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Mcl-1-Mediated Impairment of the Intrinsic Apoptosis Pathway in Circulating Neutrophils from Critically Ill Patients Can Be Overcome by Fas Stimulation

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The systemic inflammatory response syndrome and subsequent organ failure are mainly driven by activated neutrophils with prolonged life span, which is believed to be due to apoptosis resistance. However, detailed underlying mechanisms leading to neutrophil apoptosis resistance are largely unknown, and possible therapeutic options to overcome this resistance do not exist. Here we report that activated neutrophils from severely injured patients exhibit cell death resistance due to impaired activation of the intrinsic apoptosis pathway, as evidenced by limited staurosporine-induced mitochondrial membrane depolarization and decreased caspase-9 activity. Moreover, we found that these neutrophils express high levels of antiapoptotic Mcl-1 and low levels of proapoptotic Bax protein. Mcl-1 up-regulation was dependent on elevated concentrations of GM-CSF in patient serum. Accordingly, increased Mcl-1 protein stability and GM-CSF serum concentrations were shown to correlate with staurosporine-induced apoptosis resistance. However, cross-linking of neutrophil Fas by immobilized agonistic anti-Fas IgM resulted in caspase-dependent mitochondrial membrane depolarization and apoptosis induction. In conclusion, the observed impairment of the intrinsic pathway and the resulting apoptosis resistance may be overcome by immobilized agonistic anti-Fas IgM. Targeting of neutrophil Fas by immobilized agonistic effector molecules may represent a new therapeutic tool to limit neutrophil hyperactivation and its sequelae in patients with severe immune disorders. The Journal of Immunology, 2009, 183: 6198–6206.

Ccritically ill intensive care patients die as a direct consequence of their severe injuries, or by the additional damage caused by subsequent deregulated immune reactions resulting in multiple organ dysfunction syndrome (MODS)1 (1). Tissue damage leads to an immediate neutrophil activation and increased cytotoxicity yielding in endothelial dysfunction through the secretion of proteolytic enzymes such as elastase or reactive oxygen species, followed by edema and tissue destruction (2, 3). Normally, neutrophils spontaneously undergo apoptosis and their life span is limited to 8–20 h in circulation and 1–4 days in tissue. However, after severe injury, the neutrophil life span is significantly prolonged and can last up to 3 wk (4, 5). Thus, deregulated apoptosis, such as resistance to proapoptotic signals in neutrophils of trauma patients, may contribute to the development of systemic inflammatory response syndrome (SIRS) associated with tissue destruction and MODS (6–8).

From a therapeutic point of view, the transient prevention of posttrauma neutrophil hyperactivation is an important challenge. In this regard, a better understanding of posttrauma neutrophil apoptosis regulation is a prerequisite for the development of neutrophil-directed therapies. Fas (CD95)/Fas ligand (FasL, CD95L) signaling has emerged as an important cellular pathway regulating the induction of apoptosis in a wide variety of tissues and plays a critical role for counterregulation of neutrophil activity (9). The Fas receptor is a member of the TNFR family and is widely expressed on cell surfaces. Activation of this receptor by its ligand FasL or a cross-linking Ab (anti-Fas IgM mAb) results in receptor oligomerization and apoptosis induction (10). The execution of apoptosis occurs by activation of cysteine proteases, called caspases. Activated Fas forms the death-inducing signaling complex, which contains the FasR, the adapter protein Fas-associated death domain protein, and multiple procaspase-8 molecules, resulting in caspase-8 activation by autoprocessing. The initiator caspase-8 triggers a caspase cascade that activates downstream effector caspses such as caspase-3 and caspase-7 (11, 12).

Liles et al. (13) have shown that inhibition of the Fas receptor results in a partial inhibition of neutrophil apoptosis. Furthermore, different expression of proteins of the Bcl-2 family as well as caspase-mediated activation of protein kinase Cδ have been suggested to modulate neutrophil cell death (14, 15). The Bcl-2 family can be divided into antiapoptotic (such as Bcl-2, Bcl-xL, Mcl-1, and A1/Bfl-1) and proapoptotic proteins (such as Bak, Bax, Bad, and Bid). The ratio of pro- and antiapoptotic proteins and their localization, conformation, and phosphorylation status can affect the function of these proteins and the decision between apoptosis and survival (16). Pro- and antiapoptotic proteins are associated

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3 Abbreviations used in this paper: MODS, multiple organ dysfunction syndrome; ICU, intensive care unit; PI, propidium iodide; PPE, polyurethane; SIRS, systemic inflammatory response syndrome; TBS-T, Tris-buffered saline containing 0.1% Tween 20.

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with the mitochondrial membrane where they compete to regulate the exit of proapoptotic factors such as cytochrome c. Once released into the cytosol, cytochrome c associates with Apaf-1 and procaspase-9 to form the apoptosome which activates caspase-3 by proteolytic cleavage (17).

In severely injured patients, elevated levels of proinflammatory cytokines such as GM-CSF are known to extend the life span of mature neutrophils by impairing Fas-mediated apoptosis and to augment neutrophil activity (13, 18). Additionally, it has been shown that GM-CSF up-regulates Mcl-1 in neutrophils in vitro (19, 20). To overcome this neutrophil apoptosis resistance, activation of the extrinsic apoptosis pathway by specific neutrophil Fas cross-linking remains an attractive strategy to transiently decrease neutrophil activity (21). To avoid the systemic application of agonistic Fas effector molecules resulting in nonspecific side effects, an extracorporeal immune therapy with agonistic anti-Fas IgM Abs immobilized on biocompatible carriers has been proposed to selectively target neutrophils within the circulating blood (22, 23).

In this study, we provide, for the first time, evidence for impaired intrinsic apoptosis sensitivity in neutrophils from severely injured patients. We show that apoptosis resistance is due to increased Mcl-1 protein level and stability and can be overcome by ex vivo cross-linking of the Fas receptor with immobilized anti-Fas Ab.

**Materials and Methods**

***Patients***

Study approval was obtained from the local ethics committee of the University of Duesseldorf (Duesseldorf, Germany). Twenty-six multiply injured patients who were admitted to the trauma center with an injury severity score > 16 and an intensive care unit (ICU) stay > 3 days were included in this study. Informed consent was obtained from the patient’s legal representative if the patient lacked consciousness. Exclusion criteria were death of the patient on the day of admission or within the first 2 days in ICU, ICU stay of < 3 days, and withdrawal of patient consent. Immediately after admission (day 0) and every 24 h over a 12-day period, venous blood samples were collected.

***Isolation and culture conditions of human neutrophils***

Heparinized blood (10 ml) was collected from healthy volunteers and daily from patients after severe trauma over a 12-day period. Neutrophils were isolated by discontinuous density gradient centrifugation on Percoll (Biochrom) as previously described (24). After removal of PBMCs, RBCs were lysed using isotonic ammonium chloride solution (155 mM NH4Cl, 10 mM KHCO3, 0.1 mM EDTA) at 4°C for 10 min. The purity and viability of neutrophils were > 95% as examined by flow cytometry analysis and trypan blue exclusion, respectively. If not mentioned otherwise, neutrophils from patients at time A after trauma (days 0 2) were used in all experiments.

Freshly isolated neutrophils were resuspended in RPMI 1640 containing 2 mM glutamine (Biochrom), supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin (RPMI 1640 medium; Invitrogen), and 1% autologous serum to a final concentration of 1 × 106 cells/ml. A neutrophil suspension of 400 μl was incubated with immobilized agonistic anti-Fas IgM (clone CH-11; MBL) on open porous polyurethane (PU) foam carriers with or without PU foam alone, without Ab. PU foam carriers with or without anti-Fas loading (4 μg/ml; clone CH-11) were provided by Leukocare. Foams were placed in cytotubes, and incubation with neutrophils was performed in a rocking system at room temperature for 1 and 4 h, respectively. Subsequently, cells were immediately frozen at −80°C or cultured overnight at 37°C in a humidified atmosphere containing 5% CO2. For Western blot analysis, freshly isolated neutrophils were resuspended in RPMI 1640 supplemented with 1% or 10% autologous/pooled patient serum or 10% FCS (PAA Laboratories) to a final concentration of 2.5 × 106 cells/ml.

***Effect of patient serum on neutrophil apoptosis***

Neutrophils isolated from healthy volunteers were incubated overnight in RPMI 1640 supplemented with 1% or 10% autologous serum and patient serum (pool of three patients, time A), respectively. In some experiments anti-human GM-CSF Ab (R&D Systems) was additionally used. The 50% neutralization dose for this Ab was determined to be ~0.3–0.5 μg/ml in the presence of 0.5 ng/ml recombinant human GM-CSF. To neutralize the biological activity of GM-CSF in human serum, anti-human GM-CSF Ab was first incubated with the serum on ice for 30 min before addition to the culture medium.

***Flow cytometric analysis of Fas and Fasl expression***

Neutrophils were obtained over time (time A, days 0–2; time B, days 3–6; time C, days 7–11) from multiple trauma patients and from healthy volunteers. Isolated neutrophils were resuspended in PBS supplemented with 5% normal goat serum (Dako) to inhibit nonspecific binding of Abs. Cells (1 × 106) were incubated with mouse anti-human Fas IgM (clone CH-11; MBL) and mouse anti-human Fasl IgG (BD Biosciences) for 20 min on ice. Mouse IgG1 and mouse IgG to Aspergillus niger glucose oxidase (Dako) were used as negative controls. After a washing with PBS, cells were incubated with a FITC-conjugated goat anti-mouse IgM or IgG, respectively (both from DiaNova) for a further 20 min on ice while protected from light. Cells were washed twice with PBS and analyzed by flow cytometry on a FACSscan instrument using CellQuest software (BD Biosciences).

***Quantification of neutrophil apoptosis***

Apoptosis of neutrophils was evaluated either immediately after neutrophil isolation or after 18 h of culture. The proportion of neutrophils that display a hypodiploid DNA peak, i.e., apoptotic cells, was determined by the protocol described by Nicoletti et al. (25). Briefly, cell pellets were suspended in 300 μl of hypotonic solution (0.1% sodium citrate plus 0.1% Triton X-100) containing 50 μg/ml propidium iodide (PI; Sigma-Aldrich) and incubated for at least 3 h at 4°C. The red fluorescence of PI was measured by FACSscan cytometer (BD Biosciences). A minimum of 10,000 events was counted per sample. Results are represented as the percentage of hypodiploid DNA (sub-G1) corresponding to fragmented DNA characteristic for apoptotic cells.

***Detection of mitochondrial membrane depolarization***

The change of mitochondrial membrane potential was examined by staining neutrophils with a mitochondrial membrane potential-dependent lipophilic dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetrachlorobenzimidazolylcarbocyanine iodide (JC-1; Sigma-Aldrich). In brief, neutrophils were stained by addition of 3 μM JC-1 for 20 min at 37°C. Thereafter, cells were harvested by centrifugation (450 × g for 5 min), washed twice in PBS, and immediately analyzed by flow cytometry (FACScan). The excitation wavelength was 488 nm, and the emission wavelengths were 530 nm (FL1 channel) for green fluorescence and 590 nm (FL2 channel) for red fluorescence. In cells with intact mitochondrial membrane potential, the dye concentrates in mitochondrial matrix and forms red fluorescence JC-1 aggregates. By contrast, in cells with disrupted mitochondrial membrane potential, the dye is dispersed throughout the cytoplasm, where it fluoresces green in its monomeric form. Results are expressed as the relative number of cells with high levels of green fluorescence (FL1), indicating mitochondrial membrane depolarization in the cells.

***Determination of caspase-9 activity***

Neutrophils incubated with staurosporine were harvested by centrifugation, and cell pellets were stored at −80°C for further investigation. Caspase-9 activity was measured by using the Caspase-Glo 9 Assay (Promega) according to the instructions of the manufacturer with some modifications. Briefly, cells were resuspended in PBS, 0.1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS followed by cell sonication. The protein concentration of the cell lysates was determined by using the DC Protein Assay (Bio-Rad). Same protein concentrations were used for the determination of caspase activity.

***GM-CSF detection by ELISA***

Blood (10 ml) was collected from healthy volunteers and daily from patients after severe trauma over a 10- to 12-day period. Sera were harvested by centrifugation and stored at −80°C until further processing. A commercially available ELISA kit (Quantikine HS Human GM-CSF; R&D Systems) recognizing recombinant and natural GM-CSF was used for quantification of GM-CSF according to the manufacturer’s instructions (minimal detectable level <0.26 pg/ml).

***RT-PCR analysis***

Total RNA from neutrophils was extracted using TRI Reagent (Sigma-Aldrich) according to the manufacturer’s instructions. One microgram of...
total RNA was reverse transcribed using oligo(dT)$_{18}$ primer, using Omniscript Reverse Transcriptase (Qiagen) and following the manufacturer’s instructions. PCR was conducted using published gene-specific primer sequences for *Fas* (26), *Fasl* (27), and *Mcl-1* and *Bax* (28). Primers for GAPDH (29) were used to control the quality of the cDNA samples. cDNA (2.5 μl) was amplified using TaqPCR Core Kit (Qiagen), and products were separated on 1.8% agarose gel and visualized under UV after SYBR Gold (Invitrogen) staining.

For real-time PCR, total RNA was isolated from cells using High Pure RNA Isolation Kit and transcribed into cDNA with Transcriptor First Strand cDNA Synthesis Kit (both Roche). Real-time PCR analysis was performed on a LightCycler 480 using RealTime Ready Human Apoptosis strand cDNA Synthesis Kit (Roche).

**Western blot analysis**

Neutrophils were resuspended in PBS, 1% Nonidet P-40, 0.5 mM sodium deoxycholate, 0.1% SDS supplemented with the complete protease inhibitor mixture (Roche). Samples were sonicated and centrifuged at 8000 g for 10 min, and protein concentration was quantified using the DC Protein Assay kit (Bio-Rad).

Protein (30–50 μg/sample) was separated on SDS-PAGE and transferred to nitrocellulose membranes. Membranes were saturated in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) and 5% w/v nonfat dry milk for 60 min at room temperature and then immune-labeled with monoclonal mouse anti-human Mcl-1 (BD Biosciences) and polyclonal rabbit anti-human Bax Ab (Cell Signaling) in TBS-T and 1% w/v nonfat dry milk or 5% BSA. After the blots were washed in TBS-T, the membranes were incubated for 60 min at room temperature with the goat anti-mouse or anti-rabbit HRP-conjugated secondary Ab (both Dako) in TBS-T and washed as described above. Bands were visualized by the ECL method. Equal loading of gels was confirmed both by Ponceau S staining of membranes and by reincubation of the filters with a polyclonal Ab for β-actin (Santa Cruz Biotechnology). The amount of specific protein was quantified by densitometry (Quantity One; Bio-Rad).

**Statistics**

All data are presented as mean ± SEM. If not mentioned otherwise, statistical analyses were performed by using one-way ANOVA, followed by the Newman-Keuls test. Alternatively, Student’s t test was performed (GraphPad Prism Program, version 5; GraphPad Software). Values of p < 0.05 were considered statistically significant.

**Results**

**Changes in gene and protein expression in neutrophils after major trauma**

Activated neutrophils exhibit attenuated apoptosis, which is suggested to be the major reason for prolonged autodestructive effector functions (3, 30). To determine the apoptotic status of neutrophils after severe trauma, we quantified DNA fragmentation, as a hallmark of apoptosis, in freshly isolated neutrophils and found up to 7-fold reduced apoptotic rate in neutrophils from multiple trauma patients (time A, days 0–2) compared with control cells. Apoptosis was significantly diminished for at least 1 wk after severe trauma (Fig. 1).

We next studied whether delayed apoptosis of neutrophils is associated with changes in the expression of proapoptotic and antiapoptotic regulator molecules (Fig. 2). Indeed, screening of 372 different apoptosis-relevant gene transcripts by means of the human apoptosis panel revealed different patterns between neutrophils of patients and of healthy donors. In Fig. 2A, the gene expression for selected major molecules representing proapoptotic (Bax, Bad, Fas receptor) and antiapoptotic (Mcl-1) regulator functions are shown. This gross examination of cDNA revealed first evidence that multiple trauma did not alter the mRNA levels for proapoptotic Bax or Bad in neutrophils. However, substantially increased levels of mRNA for the Fas receptor, which promotes activation of the extrinsic apoptotic pathway (12), and for Mcl-1, an antiapoptotic protein that has been implicated in neutrophil survival (20), was detected. Because of the potential key roles of Mcl-1 and Bax expression in the regulation of apoptosis-signaling pathways in neutrophils, we chose the systematic analysis of these molecules in terms of mRNA and protein expression by semiquantitative PCR (Fig. 2B) and Western blot (Fig. 2C). Data are shown from experiments conducted with neutrophil samples from up to 13 healthy controls and 11 patients, and they confirm the results obtained by the real-time PCR analysis.

Overall, Mcl-1 gene expression was increased 2-fold in neutrophils after trauma, whereas Bax gene expression remained unchanged (Fig. 2B). Nevertheless, as two previous reports showed a depletion of Bax protein in neutrophils with delayed apoptosis (30, 31), we studied Bax protein expression in neutrophils after trauma and found a significant 2.3-fold reduction in Bax levels compared with control cells. In contrast, Mcl-1 protein expression in patients was 2.5-fold increased relative to neutrophils isolated from healthy volunteers (Fig. 2C).

These findings clearly demonstrate that neutrophils of patients after multiple trauma exhibit increased amounts of Mcl-1 and decreased amounts of Bax protein, both likely to be associated with prolonged neutrophil survival and unappreciated activity.

**Effects of serum from severely injured patients and GM-CSF on neutrophil apoptosis**

As previously noted, GM-CSF significantly reduces neutrophil apoptosis by increasing Mcl-1 protein levels (19, 20). To investigate whether GM-CSF levels are increased after trauma, we measured GM-CSF concentration in serum of healthy volunteers and severely injured patients over time by quantitative ELISA (Fig. 3). GM-CSF levels were significantly elevated and highest at time A (days 0–2) after trauma compared with concentrations determined in serum of healthy volunteers. Conversely, no significant increase in GM-CSF concentrations could be measured at time B (days 3–6) and time C (days 7–11) after trauma.

We therefore hypothesized, that elevated concentrations of GM-CSF in the serum of patients might be responsible for the attenuation of neutrophil apoptosis. When neutrophils isolated from healthy volunteers were incubated in medium supplemented with 1% or 10% pooled patient serum (time A), we found neutrophil apoptosis to be strongly down-regulated compared with apoptosis measured in cells cultured in medium containing autologous serum (Fig. 4A). The addition of neutralizing anti-GM-CSF Ab to 1% or 10% autologous serum did not significantly increase apoptosis of cultured cells (Fig. 4B). In contrast, when anti-GM-CSF Ab was added to pooled patient serum, a significant raise of apoptosis could be measured, indicating that elevated levels of GM-CSF measured in the serum of patients indeed lead to the impairment of neutrophil spontaneous apoptosis (Fig. 4C).

The antiapoptotic property of patient serum was found to correlate with marked increased Mcl-1 protein expression in neutrophils cultured in medium supplemented with 1% and 10% serum.
However, this increase in Mcl-1 protein levels was abolished in the presence of neutralizing anti-GM-CSF Abs (Fig. 4D). Taken together, GM-CSF was identified to be a key factor in maintaining neutrophil viability after multiple trauma by the up-regulation of prosurvival molecules such as Mcl-1.

The intrinsic pathway of apoptosis is impaired in trauma neutrophils

We hypothesized that reduced spontaneous apoptosis seen in neutrophils isolated from trauma patients is due to increased levels of Mcl-1 protein, which is known to prevent proapoptotic factors such as Bax from triggering mitochondrial membrane depolarization. To explore whether the intrinsic pathway of apoptosis is impaired in activated neutrophils, we used staurosporine to induce apoptosis in cells isolated from healthy volunteers and trauma patients (Fig. 5). Fig. 5A shows that incubation of cells with staurosporine induces a significant concentration-dependent increase in mitochondrial membrane depolarization in control cells, but not in cells from trauma patients or cells pre-incubated with pooled patient serum. The loss of mitochondrial membrane potential in controls was associated with increased apoptosis (Fig. 5B). Conversely, staurosporine-induced apoptosis increase in patient neutrophils and in control cells preincubated with patient serum was less prominent. To verify whether changes initiate apoptosis by caspase activation, we further measured caspase-9 activity after staurosporine challenge. In general, caspase-9 is activated through the formation of the apoptosome involving procaspase-9, Apaf-1 and cytochrome c (32). Activation occurs in the cytosol following the translocation of mitochondrial cytochrome c, a process that is facilitated by mitochondrial membrane depolarization (33). Consistent with our findings concerning mitochondrial membrane depolarization, patient neutrophils showed decreased caspase-9 activity after staurosporine treatment compared with the control (Fig. 5C).

As depicted in Fig. 5D, the level of the antiapoptotic protein Mcl-1 declined in control neutrophils undergoing apoptosis after treatment with 0.2 μM staurosporine, whereas in patient cells and in neutrophils incubated in the presence of patient serum, this decline was partially prevented. These data again indicate that GM-CSF is responsible for reduced apoptosis and constant levels of Mcl-1, presumably by delaying Mcl-1 protein turnover. To verify this assumption, we again performed GM-CSF blocking studies (Fig. 6). When control cells were preincubated with pooled patient

**FIGURE 2.** Expression of Fas and Bcl-2 family members. A, Quantitative real-time PCR evaluation of gene expression in control and patient neutrophils. Gene expression changes in patients are represented as fold increase as compared with controls (---). The expression of four genes is depicted. Data were obtained from three healthy controls and three patients. B, Mcl-1 (n ≥ 8) and Bax (n ≥ 8) gene expression in neutrophils isolated from healthy volunteers (control) and patients analyzed by semiquantitative PCR. Amplification levels were related to the housekeeping gene GAPDH. ***, p < 0.01 vs control (Student’s t test). C, Western blot analysis showing Mcl-1 (n ≥ 11) and Bax (n ≥ 7) expression in neutrophils isolated from healthy volunteers (control) and patients. Representative blots of three controls and patients are depicted. The normalized values for Mcl-1 and Bax, obtained by densitometric analysis, are reported as arbitrary units. *, p < 0.05 vs Control (Student’s t test).

**FIGURE 3.** GM-CSF concentrations in serum. Sera of multiply injured patients (n = 22) were analyzed using a commercially available GM-CSF ELISA kit at time A (days 0–2), time B (days 3–6), and time C (days 7–11) after trauma and compared with healthy volunteers (n = 8). GM-CSF concentrations at time A (days 0–2) after trauma were significantly increased compared with GM-CSF levels in controls. ***, p < 0.01 vs control.
serum supplemented with neutralizing anti-GM-CSF Ab, we observed significantly increased mitochondrial membrane depolarization (Fig. 6A) as well as apoptosis (Fig. 6B) following staurosporine treatment when compared with cells treated in the absence of anti-GM-CSF Ab. Altogether, these data confirm that apoptosis resistance in patient neutrophils after induction of the mitochondria-dependent intrinsic pathway is mediated by high levels of Mcl-1 protein, which was found to be stabilized in a GM-CSF-dependent way.

Expression of Fas and FasL on neutrophils

Neutrophil apoptosis may be accelerated by many extracellular stimuli and is often mediated by cell surface death receptors such as Fas. Neutrophil death in vitro can be partially blocked by Fas/FasL pathway antagonists, as previously reported (13, 34).

To determine whether Fas/FasL mRNA and protein are expressed by neutrophils from severely injured patients, we analyzed their expression by semiquantitative PCR and flow cytometric analyses (Fig. 7). As a control, mRNA and protein expression in neutrophils from healthy donors was examined. As depicted in Fig. 8A (top), no significant difference in apoptosis was observed between control cells incubated with PU alone (−CH-11) or with Ab-loaded PU (+CH-11) for 1 h. Stimulation of patient neutrophils with immobilized CH-11 for 1 h induced an increase in the relative amount of fragmented DNA (Fig. 8A, bottom). Apoptosis induction was significantly increased after 4 h of incubation with immobilized CH-11 (Fig. 8, A and B). Prolonged Fas stimulation therefore seems to determine the extent of apoptosis. As shown in Fig. 8C, neutrophils from both controls and patients undergo mitochondrial membrane depolarization following

FIGURE 4. Effect of patient serum on neutrophil apoptosis and Mcl-1 protein expression. A, Neutrophils isolated from healthy volunteers were incubated for 18 h in medium containing 1% or 10% autologous serum or serum pooled from three severely injured patients (time A, days 0–2), respectively. Neutrophil apoptosis was determined by PI staining. *p < 0.05; **p < 0.01; n = 13. In some experiments, serum was preincubated with 0.5 or 5 μg/ml anti-human GM-CSF Ab which was shown to neutralize the biological activity of human GM-CSF. Culture medium of neutrophils (10^6/ml) isolated from healthy volunteers was supplemented with autologous serum (B) or pooled patient serum (C) with or without Ab, and cells were incubated for 18 h before determination of DNA fragmentation. *p < 0.05; **p < 0.01; ***p < 0.001. n = 8. D, Neutrophils (2.5 × 10^6/ml) from healthy donors were incubated overnight with medium supplemented with 1% or 10% autologous serum or pooled patient serum (time A, B). In addition, cells were incubated with 1% patient serum containing 0.5 μg/ml anti-GM-CSF Ab or 10% patient serum with 5 μg/ml anti-GM-CSF Ab, respectively. Mcl-1 protein expression was verified by Western blot. Blots were analyzed by densitometry and normalized to β-actin. Relative protein expression in cells incubated in medium supplemented with 1% autologous serum was set as 1. *p < 0.05. n ≥ 4.
Fas activation. This loss of mitochondrial membrane potential has been found to correlate with an increase in caspases activity (data not shown). However, when cells were preincubated with the broad-range caspase inhibitor Boc-aspartyl(OMe)fluoromethylketone, this increase was abrogated, which indicated that the activity of caspases is essential for Fas-mediated mitochondrial membrane depolarization. In accordance with previously published work, our experiments indicate caspase-dependent intracellular Mcl-1 degradation after activation of the FasR (Fig. 8D) (37).

Overall, our results demonstrate that apoptosis resistance in neutrophils from multiple trauma patients may be overcome by stimulation of the FasR and the downstream extrinsic signaling pathway.

FIGURE 5. The intrinsic apoptosis pathway is impaired in patient neutrophils. A, Freshly isolated neutrophils from healthy volunteers (control) and from patients were incubated in the presence of 0.1 or 0.2 μM staurosporine for 4 h. In parallel experiments, control cells were preincubated with 1% pooled patient serum (time A) (control + serum) for 1 h before staurosporine treatment. After 4 h of incubation, mitochondrial membrane depolarization was quantified by JC-1 staining (left). Dot-plots of one representative experiment for control, patient, and control + serum are depicted. (right). Dot-plots show the increase in neutrophil numbers with high levels of green (FL1) fluorescence. ***, p < 0.001 vs medium. n = 5. B, Freshly isolated neutrophils were incubated in the presence of staurosporine for 18 h before being assessed for apoptosis. ***, p < 0.001 vs medium. n = 6. C, Caspase-9 activity in control and patient neutrophils after staurosporine treatment. *, p < 0.05 vs control; **, p < 0.01 vs control; n = 3. D, Mcl-1 protein expression analyzed by Western blot after incubation of cells with staurosporine for 4 h. β-Actin was used as a loading control to normalize densitometry values. Relative protein expression in cells incubated in medium was set as 1. Western blot of one representative experiment (control and control + serum) is shown. *, p < 0.05 vs control; #, p < 0.05 vs medium. n = 5. FL2, red fluorescence.

FIGURE 6. The impairment of the intrinsic apoptosis pathway depends on GM-CSF. A, Freshly isolated neutrophils from healthy volunteers (control) were preincubated with 1% pooled patient serum supplemented with 0, 0.5, or 1 μg/ml neutralizing anti-GM-CSF Ab for 1 h before treatment with 0.2 μM staurosporine. After 4 h of incubation mitochondrial membrane depolarization was quantified by JC-1 staining. *, p < 0.05 vs control sample (0 μg/ml). n = 9. B, Neutrophils from healthy volunteers (control) were cultured in the presence of pooled patient serum, supplemented with neutralizing anti-GM-CSF Ab and 0.2 μM staurosporine for 18 h before quantification of apoptosis. *, p < 0.05; **, p < 0.01 vs 0 μg/ml. n = 9. FL1, green fluorescence.

Overall, our results demonstrate that apoptosis resistance in neutrophils from multiple trauma patients may be overcome by stimulation of the FasR and the downstream extrinsic signaling pathway.

FIGURE 7. Analysis of Fas and FasL expression. Relative Fas (A; n ≥ 7) and FasL (B; n ≥ 7) gene expression in neutrophils isolated from healthy donors (control) and patients at time A (days 0–2), time B (days 3–6), and time C (days 7–11) and analyzed by semiquantitative PCR. ***, p < 0.001; *, p < 0.05 vs control. C, Indirect immunofluorescence staining and flow cytometry were performed to detect cell surface Fas and FasL expression on freshly isolated neutrophils from healthy volunteers and patients (time A, days 0–2; time B, days 3–6; time C, days 7–11). Representative expression of Fas and FasL on neutrophils from one healthy person (control) and patient in the form of a fluorescence histogram (FL1-H) overlay depicting specific staining (solid) vs IgM and IgG isotype control (open) is shown.
Discussion

Extended neutrophil survival has been implicated in a variety of inflammatory diseases, and has been described in septic patients with burns, traumatic injuries, and pneumonia (6, 38–40). Deregulation of neutrophil apoptosis can result in the progression of posttraumatic SIRS and MODS, and prolonged neutrophil viability may continue to exacerbate inflammation because of the release of proteases, reactive oxygen species, and proinflammatory mediators (41, 42). Therefore, for the development of therapeutic strategies to limit unwanted outcomes of neutrophil activation a better understanding of neutrophil apoptosis resistance in critically ill patients is required. In this study, we demonstrate for the first time that apoptosis resistance in neutrophils isolated from severely injured patients is due to impaired activation of the intrinsic apoptosis pathway and may be overcome by Fas cross-linking with immunoglobulin anti-Fas Abs.

Because changes in apoptosis during inflammatory conditions may correlate with different expression of apoptotic proteins (15, 19, 20), the expression of a broad range of genes involved in neutrophil apoptosis was examined. Although numerous genes were found to be deregulated in severely injured patients compared with controls, the antiapoptotic Mcl-1 was one of the most prominent persistently up-regulated genes. It is well recognized that Mcl-1 gene expression may be triggered by agents that delay neutrophil apoptosis.
apoptosis, e.g., cytokines and many signaling pathways including MEK/ERK, p38 MAPK, PI3K/Akt, and JAK/STAT3 (18–20, 43, 44). Mcl-1 is a short-lived protein with a half-life of <3 h because of its PEST (proline, glutamate, serine, and threonine) motifs, which are targets for ubiquitination and which mark the protein for degradation by the proteasome (20). Because Mcl-1 has a rapid turnover, it is an ideal protein for apoptosis regulation. Indeed, cellular levels of Mcl-1 in human neutrophils closely correlate with their survival kinetics (45). Mcl-1function may also be regulated by posttranslational events, such as phosphorylation, that leads to Mcl-1 inactivation (46).

However, contrary to the findings by Ertel et al. (6), we now provide data demonstrating that increased serum concentrations of GM-CSF measured in patients early after trauma (days 0–2) strongly correlate with increased intracellular Mcl-1 levels and a reduced apoptosis rate of neutrophils. Mcl-1 protein levels declined over time after trauma, thus showing a strong correlation with the GM-CSF concentrations measured in patient serum. Because neutrophil spontaneous apoptosis was significantly reduced until at least day 11 after trauma, we concluded that Mcl-1 might not be the only antiapoptotic factor involved in the regulation of neutrophil apoptosis but nevertheless inhibits apoptosis efficiently at higher GM-CSF serum concentrations as determined at days 0–2 after trauma (unpublished results). Although our blocking experiments clearly confirmed the Mcl-1-dependent antiapoptotic role of GM-CSF, these results also indicate that other yet undefined serum factors may also partly contribute to neutrophil apoptosis resistance. For instance, besides GM-CSF, factors such as TNF-α, IL-1β, IL-6, IL-8, IFN-γ, and G-CSF have been shown to prolong neutrophil lifespan (47). In addition, synergistic effects of GM-CSF and G-CSF or IL-8, respectively, have been already described (48, 49).

A critical finding of our study is the identification of limited mitochondria disruption and caspase-9 activation following activation of the intrinsic apoptosis pathway by staurosporine in patient neutrophils. Mitochondria lose their membrane potential due to the opening of permeability transition pores. Membrane depolarization may be prevented by antiapoptotic Bcl-2 members such as Mcl-1. Conversely, a reduced intracellular Mcl-1 level during neutrophil death leads to Bax release from the Mcl-1–Bax complex and a subsequent translocation to the mitochondrial membrane (45).

In this study, we clearly demonstrate that patient neutrophils which overexpress Mcl-1 protein are resistant to membrane depolarization in response to staurosporine. Although the amount of Mcl-1 protein declined in staurosporine-treated cells from healthy donors, we found stable Mcl-1 protein levels in patient neutrophils and in control neutrophils which were preincubated with pooled patient serum. These experiments indicate that the increase in Mcl-1 protein level and stability detected in patients 48 hours after major trauma is mediated by serum factors.

We therefore assume that a significant increase in the Mcl-1 level in response to GM-CSF and possibly other proinflammatory agents prevents cytochrome c exit and caspase-9 activation and therefore the initiation of apoptosis by maintaining the mitochondrial transmembrane potential. Our hypothesis is supported by findings by Derouet et al. (43), who showed enhanced neutrophil survival and Mcl-1 stability after incubation of cells with recombinant GM-CSF. Moreover, our experiments reveal that GM-CSF in the serum of patients as well as increased amounts of the Mcl-1 protein are responsible for the maintenance of the mitochondrial membrane potential in staurosporine-treated neutrophils. Therefore, this study provides an important insight into the mechanisms yielding neutrophil apoptosis resistance and thus contributing to the development of SIRS. Nonetheless, additional studies are needed to address the question of whether the ability of GM-CSF to inhibit neutrophil apoptosis is exclusively mediated by Mcl-1.

Presently, it was unknown whether this intrinsic apoptosis resistance in neutrophils may be overcome by ex vivo stimulation of death receptors and thus by activation of extrinsic apoptosis pathways. In general, neutrophils are highly susceptible to rapid apoptosis after Fas stimulation, and blocking of the Fas/FasL pathway partially inhibits neutrophil death in vitro (13, 34, 50).

In this study, we demonstrate for the first time that immobilized agonistic anti-Fas Abs induce ex vivo apoptosis in neutrophils from severely injured patients. In general, similar results were also obtained in control experiments when the same Abs were used in soluble form (data not shown). The proapoptotic effect of immobilized agonistic anti-Fas IgM was more pronounced after 4 h of incubation in neutrophils from both controls and patients, when compared with the apoptotic values after 1 h of incubation. This suggests that the effect of immobilized agonistic anti-Fas IgM overcoming the apoptosis resistance is time dependent. Recently, caspase-dependent Mcl-1 degradation during sodium salicylate- and TNF-α-induced neutrophil apoptosis have been described (51, 52). Here, we found that Fas-induced mitochondrial disruption is accompanied by increased Mcl-1 turnover which depends on caspase activity.

In summary, our data demonstrate for the first time that the intrinsic apoptosis pathway in neutrophils from severely injured patients is blocked at least in part by elevated Mcl-1 levels induced by GM-CSF. However, the cells remain sensitive toward activation of the extrinsic pathway. Taken together, our findings presented herein are important for the design of better therapeutic approaches on the basis of biofunctional medical devices to prevent and control neutrophil activity during aberrant inflammatory conditions such as SIRS.

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