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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

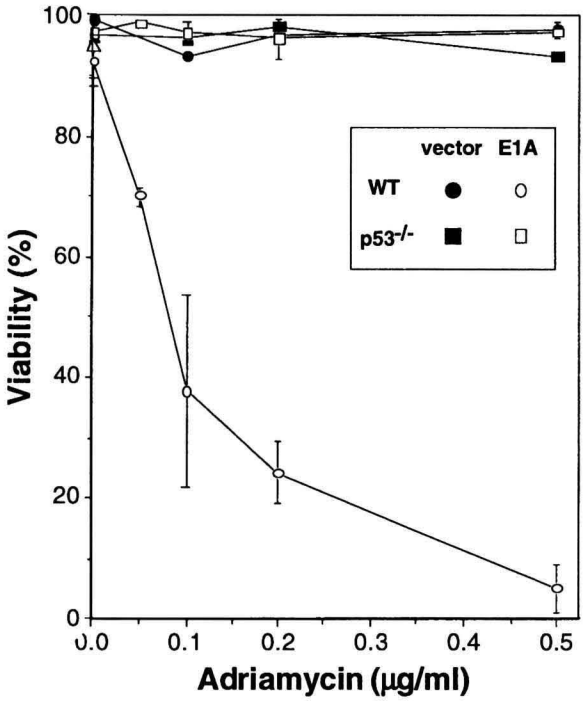

Fig. 1. E1A confers chemosensitivity. Mouse embryo fibroblasts derived from wild-type (wt) and p53-deficient mice were infected with a control retrovirus (vector) or one expressing an adenovirus-5 12S E1A cDNA. Cell populations were treated with various concentrations of the chemotherapeutic drug adriamycin, 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

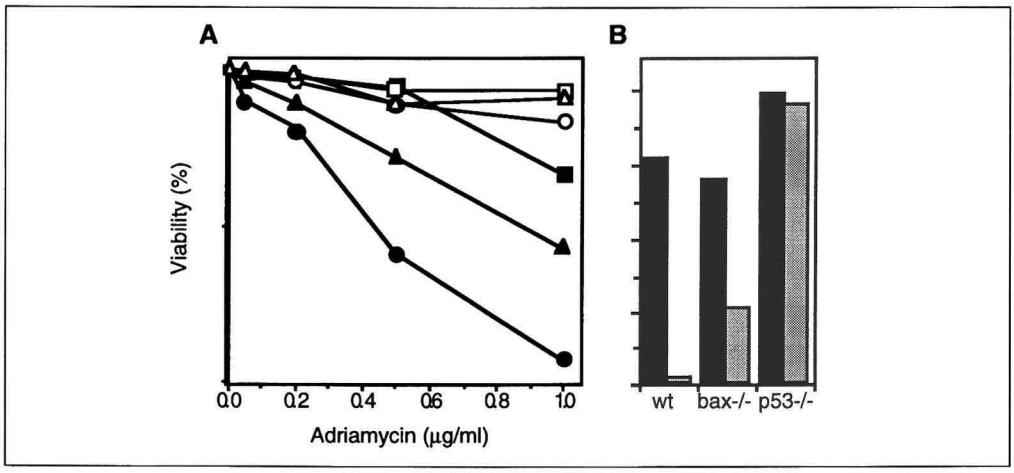

Fig. 2. Bax participates in a p53-dependent apoptotic program.
A. E1A was introduced into wild type (circles), bax-/- (triangles) or p53-/- (squares) MEFs by retroviral-mediated gene transfer. The resulting E1A-expressing cell populations were infected with a Bcl-2 expressing retrovirus (open symbols) 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

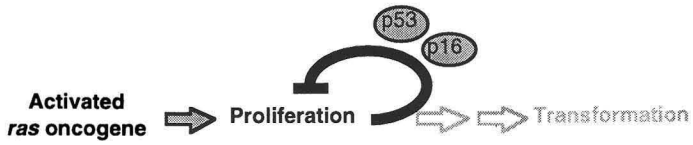

Fig. 3. p16 and p53 suppress transformation by oncogenic ras.
Prolonged expression of an activated ras oncogene induces a permanent cell cycle arrest with features of senescence. Both p53 and p16 levels increase in response to oncogenic ras and contribute to the arrested state. Escape from ras-induced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