Comment

Apoptosis and Sphingomyelin Hydrolysis: The Flip Side

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Apoptosis, a form of cell death that is coordinated by a set of defined biochemical pathways, exists throughout the animal kingdom. Its critically important roles in development, homeostasis, and disease have made this a topic of intensive research and sometimes even more intensive controversy. One of these areas of controversy concerns the role of ceramide, produced by the hydrolysis of sphingomyelin. In this issue, Tepper et al. (2000) add to this debate with an interesting new theory.

When we consider the central mechanisms of apoptosis, we have two general questions to ask: first, what determines whether a cell will live or die? And if a cell is to die, what determines the precise form of this cell death? The second question is often as important as the first, as we'll see.

Evidence from early experiments showed that ceramide is produced under a variety of conditions leading to apoptosis (Kolesnick and Kronke, 1998). The knowledge that synthetic ceramide was able to induce apoptosis, led to the unfortunately too simple conclusion that the production and action of ceramide is an obligatory step in the apoptotic process. Therefore, several studies (based predominantly on correlations) put forth the idea that sphingomyelin hydrolysis and signaling via ceramide are essential in the decision of whether a cell dies. This conclusion fell from favor as evidence accumulated against ceramide production as a major determinant of the life/death decision. Now, a new study by Tepper and colleagues in this issue (Tepper et al., 2000) has perhaps defined a new role for sphingomyelin hydrolysis in apoptosis, determining not whether but how a cell dies.

To understand this role in the context of the apoptotic process (as it is currently understood), we will first review the process itself and examine the different roles that have been proposed for sphingomyelin hydrolysis in the different pathways to apoptotic cell death.

When cells die via apoptosis, they undergo a number of morphological and biochemical changes that are stereotypical for this type of death. These changes are orchestrated by a set of cysteine proteinases that become active during apoptosis, the caspases (cysteine proteinases with specificity for aspartic acid residues; reviewed in Wolf and Green, 1999). The final throes of cell death (and the associated changes) have been named execution and the caspases responsible for coordinating these changes are the executioner caspases. Caspase-3 is the major executioner caspase, together with caspases-6 and -7.

The executioner caspases, once activated, cleave key substrates in the cell that produce the apoptotic phenotype. For example, caspase-3 cleaves iCAD (inhibitor of caspase-activated DNase), releasing CAD, which in turn cuts the chromatin between nucleosomes to produce the DNA ladder seen in many cell types as they undergo apoptosis. Another protein, acinus, is activated by caspase cleavage to cause chromatin condensation. Similarly, caspase-3 cleaves and activates p21-activated kinase-2 and gelsolin, both of which appear to participate in contortions of the plasma membrane, referred to as blebbing.

Other effects of the substrates of activated caspases are inferred from the effects of caspase inhibitors or by the use of cells lacking one or more caspases. These include loss of mitochondrial membrane potential, cell shrinkage, loss of cell adhesion, and loss of plasma membrane integrity, among others.

One of the most important caspase-mediated changes in the cell is the loss of plasma membrane lipid asymmetry. That is, the lipids of the planar membrane are normally nonrandomly distributed, some are predominantly localized to one or the other side. For example, sphingomyelin is mostly found on the outer leaflet, while phosphatidylserine and phosphatidylethanolamine are mostly on the inner leaflet of the plasma membrane. This is due to the action of phospholipid translocases that maintain the orientations of some of the lipids (Bevers et al., 1999). During apoptosis, the plasma membrane scrambles, and this involves both loss of the translocase activity and the action of an uncharacterized scramblase. Although this is often (but not always, as I'll discuss) caspase dependent, we do not know the molecular mechanism of this event.

The loss of lipid asymmetry can be readily monitored by the use of fluorescence-conjugated annexin V, which specifically binds to phosphatidylserine (PS)[1] as it externalizes on apoptotic cells (Martin et al., 1995). The appearance of PS on the outer leaflet of the plasma membrane is especially relevant, because of all the changes we have so far mentioned, it is the one with the most defined function. PS is recognized and bound by a receptor (PSR) on macrophages and other phagocytic cells, and this results in the rapid clearance of the dying cell from the body (Fadok et al., 2000). One could even argue that this is the only change that a cell needs to go through to be functionally

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[1]*Abbreviations used in this paper:* PS, phosphatidylserine; PSR, PS receptor.

dead, since it will be phagocytosed and disposed of once this has occurred. Binding of PSR influences the behavior of the phagocyte as well, acting to ensure that the dead cell is cleared without the induction of an inflammatory response, an effect that stands in contrast to the consequences of necrotic cell death.

Another biochemical change in the cell as it undergoes apoptosis is, in many cases, the production of ceramide via the hydrolysis of sphingomyelin, as mentioned above, and this also seems to often be dependent on caspase activity. Caspase inhibitors can block ceramide production during apoptosis (Brenner et al., 1998; Boesen-de Cock et al., 1999), and an intriguing reason for this has now been offered. Tepper et al. (2000) propose that as lipid asymmetry is lost and sphingomyelin is brought into contact with the cytosol, sphingomyelinases (mostly the uncharacterized neutral sphingomyelinase, but acidic sphingomyelinase can also be found in association with the inner leaflet of the plasma membrane) act to hydrolyze it to phosphatidylcholine and ceramide. But here is where their model further differs from that of other labs, they propose that it's the loss of sphingomyelin per se, and not the production of ceramide, that produces part of the apoptotic phenotype. They show that as the sphingomyelin is lost from the membrane, cholesterol (which is usually tightly associated with it) is released, thus profoundly altering the fluidity of the plasma membrane. In support of this, they show that cells that fail to externalize PS during apoptosis do not produce ceramide. Cells that do produce ceramide show more profound blebbing and membrane release (Zhang et al., 1998) that can be inhibited by addition of more sphingomyelin to the membrane.

The proposed role for sphingomyelin hydrolysis is, therefore, at the point that the fate of the cell is sealed. Is this, then, the only role for sphingomyelin hydrolysis in apoptosis? Perhaps, but to speculate about additional roles we will have to extend our discussion of the apoptotic process. So far, all of our discussion has focused on the last stages of apoptosis, and the reader may have noticed that no mention was made as to how the caspases become active to orchestrate these events. The executioner caspases reside as inactive zymogens in cells, and are activated by proteolytic cleavage, usually by another caspase. But how does this get started? The key to this is the existence of another type of caspase, called initiator caspases, which unlike the executioner caspases can be activated by binding to adapter molecules via protein-protein interaction domains (reviewed in Wolf and Green, 1999). Once activated, the initiator caspases cleave and activate executioner caspases and the apoptotic execution proceeds.

The interactions of adapter molecules with initiator caspases define two distinct (but often interconnected) pathways of apoptosis. One of these involves so-called death receptors which are a subset of the tumor necrosis factor receptor family (including TNFR1, Fas/CD95, and the TRAIL receptors, among others). Activation of a death receptor recruits a complex of adapters and initiator caspases to the intracellular region that results in the activation of the caspase at the plasma membrane (see Fig. 1). The second pathway is the mitochondrial pathway. Pro-apoptotic cell stimuli induce the activation of molecules in the Bcl-2 family (which includes both pro- and anti-apoptotic members; here we are concerned with the proapoptotic members), which target the mitochondria and induce the release of proteins that reside in the space between the inner and outer mitochondrial membranes. One of these, cytochrome c, catalyzes the activation of another adapter protein, which binds and activates another initiator caspase (see Fig. 1). Again, the activated initiator caspase cleaves and activates the executioner caspases and death proceeds. Much more detailed descriptions of these processes can be found elsewhere (e.g., Green, 1998).

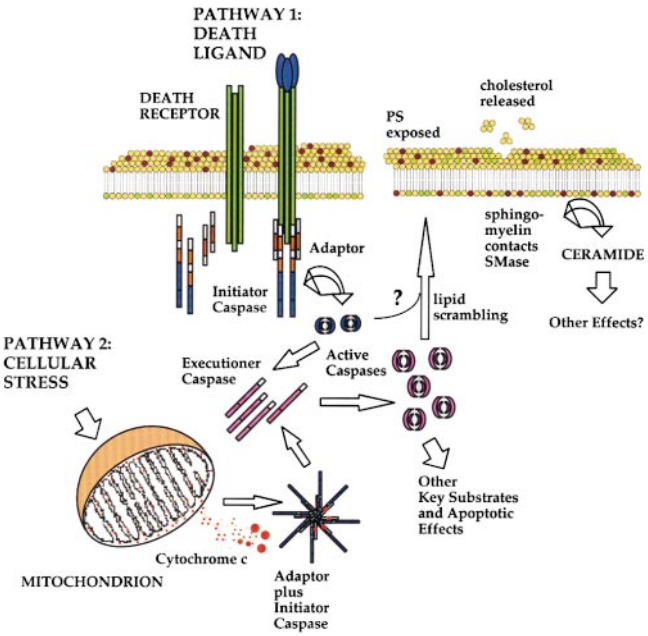

Figure 1. Apoptotic pathways leading to caspase activation, loss of plasma membrane lipid asymmetry, and sphingomyelin hydrolysis. Two pathways for apoptosis are shown. In Pathway 1, activation of death receptors, such as Fas/CD95, recruits a complex composed of adapter molecules (FADD) and the proform of an initiator caspase. This activates the caspase, which in turn cleaves and activates executioner caspases. These, or possibly the initiator caspase itself (see text) trigger scrambling of the plasma membrane via an unknown mechanism. This brings sphingomyelin to the inner leaflet, where it is hydrolyzed by sphingomyelinases in the cell. This results in ceramide generation and in a loss of sphingomyelin from the membrane. The latter, in turn, causes a loss of cholesterol, which alters plasma membrane fluidity. Ceramide may also perform signaling functions, although the importance of this aspect is untested. In Pathway 2, cellular stress (or other proapoptotic signals) trigger proapoptotic Bcl-2 family members to target mitochondria and cause the release of cytochrome c. The latter catalyzes the activation of adapter molecules (Apaf-1) which recruit and activate initiator caspases. These, in turn, cleave and activate executioner caspases. Again, the caspases trigger lipid scrambling, sphingomyelin internalization and hydrolysis, and the resultant membrane changes.

The anti-apoptotic members of the Bcl-2 family block the release of cytochrome c from the mitochondria (e.g., Kluck et al., 1997) and also can inhibit ceramide production in cells treated with DNA damaging agents (Tepper et al., 1999), consistent with the model shown in Fig. 1. Things get more confusing, however, when we look at the effects of caspase inhibitors. Tepper et al. (2000) found

that although a pan-caspase inhibitor blocked ceramide production as described previously (Brenner et al., 1998; Boesen-de Cock et al., 1999), a slightly more specific and less effective inhibitor could nevertheless separate PS externalization and ceramide production from nuclear condensation, another caspase-dependent change in the cell. Similarly, Grullich et al. (2000) found that induction of ceramide by ectopic expression of FADD (a death receptor associated adapter molecule) was caspase dependent even under conditions where apoptosis did not proceed. These results suggest that ceramide production (and perhaps lipid scrambling leading to ceramide production) can be a consequence of the action of an initiator caspase rather than only executioner caspases.

If so, then the production of ceramide may still contribute to the ultimate demise of the cell. For example, ceramide has been shown to disrupt mitochondrial respiration (Di Paola et al., 2000) which can be maintained even after cytochrome c release (Goldstein et al., 2000). It is also capable of blocking some anti-apoptotic mechanisms involving Akt (Basu et al., 1998; Zhou et al., 1998) and this too may ultimately contribute to apoptosis. If sphingomyelin hydrolysis happens at a sufficiently early point in the pathway, therefore, the production of ceramide may influence either the rate and form of cell death or work to release blocks on downstream events.

Loss of lipid asymmetry is not unique to caspase-mediated apoptosis. For example, a transient externalization of PS can occur in response to calcium flux, and Tepper et al. (2000) have shown that this can also result in ceramide production. Whether this contributes to any apoptotic pathways is an important question. Further, and intriguingly, membrane blebbing (McCarthy et al., 1997; Mills et al., 1998) and PS externalization (Vanags et al., 1996) are not always caspase dependent, even during apoptosis. We might expect that in such cases, if these ideas are correct, sphingomyelin hydrolysis and ceramide production will proceed independently of caspase activation. If so, the contribution of these effects to the life/death decision will have to be considered.

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