Neutrophil apoptosis in rheumatoid arthritis is regulated by local oxygen tensions within joints

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Abstract: Neutrophils are normally short-lived cells and die by apoptosis, but when recruited into tissues, their apoptosis is delayed, and they survive for much longer time periods. In inflammatory diseases, such as rheumatoid arthritis (RA), this delayed apoptosis may lead to increased tissue damage and a failure of the inflammation to resolve. However, there are conflicting reports in the literature as to whether neutrophil apoptosis is delayed or accelerated in rheumatoid joints. In this report, we show that neutrophils isolated from the synovial fluid (SF) of patients with RA show accelerated rates of apoptosis when incubated ex vivo and that SF, despite containing a variety of antiapoptotic cytokines, is proapoptotic. Paradoxically, levels of the key neutrophil survival protein Mcl-1 are elevated in freshly isolated SF neutrophils compared with matched peripheral blood samples from the same patients, indicating that delayed neutrophil apoptosis has been signaled in vivo as the cells enter the joints. However, when SF was added to neutrophils and incubated under hypoxia (1% O₂), conditions known to exist in vivo within joints, the SF was antiapoptotic. These data reveal that the rheumatoid synovial joint contains a complex mixture of pro- and antiapoptotic factors and that the low, local oxygen tensions that exist within these joints can exert profound effects on neutrophil survival. These experiments also highlight the importance of performing in vitro experiments under laboratory conditions that closely mimic those that occur in vivo; otherwise, misleading conclusions may be drawn. J. Leukoc. Biol. 80: 000–000; 2006.

Key Words: Mcl-1 · Bax · inflammation · hypoxia

INTRODUCTION

In inflammatory diseases such as rheumatoid arthritis (RA), many different cells of the immune system are activated, together with the production of a range of inflammatory cytokines [1–5]. Neutrophils are the most abundant cell, present in the joints of patients with RA, and have the greatest capacity of all immune cells to inflict tissue damage via their release of toxic products such as proteases [4, 6–11]. Inflammatory neutrophils can also secrete a large number of cytokines and chemokines, which can have a major influence on the progress of events within diseased joints [12–17]. Recent evidence using animal models has shown that neutrophils play a key role in the initiation and progression of experimental arthritis [18]. They can also be found in large numbers at the pannus: cartilage interface in early stages of human RA [19, 20]. Neutrophils normally have a short half-life in the circulation (8–12 h), as they constitutively undergo apoptosis [21–23]. However, this normally short half-life can be extended to several days, and a number of events that occur during inflammation, such as adhesion, transmigration, exposure to proinflammatory cytokines, and hypoxia, have been shown experimentally to delay neutrophil apoptosis [24–30]. Previous work has shown that neutrophils express a number of proapoptotic members of the Bcl-2 family of proteins (such as Bax, Bad, Bak, and Bid) and that the antiapoptotic protein Mcl-1 plays a central role in the survival kinetics of these cells [31, 32]. Thus, cytokines that delay neutrophil apoptosis result in increased expression of Mcl-1, and antisense disruption of Mcl-1 results in greatly accelerated rates of neutrophil apoptosis [27, 33].

Neutrophils that enter rheumatoid joints will be exposed to a number of these antiapoptotic processes as they are signaled to leave the circulation, adhere to capillary endothelial cells, and transmigrate into the joint spaces. Once in the joint, they are then exposed to the range of proinflammatory cytokines, typically present in synovial fluid (SF), such as granulocyte macrophage-colony stimulating factor (GM-CSF), interleukin (IL)-1β, tumor necrosis factor (TNF), and interferon-γ (IFN-γ) [34–36]. It would thus appear that then conditions within the rheumatoid joint are conducive for delayed neutrophil apoptosis, thereby extending the period of survival of these cells and in so doing, increasing their potential to cause damage and promote inflammation. However, there have been surprisingly few studies about the control of neutrophil apoptosis in RA, and a number of conflicting findings have been described. For example, one study showed that SF neutrophils cultured ex vivo had accelerated rates of apoptosis and that rheumatoid SFs, including those containing high levels of antiapoptotic cytokines, were proapoptotic [37]. In contrast, another report indicated that SF neutrophils had delayed apoptosis and that rheumatoid SF was antiapoptotic [38]. The conclusion from
these apparently contradictory reports is that rheumatoid SF must contain pro- and antiapoptotic factors, the net effect on neutrophil cell death or survival reflecting a balance between the activities of such factors. In the present study, we show that local O₂ concentrations within SF have a profound effect on neutrophil apoptosis. Under conditions of normoxia (approximately 21% O₂) of typical laboratory experiments, the activity of the proapoptotic factors within SF predominates, whereas under hypoxia (1% O₂), typically found within rheumatoid joints, antiapoptotic pathways are triggered.

MATERIALS AND METHODS

Materials

Neutrophil isolation medium (NM) was from Cardinal Associates (Sante Fe, NM), Ficoll-Paque was from Amersham Pharmacia (Uppsala, Sweden); RPMI-1640 medium was from Gibco-BRL (Paisley, UK), and fetal bovine serum (FBS) was from Sigma (Poole, UK). Cytokines used were GM-CSF (Glaxo, Greenford, UK) and IFN-γ (Boehringer Mannheim, Germany). Fluorescein isothiocyanate (FITC)-conjugated CD15 and CD16 antibodies, McI-1 and Bax antibodies, and FITC-labeled goat antionmouse antibodies were from Becton Dickinson (San Jose, CA), and peroxidase-linked donkey antirabbit antibodies, enhanced chemiluminescence (ECL) detection kit, and Amplify antibodies were from Amersham (UK). FITC-labeled annexin V was from Biosource (Camarillo, CA). The hypoxia chamber was purchased from Billups Rothenberg Inc. (Del Mar, CA).

Cell isolation and culture

The local Institutional Review Board, South Sefton Research Ethics Committee, approved this study. Peripheral blood cells were obtained from heparinized venous blood of healthy donors and patients with RA (fulfilling the 1987 revised American Red Cross criteria) and were separated into neutrophil and mononuclear cell fractions using NIM (as described in the manufacturer’s instructions) [39–40]. Contaminating erythrocytes were removed by hypotonic lysis. Neutrophils from the SF of RA patients were isolated soon after aspiration using Ficoll-Paque in a one-step centrifugation method [41, 42]. Neutrophils were examined routinely for purity and viability using trypan blue exclusion, which was >97% and >98%, respectively, immediately after isolation. Purity was confirmed using morphological analysis of cytopsins and CD15 and/or CD16 expression. Purified neutrophils were resuspended in RPMI 1640, supplemented with 10% FBS and 1 mM L-glutamine at 5 × 10⁶ cells/mL, and cultured at 37°C in a humidified CO₂ chamber. Cytokines were added as indicated.

Morphological estimation of apoptosis

Following culture, a 20-μL aliquot of suspension (1 × 10⁵ cells) was made up to 200 μL with RPMI 1640, and cells were cytocentrifuged using a Shandon Cytopsin3 (Runcorn, Cheshire, UK). Romanowsky staining of cytopsins allowed apoptosis to be scored by morphology, as described in ref. [32]. This method correlates well with other markers of apoptosis [21].

Annexin V-FITC staining

Neutrophils (10⁵) were removed from culture and resuspended in annexin V binding buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂) before the addition of annexin V-FITC at a 1:100 dilution. After 10 min in the dark, cells were pelleted at 400 g and resuspended in annexin V binding buffer before analysis by flow cytometry using a Coulter-EpicAltra flow cytometer. Cells/sample (10,000) were analyzed.

Reactive oxygen metabolite production

Chemiluminescence was measured at 37°C in neutrophil suspensions (1 × 10⁶/mL) in Hanks’ balanced saline solution medium, which was supplemented with 10 μM luminol using an LKB 1251 luminometer [43]. Cells were stimulated by the addition of phorbol 12-myristate 13-acetate (0.1 μg/mL).

CD16 staining and flow cytometry

Neutrophils were incubated with anti-CD16 antibodies [to detect surface Fc receptor for immunoglobulin G (IgG; FcγRIIIb expression], as described previously [39], with some modification. Briefly, neutrophils were labeled with FITC-conjugated anti-CD16 antibody (3G8) using a standard immunofluorescence technique. Cells were suspended in phosphate-buffered saline (PBS)/1% bovine serum albumin (BSA); globulin-free 0.1% sodium azide (pH 7.2) and incubated with antibody for 30 min at 4°C. Antibodies were used at saturating concentrations, and nonimmune mouse IgG of the appropriate subclass was used as a control. Stained cells were washed twice with cold PBS/1% BSA/0.1% azide and then analyzed by flow cytometry. Fluorescence distributions represent a total of 5000 gated events.

Western analysis

Following culture, 10⁶ cells were lysed rapidly in boiling, reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing aprotinin (20 μg/mL), leupeptin (20 μg/mL), pepstatin (10 μg/mL), and phenylmethylsulfonyl fluoride (400 μg/mL); Samples were boiled immediately for 5 min with occasional vortexing and stored at −80°C until use. SDS-PAGE and electrophoresis to polyvinylidene diffuoro membranes were performed as described [31]. Primary antibodies used were anti-Mel-1, anti-Bax, and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and horseradish peroxidase-conjugated secondary donkey antirabbit IgG was used. Bound antibodies were detected using the ECL system. Densitometry on carefully exposed blots (to avoid film saturation) was performed with Image 1.44 VDM software (National Institutes of Health, Bethesda, MD). Ponceau S-stained actin on membranes after electrottransfer was used to confirm equivalence of loading of neutrophil samples.

Statistics

Data sets were analyzed using the Student’s t-test.

RESULTS

Effects of rheumatoid SF on neutrophil apoptosis

Cell-free SF from patients with RA was incubated for 22 h in vitro with neutrophils isolated from the blood of healthy controls. Analysis of cell morphology showed that at concentrations of SF of 25% (v/v) and greater, apoptosis of neutrophils was accelerated significantly. For example, in the absence of SF, apoptosis was 40% (±4%), whereas at 75% (v/v) SF, this value had increased to over 90% (Fig. 1). Similar results were obtained with five SFs from different patients and with neutrophils from five different donors. These data were confirmed when neutrophil apoptosis was measured by phosphatidylserine exposure on the cell surface (i.e., ability to bind FITC-annexin V), which increased in parallel with the increase in apoptotic morphology (data not shown). Expression of CD16 (FcγRIIIb) on the cell surface (which decreases as cells become apoptotic) and cell function (the ability to mount a respiratory burst of reactive oxidant production) declined in parallel with the appearance of apoptotic morphology (data not shown).

Apoptosis of control and rheumatoid neutrophils

Neutrophils were purified from the blood of healthy controls and patients with RA and also from the SF of rheumatoid patients. Cells were incubated in the absence and presence of...
GM-CSF (50 U/mL) + IFN-γ (100 U/mL), two agents known to delay neutrophil apoptosis. Unfractionated, SFs, containing SF factors and other immune cells (e.g., macrophages), which could influence the rate at which neutrophils undergo apoptosis ex vivo, were also incubated alongside these purified cell preparations. Apoptotic neutrophils were not detected in freshly isolated preparations from the blood of healthy controls or patients with RA, whereas low numbers of apoptotic cells (1–3% of the total) were detected in preparations isolated from SF (Fig. 2). After 22 h incubation in culture, apoptosis in control neutrophils had increased to 40% (±5%), and GM-CSF + IFN-γ treatment (P < 0.01) decreased this value significantly to 15% (±3%, n = 18). Neutrophils isolated from the blood of rheumatoid patients had rates of apoptosis similar to those of healthy controls, and again, GM-CSF + IFN-γ treatment significantly protected against apoptosis. Greater rates of apoptosis were seen after ex vivo culture of SF neutrophils, and although cytokine treatment partly protected against apoptosis, this protective effect was not as great as that seen for blood neutrophils from healthy controls or rheumatoid patients. When neutrophils were incubated in unfractionated SF, >95% of the cells were apoptotic, and cytokine treatment was virtually ineffective. This finding indicates that the antiapoptotic activity of the externally added cytokines was blocked by the activity of proapoptotic factors within the SFs.

These experiments demonstrate that SF neutrophils show accelerated rates of apoptosis when cultured ex vivo (compared with blood neutrophils) and that factors within SF accelerate the rate of apoptosis during this culture. We were then determined to identify the processes that may regulate cell death and survival.

Changes in the levels of Bax and Mcl-1

Previous work has shown that Mcl-1 levels are cytokine-regulated in neutrophils and that this protein plays a key role in the survival of these cells [31, 32].

We then measured cellular levels of Mcl-1 and Bax in freshly isolated neutrophils from controls and the blood and SF of patients with RA. Figure 3A shows the levels of these two proteins (plus the housekeeping gene GAPDH) in paired neutrophil samples isolated from the blood and SF isolated from four patients with RA. This indicates levels of Mcl-1 were elevated significantly in neutrophils isolated from the SF, whereas there were no significant changes in the levels of Bax.
Figure 3B shows summary data from 15 patients with RA. Levels of Bax protein were not significantly different in freshly isolated control blood neutrophils, rheumatoid blood neutrophils, or in SF neutrophils and were not significantly altered after 22 h culture in vitro in the absence or presence of GM-CSF and IFN-γ (Fig. 3B).

In contrast, much greater changes in levels of expression of Mcl-1 were observed in these experiments. For example, levels of Mcl-1 protein decreased after culture of neutrophil for 22 h in vitro to 25% of values measured in freshly isolated cells (Fig. 3C). Levels of Mcl-1 protein in blood neutrophils from rheumatoid patients were similar to those measured in freshly isolated control blood neutrophils, and again, levels of this protein declined as cells were cultured in vitro. The addition of GM-CSF partially protected against this decline in Mcl-1 levels, and levels of Mcl-1 protein in freshly isolated SF neutrophils were elevated significantly above those in peripheral blood neutrophils from rheumatoid patients. However, closer examination of data from paired blood and SF neutrophils from individual patients revealed considerable heterogeneity in that Mcl-1 protein levels were elevated in 19/25 (76%) SF neutrophil samples, compared with the paired peripheral blood samples. During culture of SF neutrophils, levels of Mcl-1 declined (as cells progressed into apoptosis), and the addition of exogenous cytokines could not protect against this decline in Mcl-1.

The above data would indicate that in the majority (76%) of SF samples, Mcl-1 protein expression is elevated, indicating that delay of apoptosis has been triggered in vivo. However, the
data from the apoptosis experiments show accelerated apoptosis during ex vivo culture in the majority of samples and that SF is proapoptotic. We then set out to investigate the reasons for this paradox. Previous work has shown that reactive oxidants can promote neutrophil apoptosis [28, 44] and that hypoxia can delay neutrophil apoptosis [45]. In view of the facts that rheumatoid SF can stimulate reactive oxidant generation by neutrophils [46, 47], and the rheumatoid joint is hypoxic, being subject to exercise-driven cycles of reoxygenation [48, 49], we decided to investigate the role of oxygen and reactive oxidants in regulation of neutrophil apoptosis by SF.

Effects of hypoxia on SF-induced neutrophil apoptosis

Neutrophils were incubated in the presence and absence of GM-CSF under normoxia (21% O₂) or hypoxia (1% O₂), and apoptosis was determined after 18 h incubation. Constitutive apoptosis was delayed significantly by hypoxic incubation in the presence or absence of GM-CSF (Fig. 4A). In parallel, we also incubated neutrophils with cell-free SFs from 13 different patients with RA, again under normoxia and hypoxia. In all cases, rates of apoptosis were lower during incubation under hypoxic conditions compared with incubation under normoxia. There was, however, considerable variation in the rates of apoptosis stimulated by these different SFs. For example, under normoxia, most but not all SFs resulted in rates of apoptosis that were greater than those observed in untreated controls. Furthermore, under hypoxia, rates of apoptosis in the presence of some SFs were significantly lower than those observed during incubation with GM-CSF.

We then determined if incubation under hypoxia affected levels of Mcl-1 protein in neutrophils treated with SF. When incubated under normoxia, levels of Mcl-1 in neutrophils were increased by GM-CSF (Fig. 4B), but five different SFs resulted in markedly differing changes in Mcl-1 protein: Most SFs resulted in decreased Mcl-1 levels, whereas one fluid resulted in elevated Mcl-1 levels. When incubated under hypoxia, four of five SFs enhanced neutrophil Mcl-1 levels above those measured in control, untreated cells. Thus, under hypoxia, most SFs contain factor(s) that can increase Mcl-1 protein levels and delay apoptosis, but these effects are only observed under conditions of oxygenation that occur in vivo.
DISCUSSION

The major findings of this study are that rheumatoid SF contains a variety of factors that can delay or accelerate neutrophil apoptosis, but local oxygen tensions within the diseased joint are a major influence on the fate of these cells. Thus, at low oxygen concentrations (hypoxia), apoptosis delay is triggered, whereas at higher oxygen concentrations, the proapoptotic activities of these SF factors predominate. As it has previously been shown that the rheumatoid joint is hypoxic, but subject to exercise-driven cycles of reperfusion [48, 49], the diseased joint is likely to be a dynamic environment, exerting subtle and cyclic changes in the rate of apoptosis of the infiltrating cells.

Previous reports have generated conflicting data about the ability of SF to promote [37] or delay [38] neutrophil apoptosis. These fluids contain complex mixtures of molecules with the potential to exert a number of effects on cells. They invariably contain large quantities of immune complexes that can interact with immune cells via their FcRs to mediate cell activation [46, 47]. Soluble, immune complexes have been shown to activate the release of reactive oxygen metabolites and granule enzymes from cytokine-primed neutrophils, which may contribute to the tissue-damaging events that occur within joints [46, 47]. Rheumatoid SFs are also a rich source of cytokines such as GM-CSF, IL-1β, TNF-α, and IFN-γ [34 –36], which have been shown to delay neutrophil apoptosis significantly. There is also considerable variation in the levels of these activating and apoptosis-delaying factors in SFs isolated from different patients. It is not surprising, therefore, that when these fluids are added to suspensions of neutrophils in vitro, they induce a variety of effects on cell function.

One important factor that is not often considered in most ex vivo experiments is the concentration of molecular oxygen used to incubate the cell suspensions. Most in vitro experiments are performed in air or air mixtures of 21% O2 (760 mm Hg) at 37°C. Rarely, if ever, will such high concentrations of oxygenation occur in vivo. The O2 concentration of arterial blood is usually within the range 2–3% O2, and in tissues, O2 concentrations are much lower [50]. Quite often, O2 gradients exist across tissues, such that O2 levels within cells are determined by their distance from capillaries and their rate of oxygen use for respiration or other purposes. The O2 tensions within SFs have been reported to be as low as 1–2% [51]. Much research has focused on the role of hypoxia in tumor cell biology, as it has been realized for some time that local oxygen tensions within tumors can be extremely low, often approaching anoxia [52, 53]. These low O2 tensions induce dramatic changes in the metabolism of tumor cells (usually limiting the rates at which these cells can undergo oxidative phosphorylation) but can also trigger differential gene expression via activation of specific classes of transcription factors, which are regulated by low O2 concentrations [52, 53].

Perhaps the most studied hypoxia-regulated transcription factors are the hypoxia-inducible factors (HIFs) and HIF-1, -2, and -3 are heterodimeric transcription factors, comprising α subunits, which are unstable under normoxia, becoming ubiquitinylated by an E3 ubiquitin ligase complex that contains the von Hippel-Lindau tumor suppressor and other proteins [54–56]. This interaction is governed by hydroxylation of a highly conserved prolyl residue within HIF, and once ubiquitinated, the protein is degraded by the proteasome. Under hypoxia, the hydroxylation cannot occur, and the α subunit is stabilized and then binds to the β subunits, which are constitutively expressed members of the aryl hydrocarbon receptor nuclear translocator family of proteins. The heterodimers then move to the nucleus, where together with cyclic adenosine monophosphate response element-binding protein binding protein (CBP)/p300 and perhaps other factors, they stimulate transcription of genes possessing hypoxia-response elements. CBP/p300 possesses histone acetyltransferase activity, which may induce chromatin rearrangements to facilitate HIF binding. Other factors have also been implicated in hypoxia-dependent increases in gene expression, including increased mRNA and protein stability and activation of other transcription factors such as activated protein-1 [53] and nuclear factor-kB [57].

A growing number of HIF-regulated genes have been identified, including vascular endothelial growth factor, glycolytic enzymes, and other proteins involved in cell proliferation, which may play roles in the complex events that occur within rheumatoid joints. The promoter of the human mcl-1 gene also possesses a HIF-1-binding site [58], and binding of this factor has recently been demonstrated during hypoxia [59]. It is interesting that prostaglandin E2 (PGE2) has been shown to stabilize HIF-1α to trigger gene activation, even under conditions of normoxia [60]. Thus, hypoxic conditions that occur within rheumatoid joints, together with locally generated PGs, can trigger the transcription of such genes, including the antiapoptotic gene mcl-1. It has also been established that cytokines such as GM-CSF can trigger mcl-1 expression, and so, cytokines and hypoxia may be responsible for the increased protein levels of Mcl-1, which were observed in the majority of neutrophil samples isolated from the joints of rheumatoid patients.

Recently, it has been shown that Mcl-1 protein levels within cells can be regulated by post-translational modifications involving phosphorylation. The protein normally has a short half-life of only 2–3 h, becoming degraded via the proteasome. However, cytokines such as GM-CSF can increase cellular levels of Mcl-1 by stabilizing the protein via Akt-Erk-mediated phosphorylation [61] and slowing its rate of turnover [26, 62]. Alternatively, some agents such as okadaic acid and Taxol can accelerate the rate of Mcl-1 turnover and increase the rate at which cells undergo apoptosis. It is not known at present if the factors present within SFs regulate Mcl-1 protein levels by altering its rate of turnover.

In conclusion, this detailed investigation has shown that the survival of inflammatory neutrophils in RA is a complex process that depends on many factors that may accelerate or delay apoptosis and that local O2 tensions appear to play an especially important role. Potential strategies to therapeutically manipulate immune cell apoptosis in RA must therefore take these mechanisms into account if they are to develop successfully.

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