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Forum Review

Redox Regulation of Apoptosis by Members of the TNF Superfamily

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ABSTRACT

Tumor necrosis factor (TNF), fibroblast-associated cell surface (Fas) ligand, and TNF-related apoptosis-inducing ligand (TRAIL), all members of the TNF superfamily, are arguably the most potent inducers of cell death. These cytokines induce cell death through sequential recruitment by the death receptors TNFR1-associated death domain protein (TRADD), Fas-associated death domain protein (FADD), FADD-like interleukin-1β-converting enzyme (FLICE), and downstream caspases. Increasing evidence indicates that mitochondria play a critical role in cytokine receptor-mediated apoptosis. There is also now ample evidence that apoptosis induced by TNF and its family members is mediated through the production of reactive oxygen intermediates (also known as reactive oxygen species). Here we review the evidence linking reactive oxygen intermediates to cytokine-induced cell death mediated by TNF-α/β, Fas, TRAIL, TNF-like weak inducer of apoptosis (TWEAK), and vascular endothelial cell growth inhibitor (VEGI). *Antioxid. Redox Signal.* 7, 482–496.

INTRODUCTION

TUMOR NECROSIS FACTOR (TNF) was the first cytokine discovered to kill tumor cells by apoptosis (programmed cell death). After the ligand binds to its receptor, this receptor–ligand complex induces several different cellular responses, consisting of cellular proliferation, differentiation, survival, apoptosis, and activation of nuclear factor-κB (NF-κB) and mitogen-activated protein kinases (MAPK) (3, 74). There are 19 ligands and 29 receptors that belong to the TNF superfamily (Table 1), including TNF-α and TNF-β (also known as lymphotoxin, LT), fibroblast-associated cell surface (Fas) ligand (FasL; also known as CD95L), TNF-like weak inducer of apoptosis (TWEAK), TNF-related apoptosis-inducing ligand (TRAIL), vascular endothelial cell-growth inhibitor (VEGI), and death receptor 6 (DR6).

Most of the TNF receptor superfamily members inducing apoptosis possess an intracellular death domain that recruits several signaling proteins for the onset of apoptotic signaling cascades via mitochondria-independent mechanisms, finally leading to caspase (cysteinyl aspartic acid-protease) activation. Deletion of the death domain abolishes apoptosis (61, 62). It has been reported that TNF-α-mediated apoptosis can be induced by multiple signals, such as phospholipases, ceramide, p53, c-myc, protein kinases, serine proteases, caspases, reactive oxygen intermediates (ROI), and cytochrome *c* release (97, 130). Today more and more evidence indicates that an intensive cross talk exists between receptor-mediated and non–receptor-mediated, and between mitochondria-dependent, and mitochondria-independent, apoptotic pathways (109).

Members of the TNF superfamily are involved in a variety of physiological and pathological processes, including chronic heart failure, sepsis (117), arthritis (83), neovascularization, tumorigenesis, allograft rejection, meningitis, cancer-induced cachexia (9), and hepatocyte regeneration (2, 25).

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TABLE 1. EXPRESSION OF THE LIGANDS (TNF-α, TNF-β, CD95L, TWEAK, VEGI, TRAIL) OF THE TNF SUPERFAMILY AND THEIR RECEPTORS

Ligand	*Cells*	*Receptor*	*Cells*
TNF-α	Macrophages	TNFR1	Most normal and transformed cells
	Natural killer cells	TNFR2	Immune cells
	T- and B-lymphocytes		Endothelial cells
TNF-β	Macrophages	TNFR1	Immune cells
	Natural killer cells	TNFR2	Endothelial cells
	T- and B-lymphocytes		Most normal and transformed cells
Fas	Activated thymocytes	FasL	Most normal and transformed cells
	Splenocytes		
	Cells of testis		
TRAIL	T-lymphocytes	DR4, DR5	Most normal and transformed cells
	Dendritic cells	DcR1, DcR2	
	Natural killer cells	OPG	
TWEAK	Monocytes	FN14	Endothelial cells
		TWEAKR2	Smooth muscle cells
			Immune cells
			Synoviocytes
			Fibroblasts
VEGI	Endothelial cells	DR3	Activated T-cells
		DcR3	Endothelial cells

APOPTOSIS

Apoptosis (cell suicide/programmed cell death) is a normal and very important event in the life cycle of most cells in the organism. It is characterized by morphological and structural features involving mitochondrial swelling, release of cytochrome *c,* cytoplasmic membrane blebbing, chromatin condensation, caspase activation, DNA fragmentation, and cell fragmentation (127). Mitochondria play a crucial regulatory role early in the proceeding of apoptosis (44, 63). A commonly accepted marker of mitochondrial engagement during apoptosis is the release of cytochrome *c* from the mitochondrial intermembrane space to the cytosol. Once released in the cytosol, cytochrome *c* forms the "apoptosome" together with apoptosis protease-activating factor-1 (Apaf-1) and caspase-9. Apoptosome formation leads to the onset and irreversible progress of apoptosis (130). Mitochondrial cytochrome *c* release has been shown to be directly regulated by caspase-8 activation. Caspase-8 and -3 cleave the proapoptotic B-cell lymphoma 2 (Bcl-2) homology domain 3 (BH3)-interfering domain death agonist protein (Bid), which belongs to the Bcl-2 superfamily, and the cleavage product of Bid promotes cytochrome *c* release from mitochondria (12) (Fig. 1).

Apoptosis is mediated by the proteolytic actions of the cysteine proteases (caspases) (23). Several reports have demonstrated that members of the TNF superfamily are potent inducers of caspase-mediated apoptosis in a variety of cells and that apoptosis may be regulated by the activation of the ubiquitous central transcription factor NF-κB (13). Activation of NF-κB by TNF leads to a proliferative response, whereas when NF-κB is inhibited, TNF induces caspase-dependent cell apoptosis (5, 13, 128). Many of the nonapoptotic effects of TNF-α, such as up-regulation of proinflammatory genes, are regulated by activator protein-1 (AP-1) and NF-κB. In many cell types such as cancer cells, NF-κB activation inhibits cell apoptosis, but it depends on the cell type (11).

Recently, NF-κB has been found in the mitochondria, where it regulates their mitochondrial mRNA expression in response to cytokines (22). As TNF-α leads to activation of NF-κB, the function of mitochondrial NF-κB remains unclear. NF-κB also regulates the production of several proteins, such as the mitochondrial regulatory proteins Bcl-2, Bcl-x_L, A1/Bfl-2, inhibitor of apoptosis protein (IAP), and TNF receptor-associated factor (TRAF), which inhibit the caspase cascade and block cytochrome *c* release from mitochondria (22, 24).

ROI

During endogenous metabolic reactions, aerobic cells produce ROI such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^\bullet$), and organic peroxides as normal products of the biological reduction of molecular oxygen (32). The electron transfer to molecular oxygen occurs at the level of the respiratory chain, and the electron transport chains are located in membranes of the mitochondria, endoplasmic reticulum, the nucleus, and the cell, but mitochondria are the major source of cellular ROI (Fig. 2) (39, 41, 106). ROI regulate signal transduction in plant and animal cells (63), but an intracellular excess ROI caused by oxidative stress leads to cell death through lipid peroxidation, alteration of DNA, and various proteins. In contrast, a low amount of ROI is involved in the defense against microorganisms (108). The intracellular damaging effects of ROI are controlled by a system of enzymatic [e.g., superoxide dismutase (SOD), glutathione peroxidase (GSH-px), glutathione reductase, catalase] and nonen-

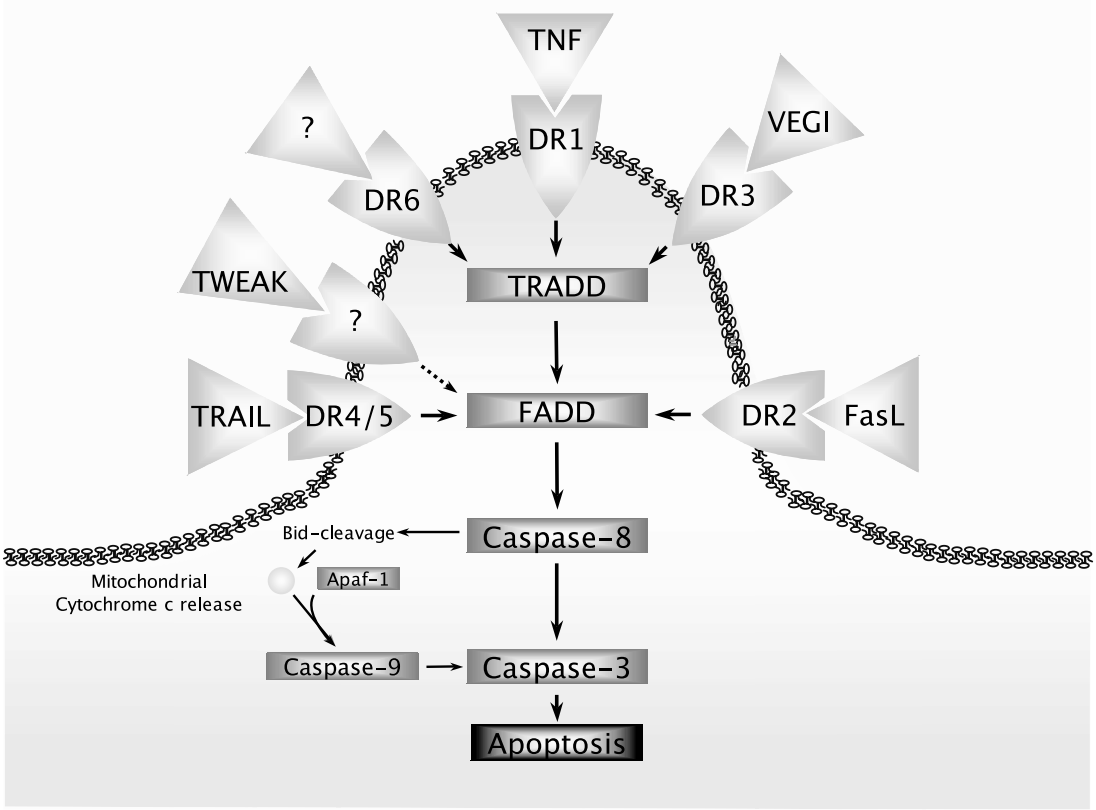

FIG. 1. Apoptosis pathway induced by the members of the TNF superfamily. Members of the TNF receptor superfamily interact with TRADD or FADD adaptor molecule and induce apoptosis through either caspase-8 activation or caspase-9 activation.

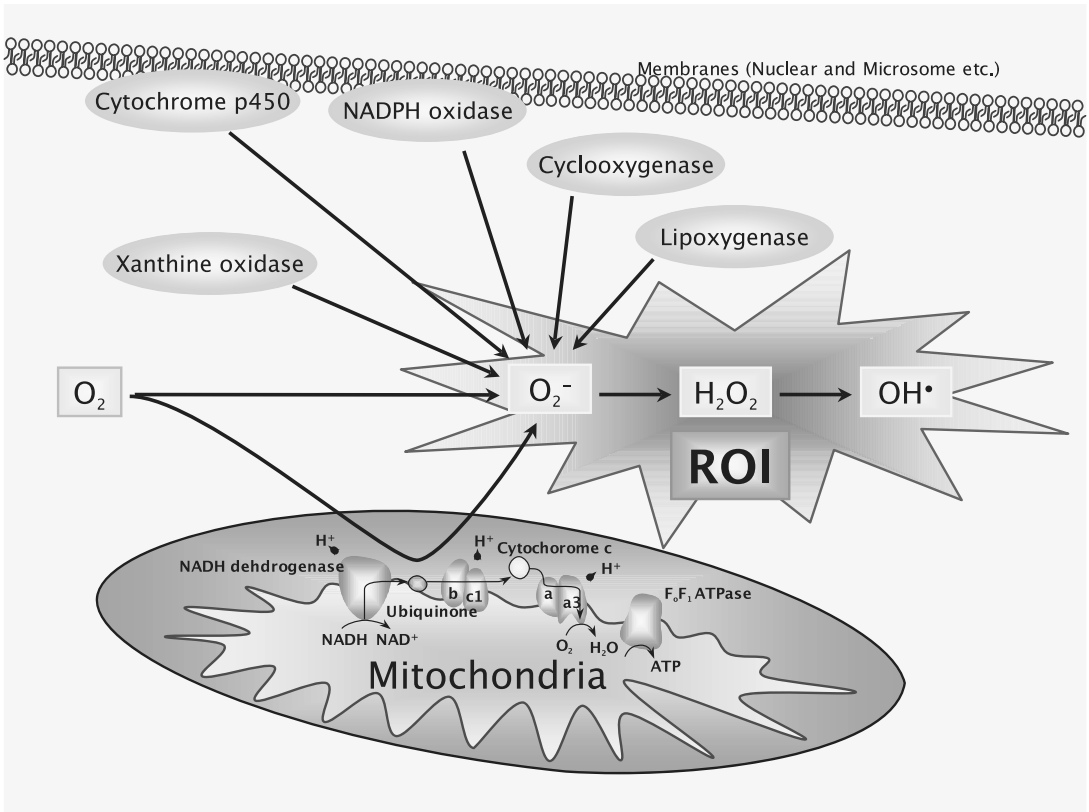

FIG. 2. Major sources of ROI in the cell.

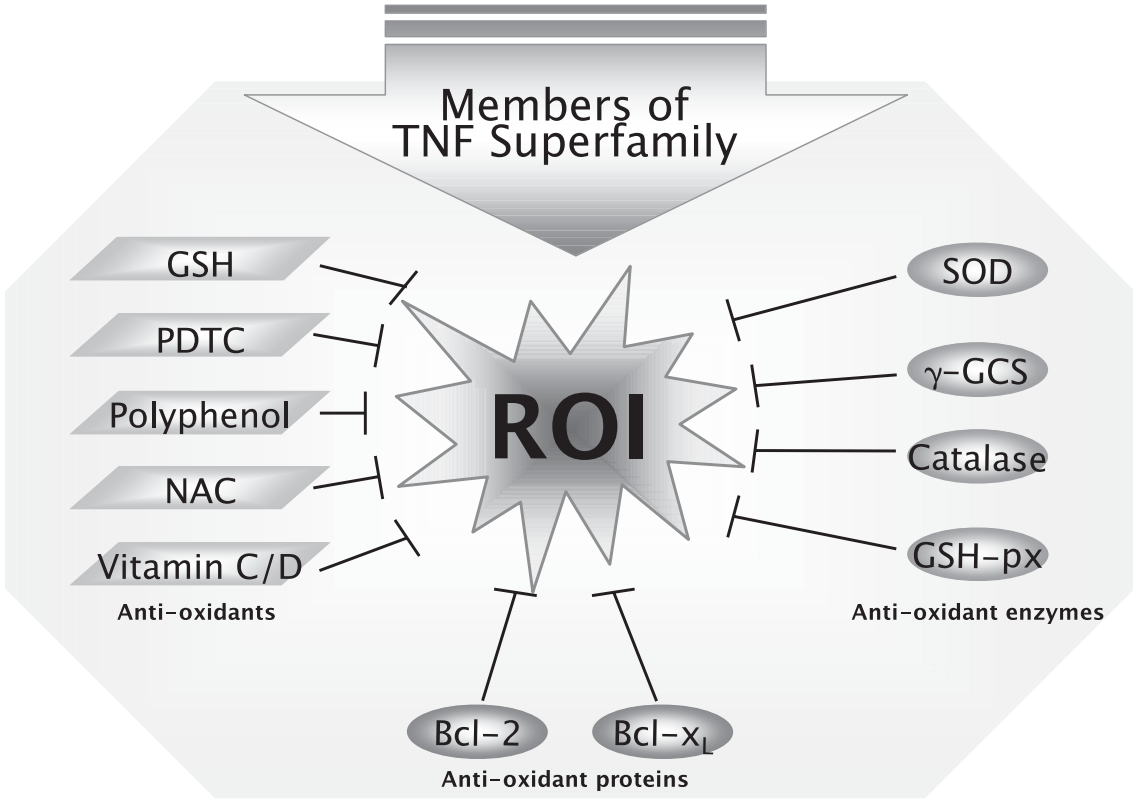

FIG. 3. Quenching of ROI by antioxidant enzymes, antioxidant proteins, and antioxidant chemicals. The intracellular damaging effects of ROI are suppressed by a system of enzymatic and nonenzymatic antioxidants, which eliminate prooxidants and scavenge free radicals. The antioxidant proteins Bcl-2 and Bcl-xL are known to suppress ROI production through inhibition of the PTPC.

zymatic antioxidants [*e.g.,* glutathione (GSH), vitamins C and D], which eliminate prooxidants and scavenge free radicals (113) (Fig. 3).

ROLE OF ROI IN CYTOKINE SIGNALING

A number of enzymes responsible for ROI synthesis have been identified (26), and a variety of stimuli, including mitogens, cytokines, and toxins, are able to activate the intracellular ROI (45, 97, 120). Several reports show that ROI release plays a role in TNF receptor-induced signaling and apoptosis (Table 2) (8, 35). Indeed, high levels of ROI have been shown in cells during apoptosis (119). Antioxidants inhibit apoptosis (75, 123), and an intracellular increase in oxidants activates apoptosis in cells (100). ROI production is an early feature of apoptosis (8). However, higher concentrations of ROI induce necrosis in cells (27, 55).

Small amounts of ROI function as intracellular messengers, mediating survival effects by, for example, increasing antiapoptotic factors (35). Taken together, oxidants and antioxidants have an important function in cellular physiology, and a delicate balance between them is needed for cellular homeostasis.

The exact TNF signal transduction pathways that activate ROI synthesis and the exact role of ROI-mediated proapoptotic and antiapoptotic signals are not fully known. Depending on cell type, several antiapoptotic effects mediated by ROI in response to TNF-superfamily ligands seem to involve NF-κB activation (Figs. 1 and 4) (35). Activation of caspase-3 and -8 and serine proteases by ROI may contribute to apoptosis (35, 64, 71, 132). TNF-induced activation of NF-κB requires the mitochondrial electron transport system, because the presence of rotenone (the protein complex I inhibitor) blocks NF-κB activation (106).

ROLE OF ROI IN CASPASE ACTIVATION

Caspases are a specialized family of proteases that execute apoptosis by destroying structural and functional cell proteins. The members of the caspase family contain a cysteine-rich residue at the active site and cleave targeted proteins after aspartic acid. They are synthesized as proenzymes and activated by removal of their NH_2-terminal prodomain. Caspase-8 and -10 are crucial proximal caspases in the caspase cascades. Caspases play a role in death receptor-mediated apoptosis because these caspases are recruited by Fas-associated death domain (FADD) (1). Caspase-3 is an important downstream executioner caspase that cleaves a wide variety of substrates important for the onset of apoptosis, such as antiapoptotic proteins of the Bcl-2 superfamily (Bcl-2, Bid), inhibitor of caspase-activated deoxyribonuclease (ICAD), and NF-κB (10).

Table 2. Role of ROI in TNF Superfamily-Mediated Cell Apoptosis

Ligands	*References*
TNF-α	
TNF-α potentiates ROI by activating p38 MAPK	31
TNF-α increases the production of ROI	121
TNF-α-induced necrosis by caspase-regulated ROI production	71
PAF-α-induced NF-κB mediated by ROI	21
Anethole inhibits TNF-α-induced ROI	16
ROI induces apoptosis	64
ROI-dependent NF-κB-mediated transcription of cytokines	86
Coronary smooth muscle cells are potential source of ROI	70
TNF-α causes hypertrophy in cardiac myocytes via ROI generation	84
TNF-α increased ROI release in macrophages	99
Combination of TNF-α and heat shock induced ROI expression and led to apoptosis	124
Mitochondrial ROI induction by TNF	40
TNF-α induces mitochondrial ROI and cytotoxicity	39
DNA damage induced by TNF-α mediated by ROI	110
Involvement of ROI in COX-2 expression by TNF-α	29
ROI mediate TNF-α gene expression	95
TNF-β	
Role of ROI release in TNF-β-mediated apoptosis has not been investigated	
TNF-β-induced cytotoxicity does not depend on ROI	94
Fas	
Fas-induced apoptosis does not depend on ROI	59
Fas-induced apoptosis leads to ROI release, disruption of $\Delta\psi_m$	8
Fas-induced pathway of cell suicide	122
Fas-induced cell death is mediated by Ras-regulated O_2^- synthesis	46
Mitochondrial transmembrane potential and ROI in Fas signaling	90
ROI and antiapoptotic factors	66
ROI is important in Fas-mediated apoptosis	4
TRAIL	
No information is available about a possible cross talk between TRAIL and ROI	
Mitochondrial amplification loop for TRAIL signaling	115
Loss of mitochondrial potential and cytochrome *c* release in response	58
TWEAK	
Role of ROI release in TWEAK-mediated apoptosis has not been investigated	
Caspase inhibition sensitizes for TWEAK-induced necrosis by ROI	85
VEGI/DR6	
Interactions of ROI have not been investigated	

During TNF-induced apoptosis, a number of intracellular proteins are activated (97), in a pathway that is mediated by ROI (Fig. 4). Reports have shown that ROI mediate both pro-apoptotic and prosurvival signals (Fig. 4) (35). ROI-mediated apoptosis has been shown in hepatocytes to be caspase-dependent or -independent, depending on the particular inducing ROI (64). Such ROI as H_2O_2 and superoxide were able to activate upstream (caspase-2 and -8) and downstream (caspase-3 and -7) caspases in hepatocytes (64). Furthermore, ROI play an important role in the TNF-signaling pathway as an upstream target in TNF-induced apoptosis and caspase activation. However, overexpression of such antioxidant enzymes as γ-glutamylcysteine synthetase (γ-GCS) and SOD inhibits TNF-induced cytotoxicity and caspase activation (76, 77), and various ROI quenchers block TNF-induced caspase activation (111) (Fig. 3).

ROLE OF ROI IN CYTOCHROME *C* RELEASE

The production of ROI and oxidative stress conditions are increased by a variety of stimuli, *e.g.,* drugs, ionizing radiation, and binding of cytokines to the cell-surface receptors (15). Several reports have revealed that the mitochondrial electron transport system and the redox potential of the cells play a key role in inducing TNF cytotoxicity by the formation of ROI (Fig. 3) (30, 37, 60, 68, 82, 87, 91, 92, 105, 110). It has been reported that TNF-α changes the redox potential of the cells (37, 92, 103) and that the altered redox potential leads to activation of transcription factors such as NF-κB to induce downstream gene expressions. This signaling mechanism is inhibited by the antioxidant *N*-acetylcysteine (NAC) and metal chelators (103, 104, 116) (Fig. 3).

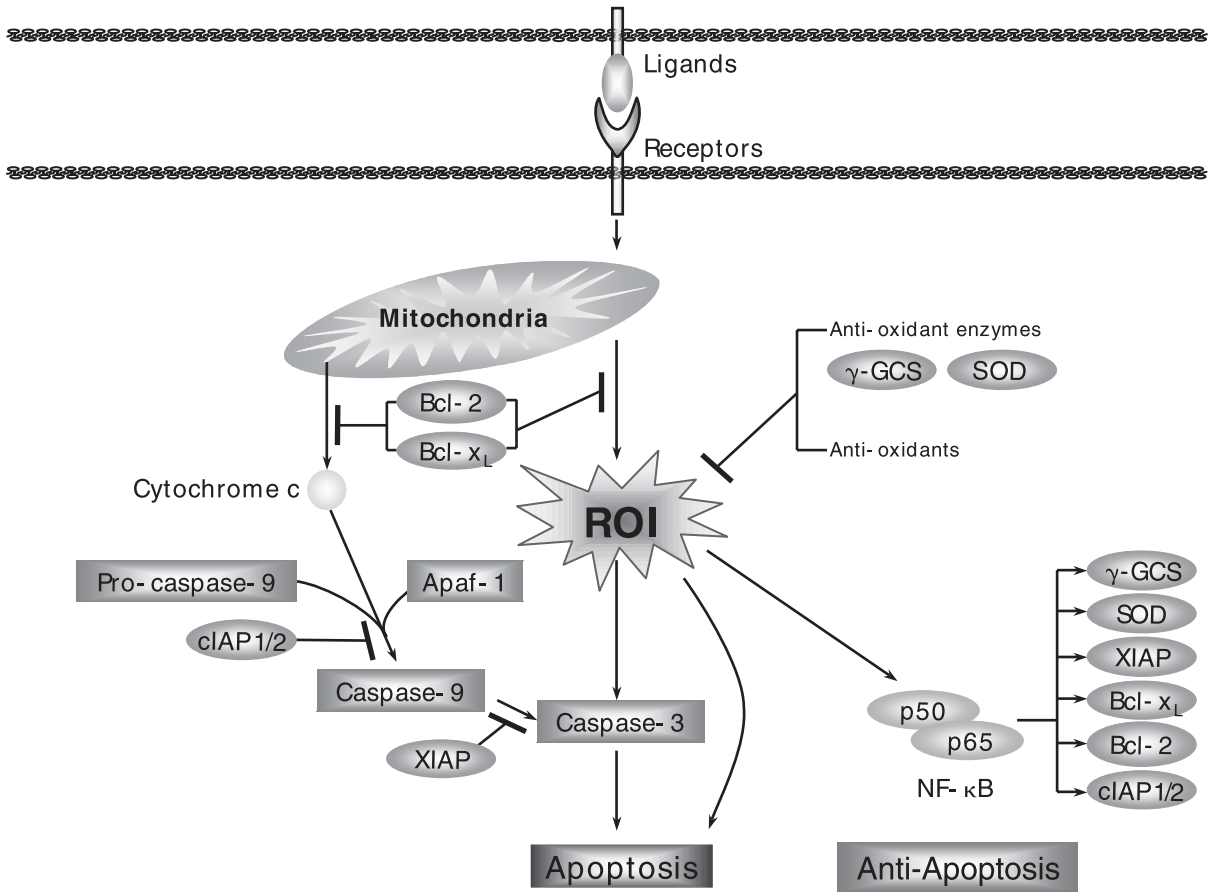

FIG. 4. ROI mediates both pro- and antiapoptotic signaling. The proapoptotic effects of ROI are mediated through caspase activation, and antiapoptotic effects are mediated via NF-κB activation.

Mitochondria are the major source of cellular ROI involved in TNF-α-induced apoptosis (39, 106). Treatment of cells with TNF-α alters mitochondrial membrane permeability, inhibits respiratory chain complex I, induces mitochondrial swelling and clustering, and leads to cytochrome *c* release. Cytochrome *c* activates caspases that kill the cell (17, 39, 89, 97, 106). Chandel *et al.* have reported that TRAF2 directs signaling generated by TNF to the electron transport mechanism in mitochondria and then to the production of ROI (17). However, the expression of manganese SOD, radical scavengers, and inhibitors of the mitochondrial electron transport chain protect the cells and provide evidence for the direct involvement of ROI in TNF signaling and cytotoxicity (105, 106, 126). The exact TNF signal transduction pathways activated by ROI synthesis and the exact role of ROI-mediated proapoptotic and antiapoptotic signals are not fully understood. The distinct role of ROI in the TNF signaling pathway that leads to apoptosis is still not elucidated and is the topic of this review.

ROI AFFECTS MITOCHONDRIAL MEMBRANE POTENTIAL

The mitochondrial membrane potential ($\Delta\psi_m$) is dependent on the electron transport chain transferring electrons from NADH to molecular oxygen and a proton transport mediated by the F_0F_1-ATPase complex (114). The energy stored in the electrochemical gradient is used by F_0F_1-ATPase to convert ADP to ATP during oxidative phosphorylation. Therefore, $\Delta\psi_m$ plays an important role for energy (ATP) production, but also in cell signaling. Many proapoptotic stimuli can affect the $\Delta\psi_m$, such as Fas, H_2O_2, p53, TNF, and staurosporine (8, 47, 72, 96, 101). These agents cause an elevated membrane potential and release of ROI. Elevation of $\Delta\psi_m$ is independent of activation of caspases and happens early during apoptosis (8, 72). Hyperpolarization of the mitochondrial inner membrane followed by increased ROI production could be shown in activated T-cells of systemic lupus erythematosus patients (36).

At the inner–outer membrane contact sites, mitochondrial pores and megachannels can be formed in response to apoptotic stimuli. This permeability transition pore complex (PTPC) consists of different transporters, channels, and outer membrane proteins. The PTPCs allow diffusion of low-molecular-mass compounds such as cytochrome *c* and lead to disruption of the mitochondrial membrane. Loss of membrane potential appears to be a point of no return in the effector phase of apoptosis (90). Inhibition of these dynamic multiprotein pore complexes can prevent loss of $\Delta\psi_m$. PTPCs induce ROI release (79). Furthermore, a specific inhibitor of PTPCs pre-

vents translocation of NF-κB (79). Mitochondrial permeability transition that leads to alteration of the cellular redox state seems to be a central coordinator of diverse ROI-dependent signaling pathways and plays a pivotal role in the induction phase of apoptosis (63).

Bcl-2 proteins were suggested to inhibit apoptosis by direct regulation of the PTPC (79) (Fig. 3). Bcl-2 is anchored in the mitochondrial outer membrane and colocalizes with the PTPC at the contact sites between the inner and outer mitochondrial membranes (63).

ROLE OF ROI IN NF-κB ACTIVATION

In the inactive state, NF-κB is present in the cytoplasm of cells in a complex consisting of two subunits and an additional inhibitory subunit IκBα. Five different subunits exist, *i.e.*, c-Rel, RelA, RelB, p50/p105, and p52/p100, which can form homo- or heterodimers in various combinations. During activation, the inhibitory subunit IκBα is phosphorylated by IκB kinase (IKK) and subsequently degraded. Once released, subunits of activated NF-κB translocate to the nucleus to bind to NF-κB recognition sites in the promotors of various genes, thus regulating gene expression (109).

The NF-κB plays a controversial role in TNF superfamily-induced signaling pathways (109). The TNF superfamily is a potent inducer of NF-κB; once activated, NF-κB inhibits TNF-stimulated apoptosis (7, 38). It regulates not only anti-apoptotic signals, but also proapoptotic signaling by the regulation of death receptors (1).

ROI have been strongly implicated in activation of NF-κB, AP-1, c-Jun N-terminal kinase (JNK), mitogen-activated protein kinase kinase (MEK), and caspase activation in response to cytokines such as interleukin-1β (IL-1β) and TNF-α (21, 70, 103, 111). ROI seem to be common mediators of the TNF gene-regulatory signaling pathways (106). But the exact mechanism for ROI-induced NF-κB activation remains unclear (86, 103). We do know that ROI mediate antiapoptotic events via NF-κB activation leading to transcription of antiapoptotic genes, including extracellular growth factor and IL-1 (34, 35, 69). In contrast to the known protective effect of NF-κB activation in TNF-α-induced hepatocyte apoptosis, NF-κB was proved to promote hepatocellular death from ROI in these cells (64).

Therefore, in the same cell type, NF-κB can promote or inhibit apoptosis depending on the apoptotic stimulus or ROI species (64, 111). The mechanism by which NF-κB promotes ROI-induced death is unknown. It has been reported that ROI are able to activate either NF-κB or caspases in ROI-mediated apoptosis (47, 55, 80). However, overexpression of the antioxidant enzymes such as γ-GCS can antagonize ROI and inhibit TNF-induced cytotoxicity and activation of caspases, NF-κB, AP-1, JNK, and MEK (77). Others have shown that some antioxidants, which inhibit NF-κB, can activate AP-1 (33, 102), demonstrating a common upstream pathway for NF-κB and AP-1, but different downstream pathways. Moreover, TNF-induced activation of NF-κB requires the mitochondrial electron transport system, because the presence of rotenone (the protein complex I of respiratory chain inhibitor) blocks NF-κB activation (106). In contrast, Higuchi *et al.* reported that both ROI-independent and -dependent pathways are possible for activation of NF-κB (56). Hayakawa *et al.* recently presented evidence that ROI do not mediate NF-κB activation (52). They showed that both NAC and pyrrolidine dithiocarbamate (PDTC) inhibit NF-κB activation independently of antioxidative function. NAC selectively blocked TNF-induced signaling by lowering the affinity of receptor to TNF. PDTC inhibited the IκBα-ubiquitin ligase activity in the cell-free system where extracellular stimuli-regulated reactive oxygen species production did not occur. Furthermore, they showed that endogenous reactive oxygen species produced through Rac/NADPH oxidase did not mediate NF-κB signaling, but instead lowered the magnitude of its activation.

ROLE OF ROI IN EFFECTS MEDIATED BY ANTIAPOPTOTIC PROTEINS

It has been proposed that ROI mediate both pro- and anti-apoptotic signaling (Fig. 3) (36). As mentioned above, ROI lead to activation of NF-κB (21, 70, 103). One group of target genes regulated by NF-κB are members of the Bcl-2 superfamily, such as Bcl-2 and Bcl-x_L (35). Members of the Bcl-2 family are known to affect the redox status of cells (78). Anti-apoptotic proteins of the Bcl-2 family, such as Bcl-2, Bcl-2-associated gene product-1 (BAG-1), and Bcl-x_L, reside in the outer mitochondrial membrane. The ratio between pro- and antiapoptotic members of the Bcl-2 family has a major influence on the onset of apoptosis. This location is also the major source for ROI release. The Bcl-2 proteins colocalize with PTPC of mitochondria (63). Despite the close proximity of ROI and Bcl-2 family members, a direct interaction between them has not been shown at the moment. It has been demonstrated that treatment of cells with oxidants leads to apoptosis that can be inhibited by Bcl-2 overexpression (57, 65). Furthermore, Bcl-x_L suppresses TNF-mediated apoptosis and activation of NF-κB, AP-1, and JNK (78). Therefore, Bcl-2 somehow seems to inhibit cell death via protection from oxidative stress, but the particular signaling pathway remains to be elucidated (Fig. 4).

ROLE OF ROI IN TNF-α-INDUCED APOPTOSIS

TNF-α interacts with TNF-α receptor 1 and 2 (TNFR1 and TNFR2) and participates in a variety of cellular responses, including antiviral activity, transcription factor activation, immune response regulation, cytotoxicity, and apoptosis (Fig. 5). Many of the proinflammatory or antiapoptotic effects of TNF-α signaling are mediated by activation of NF-κB (35). Recently, a direct effect of TNF-α signaling on the mitochondrial electron transport mechanism resulting in ROI release from mitochondria has been shown (17, 39). A number of reports have shown that ROI is involved in TNF-induced signaling in different cell types (54, 106, 111, 112).

TNF-induced mitochondrial superoxide production leads to cell cytotoxicity, and this effect could be inhibited by ROI

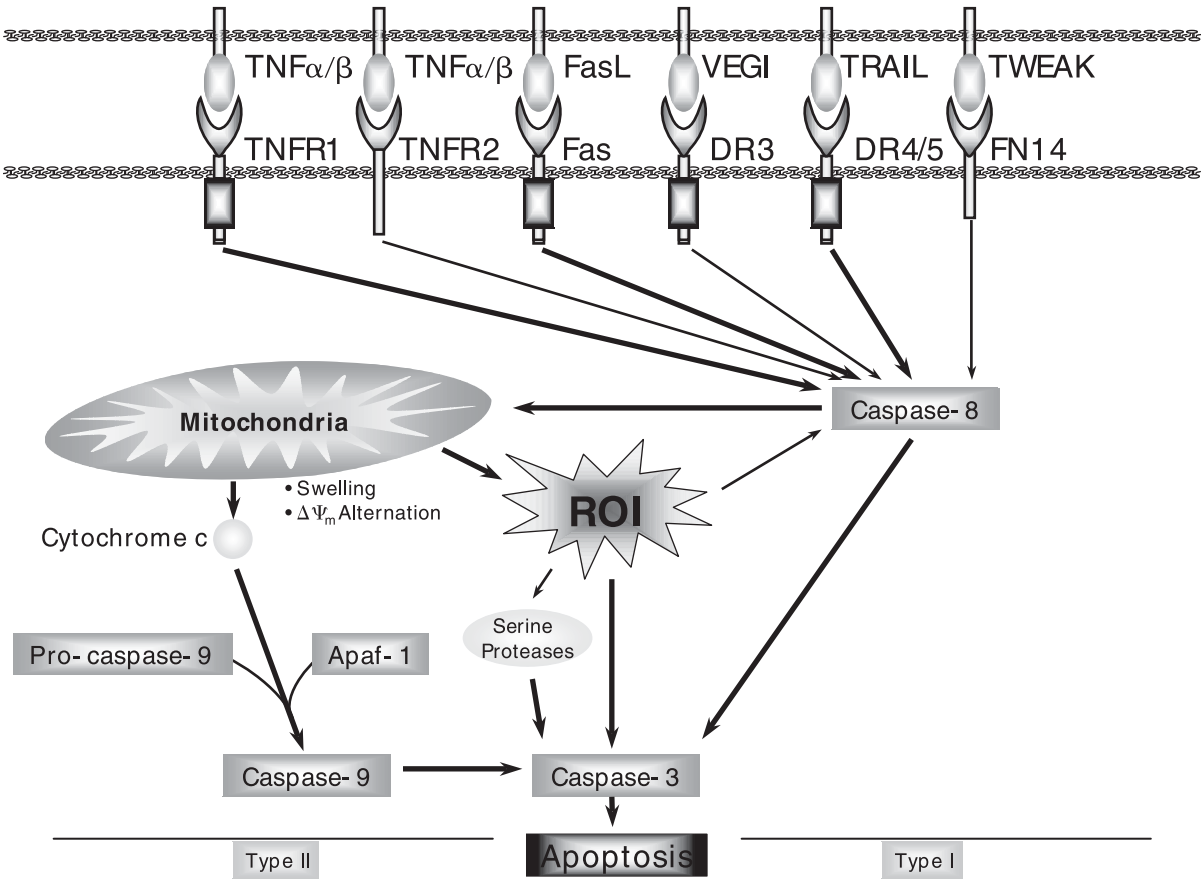

FIG. 5. Members of the TNF superfamily induce apoptosis via receptor-dependent (type I) and mitochondria-dependent (type II) pathways.

scavengers, such as butylated hydroxyanisole (39, 111). A potent antioxidant, glutathione inhibits TNF-induced apoptosis by blocking neutral sphingomyelinase in human breast cancer cells, and an increase in glutathione levels leads to inhibition of the activation of NF-κB (30, 39, 68, 73, 82, 87, 103, 110, 116). A decrease in glutathione levels leads to activation of TNF-induced apoptosis (54, 87, 107). Furthermore, a mitochondrial respiration inhibitor blocks TNF-induced cytotoxicity and differentiation (54). ROI serve as second messengers in cell signaling, and ROI scavengers can effectively block TNF-induced cytotoxicity, underlining the crucial role of ROI in cytotoxicity (39, 106). Formation of ROI was found to lead to DNA damage after TNF treatment (110).

Some other reports have focused on ROI as a common upstream signaling target in TNF-α-induced apoptosis (35). On the other hand, ROI have also been implicated in antiapoptotic signaling in response to TNF-α. TNF-α induces activation of NF-κB whose activation blocks TNF-induced apoptosis (35). Furthermore, ROI were shown to mediate TNF-induced gene transcription via NF-κB and AP-1 activation (35). Therefore, ROI appear to play a role in both pro- and antiapoptotic signaling in response to TNF-α. ROI are also implicated in the induction of TNF-α mRNA in response to UVB radiation, thus enhancing TNF-α signaling (95). Newman *et al.* found that ROI formation in smooth coronary muscle cells was accompanied by release of TNF-α (86). Gossart *et al.* found that ROI regulate TNF-α production in alveolar macrophages (42). NF-κB mediates the transcription of both antiapoptotic and proapoptotic genes (35). It has been shown that anethole, a constituent of anise, camphor, and fennel, through suppression of ROI generation inhibits NF-κB activation. Anethole suppressed TNF-induced inflammatory effects mediated by AP-1, JNK, and MEK and suppressed TNF-induced apoptosis (16), emphasizing that activation of NF-κB by ROI also mediates proapoptotic effects. It is not clear which effects disturb the balance between pro- and antiapoptotic effects induced by ROI via NF-κB (Fig. 4). The net effect may depend on cell type, the microenvironment of cells such as culture conditions, and other interacting factors.

ROLE OF ROI IN TNF-β-INDUCED APOPTOSIS

TNF-β binds to TNFR1 and TNFR2, but TNFR2 lacks a death domain (10). As a result, TNFR2 does not usually mediate apoptosis, but overexpression of TNFR2 leads to apoptosis (50). The apoptotic signaling pathway of TNFR2 is not understood at the moment (10). Powell *et al.* found that TNF-β-induced cytotoxicity could not be inhibited by the oxygen

radical scavenger gluthathione, and therefore concluded that this cytotoxicity does not depend on oxygen radicals in human ovarian and cervical carcinoma cells (94). The direct effect of TNFR2 signaling on ROI release remains to be investigated.

ROLE OF ROI IN FAS-INDUCED APOPTOSIS

The cell-surface receptor Fas (also known as DR2 or CD95/Apo-1 receptor) contains a death domain that mediates cell apoptosis via interaction with FADD. The Fas-FADD complex associates further with caspase-8 [FADD-like IL-1β-converting enzyme (FLICE)] and caspase-3 activation (1, 10). An alternative mitochondria-mediated pathway has also been described for Fas signaling, consisting of cleavage of Bid protein by caspase-8 (10).

ROI plays a crucial role in mediating Fas-dependent apoptosis (4, 122) because the Fas-induced apoptosis was completely abolished by antioxidants such as NAC and glutathione (46, 122). The Fas receptor activates ras in response to ligand binding, and activated ras leads to generation of ROI (46). Furthermore, ras was shown to be activated via sphingomyelinases or ceramide in lymphocytes. Disruption of mitochondrial transmembrane potential ($\Delta\psi_m$) and ROI release occurs early in Fas-mediated apoptosis (8). The initial increase in ROI is followed by elevation of $\Delta\psi_m$, externalization of phosphatidylserine, and later disruption of $\Delta\psi_m$, which together mediate apoptosis. Cell death, externalization of phosphatidylserine, and disruption of $\Delta\psi_m$ could be inhibited by caspase inhibitors, suggesting that these events depend on caspase activation. But elevated ROI and elevated $\Delta\psi_m$ levels persisted (8). Taken together, the balance between mitochondrial ROI, reducing agents, and other factors regulates the susceptibility to Fas-induced apoptosis. Protein phosphatase 2, for example, has been shown to be an essential factor for survival and growth of cells via regulation of intracellular ROI and antiapoptotic factors in Fas-mediated apoptosis (66). There are other reports, however, that show lack of requirements of ROI in Fas-mediated apoptosis (59). The latter studies were based on the use of antioxidants.

ROLE OF ROI IN TWEAK-INDUCED APOPTOSIS

TWEAK is the ligand that binds to fibroblast growth factor inducible-14 receptor [FN14; also known as TWEAK receptor (TWEAKR)] and was initially described in 1997 (19). TWEAK is a cell surface-associated type II transmembrane protein of the TNF superfamily, but a smaller, biological active form of TWEAK also exists that can be shed in the extracellular milieu (125). TWEAK is implicated in apoptosis, proliferation, migration, inflammation, and angiogenesis (93, 98). It has proinflammatory activity by up-regulating proteins such as prostaglandin E2, matrix metalloproteinase-1, IL-6, cell adhesion molecule (ICAM-1), and E-selectin, and induces secretion of some chemokines such as IL-8 (20, 49). TWEAK signaling has been shown in monocytes, macrophages, smooth muscle cells, fibroblasts, synoviocytes, and endothelial cells. Recently, signaling by a second TWEAK (TWEAKR2) receptor has been reported (93).

TWEAK binding to TWEAKR2 activates NF-κB, JNK, and MEK signaling cascade, as is the case for FN14. In contrast to FN14, the MAPK activity was stronger when binding to TWEAKR2 (93). Depending on cell type, TWEAK induces multiple pathways of cell death, including caspase-dependent apoptosis, cathepsin B-dependent necrosis, and endogenous TNF-α-dependent cell death. These multiple death pathways seem to be solely mediated by FN14 even though this receptor lacks an intracellular death domain (85). Nakayama's group was able to show in FN14 transfectants that the pan-caspase inhibitor sensitized the transfectants to TWEAK-induced death by necrosis via ROI and cathepsin B-dependent pathways (85). Therefore, one can suggest that some of these death pathways, *e.g.,* by ROI and cathepsin B, are caspase-independent. In contrast, TWEAK-induced apoptosis was associated with caspase-8 and -3 activation (85). Furthermore, TWEAK has been identified as an effective inducer of constitutive NF-κB activation (98). Unlike other ligands of the TNF family, TWEAK leads to prolonged NF-κB (8–24 h) activation. TWEAK-induced NF-κB activation depends on the adaptor molecules TRAF2 and TRAF5 (48, 98).

Upon TWEAK binding, the cytoplasmic tail of FN14 interacts and associates with the adaptor molecules TRAF1, 2, 3, and 5 for activation of the NF-κB signaling pathway (14, 48). The question arises whether ROI play a role in TWEAK-induced death signaling because it is known that ROI are downstream products of TRAF-mediated signal transduction (17).

ROLE OF ROI IN TRAIL-INDUCED APOPTOSIS

TRAIL is the ligand that binds to the death receptors DR4 and DR5, but also to the decoy receptors DcR1 and DcR2, as well as osteoprotegrin (OPG) (1). Decoy receptors allow ligand binding without transducing a signal, thus regulating TNF signaling. DcR1 and DcR2 lack an intracellular death domain or contain a nonfunctional death domain. DR4 and DR5 lead to apoptosis by activating TNFR1-associated death domain protein (TRADD) followed by activation of caspase-8 and -3 (1). Caspase-8 can cleave proapoptotic Bid, and the cleaved Bid leads to release of mitochondrial cytochrome *c.* TRAIL-induced apoptosis is negatively regulated by several cellular factors (1). NF-κB regulates TRAIL expression, and like all members of the TNF superfamily, TRAIL is able to activate NF-κB (1).

TRAIL is considered to induce apoptosis in a variety of cancer cells, but not in normal cells, so it is under investigation as a potentially powerful cancer therapeutic (6, 58). The susceptibility to TRAIL-mediated apoptosis depends further on the differentiation state of cells. Blocking of the function of NF-κB has been shown to lead to enhanced susceptibility of cells for TRAIL-induced apoptosis (28). The apoptosis in differentiating cells induced by TRAIL was suggested to be independent from mitochondrial pathways (53). In combina-

tion with chemotherapeutics, a synergistic effect on mitochondria (loss of mitochondrial potential, release of cytochrome *c*) has been shown (58, 81). In contrast, Soderstrom *et al.* described a mitochondrial amplification loop for TRAIL signaling evident by mitochondrial depolarization and cytochrome *c* release in response to TRAIL stimulation (115). The mitochondrial effects of TRAIL could be inhibited by MAPK signaling (115). Taken together, the evidence shows that TRAIL-induced apoptosis depends on mitochondria-dependent and -independent signaling pathways (118), but at the moment no information is available about the role of ROI in TRAIL-mediated apoptosis.

ROLE OF ROI IN VEGI-INDUCED APOPTOSIS

VEGI belongs to the TNF superfamily and binds to the DR3 and DcR3 (10). It inhibits proliferation and neovascularization by endothelial cells (131) and seems so far to be endothelial cell-specific. VEGI leads to growth arrest and programmed cell death in proliferating cells, but not in nonproliferating cells as shown by caspase-3 activation and annexin V labeling (129). The expression of VEGI is regulated by inflammatory cytokines such as TNF-α (18).

DR3, the receptor for VEGI, interacts with TRADD and forms a complex with caspase-8. VEGI mechanisms are similar to those of other members of the TNF superfamily such as NF-κB (51). VEGI also activates JNK (51). Nothing is known at present about the role of ROI in VEGI-induced apoptosis, however.

ROLE OF ROI IN DR6-INDUCED APOPTOSIS

DR6 is expressed in most human tissues. It contains a cytoplasmic death domain and four extracellular cysteine-rich motifs. DR6 interacts with TRADD (10). TNF induces the expression of DR6 by activation of NF-κB (67). Ectopic expression of DR6 in mammalian cells has been shown to induce apoptosis and to activate NF-κB and JNK (88). The ligand for DR6 is unknown at the moment, as is the role of ROI in DR6-induced apoptosis.

CONCLUSIONS

The regulatory role of mitochondria in apoptosis has been underestimated for a long time. Formerly, two separate death pathways had been hypothesized: death receptor-mediated apoptosis and apoptosis induced by mitochondrial alterations. But it has become more and more evident that separate pathways do not exist. In the last few years, several death receptor-mediated pathways were proven to cross-talk intensively with mitochondrial signaling pathways. Mitochondria play a crucial regulating and enhancing role in death and other signaling events.

The precise redox regulation of cells orchestrated by mitochondrial enzyme complexes contributes significantly to cell survival. Alterations in the redox potential of cells can change signaling leading to apoptosis. The balance between ROI and antioxidants and between pro- and antiapoptotic factors commits the cell to survival or apoptosis or necrosis. The converse roles of ROI and NF-κB in cell-death and -survival signaling depend on multiple interacting pathways that involve redox regulation of mitochondria. Therefore, ROI seem to be not only by-products of metabolic reactions of the organism, but also important signaling messengers that represent a target upstream of NF-κB activation. ROI may represent an early and very common signaling target upstream of NF-κB activation at a cross-point of multiple interacting pathways. Much work remains to be done to elucidate further its precise role in the signaling cascades of TNF receptors.

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ABBREVIATIONS

AP-1, activator protein-1; Apaf-1, apoptosis protease-activating factor-1; Bcl-2, B-cell lymphoma 2; Bid, BH3-interacting domain death agonist; caspase, cysteinyl aspartic acid-protease; DcR, decoy receptor; DR, death receptor; FADD, Fas-associated death domain protein; Fas, fibroblast-associated cell surface; FasL, Fas ligand; FLICE, FADD-like interleukin-1β-converting enzyme; FN14, fibroblast growth factor inducible-14 receptor; γ-GCS, γ-glutamylcysteine synthetase; H_2O_2, hydrogen peroxide; IAP, inhibitor of apoptosis protein; IL, interleukin; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; NAC, *N*-acetylcysteine; NF-κB, nuclear factor-κB; OPG, osteoprotegerin; PDTC, pyrrolidine dithiocarbamate; PTPC, permeability transition pore complex; ROI, reactive oxygen intermediates; SOD, superoxide dismutase; TNF, tumor necrosis factor; TNFR, TNF receptor; TRADD, TNFR1-associated death domain protein; TRAF, TNF receptor-associated factor; TRAIL, TNF-related apoptosis-inducing ligand; TWEAK, TNF-like weak inducer of apoptosis; TWEAKR, TWEAK receptor; VEGI, vascular endothelial cell growth inhibitor; $\Delta\psi_m$, mitochondrial membrane potential.

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