Role of Granulocyte-Macrophage Colony-Stimulating Factor and Its Receptor in the Genesis of Acute Respiratory Distress Syndrome Through an Effect on Neutrophil Apoptosis

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Hypothesis: That granulocyte-macrophage colony-stimulating factor (GM-CSF) and its receptor modulate the suppression of apoptosis (Ao) of normal neutrophils incubated in the plasma of patients with posttraumatic acute respiratory distress syndrome (ARDS).

Design: Experimental study using cultured human neutrophils.

Setting: University hospital, level I trauma center.

Participants: Plasma was obtained from 14 patients with early, fulminant posttraumatic ARDS (mean Injury Severity Score, 22). All samples were drawn within 24 hours after injury. Plasma was also taken from up to 21 healthy control subjects. These volunteers were also used as sources of polymorphonuclear leukocytes (PMNs).

Main Outcome Measures: (1) Effect of early, fulminant ARDS and normal plasma on spontaneous Ao and GM-CSF receptor expression in PMNs in vitro. (2) Effect of ligation of either GM-CSF or its receptor with a neutralizing monoclonal antibody (mAb) on PMN Ao in ARDS and normal plasma. (3) Correlation of extracellular GM-CSF concentration with rate of PMN Ao. (4) Levels of GM-CSF in ARDS and normal plasma and in culture supernatant of normal PMNs incubated in early, fulminant ARDS and normal plasma.

Results: Plasma from patients with ARDS enhanced PMN viability at 24 hours (data are given as mean ± SEM) 52% ± 3% control vs 60% ± 3% ARDS, P<.05). Binding of the GM-CSF receptor with a neutralizing mAb significantly reduced PMN viability in ARDS plasma, but not in normal plasma (60% ± 3% ARDS vs 53% ± 3% ARDS + mAb, P<.05). Ligation of GM-CSF with mAb had no significant effect on PMN viability in either plasma. Only 1% of PMNs expressed detectable levels of the GM-CSF receptor when incubated for 24 hours in either ARDS or normal plasma. The GM-CSF levels were undetectable (>7 pg/mL) in both ARDS and normal plasma and in culture supernatants taken after 24 hours of incubation in both plasma types. Levels of GM-CSF ranging from 0 to 50 000 pg/mL had no effect on PMN Ao in plasma-free medium.

Conclusions: The antiapoptotic effect of ARDS plasma appears to be mediated by the GM-CSF receptor. This effect occurs at both low levels of plasma GM-CSF and surface expression of its PMN receptor. Ligation of GM-CSF had no effect of PMN Ao, suggesting that Ao is triggered by Fc portion-mediated receptor cross-linking. These results provide the theoretical basis for a GM-CSF receptor mAb therapy as a novel modality of treatment for ARDS.

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Acute respiratory distress syndrome (ARDS) appears to be an inflammatory process mediated by polymorphonuclear leukocytes (PMNs).1 Large numbers of intra-alveolar PMNs accumulate in the early phase of ARDS, such that PMNs constitute 80% or more of the total cells obtained by bronchoalveolar lavage (BAL), compared with approximately 3% in normal subjects. Stimulation of PMNs to secrete elastase, myeloperoxidase, and toxic oxygen metabolites leads to the observed lung injury.1 Elimination of PMNs through the induction of programmed cell death or apoptosis (Ao) is thought to help modulate the inflammatory response in the lung after major trauma. Inhibition of Ao may have beneficial effects by perpetuating the phagocytic function of PMNs in inflammatory foci. It can, however, also increase the risk of ARDS by causing the abnormal persistence of inflammation and tissue destruction within the lung.

Many cytokines, including interleukin (IL) 8, granulocyte-macrophage colony-stimulating factor (GM-CSF), and growth-related oncogene-α, have been implicated in the modulation of PMN activity and survival in the lung in ARDS.2-6 We previously hypothesized that soluble factors in...
MATERIALS AND METHODS

All research was conducted and all patient samples were collected in accordance with the requirements of the Institutional Review Board of University of Medicine and Dentistry of New Jersey, Newark, NJ.

NEUTROPHIL SEPARATION

The PMNs were prepared from minimally heparinized whole blood of healthy volunteer donors. Specimens were spun at 1500 rpm for 15 minutes. Plasma was removed and kept at 4°C until cells were prepared for incubation. The PMNs were isolated by sequential separations using the Ficoll-Hypaque technique and dextran sedimentation. Osmotic lysis with 0.14-mol/L ammonium chloride was used to remove contaminating red blood cells. The cells were washed twice and resuspended in RPMI 1640 at 106 cells/mL. The PMN purity was assessed by flow cytometric staining for the PMN surface marker CD15: purity was typically found to be greater than 95%. Only samples that showed viability greater than 85% (as determined by trypan blue dye exclusion) were used for the experiments.

PLASMA PREPARATION
(E/F ARDS AND VOLUNTEER)

Blood was obtained from 14 patients with clinical and radiological evidence of early posttraumatic ARDS (as defined by the following: PaO2/FiO2 [fraction of inspired O2] <150 mm Hg, diffuse parenchymal infiltrates on chest x-ray film, pulmonary capillary wedge pressure <18 mm Hg, and no evidence of sepsis) and from healthy volunteers.

RESULTS

Plasma from patients with ARDS enhanced PMN viability at 24 hours when compared with control plasma (all data are given as mean ± SEM unless otherwise indicated) (60% ± 3% ARDS vs 52% ± 3% control, P<.05; analysis of variance followed by Duncan multiple range test) (Table 1). The Ao was suppressed by ARDS plasma (37% ± 2% ARDS vs 47% ± 3% control, P<.05; analysis of variance followed by Duncan multiple range test) (Table 1). In ARDS plasma, binding of the GM-CSF receptor with a neutralizing mAb both significantly reduced PMN viability (60% ± 3% ARDS vs 53% ± 3% ARDS + mAb, P<.05; analysis of variance + Duncan multiple range test) and enhanced Ao (37% ± 3% ARDS vs 47% ± 2% ARDS + mAb, P<.05; analysis of variance + Duncan multiple range test). It had no effect on viability or Ao in normal plasma (Table 1). Neutralization of GM-CSF had no effect on either viability or Ao in both ARDS and normal plasma (Table 2).

COMMENT

Prevention or termination of PMN-driven inflammatory processes such as ARDS may be effected through PMN Ao. Induction of PMN Ao with the subsequent rapid

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Blockade of GM-CSF Receptor Activity

Two micrograms of anti–GM-CSF receptor blocking mAb (Pharmingen Inc, San Diego, Calif) were added to 100,000 PMNs in 80 µL RPMI 1640 sixty minutes prior to the addition of 20 µL of ARDS or control plasma. The mAb (clone 17-2) is a mouse IgG2a, specific for the α chain of the human receptor. This concentration of mAb (20 µg/mL) provides more than 90% neutralization of the receptor, based on inhibition of the GM-CSF dependent proliferation of the erythroleukemic cell line TF-1. Cells were incubated at 37°C for 24 hours and then stained and analyzed as previously described.

Blockade of GM-CSF Activity

In those experiments in which GM-CSF itself was ligated, 0.15 µg of mAb was added to 20 µL of ARDS and control plasma prior to its addition to 80 µL of PMN suspension (10⁶ cells/mL). The BVD2-23B6 clone is a rat IgG2a, that neutralizes human GM-CSF bioactivity: 40 ng/mL has been found to neutralize the proliferative effect of 100 pg/mL of GM-CSF. The 1500 ng/mL concentration of mAb used in this experiment was therefore more than enough to neutralize the GM-CSF in both ARDS and volunteer plasma and culture supernatant.

GM-CSF Dose Response Curve

One hundred thousand donor PMNs were suspended in 95 µL of RPMI 1640, to which 5 µL of sterile phosphate buffered saline (Gibco BRL, Grand Island, NY) containing varying doses of recombinant human GM-CSF (R&D Systems, Minneapolis, Minn) was added. Final GM-CSF concentrations ranging from 0 to 50,000 pg/mL were chosen because they more than encompass the range of levels detected in clinical samples of plasma and BAL fluid (BALF) taken from patients with ARDS. Cells were incubated at 37°C for 24 hours and then stained and analyzed as previously described.

Surface Expression of GM-CSF Receptor

Granulocyte-macrophage colony-stimulating factor receptor expression was determined by adding 0.5 µg of FITC-labeled anti–GM-CSF receptor mAb to 100,000 donor PMNs, which were then incubated in the dark at room temperature for 20 minutes. Cells were washed twice and fixed with 1% methanol-free formaldehyde (Polysciences, Warrington, Pa). They were stored in the dark at 4°C until analyzed via flow cytometry within 36 hours.

Plasma and Supernatant GM-CSF Levels

The GM-CSF levels in ARDS and volunteer plasma samples and in supernatants of PMN suspensions incubated in ARDS and volunteer plasma over 24 hours were determined by means of a sandwich enzyme-linked immunosorbent assay kit (R&D Systems) as described by the vendor. The range of detectable levels was 7 to 500 pg/mL.

DATA ANALYSIS

Mean values and SEs of the mean were calculated for cell viability, Ao, GM-CSF receptor surface expression, plasma, and supernatant GM-CSF concentration. The Mann-Whitney test was used to analyze the differences in viability and Ao.

### Table 1. Comparison of Normal PMN Viability and Apoptosis After 24 Hours of Incubation in ARDS and Normal Plasma With and Without Blocking Anti–[α] GM-CSF R Monoclonal Antibody

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<th>Group (n)</th>
<th>Viability</th>
<th>Apoptosis</th>
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<tbody>
<tr>
<td>Control without × GM-CSF R (21)</td>
<td>52 (3.4)†</td>
<td>47 (3.1)†</td>
</tr>
<tr>
<td>Control with × GM-CSF R (17)</td>
<td>54 (3.9)†</td>
<td>46 (3.9)†</td>
</tr>
<tr>
<td>ARDS without × GM-CSF R (38)</td>
<td>60 (2.5)†</td>
<td>37 (2.2)†</td>
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<tr>
<td>ARDS with × GM-CSF R (28)</td>
<td>53 (3.2)†</td>
<td>47 (3.2)†</td>
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*Data expressed as mean percentage (± SEM). PMN indicates polymorphonuclear leukocyte; ARDS, acute respiratory distress syndrome; GM-CSF, granulocyte-macrophage colony-stimulating factor; and R, receptor. †P = .38 (viability) and P = .20 (apoptosis). ‡P = .002 (viability) and P = .02 (apoptosis).

The mechanisms by which PMN Ao is suppressed after injury are still unknown. It is probably in part inhibited by soluble factors. Several studies have attempted to correlate circulating and BALF cytokine levels with risk of ARDS, systemic inflammatory response syndrome, or multiple organ failure. Bronchoalveolar lavage fluid levels of G-CSF, GM-CSF, IL-1β, and IL-8, are all significantly elevated in patients with ARDS after trauma, intra-abdominal sepsis, or pneumonia. Survival after ARDS is typically associated with a rapid reduction in BALF cytokine levels, whereas persistent elevations portend a poor outcome.

Plasma from patients with systemic inflammatory response syndrome and major burns suppresses Ao of normal cultured PMNs. Neutralization of GM-CSF in burn plasma suppresses this ant apoptotic effect. Similarly, BALF from patients with ARDS inhibits normal PMN Ao when compared with volunteer lavage fluid. This effect can be blocked by immunodepleting BALF of G-CSF and GM-CSF, the alveolar levels of which are greatly increased in ARDS. High levels of G-CSF and GM-CSF have also been isolated from the supernatants of colonic mucosal specimens taken from patients with inflammatory bowel disease. These supernatants inhibited removal of the PMNs by alveolar macrophages provides the lung with a mechanism by which it can minimize PMN-mediated damage to the surrounding tissue. Normal PMNs undergo Ao after 24 hours in the peripheral circulation, but Ao is delayed in PMNs migrating into inflammatory foci. This inhibition is mediated through cytokines such as granulocyte colony-stimulating factor (G-CSF), GM-CSF, IL-1β, and IL-8. Prolongation of PMN survival through the suppression of Ao has been identified in circulating PMNs isolated from patients with severe burns. This dysregulation of Ao may enhance the tendency of these patients to develop ARDS.
normal PMN Ao in vitro, an effect that could be abrogated with preincubation of the supernatants with an anti-G-CSF mAb (but not with anti–GM-CSF mAb). Thus, high levels of proinflammatory cytokines such as G-CSF and GM-CSF within inflammatory foci in the lung and bowel may potentiate PMN-mediated tissue injury in ARDS and inflammatory bowel disease, respectively, by suppressing normal PMN Ao.

The GM-CSF is a proinflammatory cytokine produced by several cell types such as monocytes,14 activated eosinophils,15 T lymphocytes,16 and even pulmonary fibroblasts.17 It supervises the maturation process of granulocyte and monocyte progenitors.18 Several of the biological activities of mature PMNs are also modulated by GM-CSF, which acts mainly by priming PMNs and making them more receptive to activation by secondary stimuli.

While the effects of GM-CSF on PMN function are well known, the underlying mechanisms by which GM-CSF works are poorly understood. The GM-CSF receptor is a high-affinity receptor composed of 2 subunits, α and β.19 The β subunit, which is unique to the GM-CSF receptor, is thought to serve mainly as a binding site for GM-CSF.20 It may also contribute to signaling. The α subunit, however, has a larger intracytoplasmic tail and is believed to be critically involved in transduction.21 The GM-CSF signal transduction seems, in part, to involve cytoplasmic tyrosine kinases (eg, Lyn).22 The physical coupling of the Lyn kinase to the GM-CSF receptor seems to be central to the inhibition of PMN Ao.23 It is unknown how kinase activation signals the inhibition of PMN Ao. It seems, however, that alterations in the expression of members of the Bcl-2 family of proteins (eg, Mcl-1, Bcl-2, A-1, Bcl-x[L], Bad, Bak, Bcl-x[S] and Bax) lead to inhibition of Ao through the maintenance of normal mitochondrial function and the suppression of cytochrome c release into the cytosol. Cytochrome c, if released into the cytosol, binds the protein Apaf-1: this complex activates the first member

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<tr>
<th>Group (n)</th>
<th>Viability</th>
<th>Apoptosis</th>
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<tr>
<td>Control without × GM-CSF (6)</td>
<td>47 (5.0)†</td>
<td>49 (7.0)†</td>
</tr>
<tr>
<td>Control with × GM-CSF (6)</td>
<td>54 (4.6)†</td>
<td>47 (5.3)†</td>
</tr>
<tr>
<td>ARDS without × GM-CSF (13)</td>
<td>58 (3.8)‡</td>
<td>38 (3.3)‡</td>
</tr>
<tr>
<td>ARDS with × GM-CSF (13)</td>
<td>56 (4.7)‡</td>
<td>40 (5.4)‡</td>
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* Data expressed as mean percentage (± SEM). PMN indicates polymorphonuclear leukocyte; ARDS, acute respiratory distress syndrome; and GM-CSF, granulocyte-macrophage colony-stimulating factor.
†P = .28 (viability) and P = .65 (apoptosis).
‡P = .06 (viability) and P = .19 (apoptosis).
Activated PMNs are widely accepted as key mediators of posttraumatic ARDS. Proinflammatory cytokines such as GM-CSF prime PMNs, thereby making them more receptive to activation by secondary stimuli. Cytokines also help to regulate PMN function by modulating the apoptotic elimination of PMNs within inflammatory foci. Changes in cytokine production within the peripheral circulation and lung after major trauma can lead to a suppression of PMN Ao, the abnormal persistence of activated PMNs, and the exaggerated production of reactive oxygen intermediates and proteases within inflammatory foci. Under these conditions, PMN activity is transformed from a controlled and beneficial antimicrobial function to an excessive and autodestructive phenomenon, which can produce the lung damage associated with ARDS.

If alterations in the normal equilibrium of cytokine activity in the plasma and lung are responsible for the abnormal persistence of activated PMNs in tissues such as the lung after major trauma, therapies aimed at restoring this equilibrium may help to reduce a patient’s risk of posttraumatic ARDS. Because previous studies have shown that GM-CSF is present at high concentrations in the lungs of patients with ARDS, our study aimed to demonstrate that GM-CSF is an important modulator of PMN apoptosis in vitro. Furthermore, we wished to see whether neutralization of GM-CSF activity in ARDS plasma could restore normal patterns of PMN Ao. Our results provide the theoretical basis for anti-GM-CSF receptor monoclonal antibody manipulation of PMN Ao as a novel modality of treatment for posttraumatic ARDS.

(caspase 9) of a cascade of cytosolic proteases (caspases), all of which act through the cleavage of vital cellular proteins both within the cytoplasm and nucleus.24 Induction of Mcl-1, Bcl-2, A-1, and Bcl-x(L) suppresses Ao, whereas Bad, Bak, Bcl-x(S) and Bax all promote cell death.25 The GM-CSF receptor may act through tyrosine kinase activation to alter the equilibrium between antiapoptotic and proapoptotic members of the Bcl-2 family of proteins: this would then stabilize mitochondrial function, suppress cytosolic cytochrome c release, and prevent caspase activation.

The caspases are all homologous to ced-3, a cell death gene described in the nematode Caenorhabditis elegans. The first mammalian homologue of ced-3 to be identified was IL-1β converting enzyme.25 The caspases have been grouped according to sequence homology as being either IL-1β converting enzyme–like proteases (caspases 1, 4, and 5) or ced-3–ones like (caspases 3, 6, 7, 9, and 10). They are all synthesized as inactive proenzymes that are activated by proteolytic cleavage. Caspase-3 has been implicated as a key enzyme that is activated during the early stages of Ao.24 Active caspase-3, found only in cells undergoing Ao, consists of a heterodimer of 17- and 12-kd subunits, which are derived from the 32-kd proenzyme. The active enzyme cleaves and activates other caspases, as well as relevant targets in the cytoplasm (eg, actin) and nucleus (eg, poly[adenosine diphosphate-ribose] polymerase).26 Thus, the caspase cascade triggers the DNA, cytoskeletal and membrane changes characteristic of Ao. No studies have yet dem-
CSF receptor with mAb triggers PMN Ao in ARDS plasma. Ligation of GM-CSF has no effect on PMN Ao in ARDS plasma, suggesting that PMN Ao is augmented by receptor cross-linking by mAb rather than simple blockade of ligand-receptor interaction. The GM-CSF, even at very high doses, has no effect on PMN Ao in plasma-free medium, confirming that regulation of PMN Ao may occur through mechanisms other than modulation of GM-CSF binding to its receptor. These results provide the theoretical basis for anti–GM-CSF receptor mAb manipulation of PMN Ao as a novel modality of treatment for posttraumatic ARDS.


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REFERENCES


DISCUSSION

Ori D. Rotstein, MD, Toronto, Ontario: The authors test the now popular hypothesis that persistent systemic inflammation in the critically ill patient might be related to delayed apoptosis of the critical injurious cell, the neutrophil. They show that drawing plasma from patients 24 hours after major trauma delays apoptosis of normal donor neutrophils. They suggest a role of granulocyte-macrophage colony-stimulating factor (GM-CSF) in this effect based on the ability of anti–GM-CSF receptor antibody to reverse the inhibition. Curiously, neutralization of plasma GM-CSF did not have a similar effect, levels of GM-CSF in patient plasma were undetectable, GM-CSF receptors were virtually undetectable, and GM-CSF itself had no effect at any concentration studied. They conclude a role for GM-CSF and its receptor in modulating apoptosis and suggest GM-CSF receptor antagonism as a potential novel therapy. I have 3 questions.

First, the authors’ main finding relates to the reversal of the antiapoptotic effect with the monoclonal antibody directed against the receptor. Did the investigators control for this nonspecific effect by using an irrelevant antibody?

Second, endotoxin also inhibits apoptosis. What evidence do the authors have that the effect is not due to endotoxin in the trauma patient serum? Have they heated the serum? Have they used specific antibodies to deplete the endotoxin?

And finally, the authors suggest that cross-linking the GM-CSF receptor may exert direct effects, but could also be a modulator of the antiapoptotic effect of plasma than implicating a role for GM-CSF as the inhibitor. Did engagement of the receptor with GM-CSF alter the effect of patient plasma on spontaneous apoptosis? Did it have an effect on Fas-induced apoptosis?

Dr Goodman: I would join Dr Rotstein in being very puzzled by the fact that whereas we were able to inhibit the apoptosis binding the receptor, we couldn’t do the same thing by binding the GM-CSF itself. We didn’t look at the irrelevant antibody, nor did we look at endotoxin. What are we hoping to do is to answer the question whether this is a crosslinking phenomenon by looking at F(ab′)2 fragments of the anti-receptor antibody, which would not be able to crosslink receptors, and see whether we can get a different effect or whether it is the same effect. Although it is a very good idea, we haven’t looked at Fas-induced apoptosis, but that obviously is a good question to ask.

Cynthia Ogle, PhD, Cincinnati, Ohio: In the transplant world, OKT3, when you treat lymphocytes with that, you get a TNF release. When you treat your neutrophil with an anti-GM-CSF receptor, do you get any other cytokine release or any stimulation of your neutrophil?

Dr Goodman: We haven’t looked at cytokine production, but, again, that would be something to look into.