Cell Death and Differentiation (2003) 10, 4–6

www.nature.com/cdd

News and Commentary

Death receptors leave a caspase footprint that Smacs of XIAP

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Cell Death and Differentiation (2003) **10,** 4–6. doi:10.1038/sj.cdd.4401176

For some time now, fierce debate has surrounded the role of mitochondria in apoptosis induced by the so-called death receptors. This issue largely focused on the ability of antiapoptotic Bcl-2 proteins to inhibit CD95 (Fas/Apo-l)-mediated cell death.

Some argued that Bcl-2 and Bcl-x_L could inhibit CD95-induced apoptosis, because they prevented the release of cytochrome *c* from mitochondria and, consequently, formation of the Apaf-1 (apoptotic protease-activating factor-1) apoptosome and activation of caspase-9.

However, it is now clear that CD95-mediated apoptosis can be prevented by the X-linked inhibitor-of-apoptosis (XIAP) protein, which binds to and inhibits active caspase-3.[1]

Therefore, in addition to preventing the release of cytochrome *c* from mitochondria, Bcl-2 and Bcl-x_L, can also inhibit apoptosis, by preventing the release of Smac/DIABLO (second mitochondrial activator-of-caspases), which promotes apoptosis by antagonizing XIAP's inhibition of caspase-3[2] (Figure 1).

There are at least two fundamental pathways by which cells can undergo apoptosis and both involve the activation of caspases. In one pathway, sometimes referred to as the extrinsic pathway, death receptors, including CD95, DR4 and DR5 (death receptors 4 and 5; also called TRAIL-R1 and TRAIL-R2), as well as TNFR1 (tumor necrosis factor receptor 1), are activated by their cognate ligands, CD95 ligand, TRAIL (TNF-related apoptosis-inducing ligand) and TNF, respectively.

Each of these receptors belongs to the TNF superfamily of receptors, and each activates the apical caspase-8 by first recruiting the adapter proteins, FADD (Fas-associated death domain) and/or TRADD (TNF receptor-associated death domain). In particular, the complex of CD95 with FADD and caspase-8 is referred to as the DISC or death-inducing signaling complex. Following its activation within the complex, processed caspase-8 can in turn activate the effector caspase-3, which cleaves critical structural and regulatory proteins, resulting in apoptosis.[3]

In the other caspase-activating pathway, often referred to as the intrinsic pathway, diverse stimuli induce the release of cytochrome *c* from mitochondria, frequently *via* a mechanism that requires the involvement of the proapoptotic Bcl-2 proteins, Bax or Bak. Once released, cytochrome c, in concert with dATP or ATP, induces oligomerization of Apaf-1 into a high molecular weight apoptosome complex.

This Apaf-1 apoptosome then serves as a positive allosteric regulator of apical caspase-9 activity. Similar to the extrinsic pathway, caspase-9 then activates caspase-3, which in turn executes the cell death program.[4] In some cell types, the extrinsic and intrinsic pathways may function entirely independent of one another. However, it appears that in other cases, death receptor-induced apoptosis cannot occur without mitochondrial involvement. Indeed, the antiapoptotic Bcl-2 proteins, Bcl-2 and Bcl-x_L, can inhibit CD95L-, TRAIL- and TNF-induced apoptosis in some cell types, but not in others. For the sake of convenience, it was suggested that cells be classified as either Type II or Type I cells, depending upon whether Bcl-2 could or could not inhibit death receptor-induced apoptosis, respectively.[5] So how does death receptor stimulation initiate the release of cytochrome *c* from mitochondria? Active caspase-8 can cleave the proapoptotic Bcl-2 protein Bid into a truncated form (tBid), which then translocates from the cytosol to the outer mitochondrial membrane, where it stimulates release of cytochrome *c*.[6] But if caspase-8 is activated in Type I and Type II cells and each contain Bid, should not cytochrome *c* be released in both situations, resulting in formation of the apoptosome? The answer is yes, but the argument has been that in contrast to Type I cells, there is not enough caspase-8 activated within the DISC in Type II cells, and therefore these cells require formation of the Apaf-1 apoptosome, so that active caspase-9 can cooperate with caspase-8 to activate caspase-3.[5] However, others have questioned the existence of Type II cells altogether, arguing that in many studies inappropriate CD95 agonistic antibodies were utilized, rather than the natural ligand.[7] In addition, and perhaps more importantly, they have astutely pointed out that cells isolated from *cytochrome c−/−, apaf-1−/−* and *caspase-9−/−* mice are not resistant to CD95-induced apoptosis. However, studies with *bid−/−* mice indicate that the mitochondrial pathway is vital for CD95-induced apoptosis in some tissues, particularly the liver.[8] So how is it possible that Bid is required for death receptor-induced apoptosis, whereas the Apaf-1 apoptosome and caspase-9 are not? In hindsight, the answer may actually be quite simple. It might be that death receptors induce the release of additional mitochondrial factors, in a Bid-dependent manner, that are necessary for promoting caspase activation and cell death, but do not necessarily involve formation of the Apaf-1 apoptosome. Smac is a protein that contains a mitochondrial targeting sequence. Following its import into mitochondria, it

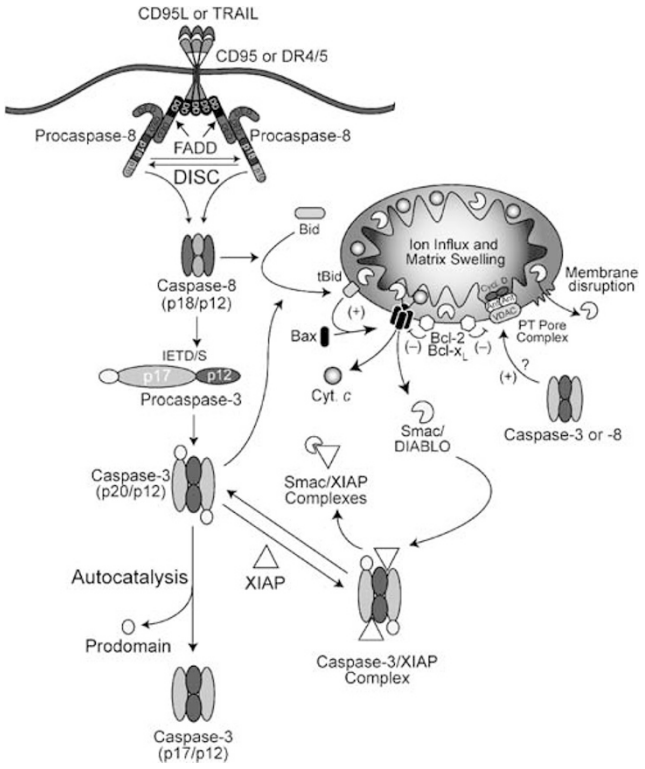

Figure 1 Mitochondrial release of Smac/DIABLO is regulated by pro- and antiapoptotic Bcl-2 family members and is required to antagonize caspase-3-XIAP interactions during death receptor-induced apoptosis in Type II cells. Ligation of death receptors (CD95 or DR4/5) with their cognate ligands (CD95L or TRAIL) results in formation of the DISC and activation of caspase-8. Active caspase-8 then (1) processes procaspase-3 to its intermediate p20/p12 form, and (2) cleaves and activates Bid, which stimulates formation of large oligomeric, channel-forming Bax pores in the outer mitochondrial membrane. XIAP initially binds to and inhibits p20/p12 caspase-3, preventing the onset of apoptosis. However, Bax channels (or perhaps caspase-activated PT pores) subsequently facilitate the release of Smac from mitochondria. This IAP antagonist promotes disruption of XIAP's interaction with caspase-3, and unrestrained p20/p12 caspase-3 then undergoes autocatalytic activation to produce its fully mature p17/p12 form. Active caspase-3 subsequently activates the downstream effector caspase-6 and induces apoptosis *via* cleavage of critical structural and regulatory proteins

is N-terminally processed by an unknown protease, which exposes an AVPIA motif that is remarkably similar to the N-termini of the *Drosophila* IAP antagonists, Reaper, Hid, Grim and Sickle.[9,10] Indeed, a number of structural and biochemical studies have confirmed that this AVPIA motif is absolutely required for Smac to interact with XIAP.[11] IAPs were first identified in baculoviruses, and each contains one or more baculovirus IAP repeat (BIR) domains and frequently possesses a C-terminal RING zinc-finger domain. Several IAPs are known to inhibit caspases *in vitro,* but XIAP, which inhibits active caspase-9 through its BIR3 domain and active caspases-3 and -7 through its Linker-BIR2 domain, is by far the most potent.[12] Therefore, since XIAP does not inhibit caspase-8, XIAP can prevent CD95-mediated apoptosis exclusively through inhibition of caspase-3.[1] However, the levels of XIAP may actually dictate whether a cell exhibits a Type I or Type II phenotype. Indeed, cells that would otherwise undergo apoptosis *via* a direct DISC→caspase-8→caspase-3 pathway (Type I cells), in the presence of high levels of XIAP, may require mitochondrial release of Smac in order to antagonize XIAP–caspase-3 interactions. Thus, the latter situation represents a novel Type II pathway, since it is the ability of Bcl-2 and Bcl-x_L to inhibit release of Smac, and not cytochrome *c,* that controls caspase activation and cell death.[2] Interestingly, the existence of this novel pathway can be visually observed by a unique footprint or pattern of caspase-3 processing. Indeed, when Bcl-2 or Bcl-x_L inhibits the release of Smac, XIAP maintains caspase-3 primarily in its partially processed p20/p12 form, whereas in the presence of Smac, p20/p12 caspase-3 disassociates from XIAP and as a result, can undergo autocatalytic processing to remove its prodomain and generate its fully mature p17/p12 form.[2] Furthermore, as already noted, in CD95-stimulated Type II cells, the total amount of processed caspase-8 is frequently much lower than in similarly treated Type I cells. However, the presence of XIAP may also provide an explanation for this result, since inhibition of caspase-3 by XIAP prevents a feed-forward caspase-3→caspase-6→caspase-8 amplification pathway. As this novel Type II pathway could potentially be very important, it is critical to understand the mechanism(s) controlling the release of Smac from mitochondria. Smac release in TRAIL-treated cells appears to be both caspase- and Bax-dependent and, compared to cytochrome *c* release, occurs with similar kinetics (unpublished data).[13–15] It is not surprising that Smac release would be caspase-dependent following death receptor stimulation, since activation of caspase-8 within the DISC is required for virtually all apoptotic signaling, including tBid-dependent release of cytochrome *c.* However, toxicant and UV treatments also induce release of both cytochrome *c* and Smac, but apparently only the latter is caspase-dependent.[13] Therefore, is it possible that Smac release is both tBid- and Bax-dependent, regardless of the apoptotic stimulus? Indeed, toxicant-induced release of cytochrome *c,* formation of the Apaf-1 apoptosome and sequential activation of caspases-9, -3 and -8 are all Bid-independent steps, but cleavage of Bid by caspases-3 and -8 might be required to induce formation of larger Bax pores, through which Smac might subsequently be released. The use of *bid−/−* mice should be helpful in testing such a hypothesis. It is also tempting to speculate that activation of the mitochondrial permeability transition (PT) pore might be required for release of Smac, since active caspases also trigger a loss in the inner mitochondrial membrane potential, resulting in mitochondrial swelling and rupture of the outer mitochondrial membrane. So what is the relative importance of the Apaf-1 apoptosome *versus* Smac, in promoting death receptor-induced apoptosis? Studies indicate that Smac can potentiate CD95L- and TRAIL-induced apoptosis and that cell death is apparently not inhibited by overexpression of dominant-negative caspase-9.[2,14,15] However, it remains to be seen, for example, if overexpression of Bcl-2 or Bcl-x_L can inhibit death receptor-induced apoptosis in *apaf-1-/-* cells. Haraguchi *et al.*[16] determined the effects of Bcl-2 over-expression on Apaf-1-independent cell death, induced by chemicals and UV radiation, but unfortunately not by death receptors. In some tumor cells, Apaf-1 expression can be inhibited *via* gene methylation, and even when Apaf-1 is

expressed, the apoptosome can be inhibited by an endogenous dominant-negative, short isoform of caspase-9 or possibly by certain heat shock proteins (Hsp-70 and Hsp-90). Therefore, in these instances, mitochondrial release of Smac may be particularly critical, since the apoptosome would be unable to contribute to caspase activation and cell death. Interestingly, Smac-deficient mice appear to be as sensitive as the wild-type mice to CD95-induced apoptosis in the liver. Moreover, cells derived from *smac* −/− mice are not resistant to TNF, agonistic CD95 antibodies or TRAIL.[17] However, the dual IAP antagonist and serine protease, Omi/HtrA2, which is also a mitochondrial protein, may be capable of compensating for the absence of Smac, although under normal circumstances its protease activity appears to mediate its primary proapoptotic effect.[18] Given the interest in developing small, Smac-like mimetics for the pharmacological treatment of cancer, additional studies should be conducted to determine the true importance of IAPs and their antagonists in preventing or promoting apoptosis, respectively.

Note Added in Proof

Recently, using wild-type and *bid*−/− mice, Li and colleagues demonstrated that Bid is required for the release of Smac and consequently the disruption of IAP-caspase-3 interactions during Fas-induced apoptosis.[19]

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