Regulation of Macrophage Migration Inhibitory Factor Expression by Glucocorticoids *in Vivo*

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Glucocorticoid hormones are important anti-inflammatory agents because of their anti-inflammatory and proapoptotic action within the immune system. Their clinical usefulness remains limited however by side effects that result in part from their growth inhibitory action on sensitive target tissues. The protein mediator, macrophage migration inhibitory factor (MIF), is an important regulator of the host immune response and exhibits both glucocorticoid-antagonistic and growth-regulatory properties. MIF has been shown to contribute significantly to the development of immunopathology in several models of inflammatory disease. Although there is emerging evidence for a functional interaction between MIF and glucocorticoids *in vitro*, little is known about their reciprocal influence *in vivo*. We investigated the expression of MIF in rat tissues after ablation of the hypothalamic-pituitary-adrenal axis and after high-dose glucocorticoid administration. MIF expression is constitutive and independent of the influence of adrenal hormones. Hypophysectomy and the attendant loss of pituitary hormones, by contrast, decreased MIF protein content in the adrenal gland. Administration of dexamethasone was found to increase MIF protein expression in those organs that are considered to be sensitive to the growth inhibitory effects of glucocorticoids (immune and endocrine tissues, skin, and muscle). This increase was most likely because of a posttranscriptional regulatory effect because tissue MIF mRNA levels were not influenced by dexamethasone treatment. Finally, MIF immunoneutralization enhanced lymphocyte egress from blood during stress-induced lymphocyte redistribution, consistent with a functional interaction between MIF and glucocorticoids on immune cell trafficking *in vivo*. These findings suggest a role for MIF in both the homeo-

static and physiological action of glucocorticoids *in vivo*. (Am J Pathol 2003; 162:47–56)

Macrophage migration inhibitory factor (MIF) is encoded by a highly conserved gene that is shared by protozoa, plants, nematodes, and vertebrates.¹ The protein activity was described first in T-lymphocyte-conditioned medium and named for its ability to inhibit the random migration of macrophages *in vitro*.^{2,3} Since those studies, a variety of cells and tissues have been identified to constitutively produce MIF. These include immune/inflammatory cells such as monocytes/macrophages, lymphocytes, and eosinophils; endocrine organs including the anterior pituitary, the adrenal, the pancreatic islets of Langerhans, the granulosa cells of the ovary, and the Leydig cells of the testis; endothelial cells; epithelial cells in skin, kidney, prostate, and the epididymis; and the central nervous system. In parallel, MIF has been shown to exhibit a number of critical proinflammatory and growth regulatory activities.⁴

Based on studies using recombinant MIF and neutralizing anti-MIF monoclonal antibodies, MIF is an important mediator in the pathogenesis of inflammatory disorders such as endotoxemia/sepsis,^{5–7} arthritis,⁸ glomerulonephritis,⁹ and inflammatory bowel disease.¹⁰ Paradoxically, MIF also is released into the supernatant of cultured macrophages,^{11,12} synoviocytes,¹³ and neurons¹⁴ that have been treated with low levels of glucocorticoids. MIF counterregulates glucocorticoid action and restores macrophage cytokine production and T-cell activation during treatment with immunosuppressive levels of glucocorticoids.^{12,15} Many of the proinflammatory properties of MIF thus have been attributed to its capacity to act as a glucocorticoid antagonist in the immune system. Indeed, in two murine models of inflammation, endotoxic shock and antigen-induced arthritis, the administration of recombinant MIF inhibits the therapeutic action of glucocorticoids.^{12,16}

MIF also has important growth regulatory properties outside of its proinflammatory spectrum of action. MIF mRNA is induced as part of the delayed early response¹⁷

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after serum stimulation of NIH-3T3 fibroblasts, and MIF expression correlates with lens cell¹⁸ and tissue specification during embryonic development.¹⁹ Functional interactions of MIF also have been described with the tumor suppressor protein p53,^{20,21} and with Jab1, an inhibitor of the cell-cycle regulator p27^{Kip1}.²²

The preclinical development of anti-MIF therapies has exploited the concept that inhibition of MIF may be additive or synergistic with the effects of therapeutic glucocorticoids. In other words, by inhibition of an endogenously produced glucocorticoid antagonist (ie, MIF), it may be possible to reduce or eliminate the requirement for glucocorticoids in patients with refractory autoimmune or severe inflammatory disease. This strategy may be particularly valuable in those clinical conditions in which glucocorticoid dependence or resistance to glucocorticoid therapy frequently develop.^{23,24}

The molecular mechanisms of MIF action have come under increasing study, and several distinct pathways for an interaction between glucocorticoids and MIF have been proposed on the basis of *in vitro* studies.^{22,25,26} At the physiological level, it is apparent that MIF is expressed constitutively in many tissues and in plasma, and that in the absence of disease, both stress and glucocorticoid administration result in an increase in circulating MIF levels.¹² Yet, relatively little is known about the functional relationship between glucocorticoids and MIF expression in tissues. In the present report, we have studied MIF protein and mRNA expression after experimental ablation of the hypothalamic-pituitary-adrenal axis and after administration of a therapeutic dose of glucocorticoids to normal rats. We report that MIF expression parallels the adaptive response of tissues to the growth-inhibitory effects of glucocorticoids such as lymphocyte apoptosis or tissue atrophy and provide evidence for a role for MIF in glucocorticoid-mediated lymphocyte redistribution.

Materials and Methods

Animals

Male Sprague-Dawley rats at 250 to 300 g were used for all studies and were obtained from Taconic Farms Inc. (Taconic, NY). Hypophysectomized (Hx) and adrenalectomized (Adx) rats were prepared at Taconic, maintained with 5% glucose in water, or physiological saline solution after Taconic's specifications, and sacrificed for the expression studies on day 10 after surgery. All animals were rested for 5 days before experimental manipulation, received normal rat chow, and were exposed to a conventional 12-hour light-dark cycle.

Expression Experiments

Dexamethasone (Elkins-Sinn Inc., Cherry Hill, NY) was injected intraperitoneally at a dosage of 10 mg/kg in 500 μ l of 0.9% sterile NaCl. The control group received an equal volume of 0.9% sterile NaCl. All injections were administered at 9 a.m., either once, or for five consecu-

tive mornings. Rats were sacrificed in groups of three at 0, 6, 12, 24, or 96 hours by CO₂ asphyxiation, rapidly perfused with ice-cold saline, and the organs were immediately harvested and frozen in liquid N₂. The effectiveness of the ablative surgery in Hx or Adx rats was verified in each animal by the reduction in testis size (Hx group), or the bilateral absence of the adrenal gland (Adx group). All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of North Shore University Hospital.

Stress Experiment

Well-rested, male adult Sprague-Dawley rats were injected intraperitoneally with 3 mg/kg of anti-MIF (III.D.9) or control-IgG1 in the morning. Four hours after the injection, the animals were placed in Plexiglas restrainers (with ample ventilation for breathing) for 2 hours starting at 12 a.m. Blood samples were collected at 0, 0.5, 1, and 2 hours of stress and at 1 and 3 hours during recovery via the tail clip method. White blood cell counts and differentials were obtained on a hematology analyzer (Sysmex, McGraw Park, IL).

Corticosterone Assay

Serum corticosterone was measured by radioimmunoassay (ICN Pharmaceuticals, Irvine, CA) following the manufacturer's instructions.

Western Blotting

Frozen tissue was homogenized in RIPA-buffer supplemented with protease inhibitors (Roche, Nutley, NJ) using a rotor-stator homogenizer (PT 3000; Brinkmann Instruments, Westbury, NY) and the protein concentration was determined by the Bio-Rad DC protein assay (Bio-Rad, Hercules, CA). The tissue samples were adjusted to equal protein concentrations and 10 μ g of protein was electrophoresed in 18% polyacrylamide gels (Ready Gel, Bio-Rad) and transferred onto Immobilon filters (Millipore, Bedford, MA). MIF was detected with the polyclonal anti-MIF antibody R102 and visualized with a peroxidase-conjugated rabbit anti-mouse-IgG antibody and enhanced chemiluminescence (ECL; Amersham Pharmacia, Piscataway, NJ). The anti-actin polyclonal antibody and the anti-signal regulatory protein (SiRP) monoclonal antibody (clone OX-41) were purchased from Chemicon International Inc., Temecula, CA. The lysates obtained from the tissues of the dexamethasone-treated rats were compared with those from the saline-treated rats for each time point. The amount of specific protein present was quantified by densitometry using a GS-710 Calibrated Imaging Densitometer supported by Quantity One 4.2.2 software (Bio-Rad) and expressed in relative units (units). Densitometric measurements were standardized relative to known quantities of rMIF electrophoresed and Western blotted as control.

Immunohistochemistry

Paraformaldehyde-fixed and paraffin-embedded tissues were cut into 5- to 6- μ m sections, mounted onto poly-L-lysine-coated glass slides, deparaffinized in xylene, and passed through decreasing concentrations of alcohol in water. The specimens then were treated in 3% H₂O₂ in phosphate-buffered saline (PBS) for 30 minutes in the dark to inactivate endogenous peroxidases. The sections were incubated in blocking solution (LSAB horseradish peroxidase kit; DAKO, Botany, Australia) for 30 minutes and stained overnight at 4°C with the anti-MIF monoclonal antibody III.D.9.²⁷ Of note, this monoclonal antibody (IgG1 subclass) was raised to recombinant mouse MIF, which differs from rat MIF by a single amino acid substitution (mouse MIF, Asn⁵⁴; rat MIF, Ser⁵⁴), and has been used successfully to quantify rat tissue MIF in previous studies.^{28,29} An IgG1-isotype control was used as negative control. After three washes in PBS/0.05% Tween 20, the bound antibody was visualized with the DAKO LSAB horseradish peroxidase kit. The sections were stained with 3-amino-9-ethylcarbazole as chromogenic substrate and counterstained with Meyer's hematoxylin.

Northern Blotting

Total RNA was isolated from frozen tissue using the RNeasy Kit (Qiagen, Valencia, CA) and eluted in nuclease-free water. Equal amounts of total RNA (10 μ g) were denatured at 65°C for 15 minutes, separated on a 1% agarose gel containing 2.2 mol/L of formaldehyde, and transferred in alkali to a positively charged nylon membrane (Hybond-N, Amersham). After baking for 1 hour at 80°C, the membrane was prehybridized at 45°C for 2 hours in prehybridization solution and subsequently hybridized overnight with a ³²P-dCTP-labeled MIF-, GAPDH-, or β -actin-cDNA probe.²⁸ Probes were labeled by the High Prime DNA Labeling Kit (Boehringer Mannheim/Roche). After two 5-minute washes with 2 $\times$ standard saline citrate/0.1% sodium dodecyl sulfate at room temperature and two 15-minute washes with 0.1 $\times$ standard saline citrate/0.1% sodium dodecyl sulfate at 45°C, the membrane was exposed to a BioMax X-ray film at -70°C for 2 to 8 hours.

In Situ Hybridization

The MIF probe was prepared by subcloning the 420-bp *Xba*I/*Bam*H1 cDNA fragment from a mouse MIF cDNA in pET11b into the Bluescript SK+ vector (Stratagene, La Jolla, CA). This MIF fragment is 100% homologous to rat MIF and shows a single mRNA species of the predicted size when used as probe in Northern blotting of total RNA.²⁸ The plasmid was linearized for the generation of MIF sense and anti-sense riboprobes. Both probes were labeled with ³⁵S-dUTP and *in situ* hybridization of formalin-fixed tissue sections was performed by Molecular Histology Inc. (Gaithersburg, MD). The expression of MIF-specific mRNA was determined by a Fuji Bas 5000 phosphor-imaging system (Fuji, Stamford, CT).

Figure 1. MIF protein expression in Hx and Adx rats. Western blotting of various tissues from Hx rats, Adx rats, and sham-operated controls ($n = 3$ per group) at day 10 after surgery. Equal amounts of total protein were loaded. Two representative examples are given for each tissue and experimental condition.

Data Analysis and Statistics

All data are given as mean $\pm$ SD. An unpaired, two-tailed Student's *t*-test was used to determine differences between groups; $P < 0.05$ was considered significant.

Results

Endogenous Glucocorticoids Do Not Regulate Constitutive MIF Expression, but Loss of Pituitary Hormones Leads to Reduced Adrenal Expression of MIF

Glucocorticoids are synthesized by the cortex of the adrenal gland, and their production is tightly controlled by adrenocorticotropin (ACTH) secreted from the hypophysis.³⁰ To address the question of whether endogenous glucocorticoids regulate MIF expression, we analyzed MIF protein levels in tissues obtained from Hx rats, Adx rats, and sham-operated controls. When compared to controls, MIF protein content in the thymus, spleen, testis, epididymis, liver, kidney, skin, and muscle was unaffected on day 10 after removal of the adrenals (Figure 1). These results indicate that endogenous glucocorticoids do not influence the constitutive expression of MIF in these tissues.

Hx rats lack pituitary hormones such as ACTH, prolactin, thyroid-stimulating hormone, follicle-stimulating hormone, or luteinizing hormone. ACTH is the major growth factor for the adrenal cortex, and its loss leads to adrenal gland atrophy. We found that MIF protein levels in the adrenal glands of Hx rats were significantly reduced on day 10 after surgery when compared to sham-operated controls (Hx, 18 ± 15 units *versus* sham, 146 ± 17 units;

Figure 2. MIF protein expression in Hx rats. Protein lysates from the adrenals of Hx and sham-operated controls were compared by Western analysis for their content of MIF and actin ($n = 3$ per group). MIF expression in adrenals at day 10 after surgery from Hx animals was significantly reduced when assessed by densitometric analysis ($P < 0.001$, Student's t -test).

$P < 0.001$; Figure 2). This finding differs sharply from the normal pattern of MIF protein content observed in the other organs examined and indicates that, like glucocorticoids, MIF production in the adrenal gland is directly or indirectly dependent on stimulation by pituitary hormones, possibly ACTH.

Exogenous Glucocorticoids Induce Tissue-Specific and Time-Dependent Changes in MIF Protein Expression

We next analyzed the effect of exogenous glucocorticoid administration on the expression of MIF protein in normal, intact rats. In the first experimental group, Sprague-Dawley rats were treated with a single dose of dexamethasone (10 mg/kg, i.p.) and the tissues were harvested at 0, 6, 12, and 24 hours after treatment. In a second experimental group, rats were sacrificed 96 hours after receiving dexamethasone (10 mg/kg, i.p.) each day for 5 days. Plasma levels of corticosterone, the endogenous glucocorticoid in the rat, were undetectable at 24 and at 96 hours after dexamethasone injection, thus verifying the biological activity of the administered dexamethasone (ie, suppression of adrenal function). By contrast, the saline-injected controls had corticosterone levels that were within normal limits (Dex-treated, <25 ng/ml; controls, 75 to 260 ng/ml; $P < 0.001$).

Several organs showed tissue-specific changes in MIF immunoreactivity when compared to saline-injected controls. These data are summarized in Figure 3 and discussed below.

Thymus and Spleen

The thymus showed a pronounced and sustained increase in MIF protein expression at 12 and at 24 hours after dexamethasone administration (24-hour Dex, $358 \pm$

Figure 3. Dexamethasone-induced changes in MIF protein expression in various rat tissues. The content of MIF protein from dexamethasone-treated and saline-treated rats was determined in a semiquantitative manner by Western blotting and densitometric analysis. Data are the mean and SD of the change of MIF protein in dexamethasone-treated rats compared to the saline controls. 1, no change; 2, twofold; 3, threefold; and so forth. *, Statistically significant with $P < 0.05$, Student's t -test.

29 units versus control, 73 ± 8 units; $P < 0.04$; Figures 3 and 4A). MIF immunoreactivity in the spleen also increased, but immunoreactivity was less prominent and the increase occurred throughout a longer time course than in the thymus (24-hour Dex, 91 ± 57 units versus control, 45 ± 2 units; $P < 0.02$; Figure 3). These increases in tissue MIF content also were transient. A 5-day course of dexamethasone led to atrophy of the thymus and significantly reduced the MIF content in lymphocyte-rich organs (thymus: Dex, 23 ± 11 units versus control, 82 ± 13 units, $P < 0.005$; spleen: Dex, 19 ± 4 units versus control, 51 ± 22 units, $P = \text{n.s.}$; Figure 3). Intracellular levels of the control protein actin were not significantly altered by dexamethasone administration, indicating that the observed changes in MIF were not because of non-specific effects on cellular protein content (Figure 4).

Circulating monocytes and lymphocytes respond to high glucocorticoid levels by a rapid but transient redistribution from the peripheral blood into lymphatic tissues and the marginal vascular pool.^{30,31} We considered that the observed increase in tissue MIF content might be attributed to the influx of MIF-containing leukocytes in organs such as the thymus or the spleen. We tested this possibility by analyzing tissue lysates for their content of signal regulatory proteins (SiRPs), which are a group of transmembrane glycoproteins specifically expressed by cells of the myeloid lineage.³² We found that at the time of maximal MIF expression in organs such as the

Figure 4. MIF protein expression in representative organs after dexamethasone administration. **A** and **B:** Rats ($n = 3$ per group) received a single injection of saline (control) or dexamethasone at 10 mg/kg (Dex). MIF, signal regulatory protein, and actin contents in organ lysates were measured by Western blotting as described in Materials and Methods. **C:** Skin and muscle show a slight increase in MIF protein content in rats treated for 5 days with dexamethasone. Representative analyses from four different animals are shown.

thymus (Figure 4A), tissue signal regulatory protein levels were not elevated when compared to controls. Thus, leukocyte redistribution does not account for the observed increase in MIF expression 24 hours after glucocorticoid treatment.

Endocrine Organs

Among the endocrine organs examined, the adrenal gland showed the greatest increase in MIF expression (Dex, 197 ± 65 units *versus* control, 41 ± 11 units; $P < 0.05$), followed by the testis (Dex, 169 ± 27 units *versus* control, 51 ± 7 units; $P < 0.02$), and the epididymis (Dex, 222 ± 35 units *versus* control, 91 ± 7 units; $P < 0.02$) (Figures 3 and 4B). The increase in MIF content in these organs was detectable at 12 hours after dexamethasone administration, but with the exception of the testis, this increase was transient and MIF levels returned to baseline at 24 hours (Figure 3). These changes in tissue MIF content also were not because of nonspecific effects on

Figure 5. Plasma MIF levels during dexamethasone treatment. Dexamethasone (10 mg/kg, Dex) or saline (control) was administered intraperitoneally to Sprague-Dawley rats ($n = 3$ per group) at 9 a.m. each morning for 5 days. MIF levels were measured by Western blotting at 0, 6, 12, 24, and 96 hours after first injection. Plasma was pooled for the analysis of each time point. rMIF (10 ng) was loaded as control.

general protein metabolism because there were no detectable changes in the tissue expression of actin.

Skin and Muscle

We found that in skin and muscle, there was a modest increase in cellular MIF only after a prolonged (5 day) course of dexamethasone (skin: Dex, 107 ± 7 units *versus* control, 58 ± 19 units, $P < 0.02$; muscle: Dex, 67 ± 6 units *versus* control, 44 ± 14 units; $P = \text{n.s.}$) (Figure 4C).

Plasma

MIF circulates normally in blood and its concentration increases after dexamethasone administration.¹² We confirmed this finding in rats treated with dexamethasone by Western blotting of plasma (Figure 5). Plasma MIF was transiently elevated in the circulation at 6 and at 12 hours after dexamethasone administration when compared to saline-treated controls. Thereafter, circulating MIF levels decreased.

Localization of Glucocorticoid-Responsive MIF Expression by Immunohistochemistry

To determine which cell types increase their content of MIF protein in response to dexamethasone, we performed immunohistochemistry on paraformaldehyde-fixed tissue sections at the time of maximal MIF protein expression. In dexamethasone-treated rats, MIF-positive cells markedly increased in the medulla of the thymus (Figure 6, A and B) and in the red and white pulp of the spleen (Figure 6, C and D). An increase in MIF immunoreactivity in the epithelial cells of the epididymis (Figure 6, E and F), in the zona glomerulosa and fasciculata of the adrenal cortex (Figure 6, G and H), and in the interstitial cells of the testis (not shown) also was apparent. Of note, this cellular distribution of MIF mimics the pattern of MIF

Figure 6. Immunohistochemical localization of glucocorticoid-induced changes in MIF protein expression. Tissues from dexamethasone-treated rats were compared with saline-treated controls. MIF protein was detected in paraformaldehyde-fixed sections by immunohistochemistry. MIF expression increased in the medullary cells of the thymus (**A, B**), in the red and white pulp of the spleen (**C, D**), in the epithelium of the epididymis (**E, F**), and in the cortex of the adrenal gland (**G, H**).

expression previously reported in normal (untreated) tissues,^{28,33,34} suggesting that the effect of dexamethasone is not to induce *de novo* MIF production, but rather to increase MIF in those cells that already express baseline levels of the protein.

Glucocorticoids Do Not Influence Tissue MIF mRNA Levels

We next examined whether the observed changes in tissue MIF content were associated with a change in the levels of MIF mRNA. Total RNA from rat tissues sampled from the time of maximal MIF induction (12 hours for adrenal, testis, and epididymis; and 24 hours for thymus and spleen) was analyzed by Northern hybridization. Although there was a slight increase in MIF mRNA in the spleen and in the adrenal gland of dexamethasone-treated rats when compared to the saline-treated controls (spleen: MIF, $30 \pm 15\%$, $P < 0.05$; adrenal: MIF, $40 \pm 3\%$, $P = \text{n.s.}$), this increase was not specific for MIF because the mRNA levels for the housekeeping genes GAPDH and β -actin were up-regulated to a similar extent

Figure 7. Northern blotting analysis of MIF mRNA expression. Total RNA from saline-treated (–) and dexamethasone-treated (+) rats ($n = 3$ per group) was analyzed using specific cDNA probes for MIF, GAPDH, and β -actin. Shown are representative blots from the adrenal (12 hours after dexamethasone administration), and from the spleen and the thymus (24 hours after dexamethasone administration). The MIF mRNA levels do not appear to be increased relative to the mRNA for GAPDH and β -actin. Protein levels of β -actin were not altered by dexamethasone treatment (Figure 4).

(spleen: GAPDH, $51 \pm 29\%$, $P < 0.04$; β -actin, $20 \pm 8\%$, $P = \text{n.s.}$; adrenal: GAPDH, $35 \pm 4\%$, $P < 0.02$; β -actin, $69 \pm 10\%$, $P = \text{n.s.}$) (Figure 7). We did not detect any significant changes in MIF mRNA levels in any of the other organs that we examined (thymus, testis, and epididymis; data not shown). To exclude the possibility that MIF mRNA was selectively up-regulated in certain cells within tissues, we also performed *in situ* hybridization with a MIF-specific probe. MIF mRNA was localized within the same cells that produce MIF protein, and we could not detect any significant increases in MIF mRNA levels within these cell types (data not shown). These results suggest that the increase in the cellular production of MIF does not seem to be because of an increase in MIF gene expression, but instead may be the result of posttranscriptional regulation of preformed, MIF mRNA.

Immunoneutralization of MIF Enhances Stress-Induced Leukocyte Redistribution

Glucocorticoids regulate the turnover and trafficking of leukocytes between immune compartments,³⁰ and selective changes in the number and/or the proportion of peripheral blood subpopulations have been shown to be a function of corticosterone levels in rodents.³⁵ To examine the physiological role of endogenous MIF in glucocorticoid-mediated lymphocyte trafficking, we studied a well-characterized rat model of mild, acute stress that leads within 1 hour to a significant (~ 80 -fold) increase in plasma corticosterone levels.³⁶ We found that in rats treated with a neutralizing dose of anti-MIF monoclonal antibody, there was a significant enhancement in the early phase of stress-induced egress of peripheral leukocytes from the circulation (Figure 8). This decrease was mainly accounted for by lymphocytes because peripheral blood neutrophil counts were unaffected during this time. The nadir in circulating lymphocyte counts during stress was lower in the anti-MIF-treated group than in the isotype control group (white blood cell: anti-MIF, $11.5 \pm 1.9 \times 10^3/\mu\text{l}$ and isotype control, $14.3 \pm 2.9 \times 10^3/\mu\text{l}$; lymphocytes: anti-MIF, $6.4 \pm 1.4 \times 10^3/\mu\text{l}$ and

Figure 8. Effect of anti-MIF treatment on stress-induced leukocyte redistribution *in vivo*. Young adult male Sprague Dawley rats were injected with 3 mg/kg of anti-MIF or an isotype control IgG ($n = 3$ per group) 4 hours before a 2-hour stress by restraint experiment. Total white blood cell and differentials were determined before, during, and after stress on a hematology analyzer. Values are mean $\pm$ SD. *, Significant with $P < 0.05$ in a Student's t -test.

isotype control, $8.1 \pm 1.5 \times 10^3/\mu\text{l}$). When compared to pre-stress values, the relative decrease in circulating lymphocytes was more pronounced in the anti-MIF-treated group than in the control group (white blood cell: anti-MIF, $40 \pm 7\%$ and isotype control, $30 \pm 12\%$; $P = \text{n.s.}$; lymphocytes: anti-MIF, $49 \pm 4\%$ and isotype control, $33 \pm 8\%$; $P = 0.038$). Thus, treatment with a neutralizing anti-MIF monoclonal antibody both accelerates and enhances lymphocyte redistribution and suggests a functional interaction between glucocorticoids and MIF *in vivo*.

Discussion

Glucocorticoids are physiological hormones that are essential for life.³⁷ They are synthesized in the adrenal cortex, secreted, and circulate in blood at a concentration that fluctuates in a circadian rhythm.³⁸ Glucocorticoids mediate many aspects of homeostasis, regulate the immune system, and play a critical role in the systemic stress response. The powerful anti-inflammatory and immunosuppressive properties of glucocorticoids also has made them an extremely valuable therapy in patients with severe inflammatory and autoimmune dis-

orders. Glucocorticoids interrupt inflammatory processes by inhibiting the activation of immune cells, by decreasing the production of inflammatory mediators and cell-surface adhesion molecules, and by inducing growth arrest or apoptosis of lymphocytes.^{39–41}

MIF has been characterized as a proinflammatory cytokine with important activities in innate and acquired immunity,⁴ and many of its immunoregulatory properties appear to be because of its action as a glucocorticoid antagonist. MIF release occurs in response to glucocorticoids both *in vitro* and *in vivo*, and recombinant MIF counteracts the glucocorticoid-induced suppression of cytokine production in macrophages and T lymphocytes.^{12,15} *In vivo*, MIF overcomes the protective effect of glucocorticoids in murine models of endotoxic shock and antigen-induced arthritis.^{12,16} Certain features of MIF however, such as its constitutive expression in plasma and in diverse cell types, its high evolutionary conservation from unicellular organisms to vertebrates, and its structural homology with bacterial isomerases,^{1,4} are remarkable for an inflammatory mediator. MIF's precise molecular mechanism of action also is not fully understood. Although several studies have emphasized the activating properties of extracellular MIF, a functional cell-surface receptor for MIF has not yet been characterized. Several intracellular MIF-binding proteins have been identified by yeast two-hybrid methodology, which suggests that these interactions may mediate certain of MIF's activities.^{20,22,42} A regulatory role of MIF in glucocorticoid action nevertheless is supported by MIF's ability to induce sustained ERK-1/2 activation and counterregulate glucocorticoid inhibition of cPLA₂,²⁵ and prevent glucocorticoid-induced expression of the nuclear factor- κ B inhibitor, I κ B.²⁶

In general, glucocorticoids suppress the expression of proinflammatory genes at the level of DNA transcription. Very few genes with functional importance in the immune system are induced by glucocorticoids. Exceptions include the phospholipase A₂-inhibiting proteins (lipocortins),⁴³ the adhesion molecule CD163,⁴⁴ and under special circumstances, interleukin-10,^{45–47} transforming growth factor- β ,^{48,49} platelet-derived growth factor,⁵⁰ granulocyte colony-stimulating factor,^{51,52} and monocyte colony-stimulating factor.⁵³ Although the promoter for murine MIF contains elements that could confer responsiveness to glucocorticoids, such as a negative glucocorticoid responsive element, a CK-1, and a nuclear factor- κ B site,⁵⁴ we did not find in the present study that MIF gene expression is dependent on glucocorticoid levels *in vivo*. Glucocorticoid deficiency in Adx rats also did not lead to altered expression of MIF. These findings are in keeping with studies in humans demonstrating that pituitary stimulation or suppression does not alter plasma MIF levels.⁵⁵ On the other hand, rats treated with a high-dose of dexamethasone showed increased MIF protein levels in glucocorticoid-sensitive tissues such as the thymus, spleen, adrenal gland, testis, epididymis, skin, and muscle. This increase in cellular MIF content was detected within 12 to 24 hours in immunological and endocrine organs, and after 96 hours in skin and muscle. Evaluation of tissue MIF mRNA levels by Northern blotting or by *in*

situ hybridization showed no changes in MIF mRNA expression despite increased protein expression, suggesting that the increase in cellular MIF content is the result of posttranscriptional regulatory mechanisms. Precedents for such an effect include the cyclin-dependent kinase inhibitor p21^{Cip1}, and thymidine kinase, the expression of which increases on glucocorticoid stimulation because of an enhancement of mRNA stability.^{56,57} Additional regulatory mechanisms such as translational control,^{58,59} decreased protein turnover,⁶⁰ and inhibition of secretion^{61–64} also may play a role in MIF expression and should be considered in future investigations.

A novel finding of the present study is that MIF expression may be influenced indirectly by glucocorticoids via the growth status of a tissue. First, we observed MIF levels in the tissues of Hx rats to be unchanged with the exception of the adrenal gland, which is the target organ for hypophyseal ACTH. Second, the tissues that we observed to respond to glucocorticoids with an up-regulation of MIF protein, such as the thymus, spleen, adrenal, testis, epididymis, muscle, and skin, also are those that are known to be sensitive to high glucocorticoid levels. Therapeutic doses of glucocorticoids produce lymphocyte apoptosis in the thymus and the spleen, atrophy of the adrenal cortex, hypogonadotropic hypogonadism, atrophic changes in skin, and a wasting myopathy.⁶⁵ Growth inhibition by glucocorticoids is a highly cell-type-specific process and can be mediated through cell-type-specific repression of growth promoting factors (eg, *c-myc*, cyclin D3, CDK4)^{66–68} or induction of growth-inhibiting factors (eg, p21^{Cip1}, p27^{Kip1}).^{56,69–71} The liver and the kidney, which also express MIF constitutively, do not atrophy during glucocorticoid therapy, and did not show changes in MIF protein levels during dexamethasone administration. The delay of MIF up-regulation in skin and muscle may reflect a different level of sensitivity of these organs to glucocorticoids. Immune cells and cells of the adrenal cortex are very sensitive to glucocorticoids,⁷² whereas keratinocytes and myocytes are less so. Clinically, suppression of the immune response or of the adrenal production of glucocorticoids occurs quickly, whereas skin atrophy and muscular wasting occur later in time.⁶⁵ Accordingly, we suggest that MIF may be involved in the process of glucocorticoid-induced tissue atrophy. Studies in MIF-deficient mice have not to date revealed any phenotype related to tissue atrophy,^{73,74} however transgenic MIF-overexpressing mice have recently been discussed to show signs of osteoporosis, which results in part from atrophic changes in osteoblasts.⁷⁵

Recent *in vitro* studies have focused attention on MIF's role in cell cycle control, proliferation and apoptosis. Kleemann and colleagues²² identified Jab-1, a co-activator of the transcription factor AP-1 and inhibitor of the cell cycle regulator p27^{Kip1}, as an intracellular binding partner of MIF. MIF overexpression was shown to increase levels of p27^{Kip1} and to reduce the growth of proliferating fibroblasts. Hudson and colleagues²⁰ identified MIF to be an inhibitor of p53 activity and showed that MIF inhibits p53-dependent growth arrest and apoptosis. Finally, studies of murine embryonic MIF^{−/−} fibro-

blast cell lines indicate that MIF is an important regulator of growth and *ras*-mediated oncogenic transformation (Petrenko O., Fingerle-Rowson G, Mitchell R, Metz CN, submitted).

We also assessed the physiological interaction between endogenous MIF and glucocorticoids *in vivo* by examining the effect of anti-MIF on stress glucocorticoid-mediated lymphocyte redistribution from blood. Treatment of rats with a neutralizing anti-MIF antibody was found to enhance this effect of glucocorticoids, a result that is consistent with MIF's previously characterized role as a glucocorticoid antagonist.^{12,16,76}

In summary, our findings provide new evidence for the glucocorticoid-mediated regulation of MIF protein expression in glucocorticoid-sensitive tissues. In particular, the pattern of intracellular MIF expression parallels the adaptive response of these tissues to the growth-inhibitory effects of glucocorticoids. Endogenous MIF also acts as a functional glucocorticoid antagonist during stress-induced lymphocyte redistribution. Studies using newly developed MIF-deficient (Fingerle-Rowson G et al, in preparation and refs. 73, 74) and MIF-overexpressing mice should prove useful in unraveling the precise functional relationship between MIF and glucocorticoids in sensitive tissues. It also will be important to use these genetically defined animal models to study the potential utility of inhibiting MIF so as to enhance the anti-inflammatory properties of glucocorticoid while sparing their dose-limiting side effects.

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