

Minireview

Apoptosis: Target of Cancer Therapy

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Abstract

Recent knowledge on apoptosis has made it possible to devise novel approaches, which exploit this process to treat cancer. In this review, we discuss in detail approaches to induce tumor cell apoptosis, their mechanism of action, stage of development, and possible drawbacks. Finally, the obstacles yet to be overcome and the perspectives for potential clinical use of apoptosis-triggering approaches in cancer therapy in the future are assessed.

Introduction

In 1972, Kerr *et al.* (1) described a distinct morphology of dying cells and called it apoptosis. The term was coined based on the fact that the release of apoptotic bodies by dying cells resembled the picture of falling leaves from deciduous trees, called in Greek “apoptosis” (1). This type of cell death has also been called programmed or physiological cell death, and is characterized by a genetic controlled autodigestion of the cell through the activation of endogenous proteases. This process results in cytoskeletal disruption, cell shrinkage, membrane blebbing, nuclear condensation, and internucleosomal DNA fragmentation (2). The major impact that the knowledge acquired on apoptosis has had on developmental biology is the realization that tissue homeostasis relies not only on factors that rule proliferation and differentiation but also on determinants that influence cell survival/death. Furthermore, being a gene-controlled process, apoptosis is susceptible to disruption by mutations (3). Hence, it soon became clear that failure to undergo apoptosis might be involved in the pathogenesis of a variety of human diseases such as viral infections, autoimmune diseases, and cancer (4, 5).

It has been known for many years that a massive cell loss takes place during carcinogenesis, because tumor growth rates can be <5% of that predicted by proliferation measurements alone. Kerr *et al.* (6) were the first to suggest that apoptosis is the process responsible for that percentage of cell loss, and this

idea was supported by subsequent studies that demonstrated a large percentage of apoptotic cells in spontaneously regressing tumors and tumors exposed to cytotoxic agents. Despite suggestions of links between apoptosis, carcinogenesis, and response to chemotherapy (7), the process of apoptosis was not additionally explored until the beginning of the 1990s when oncogenes and tumor suppressor genes involved in this process were identified; they provided the molecular link that was missing, and this boosted enormously the interest in apoptosis (8).

The growing knowledge on the relation between apoptosis and carcinogenesis has led to the identification of several gene alterations. A detailed description of these alterations is out of the scope of this review, and some comprehensive overviews of the pathways involved in the regulation of apoptosis published over the last 5 years can be helpful at this end (9–17). However, what should be kept in mind is that if the relation between carcinogenesis and dysregulation of apoptosis is so intimate, any therapeutic strategy aimed at specifically triggering apoptosis in cancer cells might have potential therapeutic effect (18).

For many years, the effect of anticancer drugs on tumor cells was attributed to their crippling action on rapidly proliferating cancer cells. The drug-target interaction would lead to irreparable damage, and tumor cell death would be a consequence of the disruption of vital metabolic functions. However, over the past few years it has become clear that anticancer drugs are able to induce apoptosis and that this process is involved in the mediation of their cytotoxic effects. Furthermore, the induction of apoptosis was found to be a common event for different classes of anticancer agents, and because apoptosis induced by distinct classes of anticancer agents converges into similar downstream mechanisms, disruption of such mechanisms can lead to broad drug resistance (19–24). Nevertheless, the lack of specificity of cytotoxic drugs for tumor cells and the resulting toxicity to normal tissue hampers an additional exploitation of their apoptotic effects.

The aim of this review is to discuss some of the strategies to exploit apoptosis to kill cancer cells, in terms of mechanism of action, stage of development, drawbacks, and potential future clinical use in cancer therapy.

Developing Apoptosis-triggering Therapeutic Strategies

Because apoptosis is a gene-controlled process, it is susceptible to genetic manipulation with therapeutic purposes. Several features make apoptotic genes and proteins attractive targets for cancer treatment. First, the growing knowledge on the apoptotic machinery certainly provides many theoretical opportunities to manipulate pathways leading to an increased tumor cell death. Second, recent technological developments enable approaches that allow the genetic and phenotypic modification of cancer cells, and several genetic alterations have been found to be cancer cell-specific, which may allow them to specifically target a tumor cell. Third, advances in combinatorial chemistry

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Table 1 Apoptosis-triggering strategies

Strategy	Target	Approach	Stage of development
Proapoptotic approaches			
Introduction of proapoptotic players	TRAIL	Recombinant protein	Clinical trials planned
	Apoptin	Gene therapy	Preclinical
Modulation of antiapoptotic genes or pathways	Caspases	Gene therapy	Preclinical
	Mitochondria:		
	Proapoptotic molecules (Bax, BCL-Xs)	Gene therapy	Preclinical
	Downregulate antiapoptotic molecules (Bcl-2, Bcl-XL)	ODNs	Phase II/III
Restoration or manipulation of tumor suppressor genes	Direct effect on mitochondria	Lonidamine, arsenite	Phase III
	Direct effect on pores	PK 11195	Preclinical
	p53	Gene therapy	Phase II/III
	Retinoblastoma	Gene therapy	Preclinical
Permissive approaches	<i>FHIT</i>	Gene therapy	Clinical trials planned
	Oncogenes		
	PI3k	LY294002	Preclinical
	Ras	Small molecules, ODNs	Phase II/III
	BCR-ABL	Small molecule (STI-571)	Phase III
	NFκB	ODNs	Phase I/II
	Proteasome inhibitors	PS-341	Phase II
	<i>c-raf</i>	ODNs	Phase II
	<i>c-myc</i>	ODNs	Preclinical
	Cell cycle modulators	UCN-01, flavopiridol	Phase III

and new methods of random screening have accelerated the pace of development of inhibitors to a selected target.

The development of several apoptosis-triggering therapeutic modalities is in the advanced preclinical or early clinical stage of testing. From a mechanistic point of view two types of approaches can be distinguished: (a) strategies that directly induce apoptosis, named here as pro-apoptotic approaches; and (b) strategies that modulate survival signaling pathways thereby facilitating the occurrence of apoptosis, called here permissive approaches. A summary of the stage of development of several strategies to induce tumor cell apoptosis is provided in Table 1.

Proapoptotic Approaches

Apoptosis can be achieved through the exploitation of existing cellular players and pathways such as death receptors and caspases, or the introduction of exogenous proapoptotic molecules such as Apoptin. Proapoptotic strategies can involve: (a) direct introduction of proapoptotic players; (b) modulation of antiapoptotic molecules; or (c) restoration of tumor suppressor gene function.

Direct Introduction of Proapoptotic Players

Activation of Death Receptor Pathways. Receptors of the TNF- α^3 superfamily can be subdivided into two groups,

based on the presence or absence of a cytoplasmic DD (25). Among the DD-containing members of the TNF superfamily (death receptors) are TNFR-1, Fas (Apo-1 and CD95), DR3, DR4, DR5, and DR6. Binding of three ligand molecules to a homotrimeric death receptor molecule leads to clustering of the receptor DDs and aggregation of signaling molecules to form a functional DISC within the cell (26). Initiator procaspase-8, recruited to the DISC by virtue of its DEDs, becomes activated by autoproteolysis and dissociates from the DISC to initiate the activation of the caspase cascade.

Death receptors have been pursued as potential targets for cancer therapy for many years. Candidates such as TNF and Fas have been extensively investigated after the observation of promising antitumor activity obtained *in vivo*. Unfortunately it became clear that TNF, besides not being effective in inducing cancer cell kill *in vivo* at concentrations achievable systemically, also induced ischemic and hemorrhagic lesions in several tissues (27, 28). Although initially shown to induce apoptosis in tumor cells and to be potentially synergistic with chemotherapy, FasL development as an anticancer drug was also discouraged by induction of massive hemorrhagic necrosis in the liver in mice (29).

The problems seen with TNF and Fas seemed overcome when the TRAIL or APO2L emerged as a potential anticancer agent (30). TRAIL was able to induce p53-independent apoptosis in a variety of tumor cell types (31–33), and it appeared not to induce toxicity in normal cells (32, 33). Moreover, TRAIL was able to suppress tumor growth of colon and breast

³ The abbreviations used are: TNF, tumor necrosis factor; DD, death domain; DISC, death-inducing signaling complex; DED, death effector domain; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; CARD, caspase recruitment domain; PTPC, permeability transition pore complex; ODN, oligodeoxynucleotide; AML, acute myelogenous leukemia; LND, lonidamine; NSCLC, non-small cell lung cancer; IAP, inhibitor of apoptosis; FHIT, fragile histidine triad; NFκB, nuclear

factor κB; IκB, inhibitor of nuclear factor-κB; PI3k, phosphatidylinositol 3'-kinase; FTase, farnesyl transferase.

xenografts without the side effects observed with TNF and FasL (34). In addition, a synergistic antitumor effect was observed when TRAIL was combined with chemotherapy or radiation (35–37). Combination therapy with TRAIL and DNA-damaging agents may be particularly appealing in the context of tumors with functional p53, because *DR5* is a p53-responsive gene (38). However, the development of TRAIL as an anticancer drug suffered a major setback when Jo *et al.* (39) reported that human primary hepatocytes were also sensitive to TRAIL-induced apoptosis. Studying *in vitro* normal hepatocytes derived from 20 individuals, the authors observed that in striking contrast with mice and nonhuman primates, >60% of the human hepatocytes underwent apoptosis within 10 h of exposure to TRAIL (39). These results on human hepatocytes were confirmed recently (40). Furthermore, TRAIL has also been shown to trigger apoptosis in human brain cells (41), although in preclinical studies TRAIL failed to induce apoptosis in the brain of laboratory animals (34, 34, 42). Altogether, these data point to an important interspecies variability in the response to TRAIL. The findings on TRAIL pinpoint once again the limitation of preclinical studies on animal cells in predicting toxicity of biological agents in humans (43). More detailed studies on TRAIL are certainly needed, and differences in the structure of different preparations of recombinant TRAIL used in different studies must be investigated as a potential cause of toxicity in humans (40, 44, 45).

Synthetic Activation of Caspases. The degradation and elimination of cells in apoptosis is dependent on the degradation of cellular proteins by caspases, a family of cysteinyl aspartate-specific proteinases (9, 46, 47). Caspases are constitutively present in the cells as zymogens (procaspases), and activation of procaspases requires their cleavage at caspase consensus sites in their proenzyme structures, implying that these enzymes can be activated either autocatalytically or that some caspases sequentially activate others in a hierarchical fashion. The caspase cascade includes “initiator” proteases, such as caspase-8, -9, and 10, which activate “machinery” proteases such as caspase-3 and caspase-7 (48). Initiator caspases are activated by proapoptotic signals at the onset of the executive phase of programmed cell death. They contain in their prodomains one or two protein modules that can physically link these proteases to adaptor molecules containing similar domains via homophilic interactions. Two types of interaction modules have been identified in the prodomains of initiator caspases: the DED in caspases-8 and -10, and the CARD in caspase-9 (49–51). The two major routes of activation of the caspase cascade, the death receptor and the mitochondria pathways, use DEDs and CARDs, respectively.

Caspases have been engineered by fusing one or more chemically inducible dimerization domains; on administration of a lipid-permeable dimerizing drug, protein aggregation occurs leading to a successive activation of downstream signals (52). These engineered molecules are named artificial death switches, and it has been demonstrated that chemical activation of caspases-1 and -3 is sufficient to trigger apoptosis in cancer cells (52).

This system of chimeric inducible caspases has been tested recently in prostate cancer cell lines. Replicative-deficient adenoviral vectors (Ad-G/iCasp1) were efficiently transduced into cancer cells, and on exposure to chemically inducible dimeriza-

tion, an important increase of number of apoptotic cells was observed (53). Moreover, no bystander cytotoxicity was observed, suggesting that this approach may be safe, although this awaits confirmation in *in vivo* model systems.

Apoptin. A promising strategy to selectively kill tumor cells while sparing normal cells is the use of Apoptin (VP3), a M_r 14,000 protein derived from chicken anemia virus (54). Recent data suggest that apoptosis induced by this molecule involves caspases (55). *In vitro* results show that Apoptin is very active against cancer cells, without inducing toxicity to normal cells (56). This tumor-specific effect might be explained by the nuclear localization of the protein in tumor cells, an absolute requirement for its activity, whereas in normal cells the protein localizes in the cytoplasm (57). Furthermore, Apoptin is equally active in genetically disrupted and potentially chemoresistant cells, such as p53-mutant, Bcl-2-overexpressing or BCR-ABL-expressing tumor cells (54, 58–60).

Gene-therapy strategies are under development to deliver Apoptin into tumor cells *in vivo* (61). In preclinical studies, multiple adenovirus injections into healthy rats and nude mice showed no toxicity (56). In addition, antitumor effects were observed in nude mice bearing s.c. human hepatoma (54). Nevertheless, these results are still preliminary and further preclinical work using human cells is required to ensure the safety and better assess the potential of Apoptin as an anticancer compound.

Modulation of Antiapoptotic Players

Targeting Mitochondria. Besides the death receptor pathways, a second route leading to caspase cascade activation and apoptosis involves mitochondria (62, 63). Mitochondria contain apoptogenic proteins that are released into the cytoplasm during apoptosis. Among these, cytochrome *c* is pivotal in the activation of the caspase cascade and the commitment to die. In addition to cytochrome *c*, Smac/DIABLO (60, 64), EndoG (65), heat shock protein-60 (involved in procaspase activation), and apoptosis-inducing factor are released (66). Moreover, some authors have suggested that intramitochondrial procaspase-2, -3, and -9 are also released from mitochondria (66–68). The presence of cytochrome *c* in the cytoplasm allows interaction with the CARD-containing adapter protein Apaf-1 (69, 70), which normally is in a dormant state, ATP, and procaspase-9 via a CARD-CARD (71) interaction forming a ternary complex, termed the vertebrate “apoptosome” (72, 73). In this holoenzyme, procaspase-9 is activated by conformational change (73). Subsequently, active caspase-9 activates downstream caspase zymogens, starting the caspase cascade (69, 71).

The role of mitochondria in apoptosis is complex and has been extensively reviewed (15, 62, 63, 66). Because the activation of mitochondria has been considered the “point of no return” in the apoptotic process (74), the manipulation of mitochondria activation with proapoptotic intentions has been envisaged as a potential therapeutic approach. Activation of mitochondria is accompanied by the translocation of cytochrome *c* from the mitochondrial intermembrane space into the cytoplasm and may involve a large mitochondrial conductance channel called the PTPC (75). Nonetheless, the role of PTPC in the process remains controversial (76–78).

Indirect mitochondria activation can be achieved via the

modulation of the players that act on mitochondria pores altering the balance between proapoptotic and antiapoptotic members of the Bcl-2 family. This can be done either by down-regulation of antiapoptotic molecules (*e.g.*, antisense ODNs against Bcl-2) or by up-regulation of the proapoptotic counterparts (*e.g.*, gene therapy with Bax). Delivery of Bax vectors by gene therapy would be a logical approach to indirectly activate mitochondria. However, constructing adenoviral vectors expressing the Bax gene driven by a constitutive promoter proved to be troublesome probably because of the proapoptotic activity of the gene product (79). Recent data, although, suggest that such difficulties can be tackled by different molecular biology approaches, and lead to an enhanced apoptosis in different tumor types (80–84). Alternatively, the introduction of Bcl-xs, another dominant-negative repressor of Bcl-2 and Bcl-XL, can also induce tumor regression in xenografts and potentiate the effects of chemotherapy (85, 86).

The balance between pro- and antiapoptotic members of the Bcl-2 family can also be altered in favor of proapoptotic players by decreasing Bcl-2 and Bcl-XL expression levels. Some ODNs targeting these molecules have been shown to be effective. An 18-mer phosphorothioate antisense ODN, G-3139, (Genta, Inc., Lexington, MA), targeting the translational initiation codon of the *Bcl-2* gene, has been shown to suppress Bcl-2 expression *in vitro* and sensitize cells to chemotherapy (44, 87). This effect appears to involve induction of apoptosis because liposomal Bcl-2 antisense oligonucleotides exerted proapoptotic function in primary AML samples (88), and antisense therapy against Bcl-2 in SCID mice was able to induce apoptosis especially when combined with chemotherapy (89). G-3139 is presently undergoing clinical development, and Phase I studies have confirmed the safety of this approach either alone or combined with chemotherapy (90–92). Moreover, antitumor activity has been observed in Phase I/II trials in melanoma patients (44), and Phase III clinical trials for malignant melanoma are under way. In addition, Phase II trials exploring G-3139 combined with chemotherapy are being performed in high-grade lymphoma, small cell lung, prostate, breast, and colorectal cancers (44, 93). The development of ODNs that target Bcl-2 and Bcl-XL simultaneously, based on the high homology shared by these molecules, are currently being developed. The bispecific compound 4625 has been shown to induce apoptosis in tumor-derived cell lines *in vitro* and *in vivo* (94).

An alternative approach to target mitochondria with proapoptotic intentions takes into consideration alterations of apoptotic pathways located upstream of mitochondria, involving p53, death receptors, or apical caspases. Alterations of these molecules have the potential to prevent mitochondria activation (75). Anticancer agents that directly and specifically target and activate mitochondria components, such as LND, arsenite, betulinic acid, and CD437 are able to overcome a potential upstream inhibition. Arsenite has been shown to act on isolated mitochondria inducing PTPC opening (75), and this agent is now being considered for acute promyelocytic leukemia. Another drug in advanced stage of development is LND. Experiments *in vivo* and *in vitro* have shown that LND enhances the induction of apoptosis by conventional anticancer drugs such as cisplatin, doxorubicin, cyclophosphamide, and paclitaxel (75). Results from Phase II and III trials of the combination of

chemotherapy and LND, in metastatic breast cancer and locally advanced NSCLC are encouraging (95–97).

The strategies to target mitochondria discussed may somewhat be limited by the fact that they are likely to be ineffective in the context of overexpression of Bcl-2. Bcl-2 and its antiapoptotic counterparts are overexpressed in several types of tumors (18, 98), being able to antagonize the effect of drugs that act directly or indirectly on mitochondria. This effect can be additionally potentiated by loss-of-function mutations and/or alterations at the transcriptional level that may decrease the expression of functional Bax (99–101). In light of the growing knowledge on mitochondria, one could envisage approaches to directly target specific PTPC components and overcome possible alterations or mutation of components of the Bcl-2 family. One strategy to circumvent Bcl-2-like effects involves ligands of another PTPC component, the peripheral benzodiazepine receptor, such as PK11195. This compound is able to overcome the resistance of Bcl-2 overexpressing cells to etoposide. Targeting PTPC may take advantage of differences in the composition and regulation of the PTPC between normal and tumor cells (75). Components of the PTPC, such as peripheral benzodiazepine receptor, Prax-1, and mitochondrial creatine kinase may be overexpressed in some tumors (102, 103). Hence, besides bypassing alterations of Bcl-2 family members, targeting PTPC components may prove to be a strategy with a therapeutic potential.

Targeting IAPs. The capacity of caspase-9 to activate downstream caspases seems to be under the control of a regulatory system based on endogenous inhibitors. IAPs were originally identified in the genome of baculoviruses on the basis of their ability to suppress apoptosis in infected host cells (104). Members of the IAP family contain one to three modules of a common 70-amino acid zinc-binding motif called the baculoviral IAP repeat domain, which is critical for the antiapoptosis function (105). Several human cellular homologues of the baculovirus IAPs have been identified such as NAIP, c-IAP1, c-IAP2, XIAP, survivin, Apollon, Livin, and others (106–109). Another player in the balance between caspases and IAPs is the molecule Smac/DIABLO (60, 110). This molecule is an apoptosis-promoting factor released by mitochondria that antagonizes the function of IAPs (60, 110). In addition, another molecule called Omi/HtrA2 has been described recently as able to antagonize the antiapoptotic function of IAPs (111).

Recent attempts to use IAPs as targets for anticancer therapy have focused on *survivin* and XIAP (MIHA; Refs. 112, 113). Experiments *in vitro* demonstrated that these proteins exert their antiapoptotic role by inhibiting caspases -3, -7, and -9. Because these caspases have been shown *in vitro* to be relevant for chemotherapy-induced apoptosis (114), targeting their natural inhibitors, the IAPs, was foreseen as a potential way to enhance chemosensitivity. Indeed, in NSCLC cells the use of the oligonucleotide 4003 inhibited up to 70% of *survivin* mRNA expression leading to sensitization of cancer cells to etoposide (113). Moreover, down-regulation of XIAP by adenoviral antisense expression induced apoptosis in ovarian cancer cells with wild-type p53 (112). These encouraging results have triggered the planning of clinical studies using antisense IAPs. However, the role of IAPs may be more complex than initially suggested by *in vitro* data. In fact, in contrast to what has been

anticipated by several *in vitro* studies, in NSCLC patients the expression of c-IAP1, c-IAP2, and XIAP did not predict response to chemotherapy (115). Moreover, no difference in response to chemotherapy between survivin-positive and -negative cases was observed in patients with non-Hodgkin's lymphoma (5, 116) and AML (117). In addition, the expression of XIAP in radically resected NSCLC patients did not correlate with the apoptotic index but did inversely correlate with tumor proliferation. Prognostically, higher XIAP expression was translated into a significantly longer overall survival in this group of patients (118). Furthermore, in a recent study in gastric cancer patients, the nuclear localization of survivin was shown to have positive effects on prognosis (119). If confirmed, these results may imply that an unrestricted inhibition of survivin in both cytoplasm and nucleus by ODNs may not be desirable.

Possible explanations for these contradictory data are that IAPs are probably not only involved in apoptosis inhibition through caspase blockade but also in other important functions, such as proliferation (115, 117, 118, 120–123). Moreover, the net effect of IAPs possibly depends on their interaction with regulatory molecules such as Smac/DIABLO (60, 110), HtrA2 (124), and XIAP-associated factor 1, an antagonist of the XIAP apoptotic activity (125). Hence, although promising on theoretical grounds, additional studies on the function and interactions of IAPs are essential to best exploit them as targets for anticancer therapy (115, 118).

Restoration of Function of Tumor Suppressor Genes

Loss or mutation of *p53* is a very common genetic abnormality in cancer (126). Among the multiple effects that *p53* alterations may have on the malignant process are changes in apoptosis and proliferation, which have been related to an altered sensitivity to radiation and chemotherapy (3, 22, 127). In line with that, initial Phase I *p53*-based gene therapy trials suggested that *p53* replacement could lead to an increase in apoptosis in tumor cells and surrounding cells as a bystander effect (128).

As suggested by preclinical data, it is likely that the combination of chemotherapy with the reintroduction of wild-type *p53* may increase the tumor cell kill. A Phase I study has been performed at the M. D. Anderson Cancer Center, Houston, TX, evaluating the safety of a sequential administration of Ad5CMV-*p53* with cisplatin in NSCLC patients (129). It remains to be seen if the combinations of Ad5CMV-*p53* and chemotherapy will result in increased apoptotic effect and tumor responses.

An alternative approach to target *p53* is via compounds that stabilize its DNA-binding domain in the active conformation (130). Besides promoting the stability of wild-type *p53*, such compounds also allow mutant *p53* to keep a active conformation (130). Additional development are awaited to provide a better idea about the clinical potential of these compounds.

Reintroduction of other tumor suppressor genes have also resulted in an increase in tumor cell apoptosis. When both *p16INK4* and wild-type *p53* were transduced into cancer cells, a synergistic apoptotic effect was observed (131). Furthermore, re-expression of the *FHIT* tumor suppressor gene has also been associated with induction of apoptosis (132). *FHIT* is a frequent target of deletions associated with abnormal RNA and protein

expression in primary tumors, and cell lines of lung, head and neck, kidney, cervix, and breast cancers (133–135). An adenoviral vector overexpressing *FHIT* inhibited cell growth and induced apoptosis in human lung, and head and neck carcinoma cells with *FHIT* gene abnormalities but not in normal human bronchial epithelial cells (136). The expression of *FHIT* in the SW480 human colon carcinoma cells inhibited growth and rendered the cells susceptible to apoptosis (137). Moreover, re-expression of this gene in H460 cells has been related with high rates of apoptosis and cell cycle alterations such as G₀/G₁ arrest (132). These results were confirmed when the *FHIT* gene was delivered at high efficiency by a recombinant adenoviral vector. Gene therapy strategies using *FHIT* are under development (138, 139).

Apoptosis-permissive Approaches

The ubiquitous distribution of the apoptotic machinery in cells requires that apoptosis be tightly controlled. Several intricate signaling pathways mediate survival messages that in normal conditions contribute to keep the cellular homeostasis. The antitumor effect achieved by the blockade of some of these pathways is commonly accompanied by an increase in apoptosis in cancer cells; this essentially ensues as a “side effect” of the blockade of a major process. Nevertheless, it has become clear that these apoptosis-triggering properties may be explored therapeutically, and the most promising examples are discussed below.

NFκB

Alterations in the NFκB pathway have an intimate relation to oncogenic transformation, because the blockade of IκB, the natural inhibitor of NFκB by oligonucleotides, favors cell transformation (140). Furthermore, tumor cells such as myeloma cells show an enhanced NFκB activity compared with normal cells (141). In addition, despite contrasting initial reports (142, 143), it soon became clear that the inhibition of this protein was associated with potentiation of apoptosis, including chemotherapy-induced cell death (144, 145). Besides the evidence supporting the targeting of NFκB as an anticancer strategy, inhibition of NFκB seems an attractive approach also because in most normal cells NFκB is sequestered in the cytoplasm and inactive. So, theoretically its blockade by therapy would not harm normal cells.

At this end, some strategies have been proposed. One is the use of antisense oligonucleotides carrying the NFκB/Rel consensus sequence, which was shown to sensitize AML cells to AraC (146). Another strategy to block this pathway may be delivery of IκB by adenoviral vectors, because normal cells already have the stable complex IκB/NFκB in their cytoplasm. Alternatively, the transcriptional activation of NFκB could be blocked by small molecules that disrupt its complex with coactivators. The enhanced activity of NFκB in cancer cells could also be targeted indirectly through blockade of Ras (147, 148) and/or PI3k (149), because these pathways may use NFκB to achieve their antiapoptotic function. Additional preclinical studies are needed to better assess the therapeutic potential of the NFκB blockade and the best strategy to explore its apoptosis-permissive effects.

Proteasome Inhibition

The 26S proteasome regulates protein turnover in eukaryotic cells. Because a large repertoire of human proteins are regulated by the ubiquitin-mediated proteasome pathway, any alteration of this machinery could favor cell transformation through disturbances in cell cycle, tumor growth, and survival (150). Compounds that inhibit the proteasome have been shown to be active in several animal models of inflammation and cancer (151).

One of these compounds is the PS-341 (Millenium Pharmaceuticals, Inc.). PS-341 induces a consistent antitumor activity against both sensitive and chemoresistant myeloma cells (141). In fact, the sensitivity to chemotherapy of resistant myeloma cells was increased up to 1,000,000-fold when combined to a noncytotoxic dose of PS-341 (141). Interestingly, this chemosensitizing effect has been described also in pancreatic cancer cells, which became more sensitive to gemcitabine-induced apoptosis when this drug was combined to PS-341 (152).

A possible explanation for the apoptosis-permissive action of PS-341 is its stabilizing effect on I κ B, thereby inhibiting NF κ B (141, 150). Alternatively this compound may have an effect on other proteins involved in apoptosis, such as Bcl-2, which was shown to be down-regulated by PS-341 in pancreatic cancer cells (152). Genomic profiling studies will certainly be helpful in identifying other molecular targets influenced by PS-341 (153). This information coupled to the results of several Phase II studies with PS-341 that are under way (151) will give a better idea of the clinical potential of this compound as an anticancer agent.

PI3k/Akt, BCR-ABL, and Ras

The PI3k pathway is one of the most extensively investigated antiapoptotic pathways and is linked to cellular transformation (154). Among the PI3k targets that have been implicated in the suppression of apoptosis, c-Akt seems to play a major role (155). This molecule has been explored in current models of oncogenesis. In some of these models, an increase of Akt activity has been associated with the loss of the negative regulator of this pathway, the tumor suppressor gene *PTEN/MMAC* (156).

The PI3k/Akt pathway comprises different classes of kinases at distinct levels, which could be potentially targeted with therapeutic purposes by small molecules. Furthermore, the fact that the PI3k/Akt pathway is much more active in cancer cells than in normal cells may provide a potential increase of therapeutic index. Preclinical studies are ongoing and should address the potential effect that the blockade of the PI3k will have on the induction of apoptosis in cancer cells (148).

Alternatively, targeting pathways placed in parallel or downstream of PI3k/Akt such as BCR-ABL and Ras may be an interesting option, because these pathways display extensive interactions. The *BCR-ABL* fusion gene is responsible for the dysregulation of the activity of the tyrosine kinase leading to the malignant phenotype of the BCR-ABL-expressing chronic myeloid leukemia and acute lymphoid leukemia blasts (157). The tyrosine kinase inhibitor STI 571 (Gleevec) can revert the tumorigenic and antiapoptotic effect of BCR-ABL. Moreover an

increase in apoptosis is observed. The explanation of the effect of STI-571 facilitating apoptosis remains unclear, but it seems to involve the down-regulation of BCL-X, followed by cytochrome *c* release and caspase activation (158), and/or inhibition of the activity of Akt and NF κ B (157).

Another interesting target is the *Ras* oncogene protein product, which not only provides proliferative signals but also restrains apoptosis (159). Therefore, inhibition of Ras protein can be devised as a strategy to commit cells to apoptosis by preventing Ras-dependent inhibition of apoptosis (159). In fact, several studies have demonstrated that, depending on the cellular context, drugs that block Ras farnesylation may trigger apoptosis (160, 161). The blockade of *Ras* can decrease NF κ B activity and favor proapoptotic signals. This facilitation of apoptosis by FTase inhibitors might be the explanation for the synergistic and/or additive effects observed when these drugs were combined with chemotherapy (160, 162, 163). Additional studies are required to capitalize on the facilitation effects of FTase on chemotherapy-induced apoptosis.

Recent evidence suggests that FTase inhibitors can partially act through blockade of the PI3k/Akt pathway (164), which might explain the induction of apoptosis by these compounds.

c-myb and *c-raf*

The down-regulation of oncogenes such as *c-myb* and *c-raf* by antisense ODNs has been shown in preclinical studies to increase apoptosis in tumor cells. The use of LR-3001, an ODN that targets *c-myb*, is able to induce apoptosis in leukemia cells *in vitro* (93). Moreover, apoptosis in epithelial cells can also be induced when the oncogene *c-raf* is targeted by the ODN ISIS 5132 (165). Phase I studies demonstrated that this drug is safe and devoid of significant myelotoxicity (166, 167), and several Phase II trials are currently testing the antitumor activity of ISIS 5132 against different tumor types (93, 168). The development of second-generation ODNs that are less susceptible to degradation and the use of ODNs in combination with chemotherapy are likely to boost the clinical development of these compounds. These combinations will allow a better comprehension of the effects of ODNs on the facilitation of tumor cell apoptosis.

Cyclin-dependent Kinase Modulators

The knowledge that the majority of human tumors possess an abnormal retinoblastoma pathway provided the rationale for the development of a "mechanism-based therapy," targeting components of the cell cycle control machinery. Preclinical data suggest that the antiproliferative effect of some of these agents such as flavopiridol, roscovitidine, and UCN-01 is accompanied by induction of apoptosis in particular cell types (169–171). In addition, a synergistic effect has been reported when UCN-01 was combined with DNA-damaging agents (170), and it might be interesting to evaluate whether this effect is a result of an increase of apoptotic cell death. The mechanistic details of the proapoptotic effects are still unclear and may depend on the cellular context. One possible explanation would be the effects of UCN-01 and flavopiridol on protein kinase C, because in preclinical studies protein kinase inhibition also resulted in

induction of apoptosis and enhancement of the effect of cytotoxic chemotherapy (172).

Conclusions

The era of targeted therapy has progressively emerged in oncology, and apoptosis-triggering strategies, either proapoptotic or apoptosis-permissive, are likely to play an important role in this context.

As far as proapoptotic strategies are concerned, toxicity may represent the potential obstacle to successful clinical development. These approaches are not necessarily based on structural differences between normal and cancer cells. Therefore, achieving tumor cell specificity, while minimizing toxicity, will probably be the major challenge in the development of this type of approach. Tumor cell specificity is not the major concern for apoptosis-permissive strategies, which mainly target cancer cell-specific alterations. However, the mechanisms by which apoptosis is facilitated are, thus far, mostly unclear, and only a better mechanistic understanding will allow a more effective exploitation of this secondary apoptotic effect during clinical trials.

In general, because of mutations/alterations in the apoptotic machinery, solid tumors have often lost the capacity to undergo instantaneous and massive apoptosis, the so-called primary response that characterizes sensitive cells such as leukemia cells (173). The direct activation of alternative pathways by proapoptotic approaches such as death receptors (e.g., TRAIL) or introduction of exogenous proapoptotic molecules such as Apoptin are nonetheless capable of inducing apoptosis even in a genetically disrupted context.

Alternatively, apoptosis-permissive approaches are also potentially interesting. Backed-up by the progressive development of methods such as cDNA microarrays and tissue microdissection that allow the genomic profiling of the tumors, the defect in the apoptotic machinery could be identified and subsequently repaired, for instance via the reintroduction of tumor suppressor genes by gene therapy. Conversely, strategies that modulate the antiapoptotic effect of oncogenes could be used to facilitate and enhance the effect of conventional chemotherapy. Particularly interesting are combinations of strategies that block survival signaling pathways such as Ras, PI3k, and NF κ B with conventional cytotoxic treatment. Moreover, combining proapoptotic and apoptosis-permissive approaches may, in theory, result in an additive/synergistic apoptotic effect and are worth exploring.

Different therapeutic avenues have certainly been opened by the knowledge acquired on apoptosis, and despite the long way ahead, before they become a therapeutic option, there is room for optimism. Provided that a careful and well-designed plan of clinical development will be followed, apoptosis-triggering strategies are likely to be integrated into the anticancer armamentarium in the next decade.

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