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Mitochondrial Redox Signaling during Apoptosis

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The regulatory role of cellular redox state during apoptosis is still controversial. Early redox signaling can transduce divergent upstream signals to mitochondria and initiate apoptosis. On the other hand, release of mitochondrial cytochrome *c* triggers generation of reactive oxygen species (ROS) and renders apoptotic cells much more oxidized. Although the sequential caspase activation does not have apparent redox-sensitive components, redox signaling provides a separate pathway that is parallel with the caspase cascade. The function of the apoptosis-associated redox change is uncertain. It could provide positive feedback mechanisms, such as activating mitochondrial permeability transition and apoptosis signaling kinase (ASK-1). Since apoptotic cells are designated to be quickly eliminated, the dramatic cellular oxidation could be involved in the final degradation of apoptotic bodies and even the termination of the proteolytic activity after phagocytosis.

KEY WORDS: Apoptosis; redox; mitochondria; E_h; ROS; ASK-1; thioredoxin.

INTRODUCTION

Apoptosis, or programmed cell death, is a tightly regulated process that is involved in many vital functions such as tissue development, carcinogensis, and immune response (Thompson, 1995). A set of precisely timed biochemical and morphological changes occur during apoptosis (Wyllie *et al.*, 1980; Kerry *et al.*, 1972). Earlier data provided strong evidence that reactive oxygen species (ROS) can induce the process and that antioxidants can prevent it (Slater *et al.*, 1995). More recent data demonstrate that activation of a proteolytic cascade involving cysteine-dependent, aspartate-specific caspases provide a central common biochemical pathway of apoptosis (Alnemri *et al.*, 1996; Thornberry and Lazebnik, 1998). Because procaspases are present constitutively in the cytosol with low, but significant, activity (Cohen, 1997; Thornberry and Lazebnik, 1998), the regulatory mechanisms to either initiate or prevent their autocatalysis appear to be the key checkpoints for apoptosis. Recent progress along several lines of research have placed mitochondria at the center of this multilevel regulatory process. While much has been focused on redox-unrelated mechanisms, mitochondria are major redox-active organelles, functioning both in energy metabolism and ROS generation. The focus of this review is on mitochondrial redox signaling during apoptosis and its possible functions in the tightly controlled multistep process of caspase activation.

CASPASE ACTIVATION BY REDOX-UNRELATED MECHANISMS

Among the 13 caspases identified so far, several are classified as "initiator caspases" (Thornberry and Lazebnik, 1998). These are upstream in the proteolytic cascade. Once activated, they cleave "effector caspases" and result in their activation. Initiation appears to involve a "recruitment–autoactivation" mechanism wherein protein complexes bring procaspases to the vicinity of each other and facilitate an autoactivation. Such protein complexes include the plasma membrane-associated death-inducing signal complex (DISC) (Scaffidi *et al.*, 1998) and the cytoplasmic apoptosome

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(Li *et al.*, 1997). Early mitochondrial changes, particularly the breakdown of the outer membranous structure (Vander Heiden *et al.*, 1997; Single *et al.*, 1998) and release of cytochrome *c* (cyt *c*) (Yang *et al.*, 1997; Kluck *et al.*, 1997), have important signaling roles in activation of apoptosis by these mechanisms.

Phosphorylation and dephosphorylation are also involved in the regulation of caspase activation. Mitochondria serve as a structural base for docking and targeting apoptosis-related proteins with different states of phosphorylation. Bcl-2 can target Raf-1 kinase to the mitochondria and Raf-1 can then phosphorylate BAD and prevent apoptosis (Wang *et al.*, 1996). The phosphorylation state of Bcl-2 itself is critical for its function. IL-3 withdrawal was found to result in dephosphorylation of Bcl-2 and activation of apoptosis (Ruvolo *et al.*, 1998; Ito *et al.*, 1997). Similar effects are also achieved by the relatively nonspecific protein tyrosine kinase inhibitors staurosporine and H-7 (Ito *et al.*, 1997).

Although it is well established that cellular redox state is a key regulator of cell proliferation and differentiation (Burdon, 1995; Cotgreave and Gerdes, 1998), its role in apoptosis is still controversial. Recent findings suggest that mitochondria serve as both an important source and a target of intracellular oxidant signal during apoptosis. However, other evidence indicates that the caspase cascade can be activated without a requirement for a redox signal. To clarify this conflicting picture, one can consider three alternatives: that redox signaling is irrelevant to apoptosis; that redox signaling is an integral component of one or more of the caspase activating mechanisms; and that redox signaling is a consequence of caspase activation and has a function in either execution or termination of the apoptotic program.

IS REDOX SIGNALING RELEVANT TO APOPTOSIS?

Redox signaling can occur by either reactive oxygen species (ROS) such as superoxide and peroxides, or by thiol-disulfide couples such as GSH/GSSG. Studies in the early 1970s showed that mitochondria are important sites of ROS generation (Boveris and Chance, 1973). Together with the finding that the antiapoptotic protein Bcl-2 is associated with mitochondria, it led to the consideration that ROS could be an important activator of apoptosis and that Bcl-2 could function as an antioxidant (Hockenbery *et al.*, 1990). Overexpression of Bcl-2 protects cells from both apoptosis and necrosis in most experimental systems (Adams and Cory, 1998). Its localization to the mitochondria puts it close to a major source of ROS (Hockenbery *et al.*, 1990). Bcl-2 has an apparent antioxidant function because it protected cells from lipid peroxidation and apoptosis that were induced by hydrogen peroxide and the redox cycling agent menadione (Hockenbery, *et al.*, 1990; Kane *et al.*, 1993). However, Bcl-2 is not a direct radical scanvenger, as it failed to inhibit cyanide insensitive oxygen consumption induced by menadione (Hockenbery *et al.*, 1990).

The hypothesis that ROS are important mediators of apoptosis was challenged by Raff and co-workers. They found that cells either lacking mitochondrial DNA (Jacobson *et al.*, 1993) or grown under nearly anaerobic conditions (Jacobson and Raff, 1995) could still undergo apoptosis. Overexpression of Bcl-2 was found to be protective in both systems. Therefore, the antiapoptotic function of Bcl-2 and its related family members can not be solely explained by an antioxidant function. However, those experiments do not rule out a role of redox signaling in apoptosis. They only indicate that apoptosis can be activated in cells lacking mitochondrial respiration and by a mechanism that does not require oxygen. Because apoptosis can be induced by a divergent array of stimuli that are coupled to different signaling mechanisms, there remains a large range of processes in activation or execution of apoptosis, which can be redox dependent without conflicting with these findings.

Of particular importance is the evidence showing that antioxidants, especially the thiol compound *N*-acetylcysteine, block or delay apoptosis in many systems (Table I). These include, but are not limited to, HIV infection (Malorni *et al.*, 1993), tumor necrosis factor (Mayer and Noblé, 1994), growth factor withdrawal (Atabay, *et al.*, 1996), and induction of p53 (Polyak *et al.*, 1997). As many of these apoptotic stimuli are less harsh and more relevant to pathological conditions *in vivo*, it is unavoidable to conclude that apoptosis does involve some general redox sensitivity, either at the level of activation or at the level of execution. However, a link between these different conditions is still missing, suggesting, perhaps, that there is not a common link. In the face of the evidence that there is a general redox sensitivity but no apparent common link, it is appropriate to consider that redox signaling occurs at different steps in the biochemical pathway of apoptosis.

Table I. Inhibition of Apoptosis by Antioxidants

Activation mechanism	Cell line	Antioxidant	Effect	Ref.
TNF-a	L 929	NAC	Inhibition	Saitoh *et al.*, 1998
P53	Smooth muscle cells	NAC, PDTC	Inhibition	Johnson *et al.*, 1996
HIV	U937	NAC	Inhibition	Malorni *et al.*, 1993
Anti-CD3	Do11-10	NAC, GSH	Inhibition	Jones *et al.*, 1995
Ischemia/reperfusion	Cardiac myocytes	Carvedilol	Inhibition	Yue *et al.*, 1998
Hyperoxia	MDCK	Vitamin E	Inhibition	Jyonouchi *et al.*, 1997
Nitric oxide	COLO 205	NAC	Inhibition	Ho *et al.*, 1997
Dexamethasone	Do11-10	NAC	Stimulation	Jones *et al.*, 1995
Staurosporine	HL 60	NAC	No effect	Cai and Jones, 1998

IS REDOX SIGNALING AN INTEGRAL COMPONENT OF ONE OR MORE OF THE CASPASE ACTIVATION MECHANISMS?

A simplified scheme integrating possible sites of redox signaling with known caspase activation mechanisms in presented in Fig. 1. Apoptosis can be directly induced by a variety of oxidants, such as peroxide (Gardner *et al.*, 1997), diamide (Sato *et al.*, 1995) and redox cycling agents (Sun and Ross, 1996). Depending upon the concentrations used, oxidants can induce either apoptosis, necrosis, or atypical apoptosis (Lennon *et al.*, 1991; Gardner *et al.*, 1997). The key switch between apoptosis and necrosis seems to be controlled by mitochondria. This is clearly important under toxicological conditions and may also be important physiologically.

Low-dose oxidants can trigger the mitochondrial permeability transition, release of both cytochrome *c* (Cai *et al.*, 1999) and apoptosis inducing factor (AIF) (Susin *et al.*, 1996), and, therefore, activate apoptosis. Thiol antioxidants block these effects of the oxidants:

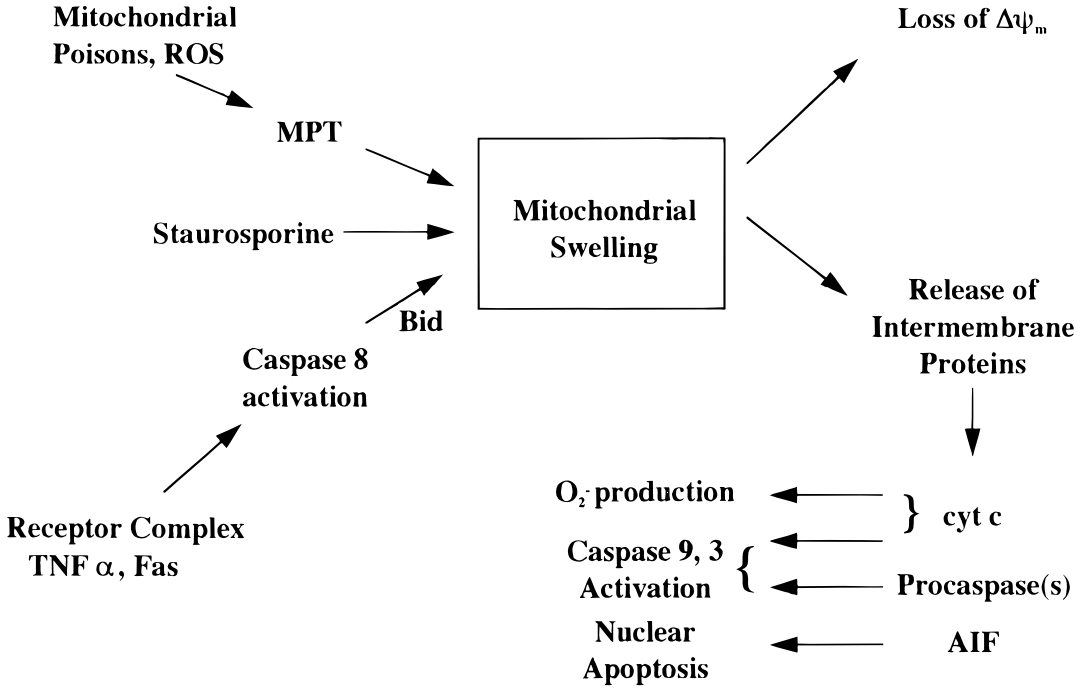

Fig. 1. Central role of mitochondria in apoptosis. Apoptosis can be induced by divergent upstream signals, which result in mitochondrial swelling and breakdown of the outer membrane. The release of mitochondrial intermembrane proteins both initiates caspase activation and triggers ROS generation.

High-dose oxidants can severely damage mitochondrial energetic function, resulting in acute energetic failure and a dramatic decrease of cellular ATP level. Since the activation of caspases by the apoptosome seems to require ATP (or dATP) (Liu *et al.*, 1996), a drop in the ATP level may delay the caspase activation and, therefore, cause the cells to undergo necrosis before apoptosis. Thus, an additional complication in interpretation of redox signaling is that there can be no simple dose response characteristic of activation of apoptosis in response to oxidants, because of the presence of the qualitatively different necrotic response.

A second mechanism for activation of apoptosis that is redox sensitive is that mediated by apoptosis signaling kinase (ASK-1). ASK-1 is inhibited by reduced thioredoxin (Saitoh *et al.*, 1998) and, thus, any oxidant that causes the oxidation of thioredoxin can be expected to activate apoptosis by activating ASK-1. While this activation mechanism has not been well established for any specific case of oxidant-induced apoptosis, the redox characteristics made it particularly attractive for playing such a role (see below).

A third mechanism for activation of apoptosis that appears to involve redox signaling is the Fas receptor/Fas ligand (Fas/Fas L) system. FasL expression in Jurkat and microglial cells can be upregulated by hydrogen peroxide, via a mechanism probably involving transcriptional regulation by NF-κB (Bauer, *et al.*, 1998; Vogt *et al.*, 1998). Antioxidants can inhibit the FasL expression after T cell receptor ligation (Bauer *et al.*, 1998). Thus, in principle, a physiologic level of oxidative stress could upregulate Fas L expression and signal an enhanced sensitivity to activation of apoptosis.

Evidence is also available for a rapid and transient generation of ROS in response to Fas activation, which may be produced by a plasma membrane-associated NAD(P)H oxidase (Suzuki *et al.*, 1998). In anti-Fas treated B cell lymphoma BJAB cells, a very early ROS generation was detected using luminol assay. The signal was detected within 20 sec and reached maximum at 5 min. Pretreatment of cells with a flavoprotein inhibitor, diphenylene iodonium chloride (DPI), inhibited its generation. These findings provide evidence for the involvement of redox regulation during the initial Fas-receptor mediated caspase activation that occurs at the plasma membrane level. However, because this is only a transient increase in ROS generation, it is not clear whether it functions in apoptosis signaling or it is an unrelated activity. Growth signaling via Ras is known to involve generation of superoxide by a membrane NADPH oxidase (Shatwell and Segal, 1996; Irani *et al.*, 1997). Thus, the ROS generation associated with Fas activation may reflect another plasma membrane-associated signaling response that is coincidentally activated by Fas oligomerization.

While redox signal seems to be important in regulating the early signaling of apoptosis, it probably has nothing to do with the autocatalysis cascade of caspases. This is supported by the finding that antioxidants, including NAC, PDTC, and spin-trapping reagents, do not inhibit apoptosis in HL 60 cells treated with staurosporine (Cai and Jones, 1998). With *in vitro* reconstitution systems, it has been shown that the redox state of cytochrome *c* was not relevant to its ability to initiate the activation of caspases (Kluck *et al.*, 1997; Hampton *et al.*, 1998).

In summary, available evidence indicates that activation of apoptosis by several mechanisms is redox sensitive. Among these, mitochondrial activation of apoptosis is involved and MPT appears to directly involve ROS and/or thiol oxidation. Oxidation of thioredoxin appears to allow activation of ASK-1 and upregulation of Fas L is signaled by oxidants. Thus, although the central caspase cascade is not redox activated, upstream signaling can be activated by oxidants and inhibited by thiol-reductants, rendering the overall process redox dependent.

IS REDOX SIGNALING A CONSEQUENCE OF CASPASE ACTIVATION?

Apoptosis-associated redox-changes can be gauged by different methods, including measurement of cellular thiol-disulfide redox change, usually a loss of reduced glutathione (GSH) (Cai and Jones, 1998; Marchetti *et al.*, 1997), lipid peroxidation using indicators such as *cis*-parinaric acid (Hockenbery *et al.*, 1993) and acridine orange (Polyak *et al.*, 1997), superoxide generation determined by luminol assay (Suzuki *et al.*, 1998), and ROS generation using ROS-sensitive dyes, such as dihydrorhodamine (Rothe *et al.*, 1991) and dichlorofluorescin (Hockenbery *et al.*, 1993).

As indicated above, an early redox signal is associated with activation of the Fas system. This signal was detected by luminol, indicating that is a ROS. Studies of changes in cellular GSH have revealed that GSH is commonly decreased during apoptosis (Malorni *et al.*, 1993; Van den Dobblesteen *et al.*,

1996). Although initially interpreted as being due to oxidation, more recent studies have suggested that the loss of GSH involves a specific export mechanism, which is downstream of caspase activation.

In anti-Fas treated Jurkat T cells, a rapid efflux of intracellular GSH into the culture medium was detected (Van den Dobbelsteen *et al.*, 1996). This process started as early as 30 min and $>$ 90% GSH was in the medium after 3 h. Under similar conditions, caspase-8 was activated within minutes after Fas receptor oligomerization (Scaffidi *et al.*, 1998). Co-treatment of cells with a general caspase inhibitor, z-Val-Ala-Asp-chloromethylketone, inhibited both GSH efflux and apoptosis, indicating the loss of GSH was secondary to receptor-mediated caspase-8 activation. Although no direct evidence is available, a plasma membrane-associated GSH specific transport mechanism was proposed to be responsible for the efflux of GSH.

Since GSH is one of the most abundant water-soluble antioxidants inside the cell, the loss of GSH will certainly decrease the cellular redox buffer capacity and sensitize the affected cells to any further oxidant signal. However, glutathione exists in both a reduced and an oxidized form (GSSG), and the relative concentrations of these forms determine the cellular GSH/GSSG redox state. An efflux of GSH, therefore, does not necessarily mean that the cells have been oxidized.

Direct evidence showing the generation of ROS from mitochondria during apoptosis has been recently obtained. In HL60 cells treated with staurosporine, mitochondrial cytochrome *c* was released well before the caspase activation and mitochondrial depolarization, which left an energized mitochondrial electron transport chain with compromised components. The efficiency of the normal electron transport is high so that usually $>$ 98% (Chance *et al.*, 1979; Hockenbery *et al.*, 1993) of the electrons are transferred with a four-electron reaction to O_2 to generate water. However when cytochrome *c* is lost from mitochondria, electron flow through the respiratory chain will be blocked at the ubiquinol:cytochrome *c* oxidase site; this results in electron transfer to oxygen by a one-electron reaction, to produce superoxide (Fig. 2). Because of the ubiquitous presence of superoxide dismutase, enhanced superoxide results in increased hydrogen peroxide and oxidation of glutathione by the glutathione peroxidase reaction.

In mitochondria isolated from HL60 cells treated with staurosporine, superoxide generation was detected after cytochrome *c* was released and substrate-stimulated oxygen consumption was inhibited (Cai and Jones, 1998). Mitochondria isolated from cells overexpressing Bcl-2 showed neither cytochrome *c* loss, nor the production of superoxide. When isolated mitochondria were treated with KCN to inhibit the electron transfer to oxygen, a much more significant generation of superoxide production was detected in both control and Bcl-2 overexpressing mitochondria. Therefore, consistent with previous reports, the apparent antioxidant function of Bcl-2 can not be attributed to a direct radical scavenger effect. Instead, Bcl-2 blocked mitochondrial superoxide production by inhibiting cytochrome *c* release.

Since cytochrome *c* release is a general mechanism in signaling of apoptosis, this mitochondria-based redox signaling mechanism could well be extrapolated to other systems. In combination with the observation that GSH efflux is stimulated in a caspase-dependent manner, these results indicate that GSH loss and enhanced mitochondrial ROS generation combine to produce a substantial, sustained oxidation in cells in a process that parallels activation of caspase cascade. The function of this signal is not clear but may be to provide a proper context for efficient execution of the later phases of apoptosis and ultimately to provide a mechanism for termination of the process (Fig. 3).

THE FUNCTION OF REDOX CHANGE DURING APOPTOSIS

The intracellular redox change can be measured and interpreted by using the redox potential E_h, as calculated from the Nernst equation, using concentration of GSH and GSSG. Normal cells have a redox potential around -240 mV. In HL 60 cells treated with staurosporine, there was a nearly 50 mV oxidation after cytochrome *c* was released (Cai and Jones, 1998). This magnitude of change principally will shift the protein vicinal dithiols (with E_0 in the appropriate range) to a much more oxidized state and, thereby, affect their function. However, little information is available regarding the altered protein functions caused by apoptosis-associated cellular oxidation.

Thioredoxin is a possible candidate. It has a cysteine-X-X-cysteine motif at its active center (Powis *et al.*, 1998). The two cysteines can form a disulfide bond and this loop structure can be opened or closed, based upon the redox state of the protein. The redox potential of thioredoxin is -260 mV and, within normal proliferating cells, is approximately half reduced and half

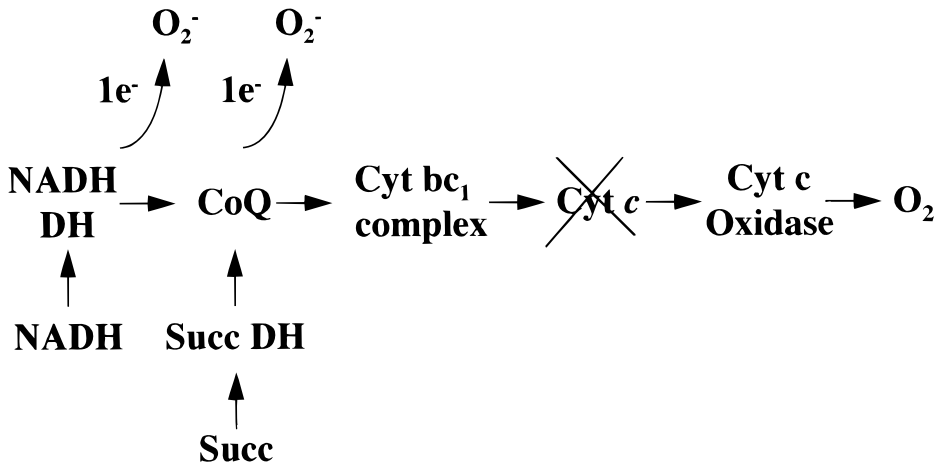

Fig. 2. Loss of mitochondrial cytochrome *c* results in stimulated superoxide production. Release of cytochrome *c* before the mitochondrial permeability transition blocks electron flow at the cytochrome *c* oxidase. Superoxide can be generated from both NADH dehydrogenase complex and coenzyme Q (CoQ) site.

oxidized. During apoptosis, where cellular redox potential is oxidized to −200 mV and below, thioredoxin will be almost fully oxidized. As a consequence, any protein whose function is regulated by the reduced form of thioredoxin will be also affected. One such protein that is relevant to apoptosis is ASK-1.

ASK-1 was genetically cloned by degenerative PCR (Ichijo *et al.*, 1997). Later it was found to play critical roles in both TNFα (Ichijo *et al.*, 1997)- and Fas (Chang *et al.*, 1998)-mediated apoptosis, as indicated by the observation that the dominant negative form of ASK-1 can block these two processes. ASK-1 has been shown under the redox control by thioredoxin (Saitoh *et al.*, 1998), which may provide an answer to how antioxidants can protect cells from TNFα. Although no data are available at the present time, one possibility is that TNFα can trigger mitochondria to generate a redox signal, which, in turn, oxidizes thioredoxin and releases its inhibition of ASK-1. A relatively long delay between TNFα treatment and caspase activation, together with the early mitochondrial change, support that possibility (Bradham *et al.*, 1998). Thus, this redox activation of ASK-1 could function in parallel with the release of mitochondrial intermembrane proteins to provide an alternative apoptosis signaling pathway.

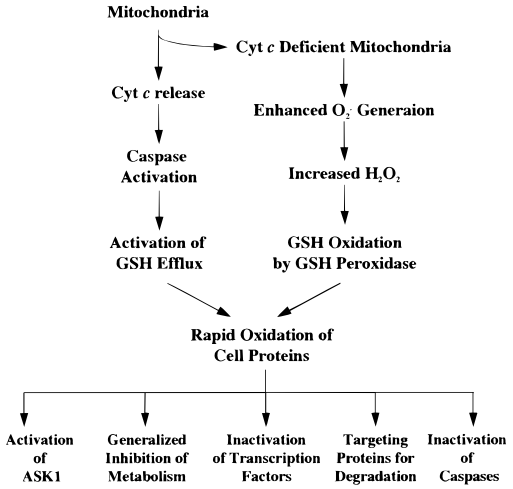

Fig. 3. Generation and possible functions of mitochondria-mediated oxidation of the cellular thiol-disulfide pools. Enhanced generation of superoxide from mitochondria, together with activation of GSH efflux, dramatically oxidizes the cellular redox state and provide a separate signaling pathway that is in parallel with the caspase cascade.

Such a pathway could explain the apparent caspase-independent apoptosis. Pretreatment of cells with zVAD-fmk prevented apoptosis induced by staurosporine, VP- 16 and actinomycin D. However, they still died very late, apparently via a process independent of caspase (Amarante-Mendes *et al.*, 1998). Although the redox change was not measured, the release of mitochondrial cytochrome *c* by these inducers should result in a dramatic oxidation. Thus, this caspase-independent atypical apoptosis could be a consequence of oxidative changes.

Besides these direct effects, the intracellular oxidation after mitochondrial cytochrome *c* release may feed back positively on the mitochondria and amplify

the initial apoptotic stimuli by triggering the mitochondrial permeability transition (MPT). MPT pore complexes are localized at the contact regions of mitochondria, where inner and outer membranes contact with each other directly (Zoratti and Szabo, 1995). With isolated mitochondria, agents that activate MPT induce nuclear changes characteristic of apoptosis (Susin *et al.*, 1998). Oxidants are well-known activators of the MPT (Zoratti and Szabo, 1995). Mitochondrial transmembrane potential is lost in cells undergoing apoptosis. Cyclosporin A, which inhibits MPT at a concentration much lower than that required for inhibition of phosphatase function of calcineurin, inhibits TNFα-induced MPT and apoptosis (Bradham *et al.*, 1998). Thus, a general function of an oxidant signal during apoptosis may be to amplify the signal by activating additional mitochondria to undergo the MPT.

Introduction of an oxidizing environment into apoptotic cells may also provide a mechanism to enhance the efficiency of late events of apoptosis. Oxidation results in targeting proteins to proteosomes for degradation (Davis and Goldberg, 1987; Grune *et al.*, 1997). Oxidation results in targeting mitochondria to lysosomes during reticulocyte maturation (Wiesner *et a1*., 1990). Oxidation of erythrocyte membrane proteins results in removal of the erythrocytes by macrophages (Sambrano *et al.*, 1994). Thus, one may surmise that oxidation is an efficient way to enhance removal of protein components in a cell targeted for removal.

Oxidation is also an efficient way to terminate metabolic pathways. About half of all enzymes have critical thiol groups and most transcription factors have critical thiols. Therefore, oxidation provides a general inactivation mechanism for much of the chemistry of the cell. Of special interest, caspases have critical cysteine groups and are inactivated by oxidants (Hampton and Orrenius, 1997; Nobel *et al.*, 1996). Thus, activation of oxidative mechanisms during the terminal phase of apoptosis provides a built-in mechanism to autoinactivate the key death proteases, thus, preventing them from damaging other cells.

SUMMARY AND CONCLUSIONS

While it is becoming clear that the central biochemical pathway for apoptosis, the caspase cascade, does not require redox signaling for execution of the death command, abundant evidence is available to indicate that apoptosis is often redox sensitive. This sensitivity is due, in part, to the presence of multiple oxidant-sensitive activation mechanisms. In addition, the mitochondrial release of cytochrome *c*, which is a common signaling event in caspase activation, triggers a switch in electron transport that results in substantial mitochondrial superoxide generation. This process results in oxidation of cellular thiols, inactivating cellular metabolic pathways, marking proteins for degradation, and providing a mechanism for ultimately inactivating the death proteases and terminating apoptosis. Thus, although redox signaling does not appear to be an obligatory component in the caspase cascade, it is of fundamental importance to certain activation mechanisms and is central to the efficient execution of the terminal phase of apoptosis.

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