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The Extrinsic Pathway of Apoptosis

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Summary

Defects in the extrinsic pathway are linked to several disease states, including cancer. Pharmacologic manipulation of the extrinsic pathway holds exciting promise for cancer treatment. This review will discuss the current understanding of the molecular signaling events that originate from extracellular sources to initiate apoptosis, how the pathway is activated by conventional chemotherapeutic agents, and novel opportunities to exploit the extrinsic pathway for cancer treatments.

Key Words: Apoptosis; TNF; Fas; TRAIL; NF-κB; chemotherapy; death receptor; decoy receptor; extrinsic pathway; autoimmunity.

1. OVERVIEW OF SIGNALING EVENTS

1.1. Introduction

Apoptosis is essential to development and maintaining a healthy life for multicellular organisms. It is a rapid, catastrophic process that is precisely regulated in both its initiation and its execution. Although the phenomena had been described for almost a century, in 1972, Kerr, Wyllie, and Currie first coined the term “apoptosis” to differentiate naturally occurring developmental cell death from the necrosis that results from acute tissue injury. They also noted that apoptosis was responsible for maintaining tissue homeostasis by mediating the equilibrium between cell proliferation and cell death in a particular tissue. Morphologic characteristics of apoptosis include cell membrane blebbing, cell shrinkage, chromatin condensation, and DNA fragmentation. Under normal circumstances, cells undergoing apoptosis are recognized by macrophages or neighboring cells that consume the cells’ fractionated carcasses. There are two distinct molecular signaling pathways that lead to apoptotic cell death: (i) the extrinsic, or extracellularly activated, pathway and (ii) the intrinsic, or mitochondria-mediated, pathway. Both pathways activate a cascade of proteolytic enzymes called caspases that mediate the rapid dismantling of cellular organelles and architecture. Caspases are a family of proteins containing a nucleophilic cysteine
residue that participates in the cleavage of aspartic acid-containing motifs (2). There are two groups of caspases, the initiator (or apical) caspases and the effector (or executioner) caspases. Initiator caspases are capable of autocatalytic activation whereas effector caspases need activation by initiating caspase cleavage. Initiator caspases mediate the primary signaling events that result in extrinsic apoptosis activation.

Dr. P. Bruns, a German physician, first hinted at the existence of the extrinsic pathway leading to apoptosis in a paper he published in 1868 (3). He reported that acute bacterial infection caused tumor regression in a subset of his patients. More than a century later, tumor necrosis factor (TNF) and lymphotoxin were isolated and found to be effective at killing cultured tumor cells (4,5). After these factors were cloned (6,7), it became evident they were highly homologous. The advent of automated DNA sequence analysis made possible the identification of previously unknown messenger RNA (mRNA) transcripts and brought with it the discovery of an entire family of homologous TNF proteins (8,9).

The TNF family acts by binding the extracellular domains of receptor proteins: there are 19 ligands in the TNF family that can bind one or more of 29 receptors belonging to the TNF receptor (TNFR) family (8,9). Members of the TNF family are primarily produced as type II transmembrane proteins, arranged in stable homotrimers. The structures of the receptors are diverse, and recent observations from crystallization analyses indicate many similarities, but also many differences, among them (10). For the purpose of this review, we will focus only on the TNFR family members that can activate caspases and elicit death signals and will pay particular attention to signaling in the context of cancer therapy.

Each member of the TNF family binds to one or more receptors in the TNFR family, and some receptors bind one or more ligands. TNFRs contain one to four copies of a conserved cysteine-rich domain that follows a hydrophobic amino terminus and precedes the transmembrane domain. Ligand binding elicits a multitude of responses (including apoptosis, proliferation, and inflammation) and the given response depends upon the adapter proteins the bound receptor recruits. TNFRs signal through two classes of adapter proteins, TNFR-associated factors (TRAFs) and “death domain” (DD)-containing proteins. The subset of TNFRs that can activate apoptosis also possesses the DDs (11). The DD is a conserved stretch of approximately 80 amino acids found to be essential for transducing the apoptotic signal. The death receptors that appear to play important roles in mediating apoptosis are those that bind TNF (TNF-α, TNFSF2), FasL (also known as CD95L, TNFSF6), or TNFR apoptosis-inducing ligand (TRAIL; also known as Apo2L, TNFSF10). There are four other DD-containing TNFR members, but there is little evidence directly coupling these receptors with caspase activation. They are the receptor for nerve growth factor (NGFR), the ectodermal dysplasia receptor (EDAR), DR3, and DR6. This chapter will focus only on the receptors for TNF, FasL, and TRAIL because of their major role in human disease and significant potential for therapeutic interventions.

1.2. Molecular Signaling Events

The death-inducing TNFRs recruit initiating caspases and in doing so can activate a cascade of caspase cleavage that rapidly lead to cell death (Fig. 1). The generalized sequence of events is as follows: after binding ligand, the receptors undergo conformational changes that result in recruitment of an assembly of proteins, termed the
The extrinsic pathway of apoptosis. Soluble tumor necrosis factor (TNF) family ligands TNF receptor (TNFR) apoptosis-inducing ligand (TRAIL), FasL, and TNF form trimers that recognize and bind their cognate death receptors. I) FasL and TRAIL: after binding ligand, DR4, DR5, and Fas undergo conformational changes resulting in assembly of the death-inducing signaling complex (DISC). Decoy receptor 1 (DcR1), DcR2, and DcR3 bind ligand with high affinity but do not induce apoptosis. DR4, DR5, and Fas then recruit Fas-associated death domain (FADD) through complementary death domains (DDs). FADD can recruit caspase 8 through their complementary death-effector domains (DEDs). Recruitment of caspases 8 to the DISC leads to its autoproteolytic cleavage, releasing two subunits that form active enzyme. In type I cells, caspase 8 cleaves and sufficiently activates effector caspases 3, 6, and 7 to fully engage the apoptotic response. In type II cells, activated caspase 8 cleaves Bid, which stimulates Bax and Bak to release factors from the mitochondria, including cytochrome c, thus activating the intrinsic pathway of apoptosis and augmenting active caspase 8. II) TNF: TNF binds TNF-R1 and recruits TNFR-associated DD (TRADD) through its DD and a complex of proteins containing receptor-interacting protein (RIP) and TNFR-associated factor 2 (TRAF2) (Complex I). Complex I can activate inhibitor of nuclear factor (NF)-κB (IKB)–kinase complex, thereby freeing NF-κB for entry into the nucleus and rapid transcription of anti-apoptotic genes, including FLICE inhibitory protein (FLIP) and cIAP1/2. Complex I then dissociates from TNF-R1 where it binds FADD and caspase 8 (Complex II). If in sufficient abundance, FLIP can block Complex II’s caspase 8 from self-activation. Otherwise, complex II triggers a caspase 8-driven apoptotic response.

death-inducing signaling complex (DISC). The DISC was first described in FasL–Fas apoptotic signaling (12). TRAIL binding to its death-inducing receptors acts in a manner similar to FasL, whereas TNF-mediated signaling is more complex and will be discussed in Section 1.3 further detail. The ligand-bound Fas or TRAIL death receptors recruit a DD-containing adapter protein, Fas-associated DD (FADD) (13).
FADD contains a second important death receptor-signaling motif, the death-effector domain (DED), and is the only protein in either the human or the mouse genome that contains both a DD and a DED. Bound FADD recruits initiator caspases 8 and 10 through complimentary DED domains (14,15). Recruitment of caspases 8 and 10 to the DISC leads to their autoproteolytic cleavage and release of two caspase subunits that form a mature active enzyme (16,17). If in sufficient abundance, activated caspase 8/10 cleaves and activates effector caspases 3 and 7, thereby fully engaging the caspase cascade. In some cells, named type I cells, activation of these effector caspases by activated caspase 8/10 alone is sufficient to induce apoptosis (18). In type II cells, activated caspase 8/10 stimulates the release of factors from the mitochondria, including cytochrome c, Smac/DIABLO, and Omi/Htr2A, thereby engaging the intrinsic pathway of apoptosis (see Chap. 1 for detailed review).

Why cells behave in a type I or a type II manner is not well understood. Gene expression analysis comparing type I and type II cells using Fas activation has been performed (19). The expression analysis of type I cell lines showed a preponderance of mesenchymal-like genes, whereas the type II cell lines preferentially express epithelium-like markers. A chemical screen for growth inhibition of these cells revealed that actin-binding compounds selectively inhibited growth of type I cells and tubulin-interacting compounds inhibited growth of type II cells. The functional significance of this observation may become useful in chemotherapeutic treatment selection for cancers with these types of gene expression profiles.

Caspase 8/10 connects the intrinsic and extrinsic pathways by cleaving Bid, a BH3-only member of the Bcl-2 family, which can mediate destabilization of the outer mitochondrial membrane by interacting with other Bcl-2 family members (20,21). To date, Bid is the only known physiologic mediator that connects the extrinsic pathway with release of apoptotic factors from mitochondria. However, a recent report showed the tumor suppressor protein RASSF1A associated with activated death receptors to contribute to Bax activation (22). The mechanism of action proposed is that RASSF1A binds to modulator of apoptosis (MAP)-1, a BH3-like protein, which can associate with Bax resulting in Bax translocation to the mitochondria. RASSF1A or MAP-1 siRNA-mediated knockdown diminished TRAIL-induced apoptosis, but this effect was shown only in the presence of cyclohexamide. RASSF1A could immunoprecipitate with TNF-R1 after a relatively long TNF treatment (2–3 h) while in the presence of cyclohexamide. Therefore, the significance of RASSF1A/MAP-1 modulation of Bax translocation in the context of a delayed death ligand response requiring protein synthesis inhibition is not clear. Nevertheless, it is interesting that a signaling pathway not involving Bid and connecting death receptors to the mitochondria has been discovered.

In humans, the CASPASE 8 gene is found on chromosome 2q33–q34 in tandem with two other highly homologous proteins, CASPASE 10 and FLICE inhibitory protein (FLIP). Caspase 10 contains both a caspase domain and a DED. Caspase 10 is also recruited to the DISC, and whether it can functionally substitute for caspase 8 is controversial (23,24). Studies of the role of FLIP recruitment to the DISC have revealed both activating and inhibitory functions depending on expression level. Although many FLIP isoforms are expressed in cells, only two are present at the protein level, a 55-kDa variant (FLIP_L) and a 26-kDa form (FLIP_S) (25). FLIP_S contains DEDs but lacks the caspase domain and acts as a direct inhibitor of caspase 8 cleavage. FLIP_L contains tandem DEDs but lacks critical residues in its caspase domain including...
the catalytic cysteine, suggesting it to be a classical dominant-negative inhibitor. However, there are reports of FLIPₐ acting as an inducer of caspase 8 autoproteolytic activation \((27\,28)\). These results were recently challenged by a study using siRNA to selectively knockdown each FLIP isoform \((29)\). Separate knockdown of either FLIPₐ or FLIPₜ enhanced DISC formation and caspase 8 activation, suggesting the endogenous role of FLIP as primarily inhibitory.

### 1.3. TNF Pathway

TNF is a pro-inflammatory cytokine produced by a wide range of immune cells, including monocytes, macrophages, T cells, B cells, and natural killer (NK) cells \((8\,9)\). Large amounts of soluble TNF are released in response to lipopolysaccharide (LPS) and other bacterial products. High concentrations of TNF induce septic shock, and prolonged exposure to low concentrations of TNF can result in cachexia, a wasting syndrome. TNF is involved in the progression of many human diseases, including autoimmune diseases (Crohn’s disease, rheumatoid arthritis), neurodegeneration, and cancer \((30)\).

There are two receptors for TNF, TNF-R1 and TNF-R2 \((31\,32)\). TNF-R1 is expressed ubiquitously and has a DD, whereas TNF-R2 has no DD and is found mainly in cells of the immune system and endothelium. TNF-R1 principally regulates the immune system by activating pro-survival signaling. TNF-R1 elicits an anti-apoptotic action by activating nuclear factor (NF)-κB, AP-1, and other transcription factor pathways. This explains why TNF-induced apoptosis using \textit{in vitro} systems often requires the inhibition of RNA or protein synthesis. TNF-binding TNF-R1 does not bind FADD directly to activate caspase 8/10 cleavage, in contrast to Fas and the TRAIL death receptors. Instead, TNF-R1 binds to the DD-containing adapter protein TNFR-associated DD (TRADD) \((33)\). The DD of TRADD binds other DD-containing proteins, including FADD and receptor-interacting protein (RIP). TRADD can also recruit one of two TRAF proteins (TNFR-associated factor), TRAF2 and TRAF5 \((34\,35)\). RIP is essential for TNF-induced NF-KB activation \((36)\). In unstimulated cells, NF-κB is held in the cytoplasm by the inhibitor of NF-κB, IκB. TNF activates NF-κB by initiating ubiquitin-mediated degradation of IκB. Phosphorylation of IκB dissociates it from NF-κB, releasing it for entry into the nucleus and initiating transcription of a large number of mostly anti-apoptotic, pro-survival genes. These include cellular-inhibitors of apoptosis (c-IAP1 and c-IAP2), FLIP, Bfl-1/A1, A20, Mn super oxide dismutase (MnSOD), and others (for a review see \((37)\)). IκB is phosphorylated by the IκB kinase (IKK) complex. IKK activity can be purified as a complex containing two kinase subunits, IKKα (IKK1) and IKKβ (IKK2), and a regulatory subunit, NF-κB essential modifier (NEMO; IKKγ). IKKβ is necessary and sufficient for phosphorylation of IκBα and IκBβ. Studies with TRAF2- and RIP-deficient murine embryo fibroblasts (MEFs) showed that both molecules are independently recruited to TNF-R1 \((38)\). It also appears that TRAF2 is sufficient to recruit the IKK complex to TNF-R1, but RIP is necessary for the activation of the IKKs \((38)\). After phosphorylation by IKKs, IκB proteins are ubiquitinated by members of the Skp1, Cullin, and F box proteins (SCF) family of ubiquitin ligases. The liberated NF-κB dimers translocate to the nucleus where they bind DNA. Activated NF-κB is then down-regulated by multiple pathways, including a negative feedback loop where newly synthesized IκBα binds to nuclear NF-κB and exports it to the cytosol. TNF can also activate other transcription
factors through c-Jun NH$_2$-terminal kinase (JNK) and p38/mitogen-activated protein kinase (MAPK). TRAF2 stimulates JNK through the MAPK kinase MKK7, promoting phosphorylation of c-Jun thereby increasing AP-1 activity.

This pathway of NF-κB activation is referred to as the classical, or canonical, pathway but is one of two major pathways that activate NF-κB. The non-canonical, or alternative, pathway results in the specific activation of two of the five NF-κB subunits, p52 and RelB. The other subunits are p50, RelA, and RelC, and these form heterodimers that are transcriptionally active. Unlike the classical pathway, the alternative pathway is based on IKKα homodimers that prefer the precursor of p52, p100. IKKα binds RelB and sequesters it in the cytoplasm; activation of IKKα results in the degradation of the carboxy-terminus of RelB and nuclear translocation of p52/RelB dimers. The alternative pathway is activated mainly by cytokines involved in development and maintenance of secondary lymphoid organs. Another pathway of NF-κB activation is independent of IKK, and receptor signaling, and is instead based on activation of casein kinase 2 (CK2). CK2 activation can induce IκBα degradation through its phosphorylation. This pathway only has a minor role in physiologic NF-κB activation.

TNF can also activate caspase-mediated apoptosis, but it appears that the NF-κB pathway must be disabled for this to occur. In vivo TNF-induced apoptosis has a minor role in comparison with its overwhelming function in regulating inflammation. TNF-mediated caspase activation occurs when TRADD binds FADD through a DD interaction. Recent evidence shows that FADD only associates with the TNF-R1 complex after it has been internalized by endocytosis. FADD-bound TRADD recruits caspase-8 and self-activates if not inhibited by NF-κB-induced anti-apoptotic proteins. The NF-κB targets, c-FLIP, TRAF1, and c-IAP1, have all been found to co-immunoprecipitate with a TNF-R1/TRADD/FADD cytosolic complex. These anti-apoptotic factors could help to dampen an apoptotic response. Therefore, apoptotic signaling through TNF-R1 includes an NF-κB-mediated rescue response that results in cell death if newly synthesized survival signals fail to be activated.

1.4. Fas Ligand

Fas plays a major role in the regulation of apoptosis of immune cells and has been implicated in immune system diseases and cancer. Fas–FasL interactions are important for regulating the immune system in several ways: Fas is involved in cytotoxic T-cell-mediated killing, destruction of inflammatory and immune cells in immune-privileged sites, and deletion of self-reacting B cells and activated T cells at the end of an immune response. Dysregulation of Fas or FasL expression is associated with several disease states. Elevated serum levels of FasL have been seen in patients with NK-cell large granular lymphocyte leukemia, systemic lupus erythematosus, rheumatoid arthritis, Sjogren’s syndrome, lymphohistiocytosis, myocarditis, and acute graft-versus-host disease. Some tumors have been reported to express FasL, which may be a mechanism they developed to evade attacking lymphocytes.

Fas-mediated cell death was identified indirectly by the generation of monoclonal antibodies that recognized cell surface antigens on a malignant human lymphoblast cell line. Once cloned, Fas was found to map to the chromosomal location of a mouse lymphoproliferative disorder known as lpr. A point mutation near the extracellular carboxyl domain of FasL gives rise to the gld phenotype. Both lpr and gld mice fail
to delete excess lymphocytes and display a lymphoproliferative phenotype including lymphadenopathy and splenomegaly. One other receptor, decoy receptor 3 (DcR3), binds to FasL. DcR3 lacks an apparent transmembrane sequence and appears to be secreted. DcR3 was found genetically amplified in several human cancers including lung and colon carcinomas and is overexpressed in several adenocarcinomas, glioma cell lines, and glioblastomas (51–53). There is preliminary data suggesting that serum DcR3 level might be a useful predictive marker for cancer diagnosis (54).

1.5. TRAIL

TRAIL was identified in silico using TNF sequence homology searches of the human genome database of expressed sequence tags (55,56). TRAIL is unique among the TNF superfamily, and most other cytokines, because it can bind five different receptors (57,58). Two of these receptors contain DD and are pro-apoptotic. They are type 1 transmembrane receptors and referred to herein as DR4 (death receptor 4/TRAIL R1) and DR5 (death receptor 5/KILLER/TRICK2/TRAIL R2). The three other TRAIL receptors lack DDs, including one that binds TRAIL very weakly at physiologic temperatures (osteoprotegerin/OPG). The remaining two receptors are also called “decoys” because they bind TRAIL with high affinity but cannot transduce the death signal. Decoy receptor 1 (DcR1/TRID/TRAIL-R3) lacks an intracellular domain (it is attached to the plasma membrane by a glycoprophatidylinositol anchor), and decoy receptor 2 (DcR2/TRUNDD/TRAIL R4) has a truncated DD in its cytoplasmic tail.

If bound to either DR4 or DR5, TRAIL and FasL exert their actions in an analogous manner. Binding of TRAIL triggers DISC formation, caspase 8/10 activation, and rapid apoptosis in sensitive cells. Similarly to Fas, apoptosis triggered by TRAIL can engage the mitochondrial pathway in type II cells or independently of the mitochondria in type I cells (59). The apoptotic signaling pathway downstream of FasL and TRAIL acts through FADD and appears to be very similar. However, one significant difference between TRAIL and FasL is their potential as chemotherapeutic agents. When administered systemically in mice, FasL induces a rapid cytotoxic effect in hepatocytes whereas TRAIL appears relatively non-toxic (60,61). This observation, plus TRAIL’s dramatic ability to kill cancer cells while leaving normal cells unharmed, opened up an exciting new opportunity for development of a “silver bullet” for cancer therapy. TRAIL and DR4- and DR5-activating antibodies recently have entered into clinical trails.

Several mechanisms have been proposed to explain why some cancer cells are highly sensitive to TRAIL-induced death. An attractive hypothesis is differential expression of the decoy and death receptors. However, the decoy receptors do not consistently appear highly expressed in normal cells or to be absent in cancer cells (62–65). Mutant death receptors or defective receptor processing has been observed in TRAIL-resistant cancer cells (64–65). Additional intracellular factors leading to TRAIL resistance affect the caspase 8/c-FLIP ratio, such as loss of caspase 8 and caspase 10 because of mutations or gene methylation (66), caspase-associated ring protein (CARP)-dependent degradation of caspase 8 (67), or high c-FLIP expression levels (62,63). Up-regulation of FLIP was detected in many tumors (68–70), and expression of FLIP in transgenic mice results in escape from T-cell immune surveillance and subsequent tumor growth (71,72). Further downstream in the TRAIL apoptotic pathway, Bax mutations or increased expression of IAP family members, in particular XIAP and survivin, can also cause resistance.
Recently, the human oncogene c-Myc was identified as a bio-marker for TRAIL sensitivity \(^{73}\). In this study, a panel of human tumor cell lines was examined, and a direct linear correlation was observed between TRAIL sensitivity and high c-Myc expression. Myc was found to bind the FLIP promoter and repress FLIP transcription \(^{73}\). Furthermore, Myc was isolated from an siRNA screen to identify modulators of TRAIL sensitivity \(^{74}\). A separate study showed Myc up-regulation of DR5 expression \(^{73}\), but how Myc regulates DR5 is not clear. Other oncogenic proteins can sensitize cells to TRAIL, including E1A \(^{76}\) and oncogenic Ras \(^{77,78}\), but Ras-mediated TRAIL sensitization has not been observed consistently \(^{73}\).

2. ROLE OF THE EXTRINSIC PATHWAY IN DISEASE

The TNF family plays important functions in innate and adaptive immunity and directly activates pathways leading to cell survival, proliferation, differentiation, and death. Dysregulation of the TNF family members that can elicit apoptosis results in diseases of the immune system, neurodegenerative disorders, and cancer. Two known genetic diseases that are associated with defects in the extrinsic pathway include Fas-linked autoimmune lymphoproliferative syndrome (ALPS) and TNF-R1-associated periodic syndrome (TRAPS) \(^{8}\). Fas-mediated apoptosis is required for normal lymphocyte homeostasis and peripheral immune tolerance \(^{79}\). In Fas-deficient lpr mice and in patients with heterozygous dominant-interfering defective Fas alleles (encoding defective Fas proteins that complex with normal Fas), abnormal accumulation of lymphocytes often results in systemic autoimmunity \(^{79,80}\). Afflicted individuals develop pathogenic autoantibodies—frequently against hematopoietic cells—that cause hemolytic anemia, thrombocytopenia, or neutropenia \(^{81}\). Mouse knockout studies show the TRAIL pathway may also be involved in autoimmune disease \(^{82}\). In TRAPS, heterozygous dominant alleles of defective TNF-R1 appear to enhance the pro-inflammatory effects of TNF. This may be due in part to a decrease in TNF-R1 shedding \(^{83}\).

TNF and TNFR families are being targeted for therapies against a wide range of human diseases such as atherosclerosis, osteoporosis, autoimmune disorders, allograft rejection, and cancer. For example, pharmaceuticals to inhibit TNF have been developed to control previously recalcitrant inflammatory conditions such as rheumatoid arthritis and inflammatory bowel disease \(^{84,85}\). Specific TNF antagonists include the TNF antibody infliximab (Remicade) and the TNFR–immunoglobulin G (IgG) fusion protein etanercept (Enbrel) \(^{86}\).

What are the physiological functions of apoptosis-inducing TNF family members? The use of inhibitory antibodies and mouse knockout models has provided great insight into this question (Table 1).

2.1. Lessons from Knockout Animals

2.1.1. TNF AS A TUMOR PROMOTER

Mouse TNF and TNF-R1 knockout studies show that TNF plays an essential role in protecting against infection by pathogenic organisms. There is also growing evidence that TNF signaling is involved in fostering tumor growth. Expression studies show abnormally high concentrations of TNF in tumors. Studies of various hematopoietic and solid tumor types found an association between TNF expression, poor survival, and
Table 1

Major extrinsic pathway proteins and their official names according to the HUGO Gene Nomenclature Committee (HGNC) are shown. Knock-out mice generated for these proteins are described.

<table>
<thead>
<tr>
<th>Molecular pathway molecules</th>
<th>Official nomenclature (HGNC)</th>
<th>Human chromosome</th>
<th>Mouse knockout phenotype</th>
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<tbody>
<tr>
<td>Death ligand</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF (TNF-α, Cachectin)</td>
<td>TNFSF2</td>
<td>6p21.3</td>
<td>Viable, highly susceptible to challenge with an infectious agent and resistant to lipopolysaccharide (LPS)-induced death following D-galactosamine treatment (188)</td>
</tr>
<tr>
<td>FasL (CD95)</td>
<td>TNFSF6</td>
<td>1q23</td>
<td>Viable but early death (50% at 4 months); FasL−/− mice exhibit splenomegaly and lymphadenopathy associated with lymphocytic infiltration into multiple organs and autoimmune disease (189); Gld mice carry mutations in FasL and suffer from autoimmune disease (50)</td>
</tr>
<tr>
<td>TRAIL (Apo2L)</td>
<td>TNFSF10</td>
<td>3q26</td>
<td>Viable; susceptible to induced and spontaneous tumorigenesis (57)</td>
</tr>
<tr>
<td>Death receptor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-R1</td>
<td>TNFRSF1A</td>
<td>12p13.2</td>
<td>Viable, resistant to LPS-induced death following D-galactosamine (190)</td>
</tr>
<tr>
<td>Fas (CD95, Apo1)</td>
<td>TNFRSF6</td>
<td>10q24.1</td>
<td>No Fas−/− mouse published; mice carrying the lymphoproliferation (lpr) mutation have defects in the Fas antigen gene. The lpr mice develop lymphadenopathy and suffer from a systemic lupus erythematosus-like autoimmune disease, indicating an important role for Fas antigen in the negative selection of autoreactive T cells in the thymus (191)</td>
</tr>
<tr>
<td>DR4 (TRAILR1, APO2)</td>
<td>TNFRSF10A</td>
<td>8p21</td>
<td>Viable, but has an enlarged thymus, defective apoptotic response to ionizing radiation (88)</td>
</tr>
<tr>
<td>DR5 (TRAILR2, KILLER, TRICK2)</td>
<td>TNFRSF10B</td>
<td>8p22–p21</td>
<td></td>
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<tr>
<td>Adapter proteins</td>
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<td></td>
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<tr>
<td>FADD (MORT)</td>
<td>FADD</td>
<td>11q13.3</td>
<td>Embryonic lethal (d11.5); mice show signs of cardiac failure and abdominal hemorrhage (84)</td>
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(Continued)
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<tr>
<th>Molecular pathway molecules</th>
<th>Official nomenclature (HGNC)</th>
<th>Human chromosome</th>
<th>Mouse knockout phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRADD</td>
<td>TRADD</td>
<td>16q22</td>
<td>Embryonic lethal (d13.5); embryos exhibit impaired heart muscle development and accumulation of erythrocytes [90]</td>
</tr>
<tr>
<td>Caspase 8 (FLICE, MACH, MCH5)</td>
<td>CASP8</td>
<td>2q33–q34</td>
<td></td>
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<tr>
<td>Caspase 10 (MCH4)</td>
<td>CASP10</td>
<td>2q33–q34</td>
<td>Embryonic lethal (d10.5); exhibit impaired heart development [192]</td>
</tr>
<tr>
<td>FLIP (1-FLICE, CASPER, FLAME1, CASH, CLARP, MRIT)</td>
<td>CFLAR</td>
<td>2q33-q34</td>
<td></td>
</tr>
<tr>
<td>RIP (RIP1)</td>
<td>RIPK1</td>
<td>6p25.2</td>
<td>Viable, but fails to thrive; displays extensive apoptosis in both the lymphoid and adipose tissues and dies at 1–3 days of age [193]</td>
</tr>
<tr>
<td>TRAF2 (TRAP)</td>
<td>TRAF2</td>
<td>9q34</td>
<td>Traf2−/− mice appear normal at birth but die prematurely; atrophy of the thymus and spleen and depletion of B-cell precursors are observed [35]</td>
</tr>
<tr>
<td>TRAF5</td>
<td>TRAF5</td>
<td>1q32</td>
<td>CD27- and CD40-mediated lymphocyte activation is substantially impaired in traf5−/− lymphocytes [194]</td>
</tr>
</tbody>
</table>

resistance to therapy [91–94]. TNFR1−/− mice show reduced levels of metastatic lung disease following intravenous tumor cell injection [95] and reduced liver metastases following intrasplenic tumor cell injection [96]. TNF itself has been proposed as a tumor promoter. Using a standard mouse model of two-step chemical carcinogenesis, it was found that TNF−/− mice developed 10 times fewer skin tumors than wild-type mice [97]. Similar results indicating a role for TNF in tumor promotion were seen by using a model of hepatic carcinogenesis in TNF-R1−/− mice and was unaffected in TNF-R2−/− mice [98].

Recent work by Karin and colleagues showed that TNF mediates an inflammatory response by LPS, causing metastatic growth to the lung of intravenously injected colon adenocarcinoma cells [99]. Inhibiting NF-κB signaling in the colon cells prevented their metastasis and resulted in their apoptosis. TRAIL expression by mouse lung tissue following LPS administration mediated the death of NF-κB-deficient cancer cells. DR5 was also found up-regulated in tumors following LPS treatment but only in cells that were NF-κB deficient. These results shed light on the interplay between TNF and...
TRAIL signaling and show that if NF-κB is blocked, TNF-mediated growth can be converted into TRAIL-mediated death.

2.1.2. FasL and Negative Selection

Higher organisms have developed several mechanisms to eliminate unwanted cells rapidly, and Fas is an important mediator of this process. FasL expression at sites of immune privilege identified an important role for FasL in the interaction between non-lymphoid tissues and the immune system. The concept of “tumor counterattack” has been proposed to explain the observation that Fas is down-regulated and FasL up-regulated in the same tumor cells (100). According to this concept, “immune cells are unable to destroy tumor cells since they are attacked by the FasL expressing cells.” In light of this observation, significant evidence has accumulated that indicates Fas may play a major role in immune suppression of tumorigenesis. FasL expression has been reported on numerous tumors of varying origin, including colon, gastric, lung, and astrocytoma (47). Disease progression is associated with increasing levels of FasL (101, 102), and FasL expression has also been found higher in some metastatic tumors than in primary ones (103, 104). Animal studies have corroborated the FasL counterattack theory. For example, subcutaneous injection of FasL-expressing murine melanoma cells into Fas-deficient lpr mutant mice resulted in delayed tumor growth compared with that in wild-type mice (105). However, conflicting results cast doubt on the Fas counterattack theory, instead suggesting that FasL can also play a growth stimulatory role (47, 106). These observations, plus those suggesting that FasL may have stimulatory effects, including induced motility of tumor cells (107) and growth signaling (108), suggest that the initial theory of tumor counterattack may be oversimplified and that FasL does more than trigger apoptosis.

2.1.3. TRAIL and Tumor Surveillance

Recent evidence from mouse knockout studies, plus TRAIL’s ability to activate apoptosis in various cancer cells, led to the hypothesis that a principal function of TRAIL is to kill transformed cells. Unlike FasL, TRAIL is not expressed at detectable levels on the surfaces of T cells, NKT cells, B cells, dendritic cells, monocytes, or most NK cells (109). The one exception is mouse liver NK cells, which constitutively express TRAIL in an interferon (IFN)-dependent manner (110, 111). Cell surface TRAIL expression increases through several pathways. Antigen-dependent activation of CD4+ and CD8+ T cells from peripheral blood induces expression of TRAIL mRNA, as does stimulation of macrophages with IFN-γ. The expression of TRAIL on liver NK cells and their anti-metastatic potential depend on the presence of IFN-γ and interleukin (IL)-12, as these effects were not observed in mice deficient in IFN-γ (87, 111). Tumor cells from cancer patients activate macrophages to produce TRAIL, and these macrophages can release molecules that induce increased expression of DR4 and DR5 on tumor cells (112).

Studies with TRAIL knockout mice confirm a role for TRAIL in anti-tumor immune surveillance by NK cells, specifically in host defense against tumor initiation and metastasis (87, 111). TRAIL−/− mice were more susceptible to experimental and spontaneous tumor metastasis and were also more likely to form tumors following exposure to the chemical carcinogen methylcholanthrene (87). There are two knockout mice for the receptor for TRAIL. Mice have only one receptor for TRAIL, mouse KILLER,
and it shares its highest homology with human DR5 \( (113) \). Two knockout models for the TRAIL receptor have been generated and both are viable \( (88,114) \). Both develop normally, but one mouse strain has an enlarged thymus \( (88) \). This study showed that DR5 has a limited role during embryogenesis and early stages of development, but plays an organ-specific role in response to DNA-damage. When exposed to ionizing radiation, certain DR5\(^{-/-}\) tissues show reduced apoptosis, including the thymus and spleen. Mice wild-type for DR5 show a selective increase in DR5 expression following IR in the thymus and spleen \( (115) \), suggesting a significant connection between IR-induced DR5 and resulting apoptosis in these tissues.

Genetic defects in TRAIL signaling have not been strongly correlated with disease states in humans. However, deletions and mutations of DR4 and DR5 have been observed in some human tumors \( (63,65,116–118) \).

An intriguing study has implicated TRAIL in mammary tissue lumen formation and suggests that dysregulation of TRAIL signaling may be a hallmark of early breast cancer lesions \( (119) \). Using an in vitro cell culture model of 3D acinar-like structures using immortalized mammary epithelial cells, TRAIL was found to partially mediate both the apoptotic and the autophagic cell death associated with lumen formation. Autophagy is a cellular process where a multi-membrane vacuole containing cytoplasmic contents fuses with the lysosome. This results in degradation of the vacuole contents by lysosomal enzymes into recyclable macromolecules \( (120) \). Recent evidence indicates that autophagy can mediate a form of programmed cell death, where there is an accumulation of vacuoles resulting in massive organelle degradation. How TRAIL is activated during mammary acinar morphogenesis is not known, but this is the first report connecting autophagy with TRAIL function \( (119) \). Little is known about how and to what degree the extrinsic pathway signaling affects autophagy, but there are reports that TNF signaling can induce autophagy and possibly contribute to TNF-induced apoptosis \( (121,122) \).

3. ROLE OF EXTRINSIC PATHWAY IN CHEMOTHERAPY AND RADIOSENSITIVITY

Chemotherapy and radiation, when used successfully, act to inhibit tumor growth. Ionizing radiation and DNA-damaging chemotherapeutics can elicit an apoptotic response that is principally mediated through activation of the p53 tumor suppressor protein. p53 is the most commonly mutated protein found in human cancers and is a potent transcriptional activator of genes that play principal roles in cell-cycle arrest and apoptosis \( (123) \). Recent evidence suggests that p53 also influences apoptosis by directly interacting with members of the Bcl-2 family \( (124) \). Members of the Bcl-2 family that p53 can activate transcriptionally include Bax, Puma, Noxa, Bnip3L, Bak, and Bid. p53 also directly contributes to activation of the extrinsic pathway. Death receptors for both TRAIL and FasL have been identified as p53 target genes \( (113,125) \).

**KILLER/DR5** was originally discovered as a DNA-damage-inducible p53 target gene \( (113) \) and is transcriptionally activated by p53 \( (126) \). Certain tissues, including the spleen, small intestine, and thymus, show large increases in DR5 expression following ionizing radiation that is dependent on transcriptionally active p53 \( (88,113,127) \). DR4 may also be regulated by p53 in a limited number of cell lines \( (128) \). Several studies have found a p53-dependent increase in Fas or FasL, which contributes to
mediating the apoptotic response after conventional chemotherapy \(125,129,131\), but Fas is not essential for mediating p53’s effects. Lymphocytes from \(lpr\) mice, or those expressing DN-FADD, are equally sensitive to chemotherapy and ionizing radiation; p53 deficiency or constitutive expression of Bcl-2 markedly increased the resistance of lymphocytes to gamma radiation or anticancer drugs, but lymphocytes were still sensitive to killing by FasL \(132\). Furthermore, apoptosis induced by chemotherapeutic drugs is not altered in embryonic fibroblasts from FADD and caspase 8 knockout mice \(89,90\), indicating only a partial role for the death receptor pathway in response to chemotherapeutic agents. Nevertheless, partial resistance of DR5-null tissues to ionizing radiation implicates the extrinsic pathway in DNA-damage-induced apoptosis.

4. EXPLOITING THE EXTRINSIC PATHWAY FOR CHEMOTHERAPY-INDUCED KILLING

Ever since the discovery of TNF, great attention has been focused on the TNF ligands as mediators of cancer cell death \(133,135\). Through the efforts of many scientists over the course of decades, clear pictures are emerging of the basic mechanisms of extrinsic pathway-signaling events. Understanding these events has led to exciting advances in using extrinsic pathway signaling for cancer therapy.

4.1. TRAIL

Despite the ability of TNF and FasL to induce apoptosis in cancer cells, severe toxic side effects preclude both ligands from use in systemic anticancer therapy. Systemic administration of TNF caused an inflammatory response resembling septic shock in humans \(136\). FasL or agonistic anti-Fas antibody caused lethal liver injuries in preclinical models \(137\). By contrast, recombinant human TRAIL showed no toxicity when systemically administered in rodents and non-human primates \(60,61,138\). Recombinant human TRAIL has apoptosis-inducing capacity in various tumor cells in culture and in tumor implants in severe combined immune deficiency (SCID) mice \(134\). Recombinant TRAIL (Genentech/Amgen) and activating DR4 and DR5 antibodies (Human Genome Sciences/Cambridge Antibody Technology) are currently under clinical investigation. Getting TRAIL into clinic trials was delayed by observations that certain preparations of recombinant human TRAIL had selective toxicity toward normal human hepatocytes \(140\). Because TRAIL was toxic to cultured human hepatocytes, and not to mouse or non-human primates, it resulted in the careful analysis of different TRAIL preparations. It became apparent that TRAIL protein fused with non-physiological amino acid tags or with preparations of native TRAIL using different stabilizing chemicals resulted in multimerized, highly potent versions of TRAIL \(142,143\). Therefore, the potential toxicity of these TRAIL versions toward normal cells can be avoided if native TRAIL is properly prepared, or if activating monoclonal antibodies specific to DR4 or DR5 are used \(139,144,145\). Another approach to minimizing off-target TRAIL toxicity is the combination of TRAIL with an inhibitor of caspase 9, which can protect normal cells but is ineffective in protection tumor cells possessing a type I signaling mechanism \(143,146\).

Like most normal cells, many cancer cells are resistant to TRAIL-induced apoptosis. However, many conventional and novel chemotherapies can act synergistically when combined with TRAIL. Chemotherapy or irradiation sensitized resistant cells to TRAIL.
in vitro and in vivo (63,147–150). Many cytotoxic chemotherapeutic agents result in DNA damage or other cellular stress that causes stabilization of the p53 tumor suppressor protein. p53 transcriptionally activates DR5 and other pro-apoptotic proteins that enhance the TRAIL signal (113). Therefore, combining TRAIL with such agents should prove to be a useful therapeutic strategy in tumors harboring functional p53. However, tumor progression and resistance to chemotherapies occur because tumors select for cells defective in p53 signaling. An exciting facet of death receptor signaling is that it can occur in the absence of functional p53. Inhibitors of histone deacetylases (HDACIs) can induce apoptosis in cancer cells and are currently in clinical trials. One action of HDACIs is the increased expression of TRAIL, DR5, Fas, and FasL in leukemic cells, resulting in selective apoptosis of these cells (151,152). HDACIs enhance synthesis of several proteins involved in TRAIL signaling including DR5 and when combined with TRAIL show the ability to sensitize TRAIL-resistant cells (153,154). Both glucocorticoids and IFN-γ also increase DR5 expression, which may enhance TRAIL activity (155). These are some of the many strategies being approached to combine TRAIL with novel agents that target proteins in both the extrinsic and intrinsic pathways, thereby increasing their sensitivity to the killing potential of TRAIL. Promising compounds identified that have been combined with TRAIL are discussed below.

4.2. TNF

Recombinant TNF was approved for isolated limb perfusion therapy against sarcomas in Europe in 1998 (156). TNF combined with chemotherapeutic agents such as melphalan shows specificity toward destruction of tumor vasculature and is very effective when used for localized treatment of sarcomas and melanomas (157,158). TNF plus melphalan is awaiting approval following phase III clinical trials for use in the US.

4.3. NF-κB

Substantial evidence indicates that NF-κB regulates oncogenesis and tumor progression. Many anticancer agents induce NF-κB nuclear translocation and activation of their target genes, which impinge on cellular resistance to anticancer agents. TNF is up-regulated by some chemotherapeutic agents, thus activating NF-κB. FasL and TRAIL also can activate NF-κB signaling (107,159). Several strategies have been investigated to block pro-survival death receptor signals so that extrinsic apoptotic signals can dominate.

NF-κB inhibitors have been identified that enhance the cytotoxic effects of many conventional chemotherapies and novel anticancer agents (160). Inhibiting the proteosome is an approach taken to block NF-κB activation through degradation of IkB proteins. The proteosome inhibitor Bortezomib (Velcade; PS-341) has recently been approved for treating multiple myeloma and is in clinical trials testing effectiveness against several other cancer types (161,162). Bortezomib also has been effectively combined with many conventional chemotherapeutic agents and radiation (for a recent review see 163). Proteosome inhibition results in stabilization of several critical regulators of apoptosis, including p53, Bid, and Bax; therefore, the effectiveness of Bortezomib may depend on partially on inhibiting NF-κB.
Strategies have been developed to inhibit NF-κB directly. Two compounds were identified, BAY 11-7082 and BAY 11-7085, that block IκBα phosphorylation and prevent its degradation (164). Bay 11-7082 was used to enhance mitochondria dysfunction induced by UCN-01, a cell-cycle checkpoint-abrogating agent (165). A recent study showed that rituximab (Rituxin), the anti-CD20 antibody approved for treatment of non-Hodgkin’s lymphoma, can inhibit IKK activity and block constitutive NF-κB signaling (166). Non-steroidal anti-inflammatory drugs (NSAIDs), including Cox-2 inhibitors, have been identified as inducing regression of adenomatous polyps of the colon, and NF-κB has been implicated in mediating NSAID action. Aspirin and sulindac have both been shown to inhibit IKK activity and may prove useful in targeting NF-κB (167,168). Many other IKK inhibitors have been identified, but further studies are necessary to determine whether they will be clinically useful (160,169).

4.4. FLIP

Whether a tumor cell is sensitive to death ligand-induced apoptosis depends on both receptor cell surface expression and an intact downstream-signaling pathway. FLIP is an important regulator of the death signal, and a compound was recently discovered that reduces FLIP expression. The synthetic oleanane triterpenoid 2-cyano-3, 12-dioxooleana-1,9-dien-28-oic acid (CDDO) was reported to have potent differentiating, anti-proliferative, and anti-inflammatory properties and reduce tumor growth in vivo (170,171). CDDO was initially constructed to mimic naturally occurring inhibitors of nitric oxide production induced by IFN-γ. The mechanism of action of CDDO and its imidizol derivative (CDDO-Im) are not fully understood, but CDDO was found to induce apoptosis involving caspase 8 cleavage (172). Later, it was observed that CDDO activates a pathway resulting in FLIP degradation (173,174). CDDO or CDDO-Im can cause apoptosis and cell death in a number of different human cancers, but it has shown potent synergy when used in combination with TNF or TRAIL (175–177). In vivo studies using nude mice bearing human breast cancer MDA-MB-435 xenografts showed CDDO-induced tumor growth arrest by using daily treatments for 25 days (178). A later study did not recapitulate tumor growth arrest using CDDO-Im in nude mice bearing MDA-MB-468 breast cancer xenografts but restricted tumor growth when combined with TRAIL (179).

4.5. Recruiting the Intrinsic Pathway to Sensitize Cells to the Extrinsic Pathway

Because the extrinsic pathway is linked to intrinsic apoptotic signaling, combining extrinsic and intrinsic pathway activators should elicit a “double whammy.” For example, TRAIL has been combined with several agents identified or specifically designed to target intrinsic signaling.

The Bcl-2 family is the major mediator of outer mitochondrial membrane depermeabilization resulting in the release of pro-apoptotic factors, such as cytochrome c, Smac/DIABLO, and Omi/Htr2A (180). Overexpression of anti-apoptotic Bcl-2 family members, such as Bcl-2, Bcl-XL, A1, or Mcl-1, is frequently observed in many tumor types and contributes to chemotherapeutic resistance. Several strategies are under investigation to target these anti-apoptotic proteins. These include (i) interfering oligonucleotides to down-regulate expression; (ii) use of BH3-only peptides or controlled Bax expression
to abrogate protection; and (iii) small molecules that can inhibit protective interactions. The only agent of these categories that is currently in clinical trials are nuclease-resistant antisense oligonucleotides targeting Bcl-2 mRNA (G3139). G3139 (Genasense) is in phase II and III clinical trials treating a wide variety of adult and childhood tumors\(^{[181]}\). However, G3139 was not approved for treatment of melanoma because results from phase III trials showed it did not extend survival\(^{[182]}\). There are no reports investigating G3139 in combination with TRAIL pre-clinically, but it was shown to sensitize Fas- and IFN-γ-resistant renal cancer cell line to IFN-γ combined with an Fas-activating antibody\(^{[183]}\). Adenoviruses that express Bax and TRAIL under control of the promoter for the human telomerase protein subunit (hTERT) were used to treat nude mice bearing ovarian tumor xenografts\(^{[184]}\). Though preliminary, these strategies (or agents) diminished tumor growth while maintaining relatively low toxicity.

Anti-apoptotic Bcl-2 family members are held in check by Bcl-2 family proteins that contain only the BH3 member of the four Bcl-2 homology domains, the so-called BH3-only proteins. A novel approach was taken to generate stabilized BH3 peptides termed SAHBs (stabilized α-helix of Bcl-2 domains)\(^{[185]}\). These peptides proved to be protease-resistant and cell-permeable molecules that bound with high affinity to multidomain BCL-2 member pockets. A SAHB of the BH3 domain from BID was effective in inhibiting growth of human leukemia xenografts in vivo in short-term assays. A small molecule BH3 mimetic, ABT-737, shows promise in the treatment of Bcl-2- or Bcl-XL-overexpressing tumors\(^{[186]}\). ABT-737 was identified using a structure-based combinatorial chemical approach to target Bcl-XL and binds Bcl-XL, Bcl-2, and Bcl-w with high affinity\(^{[186]}\). ABT-737 synergized with paclitaxel and the activated BH3-only protein Myr-Bid to cause apoptotic cell death. Because overexpression of Bcl-2 and Bcl-XL is the key to many cancers’ resistance to apoptotic stimuli, Bid SAHBs or ABT-737 will very likely synergize with other chemotherapeutic agents, including TRAIL and other extrinsic pathway activators. Whether Mcl-1 overexpression will mediate resistance of tumors remains to be determined in clinical trials.

Other classes of apoptotic targeting agents have also been combined with TRAIL resulting in significant tumor regression. A small molecule SMAC mimic potentiates TNF- and TRAIL-induced death\(^{[187]}\). Treatment of glioblastoma cells with a combination of TRAIL and the SMAC mimic resulted in apoptosis of tumor cells, but normal cells were not harmed. Data from animal studies have not been published yet.

5. CONCLUDING REMARKS

Despite being investigated for decades, the TNF and the TNFR family of proteins continue to provide important insights into human health and disease. Here, we focused on the current understanding of their role in activating the extrinsic pathway of apoptosis, how this affects oncogenesis, and how this knowledge can be used for targeted chemotherapeutic design. Several promising cancer-killing agents that engage the extrinsic signaling pathway are in clinical trials, and several more appear promising in preclinical studies. The next stage of clinical research must include rational combination of chemotherapeutic agents that both activate apoptotic signaling pathways and block pro-survival mechanisms, while minimizing off-target toxicities. A major challenge to be overcome is determining whether a patient will respond to agents that activate the extrinsic or intrinsic pathways of apoptosis prior to their treatment.
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