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REVIEW

More than one way to die: apoptosis, necrosis and reactive oxygen damage

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Cell death is an essential phenomenon in normal development and homeostasis, but also plays a crucial role in various pathologies. Our understanding of the molecular mechanisms involved has increased exponentially, although it is still far from complete. The morphological features of a cell dying either by apoptosis or by necrosis are remarkably conserved for quite different cell types derived from lower or higher organisms. At the molecular level, several gene products play a similar, crucial role in a major cell death pathway in a worm and in man. However, one should not oversimplify. It is now evident that there are multiple pathways leading to cell death, and some cells may have the required components for one pathway, but not for another, or contain endogenous inhibitors which preclude a particular pathway. Furthermore, different pathways can co-exist in the same cell and are switched on by specific stimuli. Apoptotic cell death, reported to be non-inflammatory, and necrotic cell death, which may be inflammatory, are two extremes, while the real situation is usually more complex. We here review the distinguishing features of the various cell death pathways: caspases (*c*ysteine proteases cleaving after particular *asp*artate residues), mitochondria and/or reactive oxygen species are often, but not always, key components. As these various caspase-dependent and caspase-independent cell death pathways are becoming better characterized, we may learn to differentiate them, fill in the many gaps in our understanding, and perhaps exploit the knowledge acquired for clinical benefit.

Keywords: apoptosis; necrosis; reactive oxygen; cell death

Introduction

In the last five years, there has been a dramatic increase in interest, and also in molecular understanding, of programmed cell death (PCD). PCD is essential in development, morphogenesis, tissue remodelling, and immune regulation, but is also involved in many pathologies. The primary paradigm of natural PCD is observed during normal embryogenesis. In careful morphological studies using electron microscopy, Schweichel and Merker (1973) identified three pathways of PCD: the first was characterized by condensation of nucleus and cytoplasm, and clearly corresponds to apoptosis, the second was characterized by abundant autophagic vacuoles and no or minimal nuclear changes, as often seen in cells dying by necrosis, while the third type was a more rare variant of necrotic death. Clearly, both apoptosis and necrosis are genetically programmed. But death of cells in tissues is difficult to study because of asynchrony, and especially because of interference and interaction with neighbouring cells. Therefore, most morphological, physiological and molecular characterizations of apoptosis and of necrosis have been done on isolated cell populations.

The typical features of an apoptotically dying cell have been amply documented and are remarkably constant for different cell types and genotypes: membrane blebbing, outer membrane leaflet inversion and exposure of phosphatidyl serine, cytosolic condensation, protein cross-linking and cell shrinkage, nuclear condensation, breakdown of nuclear DNA, first in large segments and subsequently in nucleosomal fragments, and finally, at least *in vitro*, the falling apart with formation of well-enclosed, apoptotic bodies (Figure 1).

None of these features is observed in cells dying by necrosis, except for annexin staining of phosphatidyl serine, but this may be due to internal access because of leakiness of the cell membrane. Rather, necrotic cells swell, round up, and then suddenly collapse like a punctured balloon, spilling their contents in the medium (Grooten *et al*., 1993). The swelling cells readily take up some dyes such as propidium iodide, suggesting a rapid loss of cell membrane integrity. It is particularly noteworthy that even in the lysing cell, there is no detectable oligonucleosomal degradation of nuclear DNA.

Already more than two centuries ago, anecdotal observations were reported regarding tumor regression as a result of spontaneous or induced infection (Hall, 1997). This was later followed by more controlled studies in experimental animals, and further narrowed to effects of endotoxin on tumour regression. This led to the seminal paper of L Old and colleagues in 1975, showing that endotoxin induces in BCG-sensitized animals a factor, now known as Tumour Necrosis Factor (TNF), which leads to rapid hemorrhagic necrosis of an established tumour (Carswell *et al*., 1975). Later studies indicated that this remarkable antitumour effect was in fact host-mediated. In the same paper it was also shown that TNF is highly toxic

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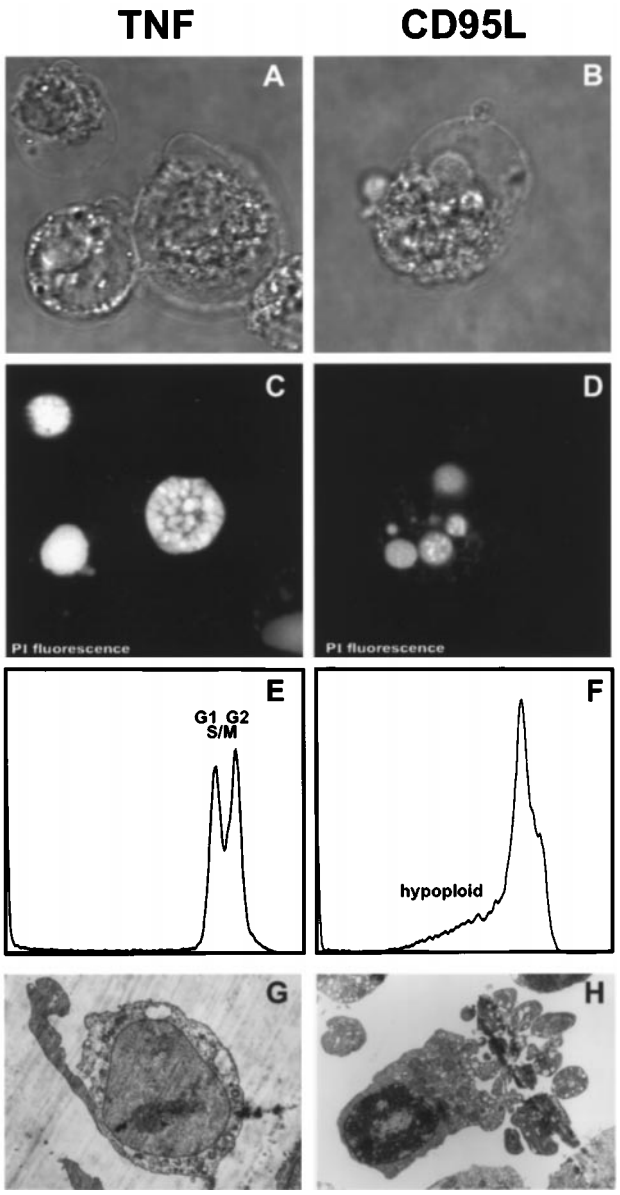

Figure 1 L929.hCD95 cells die by necrosis or by apoptosis after treatment with TNF or CD95L, respectively. L929.hCD95 are L929 cells transfected with the human CD95 (Fas) gene. Treatment with TNF leads to typical necrosis (swelling, **A**; no evident change in the nucleus, **C** and **G**; no obvious degradation of nuclear DNA, **E**). Treatment of the same cells with CD95L (in fact, a monoclonal antibody) leads to typical apoptosis (membrane blebbing, **B**; formation of apoptotic bodies, **D** and **H**; pronounced degradation of nuclear DNA, **F**). The same cells are shown by phase contrast microscopy (**A** and **B**), and by confocal microscopy after staining with propidium iodide (**C** and **D**). Degradation of nuclear DNA was measured by FACS analysis (**E** and **F**) (Vercammen *et al*., 1998a,b). Electron microscopy confirms the typical necrotic and apoptotic features (**G** and **D**) (by courtesy of Dr JR Bradley, Cambridge)

to L-cells (NCTC clone 929), derived from a C3H mouse fibrosarcoma. When pure, recombinant TNF became available, it was soon confirmed that TNF, especially in combination with interferon-γ, was cytotoxic for many transformed cell lines, while the viability of normal, euploid cell lines was not affected (Sugarman *et al*., 1985; Fiers *et al*., 1986). Which of these cell lines dies by necrosis or by apoptosis was not investigated at the time.

Another major natural inducer of cell death is undoubtedly CD95L, the ligand which clusters and thereby triggers its receptor CD95 (also known as Fas or Apo-1) (Nagata, 1997; Krammer, 1999). TNF can exist both in a membrane-bound form and in a soluble form, acting in a paracrine or in a systemic manner, respectively, while CD95L is mainly or exclusively cell membrane-bound and operational via autocrine or paracrine effects (Tanaka *et al*., 1998).

There are, of course, many other effector systems of cell death, some physiological, such as deprivation of soluble survival factors or of a compatible adhesion substratum (Raff, 1992) or encounter of activated cytotoxic T-cells, some physical or chemical, such as irradiation or chemotherapeutic drugs. But most of these involve processes which connect to pathways first revealed by study of the prototype cell death inducers TNF and CD95L, and the present review will mainly focus on their effects.

Briefly, the following cell death pathways initiated by death receptors can be distinguished (summarized in Table 1). (i) Activation of an initiator caspase (caspase-8) which directly activates the effector caspases (especially caspase-3 and -7). The latter are then responsible for the diverse phenomena which characterize apoptotic cell death. (ii) The initiator caspase (caspase-8) produces in the cytosol a processed molecule (Bid/CAF) which acts on the mitochondria, resulting in the release of cytochrome c (cyt c) and other proteins from the intermembrane space. Cyt c then leads to activation of caspase-9 and downstream effector caspases. (iii) TNF triggers a pathway resulting in enhanced production of reactive oxygen species (ROS) in the mitochondria. The cells swell and then suddenly collapse with extensive leakage of their content. This is typical necrotic cell death, as observed in fibrosarcoma cells. There is no involvement of caspases, on the contrary, constitutively active, caspase-like enzymes interfere with the necrotic process. (iv) TNF or other stimuli can lead to oxidative stress, usually involving ROS production by activated mitochondria. The ROS then cause release of cyt c from the mitochondria, which in turn brings about the activation of the effector caspases. It is likely that this category in fact includes several pathways or variations; in some systems, the process can be inhibited by mitochondrial manganese superoxide dismutase (MnSOD), in others ROS act primarily in the cytosol, possibly causing directly the release of mitochondrial intermembrane proteins. (v) (Not really an alternative pathway): TNF and CD95L triggering can activate the MAP kinase kinase kinase (MAPKKK)-type apoptosis signal-regulating kinase ASK1; this activation is prevented by common reducing agents. ASK1, via JNK and p38 MAPK, leads to activation of transcription factors, which results in expression of CD95L. Autocrine and paracrine effects then cause cell death via the pathways described in (i) or (ii).

The prototype apoptotic cell death pathway

In recent years, considerable progress has been made towards a molecular understanding of the processes underlying apoptotic cell death pathway(s) (for recent

Table 1 Pathways to cell death

Pathway[a]	*Stimulus*[b]	*Initiator*	*Mediator*	*Effector*	*Inhibitor*[c]	*Outcome*
I	TNF/CD95L	caspase-8	–	caspases (−3, −6, −7)	CrmA[d] zVAD-fmk[e]	apoptosis
II	TNF/CD95L	caspase-8 (→Bid)	mitochondria (→cyt c)	caspases (−9, −3, −6, −7)	CrmA[d] zVAD-fmk[e] Bcl-2[f]	apoptosis
III[g]	TNF	?	mitochondria (→ROS)	?	BHA[h]	necrosis
IV[i]	TNF[j]	?	ROS (mitochondria)[k]	(caspases −9, −3, −6, −7)	MnSOD[k] zVAD-fmk[k] NAC[k,l]	apoptosis/necrosis
V[m]	TNF/CD95L[j]	ASK1	JNK, p38 MAPK	CD95L	NAC[l]	(apoptosis)

[a]Some pathways are cell type-specific, but more than one pathway can be (latently) present in the same cell, and be switched on specifically, depending on the stimulus. [b]This review focuses on TNF and CD95 effects, but other cell death stimuli (e.g. cytotoxic T-cells, chemotherapeutic drugs, UV- or γ-irradiation, growth factor or substratum withdrawal) directly or indirectly connect to the same pathways. [c]Only some particularly characteristic examples are given. [d]CrmA is derived from a cowpox-virus gene; it inhibits specifically caspase-8 (and caspase-1). [e]zVAD-fmk is a broad-spectrum caspase inhibitor. [f]Bcl-2 and Bcl-x_L are the most studied members of a large family of related, membrane-associated, anti- and pro-apoptotic proteins. [g]This pathway has been demonstrated so far only in fibrosarcoma cells. [h]Butylated hydroxyanisole. [i]Presumably several (partially) different pathways are grouped here. [j]Many other physiological, chemical or physical stimuli/insults can switch on this pathway (cf. main text). [k]This may not apply to all systems which are classified in this group. [l]N-acetylcysteine; also many other reducing agents such as PDTC, reduced glutathione, etc. [m]This is not a real pathway, but is often mistaken for one; it connects to pathways I or II

reviews, see Raff, 1992; Nagata, 1997; Ashkenazi and Dixit, 1998; Baker and Reddy, 1998; Dragovich *et al*., 1998; Green and Reed, 1998; Mignotte and Vayssière, 1998; Schulze-Osthoff *et al*., 1998; Singh *et al*., 1998; Thornberry and Lazebnik, 1998; Krammer, 1999; Los *et al*., 1999; Vaux and Korsmeyer, 1999). This became possible, on the one hand, by taking clues from the genetically thoroughly characterized apoptotic cell death pathway in *Caenorhabditis elegans* (Ellis and Horvitz, 1986; Metzstein *et al*., 1998) and, on the other hand, by making clever use of new molecular biology tools such as yeast two-hybrid screening (Wallach *et al*., 1998) and sequence homology searches. But also classical biochemical fractionation experiments contributed essential findings. CD95L and TNF kill apoptotically many cell types (e.g. KYM, MCF-7, PC60) by an almost identical mechanism (Tewari and Dixit, 1995).

Briefly (and oversimplifying), the apoptotic cell death pathway triggered by CD95L is believed to proceed as follows: the membrane-bound ligand clusters its receptor CD95, the intracellular part of which contains a characteristic 85-amino-acids region known as 'death domain' (DD). The receptor DD cluster recruits several copies of another DD protein, named FADD, the amino-terminal part of which is a death effector domain (DED). The latter now engages in a homophilic interaction with the caspase-8 zymogen prodomain consisting of two DEDs (Boldin *et al*., 1996; Muzio *et al*., 1996). The induced proximity of two (or more) zymogen molecules with a low intrinsic activity leads to autoactivation, with release in the cytosol of fully active caspase-8 (Salvesen and Dixit, 1999). Possibly, the activation of the caspase-8 zymogen is more complex and may involve an additional protein, such as FLASH (Imai *et al*., 1999). The role of this FLASH protein in CD95 signalling, however, is still controversial (Koonin *et al*., 1999). Active caspase-8 is a key molecular trigger, and is also referred to as an initiator caspase. It acts on various cytosolic substrates, such as CAF or Bid, a C-terminal fragment of which leads to loss of mitochondrial transmembrane potential ($\downarrow\Delta\Psi_m$) and release of cyt c into the cytosol (Li *et al*., 1998; Luo *et al*., 1998; Steemans *et al*., 1998). Presumably, CAF and Bid are identical (M Steemans and J Grooten, personal communication). Bid requires Bax, a pro-apoptotic Bcl-2 family member, for its action on mitochondria (Desagher *et al*., 1999). Note, however, that bid$^{-/-}$ mice are viable and apparently healthy, and that derived cell lines reveal only a reduced sensitivity to TNF or CD95L (Yin *et al*., 1999). Cyt c release can also occur without $\downarrow\Delta\Psi_m$ (Hakem *et al*., 1998; Luo *et al*., 1998). The release of cyt c without morphological changes of the mitochondria may involve interaction of apoptogenic factors (for example, the Bcl-2 family members Bax or Bak) with the outer membrane voltage-dependent anion channel (VDAC), and enlargement of its pore (Shimizu *et al*., 1999). More profound effects would involve the 'permeability transition pore' as well (Martinou, 1999). The latter corresponds to sites where adenine nucleotide translocator (ANT), located in the inner mitochondrial membrane, interacts with VDAC, located in the outer mitochondrial membrane. In fact, a decreased ANT function is an early event in the apoptogenic pathway (Vander Heiden *et al*., 1999). The release of cyt c is not specific, as other intermembrane proteins appear in the soluble cell fraction as well (Single *et al*., 1998; Samali *et al*., 1999; Susin *et al*., 1999).

Cyt c in the presence of dATP or ATP induces a conformational change of the adaptor protein Apaf-1 and/or release from a Bcl-2 family member, such that Apaf-1 not only binds procaspase-9, but also dimerizes, which allows the juxtaposition of two procaspase-9 molecules resulting in autoactivation and release of mature caspase-9 (Hu *et al*., 1998; Pan *et al*., 1998; Srinivasula *et al*., 1998; Yang *et al*., 1998; Stennicke *et al*., 1999). There is, in fact, a parallel between the presumed role of FLASH in the activation of procaspase-8 and that of Apaf-1 in the maturation of procaspase-9. The latter then processes and thereby activates effector caspases such as caspase-3 and caspase-7 (Slee *et al*., 1999). These effector caspases act on a multitude of substrates, affecting the integrity of the cell skeleton, the nuclear lamin structure, the DNA, etc., while repair systems become inactivated (Stroh and Schulze-Osthoff, 1998). The net result of these proteolytic cascades is the well-known, characteristic morpholo-

gical and biochemical features of an apoptotically dying cell. The effector caspases may also act on substrates upstream of the mitochondria, thus creating a positive feedback loop (and a rapid decision of no-return), *viz* caspase-3-mediated cleavage of procaspase-8 (Van de Craen *et al*., 1999). Another positive feedback effect consists of caspase-3 processing of Bcl-2, generating a Bax-like effector fragment (Cheng *et al*., 1997).

In some cell types, referred to as type I, caspase-8 can directly activate the effector caspases without requirement of mitochondrial cyt c release (Scaffidi *et al*., 1998). These type I cells are distinguished from type II by the formation of large amounts of active caspase-8 at the 'death-inducing signaling complex' (DISC) and by the absence of an inhibitory Bcl-2 (or family member) effect. Not unexpectedly, apaf-$1^{-/-}$ mice hardly survive until birth, but thymocytes and T-cells from the survivors could still be killed by CD95 ligation, again supporting the existence of alternative pathways (Yoshida *et al*., 1998).

Largely the same caspase-dependent pathway is undoubtedly involved in cells such as KYM, MCF-7, or PC60, which are apoptotically killed by TNF (incidentally, caspase-3 is absent in MCF-7 cells, but TNF-induced cell death still occurs, although without some typical features such as oligonucleosomal DNA fragmentation and membrane blebbing; Jänicke *et al*., 1998). Triggering occurs by clustering of the TNF receptor I (TNF-RI, also known as TNF-R55); ligand binding leads to release of SODD, a protecting (or silencing) protein normally bound to the intracellular domain (Jiang *et al*., 1999; Tschopp *et al*., 1999). The TNF-RI intracellular part contains, in addition to a C-terminal DD, also an extensive, membrane-proximal domain, which can bind specific adaptors. The role of this membrane-proximal domain in cell death is limited, but it is known to affect several other physiological cell functions (Tartaglia *et al*., 1993; Adam-Klages *et al*., 1996; De Vos *et al*., 1998; Hildt and Oess, 1999; Peppelenbosch *et al*., 1999). The clustered TNF-RI DDs recruit the DD-containing adaptor protein TRADD. This is a multi-faceted protein; it binds FADD, and in this way initiates a caspase-8-dependent apoptotic cell death pathway described above. But TRADD also interacts with TRAF2, and the DD-containing RIP. The latter proteins are involved in activation of p38 MAPK, jun N-terminal kinase (JNK) and, perhaps even more important, NF-κB, responsible for rapid expression of a specific set of genes. Some of these code for proteins protecting against cell death, for example A20 (De Valck *et al*., 1999), TRAF1, TRAF2 and the 'Inhibitor of Apoptosis Proteins' c-IAP1 and c-IAP2 (Wang *et al*., 1998a). The rapid synthesis of these rescue proteins explains at least in part why actinomycin D or cycloheximide are powerful sensitizers of the TNF-RI-induced cell death pathway(s) (Vercammen *et al*., 1997). In fact, prior induction of NF-κB or its constitutive activation in some tumour cells render these targets TNF-insensitive (Manna and Aggarwal, 1999). Conversely, inactivation of NF-κB in tumour cells renders them sensitive to TNF and to chemotherapy (Wang *et al*., 1999).

L929 cells die by necrosis as a result of ROS formation in the mitochondria

Because of its exquisite sensitivity to TNF, the cell line L929, already mentioned in the introduction, has often been used for studies on the mechanism of action of TNF, as well as for TNF assays and standardization.

What is the evidence that TNF-induced necrosis of L929 cells is due to ROS formation in the mitochondria? (i) Early studies, mainly by electron microscopy, already indicated that the only cell component or organelle revealing morphologic changes following TNF treatment, were the mitochondria (Matthews, 1983). Stacking of cristae and even formation of weird, multilamellar, onion-like structures were observed (Schulze-Osthoff *et al*., 1992). (ii) Reducing the relative oxygen partial pressure (pO_2) protects the cells (Neale *et al*., 1988). Note that Jacobson and Raff (1995) reported that PCD was independent of pO_2, but this conclusion applied to the particular cell system, cytotoxic stimuli and undoubtedly the cell death pathway under study. (iii) Selection of L929 cells for enhanced resistance to H_2O_2 generates cells which are also resistant to TNF (Park *et al*., 1992). (iv) Specific inhibition of the mitochondrial electron transport complex I or II protected, and simultaneous inhibition of both complex I and complex II protected very effectively the L929 cells from TNF-induced death (remember that both mitochondrial complex I and complex II feed electrons, derived from oxydation of NADH and succinate, respectively, to the downstream electron transport system) (Schulze-Osthoff *et al*., 1992). Inhibitors that act further down the oxido-reduction pathway were either not protective or even synergized with TNF. These observations suggest that as a result of TNF action somewhere between complex I/II and complex III, presumably at the ubiquinone site, electrons are shunted away towards excessive ROS formation (Schulze-Osthoff *et al*., 1992). It is also noteworthy that inhibitors such as dinitrophenol, which disrupts the proton gradient, or ionomycin, which blocks the F1-ATPase, have little or no effect on TNF susceptibility. (v) Using direct measurements, Lancaster *et al*. (1989) showed that TNF treatment rapidly impaired the mitochondrial electron transfer in digitonin-treated fibroblast cells. (vi) L929 cell lines were selected for loss of mitochondrial DNA, and therefore these sublines have an impaired oxidative phosphorylation pathway. As a result, these respiration-deficient mitochondrial DNA-minus cells became largely resistant to TNF cytotoxicity (Schulze-Osthoff *et al*., 1993). (vii) L929 cells are efficiently protected from TNF-induced killing by the reducing agent butylated hydroxyanisole (BHA) (Brekke *et al*., 1992; Schulze-Osthoff *et al*., 1992). Note that BHA has no effect whatsoever on TNF-induced apoptosis in cells such as PC60, KYM or MCF-7. It is also remarkable that many other, non-toxic, reducing agents, such as N-acetylcysteine, ascorbic acid or pyrrolidine-dithiocarbamate (PDTC), are not protective against TNF-induced L929 killing. A plausible explanation is that ROS are formed and act in a hydrophobic environment, e.g. at mitochondrial membranes where BHA can penetrate but not most other, more hydrophilic, reducing agents. This result also implies that the key oxidative step, which commits the cell to necrotic

death, is specific and localized, and is not due to accumulative, generalized oxidative damage. (viii) ROS formation by the mitochondria can be directly and semi-quantitatively followed on a per-cell basis, using appropriate fluorogenic markers and confocal microscopy or flow cytometry (Goossens *et al*., 1995). All cells, including L929, have a basic, homeostatic, mitochondrial ROS production, estimated at 1–2% of total O_2 consumption (Boveris and Chance, 1973). But soon after TNF treatment, the mitochondrial ROS formation becomes accelerated. When at any time after the start of the experiment BHA is added, the excessive ROS formation stops, and no further cell deaths occur. Normally, most ROS are neutralized by the reducing environment of the mitochondria, mainly reduced glutathione (GSH). An agent such as diethylmaleate (DEM) reacts irreversibly with all SH groups, and obviously is quite toxic. Hence, it cannot be added together with TNF, as it might inhibit all signaling pathways. But added a few hours after TNF addition, DEM blocks rapidly all SH groups, including the mitochondrial GSH reservoir, and therefore eliminates the electron sink; under these conditions, a dramatically enhanced TNF-induced ROS production can be measured (Goossens *et al*., 1995). It may be added that the mitochondrial, reduced GSH only neutralizes the ROS overflow. Presumably, this occurs downstream from the ROS activity responsible for the critical step in necrotic cell death. MnSOD, a mitochondrial matrix enzyme, is upregulated by TNF in many cell types (Jones *et al*., 1997). Moreover, it was shown that constitutive expression of MnSOD in HEK 293 cells or in ME-180 cells protected against TNF-induced cell death, while antisense MnSOD mRNA expression sensitized the cells (Wong *et al*., 1989). These results implicate mitochondrial superoxide in the cytotoxic pathway in these systems. However, TNF does not induce MnSOD in L929 cells and constitutive expression following transfection of the MnSOD gene did not protect (Goossens *et al*., 1999); also catalase, expressed in the mitochondrial matrix by means of an appropriate addressing sequence, had no effect (Goossens *et al*., 1999). These data suggest that in L929 cells ROS acts at or close to its site of production, while only excess ROS diffuses to the mitochondrial matrix, where GSH functions as a sink.

What could be the pathway of TNF-induced necrosis? Unfortunately, we only have a number of pieces of the puzzle, and unlike for the apoptotic pathway, these pieces cannot yet be connected to provide a plausible general pathway. We do know that, as for the apoptotic pathway, trimerization of only the DD of the TNF-RI is sufficient to initiate the necrotic pathway (Tartaglia *et al*., 1993; Vandevoorde *et al*., 1997). The DD cluster then recruits TRADD, which in turn can bind TRAF2 and RIP, as mentioned in the previous section for apoptotically dying cells. Little is known regarding the step(s) downstream from TRADD or FADD to the mitochondria in L929 cells, except that it is not caspase-8-mediated (see below). But (an)other protease(s) is (are) presumably involved, as this pathway is particularly sensitive to inhibitors such as TLCK, which have almost no effect on the apoptotic pathway (Suffys *et al*., 1988; Vercammen *et al*., 1997). Many open questions remain regarding the molecular events further down the pathway. What mechanism is responsible for shunting electrons away towards excess ROS production at a step beyond mitochondrial complex I/II? What is the nature of the key sensor/executioner referred to above, which becomes oxidized by the mitochondrial ROS; a mitochondrial membrane protein with vicinal SH groups? How does an oxidized sensor provoke cell lysis? Do activated lipases play a crucial role in necrosis (De Valck *et al*., 1998; Hofmann and Dixit, 1998; Belaud-Rotureau *et al*., 1999)? Some of these questions can be further defined, albeit based on negative evidence: as there is no need for transcription, all macromolecular components of the lytic pathway must be preformed and present in the cell, ready for activation. Excess ROS production is not a result of cyt c release, and right until the moment of death there is no decrease of $\Delta\Psi_m$ or of cellular ATP (Sánchez-Alcázar *et al*., 1997; G Denecker, 1999 unpublished results).

Although not as extensively studied as in L929 cells, nearly all characteristics of the necrotic cell death pathway discussed above have also been observed with WEHI 164 clone 13, another mouse fibrosarcoma cell line (Espevik and Nissen-Meyer, 1986). It is also of interest that TNF has a mitogenic effect on human dermal fibroblasts, and it decreases the synthesis of type I and type III collagen. Remarkably, also these activities are blocked by BHA, implicating mitochondrial ROS in these mitogenic and gene-regulatory effects (Taniguchi *et al*., 1996).

Finally, one should be careful neither to generalize nor to simplify. Necrotic cell death is not always the result of excess ROS damage; vice versa, a cell which dies as a result of excessive ROS formation, does not necessarily necrotize. Moreover, the phenotype of a cell line is often subject to subtle influences; it is easily possible to isolate L929 sublines with an increased susceptibility to TNF-induced necrosis (e.g. the subline L929s; Vanhaesebroeck *et al*., 1991), or sublines which are killed by TNF in a typical apoptotic pathway. This suggests a delicate balance between both pathways to cell death, perhaps based on the relative expression level of a few genes, and which can be tipped one way or the other.

Apoptosis and necrosis can occur in the same cell

As mentioned already, TNF induces typical apoptotic death in many cell types (KYM, MCF-7, PC60), while in L929 and other fibrosarcoma cells TNF causes typical necrosis. The simplest explanation for these different TNF-activated pathways would be that in the latter cell types a critical component of the apoptotic pathway, e.g. FADD or caspase-8, is missing. But when this hypothesis was tested, came a first surprise. L929.hCD95 is a transfected L929 derivative in which the human CD95 gene was expressed constitutively; upon treatment with monoclonal anti-CD95, these cells died rapidly by typical apoptosis (Vercammen *et al*., 1998b) (Figure 1). Hence, all the machinery necessary for the apoptotic pathway is present in the cells. The introduction of the human CD95 gene did not affect at all the necrotic response to TNF.

When the L929.hCD95 cells were treated with an agonistic anti-CD95 antibody and in addition a broad-

spectrum caspase inhibitor such as benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone (zVAD-fmk) was added, as expected, the apoptotic pathway was blocked. But remarkably, the cells now died by necrosis; this is an active process, involving again enhanced ROS production, which could be inhibited by BHA (in the presence of both zVAD-fmk and BHA, the cells no longer proliferated and slowly disappeared). So we can conclude that CD95 triggering can lead to a vigorous apoptotic response, but when the latter is blocked, at least in some cell types, an alternative, slower process leading to necrosis becomes apparent (Vercammen *et al*., 1998b). Kawahara *et al*. (1998) recently described another Jurkat cell-derived system in which apoptotic death could be replaced by necrosis. They made a construct coding for a fusion protein consisting of an inducible oligomerization domain followed by a FADD domain. As expected, upon addition of an oligomerizing agent, typical apoptosis was observed. Remarkably, however, when the caspase-dependent pathway was blocked, either by using a caspase-8-negative Jurkat subline or by addition of a potent caspase inhibitor, induced oligomerization of FADD still led to cell death. But characteristic apoptotic features were not observed (no internucleosomal DNA cleavage or lamin breakdown, etc.); rather, the cells seemed to die by necrosis! In fact, treatment of Jurkat cells or peripheral blood lymphocytes with CD95L results in increased ROS production and slightly enhanced $\Delta\Psi_m$, even in the presence of the broad-spectrum caspase inhibitor zVAD-fmk (Banki *et al*., 1999). These events are reminiscent of those occurring in L929 cells treated with TNF, and suggest a similar necrotic pathway. In fact, a number of cell systems have been reported in the literature, where in the presence of a broad-spectrum caspase inhibitor, necrotic cell death features were observed (reviewed by Kitanaka and Kuchino, 1999). A typical example was a transfected glioma cell where induction of an introduced activated H-ras gene led to necrotic death characterized by extensive autophagy (Kitanaka and Kuchino, 1999). It is of interest that in several of these caspase-independent systems the normally apoptogenic Bax protein was implicated. Furthermore, induced expression of mammalian Bax in yeast cells leads to cell death due to mitochondria-derived ROS (Madeo *et al*., 1999).

Similar remarkable findings have even been described for an *in vivo* system. Death and removal of interdigital cells in the mouse embryo is a prototype example of developmental PCD. Studying this system, Chautan *et al*. (1999) found that blocking of apoptosis, either by addition of a caspase inhibitor or by using apaf-$1^{-/-}$ mice, did not prevent interdigital cell death or removal. In the absence of caspase activity, these cells revealed features strongly suggesting ongoing necrosis. Even under normal conditions, a fraction of interdigital cells apparently died by necrosis rather than by apoptosis. These results are in agreement with earlier studies on PCD in embryogenesis, where necrotic cell death has often been observed (Schweichel and Merker, 1973; Kitanaka and Kuchino, 1999). It seems likely, therefore, that in many cell types both an apoptotic and a necrotic pathway are latently present. When upon stimulation, the caspase-dependent pathway is non-functional or blocked, an underlying, slower pathway becomes in evidence, which leads to the cell's demise by necrosis.

However, one should not equate caspase-independent cell death with necrosis. There are many examples of apoptotic cell death occurring even in the presence of broad-spectrum caspase inhibitors such as zVAD-fmk (Borner and Monney, 1999). For example, one mechanism may involve an apoptosis-inducing factor (AIF). Various triggers of cell death cause release of AIF from the mitochondrial intermembrane space; AIF then moves to the nucleus, where it causes apoptotic features (Lorenzo *et al*., 1999).

Does a caspase also play a role in mitochondrial turnover?

As mentioned above, the cell line L929.hCD95 can be killed either necrotically by treatment with TNF, or apoptotically by addition of CD95L, and this pathway is blocked by zVAD-fmk. So, what happens when a caspase inhibitor is added to TNF-treated L929 cells? This provided the second surprise. Not only did the caspase inhibitor zVAD-fmk, as expected, not block the TNF-triggered necrotic pathway, but unexpectedly it dramatically synergized (Vercammen *et al*., 1998a)! In the presence of the inhibitor, the cells became at least 1000-fold more sensitive to TNF. This corresponded to an enhanced ROS production. A similar sensitization was obtained by constitutive expression of CrmA (CrmA is a cowpox virus-derived gene; CrmA protein inhibits efficiently caspase-8 and -1, but not effector caspases; Zhou *et al*., 1997). An inactive mutant of CrmA (T291R) did not synergize (G Van Loo, 1999 unpublished results). These results suggest that a caspase, most probably caspase-8, or another protease similarly sensitive to the caspase-inhibitors tested, and which is either constitutively active or becomes activated by the trigger, counteracts the TNF-induced signaling to necrosis. Blocking this protective mechanism by a caspase inhibitor results in strong sensitization (Vercammen *et al*., 1998a).

What could be the mechanism of this dramatic sensitization of the TNF-induced necrotic pathway by caspase inhibitors? Of course, here we come into the domain of pure speculation. But models, albeit highly hypothetical, at least may suggest avenues for further experimentation. As mentioned above, cells have a basic rate of mitochondrial ROS production; this may be an inherent leakiness of the system. But it is a stochastic process, and a cell contains many mitochondria (e.g. there are about 500 to 2500 mitochondria in a rat liver cell). Hence, in any cell some mitochondria produce more ROS than others. It may even be a self-amplifying process, in the sense that mitochondria damaged by ROS may be impaired in their normal electron flow, and therefore continue to deviate even more electrons into ROS formation. Suppose now that there exists a surveillance system which recognizes and removes the most damaged mitochondria; and suppose that in this surveillance system a caspase or a caspase-like protease plays a critical role (Figure 2). Autophagy has indeed been observed in TNF-treated cells (Prins *et al*., 1998). Under normal conditions this surveillance system mobs up an occasional, functionally impaired mitochondrion. Upon treatment with TNF, however,

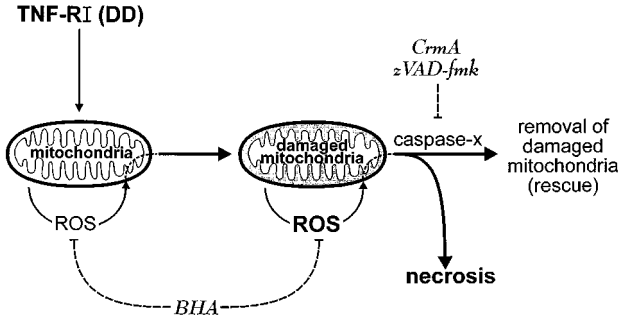

Figure 2 TNF-induced necrosis in L929 cells. Clustering of TNF-RI leads via unknown intermediates to increased formation of ROS in the mitochondria. The site of production is presumably at the ubiquinone step. BHA (butylated hydroxyanisole) is the only reducing agent which effectively blocks the excessive ROS production and the resulting necrosis. The ROS damage the respiratory chain, which becomes even less efficient in transferring electrons to O_2, which means further enhanced ROS production (a positive feedback loop). When too many mitochondria are damaged, the cells swell and finally die by necrosis. A hypothesis to explain the strong enhancement by caspase inhibitors: Suppose a surveillance system removes the most damaged mitochondria. An essential component of this system would be a caspase-like enzyme (caspase-x), most probably caspase-8. Blocking of this caspase inactivates the rescue system, resulting in accumulation of high ROS-producing mitochondria and more rapid demise of the cell (after Vercammen *et al*., 1998a)

ROS production becomes accelerated, and now many more mitochondria sustain ROS-induced damage, and need to be removed by the surveillance system; occasionally, a cell can no longer cope and dies. In the presence of the caspase inhibitor, the surveillance system is blocked; therefore, damaged – this means higher ROS-producing – mitochondria accumulate, causing the rapid demise of the cell. There is perhaps some evidence for such a system. Autophagy, which here refers to removal of mitochondria, has been described in yeast and involves a proteasome-like complex (Mizushima *et al*., 1998); remarkably, one component of this complex is closely related to an 'apoptosis-specific protein' in mammals (Hammond *et al*., 1998).

A scenario as proposed here would imply that a caspase (or a caspase-like enzyme) plays an important repair role in maintaining homeostasis of the cell. If so, would a caspase inhibitor not be toxic for healthy cells? Perhaps it is; zVAD-fmk, although efficient in protecting CD95L- or TNF-treated cells from apoptotic death, may affect cell survival in the long term. The hypothesis concerning caspase- or protease-mediated surveillance of damaged mitochondria merits further investigation, not the least because various caspase inhibitors are being developed for preventing inflammatory responses and for inhibiting neuro- and other degenerative diseases.

Apoptosis vs necrosis: not the two sides of a coin

The neat features of apoptotic cell death have been extolled in numerous reviews. The cells die rapidly, without any spilling of their content, and the corpses are removed without delay. In short, the perfect suicide/murder. This may be ideal for development, for tissue remodelling, or for termination of a vigorous immune response. On the other hand, when a cell is attacked by a pathogen or sustains chemical or physical insults, it is of advantage to the organism that signals are sent out, such that proper specific and non-specific defences are set up, in short an inflammatory response. This is best achieved by necrotic cell death. Although in necrosis, by definition, the cell content is released, this does not necessarily mean that the patient or organism mounts an auto-immune response.

In reality, the aforementioned alternative pathways of apoptotic and necrotic cell death corresponding to non-inflammatory and inflammatory outcomes, respectively, are extreme examples of a wide spectrum of cellular responses. CD95L is the best known inducer of bona fide apoptosis. Yet, tumour cells expressing a stable form of CD95L on their surface, when injected into mice, elicited a vigorous inflammatory response, which was mediated by IL-1 (Miwa *et al*., 1998). Another example is ischemia-reperfusion injury, which is characterized not only by extensive apoptotic cell death, but also by damaging inflammation; blocking apoptosis also alleviated the inflammation (Daemen *et al*., 1999). Cells which are recognized as non-self, are killed by cytotoxic T-cells in a typical apoptotic process initiated either by CD95 clustering or granzyme B/perforin action. Yet, for many years immunologists have been following the cytotoxic T-cell reactivity by release into the medium of ^{51}Cr from the target cells! In fact, more detailed studies have revealed that when a cell dies by a typical apoptotic process, usually there occurs a late-phase necrosis, characterized by spillage of cell content (Dive *et al*., 1992; Geier *et al*., 1995; Slater *et al*., 1995). In the studies of Chautan *et al*. (1999) on the removal of interdigital cells in the developing mouse embryo referred to above, when apoptosis was prevented, the cells died by necrosis, and even under normal conditions a certain percentage of the cells are removed by necrosis; yet, in neither condition was there evidence for an inflammatory response.

In Jurkat and in HeLa cells it has been observed that when intracellular ATP levels drop below a critical value, CD95 triggering no longer elicits apoptotic cell death, but a pathway leading to necrosis becomes in evidence (Eguchi *et al*., 1997; Leist *et al*., 1997). It is tempting to believe that this observation is related to the known requirement for dATP, in direct equilibrium with ATP, for the Apaf-1-mediated activation of procaspase-9. It is not known whether the necrotic pathway observed under low ATP conditions is caspase-independent, as in the studies of Kawahara *et al*. (1998) and the other examples referred to above. In those cells where both pathways can occur, apoptosis fails when ATP levels drop below 15–25% of control cells (Lieberthal *et al*., 1998; Lelli *et al*., 1998). But, as mentioned above, in fibrosarcoma cells, TNF treatment results in necrosis without a decrease of the ATP level (G Denecker, 1999 unpublished results). AKR-2B fibroblasts die upon withdrawal of serum; the intracellular ATP content remained high, yet detailed analysis revealed features both of necrosis (rapid membrane leakage, no oligonucleosomal DNA fragmentation) as well as of apoptosis (membrane

blebbing, strong nuclear condensation) (Simm *et al*., 1997). Although dATP/ATP is required for apoptosis, absence or blockage of the latter pathway apparently reveals a slower, necrotic process, independent of the ATP level.

Other conditions, such as relative abundance of Bax and Bcl-x_L, may also determine whether a cell will die by apoptosis or by necrosis (Shinoura *et al*., 1999).

Involvement of ROS in other cell death pathways

As briefly mentioned above, cell death pathways initiated by stimulation of CD95 or TNF-RI, or even following encounter with an aggressive cytotoxic T-cell, are usually independent of ROS or pO_2. Indeed, in the typical pathway involving initiator caspases and effector caspases, there is no need to invoke an oxido-reduction sensitive step. On the other hand, as described above, TNF-induced necrosis, which occurs in several fibrosarcoma cell lines, is caused by ROS formed in the mitochondria; unlike the systems described below, this process can be inhibited by BHA, but not by most other commonly used, reducing agents.

A TNF effect which has confused some investigators, is the strong priming for an oxidative burst (Berkow *et al*., 1987). It involves translocation from the cytosol to the cell membrane of components of the NADPH oxidase complex (Dusi *et al*., 1996). The release of superoxide radicals in the medium is dramatic in the case of monocytes and neutrophils, but many other cell types, especially transformed cells, can also produce some response. This property is clearly part of the anti-microbial function of TNF. The enzymatic reaction can be distinguished, as it is blocked by specific inhibitors such as diphenylene iodonium.

But there are many reports on other cell death systems where an involvement of ROS has been demonstrated or surmised. However, the situation can be quite complex, as ROS may induce cellular defences against oxidative stress, while on the other hand (perhaps a different reactive oxygen species) it may trigger apoptotic cell death. As mentioned above, TNF induces mitochondrial MnSOD in many cells, albeit the induction is often conspiciously absent or less efficient in cancer-derived cells (Kawaguchi *et al*., 1990; Wong, 1995; Xu *et al*., 1999). ROS are involved in MnSOD induction, as the process can be inhibited by reducing agents such as N-acetylcysteine (Warner *et al*., 1996). The fact that MnSOD protects against TNF-induced death in cells as diverse as kidney cells (HEK293), melanoma cells (ME-180 and A375) and breast tumour cells (MCF-7) strongly suggest that superoxide, locally produced in the mitochondria, is another mechanism leading to cytotoxicity (Wong *et al*., 1989; Hirose *et al*., 1993; Li and Oberley, 1997). It has also been suggested that MnSOD alleviates, at least to some extent, the damage due to oxidative stress in the case of ischemia/reperfusion (Eddy *et al*., 1992). Injury after ischemia/reperfusion can be explained as follows: upon deprivation of O_2, ATP levels in the cells drop and this causes translocation of Bax from the cytosol to the mitochondria, where it induces cyt c release. Upon reoxygenation, ATP (and dATP) is formed, which causes activation of the effector caspases-3 and -7 leading to cell death (Saikumar *et al*., 1998a). Note, however, that also caspase-independent injury occurs in ischemia-reperfusion (Saikumar *et al*., 1998b).

At least in some cell types, mitochondria-derived ROS cause the release of cyt c, followed by activation of the effector caspases. For example, in the myelogenous leukemia cell line ML-1a, TNF treatment leads to mitochondrial ROS production, a process which is inhibited by reducing agents such as PDTC (this means a mechanism different from that observed in L929 cells). The mitochondrial ROS then causes cyt c release and, via caspase-3 activation, apoptosis (Higuchi *et al*., 1997, 1998). A similar mechanism may occur in activated T-cells, destined to die, independently of TNF or CD95L; endogenously generated ROS lead to caspase-dependent apoptosis (Hildeman *et al*., 1999). In HeLa cells, TNF can switch on two different pathways leading to cell death. One, which involves mitochondrial ROS production, and the other not; but both finally result in the activation of effector caspases (Sidoti-de Fraisse *et al*., 1998). Treatment of Jurkat cells with oxidants such as H_2O_2 or with tributyltin rapidly leads to cyt c release, followed by effector caspase activation and apoptosis (Stridh *et al*., 1998). These various results suggest that there exists indeed an alternative mechanism for cyt c release and which is perhaps a direct effect of ROS on the mitochondria. Indeed, treatment of isolated mitochondria with tert-butylhydroperoxide leads to opening of permeability transition pores and release of mitochondrial intermembrane proteins (Susin *et al*., 1999).

There are many observations which indicate that the redox status of a cell plays a dominant role for survival following an insult. Oxidative stress is a potential threat for most cells, prokaryotic as well as eukaryotic, and is universally sensed by disulfide bond formation (Åslund and Beckwith, 1999). The main redox buffers in cells are glutathione and thioredoxin. In fact, thioredoxin is overexpressed in many types of human cancer, presumably as a result of anti-apoptotic selection (Baker *et al*., 1997). Thioredoxin, in combination with thioredoxin peroxidase, very efficiently inactivates ROS and protects cells from apoptosis induced by serum deprivation, ceramide or etoposide treatment (Zhang *et al*., 1997). Thioredoxin also prevents loss of pancreatic β-cells in diabetes mellitus model systems (Hotta *et al*., 1998). As mentioned above, in some cell types H_2O_2 induces cyt c release, resulting in caspase activation and apoptosis. The same outcome can be achieved by treatment with sulfhydryl-blocking reagents, such as diamide; as expected, this process is inhibited by thioredoxin (Ueda *et al*., 1998). It is of considerable interest that higher concentrations of diamide no longer result in caspase activation, but the cells now die by necrosis. This is reminiscent of the results referred to above (Kawahara *et al*., 1998; Vercammen *et al*., 1998b; Kitanaka and Kuchino, 1999). Presumably, when the level of cytosolic ROS becomes too high, the essential cysteine in the active center of the caspases becomes oxidized or blocked (Hampton and Orrenius, 1998; Ueda *et al*., 1998). Could this modification of the essential caspase cysteine also play a role under more physiological

conditions? ROS can react with nitric oxide, an important signal molecule; the resulting nitric superoxide radical can then nitrosylate sulfhydryl groups. In fact, intracellular caspase-3 zymogen was found to be nitrosylated on its catalytic site cysteine, and, remarkably, CD95 triggering induced both denitrosylation and processing, resulting in active enzyme (Mannick *et al*., 1999).

Death of neuronal cells is an important process in development, but moreover it is also crucial in many pathologies. Neuronal cells easily die by oxidative stress or by withdrawal of growth factors (e.g. neurotrophic growth factor) or lack of trophic support (Greenlund *et al*., 1995; Castagne and Clarke, 1996; Mignotte and Vayssière, 1998). Both lack of growth factor or of trophic support leads to ROS production, which finally results in apoptotic death, although also necrotic features have been observed, for example in Alzheimer's and Parkinson's diseases (Kane *et al*., 1995; Kitanaka and Kuchino, 1999). The production of ROS, and hence of apoptosis, can be inhibited by reducing agents such as N-acetylcysteine. But also expression of Bcl-2 is protective. Remarkably, this is not (only) by inhibiting the activation of effector caspases, but by enhancing the reducing capacity of the cell (Hockenbery *et al*., 1993; Kane *et al*., 1993; Ellerby *et al*., 1996). Even in yeast, human Bcl-2 protects against cytosolic oxidative stress (Longo *et al*., 1997).

It is intriguing that caspase-1, generally believed to be only involved in inflammation, may also play a role in neuronal cell death as a result of trophic factor withdrawal, or ischemia/reperfusion, or some neurodegenerative diseases, presumably by producing interleukin-1β (Friedlander *et al*., 1997b; Hara *et al*., 1997; Friedlander and Yuan, 1998). Activation of caspase-1 is enhanced by ROS. In familial amyotrophic lateral sclerosis (ALS), a mutant CuZnSOD may produce more of a particular ROS in the cytosol; indeed, caspase-1 which results in interleukin-1β activation, is implicated in the apoptosis of motor neurons, characteristic of this disease (Friedlander *et al*., 1997a; Pasinelli *et al*., 1998).

Finally, we should also mention here a system which superficially appears like oxidative stress-induced cell death, but which relies in fact on the CD95L-CD95 pathway. This system is regulated by the 'apoptosis signal-regulating kinase' or ASK1 (Ichijo *et al*., 1997). ASK1 is a MAPKKK that, via two different kinase kinases, leads to activation of JNK and p38 MAPK. ASK1 is a crucial sensor in many types of cells. It becomes activated by TNF via interaction with TRAF2 (or TRAF5) (Nishitoh *et al*., 1998), but is also linked to CD95 by Daxx (Chang *et al*., 1998, 1999), becomes activated by agents interfering with the microtubuli (Wang *et al*., 1998b) and by genotoxic compounds such as cisplatinum (Sanchez-Perez *et al*., 1998; Chen *et al*., 1999), and last but not least, it is responsible for the cellular response to oxidative stress (Gotoh and Cooper, 1998; Saitoh *et al*., 1998). Under normal conditions, ASK1 is bound in an inactive form to thioredoxin, from which it can be released by oxidative stress, for example, treatment of the cells with H_2O_2. The released ASK1 then dimerizes and as a result becomes activated. Common reducing agents, such as N-acetylcysteine, prevent the activation. But how could transcription activators such as JNK and p38 MAPK enhance cell death? The most likely explanation is that they induce rapid synthesis of CD95L, such that the cells become killed by autocrine or paracrine effects. Such oxidative stress-induced CD95L expression has been observed in Jurkat (Bauer *et al*., 1998; Faris *et al*., 1998), microglial (Vogt *et al*., 1998), PC12, primary neuronal (Le-Niculescu *et al*., 1999), and hepatoma cells (Hug *et al*., 1997), and hence may be relatively common. Many chemotherapeutic drugs upregulate CD95L in tumor cells (Müller *et al*., 1998), possibly via ROS-mediated activation of ASK1. It is of interest that this enhanced expression of CD95L is p53-independent, while the same drugs also upregulate CD95, but only in p53 wild-type cells. Finally, it may be noted that JNK can also have an anti-apoptotic effect, for example in fibroblasts, where activated Jun cooperates with NF-κB in the induction of anti-apoptotic proteins (Wisdom *et al*., 1999). Also studies with sek1$^{-/-}$ (JNK kinase) and traf2$^{-/-}$ mice support a protective role of JNK in development; the enzyme may be required for anti-apoptotic Bcl-x_L expression (Yeh *et al*., 1997; Nishina *et al*., 1998, 1999).

It is fair to conclude that endogenously produced ROS play a key role in PCD pathways of many cell types. But our molecular understanding of the mechanisms involved is still very fragmentary, both regarding the formation as well as the action of ROS. Also one should not overlook that the term ROS covers in fact a number of quite different oxidative species. In some experimental systems, ROS formed by mitochondria can only be neutralized by the hydrophobic BHA, and may lead to caspase-independent cell death. In other systems, potentially lethal superoxide accumulates in the mitochondrial matrix, where it can be inactivated by MnSOD. In still other systems, ROS leads to release of cyt c from the mitochondria, followed by caspase-mediated cell death. Finally, the action of ROS may be explained by ASK1 activation. These observations in different cell systems indicate the existence of several alternative cell death pathways, and the molecular mechanisms involved need to be further elucidated.

Various pathways which are believed to lead to cell death, are summarized in Table 1.

Abbreviations
BHA, butylated hydroxyanisole; cyt c, cytochrome c; CD95L, CD95 ligand; DD, death domain; DED, death effector domain; DEM, diethylmaleate; MAPKKK, MAP kinase kinase kinase; MnSOD, manganese superoxide dismutase; NAC, N-acetylcysteine; PCD, programmed cell death; PDTC, pyrrolidine-dithiocarbamate; ROS, reactive oxygen species; TNF, tumour necrosis factor; TNF-RI, TNF receptor I; zVAD-fmk, benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone.

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