Apoptosis control by death and decoy receptors

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The death receptors Fas and tumor necrosis factor receptor 1 (TNFR1) trigger apoptosis upon engagement by their cognate death ligands. Recently, researchers have discovered several novel homologues of Fas and TNFR1: DR 3, 4, 5, and 6 function as death receptors that signal apoptosis, whereas DcR 1, 2, and 3 act as decoys that compete with specific death receptors for ligand binding. Further, mouse gene knockout studies have enabled researchers to delineate some of the signaling pathways that connect death receptors to the cell's apoptotic machinery.

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Abbreviations

CAD	caspase-activated DNase
CRD	cysteine-rich domain
DcR	decoy receptor
DR	death receptor
EST	expressed sequence tag
FADD	Fas associated death domain
FasL	Fas ligand
ICAD	inhibitor of CAD
JNK	Jun amino-terminal kinase
OPG	osteoprotegerin
NF	nuclear factor
RAG	recombinase-activating gene
RIP	receptor interacting protein
TNF	tumor necrosis factor
TNFR	TNF receptor
TRADD	TNFR associated death domain
TRAIL	TNF-related apoptosis-inducing ligand

Introduction

Apoptosis is a cell-suicide mechanism that plays a crucial role in development and homeostasis of metazoans (for reviews see [1,2]). Apoptosis eliminates individual cells when they are no longer needed or have become seriously damaged. Metazoan cells appear to be programmed to die by default and they execute apoptosis if they do not receive appropriate survival cues from their environment (for reviews see [3,4••]). In addition, metazoan cells have internal sensors for well-being that can initiate apoptosis if the cell is unable to repair defects such as DNA damage.

Higher metazoans have evolved an additional signaling mechanism that actively directs cells to die by apoptosis; this process, which we refer to as 'instructive apoptosis', is critical particularly in the immune system. The best-studied paradigm for instructive apoptosis is that of the death receptor Fas (also called Apo-1 or CD95) and its ligand (FasL/CD95L) (for reviews see [3,4••]). Ligation of cell-surface Fas by FasL delivers a powerful signal that rapidly commits the cell to apoptotic death. FasL-dependent apoptosis plays a critical role in peripheral deletion, a process that eliminates activated lymphocytes at the end of an immune response. In addition, FasL-mediated apoptosis contributes to elimination of virus-infected cells or cancer cells by cytotoxic lymphocytes. FasL-dependent instructive apoptosis also helps to eliminate infiltrating lymphocytes that can perturb normal function in 'immune-privileged' tissues such as the eye or testis.

In the past five years, biochemical and genetic studies have helped researchers in this field to elucidate the intracellular signaling pathways that mediate instructive apoptosis; surprisingly, these experiments also reveal that certain components of the instructive cell-death pathway have roles not only in apoptosis but also in embryonic development, as well as in control of antigen-induced lymphocyte proliferation in the adult animal. The advent of human genome sequencing has facilitated the discovery of several gene products that regulate instructive apoptosis; some of these molecules have exciting potential for therapeutic application, particularly in cancer treatment. Here, we review some of these developments.

Novel death ligands

FasL belongs to a family of proteins that have structural homology to tumor necrosis factor (TNF) (for review, see [5]). FasL and TNF define a subset of TNF-family members that have apoptosis-inducing activity (for reviews, see [3,4••]). *In vitro* studies show that FasL is critical for activation-induced T-cell apoptosis; furthermore, mice or people that carry spontaneous mutations in the genes encoding FasL or Fas, and Fas gene knockout mice, accumulate lymphocytes resulting in a massive, lethal enlargement of lymph nodes. These findings indicate that the main biological role of FasL is to signal instructive apoptosis during peripheral deletion of lymphocytes. TNF or TNF receptor (TNFR) knockout mice display an increased susceptibility to microbial infection and a suppressed inflammatory response when challenged with bacterial endotoxin. These findings together with *in vitro* results indicate that the main biological role of TNF is the induction of inflammatory-response and stress-response genes through the transcription factors AP-1 and nuclear factor (NF)-κB. Researchers have not yet defined the physiological context in which TNF initiates apoptosis.

Through screening of DNA databases for expressed sequence tags (ESTs) with similarity to TNF, two groups discovered another death ligand called Apo2 ligand (Apo2L) [6] or TNF-related apoptosis-inducing ligand (TRAIL) [7]. Apo2L's closest sequence homologue is FasL. *In vitro* studies show that like FasL, Apo2L potently induces apoptosis in tumor cells (for review see [4••]).

Apo2L mRNA is expressed constitutively in many tissues, and transcript levels increase in T cells upon stimulation with phytohemagglutinin [8–10]. Resting peripheral T-cells are resistant to apoptosis induction by Apo2L; however, interleukin-2-stimulated T-cells acquire sensitivity to Apo2L [9,11,12], which suggests a role for this ligand in peripheral deletion. Apo2L also might contribute to instructive apoptosis of virus-infected cells because T cells from human immunodeficiency virus (HIV)-infected patients are more susceptible to apoptosis induction by this ligand than are uninfected cells [10]; further, Apo2L appears to be involved in tumor cell killing by CD4-positive cytotoxic T-cells [13].

EST database screening revealed yet another death ligand, called Apo3L [14], or TWEAK [15]. Apo3L's closest sequence homologue is TNF. Like TNF, Apo3L activates apoptosis in certain tumor cell lines and inhibition of protein synthesis enhances this effect. Apo3L also activates NF-κB [14] and induces expression of interleukin-8 [15]. Unlike TNF's restricted expression in activated lymphoid and endothelial cells, Apo3L's mRNA expression is constitutive in many tissues.

Novel death and decoy receptors

FasL and TNF interact with receptors that belong to the TNFR gene superfamily (for review see [16]). The receptors in this family have several cysteine-rich domains (CRDs) in their amino-terminal region. The cytoplasmic sequence divides the TNFR superfamily into two main subgroups of receptors that either possess or lack a so called 'death domain' [17,18]. The death-domain-containing receptors, or 'death receptors' (DR) (for review see [4••]) include TNFR1, Fas, and the more recently discovered DR3 (also called Apo3, WSL-1, TRAMP or LARD) [19–23], DR4 [24], DR5 (also called TRAIL-R2, TRICK2, or KILLER) [8,25–28], and DR6 [8,25–28]. The death domain couples each receptor to caspase cascades that induce apoptosis or to kinase cascades that turn on gene expression through NF-κB and AP-1. Yet another subgroup of TNFR-homologues consists of decoy receptors, which function as inhibitors, rather than transducers of signaling. This subgroup includes decoy receptor (DcR)1 (also called TRID, LIT, or TRAIL-R3 [24,26,29–32]), and DcR2 (also called TRUNDD or TRAIL-R4) [33–35] — both of which are cell-surface molecules — as well as osteoprotegerin (OPG) [36] and DcR3 [37••] — both of which are secreted, soluble proteins.

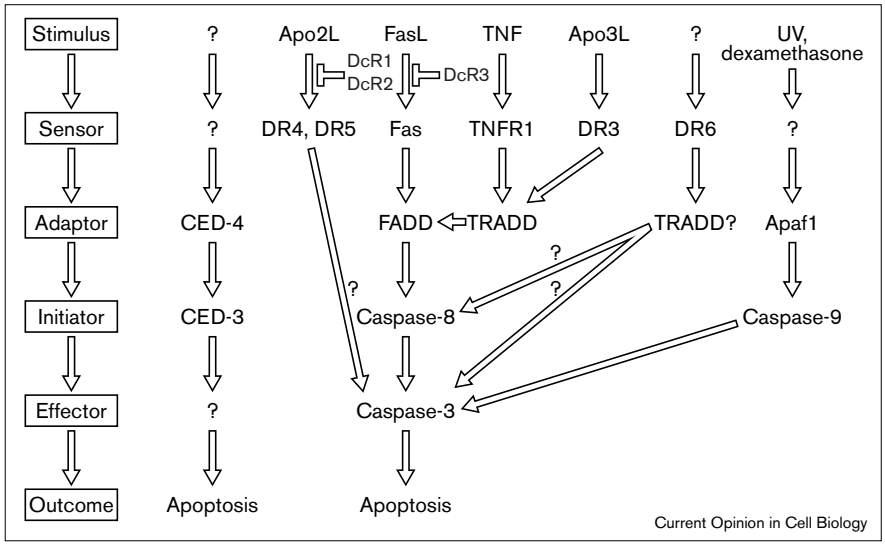
DR3, was discovered on the basis of ESTs with homology to death domains, as well as through its interaction with TNFR1's death domain in the yeast two-hybrid system. DR3 has four extracellular CRDs and its closest sequence homologue is TNFR1 [19–23]. Overexpression of DR3 stimulates apoptosis and activates NF-κB through the same signaling pathways that TNFR1 engages. The expression of the two receptors differs: TNFR1 is ubiquitous, whereas DR3 mRNA is expressed mainly in lymphocyte-rich tissues. Notably, this expression pattern seems to be inversely related to that of TNF and Apo3L, the ligands of TNFR1 and DR3, perhaps providing a clue to the specific biological role of DR3 which could also be in the regulation of lymphocyte function.

Four of the novel cellular receptors bind to Apo2L and are structurally related: DR4, DR5, DcR1, and DcR2 (for review see [4••,38]). Each has two extracellular CRDs, and shows closer homology to the other Apo2L receptors than to the rest of the TNFR superfamily. DR4 and DR5 have a cytoplasmic death domain. DcR1 lacks a cytoplasmic region, and appears to be attached to the cell surface through a glycophospholipid anchor. DcR2 has a cytoplasmic death domain, but this is two thirds shorter than a typical death domain and does not signal apoptosis. The extracellular domains of DcR1 or DcR2 compete with those of DR4 or DR5 for ligand binding. Cell transfection with DcR1 or DcR2 inhibits apoptosis induction by Apo2L; furthermore, removal of DcR1 from the cell surface by enzymatic cleavage of its lipid anchor sensitizes cells to Apo2L. Thus, DcR1 and DcR2 can act as decoys that prevent Apo2L from inducing apoptosis through DR4 and DR5. Hence, cells that express DcR1 and/or DcR2 at high levels relative to DR4 or DR5 may use the decoys as protection against Apo2L's cytotoxic action. Consistent with this model, DcR1 levels drop in T cells upon stimulation [32], whereas DR5 levels rise [8], concomitant with an increase in sensitivity to Apo2L [9,11]. In addition to these four cell-associated receptors, the secreted TNFR-homologue OPG can bind to Apo2L, albeit with lower affinity [39].

Even though recombinant TNF and FasL trigger apoptosis in many tumor cells, the severe toxicity of these ligands toward normal tissues impedes their therapeutic application in cancer treatment. Systemic injection of TNF causes a lethal vascular inflammatory syndrome that resembles septic shock, whereas FasL administration causes lethal liver damage through hepatocyte apoptosis [3]. In contrast, injection of Apo2L causes little toxicity in experimental animals (A Ashkenazi, R Pai, S Fong, S Leung, S Marsters *et al.*, unpublished data). *In vitro*, Apo2L triggers apoptosis in a wide spectrum of tumor cell lines, but it is not cytotoxic toward many normal cell types. *In vivo*, treatment of athymic nude mice carrying human tumor xenografts with Apo2L substantially inhibits tumor progression (A Ashkenazi, R Pai, S Fong, S Leung, S Marsters *et al.*, unpublished data). One mechanism that may contribute to the selectivity of Apo2L's cytotoxicity against tumors is the differential expression of its death and decoy receptors (for review see [4••]). Other mechanisms may involve modulation of apoptosis-signaling downstream of DR4 or DR5. Thus, unlike TNF or FasL, Apo2L may be useful for killing tumor cells without damaging normal tissues.

DR6 has four extracellular CRDs and a cytoplasmic death domain [40••]. An unusual feature of DR6 is the presence of a putative leucine-zipper sequence that overlaps with a proline-rich motif in the cytoplasmic region. The proline-rich motif resembles sequences that bind to Src-homology-3

Figure 1



The apoptosis-signaling pathways of death ligands and receptors share essential features with those of other apoptotic stimuli but the specific molecular components of these pathways differ. The death receptors Fas, TNFR-1, DR3, DR4, and DR5 function as sensors for apoptotic stimulation by their cognate ligands. Decoy receptors modulate apoptosis-induction by diverting specific death ligands from their death receptors; DcR1 and DcR2 bind to Apo2L, whereas DcR3 binds to FasL. The death adaptor FADD couples Fas to capase-8. FADD also couples TNFR1 to capase-8, although this occurs indirectly through the adaptor TRADD. DR6 seems to bind to TRADD but not to FADD; however, the pathway from DR6 to capase activation is unclear. DR4 can signal apoptosis without FADD; whether DR5 can do the same is yet unknown. Capase-3 is one of several capases that execute the apoptotic program downstream to capase-8.

(SH3) domains, which are found in many intracellular signal-transducing molecules. The DR6 mRNA is expressed widely in human tissues and in various tumor cell lines. Overexpression of DR6 induces apoptosis in HeLa S3 ovarian carcinoma cells but not in MCF-7 breast carcinoma cells. In addition, overexpression of DR6 in embryonic kidney 293 cells activates both the NF-κB and the Jun amino-terminal kinase (JNK)/AP-1 pathways. The ligand interactions and physiological function of DR6 are yet to be elucidated.

DcR3 has four CRDs and it is a secreted, soluble protein [37••]; its closest sequence relative is OPG, which also is a secreted, soluble molecule [36]. DcR3 binds to FasL with an affinity that equals that of Fas and it competes with Fas for FasL-binding; further, DcR3 inhibits apoptosis induction by FasL, which suggests that it is a decoy receptor for FasL. There is DcR3 mRNA expression in spleen, colon and lung, but the physiological role of this receptor is unclear. Studies with 35 primary lung and colon tumors show substantial amplification of the DcR3 gene in about half of the tumors examined and frequent DcR3 mRNA overexpression in malignant tissue. Given that FasL is an important mediator of tumor cell killing by natural killer cells and cytotoxic T cells, the genomic amplification of DcR3 suggests that certain tumors may escape immune cytotoxic attack by means of DcR3 overexpression. If this model is correct, then therapeutic strategies could be devised to inhibit the interaction of DcR3 with FasL and thus potentially to enhance the antitumor immune response of cancer patients whose tumors overexpress DcR3.

New insights into the signaling of instructive apoptosis

Biochemical studies delineate the key intracellular signaling pathways that lead from Fas or TNFR1 to initiation of apoptosis (for reviews see [3,4••]). These pathways typically involve adaptor molecules that recruit an initiator caspase into the apoptosis signaling complex through serial homophilic interactions of death domains and of death effector domains (Figure 1). The initiator caspase activates itself by proteolytic processing and stimulates downstream effector caspases that carry out the apoptosis program. In essence, this pathway is similar to death pathways triggered by stimuli such as UV irradiation or glucocorticoids and to the developmental cell death pathway of *Caenorhabditis elegans* although the particular signaling molecules differ (Figure 1).

Engagement of Fas by FasL recruits the death-domain-containing adaptor FADD (Fas associated death domain)/Mort-1 to the receptor's death domain [3,4••]. FADD in turn recruits the protease procaspase-8 through homophilic interaction of death effector domains. Juxtaposition of procaspase-8 molecules leads to activation by self-processing; caspase-8 then initiates apoptosis by activating downstream effector caspases such as caspase-3. The effector caspases cleave many cellular substrates including structural proteins, signaling proteins, and regulators of DNA replication or transcription (for review see [41]); cleavage of these substrates underlies many of the biochemical and morphological events of apoptosis. One such caspase substrate is the inhibitor of caspase-activated DNase (ICAD), also called DFF [42–44]. ICAD binds to CAD (caspase-activated DNase) and keeps it in the cytosol; cleavage of ICAD by caspases allows CAD to migrate to the nucleus where it executes internucleosomal digestion of DNA [42,43].

The apoptosis signaling pathway that TNF triggers is similar though not identical to that of FasL (for review see [4••]). Ligation of TNFR1 by TNF recruits the death-domain-containing adaptor TRADD (TNFR associated death domain) to the receptor's death domain. TRADD in turn recruits FADD and FADD recruits procaspase-8.

TRADD also serves to recruit the serine/threonine kinase RIP and the adaptor TRAF2, which are implicated in activation of the NF-κB and the JNK/AP-1 pathways. Gene knockout experiments demonstrate that RIP is essential for the activation of NF-κB by TNFR1 [45], whereas TRAF2 is critical for engagement of the JNK/AP-1 pathway [46,47]. Two cascades of mitogen-activated protein kinase (MAPK)-like enzymes connect the TNFR1 signaling complex to these latter pathways. NIK [48] activates a kinase complex that phosphorylates the inhibitor I-κB — I-κB kinase (IKK) — leading to its degradation and thereby to activation of NF-κB (for review see [49]). Similarly, ASK1 activates the JNK/AP-1 pathway in response to TNF [50].

Experiments with Apo3L and DR3-overexpression studies implicate TRADD and FADD in activation of apoptosis, and TRADD, RIP, and TRAF2 in induction of NF-κB and JNK/AP-1 by Apo3L and DR3 (for review see [4••]). The results of studies with Apo2L and with overexpression of DR4 or DR5 are inconclusive with respect to the importance of FADD in apoptosis signaling by these receptors (for review see [4••]); however, FADD gene knockout experiments show that DR4 can induce apoptosis independently of FADD [51••]. Cotransfection of adaptor molecules with DR6 suggest that this receptor binds to TRADD, but not to FADD [40••]. Hence, DR6 may use a TNFR1-like mechanism to activate NF-κB and JNK, but its pathway to apoptosis activation may be FADD-independent.

Experiments with transgenic mice expressing a dominant-negative mutant of FADD in T cells [52••,53••], and studies with FADD gene-knockout mice [51••,54••] confirm that FADD has an essential role in apoptosis activation by Fas, TNFR1, and DR3. One unexpected finding is that FADD-deficient mice die *in utero*, which suggests that FADD is essential for proper development. Another surprising result is that T cells expressing the dominant-negative FADD transgene [52••,53••], or FADD-deficient T-cells (rescued in a recombinase-activating gene [RAG]-1-deficient mouse background) [54••] do not proliferate as well as wild-type T-cells do in response to antigenic stimulation. These observations suggest that FADD is not solely a mediator of apoptosis but also signals survival and proliferative functions through mechanisms that as yet are poorly defined.

Caspase-8 knockout studies indicate that this enzyme is essential for apoptosis initiation by Fas, TNFR1 and DR3, but not for activation of the NF-κB or JNK/AP-1 pathways by these receptors [55••]. Caspase-8-deficient embryos die at day 11 or 12 of gestation, as do FADD-deficient embryos. In both cases the predominant pathologic outcome is impaired heart muscle development and accumulation of erythrocytes. This raises the intriguing possibility that the phenotypic similarity of FADD and caspase-8 knockout stems from perturbation of a common signaling pathway that involves both molecules. Studies with a mutant human Jurkat T leukemia cell line that is deficient in caspase-8 confirm the requirement for this caspase in Fas-mediated apoptosis but show only partial attenuation of TNF-induced cell death [56••]. This result suggests that, in some cell types, TNF may use an alternative mechanism to initiate apoptosis. Another notable difference is that Fas-mediated activation of the JNK/AP-1 pathway is abolished in the mutant Jurkat cells but is not impaired in caspase-8 knockout embryonic fibroblasts, which suggests more than one mechanism of JNK/AP-1 activation by FasL.

Which factors propagate apoptosis downstream of caspase-8? One effector caspase that is stimulated after caspase-8 activation is caspase-3 (for review see [41]). Experiments with caspase-3 knockout mice [57•] indicate that this caspase is critical for induction of DNA fragmentation by FasL and TNF. In contrast, non-nuclear apoptotic events initiated by these death ligands do not require caspase-3. Hence, caspase-3 is one of several effector caspases that act downstream of caspase-8.

Another event implicated downstream of caspase-8 activation is release of cytochrome c from mitochondria. Cytochrome c appears to be important for apoptosis initiation by stimuli such as DNA-damage or glucocorticoids; however, its importance for apoptosis induction by death ligands is controversial. Cytochrome c binds to Apaf1, the mammalian homologue of *C. elegans* CED-4, which in turn activates caspase-9, a mammalian homologue of *C. elegans* CED-3, thereby initiating apoptosis (for review see [41,58]). Caspase-8 activation leads to cleavage of Bid, a Bcl-2-interacting protein that induces release of cytochrome c from mitochondria [59–61]. This is consistent with a model in which Bid is a downstream mediator of apoptosis-induction by FasL and TNF; however, gene knockout of the targets of cytochrome c, namely, Apaf1 [62,63] or caspase-9 [64,65], does not affect cellular sensitivity to Fas-mediated apoptosis. Thus, Bid, mitochondria, and cytochrome c do not have an obligatory role in apoptosis signaling by FasL, although they may contribute to this function in some yet undefined context.

DEDD is one more intriguing molecule that was identified through EST database screening [66•]. The DEDD mRNA is expressed ubiquitously and the protein contains a death effector domain similar to those of FADD and caspase-8. In addition, it has two nuclear-localization sites and a carboxy-terminal sequence that bears homology to histones. Overexpression of DEDD induces weak apoptosis through interaction with FADD and caspase-8. Perhaps more importantly, Fas-ligation induces translocation of DEDD from cytoplasm to nucleus. Further, recombinant DEDD binds to DNA and inhibits transcription in a reconstituted *in vitro* system. These observations suggest that DEDD may serve to shut down transcription during Fas-mediated apoptosis, although this model requires verification in a more physiological system.

Conclusions and future prospects

Biochemical and genetic studies have unraveled many of the signaling mechanisms that mediate induction of instructive

apoptosis by FasL and TNF. It is likely that the novel death ligands and receptors use similar pathways, although the molecular details of this are still obscure. Important questions that emerge from gene knockout studies concern the roles of FADD and caspase-8 in nonapoptotic signaling pathways, and the identity of receptor(s) that control those functions. Genome sequencing has revealed a plethora of new death ligands, death receptors and decoy receptors, and an unexpected complexity in the TNF ligand and receptor superfamilies. What are the unique biological roles of these ligand-receptor systems? What are the mechanisms that integrate their functions within the immune system? Do any of them have roles outside the immune system? We hope that some of these exciting new molecular discoveries will lead to new cancer treatments.

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