Corticoids Normalize Leukocyte Production of Macrophage Migration Inhibitory Factor in Septic Shock

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Background. A regulatory loop between macrophage migration inhibitory factor (MIF) and glucocorticoids has been characterized in animal models. Renewed interest in glucocorticoid treatment for septic shock offers an opportunity to analyze this regulatory loop in humans.

Methods. We investigated the ex vivo release of MIF by peripheral blood mononuclear cells (PBMCs) sampled from glucocorticoid-treated and -untreated patients with septic shock. Blood was obtained, before glucocorticoid treatment, and within the first day of treatment, from patients with septic shock who required treatment with moderate doses of hydrocortisone and fludrocortisone.

Results. PBMCs from patients contained significantly higher amounts of MIF than cells from healthy control subjects. In culture, spontaneous release of MIF and release induced by lipopolysaccharide (LPS), heat-killed *Escherichia coli*, and red blood cell lysates were significantly higher in patients than in control subjects. PBMCs from patients treated with glucocorticoids showed a lower release of MIF in response to LPS, heat-killed *Escherichia coli*, and peptidoglycan than did PBMCs from untreated patients and showed levels similar to PBMCs from healthy control subjects.

Conclusion. To our knowledge, MIF is the first proinflammatory cytokine in which ex vivo release by circulating cells is enhanced during sepsis. Glucocorticoid treatment normalized the release of MIF by circulating PBMCs from patients with septic shock.

Septic shock accounts for ~9% of intensive care unit (ICU) admissions, and its mortality rate remains ~50% [1], although successful therapies have been reported recently [2]. Among these promising recent therapeutic approaches, moderate doses of corticosteroids have been investigated, although the exact underlying mechanisms remain unclear [3, 4].

Macrophage migration inhibitory factor (MIF) was one of the first reported cytokines. It was discovered in 1966 as a T cell–derived mediator involved in delayed-type hypersensitivity [5, 6]. Then it was shown to also be produced by a variety of cells, including B lymphocytes [7], macrophages [8], endothelial cells [9], epithelial cells [10], fibroblasts [11], dendritic cells [12], and pituitary cells [13]. MIF is also expressed in thymus, spleen, testis, muscle, skin, and adrenal glands [14, 15]. First identified as a mediator that inhibits the random migration of monocytes and macrophages, its activity was shown to synergize with lipid A [16] and extended to the inhibition of chemokine-induced migration [17]. However, most of its recently ascribed properties have been associated with its capacity to enhance the inflammatory response. MIF favors the expression of inflammatory cytokines [18, 19] and counteracts the effects of glucocorticoids [18, 20]. Accordingly, MIF plays a major role in various inflammatory disorders, particularly sepsis. MIF potentiates lipopolysaccharide (LPS)–induced lethality—indeed, blocking MIF is protective against the lethal effects of LPS [13], LPS-induced lung injury [21], bacille Calmette-Guérin–LPS–induced liver failure [22], and experimental peritonitis [23]. In addition, MIF up-regulates the expression of Toll-like receptor–4, the surface molecule involved in LPS-induced signaling [24].
Targeted disruption of the MIF gene in mice has confirmed its role in LPS-induced lethal shock [19]. MIF induces signal transduction via the CD74 molecule, the cell-surface form of the major histocompatibility class II–associated invariant chain [25]. In clinical settings, high levels of circulating MIF have been found in patients with severe sepsis and septic shock [23], but MIF is present at homeostasis in human plasma and exists as a preformed molecule within cells. Its release can be induced by numerous pathogen-associated molecular patterns and by inflammatory cytokines [8], as well as by low concentrations of glucocorticoids [14, 18, 26]. Given the theoretical cross-talk between MIF and glucocorticoids, we investigated, in patients with septic shock, the ex vivo production of MIF by peripheral blood mononuclear cells (PBMCs) in relation to glucocorticoid treatment.

**SUBJECTS, MATERIALS, AND METHODS**

The protocol was approved by the Comité de Protection des Personnes se Prettant à la Recherche Biomédicale de Saint Germain en Laye ethics committee, and written informed consent was obtained from patients or their proxies.

**Participants**

Consecutive patients admitted to the ICU at Raymond Poincaré hospital (Garches, France) were included if they met common criteria for septic shock [27]. Patients <18 years old, pregnant women, and patients who had received any dose of glucocorticoids within 1 month before study entry or who had known underlying conditions that may have altered the hypothalamic-pituitary-adrenal axis [28] were excluded. Control subjects included volunteers (15 men; mean ± SD age, 39 ± 11 years) from the health staff who were deemed to be healthy on the basis of a thorough medical examination and standard laboratory test results.

**Investigated Parameters**

**General patient characteristics.** Demographic data, a Simplified Acute Physiology Score II (SAPSII) [29], vital signs, organ dysfunction [30], the source of infection, and the type of pathogen were systematically recorded at baseline. Interventions were left to the physicians’ discretion and included the administration of antibiotics, fluids, vasopressors, mechanical ventilation, and glucocorticoid treatment (50 mg of hydrocortisone every 6 h, combined with 50 μg of oral fludrocortisone once per day, for 7 days). Blood samples were drawn, during working hours of the laboratory, either within the first 12 h of glucocorticoid treatment or immediately before the administration of glucocorticoids in patients who met inclusion criteria. Ex vivo investigations required fresh blood, not frozen samples. At the time of sampling, all patients were receiving catecholamines.

**Cell preparation and cell cultures.** A sample of 20 mL of human peripheral blood was obtained on citrate. A mean of 2 h elapsed between blood sampling in the ICU and its handling in the laboratory. In the meantime, blood samples were kept at room temperature. A similar timing was achieved with blood from healthy control subjects. Preliminary experiments showed that the length and conditions of storage did not significantly modify the production or release of MIF.

Blood diluted 1:2 in RPMI 1640 ultraglutamine medium (BioWhittaker) was layered on Ficoll (MSL; Eurobio) and centrifuged, as described elsewhere [31]. PBMCs were recovered, washed, and resuspended at 2 × 10⁶ cells/mL in RPMI 1640 medium supplemented with 100 IU/mL penicillin, 100 μg streptomycin, and 5% decomplemented normal human serum (BioWhittaker). PBMCs were activated for 20 h at 37°C in a 5% CO₂ incubator in 5-mL polypolyne tubes (Becton-Dickinson) with the following agents: 100 ng/mL or 1 μg/mL Escherichia coli 0111:B4 LPS (Sigma), 100 μg/mL heat-killed staphylococci (SAC; Pansorbin cells; Calbiochem), 2 × 10⁶ cells/mL heat-killed E. coli (a gift from J. Rabillon, Institut Pasteur), a mixture of 1 ng/mL phorbol myristate acetate and 5 ng/mL ionomycin (Sigma), and 10 μg/mL staphylococcal peptidoglycan (Toxin Technology) or human red blood cell lysate of 1.2 × 10¹⁰ cells/mL obtained after osmotic shock.

**Cell lysates.** PBMCs were centrifuged, and pellets were resuspended in fresh RPMI 1640 medium and put through 3 freeze-thaw cycles. Tubes were centrifuged, and supernatants containing cell lysates were collected.

**MIF and tumor necrosis factor (TNF) ELISA.** Cell-culture supernatants and cell lysates were tested for MIF content by use of a specific ELISA (R&D Systems), according to the manufacturer’s instructions. Cell-culture supernatants were tested for their TNF content by use of the duoSet specific ELISA (R&D Systems).

**Statistical Analyses**

Continuous variables are reported as medians and ranges, and categorical variables are reported as number and percentage. Comparisons between groups were performed by use of non-parametric analysis of variance (Friedman tests) or the Mann-Whitney U test. P < .05 indicated significant statistical differences. All analyses were performed with StatView (version 4.51.1; Abacus Concepts) software.

**RESULTS**

**Participants.** Nineteen patients were included (14 men and 5 women; mean ± SD age, 71 ± 3 years; mean ± SD SAPSII score, 51 ± 5). The origins of infection were lungs (n = 13), urinary tract (n = 3), and bone (n = 1). Six patients had positive blood-culture results. Fifteen had gram-negative infection, 11 had gram-positive infection, and no pathogen could be identified in 3 patients. Organ dysfunctions included respiratory...
Figure 1. Levels of macrophage migration inhibitory factor (MIF) in lysates of peripheral blood mononuclear cells (PBMCs) isolated from healthy control subjects (white bars) and from patients with sepsis (grey bars) either immediately after isolation (T0) or after 20 h of culture in the presence of the indicated stimuli. The data are expressed as median, 25th–75th percentile, and 5th–95th percentile. $P$ corresponds to the comparison between healthy donors and patients with sepsis. *E. coli*, *Escherichia coli*; *iono*, ionomycine; LPS, lipopolysaccharide; PGN, peptidoglycan; PMA, phorbol myristate acetate; SAC, heat-killed staphylococci.

$(n = 18)$, cardiovascular $(n = 15)$, renal $(n = 7)$, central nervous system $(n = 7)$, liver $(n = 3)$, and hemato logic $(n = 3)$ failure. All patients were receiving inotropic drugs or vasopressors and required mechanical ventilation and hydrocortisone in addition to fludrocortisone. Blood samples were obtained before the initiation of glucocorticoid treatment in 9 patients. In these patients, the median time between the onset of shock and blood sampling was 3 h (range, 2–3 h). In patients treated with glucocorticoids $(n = 10)$, the median time between the onset of shock and blood sampling was 4 h (range, 3–6 h). The difference in delay between the onset of shock and blood sampling between the 2 groups was not statistically significant ($P = .14$). All treated patients were studied after a single dose of hydrocortisone and fludrocortisone. Five patients died in the hospital.

**MIF in cell lysates.** At homeostasis, circulating leukocytes contain large amounts of MIF. As shown in figure 1, the amounts of MIF within circulating PBMCs from patients with sepsis were significantly higher than in those in healthy control subjects. In contrast, after culture, in the absence or presence of activating agents, the levels of MIF in cell lysates were similar between healthy control subjects and patients. However, MIF levels measured in cell lysates after we cultured PBMCs from patients with sepsis who did not receive corticoids were significantly higher than those from healthy control subjects (table 1). The addition of agonists did not modify levels in this group of patients (data not shown).

**MIF in cell-culture supernatants.** Because very limited information on the in vitro induction of MIF with human leukocytes was available at the beginning of the study, we performed preliminary experiments to determine the most appropriate culture conditions. Low levels of MIF were obtained in whole blood samples (median, 675 pg/mL; range, 128–1372 pg/mL), and high levels that were insensitive to triggering agents were obtained in isolated neutrophils (median, 2327 pg/mL; range, 1878–6444 pg/mL). Enhanced release of MIF could be achieved with PBMCs in response to some activators. Surprisingly, in contrast to findings in rodent cells, we could not induce MIF release by PBMCs from healthy control subjects in the presence of dexamethasone (data not shown). Heat-killed bacteria and pathogen-associated molecules like LPS and peptidoglycan induced a low but significant release of MIF in PBMCs from healthy control subjects. Accordingly, MIF was studied in supernatants of PBMCs cultured for 20 h in the absence or presence of activators. Of note, ex vivo spontaneous MIF release by PBMCs from patients with sepsis was inversely correlated with the cortisol response to adrenocorticotropic hormone (ACTH; $r = -0.55; P = .02$). As shown in figure 2, the spontaneous and activated release of MIF was higher in patients than in healthy control subjects. The differences reached sta-
Figure 2. Levels of macrophage migration inhibitory factor (MIF) in supernatants of peripheral blood mononuclear cells (PBMCs), isolated from healthy control subjects (white bars) and all patients with sepsis (gray bars), cultured for 20 h in the presence of the indicated stimuli. The data are expressed as median, 25th–75th percentile, and 5th–95th percentile. P corresponds to the comparison between healthy donors and patients with sepsis. E. coli, Escherichia coli; iono, ionomycine; LPS, lipopolysaccharide; NS, not significant; PGN, peptidoglycan; PMA, phorbol myristate acetate; SAC, heat-killed staphylococci.

Table 2. Production of tumor necrosis factor by lipopolysaccharide (LPS)–activated peripheral blood mononuclear cells from healthy control subjects (n = 10) and patients with sepsis (n = 7).

<table>
<thead>
<tr>
<th>Concentration of Escherichia coli LPS used</th>
<th>TNF level, pg/mL</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Healthy control subjects</td>
<td>Patients with sepsis</td>
<td>P</td>
<td></td>
</tr>
<tr>
<td>100 ng/mL</td>
<td>4979 (2057–9829)</td>
<td>1990 (496–4956)</td>
<td>.008</td>
<td></td>
</tr>
<tr>
<td>1 μg/mL</td>
<td>5250 (1961–9633)</td>
<td>1726 (32–4145)</td>
<td>.005</td>
<td></td>
</tr>
</tbody>
</table>

**NOTE.** Data are median (range).

MIF production in sepsis

MIF shares with glucocorticoids a regulatory loop [14, 18, 20, 26], and MIF has been clearly characterized as a proinflammatory cytokine [13, 18, 19, 21, 23]. Accordingly, it was of interest to investigate the capacity of PBMCs from patients with sepsis to release this cytokine and to analyze the effect of glucocorticoid treatment.

In contrast to most other proinflammatory cytokines, MIF exists at homeostasis as a preformed molecule within circulating leukocytes, and it is present in plasma as a circulating cytokine. We have shown that MIF levels within the PBMCs were higher in patients with septic shock than in healthy control subjects, which suggests enhanced synthesis and an intracellular accumulation of this cytokine during septic shock. No difference was observed in the numbers of lymphocytes and monocytes between the 2 groups, ruling out the possibility that normalized MIF production reflects a modification of the relative number of cells. Further analysis would be required to analyze the exact nature of cell-associated MIF. Given that it is well known that MIF accumulates within cells, our technique did not allow us to discriminate between intracellular MIF and that bound to the cell surface.

The analysis of MIF in supernatants of PBMCs cultured in

**DISCUSSION**

MIF was significantly lower in treated patients than in untreated patients when responsiveness to LPS, heat-killed E. coli, and peptidoglycan was analyzed. Samples from glucocorticoid-treated patients had a capacity to release MIF in response to different stimuli that was very close to that obtained with PBMCs from healthy control subjects (figure 3).
Figure 3. Levels of macrophage migration inhibitory factor (MIF) in supernatants of peripheral blood mononuclear cells (PBMCs), isolated from healthy control subjects (white bars) and patients with sepsis treated (gray bars) or untreated (hatched bars) with glucocorticoids, cultured for 20 h in the presence of the indicated stimuli. $P$ corresponds to the comparison between treated and untreated patients with sepsis. $E. coli$, *Escherichia coli*; iono, ionomycine; LPS, lipopolysaccharide; NS, not significant; PGN, peptidoglycan; PMA, phorbol myristate acetate; SAC, heat-killed staphylococci.

the absence or presence of various activators (100 ng of LPS, SAC, and red blood cell lysate) revealed that cells from patients with septic shock had an increased capacity to release MIF. This observation stands in contrast to findings obtained for all inflammatory cytokines that have been studied so far. Indeed, the ex vivo production of TNF, interleukin (IL)–1α, IL-1β, IL-2, IL-6, IL-8, IL-12, and interferon-γ is reduced during sepsis [31, 33–38]. To our knowledge, MIF is the first proinflammatory cytokine that has been shown to have enhanced ex vivo production during sepsis. Accordingly, it can be suggested that enhanced levels of circulating MIF in plasma from patients with septic shock [23] may reflect the up-regulation of MIF synthesis by PBMCs. However, other sources—including the pituitary gland and other circulating cells, like neutrophils—may contribute to the enhanced plasma level of MIF observed during sepsis.

It is worth mentioning that MIF, similar to IL-10, is regulated by the Sp-1 transcription factor: there are 4 Sp-1 sites within the promoter of the MIF gene [39]. We have shown that the presence of this transcription factor, in contrast to NF-κB, was enhanced in the nucleus of PBMCs from patients with systemic inflammatory response syndrome. This observation partially explains the enhanced ex vivo release of IL-10 observed in these patients [40, 41]. One may therefore hypothesize that a similar enhancement of Sp-1 in patients with sepsis explains the enhanced release of MIF.

Lysates of red blood cells led to significantly higher levels of MIF in the culture supernatants of PBMCs from patients with septic shock [23] and may enhance susceptibility to infection [43]. Hemoglobin enhances complement activation [44] and increases mortality caused by bacterial endotoxin [45]. Indeed, during sepsis, the induction of MIF by products derived from hemolysis may contribute to systemic inflammation. CD163 has been identified as the receptor for the haptoglobin-hemoglobin complex [46]; its expression on the surface of circulating monocytes has been shown to be markedly increased by the intravenous injection of LPS in human volunteers [47], and it contributes to the production of IL-10 as induced by the haptoglobin-hemoglobin complex [48]. Further studies concerning the role of MIF in human sepsis should investigate the expression of CD163 on circulating leukocytes and its involvement in the induction of MIF production by hemoglobin, as well as its CD74 receptor expression on various cells [25].

In contrast to studies of rodent cells and tissues [14, 18, 26], our study did not involve the induction of MIF neosynthesis and release by the addition of glucocorticoids to whole blood samples, isolated neutrophils, or PBMCs (data not shown). In septic shock, glucocorticoid treatment was associated with a down-regulated level of cell-associated MIF and MIF release by various activators and yielded MIF levels similar to those in healthy control subjects. Although the number of patients in the present study was relatively low, the results of our statistical analysis reinforce this observation. The inclusion of a higher number of patients would likely have allowed statistically significant differences with a higher number of the activators. Further analysis would be required to address whether (1) a relative decrease in the presence of an MIF-producing cell subpopulation has been induced by the glucocorticoid treatment and (2) the effect of the treatment reflects a direct consequence
of glucocorticoid on PBMC function or of intermediate mediators. Furthermore, in another study that enrolled 220 patients, plasma levels of MIF have been analyzed: a reduced level of plasma MIF was observed on day 3 and 7 in the corticoid-treated group, compared with the placebo group (D.A., unpublished data). These data parallel our findings observed at the cellular level soon after treatment. The apparent discrepancy between these findings and the current dogma of cross-talk between glucocorticoids and MIF emphasizes the fact that, in patients with septic shock, glucocorticoid treatment reduces the overwhelming production of proinflammatory cytokines and normalizes MIF production. Furthermore, the cortisol response to ACTH was inversely correlated with ex vivo spontaneous MIF release by PBMCs from patients with sepsis ($r = -0.55; P = .02$). This finding suggested that, in septic shock, adrenal insufficiency exaggerates the up-regulation of MIF, contributing to overwhelming systemic inflammation.

In conclusion, in humans, septic shock is associated with an up-regulation of MIF synthesis, accumulation, and release by PBMCs in response to the release of various activators. Moderate doses of hydrocortisone and fluorocortisone, which are known to contribute to reduced mortality in patients with septic shock, normalize the production of MIF.

**Acknowledgments**

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