Insulin-like growth factor-1 delays Fas-mediated apoptosis in human neutrophils through the phosphatidylinositol-3 kinase pathway

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Abstract

Apoptosis of human neutrophils is a crucial mechanism for the resolution of inflammation. We previously showed that insulin-like growth factor-1 (IGF1) delays spontaneous neutrophil apoptosis without influencing the secretion of cytokines by these cells. In the present study, we further addressed the role of IGF1 in regulating neutrophil survival in the presence of other factors present during inflammation, and the mechanism involved in delaying apoptosis. We show that IGF1 delays neutrophil apoptosis triggered by the agonistic anti-Fas antibody CH11 and that the effect of IGF1 is comparable in magnitude to that of the acknowledged anti-apoptotic cytokines interferon- γ (IFNG) and granulocyte-macrophage colony-stimulating factor (GM-CSF; now known as CSF2). Furthermore, IGF1

exerted additional effects on cell survival in the presence of these cytokines. IGF1 did not affect Fas expression or activation by anti-Fas of caspase-8, but inhibited the depolarization of the mitochondrial membrane. Inhibitor studies indicate that the phosphatidylinositol-3 kinase (PI3K) pathway, but not the MEK-ERK pathway, mediates the effects of IGF1. However, in contrast to CSF2, IGF1 did not induce phosphorylation and translocation to the membrane of AKT, the canonical downstream target of PI3K. We therefore speculate that other downstream targets of PI3K are involved in the delay of neutrophil apoptosis by IGF1, possibly through stabilization of the mitochondrial membrane.

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Introduction

Neutrophils are pivotal effector cells in the innate immune response. They are phagocytic cells that participate in inflammatory reactions as a first line of defense against invading micro-organisms. Neutrophils are recruited to sites of inflammation by chemoattractants such as interleukin-8 (IL8). This inflammatory cytokine is produced by monocytes and macrophages, and is also one of the most abundant cytokines produced by neutrophils, which triggers degranulation and oxidative burst, both of which contribute to the destructive properties of these cells (Cassatella 1999). Regulation of the neutrophil life span by apoptosis is a crucial process in the resolution of inflammation, and alterations of apoptosis in neutrophils or their precursors are associated with a number of disorders (Maiani *et al.* 2004), including neutropenia (Aprikyan *et al.* 2000, 2001, 2002) and chronic neutrophilic leukemia (Kobayashi *et al.* 2002).

Neutrophils are programmed for spontaneous apoptosis (the intrinsic apoptotic route). In these cells, this mechanism of apoptosis has special features, probably due to peculiarities of neutrophil mitochondria, which are pivotal for apoptosis (Simon 2003). Importantly, neutrophil apoptosis is highly regulated. Spontaneous apoptosis can be inhibited by inflammatory cytokines, including granulocyte macrophage colony-stimulating factor 2 (GM-CSF; now known as CSF),

granulocyte colony stimulating factor 3 (CSF), interferon- γ (IFNG) and IL8 (Colotta *et al.* 1992, Kettritz *et al.* 1998). On the other hand, the apoptotic process can be stimulated through stimulation of the extrinsic route. This pathway is mainly stimulated by death-inducing receptors belonging to the tumor necrosis factor (TNF)/nerve growth factor (NGF) receptor super-family, such as Fas, TNF-related apoptosis-inducing ligand (TNFSF10) receptors, TNFRSF9 (CD137), and the type I TNF receptor (Simon 2003).

In a previous study, we showed that insulin-like growth factor-I (IGF1) delays spontaneous neutrophil apoptosis in serum-free medium (SFM), and that this effect was not mediated by the modulation of cytokine secretion by neutrophils (Kooijman *et al.* 2002). These findings indicate that IGF1 may be involved in the regulation of neutrophil apoptosis, either through endocrine effects of liver-derived IGF1 under the control of growth hormone (GH1) or via autocrine or paracrine effects. Indeed, IGF1 is produced by cells of the immune system including macrophages (Rom *et al.* 1988), mitogen-activated peripheral blood mononuclear cells (PBMC) (Nyman & Pekonen 1993), and stromal cells in bone marrow stromal (Abboud *et al.* 1991). Since IGF1 production in the immune system is predominantly regulated by cytokines instead of GH1 (Kirstein *et al.* 1992, Noble *et al.* 1993, Arkins *et al.* 1995, Fournier *et al.* 1995), it is possible that IGF1 is part of a cytokine network that modulates the resolution of apoptosis

through regulation of neutrophil survival. In addition, IGF1 may also be involved in the regulation of inflammatory reactions via effects on neutrophils, because it stimulates superoxide anion production (Fu *et al.* 1991), the uptake of immunoglobulin (IgG) opsonized *Staphylococcus aureus*, the oxidative burst and degranulation in neutrophils (Bjerknes & Aarskog 1995). During inflammation, neutrophils are exposed to many factors that can modulate apoptosis, including other cells expressing Fas-ligand, soluble Fas-ligand, and inflammatory cytokines. Some of these factors are able to modulate each other's effects. For instance, it has been shown that CSF2 is able to delay Fas-mediated apoptosis by suppression of Fas-associated protein with dead domain (FADD) recruitment (Kotone-Miyahara *et al.* 2004) and that activation of TNFRSF9 abrogates the anti-apoptotic effects of CSF2 in neutrophils (Heinisch *et al.* 2000). It has also been shown that TNF down-regulates TNFRSF9 expression on neutrophils, indicating that the extrinsic route for apoptosis can be inhibited by cytokines (Heinisch *et al.* 2000). Furthermore, IGF1 also inhibits Fas-mediated apoptosis in T-cells (Walsh & O'Connor 2000) and neutrophils (Wipke & Allen 2001), and T-cells (Isaacs 2008) contribute to the pathology of inflammatory diseases like rheumatoid arthritis (RA).

Therefore, we further addressed the role of IGF1 in regulating neutrophil survival in the presence of other factors present during inflammation. We assessed the effects of IGF1 on Fas-mediated induction of apoptosis in the presence or absence of inflammatory cytokines (IFNG or CSF2). We show that IGF1 delays Fas-mediated neutrophil apoptosis via the phosphatidylinositol-3 kinase (PI3K) pathway. In addition, IGF1 inhibits depolarization of the mitochondrial membrane in the presence of CH11, but does not affect Fas expression and the activation of caspase-8. The anti-apoptotic effect of IGF1 was also observed in the presence of anti-apoptotic cytokines, indicating that IGF1 could play a role in modulating neutrophil survival during inflammation.

Materials and Methods

Reagents

Recombinant human (rh) IGF1 was kindly provided by Lilly Research Laboratories (Indianapolis, IN, USA). Contamination with endotoxin was not detectable using the limulus amebocyte lysate assay (detection limit 1.25 pg/μg; Biowhittaker, Walkersville, MD, USA). The monoclonal antibody against the IGF1 receptor (αIR3; GR11L) was obtained from Calbiochem (VWR, Leuven, Belgium). CSF2 and IFNG were from PeproTech (London, UK). RPMI, penicillin, and streptomycin were purchased from Invitrogen Life Technologies. U0126, anti-caspase-8 (12F5) and a fluorescein-isothiocyanate (FITC)-conjugated IgG3 monoclonal antibody against Fas (APO-1-3) were obtained from Alexis Biochemicals (Zaventem, Belgium). A FITC-labeled isotypic control antibody was obtained from BD Biosciences Pharmingen (San Diego, CA, USA). The agonistic

anti-Fas immunoglobulin (IgM) monoclonal antibody CH11 was from Upstate (Milton Keynes, UK). BSA (A-2153, Sigma) was of standard grade quality and selected by us because it contained undetectable levels of IGF1 (<0.05 ng/mg). Wortmannin, cycloheximide, 3-aminopropyltri-ethoxysilane (APES), and JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide) were also obtained from Sigma. Horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG and sheep anti-mouse IgG were obtained from Amersham Pharmacia Biotech. Anti-actin was from Santa Cruz Biotechnology (Boechout, Belgium). Recombinant human TNF and antibodies against phosphorylated AKT (pS⁴⁷³), phosphorylated ERK (pTpY^{185/187}), and total-AKT were obtained from Biosource (Nivelles, Belgium).

Cell preparation and cell culture

Human neutrophils were purified from heparinized venous blood drawn from healthy donors as described previously (Kooijman *et al.* 2002). Informed consent was obtained from all blood donors and the research protocol has been approved by the local ethical committee. Freshly isolated neutrophils were suspended in SFM (RPMI 1640 with glutamax-I, supplemented with 0.1% BSA (A-2153), 100 U/ml penicillin, and 100 μg/ml streptomycin).

For assessment of apoptosis, neutrophils were cultured in 5 ml polystyrene Greiner tubes (Greiner Bio-one, Wemmel, Belgium) for indicated periods of time in a humidified 5% CO₂ atmosphere at 37 °C. IGF1 was added 30 min before CH11 and inhibitors were always added 30 min before the addition of IGF1. At the end of the culture period, 1 × 10⁵ cells were brought onto APES-coated slides and left at room temperature for 30 min to attach.

For phosphorylation and AKT translocation experiments, neutrophils were treated in eppendorf tubes (Eppendorf AG, Hamburg, Germany) in a prewarmed (37 °C) water bath. Kinase inhibitors or vehicle (dimethylsulfoxide) were always applied 30 min before the addition of IGF1 or CSF2.

Assessment of apoptosis

Neutrophils attached to APES-coated slides were fixed and stained with Diff-Quick according to the manufacturer's procedure (Dade Behring S.A., Brussels, Belgium). The proportion of apoptotic neutrophils was determined by light microscopy. Apoptotic neutrophils were defined as cells displaying pyknotic nuclei and diminution of cell volume (Murray *et al.* 1997). We have previously demonstrated that the effects of IGF1 on apoptotic morphology are in line with DNA fragmentation assay and annexin-V binding assays (Kooijman *et al.* 2002).

Flow cytometric analysis of Fas expression

Neutrophils were suspended in 50 μl PBS/1% BSA containing 0.25 μg anti-Fas antibody (APO-1-3) or an

isotype-matched control antibody. After 30 min of incubation at 4 °C, neutrophils were washed twice in PBS and suspended in 50 µl PBS. Stained neutrophils were analyzed on a FACS Vantage flow cytometer with a 488 nm argon laser (Becton Dickinson, Erembodegem, Belgium). For each sample forward light scatter, side scatter and fluorescence of 10 000 cells were acquired. Debris and remaining erythrocytes were excluded by gating on the basis of forward and side scatter. Data analysis was performed using WinMDI 2.8 software (URL: <http://facs.scripps.edu>).

Cytokine measurements

Neutrophils were separated from the culture medium by centrifugation for 10 min at 400 g at room temperature. Culture media were collected and stored at -20 °C until use. The levels of IL8, IL1B, TNF, or IL6 were quantified by ELISA using commercial antibody pairs (Cytosets) from Biosource International as described previously (Kooijman *et al.* 2002). The sensitivities of the ELISA's for IL8, IL1B, TNF, or IL6 were 3, 10, 4, and 10 pg/ml respectively.

Western blotting analysis

Neutrophils (1.5×10^6) were lysed in 20 µl ice-cold distilled water containing protease inhibitors (1 tablet in 5 ml H₂O, Roche Diagnostics). Next, 20 µl boiling 2× concentrated sample buffer was added to the total cell lysate and the samples were boiled for 10 min. SDS-PAGE and transfer were performed as previously described (Kooijman *et al.* 1997). After blocking for 1 h at room temperature in blocking buffer (PBS/5% non-fat dry milk/0.1% Tween-20), blots were probed with primary antibodies (1/1000 in PBS/5% BSA/0.1% Tween-20) for 18 h at 4 °C. Blots were washed three times for 10 min in PBS/0.1% Tween-20 and incubated with a species-specific HRP-labeled IgG (1/5000 in blocking buffer) for 1 h at room temperature. After washing, immunoreactive bands were detected using enhanced chemiluminescence (Perkin-Elmer, Boston, MA, USA). To check for equal loading, blots were stripped by incubation in 0.25 M NaOH at room temperature and incubated with polyclonal antibody against actin. Densitometric analysis was performed using Scion Image (Scion Corporation, Frederick, MO, USA).

Depolarization of the mitochondrial membrane

After cell culture in SFM, JC-1 was added in a final concentration of 9 µM. After 5 min incubation at 37 °C, the cells were washed with PBS and centrifuged (200 g) for 5 min. Subsequently, the cells were resuspended in PBS and analyzed with the FACS Vantage flow cytometer. For each sample forward light scatter, side scatter, FL1 and FL2 of 10 000 cells were acquired. Debris and remaining erythrocytes were excluded by gating on the basis of forward and side scatter. The cells were analyzed with WinMDI 2.8 software.

AKT translocation assays

Neutrophils were isolated and resuspended at a final concentration of 40×10^6 in 500 µl SFM. After 30 min pre-incubation at 37 °C, the cells were treated with SFM, 300 ng/ml IGF1, or 50 ng/ml CSF2 for indicated periods of time. The reaction was stopped by adding ice cold buffer (100 mM KCl, 50 mM HEPES, 5 mM NaCl, 1 mM MgCl₂, 0.5 mM EDTA, 5 µg/ml aprotinin, 5 µg/ml leupeptin, 1 mM activated orthovanadate, 0.25 mM (4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride). The suspension was sonicated for 20 s and centrifuged for 10 min at 15 000 g. Supernatants were collected and centrifuged for 2 h at 50 000 g in a Beckman Avanti J25 centrifuge with a JA20 rotor. All membrane material in the pellets, obtained from 40×10^6 cells, was analyzed by western blotting using an 8.5% gel. Unequal loading of the gel was corrected by quantifying the amounts of actin on the western blot.

Statistical analysis

To determine statistical significance, repeated-measures ANOVA with a Bonferroni multiple comparison post-test was used (Figs 1 and 5C). The one-sample *t*-test with a Bonferroni correction for multiple comparisons was used to assess whether relative increase in the percentage of apoptotic neutrophils was significantly different from the control values, which were put at 100% (Fig. 2). In other experiments, the two-tailed unpaired *t*-test was used. Means were considered to be statistically different at *P* values < 0.05.

Results

Inhibition of spontaneous and Fas-mediated apoptosis

We measured apoptosis in freshly isolated neutrophils by morphological evaluation. After 5 h of culture, the percentage of apoptotic neutrophils increased with 12.1% from 2.7% at *t*=0 to 14.8% (spontaneous apoptosis) at 5 h. To test the effects of IGF1 on Fas-mediated apoptosis, we incubated neutrophils with 100 ng/ml of the anti-Fas antibody CH11. This concentration of CH11 has been shown to stimulate Fas-mediated apoptosis in neutrophils. It activates caspase-8 (Kotone-Miyahara *et al.* 2004, Iwase *et al.* 2006) and it evokes the recruitment of FADD to Fas (Kotone-Miyahara *et al.* 2004). Indeed, stimulation of the extrinsic pathway by CH11 further increased the percentage of apoptotic neutrophils to 39.6% in the absence of IGF1 and to 18.5% in the presence of IGF1 (Fig. 1C). Although CH11 activates the extrinsic apoptotic pathway, one may not exclude the possibility that the intrinsic route, involved in spontaneous apoptosis, is still operative. However, since the anti-apoptotic effects of IGF1 in the absence of CH11 are much smaller than those in the presence of CH11, the effect of IGF1 on CH11-treated neutrophils is at least in part due to the inhibition of

Fas-mediated apoptosis. After 18 h of incubation (Fig. 1D), the effects of IGF1 on spontaneous and Fas-mediated apoptosis are relatively less compared with the effects after 5 h. To assess whether IGF1 can also delay the effects of other death-inducing signals, we tested the effects of IGF1 in the presence of TNF. However, we found that TNF augmented spontaneous apoptosis during the first 3 h of the culture period (Fig. 1A and B), although these effects did not reach significance. In contrast, at 18 h spontaneous apoptosis was significantly reduced by TNF. Addition of IGF1 resulted in a further reduction in the increase of apoptotic cells (Fig. 1D). The differential effects of TNF on early time points compared with 18 h are in line with observations from others (Murray *et al.* 1997). In contrast, the rate of neutrophil apoptosis in our culture system is higher than that shown by Murray *et al.* (1997), probably due to the use of SFM instead of serum-containing media.

The dose-response curve in Fig. 1E shows that physiological concentrations of IGF1 effectively inhibit Fas-mediated apoptosis. The inhibitive effects of a blocking antibody against the IGF1R, and our finding that insulin was without effect (data not shown) indicate that the effects of IGF1 are mediated by the IGF1R (Fig. 1F).

Anti-apoptotic effects of IGF1, IFNG and CSF2

The anti-apoptotic effects of cytokines have predominantly been assessed in the presence of serum, and thereby in the presence of IGF1 and other factors. To evaluate their independent effects and possible synergistic interactions with IGF1, we cultured neutrophils in SFM with IGF1, CSF2, IFNG or combinations thereof. Figure 2 reveals that IGF1 and both cytokines significantly inhibit Fas-mediated apoptosis, and it appeared that the anti-apoptotic effects of IGF1 were comparable to those of CSF2 and IFNG. The average inhibition of apoptosis by 300 ng/ml IGF1, 1 ng/ml CSF2 (Fig. 2A), and 2 ng/ml IFNG (Fig. 2B) in seven independent experiments was 44, 51, and 47% respectively. It also appeared that IGF1 has the potential to inhibit Fas-mediated apoptosis when neutrophils are exposed to CSF2 or IFNG at concentrations that effectively inhibit apoptosis. In the presence of 2 ng/ml IFNG or 1 ng/ml CSF2, IGF1

significantly reduced apoptosis by 58 and 44% respectively. Remarkably, at higher concentrations of IFNG or CSF2 the effects of IGF1 were smaller and not significant.

IGF1 does not modulate cytokine production by neutrophils

Pro-inflammatory cytokines such as IL8, IL6, TNF, and IL1B could mediate the anti-apoptotic effect of IGF1. Recently, it has been shown that the inhibition by TNF of spontaneous neutrophil apoptosis was mediated by the increase in IL8 production (Cowburn *et al.* 2004). To address this issue, we measured the effect of IGF1 on the secretion of cytokines by neutrophils exposed to SFM (vehicle), CH11, and TNF (10, 100 ng/ml). CH11 and IGF1 had no effect on the secretion by neutrophils of IL8 (Fig. 2C), TNF, or IL1B (data not shown) during the first 5 h of culture. After 18 h, however, IL8 levels in the culture medium of CH11-stimulated cells were reduced by IGF1. IL6 levels in the conditioned media were below the detection limit.

IGF1 does not affect Fas expression and caspase-8 activation in neutrophils

Having established anti-apoptotic effects of IGF1 on Fas-mediated neutrophil apoptosis, we next sought to investigate whether these effects are mediated by the modulation of Fas expression. Indeed, IGF1 has been shown to modulate Fas expression on the cell surface of several cell types. For instance, in osteoblasts IGF1 stimulates Fas expression (Kawakami *et al.* 1998), whereas IGF1 down-regulates the expression of Fas in PHA-activated cord blood T cells (Tu *et al.* 2000). We assessed the effects of IGF1 on Fas expression in neutrophils by flow cytometry. The histogram in Fig. 3A confirms the observation of others (Iwai *et al.* 1994, Liles *et al.* 1996, Kotone-Miyahara *et al.* 2004) that neutrophils express Fas. As shown in Fig. 3B, IGF1 does not influence cell surface expression of Fas on neutrophils. IGF1 has been shown to upregulate Flice (caspase-8) inhibiting protein (Flip), which interferes with the signaling of death-inducing receptors through blocking the association of caspase-8 with the intracellular domain of these receptors (Mitsiades *et al.* 2003). Therefore, it is conceivable that IGF1 interferes with

Figure 1 IGF1 delays neutrophil apoptosis. Apoptotic cells were assessed morphologically directly after isolation of neutrophils ($t=0$) and after indicated culture periods in SFM. Values in the histograms represent the increase in the percentage of apoptotic cells during the culture period (A–D). Cells were cultured in the absence of stimuli, in the presence of either IGF1 (300 ng/ml), CH11 (100 ng/ml), or TNF (10, 100 ng/ml), or in the presence of CH11 and IGF1 or TNF and IGF1. Vehicle (SFM) or IGF1 was added at the start of the culture. After 30 min, CH11 or TNF (10, 100 ng/ml) were added and the cells were further cultured for indicated periods of time. Values are mean $\pm$ S.E.M. from quadruplicate incubations. This experiment is representative of three independent experiments using different blood donors. To determine the statistical significance of IGF1 effects, a two-tailed unpaired t -test was used. * $P<0.05$; ** $P<0.01$; *** $P<0.001$. To determine the effect of CH11 or TNF alone versus vehicle, an ANOVA with a Bonferroni multiple comparison post-test was used. *** $P<0.001$ (E). Dose-response curve for inhibition of Fas-mediated apoptosis by IGF1. The cells were treated with CH11 and IGF1 as described in Fig. 1A–D and cultured for 5 h after addition of CH11. Values are mean $\pm$ S.E.M. from quadruplicate incubations and representative of four independent experiments. # $P<0.05$; *** $P<0.001$ significantly different from the vehicle. (F) Abrogation of the anti-apoptotic effect of IGF1 by an antibody against the IGF1 receptor (α IR3). Neutrophils were treated with CH11 and IGF1 as described for Fig. 1A–D and cultured for 5 h. Part of the cells were pre-incubated with 1 μ g/ml α IR3 for 30 min prior to the addition of IGF1 or vehicle. Values are mean $\pm$ S.E.M. from quadruplicate incubations and representative of three independent experiments using different blood donors. * $P<0.05$.

Figure 2 Inhibition of Fas-mediated apoptosis by 300 ng/ml IGF1 in the absence or presence of (A) CSF2 or (B) IFNG. IGF1, CSF2, IFNG, or combinations thereof were added 30 min before the addition of 100 ng/ml CH11. Subsequently, cells were incubated for 5 h in the presence of CH11, after which apoptosis was assessed morphologically. The increase in the fraction of apoptotic cells is expressed as a percentage of the control (no cytokine, no IGF1). # = Statistically different from the control (100%, $P < 0.05$). Data represent mean $\pm$ S.E.M. of seven independent experiments using different blood donors. * = Significant effect of IGF1 ($P < 0.05$); NS = not significant. (C) Effect of IGF1 on IL8 production. Neutrophils were incubated for indicated periods of time with vehicle, IGF1 (300 ng/ml), CH11 (100 ng/ml), or CH11 and IGF1. Values are mean $\pm$ S.E.M. of quadruplicate incubations. This experiment is representative of three independent experiments using different blood donors. *** $P < 0.001$.

the extrinsic apoptotic pathway through inhibition of caspase-8 activation. Binding of CH11 to Fas results in the binding and cleavage of full caspase-8, leading to the expression of the small active caspase-8 fragment (p18) that can be detected by western blotting. Figure 4A shows that caspase-8 cleavage is stimulated by CH11. In contrast to CSF2 that is known to inhibit caspase-8 cleavage (Watson *et al.* 1999, Kotone-Miyahara *et al.* 2004), IGF1 did not inhibit the cleavage of caspase-8 (Fig. 4A).

IGF1 partially blocks the destabilization of the mitochondrial membrane

Depolarization of the outer mitochondrial membrane is key to the activation of the intrinsic apoptotic pathway triggered by

stress factors. In addition, depolarization may also occur in Fas-mediated apoptosis via truncation of the B-cell lymphoma 2 (BCL2) family protein Bid by activated caspase-8. We therefore assessed the effect of IGF1 on membrane depolarization by flow cytometric analysis using the fluorescent indicator JC-1. At a high mitochondrial membrane potential, JC-1 forms aggregates in the mitochondria and emits red fluorescence with a maximum at 590 nm. Monomers emitting green fluorescence (maximal emission at 527 nm) are formed at low membrane potential. It appeared that, cultured neutrophils comprise two fractions, one with an intermediate level of green fluorescence and then the other with high-level representing cells with a normal mitochondrial membrane potential and cells with a depolarized membrane respectively. As depicted in Fig. 4C, stimulation with CH11 (100 ng/ml) for 3 h increased the

Figure 3 IGF1 does not affect cell surface expression of Fas on neutrophils. (A) Histogram of APO-1-3-stained freshly isolated neutrophils superimposed over the histogram of neutrophils stained with an irrelevant isotopic matched antibody. (B) Neutrophils were incubated for indicated periods of time with vehicle or 300 ng/ml IGF1, and subsequently labeled with a fluorescein-conjugated antibody against Fas (APO-1-3) for flow cytometric analysis. This graph shows the effects of IGF1 on the specific mean fluorescence intensity (MFI) of APO-1-3-stained neutrophils. Specific MFI was calculated by subtracting the MFI of controls from the MFI of APO-1-3-stained neutrophils. Values are mean $\pm$ S.E.M. of quadruplicate incubations. This experiment is representative of four independent experiments using different blood donors.

percentage of cells with a high level of green fluorescence. Figure 4D shows the effects on the level of green fluorescence after stimulation for 5 h with SFM, IGF1, CH11 or CH11 and IGF1. CH11 increased the percentage of cells showing mitochondrial membrane depolarization, after 3, 4 and 5 h of culture. In addition, IGF1 decreased the fraction of these cells in the presence of CH11 (Fig. 4B). Taken together our results suggest that the anti-apoptotic effect of IGF1 in CH11-treated neutrophils is mediated, at least in part, by stabilization of the mitochondrial membrane.

IGF1 signaling pathways

To address the role of two principal IGF1R signaling routes, the PI3K pathway and the MEK-ERK pathway, we incubated neutrophils with different kinase inhibitors. Figure 5A shows that the inhibition of apoptosis by IGF1 is completely abrogated in the presence of the PI3K inhibitor wortmannin, whereas the effect of IGF1 is hardly or not affected by the MEK inhibitor U0126. Phosphorylation studies revealed that IGF1 does not influence the phosphorylation of MAPK1 nor that of the PI3K downstream target AKT, whereas CSF2 augmented the phosphorylation of these kinases markedly (Fig. 5B and C). These results confirm that the MEK-ERK pathway is not involved and indicates a role for PI3K targets other than AKT. Activation of PI3K leads to the generation of phosphatidylinositol triphosphate (PIP3), which functions as a docking site for AKT and other proteins through their pleckstrin homology (PH) domains (Takesono *et al.* 2002). To confirm that AKT is not involved in IGF1 signaling, we assessed the translocation of AKT in response to IGF1 and CSF2. Since translocation of AKT precedes its activation through phosphorylation at the

membrane, we assessed translocation at several time points between 20 s and 10 min. We found CSF2 to evoke the recruitment of AKT to the membrane, while IGF1 had no effect (Fig. 5D and E).

Discussion

Apoptosis in neutrophils is pivotal to the resolution of inflammation (Savill 1997). Interestingly, neutrophils display several special features with respect to apoptosis. The mitochondrion, which does not seem to be required for energy supply, appears to be specialized in the regulation of apoptosis, and glucocorticoids inhibit apoptosis in neutrophils, whereas in other cell types, including eosinophils, they induce apoptosis. Another special feature of neutrophils is that these cells are subject to spontaneous apoptosis. When neutrophils leave the bone marrow environment, they have a circulating half-life of 6–10 h. However, it should be stressed that neutrophil apoptosis during inflammation is highly regulated by several pro- and anti-apoptotic factors in the surrounding milieu. It has been described by many groups that inflammatory cytokines inhibit apoptosis (Maianski *et al.* 2004), which may lead to enhanced neutrophils survival during inflammation. On the other hand, ligands engaging so-called death receptors of the TNF/NGF receptor superfamily are able to stimulate neutrophil apoptosis (Simon 2003). Although the effects of many apoptosis-regulating agents are known, the molecular mechanism involved in the orchestration of neutrophils survival remains to be established.

In a previous study, we showed that IGF1 delays spontaneous apoptosis of human neutrophils in SFM (Kooijman *et al.* 2002). Although neutrophils secreted low

Figure 4 IGF1 does not affect caspase-8 activation but partially blocks the destabilization of the mitochondrial membrane. (A) Effects of IGF1 and CSF2 on caspase-8 activation. Cells were pre-incubated for 30 min with 20 μ g/ml cycloheximide (CHX) to facilitate the activation of caspase-8. Subsequently, the cells were treated for 30 min with vehicle, IGF1 (300 ng/ml) or CSF2 (50 ng/ml), after which they were cultured for 5 h in the presence or absence of 100 ng/ml CH11. Activation of caspase-8 was measured by assessing the cleavage of full caspase-8 by western blot analysis of 1.5×10^6 cells. The immunoblot of the cleaved fragment (p18) was exposed to the chemiluminescence reaction for a longer period (60 min) than the immunoblot of full caspase-8 and the intermediate fragments p43/p41 (5 min). To check for equal loading, blots were stripped and incubated with the polyclonal antibody against actin (lower panel). This result is representative of three independent experiments using different blood donors. (B) Neutrophils were pre-incubated for 30 min with vehicle or 300 ng/ml IGF1 and subsequently cultured for 3, 4, and 5 h in the presence or absence of CH11 (100 ng/ml). Mitochondrial membrane depolarization was assessed by flow cytometry using JC-1 as a membrane potential sensitive dye. The percentage of cells with high levels of green fluorescence (FL1) was determined. Treatment with the K^+ -ionophore valinomycin resulted in loss of membrane potential in virtually all cells (not shown). Values are mean $\pm$ S.E.M. of quadruplicate incubations. This experiment is representative of three independent experiments using different blood donors. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. (C) Dot-plots of the primary data from a representative experiment. The dot-plots and corresponding histograms show the increase in neutrophils with high levels of green fluorescence after stimulation with CH11 (100 ng/ml) for 3 h. (D) Dot-plots representing primary data from neutrophils incubated for 5 h with vehicle, IGF1, CH11 or CH11 and IGF1. The numbers in C and D indicate the percentage of cells with intermediate and high FL1 levels respectively.

levels of inflammatory cytokines, they did not mediate the effects of IGF1. However, during inflammation, neutrophils are exposed to large amounts of cytokines produced by monocytes and lymphocytes, and to FASLG (Fas-ligand) expressed on activated T cells or secreted into the milieu by macrophages after ingestion of apoptotic neutrophils. It has been shown that IGF1 inhibits Fas-mediated apoptosis in T-cells (Walsh & O'Connor 2000) and that there is a reduced susceptibility to Fas-mediated apoptosis in peripheral blood

T-cells from patients with RA (Szodoray *et al.* 2003). Removal of neutrophils protects mice from joint-specific inflammation in a murine model of RA showing the important role of neutrophils (Wipke & Allen 2001) in RA. Elevated levels of IGF1 are reported in synovial fluid from patients with osteoarthritis, RA (Matsumoto *et al.* 1996) or in lung tissues from patients with acute respiratory distress syndrome (Krein *et al.* 2003), all diseases where delayed neutrophil apoptosis contributes to the disease. To establish

Figure 5 IGF1 signaling pathways. (A) Inhibition of the anti-apoptotic effects of IGF1 by the PI3K inhibitor wortmannin. Neutrophils were pre-incubated for 30 min with vehicle (DMSO), 0.03 μ M wortmannin, or 1.0 μ M U0126, and subsequently treated with vehicle or IGF1 for 30 min. Next, CH11 (100 ng/ml) was added and cells were cultured for 5 h in the presence of CH11. Apoptosis was analyzed morphologically. Values are mean $\pm$ S.E.M. of quadruplicate incubations. This result is representative of four independent experiments using different blood donors. $^{**}P < 0.01$; $^{***}P < 0.001$; NS = not significant. (B) Effects of IGF1 and CSF2 on phosphorylation of AKT and MAPK1 were assessed by immunoblotting using phospho-specific antibodies. Before each phosphorylation experiment, neutrophils were pre-incubated for 60 min in SFM. Then, neutrophils were stimulated for indicated periods of time (minutes; h = hour) with vehicle (V), 300 ng/ml IGF1 (I), or 50 ng/ml CSF2 (G). Phosphorylation of kinases was assessed using phospho-specific antibodies. To check for equal loading, blots were stripped and incubated with the polyclonal antibody against actin (lower panel). This experiment is representative of three independent experiments using different blood donors. (C) The histogram represents the fold change in the phosphorylation level of AKT versus vehicle 1 min, normalized against actin. Values are mean $\pm$ S.E.M. from three (IGF1) or four (CSF2) independent experiments using different blood donors. $^{##}P < 0.01$. (D) Effects of IGF1 and CSF2 on the recruitment of AKT to the membrane. Neutrophils were pre-incubated for 30 min in SFM and subsequently stimulated with vehicle (V), 300 ng/ml IGF1 (I), or 50 ng/ml CSF2 (G). Membranes were isolated by differential centrifugation, after which the amount of AKT was measured by western blot analysis. To confirm equal amount of loading, the membranes were reblotted with a polyclonal antibody against actin. (E) The histogram represents the fold-change in membrane-bound AKT normalized against actin. Values are mean $\pm$ S.E.M. from three independent experiments using different blood donors. To determine the effect of CSF2 versus vehicle (20 s), a one-sample *t*-test with a Bonferroni multiple comparison post-test was used. $^{*}P < 0.05$.

the potential role of IGF1 in the regulation of neutrophil apoptosis during inflammation, we studied the effects of IGF1 in the presence of pro- or anti-apoptotic signals. Inhibition of Fas-mediated apoptosis by IFNG, CSF2, CSF3, and TNF has been previously reported (Liles *et al.* 1996, Watson *et al.* 1999, Kotone-Miyahara *et al.* 2004). To explore the possible interactions between IGF1 and anti-apoptotic cytokines, we assessed the effects of IGF1 on Fas-stimulated apoptosis in the presence of CSF2 or IFNG. Our results demonstrate that the inhibitory effects of CSF2 and IFNG do not depend on the presence of IGF1. Since IGF1 delays apoptosis not only in SFM but also in the presence of the established anti-apoptotic cytokines CSF2 or IFNG, we believe that IGF1 has the potential to inhibit neutrophil apoptosis during inflammation. However, at high concentrations of IFNG or CSF2, the inhibitive effect of IGF1 was partially abrogated. Especially, high concentrations of IFNG, which were also less effective in reducing apoptosis in the absence of IGF1, almost completely blocked the effect of IGF1. The role of this phenomenon during inflammation and the mechanism involved remain to be established. An intriguing question is whether IGF1 is involved in the orchestration of neutrophil apoptosis during inflammation. IGF1 may mediate certain effects of cytokines during inflammation, because it can be produced by macrophages (Rom *et al.* 1988, Arkins *et al.* 1993) and regulated by inflammatory cytokines including TNF (Noble *et al.* 1993) and IL1B (Kirstein *et al.* 1992). Furthermore, several authors demonstrated that the Th1 cytokine IFNG inhibits IGF1 production in macrophages (Kirstein *et al.* 1992, Arkins *et al.* 1995), whereas the Th2 cytokines IL4 and IL13 stimulate IGF1 production in these cells (Wynes & Riches 2003). The last observation prompted the authors to speculate that Th1 cytokines inhibit IGF1 expression in macrophages, while Th2 cytokines enhance IGF1 expression. However, the specific role of IGF1 in regulating neutrophil apoptosis during Th1 and Th2 immune responses remains to be established. Another argument for a regulatory role of IGF1 in neutrophil apoptosis is provided by the observation that two neutrophil proteases, cathepsin G and elastase, effectively cleave all six well-characterized IGF-binding proteins (IGFBPs; Gibson & Cohen 1999). Proteolytic cleavage of IGFBPs leads to the release of sequestered IGF1, increasing the concentration of free IGF1 (Firth & Baxter 2002).

We show that IGF1 delays Fas-mediated apoptosis and that IGF1 and TNF have the same potency to delay spontaneous neutrophil apoptosis. Moreover, the additive effect of IGF1 on TNF-inhibited spontaneous apoptosis can be interesting because several inflammatory diseases are now treated with an anti-TNF antibody to prevent TNF binding to its receptor. This could also block the anti-apoptotic action of TNF, leading to increased neutrophil apoptosis helping to fasten the removal of neutrophils. It would be interesting to study the additive effect of TNF- and IGF1-blocking antibodies.

Since IGF1 did not modulate cell surface expression of Fas or Fas-mediated cleavage of full caspase-8, we conclude that

IGF1 does not act at the level of Fas signaling at the plasma membrane. In contrast, IGF1 delays the destabilization of the mitochondrial membrane in the presence of CH11. Depolarization of the mitochondrial membrane potential is regulated by BCL2 family proteins. Anti-apoptotic members of this family interact with the outer membrane serving to keep its integrity. In contrast, pro-apoptotic members trigger membrane depolarization, in part, through sequestration of anti-apoptotic BCL2 proteins. We hypothesize that IGF1, at least in part, inhibits neutrophil apoptosis through regulation of BCL2 family proteins. It should be noted that the relative effect of IGF1 on the fraction of cells exhibiting mitochondrial membrane depolarization is smaller than the effect on the fraction of apoptotic cells (Figs 1C and 4B). Therefore, the possibility exists that IGF1 also affects apoptosis through regulation of other processes, e.g. activation of inhibitors of apoptosis such as X-linked inhibitor of apoptosis (XIAP), cellular inhibitor of apoptosis protein 1 (BIRC2), cellular inhibitor of apoptosis protein 2 (BIRC3) or survivins that are present in neutrophil (Hu & Sayeed 2005, Martinelli *et al.* 2006). These molecules are able to inhibit caspase-3 activation without affecting caspase-8 and mitochondrial membrane potential.

Several protein kinase cascades that can be activated upon IGF1 binding to the IGF1R have been defined (Le Roith *et al.* 2001). Ligand binding to the extracellular α -subunits leads to phosphorylation of the β -subunits and subsequent association of adapter molecules such as insulin receptor substrates and the Src homology 2 domain containing transforming protein SHC, which serve to recruit other signaling molecules. Recruitment of the 85 kDa regulatory subunit of PI3K results in the activation of the enzyme to phosphorylate phosphatidylinositol biphosphate (PIP2) at the 3-position to generate PIP3. Another pathway that emerges after tyrosine phosphorylation of the IGF1R involves the activation of MAPK3 and MAPK1, through the recruitment of Grb2 and subsequent activation of ras, raf, and MEK. Although it has been established that the PI3K is an important player in regulating neutrophil apoptosis (Cowburn *et al.* 2002, Yang *et al.* 2003), the MEK–MAPK1 pathway has also been implicated in this process (Klein *et al.* 2001, Sheth *et al.* 2001, Gardai *et al.* 2004, Kooijman 2006). We therefore established the effects of IGF1 in the presence of the MEK inhibitor U0126 and the PI3K inhibitor wortmannin. It appeared that the MEK–MAPK1 pathway was not involved, whereas inhibition of the PI3K pathway completely abrogated the inhibitive effect of IGF1. Remarkably, IGF1 did not stimulate the phosphorylation or translocation to the membrane of AKT, indicating IGF1 delays neutrophil apoptosis through other PI3K-dependent molecules. In contrast, CSF2, which also delays neutrophil apoptosis via the activation of PI3K (Klein *et al.* 2000), did induce phosphorylation and translocation of AKT.

We also showed that CSF2 inhibits caspase-8 degradation, which is in line with the observation that CSF2 attenuates the recruitment of FADD to the DISC of Fas (Kotone-Miyahara *et al.* 2004) and subsequent activation of caspase-8

(Watson *et al.* 1999). This process was also shown to be PI3K dependent. These findings implicate that the inhibition of apoptosis by CSF2 involves in part other signaling pathways than those used by IGF1. This could explain additional effects of CSF2 and IGF1 on neutrophil apoptosis presented in Fig. 2A. PI3K has several downstream targets other than AKT which may be involved in the inhibition by IGF1 of neutrophil apoptosis, including protein kinase C delta (PRKCD) and TEC kinases (Takesono *et al.* 2002). It has been demonstrated that IFNB1 inhibits apoptosis by PRKCD activation through the PI3K pathway (Wang *et al.* 2003). However, the protein kinase C inhibitors GFX109203 and rottlerin did not affect the anti-apoptotic effects of IGF1 (data not shown). Identification of PI3K downstream targets involved in neutrophil apoptosis may lead to the discovery of new compounds able to regulate inflammatory processes through the modulation of neutrophil survival. Alternatively, neutrophil survival could be influenced by the components of the IGF1 system or IGF1R antagonists.

Declaration of interest

The authors have nothing to disclose.

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