepfer and Haigler, 1987). The fraction of PS in such membranes determines together with the Ca^{2+}-level the number of Annexin V binding sites (Andree et al., 1990; Tait and Gibson, 1992). Bound to the phospholipid surface Annexin V forms two-dimensional lattices, which are stabilised by protein-protein interactions (Andree et al., 1992; Mosser et al., 1991). Probably, the first Annexin V molecule associates with its binding site comprising PS and acts as an initiator from which crystallisation on the surface may proceed (Andree et al., 1992). Binding of Annexin V to phospholipid membranes is reversible when calcium ions are chelated and the velocity at which association and dissociation occur suggests that Annexin V does not penetrate the membrane and behaves as an extrinsic membrane protein (Andree et al., 1990; Meers and Mealy, 1993. Altogether these binding features bestow Annexin V with properties par excellence to study cell surface exposure of PS.

Annexin V and Cell Surface Exposed Phosphatidylserine, a Revealing Pas de Deux of Apoptosis

During the last five years apoptosis is enjoying a steep increase of interest mainly because it represents a new concept how multicellular organisms from worms to mammals regulate their cell number. Paradoxically, this concept depicts this form of cell death to be crucial for life in many ways. It became clear that apoptosis is a process, which is accurately orchestrated and organized inside the cell by gene products. To date it is accepted that every cell type carries the machinery to commit suicide by apoptosis. The molecular biology and biochemistry of this death machinery are at the beginning of being unravelled and show already great diversity for the various cell types and the signals by which apoptosis is induced. Beyond this diversity three functionally distinct phases of apoptosis can be distinguished (Thompson, 1995; Kroemer et al., 1995). The initiation phase is the most heterogeneous one in which death-inducing signals like Fas Ligand and TNFα, a lack of growth and survival signals, or DNA damage may induce the cell to prepare for suicide in a private

way. This preparation results in the activation of a more general effector phase, in which the cell still is able to make the decision to die. This phase is characterized by the activation of proteases of the ICE/ICE-like family, the caspases (Nagata, 1997), which build a cascade amplifying the death signals (Martin and Green, 1995; Nataga, 1997). The death signals likely target on the mitochondrion which subsequently releases the Apoptosis Inducing Factor (AIF), a protease which is inhibited by the broad spectrum caspase inhibitor Z-VAD.fmk. This event marks the point-of-no-return and the cell has entered the degradation phase in which 'death' of cytoplasm and nucleus are executed by a thousand cuts in a way that seems common to all cells (Martin and Green, 1995; Susin et al. 1996).

In 1992 Valerie Fadok and co-workers published that apoptotic leukocytes expose PS at their surface likely to serve the physiological need to remove the dying cell by phagocytosis (Fadok et al., 1992). At that time Annexin V was known as a PS binding protein (see section above) and appreciated for its ability to bind to PS exposing cells like activated platelets (Thiagarajan and Tait, 1990). The publication of Fadok and co-workers triggered us to investigate the binding of Annexin V to apoptotic cells. Using firstly leukocytes it was demonstrated that Annexin V exhibits low affinity for the cell surface of the leukocyte unless apoptosis is being executed (Homburg et al., 1995; Koopman et al., 1994; Vermes et al., 1995). Next it was shown that also tissue embedded cell types express this phenomenon (Van Engeland et al., 1996; figure 2). Combination of the vital dye propidium iodide with fluoresceinated Annexin V revealed that the apoptotic cell generates Annexin V binding sites at its surface while maintaining the plasma membrane integrity intact (Homburg et al., 1995; Koopman et al., 1994; Vermes et al., 1995).

Competition experiments using phospholipid vesicles demonstrated that the binding site for Annexin V on the apoptotic cell comprises PS (Martin et al., 1995). The Annexin V assay to measure cell surface exposure of PS (Martin et al., 1996b) increased our knowledge about the regulation of PS exposure during apoptosis rapidly. Apoptosis associated cell surface exposure of PS happens during the effector phase, likely downstream of the point where the mitochondrion gets involved (Castedo et al., 1996) and releases AIF into the cytoplasm (Susin et al., 1996). Indirect evidence for this notion comes from experiments with Bcl-2, which is an anti-apoptotic protein. Bcl-2 inhibits both the release of AIF from mitochondria (Susin et al. 1996) and cell surface PS exposure of cells treated with pro-apoptotic agonists (Castedo et al., 1996; Martin et al., 1996a). Studies with Jurkat cells showed that Fas mediated apoptosis activates the executioner, which subsequently turns on the machinery for exposing PS at the cell surface (Martin et al., 1996a). This PS exposing machinery is likely a scramblase (Verhoven et al., 1995), which probably resides constitutively in the plasma membranes of the viable cell (Martin et al., 1996a) as is the case for platelets and erythrocytes. Comparable to platelets and erythrocytes activation of the scramblase during apoptosis does not require the involvement of the nucleus (Castedo et al., 1996; Martin et al., 1996a). Whether the scramblase of nucleated cells is identical to the scramblase of platelets and erythrocytes remains to be answered.

Using Annexin V it turned out that cell surface exposure of PS is a general phenomenon of apoptosis occurring in hemopoietic (Homburg et al., 1995; Koopman et

Fig. 2. Life and death in an MR65 cell population as visualized by Annexin V-Oregon Green and propidium iodide. MR65 cells were incubated with Annexin V-Oregon Green (green), washed and fixed by methanol. The fixed cells were then stained by propidium iodide (red) and analyzed by confocal scanning laser microscopy. On the left a cell in the mitotic anaphase is seen. The propidium iodide stain is encircled; the Annexin-V stain is not encircled. Note the absence of fluorescence outside the nuclear region indicating that Annexin V does not bind to a cell which is in cell cycle. On the right an apoptotic cell is visualized by the plasma membrane staining and showing in a condensed and fragmented nucleus.

Table 1. Ubiquity of cell surface exposure of PS during apoptosis.

Cell type	Apoptosis initiating stimulus
Leukocytes	*Plasma membrane receptor/ligand*
neutrophil	lack of growth factor
T-lymphocyte	Fas/Fas ligand interaction
B-lymphocyte	TNFR/TNFα interaction
monocyte	*Intracellular receptor/ligand*
Tissue cells	glucocorticoid
endothelial cell	*Intracellular signalling*
smooth muscle cell	C2-ceramide
fibroblast	staurosporine
neuron	olomoucine
Tumours	*Macromolecular synthesis*
leukemic cell	actinomycin D
carcinoma cell	cycloheximide
	DNA
Mouse and avian embryos and insect pupae	etoposide
All cell types during development	camptothecin
Plant cells	
Nicotiana plumbaginifolia	

al., 1994; Martin et al., 1995; Vermes et al., 1995) and tissue embedded cells (Bennett et al., 1995; Van Engeland et al., 1996) regardless of the initiating stimulus (table 1). This ubiquitous phenomenon appeared also to be part of apoptosis *in vivo* as was shown by van den Eijnde and co-workers by injecting Annexin V-Biotin into the bloodstream of living mouse embryos (Van den Eijnde et al., 1997).
A recent study shows that plant cells expose PS at their cell surface during execution of apoptosis (O'Brien et al., 1997). Together with the findings that PS exposure also occurs during apoptosis of avian and insect cells (see contribution of Van den Eijnde et al.) this indicates that like the apoptotic concept, the mechanism to regulate PS asymmetry during life and death is conserved through evolution and constitutes, apparently, an absolute necessity for the multicellular life forms as we know them.

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Phosphatidylserine Exposure by Apoptotic Cells; a Phylogenetically Conserved Mechanism

Abstract

Tolerance of the existence of the individual cell in multicellular organisms is mediated by the distribution of the various phospholipid species across the bilayer of the plasma membrane. This concept arises from *in vitro* studies, which show that cell-surface exposed phosphatidylserine on ageing erythrocytes and apoptotic leukocytes triggers elimination of these cells by phagocytosis. In contrast, blood cells are inert in this respect when this aminophospholipid is predominantly residing in the plasma membrane leaflet facing the cytoplasm. We have studied the *in vivo* distribution of cell surface-exposed phosphatidylserine by injecting biotinylated AnxV, a Ca^{2+}-dependent phosphatidylserine binding protein, into viable mouse and chick embryos and Drosophila pupae. The apparent binding of (Annexin V) to cells that were present in regions of developmental cell death and that were exhibiting the morphology which is characteristic of apoptosis indicates that phosphatidylserine exposure by apoptotic cells is a phylogenetically conserved mechanism.

Introduction

Degenerating cells following specific spatio-temporal patterns were observed in developing specimens as early as the mid 19th century (Clarke and Clarke, 1996). After being 'forgotten' by the scientific community for more than a century, this 'shrinkage necrosis' regained interest by developmental biologists after Glücksmann's review, showing that this kind of cell death with its peculiar cell morphology was intimately linked to the normal development of both invertebrate and vertebrate species (Glücksmann, 1951). It is, however, to the credit of Kerr, Wyllie and Currie that the concept of a *physiological* kind of cell death reached a wide scientific community (Kerr *et al.*, 1972). These scientists revealed that degenerating cells that shrunk, showed chromatin condensation, margination and pyknosis and eventually fragmented into separate units were an integral part of both developmental processes and adult tissue homeostasis. These, so-called, apoptotic cells are not only morphologically distinct from the ballooning necrotic cells, but also differ with regard to

how they are cleared. Namely, a necrotic cell looses its plasma membrane (PM) integrity, spills its contents to the surroundings and elicits an inflammatory reaction whilst the remainders become removed through phagocytosis. In contrast, the apoptotic cell is subsequently ingested and digested by neighbouring cells or specialised macrophages, while retaining its PM intact and without eliciting any inflammatory response (Wyllie *et al.*, 1980).

It is probably this silent disappearance which makes apoptosis a physiological kind of cell death. In *in vitro* studies of adult mammalian apoptotic neutrophils and lymphocytes, three kinds of structural changes of the PM have been identified that lead to phagocyte recognition: i) formation of thrombospondin binding sites, ii) exposing side chain sugars after loss of sialic acid, and iii) cell surface exposure of phosphatidylserine (PS) (Savill *et al.*, 1993). Results from an *in vitro* study by Ratner and co-workers (1986) have indicated that the involvement of PS exposure in cell removal may not be limited to mammalian species. Namely, like mouse peritoneal macrophages, insect phagocytes obtained from the moth *Heliothis virescens* specifically recognise and ingest phospholipid vesicles composed of both phosphatidylcholine and PS and not vesicles composed of phosphatidylcholine only. It was this study that prompted us to test whether the phenomenon of PS exposure on the PM of apoptotic cells *in vivo* is not restricted to mammals.

To test the hypothesis that the phenomenon of PS exposure on the PM of apoptotic cells is a species-spanning process, we have injected biotinylated AnxV (AnxV-biotin) intracardially into mouse and chick embryos, and into the haemolymph of *Drosophila pupae*. The localisation of this Ca^{2+}-dependent PS, binding protein, which is indicative for cell surface exposure of PS, was studied at the cellular level in semithin sections. The AnxV-biotin spatio-temporal binding patterns were evaluated in paraffin sectioned specimens.

Materials and Methods

AnxV-Biotin Binding in Mouse Embryos

Experimental animals

Pregnant FVB-mice, from 10-14 days postcoitum (PC; plug = day 0), were sacrificed by cervical dislocation after ether anaesthesia. The uteri were dissected out and from the embryos collected, 60 were used for detection of cell surface exposed PS by microinjection of AnxV-biotin according to procedures recently described by van den Eijnde et al. (1997a) Another 16 embryos were used for control experiments using AnxV-biotin, which was inactivated by heating it for 10 minutes at 56°C (Reutelingsperger *et al.*, 1985). Non-specific binding of AnxV and quenching of endogenous peroxidase activity was tested for in this manner (van den Eijnde *et al.*, 1997a).

Microinjection of AnxV-Biotin

Embryos were perfused by microinjection using a Hamilton-Syringe pipetting system with glass needles (tip diameter ~ 20 µm). Per embryo, a volume of approximately 3

µl AnxV-biotin (500 µg/ml), purchased from NeXins Research BV, Hoeven, The Netherlands, (APOPTEST™-biotin kit), was injected through the ventricle of the heart under a preparation microscope while the embryo was kept in HEPES buffer (20 mM Hepes (pH 7.4), 132 mM NaCl, 2.5 mM $CaCl_2$, 6 mM KCl, 1 mM $MgSO_4$, 1.2 mM K_2HPO_4, 5.5 mM glucose, 0.5% BSA) at 37°C. When injected, a temporary blanching of the umbilical vein could be seen. Successfully injected embryos that showed heart activity after 30 minutes of incubation were fixed and further processed for light microscopy (LM).

DETECTION OF ANXV-BIOTIN BINDING

The morphology of AnxV-biotin binding cells was studied in semithin sections through day 12 embryos. To obtain these sections, embryos were microinjected with AnxV-biotin as described above. After removing the placenta, the embryos were subsequently intracardially perfused with 0.5 ml 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer. The tissues were postfixed overnight in the same fixative, and cut on a Vibratome into 50 µm sections. Endogenous peroxidase activity was blocked by incubating the sections in methanol/H_2O_2 (9:1 v/v) for 20 minutes. Sections were washed in phosphate buffered saline (PBS). Bound AnxV-biotin was visualised using the avidin-biotin complex method with horseradish peroxidase-conjugated avidin (ABC Elite kit, Vector Laboratories, USA) at room temperature. After washing with PBS, staining was developed with 3,3'-diaminobenzidine tetrahydrochloride (DAB) (0.05%). After the reaction with DAB, sections were postfixed in 1.5% OsO_4 in a 8% glucose solution, rinsed in aquadest, stained *en bloc* in 3% uranyl acetate, dehydrated in dimethoxypropane and embedded in Durcupan. Semithin tissue sections were cut on an ultratome (Ultracut S, Reichert Jung, Germany) and counterstained with Toluidine blue.

Day 10-14 mouse embryos were used for the study of AnxV-biotin binding patterns. These embryos were fixed overnight at 4°C in HEPES buffer containing 4% formalin, dehydrated and embedded in paraffin and serially sectioned at 3 µm. The sections were processed to visualise the biotinylated AnxV as described for the semithin sections and were counterstained with Hematoxylin.

AnxV-Biotin Binding in Chick Embryos

Eggs from white leghorn chick, obtained from Drost, Loosdrecht, The Netherlands, were incubated at 37°C, 80% relative humidity. The eggs were removed from the incubator when the embryos were expected to have reached the Hamburger-Hamilton stage 18 (Hamburger and Hamilton, 1951). After windowing the eggs, the embryos were staged. Embryos at HH17-HH19 were intracardially injected with AnxV-biotin (n=10) or heat-inactivated AnxV-biotin (n=4) and incubated for 30 minutes, at 37°C. After incubating, the embryos were removed from the egg, and processed for AnxV-biotin visualisation in paraffin sections as described above.

AnxV-Biotin Binding in Drosophila Pupae

Drosophila melanogaster larvae were collected when they had reached the prepupal stage (Bainbridge and Bownes, 1981). After 30 hours of incubation at 22°C, 26

Fig. 1. Semithin sections through day 12 mouse embryo showing early (A; arrow) and late (B; arrow) apoptotic cells that are AnxV-biotin positive at the PM, amidst unlabelled viable cells. Also, labelled cells were phagocytosed (C; arrow). See also van den Eijnde 1998.

pupae were removed from the cuticle under a preparation microscope. At room temperature, pupae were microinjected with 1 µl AnxV-biotin (1 mg/ml) (n=20) or heat inactivated AnxV-biotin (n=6) into the haemolymph. After 2 hours of incubation at room temperature, the pupae were rinsed with HEPES buffer and subsequently processed for AnxV-biotin visualisation. The procedures were similar to those used for the mouse embryos except for exchanging a 100% ethanol for pentane/100% ethanol (1:1 v/v) as the last step of dehydrating the specimens.

Results

While the mouse and chick embryos and *Drosophila* pupae that were injected with heat-inactivated AnxV-biotin, i.e. AnxV-biotin with a destroyed phospholipid binding activity (Reutelingsperger *et al.*, 1985), did not show any cell labelling, those that were injected with active AnxV-biotin showed cell labelling at specific locations[1].

AnxV-Biotin Binding in Mouse Embryos

Semithin Sections

In semithin sections of day 12 mouse embryos, AnxV-biotin was observed to specifically bind the PM of cells that were showing the features of apoptosis. Compared to the surrounding viable cells, the labelled apoptotic cells were more rounded-off and also were having a more condensed chromatin structure (Fig. 1A) reaching up to clear pyknosis (Fig. 1B). In addition, early and late apoptotic cells that were AnxV-biotin positive have been ingested by phagocytes. Figure 1C shows an example of a phagocytosed cell that appears to be in the early phase of apoptosis, exhibiting only the first signs of chromatin condensation.

Paraffin Sections

In the paraffin embedded and serially sectioned mouse embryos, AnxV-biotin labelling was observed at specific locations throughout the embryos. Examples of such spatial and temporal specific cell death patterns (Glücksmann, 1951) visualised with AnxV-biotin staining are given in figure 2. In day 10 embryos cell death is shown in the

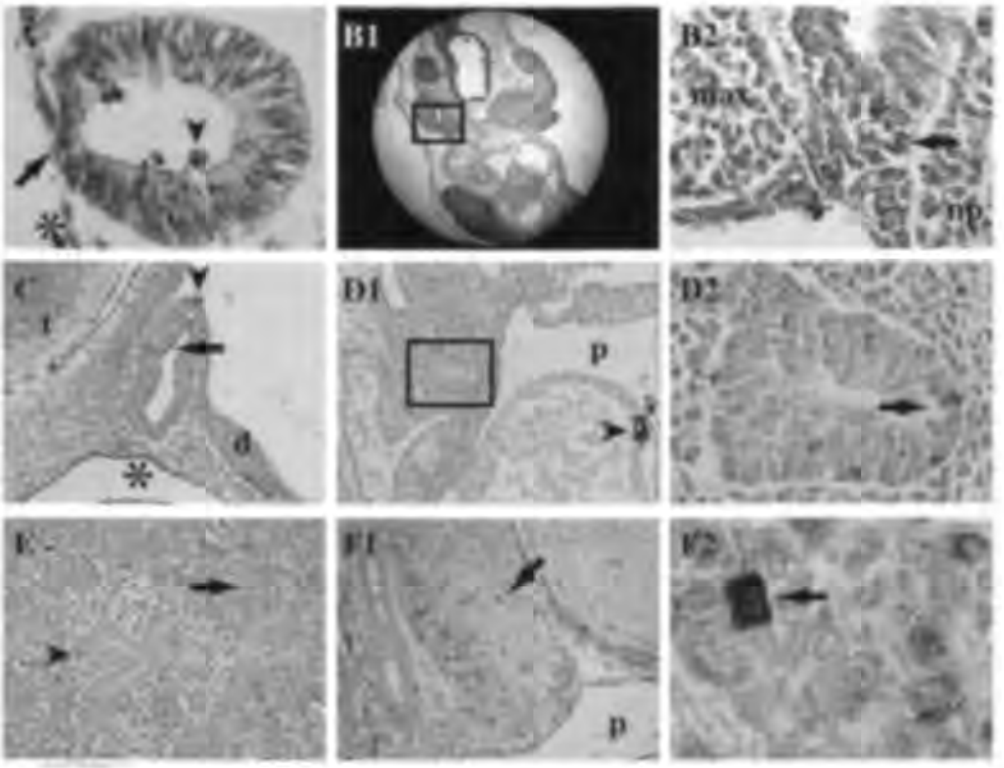

Fig. 2. Paraffin sections through day 10 (A, B1-B2), day 11 (C, D1-D2), day 13 (E) and day 14 (F1-F2) mouse embryos. Examples are shown of AnxV-biotin binding cells in the lens vesicle (A), both in the epithelium of the developing lens (arrow), and in its lumen (arrowhead), the overlaying ectoderm is marked with an asterix. At another location in the same day 10 embryo (B1; overview), AnxV-biotin has stained cells (B2; arrow) at the fusion site of the maxillary (B2; max) and nasal prominences (B2; np). In a one day older embryo, in the head region, AnxV-biotin binding cells are shown in the developing pituitary (C; t = telencephalon, d= diencephalon); cells were stained in the epithelium of the pharyngeal pituitary (C; arrow), in the underlying ectoderm of the stomodeum (C; asterix) and in the future posterior pituitary (C; arrowhead). Directly above the heart in another day 11 embryo (D1; p = pericardial cavity) cells were labelled in the developing thymus (D2; arrow). Note the labelled cells in the myocardial wall of the atrium (D1; arrowhead), indicating the site of injection of AnxV-biotin. In day 13 metanephros, cells were stained in the developing glomeruli (E; arrow) and at the epithelial-mesenchymal interface (E; arrowhead). Figure 2F shows cells in a day 14 thymus (F1; overview, p = pericardial cavity), at a higher magnification pyknotic labelled cells could be recognised (F2; arrow). See also van den Eijnde 1998.

developing lens vesicle (Fig. 2A) and in the fusing maxillary and nasal prominences (Figs. 2B1-B2). In figure 2C examples are presented from day 11 mouse embryos. AnxV-biotin labelling is shown in Rathke's pouch, i.e. the presumptive pharyngeal pituitary, as well as in the ectoderm of the stomodeum directly underneath Rathke's pouch, and in the overlying neuro-epithelium of the infundibulum. Endoderm derived apoptotic cells were present in the thymus anlagen (Figs. 2D1-D2). In day 13 metanephros (Fig. 2E) apoptosis is located in areas of epithelial mesenchymal

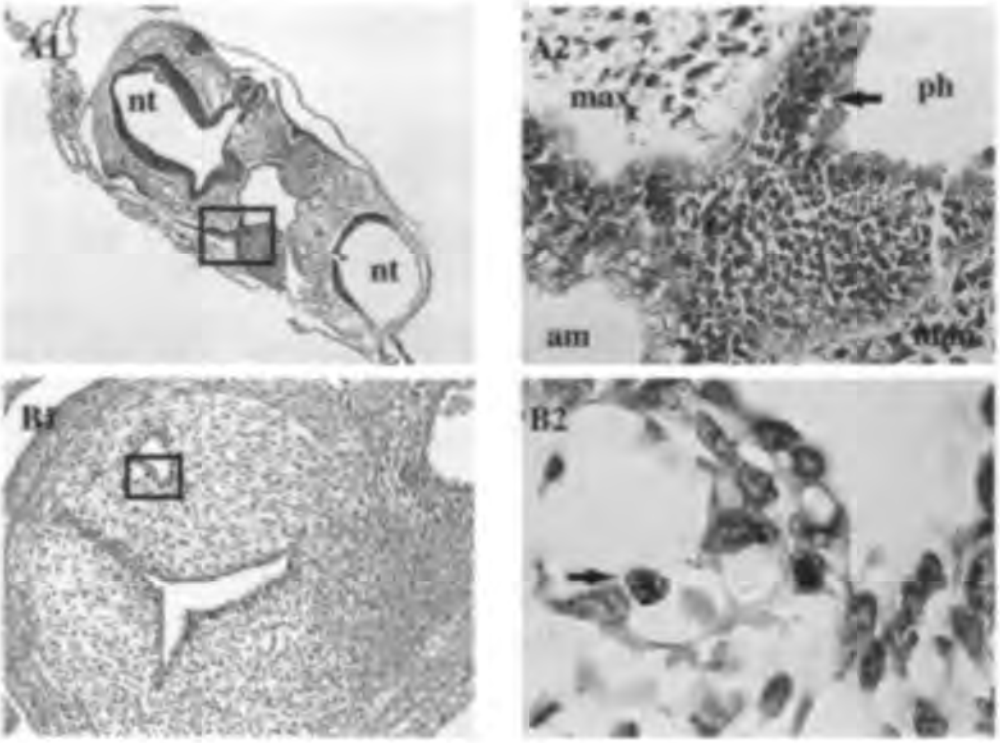

Fig. 3. Examples from chick, showing labelled cells in the head region of an HH16 embryo (A1; overview, nt = neural tube) where apoptosis functions in the fusion of the maxillary (A2; max) and mandibular prominences (A2; man, ph = pharynx, am = amniotic cavity). Figure 3B shows cell death in a aorta valve leaflet of a stage HH18 embryo (B1; overview). At a higher magnification the labelled cells were clearly pyknotic (B2; arrow). See also van den Eijnde 1998

interaction and in the developing nephrones. In figures 2F1-F2 labelled cells are shown in day 14 thymus.

AnxV-Biotin Binding in Chick Embryos

Similar to our findings in mouse embryos, also the AnxV-biotin binding cells in chick were located in regions where apoptotic cell death serves developmental processes. Examples are shown of staining in the fusion area of the maxillary and mandibular prominences in an HH17 embryo (Figs. 3A1-A2) and in the aortic valve leaflet of an HH19 embryo (Figs. B1-B2).

AnxV-Biotin Binding in Drosophila Pupae

Examples of AnxV-biotin stained cells in areas where apoptotic cells are present during *Drosophila* pupariation are depicted in figure 4. Labelled pyknotic cells (compare with Fig. 1B) are shown in the proboscis of a P5 pupa (Figs. A1-A2). Similar to our

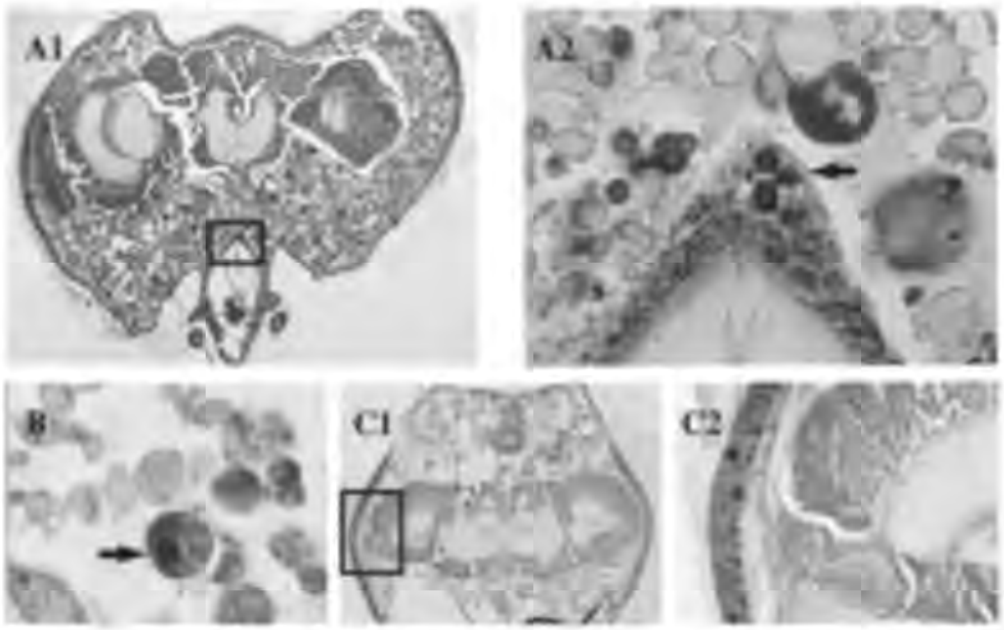

Fig. 4. Examples from insect showing labelled apoptotic cells in the proboscis of an stage P5 *Drosophila melanogaster* pupa (A1; overview), at a higher magnification these AnxV-biotin cells were observed to be pyknotic (A2; arrow). A haemocyte in a P6 pupa that has ingested a labelled and presumably apoptotic cell (B; arrow) is depicted in figure B. In a transverse section through a P7 pupa (C1; overview) cells are intensely labelled in the eye. This is shown at a higher magnification in figure C2. See also van den Eijnde 1998.

findings in mouse embryos (see Fig. 1C) also labelled apoptotic cells were found ingested by phagocytes/haemocytes in *Drosophila* (P6: Fig. 4B). Figure 4C shows an intense labelling of cells in a stage P7 *Drosophila* eye. The timing of this is consistent with previously described developmentally regulated cell death in the eye (Wolff and Ready, 1991).

Discussion

After receiving the signal to die, or after loss of the stimulus to survive, in the apoptotic cell an energy consuming death machinery is initiated (Collins *et al.*, 1994; Steller, 1995). This machinery entails biochemical and morphological changes in the nucleus (Caelles *et al.*, 1994; Falcieri *et al.*, 1994; Oberhammer *et al.*, 1994) and cytosol (Wyllie *et al.*, 1980; Zhang *et al.*, 1996). The PM also plays an active role in the process of apoptosis by expressing specific epitopes (Rotello *et al.*, 1994) that may trigger the phagocytic removal of the cell by phagocytes (Savill *et al.*, 1993). One such an epitope appears to be the PS molecule (Bennet *et al.*, 1995; Fadok *et al.*, 1993; Fadok *et al.*, 1992; Shiratsuchi *et al.*, 1997).

Phospholipids are not equally distributed across the two leaflets of the PM. In viable cells, aminophospholipids, like PS are mainly located in the PM leaflet that faces the cytosol. This PS asymmetry of the PM is achieved by the action of so-called flippases (Diaz and Schroit, 1996; Higgins, 1994), which translocate PS from the outer PM leaflet to the inner layer in an ATP- and Mg^{2+}-dependent manner (Martin and Pagano, 1987; Seigneuret and Devaux, 1984; Tang *et al.*, 1996; Zachowski *et al.*, 1989). Under specific conditions, the PS out-in translocase(s) are inhibited and a PS scramblase is activated, leading to a symmetrical distribution of PS across the two leaflets of the PM, effecting cell surface exposure of PS.

In *in vitro* assays, PS exposure at the outer PM leaflet has been described to occur in receptor/ligand activated platelets (Williamson *et al.*, 1995), ageing erythrocytes (Basse *et al.*, 1996; Connor *et al.*, 1994), and during apoptosis in a variety of cell types, i.e. smooth muscle cells (Bennet *et al.*, 1995), spermatogenic cells (Shiratsuchi *et al.*, 1997) and blood cells (Homburg *et al.*, 1995; Koopman *et al.*, 1994; van Engeland *et al.*, 1996; Vermes *et al.*, 1995). Recently we have established that PS-PM asymmetry is also tightly regulated in the developing day 11-13 mouse embryo *in vivo*. Through intracardial injections using AnxV-biotin as a probe (van den Eijnde *et al.*, 1997a; van den Eijnde *et al.*, 1997b) we have shown that binding of this Ca^{2+}-dependent PS binding protein was very much restricted to apoptotic cells. Apoptotic cells throughout the embryo appeared to carry PS in their outer PM leaflet, irrespective of the cell's lineage. In line with the *in vitro* studies (Martin *et al.*, 1995), cells binding AnxV-biotin were in the process of apoptosis, spanning the process from early, when only distinguishable from viable cells at the electron microscopical level, until after fragmentation into apoptotic bodies (van den Eijnde *et al.*, 1997a). Furthermore, this PM alteration appeared to precede apoptosis associated DNA-fragmentation so far as could be detected via the TUNEL procedure (van den Eijnde *et al.*, 1997b).

In the present study we have probed for PS exposure in day 10-14 mouse embryos, but of greater importance, we have also determined whether apoptotic cells from non-mammalian species expose PS, like their mammalian counterparts. The presence and extent of PS at the outer PM leaflet was assessed by intracardial injections of biotinylated AnxV-biotin in mouse and chick embryos, and via injection into the haemolymph in *Drosophila* pupae. This study was conducted in developing animals, because of their reproducible, strict spatio-temporally regulated apoptosis patterns, facilitating the evaluation of the AnxV binding patterns. The interaction of AnxV-biotin with the embryonic/pupal cells relies on its phospholipid binding property, as was shown from absence of labelling in the species that had been injected with heat-inactivated AnxV-biotin, i.e. AnxV with a destroyed phospholipid binding capacity.

In mouse embryos, in line with previous results (van den Eijnde *et al.*, 1997a; van den Eijnde *et al.*, 1997b), AnxV-biotin appeared to bind to apoptotic cells in early and late apoptotic stages, as well as to cells that were in the process of becoming phagocytosed. In addition to the study of cell morphology in semithin sections, we also studied the AnxV binding patterns in embedded serially sectioned mouse embryos. Specific patterns of AnxV-biotin labelling were observed in tissue areas where cell death serves developmental processes, throughout the embryos. This labelling appeared to be independent of the cell's lineage, i.e. ectoderm, endoderm

and mesoderm. Also a subpopulation of circulating cells was observed to be AnxV-biotin positive (data not shown). We consider these large embryonic red blood cells with a pyknotic and often fragmented nucleus as early erythroblasts derived from the yolk sac. In embryos that are in the transitional phase from having a nucleated to an anucleated red blood cell population this cell type is destined to be removed (Kaufmann, 1992). The embryos under study were in this transitional period. Both the adherence of these cells to endothelial cells as well as the presence of many cell fragments in endocardial cells and endothelial cells throughout the embryos suggest that these embryonic red blood cells expose PS alike adult ageing erythrocytes (McEvoy *et al.*, 1986) that should be cleared from the circulation. However, the restriction of adult ageing red blood cells to be cleared only by specialised endothelial cells, i.e. the reticuloendothelial system (Allen *et al.*, 1988; Schroit *et al.*, 1985) probably does not account for the embryonic counterparts.

In chicken embryos and *Drosophila* pupae we observed specific binding of AnxV-biotin to cells in regions where apoptosis plays a developmental role, similar to our findings in mouse embryos. The morphology of AnxV-biotin binding cells could be evaluated in the paraffin sections to some extent. Often AnxV-biotin appeared to have bound to cells that were pyknotic or fragmented. We consider this as a further indication of the binding of AnxV-biotin to apoptotic cells in these species, in addition to the observed AnxV-biotin binding patterns during development.

In conclusion, utilising AnxV's specific Ca^{2+}-dependent binding to PS, we have studied for the existence of PS exposure by apoptotic cells *in vivo* during mouse, chick and *Drosophila* development. The results indicate that the regulation of PS asymmetry is shared by mammals, avians and insects and appears to be a phylogenetically primitive concept of cellular existence. The general commitment of cells to PS exposure when in the apoptotic state leads to the assumption that it is an important factor in phagocytic removal in the developing mammal, avian and insect. Evidence for such a role, as has been given for adult mammalian cells (Bennet *et al.*, 1995; Fadok *et al.*, 1992; Shiratsuchi *et al.*, 1997) has, however, yet to be established.

Acknowledgements

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The Role of Bax/Bcl-2: From T Cell Development to the Cell Death-cell Cycle Interface

Abstract

Transgenic mice were generated overexpressing *baxα* gene in T cells. Thymocytes from the *baxα* transgenics show accelerated apoptosis in response to multiple stimuli. However, while *baxα* transgenic thymocytes in a p53-/- background still show accelerated apoptosis in response to glucocorticoid treatment they do not exhibit a restored response to DNA damage induced apoptosis. We show that Baxα overexpression can lead to a defect in T cell maturation which reflects a perturbation of T cell development and that the Baxα protein is involved in distinct apoptotic pathways. We further demonstrate that Baxα overexpression can facilitate the entry of T cells into S phase whereas Bcl-2 delays this entry via modulation of the level of $p27^{Kip1}$ protein.

Introduction

Apoptosis, or programmed cell death, is a widespread process used to eliminate unwanted or damaged cells from multicellular organisms. For instance, apoptosis serves to ensure the selection of appropriate lymphoid populations during thymic T cell development (Surh and Sprent, 1994). Apoptosis of immature T cells and T cell hybridomas can be readily induced by DNA damaging agents such as ionising radiation and etoposide (Clarke *et al.*, 1993) as well as glucocorticoids (Wyllie, 1980) and various agents that induce activation (Green and Scott, 1994). Much effort is now being expended to elucidate the genetic and biochemical mechanisms of apoptosis. Prominent among the genes already discovered to be involved is the *bcl-2* oncogene.

Bcl-2 was first isolated by virtue of its translocation into the immunoglobulin gene locus in some follicular B cell lymphomas (Cleary *et al.*, 1986; Tsujimoto and Croce,1986). Bcl-2 overexpression was subsequently shown to block cell death in an IL-3 dependent cell line upon withdrawal of the cytokine (Vaux *et al.*, 1988). Bcl-2 and its relative $Bcl\text{-}X_L$ (Boise *et al.*, 1993) are potent inhibitors of apoptosis induced by various stimuli such as irradiation, glucocorticoid treatment, calcium ionophores or cytotoxic drugs (Sentman *et al.*, 1991; Strasser *et al.*, 1991; Chao *et al.*, 1995).

Another member of the *bcl-2* gene family is *bax*. The Bax protein was isolated by virtue of its interaction with Bcl-2 (Oltvai *et al.*, 1993). Alternate splicing within the

bax gene produces three classes of transcript of which the α mRNA encodes the 21 KDa protein that can heterodimerise with Bcl-2 (Oltvai *et al.*, 1993). In this same study, the overexpression of Baxα in an IL-3 dependent cell line is seen to accelerate apoptosis upon removal of the cytokine. Furthermore, the ratio of Baxα to Bcl-2 appears to be critical since a predominance of Baxα accelerates apoptosis in response to factor withdrawal whereas overexpressed Bcl-2 greatly diminishes apoptosis.

As mentioned above, DNA damaging agents can exert an apoptosis response in many cell types, particularly lymphocytes. A critical regulator of the cellular response to DNA damage is the transcription factor encoded by the p53 tumour suppressor gene (Cox and Lane, 1995). When non-irradiated cells are forced to express high levels of p53 they undergo either growth arrest (Michalovitz *et al.*, 1990) or apoptosis (Yonish-Rouach *et al.*, 1991). Analysis of mice in which both p53 alleles have been inactivated (p53-/-) by gene targetting have demonstrated that p53 is required for the induction of apoptosis in the thymocytes by γ-radiation and by some DNA damaging drugs (Clarke *et al.*, 1993; Lotem and Sachs, 1993; Lowe *et al.*, 1993). The way in which p53 induces apoptosis is not clear. However, evidence has recently been obtained that implicates Bax/Bcl-2. Restoration of p53 in a murine leukaemia cell leads to increases in *bax* mRNA and protein levels accompanied by lower steady state levels of *bcl-2* mRNA and protein (Miyashita *et al.*, 1994). Moreover, p53 has been shown to directly activate transcription of the *bax* gene (Miyashita and Reed, 1995).

The development and proper function of an effective immune system requires apoptosis (Cohen *et al.*, 1992). Immature T cells undergo random rearrangement of their antigen receptor genes (reviewed in Davis, 1990). Only those cells bearing T cell receptors (TCRs) of appropriate specificity are positively selected for survival and further differentiation. The rest, which is at least 95% of the CD4+CD8+ T cell precursors, undergo apoptosis (Surh and Sprent, 1994). Those apoptosing cells are either deleted due to expression of self-reactive receptors (negative selection) or die because they failed to receive a surviving signal (death by neglect) (von Boehmer, 1994). The cellular mechanisms involved in positive or negative selection are not yet fully outlined nor is the role of apoptosis related molecules, if any, in the process. However, for example, bcl-2 expression has been shown to be upregulated during or directly following positive selection (Linette *et al.*, 1994).

Results

T cells from Baxα Transgenics show Accelerated Apoptosis in Response to Certain Apoptotic Stimuli

Several transgenic lines of mice were produced carrying a construct containing the human CD2 promoter and LCR element as well as the mouse *baxα* cDNA with a haemagglutinin (HA) epitope attached. The HA epitope was added to the N-terminus of murine *baxα* using PCR followed directly by the cDNA coding for mouse *baxα* so that HABaxα protein produced by the transgene can be distinguished from endogenous Baxα. The CD2 HA*baxα* transgene is shown in Figure 1.

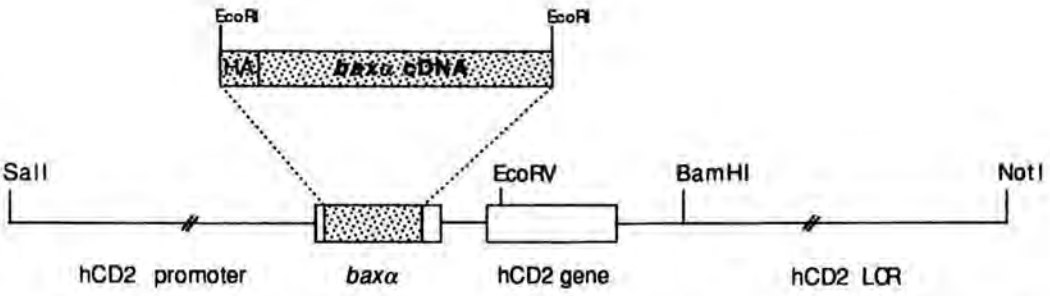

Fig. 1. *Baxα* transgene. *Baxαα* transgene construction. The HA tagged *baxα* insert flanked by EcoRI sites was generated by PCR from the murine *baxα* cDNA. The insert was cloned into the EcoRI site in exon 1 of the human CD2 expression cassette containing the CD2 promoter, shortened CD2 coding region and 3' LCR. The SalI-NotI fragment was isolated for injection into fertilised mouse oocytes.

DNA from the transgenic lines generated was analysed by Southern blot analysis and subsequent studies focussed on the two lines with the highest copy numbers of transgene. These lines of *baxα* transgenic were designated Bax 18 and Bax 25. Western blot analysis was used to examine the level of HABaxα expression in the T cells of the transgenic mice. Baxα expression was detected using a rabbit polyclonal antibody against Baxα and the 12CA5 mouse monoclonal antibody (mAb) against the HA epitope. The addition of the 11 amino acid HA epitope means that the HABaxα protein is larger than the endogenous Baxα. The ratio of HABaxα to endogenous Baxα was determined by phosphorimaging using the actin control to normalise each track. The ratio HABaxα: Baxα was 145% for Bax18 and 155% for Bax25 (Brady *et al.*, 1996a).

To assess the effect of Baxα on survival *in vitro*, thymocytes from *baxα* transgenic mice and control littermates were cultured in medium. Samples were harvested at various time points and the percentage of apoptotic cells determined using a flow cytometry-based technique which measures propidium iodide staining of DNA after cell lysis in a hypotonic buffer (Nicoletti *et al.*, 1991). Over a 3 day period substantially more apoptosis is seen in the baxα transgenic thymocytes than in control thymocytes (Figure 2A). By 72 h 40-50% of control thymocytes had still diploid DNA content whereas virtually all the *baxα* thymocytes were apoptosed. Similarly, splenic T cells from baxα transgenic mice showed accelerated apoptosis compared with splenic T cells from control littermates (data not shown).

The glucocorticoid dexamethasone has been shown to induce apoptosis in thymocytes (Wyllie, 1980). The result of dexamethasone treatment of thymocytes from the Bax 18 and 25 transgenic as well as non-transgenic littermates is shown in Figure 2B. The cells were harvested after 4-5 hours incubation with 2μM dexamethasone. Greater than 60% of the Bax 18 transgenic thymocytes are in apoptosis whereas only 30% or so of those from non-transgenic thymocytes are apoptotic. Similarly approximately 60% of the Bax 25 transgenic thymocytes are apoptotic as opposed to less than 30% for the non-transgenic thymocytes.

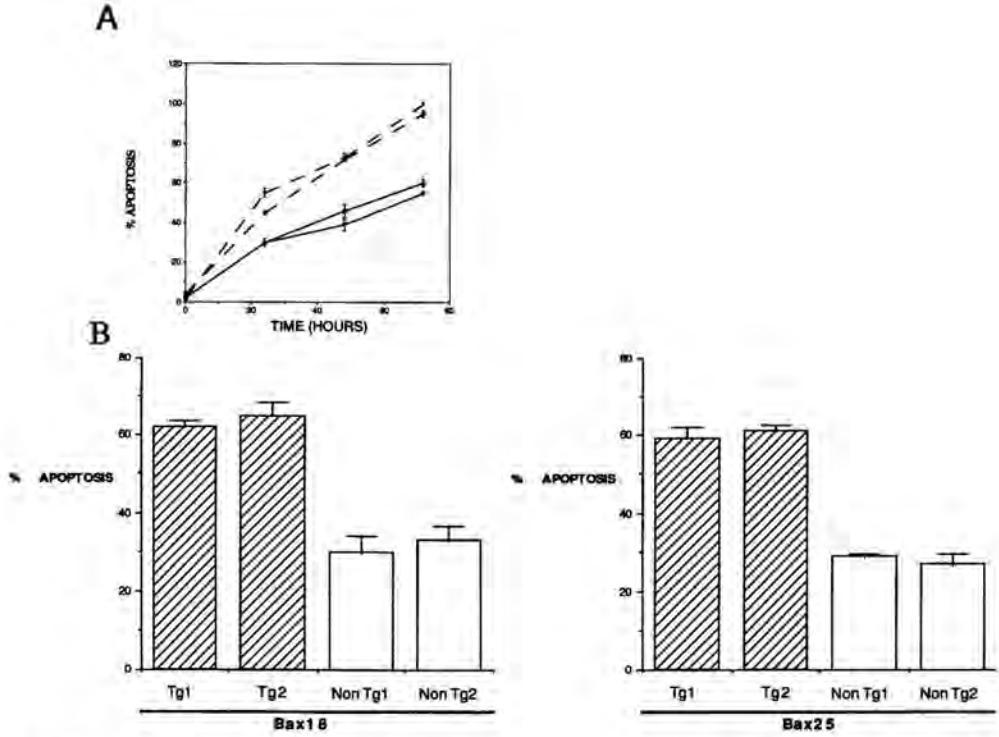

Fig. 2. Baxα accelerates thymocyte apoptosis.
(A) Thymocytes from *baxα* transgenic mice die faster during *in vitro* culture. Thymocytes were isolated from *baxα* transgenic mice and non-transgenic littermates and cultured in medium at 1×10^6 cells/ml. The percentage of apoptosis of *baxα* transgenic thymocytes (open circles and solid squares) is represented by broken lines and control littermate thymocytes (open squares and solid circles) by continous lines. Each data point represents the mean range of duplicate determination of apoptosis in 10,000 cells.
(B) Percentage of apoptosis in *baxα* transgenic thymocytes dexamethasone. Thymocytes were isolated from transgenic (Tg1,2) and non-transgenic littermates (Non Tg 1,2) of the Bax 18 and Bax 25 lines and treated with 2μM dexamethasone. 4.5 hr after treatment the cells were harvested and prepared for FACS to measure the DNA content of the nuclei by propidium iodide staining. The percentage of apoptotic cells shown is normalised to the amount of apoptotic cells found in untreated cultures for the same animal at the same time point.

The *baxα* transgenic thymocytes were further assessed for their sensitivity to apoptosis induced by DNA damage. Certain cell types, particularly lymphocytes are known to be highly sensitive to DNA damage induced apoptosis caused by γ-radiation or cytotoxic drugs, as used in chemotherapy (Cohen *et al.*, 1992). This sensitivity is known to be greatly reduced by Bcl-2 overexpression (Sentman *et al.*, 1991; Strasser *et al.*, 1991). Low doses of γ-radiation were administered to the Bax 18 and Bax 25 thymocytes. The doses were in the range 50 cGy to 200 cGy. For both lines the transgenic thymocytes are substantially more sensitive to γ-radiation than non-transgenic littermates. After 8 hr greater than 30% apoptosis is found in Bax 18 mice irradiated with only 100 cGy (Brady *et al.*, 1996a).

Baxα Overexpression in p53 Null T Cells Still Accelerates Glucocorticoid Induced Apoptosis but does not rescue DNA Damage Induced Apoptosis

The *baxα* mice were crossed so as to produce mice homozygous for the p53 null mutation while being *baxα* transgenic. Thymocytes from p53 null mice have been shown to be resistant to apoptosis induced by DNA damaging agents such as γ-radiation or etoposide (Clarke *et al.*, 1993; Lowe *et al.*, 1993). Thymocytes were analysed from mice heterozygote and homozygote for the p53 null allele and then plus or minus the *baxα* transgene. The result of dexamethasone treatment is shown in Figure 3A. High levels of apoptosis were found in the mice transgenic for *baxα* regardless of p53 status. Much lower levels of apoptosis are seen in thymocytes from mice heterozygote or homozygote for the p53 null allele without *baxα*.

A different situation pertains in thymocytes treated with the DNA damaging agent etoposide (Figure 3B) or γ-radiation (Figures 3C and D). High levels of etoposide induced apoptosis are seen in p53 heterozygote thymocytes, somewhat higher in those also expressing HABaxα. There are only very low levels seen in p53 null homozygotes regardless of the presence or absence of the *baxα* transgene. The same situation applies to thymocytes irradiated with 100 cGy (Figure 3C) or 500 cGy (Figure 3D) of γ-radiation. Irradiated p53 null thymocytes showed a slightly increased level of apoptosis with *baxα* than without but we do consider this significant outside of inherent experimental variation. Therefore, it is clear that Baxα overexpression accelerates apoptosis due to glucocorticoid treatment in a p53 independent manner. However, while Baxα overexpression can accelerate apoptosis due to DNA damage this process is dependent on p53 status since, it is not effective in the absence of p53.

Baxα acts in Distinct Apoptotic Pathways

Given that Baxα overexpression accelerates apoptosis in thymocytes in response to DNA damage as well as glucocorticoid treatment, we then investigated whether this also holds true for other pathways leading to cell death. Fas/APO-1 is a cell surface protein that induces apoptosis in immature thymocytes treated with an anti-Fas antibody (Ogasawara *et al.*, 1995). We incubated thymocytes from baxα transgenic mice and control littermates with 1μg/ml anti-Fas antibody (Jo2) for 20h. As on thymocytes from normal mice (Ogasawara *et al.*, 1995), virtually all thymocytes from baxα transgenic mice express Fas/APO-1 (data not shown). No accelerated apoptosis was found in baxα transgenic thymocytes in response to anti-Fas antibody, either with or without cycloheximide (Brady *et al.*, 1996b). A similar result was found when baxα and control thymocytes were incubated with 10 ng/ml of TNFα for 20 h. These data show that overexpression of Baxα does not accelerate the cell death pathways initiated in thymocytes by activating Fas with an agonistic antibody or treatment with TNFα.

Baxα Transgenics have decreased Numbers of Mature T Cells in vivo

Having established that baxα transgenic T cells show accelerated cell death *in vitro*, the constitution of T cell compartments *in vivo* was also scrutinised. Thymocytes from baxα transgenic mice and control littermates were analysed by two colour flow cytometry using antibodies against CD4 and CD8. As shown in Figure 4, a

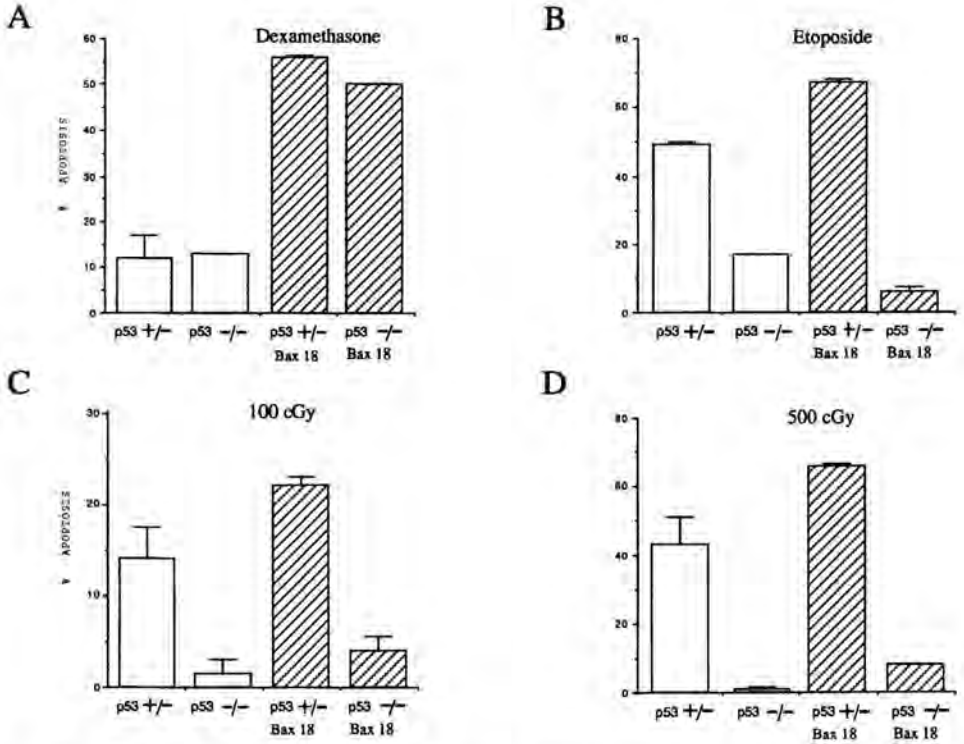

Fig.3. Percentage apoptosis in thymocytes from mice with or without p53 and/or the *baxα* transgene. (A) Thymocytes were isolated from mice heterozygote for a p53 null allele (p53+/-) or homozygote for p53 null alleles (p53-/-) and either containing the *baxα* transgene from the Bax 18 line or not. The cells were treated with 2μM dexamethasone for 4.5 hr, harvested and prepared for DNA content measurement. Thymocytes were also treated with 50 μM etoposide for 7hr (B) or, cultured for 8 hr following 100 cGy of γ-radiation (C) or cultured for 8 hr following 500 cGy of γ-radiation (D), before harvesting and analysis. The percentage of apoptotic cells is shown.

considerable decrease in the number of CD4+ single positive (SP) and CD8+ SP cells was seen in the baxα transgenic mice in comparison to control littermates. The baxα transgenic mice have only 25 to 30% of the control levels of SP cells in the thymus. This was consistent for both transgenic lines studied, Bax18 and Bax25 and could be observed at all the ages studied i.e. 2 to 8 weeks. This change is also reflected in a decrease in the absolute number of SP thymocytes as the total number of thymocytes did not significantly vary between baxα transgenic and control littermates in the age range studied. Concomitant with the decrease in SP T cells in the baxα transgenic thymus is an increase in the number of CD4+CD8+ double positive (DP) immature thymocytes. A similar analysis was carried out on red cell-depleted splenocytes (Brady *et al.*, 1996b). As for thymocytes, a substantial reduction in CD4+ and CD8+ SP T cells was seen in the spleen of baxα transgenic mice. These results show that a consequence of the expression of the baxα transgene in T cells is a major reduction in the numbers of mature T cells *in vivo*.

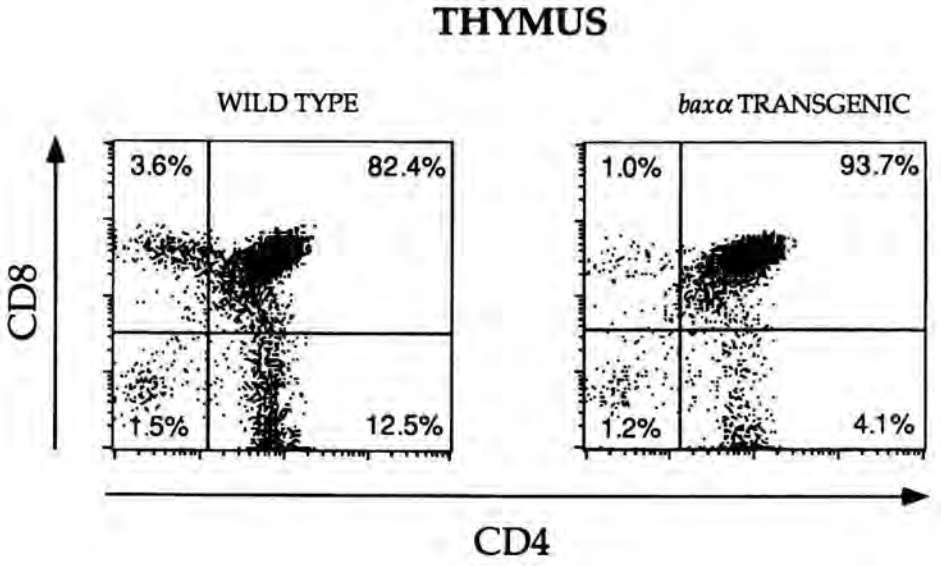

Fig. 4. *Baxα* transgenics have decreased numbers of mature T cells in vivo.
CD4 and CD8 expression on thymocytes and splenocytes from *baxα* transgenics and control littermates. The percentage of cells in each population is indicated.

Baxα Transgenics have an Increased Number of Cycling Cells in the Thymus

In light of the reduced numbers of mature T cells in baxα transgenic mice described above but the unchanged number of thymocytes relative to control littermates, we examined whether this had implications for the number of cycling cells in the thymus. To investigate this *in vivo* we injected the thymidine analog 5-bromo-2'-deoxyuridine (BrdU) intraperitoneally into baxα transgenic mice and control littermates. Four hours later the thymus was removed from each mouse and the BrdU content of the thymocytes measured using a BrdU specific antibody with propidium iodide counterstaining to assess DNA content as an indicator of position in the cell cycle (Begg *et al.*, 1988). We observed that at least twice the number of thymocytes in baxα transgenic compared to control littermates, are in the S phase of the cell cycle (Brady *et al.*, 1996b). *baxα* thymocytes show a concomitant decrease in the number of cells in G0/G1.

Baxα accelerates the Entry into S Phase of Cycling T Cells whereas Bcl-2 delays it

In order to investigate if Baxα can directly affect S phase entry we had to look at a situation in which a synchronised population of primary T cells was allowed to re-enter the cell cycle following an exogenous stimulus. To do this, we incubated splenocytes with ConA for 72 hours. We then starved the resulting T cell blasts of IL-2 for 14 h which resulted in a completely arrested synchronised population, confirmed by propidium iodide staining and flow cytometry (data not shown). Subsequent addition of

IL-2 stimulates the arrested cells to re-enter the cell cycle. Samples were taken at various time points after the addition of IL-2 to allow analysis of cell cycle status as monitored by the kinetics of $p27^{Kip1}$ downregulation as well as BrdU incorporation. T cells induced to leave a quiescent state by IL-2 show a decrease in the level of $p27^{Kip1}$ allowing CDK2 activation and entry into S phase (Nourse *et al.*, 1994; Firpo *et al.*, 1994). As shown in Brady *et al.*, 1996b, the level of $p27^{Kip1}$ decreases with time upon addition of IL-2 to the arrested T cell blasts. However, the overall level of $p27^{Kip1}$ is lower in the baxαtransgenic T cell blasts than in those from control littermates. To confirm that the lower levels of $p27^{Kip1}$ were reflected in an accelerated entry into S phase we also analysed the IL-2 restimulated blasts for BrdU incorporation. The baxα transgenic T cell blasts enter S phase faster than T cell blasts from control littermates as measured by their increased level of BrdU incorporation in the first 18 h after IL-2 stimulation (Brady *et al.*, 1996b).

The implication of the above is that overexpression of the apoptotic effector molecule Baxα can influence the rate at which cycling cells enter S phase. To test whether this effect has a wider context than Baxα alone we tested whether or not Bcl-2 would exert an opposing effect. We also generated ConA stimulated T cell blasts from the spleens of Eμbcl-2 transgenic mice (McDonnell *et al.*, 1989) and control littermates. The levels of $p27^{Kip1}$ were higher in the bcl-2 transgenic T cell blasts and the rate of decline of these levels was also slower than in controls following IL-2 stimulation (Brady *et al.*, 1996b). The CDK inhibitor $p21^{Cip1}$, is induced upon IL-2 stimulation (Nourse *et al.*, 1994). The kinetics of $p21^{Cip1}$ induction for both the bcl-2 and baxα T cell blasts upon IL-2 addition were measured and remained unchanged in comparison to control blasts (data not shown). To verify that the slower decline in $p27^{Kip1}$ levels in the bcl-2 T cell blasts correlated with a delayed entry into S phase we measured the level of BrdU incorporation after IL-2 addition. As seen in Brady *et al.*, 1996b, the entry of Bcl-2 transgenic T cell blasts into S phase is substantially delayed in comparison to that in control littermates. These data demonstrate that overexpression of a molecule which accelerates apoptosis, such as Baxα, or a molecule that blocks apoptosis, such as Bcl-2, can directly influence the rate at which cells enter the cell cycle: an accelerated rate in the case of Baxα and a delayed rate in the case of Bcl-2.

Discussion

We have demonstrated that the overexpression of Baxα in primary T cells can accelerate apoptosis in these cells in response to different stimuli. Comparing protein expression we find that thymocytes from both Bax 18 and Bax 25 have transgenic HA Baxα levels of approximately 150% that of endogenous Baxα. This level of HABaxα is sufficient to substantially accelerate apoptosis in thymocytes in response to low doses of γ-radiation, dexamethasone and etoposide. This is in direct contrast to thymocytes from *bcl-2* transgenic mice which become more resistant to these apoptotic stimuli (Sentman *et al.*, 1991; Strasser *et al.*, 1991). Our data and the previous work of others point to a situation in T cells where the ratio of Baxα to Bcl-2 is very important for determining response to apoptotic stimuli.

Clearly, p53 has a central role in the regulation of apoptosis in response to DNA damaging agents (Clarke *et al.*, 1993; Lotem and Sachs, 1993; Lowe *et al.*, 1993). p53 also directly activates transcription of the *bax* gene (Miyashita and Reed, 1995). Taken together these findings suggested that Baxα may act downstream of p53 in a p53 dependent apoptosis pathway. The presence of the *baxα* transgene accelerated apoptosis in thymocytes from both p53-/- and p53+/- mice in response to dexamethasone.

In contrast with the response to dexamethasone the presence of the *baxα* transgene accelerated apoptosis in only thymocytes from p53+/- mice after exposure to γ-radiation and etoposide. Thymocytes from p53-/- mice with the *baxα* transgene showed similar resistance to apoptosis by these DNA damaging agents as p53-/- mice without the *baxα* transgene. Therefore, the suggestion that p53 acts to induce apoptosis following DNA damage by altering the Baxα:Bcl-2 ratio in favour of Baxα to accelerate cell death (Miyashita *et al.*, 1994; Cox and Lane, 1995; Miyashita and Reed, 1995) is a part of but clearly not the complete picture. Presumably, p53 is required to induce other necessary factor(s) apart from Bax to hasten cell death or perhaps directly activate such factor(s) by a protein-protein interaction.

There are multiple pathways leading to apoptosis which are subject to distinct regulation (Strasser, 1995). We find that the cell death pathway activated via the Fas receptor is not enhanced by overexpression of Baxα. This suggests that signalling through Fas occurs via an alternative pathway than the one involving Baxα. This correlates with the recent finding that overexpression of Bcl-2 in the lymphoid cells of transgenic mice does not inhibit Fas induced apoptosis (Strasser *et al.*, 1995). Similarly, we find that TNFα induced apoptosis is not accelerated in Baxα transgenic T cells which again tallys with earlier data that Bcl-2 is a poor inhibitor of TNF-induced apoptosis (Vanhaesebroeck *et al.*, 1993).

The Baxα mice have only 25-30% the level of mature SP T cells in thymus and spleen as compared to control littermates whereas the number of DP thymocytes is slightly increased. This contrasts with the lymphoid hyperplasia reported in bax (-/-) mice (Knudson *et al.*, 1995). The instance of a slightly increased number of baxα DP thymocytes indicates that until the point of selection there is probably no stimulus to induce apoptosis. When, however, the thymocytes are cultured *in vitro* the baxα DP thymocytes die faster than the control thymocytes.

The depletion of mature T cells *in vivo* suggests that the baxα mice may have a defect in selection. Immature thymocytes that are not positively selected are thought to die via negative selection or neglect. It has been shown that apoptosis is the major process whereby thymocytes die and that the vast majority of apoptotic cells in the thymus seem to be a reflection of failure to undergo positive selection (Surh and Sprent, 1994). Our data indicate that overexpression of an apoptosis accelerating molecule, such as baxα, can directly cause failure of selection.

A further aspect of the thymi of baxα transgenic mice which we observed was the doubling of the number of thymocytes in the S-phase of the cell cycle. This could be attributed to the influence of a homeostatic process within the thymus whereby the depletion of mature T cells, mediated by the baxα transgene, is counteracted by

an increased recruitment of cells into cell cycle within earlier compartments in an attempt to fill up the void. Such a process may exist and result in increased cycling but we were interested whether Baxα overexpression itself could influence T cell entry into cell cycle. To assess this we stimulated splenocytes with ConA to generate T cell blasts, and incubated the cells in medium without IL-2 for 14 h before restimulation by adding IL-2. It has been demonstrated that IL-2 allows CDK activation by causing the elimination of the CDK inhibitor protein p27Kip1 (Nourse *et al.*, 1994; Firpo *et al.*, 1994). p27Kip1 governs CDK activity during the transition from quiescence to S phase in T lymphocytes. We find that Baxα overexpression correlates with overall decreased levels of p27Kip1 after IL-2 stimulation and, furthermore, that this correlates with accelerated entry into S phase as measured by BrdU incorporation and propidium iodide counterstaining. Further confirmation of the finding, that apoptosis regulatory molecules can have an influence on cell cycle, comes from our observation of the opposite effect in bcl-2 transgenic T cells namely, that Bcl-2 delays the downregulation of p27Kip1 after IL-2 stimulation with a concomitant delay in S phase entry. We also observe that the absolute levels of p27Kip1 are higher in the bcl-2 blasts compared to controls.

These data pose the questions how apoptosis regulatory molecules can influence the cell cycle machinery and with what purpose. The two main possibilities for how the interaction can occur are either directly or indirectly. Several analyses (e.g. Makela *et al.*, 1994; Harper *et al.*, 1993) have already taken place for proteins interacting with members of the cell cycle machinery but to date no interaction with Bcl-2 family members has been reported. However, such an interaction cannot be excluded. The indirect association could be via proteases downstream of Baxα/Bcl-2 whose activities might modulate components of the cell cycle. Thus, the activation of these downstream proteases could be a necessary event contributing to the process of downregulation of cell cycle inhibitory proteins like p27Kip1 necessary for cell cycle progression.

Although, our data do not unequivocally position the Baxα/Bcl-2 effect within the cell cycle machinery, we have demonstrated that their overexpression results in modulation of p27Kip1 levels. As a consequence of this Baxα/Bcl-2 levels could influence the timing of the activation of cyclin E/CDK2 and cyclin A/CDK2 complexes. We have shown here the effects of Baxα/Bcl-2 on cell cycle and T cell selection. These may be separate functions of the same proteins or they could be interconnected. One way to explain both effects is based upon the observation that nondividing immature T cells, in particular, are positively selected (Huesmann *et al.*, 1991). Baxα overexpression would lead to more T cells in cycle whereas Bcl-2 would do the reverse.

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Control of Neuronal Survival by Neurotrophins

Abstract

Neurotrophins are related secretory proteins that control cell survival in the nervous system. All can prevent programmed cell death by binding to specific cell surface receptors belonging to a family of tyrosine kinase receptors. As these receptors are expressed in subgroups of developing neurons, interference with the function of these receptors or of their ligands leads to selective neuronal deficits in the nervous system. All neurotrophins also bind to another receptor designated the neurotrophin receptor p75.

This member of the tumor necrosis factor receptor family can be activated by nerve growth factor, leading to the death of neurons in the developing nervous system. Thus, the neurotrophin nerve growth factor controls cell numbers in opposite ways by its ability to activate 2 different receptors.

Introduction

In multicellular organisms, programmed cell death is now widely recognised as an important mechanism contributing, like cell division, to the control of cell numbers (Raff, 1992). In the developing nervous system, the death of cells can already be observed during the earliest stages of development, in proliferating neuroepithelia such as the neural tube or the retina (Glücksmann, 1951; Cuadros and Rios, 1988; Homma et al., 1994). Later, when identifiable groups of neurons cease to divide and begin to make contact with their target cells, the extent of cell death can be better appreciated by counting the total number of cells before and after target innervation. During this phase, neuronal death can be readily quantified, and in a variety of neuronal populations, losses of 50% or more have been reported (for review, see Oppenheim, 1991). However, the real extent of cell death during early neurogenesis is still difficult to appreciate quantitatively. The fate of individual cells cannot be traced on a large scale, and dying cells are rapidly eliminated. By contrast, in the nematode *Caenorhabditis elegans*, the total number of cells is so small that it is possible to monitor the fate of every cell during development. These quantitative studies have revealed that out of the 1090 originally somatic cells generated, 131 die, 105 of which being neurons (for review see Ellis et al., 1991).

Studying the mechanisms controlling developmental cell death is of special significance in the nervous system, as unlike other cells, neurons typically become postmitotic early and never divide subsequently. In vertebrates, several molecules regulating the survival of neurons have been identified, including in particular a small gene family known as the neurotrophins, which consist of 4 members in mouse and human. These genes encode very basic, secretory proteins named nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin3 (NT3) and neurotrophin4/5 (NT4/5). The neurotrophins are non-covalently bound dimers, and even though the existence of soluble monomers has been demonstrated, these monomers spontaneously reform dimers (Kolbeck et al., 1994). Crystal structure data indicate that the neurotrophins have very similar, elongated shapes, with the monomers forming a large hydrophobic interface (McDonald et al., 1991; Robinson et al., 1995). The known biological properties of the 4 neurotrophins can be accounted for by their ability to bind to 2 distinct receptors, one designated the neurotrophin receptor p75 ($p75^{NTR}$), the other consisting of a group of closely related receptor kinases referred to as the *trk*s.

The interaction of all neurotrophins with their *trk* receptors prevents neuronal death, whereas recent results indicate that the interaction of one of them, NGF, with $p75^{NTR}$ causes cell death.

II. Neurotrophins and their Tyrosine Kinase Receptors

A. Binding of Neurotrophins to the trk Receptors

A group of 3 closely related receptor tyrosine kinases, referred to as *trk*s, has been identified in higher vertebrates. The *trk*s bind neurotrophins specifically with nanomolar affinities (for review, see Barbacid, 1994). NGF binds to *trk*A, BDNF and NT4/5 to *trk*B and NT3 to *trk*C. Neurotrophin binding induces dimerisation of the receptors, and the activation of their kinase domains results in tyrosine phosphorylation (for review, see Kaplan and Stephens, 1994). Two recent findings help to explain why BDNF and NT4/5 have biological effects which are not always identical, even though both bind to *trk*B with identical affinities. An extracellular splice variant of *trk*B has been discovered which preferentially interacts with BDNF over NT4/5 (Strohmaier et al., 1996). Also, the participation of $p75^{NTR}$ in a (still hypothetical) p75/*trk*B complex seems to be more critical for NT4/5 binding, including retrograde transport, than it is for BDNF (Curtis et al., 1995; Rydén et al., 1995).

B. Activation of trk Receptors prevents Neuronal Death

The generation of mouse mutants with functional deletions in the *trk*A, B and C genes has allowed comparisons between the phenotype of such animals and that of mice with deleted neurotrophin genes (for reviews, see Barbacid, 1994; Snider, 1994). This work led to the conclusion that the *trk*s are essential for transducing the neuronal survival activity of neurotrophins. Thus for example, when the peripheral sympathetic or sensory system of *trk*A -/- animals is compared with that of NGF -/-

animals, the same neuronal subpopulations are absent (Snider, 1994). This suggests that in order to prevent programmed cell death, activation of tyrosine kinase receptors by neurotrophins is necessary.

How the activation of receptor tyrosine kinases interferes with programmed cell death is still not well understood. Presumably, a phosphorylation cascade prevents at some point the activation of intracellular proteases. Indeed, genetic experiments using *C. elegans*, made possible because of the small number of cells in this nematode (see above), led to the crucial observation that a null mutation in the *ced-3* gene blocks programmed cell death, and that *ced-3* acts cell autonomously (Yuan and Horvitz, 1990). Subsequent work revealed that *ced-3* encodes a protease related to a previously identified vetebrate protease with specificity for aspartate residues (Yuan et al., 1993). Inhibition of such proteases prevents programmed cell death in neurons (Gagliardini et al., 1994). Also, injections into the cytoplasm of cultured neurons of plasmids encoding *bcl-2* block programmed cell death in neurons (Garcia et al., 1992). *Bcl-2* was discovered because of a chromosomal translocation in human (positioning *bcl-2* under the inappropriate control of an immunoglobulin promoter), resulting in excesses of B-cells (and of lymphoma), explained by a lack of cell death (Tsujimoto and Croce, 1986). Significantly, the nematode gene *ced-9*, the function of which is necessary to prevent programmed cell death, encodes a protein clearly related to bcl-2 (Hengartner and Horvitz, 1994; for review, see Horvitz et al., 1994). Thus, it appears that the intracellular mechanisms controlling programmed cell death in neurons are basically similar to those uncovered in *C. elegans* using a genetic approach.

C. Specificity of trk Expression and of Neurotrophin Action

The discovery that members of the *trk* family are functional receptors for the neurotrophins has been important and useful, as the pattern of expression of these genes in the nervous system helps to predict which neuronal populations are likely to respond to which neurotrophin (Tessarollo et al., 1993).

The specificity of the neurotrophin-*trk* system is especially well documented in the peripheral sensory system. In particular, it has been possible to generate animals lacking specific types of sensory inputs such as those involved in nociception or proprioception (for a recent review, see Lewin and Barde, 1996). This can be achieved either by injecting antibodies neutralising the neurotrophins or their *trk* receptors, or by deleting the corresponding genes. To a large extent, these results can be explained by the fact that many small, unmyelinated sensory neurons and axons express *trk*A, but not *trk*C, which is expressed by large sensory neurons, including the myelinated Ia afferents contacting ventral horn motoneurons (reviewed in Snider, 1996; Lewin and Barde, 1996). The specificity of the resulting phenotype is likely to be of considerable use to understand the molecular diversity of sensory neurons involved in the perception of various stimuli. In the CNS, while the expression of *trk* receptors (at least in rodents) is less obviously correlated with the survival of specific neuronal populations than is the case in the PNS, the pattern of *trk* expression remains a very useful predictor of neurotrophins' actions, such as for example effects on gene expression or dendritic arborisation (Ip et al., 1993; McAllister et al., 1995).

D. Significance of Neurotrophins in the Developing CNS

In chick embryos, antibody deprivation experiments have indicated that neurotrophins also control the development of CNS neurons (Bovolenta et al., 1996). In this regard, the retina has been a particularly useful structure to study, both because of its rapid development (millions of cells being generated over only few days), and its accessibility to experimental manipulations. Many cells in the neural retina express the catalytic NT3 receptor *trk*C, and some $p75^{NTR}$ (see below). Also, NT3 is present in the retina, mostly in the pigmented epithelium early in development. Acute deprivation of NT3 results in profound modifications affecting the development of most cell types (Bovolenta et al., 1996). In particular, as soon as the optic nerve forms, about half of the axons are missing in the optic nerve of antibody-treated embryos. This deficit does not reflect a developmental retardation, as it persists for as long as the optic nerves have been examined. Also, a general reduction in the size of all retinal layers indicates that NT3 is an essential retinal signal influencing the fate of many cells. Detailed examination of such embryos has revealed that NT3 must be more than an essential survival factor for developing retinal cells. Indeed, NT3 seems to play a role as a differentiation factor, needed to take retinal neuroblasts out of the cell cycle. Thus in the absence of NT3, cell division has been shown to be abnormally high in the retina (Bovolenta et al., 1996).

It thus appears that neurotrophins are essential survival factors for a variety of developing vertebrate neurons, that they need to activate tyrosine kinases to prevent programmed cell death, and that at least NT3 is also an essential differentiation factor early in the development of CNS neurons.

III. The Neurotrophin Receptor $P75^{NTR}$

A. Neurotrophin Binding to $p75^{NTR}$, a Member of a Gene Family

When $p75^{NTR}$ is expressed in fibroblastic cell lines, it binds all neurotrophins with similar, nanomolar affinities, though when binding parameters are examined in detail, different kinetics and degrees of positive co-operativity are observed (Rodríguez-Tébar et al., 1990, 1992; for review and discussion, see Bothwell, 1995; Dechant and Barde, 1997). This suggests that differential conformational changes are induced by neurotrophin binding. Indeed, the results of recent functional experiments have indicated that specific effects can be elicited by the neurotrophins through $p75^{NTR}$ (see below).

$P75^{NTR}$ was the first member to be isolated of a still growing family of structurally related, non-catalytic receptors (for review, see Chao, 1994). These include in particular the TNF receptors I and II (TNFRI and TNFRII), and the Fas antigen (also referred to as Apo-1 or CD95). These receptors are characterised by an extracellular domain containing usually 3 or 4 repeated cysteine-rich subdomains. However, their intracellular sequences are not obviously related and lack any motifs providing clues as to the type of biochemical reactions they may catalyse. The only clear intracellular

homology is a sequence of about 60 amino acids in Fas and TNFRI. This segment has been coined the 'death domain', as these 2 receptors have clearly been shown to induce programmed cell death in a variety of cell types (Itoh and Nagata, 1993; Tartaglia et al., 1993; Nagata and Golstein, 1995). This death domain also shows some amino acid identities with the sequence of the *reaper* gene (White et al., 1996), known to cause programmed cell death in *Drosophila* (Golstein et al., 1995).

B. Functional Consequences of Neurotrophin Binding to p75^{NTR} in vitro

One of the functions of p75^{NTR} that has received much attention is its ability to increase the affinity of NGF binding to *trk*A, and to form a high affinity receptor on cells expressing both *trk*A and p75^{NTR} (see in particular Mahadeo et al., 1994). But many cells in the nervous system and elsewhere express p75^{NTR} and not *trk*A, which has a comparatively restricted pattern of expression. So an intriguing question has been, for a long time, whether neurotrophin binding to p75^{NTR} on cells not expressing catalytic forms of *trk*-receptors would have any measurable biochemical or biological consequences.

One of the first clear answer came from experiments performed with p75^{NTR}-expressing T9 glioma cells, where neurotrophin binding was shown to transiently activate a sphingomyelinase, resulting in the production of the lipid second messenger ceramide (Dobrowsky et al., 1994; Dobrowsky et al., 1995). Evidence that p75^{NTR} can signal in non-transformed cells was subsequently provided using cultured rat Schwann cells, and the addition of NGF was shown to lead to the translocation of the transcription activator NF-κB to the nucleus (Carter et al., 1996). This activation of NF-κB occurs through the binding of NGF to p75^{NTR}, as Schwann cells isolated from p75^{NTR} -/- mice did not show translocation of NF-κB. Interestingly, neither BDNF nor NT3 activate NF-κB (Carter et al., 1996). While the biochemistry of this selectivity is not yet understood, substantial differences in the p75^{NTR} binding parameters have been noted when NGF, BDNF and NT-3 are compared (see above). Also, differences were observed in the circular dichroism spectra of p75^{NTR} when mixed with each of the neurotrophins, suggesting different conformational changes accompanying binding (Timm et al., 1994). Finally, in recent experiments, p75^{NTR} was shown to form high affinity, specific binding sites for NT3 when expressed in neurons, as opposed to fibroblastic cell lines (Dechant et al., 1997). This suggests that p75^{NTR} may associate with cytoplasmic proteins, opening the possibility that the cellular context in which this receptor is expressed could dramatically affect the ability of p75^{NTR} to signal (see also Dechant and Barde, 1997 for review).

Recent work with cultured oligodendrocytes indicates that binding of NGF to p75^{NTR} by NGF activates jun kinase and initiates programmed cell death (Casaccia-Bonnefil et al., 1996). Again, this effect could not be seen with NT3 or BDNF. When ceramide production was measured following exposure of oligodendrocytes to the various neurotrophins, only NGF, but neither BDNF nor NT3, triggered a sustained release of ceramide. Taken together, these results suggest that, as with the activation of NF-κB, only NGF is able to activate p75^{NTR} to cause cell death.

C. *Functional Consequences of Neurotrophin Binding to p75^{NTR}* in vivo

It is clear that the expression of p75^{NTR} is, as such, not sufficient to predict an activation of NF-κB or cell death by NGF. As with the other receptors of the p75^{NTR} family, interaction with cytoplasmic proteins is likely to be required to transduce any effects of ligand binding, and until such interactors have been identified, it will be difficult to predict signalling by p75^{NTR} merely by looking at the tissue distribution of this receptor. This raises the important question of whether or not any of the observations with cultured cells is relevant to the situation *in vivo*, and recent experiments indicate that this seems to be the case. Early in development in the avian retina, substantial cell death is observed, mostly in the central retina (Cuadros and Rios, 1988). It has been hypothesized that this might relate to the formation of the optic nerve, as all the axons of the retinal ganglion cells converge to the central retina to form the optic nerve (Cuadros and Rios, 1988). In addition, previous work has indicated that p75^{NTR} is expressed by many cells in the central avian retina (Von Bartheld et al., 1991). As expression of the NGF gene is detected very early in the chick retina in the absence of *trk*A expression (Frade et al., 1996), it was of interest to see if removal of NGF would decrease cell death. Such turned out to be the case, and to a degree indicating that most early cell death is actually mediated by NGF (Frade et al., 1996). In the absence of *trk*A, p75^{NTR} is *a priori* likely to be the receptor mediating the death action of NGF. Direct evidence for the involvement of p75^{NTR} could be provided by the intraocular administration of antibodies blocking the binding of NGF to p75^{NTR}, which prevented cell death much like the administration of antibodies to NGF (Frade et al., 1996).

D. *Targeted Deletion of the p75^{NTR} Gene*

The gene coding for p75^{NTR} has been deleted in the third exon, coding for 3 of the 4 cysteine-rich repeats (Lee et al., 1992). These mice present sensory deficits in the peripheral nervous system (Lee et al., 1992), as well as subtle signs of hypoinnervation in the peripheral sympathetic system (Lee et al., 1994). This result fits well with the notion that when co-expressed with trkA, p75^{NTR} increases the affinity of this receptor, enhancing in vivo the detection of low levels of NGF. Interestingly, these mice also show increased numbers of cholinergic forebrain neurons, as a *trk*A-negative population of such neurons which is normally eliminated in wild-type animals fails to disappear in mutant animals (Van der Zee et al., 1996). This result is in agreement with the suggestion that NGF can eliminate neurons through activation of p75^{NTR} during normal development, in cells not expressing *trk*A (see above). However, compared with any of the *trk*-deficient animals, the phenotype of the p75^{NTR} is considerably less spectacular. In particular, these mice not only survive the mutation, but they are also able to breed. Given the increasing number of similarities in the biological effects of NGF mediated by p75^{NTR} and those of TNF mediated by TNFRs, it might be more useful to compare the p75^{NTR} mouse mutant with those lacking TNFRs, rather than *trk* receptors. Mice lacking either TNFR receptor show a phenotype that becomes obvious when they are challenged with infectious agents, such as *Listeria monocytogenesis*. Also, these mice are actually more resistant to endotoxic shock than control mice (Pfeffer et al., 1993; Rothe et al., 1993).

As NGF has the potential to kill neurons expressing $p75^{NTR}$, it will be interesting to see if such mechanisms are also used in physio-pathological situations. For example, motor neurons are known to dramatically upregulate $p75^{NTR}$ after axotomy (Raivich and Kreutzberg, 1987; Yan and Johnson, Jr. 1988; Ernfors et al., 1989), and the administration of NGF has been show to increase the death of axotomised motor neurons (Miyata et al., 1986; Sendtner et al., 1992), while it can be prevented by the administration of BDNF (Sendtner et al., 1992).

Conclusions

With regard to neuronal survival, the physiological significance of the neurotrophins is well established. Their ability to prevent programmed cell death by activating specific receptor kinases accounts for their long known survival effects, both *in vitro* and *in vivo*. The specificity of many of their actions on various subpopulations of neurons can be explained by the patterns of expression of their specific *trk* receptors. But it is now apparent that the best known neurotrophin -NGF- can also cause cell death through its apparently unique ability to activate $p75^{NTR}$, when this receptor is expressed on cells not expressing *trk*A. How widespread this killing action of NGF is will be interesting to investigate in future experiments. Also, the molecular mechanisms linking NGF and $p75^{NTR}$, but not the other neurotrophins, with the cell death machinery need to be understood.

While this review deals with the regulation of neuronal survival during development, this should not be taken to imply that the control of programmed cell death represents the only biological function of the neurotrophins. In the CNS in particular, there is mounting evidence that neurotrophins change the morphology of neurons, and regulate the growth of dendrites and axonal terminals (see for example Cohen-Cory and Fraser, 1995; McAllister et al., 1995). As neurotrophins are synthesised in, and released by, CNS neurons as a function of neurotransmitter input, they might trigger morphological changes in relation with neuronal activity (for review, see Thoenen, 1995). So far, evidence has been mostly presented for positive or enhancing actions of neurotrophins on neuronal morphology (Snider and Lichtman, 1996). However, the theoretical possibility also exists that regressive events might be initiated by the activation of $p75^{NTR}$ by NGF. While purely speculative at this stage, one could envisage that local retraction of dendritic or axonal branches utilises mechanisms similar to those involved in cell death.

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Characterization of Apoptosis-resistant Jurkat T Cell Clones

Abstract

The T-acute lymphoblastic leukemia cell line Jurkat is sensitive to various apoptotic inputs, including stimulation of the T cell antigen receptor and CD95, receptor systems essential for the regulation of the immune response. Characterization of apoptosis-resistant variant clones from this cell line indicates that the T cell receptor can induce apoptosis by both CD95-dependent and -independent mechanisms and reveals the existence of a protein that regulates CD95 sensitivity upstream from CPP32 (caspase-3) activation.

Introduction

The T cell antigen receptor (TcR) recognizes antigenic peptides in the context of molecules of the major histocompatibility complex. As a result, resting lymphocytes are activated to divide and differentiate into helper- or cytolytic effector cells. However, renewed TcR triggering on activated T cells can induce apoptosis, which most likely serves to attenuate the immune response (Russell et al., 1991). Also, during T cell development in the thymus, TcR triggering can either rescue thymocytes from programmed cell death and induce further maturation (positive selection), or induce apoptosis and consequently delete potentially autoreactive cells (negative selection). These observations raise the question how the same receptor system can induce such vastly different responses: cellular activation or death?

Stimulation of the TcR/CD3 complex activates cytosolic protein tyrosine kinases, allowing recruitment and activation of Src-homology 2 (SH2)-domain containing molecules (Weiss and Littman 1994). Tyrosine kinase substrates include phospholipase C (PLC)-γ1 and the Shc-Grb2/Sos complex (Ravichandran et al., 1993), which initiate relatively well known signalling cascades. PLC-γ1 generates inositol trisphosphate and diacylglycerol. Inositol trisphosphate mobilizes Ca^{2+} ions from intracellular stores, leading to activity of the phosphatase calcineurin, which plays a role in the activation of the NFAT transcription factor complex (Rao 1994, Crabtree et al., 1994). Diacylglycerol is a cofactor for PKC, the enzyme thought to be required for activation of the NFκB transcription factor (Siebenlist et al., 1994). Formation of the Shc-Grb2/Sos complex induces the Ras pathway, resulting in activation of Erk 2

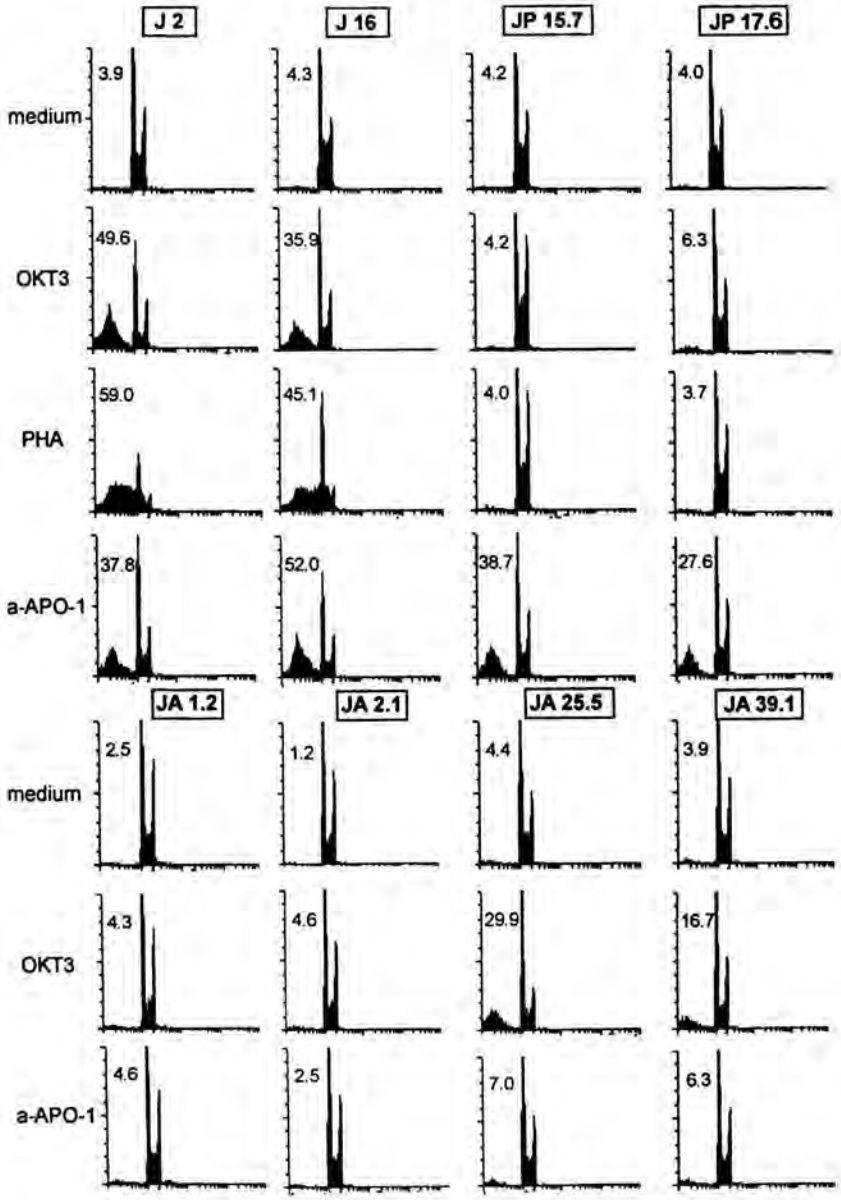

Fig. 1. Sensitivity to TcR- and CD95-induced apoptosis of wild type and variant Jurkat clones. Clones were cultured for 24 h in the presence or absence of the indicated stimuli. To stimulate the TcR/CD3 complex, OKT3 mAb was coated onto the tissue culture wells at 10 µg/ml or PHA was used in soluble form at 2 µg/ml. To stimulate CD95, soluble anti-APO-1 mAb was used at 1 µg/ml. Cells were harvested and lysed in a hypotonic buffer, which leaves the nuclei intact. DNA was stained with propidium iodide and fluorescence intensity of intact or apoptotic nuclei was measured using a FACScan. The numbers indicate the percentage of apoptotic nuclei in each sample, recognizable as a subdiploid DNA peak. J 2 and J 16 are wild type clones; JP 15.7 and JP 17.6 were selected for resistance to PHA-induced apoptosis and JA 1.2, JA 2.1, JA 25.5 and JA 39.1 were selected for resistance to anti-APO-1-induced apoptosis.

Table 1. Sensitivity of Jurkat and variant clones to TcR- and CD95-induced apoptosis

	medium	OKT3	anti-APO-1
Jurkat	4.8[a]	28.0	40.9
J 2	3.9	49.6	37.8
J 16	5.3	39.3	59.1
JP 15.7	5.4	6.6	55.0
JP 17.6	5.9	6.2	53.5
JA 1.2	2.5	4.3	4.6
JA 2.1	1.2	4.6	2.5
JA 9.3	1.4	2.4	5.7
JA 10.2	3.2	5.2	13.9
JA 16.6	1.7	7.2	3.8
JA 21.3	1.1	2.7	2.1
JA 22.3	1.7	5.7	1.9
JA 25.5	4.4	29.9	7.0
JA 35.2	6.1	20.9	10.7
JA 39.1	3.9	16.7	6.3

[a] Percentage of apoptosis 24 h after addition of stimulus, as determined according to Nicoletti et al. (22). Data are representative of four independent experiments.

mitogen activated protein kinases (MAPK) and the Fos transcription factor (Izquierdo et al., 1993). In addition, in concert with the costimulatory molecule CD28, the TcR induces Jun phosphorylation by the stress-activated protein kinases or Jun kinases (Su et al., 1994). The various transcription factor complexes can bind to promoter sites in a great variety of genes, encoding products which drive cell proliferation and differentiation (Crabtree et al., 1994).

Like TcR-induced proliferation and differentiation, TcR-induced apoptosis is also dependent on mRNA and protein synthesis (Ucker et al., 1989). Recent studies (Ju et al., 1995, Brunner et al., 1995, Dhein et al., 1995) have indicated that the TcR does not directly activate the apoptotic machinery but can induce apoptosis via the CD95 receptor system. Stimulation of the TcR/CD3 complex appeared to increase CD95 ligand (L) mRNA levels and protein expression, while TcR-induced apoptosis could be inhibited by soluble CD95 molecules. From these observations, one can conclude that TcR triggering induces signal transduction events leading to upregulation of CD95L mRNA levels. Resulting increased CD95L protein production is most likely responsible for the induction of apoptosis via the CD95 molecule.

CD95 was originally identified by anti-Fas (Yonehera et al., 1989) and anti-APO-1 (Trauth et al., 1989) mAbs selected for the capacity to induce cell death. CD95 belongs to the TNF receptor family, which includes a number of molecules implicated in the positive and/or negative regulation of cell survival (Bazan 1993). These receptors have no intrinsic enzymatic activity and employ novel signal transduction mechanisms. Apoptosis-inducing members of the TNF receptor family appear to couple directly, in a protein synthesis-independent manner to the caspase family of cysteine proteases that are thought to constitute the apoptotic machinery (Yuan 1997). These

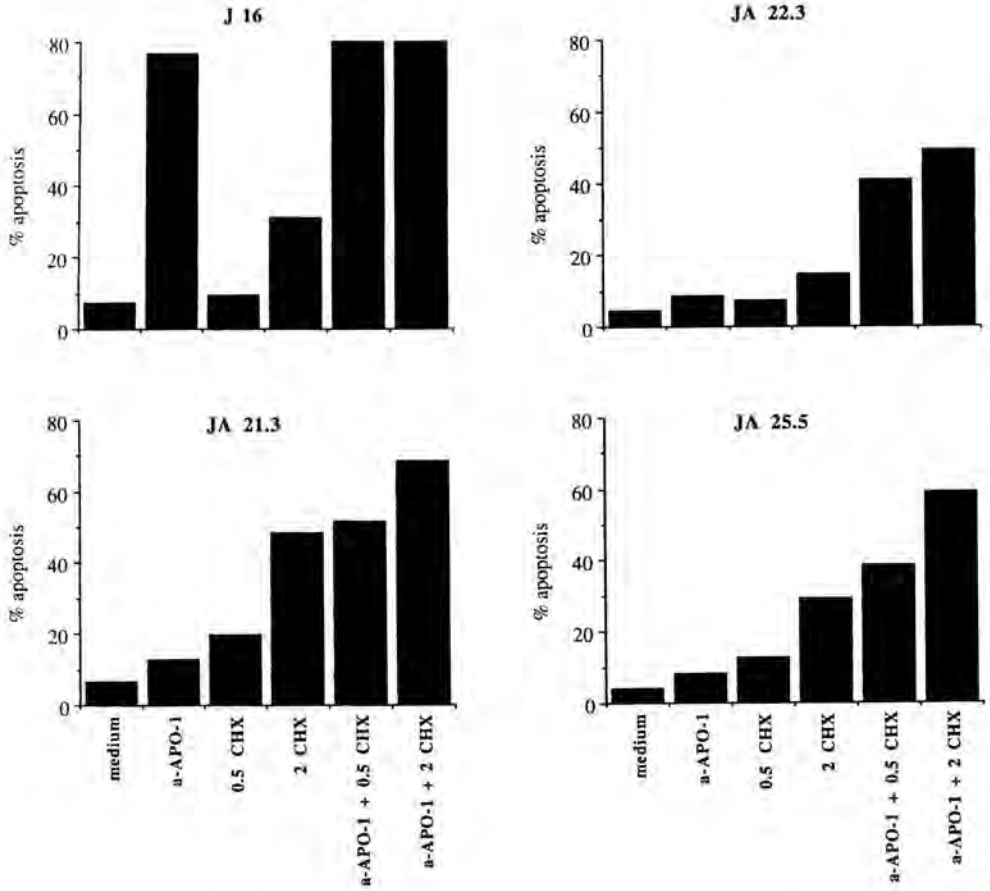

Fig. 2. The CD95 signalling pathway in JA clones is inhibited at, or upstream from CPP32 activation. Resistance can be alleviated by protein synthesis inhibition. (A) Proteolytic processing of the CPP32 protease as determined by immunoblotting of total lysates of J 16 and JA cells. Cells were preincubated with 0.5 μg/ml CHX for 4 h, CH-11 anti-CD95 mAb was added at 1 μg/ml and cells were further incubated for 1-6 h. Indicated by a dash is the precursor CPP32 molecule at 32 kDa and indicated by an arrow is its degradation product at about 20 kDa. (B) Percentage apoptosis after 20 h incubation of wild type J 16 and three JA clones with 1 μg/ml anti-CD95 mAb (anti-APO-1), CHX at 0.5 or 2 μg/ml, and anti-CD95 mAb in combination with eithr concentration of CHX. Cells were preincubated with CHX for 4 h prior to addition of anti-CD95 mAb or control stimulus.

receptors share a so-called 'death domain' in their cytoplasmic tail. In case of CD95, this domain allows interaction with a homologous region in the FADD adaptor protein upon receptor stimulation (Chinnayian et al., 1996a). With its amino-terminal 'death effector domain' FADD recruits the FLICE protease (caspase-8) to the receptor system, which allows its proteolytic activation ((Muzio et al., 1996, Boldin et al., 1996). CD95 can also activate CPP32 (caspase-3) and ICE-LAP-3 (caspase-7), which most likely operate downstream from FLICE (Chinnayian et al., 1996b).

To gain insight into the requirements for TcR- and CD95-mediated apoptosis, we have characterized a number of clones, derived from the human T- cell line Jurkat, which were selected for resistance to either TcR- or CD95-induced apoptosis. We conclude that the TcR/CD3 complex can induce apoptosis in a CD95-dependent manner, but also reveal that a CD95-independent mechanism exists. We find evidence for a protein of high turnover, that regulates sensitivity to CD95-mediated apoptosis upstream from caspase-3 activation. Given the connection between TcR- and CD95-mediated apoptosis, this protein may also play a role in life/death decisions upon TcR stimulation.

Materials and Methods

Antibodies and immunofluorescence. Purified anti-APO-1 mAb (IgG3, κ) (Trauth et al, 1989) was provided by dr. P. Krammer (DKFZ, Heidelberg, FRG). The CH-11 anti-CD95 mAb was purchased from Immunotech (Marseille, France). Mouse anti-human CD3 mAb OKT3 (IgG2a, κ) (Reinherz and Schlossman 1980) was a gift from Ciba Geigy, Basel, Switzerland. Anti-phosphotyrosine mAb 4G10 was from Upstate Biotechnology (Lake Placid, NY) and used in biotinylated form for immunoblotting. Streptavidin-conjugated horse radish peroxidase was used as second step reagent. Polyclonal antiserum to CPP32 was prepared in our institute by dr. G. Gil-Gomez. A rabbit was immunized with a glutathione-S-transferase fusion protein of human CPP32, amino acids 29-176. For immunofluorescence analysis, cells were incubated with OKT3 or anti-APO-1 mAb at 10 μg/ml, followed by FITC-conjugated goat anti-mouse $F(ab')_2$ fragments. Fluorescence intensity of 10.000 cells per sample was measured on a FACScan (Becton Dickinson, Mountain View, CA). All antibodies were used as purified Ig.

Cell culture. The human T- acute lymphoblastic leukemia cell line Jurkat and clones derived from it were cultured in Iscove's modified Dulbecco's medium with 10% FCS, 2 mM glutamine and antibiotics. Wild type clones were derived from Jurkat by limiting dilution and selected for TcR/CD3 expression. JP variant clones were derived by culturing the parental Jurkat line in the presence of 0.4 μg/ml phytohemagglutinin (PHA; HA 16, Murex Diagnostics Ltd., Dartford, U.K.) for about one month. Surviving cells were cloned and subcloned by limiting dilution, selected for expression of all TcR/CD3 components at both mRNA and protein level and for resistance to OKT3-induced cell death. JA variant clones were generated by culturing Jurkat cells in the presence of 1 μg/ml anti-APO-1 mAb for about five weeks and subsequent cloning and subcloning of surviving cells. All cells in the bulk culture resistant to anti-APO-1 mAb had maintained CD95 expression as determined by FACS analysis. Clones were selected on the basis of TcR/CD3 expression and high resistance to CD95-induced apoptosis. TcR/CD3 and CD95 expression were routinely checked by immunofluorescence.

Apoptosis assay. For induction of apoptosis, OKT3 mAb was coated onto flat-bottom microtiter plates at 10 μg/ml in PBS overnight at 4°C. Culture vessels were washed before use to remove free mAb. Anti-CD95 mAb and PHA were added in soluble form, at 1 μg/ml and 2 μg/ml respectively. Cells were seeded at 1×10^6/ml,

Table 2. Sensitivity of Jurkat-cells and variant clones to PHA-induced apoptosis

	medium	OKT3	anti-APO-1
Jurkat	4.1[a]	46.7	62.9
J 2	3.1	59.1	37.2
J 16	5.4	47.0	68.0
JP 15.7	5.3	6.3	55.0
JP 17.6	6.2	8.9	58.8
JA 1.2	1.3	22.3	5.6
JA 2.1	1.3	14.2	8.1
JA 16.6	6.8	33.3	13.8
JA 21.3	2.1	39.0	6.4
JA 22.3	2.4	31.1	6.5
JA 25.5	3.3	37.0	8.0
JA 39.1	3.8	42.9	7.0

[a] Percentage of apoptosis 24 h after addition of stimulus, as determined according to Nicoletti et al. (22). Data are representative of five independent experiments.

100 µl/well in flat- or round bottom 96 well microtiter plates in culture medium, stimulus was added and cells were incubated at 37°C, 5% CO_2 for 24 h. To detect apoptotic cells, cells were washed with PBS after incubation and lysed in hypotonic buffer consisting of 0.1% sodium citrate, 0.1% Triton X-100 and 50 µg/ml propidium iodide (Nicoletti et al., 1991). In this lysis buffer, nuclei remain intact and propodium iodide can diffuse in to stain the DNA. Fluorescence intensity of nuclei was determined on a FACScan (Becton Dickinson) and data were analysed using Lysys software. Segmented, apoptotic nuclei are recognizable in this assay as a population with diminished and rather heterogeneous fluorescence intensity as compared to intact diploid nuclei (Nicoletti et al., 1991). The percentage of nuclei present in this sub-diploid peak are considered to reflect the percentage of apoptotic cells.

Ca^{2+} mobilization assay. For measurement of the intracellular Ca^{2+} ion concentration, Jurkat clones were incubated at 10×10^6/ml with 3 µM Indo-1-AM (Molecular Probes, Eugene, OR) for 30 min. at 37°C in medium with 0.1% BSA, followed by dilution to 2×10^6/ml and a further incubation for 30 min. Cells were washed and resuspended at 2×10^6/ml in the same medium and stored on ice until analysis. After equilibration at 37°C, cells were stimulated with 1 µg/ml OKT3 mAb and mobilization of Ca^{2+} ions was determined with a FACStar flow cytometer (Becton Dickinson) using Lysys software for analysis as described (Ossendorp et al., 1992).

Detection of CD95L mRNA. Cells at 4×10^6/ml were stimulated in culture flasks coated with OKT3 mAb for 2, 4 or 6 h periods at 37°C, 5% CO_2, washed in PBS and snap frozen in liquid nitrogen. 40×10^6 cells were used per time point. Unstimulated control cells were incubated for 2 h at 37°C RNA was isolated by NP-40 lysis (Auffray and Rougeon 1980). Reverse transcription for synthesis of first strand cDNA was performed with SuperScript II (Gibco BRL, Technologies) and random hexamer

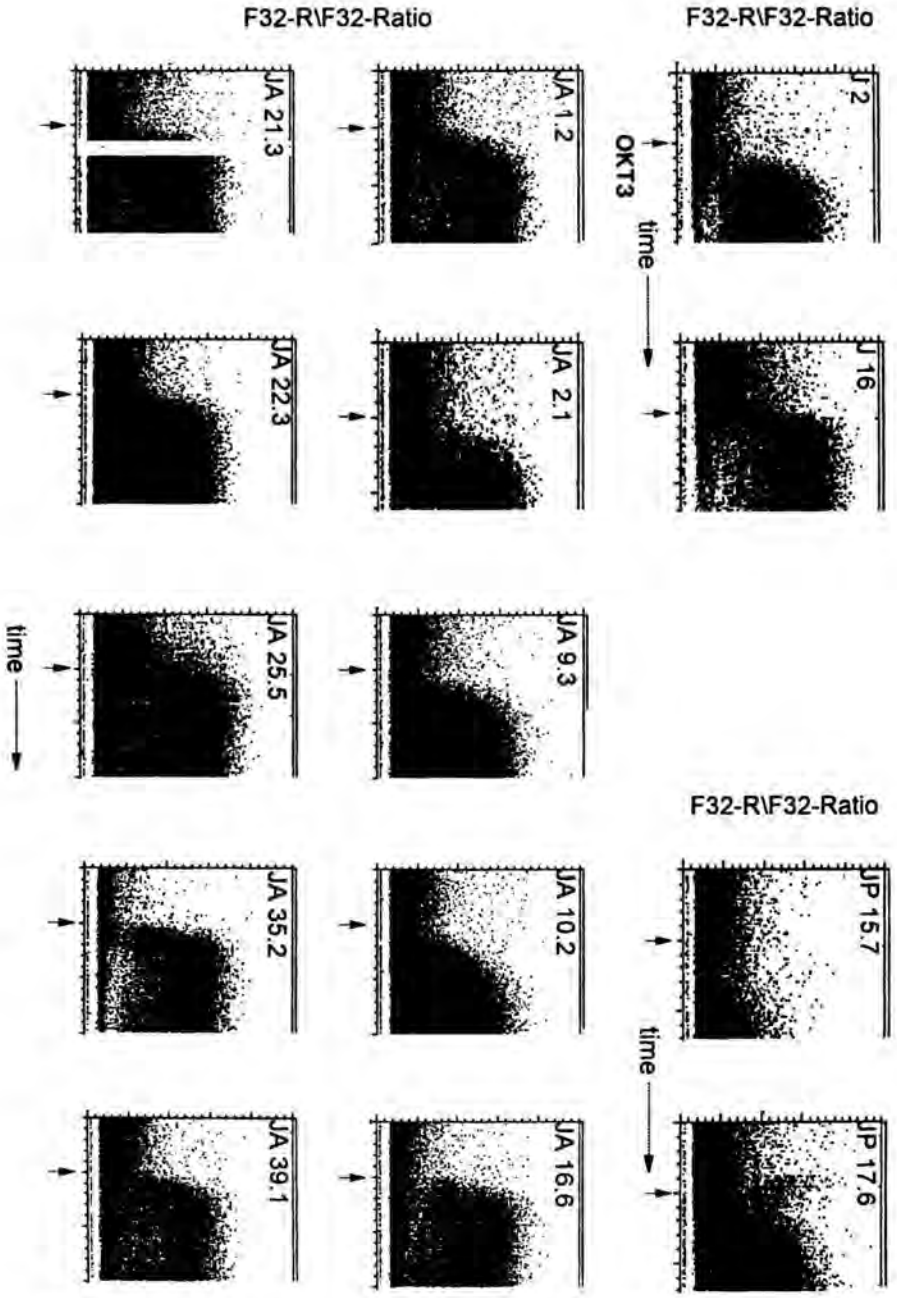

Fig. 3. Triggering of the TcR/CD3 complex mobilizes intracellular Ca^{2+} ions in all wild type and variant clones, except JP 15.7. Cells were loaded with Indo-1-AM and equilibrated in medium at 37°C for 3 min in a chamber attached to the FACStar. Measurement started in the absence of stimulus and was followed for 100 seconds; at this timepoint (arrow), OKT3 mAb was injected to a final concentration of 0.5 μg/ml and measurement was continued for another 50-100 seconds.

primers (Perkin Elmer). For PCR amplification of CD95L cDNA the forward primer 5'-TGGCAGAACTCCGAGAGTCTA and the reverse primer 5'-CACTGGTAA-GATTGAACACTGC were used. As a control, expression of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was determined using the forward primer 5'-GACCCCTTCATTGACCTC and the reverse primer 5'-CCAAAGTTGTCATG-GATG. PCR was carried out at 58°C for 25 cycles in case of CD95L and 20 cycles for GAPDH. Products were electrophoresed in agarose gels, transferred to Hybond membranes and detected with random primed ^{32}P-labeled probes. The CD95L PCR product was detected with an EcoRI-StyI fragment of the full-length cDNA clone of human CD95L. This cDNA was isolated in the laboratory of Dr. J. Tschopp (University of Lausanne, Epalinges, Switzerland). The GAPDH PCR product was detected with a rat GAPDH cDNA (Schuuring et al., 1992). Signals on the autoradiographs were quantitated with a Fuji BAS 2000 TR phosphorimager.

Immunoblotting. For analysis of CPP32 processing, cells were suspended at 10×10^6/ml in a 24 well culture plate, stimulated with medium or CH-11 mAb for various time periods at 37°C, 5% CO_2, and lysed with 50 µl 1% Nonidet P-40 in 0.01 M triethanolamine-HCl pH 7.8, 0.15 M NaCl, 5 mM EDTA, 1 mM PMSF, 0.02 mg/ml trypsin inhibitor and 0.02 mg/ml leupeptin. Lysates were centrifuged at 14.000 g for 15 min and the supernatants were mixed with concentrated reducing SDS sample buffer. Equivalents of 10^6 cells per lane were separated on a 10% SDS polyacrylamide minigel. Proteins were transferred to nitrocellulose membrane (Schleicher and Schüll, Dassel, FRG). Blots were blocked with 5% non fat dry milk in PBS, 0.1% Tween 20, incubated with purified anti-CPP32 Ig at 10 µg/ml in PBS, 0.1% Tween 20, 1% non fat dry milk, followed by a 1: 7500 dilution of horse radish peroxidase-conjugated swine anti-rabbit Ig (DAKO A/S, Glostrup, Denmark) and developed by enhanced chemiluminescence (Amersham, U.K.). For anti-phosphotyrosine immunoblotting, cells at 10^7/ml in medium were stimulated with medium or 10 µg/ml OKT3 mAb at 37°C, lysed at 4°C with 1% Nonidet P-40 in 50 mM Tris.HCl pH 7.6, 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, 1 mM NaF, and 1 µg/ml each of leupeptin, aprotonin, pepstatin and chymostatin. Lysates were centrifuged at 14.000 g for 15 min. Supernatants were mixed with SDS sample buffer and separated on a 10% SDS polyacrylamide gel. Proteins were transferred to nitrocellulose membrane. Blots were blocked with 5% BSA in PBS, 0.1% Tween 20, incubated in PBS with 0.1% Tween 20 with biotinylated 4G10 mAb, followed by streptavidin-conjugated horseradish peroxidase and developed by enhanced chemiluminescence.

Results

Generation of Apoptosis-resistant Jurkat Clones.

The Jurkat T cell line is susceptible to apoptosis-induction via both the TcR/CD3 complex and CD95 (Table 1). Since about 10-20% of cells within the Jurkat line lack TcR/CD3 expression, we have derived wild type clones by limiting dilution. These clones were selected for TcR/CD3 and CD95 cell surface expression by

immuno-fluorescence. The wild type clone J 16 was selected for high sensitivity to TcR- and CD95-induced apoptosis.

JP variant clones were derived by limiting dilution from Jurkat cells cultured for about 1 month in the presence of 0.4 μg/ml PHA, a lectin which binds to the TcR (Chilson et al., 1994) and induces both IL-2 production and death in Jurkat cells (Gillis and Watson 1980). Only five out of fifteen JP clones thus generated contained functional TcRβ mRNA and expressed the TcR/CD3 complex at levels comparable to the wild type clones. JP 15.7 and JP 17.6 were selected from these five PHA-resistant clones for concomitant resistance to apoptosis-induction by anti-CD3 monoclonal antibody. These two subclones express TcR/CD3 and CD95 at levels comparable to wild type clones.

JA variant clones were derived by limiting dilution from the Jurkat line, cultured for about five weeks in the presence of 1 μg/ml anti-APO-1 mAb. According to limiting dilution analysis, about 1 in 10^4 cells survive this treatment. Ten clones were selected for use in further experiments on the basis of TcR/CD3 expression and resistance to growth inhibition with anti-APO-1 mAb. To ensure clonality, JA clones were sub-cloned by limiting dilution. Like JP clones, they were maintained in medium without selecting stimulus after cloning from the apoptosis resistant bulk culture. Although TcR/CD3 plasma membrane expression varies between JA clones, all are positive and give rise to intracellular Ca^{2+} mobilization upon TcR/CD3 stimulation, while CD95 expression is within the wild type range (results not shown).

Sensitivity of Wild Type and Variant Jurkat Clones to TcR- and CD95-Induced Apoptosis

Apoptosis sensitivity is illustrated for wild type, JP and JA clones in Fig. 1 and listed in full in Table 1. Like the Jurkat line, wild type clones J 2 and J 16 are sensitive to apoptosis-induction with immobilized OKT3 mAb, which triggers the TcR/CD3 complex. Also, PHA induces apoptosis in the wild type clones, whereas the JP 15.7 and JP 17.6 clones, derived from the PHA-resistant Jurkat bulk culture, are fully resistant to both PHA- and OKT3-induced apoptosis. Despite their resistance to TcR-induced apoptosis, JP 15.7 and JP 17.6 are sensitive to CD95-induced apoptosis at wild type levels (Fig. 1, Table 1).

All ten JA clones repeatedly showed greatly diminished (JA 9.3 and JA 10.2) or no sensitivity to apoptosis-induction by anti-APO-1 mAb (Fig. 1, Table 1), indicating that the pathway leading to apoptosis from the CD95 molecule is inhibited in these clones. The CD95 signalling pathway is blocked upstream from, or at the level of CPP32 (caspase-3) activation, since this occurs in wild type, but not in resistant JA clones in response to CD95 stimulation (Fig. 2A). The cytoplasmic tail of the CD95 molecule in all clones is wild type, according to nucleotide sequence analysis (results not shown). Interestingly, apoptosis-sensitivity can be restored in JA clones by inhibition of protein synthesis with cycloheximide (Fig. 2B). This same treatment also allows CPP32 processing in response to CD95 stimulation (Fig. 2A). These findings indicate that in JA clones the CD95 signalling pathway and the apoptotic machinery are intact. Most likely, the CD95 signalling pathway is inhibited by a protein of high turnover that acts in between the receptor system and CPP32.

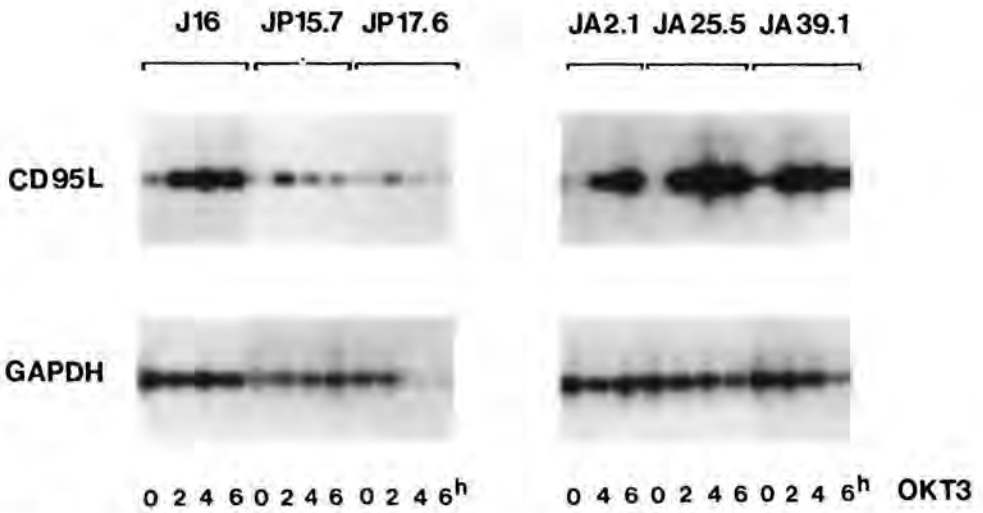

Fig. 4. Detection of CD95L mRNA after TcR stimulation in wild type J 16, TcR-resistant JP clones and JA clones. Cells were incubated in medium alone, or stimulated with OKT3 mAb for 2, 4 or 6 h at 37°C, 5% CO_2. RNA was isolated and reversed transcribed. cDNA was used as template for PCR with primers specific for CD95L and GAPDH. PCR products were run on gel, transferred to Hybond and hybridized with ^{32}P-labeled cDNA probes specific for CD95L and GAPDH.

The majority of JA clones displayed concomitant resistance to apoptosis-induction by TcR/CD3 stimulation with OKT3 mAb (Fig. 1, Table 1). In all JA clones, the TcR/CD3 complex can generate intracellular signals, since triggering with OKT3 mAb gave rise to Ca^{2+} ion mobilization (Fig. 3). The cross-resistance indicates that TcR-mediated signalling to apoptosis depends on a functional CD95 signalling pathway. Out of twenty TcR/CD3$^+$ wild type clones tested, none was resistant to TcR-induced apoptosis, indicating that the correlation between CD95- and TcR resistance in the JA clones is significant.

Connection Between TcR- and CD95-Induced Signalling Pathways Leading to Apoptosis

Recently, it was found that, at least in certain human and murine T cell lines, the TcR signalling pathway does not directly activate the apoptotic machinery, but operates via CD95. TcR stimulation led to elevation of CD95L mRNA levels and TcR-induced apoptosis could be inhibited by soluble, recombinant CD95. In one of these studies, the J 16 Jurkat wild type clone described here was used (Dhein et al., 1995). We have investigated whether the two JP clones, which are resistant to TcR-induced apoptosis, but have a functional CD95 pathway, are defective in inducing CD95L mRNA expression. Wild type clones J 2 and J 16, as well as TcR signalling defective clones JP 15.7 and JP 17.6 were stimulated with immobilized OKT3 mAb for 2, 4 or 6 h. Analysis by PCR showed clear induction of CD95L mRNA after TcR stimulation in the wild type clones, whereas in both JP clones CD95L mRNA levels did not increase significantly (Fig. 4).

The TcR/CD3 complex on the JP clones is functional, since stimulation with OKT3 mAb induces protein tyrosine kinase activity, revealed by anti-phosphotyrosine blotting of overall intracellular substrates. In wild type cells, as well as in both JP 15.7 and JP 17.6, the 42 kDa Erk 2 MAP kinase was phosphorylated upon TcR triggering (Fig. 5), as confirmed by anti-phosphotyrosine blotting of anti-Erk 1, 2 immunoprecipitates (results not shown). However, JP 15.7 is defective in TcR-induced tyrosine phosphorylation of a 150 kDa substrate (Fig. 5), and does not give rise to PLCγ-mediated mobilization of intracellular Ca^{2+} ions (Fig. 3). JP 17.6 gives a wild type response in both assays. We conclude that TcR-induced Erk 2 MAP kinase activation is not sufficient to induce elevation of CD95L mRNA levels and that PLCγ activation is most likely required.

In response to TcR stimulation, JA clones give rise to Ca^{2+} ion mobilization (Fig. 3) and CD95L mRNA upregulation (Fig. 4) indicating that the TcR signalling pathway is functional and can give rise to the production of CD95L. Resistance to TcR-induced apoptosis in the JA clones is therefore best explained by inhibition of the apoptotic CD95 signalling pathway.

Residual TcR-Mediated Apoptosis in CD95-Resistant Clones

The majority of JA clones were concomitantly resistant to CD95- and TcR-induced apoptosis. However, some CD95-resistant JA clones, particularly JA 25.5 and JA 39.1, repeatedly displayed significant sensitivity to apoptosis induction by OKT3 mAb (Fig. 1, Table 1). This finding suggests that the TcR can also induce apoptosis by a CD95-independent mechanism. When JA clones were stimulated with PHA, rather than with anti-TcR/CD3 mAb, the majority of clones was sensitive to apoptosis-induction (Table 2), even though clones like JA 1.2 and JA 16.6 do not respond even to high concentrations of strongly agonistic CH-11 anti-CD95 mAb, or recombinant CD95 ligand (data not shown). These data indicate that triggering of the TcR, possibly in concert with co-stimulatory molecules activated by PHA can activate an apoptotic pathway that does not involve CD95.

Discussion

In this paper, we describe a number of variant clones isolated from the Jurkat T cell line, which were selected for resistance to apoptosis-induction by the TcR or CD95. The JP clones, selected for TcR-resistance, undergo apoptosis in response to CD95 triggering and other stimuli known to induce apoptosis in wild type Jurkat cells, such as cycloheximide and certain anti-cancer drugs (not shown). This indicates that these cells have no defect in the apoptotic mechanism and suggests that they are specifically disturbed in signalling induced by the TcR. TcR stimulation in JP 15.7 and JP 17.6 fails upregulate CD95L mRNA, which is most likely the cause of their resistance to TcR-induced apoptosis. These clones are useful to delineate which TcR-induced signalling events are required to increment CD95L mRNA levels. In both clones, TcR stimulation induces tyrosine phosphorylation of multiple intracellular

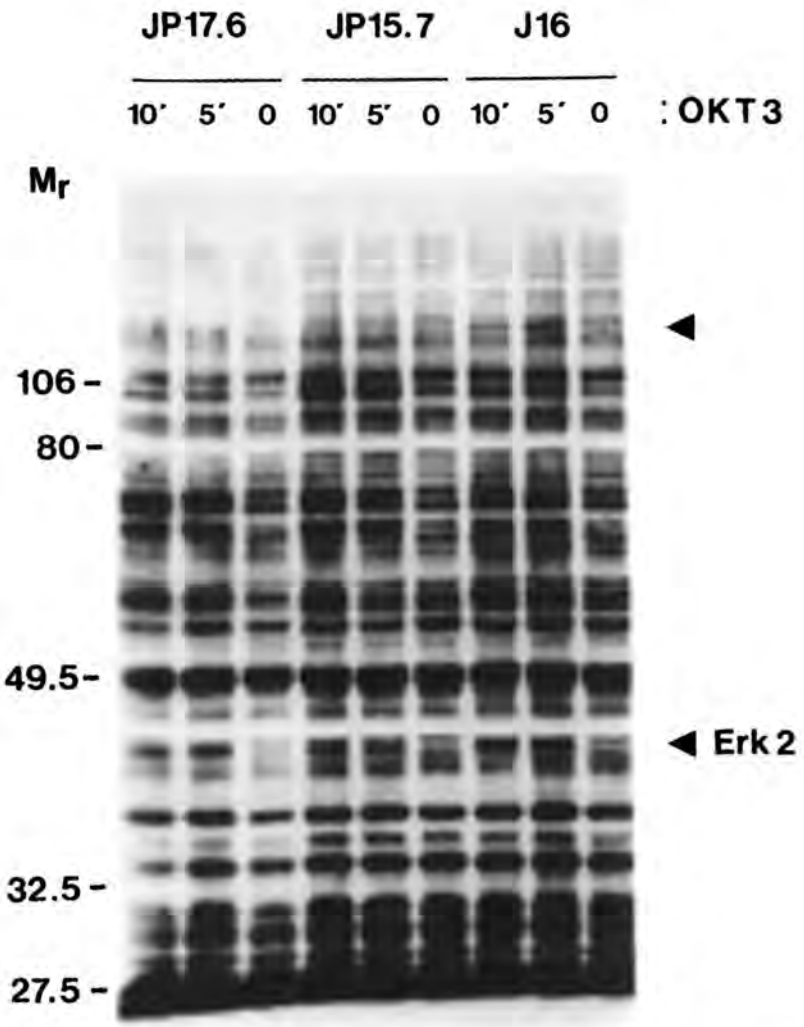

Fig. 5. TcR-induced tyrosine phosphorylation of intracellular substrates in wild type J 16 and TcR-resistant JP 15.7 and JP 17.6 clones. Cells were stimulated with OKT3 mAb and lysed with 1% NP-40. After centrifugation, lysates were mixed with reducing SDS sample buffer and separated by SDS-PAGE. Proteins were transferred to nitrocellulose and subjected to anti-phosphotyrosine immunoblotting with 4G10 mAb. The position of Erk 2 MAP kinase, which was confirmed by parallel immunoblotting with anti-Erk 1, 2 serum (not shown), is indicated. The arrow indicates the position of a substrate for TcR-induced tyrosine kinase activity of about 150 kDa, which is lacking in JP 15.7. M_r are indicated in kDa.

substrates, indicating that receptor-proximal Src-related tyrosine kinases and ZAP 70 are most likely not defective. However, JP 15.7 virtually lacked TcR-induced phosphorylation of a 150 kDa substrate, which is PLC-γ1 according to preliminary

immunoblot analysis (results not shown). Moreover, JP 15.7 is defective in TcR-induced Ca^{2+} mobilization and degradation of the IκB inhibitor of NFκB (not shown), supporting a defect in PLC-γ1 activation. Inhibition studies using cyclosporin A (CsA) have already indicated that PLCγ-induced signalling events are required for CD95L mRNA upregulation. CsA inhibits the PLCγ pathway downstream from Ca^{2+} ion mobilization and impedes activation of calcineurin and NFAT (4, 5). CsA was shown to block TcR-induced CD95L mRNA upregulation (Dhein et al., 1995, Anel et al., 1994). The second TcR-resistant clone, JP 17.6, is not defective in TcR-induced Ca^{2+} mobilization. However, preliminary evidence indicates that JP 17.6 is defective in TcR-induced degradation of IκBα, the inhibitor of NFκB. This would suggest a role for PKC-induced events, possibly NFκB activation, in CD95L mRNA upregulation. In both JP 15.7 and JP 17.6, Erk 2 MAP kinase was phosphorylated on tyrosine upon TcR stimulation and therewith presumably activated. Apparently, Erk 2 activation is not sufficient to induce CD95L mRNA elevation.

The JA clones were selected for resistance to CD95-mediated apoptosis, as induced by anti-APO-1 mAb. Clonogenic assays have indicated that about 1 in 10^4 cells survives prolongued culture with this antibody. Since the line was not mutagenized, we have selected Jurkat variant clones, which were present in the starting population. The JA clones do not display loss of function alterations, since CD95-mediated apoptosis could be restored by concomitant incubation with inhibitors of protein or mRNA synthesis. In the JA clones, the CD95 signalling pathway is inhibited in between the receptor and CPP32. The presumed inhibitory factor most likely does not act on CPP32, since this caspase can be activated by exogenous ceramide, which bypasses apoptosis-resistance in the JA clones (results not shown). Known components of the CD95 pathway upstream from CPP32 are FADD and FLICE (caspase-8). FADD variants lacking the death effector domain, which is required for coupling to FLICE, and FLICE variants lacking a functional protease domain can act as dominant negative signalling molecules which inhibit receptor mediated apoptosis (Boldin et al., 1996, Chinnayian et al., 1995, Hsu et al., 1996). It will be of interest to determine whether overexpression of endogenous versions of such molecules is responsible for inhibition of the CD95 signalling pathway in the JA clones.

The majority of CD95-resistant JA clones are resistant to TcR-induced apoptosis, in line with the mechanism proposed earlier by various groups (Ju et al., 1995, Brunner at al 1995, Dhein et al., 1995). CD95 can play a role in TcR-mediated, so-called 'activation-induced cell death' in peripheral T lymphocytes, which serves to attenuate the immune response. This follows from *in vitro* and *in vivo* analysis of CD95-deficient murine T cells (Russel et al., 1991, Singer and Abbas 1994). However, it has been evident for quite some time that the TcR can also induce apoptosis in a CD95-independent fashion, at least in thymocytes, since negative selection of autoreactive thymocytes is not impeded in CD95-deficient *lpr/lpr* mice (Singer and Abbas 1994, Sidman et al., 1992). In a TcR transgenic model, it was recently found that activation-induced cell death in peripheral T cells induced by the specific antigen influenza hemagglutinin, was independent of CD95 (Sytwu et al., 1996). Whereas a role for TNF was found in another system (Zheng et al., 1995), blocking of TNF receptors did not interfere with TcR-mediated apoptosis of peripheral T cells in the

influenza hemagglutinin system (Sytwu et al., 1996). Also in Jurkat cells TNF does not play a role in TcR-induced death, since Jurkat cells are TNF-resistant unless they are treated with inhibitors of *de novo* protein synthesis (results not shown). Possibly, other apoptosis-inducing members of the TNF receptor family can play a role in activation induced cell death in peripheral T cells and deletion of autoreactive thymocytes, such as TRAMP/Wsl-1/Apo-3/DR3 (Chinnayian 1996c, Kitson et al., 1996, Marsters et al., 1996, Bodmer ey al 1997) or the Trail receptor (Pan et al., 1997). Alternatively, no second receptor system is involved and the TcR directly activates the apoptotic machinery.

The CD95-resistant clones, with the exception of JA 9.3 and JA 10.2, are cross-resistant to apoptosis-induction by the anti-cancer drug etoposide (Boesen et al., submitted). Etoposide is a topoisomerase II inhibitor, which induces double strand DNA breaks. Friesen et al., have recently provided evidence that the DNA damaging drug doxorubicin can induce CD95L upregulation and CD95-mediated apoptosis in a human leukemic T cell line (Friesen et al., 1996). This mechanism would explain the cross-resistance as observed in the JA clones. However, in wild type Jurkat cells, nor in primary thymocytes, do we find evidence for role of CD95 in etoposide-induced apoptosis (Boesen et al., submitted). Most likely, as for TcR-induced apoptosis, there are CD95-dependent and -independent mechanisms to induce apoptosis in response to DNA damaging drugs.

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Bob van de Water, Hong Liu and James L. Stevens

ER Stress Response Proteins and Modulation of Cell Death

Abstract

Severe environmental stress induces cell death. This is often associated with the upregulation of stress response proteins including endoplasmic reticulum (ER) stress proteins like the glucose regulated proteins (GRPs) GRP78 and GRP94. Upregulation of these proteins provides a tolerance phenotype against a variety of subsequent lethal conditions. The exact mechanism of the upregulation of these ER stress proteins, their role in protection against cell death and the mechanism of their protection will be discussed.

Introduction

Cellular exposure to environmental stresses such as ischemia/reperfusion, drugs, heat shock, environmental pollutants or toxic chemicals generally causes the upregulation of stress proteins in various intracellular compartments including the cytosol, the nucleus and the endoplasmic reticulum (ER). For example, a variety of cellular stresses upregulates the expression of transcription factors such as c-Myc, c-Jun, and c-Fos, and also members of the heat shock family, including heat shock proteins HSP70, HSP 90 and HSP110 and the glucose regulated proteins GRP78 and GRP94. Different stress conditions can lead to an increased expression of different sets of stress proteins. It seems likely that the pattern of stress proteins expression and the severity of the insult determines the outcome after a toxic insult: i.e. cell recovery or death. Therefore, it is important to understand the induction and the role of different stress proteins following cellular stress.

A variety environmental stress conditions lead to induction of ER stress proteins, including GRP78 and GRP94. Here we will describe the function and role of ER stress proteins in cellular protection in relation to both apoptotic and necrotic cell death. Particular attention will be given to the discrete signaling pathways through which the ER can regulate different modes of cell death caused by different toxicants.

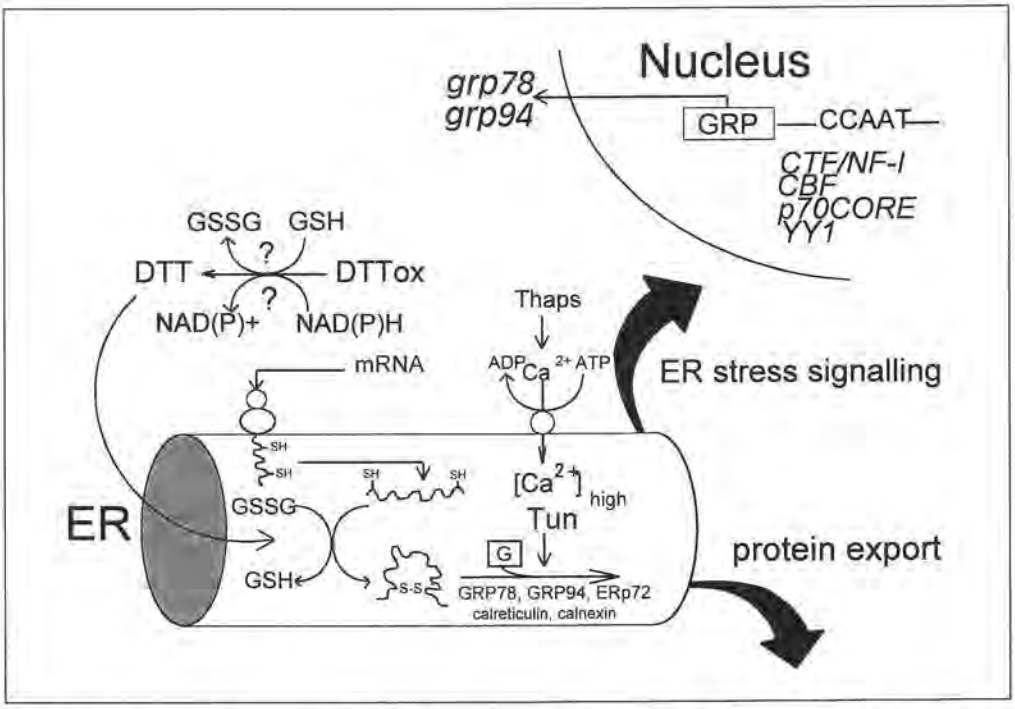

Fig. 1. Mechanisms of the induction of ER stress response proteins by different stress conditions. Perturbations of ER protein processing by DTTox, DTT, thapsigargin or tunicamycin, cause an accumulation of malfolded proteins in the ER. This results in the activation of different transcription factors that can activate specific elements in the promoter region of the genes ER stress protein. This leads to increased transcription and translation of ER stress proteins, including GRP78, GRP94, ERp72, calreticulin and PDI For further discussions see text.

ER Stress Response Proteins

ER Stress Proteins are Molecular Chaperones and Calcium Binding Proteins.

The ER is important in the processing of newly synthesised proteins made in the rough ER; proteins are folded and glycosylated prior to transport to the Golgi. The ER is also a major cellular free calcium store. $[Ca^{2+}]$ in the ER can be as high >1 mM; this is important for proper hormone responsive Ca^{2+} release as well as for normal ER protein processing.

Several proteins are involved in the processing of newly synthesised proteins in the ER. These include resident ER proteins such as GRP78/BiP and GRP94/endoplasmin, calreticulin, calnexin, protein disulphide isomerase (PDI), and Erp72 (Gething, 1994; Helenius, 1997). These ER proteins are retained in the ER due to their KDEL/HDEL retention motif (Fig. 1). They function as molecular chaperones and bind to partially folded newly synthesised, transmembrane and secretory proteins including integrins, viral proteins MHC class I and II (Gething, 1994; Helenius, 1997; Melnick,

1994). The different ER chaperones bind the unfolded proteins at discrete steps of the protein processing. For example, GRP78/BiP is involved in translocation of newly synthesised polypeptides across the ER membrane and stabilisation of partially folded or partially assembled proteins; PDI and ERp72 are involved in formation and rearrangement of disulphide bonds; calnexin and calreticulin bind predominantly (mono)glycosylated proteins, thereby preventing aggregation and promoting protein folding (Gething, 1994; Baksh, 1995; Herbert, 1995; Helenius, 1997).

Besides the chaperoning function most, if not all, of the ER chaperones are also Ca^{2+}-binding proteins (Michalak, 1992; Nigam, 1994). It is not entirely clear whether the calcium-binding capacity is necessary for the chaperoning function of ER stress proteins. However, perturbation of ER calcium by calcium ionophores or inhibition of the ER Ca^{2+}-ATPase with thapsigargin, interferes with the ER protein processing and causes ER stress protein induction (Gething, 1994). This indicates that there is a critical role for ER calcium homeostasis in ER protein processing.

Mechanism of ER Stress Protein Response

A variety of conditions induce an ER stress reponse, resulting in the upregulation of ER proteins including the GRPs, calreticulin, ERp72 and PDI (Dorner, 1991; Gething, 1994; Wenfeng, 1993). Expression of GRP78/BiP — the most abundant ER stress protein — is increased after glucose deprivation, perturbation of the ER calcium pool by calcium ionophores or thapsigargin, disturbance of the ER thiol-redox status with dithiothreitol (DTT) or β-mercaptoethanol, inhibition of protein glycosylation with tunicamycin and inhibition of vesicular transport with brefeldin A (Gething, 1994; Halleck, 1997; Kyu Kim, 1987; Wenfeng, 1993). All ER stress conditions have in common that they cause an accumulation of malfolded or unassembled proteins in the ER. Binding of GRP78 to these proteins, thereby lowering the amount of unbound GRP78, is believed to be the trigger for the ER stress response (Gething, 1994; Kozutsumi, 1988).

Although most of the studies on ER stress protein induction have been done *in vitro*, recent evidence indicates that also stressful conditions *in vivo* lead to an increase of GRP78. For example, ischemia/reperfusion injury of the brain in rats results in increased upregulation of *hsp70* and also *grp78* mRNA (Lowenstein, 1994). Moreover, we found that exposure of rats to nephrotoxic cysteine conjugates causes an upregulation of GRP78 protein in the rat renal cortex (Asmellash and Stevens, unpublished observation).

The signal transduction pathway involved in transmitting ER stress to the nucleus and activation of *grp78* transcription most likely involves several steps. First of all, the decrease in unbound GRP78, and possibly other chaperones, must be noticed by a sensor. Second, a mechanism should be present to transduce the signal from the sensor across the ER membrane to certain signal molecules in the cytoplasm; and, third, these signalling molecules should be able to either function as transcription factors themselves or activate other transcription factors that, in turn, will modulate transcription of ER stress proteins genes. In yeast an ER transmembrane protein kinase named IRE1, is required for the induction of the expression of ER stress proteins

(Cox, 1993). As yet, the mammalian homologue of IRE1 has not been identified. However, ER stress-induced upregulation of GRP78 does require both Tyr- and Ser/Thr-kinase activity and phorbol ester and cAMP do stimulate grp78 gene transcription (Cao, 1995; Hou, 1993; Prostko, 1991a).

Regulation of the promoter of GRP78 involves several transcription factors. CTF/NF-I is the best characterised and binds CCAAT or CCAAT-like motifs in the promoter of which the most proximal is important for transcriptional regulation of more upstream regulatory elements (Wooden, 1991) and is activated after exposure to tunicamycin, thapsigargin, A23187 and β-mercaptoethanol. Other transcription factors that bind the grp78 promoter have recently been identified and include CBF, p70CORE and YY1 (Li, 1994, 1997a; Roy, 1995). These interact with different stress regulatory elements of the GRP78/BiP core promoter region and binding results in transcriptional activation.

Suppression of stress-induced transcriptional activation of the grp78 promoter can also occur. The cold shock domain proteins dbpA and dbpB/YB-1 can prevent binding of the transcription factor YY1 to the stress inducible change region (SCIR) of the grp78 promoter, and thereby block the induction of the grp78 core element mediated by treatment of cells with A23187, thapsigargin or tunicamycin (Li, 1997b).

The promoter region of other ER stress proteins including GRP94, PDI, calreticulin, shares homology with the GRP78/BiP promoter and contain the Sp1 and CCAAT consensus sequences (Chang, 1989; McCauliffe, 1992). Since the promoter regions of different ER stress proteins share homology one might expect that other ER stress proteins are upregulated at the same rate as GRP78. This is however not the case. After ER stress GRP78 is the predominant ER stress protein upregulated. Therefore, it is likely that other factors also determine the pattern of upregulation of ER stress proteins.

ER Stress Response and Control of Cell Death

ER Stress Protein are Upregulated by Conditions that cause Cell Death

A variety of conditions that cause the induction of cell death also induce expression of ER stress proteins. For example, cytotoxicants, including, ionomycin, A23187 and thapsigargin, as well as deprivation of growth factors such as neuronal growth factor (NGF), or hypoxia/reoxygenation cause cell death in association with upregulation of GRP78 (Liu, 1997; McCormick, 1997; Aoki, 1997; Hori, 1996). These data suggest a relationship between cell death/survival and ER stress response. In some models induction of apoptosis by ER stress is associated with a lack of upregulation of ER stress proteins: thapsigargin-induced apoptosis in mouse lymphoma cells is associated with a lack of upregulation of GRP78 and GRP94; in contrast, cells that are resistant to thapsigargin have normal upregulation of GRPs (McCormick, 1997). Also competitive inhibition of a set of ER protein genes makes cells more sensitive to calcium ionophore treatment (Li, 1991). In the pig kidney renal epithelial cell line LLC-PK1, we also observed that thapsigargin causes apoptosis when the ER stress response is perturbed (see below). These data suggest that upregulation of GRPs

and/or other ER stress proteins is associated with cell survival, and that an insufficient upregulation will lead to cell death. Thus the upregulation of ER stress response proteins is critical for the eventual fate of the cell after a harmfull insult: survival or death.

Upregulation of ER Stress Protein provides Cellular Tolerance.

Various studies indicate that ER stress protein upregulation provides tolerance against a subsequent lethal insult. For example, upregulation of ER stress proteins by mild treatment with A23187, thapsigargin or tunicamycin provides a tolerant phenotype against subsequent lethal insults such as doxorubicin (Shen, 1987), etoposide (Chatterlee, 1994), oxidative stress (Gomer, 1991) and T-cell mediated cell killing (Sugawara, 1993). Although these harmful conditions may cause apoptosis, it has not been investigated whether preER stress protects by blocking necrosis, apoptosis or both. Moreover, the exact molecular mechanism by which ER stress proteins protect against otherwise lethal cell injury is largely unclear.

We have determined the mechanism underlying protection by ER stress against necrosis and apoptosis using different models. Ultimately, our goal is to understand how stress protein upregulation affects cell death after chemical damage *in vivo* and in the kidney in particular. In the first model, LLC-PK1 cells are treated with the alkylating agent iodoacetamide (IDAM). IDAM rapidly causes an upregulation of GRP78 in these cells (Liu, 1997). Also, IDAM depletes glutathione (GSH) in the cell and thereby increases the level of oxidative stress, followed by lipid peroxidation; the latter is involved in cell death by necrosis (Chen, 1991). Although prevention of lipid peroxidation with the lipophilic antioxidant N,N'-diphenyl-*p*-phenylenediamine (DPPD) inhibits necrosis it allows the onset of apoptosis (Chen, 1991; Liu, 1996; Van de Water, 1996). Because IDAM causes a rapid upregulation of GRP78 (Liu, 1997), indicating ER stress, we wanted to investigate the role of ER stress proteins in modulation of IDAM-induced cell death. Therefore, we pretreated cells with a variety of agents that induce an ER stress response. Upregulation of ER stress proteins by oxidized DTT (DTTox), thapsigargin or tunicamycin blocked both IDAM-induced necrosis and apoptosis in LLC-PK1 cells (Liu, 1997; Van de Water and Stevens, submitted).

In a second model we investigated the mechanism of thapsigargin-induced cell death in LLC-PK1 cells. In these cells thapsigargin itself does not cause apoptosis; however, thapsigargin treatment causes the upregulation of several ER stress proteins these conditions both GRP78, GRP94 but also calreticulin. When protein synthesis is inhibited with cycloheximide, thereby preventing upregulation of ER stress proteins, apoptosis is initiated (Van de Water and Stevens, submitted for publication). Cycloheximide itself did not cause cell death. Upregulation of ER stress proteins by tunicamycin, DTTox, A23187 or thapsigargin itself prior to thapsigargin/cycloheximide treatment, blocked apoptosis. These observations indicate that thapsigargin-induced ER stress activates different diverging signals: those that leading to the induction of ER stress proteins and those that are linked to the activation of the apoptotic machinery. Increased expression of ER stress proteins can modulate the signals that activate the apoptosis pathway.

Role of GRP78 in ER Stress-mediated Cytoprotection.

What are the ER stress proteins that modulate the apoptosis? GRP78 is the most abundant ER stress protein. The role of GRP78 in ER stress-mediated tolerant phenotype has been addressed in several studies. For example, overexpression of a targeted ribozyme directed to *grp94*, suppresses the expression levels of both GRP78 and GRP94 (Little, 1995). As a consequence, these cells are more sensitive against A23187, thapsigargin and tunicamycin-induced cell death. Similar observations were made in CHO cells that expressed a *grp78* antisense transcript (Li, 1992); and antisense *grp78*- mediated suppression of ER stress-induced upregulation of GRP78 eliminates the resistance to cell mediated cytotoxicity in fibrosarcoma B/C10ME cells (Sugawara, 1993). Importantly, in the latter case, these cells show less pronounced tumour progression in mice (Jamora, 1996).

We have analysed the role of GRP78 in IDAM-induced necrosis and apoptosis. For this purpose we have created LLC-PK1 cells that stably overexpress a 0.5 kbp *grp78* antisense (AS) transcript. In these cells the ER stress mediated upregulation of GRP78 is attenuated; this results in an attenuation of ER stress-mediated protection against IDAM-induced necrosis (Liu, 1997). However, in sharp contrast, AS*grp78*-LLC-PK1 cells are resistant against IDAM/DPPD-induced apoptosis; NEO vector control LLC-PK1 cells were killed at the same rate as normal LLC-PK1 cells. Interestingly, we found that these cells have increased levels of GRP94. In the AS*grp78* LLC-PK1 cells DTTox- induced upregulation of GRP78 is blocked. However, DTTox pretreatment is able to provide significant cytoprotection against apoptosis induced by high doses of IDAM (Table 1). This suggests that upregulation of ER stress proteins different from GRP78, also have a role in the cytoprotection against cell death. Moreover, our observation that DTTox pretreatment does not mediate cytoprotection in IDAM-induced necrosis but provides cytoprotection in IDAM-induced apoptosis indicates the involvement of distinct molecular pathways in ER stress-mediated tolerance against necrosis and apoptosis.

Altogether, these data indicate an important role for GRP78 in the ER stress mediated tolerance phenotype. However, other ER stress proteins are also important and may play a role in separate ER stress signaling pathways.

Mechanism of ER Stress Mediated Protection

GRP78-mediated Cytoprotection requires a Functional ATPase Domain.

The ATPase activity of GRP78 is important for cytoprotection. Like other hsp70 family members, GRP78 has ATPase activity (Gething, 1994). This activity is required for the release of associated proteins, as determined in vitro with recombinant, ATPase deficient, GRP78 (Wei, 1995). In line with this, expression of ATPase mutant GRP78 in COS cells causes disruption of the ER, which is dependent on the protein binding capacity of GRP78 (Hendershot, 1995). Overexpression of GRP78 itself is sufficient to protect against calcium ionophore induced cell death and is associated with an inhibition of ER stress-induced upregulation of GRP78 (Morris, 1997).

Table 1. Effect of antisense grp78 on DTTox-mediated cytoprotection against IDAM-induced cell death.

Cell line	Treatment	% Cell Death			
		IDAM			
		0 μM	100 μM	150 μM	200 μM
pkNEO (clone 10)	**IDAM**	6.3 ± 3.3^{A}	19.1 ± 0.6^{B}	$33.7 \pm 3.1^{C,D}$	39.7 ± 4.8^{D}
pkNEO (clone 10)	**preDTTox/IDAM**	7.3 ± 1.4^{A}	10.6 ± 3.6^{A}	17.5 ± 0.5^{B}	26.4 ± 1.3^{C}
pkASgrp78 (clone 10)	**IDAM**	3.3 ± 0.3^{A}	10.0 ± 0.4^{B}	12.3 ± 1.2^{B}	39.7 ± 4.4^{D}
pkASgrp78 (clone 10)	**preDTTox/IDAM**	4.2 ± 0.9^{A}	$7.9 \pm 1.5^{A,B}$	12.0 ± 1.5^{B}	25.2 ± 3.7^{C}

LLC-PK1 cells overexpressing an antisense *grp78* construct (pkASgrp78) or NEO-resistant control (pkNEO) were treated with 200 μM IDAM plus 20 μM DPPD for 45 min in EBSS. Thereafter, cells were allowed to recover in complete medium containing 20 μM DPPD. After 24 hr the % cell death was determined as described in Methods. Data are mean ± S.D. (n=3). Data are representative for two independent experiments. Significant differences were determined by ANOVA as described in Methods. Means with a different letter designation are significantly different ($p<0.05$).

In contrast, overexpression of the ATPase mutant GRP78, does not provide cytoprotection and does not block ER stress-mediated induction of *grp78* mRNA, indicating that the ATPase activity of GRP78 is necessary for the induction of a tolerant phenotype (Morris, 1997).

ER Stress Proteins as Calcium-binding Proteins

The ER stress proteins GRP78, GRP94, calnexin and calreticulin possess calcium binding capacity (Michalak, 1992; Nigam, 1994; Villa, 1991; Macer, 1988). Yet, little is known about the contribution of the calcium binding function in cytoprotection. Because cellular calcium perturbation is a critical factor in mechanisms of cell death in a variety of models (Liu, 1997; Van de Water, 1994), it is possible that ER stress-mediated cytoprotection is a result of a modulation of cellular calcium homeostasis.

Calreticulin is the best studied ER calcium binding protein. It has one high-affinity/low capacity (K_d=1.6 μM and B_{max} = 1 mol/mol) and several low capacity/high capacity (K_d=0.3-2.0 mM and B_{max}=20-50 mol/mol protein) Ca^{2+}-binding sites (Michalak, 1992). Overexpression of calreticulin in cells increases the calcium buffering capacity of the ER and blocks several hormone-mediated cellular calcium responses (Bastianutto, 1995; Camacho, 1995). ER stress increases the expression of calreticulin (Llewellyn, 1996; Plakidou-Dymock, 1994; Van de Water and Stevens, submitted for publication). We have used overexpression of calreticulin to study the role of ER calcium perturbations in the induction of both IDAM-induced necrosis and apoptosis. Overexpression of calreticulin blocks the IDAM-induced necrosis but has no effect on IDAM/DPPD-induced apoptosis (Liu et al, in press; Van de Water and Stevens; submitted). In contrast, calreticulin blocks apoptosis caused by thapsigargin treatment in the presence of cycloheximide. In the latter model buffering of intracellular calcium with the EGTA acetoxymethylester or removing extracellular calcium has no effect on thapsigargin/CHX-induced apoptosis of LLC-PK1 cells

(Van de Water and Stevens, submitted). These observations strongly suggest that emptying of ER calcium pools, and an increase of cytosolic Ca^{2+}, is primarily responsible for the initiation of thapsigargin- induced apoptosis. Yet, increases in cytoplasmic free Ca^{2+} related to ER calcium perturbations, may be linked to cytotoxicant-induced necrotic cell death (Liu, 1997; Van de Water, 1994).

Protection of Bcl-2 against apoptosis has been linked to modulation of the ER calcium pool (Distelhorst, 1996a, 1996b). Forced overexpression of Bcl-2 blocks thapsigargin-induced apoptosis in lymphoma cells (Distelhorst, 1996a; Lam, 1994), and Bcl-2 delays the emptying of the ER calcium pool after both thapsigargin or hydrogen peroxide treatment (Baffy, 1993; Lam, 1994). However, more recent studies indicate that Bcl-2 protection is independent of modulation of intracellular calcium stores (Reynolds, 1996). In our hands, overexpression of Bcl-2 has no effect on thapsigargin-induced cell death in LLC-PK1 cells. Yet, the ER calcium binding protein calreticulin blocks thapsigargin-induced apoptosis in these cells, indicating a direct role for ER calcium perturbations in thapsigargin-induced cell death (Van de Water and Stevens, submitted). These data indicate that Bcl-2 function is not simply related to calcium-perturbations; it may well be that Bcl-2 has different functions in different cell systems.

In summary, these data indicate that fundamental differences exist in the physiological role of ER calcium perturbations in necrosis and apoptosis. In addition, perturbations of ER calcium plays different roles in different models of apoptosis in different cell types.

Translational tolerance

Protein synthesis is controlled by a variety of translation initiation factors. Calcium-induced stress by calcium ionophore ionomycin or A23187 causes inhibition of protein synthesis, which is associated with phosphorylation of the eukaryotic translation initiation factor eIF2α at serine-51 (De Haro, 1996). Also other ER stresses including DTT, thapsigargin and EGTA cause a phosphorylation of eIF2α that is associated with inhibition of eIF2B (Brostrom et al., 1995; Prostko, 1992). eIF2B is a guanine nucleotide exchange factor which activity is closely linked to recycling of eIF2 (De Haro, 1996). The latter mediates the binding of Met-tRNA to the ribosome in a GTP-dependent manner. Phosphorylated eIF2α binds eIF2B thereby blocking its activity. After ionophore treatment the predominant stress protein upregulated is GRP78. Prior upregulation of ER stress proteins protects against subsequent ER stress-induced phosphorylation of eIF2α, and, thereby, provides translational tolerance (Brostrom, 1995). Also forced overexpression of GRP78 blocks the calcium ionophore-induced translational inhibition in association with a cytoprotective phenotype (Morris, 1997). Thus, these data suggest a direct link between ER stress mediated cytoprotection and translational control.

We also found that ER stress mediated cytoprotection against IDAM/DPPD-induced apoptosis is associated with translational tolerance phenotype. IDAM/DPPD treatment causes a drastic decrease in stress protein synthesis and protein synthesis in general. Pretreatment of cells with DTTox, thereby upregulating ER stress proteins,

did inhibit the IDAM/DPPD-induced block of protein synthesis (Van de Water and Stevens, submitted).

Recent data also suggest a link between translation regulation and the modulation of cell death. eIF4E is a cap binding protein that is involved in the transfer of mRNA to the 40S ribosomal subunit, a rate limiting step in translation initiation. Overexpression of eIF4E blocks apoptosis in growth factor restricted c-Myc overexpressing fibroblasts (Polunovsky, 1996). This protection is not linked to an increased expression of anti-apoptotic proteins such as Bcl-2 or Bcl-X_L. Intriguingly, eIF4E overexpression is associated with a transformed phenotype including the ability to form colonies in soft agar and tumorigenicity in nude mice (Polunovsky, 1996). Also, DAP-5, a recently cloned novel homologue of eukaryotic translation initiation factor 4G (p220) that binds eIF4E and promotes cap-binding independent translation, protects against cell death induced by interferon-γ when overexpressed in cells (Levy-Strumpf, 1997). These data indicate that modulation of translation might be a central event in the induction of apoptosis by diverse stimuli.

Activation of other Signalling Pathways after ER Stress

Activation of RNA Dependent Kinases

Transcriptional activation of ER stress protein genes is one signalling pathway that is activated after ER stress. However, other pathways are also activated. After ER stress, eIF2α is phosphorylated (see above). Phosphorylation of eIF2α occurs via a specific kinase. Different eIF2α kinases have been cloned, including the rabbit heme regulated protein kinase HRI, the human double stranded RNA dependent protein kinase, PKR, and the protein kinase GCN2 cloned from yeast (De Haro, 1996). Overexpression of dominant negative PKR prevents ER stress-induced phosphorylation of eIF2α and translational inhibition; this indicates a direct link between ER stress and PKR activation (Brostrom, 1996). The fact that eIF2α is phosphorylated after ER stress in association with apoptosis (Alcazar et al., 1995), suggest a role for eIF2 alpha kinases in modulation of cell death after ER stress.

Recent data indicate that activation of PKR by different conditions is linked to apoptosis. Thus, viral infection, interferon gamma exposure and dsRNA activate PKR and induce apoptosis (Takizawa, 1996). Moreover, overexpression of PKR itself causes a Bcl-2 dependent form of apoptosis that is mediated by ICE-like proteases (Lee, 1994, 1997). Bcl-2 does not block the PKR mediated inhibition of translation. Recent data indicate a direct role for PKR in mediating some forms of apoptosis. For example, PKR deficient fibroblasts from PKR null mice are resistant to apoptosis induced by dsRNA, TNF and lipopolysaccharide (Der, 1997). Similar observations were made in other cell types using overexpression of mutant PKR or using PKR antisense transcript (Takizawa, 1996; Yeung, 1996).

As yet, the relationship between ER stress and PKR activation in relation to cell death is unclear. Moreover, it will be interesting to see if ER stress proteins can modulate ER stress induced activation of PKR and thereby block the apoptosis.

NFκB Activation

NFκB is an important transcription factor involved in a variety of stress responses. It is present in the cytosol where it is inactive due to complex formation with IκB. Phosphorylation of IκB as a result of stimuli such as UV-radiation, inflammatory cytokines, and oxidative stress causes dissociation of the NFκB/IκB complex. NFκB is then translocated to the nucleus where it can transcriptionally activate specific target genes (Pahl, 1997).

ER stress can also induce activation of NFκB (Pahl, 1995, 1997). Thus, various stimuli such as tunicamycin, deoxyglucose, thapsigargin and brefeldin A cause NFκB activation. However, ER stress does not universally lead to NFκB activation. Thus, although DTT and mercaptoethanol upregulate GRP78, they do not activate NFκB, but, in contrast, inhibit its activation caused by other stimuli. Given that a variety of antioxidants block NFκB activation, it may be that thiol-based reductants that perturb the ER, fail to activate NFκB since they inhibit ROS production. On the other hand TNF is a good activator of NFκB, however, TNF does not upregulate GRP78 (Pahl, 1997a, 1997b).

These data indicate another potentially important route for ER stress related signalling in mechanisms that lead to a tolerant phenotype. it will be interesting to determine the role of ER stress proteins in the modulation of NFκB, and whether ER stress mediated NKκB activation is involved in the induction of a ER stress mediated tolerant phenotype against a variety of harmful conditions that lead to apoptosis or necrosis.

Summary and future directions

The above data provide a convincing link between ER stress response proteins and modulation of both necrosis and apoptosis. Most of the information on ER-mediated cytoprotection has been investigated in simple cell lines such as CHO cells or fibroblasts. Future effort should focus on the role of ER stress in cytoprotection against a different stresses in other more physiological relevant cells and with clinically relevant agents. For example, it will be important to investigate the mechanism of ER mediated cytoprotection against etoposide- and doxorubicin-induced cell death in more detail. In general, solid tumours are hypoxic, which causes upregulation of ER stress proteins. This, on its turn, can provide protection against cell death induced by etoposide, doxorubicin or cell-mediated toxicity.

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Induction of Apoptosis by Anticancer Drugs

Abstract

The identification of targets for novel anticancer drug therapy demands that they are a feature of the molecular pathology of the disease. Tumors have survived and tolerated genomic damage. Gene expression which promotes the tolerance of genomic damage defines the survival threshold of the cell: identifying gene products controlling survival and death may permit selective drug design for the initiation of apoptosis.

Cancer Therapies: the Search for Rational Approaches

Current but unsatisfactory therapies for disseminated cancer utilise the blunt instruments of cytotoxic drugs and radiation. The current approach to treatment has little rational basis with respect to the molecular pathology of the disease. Cytotoxicity to tumor cells is imposed at the expense of toxicity to some normal tissues. The efficacy of antiproliferative cytotoxins is limited to tumor types which either have a high growth fraction in comparison to normal tissues or which, as described in this chapter, have an inherently low threshold of survival after the imposition of cytotoxic damage. These are properties shared by many normal tissues like the bone marrow and small intestine. Although new cellular poisons such as Taxol are creating excitement in the oncology community, the effects of these types drugs are limited to inducing short term responses, with little impact on long-term survival (Aapro, 1996). Modest increases in survival do have a significant human impact but the goal of the drug-hunting community must be to affect long-term changes on the progression of diseases such as breast and colon cancer.

As an understanding of the molecular basis of carcinogenesis improves, novel drug targets are emerging which are based on molecular pathology. It will soon be possible to test hypotheses regarding the possibilities of inhibiting the growth or the survival of tumor cells when specific perturbations are imposed on oncogene function or restoration is made of a tumor suppressor gene (although restoration of function is a difficult strategy). Whether, in a disease which involves multiple lesions, there is the possibility to inhibit growth or bring about cell death by targeting single genetic abnormalities, such as the activity of mutated *ras* for example, is open to question.

Efficacy would depend upon there being some hierarchy amongst the genetic lesions which drive oncogenesis: is the growth or survival of a particular tumor highly dependent upon a limited number of lesions, with others being secondary? Does a lesion responsible for tumorigenesis retain its dominance, particularly under conditions of increasing genetic instability which characterizes the heterogenous cell biology of advanced tumors? Answers to these central questions which impact on the direction of possible therapeutic strategies will, hopefully, emerge in the next decade.

Cancer: The Consequences of Survival with Genomic Damage

A central question for the drug-discoverer, which relates directly to the molecular pathology of cancer, is the nature of the disease itself. It is here that the concept of apoptosis, a programmed or regulated cell death, plays a central role and is one which should change perceptions of the types of programmes of drug discovery that might yield significant impact. Cancer is characterised as a disease arising from the consequences of genetic lesions: these lesions result in the activation of cellular protooncogenes and/or the loss of tumor suppressor gene function. Despite the many and varied ways of becoming a tumor cell, a single common feature of all tumors is that they are composed of cells which have accommodated or tolerated genetic damage. On the other hand, the rate of proliferation and the size of the growth fraction is a feature which is variable across tumor types and even within tumor types. The solid tumors are not normally characterized by cells which are promiscuously cycling and dividing rapidly, despite enthusiasms regarding changes in the control of the cell cycle which occur in some cancers (Hunter and Pines, 1994). Strategies of chemotherapy targeted to the cell cycle machinery may thus have only limited impact and may affect normal dividing cells. Cancer is a disease of survival with genetic damage and the *potential* to divide, a process which in some cases may be promoted by the specific nature of some of the genetic lesions suffered, such as oncogene activation. Genomic damage to cells initiates a cellular response, epitomised by the activation of the p53 tumor suppressor gene (Hansen and Oren, 1997) or the activation of DNA repair processes such as mis-match repair (Perucho, 1996, Fishel and Wilson 1997). In both of these cases the outcome of activation is cell type and context dependent: DNA damage initiates processes which are either permissive for survival with repair (of unknown fidelity) or it activates the deletion of the cell. Loss of p53 function or of mismatch repair capability results, in some cell types, in a loss of the ability to engage cellular deletion. Deletion of a genomically damaged cell is the most certain way of preventing tumorigenesis. Therefore, it seems reasonable to suggest that the attenuation of DNA damage-induced cell deletion will dispose towards cancer. Identification of gene products which regulate the engagement of damage-induced cell deletion may be considered to be critical targets for new anticancer drugs. Because tumors and premalignant cells have DNA lesions, it is possible that in some cases they are still 'sensed' by molecules which guard genomic fidelity, then signalled. For some reason, the signal is not engaged to the appropriate response (apoptosis or a checkpoint). 'Signals' from these lesions could be harnessed to drive the self-deletion of

the cells by lowering the threshold for survival, for example by inhibition of *bcl*-2. This should be a selective strategy, as only tumors, and not normal cells, have genomic damage. One such strategy might be to drive the expression of a gene which promotes cell death. 'Hierarchies' are important here also: are there a limited number of features in a tumor cell which permit its accommodation or tolerance of the genetic lesions, including considerable genetic instability, so as to prevent DNA damage 'signals' from initiating cell deletion? If these are few in number then inhibiting them may indeed permit the lesions which characterise the tumor to selectively drive its death. In what follows, the paradigm of 'survival thresholds' (Dive and Hickman 1991, Fisher 1994) — the ease by which a cell can survive insult — and their molecular determinants, will be illustrated by reference to the epithelia of the gut. Death in the small intestine is described. This site has a low survival threshold and is not cancer prone (Goligher, 1980). This is compared to the colon and rectum, where stem cells appear to have a higher survival threshold and are cancer prone, giving rise to inherently chemoresistant tumors.

How do Cells die after Genomic or other Perturbations?

If it is the purpose of the drug therapy to kill cancer cells then the question of how they die is important because it raises the question of whether this death is genetically modulatable. Is death active or passive? Radiobiologists generally consider that irradiated cells die because of the damage imposed to chromosomal material, rendering them sterile, a passive type of death (mitotic cell death). *In vitro*, these cells are unable to replicate to form colonies (non-clonogenic) (Hendry and West, 1997). If cells undergo apoptosis in response to radiation or other perturbations, it is reasonable to suggest that genes which promote or suppress apoptosis would have a significant effect on long-term survival. This is a subject of some controversy because a number of studies have shown that after drug or radiation treatment, *in vitro* measurements of colony forming ability are *not* affected by the loss of a proapoptotic gene like p53 from primary fibroblasts (Slichenmeyer et al, 1993) or the enforced expression of a suppressor of apoptosis such as *bcl*-2 (Yin and Schimke, 1995). In the study of *bcl*-2 transfected HeLa cells the kinetics of the onset of apoptosis was changed but the ultimate outcome, measured by the capability of cells to divide and form colonies, is that none of the cells with a reduced apoptotic response maintain long-term survival. Although this data, and that from many other such studies, arises from rather artificial systems *in vitro*, it does raise the question as to whether all cell deaths are by apoptosis? Or rather, does a loss of cell proliferative capacity necessarily invoke cell loss by apoptosis? The treatment of tumors demands that cells are irreversibly sterilized, not necessarily that they are made to die acutely, nor that they necessarily engage morphological and/or biochemical features of apoptosis. The central question is whether the commitment to die is genetically controlled and open to intervention.

Apoptosis was first defined by its morphology and later by some conserved biochemical features, such as protein and DNA cleavage. The very short half life of an

apoptotic cell, its fragmentation and phagocytosis by neighbouring cells presents almost insurmountable problems in terms of quantitation and the establishment of the kinetic yield of apoptotic cells, a problem commented on by one of us (Potten, 1996). Nevertheless, careful studies have raised the question of whether radiation-induced death can be accounted for by the incidence of apoptosis (eg. Aldridge *et al*, 1996). In this study the fibroblastic cell line 208F was transfected with either Ha-*ras* or c-*myc*, and cells were irradiated. Although the transfects differed in the amount of apoptosis observed and in its kinetics of onset, clonogenic survival was not significantly changed by the expression of these oncogenes. Is this surprising?

After damage, cells may commit to a number of fates. These fates are contextual. Thus in response to a wide variety of totally disparate compounds working at different pharmacologic loci, many (but not all) HL-60 human promyelocytic leukemia cells in a population terminally differentiate, losing their clonogenicity (Beere and Hickman, 1993). Terminal differentiation necessarily commits these cells to death, as short-lived neutrophils. Their death by apoptosis is not an acute response but follows some six days after maturation (Martin et al 1990). This is a genetically controlled, programmed cell death and is initiated by drug treatment but the primary response to the drugs is not apoptosis. Instead, the differentiated neutrophils undergo apoptosis when they senesce. When fibroblasts are irradiated they may undergo a prolonged cytostasis, initiated by a p53 -dependent expression of the *waf*-1/*cip*-1 gene (Di Leonardo *et al*, 1997), and this is another example of a genetically controlled loss of clonogenicity. It is therefore not surprising that the commitment of a cell to death (with a loss of functionality, including clonogenicity) need not necessarily be the same as commitment to apoptosis, either acutely or chronically. The most important question is whether it is genetically controlled. Models in which to test this hypothesis stringently are discussed below.

Death and Apoptosis are Separable Events. Implications for the Understanding of Events Which 'Commit' a Cell to Die

The engagement of a genetically controlled mechanism of cell death, like that used during development and homeostasis, described elsewhere in this monograph, appears to be an economical use of elements of a developmental programme which deletes unwanted, undamaged cells. The morphological correlate of the programmed cell death is apoptosis. Apoptosis is also characterized by a series of discrete biochemical events which result in the cleavage of cellular proteins and DNA (see elsewhere in this monograph). If it is the goal of future tumor therapies to modulate the mechanisms that commit a cell, irreversibly, to death, using signals that arise from the cancer cell-unique events of genomic damage then it is important to discover what the mechanisms of committing a cell to die are. Are the events which characterize apoptosis, essentially the 'funeral' of the cell, the same as those which commit it to die (a 'morbidity'). It has been suggested that activation of the caspases (see Nicholson, this monograph) and the consequent proteolytic cleavage of key cellular proteins, one of the consequences of which is then DNA cleavage, may be the event

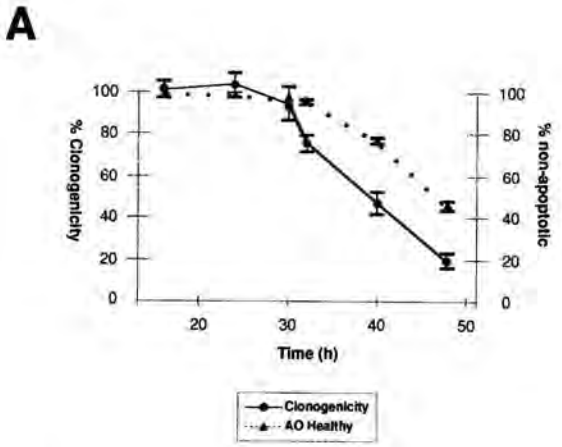

Fig. 1. The seperation of a loss of clonogenic potential from apoptosis. A: Full line shows the kinetics of a fall in clonogenicity after treating human CEM C7A cells continuously with 5μM dexamethasone. The dotted line shows the numbers of non-apoptotic cells, scored by observation of chromatin condensation using acridine orange staining. B: shows a western blot of polyADPribose polymerase (PARP) protein from cells treated as in A (the lower panel shows an overexposed blot) demonstrating that at 32h there was no detectable cleavage of PARP despite a 20% fall in clonogenicity (see A).

which commits a cell to die. The caspases are therefore considered to be important targets for therapy. Is this always so?

We have been studying the death by apoptosis of the human lymphoid cell line CEM C7A after treatment with dexamethasone. The time dependent accumulation of cells with a typical apoptotic morphology (condensed chromatin etc) was well defined, relatively synchronous and could be separated, on the basis of the timing of the withdrawal of dexamethasone from the cultures into different phases. The first where cells underwent changes, inhibitable by cycloheximide, which lead to a commitment to die. In this first phase, dexamethasone withdrawal prior to appearance of an apoptotic morphology prevented subsequent apoptosis. Secondly, we believe, there is a period where cells commit to die and thirdly a phase where execution was enacted: this produces cells with an apoptotic morphology. Whereas the events leading to commitment were sensitive to cycloheximide those of apoptosis were not (Wood et al, 1994). Recently we have performed kinetic studies of the loss of clonogenicity of these cells, using a medium where every single cell is able to form a colony (Brunet *et al*, submitted). Interestingly, the kinetics of the loss of clonogenecity precedes the

appearance of an apoptotic morphology by some 8 hours (figure 1A). All of the dexamethasone treated cells ultimately showed an apoptotic morphology. This suggested that a loss of clonogenicity was not a commitment to a state of permanent cytostasis.

Cells harvested at 32h, where there was a significant fall in clonogenicity, showed no cleavage of PARP (polyADPribose polymerase, a substrate of the caspases (figure 1B) — see elsewhere in this monograph) — and no DNA fragmentation to high molecular weight fragments or internucleosomal integers of 200 base pairs (the ladder). Furthermore, addition of the caspase inhibitor zVADfmk completely inhibited the cleavage of PARP and, most importantly, prevented the appearance of the classical apoptotic morphology. However, zVADfmk did not inhibit the fall in clonogenicity brought about by dexamethasone treatment. This suggested that the events which committed these cells to die were not the same as those events which were associated with the initiation of apoptosis and that apoptosis was an event subsequent to a commitment to cell death. The initiation of apoptotic events was prevented by caspase inhibitors. This was supported further by our finding that transfection of CEM C7A cells with *bcl*-2 rescued them from the loss of clonogenicity brought about by dexamethasone treatment and from the subsequent onset of apoptosis. This strongly suggests that bcl-2 acts at the commitment phase and is 'up-stream' of those events which are associated with the initiation of apoptosis. Our data is similar to that of Korsmeyer and colleagues (Xiang et al, 1996) who showed that expression of the pro-apoptotic bax protein induced a morphological apoptosis which was inhibited by caspase inhibitors but that the caspase inhibitors did not prevent the death of the cells, and to that from an elegant time-lapse study of oncogene or toxin-induced rat fibroblast apoptosis, where again inhibition of the caspases prevented apoptosis but not death (McCarthy et al, 1997).

Pro- and Anti-apoptotic Genes: Do they Influence Long Term Survival and Proliferative Capacity?

Why may these results be of significance? Because they suggest that commitment to death is different, and separable, from the commitment to apoptosis, and that genes such as *bax* and *bcl*-2 affect the commitment to death as well as that to apoptosis, the latter including the events associated with protein and DNA cleavage. This again may help to resolve the debate as to whether all cell deaths are apoptotic, that is, death accompanied by the classic morphology of condensed chromatin and the biochemical events of non-random DNA cleavage and proteolysis. Our data and that of Xiang *et al* (1996) permit one to propose that indeed, not all deaths need be apoptotic, but that death *is* genetically controlled and may subsequently be realised as apoptosis. It is possible, in some cell types, that cells undergo a programmed cell death but do not initiate the events of apoptosis. An example might be the differentiated cells at the tip of small intestinal villus tips (Ansari et al, 1993). The debate about the extent of involvement of genes like p53 and *bcl*-2 in determining drug sensitivity is, as stated above, contradictory, but different cell types have been compared *in vitro* under very artefactual conditions. Furthermore, it is impossible to measure,

and integrate with time, the apoptotic events that are taking place (Potten, 1996): the only 'read-out' from this integral is long term survival, and in the case of tumors and normal stem cells, this means their ability to form daughter cells, measured by some type of clonogenic assay.

Bcl-2 Promotes Clonogenic Survival after Drug Treatment in vitro when the in vivo Environment is Modelled

In asking questions about the importance of gene products like bcl-2 to suppress the response to current therapies (Reed, 1995), and to try to resolve some of the contradictions arising from *in vitro* system, discussed briefly above, we have performed experiments where the *in vivo* environment of a tumor has been modelled *in vitro*. Burkitts lymphoma cells transfected with *bcl-2* were treated with chlorambucil, a DNA cross-linking agent used in the treatment of follicular lymphomas, either as isolated cells or when cultured under conditions where the survival signal provided by the ligation of CD40, together with stromal signals, was mimicked. Whereas expression of bcl-2 alone provided the commonly observed delay in apoptosis but an insignificant clonogenic survival advantage (figure 2) the provision of an environment resembling the milieu of the germinal center for B cells provided a significant clonogenic survival (Walker et al, 1997). Therefore, a single promoter of survival, such as bcl-2, may either be insufficient to modulate the response of these cells to DNA damage or such molecules may only work efficiently when in the context of the normal environment of the cell. This data suggests that formal tests of whether gene which modulate the response to genomic damage, either inherent or imposed, must be made in proper cellular contexts. We have used *in vivo* models to test these hypotheses further.

Apoptosis in small intestine and colon in vivo: a model system for analysis of the importance of gene expression in determining the outcome of drug therapy

There is an important hierarchy of cellular survival following genomic damage to the epithelial cells of the crypts of the murine small intestine and colon (Potten, 1992, 1995). We have shown that following 1 Gray (Gy) of γ-irradiation to a mouse, the stem cell region of the small intestinal crypts undergoes significantly more apoptosis than the colon (Merritt *et al*, 1994) (figure 3). This hierarchy of deletion has been suggested to protect the small intestine from tumors, where incidence is minimal in comparison to the colon and rectum (Potten et al, 1992). From experiments with wild-type and p53 null ('knockout' animals), at 1 Gy of radiation the deletion of small intestinal epithelia is totally dependent upon the expression of p53 (Merrrit et al, 1994, 1997) whereas p53-independent mechanisms bring about a late wave of apoptosis at doses above 5 Gy (Clarke *et al*, 1997, Merritt *et al*, 1997). These p53-independent cell deaths after 8 Gy appeared to be mitotic catastrophes. Many of the cells were large, with evidence of mitotic events superimposed upon an apoptotic morphology.

In order to establish whether the presence or absence of p53 provided any clonogenic survival changes after radiation, a standard protocol was used where crypts are ablated by giving high doses of radiation (above 10 Gy) and the subsequent

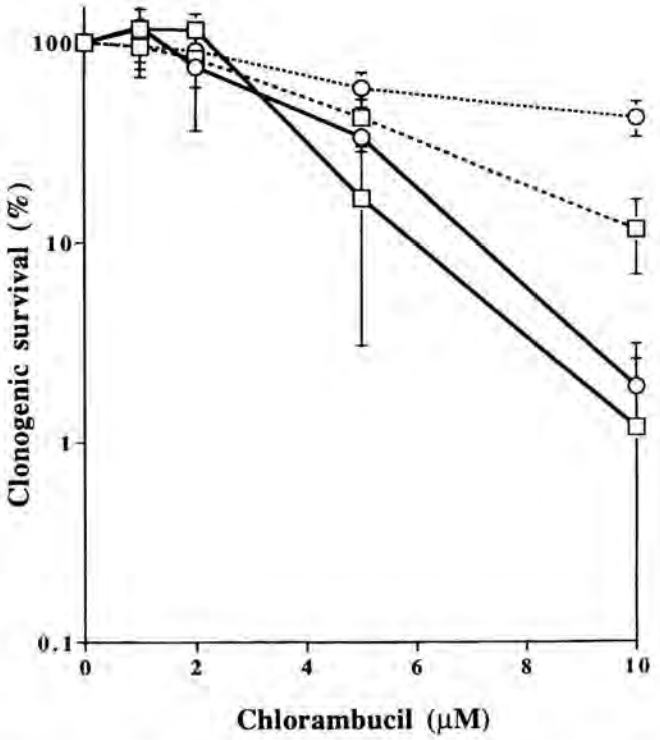

Fig. 2. The clonogenic survival of Burkitts lymphoma cells, transfected (squares) or not (circles) with bcl-2, treated with various concentrations of the alkylating agent chlorambucil either on plastic (full lines) or in an environment which attempts to reconstitute the germinal center (dotted lines). (Taken from Walker et al, 1997 and reproduced with permission).

regrowth of crypts scored as representing the survival of clonogenic cells (Potten and Hendry, 1995). The problem with this method is that it uses high doses of radiation where p53 independent mechanisms of apoptosis are engaged. Our experiments showed that, under these conditions, the loss of p53 provided no clonogenic survival advantage to small intestinal epithelial stem cells in comparison to those animals with p53 (Merritt et al, 1997). Surprisingly, in the murine colon, high doses of radiation, which invoke p53-independent modes of cell death, killed more clonogenic cells in the colonic crypts of the p53 null animals (Hendry et al, 1997). One might speculate that in the mouse without a p53 genotype, either adaptive changes had occurred which reduced the survival potential of these cells, or that endogenous gene expression in the absence of p53 was such as to sensitise the cells. This implies that non-p53-driven mechanisms of apoptosis may be suppressed in the presence of p53 expression. Such an idea is compatible with the finding that the majority of colon cancers lose p53 expression late in tumor development: its loss at an early stage from

the colon would sensitise the cells to further genomic damage, enhancing the possibilities of their deletion. The absence of p53 may release the cells from the imposition of a *waf*-1/*cip*-1 G1 checkpoint, allowing cells to progress into S-phase and the engagement of some checkpoint coupled to cell death. Presumably this is absent from small intestinal epithelia (where the loss of p53 did not sensitize to irradiation). There is a precedent, for a loss of a p53-checkpoint leading to sensitization, in the work of O'Connors group but using cell lines *in vitro* (Fan et al, 1997). Data presented below, using the drug 5-FU at lethal but not supralethal doses, provides a different result, where p53 clearly determines the extent of toxicity to the gut (Pritchard *et al*, in preparation).

Interestingly, in the elegant study by Clarke et al (1997) of the role of p53 as the 'guardian of the genome' in small intestinal epithelia *in vivo*, it was found that mutation rates, assayed by analysis of the Dlb-1 locus, which determines lectin binding, were only affected at doses of radiation above 6 Gray. At 2 Gray, when apoptosis did not occur in the small intestinal cells of the p53 null animals, deleting damaged cells, there was no higher incidence of mutation. This suggests that repair with fidelity is taking place in those cells that have not been deleted by a p53-dependent apoptosis and that, in considering the origins of malignancy in the intestine, that other mechanisms of detecting damage and initiating repair are absent. This is congruent with a provocative recent study showing that human colonic tumors show characteristics of considerable genomic instability prior to any mutation or loss of p53 (Kahlenburg et al 1996). One of the alternate mechanisms permissive for mutation and the failure to undergo apoptosis may be mismatch repair (Kinzler and Vogelstein, 1996).

bcl-2: A Critical Determinant of the Survival of Stem Cell Region of the Colonic Crypt

A major difference in gene expression between the small intestinal epithelia and those of the colon is the expression of the suppressor of apoptosis *bcl*-2 (Merritt *et al*, 1995). We showed by immunohistochemistry that bcl-2 protein was restricted in expression to the base of the colonic crypts. There was no expression of *bcl*-2 in the epithelia of the small intestine. In *bcl*-2 null animals spontaneous apoptosis, which is normally minimal, was increased specifically at the base of the colonic crypt. Treatment of the *bcl*-2 null animals with either γ-radiation (Merritt *et al*, 1995) or the cytotoxic drug 5-fluorouracil (Pritchard *et al*, submitted) initiated significant amounts of apoptosis at the base of the crypts in comparison to *bcl*-2 +/+ (wild-type) mice. The cells at the base of the colonic crypt are considered to harbor the stem cell population (Potten, 1992, 1995) and it may be the survival advantage imparted by *bcl*-2 that permits these cells to accumulate genomic damage. Colonic and rectal tumors in their early stage of development stain positively for bcl-2 protein, and it seems reasonable to suggest that these tumors and adenomas have arisen from normal cells which expressed *bcl*-2. We have shown that kinetics of the onset of apoptosis initiated by 5-fluorodeoxyuridine, a drug used in the treatment of colon carcinoma, is significantly delayed by *bcl*-2 expression (Fisher 1993). A delay in the onset of apoptosis initiated by all the classes of anticancer drugs in current use has been reported in many studies (reviewed by Reed, 1995). The question of whether *bcl*-2 provides a

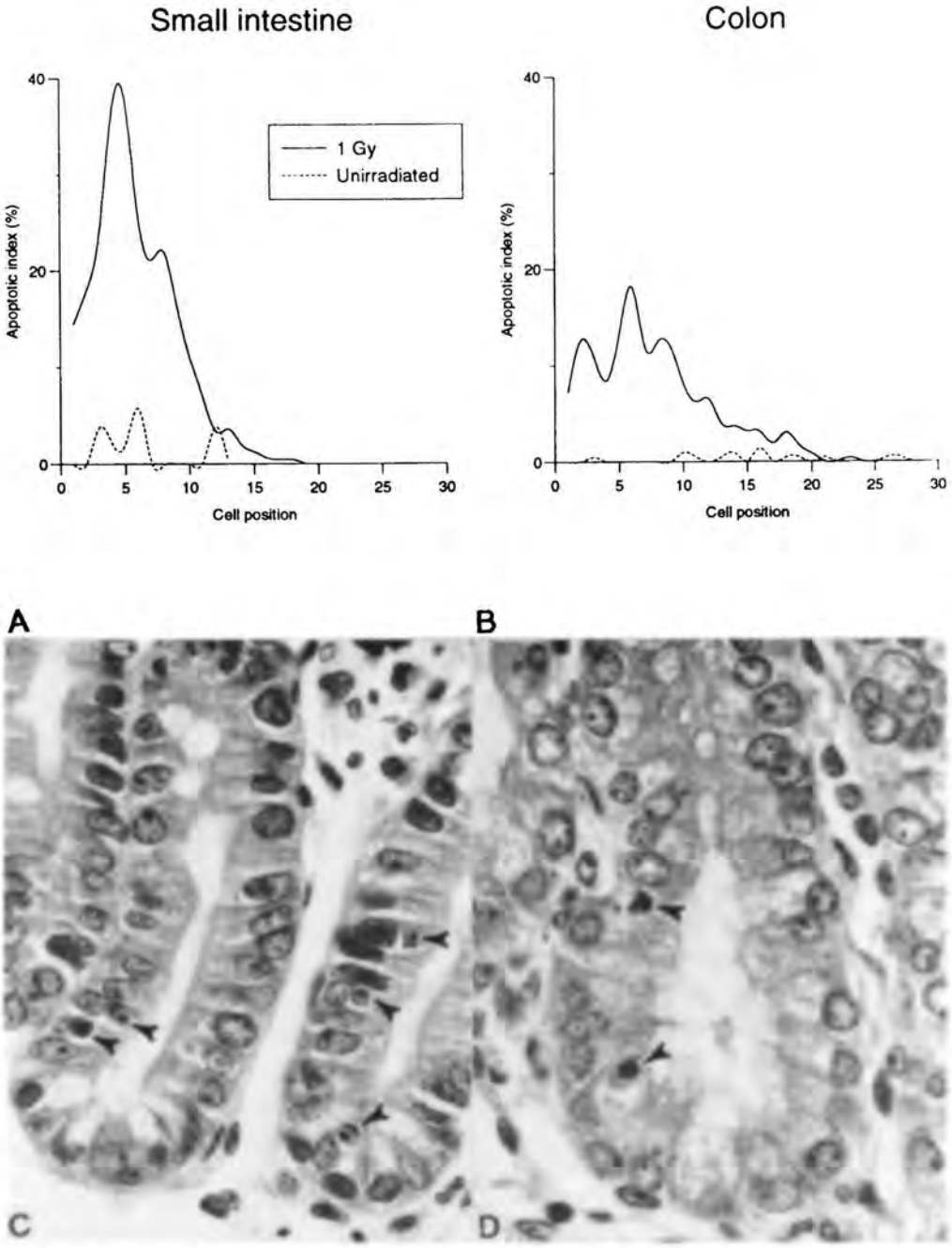

Fig. 3. Differences in the position and quantity of apoptotic events in murine small intestine and mid-colon 4.5h after 1Gy of γ-irradiation. A & B show the amount of apoptosis by cell position in the crypt, numbering from the base. C & D show sections of the small intestinal (C) and midcolonic (D) crypts. Arrowheads indicate apoptotic events. (From Merritt et al, 1995, with permission).

real survival advantage was discussed above. However, of more importance with respect to colon cancer is the finding that as tumors become more advanced the expression of *bcl*-2 was lost (Watson *et al*, 1996). In the majority of tumors this loss was coincident with the expression of stablised p53 protein in the tumors, suggesting mutation, and thus loss of function. The question arises as to what substitutes for *bcl*-2 in providing survival as the tumor advances: is it that p53 function is lost or are there other changes. For example, it was recently reported that the pro-apoptotic partner for bcl-2, bax, was mutated in advanced colonic cancers of the microsatellite mutator phenotype (Rampino et al, 1997).

5-Fluorouracil Toxicity-Dependance on p53 in vivo

5-Fluorouracil (5-FU) is an pyrimidine antimetabolite designed to prevent the *de novo* synthesis of thymidine by inhibition of the activity of the enzyme thymidylate synthase. It is used as one of the principle agents in the chemotherapy of colon carcinoma (Sobrero et al, 1997). Its mechanism of action is, however, complicated by its incorporation into RNA and, surprisingly, the perturbation of RNA metabolism may be responsible for its toxicity to some tumor cells and to normal proliferating cells, particularly in the gut. We recently showed that both small and large bowel crypt epithelia from mice underwent apoptosis *in vivo* with a pattern of distribution quite distinctive from that observed after irradiation (Pritchard *et al*, 1997). In the small intestine the stem cell area (positions 4-6, see figure 3) were spared but the transition cells, which are highly proliferative and have a shorter cell cycle time, underwent significant apoptosis. In the mid-colon, cells at the base of the crypt, in the region of the putative stem cells, underwent apoptosis. The initiation of apoptosis in the crypts was apparently due to 5-FU incorporation into RNA and, again surprisingly, this was p53-dependent. Thus we showed that in p53 null mice, the pattern of apoptosis after 40mg/kg of 5-FU was almost completely abrogated, suggesting the possibilities that p53 was either detecting RNA perturbation directly or that some change in RNA brought about DNA damage (Pritchard *et al*, 1997). Thus far, using cell lines, we have been unable to detect DNA damage at the time that p53 is activated. Despite these mechanistic nuances, the dependence on p53 for the initiation of crypt epithelial apoptosis suggested that we could test the hypothesis that toxicity to the gut was dependent upon apoptosis by comparing 5-FU-induced changes in gut morphology and function in p53 wild-type and p53 null mice where patterns of acute apoptosis (0-36h) were significantly different according to genotype.

In these experiments, it was first established that, in p53 wild-type animals, above 40mg/kg of 5-FU the numbers of apoptotic cells observed in the crypts did not increase over a 36h period (for example at 400mg/kg) (Pritchard *et al*, 1997). This compares with profound differences in morbidity over this dose range: 40mg/kg of 5-FU caused no weight loss or mortality in the p53- wild-type animals and analysis of crypt morphology over a four day period showed no histopathological changes (Pritchard *et al*, in preparation) whereas 400mg/kg induced a small weight loss and a mortality of 50% at 12 days. To assess histopathology and the effect of 5-FU on other functions of the crypt epithelia, mitotic indices were recorded over a four day

period, together with thymidine labelling *in vivo* so as to measure proliferation. Crypt integrity was scored by counting the numbers of cells in crypts, their height and, in the case of the small intestine, villus cellularity and height.

Scoring apoptosis intermittently over a 24-48h period cannot provide an accurate, integrated assessment of the total number of apoptotic events that may have taken place in this period (a so-called 'apoptotic index') (see commentary by Potten, 1996) but we assume that because at the times when peak apoptosis was observed at 40 mg/kg (24h in mid-colon) there was little significant increase after 400 mg/kg, that events in addition to apoptosis may be contributing to the pathological changes observed after the higher dose in the p53-wild-type animals. These other events, occurring presumably as a consequence of p53's ability to cause a G1 checkpoint, were scored in the remaining viable cells as profound falls in mitotic index at 24h with slight recovery at 72h, and a fall in the thymidine labelling index at 24h with recovery after 48h, in both small intestine and mid-colon. The consequences of cell loss by apoptosis, of a profound inhibition of proliferation and mitosis, were to significantly decrease crypt cell numbers in both the small intestine and the mid-colon (figure 4). Cellularity reached a nadir at 96h and was followed by a rebound recovery (greater cellularity than controls) 6 days after drug treatment. The loss of cellularity translated to a profound change in crypt morphology (figure 4) indicative of the imposition of significant gut toxicity (Pritchard *et al*, in preparation).

These results contrast significantly with those obtained from the p53 null animals: here, apoptosis was suppressed (Pritchard *et al*, 1997), although we found, at 400 mg/kg, low levels of a late, non-p53 apoptosis were observed at 54h in the small intestine. As with our experiments where p53 null mice were irradiated (Merritt *et al*, 1997) the loss of p53 permitted 5-FU epithelia from the crypts to maintain their proliferative capacity and mitotic activity. Whereas small intestinal crypt cell number fell to a nadir after 96h no change in crypt number was observed in the p53 null animals from 30 to 96h. This essentially maintained crypt integrity (figure 4). The loss of p53 function, *in vivo* where, naturally 'context' is maintained, effectively abrogated the toxicity of 5-FU to normal cells. This experiment contrasts with those we performed using high doses of radiation to determine crypt recovery, and 'clonogen' survival (Merritt *et al*, 1997) in that we performed measurements of crypt cellularity without ablating the majority of the crypt.

Does this unambiguous demonstration that 5-FU toxicity, not acute apoptosis, but acute toxicity as defined by histopathology, is dependent on p53 function support the notion that toxin-induced death is dependent upon apoptosis, since apoptosis and toxicity are reduced in the p53 null animals? Because of the impossibilities of integrating the total yield of apoptotic cells and because the peak yield of apoptosis was not increased in proportion to the histological damage imposed as the dose of 5-FU increased, we consider it unlikely. Rather, as outlined above, the fate of the crypt is determined by the multiple functions resulting from p53 activation: apoptosis and checkpoints (of various duration) being the major ones. The loss of crypt integrity reflects not only a loss of cells by apoptosis but also a fall in replicative capacity. And is what is observed here in normal cells likely to be relevant to tumors? Since we have been arguing that context is critical in determining response this is difficult to predict.

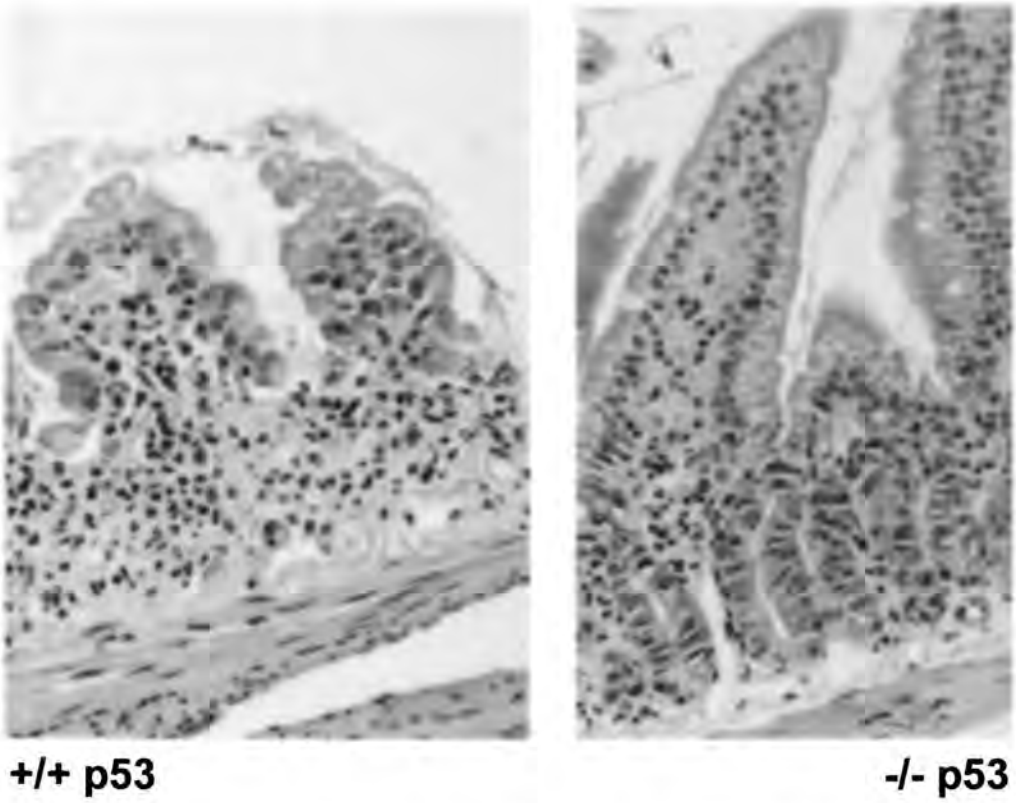

Fig. 4. Photomicrographs of H&E stained sections of murine intestines 102h following 400mg/kg 5-fluorouracil in p53 -/- animals (left panel) or p53 +/+ animals (Right panel) (×400).

Conclusions

The aim of the drug developer is to discover targets for the discovery of effective molecules which will inhibit the growth of disseminated, metastatic cancer. Because of the possibilities of escape from cytostatic regimes it is better to promote a permanent loss of proliferative capacity. Commitment to death is a secure way of achieving this. This commitment is, we suggest, genetically regulated. Death may be acute, by apoptosis, or more long term, after commitment to terminal differentiation or to senescence, when it may also have the morphology of apoptosis. We suggest here that commitment to death may be an event separable from the engagement of apoptosis, which is a well-defined process characterised both morphologically and biochemically. The events of commitment are undefined. In the lymphoid cell model we describe, commitment to death is inevitably followed by engagement of apoptosis. But, the fact that these events appear to be separable suggests that an apoptotic morphology need not inevitably follow commitment. We also suggest that gene products which

determine the threshold for survival or commitment to death operate strictly in a contextual manner; ignoring this in certain *in vitro* models may create spurious results, for example after ectopic over-expression or deletion of genes which modulate death. We caution here against the creation of generalised models which, because they are flawed by the limitations of non-contextual biology, may create unnecessary controversy. Perhaps most importantly their validity is critical for establishing strategies for drug discovery.

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S.W. Lowe

Tumor Suppressor Gene Action in Chemosensitivity and Oncogenic Transformation

Abstract

Our research is based on the premise that apoptosis, a genetically-controlled form of cell death, provides a natural defense against tumor development and underlies the cytotoxicity of most current anticancer drugs. Consequently, mutations that disrupt apoptotic programs can cause tumor progression and resistance to cancer therapy. Earlier studies identified the p53 tumor suppressor as an important regulator of apoptosis and demonstrated that *p53* mutations could promote oncogenic transformation, tumor progression, and resistance to cytotoxic agents by reducing a cell's apoptotic potential (Lowe et al., 1994; Lowe et al., 1994; Lowe et al., 1993; Lowe et al., 1993; Symonds et al., 1994). Our current research is aimed at elucidating how p53 promotes apoptosis and characterizing p53-independent apoptotic pathways that might function in tumor cells. We are also investigating how p53 and other cell death regulators affect caspase activation, the enzymes directly responsible for apoptosis. Finally, in a separate direction, we are using simple models of oncogenic transformation to investigate how cells respond to *ras* oncogenes and the consequences of this response for multistep carcinogenesis.

Modulation of chemosensitivity

Anticancer agents are effective only when tumor cells are more readily killed than normal tissue. If the cytotoxicity of these agents is partly determined by their ability to induce apoptosis, then tumor cells must be more susceptible to apoptosis than the normal tissue from which they arose. Evidence is emerging to suggest that increases in cellular susceptibility to apoptosis are tightly linked to tumorigenesis itself. For example, the adenovirus *E1A* oncogene and *c-myc* both deregulate cellular proliferation and simultaneously promote apoptosis in response to a variety of physiologic and toxic agents (Evan et al., 1992; Lowe et al., 1993). Since analogous changes are found only in neoplastic cells, their ability to enhance apoptosis provides an intriguing explanation to account for the tumor-specific action of cytotoxic agents (Lowe et al., 1993). By contrast, since apoptosis is a genetically-programmed form of cell death, mutations in genes that are required for apoptosis can produce resistance to

therapy. Together, these data support the view that tumor cell chemosensitivity is determined, at least in part, by the combined effects of oncogenic mutations on apoptosis (Lowe, 1995).

Primary fibroblasts provide a tractable model that mimics the predicted changes in chemosensitivity accompanying tumorigenesis. Primary fibroblasts are normally resistant to apoptosis induced by a variety of toxic agents, but become extremely susceptible to apoptosis by expression of E1A oncogene, or by co-expression of the E1A/ras oncogenes (Lowe et al., 1993). If these tumorigenic cells have *p53* mutations, however, cytotoxic agents are no longer able to induce apoptosis efficiently (Lowe et al., 1994; Lowe et al., 1993).

We have continued to explore the E1A/ras transformation paradigm in an attempt to better understand factors which modulate chemosensitivity. Studies by others have studied E1A in the context of adenovirus infection or in immortal or tumor-derived cell lines. However, adenovirus has a number of genes that affect apoptosis, including the E1B 19K and 55K gene products (Rao et al., 1992), and others (Marcellus et al., 1996). Furthermore, immortal or tumor derived cell lines may have acquired unknown mutations that affect apoptosis. To eliminate these problems, we have developed methodology to study the effects of E1A alone in primary cells (i.e. genetically normal cells). Our approach uses high titer retroviruses to introduce certain genes (e.g. E1A or E1A mutants) into whole populations of cells for analysis of their affect on apoptosis without substantial growth in cell culture, where other mutations might arise. For example, retroviral transduction of E1A rapidly converts chemoresistant fibroblasts into a highly chemosensitive cell (Figure 1).

The E1A oncoprotein is a multidomain protein that associates with a number of cellular proteins. In order to understand how E1A enhances chemosensitivity, we have begun to dissect the functional regions of E1A important for this activity. We introduced a variety of E1A mutants into primary human and mouse fibroblasts using high titer recombinant retroviral vectors. Our experiments identified two regions of E1A essential for its ability to promote chemosensitivity. The first is an N-terminal domain, and the second is in the center (designated conserved region 2). This region has been shown to physically interact with the retinoblastoma protein (pRb), and the related p107 and p130 proteins; consequently, mutants lacking this domain are unable to bind pRb. Unlike full-length E1A, E1A mutants with the N-terminal deletion (E1A ΔN) are unable to enhance chemosensitivity when expressed in normal cells despite normal levels of expression. Likewise, an E1A mutant lacking conserved region 2 (E1A ΔCR2) is also unable to promote chemosensitivity. Remarkably, cells that co-express *both* mutant proteins (E1A ΔN and E1A ΔCR2) behaved like cells expressing full-length E1A — they readily undergo apoptosis following treatment with chemotherapeutic agents. This result indicates that two functionally distinct regions of E1A are essential for chemosensitization of normal cells.

To determine whether the E1A ΔCR2 mutant is defective for apoptosis and chemosensitivity because it is unable to bind and inactivate either Rb, p107, or p130, we introduced this mutant into fibroblasts lacking each of these proteins. In principle, if CR2 contributes to apoptosis and chemosensitivity because they functionally inactivate pRb, then the otherwise defective E1A ΔCR2 mutant should be fully able to

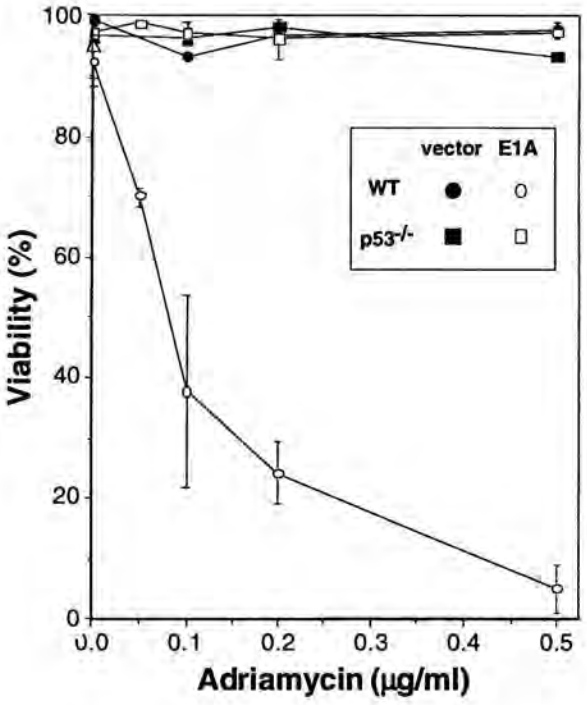

Fig. 1. E1A confers chemosensitivity. Mouse embryo fibroblasts derived from wild-type (wt) and p53-deficient mice were infected with a control retrovirus (vector) or one expressing an adenovirus-5 12S E1A cDNA. Cell populations were treated with various concentrations of the chemotherapeutic drug adriamycin, and cell viability was determined after 24 hours. E1A makes cells extremely sensitivity to the induction of apoptosis by anticancer agents. Cell death in E1A-expressing cells is potentiated by p53, since E1A-expressing cells lacking p53 do not undergo apoptosis at these drug concentrations.

promote chemosensitivity in cells lacking the retinoblastoma gene (and hence already have no functional Rb). Indeed, while the E1A ΔCR2 mutant did not promote chemosensitivity in normal, p107-deficient, it enhanced the chemosensitivity of Rb-deficient cells to the levels observed with full-length E1A. This indicates that CR2 contributes to chemosensitivity by virtue of its ability to bind and inactivate pRb; consequently, mutant forms of E1A unable to bind Rb are defective at promoting chemosensitivity in normal cells, but remarkably enhance apoptosis in cells lacking Rb.

We have previously shown that p53 is metabolically stabilized in cells expressing E1A, and that this is associated with E1A's pro-apoptotic activity. Our mutational analysis of E1A show that p53 induction precisely correlates with the ability of E1A to promote apoptosis. Of note, the regions of E1A that promote apoptosis are similar, if not identical, to the regions involved in S phase induction and transformation. Consequently, we believe that p53 accumulation and apoptosis are a cellular response to aberrant proliferation rather than a direct consequence of E1A on p53.

E1A and Apoptosis in vitro

The pro-apoptotic signal generated by E1A persists in extracts derived from E1A-expressing cells. Specifically, E1A produces an activity that promotes spontaneous caspase activation in cell extracts (Fearnhead et al., 1997). This activity is not present

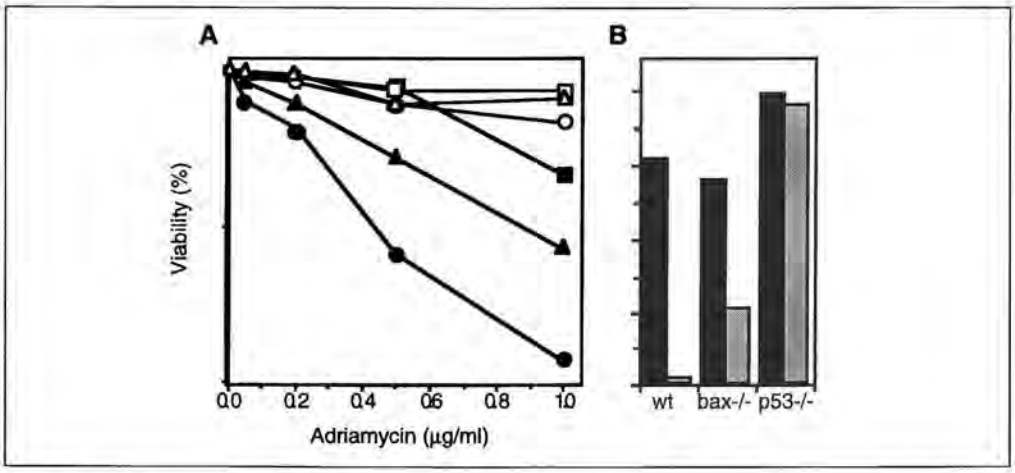

Fig. 2. Bax participates in a p53-dependent apoptotic program.
A. E1A was introduced into wild type (circles), bax-/- (triangles) or p53-/- (squares) MEFs by retroviral-mediated gene transfer. The resulting E1A-expressing cell populations were infected with a Bcl-2 expressing retrovirus (open symbols) or a vector control (closed symbols). Cell populations were treated with adriamycin and viability was assessed at 24 hours. Bax-deficiency attenuates apoptosis under circumstances where p53 is required for apoptotic cell death. Bcl-2 can suppress p53-dependent apoptosis in the absence of Bax. B. Bax (gray columns) or a control vector (black columns) was introduced into E1A-expressing cells (see A) and cell viability was assessed at 48 hours. Bax efficiently induces apoptosis in wild-type and bax-/- E1A-expressing cells, but not E1A-expressing cells lacking p53. Presumably, bax required some other p53 controlled factor to efficiently induce cell death.

in normal fibroblasts, but is present in cells expressing E1A, and hence was named Oncogene Generated Activity (OGA). Partial purification of OGA from 293 cells (human cells expressing adenovirus E1A and E1B) identified a fraction capable of mimicking E1A action by activating caspases in extracts from normal fibroblasts. This fraction did not contain E1A itself, nor CPP32 or cytochrome c. Hence, despite the global effects of E1A on cellular transcription, a component of its pro-apoptotic activity can be attributed to a discrete factor present in cell extracts.

Both the E1B 19K and Bcl-2 oncoproteins suppress apoptosis in cells expressing E1A (Chiou et al., 1994; McCurrach et al., 1997; Rao et al., 1992). Unlike cells expressing E1A alone, cells co-expressing E1A with either E1B 19K or Bcl-2 fail to activate caspases in response to anticancer drugs. However, caspases are spontaneously activated in *extracts* from these cells, indicating that OGA is retained in a latent state. If drug-resistant tumor cells also retain OGA, these data may have important implications for the development of novel cancer therapies.

p53 and Apoptosis

Inactivation of p53-dependent apoptosis promotes oncogenic transformation, tumor development and resistance to many cytotoxic anticancer agents; however, the mechanism

whereby p53 promotes apoptosis is poorly understood. p53 is a sequence-specific binding protein that can regulate transcription (Ko and Prives, 1996). One transcriptional target of p53 that may be important for apoptosis is Bax, a cell death agonist with homology to the anti-apoptotic Bcl-2 protein. Forced overexpression of p53 increases Bax expression in several cell types, and this increase correlates with the induction of apoptosis. In addition, the Bcl-2 and E1B 19K proteins, which can inhibit apoptosis induced by p53 overexpression, can physically associate with Bax. This raises the possibility that these oncoproteins interfere with p53-dependent apoptosis by antagonizing Bax function.

All of the studies mentioned above have relied on forced overexpression of p53 or Bax to induce apoptosis — circumstances that may not faithfully reproduce their normal activities. To determine whether *bax* is required for p53-dependent apoptosis, the effects of *bax*-deficiency were examined in primary mouse embryo fibroblasts (MEFs) expressing the E1A oncogene, a setting where apoptosis is strictly dependent on endogenous p53 (Lowe et al., 1993) (see Figure 1) Using this approach, we demonstrated that *bax* can function as an effector of *p53* in chemotherapy-induced apoptosis, and contributes to a *p53* pathway to suppress oncogenic transformation (McCurrach et al., 1997). Furthermore, we showed that additional p53 effectors participate in these processes. These p53-controlled factors cooperate with Bax to promote a full apoptotic response and their action is suppressed by the Bcl-2 and E1B 19K oncoproteins. These studies demonstrate that Bax is a determinant of p53-dependent chemosensitivity and illustrate how p53 can promote apoptosis by coordinating the activities of multiple effectors.

p53 Mutations and Treatment Outcome

Our studies on p53 and chemotherapy-induced apoptosis have strongly suggested that p53 mutations might promote drug resistance in human cancer. A number of recent clinical studies support this view. For example, p53 mutations are associated with a much reduced probability of remission in patients with several hematological malignancies, including B-cell chronic lymphocytic leukemia (B-CLL), myeloid dysplastic syndrome (MDS), *de novo* acute myeloid leukemia (AML), and chronic myelogenous leukemia (CML) (El Rouby et al., 1993; Wattel et al., 1994). p53 mutations are associated with relapse in both T and B cell acute lymphoblastic leukemia (ALL) (Hsiao et al., 1994), consistent with the notion that cells acquiring p53 mutations are predicted to more readily survive chemotherapy. Remarkably, patients with T-ALL that relapse with p53 mutations are much less likely to enter a second remission compared to relapse patients with normal p53 (Diccianni, 1994).

In certain solid tumors, p53 mutations are associated with reduced apoptosis in tumors that are often refractory to therapeutic intervention. For example, p53 mutations are tightly linked to reduced apoptosis in anaplastic Wilms' tumor, an aggressive subtype that responds poorly to chemotherapy (Bardeesy et al., 1995; Bardeesy et al., 1994). Similarly, p53 mutations occur at more advanced stages of colon cancer, a period when tumors display dramatic decreases in apoptosis. Finally, p53 mutations

are strongly associated with resistance to doxorubicin and certain other therapies in breast cancer (Aas et al., 1996; Bergh et al., 1995).

Not all studies have identified correlations between p53 mutations and drug resistance in human tumors. In fact, one recent study suggested that tumors with p53 mutations might be *more* responsive to therapy then tumors with normal p53 (Cote et al., 1997). At present, the reason for these discrepancies is unknown, but could arise from methodological differences between various studies (Lowe and Jacks, 1997). Alternatively, the role of p53 in chemosensitivity may be context dependent (see (Lowe and Jacks, 1997) for discussion). *p53* affects a remarkable number of cellular processes, including apoptosis, cell-cycle checkpoints, DNA repair, differentiation, and senescence. Perhaps defects in damage-induced checkpoints enhance chemosensitivity, whereas defects in apoptosis promote drug resistance. Consequently, the clinical impact of *p53* mutation may be determined by which effect predominates. This, in turn, may be influenced by tumor type, chemotherapeutic agent, or by other mutations occurring in the tumor cells. The resolution of this issue will require better methodologies for identifying p53 mutations, and a more complete understanding of the pathways that regulate — and are regulated by — *p53*.

Caspases and Chemosensitivity

Proteases related to interleukin-1β converting enzyme, called caspases, are thought to be essential components of the apoptotic 'machinery' (i.e. the molecules directly responsible for apoptotic cell death). The importance of apoptosis in cancer argues that components of this machinery may be tumor suppressors and/or drug sensitivity genes. Since proteolytic cleavage is essentially irreversible, caspase activation may represent the last regulated step in apoptosis. In this view, the endpoint of most, if not all, cytotoxic anticancer drugs is caspase activation. Consequently, most mutations that limit drug cytotoxicity act *upstream* of these proteases. If true, the caspases represent attractive drug targets.

Our laboratory is to investigating how cell death regulators like p53 affect caspase activation. Using the E1A/ras transformation system described above, we have taken a number of experimental approaches to study caspase regulation during p53-dependent and independent forms of apoptosis. First, we have shown that multiple caspases are activated during chemotherapy-induced apoptosis in MEFs, and that inactivation of p53 or overexpression of cell death inhibitors such as E1B 19K or Bcl-2 prevent caspase activation. Furthermore, we are using a variety of knockout fibroblasts harboring deletions in pro-apoptotic genes to determine how they affect caspase activation. These studies underscore the importance of caspases in cancer therapy, and are beginning to provide insights into how cell death regulators interface with the apoptotic machinery.

Multistep carcinogenesis

Cancer is a multistep process involving a series of genetic changes that each enhance the growth or survival of developing tumor cells. Considerable progress has been

Fig. 3. p16 and p53 suppress transformation by oncogenic ras.
Prolonged expression of an activated ras oncogene induces a permanent cell cycle arrest with features of senescence. Both p53 and p16 levels increase in response to oncogenic ras and contribute to the arrested state. Escape from ras-induced arrest allows transformation of rodent cells, and may contribute to the transformation process in human cells.

made in identifying tumor-specific mutations, and how these mutations alter normal gene function. For example, mutational activation of *ras* results in a protein that constitutively transmits mitogenic signals, and inactivation of p53 disrupts cell-cycle arrest or apoptosis. However, much less is known about how these mutations interact to produce the malignant phenotype (Why are these mutations often found in the same tumors? Why does *ras* mutation often precede *p53* mutation?).

Perhaps the simplest model for studying the multistep nature of cancer involves oncogenic transformation of primary cells. Primary cells are genetically normal and capable a limited number of cell divisions in culture, after which they permanently arrest by a process known as senescence. At low frequencies, primary cells acquire mutations that disrupt senescence, allowing these variants to be established into 'immortal' cell lines. Expression of oncogenic *ras* typically 'transforms' immortal cells to a tumorigenic state, but cannot transform primary cells. However, primary cells are made tumorigenic if *ras* is co-expressed with second oncogenes such as E1A (a phenomenon known as 'oncogene cooperation'), or when expressed in the absence of tumor suppressors such as p53 and p16. When expressed alone, these cooperating mutations (E1A, p53 loss, p16 loss) facilitate the immortalization process.

We recently demonstrated that prolonged expression of oncogenic *ras* in primary fibroblasts induces a permanent cell-cycle arrest involving p53 and p16 (Serrano et al., 1997). Remarkably, this arrest is indistinguishable from senescence. Inactivation of either p53 or p16 prevents *p53*-induced arrest in rodent cells, and E1A achieves a similar effect in human cells. These observations suggest that the onset of cellular senescence can be prematurely activated in response to an oncogenic stimulus. Inactivation of this senescence program, by a cooperating oncogene or as a result of spontaneous mutation, allows proliferation to continue unabated and facilitates oncogenic transformation. Consequently, premature senescence may be an important safeguard against neoplasia.

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G.J. van Steenbrugge, M.H.A. Oomen and W.M. van Weerden

Hormone Action and Apoptosis in Human Prostate Cancer Models

Abstract

Androgen ablation therapy aiming at reducing tumor burden by inhibition of proliferative activity and inducing programmed cell death in the tumor tissue, is the current front-line therapy for advanced prostate cancer. A panel of human prostatic xenograft models, representing various stages of clinical prostate cancer, was used to study the effect of hormonal manipulation on tumor cell proliferation, occurrence of apoptotic cell death, and on some functional markers. High-dose androgen-induced cell loss in the androgen sensitive LNCaP cell line in vitro was demonstrated to be apoptotic cell death. The results obtained with this cell line subtantiated the dual action of androgens in this target cell: depending on the concentration applied, proliferation can be stimulated, and apoptotic cell death may be induced.

Introduction

Prostate Cancer

The occurrence of prostate cancer has increased dramatically over the past decade (Severson *et al*, 1995). It is at present the second leading cause of cancer death in the Western male population. On average, patients are now diagnosed at an earlier stage of the disease. Most patients with tumors that are clinically confined to the prostate are treated with curative intention, either by radical prostatectomy or radiotherapy (Chodak *et al*, 1994). Patients with advanced (metastatic) disease are usually hormonally treated, i.e. by medical or surgical castration, leading to a reponse in about 40-50% of the patients. This androgen ablation therapy aims at reducing tumor burden by inhibition of proliferative activity (Walsh, 1975) and by inducing programmed cell death (apoptosis) in the tumor tissue (Szende *et al*, 1993; Denmeande *et al*, 1996). After an initial response, however, tumor relapse occurs due to the growth of androgen-independent prostate cancer cells. This relapse towards androgen refractory disease develops even if complete androgen blockade is used, and as a consequence androgen ablation is rarely curative. Prostate cancer is resistant to most cytotoxic drugs (Manni and Vogelzang, 1996), which makes advanced disease hardly treatable.

Xenograft Models of Human Prostate Cancer

The transition of androgen-dependent prostate cancer to an androgen-independent state is a process which is still poorly understood and which can only adequately be studied in experimental model systems which can easily be (hormonally) manipulated (Isaacs and Coffey, 1981). Much of the knowledge of the cellular aspects of androgen regulated growth and progression of prostatic cancer stems from investigations with the normal rat (ventral) prostate and the Dunning R3327 rat prostate cancer system (Isaacs *et al*, 1978). Extrapolation of the results obtained with such systems in the rat is, however, limited by their non-human origin and restricts the direct applicability of such models to the study of human prostate cancer.

Establishment of human prostate cancer cell lines in culture and *in vivo*, as heterotransplant in athymic nude mice, is difficult and generally a very low rate of success has been recognized (Otto *et al*, 1988). All in all, no more than ten *in vitro* cell lines, including the androgen responsive LNCaP model, and approx. 15 *in vivo* xenograft models of human prostate cancer have been described. In our institution the hormone-dependent PC-82 and two independent tumor lines, PC-133 and PC-135 were established more than ten years ago. In particular the PC-82 has been used for many studies focussing on various aspects related to endocrine dependence and androgen-regulated functions of human prostatic carcinoma (Van Steenbrugge *et al*, 1984a).

More recently, our institution was successful in developing a new series of seven permanent prostate cancer models *in vivo*. By using athymic nude mice of the NMRI strain in stead of the Balb/c strain, a substantial increase in the take rate of human prostate cancer tissues was achieved. Accordingly, during the last three years seven new prostatic xenograft models were established, originating from primary tumors (prostatectomy and transurethral resection material) as well as metastatic lesions (Van Weerden *et al*, 1996). These tumors represent various stages of clinical prostate cancer, as appears from differences in the pattern of androgen responsiveness, histological grade of differentiation, expression of the androgen receptor (AR) and of prostate specific markers, prostatic acid phosphatase (PAP) and prostate specific antigen (PSA).

Apoptosis in the Prostate

Growth rate of tumors in general is related not only to cell proliferation but also to the rate of (apoptotic) cell death (Dive and Wyllie, 1993). Apoptosis or programmed cell death (Wyllie, 1980) is a process of major interest and a subject of an increasing number of studies in a variety of normal and malignant tissues, including (hormone-responsive) prostate cancer (Howell, 1997). Androgens, besides having the well-established agonistic ability to stimulate prostate cell proliferation, also have an antagonistic ability to inhibit prostatic cell death (Isaacs, 1984). Apoptosis is important with regard to prostate cancer because this is the mechanism by which prostate cancer cells die in response to androgen withdrawal, irradiation, or chemotherapy. Following castration-induced androgen deprivation the rat ventral prostate rapidly involutes with as many as 80 percent of cells being lost within the first 10 days after castration (Lee, 1981). Androgen ablation also inhibits cell proliferation and promotes apoptotic

death in experimental human prostate cancer (Van Weerden *et al.*, 1993), and increases the apoptotic index in clinical prostate cancer (Westin *et al.*, 1995).

The death of androgen-dependent ventral prostatic glandular epithelial cells involves a cascade of biochemical changes characteristic for apoptosis (Kyprianou and Isaacs, 1988). A similar series of events, leading to (programmed) cell death in human prostate cancer tissue, has been described for the human prostate cancer xenograft, PC-82 (Kyprianou *et al.*, 1990). The molecular mechanism(s) for androgen ablation-induced glandular epithelial cell death is not very clear, but cell proliferation, DNA repair, and p53 function do not appear to be involved (Berges *et al.*, 1993). Although conflicting data were presented by Colombel (1992) with respect to the role of p53.

Bcl-2 and Prostate Cancer

Among the varies genes that are demonstrated to be implicated in the apoptotic program, also termed as the 'reactive cascade' (Buttyan *et al*, 1988) is also bcl-2 known to be a inhibitor of apoptosis (Korsmeyer, 1992). Bcl-2 is expressed in approximately 1 of 4 primary prostate cancers (McDonell et al., 1992; Bauer et al., 1996), although higher figures (up to 60% of positive cancers) have been reported by Colombel (1993). Increased expression of bcl-2 was found in androgen independent disease, implying that prostate cancers that express bcl-2 may be less responsive to endocrine treatment. It is tempting to speculate that the resistance of prostate cancers to undergo apoptosis and to be treated effectively by cytostatic drugs is determined either by increased expression of cell death blocking factors like bcl-2, or by decreased expression of death-promoting gene products.

Scope of this Chapter

This paper describes the some functional characteristics of a series of androgen-dependent and independent human prostate xenografts, and it provides information on the effect of androgen manipulation on proliferation, the occurrence of apoptosis models and on the expression of bcl-2 in these model systems.

In addition, data are presented about androgen-induced cell proliferation and cell death in the hormone-sensitive human prostatic tumor cell line LNCaP in vitro. We previously showed that androgens exert a biphasic response on growth of the LNCaP cell line and that relatively high dosages of androgen inhibited growth of LNCaP cells (Langeler *et al*, 1993). The present in vitro study showed that this androgen-induced growth inhibition has the characteristics of apoptosis. The origin of an 'apoptosis-resistant' subline of the parental LNCaP cell line is described.

Materials and Methods

In vivo Studies with Prostatic Xenografts

Transplantation of tumor tissue is routinely carried out by implanting of small tissue fragments derived from freshly obtained prostate tumor specimens into athymic nude mice (Van Steenbrugge *et al*, 1984). Tumor tissue was grafted subcutaneously

in athymic nude mice of the Balb/c or NMRI strain (derived from the breeding colony of the central animal facilities of Erasmus University). Details about the technique of transplantation, performed under light ether anesthesia, and the way tumor growth was monitored were described previously (Van Steenbrugge *et al*, 1984), as were the properties of the extensively studied PC-82 and PC-EW tumors (Hoehn *et al*, 1980; Hoehn *et al*, 1984; Van Steenbrugge *et al*, 1984a).

Hormonally manipulated mice received Silastic implants (Talas, Zwolle, The Netherlands) filled with crystalline steroid, providing constant levels of hormone for longer periods of time (Van Steenbrugge *et al*, 1984b). This method also facilitates hormonal withdrawal and (re)substitution of androgens in tumor-bearing castrated male or female mice. Castration was carried out via the scrotal route under anesthesia with tribromoethanol (Aldrich, Beerse, Belgium).

Immunohistochemistry

Tissue sections of formalin-fixed paraffin-embedded tumor specimens were used for routine histological examination as well as for immunohistochemical staining using an indirect peroxidase-antiperoxidase method with the following monoclonal antibodies, p53 (antibody DO-7 reactive with wild-type and mutant protein) and bcl-2 (clone 124), both purchased from Dakopatts, Denmark; F39.4.9 an anti-human androgen receptor (AR) monoclonal antibody provided by the Dept. of Pathology, Rotterdam. For staining of p53, bcl-2 and AR the technique of antigen retrieval using a microwave equipment was applied. The frequency of apoptotic events occurring in xenografts was estimated in tissue sections using the TUNEL method described by Wijsman et al. (1993).

The LNCaP Cell Line and Apoptosis Detection in vitro

The androgen dependent human prostate cell line LNCaP-FGC, which was derived from an early passage of the LNCaP cultures (Horoszewicz *et al*, 1980), was obtained from Dr. Julius Horoszewicz. The cells were routinely cultured in RPMI-160 medium (Life Technologies, Breda, The Netherlands) supplemented with 7.5% fetal bovine serum (Hyclone, Logan, Utah, U.S.A.) and glutamine, penicillin and streptomycin (Van Steenbrugge *et al*, 1991). Androgen-depleted medium contained 5% dextran-coated charcoal (DCC)-treated serum. For the present experiments LNCaP cells of passage 70-80 were used. The growth characteristics of these (p70) cells under various hormonal conditions have previously been described (Langeler *et al*, 1993). Androgenic effects on LNCaP growth were tested with the synthetic, non-metabolizable androgen R1881 (methyltrienolon; New England Nuclear, Boston, MA, U.S.A.).

Apoptosis in LNCaP cell cultures was determined by application of the assay of cell viability based on simultaneous staining of LNCaP cell cultures *in situ* with propidium iodide (PI) and Hoechst 33342 (Polysciences, Warrington, U.S.A.). This method provides a means to discriminate between live, necrotic, early- and late apoptotic cells (Pollack and Ciancio, 1991). In addition, a 'Cell Death Detection Elisa' (Boehringer, Mannheim, Germany) was applied in an attempt to quantify the occurrence

of apoptosis in LNCaP cell cultures. This assay is based on the quantitative *in vitro* determination of cytoplasmatic histone-associated DNA fragments (mono- and oligonucleosomes). For both methods, camptothesin (CAM) treated cells of the human myelogenous leukemic cell line HL-60 were used (Del Bino *et al*, 1990) as control, apoptotic cells. Finally, LNCaP cell cultures treated with high dosages of R1881 were processed for transmission microscopy studies.

Results and Discussion

A Panel of Human Prostate Tumor Xenograft Models

In our institution during a period of more than ten years (1977-1990) almost 200 clinical specimens were transplanted in Balb/c nude mice, resulting in a very low number of permanent tumor models: the hormone-dependent PC-82 model, two hormone-independent tumors: PC-133 and PC-135 and more recently, the hormone dependent PC-295 tumor. The PC-82 was established about 20 years ago, and was the first hormone-dependent xenograft model described in literature. Another hormone-dependent model, PC-EW, was founded by Hoehn and coworkers (Hoehn *et al*, 1984) and was also included in the panel of models used.

About three years ago, for technical reasons, we started to use the NMRI strain of nude mice as host animal for heterotransplanting prostatic carcinomas. Remarkably enough, this resulted in a considerably increased take rate: within a period of two years 6 out of 19 transplants had a positive take, leading to the development of permanent tumor lines. These models originated from primary prostatic carcinomas (derived from prostatectomy specimens), from progressive, hormone refractory tumors (tissues derived from transurethral resection material) and from metastatic lesions (lymph nodes as well as skin).

Among this group of seven (including PC-295) newly established xenografts, clearly two subgroups of models can be recognized: differentiated, androgen-dependent tumors which express the androgen receptor (AR) and which express and secrete Prostate Specific Antigen (PSA) (PC-295, PC-310 and PC-295) and two undifferentiated, androgen-independent tumors which are devoid of androgen receptors and did not express or secrete PSA. The newly established androgen dependent tumor models PC-295, PC-310 and PC-329 all showed a reduction in tumor volume after androgen ablation. The hormone independent PC-324 and PC-339 models grow equally well in male and female mice. Although not applied in the present study, tumor PC-346 is a very interesting model as it continued to grow after an initial period of tumor regression following androgen withdrawal. Therefore, it is the first human xenograft model showing the clinically so important relapse phenomenon.

Cell Death in the Androgen Dependent PC-82 and PC-EW Models

Volumes of PC-82 and PC-EW tumors declined after castration of tumor-bearing mice, the regression of the PC-EW tumor being faster than that of the PC-82 tumor (half-life of 6 and 18 days, respectively (Figure 1). The resultant decline of the

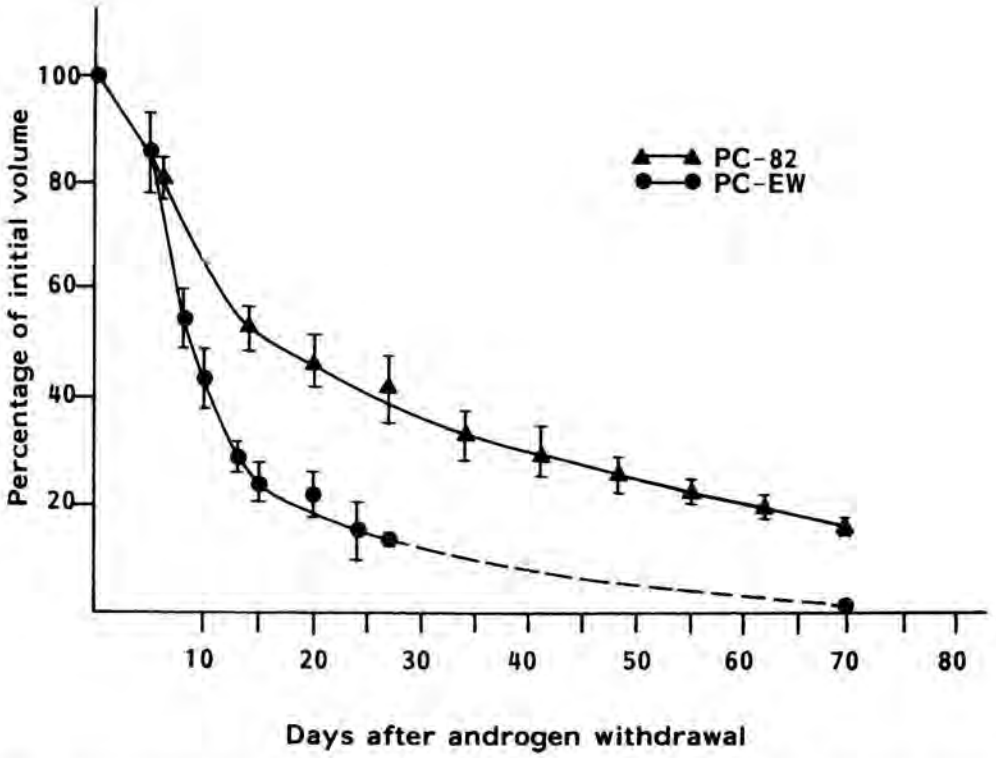

Fig. 1. Patterns of regression after androgen withdrawal from PC-82 (n=8) and PC-EW (n=7) tumor-bearing nude mice.

PC-82 tumor nodules after castration was associated with a decrease in cells incorporating BrdU (Van Weerden *et al*, 1993). Concomitantly, an increase in the number of apoptotic bodies (scored on basis of their morphological appearance) could be observed, which reached its maximum at 4 days post-castration (Table I). These observations are in agreement with previously reported results of the PC-82 tumor (Kyprianou *et al.*, 1990). The regressing PC-82 tumors did not show any sign of necrotic death, whereas androgen depletion of the androgen-dependent PC-EW tumor induced both apoptosis and necrosis. Regressing PC-EW tumors showed widespread necrosis with clusters of apoptotic cells (Table I). As a consequence of the necrotic cell death, the PC-EW tumor regressed completely (within 3-4 weeks), leaving small nodules consisting of fibrotic tissue. Whether androgen depletion-induced regression of PC-EW represents a type of secondary necrosis following an apoptotic response could not be assessed as yet. Regressing PC-EW tumors could not be restimulated by androgens even after a relatively short period (2 weeks) of androgen depletion. By contrast, in regressing PC-82 tumors viable cells remain present, which could be restimulated to grow by androgens even after long-term (over 6 months) androgen depletion (Van Weerden *et al*, 1993). Spontaneous, i.e. androgen-independent, regrowth

Table I. Cell death in regressing PC-82 and PC-EW tumors.

Time after castration	PC-82 % apoptosis	PC-82 % necrosis	PC-EW % apoptosis	PC-EW % necrosis
0 (days)	1.3 ± 0.6 (5)	< 10	1.6 ± 0.9 (5)	10
4	10.8 ± 3.0 (5)	< 10	5.4 ± 2.6 (5)	30
7	7.2 ± 3.0 (4)	< 10	2.9 ± 1.1 (4)	30
15	2.8 ± 0.2 (3)	< 10	2.4 ± 1.0 (5)	50
30	1.9 ± 1.0 (5)	< 10	2.2 ± 1.3 (4)	80

Data are expressed as mean ± SD, with number of samples in parentheses.

of these long-term androgen deprived PC-82 tumors has never been observed, however. This androgen sensitive but 'apoptosis-resistant' PC-82 tumor cell population possibly represents an intermediate step between androgen-dependence and independence.

Effects of Hormonal Manipulation on Proliferation and Cell Death in Prostate Xenografts

An androgen depletion-repletion protocol (i.e. castration for 14 days and testosterone resubstitution for 7 days) was applied to two androgen dependent (PC-295 and PC-310) and two androgen independent (PC-324 and PC-339) xenografts. In tumors PC-295/310 a clear apoptotic response (detected by the TUNEL method) was seen, concomitantly with diminished proliferative activity (table II). In these models the expression of immunohistochemically determined prostatic markers, AR and PSA was completely absent at 14 days after androgen withdrawal, whereas re-expression was seen after 7 days of androgen resubstitution. In contrast to tumor PC-295, irrespective the hormonal status of the host animal, no bcl-2 expression was observed in PC-310 (table II). Androgen withdrawal in PC-295 resulted in a increased overall expression of bcl-2. Neither the proliferative activity nor occurrence of apoptotis were significantly affected in the androgen independent PC-324 and PC-339 tumors (table II). These tumors were devoid of AR and PSA expression. In addition, these tumors demonstrated overexpression of *p53* at the immunohistochemical level, that is nuclei of over 90% of the cells were intensely stained. Interestingly enough, the same two hormone-independent tumors with *p53* overexpression were demonstrated to be also strongly immunoreactive for *bcl-2*. Although the *bcl-2* expression may be a differentiation related phenomenon, in these tumors with *p53* overexpression the intense *bcl-2* staining is most likely the result of a constitutive *bcl-2* expression. Based on these results, which were in agreement with literature data, it was concluded that tumors PC-324 and PC-339 represent late stage, hormone refractory disease.

Androgen-induced Cell Death an in vitro Prostatic Cell Line

In steroid-depleted (DCC) medium androgen dependent LNCaP-FGC (FGC) cells continued to grow at a decreased rate. Maximal growth was induced with the addition of 0.1 nM of the synthetic, non-metabolizable androgen R1881, whereas the

Table II. Effect of androgen manipulation on proliferation, apoptosis, and some functional markers in human prostate cancer xenografts

Tumor model	AD	STATUS	AR (+/-)	PSA (+/-)	BCL-2 (+/-)	APOP (%)	BrdU (%)
PC-295	+	control:	+	+	+/-	5	17
		castr:	-	-	+	25	0
		castr+T:	+	+	+	13	14
PC-310	+	control:	+	+	-	9	13
		castr:	-	-	-	16	0
		castr+T:	+	+	-	8	13
PC-324	-	control:	(+)	-	+	9	48
		castr:	-	-	+	7	42
		castr+T:	(+)	-	+	7	41
PC-339	-	control:	-	-	+	6	23
		castr:	-	-	+	11	20
		castr+T:	-	-	+	8	46

The post-castration period was 14 days (PC-295: 4 days), whereafter androgen (testosterone (T)) was resubstituted for 7 days. Data were based on 4 animals per treatment group. Immunohistochemical scores were semiequantitative estimates. Proliferative (BrdU) and apoptotic (APOP) indices were based on scores of 1000 cell count in random high power field.
AD: androgen dependence; AR: androgen receptor; PSA: prostate specific antigen.

addition of 100-fold (10 nM) of R1881 to medium with DCC led to a decrease in cell number (Langeler *et al*, 1993). In these high dose R1881-treated cultures a high rate of cell death was found. It appeared that all dying cells were detached and that attached cells were vital and continued to grow. Staining of such cultures *in situ* with a combination of the DNA binding dye, Hoechst 33324, and propidium iodide (PI), clearly demonstrated that the detached cells had features indicative for apoptosis. Specifically, cytoplasmic contraction, condensation of nuclear chromatin and the formation of membrane-bound 'apoptotic bodies' were observed. Apoptotic events were also confirmed by electron- microscopical examination of high dose R1881-treated cells. Interestingly, in the R1881-treated cultures initially blue (i.e. intact), PI excluding apoptotic cells were found, whereas gradually cultures also showed increasing amounts of cells with red luminescence that lost their membrane integrity, but which also had apoptotic features. Apparently, with this method early and late apoptotic cells could be detected.

Estimates were made of the percentage LNCaP cells that were recognized as being apoptotic based upon their pattern of DNA Hoechst stain and the number of death cells that had a positive stain with PI. From the time-course experiment shown in Figure 2, it can be seen that androgen-induced apoptosis is a relatively slow process. The peak of 18 percent apoptotic cells in 10 nM R1881-treated cells was reached about 6 days after start of treatment (Figure 2A). From this graph it can be read that in cultures grown with 0.1 nM R1881, a dosage which optimally stimulates growth of LNCaP cells, also a relatively high percentage of apoptotic cells was found. By

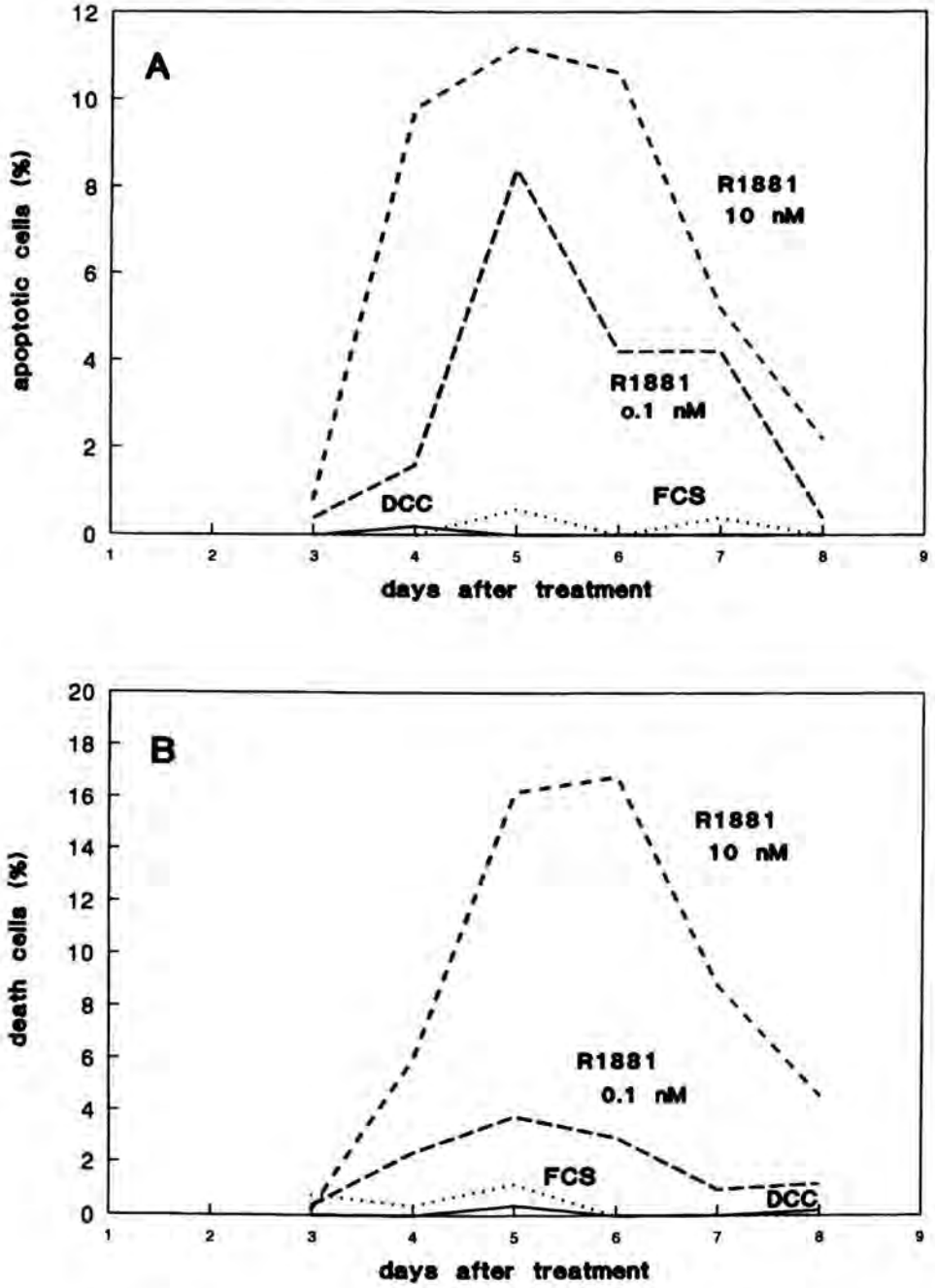

Fig. 2. Apoptotic cell death in LNCaP cells cultured in medium with complete serum (FCS), androgen-depleted serum (DCC) and DCC supplemented with 0.1 and 10 nM of the synthetic androgen R1881. Cultures were stained with propidium iodide (A) and the DNA binding dye, Hoechst 33324 (B), respectively.

Table III. Androgen (R1881)-induced cell death in the LNCaP prostatic cell line.

Conc. R1881	Enrichment Factor* (DCC = 0)		
	Day 5	Day 6	Day 7
100 nM	12.9	37.0	37.7
10 nM	2.7	59.7	73.1
1 nM	1.3	29.7	21.9
0.1 nM	2.9	43.0	19.3

* Enrichment factor of nucleosomes in the cytoplasma of LNCaP cell treated with different concentrations of the synthetic androgen R1881.

contrast, cultures grown in medium with either complete serum or androgen-depleted (DCC) serum had a very low frequency of apoptotic cells. This should be considered as spontaneous cell death occurring in all cell cultures. As described in one of the previous sections, withdrawal of androgens from the rat ventral prostate or from the androgen-dependent human PC-82 tumor in vivo, resulted in the induction of apoptosis (Kyprianou and Isaacs, 1988; Kyprianou *et al*, 1990). This apparently does not apply to the androgen- sensitive human LNCaP cell line in vitro, as in androgen-depleted medium, growth of the cells was only retarded and no increase of apoptosis was observed (figure 2).

The patterns of occurrence of PI positive cells under the different hormonal conditions (Figure 2B) paralleled those found for apoptotic cell with yet intact membranes (Figure 2A), except that the level of death cells found in cultures treated with 0.1 nM R1881 was considerably lower compared to that observed in cells cultured in the high concentration of 10 nM R1881.

The occurrence of apoptosis in the LNCaP cell cultures could also be quantified by the use of a photometric enzyme immunoassay (Cell Death Detection Elisa). This test is based upon the determination of cytoplasmatic histone-associated DNA fragments present in cells that undergo the process of apoptosis. Samples of the time course experiment with LNCaP cultures were measured in this Elisa. The nucleosome enrichment factor in androgen treated cell cultures, relative to that found for the DCC cultured cells, clearly increased in time and in a dose dependent manner (Table III). Although also in the cultures with low androgen concentrations, increased nucleosome concentrations, i.e. DNA fragmentation, was found, also with this method the highest rate of apoptotic cell death was observed with 10 nM R1881.

The observed effect of high androgen-induced apoptosis was not restricted to the synthetic androgen R1881. Although the natural androgen Dihydrotestosterone (DHT) was rapidly metabolized by the LNCaP cells, daily exposure of high dosages (1uM) of DHT resulted in similar patterns of cell death as observed for R1881. DHT-induced cell death was clearly observed after 14 days, which was even later when compared to R1881-treated cell cutures. Apparently, the cell death promoting effect of high dose androgens is a real androgenic effect, which is not restricted to synthetic

androgens. It is not yet known whether this phenomenon is mediated by the androgen receptor, or that the membrane is involved.

Not the entire culture of high dose R1881-treated cells died, and a small surviving fraction of cells continued to growth after 3 weeks of exposure. The resulting cell culture developed into a fast growing cell line with a distinct cellular morphology compared to the parental line. The cells of this subline could not be driven into apoptose by high dosages of androgen, and with this regard were considered 'apoptosis-resistant'. In contrast to the original LNCaP cells, which are poorly transplantable in athymic nude mice, cell of the developed subline were highly tumorigenic.

Conclusions

Further studies of this panel of prostatic xenograft models may result in a better understanding of the biochemical pathway involved in programmed cell death in prostate cancer. In addition it may yield information about the importance of apoptosis as a mechanism implicated in the progression of clinical prostate cancer, i.e. the transition of androgen-dependent towards androgen-resistant prostate cancer.

Androgen-induced cell death in the LNCaP prostatic carcinoma cell line was demonstrated to be apoptotic cell death. The present results obtained with LNCaP cultures subtantiate the proposed dual action of androgen: depending on the concentration applied, cell proliferation can be stimulated and apoptotic cell death may be induced.

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Bcl-2 Family Members and p53 in the Regulation of Apoptosis in Invasive Breast Cancer

Introduction

The administration of chemotherapy to women with metastatic breast cancer (BC) has significantly prolonged disease-free survival, but its impact on overall survival has been disappointing (Early Breast Cancer Trialist's Collaborative Group 1996). Women who at the time of diagnosis have no detectable metastases in axillary lymph nodes and/or at distant sites — generally refered to as node-negative patients — have a high probability of being cured by surgery alone. However, in about one-third of these patients the disease will recur due to the outgrowth of previously undetected micro-metastases. Adjuvant chemotherapy can significantly prolong the mean disease-free survival of node-negative patients, but the dilemma is that treating all these patients with chemotherapeutic drugs results in serious overtreatment.

It is well-established that tumor size and certain histological characteristics (size of the nuclei, differentiation grade and proliferative activity) are sufficiently compelling to make treatment decisions relatively straightforward in approximately half of node-negative BC patients — these patients have either an excellent or a very poor prognosis. But, in the intermediate group, recurrence rate is still 30% and more prognostic information is needed here to identify the high-risk patients (Rosen et al., 1993). With this goal in mind, many studies have attempted to determine which (combination of) tumor characteristics would allow reliable identification of these patients. At present, at least 80 putative prognostic markers have been reported, but most factors are relatively new and from many of these the value has not been fully established. In fact, many factors are somewhat related to the classical histopathological parameters (viz. tumor size, tumor grade and lymph node-status) and to each other and therefore add little new information.

A problem of equal clinical importance is that approximately one-third of BC patients treated with adjuvant chemotherapy does not respond to this treatment. It is obvious that, in order to design optimal treatment strategies for individual patients (whether they have positive nodes or not), it is essential to be able to predict a tumor's response to a specific therapy. Patients likely to be resistant to (a certain combination of) chemotherapeutic drugs might then be selected for treatment with a different and/or more aggressive regimen. But whereas the presence of estrogen and progesteron receptors in BC is widely used to decide whether or not patients should

be given (anti)hormonal therapy, at present no such markers are used to predict response to specific forms of chemotherapy; reliable predictive factors clearly would be of great value for the management of both node-positive and node-negative patients.

Thus, important clinical questions concerning the treatment of BC patients are: (i) which prognostic factors can properly separate the majority of low risk subsets of node-negative patients from those at high risk? (ii) what is the minimal level of risk that justifies systemic adjuvant therapy for node-negative patients? (iii) which factors predict response to chemotherapy and/or hormone therapy or to novel therapeutic approaches in both node-positive and node-negative patients.

Because the development of most clinically used anticancer drugs has been empirical, the molecular mechanisms that determine treatment efficacy remain largely unknown. A more complete understanding of cellular sensitivity c.q. resistance to anti-cancer therapy requires elucidation of mechanisms by which anticancer drugs cause tumor cell death. As outlined by Hickman and co-authors elsewhere in this volume, cellular damage caused by cytotoxic treatments seems insufficient to explain observed anti-tumor effects. Together with the discovery that transformed cells are inherently sensitive to induction of apoptosis by anticancer treatments this has lead to a paradigm shift in our thinking on drug-sensitivity: the majority of drugs used in chemotherapy are now believed to act by inducing a cell death program within tumor cells rather than by disrupting DNA replication or inflicting cellular damage incompatible with cell survival. The sensitivity to induction of apoptotic cell death of certain transformed cell types relative to normal cells may be the main reason for selective killing by radiation or cytotoxic drugs; if this sensitivity is reverted by mutations that disrupt certain apoptotic pathways, tumors may become resistant to a particular type of treatment (Kerr et al., 1994; Reed, 1994). Thus, a detailed understanding of the molecular mechanisms that determine the commitment of a cell to the induction of apoptosis may be crucial for the prediction of treatment responses.

An important reason for the relative paucity of clinical data regarding tumor responsiveness to chemotherapy is that most clinical trials do not contain a control group of untreated patients. When all treatment-arms in a trial include some form of adjuvant therapy, the association of a given marker with poor survival can be an indication of aggressive tumor growth or of increased chemoresistance or both. Our studies on material from EORTC Trial 10854 gave us the opportunity to circumvent this problem by selecting a subset of premenopausal node-negative patients randomized to receive either one course of peri-operative chemotherapy with a combination of 5-fluorouracil, epirubicin and cyclophosphamide (1xFEC) or no such adjuvant treatment (Van Slooten et al., 1996; Clahsen et al., in press). In a second study we used material from EORTC Trial 10902 in which patients with operable BC were randomized to receive either pre- or post-operative chemotherapy. This enabled us to directly assess primary tumor response (defined by clinical measurements as well as a mammographical assessment of response) and correlate this response with the expression of a number of molecular markers, including two factors involved in regulation of apoptotic cell death, Bcl-2 and p53.

The Bcl-2 Gene Family in the Genesis of Breast Cancer

The Bcl-2 proto-oncogene, which was initially identified on the basis of the t(14:18) chromosomal translocation found in human B-cell follicular lymphomas (Tsujimoto et al., 1984), is the founding member of a family of proteins comprising proteins that can either repress (e.g. Bcl-2, Bcl-Xl, CED-9, Mcl-1, Bcl-W, and A1) or promote apoptosis (e.g. Bax, Bcl-Xs, Bad, and Bak) (Korsmeyer, 1995). The members of this family possess one or more of four homologous domains – invariant residues within these regions are essential for their respective functions (Yin et al., 1994).To date, most research has been focussed on Bcl-2 demonstrating that overexpression protects cells against induction of apoptosis by a variety of stimuli, including irradiation and most clinically used chemotherapeutic drugs (Campos et al., 1993).

Many Bcl-2 family proteins can physically interact with each other, forming a complex network of homo- and heterodimers. Recent data have shown that homodimerization of Bax, and not heterodimerization with Bcl-2, seems essential for its apoptosis-inducing capacity and that Bcl-2 can rescue cells from the lethal effects of Bax without heterodimerizing with it (Zha and Reed, 1997). While an *in vivo* competition exists between Bcl-2 and Bax — as will be discussed in more detail in chapter 15, the ratio of these proteins ultimately determines the cellular sensitivity to the induction of cell death — each seems to be able to regulate apoptosis independently (Knudson & Korsmeyer, 1997) and also Bak can accelerate chemotherapy-induced cell death independently of its heterodimerization with Bcl-Xl and Bcl-2 (Simonian et al., 1997).

A number of Bcl-2 family members may function as pore-forming proteins, reminiscent of bacterial toxins like diphteria toxin (Schendel et al., 1997). One concept is that Bcl-2 and Bcl-Xl directly or indirectly block the mitochondrial release of cytochrome c (Kharbanda et al., 1997; Yang et al., 1997; Kluck et al., 1997, Kim et al., 1997), which is involved in the activation of proteases (caspases) required for the execution/degradation phase of the apoptotic process (Zou et al., 1997). But although inhibition of the cytochrome c-induced cascade of caspase activities could be an important function of anti-apoptotic Bcl-2 family members, Li et al. recently provided evidence that in some cells cytochrome c release is not required to induce cell death and that in these cells Bcl-Xl is still able to inhibit cell death induced by Fas or tumor necrosis factor (Li et al., 1997b).

Nevertheless, the relative expression levels of pro- and anti-apoptotic Bcl-2 family members may regulate the level of caspase activity and by doing so determine the cells' sensitivity to apoptotic stimuli. However, Bcl-2 may not only act upstream of caspases, but can also be a target of these enzymes: a remarkable recent finding is that caspase-3 can convert Bcl-2 into a Bax-like death effector (Cheng et al., 1997). Moreover, Bcl-2 can also bind to a variety of cytoplasmic proteins (Reed, 1994), including the calcium-binding protein calcineurin. As a result of latter interaction, calcineurin is unable to promote the nuclear translocation of transcription factor NF-AT, which is required for expression of interleukin-2 and other cytokines involved in T-cell proliferation (Shibasaki et al., 1997).

Under certain conditions, induction of apoptosis seems to depend on cell cycle progression and the activity of proteins involved in cell cycle regulation: e.g., cyclins

and cyclin-dependent kinases (Kranenburg et al., 1996) or their inhibitors (Wang et al., 1997). In that context it is intriguing that overexpression of Bcl-2 has been found to promote the departure of cells from the cell cycle, and to inhibit the transition from a resting to a cycling state (Pietenpol et al., 1994; Mazel et al., 1996). This property has also described for Bcl-X_L and E1B19kD and seems to be molecularly separable from the anti-apoptotic function of these proteins (Huang et al., 1997).

Recent data suggest a possible link between Bcl-2 and Bax and $p27^{KIP}$, an inhibitor of cyclin dependent kinases that functions as a 'brake' in the cell cycle. In thymocytes, which are normally quiescent, $p27^{KIP}$ needs to be degraded in order to get apoptosis — this degradation seems to be enhanced by Bax, whereas Bcl-2 does the opposite (Gil Gómez, personal communication).

Because of its importance as an inhibitor of apoptosis, we hypothesized that Bcl-2 might be useful as a molecular marker for both prognosis and treatment response in BC. In normal human breasts, high Bcl-2 expression is observed in the lobular ducts, whereas intralobular ducts show remarkable variability of Bcl-2 expression, possibly reflecting the hormonal regulation of Bcl-2 (Van Slooten et al., 1996). Bcl-2 is preferentially expressed in well-differentiated, estrogen receptor-positive breast tumors and associated with good prognosis (Bhargava et al., 1994; Gee et al., 1994; Joensuu et al., 1994; Leek et al., 1994; Gasparini et al., 1995; Hellemans et al., 1995; Silvestrini et al., 1996). In invasive BC, we and others observed a strong inverse correlation between Bcl-2 and proliferative activity (Joensuu et al., 1994; Silvestrini et al., 1994; Van Slooten et al., 1996; Van Slooten et al., in press). In line with these findings our data show that, both in invasive BC and ductal carcinoma *in situ*, lack of Bcl-2 expression is strongly associated with increased levels of apoptosis, high proliferative activity and high tumor grade, suggesting the existence of high levels of cell turnover in tumors of patients with poor prognosis.

Also in other tumor types a correlation seems to exist between high proliferative activity and high apoptotic activity and high tumor grade (Lipponen and Aaltomaa, 1994; Aihara et al., 1995; Du et al., 1996; Isacson et al., 1996; Koshida et al., 1996; Shoji et al., 1996). It is tempting then to hypothesize that these data reflect the connection between Bcl-2 expression, decreased growth fraction and tumor cell differentiation. In theory, a direct link between protection against apoptosis and a reduction of proliferation could function as a hardwired defense protecting cells against oncogenic transformation, complementary to the induction of apoptosis/senescence by oncogenic mutations. The existence of such a dual mechanism could explain the finding that in many cancer types Bcl-2 is preferentially expressed in slowly proliferating, well-differentiated tumors associated with good prognosis. It would also provide an explanation for the counter-intuitive finding that in many cancer types, loss of Bcl-2 expression — in spite of the resulting increase in tumor apoptosis — is associated with tumor progression. Long-lived cells with increased resistance to apoptosis pose a potential hazard to a multicellular organism — they are much more likely than short-lived apoptosis-sensitive cells to sustain and survive the multiple mutations needed for transformation into a cancer cell.

The importance of reducing the proliferative potential of long-lived cells may be demonstrated by the high rate at which tumors develop from breast epithelial

cells. These cells are present in the body over a long period of time, but also have to retain significant proliferative potential and may therefore be more susceptible to transformation than other long-lived cells.

P53 and Clonal Evolution in Breast Cancer

The p53 tumor suppressor gene is among the most frequently mutated genes in human cancer (Hainaut et al., 1997). In BC, mutations have been reported to occur in 15-50 % of cases, depending on the stage of the disease and the method of detection (Pietilainen et al., 1995). The p53 gene encodes a phosphoprotein which has been shown to play a critical role in controlling cell proliferation, cellular differentiation and apoptosis (Ko and Prives, 1996). The levels and activities of wildtype (wt) p53 have been shown to increase in response to irradiation and DNA damaging agents — high levels of p53 may induce apoptosis, whereas relatively low levels cause cell cycle arrest (Chen et al., 1996).

As was discussed in a previous chapter, loss of p53 function is an important factor in tumor development and progression, presumably through reduced tumor apoptosis and/or reduced induction of senescence (Howes et al., 1994; Symonds et al., 1994; Serrano et al., 1997). In response to DNA damage, p53 is involved in activation of DNA repair mechanisms and may prevent expansion of cells with damaged DNA by inducing apoptosis or senescence. The latter has been found to be dependent on elevated expression of the p53-inducible gene p21/WAF1 (Brown et al., 1997).

Animal work clearly demonstrated that inactivation of p53 is associated with treatment resistance (Lowe et al., 1993a and 1993b; Merrit et al., 1994), but at present no unequivocal evidence has been obtained of the existence of a similar association in invasive BC.

The mechanisms by which wt-p53 protein induces apoptosis are not completely understood, but p53-mediated upregulation of Bax, downregulation of Bcl-2 and activation of caspase-3 are likely to be involved (Selvakumaran et al., 1994; McCurrach et al., 1997). Bax seems to be an important downstream-effector of p53 (Yin et al., 1997) and its importance is highlighted by the discovery of frameshift mutations within the *bax* gene in subsets of colorectal and gastric cancers (Rampino et al., 1997; Yamamoto et al., 1997). In invasive BC, mutation of p53 is negatively correlated with immunodetected Bcl-2, but no significant correlation exists between Bax and p53 status (Van Slooten et al., in press).

Increased genetic instability leading to accumulation of genetic alterations may be one of the most important factors driving tumor progression in BC. In colon cancer, inactivation of p53 seems to take place prior to the occurrence of major DNA rearrangements and aneuploid clonal divergence, which is in line with the concept that inactivation of p53 allows proliferation of cells with a damaged genome, and enables the genesis of aneuploid tumor subclones. Also in BC, loss of p53 function is associated with rapid tumor growth, aneuploidy, high tumor grade and with poor prognosis (Thor et al., 1992; Allred et al., 1993; Silvestrini et al., 1996). Moreover, mutations affecting the zinc-binding domains of the gene, critical for DNA binding

of p53 (L2-L3), where reported to be associated with a particularly poor prognosis (Børresen et al,. 1996) and possibly with increased chemoresistance (Aas et al., 1996). We hypothesized that such an aggressive tumor phenotype would be characterised by increased levels of proliferation and/or reduced levels of apoptosis. However, no such phenotypical differences existed between p53 mutations inside or outside the zinc-binding domains (Van Slooten et al., submitted). Unexpectedly and in contradiction with its proposed function as 'guardian of the genome', in BC loss of p53 function was associated with increased rather then decreased levels of apoptosis. The presence of a simultaneous increase in the level of mitosis suggests the existence of increased cell turnover in p53-mutated tumors.

In line with these results it has been found that after crossing p53-deficient mice with mammary tumor-susceptible Wnt-1 transgenic mice, the off-spring all developed mammary tumors, and did so much sooner than their $p53^{+/+}$ counterparts. p53-deficient tumors had high average growth rates, i.e., showed much higher percentages of mitotic figures, but, remarkably, also no decrease in percentages of apoptotic cells compared to $p53^{+/+}$ tumors (Jones et al. 1997). Together with our own data this suggests that p53 mutations are associated with the development of a phenotype with accelerated cell turnover. The possibility that p53-deficient tumors are the end-result of many more cell divisions (thereby enhancing the mutation rate), combined with the supposed role of p53 in preventing chromosomal abberations, may contribute to rapid clonal evolution and thereby the generation of aggressive and/or treatment-resistant variants. Our finding of similar associations between p53, proliferation and apoptosis in ductal carcinoma *in situ* suggests that the development of such a dangerous phenotype may be an early event in BC. Whereas the observed increase in p53-independent tumor apoptosis may result from a loss of cell cycle control, these tumors may in fact be quite resistant to induction of p53-dependent apoptosis, e.g. as induced by genotoxic treatments.

Interestingly, this situation could be very similar for colon cancers; Fazeli and coworkers recently reported that the appearance of highly dysplastic cells in human late-stage colon adenomas, which usually coincides with loss of heterozygozity at the p53 locus, did not correlate with any reduction in the incidence of apoptosis and it was proposed that wt-p53 retards the progression from benign adenoma to malignancy by mechanism(s) other than the promotion of apoptosis (Fazeli et al. 1997). It seems relevant in this context that very low levels of wt-p53 protein have been shown to protect cells from apoptosis rather than facilitate this process (Lassus et al. 1996). It has been found that a link may exist between loss of estrogen receptor, genomic lesions in the p53 gene as well as in genes involved in hereditary BC (BRCA1 and BRCA2 respectively), and high fractional allelic loss (a measure of genetic instability), being all associated with poor prognosis (Sato et al., 1991; Schmutzler et al., 1997). Also, molecular changes in the signaling pathway from tyrosine kinase receptors (like epidermal growth factor receptor and c-erbB-2), which involves oncogene products such as Ras and the Raf-1 protein kinase, can induce estrogen-independent growth and, in the case of Raf-1 activation, apoptosis (El-Ashry et al., 1997). In a mouse model of mammary tumorigenesis, c-*erb*B-2/neu has been demonstrated to cooperate with mutant p53, resulting in the development of high grade, aneuploid

Table I. Four-year Disease-Free Survival and Hazard Ratios for Treatment Effect by p53 and Bcl-2 scores.

Parameter	PeCT		Control		HR (95% CI)	P value
	N/O	DFS (%)	N/O	DFS(%)		
p53 Negative	191/28	88.0	168/43	77.0	0.51 (0.31-0.82)	<0.01
Positive	39/10	76.0	42/10	80.0	1.12 (0.47-2.70)	0.80
Bcl-2 Low score (0-2)	60/14	79.5	52/18	67.6	0.55 (0.27-1.12)	0.09
High score (3-6)	161/21	87.0	150/31	81.0	0.61 (0.35-1.06)	0.07

N = total number of patients; O = number of events; DFS, disease-free survival; HR, hazard ratio; CI, confidence interval; PeCT, peri-operative (poly)chemotherapy.
(P53 data: Clahsen et al., 1998; Bcl-2 data: Van Slooten et al., 1996).

tumors, exhibiting increased proliferation and apoptosis, in contrast to tumors arising in p53-null mice, which show reduced apoptosis (Li et al., 1997). In addition, in Non-Hodgkin lymphomas increased levels of apoptosis and proliferation were reported to exist in the subset of Bcl-2 negative lymphomas with mutated p53, which are associated with poor prognosis (Takano et al., 1997).

Therefore, in BC, loss of p53 function and loss of expression of estrogen receptors and Bcl-2 may be associated with an aggressive phenotype, already present in the pre-invasive stage and characterized by increased cell turnover, increased genetic instability and rapid clonal evolution.

Predicting Response to Pre-Operative Chemotherapy in Breast Cancer

Unless triggered by an appropiate stimulus (e.g. DNA damage), wt-p53 protein is rapidly degraded, has a short half-life and low intracellular levels. Stabilization of p53 protein in the absence of a stimulus is always a hallmark of loss of function secondary to a mutation or interaction with viral or cellular oncoproteins (Blagosklonny, 1997).

In the premenopausal node-negative patients entered into EORTC Trial 10854, p53 protein accumulation, in contrast to Bcl-2, predicted a lack of response to 1xFEC as defined by disease-free survival time (see table I). Surprisingly, p53 seemed to predict treatment resistance in patients who developed distant metastases, but not in patients who developed local recurrence (van Slooten et al., unpublished data).

Material from patients treated with preoperative polychemotherapy offers the unique possibility to directly monitor the response of the primary tumor. For that reason, a

project was started to assess in breast tumors from patients, who had recieved four courses of FEC preoperatively (EORTC trial 10902), the relationship between a number of molecular markers and treatment response. Both biopsies taken prior to chemotherapy and surgically removed tumor material were collected, and mammographies were taken before and after chemotherapy and scored according to WHO criteria, as well as to a system that takes into account changes in the mammographical density of the lesion. It was found though that neither p53 accumulation nor expression of Bcl-2 was significantly associated with mammographical response of the primary tumor to chemotherapy.

The failure of Bcl-2 expression to predict response to chemotherapy, although in conflict with *in vitro* data, seems to be in line with its failure to predict response to disease-free survival after 1xFEC (see table I). Moreover, the complex interactions between the various anti- and pro-apoptotic members of the *bcl-2* gene family may require assessment of the ratio between various family members for an adequate prediction of treatment response.

However, because of the large body of experimental evidence highlighting the important role of p53 in treatment resistance (see also the contribution of Lowe et al. and Hickman et al to this volume), and the clinical finding of an association of p53 accumulation with lack of response to 1xFEC in BC patients, the absence of an association between p53 accumulation and mammographical response to pre-operative chemotherapy is difficult to explain. Sjögren and colleagues reported that although in their series of 316 BC patients confirmed p53 mutations were associated with treatment resistance, p53 accumulation as detected by immunohistochemistry was not Sjörgen et al., 1996). But the strong correlation we observed between p53 mutations detected by DNA analysis and immunohistochemistry respectively makes it unlikely that the lack of any significant association between tumor response and p53 mutations was solely due to 'false positive' and/or 'false negative' staining results.

As mentioned above, in BC loss of p53 function seems to be associated with reduced efficacy of polychemotherapy in preventing the outgrowth of distant metastases, but not in reducing the outgrowth of tumor cells at the site of the primary tumor. One can thus not exclude the possibility that p53 is a poor predictor of the response of a primary tumor to pre-operative chemotherapy. In fact, the magnitude of the response of the primary tumor may have little value in predicting the effect of chemotherapy on the overall survival of BC patients. Supporting this view is the finding of Linn and coworkers, that whereas coexpression of p53 and P-glycoprotein did not predict response to pre-operative chemotherapy, it strongly predicted overall survival (Linn et al., 1996).

It should be mentioned that evaluation of primary tumor response (e.g. decrease in tumor size assessed by mammography or by using calipers is difficult. In fact, several investigators found that this type of evaluation did not correspond to pathological response (e.g. as assessed by microscopical evaluation) or local recurrence-free survival. In contrast, a complete pathological response was significantly associated with improved local recurrence-free survival and overall survival (Vinnicombe et al., 1996; Brain et al.. 1997). Thus, evaluation of tumor response by an experienced pathologist seems to be the most reliable predictor of response in patients treated

with pre-operative chemotherapy. For these reasons, an important question to answer in the POCOB study is which associations exist between p53 status, initial tumor response assessed either by mammographical, clinical or pathological parameters and overall survival.

The finding that p53 activation can induce apoptosis as well as senescence may result in different types of tumor response in chemotherapy. Of note, in this respect, is the finding in colon cancer cells growing *in vitro* that loss of p21-mediated cell cycle arrest in response to irradiation resulted in an increase in apoptotic cells and a decrease in senescent cells. Moreover, in immune-deficient mice p21-/- cells were found to be more sensitive to irradiation then their p21+/+ counterparts (Waldman et al., 1997). Because p21 expression is mediated by p53, it is not unlikely that p53 mutated tumors initially respond to genotoxic treatment, but with time, due to the failure to undergo permanent cell cycle arrest, resume growth. In a wt-p53 tumor on the other hand, many cells may respond to therapy by undergoing senescence, resulting in a (temporary) decrease in tumor growth rate rather than a measurable decrease in tumor volume.

The finding that p53 mutated tumors may be associated with increased clonal evolution may have implications for the use of pre-operative CT in the treatment of BC. Experimental evidence indicates that following irradiation, in line with the function of p53 as 'guardian of the genome', the rate at which mutations accumulate in cell populations lacking functional p53 is dramatically increased compared to cell populations with wildtype p53 (Phillips et al., 1997). Thus, in theory, treatment of a large population of p53-mutated tumor cells with DNA-damaging drugs (as is the case with pre-operative (poly)chemotherapy) may increase the likelihood that unfavourable mutations accumulate, eventually resulting in a very poor treatment outcome. Future investigations will have to determine whether in BC patients with p53-mutated tumors the pre- or postoperative timing of chemotherapy affects clinical outcome.

Summary

Whereas in lymph-node negative, premenopausal BC patients high levels of Bcl-2 expression are clearly associated with reduced levels of apoptosis, reduced proliferation, and good prognosis, they do not predict response to chemotherapy (1xFEC). p53 mutations (both in- and outside the zinc-binding domains) are associated with increased levels of both apoptosis and proliferation, indicating increased cell turnover. p53 accumulation seems to predict response to chemotherapy in premenopausal, lymph-node negative patients, but only in patients who ultimately develop distant metastasis. Neither Bcl-2 expression nor p53 accumulation predicted response to pre-operative CT.

Our data suggest that p53 has little impact on the apoptosis rate in breast cancers. One possibility is that wildtype p53 induces senescence in cells with mutations in growth regulatory genes and that p53 inactivation permit cells to proceed crisis and immortalization. Mutations leading to increased cell divisions also increase the rate of (p53-independent) apoptosis and Bcl-2 counteracts both apoptosis and cell division activities. As outlined in figure 1, loss of p53 function and loss of Bcl-2 may be

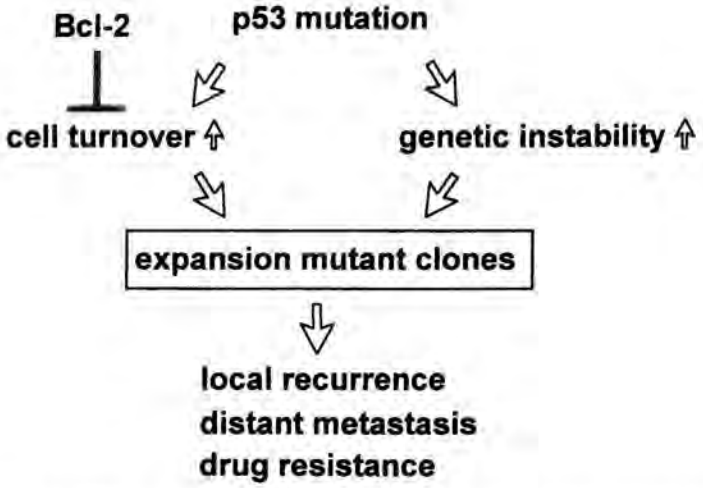

Fig. 1. P53 as an important mediator of the rate at which mutant cell clones expand within a tumor cell population, thereby affecting the rate of tumor progression. Inactivation of p53, by removing cell cycle blocks, increases proliferation rate and the rate of p53-independent (proliferation associated) apoptosis, leading to an increase in cell turnover. On the other hand, failure to delete cells with damaged DNA from the proliferating population through p53-dependent apoptosis/senescence as well as aberrations in mitotic checkpoints result in increased genetic instability. These two mechanisms may synergistically enhance the generation and expansion of mutant clones within a tumor cell population. Finally, the increased clonal evolution in tumors with inactivated p53 leads to increased rate of local recurrence, distant metastasis and drug resistance.

associated with an aggressive phenotype, already present in the pre-invasive stage and characterised by an increase in both cell turnover and genetic instability. This may result in rapid clonal evolution, which in turn may enhance tumor progression and treatment resistance.

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Bcl-2 and Family Members in Childhood Acute Lymphoblastic Leukemia

Abstract

The Bcl-2 family is emerging as a cell death family and the balance of apoptotic inducers and inhibitors within this family may regulate whether or not a cell will go into apoptosis upon appropriate stimuli. Previously we have shown that the Bax to Bcl-2 ratio in leukemic cells correlated with sensitivity to dexamethasone (DEX) in vitro. Our data suggest that Bax:Bcl-2, rather than Bcl-2 alone is important for the survival of drug-induced apoptosis in leukemic cell lines. We are now studying the relevance of these apoptotic-regulating gene products in childhood acute lymphoblastic leukemia (ALL) in a national study ALL-VII, in collaboration with the Dutch Childhood Leukemia Study Group (DCLSG). This study involves a large group of patients who received homogeneous treatment and from whom long term follow-up data have been collected.

Bcl-2: A New Category of Drug-Resistance Genes

The bcl-2 gene was identified because of its involvement in the chromosomal translocation t(14;18). bcl-2 encodes for a 26 kDa protein (Tsujimoto and Croce, 1986; Cleary *et al.*, 1986) that predominantly resides in the outer mitochondrial membrane, nuclear envelope and parts of the endoplasmatic reticulum (Krajewski *et al.*, 1993). Bcl-2 overexpression was shown to inhibit apoptosis (Vaux *et al.*, 1988; Reed, 1995).

Moreover, gene transfer methods to overexpress bcl-2 in leukemic cell lines showed that levels of Bcl-2 correlated with relative insensitivity of cells to a variety of anticancer drugs, especially those used in the chemotherapy of childhood ALL, notably glucocorticoids (GC) (Miyashita and Reed, 1992; Sentman *et al.*, 1991; Smets, 1994). Bcl-2 may therefore define a new category of drug-resistance genes, that regulate the physiological cell death pathway (Reed, 1995). Accordingly, increased levels of Bcl-2 may be associated with poor prognosis in leukemia by a dual action. First, it could expand the population of circulating blasts by promoting their survival, and second, it could antagonize response to chemotherapy by inhibiting steroid hormone- and cytostatic drug-induced apoptosis.

Bcl-2 and Bax in Clinical Studies

An increasing number of studies have been performed to correlate either Bcl-2 expression, the presence of t(14;18) translocation and to a lesser extent the Bax levels or the Bax:Bcl-2 ratio, with clinical outcome in patients with various types of cancer. The role of Bcl-2 seems to differ in various tissues. For example, high expression of Bcl-2 has been associated with a high white blood cell count (WBC) and poor prognosis in adult acute myeloid leukemia (Campos *et al.*, 1993), stressing that the main function of Bcl-2 is to inhibit apoptosis in these cells. However, high levels of Bcl-2 in non-small cell lung cancer, colon carcinoma and childhood ALL appears to associate with slowly progressing, indolent disease (Pezzella *et al.*, 1993; Sinicrope *et al.*, 1995; Salomons *et al.*, 1997), suggesting that in the affected tissues the proliferation inhibitory effects of Bcl-2 dominate over its survival promoting capacity. It would seem that the prognostic impact of Bcl-2 is diverse and probably context-dependent i.e. depending on diagnosis, type of treatment and expression levels of other apoptosis regulating genes.

Bcl-2 and Bax in ALL

In childhood ALL the role of Bcl-2 and its family members is not yet resolved. Published studies (Campana *et al.*, 1993; Gala *et al.*, 1994; Coustan-Smith *et al.*, 1996) agree that high Bcl-2 expression is the rule in ALL blasts, compared with their normal precursors, while no study has found a relation between Bcl-2 levels and the presenting features of immunophenotype, DNA ploidy and WBC. This is in contrast with our study revealing that in bone marrow aspirates from patients with childhood ALL (n=48), Bcl-2 levels were lower in T- than in B- lineage ALL (p=0.014) as assessed by Western blot analysis. This correlation could be ascribed to a simultaneous inverse relationship between Bcl-2 and WBC (p=0.023) (Salomons *et al.*, 1997). Moreover, Bcl-2 levels were higher in common ALL than in pre-B ALL (p=0.017). By contrast, similar analysis for Bax and Bax:Bcl-2 did not show a correlation with immunophenotype or WBC. Bax or Bax:Bcl-2 might therefore prove to have potential independent prognostic value. Nonuniform treatment of this patient sample prevented a meaningful comparison between Bcl-2/Bax levels and follow-up data. However, such an analysis would most likely reveal a worse outcome for patients with low Bcl-2 because of its association with high WBC and T- lineage ALL. This would be in agreement with the report of Coustain-Smith *et al.* 1996 showing that elevated levels of Bcl-2 were associated with improved survival and the absence of chromosomal abnormalities Coustan-Smith *et al.*, (1996). However, another study showed that increased expression of Bcl-2 was not found to correlate with prognosis (Gala *et al.*, 1994) while in the report of Maung *et al.* (1994) a correlation with a poor initial response to chemotherapy was found. These conflicting data may be due to the small size of patient populations studied and the limitations in quantifying Bcl-2 by immunocytochemistry.

On the other hand, it may be insufficient to study only Bcl-2. It is becoming clear that Bcl-2 and related anti-apoptotic proteins ($Bclx_L$, Mcl-1, Bag-1, Bad, A1) interact

with apoptosis-promoting proteins (Bax, $Bclx_s$, Bak, Bik/Nbk) to form hetero- and homodimers and that the relative amount of apoptosis inducers and inhibitors is more important than Bcl-2 alone (Boise *et al.*, 1993; Takayama *et al.*, 1995; Yang *et al.*, 1995; Oltvai *et al.*, 1993; Chittenden *et al.*, 1995; Kiefer *et al.*, 1995; Boyd *et al.*, 1995). For instance, a relative increase of Bax over Bcl-2 in primary thymocytes from transgenic mice as well as in leukemic cells accelerates apoptosis in response to DEX treatment (Brady *et al.*, 1996; Salomons *et al.*, 1997). Accordingly, the clinical relevance of Bcl-2 levels in ALL may be obscured by simultaneous variations in Bax and other family members.

Bcl-2 Family Members in Cell Lines and Peripheral Blood Cells (PBL)

To investigate the expression levels of the Bcl-2 family members Mcl-1, Bcl-x, Bax, Bak and Bad in PBL of healthy volunteers and in human cell lines (the leukemic T cell lines: CEM C7 and Jurkat, Daudi: a Burkitt B cell lymphoma, DoHH2: a follicular non-Hodgkin's B cell line and HL60: a promyelocytic leukemic cell line) we analyzed lysates of 200,000 cells by horizontal polyacrylamide gradient gel electrophoresis and Western blotting. Mcl-1, Bcl-x, Bax, Bak, Bcl-2 and Bad migrate as a 40, 29, 22, 25, 26, and 24 kDa protein, respectively. In figure 1 it is apparent that the levels of all proteins are variable in the cell lines tested and that these levels are well above those detected in normal PBL. This suggests that these proteins might also be expressed at elevated levels in childhood ALL. We, therefore, sought to study the relevance of these apoptotic regulating gene products in a large population of 86 leukemia patients, who received uniform treatment regimens and of whom follow-up data are being collected.

Bcl-2 Family Members in Childhood ALL (National Study ALL-VII)

Treatment Protocol ALL-VII

In collaboration with the Dutch Childhood Leukemia Study Group (DCLSG), patient material is being studied in the national study ALL-VII. Clinical specimens consist of archival bone marrow (BM) aspirates or peripheral blood cells derived from patients with childhood ALL. Patients were enrolled in this study when the children were diagnosed with ALL as first or second malignancy, the diagnosis was performed by the DCLSG, the age was 0-15 years, and treatment with corticosteroids or cytostatics prior to the diagnosis was less than 4 weeks. The patients received uniform treatment regimens according to protocol.

This study specifically allows the unraveling of the roles of apoptosis regulating proteins on resistance to glucocorticoids because the children were pre-treated with GC monotherapy and response monitoring was performed at day 8. Furthermore, standard karyological analysis has been performed, as well as the percentage of S-phase and DNA ploidy. The patients were divided into a standard risk group, a risk group

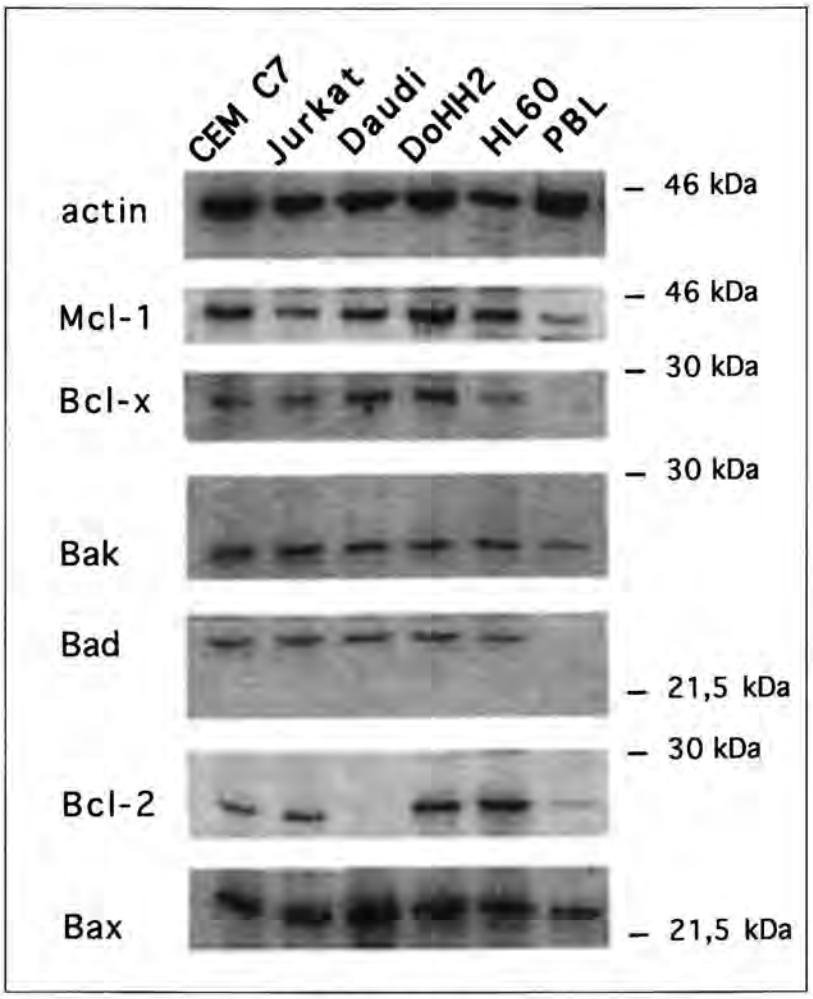

Fig. 1. Expression levels of Bcl-2 related gene products in hematological cell lines and PBL of a healthy volunteer. Proteins were separated on 12-14% polyacrylamide gradient gels by horizontal SDS electrophoresis (Pharmacia Biotech). Duplicate gels were blotted and probed with antibodies specific for Mcl-1, Bcl-x, Bax, Bak, Bad, or Bcl-2 (Santa Cruz Biotechnology: sc-819, sc-634, sc-493, Calbiochem: Ab-2, Transduction Laboratories: 31420, Dako clone 124) followed by the appropriate ^{125}I-linked secondary antibody (Amersham). The membranes were also probed with a monoclonal antibody to actin as loading controls (Boehringer Manheim). Band intensities were calculated and visualized by phosphorimaging. The size and location of the molecular weight bands are depicted in the figure.

and an experimental group according to the criteria of the DCLSG. No exclusion criteria for high or low risk were used allowing the study of relationships with factors associated with both favorable and unfavorable prognosis.

Bcl-2, Bcl-x, Bax, Mcl-1, Bak and Bad levels in ALL

The DoHH2 cell line, a follicular non-Hodgkin's B cell lymphoma that expresses Bcl-2 at high levels as a result of the t(14;18), was used as a reference throughout this study. This cell line also expresses relative high levels of Mcl-1, Bax, Bak, Bad and Bcl-x (Fig. 1) and could, therefore, be used as a standard for the detection of these proteins as well. Bcl-2 and Bax were highly variable between ALL patients as described previously. Preliminary data show that Bak and Bcl-x were also similarly variable, whereas Bad and Mcl-1 showed less heterogeneity. In order to draw conclusions as to whether some proteins of this family have clinical value, we will correlate these levels with presenting features and treatment results.

(De)regulation of Bcl-2 and Bax

The molecular basis for the large range in expression levels of both Bcl-2 and Bax in ALL is unknown. The classic cause of overexpression of Bcl-2 in follicular NHL — the translocation t(14;18) — is very rare in childhood leukemia (Campos *et al.*, 1993). The fact that high levels of Bcl-2 are also observed in follicular lymphomas in the absence of t(14;18) hints to the existence of other mechanisms that deregulate *bcl-2* (Pezzella *et al.*, 1990). In AML another translocation is found; This t(8:21) results in AML/ETO fusion protein that activates transcription of Bcl-2 (Klampfer *et al.*, 1996). The high range in Bcl-2 and Bax expression levels in ALL might also reflect different development stages in B and T cells. We found that the Bcl-2 levels were significantly higher in common ALL than inpre- B ALL, originating from pre-pre-B cells and pre-B cells, respectively, suggesting effects of differentiation on Bcl-2 levels. However, Bcl-2 levels in pre-B ALL are still much higher than in normal pre-B cells (Campana *et al.*, 1993). Thus differentiation state is probably not the sole determinant of Bcl-2 levels in ALL.

p53 is known to be able to regulate both Bcl-2 and Bax expression (Miyashita *et al.*, 1994; Miyashita and Reed, 1995) and is therefore a candidate for the deregulation of these genes. However, in childhood ALL, inactivation of p53 by mutation or mdm2 overexpression is infrequently found, although a high incidence of potential p53 inactivations at diagnosis in poor outcome leukemia has been recently reported (Marks *et al.*, 1996). In addition, other transcription regulators may deregulate the expression of Bcl-2 and related genes because of their inappropriate expression levels or the presence of functional mutations. For instance, the Gfi proto-oncogene is able to repress Bax and Bak expression in IL-2-dependent T-cell lines and inhibits death upon cultivation in IL-2-deficient media (Grimes *et al.*, 1996).

Recently, bax mutations have been reported in cell lines derived from hematological malignancies and in colon cancers of the microsatellite mutator phenotype with a frequency of approximately 30 and 50%, respectively (Meijerink *et al.*, 1995; Rampino *et al.*, 1997). The first report showed point mutations that could have an effect on the hetero- and or homo-dimerization, while the second report identified frameshift mutations that results in truncated Bax. Deregulation of Bax as result of a mutation could be important in ALL, but so far we have not found mutations in this gene in ALL.

In conclusion, the mechanism of Bcl-2 and Bax overexpression, encountered in our previous study in approximately 40% and 34% of the patients respectively, remains unclear, one or more of the above mentioned mechanisms are likely to contribute.

Future Perspectives

We hope to elucidate the relevance of Bcl-2 family members in treatment outcome of ALL. Interpatient variability in Bcl-2 and Bax levels have been shown to be high. Preliminary data show that Bak and Bcl-x are also highly variable between patients, whereas Bad and Mcl-1 showed less heterogeneity. Because Bcl-2 correlates with the favorable prognostic markers of low WBC and B-lineage, while the ratio of Bax:Bcl-2 or Bax levels do not, these latter two are potential candidates for being useful independent clinical markers.

Although Bcl-2 overexpression has been shown to render cells more resistant to various cancers drugs, elevated Bcl-2 levels are not correlated with a poor outcome of cytostatic treatment of ALL. Recent studies suggest that the balance of apoptosis-inducers versus apoptosis-suppressors is likely to contribute to drug-resistance in cancer cells, providing more relevant prognostic information. Ideally it might eventually be possible to develop new treatment regimens based on modulation of the ratio of apoptosis inducers versus apoptosis inhibitors.

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Donald W. Nicholson

Molecular Events Mediating Apoptotic Cell Suicide: Central Role of the Caspases

Apoptotic cell suicide contributes to or accounts for the pathogenesis of several human diseases. We now know that the process of cell death which is manifest as the apoptotic phenotype proceeds through a highly organized and evolutionarily-conserved biochemical pathway. Many of molecular components of this biochemical pathway have been identified only recently, lending this field to intense research investigation and the exciting prospect that therapeutic modulation of cell death may be a viable means for the treatment of human diseases where inappropriate apoptosis occurs (Nicholson and Thornberry 1997, Thompson 1995).

Caspases are Central Components of a Conserved Cell Death Pathway

A central component of the cell death pathway are the caspases, a family of cysteine proteases related to mammalian interleukin-1ß converting enzyme (ICE, caspase-1) and the *C. elegans ced-3* gene product. (The name caspase (for *c*ysteinyl *asp*artate-specific prote*ase*) (Alnemri et al; 1996) reflects two principle features of these enzymes: that they are cysteine proteases and that they have a strict requirement for Asp in P_1 of the scissile bond). Deletion or mutation of *ced-3* results in the abolition of all apoptotic deaths that would otherwise occur during *C. elegans* hermaphrodite development (Yuan et al; 1993). When *ced-3* was sequenced, it was found to encode a protein that was homologous to ICE (caspase-1) whose only known function was the proteolytic maturation of the 32 kDa IL-1ß precursor to the pro-inflammatory 17 kDa IL-1ß cytokine (Thornberry et al; 1992, Cerretti et al; 1992). Although it is now clear that ICE (caspase-1) itself does not play a major role in mammalian apoptosis, it is well substantiated that related caspase family members do. First, all of the known proteolytic cleavage events that occur during apoptotic cell death are mediated by caspases (Nicholson and Thornberry 1997, Rosen and Casciola-Rosen 1997). Second, both macromolecular (CrmA, p35, XIAP) and synthetic caspase inhibitors (peptide-electrophiles) prevent apoptosis *in vitro* and *in vivo* (Nicholson et al; 1995, Zhou et al; 1997, Xue and Horvitz 1995, Bump et al; 1995, Liston et al; 1996, Devereaux et al; 1997, Hara et al; 1997, Loddick et al; 1996, Yakovlev 1997). Finally, caspase-3 deficient mice have a striking defect in neural apoptosis (Kuida et al; 1996)

(akin to the more global defects in CED-3-deficient nematodes). Collectively, this and other evidence provides compelling evidence that caspases are key and central proteolytic mediators of apoptotic cell suicide in eukaryotes.

Function of Caspases

During apoptotic cell death, a discrete and highly limited subset of cellular polypeptides are cleaved by caspases of the CED-3 subfamily (Fig. 1). Each of these proteolytic 'victims' is specifically cleaved at an Asp-x bond and results in the disabling of a key cellular function that should not proceed during cell death (e.g. genomic repair) or the activation of auxiliary process which aid the cell death process (e.g. genomic disassembly). Caspase substrates are generally homeostatic, repair or structural proteins and the cleavage of each one appears logical for what is required to facilitate the highly ordered, deliberate and systematic process of apoptosis (Nicholson and Thornberry 1997, Rosen and Casciola-Rosen 1997). The collective effect of these cleavage events is to a) halt cell cycle progression and cell division, b) disable homeostatic and repair mechanisms, c) initiate the detachment of the dying cell from surrounding cells within organized tissue structures, d) disassemble structural components of cell skeleton, and e) mark the dying cell for engulfment by other cells. With few exceptions, most of these cleavage events occur at DxxD motifs (P_4Asp-x-x-AspP_1), implicating caspases with specificities similar to CED-3 or its most related human counterpart, caspase-3 (see below).

The Caspase Family

Thus far, ten mammalian caspase family members have been identified (Fig. 2) Phylogenetically they break down into two distinct subfamilies: those related to ICE (caspases-1 (ICE), -4, -5) and those related to the nematode 'death gene' product CED-3 (caspases-3, -6 to -10). Whereas members of the ICE subfamily account for the proteolytic maturation of pro-inflammatory cytokines (including interferon gamma inducing factor (IGIF, IL-18) as well as pro IL-1ß), members of the CED-3 subfamily are the dominant proteases that mediate apoptotic cell death. Each of the caspases is synthesized as a catalytically dormant proenzyme which requires proteolytic maturation and multiple Asp-x bonds to liberate the subunits which form the heterodimeric active form of the protease (Thornberry et al; 1992, Cerretti et al 1992, Nicholson et al; 1995). The proenzyme is comprised of three principle components: an amino-terminal prodomain which is followed by the large and small subunits of the active enzyme (Fig. 1). The large subunit (17-21 kDa) contains the principle residues necessary for proteolytic catalysis, including the catalytic cysteine residue which is harbored within the absolutely conserved QACxG pentapeptide motif, whereas the small subunit (10-13 kDa) contains the determinants which dictate substrate specificity (Wilson et al; 1994, Walker et al; 1994, Rotonda et al; 1996). Both subunits contribute residues which form the S_1 subsite to stabilize the P_1 Asp at the scissile bond of caspase substrates.

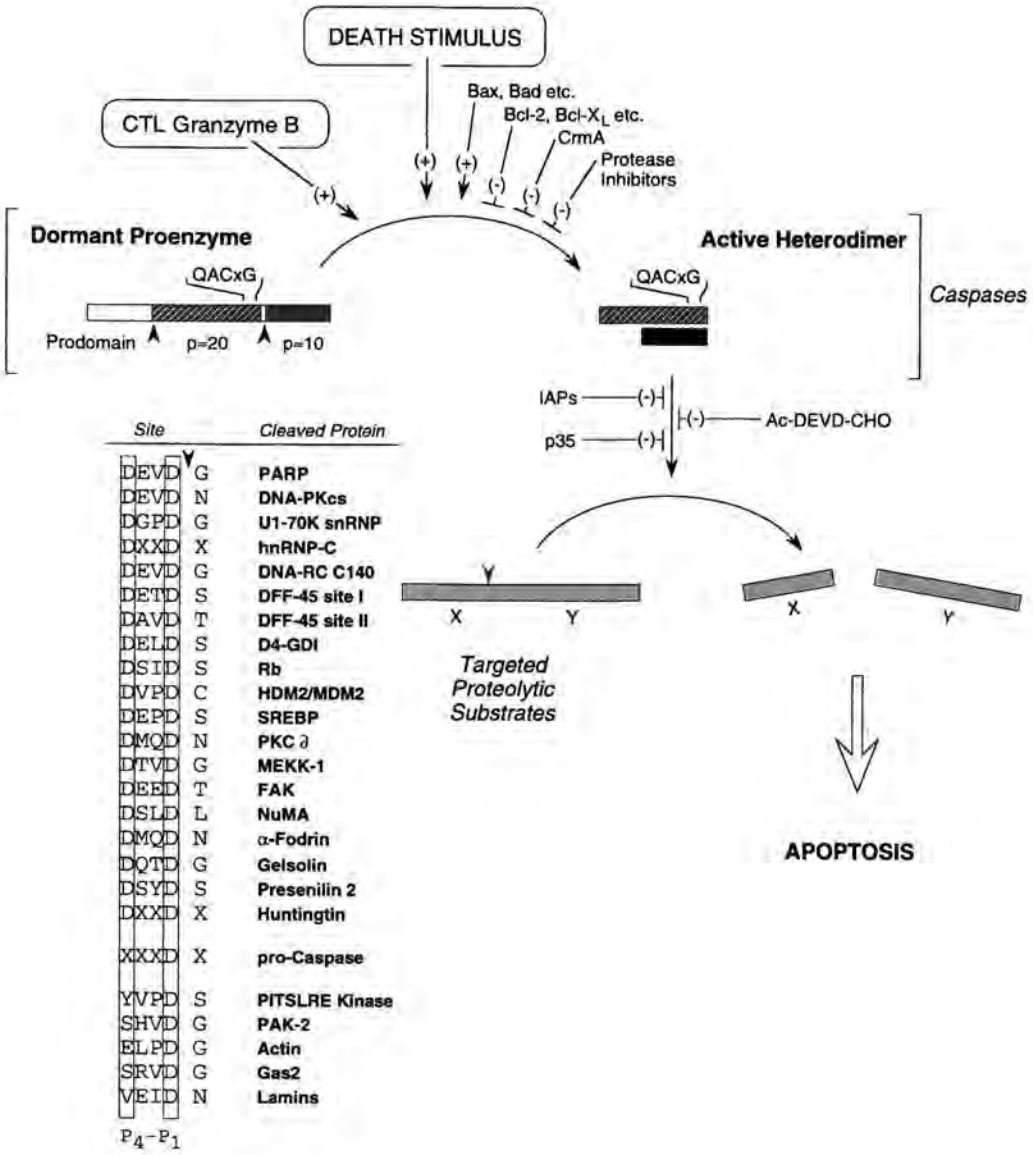

Fig. 1. Caspases play a central role in the biochemical events that result in the apoptotic phenotype. The proteolytic activation of dormant caspase proenzymes can be triggered by multiple pro-apoptotic stimuli and is under strict regulation by other endogenous and exogenous (e.g. viral) macromolecules. Once activated, caspases cleave a discrete subset of targeted cellular polypeptides at Asp-x bonds and the collective effects of these cleavage events results in the orderly process of apoptotic cell death.

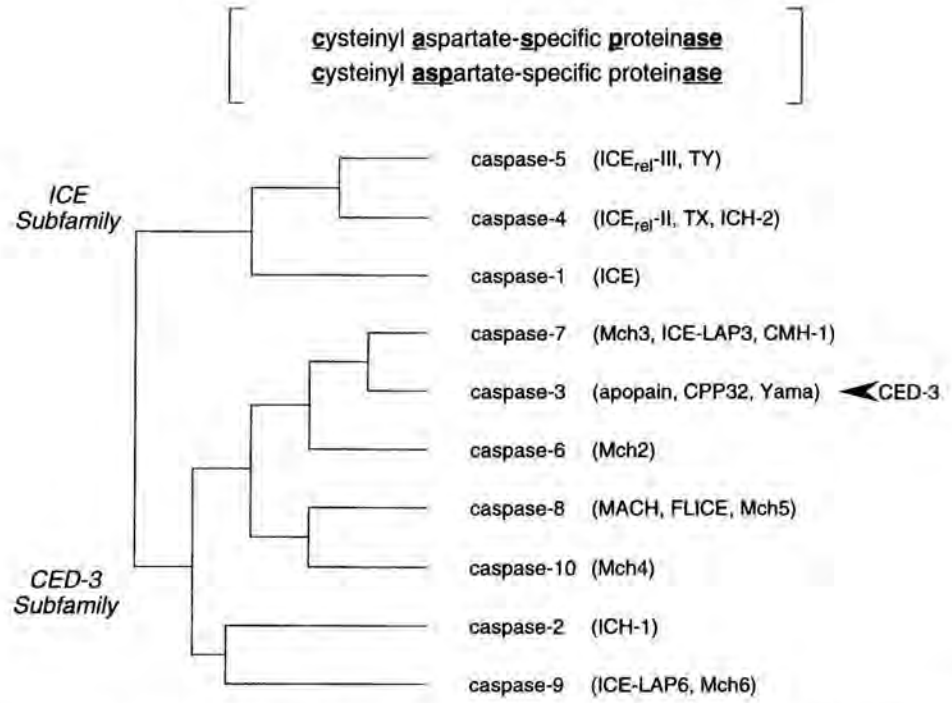

Fig. 2. The human caspase family. Caspases divide phylogenically and functionally into two distinct sub-families. The known human caspase family members are shown and their former acronyms. Caspase-3 is the most related human caspase to *C. elegans* CED-3.

While there is a high degree of homology amongst caspase family members in regions containing these subunits, the prodomains are structurally diverse and, with few exceptions, of unknown function. It appears likely that prodomains regulate or facilitate the activation of caspase proenzymes. This is best exemplified by the 'death effector domains' (DEDs) contained within the caspase-8 prodomain which facilitate pro-caspase-8 recruitment, oligomerization and activation via the CD-95 (Fas/ APO-1) receptor system (Boldin et al; 1996, Muzio et al; 1996, Medema et al; 1997).

Distinguishing Features of Caspases as Proteases

The caspases constitute a new and distinct family of proteolytic enzymes. They employ a typical cysteine protease mechanism (Wilson et al; 1994, Walker et al; 1994, Rotonda et al; 1996) involving a catalytic diad composed of the sulphydryl group of $Cys^{ICE:285}$ and the imidazole group of $His^{ICE:237}$. Like other cysteine proteases, they stabilize the oxyanion of the tetrahedral transition state through hydrogen bonding interactions with backbone amide protons ($Cys^{ICE:285}$ and $Gly^{ICE:238}$). A unique feature

of this protease family, however, is the near absolute requirement for Asp in the P_1 position of substrates. Four residues within the protease, two from each subunit, participate in the stabilization of the carboxylate side chain of the P_1 Asp ($Arg^{ICE:179}$, $Gln^{ICE:283}$, $Arg^{ICE:341}$, $Ser^{ICE:347}$). All of the residues involved in catalysis and in forming the S_1 subsite are absolutely conserved in all functional caspases in all species studied thus far. Another key feature of the caspases is that sufficient information for substrate recognition and high affinity binding is contained within the tetrapeptide sequences corresponding to the P_4-P_1 positions of proteolytic substrates (the four amino acids proximal to the scissile bond). In particular, the S_4 subsite of the caspases (which accommodates the P_4 substrate residue) is the dominant specificity determinant. Collectively, these features have facilitated the design of suitable fluorogenic and colorimetric substrates (e.g. tetrapeptide-aminomethylcoumarins and tetrapeptide-paranitroanilides, respectively) as well as extremely potent reversible (Ki < 1 nM) and irreversible inhibitors (e.g. tetrapeptide-electrophiles, where the electrophile can be an aldehyde, ketone or nitrile). For example, the acetylated tetrapeptide aldehyde corresponding to the pro IL-1ß cleavage site (Ac-YVAD-CHO) is a potent inhibitor of ICE (caspase-1; Ki = 0.76 nM)(5), the protease responsible for its maturation *in vivo*, while inhibitors corresponding to the site of cleavage within several polypeptides during apoptosis (Ac-DEVD-CHO) are very potent against caspase-3 (Ki = 0.35 nM) (Nicholson et al; 1995) and related family members. Although these and other peptide-based inhibitors have been extremely useful *in vitro*, their poor membrane permeability and metabolic instability have severely limited their utility in intact cells and *in vivo* models.

The Proteolytic Specificity of the Caspases Defines Three Functional Sub-Groups

Given the presence of at least ten caspase family members in human cells, there are several possible ways in which they each might contribute to the known roles for caspases *in vivo*. One possibility, for example, is that they might all participate in a complex interdependent proteolytic network which ensures both redundancy and amplification of the cell death cascade. Another possibility is that each enzyme performs a unique and specific task (e.g. each with different subsets of proteolytic victims) and it is the collective 'efforts' of multiple enzymes that mediates the events necessary for cell death. A simpler but equally feasible possibility is that caspases are tissue- or activation-specific isozymes that perform similar catalytic functions.

This has been resolved, at least in part, by the determination of the precise substrate specificities of all of the known mammalian caspase family members, *C. elegans* CED-3 and the serine protease granzyme B, an Asp-selective caspase activator (Rano et al; 1997, Thornberry et al; 1997). The approach taken took advantage of three key features of caspase proteolysis: a) that all require Asp in P_1, b) that the four amino acids corresponding to P_4-P_1 are necessary and sufficient for caspase recognition, and c) that the prime side of caspase active sites (C-terminal to the scissile bond) are highly promiscuous and can accommodate fluorogenic leaving groups such

as aminomethylcoumarin. A combinatorial approach was used to generate a positional-scanning substrate library comprised of all 8000 permutations of P_1 Asp tetrapeptide-AMCs (Fig. 3). Each caspase could therefore be tested for its preference for the 20 proteinogenic amino acids in each of the different subsites (S_4-S_2; S_1 being fixed with Asp) in the context of all 400 permutations of adjacent amino acids in the neighboring subsites. This method allows for the definition of the optimal tetrapeptide sequence amongst all 8000 possible permutations and the relative ability of the enzyme to accommodate alternative amino acids in each position.

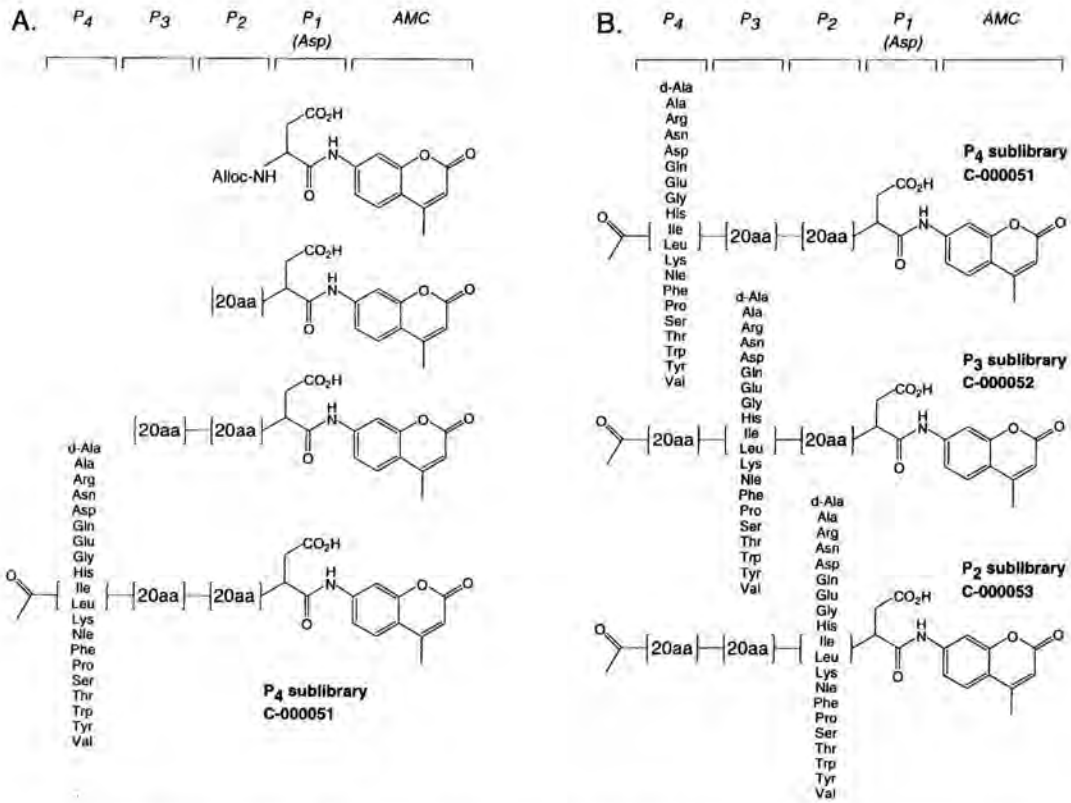

Fig. 3. Strategy for building a positional-scanning combinatorial substrate library for caspases. Asp-aminomethylcoumarin (AMC), tethered to a solid support, was combined with an isokinetic mixture of proteinogenic amino acids ([20aa]) to establish all 20 amino acids in P_2 coupled to the P_1 Asp-AMC in a single reaction mixture (panel **A**). The mixture was then re-combined with a fresh isokinetic mixture of the same amino acids to establish all 20 amino acids in P_3, resulting in a single solid-phase reaction mixture containing all 400 permutations (20 × 20) of P_3-P_2-Asp-AMC tripeptides. This mixture was then separated into 20 individual aliquots and coupled with a known single amino acid in P_4 (the positionally-defined amino acid). Thus, after release from the solid support, each well of the P_4 sublibrary contains a known amino acid in P_4 coupled to all 400 possible permutations of adjacent amino acids (in P_3-P_2) linked to the P_1 Asp-AMC (a total of 8000 fluorogenic tetrapeptides). Similar strategies were used to generate the equivalent P_3 and P_2 sublibraries (panel **B**).

Analysis of the human caspase family indicates that these proteases fall into only three specificity subgroups (arbitrarily designated group I, II and III) with remarkable similarities within each subgroup. Group I caspases, which include all of the ICE-like family members (caspases-1, -4 and -5), prefer bulky hydrophobic amino acids in the specificity-determining S_4 subsite (e.g. Tyr or Trp) but are somewhat promiscuous. This promiscuity accounts for the ability of caspase-1 to cleave some apoptotic substrates *in vitro*, although bulky hydrophobic residues have not been found in the P_4 position of any caspase substrate with the exception of the cytokine precursors cleaved by caspase-1. Group II caspases, on the other hand, are remarkable stringent for Asp in P_4 which corresponds to the P_4 residue found in most of the polypeptides cleaved by caspases during apoptosis (see Fig. 1). These enzymes, which include caspases-2, 3 and 7, thus appear the be the principle effectors of apoptosis and are highly specific for substrates containing DxxD motifs. Not surprisingly, *C. elegans* CED-3 has the same proteolytic specificity as these group II effector caspases. The third specificity subfamily, the group III enzymes which include caspases-6, -8, -9 and -10, prefer branched chain aliphatic amino acids in P_4, such as Ile, Val or Leu. Their specificity matches the maturation site found between the large and small subunit of other caspase family members, including all the group II effector enzymes. This and other evidence indicates that the principle role of group III caspases is to activate the group II effector enzymes in a process that amplifies the death signal (Fig. 4), although they may also cleave the few non-DxxD polypeptides known to be targeted during apoptosis (e.g. caspase-6 mediated cleavage of the nuclear lamins has been demonstrated (Takahashi et al; 1996)). The specificity of CTL-derived granzyme B has a similar (although apparently more stringent) specificity, consistent with its role as a caspase activator during cytotoxic granule-mediated cell death. All caspases have a preference for Glu in the S_3 subsite with some caspases (e.g. caspase-5) being remarkably stringent.

The three dimensional structures of two of the caspases has been determined (caspase-1 and -3) providing a structural basis for the relative promiscuity of group I caspases (exemplified by caspase-1) versus the high degree of substrate specificity observed with group II caspases (e.g. caspase-3) (Wilson et al; 1994, Walker et al;1994, Rotonda et al; 1996). In both cases, tetrapeptide inhibitors lie in an extended conformation in a groove on the surface. The S_4 subsite of caspase-1 is a shallow depression which easily accommodates bulky residues such as Trp, Tyr and others. The S_4 subsite in caspase-3, on the other hand, is highly constrained by the presence of an additional surface loop not present in caspase-1 and by a Trp residue which forms an additional 'wall' to limit the physical size of the S_4 pocket. Both the geometry and electrostatic character of this subsite highly favor Asp and account for the specificity of this enzyme subgroup. The structural basis of group III enzymes must await an X-ray crystal structure of one of these enzymes.

Activation and Modulation of Caspase Activity

Cells engage the death pathway via the activation of caspase proteolytic activity. Multiple 'sensors' must clearly exist to continuously survey the status of cell and determine

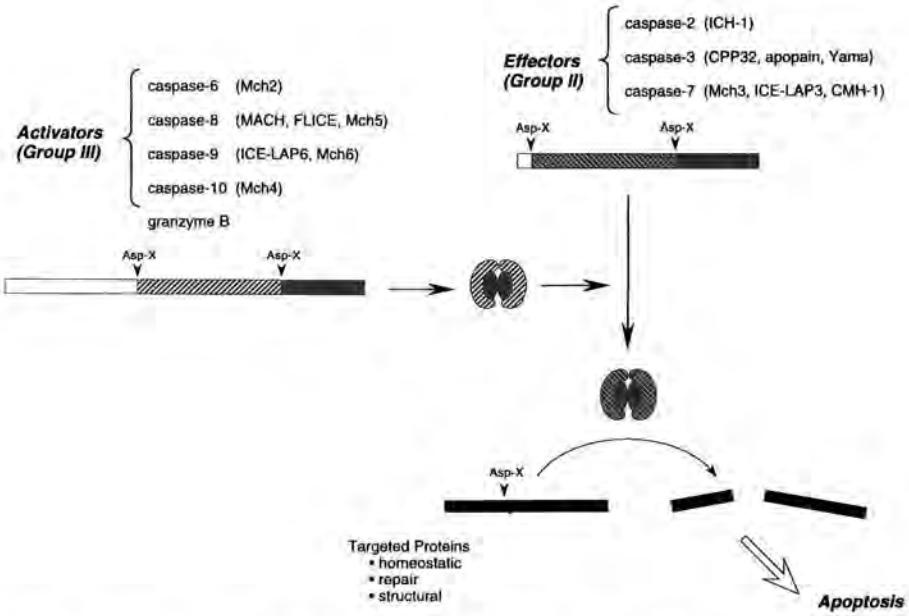

Fig. 4. Molecular ordering of the cell death pathway. The proteolytic specificity of the CED-3 subfamily of caspases indicates that they fall into two sub-groups. Group II caspases have a specificity that corresponds to the cleavage site within the majority of polypeptides that are cleaved during apoptosis. Group III caspases have a proteolytic specificity that is consistent with them being upstream activators of the Group II enzymes.

when it is appropriate to launch an apoptotic response, but how the 'decision' to die is communicated to caspase activating mechanisms is poorly understood. A key feature that probably plays an important role in several avenues of caspase activation is the ability of caspase proenzymes to undergo autocatalytic maturation when dimerization (or oligomerization) occurs. In the CD95 (Fas/ APO-1) system, for example, multiple pro-caspase-8 polypeptides are recruited to a common site following receptor ligation and they subsequently become activated and launch an apoptotic response (Boldin et al; 1996, Muzio et al; 1996). Recruitment is mediated by 'death effector domains' contained within the prodomain of pro-caspase-8 that bind to homologous DEDs in the FADD/ MORT1 adapter protein which itself is recruited to the cytoplasmic tail of the CD95 receptor upon ligand binding. Modulation of this process can occur by a caspase-like polypeptide lacking catalytic features (Usurpin, CASH, Casper, CLARP, FLAME-1, FLIP, I-FLICE and MRIT) (Rasper et al; 1997) that binds to pro-caspase-8 and precludes its recruitment to the CD95 receptor complex. (This, expectedly, attenuates cell death but in some systems this molecule has been shown to initiate apoptosis.)

Another route for caspase activation occurs through the mitochondrion, although the biochemical mechanics of this process are not entirely clear. Caspase-3 activation can be stimulated by the mitochondrial protein, cytochrome *c*, and by APAF-1 which

recruits mitochondrial proteins following cell death initiation (Zou et al; 1997). A pool of pro-caspase-3 is present in the mitochondria of many human cells and it becomes activated much earlier that the cytoplasmic pool under most circumstances (Mancini et al; 1997). These and other components may constitute a central pathway for caspase-3 activation emanating from this organelle, probably under the regulatory control of the Bcl-2/ Bax family of proteins.

A third route of caspase activation comes at the hands of 'third party' proteases, such as granzyme B (Darmon et al; 1995). In this case, cytotoxic T lymphocytes 'trick' target cells into committing suicide by extruding granzyme B and the pore-forming protein, perforin, at the surface of the target cell. Granzyme B proteolytically cleaves and activates the caspases thus launching the endogenous cell death pathway. Other activation mechanisms undoubtedly exist, and each is likely to be under the regulation of sophisticated biochemical controls to ensure the appropriateness of cell survival or death. Endogenous regulators, such as Bcl-2/ Bax family members, Usurpin-like molecules and the mammalian IAPs clearly play an important role in these processes.

Summary

Caspases are central to the apoptotic process. Approximately 20 of the limited subset of their proteolytic substrates have been identified and this has contributed to our understanding of how these proteases manifest the apoptotic phenotype. The redundancy of proteolytic specificities within the caspase superfamily coupled with the diversity of their distribution *in vivo* suggests several therapeutic opportunities that have not escaped the attention of the scientific community. As a biochemical pathway, the cell death mechanism and its many layers of molecular control still contains many secrets that are yet to be revealed.

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Apoptin-Induced Apoptosis: Antitumor Potential?

Abstract

Radiation and chemotherapy for the treatment of cancer in a number of cases have been found to exert their cytotoxic effect via the induction of apoptosis. The success of these treatments is often dependent on the presence of functional p53 and the absence of overexpression of the oncogene Bcl-2. Unfortunately, in a large number of tumors functional p53 is lacking, and/or Bcl-2 is overexpressed, which often results in resistance to anti-cancer therapy. We have previously shown that a protein derived from the chicken anemia virus (CAV) which we have named Apoptin©, can induce apoptosis in various human tumor cells, also those lacking p53 and/or overexpressing Bcl-2. Apoptin-induced apoptosis is not inhibited by downstream inhibitors of the p53-pathway like Bcl-2 or CrmA. Bcl-2 even accelerates Apoptin activity, which indicates that Apoptin induces cell death via an unusual pathway. Interestingly, Apoptin fails to induce apoptosis in human primary lymphoid, dermal, epidermal, endothelial and smooth muscle cells, but when normal primary cells are transformed or immortalized, they become susceptible. The fact that Apoptin induces a p53-independent, Bcl-2-stimulated type of apoptosis in human tumor cells but not in normal diploid cells, renders Apoptin a potential anti-tumor agent. Viral vectors carrying the Apoptin gene are currently being developed to test its applicability for cancer gene therapy.

Introduction

The development of tumors not only depends on the rate of cell proliferation, but has also been found to depend on the rate of cell death. An excess of dividing cells, or insufficient apoptosis, will both lead to accumulation of cells (Thompson, 1995). Several oncogenes and tumor suppressor genes have been found to play a role in either induction or inhibition of apoptosis. The tumor suppressor p53, for example, can either induce cell cycle arrest or apoptosis in response to DNA damage. In many tumors the p53 function is lost as a result of mutation or deletion. In such tumor cells

[1] Apoptin© is a registered trademark by Leadd BV, The Netherlands.

cytotoxic agents that act via p53 can no longer induce apoptosis, although the apoptotic machinery itself is still functional (Levine, 1997). The proto-oncogene bcl-2, which inhibits apoptosis, has been found to be activated in several lymphomas due to a translocation. Another proto-oncogene, c-myc, can either stimulate cell proliferation or induce apoptosis, depending on its expression level and the proliferation conditions. The tumor suppressor protein Rb, which can inhibit cell cycle progression by binding to E2F transcription factors, has also been implicated in the inhibition of programmed cell death (Canman and Kastan, 1995; White, 1996).

Apoptosis not only plays a role in the development of tumors but also in the treatment of cancer. Conventional therapies using radiation and cytotoxic agents often act via induction of apoptosis. In many instances apoptosis induction requires functional p53, and consequently loss of p53 function, which is a common feature in human tumors, correlates with resistance to these therapies. For example, melanomas, lung-, prostate- and colon cancers often have mutated p53, resulting in a poor response to radiation and chemotherapy (Lowe et al., 1994; Levine, 1997). Overexpression of the bcl-2 gene due to the t(14;18) translocation in lymphomas or formation of the Bcr-Abl fusion gene by the t(9;22) translocation in chronic myelogenous leukemia leads to enhanced protection against apoptosis, which has also been shown to result in resistance to chemotherapy or radiation (McDonnell et al., 1995). This has led to a search for new therapies, leading, for example to restoration of the function of p53. Adenovirus- and retrovirus-mediated transfer of p53 has been shown to inhibit the growth of certain tumor cells in tissue culture and in mouse models (Sandig et al., 1997; Gomez-Manzano et al., 1996; Yang et al., 1995). However, restoring p53-function may not be effective in tumors which have a block in the apoptosis pathway downstream of p53, e.g. tumors which overexpress Bcl-2. Therefore, an effective approach would be to induce apoptosis through a pathway that is independent of p53, and not inhibited by Bcl-2. A potential candidate for such a strategy is Apoptin, which has been shown to induce apoptosis in several human tumor cell lines, also those lacking p53 and/or overexpressing Bcl-2.

In this chapter we discuss the history of Apoptin, and outline our current knowledge of its mechanism of action. Future studies in animal models will reveal the potential of Apoptin for antitumor therapy.

Chicken Anemia Virus Induces Apoptosis

Chicken anemia virus (CAV) is a small non-enveloped virus, containing a single-stranded circular DNA-genome of 2.3 kb (reviewed by Noteborn and Koch, 1995). CAV constitutes a unique type in the newly established virus family of Circoviridae (Studdert, 1993). From its genome, a single polycistronic mRNA is transcribed, with three open reading frames (ORFs) which partially or completely overlap. As three different reading frames are used, they encode the completely different proteins VP1 (51.6 kDa), VP2 (24.0 kDa) and VP3 (13.6 kDa). These products show no homology to each other or to any other known proteins. VP1 is most likely the only protein present in the viral capsid, whereas VP2 seems to be required at some stage in the virus

assembly but has sofar not been detected in the viral particles (Todd et al., 1990; Noteborn and Koch, 1995; Koch et al., 1995).

The clinical signs of CAV infection in new-born chickens are loss of body weight, hemorrhages and increased mortality. Also severe depletion of cortical thymocytes and erythroblastoid cells in the bone marrow is observed, leading to immunodeficiency and anemia. When the animals survive the infection, repopulation with hematopoietic cells and thymocytes usually occurs 3-4 weeks after infection (McNulty, 1991). In chickens older than 3 weeks, CAV only causes subclinical signs (McNulty et al., 1991; Coombes and Crawford, 1996).

The depletion of thymocytes observed after CAV infection has been shown to be caused by apoptosis (Jeurissen et al., 1992; Noteborn et al., 1993). DNA isolated from the thymus of infected chickens shows the apoptosis-specific laddering pattern on agarose gels, which is not observed in DNA isolated from the thymus of non-infected chickens. Electron-microscopic analysis of the cortex ten days after infection shows cells containing condensed chromatin next to the nuclear membrane, and apoptotic bodies in the cytoplasm of epithelial cells.

In general, induction of early apoptosis in virus-infected cells will limit viral replication and reduce or prevent further spread. Several viruses encode proteins that inhibit or delay apoptosis, which may enable them to circumvent the cellular defense mechanism and produce large amounts of viral particles, ending in cell lysis and spread of progeny virus. This idea is supported by the fact that apoptosis inhibitors encoded by baculovirus, p35 and IAP, are necessary for full virulence of this virus (Clem and Miller, 1993). Some viruses encode both apoptosis inhibiting and inducing proteins, e.g. adenovirus and SV40. It can be reasoned that in these viruses the inhibiting factors first delay apoptosis, but eventually the apoptosis inducing proteins overcome this inhibition and cell death is induced, enabling viral spread. The effects of viral proteins on programmed cell death have been reviewed by several authors (Shen and Shenk, 1995; Teodoro and Branton, 1997).

The question that arises now is why CAV causes apoptosis. It is possible that apoptosis is an unplanned side effect of CAV infection, or a cellular defense mechanism to prevent virus spread by premature host-cell death. CAV may simply not have developed an apoptosis evasion mechanism, possibly because if the virus replicates fast enough, it may still be able to produce sufficient amounts of new viral particles before apoptosis occurs. Another possibility is that CAV needs its apoptosis-inducing product for the viral replication cycle. Mutation of the CAV protein involved in apoptosis induction (see below) prevents replication (Noteborn et al., unpublished data). Apoptosis may also be used as a means for virus spread. CAV-like particles have been detected in the remains of CAV-infected cells, apoptotic bodies, that were phagocytized by epithelial cells (Jeurissen et al., 1992).

A Single CAV-Protein is Sufficient for Induction of Apoptosis

To establish which CAV protein is responsible for the induction of apoptosis, plasmids encoding either VP1, VP2 or VP3 were transfected into cultured chicken mononuclear

cells. The cells were analyzed by indirect immunofluorescence using specific antibodies, and the DNA was stained with propidium iodide (PI). Expression of VP3 alone was sufficient for the induction of apoptosis as observed in CAV infection. Therefore we renamed VP3 as Apoptin©. Early after transfection (2 days) Apoptin is dispersed throughout the nucleus. Somewhat later, when the cells become apoptotic, Apoptin becomes aggregated. At this time, nucleosomal laddering can be seen in the DNA from Apoptin-expressing cells, but not in the DNA from cells transfected with a control plasmid (Noteborn et al., 1994).

Preliminary results indicate that VP2 also has some apoptotic activity, although much weaker than Apoptin. Expression of VP1 did not result in apoptosis (Noteborn et al., unpublished data).

The protein Apoptin consists of 121 amino acids. It is rich in prolines, and contains a hydrophobic region and two positively-charged regions. The latter two may encode nuclear localization signals and/or DNA-binding domains (Noteborn et al., 1987), whereas the hydrophobic region resembles the nuclear export signals described in other proteins (Wen *et al.*, 1995; Fischer *et al.*, 1995). A mutant Apoptin protein in which one of the two putative nuclear localization signals was deleted, had a significantly reduced apoptotic activity, and was localized mainly in the cytoplasm (Zhuang *et al.*, 1995a; Zhuang *et al.*, 1995c). Apoptin was found to co-localize with the chromatin in non-apoptotic cells, and with the condensed DNA in apoptotic cells. The basic regions of Apoptin may allow interaction with nucleic acids, explaining the nuclear localization. The presence of Apoptin in the chromatin structure, and its high proline content, may cause disturbance of the supercoil organization, thereby interfering with DNA replication and transcription, which could then result in apoptosis. Another possibility is that Apoptin acts as a transcriptional activator of genes, which directly or indirectly mediate apoptosis.

Apoptin-Induced Apoptosis is p53-Independent and not Inhibited by Bcl-2

Apoptin was found to induce apoptosis not only in transformed chicken thymocytes, but also in human tumor cells. The next step was to investigate the pathway by which Apoptin induces cell death.

To determine whether p53 is involved in Apoptin-induced cell death, Apoptin was transiently expressed in human osteosarcoma cells (Saos-2) lacking p53, expressing a mutant form, or expressing wild type p53. In all three cell lines Apoptin was able to induce apoptosis to the same extent, indicating that it does not need functional p53 (Zhuang et al., 1995c). Consistent with this was the finding that an inhibitor of p53, the adenovirus E1B-55K protein, did not decrease the apoptotic activity of Apoptin (Zhuang et al., unpublished data).

In human hematologic malignant cells expressing high levels of Bcl-2 or the fusion oncoprotein BCR-ABL, both inhibitors of cell death, Apoptin could still induce apoptosis. In the human immunoblastic B-cell lymphoma cell line (DoHH-2), expressing a high level of Bcl-2, Apoptin even induced cell death faster than in human acute myeloid leukemia cells (K562), expressing a low level of Bcl-2 (Zhuang et al., 1995a).

The adenoviral homolog of Bcl-2, E1B-19K, inhibits apoptosis by binding to Bax, a death-promoting member of the Bcl-2 family (Han et al., 1996; Rao and White, 1997). Adenovirus E1B-19K, but not Bcl-2, could inhibit Apoptin-induced apoptosis in some cell types to a certain extent, i.e. the human osteosarcoma cell lines Saos-2 and U2OS. In other tumor cell lines, E1B-19K had no effect on Apoptin activity. In Saos-2 and U2OS cells co-expressing E1B-19K, Apoptin was located mainly in the cytoplasm, whereas in other cells co-expressing E1B-19K, Apoptin is found in the nucleus. However, p53-induced apoptosis was inhibited by both Bcl-2 and E1B-19K in all tumor cell lines that were examined, without changes in the nuclear localization of p53 (Zhuang et al., 1995b). The finding that Apoptin-induced apoptosis is not inhibited by Bcl-2 and E1B-19K in most cell lines, whereas p53-induced apoptosis is inhibited, indicates that p53 and Apoptin use distinct pathways. The inhibition of both pathways by E1B-19K in some cells is difficult to explain, but suggests that E1B-19K might interact with different factors in the two routes.

Another inhibitor of apoptosis is BAG-1, a Bcl-2 binding protein that shows functional but no sequence homology with Bcl-2 (Takayama et al., 1995). In some cell types, Bcl-2 and BAG-1 cooperate to inhibit apoptosis, whereas Bcl-2 alone has only a minor effect. Like Bcl-2, BAG-1 can associate with and activate the kinase Raf-1, which in turn phosphorylates the Bcl-2 homologue BAD (Wang et al., 1996). Binding of BAD to Bcl-2 or Bcl-xL inhibits their anti-apoptotic activity. Upon phosphorylation, BAD dissociates from Bcl-2/Bcl-Xl, which could then inhibit apoptosis. BAG-1 also interacts with hepatocyte and platelet derived growth factor receptors, and several steroid hormone receptors, further indicating a role in the regulation of apoptosis. We therefore tested the effect of BAG-1, alone or in combination with Bcl-2, on Apoptin-induced apoptosis (Danen-van Oorschot et al., 1997a). Like Bcl-2, BAG-1 could not inhibit cell death induced by Apoptin, and neither could the combination of Bcl-2 and BAG-1. In parallel experiments BAG-1 did inhibit p53-induced apoptosis. Apoptin-induced apoptosis even seemed to be enhanced by Bcl-2, and by BAG-1 to a lesser extent. This confirms the observation mentioned above that Apoptin induces cell death more rapidly in cells expressing a high level of Bcl-2 than in cells expressing a low level of Bcl-2.

Caspases are Differently Involved in Apoptin- Versus p53-Induced Apoptosis

The different apoptotic pathways have been shown to culminate in the activation of a number of caspases (formerly called ICE-like proteases), involved in transduction of apoptotic stimuli and in the degradation phase.

To determine the involvement of caspases in Apoptin-induced cell death, we used different inhibitors. The cowpox virus serpin CrmA is a potent inhibitor of caspase 1(ICE) and caspase 8 (FLICE/Mach), but it can also with much lower affinity block the activity of caspase 3 (CPP32/Yama/apopain) (Nicholson et al., 1995; Zhou et al. 1997). Co-expression of CrmA with Apoptin, however, could not prevent apoptosis, whereas CrmA partially suppressed p53-induced cell death (Danen-van Oorschot et al., unpublished data). This suggests that caspases 1 and 8 do not play an essential role in Apoptin-induced apoptosis.

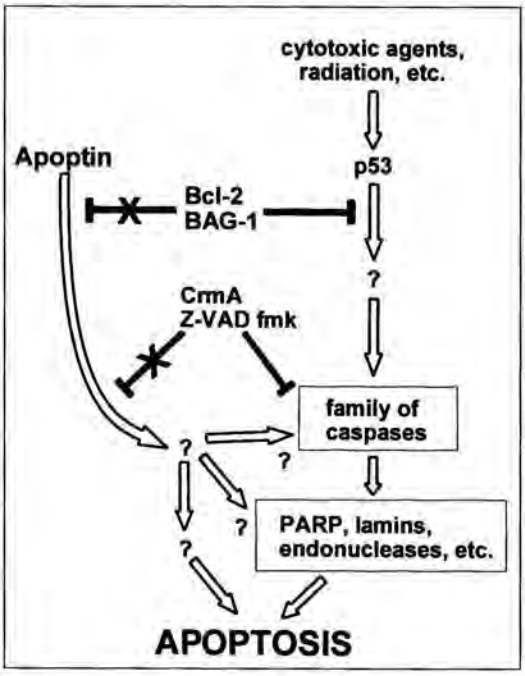

Fig. 1. Schematic representation of the Apoptin-pathway compared with the p53-pathway of apoptosis induction. In contrast to the p53-pathway, the Apoptin-pathway is not inhibited by Bcl-2, BAG-1, CrmA or Z-VAD-fmk. The more downstream part of the Apoptin-pathway is still unknown, three possible routes are shown.

Another inhibitor of caspases is the synthetic peptide N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk). The amino acid sequence VAD is similar to the cleavage site for caspase 1, and this tripeptide is regarded as a broad spectrum inhibitor of caspases. In a dose-response experiment, Saos-2 cells were transfected with plasmids encoding Apoptin or p53, and incubated with 0-100 μM Z-VAD-fmk in the medium, which was changed every 24 hours. Four days later, the cells were fixed with acetone and analyzed by indirect immunofluorescence as described previously (Danen-van Oorschot et al., 1997b). Incubation with 0-50 μM Z-VAD-fmk had no effect, but 100 μM inhibited Apoptin-induced apoptosis by about 40%. A similar inhibition was reached for p53-induced cell death at 50 μM, whereas 65% was reached at 100 μM of Z-VAD-fmk (Danen-van Oorschot et al., unpublished data). In comparison, incubation of human breast carcinoma cells with 20 (M Z-VAD-fmk could reduce caspase 8 (FLICE)-induced apoptosis by about 80%, and incubation of Drosophila Schneider cells with 50 (M Z-VAD-fmk completely blocked apoptosis induced by overexpression of Reaper (Muzio et al., 1996; Pronk et al., 1996).

These data indicate that at least caspase 1 is not necessary in Apoptin-induced apoptosis, but the involvement of other caspases that are downstream of caspase 1 (e.g. caspase 3) cannot be excluded. Alternatively, Apoptin may even act downstream of the caspases. As Apoptin was found to co-localize with the chromatin, it may bind to DNA and disturb its structure, or act as an endonuclease. Another possibility is that Apoptin associates with DNA-binding proteins, and indirectly influences

Table 1. Transformed, tumorigenic and immortalized cell lines in which Apoptin induces apoptosis.

Cell line	Type	Source
	human	
Saos-2	osteosarcoma derived cells, $p53^-$, Rb^-	7
U2OS	osteosarcoma derived cells, $p53^+$	7
Hep3B	hepatoma derived cells, $p53^-$	41
G401	kidney rhabdoid tumor derived cells, $p53^+$	10
KG-1	acute myeloid leukemia derived cells	21, 25
K562	acute myeloid leukemia derived cells, Bcr-Abl	21, 25
DoHH-2	immunoblastic B-lymphoma derived cells, high Bcl-2	19
Jobo-0	Epstein-Barr virus immortalized lymphoblastoid B cells	63
NW-18	SV40-transformed tumorigenic fibroblasts	58
Pre	SV40-transformed pre-crisis VH10 fibroblasts	18
Post	SV40-transformed post-crisis VH10 fibroblasts	18
SCC-15	squamous-cell carcinoma derived keratinocyte cells	43
HaCaT	spontaneously transformed keratinocytes	1
SVK14	SV40-transformed keratinocytes	51
911	adenovirus-5-transformed embryonal retinoblasts	8
293	adenovirus-5-transformed embryonal kidney cells	14
HT29	colon carcinoma derived cells	56
	non-human	
N1E-115	murine neuroblastoma cells	32
P19	murine embryonal teratocarcinoma cells	28
BRK-Xho	adenovirus-5-transformed baby rat kidney cells	47
cc531	rat colon carcinoma derived cells	27
cos-7	monkey SV40-transformed kidney cells	12

the chromatin structure. We are currently trying to identify proteins that associate with Apoptin by applying the yeast-two-hybrid system.

A schematic overview of the pathway via which Apoptin and p53 induce apoptosis, is given in figure 1.

Apoptin Induces Apoptosis in Tumor Cells, but not in Normal Cells

The effect of Apoptin has been examined in many human and other mammalian tumor cell lines of different origins, and in all of these apoptosis was induced (see table 1). In some tumor cell lines apoptosis was induced faster than in others, but always reaches 90-100 % at 5-6 days after transfection.

Surprisingly, Apoptin did not induce apoptosis in normal, diploid cells. We have examined human keratinocytes, vascular endothelial cells (HUVECs) and smooth-muscle cells (HSMC) at passage 3-5 after isolation, human primary T cells, and human diploid fibroblasts (VH10) at a low passage number (10-14) in tissue culture. Also, murine and rat embryonal fibroblasts were studied, but in none of these cells Apoptin was able to induce apoptosis (see table 2).

Table 2. Normal, diploid cells in which Apoptin does not induce apoptosis.

Cell line	Type	Source
	human	
T cells	phytohemagglutinin stimulated primary T cells	26
HUVEC	umbilical-cord vascular endothelial cells	16
HSMC	smooth muscle cells	16
FSK-1	epidermal keratinocytes	44, 39
VH10	diploid fibroblasts	18
	non-human	
REF	rat embryonal fibroblasts	47
MEF	mouse embryonal fibroblasts	47

Next, the effect of Apoptin was studied in transformed cells derived from these normal cells. Apoptin was found to induce apoptosis in VH-10 cells and keratinocytes that were transformed with SV40, and in spontaneously immortalized keratinocytes. Upon transformation, these cells apparently became susceptible to Apoptin-induced cell death (Danen-van Oorschot et al., 1997b). This indicates that Apoptin specifically induces apoptosis in transformed and tumorigenic cells, but not in normal diploid cells. These experiments have all been performed in cell culture, and it would therefore be of great interest to determine whether Apoptin shows the same specificity in vivo.

A clue as to why Apoptin specifically induces apoptosis in transformed and tumorigenic cells came from analysis of its localization by indirect immunofluorescence. In tumor cells Apoptin is localized in the nucleus, first in finely dispersed form, and when the cell becomes apoptotic it starts to aggregate. However, in normal diploid cells Apoptin is found predominantly as small granules in the cytoplasm (fig 2). As mentioned above, a truncated version of Apoptin, lacking one of the putative nuclear localization signals, is also localized mainly in the cytoplasm, but in more diffused form. This truncated Apoptin has a strongly reduced apoptotic activity. These observations suggest that the nuclear localization of Apoptin is an important factor for its ability to induce apoptosis. However, it is possible that nuclear localization alone is not sufficient, Apoptin may also need to be modified to become active.

Apoptin: Therapeutic Applications?

Treatment of tumors with cytotoxic agents or radiation has been shown to kill tumor cells often via the induction of apoptosis. However, many tumors are resistant to apoptosis induction by these treatments due to loss of p53 function or to overexpression of Bcl-2. For these categories of tumors, the development of new, effective therapies would be very welcome.

The results we have obtained sofar with Apoptin suggest that this viral protein might be a promising candidate for the treatment of tumors, including those that lack

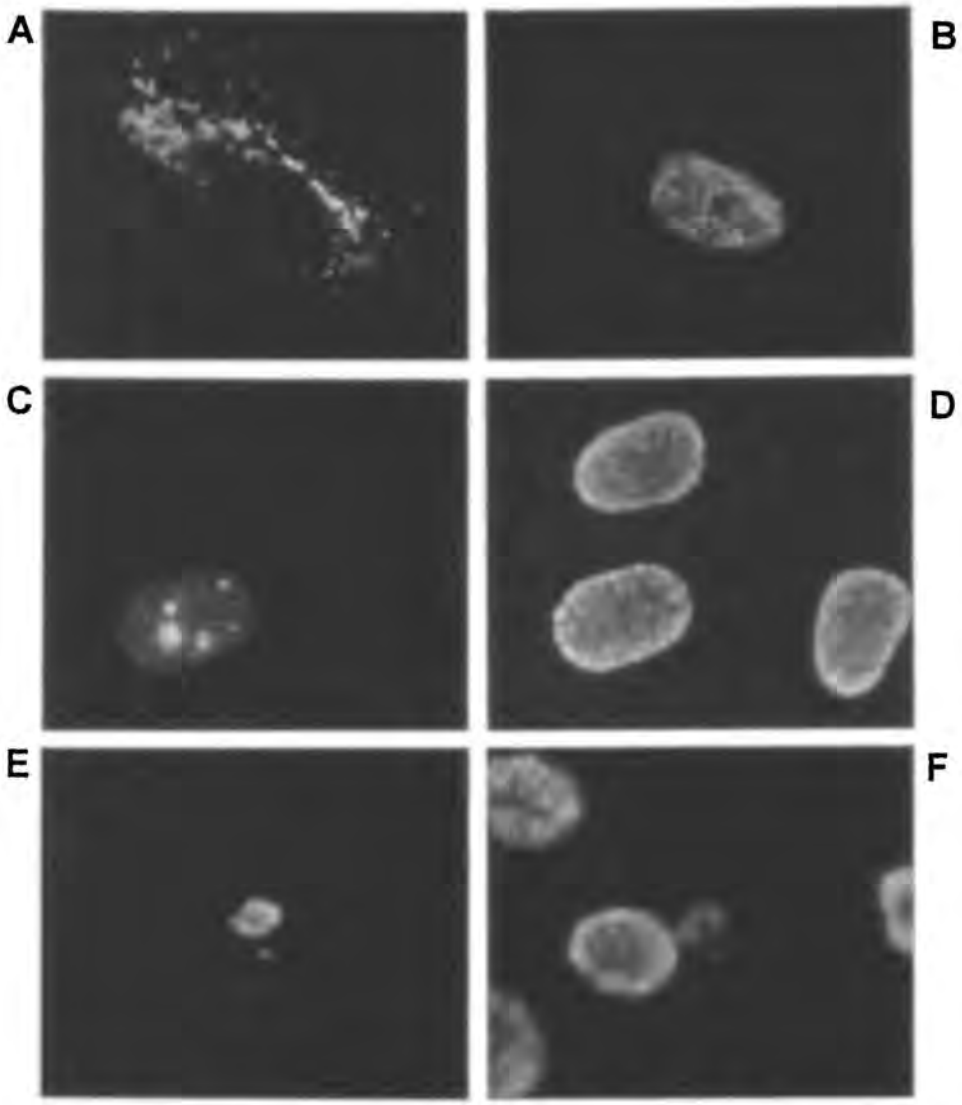

Fig. 2. Localization of Apoptin in normal diploid vs. transformed/tumor cells. Cells were transiently transfected with an expression plasmid encoding Apoptin (pCMV-VP3) and fixed with 80% acetone 2-4 days after transfection. Expression of Apoptin was determined by indirect immunofluorescence using specific antibodies 85.1 (C,E) or 111.3 (A). The DNA was stained with DAPI (B,D,F), which stains intact DNA strongly but apoptotic DNA weakly or irregularly (see also Danen-van Oorschot et al., 1997b).

A,B: normal diploid smooth muscle cell, four days after transfection, with Apoptin in the cytoplasm, non-apoptotic. C,D: tumorigenic fibroblast (NW18), two days after transfection, Apoptin is in the nucleus, non-apoptotic. E,F: NW18, four days after transfection, Apoptin is more aggregated, the cell expressing Apoptin has become apoptotic. Magnification: 1000×.

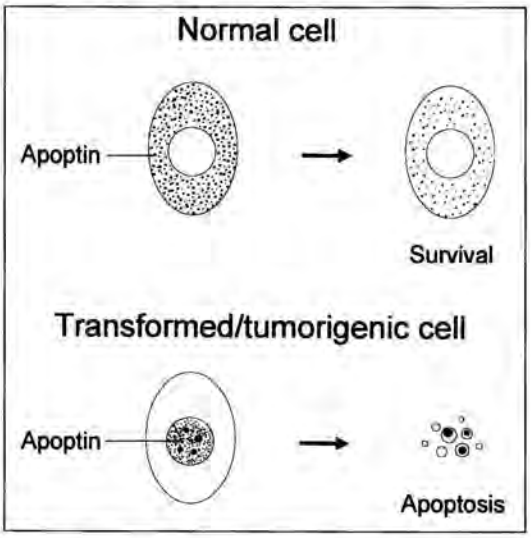

Fig. 3. Schematic overview of differential effect of Apoptin in normal vs. transformed/tumor cells. A: in a normal cell, Apoptin is localized mainly in the cytoplasm as granules of different sizes. This cell will not undergo apoptosis, and our observations (data not shown) indicate that Apoptin is eventually degraded. B: in a transformed/tumorigenic cell, Apoptin is predominantly seen in the nucleus, finely dispersed with some larger aggregates/granules. This cell undergoes apoptosis, and apoptotic bodies are formed in some of which Apoptin can still be found.

functional p53 or overexpress Bcl-2 or BCR-ABL. The apoptosis inhibitor Bcl-2 not only fails to block, but even seems to enhance Apoptin-activity (Danen-van Oorschot et al., 1997a). In cells over-expressing Bcl-2, Apoptin induces cell death more rapidly than in other cells. Apoptin can also cause programmed cell death in cells expressing the apoptosis inhibitor BCR-ABL (Zhuang et al., 1995a).

Interestingly, Apoptin does not induce apoptosis in normal diploid cells grown in vitro. Upon transformation, these cells become susceptible to Apoptin-induced cell death, indicating that this protein can differentiate between normal and transformed/tumor cells (schematically depicted in fig. 3). Taken together, these observations indicate that Apoptin is a promising candidate as an anti-tumor agent.

In order to use Apoptin as an anti-tumor agent, gene-therapy strategies are needed. A major problem that needs to be overcome is the efficient delivery of Apoptin throughout the entire tumor and to all metastases. Presently, the most commonly used gene delivery systems are viral vectors because of their high infection efficiency.

Retroviral vectors are used in the majority of approved gene-transfer protocols (reviewed by Roth and Cristiano, 1997). The advantage of this system is that the viral DNA can stably integrate into the host genome, which ensures long term expression of the introduced gene. However, this may also be disadvantageous because of the risks associated with random integration. Nevertheless, retroviral vectors can only infect dividing cells, and they are, therefore, appropriate for the treatment of cancer (Vile et al., 1996; Roth and Cristiano, 1997).

Other commonly applied vectors are based on adenovirus 5 (Ad5). Adenoviral vectors can infect many different cell types and do not require cell division. They are specifically targeted to the respiratory and gastrointestinal epithelia, as well as to the liver. This system is less suitable for long-term expression as adenoviral DNA does not integrate into the host genome. This has the advantage that there is no risk of insertional mutagenesis. These properties, and the fact that adenoviral vectors have a

high transduction efficiency and can be produced at high titers (1011), renders these vectors particularly suitable for cancer gene therapy (Ginsberg, 1996; Kozarsky and Wilson, 1993; Roth and Cristiano, 1997).

Both a retroviral and an adenoviral vector are currently being developed for the delivery of Apoptin in vivo. The preliminary results of toxicity tests in rats are promising.

It was unclear, initially, whether viral vectors expressing the Apoptin gene could be produced at all, since the helper cells that are used to grow the defective vectors might be susceptible to apoptosis induction by the insert gene. Indeed, the murine packaging cell lines PA317 and Ψ-crip which we intended to employ for the production of the retroviral vectors, were killed within 4 days after the onset of virus production (Noteborn et al., unpublished data). Hence, retroviral vectors carrying the Apoptin gene can (possibly) be produced only in a batchwise fashion, at least if no precautions are taken to prevent expression of the insert during virus replication. The helper cells used to grow adenoviral vectors, 293 cells and 911 cells, as expected were also susceptible to the killing effect of Apoptin. However, in this case the lytic cycle of the adenovirus vector proceeded faster than the induction of apoptosis, so that viral stocks with high titres could be obtained (Pietersen et al., unpublished data). Further studies on the effects of these Apoptin-expressing vectors in experimental animals will reveal whether in vivo expression of the gene will have toxic side effects.

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Epilogue

The decade-long debate continues over whether the concept of apoptosis is a milestone in the evolution of 21st century medicine or perhaps another so-called bandwagon, a transiently popular scientific idea that ultimately fails to be productive and eventualy falls by the wayside. As is reflected in the title of these Proceedings, at the International Workshop funded by the Royal Netherlands Academy of Arts and Sciences the concept of apoptosis was discussed in terms of its potential value for drug discovery and development rather than as a novel molecular mechanism alone. The general feeling emerging from the exciting discussions was that the impact of the concept on medicine is only beginning to be realized and appreciated.

The evolution of rational and novel drug development strategies may in fact ultimately prove to be the strength of the concept of apoptosis. Molecular mechanisms, regulatory genes, and the need to organize and classify biological processes clearly suggest that apoptosis is a convergent array of metabolic pathways leading to the deletion of individual cells from a population. Much has been learned about the divergent molecular mechanisms that in concert bring about macroscopic changes in tissues and organs that range from neoplastic transformation to the appearance of frank tissue and organ damage. Simple mechanisms of a few years ago have given way to multiple complex regulatory systems that comprise nonlinear feedback control of not only the death of individual cells, but also the survival and function of entire tissues. Few specific processes have been uniquely associated with apoptotic death of cells and many key processes have been enlisted for other purposes. In this emergent view, apoptosis is clearly not a single set of molecular events but rather a general category of regulatory processes that are closely integrated with many others.

Whereas the term 'apoptosis' originally signified a special form of programmed or gene-directed cell death, the whole concept has undergone an evolution as the complexity of the regulatory pathways have become more clear. It is now understood that the vast majority of cells that die in the body do so through a programmed cell death process. As the concept began to emerge, it was commonly thought that necrosis was the most likely form of death whereas evidence of apoptotic cell death was believed to be limited to developmental processes primarily during embryogenesis. Today, dysfunctional or inappropriate apoptosis is widely recognized as a key element in pathogenesis of disease. Furthermore, it is known that once the molecular cascade leading to apoptotic morphology has been initiated, it can be terminated at some time prior to the first irreversible step. New evidence has shown that cells not

only do not have to die, but they can either suppress or enhance the likelihood that neighbouring cells will die by the same process. Together, these observations make it increasingly difficult to adhere to a simple view of apoptosis; perhaps it is more accurately viewed as a program for maintenance of phenotypic fidelity and as a defence mechanism towards potentially fatal infections.

There is little value in simply calling cell death in the body by a new name. Therefore, it is of considerable value to examine the advantages of the concept of apoptosis in terms of the capacity to provide counterintuitive insights into normal physiology and the pathogenesis of disease states. Perhaps the most profound effect of the application of apoptosis is in the field of therapeutics where understanding of molecular mechanisms of disease development infers to intervene through the action of drugs. However, some may argue that the understanding of the molecular basis of disease may always be limited to the penultimate mechanism described in current literature. In the case of apoptosis, it may be difficult to convincingly prove that intervention into the control of apoptosis is the basis for a positive outcome. For the present, therapeutic efficacy of apoptosis modulation is an empirical test of hypotheses born from simple *in vitro* experiments. If apoptosis dysfunction is a valuable concept in medicine, then it will uniquely lead to discovery of effective drugs that otherwise may never have been recognized.

But it is clear that the potential for realization of a new generation of drugs is in fact rapidly approaching. New apoptosis modulating drugs are being discovered while the first ones complete preclinical animal studies and enter human trials. This new class of drugs has led researchers to counterintuitive discoveries of drug action in the intact organ and animal models used to assess potential medical value in humans. It is that counterintuitive nature that makes the concept of apoptosis so novel and valuable in the field of molecular pharmacology.

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