2 Resistance to Apoptosis in Cancer Therapy

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SUMMARY

An enormous body of work has established that conventional cancer therapies induce apoptosis in tumor cell lines and preclinical models of human disease. Parallel efforts have defined the evolutionarily conserved components of the apoptotic pathway, inspiring efforts to characterize the functional status of these components in human tumor cells. Although core pathway defects have been documented, the apoptotic pathway appears to be intact in most tumors, perhaps because disruption of the core machinery inhibits cell proliferation or interferes with other processes that are essential for tumor progression. On the other hand, it is clear that the selective pressures encountered by tumor cells do increase apoptosis resistance, most often via disruption of upstream mechanisms that produce parallel decreases in apoptosis sensitivity and increases in cell proliferation. Two of the most common examples of this type of disruption are inactivation of the p53 pathway and activation of PI-3 kinase/AKT/NF-κB, and aggressive efforts to specifically target these defects are underway. A major challenge for the immediate future is to validate the concept that apoptosis plays a crucial role in tumor response to therapy in patients receiving conventional and "designer" drugs, and more specifically, to confirm that the latter effectively hit their targets and produce the desired biological responses.

APOPTOSIS AND TUMOR PROGRESSION

Most conventional cancer therapies kill tumor cells by directly or indirectly damaging DNA, and DNA "breaks" are clearly important in triggering tumor cell death *(1)*. Thus, many investigators have assumed that the preferential cell killing observed in tumor cells as compared to their normal counterparts (the "therapeutic window") is attributable to the higher rates of cell proliferation displayed by the former. This should make the tumor cells more vulnerable to agents that interrupt DNA synthesis and/or other processes

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associated with cell division (i.e., cytokinesis), and tumor cells might die as a result of either permanent mitotic arrest or mitotic "crisis" caused by the failure to successfully complete division. This model also provides an explanation for why the death of normal cells with relatively high rates of proliferation (bone marrow hematopoietic precursors and epithelial cells in the gastrointestinal tract) is usually responsible for the dose-limiting toxicities associated with these agents. In addition, this model would predict that drug resistance would be due largely to increased drug efflux and/or DNA repair capacity.

The recognition that most (if not all) cancer therapies trigger apoptosis in models of human cancer *(1–4)* has prompted a reappraisal of this paradigm. Apoptosis is an energy-dependent evolutionarily conserved pathway that is regulated by a very diverse array of factors *(5)*, any of which could be disrupted by selection mechanisms associated either with natural tumor progression or prior exposure to therapy. At the core is a family of cysteine proteases known as caspases *(6)*, whose activation is controlled by pro- and antiapoptotic members of the BCL-2 family *(7)*. Thus, as will be described in more detail below, indirect interference with caspase activation, most likely via indirect effects on the BCL-2 family, now appears to be a common theme in the development of therapeutic resistance in cancer *(8,9)*. However, data accumulated over the past decade strongly suggest that direct inactivation of the core machinery for apoptosis (for example, via massive overexpression of BCL-2 or mutational inactivation of caspases) is not nearly as common indirect inactivation of apoptosis, commonly in a pathway-specific manner.

Why is this the case? Returning to the idea that tumor cell susceptibility to cancer therapy is associated with cell proliferation, work conducted over the past decade has also shown that apoptosis and cell division are tightly coupled *(10,11)*. The earliest report of this phenomenon came from Evan, Wyllie, and their colleagues, who showed that enforced expression of the *myc* oncogene increased levels of apoptosis in normal fibroblasts deprived of growth factors *(12)*; Jacks and Lowe refined this observation by showing that normal fibroblasts transfected with proliferation-driving oncogenes were also dramatically sensitized to cell death induced by conventional cancer chemotherapeutic agents and ionizing radiation *(13,14)*. Loss of expression of certain cell-cycle-regulating tumor suppressor proteins (for example, the Rb protein *[15,16]*) can also sensitize cells to apoptosis via deregulated activation of E2F-1 *(17,18)* (*see also* refs. *19–21* for more recent interpretations of the effects of loss of Rb on neurological development) . Indeed, recent studies have demonstrated that normal cells can be protected from the toxic effects of cancer therapy by pretreating them with cell-cycle inhibitors, thereby "parking" them in a state of reduced sensitivity to apoptosis *(22)*. Thus, a new explanation for the therapeutic window observed in cancer therapy is that early stage tumor cells display increased apoptosis sensitivity due to cell-cycle deregulation.

Considering these findings, it is easier to understand why tumor cells tend to almost always disrupt certain apoptosis regulatory mechanisms and not others. Just as deregulated cell-cycle control increases sensitivity to apoptosis, many apoptosis inhibitors also tend to inhibit cell-cycle progression. Perhaps the best example of this phenomenon has come from studies with BCL-2 itself, which delays cell-cycle progression in normal cells *(23)* as well as many different tumor cell lines *(24,25)*. Thus, BCL-2-mediated resistance to apoptosis comes at a cost in terms of cell proliferation. Even caspases themselves have been recently implicated in cell proliferation *(26–28)*, which may explain why tumor cells (and especially tumor cell lines) rarely display loss of caspases by mutation. Tumor

cells that select for this type of cell death resistance must therefore also specifically overcome the inhibition of proliferation by acquiring a second genetic or epigenetic defect *(11)*. Therefore, the most common examples of acquired apoptosis resistance in tumors simultaneously promote both apoptosis resistance and cell-cycle progression *(11)*.

LOSS OF FUNCTIONAL P53 AND RESISTANCE TO CANCER THERAPY

The fact that the p53 pathway is inactivated in the vast majority of human cancers is one of the most familiar concepts in cancer biology. p53 functions as part of an increasingly complex network of proteins that mediate cellular responses to DNA damage and a variety of other stimuli *(29,30)*. It is clear that p53-induced cell-cycle arrest is mediated largely via transcriptional activation of the gene encoding p21/WAF-1 *(31,32)*, a polypeptide inhibitor of the cyclin-dependent kinases. However, p21 does not appear to play a direct role in promoting p53-mediated cell death *(33–36)* (although it does play an important indirect role, as will be discussed in more detail below). The precise mechanisms that underlie p53-mediated cell death are complex, but there is general consensus that members of the BH3-only subfamily of BCL-2 proteins *(37,38)* and death receptor pathway components *(39,40)* play predominant roles. Most of the available evidence indicates that the p53-mediated transcriptional activation is required for cell death, but there are prominent recent examples of transcription-independent effects in the literature *(41–48)*.

Other recent studies have identified some of the pressures associated with tumor progression that select for cancer cells that possess defects in the p53 pathway. Of particular importance is the observation that p53 is activated by oncogenes via p19ARF, a protein encoded within the p16 locus that inhibits MDM-2/HDM-2-mediated ubiquitination and degradation of p53 *(49,50)*. Thus, the apoptosis that results from overexpression of Myc *(51)* or inactivation of Rb *(52)* is p53-dependent in many normal cells and presumably in early-stage tumors, and loss of p53 function reverses apoptosis sensitization.

Tumor hypoxia is another important factor that selects for loss of p53 function *(53)*. As tumors grow beyond the diffusion limit of oxygen, they must acquire the capacity to stimulate new blood flow, or a crisis caused by hypoxia and hypoglycemia ensues *(54,55)*. Hanahan has termed this transition the "angiogenic switch" *(56)*, and his laboratory and others have shown that it is associated with increased expression of proangiogenic cytokines (VEGF, bFGF, IL-8) *(56–58)* and decreased cell death *(59)*. For reasons that are not entirely clear, hypoxia-induced cell death is highly sensitive to cellular p53 status, and the hypoxic microenvironment within growing solid tumors actually selects for mutational inactivation of p53 *(53)*. This phenomenon has important implications for antiangiogenic therapy, in that tumors that retain wild-type p53 appear to respond much more impressively to therapy than do cells with mutant forms of the protein *(60)*. Other tumor progression-associated mechanisms associated with the angiogenic switch include upregulation of BCL-2 family proteins (BCL-2, BCL-X_L) *(59)*.

The molecular mechanisms underlying p53-mediated apoptosis are complex and likely depend on the cell type in question. Early work demonstrated that p53 binds to and activates the Bax promoter *(61)*, leading to increased expression of this proapoptotic

member of the BCL-2 family. However, it is now thought that Bax activation is a complex process involving translocation of the protein from the cytosol to the mitochondrion *(62,63)* and subsequent oligomerization of the protein within the mitochondrial membrane *(64–66)*. These events are sensitive to amino acids within the C-terminal "transmembrane" domain of Bax *(67)* and may be driven by members of the so-called "BH3-only" subfamily of BCL-2-related polypeptides *(66,68)* or p53 itself *(48)*. Indeed, recent studies have demonstrated that p53-mediated apoptosis is dependent on PUMA and/or NOXA, two BH3-only proteins identified in a screen for p53 targets *(38)*. Other work has shown that expression of the death receptor-associated protein Bid is also driven by wild-type p53, and still other studies suggest that p53-dependent induction of the BH3-only protein Bik drives apoptosis-associated disruption of the endoplasmic reticulum. Finally, very recent work from Green's laboratory demonstrated that cytosolic p53 can directly drive Bax activation *(48)*, an observation that is probably related to previous work showing that p53 localizes to mitochondria to promote cytochrome c release *(47)*.

p53 activation also increases the expression of critical components of death receptor-mediated pathways of apoptosis. Early work from Owen-Schaub's laboratory demonstrated that wild-type p53 drove expression of Fas in human osteosarcoma cells *(40)*, an observation that was subsequently confirmed in a variety of other tumors as well as normal tissues *(69)*. These effects appear to be mediated by p53 response elements located within the first intron of the Fas gene *(70)* as well as elements located >1.5 kb upstream of the transcriptional initiation site (L. Owen-Schaub, personal communication). Debatin's group extended these observations by showing that conventional chemotherapeutic agents kill some tumor cell lines via Fas-sensitive mechanisms *(71,72)*, and other work has shown that p53-induced Fas-Fas ligand interactions are required for the appearance of apoptotic "sunburn cells" in the skin *(73)*. Cells containing mutations in p53 accumulate in Fas ligand$^{-/-}$ animals (H. Ananthaswamy, personal communication), demonstrating the importance of this pathway in the elimination of damaged, potentially transformed cells.

More recent work has demonstrated that tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptor-2/death receptor (DR)5 is also an important transcriptional target for p53 *(74)*. Interestingly, as is true for Fas, the effects of p53 on DR5 are mediated by a p53 response element localized to the first intron of the DR5 promoter *(75)*. The effects of p53 on DR5 expression are certainly involved in the synergistic effects of DNA-damaging agents and TRAIL on apoptosis *(76)*, although p53-independent mechanisms also appear to contribute to enhanced cell killing *(77)*. That synergy is sensitive to cellular p53 status has important implications for planned human clinical trials of combination therapy with conventional agents and TRAIL, in that p53 status could prove to be an important predictor of response.

Although studies in knockout mice have established that p53 is required for DNA damage-induced apoptosis in many normal tissues *(78–80)*, loss of wild-type p53 clearly does not engender absolute insensitivity to DNA damage-induced apoptosis in tumors. There are many examples of DNA damage-induced, p53-independent apoptosis in the literature, but one of the most informative studies of the relative importance of p53 status in dictating sensitivity to chemotherapy comes from a study of relative sensitivity to 5-fluorouracil (5-FU) in the National Cancer Institute's panel of 60 tumor cell lines *(81)*. The results of this work confirmed that loss of wild-type p53 was associated with

resistance to 5-FU, but the mean IC_{50} observed in p53 wild-type cells was only about 3- to 10-fold lower than the mean IC_{50} observed in tumor cells lacking wild-type p53. Furthermore, since the assay employed in the screen cannot distinguish growth arrest from apoptosis, it is likely that p53-mediated cell-cycle arrest contributed significantly to the effects observed. Whether or not other members of the p53 family (p63, p73) can compensate for loss of p53 remains to be determined.

Reconstitution of wild-type p53 by gene transfer is the most direct means of restoring p53 pathway function in tumors *(82,83)*. Effective replication-incompetent adenoviral systems for p53 gene therapy were among the first gene transfer systems developed *(84)*. Promising results were obtained in several different preclinical (xenograft) models where Ad-p53 was combined with conventional modalities (radiation, DNA-damaging agents, taxanes *[85–89]*) *(90)*. However, obtaining high-level expression of p53 throughout the tumor has been a major obstacle to optimization of these approaches, and in the clinical trials performed to date, protein expression was largely confined to regions immediately adjacent to the needle entry site. The development of replication-competent viruses may provide one means of overcoming this pitfall. For example, Onyx Pharmaceuticals, Inc., developed a p53 adenovirus that selectively replicates in cells lacking p53 *(91,92)* by taking advantage of the fact that the adenoviral E1A protein (like Myc) drives both cell-cycle progression and p53-mediated apoptosis, effects that must be counteracted by two proteins encoded by the E1B locus *(93)*. Thus, by deleting E1B, Onyx produced a virus that can propagate efficiently only within a p53-null background. Clinical trials with the Onyx virus in combination with conventional therapy *(86,94)* are ongoing at present, but promising clinical activity has been observed in trials performed to date *(95–100)*. However, work still needs to be done to improve methods for systemic gene delivery to combat disseminated (metastatic) disease *(101)*, which is really the relevant target of adjuvant therapy in patients with most forms of cancer.

APOPTOSIS RESISTANCE MEDIATED BY AKT/PKB

AKT/PKB is a protein serine/threonine kinase that functions downstream of phosphoinositol 3' (PI-3) kinase to regulate cell proliferation, glycolytic metabolism, and survival *(102–105)*. Recent studies have demonstrated that AKT interferes with apoptosis at multiple levels via phosphorylation of survival-associated substrates including the BH3-only protein BAD, the forkhead family of transcription factors, the transcription factor NF-κB, and possibly caspases themselves *(103)*. In addition, AKT simultaneously promotes cell-cycle progression and apoptosis resistance by phosphorylating and activating another kinase, the molecular target of rapamycin (mTOR). mTOR drives proliferation via activation of ribosomal S6 kinase *(106)* and recent studies indicate that AKT-induced, mTOR-dependent increases in glucose, iron, and cholesterol uptake function to promote cell survival under conditions of growth factor withdrawal *(107,108)*. Thus, as is true for inactivation of p53, activation of AKT simultaneously promotes cell division and survival, making it an attractive target for disruption during tumor progression.

Two of the most common progression-associated molecular events that mediate tumor cell acquisition of active AKT are loss of the MMAC/PTEN tumor suppressor and overexpression of growth factor receptors (EGF-R, HER-2, and IGF-IR) *(109)*. PTEN is

a lipid phosphatase that functions as an enzymatic antagonist of PI-3 kinase, and it is commonly inactivated at a relatively late stage in solid tumor progression, often at the point where the cells acquire an invasive and/or metastatic phenotype *(110)*. Thus, loss of PTEN interferes with detachment-induced apoptosis (anoikis), a process observed in normal epithelial and other adherent cells when they are dissociated from basement membrane or extracellar matrix *(111–113)*. Although PTEN may have other biological functions unrelated to AKT, overexpression of constitutively active forms of AKT suppresses anoikis and enhances metastasis, whereas reintroduction of wild-type PTEN into tumor cells that lack it promotes sensitivity to detachment-induced cell death and loss of metastatic potential *(114–117)*. In addition, one group demonstrated that metastatic potential and AKT activation were enhanced in squamous cell carcinomas of the oral tongue selected for an aniokis-resistant phenotype via repeated short-term suspension culture *(118)*.

The acquisition of growth factor independence is a second pressure that selects for tumor clones containing active AKT *(109,119)*. Like anoikis, apoptosis associated with growth factor withdrawal probably functions physiologically to prevent the inappropriate migration of cells away from their normal microenvironment. Tumor cells subvert this regulatory mechanism by overexpressing growth factor receptors and their ligands, thereby establishing autocrine pathways that render the tumor cell less dependent upon growth factors provided by the microenvironment. In addition, tumors may induce stromal cells within the microenvironment to express the specific growth factors the tumor requires for proliferation and survival. Wherever the mechanisms have been carefully interrogated, AKT activation plays a central role in these responses.

The growth-factor receptors that have received the most attention in studies of tumor progression to date are the members of the erbB family *(120)*. This family consists of four homologous transmembrane polypeptides (erbB1–4) that contain tyrosine kinase domains within their cytoplasmic tails *(121)*. The most familiar members of the family are erbB1 (the EGF receptor) *(122)* and erbB2 (HER-2/Neu) *(123)*, both of which are overexpressed in most solid tumors as a function of progression. Furthermore, all members of the erbB family can dimerize with one another to form signaling complexes that probably mediate distinct effects on tumor cell biology *(121)*.

Studies with primary tumors have demonstrated that the EGFR and HER-2 and their ligands (EGF, TGF-α) are overexpressed in almost all solid malignancies *(109,124–126)*. In human tumor xenograft models, selection for increased metastatic potential via orthotopic "recycling" results in increased EGFR and/or HER-2 in colon, prostate, breast, bladder, and pancreatic cancer *(127–129)*. Overexpression of HER-2 in the HER-2-negative human breast line MDA-MB-435 results in increased metastatic potential and decreased sensitivity to chemotherapy-induced apoptosis *(130–132)*, whereas down-regulation of HER-2 inhibits metastasis *(133)*. Ligand-mediated activation of EGFR and HER-2 activates AKT *(123,134–136)* and other kinases involved in cell proliferation *(137)*.

Although many different targets appear to contribute to AKT-mediated apoptosis resistance, one of the best studied within the context of therapeutic resistance is the transcription factor, NF-κB. Early studies in NF-κB (p65) knockout mice demonstrated that the transcription factor is required for a critically important cellular survival pathway that is activated by tumor necrosis factor (TNF) and other proinflammatory cytokines *(138)*. Subsequent studies revealed that NF-κB is also commonly activated in tumor cells

exposed to conventional chemotherapeutic agents, and suppression of this NF-κB activation via overexpression of a molecular inhibitor of NF-κB (IκBαM) dramatically enhances tumor cell killing *(139)*. At the core of the upstream signal transduction pathway leading to NF-κB activation is IκB kinase (IKK), a large multisubunit complex that is responsible for phosphorylating IκBα and targeting it for ubiquitination and degradation by the 26S proteasome *(140,141)*. However, optimal NF-κB activation is also dependent on AKT in many different cellular systems *(142)*, either because AKT also participates in phosphorylation of IκBα *(143)* or because it directly modifies the NF-κB subunit(s) themselves (p50, p65) via serine phosphorylation.

The transcriptional targets of NF-κB are quite diverse, but its effects on cell survival appear to involve upregulation of antiapoptotic BCL-2 family proteins (BCL-2, BCL-X_L, A1) *(144–148)* and inhibitor of apoptosis proteins (IAPs) *(149)*. BCL-2 family proteins regulate caspase activation upstream of mitochondrial events leading to cytochrome c release *(147,148,150)*, whereas the IAP proteins bind directly to caspases and inhibit their activities *(151)*. These cell death inhibitory events are complementary and probably act to reinforce resistance to cell death in tumor cells that express constitutively active NF-κB. However, no systematic evaluation of the relative importance of each target in NF-κB-mediated cell death resistance in any tumor model has been performed to date. Thus, most current therapeutic strategies are aimed at inhibiting tumor cell AKT or NF-κB rather than their downstream targets.

There are several potential strategies that could be employed to block AKT activation in tumor cells, and all of them are being aggressively pursued. Excellent inhibitors of EGFR and HER-2 have been developed, including blocking antibodies and small-molecule tyrosine kinase inihibitors, which display very promising activity in preclinical models and clinical trials *(122,152)*. Furthermore, preclinical studies have demonstrated that these agents act in an additive or synergistic fashion with cytotoxic agents, although these effects are sensitive to schedule. Small-molecule inhibitors of PI-3 kinase and AKT are also being developed for human cancer therapy, and where tested preclinically, they too have displayed very promising bioactivity *(153,154)*. Trials with an inhibitor of mTOR (a rapamycin analog) are already underway in patients with prostate cancer and certain other malignancies *(155,156)*.

One fascinating approach to chemosensitization involves using proteasome inhibitors to block NF-κB activation. The first of these compounds (bortezomib, formerly PS-341, also known as Velcade*(157)*) recently received FDA approval for the treatment of patients with multiple myeloma *(158–160)*, and it is also displaying promising activity in other hematological and solid malignancies. Bortezomib prevents chemotherapy-induced activation of NF-κB in cell lines and xenograft models, and synergizes with several different agents (gemcitabine, camptothecin, radiation) to promote tumor growth inhibition *(161–164)*. Inhibition of NF-κB may also decrease invasion, metastasis, and angiogenesis by downregulating matrix metalloprotease, integrin, and angiogenic factor (VEGF, IL-8) expression by tumor cells *(165–167)*. Importantly, because the proteasome is involved in such a large number of different cellular processes, bortezomib's effects go well beyond inhibition of NF-κB. Thus, a recent side-by-side comparison study concluded that bortezomib was more active than a chemical IKK inhibitor (PS-1145) in a preclinical multiple myeloma model *(168)*. Bortezomib is a very potent inhibitor of cell-cycle progression and causes endoplasmic reticular stress by interfering with the clear-

ance of misfolded or damaged proteins within the cell. These effects probably contribute to make bortezomib a more effective anticancer agent than compounds that are designed to exclusively target NF-κB itself.

LOSS OF DEATH RECEPTOR RESPONSIVENESS

Death receptors are a family of transmembrane proteins homologous to the tumor necrosis factor receptor 1 (TNFR1) that play key roles in the maintenance of immune homeostasis and host defense *(169)*. The most familiar members of the family are the TNFR1 itself, CD95/Fas, and two of the receptors for TRAIL (DR4 and DR5). The importance of death receptors in immunity was first demonstrated in studies of the mechanism of apoptosis induced by T-cell receptor engagement of previously activated mature T-cells, a phenomenon known as "activation-induced cell death" (AICD). These studies demonstrated that AICD is mediated by increased expression of Fas and Fas ligand *(170,171)*. As noted earlier, Fas-Fas ligand interactions also mediate apoptosis in various normal cells exposed to DNA-damaging agents, and Fas expressed by tumor cells is recognized by activated cytotoxic T-cells and natural killer cells that express Fas ligand, resulting in specific tumor cell killing. Tumor cell sensitivity to Fas is further increased by cytokines (particularly interferons) that are produced by activated T-cells and components of the innate immune system (macrophages). Antibodies to Fas are also highly cytotoxic to a subset of human tumor cells in vitro *(172)*, which at one time prompted enthusiasm for their potential use in cancer therapy. Unfortunately, normal hepatocytes are highly sensitive to Fas-mediated apoptosis *(173)*, making a systemic approach based on anti-Fas antibodies or Fas ligand itself infeasible.

Although there are substantial concerns about using TNF or Fas ligand in cancer therapy, the prospects for TRAIL appear much more promising. Initial studies demonstrated that TRAIL triggered apoptosis in most human cancer cell lines but not in their normal counterparts *(174)*, and initial in vivo studies with recombinant murine or human TRAIL in rodents or primates demonstrated little to no toxicity *(175)*. These findings prompted academic centers and industry to develop recombinant TRAIL and agonistic anti-DR4 and -DR5 antibodies for use in cancer therapy *(176)*, and clinical trials with these agents are underway. Unfortunately, the clinical development of TRAIL encountered a setback with a prominent report demonstrating that isolated human hepatocytes underwent apoptosis when they were exposed to certain preparations of recombinant TRAIL in vitro *(177)*. These concerns have been alleviated somewhat by arguments that the effects were preparation-specific and that the recombinant TRAIL and the anti-death-receptor antibodies are not cytotoxic to normal cells.

Given their central roles in host defense, it is not surprising that tumors disrupt death receptor pathways as a function of progression. The earliest examples of this phenomenon came from studies with metastatic melanoma cells, which short-circuit the Fas pathway by downregulating surface expression of the receptor *(178)* and by expressing a soluble "decoy" form of Fas that competes with the full-length molecule for binding to Fas ligand *(179)*. Subsequent studies demonstrated that colon cancer cells also downregulate Fas expression and sensitivity with progression *(180,181)*, and similar processes have been described in other solid tumor models. The selective pressures that enrich for Fas resistance are associated with innate and adaptive antitumor immunity as well as the high-level expression of Fas ligand in endothelial cells within "immune

privileged sites" (lung, brain), which probably serves to prevent the inward migration of activated T-cells and tumor cells that express Fas *(182)*. Because DNA-damaging agents appear to kill certain tumor cells via p53-dependent, Fas-mediated mechanisms, it is likely that the same pressures that induce loss of p53 select for loss of Fas sensitivity as well.

Like Fas, the receptors for TRAIL are also upregulated via p53-dependent mechanisms, and the loss of TRAIL receptor inducibility that accompanies loss of functional p53 also probably contributes to chemoresistance in tumors. However, given current enthusiasm for TRAIL-based cancer therapy, the observation that many tumor cells display baseline resistance to TRAIL presents an additional challenge. The mechanisms underlying TRAIL resistance in tumors are complex and probably include expression of decoy receptors that compete for TRAIL binding and expression of cellular FLICE-inhibitory proteins (c-FLIPs) *(169)*, which are enzymatically inactive homologs of caspase-8 that compete for binding to the death-inducing signaling complex (DISC). Furthermore, recent studies have demonstrated that AKT desensitizes tumor cells to death receptor-mediated suicide, in part via effects on the FKHD family of transcription factors (which promote death receptor/ligand expression) *(183)* and in part via more direct effects on the death receptor-mediated signaling pathways that precipitate cell death.

Recent work from our laboratory has identified another tumor progression-associated event that selects for loss of TRAIL and Fas sensitivity. These studies have been conducted in bladder cancer cells, which appear to be particularly sensitive to innate immune defense systems, as exemplified by the fact that the immune modulator BCG remains frontline therapy for invasive disease, producing complete responses in a majority of patients. Thus, it is likely that inflammatory cytokines expressed by stromal elements within the bladder apply constant pressure to transformed bladder epithelial cells. We have found that interferons induce high-level TRAIL expression in approx 50% of human bladder cancer cell lines (from a panel of over 20), and a subset of these are directly sensitive to interferon (IFN)-induced apoptosis (A. Papageorgiou et al., manuscript submitted). Importantly, specific inhibitors of caspase-8 and blocking anti-TRAIL antibodies attenuate cell death, strongly suggesting that IFN-induced apoptosis is mediated in part via autocrine production of TRAIL. However, some of the lines that secrete high levels of TRAIL are not sensitive to TRAIL-induced apoptosis. Thus, the emergence of TRAIL resistance appears to be a common event in bladder cancer progression. Even though IFNs can activate p53, loss of wild-type p53 does not explain the emergence of TRAIL resistance in our panel.

Although TRAIL resistance appears to be a fairly common problem in cancer cell lines, exciting new data indicate that this resistance can be overcome by combining TRAIL with certain conventional and investigational agents. A key feature of TRAIL-mediated cell death is that, unlike many of the other pathways described above, cell-cycle arrest appears to sensitize tumor cells to TRAIL *(184,185)*. For example, some TRAIL-resistant cell lines can be dramatically sensitized with DNA-damaging agents *(186–189)*, effects that may be sensitive to wild-type p53 and its transcriptional targets *(76)*. Furthermore, a variety of different investigational agents (flavopiridol *[190]*, histone deacetylase inhibitors *[191,192]*, bortezomib *[189,193,194]*) also synergize with TRAIL to promote killing of otherwise resistant tumor cells. In our recent studies we have linked

these effects to an inhibition of cyclin-dependent kinases (L. Lashinger, manuscript in preparation), and we suspect that any therapy that blocks cell-cycle progression at the G_1 to S transition *(184)* will sensitize tumor cells to TRAIL. An excellent example of this is found with our studies with EGFR antagonists, which appear to promote TRAIL sensitization in bladder cancer cells via a p27-dependent mechanism *(195)* (M. Shrader, manuscript in preparation).

OTHER RESISTANCE MECHANISMS

As discussed above, given that the core molecular machinery for apoptosis is evolutionarily conserved, one might predict that the downstream elements would represent common targets for inactivation during tumor progression. Current thinking holds that caspase activation is the key rate-limiting step in apoptosis and that in mammalian cells activation of the so-called execution-phase caspases (3, 6, and 7) correlates with irreversible commitment to death. Members of the BCL-2 and IAP families promote or inhibit cell death via direct or indirect effects on caspase activation, and in mammalian cells, mitochondrial factors (cytochrome c, SMAC) and the cytosolic adaptor protein Apaf-1 also cooperate to promote caspase activation. Thus, defects in the caspases themselves or in the regulation of BCL-2 family proteins, IAPs, SMAC, cytochrome c, or Apaf-1 would be expected to be commonly associated with drug resistance in human tumors.

There are data consistent with this hypothesis, although it is not clear why these central pathways are not disrupted even more frequently than they are. Mutational inactivation of caspase-3 has been documented in only one human cancer cell line (the breast adenocarcinoma MCF-7) *(196,197)*, and caspase-7 mutations have been identified in only a relatively small subset of human solid tumors (<3%) *(198)*. Mutational inactivation of Apaf-1 has recently been described in melanoma *(199)*, and downreglation of Apaf-1 has also been reported in ovarian cancer *(200)*. There are no reports of mutational inactivation of SMAC or cytochrome c, although disruption of the latter's ability to participate in electron transport might select against cytochrome c modification. Importantly, early studies conducted by Raff's laboratory failed to identify any mammalian tumor cell line (or for that matter primary cell type) that could not be induced to undergo apoptosis following treatment with the protein kinase inhibitor staurosporine *(201)*. (The only exception to this rule was human embryonic cells isolated before the blastomere stage of development.) Together, these observations strongly suggest that disruption of the core apoptotic machinery for apoptosis does not commonly occur in human cancer cells.

There is much more evidence available implicating alterations in BCL-2 family proteins or the IAPs in tumor progression, but the results of these studies are mixed. Early work demonstrated that follicular B-cell lymphoma is driven by a chromosomal translocation that mediates high-level expression of BCL-2 in follicular B-cells *(202)*, and subsequent work showed that the acquisition of androgen independence in prostate cancer is associated with increased BCL-2 levels *(203)*. However, in other common solid tumors the role of BCL-2 in tumor progression and response to therapy is less clear. For example, the majority of early studies showed that expression of BCL-2 correlated with expression of the estrogen and progesterone receptors and a well differentiated phenotype in primary breast adenocarcinomas *(204)*, although more recent work suggests that higher levels of BCL-2 may correlate with poor response to neoadjuvant therapy *(205)*.

There is also some evidence that BCL-2 levels increase in metastatic melanoma, but once again reports arguing against this conclusion can also be found in the literature *(206)*. Perhaps even more paradoxically, our laboratory and another group independently demonstrated that basal levels of apoptosis were significantly elevated in matched liver metastases relative to the levels observed in primary colorectal adenocarcinomas *(207,208)*, effects that were associated with significant decreases in BCL-2 expression in the metastases. Frameshift mutations in Bax or Bak have been identified in subsets of gastric and colorectal adenocarcinomas (mostly with microsatellite instability) *(209–212,213)* and in human DU-145 prostate adenocarcinoma cells, but simultaneous inactivation of both Bax and Bak has not been reported. Finally, there are numerous studies that have linked increased expression of the IAP family member survivin to progression in breast and prostate cancer and various other solid malignancies *(214)*. However, survivin's function as a caspase inhibitor appears to be more restricted than the spectra of activity of other IAP family members, whereas it appears to function as a mitotic cofactor in most cells *(214)*, raising questions about whether tumors select for increased survivin in order to block apoptosis or to increase proliferation. Even the other IAP family members display diverse intracellular functions ranging from ubiquitin ligase activity to the regulation of death receptor function. As discussed above, one attractive explanation for these observations is that BCL-2 family proteins and the IAPs also play important roles in cell division or other critical homeostatic mechanisms. Thus, a tumor that gains apoptosis resistance by disrupting one of these pathways may do so at a cost. It is possible that posttranslational modifications of the core machinery that selectively promote resistance to cell death will be more common in cancer. If this proves true, then it may be possible to target these events to reverse cell death resistance.

APOPTOSIS RESISTANCE AND CANCER THERAPY: IMPLICATIONS FOR CANCER PATIENTS

An important caveat associated with almost all of the work described above is that the experiments were conducted in models of human cancer rather than in primary human tumors themselves. Recent studies employing microarrays to survey patterns of gene expression have cast doubt concerning how accurately human cell lines reflect primary tumors. Furthermore, most of the popular transgenic mouse models rely on tissue-specific viral inactivation of key pathways (i.e., p53 and Rb) to produce solid tumors, and in most cases they do not accurately model specific processes associated with spontaneous tumorigenesis in humans. Even more important to the present discussion, apoptosis is not the only cell-death pathway that can be activated by conventional and investigational cancer therapies, and where interrogated, the effects of strong apoptosis inhibitors (overexpression of BCL-2, exposure to peptide caspase antagonists) on clonogenic survival have not been particularly impressive. It is formally possible (and many prominent investigators remain convinced) that induction of permanent growth arrest (senescence) and necrosis are just as important as apoptosis in promoting tumor regression in patients receiving cancer therapy *(1)*.

Our laboratory and others have begun to address this gap in knowledge by measuring apoptosis before and at various times after neoadjuvant or investigational therapy in patients, with the aim of determining whether or not rates of apoptosis are predictive of clinical response. A major challenge presented by these studies is that current methods

for measuring apoptosis require tissue (i.e., biopsies), and in many cases acquiring these tissues may present significant risk to the patient. Furthermore, tumors are thought to be very heterogeneous, so the selection of site(s) for biopsy could have significant effects on the results obtained. Apoptosis is also a very dynamic process, and steady-state measurements of cell death at arbitrarily selected time points might not accurately reflect rates of death within the tumor as a whole.

In spite of these concerns, our recent experience strongly suggests that early, drug-induced increases in apoptosis are critically important for overall clinical response in patients receiving neoadjuvant doxorubicin and/or taxanes for advanced breast cancer *(205,215)*. In this study we obtained 18-gauge core biopsies from 14 breast tumors just prior to and 24, 48, or 72 h after the first dose of chemotherapy. We stained the tumors using a fluorescent TUNEL method and quantified percentages of positive cells using a laser scanning cytometer (LSC). The LSC functions much like a flow cytometer to quantify immunofluorescence intensities at the single-cell level using tissue sections rather than cells in suspension. Compared to manual counting methods, the LSC provides greater sensitivity and objectivity, and using combinations of fluorescent probes one can interrogate events within subpopulations of tumor cells (i.e., epithelial vs endothelial cells). Furthermore, with the LSC we are able to measure percentages of apoptosis in very large numbers of tumor cells (typically over 10,000), providing much greater accuracy. The results of our first study revealed a strongly significant correlation ($p = 0.003$) between chemotherapy-induced apoptosis and the clinical (pathological) responses that were observed 3 mo later, in spite of the relatively small sample size *(215)*. If these preliminary observations can be validated in a larger patient population, they confirm that apoptosis is of central importance to therapeutic outcome.

In other studies we are investigating the role of apoptosis in tumor responses to antiangiogenic agents. The tumor vasculature has become a popular therapeutic target with the appreciation that tumors absolutely depend on angiogenesis for continued growth (see above) and that endothelial cells do not appear to acquire a drug-resistant phenotype following repeated exposure to antiangiogenic agents *(216)*. By first labeling tumor sections with a marker for endothelial cells (i.e., anti-CD31), one can simultaneously determine rates of apoptosis in tumor cells as well as tumor-associated endothelial cells. In preclinical models we showed that a variety of different biology-based investigational approaches (EGFR antagonists, interferons, VEGF receptor antagonists) induce a wave of apoptosis in tumor-associated endothelial cells that then leads to increases in epithelial cell death, presumably as a consequence of hypoxia and nutrient deprivation *(217–219)*. Thus, we have argued that increased endothelial cell apoptosis represents an excellent surrogate for effective antiangiogenic therapy *(220)*. However, endothelial cell death will result in tumor growth inhibition or regression only when it triggers a subsequent wave of tumor cell death. Because susceptibility to hypoxia nutrient deprivation-induced apoptosis is determined by tumor cell properties (p53 status), some tumors will be more sensitive to vascular targeting agents than others.

Based on this preclinical experience we designed a study to determine the effects of the antiangiogenic agent endostatin on parameters of angiogenesis inhibition and apoptosis in biopsies obtained from patients enrolled on a Phase I dose escalation trial *(221,222)*. In this study, excisional biopsies were obtained just prior to initiation of therapy and again after 56 d of treatment. No objective clinical responses were observed

in our trial or the two other trials performed at different institutions. However, LSC-mediated quantification of apoptosis in endothelial (CD31+) cells revealed that endostatin induced significant increases in cell death at intermediate dose levels (180–300 mg/m^2) *(223)*. Furthermore, quantitative analysis of several other markers of angiogenesis inhibition (MVD, endothelial BCL-2 content, nuclear HIF-1α localization) all revealed maximal biological effects within the same dose range *(223)*. Importantly, however, tumor cell death did not increase in parallel, strongly suggesting that the extent of angiogenesis inhibition was not sufficient to produce a meaningful therapeutic effect. We are currently performing similar studies with biopsies obtained from patients enrolled in clinical trials with other antiangiogenic agents, some of which have generated a significant number of clinical responses. Many of these compounds target growth factor receptors, so quantitative analysis of their effects on proximal and distal signal transduction pathways is an integral component of the studies.

The results of the two examples outlined above strongly suggest that meaningful biological information can be obtained from primary patient tumor tissue via the use of sensitive analytical methods. Thus, one of the major challenges for the near future is to directly test some of the conclusions generated in preclinical studies summarized above. For example, there is general consensus that DNA-damaging agents trigger apoptosis via a p53-sensitive pathway. Thus, one prediction from this model is that effective therapy with conventional DNA-damaging agents should be associated with p53 activation and perhaps accumulation of relevant p53 target proteins (BH3-only proteins, Fas, DR5). Similarly, effective inhibition of AKT activation can be readily confirmed using phospho-specific antibodies designed to detect the active form of the kinase. Thus, as PI-3 kinase/AKT inhibitors enter clinical trials in patients, it will be important to confirm that these compounds "hit" their targets, leading to decreased AKT phosphorylation and inhibition of downstream AKT targets (mTOR, NF-κB, and so on). Similar strategies should be used to confirm that inhibitors of the erbB family of receptors (EGFR, HER-2) hit their targets in tumors. Phosphospecific antibodies are commercially available that recognize the active forms of the EGFR and HER-2, and preclinical studies have demonstrated that they can be used to monitor receptor activity in tumor tissues. Therefore, the hypothesis that erbB family members drive AKT activation in some tumors can now be directly tested in primary patient tissues. Finally, as TRAIL and anti-death-receptor antibodies are developed, it will be important to measure the markers of TRAIL susceptibility or resistance identified in the preclinical studies and to monitor markers of TRAIL sensitization and apoptosis in patients treated with TRAIL-based combination therapies. Related to this issue, we are starting a clinical trial with recombinant interferon-α in which we will measure IFN-induced signal transduction (phosphorylation of STAT-1, accumulation of IRF-1), accumulation of TRAIL, and apoptosis in biopsies obtained just prior to and 24–72 h after the initiation of therapy. If these studies are not performed, it is likely that molecular targets will be discarded because inactive compounds failed to produce clinical responses in patients treated with them.

Although biopsy-based approaches for measuring apoptosis and apoptosis-associated molecular mechanisms are currently the most feasible, a major priority for ongoing research is to develop noninvasive strategies to measure these pharmacodynamic processes. There are already good methods available to measure blood flow and glucose metabolism by positron emission tomography or magnetic resonance (MR) imaging

(222), and it appears that the development of new contrast agents will allow for MR-based assessment of apoptosis in real time in the near future *(224)*. For example, it is possible to detect apoptotic cells by staining them with annexin V, which binds to surface-exposed phosphatidylserine on the dying cell, and it is also possible to couple annexin V to contrast-enhancing agents for in vivo imaging of cell death *(225)*. It is anticipated that new contrast agents capable of directly monitoring caspase activation and the signal transduction events that regulate it will be available soon *(226)*. Ultimately, we hope that these methods will be employed to monitor therapeutic effects long before overt tumor regression can be observed and will allow physicians to rapidly tailor therapy to optimize patient benefit.

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