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REVIEW

TNFα and reactive oxygen species in necrotic cell death

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Death receptors, including the TNF receptor-1 (TNF-RI), have been shown to be able to initiate caspase-independent cell death. This form of "necrotic cell death" appears to be dependent on the generation of reactive oxygen species. Recent data have indicated that superoxide generation is dependent on the activation of NADPH oxidases, which form a complex with the adaptor molecules RIP1 and TRADD. The mechanism of superoxide generation further establishes RIP1 as the central molecule in ROS production and cell death initiated by TNFα and other death receptors. A role for the sustained JNK activation in necrotic cell death is also suggested. The sensitization of virus-infected cells to TNFα indicates that necrotic cell death may represent an alternative cell death pathway for clearance of infected cells.

Keywords: TNF, necrosis, Nox1, TRADD, RIP1, ROS, JNK

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ROS and death receptors

Levels of cellular reactive oxygen species (ROS) are modulated by the balance between the cellular processes that produce ROS and the processes that eliminate them [1]. In most situations, ROS are attenuated by systems of antioxidant enzymes, including superoxide dismutases, catalase, thioredoxin reductase, glutathione peroxidases, and peroxiredoxins, as well as non-enzymatic antioxidants [1, 2]. ROS are produced primarily by three sources within the cell. First, and foremost in ROS production is the mitochondria, from which some electrons leak at a rate that has been estimated to be between 2 to 5% as they are transferred between electron transport chain complexes [3, 4]. A second major source of ROS in the cell are the NADPH oxidase complexes, which use NADPH as a substrate to bring about the direct reduction of molecular oxygen to produce superoxide anions [5] which play an important role in defense against environmental pathogens [6] as well as in signal transduction pathways. Third, many other enzymes produce ROS in many different cell types, although less robustly, including xanthine oxidase, cyclooxygenases, lipoxygenases, myeloperoxidases, heme oxygenase, monoamine oxidases, and aldehyde oxidase, as well as cytochrome P450-based enzymes. Cytokines and growth factors that bind to receptors of different classes have also been reported to lead to the generation of ROS that function as second messengers in their signal transduction pathways [7, 8], however, the production of ROS may not be intrinsic to the cytokines and growth factors and may actually be achieved by the activation of other enzymes, such as NADPH oxidases. This appears to be the case in signaling and death induced by death receptors.

The death receptors are a subfamily of receptors in the TNF Receptor superfamily (for reviews, [9, 10]). These receptors contain an intracellular "death domain" through which they mediate their downstream signaling pathways by means of homotypic interactions with death domain-containing adaptor proteins, such as FADD, TRADD, and RIP1. These death receptors, including TNF-R1 (Figure 1), Fas, Death Receptor 3, and the TRAIL receptors, DR4 and DR5, induce apoptosis in many cell types through FADD-dependent activation of caspase-8. When activated, caspase-8 may act in the apoptotic process by directly cleaving cellular proteins, or may act indirectly to induce apoptosis through cleavage of the BH3-only protein BID. Truncated Bid protein acts on the mitochondria to cause the release of cytochrome c from the mitochondria, which activates further downstream caspases through an Apaf-1/caspase-9 mediated pathway.

While the pathways leading to caspase-dependent apoptosis are reasonably well understood, death receptors also have been shown under some circumstances to initiate cell death through less-defined pathways that are not dependent on caspase activity [11]. In the case of the TRAIL receptors, caspase-independent cell death has been reported to take the form of autophagic cell death [12, 13], a variant of programmed cell death that requires specific

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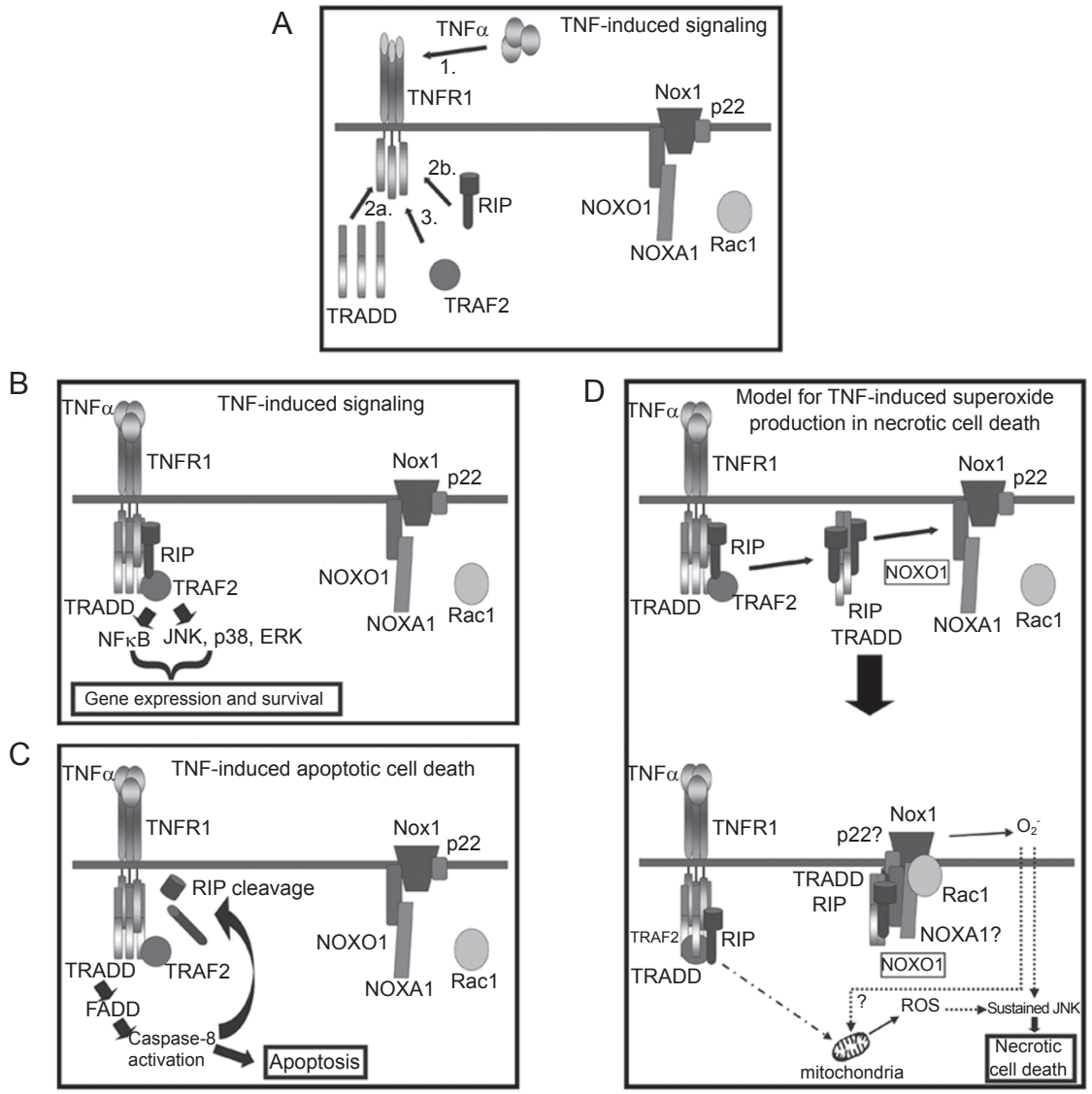

Figure 1 (A) TNFα signaling proceeds by the binding of the TNFα trimer to the TNF-R1 receptor. This initiates a change in the receptor that brings about the binding of the TRADD and RIP1 adaptor proteins to the death domain of the receptor. These adaptor proteins are then able to recruit TRAF2 and the IKK machinery (not shown) to the complex. **(B)** Recruitment of TRADD, RIP1, TRAF2, and the downstream machinery causes the degradation of the IκBα protein and activation of MAP kinase kinase kinases (MAP3Ks). The proteolytic degradation of IκBα, which typically retains NF-κB in the cytoplasm, allows NF-κB to translocate to the nucleus where it acts as a transcription factor. The MAP3Ks phosphorylate and activate downstream kinases that result in the activation of the MAP Kinases, JNK, p38 and ERK. Activation of these kinases in combination with NF-κB activation results in the transcription of pro-inflammatory and pro-survival genes. **(C)** When NF-κB activation is diminished or prevented, TNFα signaling results in the recruitment of FADD and caspase-8 to a secondary complex that triggers caspase-8 activation, which leads to apoptosis. One of the substrates for caspase-8 is RIP1. Cleavage of RIP1 further potentiates apoptosis. **(D)** When caspases are inhibited, apoptosis is prevented and the RIP1 protein is stabilized. RIP1 and TRADD form a complex with the NOXO1, which recruits Nox1 and Rac1 to form an active superoxide producing complex. RIP1 is proposed to be important for NOXO1 recruitment, while TRADD may be important in the activation of the complex through its interaction with the SH3 domain of NOXO1. The presence of NOXA1 as an activator is predicted, but has not yet been shown. The presence of p22 in the complex is unknown. Production of superoxide is proposed to promote the sustained activation of JNK, which leads to necrotic cell death.

genetically defined pathways and which occurs following a failed attempt by the cell to survive by meeting its energetic needs through the lysosomal degradation of cellular proteins and/or organelles [14, 15]. In most cases, however, caspase-independent cell death induced by death receptors does not appear to be the result of autophagy, as autophagy inhibitors such as 3-methyladenine, chloroquine, and PI 3-kinase inhibitors, or energy supplementation with methyl pyruvate fail to inhibit cell death. The morphology of this form of death resembles that of necrosis, with organelle and cellular swelling, large-scale mitochondrial damage and loss of membrane integrity [15, 16]. Therefore, we (and others in the field) have referred to caspase-independent cell death induced by death receptors as "necrotic cell death", although this is clearly not the passive, non-programmed process that necrosis is usually depicted as [17]. Although necrosis has been described classically as accidental cell death occurring only in cases of severe pathological damage, there is now evidence that necrotic cell death may also play a role in normal physiology and development [11, 14].

Necrotic cell death induced by death receptors has been proposed to involve the generation of ROS derived from either mitochondrial or non-mitochondrial sources [14, 16]. Caspase inhibition under these circumstances actually leads to an enhancement of cell death, rather than its inhibition [18]. This may be due, in part, to caspase-8 cleavage of RIP1 during the apoptotic process (Figure 1C, [19]), and may imply a certain level of basal cleavage of RIP1 in some cell types [20]. From the data of many groups, RIP1 appears to be a central molecule in the initiation of caspase-independent cell death by death receptors, as well as by other stimuli [11, 18, 21, 22]. The RIP1 protein is necessary for the generation of ROS by TNFα and is required for the initiation of caspase-independent cell death [22, 23].

FasL, TRAIL, and TNFα have been shown to lead to ROS generation in response to receptor stimulation [16, 24-28]. The production of ROS in many cases has been implicated to come from downstream events involving mitochondria [16]. It is now becoming clear, however, that both TNFα and FasL can stimulate the production of superoxide by activating NADPH oxidases [25, 29-31].

NADPH oxidases

NADPH oxidases are one of the few enzymes specifically dedicated to the production of ROS. Originally thought to be a single enzyme, NADPH oxidases have now been found to comprise a large family of enzymes, with the expression of different isoforms being tissue specific [5]. Activated neutrophils and macrophages produce large quantities of superoxide for host defense via the phagocytic form of NADPH oxidase, Nox2 (previously known as gp91phox). A number of other NADPH oxidase family members (Nox1, Nox3, and Nox4, Nox5, DUOX1, DUOX 2) have been characterized [5] in non-phagocytic cell types, including fibroblasts, vascular smooth muscle cells, cardiac myocytes, and endothelial cells. These cell types produce small amounts of ROS by NADPH oxidases to regulate intracellular signaling cascades [5, 6]. NOXs 1-4 exist as heterodimers with a 22 kDa subunit (p22phox), and require the presence of this small subunit for activity. In the case of Nox2, two other activator subunits are required for activity. Upon phosphorylation in response to stimulus, the p47phox subunit binds to membrane phospholipids, interacts with p22phox, and recruits the p67phox subunit to the complex [5]. The p67phox activator binds and stabilizes an interaction of the complex with the small GTPase Rac (Rac1 or Rac2, depending on cell type), resulting in an active enzyme complex [5]. The regulatory p41NOXO1 and p51NOXA1 subunits may function in other oxidase complexes, such as Nox1, similarly to p47phox and p67phox, respectively, with some small differences [32-34]. NOXO1, unlike p47phox, lacks an autoinhibitory region, and does not require phosphorylation for membrane translocation. Hence it requires no new activation stimulus for activity. Its PX domain also has a slightly different lipid preference [35]. NOXA1, unlike p67phox, has a single SH3 domain rather than two SH3 domains. In overexpression studies, when p47phox and/or p67phox were exchanged for NOXO1 and/or NOXA1 and vice-versa in the Nox1 and Nox2 complexes, the complexes were still functional, though the superoxide generation was less potent [32-34]. It is unclear as to whether this actually occurs in endogenous complexes, but, if true, it may indicate that there is some redundancy in the use of subunits by Nox enzymes, or that the amount of superoxide produced may be modulated by subunit expression in a cell type specific manner.

TNFα has long been known to potentiate NADPH oxidase activity in macrophages, monocytes, and neutrophils, however, a second stimulus with another agonist, such as PMA was considered necessary to actually activate the oxidase. While it has been clear that TNFα is a powerful enabler of Nox2 activation, the mechanism by which this occurs is uncertain. Several reports have shown that TNFα leads to increased expression of various NADPH oxidase components [36-38], and this appears to contribute to increased oxidase activity, especially in the long term. However, more direct potential mechanisms for potentiation of superoxide production have been proposed, some of which involve the phosphorylation of p47phox by different kinases, including PKCζ [39], a tyrosine kinase [40-42], or p38 MAPK [43]. TNFα has also been proposed to regulate Nox2 activity though regulation of the proposed associated

hydrogen ion channel that balances the electronic charge translocated by NADPH oxidase [44].

In contrast to the indirect activation of Nox2, we have recently shown that TNFα is a direct activator of the Nox1 NADPH oxidase in the L929 murine fibrosarcoma cell line, as well as in $p65^{-/-}$ MEFs subjected to caspase inhibition [31]. Treatment of L929 cells with TNFα alone is sufficient to induce superoxide production within 30-45 minutes of treatment, and the superoxide generated by Nox1 contributes substantially to TNFα-induced necrotic cell death. Knockdown of Nox1 with siRNA prevents both superoxide generation and cell death in response to TNFα. Therefore, Nox1 appears to be the primary source of ROS involved in TNFα-induced cell death in L929 cells.

Nox1, NOXO1 and Rac1 form a signaling complex with components of the TNF receptor complex in a TNF-dependent manner [31]. The mechanism of activation of Nox1 by TNFα in these cells is suggested by protein interactions between the death domain-containing TNF-R1 adaptor proteins RIP1 and TRADD, and the Nox1 organizer protein, NOXO1 (Figure 1D). RIP1 interacts strongly with NOXO1 protein through an as yet undefined domain, while the polyproline-rich region of TRADD interacts weakly with the SH3 domain of NOXO1. RIP1 is required for formation of the induced signaling complex, as Nox1 fails to immunoprecipitate with TRADD in $RIP^{-/-}$ MEFs under TNFα-mediated necrotic conditions. Overexpression of a mutant TRADD protein with a mutation in its proline region that abrogates binding to NOXO1 diminishes superoxide formation and cell death in response to TNFα, and thus the mutant protein functions as a dominant negative molecule with regard to Nox1 activation, without affecting other TNFα signaling pathways. Based on the relative affinities of RIP1 and TRADD for NOXO1, we have proposed a model in which RIP1 recruits NOXO1 and Nox1/NOXO1/Rac1 to the complex, where a second, lower affinity interaction, between NOXO1 and TRADD is required to promote oxidase activation [31].

Consistent with the hypothesis that NOXO1 but not p47phox was involved in oxidase activation, we found that the tyrosine kinase inhibitor genestein, the general PKC inhibitor, bisindolymaleimide, or a specific PKCζ peptide inhibitor had no effect on the superoxide production in L929 cells in response to TNFα. Therefore it appears that induction of this protein complex is a completely novel mechanism by which TNFα affects oxidase activation.

Although previous work has established a role for ROS in the pathway of TNFα–induced necrotic cell death [23, 45-48], some previous data have suggested that the process involves mitochondrial derived ROS [16, 45, 49, 50]. While we have established that the non-mitochondrial oxidase Nox1 appears to be involved, the source of ROS in TNF-stimulated cell death is by no means exclusive. In other experiments, we have detected a large amount of what appears to be mitochondrial-derived ROS generated several hours after the addition of TNFα, and at a much later time point than the earliest appearance of superoxide (unpublished data). While this could mark the appearance of non-specific ROS as a result of the beginning of cell death, it could also suggest the participation of both Nox1-produced superoxide and mitochondrial-produced ROS in the process. As the mechanism of production of mitochondrial-derived ROS has not yet been established, it may be that the superoxide produced by non-mitochondrial oxidases leads to the generation of mitochondrial-derived ROS. Oxidative radical stress itself is known to further amplify the production of ROS within the mitochondria through protein damage [51], which may act as a positive feedback loop. One other group has suggested that Nox1 plays a role in necrotic cell death induced by serum withdrawal. In this case, they propose that Nox1 activation is actually downstream of the production of mitochondrial ROS [52]. Though we cannot at this time eliminate this possibility, the kinetics of complex formation suggests to us that the Nox1-mediated superoxide generation is an early event.

Contribution of ROS to cell death

How do the superoxide and other ROS generated downstream of TNF stimulation contribute to cell death? The molecular targets of ROS have begun to be defined, but much is not yet known. ROS production may have various roles in cell death, namely, ROS may directly oxidize cellular proteins, lipids, or nucleic acids and cause general damage and dysfunction, or ROS may initiate cell death processes through affecting various signaling cascades [17]. One of the major ways by which ROS affect signaling pathways is through the direct reaction of these compounds with the catalytic sites of phosphatases. Classical protein tyrosine phosphatases have long been known to be inactivated by ROS-mediated oxidation of their catalytic cysteine [53, 54]. More recent data suggest that ROS are also capable of inactivating dual specificity phosphatases [55], which can dephosphorylate tyrosine and serine/threonine residues, as well as phospholipids. Depending on the oxidation state of the catalytic cysteine, inactivation of phosphatases by ROS may be reversible or irreversible [54, 56]. Having a prolonged phosphorylation status affects the activity of many proteins within the cell, including the stress-activated MAP kinases, p38 and JNK [55].

JNK activity has been reported to be positively regulated by ROS in a number of ways. Primarily, JNK is positively affected by the direct inactivation of JNK phosphatase

activity by ROS [55]. The monomeric form of glutathione S-transferase Pi (GSTπ) binds directly to the C-terminus of JNK and inhibits its activation [57]. ROS induces oligomerization of GSTπ and causes its dissociation from JNK, resulting in JNK activation [58]. ASK1 is a MAP3K that has been reported to be important in promoting downstream sustained phosphorylation of JNK under conditions of oxidative stress [59]. The reduced form of thioredoxin binds to ASK1 and blocks its kinase activity in a large complex, which has been termed the ASK1 signalosome [60-62]. Upon oxidation, thioredoxin is released and TRAF2 and/or TRAF6 are recruited to the signalosome along with downstream kinases, which then are involved in the activation of JNK [62].

JNK has many different functions in cell death [63]. Sustained JNK activation is seen in L929 cells after TNF treatment, which appears to correlate with superoxide generation and necrotic cell death [31]. Suppression of JNK by genetic or pharmacological approaches offers significant protection against cell death induced by ROS and TNF [64], and inhibition of JNK completely blocks TNF-induced necrotic cell death in L929 cells [31]. This is consistent with the finding that exogenously applied ROS induce necrotic cell death via JNK activation [21]. Therefore, JNK is proposed as a critical mediator of necrotic cell death [64], and sustained JNK activation is thought to be one of the key events in necrotic cell death induced by TNF-α [31, 47, 55].

Physiological relevancy of TNF-induced necrotic death

In most cell types under physiological conditions, TNFα treatment does not result in cell death (Figure 1B). This is due in large measure to the activation of NF-κB, which stimulates the transcription of genes for pro-survival proteins [65], such as the caspase-8 inhibitory protein cFLIP, the cytoplasmic zinc finger protein A20, the antiapoptotic Bcl-2 family protein A1, and the cellular inhibitor of apoptosis proteins (cIAPs). Other NF-κB regulated genes have been reported to affect JNK activation, such as GADD45β and XIAP [66, 67]. TNF-α-induced NF-κB activation can reduce ROS levels via increased expression of the antioxidant proteins FHC and MnSOD [68, 69]. Thus, NF-κB prevents both apoptosis and sustained JNK activation [70-73]. Therefore, under normal conditions, both apoptotic and necrotic cell death are inhibited; and under most circumstances where NF-κB is inhibited, apoptosis generally prevails due to the activation of caspases (Figure 1C). Under what conditions, then, does TNFα initiate necrotic cell death? The key to this question may lie in the increased sensitization of cells to necrotic cell death in the presence of caspase-inhibitors, as it has long been known that many pathogens encode proteins that inhibit caspase activation. They consequently prevent apoptosis of an infected cell in response to TNFα, which, as an inflammatory cytokine, would be present at the highest amounts during an infection. Thus, it has been proposed that necrotic cell death may represent an alternative pathway to eliminate infected cells from the body [11]. This hypothesis is borne out by the observation in several cases that cells infected with virus are actually sensitized to TNFα-induced killing, and that viruses lacking the caspase-inhibitory proteins do not lead to sensitization [74, 75]. RIP1 deficiency, however, rescued infected cells from TNFα-induced cytotoxicity [75]. These reports do suggest that necrotic cell death induced by TNFα is likely to be physiologically relevant under conditions of infection and that RIP1 is a central molecule in initiation of the TNFα-induced necrotic cell death pathway.

Conclusion

In conclusion, TNFα induces a necrotic-like cell death when caspases are inhibited within the cell. This death is mediated, at least in part, by ROS generated by the activation of the NADPH oxidase, Nox1, through complex formation between Nox1 subunits and the TNF receptor adaptors RIP1 and TRADD [31]. The model we have proposed (Figure 1D) is that RIP1 recruits NOXO1 and Nox1/NOXO1/Rac1 to a complex where TRADD is required to promote oxidase activation. ROS likely mediate some of their downstream effects on necrotic cell death through the sustained activation of JNK. TNFα-induced necrotic cell death is most likely to play a physiological role in the clearance of infected cells during an immune response.

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