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Review

Regulation of neutrophil apoptosis via death receptors

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Abstract. Human neutrophils constitutively undergo apoptosis, process which is critical for the successful resolution of inflammation by the safe removal of effete cells. A wide variety of agents can modulate neutrophil apoptosis and these act through multiple and complex receptor-signalling pathways. Whilst these pathways can be initiated via distinct cell surface receptors, many downstream intracellular pathways can converge, use common molecules or trigger similar cellular activities, such as activation of caspases and transcription factors. The cell surface receptors, TNFR and Fas both trigger apoptosis in certain cell types, including neutrophils. However, TNF receptors also activate survival mechanisms in human neutrophils. This review summarises current knowledge about the regulation of neutrophil apoptosis via death receptors, the molecular components involved in signalling and potential therapeutic targets that are based on death receptors or their signalling pathways.

Key words. Neutrophil; apoptosis; death receptor; TNF-α; Fas; protein kinase; anti-TNF-α.

Introduction

Neutrophils are polymorphonuclear leukocytes that are critical components of the cellular immune system. They are generally regarded as terminally differentiated and are the most abundant type of immune cell in the circulation [1], being the first line of defence against bacterial and fungal infections. They are produced in bone marrow from myeloid stem cells and are then released into the circulation where they spend their short life-span (8–20 h). However, their survival time can increase significantly once they migrate out of the circulation and into the site of infection or inflammation where they become exposed to pro-inflammatory signals [1]. Aged neutrophils undergo constitutive apoptosis in the absence of survival factors and are removed by macrophages or other cells (e.g. fibroblasts) by phagocytosis [2]. This mechanism of removal of apoptotic neutrophils is essential for the resolution of inflammation, because it prevents neutrophils from releasing their cytotoxic contents into their surroundings, as would happen if death occurred by necrosis [2]. Whilst neutrophils have a very short life-span in the circulation, a wide range of extracellular agents can modulate both neutrophil apoptosis and survival under different pathophysiological conditions. Cell surface receptors play essential roles in sensing the environmental conditions of neutrophils, and multiple intracellular pathways are involved in the mechanisms conveying the signals from receptors to intracellular effector molecules. Protein kinases play critical roles in transducing signals for both apoptosis and survival. Whilst extended neutrophil survival is essential to fight infections, this extended survival is only temporary, and death by apoptosis followed by safe removal by phagocytic cells prevents tissue damage during the resolution of inflammation [3]. On the other hand, inappropriate delay of neutrophil apoptosis within tissues may be responsible for the tissue damage seen in a number of inflammatory diseases: neutrophils with an ex-

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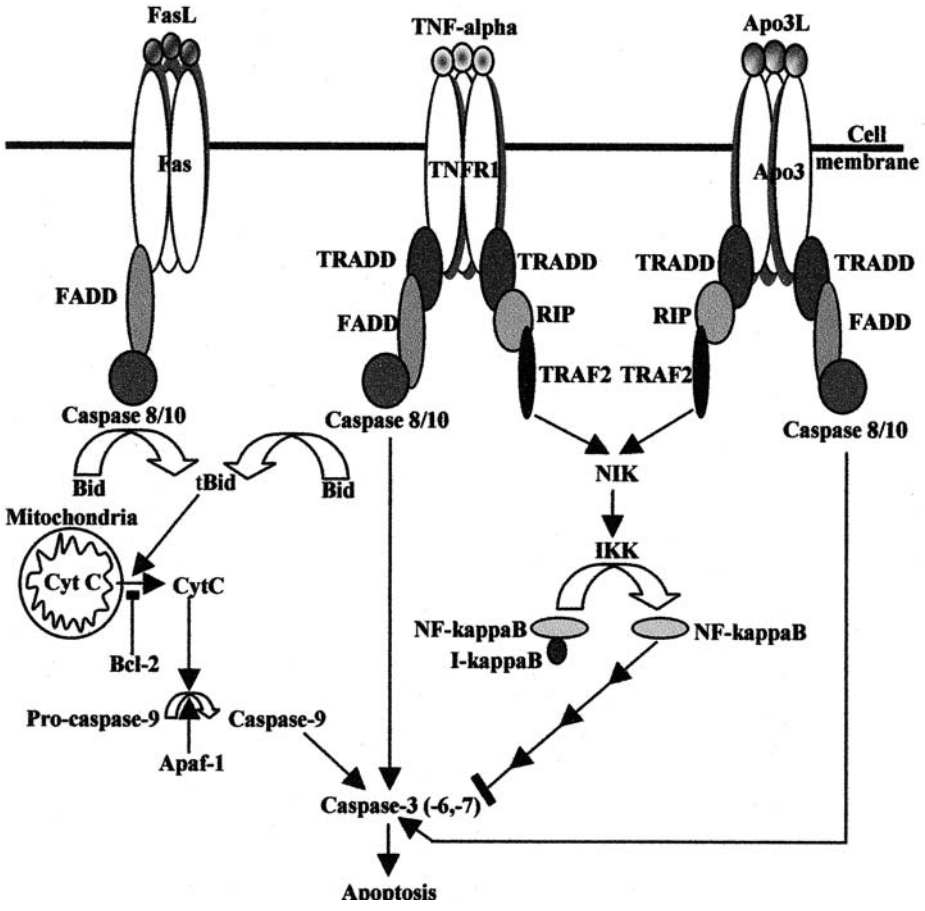

Figure 1. Apoptosis signalling through death receptors. Receptors and intracellular molecules involved in death receptor-mediated apoptosis are shown. Whilst Fas, TNF-α and Apo3L induce apoptosis by activating caspase cascades, TNF-α and Apo3L can also activate specific transcription factors (e.g. NF-κB) to delay apoptosis. Mechanisms are discussed in detail in the text.

tended lifespan will have an enhanced capacity to cause tissue damage via the release of their toxic molecules.

Two major pathways that regulate apoptosis have been defined in a number of different cell types. The first are the death receptor pathways, initiated mainly by tumour necrosis factor receptors (TNFRs) and Fas that can directly activate a caspase cascade via activation of caspase-8 as an initiator caspase [4]. The second is termed the intrinsic apoptosis pathway that involves mitochondria and the Bcl-2 family members, and results in activation of a caspase cascade via activation of caspase-9 as an initiator caspase [5] (fig. 1). The release of mitochondrial cytochrome c and the relative levels and activities of pro- and anti-apoptotic Bcl-2 family members control the activation of procaspase-9 [5]. Apoptotic signals mediated by death receptors can usually bypass the mitochondrial pathway and thus escape the regulation points controlled by the Bcl-2 family proteins. However Bid, one of the pro-apoptotic members, can be cleaved by caspase-8, which is usually activated through death receptors [6]. Activated Bid is then translocated to mitochondria and can induce cytochrome c release [6] (fig. 1). Bid activation is the best-characterised cross-talk between the two apoptosis pathways involving death receptors and mitochondrial components.

Apoptosis mechanisms activated by death receptors

Death receptors are cell surface receptors that transmit apoptosis signals initiated by specific death ligands (fig. 1). They include TNFRs, Fas (Apo-1/CD95), DR3 (Apo-3/TRAMP), DR4 (TRAIL-R1), DR5 (TRAIL-R2) and DR6. The two best-characterised death receptors, TNFR1 and Fas, are both transmembrane protein members of an expanding TNF nerve growth factor (NGF) family that signals for apoptosis in many cell types [4], including neutrophils [7, 8].

Each death receptor contains cysteine-rich extracellular domains and a motif in the cytoplasmic region termed a 'death domain (DD)' [4, 9]. Death domains are mainly involved in protein-protein interactions and connect the receptors with the components of the intracellular apoptosis machinery. Associations between

death domains occur upon receptor-ligand binding and these interactions are necessary for initiation of apoptosis [4, 9].

FasL is the ligand for Fas and is a homotrimeric molecule. Binding of Fas with trimeric FasL leads to cross-linking of three receptor molecules resulting in clustering of intracellular death domains. The association of receptor death domains induces the recruitment of adaptor proteins [4, 9]. The major adaptor protein is the Fas-associated death domain-containing protein (FADD), and recruited FADD associates with the activated receptor through its own death domains. FADD also contains a 'death effector domain' (DED) that allows its interaction with pro-caspase-8 via its respective DEDs. Fas/FADD/pro-caspase-8 together form a protein complex called the 'death-inducing signalling complex' (DISC) [10]. Pro-caspase-8 has been suggested to be activated according to the 'induced proximity model' in which caspase precursor aggregation mediated by FADD induces autoprocessing and autoactivation through cross-cleavage [11]. Indeed, gene knock-out experiments in mice in which FADD is deleted have shown that FADD is one of the essential components of the apoptosis machinery induced by FasL and TNF-α [4].

TNF-α, a trimeric molecule, is the ligand for TNF receptors. Exposure of cells to TNF-α can induce multiple effects including cell differentiation, proliferation, apoptosis and other pro-inflammatory effects. TNF binding induces trimerisation of TNFR1 bringing the death domains of the receptors into close proximity [4, 9]. Subsequently, TNFR-associated death domain-containing proteins (TRADDs) bind to clustered receptors via their respective DDs. TRADD can also associate with other secondary adaptor molecules including TNFR-associated factor-2 (TRAF2) and receptor-interacting protein (RIP) leading to the activation of the transcription factors, NF-κB and AP-1 [4]. However, TRADD can also associate with FADD, thereby inducing the activation of pro-caspase-8 which leads to apoptosis [4, 9]. Whilst TNF-α can induce apoptosis through TNFR1 in some cell types including neutrophils [8], triggering the activation of NF-κB and AP-1 may induce the expression of survival factors, thereby providing resistance against apoptosis [12, 13]. In this respect, TNF-α is a bifunctional molecule and the response of a cell to this agent probably depends upon interplay between pro- and anti-apoptotic signalling events.

Signalling pathways initiated by Fas (CD95) in human neutrophils

Fas is widely expressed on several cell types, whereas constitutive expression of FasL is relatively limited. Although constitutive Fas expression by human neutrophils has been confirmed by many groups [7, 14, 15], there are conflicting reports of FasL expression by human neutrophils [7, 14–16]. Differences in neutrophil purification techniques, culture conditions, gene expression detection techniques and sources of antibodies may be potential reasons for these conflicting results.

Both pro- and anti-inflammatory roles for FasL have been reported and, in part, these discrepancies may be related to whether the FasL is soluble or membrane bound. Indeed, the membrane-bound form of FasL may be a stronger inducer of apoptosis than the soluble FasL (sFasL). SfasL derived by specific cleavage of the extracellular domain of membrane-bound FasL by metalloproteinases (MMPs) has been shown to act as a chemotactic factor for human neutrophils in in vitro migration assays [17, 18]. However, the signalling pathways activated by sFasL responsible for its chemotactic properties have been suggested to be independent from the known death domain-interacting molecules of the receptor [17]. This may represent an indirect, autocrine chemotactic mechanism activated by sFasL, because FasL induces the secretion, by neutrophils, of interleukin (IL)-1β, itself a neutrophil chemoattractant [19]. Neutrophils undergoing spontaneous apoptosis have also been shown to release sFasL and thereby induce apoptosis of co-cultured cells in a paracrine fashion [7, 16]. Whilst an interaction between Fas and FasL was initially suggested as a mechanism to explain constitutive neutrophil apoptosis [7], neutrophils from Fas-deficient mice were found to undergo spontaneous apoptosis at the same rate as wild-type mice, thereby arguing against a role for the Fas/FasL system in constitutive apoptosis [20]. In agreement with this idea, Fas blockade with a neutralising anti-monoclonal antibody had no effect on constitutive neutrophil apoptosis [14]. When Fas was ligated in the presence of granulocyte/macrophage-colony-stimulating factor (GM-CSF), the cytokine did not prevent apoptosis, suggesting that inflammatory neutrophils retain the ability to respond to death signals even in the presence of survival signals [15].

The role of reactive oxygen species (ROS) in the Fas-triggered apoptosis pathway in many cell types, including neutrophils, remains controversial. ROS generated by the NADPH oxidase are not involved in the activation of caspases in neutrophils undergoing spontaneous and Fas-triggered apoptosis, but high levels of ROS produced by activated neutrophils can prevent caspase function [21]. However, caspase-mediated proteolysis and activation of protein kinase C-δ (PKC-δ) has been reported to act as a molecular link between the Fas receptor and the NADPH oxidase system and plays a central role in regulating neutrophil apoptosis [22]. Neutrophils from patients with chronic granulomatous disease (CGD) exhibit delayed apoptosis in vitro and are also resistant to anti-Fas

monoclonal antibody treatment, indicating the possible involvement of ROS in the Fas-mediated signalling system [23].

The involvement of mitogen-activated protein kinases (MAPKs) in Fas-triggered neutrophil apoptosis has also been investigated. Whilst the role of p38 MAPK in spontaneous neutrophil apoptosis is undefined, incubation of neutrophils with anti-Fas antibody does not affect the phosphorylation and activation of p38 MAPK, c-Jun N-terminal kinase (JNK) or extracellular signal-related protein kinase (ERK) [24, 25]. However, Fas has been shown to activate p38 MAPK and JNK in Jurkat T lymphocytes [26]. p38 MAPK activity was recently reported to generate a survival signal that is transiently inactivated during both spontaneous and Fas-induced neutrophil apoptosis, whereas Fas-activated phosphatidylinositol 3-kinase (PI3K) generates a pro-apoptotic signal in human neutrophils [27].

Fas activation decreases phorbal myristate acetate (PMA)-stimulated neutrophil adhesion to endothelial cells that is required prior to their migration from the capillary bed to the site of infection [28]. This is partially due to the effects of Fas on the localisation of PKC-δ. Whilst PMA stimulation results in the membrane association of PKC-δ, Fas activation has been shown to result in the cytosolic localisation of PKC-δ [29]. Cytoplasmic PKC-δ is cleaved by caspase-3 during Fas-induced apoptosis to generate a catalytically active fragment. This fragment phosphorylates phospholipids resulting in phosphatidylserine externalisation [29].

Whilst Fas engagement by an agonistic anti-Fas antibody results in enhanced activities of caspases-8 and -3 and increased mitochondrial permeability, mitochondrial stabilising agents significantly inhibit Fas antibody-induced apoptosis, indicating that mitochondrial disruption is necessary for the induction of apoptosis by Fas engagement [30]. The pro-apoptotic protein Bid, itself activated by caspase-8, can provide this cross-talk between the two apoptosis pathways.

Signalling pathways initiated by TNF receptors in human neutrophils

In contrast to the well-known apoptotic effect of Fas, the effects of TNF-α on neutrophils are more complicated, because this cytokine rapidly accelerates apoptosis in a subpopulation of cells, but can stimulate an anti-apoptotic pathway in the surviving cells [8]. Neutrophils express more than twice as many TNFR75 as TNFR55 and, furthermore, TNFR75 has a higher affinity for TNF-α [8]. However, selective mutational analysis of the two TNF receptors (TNFR55 and TNFR75) has indicated that TNFR75 may function to facilitate a death signal primarily initiated via TNFR55 [8, 31]. The precise nature of the interactions between these two receptors and the molecular processes that regulate their function remain to be elucidated. Whilst TNFR75 has a relatively short cytoplasmic domain with no intrinsic kinase activity or death domain (as is found in TNFR55 and Fas), the discovery of a TNFR75-associated kinase that phosphorylates both receptors provides a potential mechanism whereby TNFR75 could facilitate TNFR55 function [4, 9]. Emerging evidence also shows that the TRAF2-binding site on TNFR75 is involved in enhancement of TNFR55-induced cell death [31]. The divergent effects of TNF-α on neutrophil apoptosis or survival have also been reported to be dose dependent [32]. Whilst survival is increased at low doses (0.1–1 ng/ml), a dominant pro-apoptotic effect was observed at higher doses (10–100 ng/ml), the latter process mediated by the production of ROS [32]. However, production of ROS may not be the only mechanism of inducing neutrophil apoptosis, as catalase did not decrease this apoptosis and TNF-α was also able to promote apoptosis in CGD neutrophils [33]. TNF-α does not increase apoptosis of neutrophils if other conventional agonists [e.g. N-formyl-methionyl-leucyl-phenylalanine (fMLP), GM-CSF] first activate them. This may be due, at least in part, to the fact that many agonists can induce TNFR shedding from the cell surface resulting in subsequent decreased sensitivity to the effects of this molecule [33–35]. Alternatively, TNF-α signalling may not be sufficient to overcome the survival signals generated by anti-apoptotic agents. Besides these reports, stimulation of death receptors has recently been demonstrated to disrupt anti-apoptosis pathways initiated by survival factors in neutrophils, and this is probably because of association of death receptors with activated Src homology domain 2 (SH2)-containing tyrosine phosphatase-1 (SHP-1) [36].

The survival signals generated by TNF-α are mainly initiated from TNFR55 and there is wide acceptance that they are mediated via activation of NF-κB and enhanced transcription of survival proteins [12, 32, 37]. A1 and Bcl-X are two possible targets whose expression is regulated by NF-κB in other cell types [37]. A1 is constitutively transcribed in human neutrophils, and expression can be further increased by TNF-α [38]. However, the full complement of survival proteins induced by activated NF-κB in human neutrophils still remains to be identified. Mcl-1, one of the main anti-apoptotic proteins expressed in human neutrophils, does not have an obvious NF-κB-binding site in its promoter [39]. TNF-α may also elicit an anti-apoptotic effect via p38 MAPK-induced expression of the anti-apoptotic chemokine IL-8, whose promoter has an NF-κB-binding site [40, 41].

Whilst TNF-α and Fas activate a caspase cascade that terminates in apoptosis, two recent studies have reported the presence of caspase-independent death pathway(s) in human neutrophils treated with TNF-α [42, 43]. Inhibition of caspases with general caspase inhibitors has been re-

ported not to prevent TNF-α-induced neutrophil death, but this treatment completely blocked Fas-induced neutrophil apoptosis. This caspase-independent cell death depends upon mitochondrial-derived ROS and protein synthesis [42, 43]. Caspase-3, but not caspase-6 and -7, is activated in TNF-α-induced neutrophils via caspase-8, and this caspase cascade down-regulates ROS production [44].

Protein kinases are the main mediators of the signals initiated from TNF receptors. Figure 2 summarises the results of studies that have been carried out to identify the intermediary components of these signalling pathways. Surprising to note is that p38 MAPK can convey signals leading to either apoptosis or survival. P38 MAPK regulating these opposing pathways may transmit signals to other downstream signalling molecules that decide cell fate. Whilst there is no doubt that p38 MAPK is activated by the signals generated from activated TNF receptors [40, 41, 45–47], there are conflicting results for activation of ERK and JNK in neutrophils following TNF-α stimulation [41, 45–47]. ERK has been reported to be weakly activated by the signals generated from activated TNF receptors [46, 47], but these findings have not been confirmed in other reports [41, 45]. Although previous studies have failed to show any JNK activation in TNF-α-activated human neutrophils [46, 47], TNF-α-activation of JNK leading to neutrophil apoptosis has been recently demonstrated [48]. Furthermore, various isoforms of PKC are also reported to be activated in TNF-α-stimulated neutrophils [49, 50].

Whilst it has been suggested that TNF-α leads to neutrophil apoptosis via the signals mediated by PKC-ζ (but not PKC-β) [49], PKC-δ induces an anti-apoptotic effect by activating NF-κB [50]. Bad phosphorylation via the PIK3/Akt pathway and its subsequent inactivation is another mechanism by which TNF-α may generate a survival signal [51]. Thus, the local environment of pro- and anti-apoptotic factors and the nature of the multiple intracellular signals that are generated following their binding to cell surface receptors may govern neutrophil fate. The timing of the generation of these multiple signals is also likely to be important, as is the past history of the neutrophils with respect to their age and exposure to other agonists.

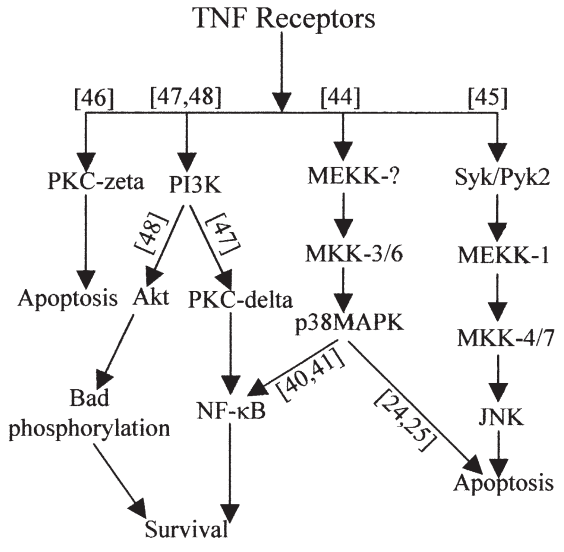

Figure 2. Protein kinase pathways in human neutrophils triggered by TNF-α binding to TNF receptors. A single signal delivered through a common receptor can produce fundamentally divergent responses. The multiplicity of the pro- and anti-apoptotic signals generated by protein kinases may also help to explain the differential effects of TNF-α on neutrophils. p38 MAPK is at the junction of pathways and its activation may lead to either survival or apoptosis of human neutrophils. Also interesting to note is that different isoforms of PKC may produce opposing effects on cell fate. (Numbers in brackets show the references cited and details of these signalling pathways are described in the text).

Other death receptors expressed by human neutrophils

Whilst TNFRs and Fas are the best-characterised death receptors expressed on human neutrophils, expression of TRAIL and TRAIL-Rs has also been reported [52, 53]. TRAIL-R1 (DR4) and TRAIL-R2 (DR5) contain a death domain and can induce apoptosis in some cells. However, TRAIL-R3 (DcR1) and TRAIL-R4 (DcR2) have no death domains capable of inducing apoptosis and have been shown instead to act as decoy receptors and to protect against apoptosis [4]. Human neutrophils express TRAIL, TRAIL-R2 and R3, and neutrophil apoptosis has been shown to be specifically accelerated by exposure to exogenous TRAIL. NF-κB is not involved in this signalling pathway and TRAIL does not induce a chemotactic response [53]. The role of TRAIL in regulating constitutive neutrophil apoptosis was excluded and TRAIL has been suggested to provide a mechanism for clearance of neutrophils from sites of inflammation [53]. TRAIL has also been suggested to limit cytokine-mediated anti-apoptotic effects under certain inflammatory conditions [52].

Future perspectives and concluding remarks

Manipulation of apoptosis via the death receptor pathways offers exciting therapeutic potential in preventing or treating inflammatory diseases [19, 54]. Fas-expressing inflammatory cells have previously been suggested to be eliminated therapeutically by delivery of FasL to the site of inflammation to promote apoptosis and thereby help resolve the inflammation. However, a pro-inflammatory effect of sFasL has been reported, but this has only been demonstrated in in vitro experimental systems [17–19]. Recent clinical trials have shown encouraging therapeutic

effects of anti-TNF-α antibodies and soluble TNF receptors in the treatment of several chronic inflammatory diseases [e. g. rheumatoid arthritis (RA), Crohn's disease and inflammatory bowel disease] indicating the key role of this molecule in disease pathology [54–58]. Etanercept (Enbrel) is a novel human recombinant version of the soluble TNFR75 that is linked to the Fc receptor of human IgG [55], whilst Infliximab (Remicade), is a chimeric IgG1k monoclonal anti-TNF-α antibody [56]. Both TNF-α inhibitors interrupt the inflammation process by binding TNF-α molecules, thereby preventing their activation of cell surface receptors [55–57]. Anti-TNF therapy in RA also decreases synovial expression of the chemokines IL-8 and MCP-1, and diminishes inflammatory cell migration into affected joints [58]. However, the role of these molecules on apoptosis or survival of immune cells, including neutrophils, has not been directly measured in the treatment of disease. Clearly, these clinical observations indicate that new therapeutics that target death receptors or death ligands will provide better and more specific treatment for chronic inflammatory diseases.

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