FLIPping the Balance between Apoptosis and Proliferation in Thyroid Cancer

Commentary on Mitsiades et al., p. 3705

Robin Mathew and Eileen White

In an article in this issue of Clinical Cancer Research, Mitsiades et al. (1) suggest that molecular alterations in thyroid cancer can divert a death receptor–mediated apoptotic pathway into a proliferative response, which poses a serious challenge to treating this cancer effectively by receptor-mediated activation of apoptosis. A clearer understanding of the molecular events that regulate apoptosis and the mechanisms by which apoptosis is sometimes altered to impart a proliferative advantage to cancer cells is fundamental in finding a better cure for this deadly disease.

Role of Apoptosis in Tumorigenesis

Tumorigenesis is the manifestation of a delicate balancing act gone awry. Under normal circumstances, tissue homeostasis is a perfectly choreographed process balancing prosurvival and death signals. When shifted in favor of proliferative signals, often as a result of genetic mutations, the imbalance leads to deregulated cell growth that contributes to malignancy. In normal cells, this dangerous possibility is kept under check by coordinate activation of a death response, such as apoptosis, that results in careful elimination of genetically unstable cells (2). Therefore, inactivating mutations in apoptosis pathways often play a causative role in tumorigenesis. Indeed, mutations that confer resistance to one or more of the apoptotic pathways are among the most commonly found alterations in human tumors.

Cell death pathways are often considered a part of a natural tumor-suppressor mechanism working around the clock to maintain tissue homeostasis. In addition to being an effective tumor-suppressor mechanism, apoptosis is also an important determinant of treatment response. Defects in apoptosis contribute to the emergence of treatment resistance in cancer therapy. Inactivation of apoptotic pathways by BCL-2 overexpression, RAS activation, HER2 overexpression, or p53 mutations are characteristic of human tumors that often correlate with worse prognosis (3). Therapeutic restoration of apoptosis in tumor cells or diverting apoptosis-resistant tumor cells to an alternate death mechanism is beginning to be achieved (4, 5).

Promoting apoptosis in tumor cells by activating death receptor–mediated apoptotic pathways or by eliminating survival signaling that inactivates these pathways are strategies waiting to be fully exploited in cancer treatment (6). An increasing number of recombinant ligands or agonist antibodies to death receptors are finding their way into our armamentarium of potential therapeutic targets. Do these proapoptotic strategies finally hold the key to curing cancer? As promising as this may sound, the picture looks much more complex. An increasing body of evidence in several cancer types suggests that molecular alterations can divert a death receptor–mediated apoptotic pathway into a proliferative response, which presents a serious challenge in treating these cancers effectively by receptor-mediated activation of apoptosis (7). Mitsiades et al. (1), in this issue, suggest that thyroid cancer is also following this paradigm. Tailoring cancer treatment to specific cancer genotypes, in which the apoptotic outcome is defined, will be necessary to achieve treatment response.

Major Apoptotic Pathways

Apoptosis, also known as type I programmed cell death, is an expedient way of eliminating the abnormal cells from the genetic pool without eliciting a host immune response. Unlike other modes of cell death, such as necrosis, the cell membrane remains fairly intact during apoptosis and the exposed phosphatidylserines on the cell surface act as signals that facilitate the removal of dead cells by phagocytes, thus preventing an inflammatory response (4). This physiologic suicide has an important role in embryonic development, organogenesis, immune function, tumor suppression, and tissue homeostasis in multicellular organisms.

In general, apoptosis is mediated by the activation of a panel of caspases (cysteine proteases) that are normally expressed as inactive precursors (zymogens) in the cytoplasm. Caspases are classified into two groups: the upstream initiator caspases with long prodomains (such as caspase-9, caspase-2, caspase-8, or caspase-10) and the downstream effector caspases with short prodomains (such as caspase-3, caspase-6, or caspase-7). When initiator caspases are activated by various death stimuli, they activate downstream effector caspases that in turn cleave a specific set of substrates, resulting in cytoplasmic fragmentation leading to cell death (8).

Apoptosis is tightly controlled by internal as well as external signals. Two major pathways control apoptosis in mammalian cells; the intrinsic pathway that is induced through the mitochondrial membrane permeabilization and the extrinsic pathway, also known as the death receptor pathway, that is

Authors' Affiliations: 1Center for Advanced Biotechnology and Medicine; 2Department of Molecular Biology and Biochemistry, Rutgers University; 3University of Medicine and Dentistry of New Jersey, Piscataway, New Jersey; and 4Cancer Institute of New Jersey, New Brunswick, New Jersey

Requests for reprints: Eileen White, Howard Hughes Medical Institute, CABM 140, 679 Hoes Lane, Piscataway, NJ 08854. Phone: 732-235-5329; Fax: 732-235-5795; E-mail: ewhite@cabm.rutgers.edu.

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induced through the specific cell surface receptors (9). A third pathway mediated by cytotoxic T lymphocytes has been identified in which granzyme B activates the mitochondrial apoptotic pathway through BID (10) or directly activates caspase-3 (11).

### The Mitochondrial Pathway of Apoptosis

The intrinsic pathway is triggered by genotoxic stress, such as DNA damage, growth factor deprivation, hypoxia, or oncogene activation, and is regulated by the BCL-2 family of proteins. Once triggered, these signals initiate a cascade of biochemical events that result in the permeabilization of the outer mitochondrial membrane and the release of cytochrome c, along with other proteins that aid in various physiologic and biochemical aspects of apoptosis. The proapoptotic BCL-2 family members, such as multidomain BAX, and BAK or BH3-only proteins, such as BIM, BID, and BAD, play a central role in mitochondrial membrane permeabilization that causes the release of cytochrome c. Although both types of proapoptotic proteins are required to initiate apoptosis, the BH3-only members of the family function upstream of BAX and BAK, making BAX and BAK the core downstream regulators of the apoptotic machinery (12). The antiapoptotic members of the family, such as BCL-2 or BCL-XL, can bind to BH3-only members upstream of BAX and BAK and prevent the release of cytochrome c, thereby blocking apoptosis (13). As these signals converge at the central hub, the mitochondria, this pathway is also known as the mitochondrial pathway of apoptosis.

### The Death Receptor Pathway of Apoptosis

In contrast, the extrinsic, or the death receptor, pathway of apoptosis is activated through the tumor necrosis factor (TNF) family of cytokine receptors. This pathway plays a prominent role in immunosurveillance and cell-mediated cytotoxicity. In the death receptor pathway of apoptosis, extracellular domains of the death receptors receive the external signals from death ligands and through adaptor proteins containing death domains that facilitate protein-protein interaction, they activate death effector caspases that destroy the cell. Death ligands characteristicy activate the receptors on binding by receptor cross-linking and oligomerization, which in turn recruits specialized adaptor proteins that lead to activation of caspase cascades. The prototype death receptors are Fas, also known as CD95 or Apo1, and TNF receptor 1 (TNFR1), and their death domain–containing adapter proteins are Fas-associated death domain and TNFR–associated death domain, respectively. Fas-associated death domain is also recruited to TNFR1 and is involved in TNF signaling, along with TNFR–associated death domain. Death receptor–mediated apoptosis can be induced through the activation of any of the family of death receptors, such as Fas, TNFR1, DR3, TNF-related apoptosis-inducing ligand (TRAIL) receptor 1 (TRAIL-R1; DR4), TRAIL-R2 (KILLER/DR5), NGFR, and EDAR by their respective ligands (14).

### Fas Signaling in Death Receptor Pathway

Fas ligand (Fas-L/CD95L/Apo 1L) is the ligand activator of Fas in TNF superfamily. Fas-L expression is highly tissue specific and often limited to activated immune cells, such as T lymphocytes or natural killer cells. An alternate way in which cells manage to evade the immunosurveillance is by up-regulating their own Fas-L expression. In fact, immunoprivileged organs use their Fas-L expression as a protective shield against immunosurveillance by inflammatory cells (15). Protection from death receptor–mediated apoptosis is implicated in tumorigenesis, as it can not only protect potentially malignant cells from apoptosis, but also can facilitate evasion of immunosurveillance by the tumor-associated immune response. Fas is transcriptionally regulated by p53 and is widely expressed in thymocytes and activated T cells and found abundantly in the liver, heart, and kidney.

Binding of the Fas-L to Fas induces the formation of the death-induced signaling complex (DISC), a complex formed by the cross-linked Fas, Fas-associated death domain, and the zymogen form of caspase-8. At the DISC, pro-caspase-8 oligomerizes and is activated by autocatalysis (16) and functions as the initiator caspase of the pathway. Activated caspase-8 then cleaves caspase-3, resulting in its activation, leading to rapid cell killing of Fas-bearing cells. The receptor-mediated caspase-8 activation itself is sufficient to result in cell killing through the activation of caspase-3 in type I cells. However, in type II cells, where apoptosis is inhibited by direct inhibitors of caspases (IAPs), apoptosis requires a secondary amplification step that involves cross-talk between the mitochondrial and death-receptor apoptotic pathways. In this step, active caspase-8 cleaves the proapoptotic BCL-2 family member BID to generate a truncated form of BID, known as t-BID, that binds to and conformationally activates both BAX and BAK. This structural activation leads to the translocation of BAX to the outer mitochondrial membrane where BAK is already located, causing BAX and BAK oligomerization and release of cytochrome c along with other apoptogenic proteins that are direct inhibitors of IAPs (17). Antiapoptotic BCL-2 family proteins can bind and inactivate BID upstream of BAX and BAK and can prevent apoptosis. Thus, Fas signaling pathway functions to mediate the host innate immune response and induce receptor-mediated apoptosis in virus- and bacteria-infected cells.

### Cellular FLICE Inhibitory Protein Flips the Balance between Apoptosis and Proliferation

Although the Fas-mediated signaling pathway is evolutionarily designed to facilitate immunomediated apoptosis in response to various stimuli, such as inflammation, this is not always the case. Fas cross-linking is also shown to activate proliferative pathways in several cell types. The essential mediator of this transition from death to proliferation is the cellular FLICE-inhibitory protein (c-FLIP), an enzymatically inactive caspase-8 homologue (18). As a result of this homology, c-FLIP exerts dominant-negative effects, inhibiting the activation of caspase-8. Fas-L has been shown to induce nuclear factor-kB (NF-kB) activation and interleukin-8 production without inducing apoptosis (19). Although there are conflicting reports on the role of c-FLIP in NF-kB activation, its role in switching apoptotic signals to proliferative signals has been shown by several studies. Increased level of c-FLIP results in the activation of extracellular signal-regulated kinase (ERK) and NF-kB signaling pathways after T-cell receptor ligation (20). c-FLIP, which itself is an NF-kB target, inhibits the...
Fas-induced NF-κB activation Fas ligation (21). The exact function of c-FLIP may be cell type specific and may depend on various isoforms of c-FLIP (7).

The NF-κB pathway is generally activated in human tumors and interestingly many tumor cells show elevated c-FLIP levels (22). Furthermore, overexpression of c-FLIP in Jurkat cells is correlated with resistance to TRAIL-induced apoptosis (23). As mediators of the switch from cell death to survival, elevated levels of c-FLIP raises the dangerous possibility that tumor cells may respond to the Fas-L on attacking T cells by switching from apoptosis to proliferation.

Overexpression of c-FLIP has been reported in a variety of cancers including melanomas, Hodgkin’s lymphoma, colon cancer, and thyroid cancers (24). Complementary to this, homozygous deletion of caspase-8 is embryonic lethal in mice and its tissue-specific deletion results in resistance to death ligand–induced apoptosis (25). Homozygous inactivation of caspase-8 is shown to result in defective apoptosis and immune homeostasis in humans (26), and caspase-8 inactivation in tumors promotes metastasis (27). When viewed together, these observations show the critical role of the caspase-8 in Fas signaling in immune system function and its modulation by the caspase-8 inhibitor molecule c-FLIP. The importance of this pathway in immunity is further illustrated by the fact that Fas and Fas-L genes undergo spontaneous mutations in mice and show lymphoproliferative and autoimmune syndrome phenotypes (28) and specific inhibition of NF-κB in T cells reverses these phenotype (29). Mutations in Fas and Fas-L genes in humans have been linked to acute lymphoproliferative disease (28). Several human tumors also show overexpression of Fas, including papillary thyroid carcinoma (30).

Thyroid cancer is a human malignancy with a strong immunoregulatory component. Overexpression of Fas and the presence of lymphocytic infiltrates are common and intriguing observations in these cancers. Despite Fas expression, thyroid cancers are not only resistant to Fas-mediated apoptosis but are also hyperproliferative in response to Fas-L-mediated cell signaling. Exactly how this pathway is altered in thyroid cancers is currently not known. Nevertheless, several genetic aberrations can be postulated to explain this abnormality, including the inhibition of caspase-8 by c-FLIP (Fig. 1).

**c-FLIP in Papillary Thyroid Carcinomas**

In the current work by Mitsiades et al., the authors report that Fas cross-linking in papillary thyroid carcinoma cells recruits c-FLIP to the DISC, and this recruitment diverts the Fas signaling pathway to promote proliferation by activating mitogen-activated protein/ERK/extracellular signal regulated kinase (MEK/ERK), activator protein, and NF-κB pathways. They have also found that the balance between procaspase-8 and c-FLIP levels is the key factor regulating this phenotype, as inhibition of c-FLIP expression resensitized thyroid carcinoma cells to Fas-mediated apoptosis. How does c-FLIP switch Fas-mediated apoptotic signaling to proliferation? One possible mechanism could be that c-FLIP inhibits caspase-8 activation and apoptosis and also recruits TRAF1, TRAF2, RIP1, and RAF-1 leading to the activation of extracellular signal-regulated kinase and NF-κB pathways similar to what is proposed for TNF-R1 (20). Fas provides costimulatory activity with T-cell receptor cross-linking by similar mechanisms (7).

**Therapeutic Implications**

How are these observations important in dealing with the inherent abnormalities in cancer cells? First, as the majority of current cancer therapeutics rely on the induction of apoptosis, these observations underscore the importance of specific molecular alterations in predicting treatment response. In tumors where one or more of the apoptotic pathways are inactive, these therapeutic agents are less likely to be effective. Second, they also call into question the efficacy of the death receptor agonists as an effective therapeutic modality in tumors where c-FLIP is overexpressed, as these cells have the potential to diverge from an apoptotic signaling onto other proliferative pathways. Currently, there are several humanized anti-TRAIL-R1 and anti-TRAIL-R2 antibodies undergoing phase I/II clinical trials (31, 32) singly and in combination with other chemotherapeutic agents. For example, the novel anticancer molecule HGS-ETR1 that specifically binds to TRAIL-R1 has been shown to be effective both *in vitro* and *in vivo* in a wide variety of human tumor xenografts (31). As the majority of human cancers express death receptors, targeting these receptors by agonist monoclonal antibodies may be an effective strategy in many tumor types. However, the current finding, along with other reports that death receptor signaling in cancer may be altered by the components of the pathway to impart survival impetus to neoplastic cells, may have a dangerous consequence. As these strategies rely on the activation of the death receptor pathway, in tumors where these pathways are hijacked to alternate
proliferative pathways, the treatment is likely to be counter-
productive. It may be prudent in those cases to use other small-molecule inhibitors of targets such as c-FLIP as an adjuvant to TRAIL and other death receptor–targeting therapeutic agents to enhance their versatility to embrace even those tumors in question. These findings also underscore the necessity to understand specific mutations in tumors so as to tailor a rational tumor therapy for individual tumor genotype. That is where the future of cancer chemotherapy belongs.

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