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FREE RADICALS AND APOPTOSIS: RELATIONSHIPS WITH GLUTATHIONE, THIOREDOXIN AND THE BCL FAMILY OF PROTEINS

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1. ABSTRACT

Cellular fate is controlled by a number of factors within the cell, including an abundance of, and defenses against, free radicals generated both endogenously and exogenously. Free radical species are involved in regulating various growth, differentiation and death processes including apoptosis. Apoptosis is a preferred form of cell death because it is highly ordered resulting in the death of a cell with minimal effects on surrounding cells or tissues. Radicals generated during apoptosis directly modulate signaling cascades by activating or inhibiting survival transcription factors (i.e. NF-kappa B and AP-1), or more indirectly affecting such signaling by changing the cellular redox status [i.e. glutathione (GSH) and thioredoxin (Trx)]. At high levels, free radicals, including reactive oxygen species and various unwanted and harmful byproducts of reactions with tissue macromolecules, particularly lipids, can cause acute injury if not hindered by cellular antioxidants. These antioxidant protective systems are not only involved in preventing stress, but also maintaining the normal functioning of specific transcription factors and the bcl proteins. This review will discuss the association of reactive oxygen species with GSH, Trx and bcl proteins in apoptosis.

2. INTRODUCTION

The possibility that reactive oxygen species (ROS) play a significant role in apoptosis has been reviewed numerous times (1,2) and a relationship between free radicals and several effectors of apoptosis has been reported. A link between apoptosis, free radicals and the bcl-2 family of protooncogenes has also been raised, and is supported by several studies indicating that the proteins encoded by some of the anti-apoptotic bcl-2 family members suppress apoptosis induced by various oxidative processes (reviewed in 3). In addition, various antioxidants and antioxidant enzymes appear capable of preventing apoptosis induced by a variety of agents (4-6).

Radical species may either directly or indirectly mediate processes that lead to altered cell growth, differentiation and death (2,7,8). Although high levels of ROS can cause acute necrotic injury, lower levels can have effects on growth factors or alter gene expression and induce apoptosis (9). Hydrogen peroxide (H_2O_2) induces apoptosis in several different human tumor cell lines (10), and fas-induced apoptosis is accompanied by the production of ROS (11). There is also extensive evidence that oxidants are formed upon the induction of apoptosis by many xenobiotics including agents that are not obviously linked to free radical production (12). The goal of this review is to examine some of the intricate pathways involved and how cells are affected by oxidant-induced apoptosis in terms of the glutathione (GSH), thioredoxin (Trx), and bcl systems.

3. APOPTOSIS

It is important to point out that the choice of *in vitro* models for examining toxicant responses may lead to very different outcomes. For example, while isolated cells are excellent and inexpensive alternative models to whole animals for examining signaling mechanisms leading to apoptosis, it is vital to consider their source when analyzing experimental data. A majority of cell lines are derived from either immortalized cancerous tissues or have been transformed to become immortal. Therefore, the toxicant/toxin treatments used to initiate cell death must first overcome the cell's inherent ability to perpetually divide. These characteristics may explain the variation that is seen between different cell lines and between cell lines and primary cells when comparing the effects of the same injurious agent.

Throughout life and death, a cell's fate is controlled by a number of triggers and signaling pathways. One of the most important paths to death is known as apoptosis.

Apoptosis involves a series of well-organized events requiring active cell participation and is the basis for normal tissue remodeling as well as the result of certain toxic insults (13). Apoptosis [characterized by cell shrinkage and localized, non-inflammatory death] differs morphologically from necrotic cell death [overall cell swelling and inflammation (14)] and several genes have been identified as controlling apoptosis in different species (15).

Apoptosis is both a harmful, as well as a beneficial process. It is necessary, for example, in controlling death during embryonic cell development, during normal cell turnover, and as the immune system responds to different stimuli. Apoptosis is also important in the termination of critically damaged cells that have been exposed to toxicants. However, excess apoptosis can have adverse consequences as occurs in neurodegenerative diseases and autoimmune disorders. Several common biochemical markers of apoptosis include phosphatidylserine externalization to the outside of the plasma membrane, nucleosomal DNA cleavage into 180 base pair fragments, and caspase-3 activation (16, 17). However, examples of atypical apoptosis exist where one or more of these markers do not occur.

4. FATTY ACID OXIDANTS AND APOPTOSIS

While a requirement for life, the metabolism of oxygen also yields reactive and partially reduced byproducts, known as ROS, which can cause unwanted stress. ROS and related free radicals can also be generated by environmental factors (cigarette smoke, automobile exhaust, chemicals in food, or drinking water), aging and a number of disease states (Alzheimer's, Parkinson's, ischemia, and amyotrophic lateral sclerosis), as well as through immune responses (via phagocytes and neutrophils) (18, 19). Major ROS in biological systems include the superoxide anion, H_2O_2, hydroxyl radical and peroxynitrite. Interestingly, there is a constant low level of free radicals in the body that seems to be important in signaling. Maintaining this low level of oxidants, a "tonus", is critical for normal function. A delicate balance of these free radicals is required since excess radicals will disrupt signaling, and at sufficient levels cause damage to DNA, lipids and protein, triggering a cascade of overt tissue damage. Cells protect themselves from such damage by using specific enzymes such as superoxide dismutase, catalase, glutathione peroxidase, and the peroxiredoxins, among others. Protection is also achieved with a number of chemical antioxidants including tocopherol (vitamin E), ascorbate, GSH and urate (20).

Fatty acids that are neither bound to proteins nor esterified may also yield oxidative species that are toxic to cells. For example, in Jurkat (a human T cell line) and Raji (a human B cell line) cells, fatty acid toxicity is related to both the chain length and the degree of unsaturation. The longer chain, more unsaturated species are the most toxic (21). In part, oxidized lipid products are thought to mediate this toxicity (22-27). A report that apoptosis induced by the lipoxygenase (LOX) inhibitor nordihydroguaiaretic acid (NDGA) is accompanied by the rapid stimulation of lipid peroxidation (28) also supports the hypothesis that the generation of lipid hydroperoxides, rather than inhibiting LOX activity, may be an important initiating stimulus to apoptosis. However, the ability of antioxidants such as pyrrolidinedithiocarbamate and N-acetylcysteine to induce apoptosis in vascular smooth muscle cells (29) indicates the complexity of redox reactions in controlling apoptosis among different cell types. Furthermore, saturated fatty acids such as palmitate (possibly mediated through ceramide), that cannot be peroxidized, can induce apoptosis (30-31).

The mechanisms by which oxidants induce apoptosis are unknown, but may involve the generation of some signaling factor in addition to more direct damage. Since polyunsaturated fatty acids are highly susceptible to oxidation, lipid messengers or lipid peroxides that can induce apoptosis are reasonable candidates to be such signaling factors (1, 27, 32, 33). Fatty acid oxidation can involve both enzymatic and non-enzymatic pathways. LOX catalyzed arachidonic acid metabolites such as leukotrienes, or hydroperoxy- or hydroxyeicosatetraenoic acid (HPETE or HETE) are potent biological mediators that regulate cell proliferation and apoptosis (34). LOX inhibitors suppress tumorigenesis and/or tumor cell proliferation (35) and induce apoptosis in many tumor cell systems. In direct contrast, other investigators have found that LOX activity and products were increased during apoptosis in three different cell lines (22).

Most of the conclusions implicating LOX and LOX products in apoptosis have relied on inhibitors with unclear specificity. More recent work has clearly shown that pathways independent of LOX are involved (36-41). The apoptosis-inducing activity of cyclooxygenase-2 (COX-2) inhibitors similarly appears to be independent of actions on COX-2 (42-44). On the other hand, LOX and COX-2 products do have effects on apoptosis, and blocking their production influences apoptosis in some systems, suggesting that not all apoptotic effects in all systems are independent of these enzymes (45, 46).

Recent data have indicated that the ability of COX-2 inhibitors to block PDK1 and thus Akt activation is important in the induction of apoptosis (47, 48), and LOX inhibitors also inhibit this kinase (Kehrer, unpublished data). Interestingly, the action of COX-2 inhibitors on Akt appears to be independent of both *bcl-2* (47) and *bcl-xL* (48). Another possible mechanism of LOX/COX-2 inhibitor-induced apoptosis is related to the fatty acid substrates of these enzymes. Fatty acid transport proteins have been postulated to participate in signal transduction pathways and in fatty acid regulation of gene expression, cell growth, and cell differentiation (49-51). Disrupting the binding of fatty acids to binding proteins, as well as to fatty acid metabolizing enzymes such as LOX and COX-2 may, therefore, increase intracellular levels of unbound fatty acids that can stimulate apoptosis. This concept is supported by studies showing an increase in unbound fatty acids after treatment with COX-2 (52) or LOX (53) inhibitors. Furthermore, arachidonic acid has been

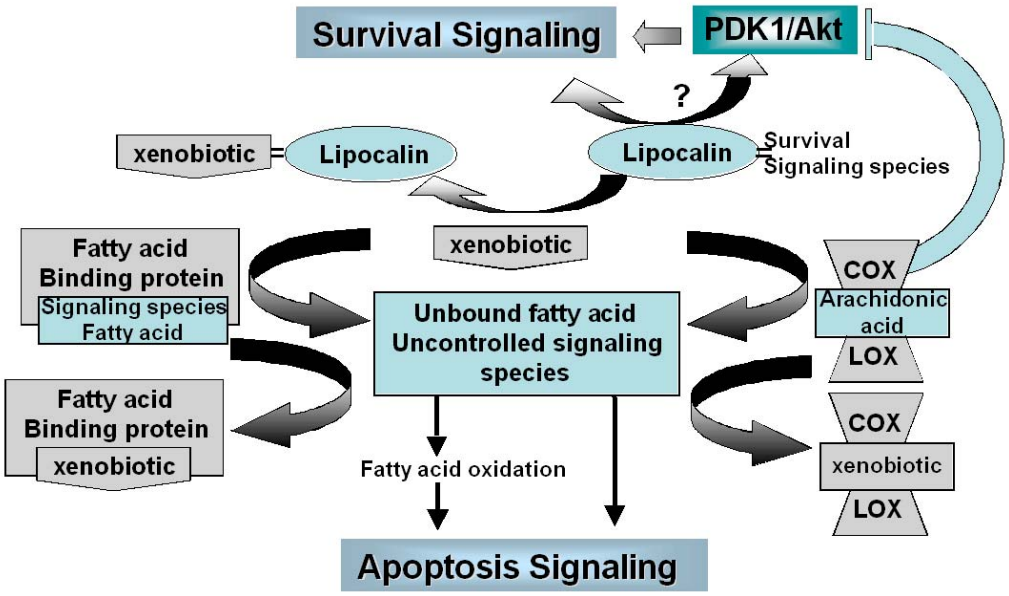

Figure 1. Apoptosis may occur through the xenobiotic-induced release of unesterified fatty acids and related species from protein binding sites. This may result in abnormal signaling, either directly by the released fatty acid or subsequent to their oxidation to bioactive species. Propagation of such abnormal signals may activate various apoptotic pathways, or inhibit survival pathways.

proposed to be a physiological mediator of apoptosis (54) and a recent study indicates that downregulating at least one extracellular fatty acid binding protein (lipocalin) increases apoptosis (55). The increase in the 24p3 lipocalin in murine FL5.12 cells treated with MK886 (39) may be explained by a blockade of this lipocalin, resulting in feedback effects, again supporting this concept. Figure I illustrates this mechanism.

Fatty acids have differential functions. While unesterified fatty acids that are not protein bound are generally toxic, some studies have shown that they can decrease apoptosis. For example, while palmitate (16:0) increases apoptosis, oleate (18:1) decreases apoptosis in breast cancer cells (56). In addition, arachidonic acid (20:4) can decrease apoptosis in W256 carcinosarcoma cells (57). These protective effects have been proposed to involve LOX and/or COX enzymes. On the other hand, as described above, it has also become clear that inhibitors of both COX-2 and LOX enzymes induce apoptosis by pathways independent of these inhibitory actions.

More recently a role for cardiolipin in apoptosis has been proposed by Iverson and Orrenius (58). In particular, these authors have suggested that cytochrome *c* associates strongly with cardiolipin in the inner mitochondrial membrane, and that release can only occur when the cardiolipin-cytochrome c association is broken through oxidative reactions. Cardiolipin contains unsaturated fatty acids as acyl moieties. These may become oxidized and contribute to the breaking of this interaction.

5. GLUTATHIONE AND APOPTOSIS

A main function of the ubiquitous tripeptide GSH (gamma-glutamyl-cysteinyl-glycine) is to protect the cell against damage induced by electrophiles, ROS, antineoplastic agents, and a variety of other stress-initiated conditions (59-61). GSH is present in low mM (0.5-10 mM) concentrations in the cytosol, and is also found to a lesser extent in the nucleus and within mitochondria. These different pools are somewhat connected, but at least in terms of mitochondria are also manipulated independently through localized glutathione reductase/peroxidase (62).

GSH is normally present in a cell 98 to 99% in the reduced form, with the remainder largely as glutathione disulfide (GSSG) or as mixed disulfides. Glutathione is mainly formed through a two-step, energy dependent *de novo* synthesis. Cysteine is taken up by a cell, where it unites with glutamate via gamma–glutamylcysteine synthetase and is then further combined with glycine via GSH synthetase to form GSH. Small variations in intracellular GSH in response to an oxidative stress will have a large impact on GSSG and can cause profound effects on thiol redox-dependent cell signaling. The regeneration of GSH from GSSG at the expense of NADPH through the action of glutathione reductase works to prevent this occurrence. Excess GSSG can be exported out of a cell to maintain the high ratio of intracellular GSH/GSSG. This exported material may also be degraded and taken up by cells for future regeneration of intracellular GSH. The gamma-glutamyl bond of GSSG is broken by gamma-glutamyltranspeptidase present on the external plasma membrane followed by cleavage of the cysteinyl-glycine bond by cell surface dipeptidases. The free amino acids are then available for import into a cell for processing and regeneration of GSH.

While important in maintaining a reduced cytosolic environment, GSH also aids in electrophile removal through glutathione S-transferase (GST)-mediated

conjugation reactions and subsequent elimination of the conjugate via export pumps through the bile or kidney (63). Several studies have shown that GSH is rapidly exported from cells stimulated to undergo apoptosis. The bcl proteins may control this process (64-67), although the mechanistic importance of GSH in apoptosis remains to be demonstrated.

Overall, GSH serves many roles in defending a cell against adverse insults. Therefore, a loss of GSH, such as seen following an electrophilic insult, may permit toxicant interaction with other important and less abundant cell entities. Similarly, by virtue of its role as a cofactor for glutathione peroxidase, GSH is involved in the reduction of lipid hydroperoxides (which induce apoptosis) to alcohols (which do not). This suggests that losing GSH may facilitate, but is not required, for apoptosis.

As mentioned earlier, antioxidants can inhibit the development of apoptosis following treatment with various initiators- including non-oxidants. There is an interesting correlation in numerous cells between the levels of GSH and resistance or induction of apoptosis. One explanation for apparent discrepancies in the role of GSH and oxidants is the report that the oxidative tonus of cells exposed to anti-fas antibody (to induce apoptosis) is increased by the stimulation of GSH efflux, not by the formation of ROS (66). Supplementing these cells with GSH did not prevent apoptosis, possibly due to rapid efflux, a concept supported by the finding that decreasing the efflux process also decreased apoptosis (67). On the other hand, antioxidants may provide some protection from apoptosis by slowing oxidative processes that proceed in the absence of adequate GSH (68). Similarly, an increased level of oxidized lipids that may accompany a loss of GSH may trigger the apoptotic machinery as the cell responds to damage that has occurred.

In addition to Jurkat T lymphocytes (66), apoptosis has been reported after depleting GSH with diethyl maleate (DEM) in polymorphonuclear leukocytes (69) and with buthionine sulfoximine in fibroblasts (70) and pro-B cells (71). A link between GSH and apoptosis has also been reported in thymocytes (72). However, while the extent of GSH depletion has been suggested as a determinant of whether or not apoptosis occurs (73), GSH levels alone do not appear to be critical for the induction of death in GT1-7 cells since depletion with DEM, which causes 100% loss of viability in control cells, does not induce any death in cells that over-express bcl-2 (74). Apoptosis *per se* was not assessed in this study and the massive death in control cells is suggestive of a gross disruption of function. DEM was also reported to induce apoptosis in L929 transformants stably overexpressing human Fas (75). Once again, the assay for viability was not specific for apoptosis and the 100% loss of viability within 3 to 6 hours after treatment with DEM is not consistent with effects solely on GSH. In fact, DEM is able to alkylate a number of sites resulting in the inactivation of NF-kappa B (76), and the loss of Trx-1 (Kehrer laboratory, unpublished data). The roles for NF-kappa B (77) and Trx (described below) in apoptosis suggest the effects of DEM on GSH may not be the critical determinant of apoptosis. Overall, depleting GSH can enhance the apoptosis seen with many xenobiotics, but alone appears to be insufficient to induce massive apoptosis unless extensive depletion is maintained for long periods of time. However, the high concentrations of GSH typically found in cells clearly demonstrate that it plays a very important protective role for numerous cellular processes.

6. THIOREDOXIN AND APOPTOSIS

Cell growth is dependent upon a reducing intracellular environment. While GSH is present within the cell in mM concentration and is a major factor in maintaining this state of reduction, Trx, another thiol essential for cell growth, is present in only μM concentrations. This large quantitative difference suggests that Trx is less significant than GSH in terms of general antioxidant activity. It is important to note, however, that Trx plays more explicit roles in the redox control of cell signaling and apoptosis than does GSH, particularly through regulating transcription factors and kinase cascades.

Trx was originally discovered in *E. coli* by Laurent *et al.* (78), and was later identified as being identical to several other redox proteins in eukaryotic cells. Interestingly, Trx is present in life forms from *E. coli* to plants to humans, demonstrating its importance in all forms of life. The Trx family is comprised of several proteins functioning in different locations inside and out of the cell (79-81). In eukaryotic species, the most abundant Trx is the cytosolic/nuclear thioredoxin-1 (Trx-1; 12 kDa). There is also an amino terminal extended mitochondrial thioredoxin-2 (Trx-2; 18 kDa), as well as minor forms including a Trx-like protein called $p32^{Trx}$ (32 kDa), carboxy terminal truncated Trx-1 which localizes to the membrane and functions as a secretory cytokine (8-10 kDa), a sperm-specific thioredoxin (SpTrx, 90 kDa) and a thioredoxin-related protein (TRP14, 14 kDa).

The Trx active site contains a redox-active dithiol that can be oxidized to the corresponding disulfide. Thioredoxin reductase (TR) converts oxidized Trx (with the assistance of $FADH_2$ and NADPH) to reduced Trx, a selenium dependent flavoprotein, endogenously found as a homodimer (Figure 2). Like the Trx family, TR family members are distributed in various locations throughout the cell. Cytosolic (TR1) and mitochondrial (TR2) forms are present to maintain this small peptide in the reduced form, which predominates in intact cells (82). A novel TR, thioredoxin and glutathione reductase (TGR), capable of reducing both oxidized Trx and GSSG, was found in the testes thus establishing a link between the Trx and GSH families (83). *In vitro*, GSH also recently has been shown to directly conjugate to Cys-73 of Trx (84), although this interaction remains to be seen in cells.

Trx-1 contains no known nuclear localization or nuclear export sequences but can translocate into the nucleus in cultured cells in response to H_2O_2 (85), hypoxia (86), phorbol esters (87-88), tumor necrosis factor (88), ultraviolet irradiation (87, 89), ionizing radiation (90),

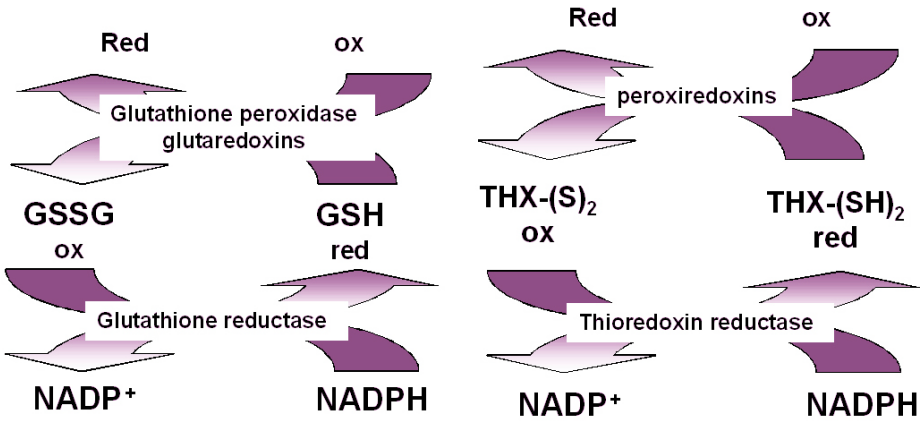

Figure 2. Both GSH and Trx contain redox dependent cysteines that are readily oxidized by endogenous or exogenous species. Enzymatic oxidation occurs through either glutathione peroxidase and glutaredoxins for GSH or the peroxiredoxins for Trx. Both GSH and Trx are reduced from their oxidized state by respective reductase enzymes that use NADPH as a cofactor.

interleukin-1beta, (91), lipopolysaccharide (91), and cisplatin (92). A similar translocation occurs in intact animals following ischemia-reperfusion injury in the brain (93) and free radical-mediated kidney toxicity (94). Interestingly, all of these stimuli involve free radicals and also can cause apoptosis. It is thus possible that nuclear Trx increases in response to stress as a compensatory or protective factor and that it may modulate various apoptosis pathways, in particular those mediated by redox-sensitive transcription factors that are affected by Trx-1.

Trx has important influences on how cells respond to toxicants, especially oxidants and electrophiles (95). Multiple mechanisms are likely involved in these responses, particularly effects on various redox-regulated signaling pathways. Direct scavenging of reactive species by Trx is certainly chemically feasible, but since Trx is present at much lower concentrations than other endogenous antioxidants and nucleophiles such as GSH, it seems unlikely to play a major role. On the other hand, Trx-1 plays a direct role in reducing peroxides as a cofactor for the peroxiredoxins (96, 97). Together with its function as a cofactor for ribonucleotide reductase and methionine sulfoxide reductase, it is clear that Trx-1 contributes to cellular repair and oxidant defense systems (79), and that these effects help determine the outcome of some toxic insults.

Besides the activities described above, Trx can serve as an electron donor in the endoplasmic reticulum to aid in the rearrangement of intra- and inter-chain disulfide linkages until proteins reach their native conformation prior to protein disulfide isomerase (PDI) completing the folding process. PDI is a homodimer consisting of a component that is homologous to, and that contains a cysteine disulfide linkage similarly to Trx, although it has not been named as a Trx family member at this time (98).

The roles of Trx appear integral to cell survival and it is not surprising that Trx-1 null mice died as embryos shortly after implantation (99) and that the downregulation of Trx-1 enhances sensitivity of cells to toxicant treatment in WEHI lymphoma cells (100). Deleting Trx-2 is also lethal to embryonic mice. Lethality appeared at the mid-point of development when oxidative phosphorylation began, clearly establishing its association with the mitochondrion (101). This led the authors to conclude that the generation of ROS was the key event in cell death in Trx-2 null mice. Further, because fibroblasts cultured from Trx-2 null embryos were not viable and showed extensive apoptosis, it is clear that Trx-2 is an important antioxidant/antiapoptotic defense system. Trx-2 heterozygous animals appeared to function normally (101), but may be more susceptible to toxicants, although this has not been tested. Using a tet-repressible Trx-2 transgene in chicken B cells, an accumulation of ROS (2- to 3-fold) after 5 days and an increase in apoptosis to 45% of total cells by day 7 has been shown (102). The Trx-2 suppressed cells were also more susceptible to apoptosis induced by serum-withdrawal than were control cells. Overall, Trx plays many different pro-growth, as well as anti-apoptotic, roles *in vitro* and *in vivo* (103). Consistent with these functions, Trx overexpression has been noted in disease states such as AIDS (104, 105), rheumatoid arthritis (106), and hypoxic tumors (107).

The protective effects of Trx-1 and Trx-2 against oxidative stress and some xenobiotic-induced toxicities is indicative of a role for its antioxidant potential in apoptosis (108-113) and provides further support for the concept that oxidants are important proapoptotic species. Overexpression of Trx-2 also makes human embryonic kidney cells resistant to etoposide (114). The toxic mechanism of etoposide is considered independent of oxidative stress suggesting that Trx-2 can modulate some basic apoptosis signaling pathway(s), although Pham *et al.* (115) and Yokomizo *et al.* (116) demonstrated mitochondrial-related oxidative stress following etoposide treatment.

Mechanisms by which Trx-2 may function as an anti-apoptotic protein are by interacting with components of the mitochondrial respiratory chain, including cytochrome *c* (102) and by modulating mitochondrial potential (114, 117). In addition, there are numerous redox-sensitive control points during apoptosis that could

be affected by Trx or GSH. These include the caspases that require a reduced cysteine to be active (118) and apoptosis signal-regulated kinase-1 (ASK1), a kinase involved in the initiation of apoptosis. The reduced form of Trx-1 binds with ASK1 creating an inactive complex (119). Various apoptosis-inducing stresses (particularly oxidative) can break this complex, activating ASK1 and leading to the activation of c-Jun amino-terminal kinase (JNK)/ p38 and apoptosis (120, 121). Overall, Trx provides reducing equivalents for more specific redox-regulated signaling cascades than does GSH and thus, may offer a more selective target for future clinical therapies.

7. REDOX TRANSCRIPTION FACTORS AND APOPTOSIS

At least 64 redox-regulated transcription factors have been identified (122, 123). Several of these affect cell division and apoptosis, and have critical redox dependent cysteines in their DNA binding domains. Trx-1, and perhaps GSH, may provide mechanisms for maintaining their reduced and functional forms (reviewed in: 7, 124). Of specific interest has been the role of Trx to specifically regulate the reduction of NF-kappa B, redox factor-1 (Ref-1), p53, glucocorticoid receptors, hypoxia inducible factor 1alpha (HIF-1alpha), and the activation of other transcription factors involved in apoptosis (79, 107).

NF-kappa B and Activator Protein-1 (AP-1) are key components in maintaining cell growth and proliferation, as well as in regulating cellular protective defenses against pathogens, ROS, cytokines, inflammation, radiation, and immunological responses (125, 126). NF-kappa B is particularly susceptible to alkylation-related modification since it contains an essential cysteine (Cys62 of its p50 subunit) in its DNA binding domain. In general, activating NF-kappa B is considered to be anti-apoptotic. Cellular oxidation can lead to NF-kappa B activation (127). However, at the nuclear level, NF-kappa B must be reduced in order to bind to DNA. Although initial studies indicated that this reduction was mediated by GSH (128), it is now evident that Trx-1 is responsible (88).

NF-kappa B is found in an inactive form in the cytosol bound by inhibitor kappa B (I□appa B) proteins. In response to appropriate stimuli, I□appa B is phosphorylated, ubiquitinated, and subsequently degraded. Trx's roles in the regulation of NF-kappa B are to block the degradation of I□appa B in the cytosol, while reducing the p50 subunit in the nucleus, thus allowing it to bind DNA (88, 129). Trx's nuclear translocation mechanism is unclear, but it is thought to bind to a groove in the p50 subunit of NF-kappa B thereby translocating with p50 as it moves into the nucleus (130, 131). Subcellular co-localization of Trx and p50 following treatment with UVB irradiation supports this concept (88).

AP-1 is a collection of protein complexes (mainly comprised of homo- and heterodimers of c-fos and c-jun) that regulate transcription and apoptosis in response to various stimuli (132). Like NF-kappa B, cellular thiol redox state affects the activation of AP-1 (128, 133). This appears to involve a direct association between Trx-1 and Ref-1 that affects the transcriptional activity of AP-1 (87). AP-1 translocation into the nucleus is also affected by the stress-activated signaling pathway, particularly through the phosphorylation of c-jun by JNK following ASK-1 activation. Since these, and other, redox-regulated transcription factors have effects on cellular growth and death (134), one might expect Trx-1 to exert at least some of its effects through these key signaling pathways.

8. THE BCL PROTOONCOGENES

The mitochondrion is a major control point for caspase activation and apoptosis. Not only is it intimately involved in the generation of and protection against ROS, but many pro- and anti-apoptotic signals are recruited to the mitochondria, particularly those in the Bcl-2 protein family. These proteins are categorized by their Bcl-2 Homology (BH) domains. The Bcl-2 family contains pro-apoptotic proteins with one BH3 (bad, bik, blk, bid, hrd, bim/bod, bmf, noxa/puma) or two to three shared BH domains (bax, bak, bcl-x_S, bok, bcl-g_L), and antiapoptotic proteins with three to four shared BH domains (bcl-2, bcl-x_L, bcl-w, mcl-1, A1, boo). The BH3-only proteins heterodimerize in the BH1, BH2 and BH3 formed hydrophobic region of the anti-apoptotic proteins. The pro-apoptotic nature of BH3 domain-only proteins has recently been emphasized and more fully studied in initiating apoptotic cascades (135). Treatment of the non-lethal bim or bmf knockout mice demonstrate the absence of a typical apoptotic response, suggesting that the involvement of bim or bmf is required for normal apoptotic signaling (136, 137).

The most well-known and widely studied Bcl family member is the bcl-2 protein. The bcl-2 gene product is a hydrophobic 26 kDa protein that resides in cellular membranes including the nuclear envelope, endoplasmic reticulum and mitochondrion (138). Overexpression of bcl-2 protects cells from apoptosis induced by numerous agents (139). A related gene, bcl-x_L, has almost identical subcellular localization and effects as does bcl-2 (140). It has been suggested that the amount of bcl-2 alone is not critical in regulating apoptosis, but rather the balance between this protein and bax, a gene that encodes a dominant-inhibitor of bcl-2 (141). Many studies support this concept, although it does not appear to apply under all conditions. Protein expression studies by Hsu and Youle suggest that the bax/bcl-2 dimer is only formed in the presence of detergent (142) calling into question the significance of this dimerization. In general, the roles of the bcl-2 family of proteins are complex and involve not just simple interactions, but also homodimerization, pore formation, and the generation of truncated forms such as t-bid that can affect apoptosis by altering the structure and function of other family member proteins (143-145).

bcl-2 acts downstream from most initiating events, possibly by blocking signal(s) for apoptosis or by interfering with the products of apoptotic genes (146). The similarity between bcl-x_L and bcl-2 and bacterial pore-forming toxins (147) indicates that they may affect cell survival by regulating the permeability of cellular membranes to ions or proteins (148, 149). Control of the

mitochondrial permeability transition by bcl proteins has also been suggested as a mechanism by which they affect apoptosis (150).

Bcl-2 and bcl-x_L may provide protection against oxidants by shifting the cellular redox potential to a more reduced state (151). Bcl-2 seems to prevent cell death by decreasing the net cellular generation of ROS in a neural cell line (74). It has also been proposed that this gene inhibits UVA-induced apoptosis by suppressing the generation of specific UVA-mediated ROS (152). However, it is apparent that the bcl genes are not antioxidants per se as they do not react directly with free radicals (153) or lipid peroxides (25, 150) and they are not induced in response to oxidative stress (154). Mitochondria appear not to be the sole source of the radicals seen during apoptosis since overexpression of bcl-2 does not affect the function of this organelle (5) and bcl-2 blocks apoptosis in human fibroblast cells lacking mitochondrial DNA even though such cells lack a functional respiratory chain (156). Nevertheless, it is interesting that inducing very high levels of bcl-2 expression in PC12 cells causes oxidative stress and cell cycle arrest (157). The mechanism is not known, but may involve mitochondrial events.

It is possible to prevent apoptosis by overexpressing bcl-2 protein without altering the production of ROS that occurs upon withdrawal of serum (119) or addition of steroids (5, 158). This suggests that either the formation of apoptotic signals by ROS is blocked, or their ability to stimulate this cell death pathway is no longer functional. This latter effect may be due to maintenance of antioxidant enzyme activity (158) through the increase in GSH routinely seen in bcl-2 overexpressing cells (64, 159). This effect appears to be related to the ability of bcl-2 to inhibit methionine-dependent GSH efflux (160), and/or some other as yet unknown activity perhaps involving sequestration of GSH into the nucleus (65). This function is interesting as it explains the localization of bcl proteins in the nuclear membrane as well as the permissive role of GSH in apoptosis.

9. PERSPECTIVE

Apoptosis initiated by a number of diverse agents can be altered by the Trx and GSH systems as well as the bcl-2 and bcl-x_L protooncogenes suggesting some mechanistic commonality. Free radicals or oxidants have been widely suggested to be important stress initiating factors. However, many questions remain regarding their role. These questions include the fact that the protein product of bcl-x_L can form a membrane pore that is possibly able to transport larger molecules, including proteins; that bcl-2 may interact with lipids (161); and extensive data on the relationships among oxidized lipids, GSH, Trx and apoptosis. These findings are suggestive that changes in the intracellular redox environment are conducive to the initiation of apoptosis.

A number of pathways involved in apoptosis signaling are clearly affected by perturbations of the cellular redox state. The pathways discussed in this article are but a small part of those active in cells. However, they are important processes in maintaining life and preventing overt oxidative stress from causing cell death. There is much already known about these pathways, yet whether these or others are critical remains unknown. The potential roles for these pathways in numerous diseases including cancer, neurological diseases, and muscle paralysis, all of which contain some aspect of thiol related losses, mitochondrial dysfunction and apoptosis (or a lack of it) indicates that by fully understanding these pathways, we may create endless possibilities to better treat, prevent or even cure these and many other diseases.

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