Characterization of the survival effect of tumour necrosis factor-α in human neutrophils

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Abstract

Granulocyte apoptosis has been proposed as a fundamental, injury-limiting granulocyte-clearance mechanism. As such, inhibition of this process may prevent the resolution of inflammation. Our previous studies have shown that TNFα (tumour necrosis factor-α) has a bi-modal influence on the rate of constitutive neutrophil apoptosis in vitro, causing early acceleration and late inhibition of this process. The pro-apoptotic effect is uniquely TNFR1 (TNF receptor 1) and TNFR2-dependent and the latter survival effect is mediated via phosphoinositide 3-kinase and NF-κB (nuclear factor-κB) activation. In the present study, we show that, in contrast with GM-CSF (granulocyte/macrophage colony-stimulating factor), the delayed addition (i.e. at 6 h) of TNFα increases its survival effect despite substantial loss of neutrophil TNFR1 and TNFR2 at that time. This paradox was resolved using PBMC (peripheral blood mononuclear cell)-deplete and 5% PBMC-replete neutrophil cultures, where the enhanced survival effect observed after delayed TNFα addition was shown to be PBMC-dependent. TNFR2-blocking antibodies had no effect on the late survival effect of TNFα, implying a TNFR1-dependent process. Finally, IκB (inhibitory κB-α) and NF-κB time-course studies demonstrated that the survival effects of both GM-CSF and TNFα could be explained by maintenance of functional NF-κB.

Introduction

Inappropriate suppression of neutrophil apoptosis has been proposed as an important mechanism contributing to the persistence of granulocytic inflammation. Hence, BAL (bronchoalveolar lavage) fluid from patients suffering from acute respiratory distress syndrome, a condition characterized by persistent neutrophilic alveolitis, inhibits neutrophil apoptosis ex vivo [1]. Similarly, persistent airway neutrophilia, which is linked with the development of chronic lung disease following the neonatal respiratory distress syndrome, is associated with inappropriately low rates of neutrophil apoptosis and a failure to generate ‘pro-apoptotic’ BAL fluid [2]. Also, a range of therapeutic strategies designed to stimulate apoptosis have resulted in accelerated resolution of granulocytic inflammation. As a consequence, we and others have aimed to determine the molecular and cellular bases of the survival effect of certain key inflammatory cytokines with a view to blocking this effect.

TNFα (tumour necrosis factor-α) was first reported to inhibit neutrophil apoptosis [3], but subsequently found to have more complex actions. Hence, we and others demonstrated the ability of TNFα to stimulate apoptosis at early time points; this effect has subsequently been shown to be caspase-dependent and requires the co-ligation of both TNFR1 (TNF receptor) and TNFR2 [4]. Interestingly, the preactivation state of the neutrophil has been found to be critical in determining the pro-apoptotic effects of TNFα and, similarly, the efficacy of TNFα killing varies widely between subjects. Apart from its dependence on phosphoinositide 3-kinase and NF-κB (nuclear factor-κB) signalling, far less is understood about the later survival effect of this cytokine.

Materials and methods

Neutrophil isolation and culture

Human neutrophils were purified from the peripheral blood of healthy human volunteers by dextran sedimentation, followed by centrifugation through discontinuous plasma/Percoll gradients [4,5]. Initial cell purity was assessed using air-dried cytocentrifuge preparations fixed in methanol and stained with May/Grunwald/Giemsa and was routinely >95% neutrophils with <1% mononuclear cell contamination. For experiments using highly purified cells, neutrophils were further purified as described previously using negative magnetic selection with a custom mixture of antibodies to CD36, CD2, CD3, CD19, CD56 and glycophorin A (StemCell Technologies, Vancouver, BC, Canada) [5]. In the PBMC (peripheral blood mononuclear cell)-addition experiments, suspensions of mononuclear-cell-deplete neutrophils were...
supplemented with 5% PBMCs derived from the initial upper platelet-poor plasma/42% Percoll interface. The purified cells were washed sequentially with platelet-poor plasma, PBS without and PBS with CaCl₂ and MgCl₂, and were suspended at 5 × 10⁶ cells/ml in MDM (Iscove’s modified Dulbecco’s medium), supplemented with 10% autologous serum, 50 µg/ml streptomycin and 50 units/ml penicillin (referred to as ‘supplemented MDM’ hereafter) [4,5]. Neutrophils were routinely cultured in supplemented MDM (referred to as ‘supplemented MDM’ hereafter) [4,5].

Assessment of neutrophil apoptosis
After gentle resuspension, neutrophils were harvested, cyto-centrifuged, and the resulting slide preparations fixed and stained as detailed above. Cell morphology was examined under oil-immersion light microscopy using a ×100 objective and apoptotic neutrophils were defined as the cells containing darkly stained pyknotic nuclei [6]. For each condition examined, cytopsins were prepared from triplicate incubations and a total of 300 neutrophils/slide counted with the observer blinded to the assay conditions. Apoptosis was also assessed by a flow cytometry-based method using fluorescein isocyanate-labelled recombinant human annexin V and propidium iodide staining [7].

Assessment of neutrophil TNFR expression
The expression of TNFR1 and TNFR2 in human neutrophils was studied by direct immunofluorescence using a primary antibody directed against TNFR55 or TNFR75 and a FITC-conjugated secondary antibody. Cells were incubated as detailed above and, at the appropriate time points, cells were transferred to pre-chilled U-bottomed flexiwell plates, washed (220 g, 90 s, 4°C) in 100 µl of ice-cold buffer (PBS containing 0.2% BSA and 0.1% sodium azide) and resuspended in 40 µl of a predetermined saturating concentration of mouse anti-human TNFR55, TNFR75 (R&D Systems, Abingdon, Oxfordshire, U.K.) or CD2 mAb as a negative control (UCHT-1 clone, IgG1; Scottish Antibody Production Unit, Carluke, Lanarkshire, U.K.). After a 60 min period on ice, the cells were washed twice and incubated for 30 min with 40 µl of FITC-conjugated goat anti-mouse immunoglobulin (Jackson Immunoresearch Laboratories, West Grove, PA, U.S.A.) diluted 1:40 with PBS/BSA/azide buffer. After further washing, the samples were analysed using a FACSCalibur (Becton Dickinson, Oxford, U.K.).

Western-blot analysis
Neutrophils (25 × 10⁶/condition) were harvested from 6-well plates and immediately quenched in ice-cold PBS. Cells were then pelleted by centrifugation (352 g, 10 min, 4°C) and resuspended in 0.5 ml of hypo-osmotic lysis buffer (10 mM Tris, pH 7.8/1.5 mM EDTA/10 mM KCI/0.5 mM dithiothreitol/1 mM sodium orthovanadate/2 mM Leu-misole/0.5 mM benzamidine/Complete® protease inhibitor cocktail). Thereafter, the cells were immediately centrifuged at 16,000 g for 5 s and then resuspended in 0.5 ml of fresh hypo-osmotic buffer. After placing for 10 min on ice, the cells were sonicated for 20 s, centrifuged (3300 g, 4°C, 15 min) and the supernatants stored at −80°C. Lysates prepared using this method are positive for both nuclear (SUMO-1, small ubiquitin-related modifier-1) and cytoplasmic (lactate dehydrogenase) markers (results not shown). Moreover, this method was designed specifically to deal with the high degrees of proteolytic activity and NF-κB degradation inherent in most extraction methods applied to human neutrophils (see [8] for a discussion).

Cell lysates (100 µl) were resolved on 12% SDS/polyacrylamide gels and proteins transferred on to PVDF membranes for 1 h at 4°C in 200 ml/l methanol, 3.03 g/l Tris and 14.4 g/l glycine. After transfer, membranes were blocked for 1 h with 5% milk powder/PBS/0.1% Tween. The membranes were then incubated overnight at 4°C with primary antibodies directed against NF-κB (Santa Cruz Biotechnology) or I-κBα (inhibitory κ B-α; Cell Signaling Technology) at 1:500 dilution in PBS/Tween 20 buffer. After three washes, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit polyclonal antibody (Dako, Ely, Cambs., U.K.) at a 1:2500 dilution for 1 h. After final washing, bands were detected by chemiluminescence using SuperSignal West Pico ECL® (Pierce, Cheshire, U.K.). Equal protein loading was ensured by initial volume correction following protein assay (Bradford assay) and confirmed by reprobing the membranes for β-actin expression.

Statistical analysis
Results are expressed as the means ± S.E.M. for n independent experiments from separate donors. Statistical analysis was performed by ANOVA using a post-test Tukey where appropriate. Differences were considered significant when P < 0.05.

Results and discussion
Delayed addition of TNFα increases its survival effect
We first confirmed our earlier observation [4] that delayed addition of TNFα (i.e. at 6 h) significantly enhanced the magnitude of the neutrophil survival effect observed at 20 h. This was evident when apoptosis was assessed either by morphology (Figure 1A) or by annexin/FITC staining (results not shown). It may be noted that such an effect was not observed with GM-CSF (granulocyte/macrophage colony-stimulating factor; Figure 1A). These results are somewhat surprising given the previously reported time-dependent loss of both TNFR1 and TNFR2 when neutrophils are cultured...
Figure 1: The enhanced survival of neutrophils observed after delayed addition of TNFα is PBMC-dependent
Neutrophils were purified using plasma/Percoll gradients alone (A) or additional negative magnetic selection to remove all the contaminating PBMCs (B, C) and then cultured at 5 × 10⁶/ml in supplemented MDM with TNFα (200 units/ml) or GM-CSF (10 ng/ml) added at either 0 or 6 h. A 5% PBMC supplementation was used for enriched cultures. After 20 h, the cells were harvested and apoptosis was assessed by morphology. Results are expressed as the means ± S.E.M. for n = 3 independent experiments, each performed in triplicate. *P < 0.05, compared with 0 h controls; #P < 0.05. NS, not significant.

in vitro [4]. Indeed, re-evaluation of this under the current incubation conditions has confirmed >95% spontaneous loss of neutrophil TNFR1 expression by 6 h.

Contaminating mononuclear cells contribute to the survival effect of TNFα
Whereas one explanation for the augmented TNFα-mediated survival response observed with delayed cytokine addition could be loss of the small early pro-apoptotic effect seen with immediate addition of TNFα, we wished initially to determine the dependence of the TNFα survival effect on the presence of contaminating PBMCs. This follows our earlier studies where the TLR4-dependent survival effect of lipopolysaccharide in a similar culture model was shown to be due to both direct and indirect (PBMC-dependent) inhibition of apoptosis [9]. Such a prediction would be supported by existing in vivo studies where monocyte depletion sufficient to cause a 70% decrease in BAL macrophage number in C57B1/6 mice resulted in a significant decrease in neutrophilic alveolitis in response to intra-tracheal and intravenous instillation of clodronate [10].

The use of antibody-coated magnetic microbeads allows neutrophil preparations to be depleted of almost all PBMCs, in particular decreasing contaminating monocytes to a monocyte/neutrophil ratio of approx. 1:3000 [5]. Importantly, this extra manipulation has no effect on basal levels of CD62-L or the responsiveness of these cells to fMLP (N-formyl-methionyl-leucylphenylalanine; ‘chemotactic peptide’). Figure 1(B) demonstrates that whereas the survival effect of TNFα is still evident in monocyte-deplete neutrophils, the magnitude of the survival effect is enhanced in the presence of 5% PBMCs. Critically, the augmented survival effect seen with delayed cytokine addition is shown to be entirely PBMC-dependent. In contrast, the survival effect of GM-CSF is unaffected by the presence of contaminating cells (Figure 1C).

These results suggest that the overall effect of TNFα on neutrophil apoptosis rates in vitro represents a composite of at least three independent factors, namely (i) a direct early pro-apoptotic effect, (ii) a direct early and sustained anti-apoptotic effect and (iii) an indirect and delayed (PBMC)-dependent survival effect. This latter effect is presumed to reflect the ability of PBMCs to secrete or express an additional survival factor(s) that displays greater efficacy compared with TNFα itself. It is quite probable that this effect is mediated by monocytes within PBMCs, although we have not formally excluded a role for other cells, such as lymphocytes, in mediating these responses.

TNFR dependence of the TNFα survival effect
To determine the TNFR dependence of the anti-apoptotic effect of TNFα, neutrophils were preincubated with rat IgG2b anti-human TNFR2 mAb or a control isotype-matched rat anti-human IL-2 receptor mAb for 30 min before the addition of TNFα. Cells were then harvested at 20 h and apoptosis was assessed morphologically.

As shown in Figure 2(A), neither incubation strategy had any effect on the survival effect of TNFα. This was not due to the loss of functional activity of the TNFR2 mAb, since flow-cytometric analysis of antibody binding to freshly isolated neutrophils was identical for antibody incubated for either 0 or 20 h in serum-supplemented medium (results not shown). Moreover, this antibody causes complete abrogation of the early pro-apoptotic effect of TNFα [4]. In equivalent experiments designed to assess the role of TNFR1 in the
Figure 2 | TNFα-mediated survival of neutrophils is independent of TNFR2 (TNFR75) and is associated with persistent expression of NF-κB and I-κBα proteins [15]

(A) Neutrophils were cultured at 5 × 10^6/ml in the presence or absence of TNFα (200 units/ml), with 28 μg/ml TNFR2-blocking mAb or control IL-2 receptor (IL-2R)-blocking mAb. Apoptosis was assessed by morphology at 20 h. Results are expressed as the means ± S.E.M. for triplicate determinations from a single representative experiment of two (*P < 0.05, compared with 0 h controls). (B) Neutrophil lysates were prepared, as detailed in the Materials and methods section, from 25 × 10^6 neutrophils per condition after culturing for 10 min, 1 h or 6 h in the presence of either TNFα (200 units/ml), GM-CSF (10 ng/ml; lane G) or supplemented MDM alone (lane N). Lysates were separated by SDS/PAGE and membranes were developed with antibodies directed against NF-κB or I-κBα. Results are representative of a series of blots from n = 4 independent experiments, with each membrane re-probed for β-actin.

TNFα prevents the normal time-dependent loss of NF-κB and I-κBα protein expression

Glutaxin, a potent and specific inhibitor of NF-κB, has been shown previously to increase the rate of constitutive apoptosis in neutrophils and also to sensitize these cells to the early pro-apoptotic effects of TNFα [11]. The present study, together with more recent observations [8,12,13], has suggested that basal and agonist-stimulated activation of NF-κB may play a pivotal role in regulating apoptotic threshold in neutrophils. To examine this hypothesis in more detail, we have determined the effects of aging alone on I-κBα and NF-κB p65/RelA expression. In addition, we have examined the effects of co-incubation with either TNFα, a well-characterized direct activator of this pathway [11], or GM-CSF, which drives NF-κB-dependent IL-8 synthesis in the absence of I-κBα loss [14]; results not shown. As demonstrated in Figure 2(B), both IκBα and NF-κB expression in neutrophils decreased markedly by 6 h, whereas the levels were preserved in cells co-incubated with TNFα or GM-CSF. This occurred even in the face of major early losses in I-κBα observed after TNFα treatment. Given the very low levels of I-κBα and NF-κB expression at 6 h, the augmented survival effect of TNFα added at this time point was probably not signalled via this pathway.

In summary, the present study has revealed an additional PBMC-dependent mechanism whereby TNFα inhibits neutrophil apoptosis. The indirect nature of this survival effect is supported by the major loss in TNFR1/TNFR2 and I-κBα/p65 NF-κB expression at 6 h when this pathway is most active. Moreover, detailed analysis of I-κBα/p65 NF-κB expression in neutrophils suggests that the major time-dependent loss in NF-κB expression probably represents the essential trigger for the onset of constitutive apoptosis. This conclusion is supported by results of recent studies showing that the introduction of a NEMO (NF-κB essential modulator)-binding domain peptide into neutrophils using an HIV–TAT transduction shuttle causes a selective inhibition of IKKγ (IκB-kinase γ; NEMO)/IKKβ interaction and NF-κB activation; as a consequence, there is a major increase in neutrophil apoptosis [8].

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References


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