Harnessing Apoptosis for Improved Anticancer Gene Therapy

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Abstract

Advances in our understanding of the mechanisms by which tumor cells detect drug-induced DNA damage leading to apoptotic death have aided in the design of novel, potentially more selective strategies for cancer treatment. Several of these strategies use proapoptotic factors and have shown promise in sensitizing tumor cells to the cytotoxic actions of traditional cancer chemotherapeutic drugs. Although antiapoptotic factors are generally regarded as poor prognostic factors for successful cancer chemotherapy, strategies that use antiapoptotic factors in combination with suicide or other gene therapies can also be considered. The introduction of antiapoptotic factors that act downstream of drug-induced mitochondrial transition delays, but does not block, the ultimate cytotoxic response to cancer chemotherapeutic drugs that activate a mitochondrial pathway of cell death. Recent studies using the cytochrome P-450 prodrug cyclophosphamide exemplify how the antiapoptotic, caspase-inhibitory baculovirus protein p35 can be combined with P-450 gene-directed enzyme prodrug therapy to prolong localized, intratumoral production of cytotoxic drug metabolites without inducing tumor cell drug resistance. This model may be adapted to other gene therapies, including those that target death receptor pathways, to maximize the production of soluble, bystander cytotoxic factors and prodrug metabolites and thereby amplify the therapeutic response.

Introduction

Aberrant regulation of cell growth has traditionally been viewed as the major underlying mechanism for tumor formation; however, it is becoming increasingly clear that cellular changes that lead to inhibition of apoptosis play an essential role in tumor development (1). Many cancer chemotherapeutic drugs activate apoptotic mechanisms of tumor cell death, suggesting that factors that impair programmed cell death contribute to the resistance of tumor cells to cytotoxic drug treatment (2). Elucidation of the apoptotic pathways that are triggered by anticancer therapies is thus an important area of study that may provide insights into the underlying causes of intrinsic and acquired drug resistance and facilitate the development of novel anticancer therapies. This review discusses recent advances in this field and highlights novel therapeutic approaches that use proapoptotic factors to increase responsiveness to classic anticancer drugs, as well as antiapoptotic factors to optimize suicide and other gene-based therapies for cancer treatment.

Role of Caspases in Tumor Cell Death

Many commonly used anticancer drugs induce tumor cell apoptosis, a process that is mediated by caspases, a ubiquitous family of cysteine proteases that includes both upstream (initiator) and downstream (effector) caspases (3, 4). Caspases are synthesized in an inactive proform that is activated by proteolytic cleavage at two or more sites. Cleavage at one site generates the large and small subunits of the mature, active protease, whereas cleavage at a second site removes the prodomain (3, 5, 6). The initiator caspases, typically caspases 8 and 9, are activated by two alternative pathways, both of which lead to apoptotic cell death. One pathway is triggered by cellular stresses that induce changes in mitochondrial function and is primarily associated with the activation of caspase 9 (“intrinsic” apoptotic pathway; Refs. 7, 8). The second (“extrinsic”) pathway activates caspase 8 and proceeds via the formation of a DISC at the cell surface, which provides a mechanism for aggregation and autocleavage (autoactivation) of the caspase (Ref. 9; Fig. 1). As discussed below, anticancer drugs with diverse mechanisms of action can activate both apoptotic pathways. Moreover, in both pathways the initiator caspase cleaves and thereby activates downstream, effector caspases, such as caspase 3, caspase 7, and others. This caspase cascade ultimately leads to proteolytic cleavage of a variety of cellular proteins and induces the broad range of morphological changes that are characteristic of cells undergoing apoptosis.

Mitochondrial Cell Death Pathway

In stress-induced cell death, signals received by mitochondria stimulate mitochondrial membrane permeabilization and release several proapoptotic factors into the cytosol (10–12). Key mitochondrial factors released in this manner include cytochrome c (13), certain caspases (14), AIF, which induces chromatin condensation and DNA fragmentation (15, 16), and Smac/Diablo, which neutralizes IAP proteins and allows caspase activation to proceed (Refs. 17–19; Table 1). Mitochondrial release of cytochrome c triggers formation of the apoptosome, an oligomeric, multiprotein complex comprising cytochrome c, ATP, caspase 9, and the scaffold protein Apaf-1, which stimulates/amplifies the activation of caspase 9 and downstream apoptotic events (20, 21).

Mitochondrial cytochrome c release and apoptosome formation are subject to regulation by proteins belonging to the Bcl-2 family, comprising at least 16 family members. The Bcl-2 family includes proapoptotic members, such as Bax and Bid, which promote mitochondrial release of proapoptotic factors; and anti-apoptotic members, such as Bcl-2, Bcl-XL, and Mcl-1, which block factor release (22). A positive correlation between the expression of Bcl-2 and a chemoresistant phenotype has been observed in many human tumors, suggesting that Bcl-2 and other family members are important determinants of the clinical responsiveness to a wide range of anticancer chemotherapeutic agents (22).

Receptor-mediated Cell Death Pathway

Receptor-mediated cell death is initiated by the binding of a death-inducing ligand to a cysteine-rich repeat region in the extracellular domain of a death receptor. This, in turn, leads to activation of trimerized death receptor at the cell surface and activation of caspase

1 The abbreviations used are: DISC, death-inducing receptor signaling complex; AIF, apoptosis-inducing factor; IAP, inhibitor of apoptosis; Apaf-1, apoptosis protease-activating factor 1; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; FADD, Fas-associated death domain-containing protein; FLIP, Fas-associated death domain-like ICE inhibitory protein; TRAF, TNF receptor-associated factor; NF-kB, nuclear factor-kB; DNA-PK, DNA-dependent protein kinase; GDEPT, gene-directed enzyme prodrug therapy; HSV-tk, herpes simplex virus-thymidine kinase.

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8-dependent cell death, as outlined below (Fig. 1). Death receptor ligands include TNF-α, Fas ligand, and TRAIL, each associated with its own specific death receptor. These death receptor-activating ligands are expressed in both membrane-bound and soluble forms and share a homologous 150-amino acid region that interacts with, and may serve to aggregate, the death receptor (23). Each death receptor contains a cytoplasmic tail “death domain” that binds the correspond-
ing COOH-terminal death domain of an adaptor protein such as FADD (24). The adaptor protein additionally contains a death effector domain (25) that binds to the NH2 terminus of the caspase 8 prodomain, thus facilitating DISC formation and proteolytic autoactivation of caspase 8.

Two classes of cells can be distinguished based upon their response to factors that induce death receptor-dependent cell death (26). In type

![Apoptotic Factors and Cancer Gene Therapy](https://cancerres.aacrjournals.org/content/63/21/8564)

Table 1  Factors involved in tumor cell apoptosis

<table>
<thead>
<tr>
<th>Factor/Complex</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apaf-1 (apoptosis protease-activating factor 1)</td>
<td>Adaptor protein of the apoptosome. Critical for assembly of complex leading to binding of caspase 9, cytochrome c, and ATP, and resulting in caspase 9 activation.</td>
<td>(15, 16)</td>
</tr>
<tr>
<td>AIF (apoptosis-inducing factor)</td>
<td>Bcl-2-regulated mitochondrial protein that induces chromatin condensation and DNA fragmentation.</td>
<td>(15, 16)</td>
</tr>
<tr>
<td>Apoptosome</td>
<td>Multi-protein complex comprising the adaptor protein Apaf-1, caspase 9, cytochrome c, and ATP.</td>
<td>(20, 21)</td>
</tr>
<tr>
<td>Bcl-2 family</td>
<td>Family of proteins comprising pro- and antiapoptotic members. Regulates release of proapoptotic factors from mitochondria.</td>
<td>(22)</td>
</tr>
<tr>
<td>Caspases</td>
<td>Cysteine proteases whose activation leads to proteolytic cleavage of various cellular proteins and induces the large number of morphological changes characteristic of cells undergoing apoptosis.</td>
<td>(3)</td>
</tr>
<tr>
<td>DED (death effector domain)</td>
<td>Protein domain that regulates programmed cell death. The DED contains a flexible coil that interacts with caspase 8 to mediate DISC formation. Some DED-containing proteins can inhibit DISC formation.</td>
<td>(25)</td>
</tr>
<tr>
<td>Death receptors</td>
<td>Cell surface proteins that bind to extracellular death-inducing ligands, aggregate, and induce an intracellular cascade of apoptotic events.</td>
<td>(3)</td>
</tr>
<tr>
<td>DISC (death-inducing signaling complex)</td>
<td>Multi-protein complex comprising a death receptor and ligand, an adaptor protein, and caspase 8.</td>
<td>(2)</td>
</tr>
<tr>
<td>FADD (Fas-associated death domain-containing protein)</td>
<td>Adaptor protein associated with death receptors. Contains both a “death domain” (DD), which interacts with the receptor, and a DED, which interacts with caspase 8.</td>
<td>(2)</td>
</tr>
<tr>
<td>FLIP (Fas-associated death domain-like ICE inhibitory protein)</td>
<td>Cellular protein structurally similar to caspase 8. Interferes with recruitment and activation of caspase 8 by death receptors.</td>
<td>(23)</td>
</tr>
<tr>
<td>IAP (inhibitor of apoptosis)</td>
<td>Protein that binds directly to and inactivates caspases.</td>
<td>(19)</td>
</tr>
<tr>
<td>Smac/Diablo</td>
<td>Bcl-2-regulated mitochondrial protein that inactivates certain IAPs.</td>
<td>(17, 18)</td>
</tr>
<tr>
<td>TRAF (tumor necrosis factor receptor-associated factor)</td>
<td>Family of adaptor proteins that couples the tumor necrosis factor receptor family and can convey either a death signal or a survival signal.</td>
<td>(39)</td>
</tr>
</tbody>
</table>
I cells, the binding of a death receptor ligand activates caspase 8 to a level that is sufficiently high to directly initiate a downstream caspase cascade. In type II cells, however, the cellular level of activated caspase 8 is too low to propagate the caspase cascade and the ensuing apoptotic response in the absence of a mitochondria-dependent amplification step. This cross-talk from the death receptor pathway to the mitochondrial apoptotic pathway is initiated by caspase 8 cleavage of the cytosolic, proapoptotic Bcl-2 family protein Bid, which translocates to mitochondrial membranes and induces cytochrome c release (Refs. 27, 28; Fig. 1). The resulting formation of the apoptosisosome, activation of caspase 9 and the downstream apoptotic responses seen in death receptor ligand-treated type II cells can be blocked by mitochondrial antia apoptotic factors such as Bcl-2 (26, 28, 29). Presumably, type II cells differ from type I cells by having lower levels of caspase 8 or one of the other factors required for formation of the DISC complex. Alternatively, the mitochondrial dependence of death receptor-induced apoptosis in type II cells may result from the expression of an apoptosis resistance factor that partially blocks caspase 8 activation, rendering the cells dependent on mitochondrial amplification of the initial death signal (28).

**Cellular IAPs**

The basal level of apoptosis is tightly controlled in mammalian cells by endogenous IAPs, several of which may become dysregulated and have been implicated in tumorigenesis and chemoresistance. IAP proteins inhibit mitochondrial- and death receptor-induced apoptosis, in some cases by binding directly to and inactivating the target caspase (19). In the case of XIAP, mitochondrial cell death is blocked by direct IAP inhibition of cytochrome c-induced caspase 9 activation. By contrast, caspase 8 is resistant to the inhibitory action of XIAP, which suppresses death receptor apoptosis at the downstream, caspase 3 activation step (30). One IAP, survivin, is frequently overexpressed in tumor cells (31). IAP activity is regulated by Smac/Diablo, which upon release from mitochondria inhibits IAP activity, thereby facilitating caspase 9 activation (17, 18). Moreover, IAPs with a RING finger domain, such as cIAP1 and cIAP2, have intrinsic ubiquitin protein ligase (E3) activity, which facilitates their auto-ubiquitination and degradation and may lead to IAP down-regulation in response to certain apoptotic stimuli (32).

FLIPs are antiapoptotic factors that contain death effector domain sequences and thus share structural similarity with caspase 8. This feature enables FLIPs to interfere with the recruitment and activation of caspase 8 by ligand-activated death receptors, thereby blocking receptor-mediated apoptosis (Ref. 23; Fig. 1). Receptor-mediated cell death can also be inhibited by the expression of decoy receptors, which bind death receptor ligands but have a truncated (or absent) cytoplasmic death domain and are therefore unable to transmit an intracellular death signal. Decoy receptors inhibit apoptosis induced by TRAIL [decoy receptors DcR1 and DcR2 (33, 34)] and Fas ligand [decoy receptor DcR3 (35, 36)]. TRAIL decoy receptors DcR1 and DcR2 are down-regulated in tumor cells but not normal cells (37, 38) and may protect normal cells from TRAIL-induced apoptosis. By contrast, the Fas decoy receptor DcR3 is overexpressed in lung and colon tumors, suggesting a role in tumor cell resistance to Fas ligand (35).

In contrast to the proapoptotic responses that are stimulated by Fas and TRAIL receptor, TNF receptor signaling often leads to enhanced tumor cell survival and proliferation. TNF receptor recruitment of TRAF adapter proteins leads to the activation of NF-κB (39), which acts as a survival factor and a suppressor of apoptosis (40, 41). NF-κB suppresses apoptosis by multiple mechanisms. These include up-regulation of anti-apoptotic factors, such as TRAFs, IAPs (42, 43), TRAIL decoy receptor (44), and FLIP (45), leading to a block in caspase 8 cleavage and conferring resistance to Fas ligand and TNF-α (45). NF-κB also induces the Bcl-2 homologue A1/Bfl-1, which blocks etoposide-induced apoptosis by inhibiting mitochondrial cytochrome c release and caspase 3 activation (46). cIAP1 bound to TNF receptor signaling complexes can induce ubiquitination and degradation of the associated TRAF adapter protein, thereby interrupting TNF induction of NF-κB and potentiating TNF-induced apoptotic responses (47).

**Induction of Apoptosis by Drug-induced DNA Damage**

The association between drug-induced DNA damage, apoptosis, and tumor remission is well-established for hematological malignancies and for some, but not all, solid tumors (48). Recent studies have provided insight into the question of how tumor cells detect nuclear DNA damage induced by cancer chemotherapeutic drugs and how this leads to the induction of apoptosis. Drugs such as methotrexate (49), betulinic acid (50), and cyclophosphamide (51) activate the mitochondrial/caspase 9 pathway of cell death, whereas other drugs, including doxorubicin (50), etoposide, teniposide (52), 5-fluorouracil (53, 54), cytarabine (55), and cisplatin (56), primarily activate the death receptor/caspase 8 pathway. In some cases the pathway of apoptotic death is tumor cell type dependent, as reported for paclitaxel (Taxol; Refs. 57, 58) and the produg ganciclovir (59, 60). Drug-induced cell death may also involve other pathways that diverge in one or more respects from the classical apoptotic pathways, as suggested for paclitaxel (61, 62) and tamoxifen in the case of estrogen receptor-positive tumor cells (63).

The sensing of DNA damage in the form of single-strand DNA and double-strand DNA breaks is an intricate process that is only partially understood. DNA-binding factors that may serve as sensors of DNA damage include the RAD17-RFC and Rad9–1–1 supercomplex for single-strand DNA breaks, and the Ku subunit of DNA–PK, the MRE11–Rad50–NBS1 complex, and the tumor suppressor BRCA1 for double-strand DNA breaks (64–67). Directly downstream of these proteins are three enzymes belonging to the phosphatidylinositol 3-kinase family: DNA–PK, ATM, and ATR. These enzymes, often referred to as transducers, relay and amplify the DNA damage signal by phosphorylation of a small group of effector kinases. These include the serine/threonine kinases Chk1 and Chk2 (65, 66) and the ubiquitously expressed tyrosine kinase c-Abl (Refs. 68, 69; Fig. 2). Phosphorylation of these effector kinases, in turn, leads to phosphorylation of various downstream targets, including the transcription factors p53, p73, and E2F-1 (70–73) and may result in DNA repair, cell cycle arrest, or apoptosis. Interestingly, several of the transducers and downstream effectors in this pathway, including ATM, ATR (66), and p53 (74), may also serve as direct DNA damage sensors, highlighting the complexity of the overall DNA damage response pathway. Phosphorylation of p53 enhances the DNA damage response by multiple mechanisms, including trans-activation of p21, a potent inhibitor of cyclin-dependent kinase and inducer of cell cycle arrest (70, 75, 76). Effector kinase-catalyzed serine phosphorylation of p53’s trans-activation domain blocks the binding of Mdm-2, an E3 ubiquitin protein ligase that targets p53 for proteasome degradation (65, 70, 76). The resulting increase in p53 protein stability increases the transcription of p53 target genes, including those encoding the proapoptotic Bcl-2 family members Bax, Noxa (77, 78), and PUMA (79). These proteins, in turn, translocate from the cytosol to the mitochondrial outer membrane and induce mitochondrial transition, apoptosis formation, and caspase 9-dependent apoptosis. p53 can also down-regulate Bcl-2 (80, 81) and stimulate expression of enzymes such as proline oxidase, which increases mitochondrial production of cytotoxic, reactive oxy-
Drug-induced Fas ligand gene expression may be induced by DNA damage in certain tumor cell types (97, 98). One proposal is based on the observation that DNA damage-inducing anticancer drugs such as doxorubicin, 5-fluorouracil, and cisplatin may induce the expression of Fas ligand and the TRAIL receptors DR4 and DR5. The mechanism by which these drugs induce Fas ligand and TRAIL receptor gene expression is not yet fully understood, but it is believed to involve the activation of caspase-dependent apoptotic responses in tumor cells treated with DNA damage-inducing agents such as doxorubicin, 5-fluorouracil, and cisplatin.

Several hypotheses have been advanced to explain the activation of caspase-dependent apoptotic responses in tumor cells treated with DNA damage-inducing agents such as doxorubicin, 5-fluorouracil, and cisplatin. One hypothesis is that DNA damage induces the expression of caspase-dependent apoptotic factors in tumor cells. Another hypothesis is that DNA damage induces the expression of proapoptotic Bcl-2 family members such as Mcl-1, which directly interacts with and catalyzes p73 tyrosine phosphorylation. These hypotheses, in turn, initiate a phosphorylation cascade (P) that involves the serine/threonine kinases Chk1 and Chk2 and the ubiquitously expressed tyrosine kinase c-Abl and their downstream targets, including the transcription factors p53, p73, and E2F-1. Phosphorylation of these molecules may alter their transcriptional activity, resulting in the transcriptional (T) activation of numerous genes, some of which are shown in this simplified scheme. Several proapoptotic molecules are downstream-regulated in response to DNA damage, including Bcl-2 and Mcl-1 (inverted arrows). Other pathways, as well as cross-talk within the pathways shown, is known to occur. See text and references therein for further details.

Proapoptotic Cancer Therapies

Several anticancer therapies are currently being developed with the goal of modulating the expression or activity of factors that contribute to tumor cell apoptosis. In one approach, enhanced chemosensitivity can be achieved by gene therapies delivering transcription factors that respond to DNA-damaging agents, such as p53 (106–108) and E2F-1 (109–111). Chemo-responsiveness can also be increased by gene therapy using the adenovirus E1A protein, which imparts a range of antitumor effects, including induction of apoptosis (112), and can be delivered in a tumor-selective manner using conditionally replicating adenoviral vectors (113). In another approach, proapoptotic genes can be introduced into tumor cells to enhance chemosensitivity. For example, cellular caspase activity can be increased by delivery of a full-length zymogen form of a caspase cDNA (114, 115) or by delivery of a mutant, inhibitory form of the IAP survivin (116). The mitochondrial cell death pathway can be selectively targeted by delivery of a proapoptotic protein such as Apaf-1 (117) or Bax, which counters the chemoresistant effects of Bcl-2 (118, 119). Alternatively, antiapoptotic proteins such as Bcl-2 or Bcl-XL can be down-regulated using antisense oligonucleotides (120–122), by intracellular expression of anti-Bcl-2 antibodies (123), or by introduction of Bcl-2 siRNA (124). These latter studies reveal a striking role for Bcl-2 in suppressing p53-dependent apoptosis, as demonstrated by the massive apoptosis of colorectal carcinoma cells that occurs upon silencing of Bcl-2 expression, even in the absence of genotoxic agent treatment (124). Novel small molecule inhibitors selective for Bcl-2 or Bcl-XL may also be useful (125, 126). Bcl-2 and Bcl-XL inhibitory strategies may be particularly effective when combined with anticancer drugs that induce a mitochondrial cell death pathway, such as cyclophosphamide (51, 121).

Gene therapies that deliver a cell death receptor ligand such as TRAIL (127, 128) may be used to enhance the death receptor-dependent cell death pathway and increase responsiveness to traditional anticancer agents (103, 129, 130). The expression of TRAIL confers bystander cell toxicity, presumably via a paracrine mechanism (128). Recombinant TRAIL induces tumor regression in preclinical models (131), and in contrast to Fas ligand and TNF-α, exhibits little toxicity to normal tissues because of the host cell protective effects of TRAIL decoy death receptors, which are selectively down-regulated in many tumor cells (37, 38). Death receptor and mitochondrial pathways of apoptosis may also be targeted simultaneously by combining TRAIL with Bax gene delivery (132). In an alternate approach, TRAIL receptor DR4-deficient tumor cells can be sensitized to TRAIL by gene therapy using p53, which up-regulates DR4 expression in tumor cells (133). Tumor cells can also be sensitized to TRAIL by overexpression of Smac/Diablo, the neutralizing inhibitor of IAPs, or by treatment with cell-permeable Smac/Diablo peptides, which induce the release of endogenous mitochondrial Smac/Diablo and lead to synergistic activation of multiple caspases (134–136).
Gene therapies can also be designed to block the antiapoptotic, tumor cell-protective NF-κB inductive response to cytotoxic drug treatment. This may be accomplished by delivery of a dominant-negative, proteolytically stable “super repressor” of IκB, which in some tumor cell types blocks drug-induced NF-κB activation and increases chemosensitivity to anticancer drugs and to TNF-α (137, 138). In the case of hepatocellular carcinoma, the IκB super-repressor also enhances TNF-α-induced apoptosis but has an undesirable, antiapoptotic effect on chemotherapy-induced cell death (139). NF-κB activation may also be blocked by certain proteasome inhibitors now under development for cancer treatment (140, 141) and by the low molecular weight protein kinase C inhibitor Go6976, which induces regression of established mouse mammary tumors by blocking NF-κB activation and reinstating an apoptotic program of gene expression (142).

GDEPT Using Cytochrome P-450

Prodrug-activation gene therapy, also referred to as suicide gene therapy or GDEPT, has been widely studied as a strategy to increase the sensitivity of cancer cells to apoptosis induced by anticancer prodrugs (143, 144). Introduction of a suicide gene using a suitable gene therapy vector provides the tumor cell with the capacity for localized prodrug activation, thereby restricting production of the toxic drug metabolite to the tumor tissue. GDEPT can impart a strong bystander cytotoxic response because of the diffusion of activated drug metabolites from a transduced tumor cell into neighboring, naïve tumor cells that do not express the prodrug-activation enzyme (145). In contrast to many other cancer gene therapies, therefore, GDEPT does not require the genetic modification of each individual tumor cell. Widely studied suicide gene therapy systems include HSV-1 tk in combination with the antiviral prodrug ganciclovir (146, 147) and the bacterial gene cytosine deaminase in combination with the prodrug 5-fluorocytosine (148, 149). Ganciclovir is ultimately activated to a cytotoxic nucleoside triphosphate, which induces caspase 8-mediated death in tumor cells that have a functional p53 (59) but proceeds via a caspase 9 apoptotic pathway in p53-deficient cells (60). 5-Fluorocytosine is activated to 5-fluorouracil, an established anticancer drug that activates the death receptor/caspase 8 pathway (53, 54), as noted above.

A third enzyme-prodrug combination useful for GDEPT treatment of cancer utilizes cytochrome P-450 enzymes, which can be combined with a variety of anticancer prodrugs, the most widely studied one being the bifunctional alkylation agent cyclophosphamide and its isomer ifosfamide (150, 151). P-450-based GDEPT has also been exemplified with certain bioreductive drugs (152, 153). This gene therapy appears to be particularly promising (154–159) and can be carried out using genes that code for endogenous human liver P-450 enzymes, such as CYP2B6, diminishing the possibility of adverse immune responses (144). P-450-activated cyclophosphamide diffuses freely across cell membranes and elicits a substantial bystander cytotoxic effect, as shown in model studies using tumor cells grown in monolayers (155) and spheroid cultures (157). The therapeutic potential of P-450-based cancer GDEPT strategies has been exemplified using retroviral and replicating herpes viral and adenoviral vectors (157, 160–162) and has shown promise in initial Phase I/Phase II trials with localized P-450 transfer using viral vectors or delivery of microencapsulated P-450-expressing cells to the tumor vasculature (163–165). Further studies on the mechanisms of tumor cell death induced by P-450 prodrugs such as cyclophosphamide (51) may help identify tumor cell types that are most responsive to this therapeutic strategy, as well as other chemotherapeutic agents and gene therapies that provide synergy when combined with P-450 GDEPT.

Antiapoptotic Strategies to Enhance P450 GDEPT and Other Gene Therapies

Proapoptotic gene therapies, such as the Bax and p53 gene therapies noted above, display intrinsic antitumor activity. Moreover, these proapoptotic therapies may confer additional therapeutic benefit by lowering the threshold drug concentration required for traditional anticancer agents to induce tumor cell death. However, the combination of a proapoptotic therapy with suicide gene therapy, such as P-450 GDEPT, is likely to be counterproductive, because it will shorten the life span of the prodrug-activating tumor cell and consequently limit net production and release of active drug metabolites (166). Agents that deplete cell-protective small molecules, such as glutathione, or strategies that decrease the expression or activity of protective enzymes, such as glutathione S-transferase (167–169) or aldehyde dehydrogenase (170) may be used to sensitize tumor cells to cyclophosphamide and other alkylating agents (170–173). However, by enhancing tumor cell chemosensitivity, these modulation strategies will necessarily undermine the effectiveness of a GDEPT strategy: the life span of tumor cells that express the suicide gene will be shortened, and net production and release of active drug metabolites will consequently be diminished, thus limiting the bystander cytotoxic effect that is vital to the overall success of the GDEPT approach. On the other hand, any effort to suppress apoptosis of those tumor cells that express the suicide gene increases the risk of generating an aggressive, drug-resistant tumor.

A general solution to this problem has been proposed based on the results of a P-450 GDEPT study using cyclophosphamide. Specifically, the baculovirus-encoded pan-caspase inhibitor p35 (174) was shown to slow down, but not block, the death of tumor cells that express P450 in a manner that substantially increased net production of bystander, cytotoxic cyclophosphamide metabolites (Ref. 166; Fig. 3). Although tumor expression of antiapoptotic factors is widely considered to be associated with chemoresistance (31, 175), caspase inhibitors such as p35 may nevertheless be useful for enhancing prodrug activation in the context of GDEPT treatments for cancer. The notion of introducing an antiapoptotic factor into a tumor cell may seem counterintuitive; however, caspase activity appears to be nonessential for tumor cell death, as revealed by the ability of caspase pathway inhibitors to delay but, ultimately, not block tumor cell death (104, 176–178). This finding is intriguing and can be explained by the observation that p35, IAP proteins, and certain other caspase inhibitors act downstream of the critical mitochondrial transition step; consequently, these antiapoptotic factors do not interfere with the upstream release of cytochrome c that is mediated by Bax and other proapoptotic Bcl-2 family members in cells undergoing stress- or DNA damage-induced cell death (179). Once the mitochondrial apoptotic pathway has been engaged and mitochondrial potential is lost, drug-induced ATP depletion occurs, and a commitment to cell death ensues, even in tumor cells that contain a strong caspase inhibitor, such as p35 (166). The commitment to cell death in tumor cells that have undergone mitochondrial transition is manifested by the irreversible loss of clonogenic activity via a necrotic process (180–182).

Mitochondrial release of the proapoptotic factor AIF may contribute to the irreversibility of drug-induced cell death in the presence of the caspase inhibitor p35, as indicated by the ability of AIF to induce caspase-independent cell death (15, 183). AIF is sequestered in the mitochondrial intermembrane space, and following its release to the cytosol in response to apoptotic stimulation, it translocates to the nucleus and causes large-scale chromatin fragmentation (16). This process is not blocked by the general caspase inhibitor benzylxycarbo-nal-Val-Ala-Asp-fluoromethylketone, although the release of AIF from mitochondria is regulated by Bcl-2 (14).
The concept that underlies the introduction of a pan-caspase inhibitor to increase the bystander activity of P-450-activated cyclophosphamide (166) is expected to be applicable to GDEPT using other anticancer P-450 prodrugs, some 10–12 of which are known (144). Other GDEPT enzyme-prodrug combinations, including those based on the prodrugs ganciclovir and 5-fluorocytosine, are also likely to benefit from strategies that delay death of prodrug-activating “factory cells” and thereby enhance bystander activity. The bystander activity of activated ganciclovir is relatively weak, and this contributes to the limited effectiveness of HSV-tk-based gene therapies. However, even in the case of cytosine deaminase gene therapy using the prodrug 5-fluorocytosine, 5-fluoracil-producing tumor cells display up to a 500-fold greater susceptibility to killing than bystander tumor cells (184), suggesting that the coexpression of p35 or other caspase inhibitors may increase bystander cytotoxic activity in this case as well. In p53-deficient tumor cells, HSV-tk GDEPT in combination with ganciclovir treatment activates the mitochondrial/caspase 9 pathway (60) and should benefit from any increase in bystander activity that antiapoptotic factors such as FLIP may provide.

Several related approaches may be used to prolong tumor cell survival and increase bystander activity in the case of GDEPT gene-prodrug combinations that activate the death receptor/caspase 8 pathway, such as cytosine deaminase-5-fluorocytosine (53, 54). For example, overexpression of XIAP may provide for the selective inhibition of effector caspases, leading to the desired delay in receptor-mediated cell death. XIAP does not block caspase 8 activation (30), and consequently, the proapoptotic factor Bid may still undergo cleaving to tBid in XIAP-transduced cells, leading to mitochondrial transition and tumor cell death. tBid-induced mitochondrial transition also leads to release of Smac/Diablo and negative feedback inhibition of XIAP, further facilitating death of the XIAP-expressing tumor cells. In an alternative approach, death receptor pathway-mediated cell death may be prolonged without blocking the ultimate cytotoxic outcome by overexpression of FLIPs, which are structurally similar to caspase 8 and interfere with the recruitment and activation of caspase 8 by death receptors. The level of FLIP expression may be an important determinant of its ability to slow down cell death but not inhibit it. Decoy death receptors may also be used to slow transmission of a death receptor signal without completely blocking it (34).

Although FLIPs have been associated with drug resistance (185, 186), the cell damage that is induced by chemotherapeutic drugs may itself be sufficient to kill the tumor cell, despite the inhibition of the caspase 8-dependent apoptotic pathway. Factors that determine the extent to which GDEPT activity can be enhanced using these approaches include the mechanism of cell death induced by the activated prodrug, the extent to which the antiapoptotic factor prolongs cell death, and the extent to which bystander killing is enhanced by expression of the antiapoptotic factor. The ability of caspase inhibitors or other antiapoptotic factors to prolong but not block prodrug-induced cell death may thus serve as a general way to enhance a broad range of suicide-based cancer treatments.

Finally, antiapoptotic factors may also be introduced into microencapsulated cell vectors (187) to protect the encapsulated cell from the cytotoxic action of a soluble proapoptotic factor that it produces. For example, FLIP or TRAIL decoy receptor could be introduced to protect cell vectors that have been engineered to secrete high levels of TRAIL. In this way the encapsulated cells may secrete TRAIL into the tumor milieu for a prolonged period of time without themselves succumbing to TRAIL-induced apoptosis.

**Antiantiapoptotic Gene Therapies: Safety Considerations**

To be effective, gene therapies that incorporate antiapoptotic factors must be carried out in a manner that avoids the induction of tumor cell drug resistance. To achieve this goal, expression of the therapeutic gene and the antiapoptotic factor should be tightly linked, e.g., by use of an internal ribosome entry sequence (188), to preclude the possibility of expressing the antiapoptotic factor in tumor cells that do not also receive a drug sensitivity gene (e.g., the suicide/prodrug activation gene). The immunogenicity of a nonmammalian antiapoptotic factor, such as the baculovirus protein p35, may further insure that all of the tumor cells that express the factor are ultimately eliminated by the immune system. The introduction of an antiapoptotic factor, such as p35, may shift the mechanism of drug-induced cell death from an apoptotic pathway to necrotic cell death, potentially leading to an enhanced systemic (immune system-based) bystander killing effect. As an added measure of safety, the antiapoptotic factor can be placed under the control of a strong, repressible promoter, such
as the “Tet-off” expression system, so that it can be switched off when necessary by treatment with the antibiotic tetracycline. Tet-off gene therapy vectors provide a high degree of control of transgene expression, which can be suppressed in a wide range of cells at doses of tetracycline that are very low and exert no significant toxic effects on cell proliferation or animal growth, even with continuous treatment (189–191). Safety could be further increased by building into the gene therapy vector the inducible expression of a factor that kills the tumor cell by an alternate pathway of cell death, one that is not blocked by the antiapoptotic factor. For example, the pan-caspase inhibitor p35 can be used to prolong the life of a P450-expressing prodrug factory cell, followed by the inducible expression of a death receptor ligand, such as TRAIL, to eliminate any p35-expressing tumor cells that remain. Similarly, a TRAIL decoy receptor can be used to protect a TRAIL-secreting tumor cell from TRAIL-induced suicide, followed by inducible expression of a prodrug-activating enzyme and prodrug treatment to insure elimination of the TRAIL decoy receptor-expressing cells.

Finally, antiapoptotic factors can safely be used in an alternative gene-based therapy, whereby the therapeutic gene and the antiapoptotic factor are incorporated into a cell-based vector, such as cellu-

References


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