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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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