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Teaching Cancer Cells to Die

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Abstract Tumor cells have evolved numerous mechanisms to thwart apoptosis. As our understanding of the machinery which regulates cell-death evolves, these apoptotic defects have fallen into the crosshairs of cancer drug developers. The issues raised in exploiting these alterations for therapeutic benefit are discussed. J. Cell. Biochem. 92: 651–655, 2004. © 2004 Wiley-Liss, Inc.

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It is now accepted dogma that the ability to circumvent apoptosis is a selected trait among tumor cells, as important as growth factor independence or escape from cell cycle control. Tumor cells harboring such alterations find themselves not only with a selective growth advantage, but also a means for evading chemotherapy and radiation-based treatments. Thus, the very process by which tumor cells are produced may select for functions that confound successful treatment.

In the last 10 years many of the regulatory mechanisms controlling programmed cell death as well as the means cancer cells use to subvert them have been flushed out. Not surprisingly, two general strategies have emerged: (1) disabling activators/facilitators of apoptosis (i.e., p53, Bax, Apaf1) and (2) upregulating repressors/antagonists of apoptosis (i.e., BCL2, BclXL, FLIP, IAPs). Discussion of all described alterations is beyond the scope of this article and has been skillfully handled by others [Johnstone et al., 2002]. As a consequence of this increased molecular understanding, a new therapeutic strategy has emerged—to target the defenses used by tumor cells to resist apoptosis. This approach raises several conceptual challenges: how tractable apoptotic signaling pathways are as drug targets, whether cancer cells are inherently vulnerable, and what is the potential clinical utility of drugs borne from this approach.

Poised to Die

Expression of growth deregulating oncoproteins such as c-Myc, E1A, and Ras in normal cells induces cell proliferation as well as apoptosis [reviewed in Evan and Littlewood, 1998]. This self-sacrifice has likely evolved to allow organismal survival in the face of cellular-level transformation. Thus, in cells with proficient apoptotic programs, transforming events may be self-limiting.

The Cory group elegantly demonstrated that the combination of an apoptosis blockade (i.e., BCL2 upregulation) with a transforming oncogene (*myc*) dramatically stimulates the efficiency and accelerates the kinetics of onset of lymphomagenesis in Eu-myc transgenic mice [Cory et al., 1999 and references therein]. This paradigm is perhaps best exemplified by the Adenovirus system. In Adenovirus infected cells, growth deregulation by the E1A oncoprotein triggers a death signal which is inhibited by the BCL2 homologue, E1B19k. E1B19k antagonizes apoptosis by binding to pro-apoptotic Bax and Bak and preventing their oligomerization [Cuconati and White, 2002]. This function is so critical that E1B19k mutant virus infection results in massive cell-death which limits virus replication. Loss of Bax and Bak was also essential for the development of a tumorigenic phenotype in mouse kidney epithelial cells

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transformed with E1A and dominant negative p53 [Degenhardt et al., 2002].

Evidence that tumor cells are "poised to die" is not limited to model systems. Pancreatic, Breast, and Colon cancer cell lines all exhibit elevated levels of Caspase 3 activity relative to normal cell controls [Yang et al., 2003a]. Elevated caspase expression has also been observed immunohistochemically in primary tumor tissue [Vakkala et al., 1999; Nakopoulou et al., 2001].

Collectively, these observations suggest that restoration of defunct cell death pathways may be an effective therapeutic strategy. The anticipated therapeutic window of such drugs would be large since healthy cells already have intact apoptotic signaling pathways whereas malignant cells, with heavy mutational loads (and consequently heavier apoptotic signal loads) may be more sensitive.

Selecting Targets

Correction of apoptotic defects could conceivably be accomplished by restoring defective proapoptotic components or by inhibiting antiapoptotic ones. The former would seem to require a gene therapy approach and this is indeed well underway for p53 [Zhang, 2002, and references therein]. Notable exceptions include recently discovered small molecules purported to reactivate mutant or repressed p53 [Foster et al., 1999; Bykov et al., 2002; Vassilev et al., 2004], and the emerging class of drugs, exemplified by histone deacetylase inhibitors (HDACIs) and DNA methyltransferase inhibitors (DNAMTIs), which may reverse epigenetic silencing via chromatin remodeling [Leone et al., 2003; Yoshida et al., 2003].

HDACIs and DNAMTIs likely achieve antitumor effect through multiple mechanisms. However, the observations that apoptotic signaling components such as Apaf1 and Caspase 8 [reviewed in Johnstone et al., 2002] are targets for epigenetic silencing, together with the observation that HDAC inhibitors induce upregulation of TRAIL, DR4, and downregulation of FLIP, XIAP, and BCL2 [Guo et al., 2004], strongly suggest that apoptosis modulation is critically involved.

Historically speaking, the pharmaceutical industry has been more successful inhibiting functional drug targets than restoring activity to defective ones. IAP proteins and BCL2/BclXL are examples of apoptosis targets, the inhibition of which might resensitize a tumor cell to apoptosis. IAP proteins are BIR (baculoviral IAP repeat) containing proteins, several of which are specific inhibitors of Caspases 3, 7, and 9. XIAP contains three BIR domains wherein the linker region between BIR1 and BIR2 is a potent and specific inhibitor of Caspases 3/7 while BIR3 is specific for Caspase 9 [reviewed in Salvesen and Duckett, 2002]. Anti-apoptotic BCL2 family members BCL2 and BclXL retard Bax and Bak-mediated release of cytochrome C and other proapoptotic regulatory molecules from the mitochondria [reviewed in Heiden and Thompson, 1999].

For both IAPs and BCL2/BCL-XL there is experimental and epidemiological data correlating expression of each with decreased sensitivity to chemotherapy. Generally speaking, both are overexpressed in a broad range of tumor types and have been mechanistically linked to a defined block in apoptotic signaling. Furthermore, ample structural information is available for select IAP and BCL2 family members to guide emerging in silico approaches for rational drug design. Pharmacological inhibition of these targets or others sharing these characteristics would in principle facilitate tumor-selective apoptosis.

Requirement for a Death Trigger?

A provocative question is whether such agents would be sufficient to induce cell death or useful merely as regimens in a cocktail with an agent capable of initiating an apoptotic signal.

Both peptidic and small molecule inhibitors of BCL2/BclXL have been identified and evaluated in cell culture models [Nakashima et al., 2000; Degterev et al., 2001; Tzung et al., 2001; La Vieira et al., 2002]. In some instances, standalone activity is described while in others, only enhancement of apoptosis triggered by a costimulus is observed. The phenomena of cell type differences and off-target effects can obscure anti-tumor activity truly resulting from pharmacological inhibition of the target. Sorting this out is a formidable challenge.

IAPs such as XIAP are unique in that a naturally occurring "small molecule" inhibitor, Smac, has been described [Du et al., 2000; Verhagen et al., 2000]. Smac is a mitochondrial protein which binds to the caspase-interaction surface of both BIR2 and BIR3 and in so doing precludes caspase inhibition. The N-terminal

seven amino acids of mature Smac are capable of neutralizing BIR3, however, full length Smac is required for neutralization of BIR2 [reviewed in Salvesen and Duckett, 2002]. An ever increasing number of studies on Smac have conceptually validated XIAP inhibition as a therapeutic approach. Transfection of a cDNA encoding Smac does not directly impact proliferation but hypersensitizes cells to UV irradiation [Du et al., 2000; Verhagen et al., 2000]. Several groups have tested peptide fusions containing from 7 to 55 amino acids of Smac fused to membrane permeabilizing moieties such as Tat and antennapaedia on tumor cell lines in vitro [Arnt et al., 2002; Fulda et al., 2002; Vucic et al., 2002; Yang et al., 2003b]. Consistently, such peptides have little effect on tumor cell proliferation when administered alone but synergize impressively with a range of cytotoxic drugs in vitro. Two groups have demonstrated synergy in vivo, one using a subcutaneous xenograft model and Cisplatin [Yang et al., 2003b], the other using an orthotopic brain tumor model and TRAIL [Fulda et al., 2002].

As therapeutics, peptides have the drawback of being highly unstable. Thus, there is considerable interest in the development of bona fide small molecule Smac mimics. A non-peptidic small molecule, TWX024, was recently identified in a high throughput screen as an inhibitor of the BIR2-Caspase 3 interaction. TWX024 synergizes with CD95 or TRAIL but does not exhibit stand-alone activity [Wu et al., 2003]. Interestingly, BIR2-specific non-peptidic inhibitors identified in other screens were found to have both synergistic and stand alone activity against a broad range of tumor cell lines [Schimmer et al., 2004]. Whether such therapies will work alone or not remains an open question. In the event that drug combinations are required, the benefit may be that the sensitivity to an apoptotic stimuli is raised and hence, lower doses of chemotherapeutic drugs might be efficacious. This would reduce toxicity and possibly also the occurrence or rapidity of onset of drug resistance.

Complexity of Signaling Pathways

Yet another complexity which challenges this approach is the variety of means tumor cells may employ to circumvent apoptosis and acquire resistance to chemotherapy. Far upstream of caspase activation, survival pathways, drug efflux pumps, mutations to drug targets, enhanced drug metabolism, and enhanced DNA repair, all impact whether cell death occurs in response to a given stress.

Using the Eu-*myc* mouse model, the Lowe group [Schmitt et al., 2000] has begun to assess the individual contribution of various apoptotic defects to chemotherapeutic responses. Lymphomas with either AKT activation or BCL2 upregulation are equally resistant to treatment with Adriamycin alone or Rapamycin alone. The two drugs in combination are effective against the AKT cells but not the BCL2 cells [Wendel et al., 2004]. Thus, the particular mechanism(s) a tumor cell employs to circumvent apoptosis profoundly impacts what chemotherapeutic drugs will succeed or fail.

In fact, it may be even more complex than this. Tumor cells are by definition heterogeneous. Thus, a solid tumor could in principle contain one population of tumor cells with a single, mild apoptotic defect and another population of cells in which apoptotic signaling is almost completely blocked. This may explain why initial responses to chemotherapy, in SCLC, e.g., are often very good but chemoresistant cancer nearly always returns following remission [Zimmermann et al., 2003]. Indeed, simultaneous blocks to intrinsic and extrinsic death signaling pathways were found in 92% of AML patients with chemoresistant disease but in only 33% of patients who were chemoresponsive [Schimmer et al., 2003].

Parting Perspectives and Unmet Needs

For this therapeutic approach to succeed, strategies will have to evolve to identify what apoptotic defect(s) occur in a given tumor type. A better understanding of how oncogene deregulation results in a proapoptotic signal will provide clues as to where apoptotic defects are likely to turn up. Apoptotic therapies will likely realize their greatest potential when incorporated into regimens tailored to the genotype of a particular tumor.

Finally, there is a need to identify new apoptosis targets. The IAP and BH3 family targets have solid rationale but are protein–protein interaction problems. The industry's success rate at targeting enzymes is far greater than for protein–protein interactions yet few enzyme targets are known. siRNA technology was recently used to identify both positive and negative modulators of TRAIL mediated

apoptosis [Aza-Blanc et al., 2003]. Hopefully genome-wide studies such as these will yield additional drug target candidates in the not too distant future.

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