Role of macrophage migration inhibitory factor in bleomycin-induced lung injury and fibrosis in mice

YOSHINORI TANINO, HIROKI MAKITA, KENJI MIYAMOTO, TOMOKO BETSUYAKU, YOSHINORI OHTSUKA, JUN NISHIHIRA, and MASAHARU NISHIMURA. Role of macrophage migration inhibitory factor (MIF) in bleomycin-induced lung injury and fibrosis in mice. Am J Physiol Lung Cell Mol Physiol 283: L156–L162, 2002. First published February 15, 2002; 10.1152/ajplung.00155.2001.—Macrophage migration inhibitory factor (MIF) is a unique cytokine that reportedly overrides the anti-inflammatory effect of endogenous glucocorticoids. MIF has been demonstrated to be involved in a variety of inflammatory diseases. In this study, we examined the role of MIF in bleomycin (BLM)-induced lung injury and fibrosis. The levels of MIF in lung tissues and bronchoalveolar lavage fluids were significantly increased in the period 5–10 days after intratracheal administration of BLM. Treatment with the anti-MIF antibody significantly reduced the mortality at 14 days and the histopathological lung injury score at 10 days. These effects were accompanied with significant suppression of the accumulation of inflammatory cells in the alveolar space and tumor necrosis factor-α in the lungs at 7 days. However, the anti-MIF antibody did not affect either the content of lung hydroxyproline or the histopathological lung fibrosis score at 21 days after BLM. These data provide further evidence for the crucial role of MIF in acute lung inflammation but do not support the involvement of MIF in lung fibrosis induced by BLM in mice.

macrophage migration inhibitory factor; bleomycin; lung fibrosis; tumor necrosis factor-α

MACROPHAGE MIGRATION INHIBITORY factor (MIF) was first described as one of the earliest cytokines that was derived from activated T cells and prevented random migration of macrophages (6, 12). Cloning of human MIF cDNA has led to extensive studies using purified recombinant MIF (36), and this protein has been postulated to function as a proinflammatory cytokine (3, 8). It was reported by Donnelly and colleagues (13) that the level of MIF in bronchoalveolar lavage (BAL) fluid was increased in patients with acute respiratory distress syndrome (ARDS). They also demonstrated that MIF augmented, and an anti-MIF antibody attenuated, the secretion of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-8 (IL-8) from alveolar macrophages. We subsequently demonstrated that an anti-MIF antibody attenuated lipopolysaccharide (LPS)-induced neutrophil accumulation in rats lungs, at least in part, by suppressing the level of the neutrophil chemokine macrophage inflammatory protein-2 (MIP-2; see Ref. 20). Although these data support the idea that MIF is a proinflammatory cytokine involved in acute lung injury, this protein may not be like other cytokines. MIF is now known to be constitutively expressed in a variety of cells, including not only immunocytes such as macrophages and T cells but also the anterior pituitary cells and bronchial epithelial cells in the lungs (2, 3, 9, 20). MIF has the unique feature of overriding the anti-inflammatory and immunosuppressive effects of glucocorticoids (8, 9). Furthermore, MIF is reported to have some catalytic activities for D-dopachrome tautomerase (30), phenylpyruvate tautomerase (29), and thiol protein oxidoreductase (18). Accordingly, it is considered that MIF is a pleiotropic peptide, functioning as a cytokine, a hormone, and/or an enzyme (34).

Bleomycin (BLM), an antineoplastic antibiotic, induces interstitial lung inflammation followed by lung fibrosis when intratracheally injected into rodents, so BLM-induced lung injury has been used as a model to investigate the cell biology and histopathology of pulmonary lesions that resemble human idiopathic pulmonary fibrosis (4, 5, 33). In this model, several inflammatory cytokines such as TNF-α (24–26), MIP-2 (17), and monocyte chemoattractant protein (MCP)-1 (2) play important roles in the pathogenesis of lung fibrosis. Neutralization of these cytokines attenuates lung inflammation and fibrotic changes as well. Based on these facts, it is anticipated that a reduction of cytokine production in BLM-induced acute inflammation may lead to attenuation of subsequent fibrosis. However, the cytokine network is so complex that the mechanism of lung fibrosis is not fully understood.

In this study, we attempted to clarify whether an anti-MIF antibody could attenuate BLM-induced acute inflammation. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
l lung inflammation/injury and subsequent fibrosis in mice. We found that the anti-MIF antibody reduced BLM-induced mortality and acute lung inflammation but did not influence lung fibrosis.

METHODS

Animals. Studies were performed on 8-wk-old male C57BL/6J mice (22.0 ± 0.1 (SD) g). They were allowed free access to water and commercial chow. This research adhered to the Declaration of Helsinki and was approved by the Animal Experiment Ethics Committee of Hokkaido University School of Medicine.

Materials. BLM hydrochloride (Bleo; kindly donated by Nippon Kayaku, Tokyo, Japan) was dissolved in pyrogen-free physiological saline just before each experiment. The following materials were obtained from commercial sources: the micro bicinchoninic acid (BCA) protein assay reagent kit from Pierce (Rockford, IL), red blood cell lysis buffer from Sigma Chemical (St. Louis, MO), Diff-Quick stain kit from Kokusai Shiyaku (Kobe, Japan), specific mouse TNF-α and MCP-1 enzyme-linked immunosorbent assay (ELISA) kits from BioSource International (Camarillo, CA), and specific mouse MIP-2 ELISA kit from R&D systems (Minneapolis, MN). All other chemicals were of analytical grade. Rat MIF was expressed in Escherichia coli and purified to homogeneity as described in our previous publication (22). Polyclonal anti-rat MIF serum was generated by immunizing New Zealand White rabbits with purified recombinant rat MIF in our laboratory (20). Because only a single amino acid residue is different between rat and mouse MIFS, the anti-rat MIF antibody immunologically cross-reacts with mouse MIF as previously described (19).

Experimental design. Experiments were designed to examine the role of MIF in BLM-induced acute lung injury and subsequent fibrosis. At first, we measured the levels of MIF in lung tissues and BAL fluids before and after intratracheal injection of BLM (2.5 mg/kg in 50 µl saline). We then evaluated the effect of the anti-MIF antibody on BLM-induced lung injury and fibrosis by comparing an anti-MIF antibody group (anti-MIF antibody + BLM) with a normal IgG group (nonimmune rabbit IgG + BLM). Mice were intraperitoneally injected with 10 mg/kg of either the anti-MIF antibody or nonimmune rabbit IgG 2 h before intratracheal injection of BLM (2.5 mg/kg) and subsequently were given it three times per week throughout the experiment. The effects of the antibody were analyzed by the following parameters: 1) the mortality at 14 days with 2) the assessment of lung injury such as the wet lung weight, wet lung weight-to-body weight ratios, total and differential cell counts in BAL fluids, total protein concentration in BAL fluids, histopathological lung injury score, and the levels of cytokines in lung homogenates at 7 or 10 days with a high dose of BLM (2.5 mg/kg), and 3) the severity of fibrosis at 21 days with a low dose of BLM (1.0 mg/kg).

Measurement of wet lung weight-to-body weight ratio. Animals were exsanguinated by direct cardiac puncture. The heart and lungs were removed en bloc. The heart, esophagus, and lymph nodes were carefully removed. Both total wet lung weight and the wet lung weight-to-body weight ratio were measured as indicators of lung inflammation (23).

Morphological evaluation of lung sections. For morphological evaluation, the lungs were excised by opening the chest. The lungs were fixed by inflation at 25 cmH₂O with a phosphate buffer (10 mM; pH 7.4) containing 10% formalin for 24 h and then embedded in paraffin for histopathological examination. A 5-µm-thick tissue section was prepared from the midportion of paraffin-embedded whole lung tissue and stained with hematoxylin and eosin. An observer who was blinded to the animals group assignment assessed 30 randomly chosen regions per tissue sample at a magnification of ×100 and determined the average score of lung inflammation or fibrosis. Severity of lung inflammation and fibrosis was semiquantitatively assessed, according to the methods described (1, 23). Briefly, the grade of lung inflammation was scored on a scale of zero to five. Criteria for grading lung injury were as follows: grade 0, normal tissue; grade 1, minimal inflammatory change; grade 2, mild to moderate inflammatory changes (no obvious damage to the lung architecture); grade 3, moderate inflammatory injury (thickening of the alveolar septae); grade 4, moderate to severe inflammatory injury (formation of nodules or areas of pneumonitis that distorted the normal architecture); grade 5, severe inflammatory injury with total obliteration of the field. The grade of lung fibrosis was scored on a scale of zero to eight. Criteria for grading lung fibrosis were as follows: grade 0, normal lung; grade 1, minimal fibrous thickening of alveolar or bronchial walls; grade 2, minimal fibrous bands or small fibrous masses; grade 3, severe distortion of structure and large fibrous areas; grade 4, total fibrous obliteration of the field. If there was any difficulty in deciding between two odd-numbered categories, the field would be given the intervening even-numbered score.

Measurement of lung hydroxyproline. Collagen deposition was estimated by determining the total hydroxyproline content of the lung. Both lungs were hydrolyzed with 6 N hydrochloric acid at 110°C for 18 h according to Wossner’s (37) method. After neutralization with sodium hydroxide, the hydrolyzates were diluted with distilled water. Hydroxyproline in hydrolyzates was assessed colorimetrically at 561 nm for p-dimethylaminobenzaldehyde. Results were expressed as micrograms of hydroxyproline per lung.

Preparation of lung homogenates. The whole lungs were homogenized and sonicated in 1.0 ml of the anti-protease-containing buffer (1× PBS with 2 µM phenylmethylsulfonyl fluoride and 1 µg/ml each antipain, leupeptin, and pepstatin A), as previously described (17). Specimens were centrifuged at 900 g for 15 min, filtered through 0.45-µm pore-size sterile filters (Toyo Roshi, Tokyo, Japan), and frozen at −70°C until use for the measurement of the levels of cytokines in lung tissues.

BAL. BAL was carried out for the measurement of MIF in BAL fluid as described elsewhere (4, 5). The lungs were lavaged three times with a total of 1.8 ml of 0.9% saline by inserting a plastic cannula in the trachea of the mouse. The recovery rate of BAL was >90% for all the animals tested. BAL fluid was centrifuged (500 g at 4°C), and the supernatant was used for the measurement of MIF and total protein concentration. The total protein concentration in BAL fluid was measured using the Micro BCA protein assay reagent kit according to the manufacturer’s protocol. The cell pellets were resuspended in 1.0 ml of red blood cell lysis buffer. The number of total cells and differential cell counts in BAL fluid were determined using a hemocytometer and cyto-spins stained with a Diff-Quick stain kit.

ELISA of MIF. The levels of MIF in lung tissues and BAL fluid were quantitated by an ELISA method, which was established in our laboratory. The details of measurement were described in our previous publication (20). The lower detection limit in this system was 1.5 ng/ml.

ELISA of TNF-α, MCP-1, and MIP-2. The levels of TNF-α, MCP-1, and MIP-2 in lung tissues were measured using
mouse ELISA kits according to the manufacturer’s protocols. The lower detection limits in these ELISA systems were 3.0 pg/ml for TNF-α, 9.0 pg/ml for MCP-1, and 1.5 pg/ml for MIP-2.

Statistics. Data are expressed as means ± SE unless otherwise stated. Statistical analyses were performed on the data through single-factor ANOVA among more than two groups and with Student’s unpaired t-test for comparisons of two groups. Survival analysis was performed using the Kaplan-Meier method with the log-rank test. P values < 0.05 were assumed to be significant.

RESULTS

Expression of MIF in BLM-induced lung injury. The levels of MIF in BAL fluids before BLM treatment were below the detection limit. For later analysis, values were assumed to be 0.75 ng/ml in BAL fluids. The levels of MIF in lung tissues (Fig. 1A) and BAL fluids (Fig. 1B) began to increase at 3 days post-BLM and reached a significant level at 5–10 days compared with the level before BLM treatment.

BLM-induced mortality. We compared the survival rate until 14 days post-BLM between the anti-MIF antibody group (n = 13) and the normal IgG group (n = 12). In the normal IgG group, 7 of 12 mice died by 14 days, and the mortality rate was 58%. On the other hand, only 2 of 13 mice died in the anti-MIF antibody group. The mortality rate in this group was 15%, which was significantly lower than that of the normal IgG group (P < 0.05, Fig. 2).

Wet lung weight and total body weight. Both wet lung weight and the wet lung weight-to-body weight ratio at 10 days post-BLM were significantly lower in the anti-MIF antibody group (n = 9) than in the normal IgG group (n = 14; P < 0.05; Table 1).

In both groups of mice, there was a decline in total body weight over time after BLM treatment. The anti-MIF antibody significantly attenuated the total body weight loss as well. The body weight in the normal IgG group decreased from 23.9 ± 0.2 before BLM treatment to 18.3 ± 0.6 g (body weight = 5.6 ± 2.5 g) at 10 days after BLM treatment, but from 23.9 ± 0.2 to 20.8 ± 0.9 g (body weight = 3.1 ± 2.3 g) in the anti-MIF antibody group (P < 0.05).

Inflammatory cells and protein in BAL fluids. Total cell numbers and differential cell counts are shown in Table 2. The anti-MIF antibody significantly attenuated total cell numbers and the number of neutrophils at 3 and 7 days post-BLM. On the other hand, there were no differences in the concentration of total protein in BAL fluid between these two groups at both 7 and 10 days post-BLM (Table 3).

Histopathological evaluation. A representative lung injury 10 days post-BLM is shown in Fig. 3. There were thickening of alveolar septae, hemorrhagic pneumonitis, and distortion of normal lung architecture (Fig. 3A). The anti-MIF antibody caused significant attenuation of these findings from 2.9 ± 0.2 to 2.4 ± 0.1 (P < 0.05) in the lung injury score (Figs. 3B and 4).

Levels of cytokines in lung tissues. The levels of TNF-α, MIP-2, and MCP-1 in lung tissues significantly increased at 7 days post-BLM compared with the pre-treatment level (P < 0.05 for all, Fig. 5). The anti-MIF

Table 1. Wet lung weight and wet lung weight-to-body weight ratio

<table>
<thead>
<tr>
<th></th>
<th>Wet Lung Wt, mg</th>
<th>Wet Lung Wt-to-Body Wt Ratio, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal IgG</td>
<td>220.3 ± 19.4</td>
<td>1.12 ± 0.16</td>
</tr>
<tr>
<td>MIF Ab</td>
<td>186.3 ± 44.1*</td>
<td>0.94 ± 0.25*</td>
</tr>
</tbody>
</table>

Data are means ± SE. MIF Ab, macrophage migration inhibitory factor antibody. *P < 0.05 in statistical comparison with normal IgG group.
antibody significantly attenuated the increase in the level of TNF-α (Fig. 5A) but not that of either MIP-2 or MCP-1 (Fig. 5, B and C).

Effect of anti-MIF antibody on BLM-induced lung fibrosis. In the experiment using a high dose of BLM, we measured the hydroxyproline content at 10 days post-BLM and failed to find any significant difference between the anti-MIF antibody group and the normal IgG group (348.6 ± 40.5 μg/lung, n = 10 vs. 330.5 ± 46.5 μg/lung, n = 9, not significant). In the experiment using a low dose of BLM, we measured the hydroxyproline content at 21 days post-BLM. However, we did not find any significant difference in the hydroxyproline content between the two groups (Table 4). There was no significant difference either in the severity of lung fibrosis between the anti-MIF antibody group and the normal IgG group (Table 4).

DISCUSSION

In this study, we demonstrated that treatment of mice with an anti-MIF antibody significantly attenuated BLM-induced mortality and acute lung inflammation. The levels of MIF in lung tissues and BAL fluids were significantly increased in the period from 5 to 10 days post-BLM compared with the baseline level. These data indicated that MIF was certainly involved in the acute inflammatory phase in BLM-induced lung injury. In addition, we found that the antibody significantly suppressed the accumulation of inflammatory cells to the alveolar space and the level of TNF-α in lung tissues. On the other hand, treatment with the anti-MIF antibody did not affect lung fibrosis at all, as evaluated by the lung hydroxyproline content and the pathological score of lung fibrosis. All these data support the idea that MIF is implicated in the pathogenesis of acute lung inflammation in this model but may not be in the subsequent fibrotic process.

MIF was initially considered to be a T cell-derived cytokine that inhibited the random migration of macrophages in vitro, contributing to delayed-type hyper-
demonstrated that MIF knockout mice were resistant to the lethal effects of a high dose of bacterial LPS and had lower plasma levels of TNF-α than wild-type mice, although another group (15), using the other MIF knockout mice, could not confirm the result. The present study provides further evidence that MIF is implicated in the acute lung injury that is induced not only by LPS but also by BLM.

In this study, we also demonstrated that the level of TNF-α in lung tissues was significantly lower in the anti-MIF antibody group than in the BLM-treated group at 7 days post-BLM. This finding indicated that the effect of the anti-MIF antibody on acute lung injury might be associated with suppression of the level of TNF-α, which is consistent with our previous study (19) and others (10, 13). On the other hand, a recent study demonstrated that an anti-MIF antibody has an anti-inflammatory effect without the presence of TNF-α because the antibody protected TNF-α-deficient mice from fulminant septic shock induced by cecal ligation and puncture (10). Thus the effect of the anti-MIF antibody is not considered to be exclusively due to suppression of TNF-α.

It is widely accepted that TNF-α plays a pivotal role in BLM-induced lung injury and fibrosis. Neutralization of TNF-α with an anti-TNF-α antibody or administration of soluble TNF receptors (TNFRs) can prevent the development of lung fibrosis resulting from BLM exposure in mice (25, 27, 34). Moreover, BLM exposure induces minimal lung inflammation or lung collagen deposition in double-TNFR (p75 and p55) knockout mice (24). Thus it could be hypothesized that suppression of TNF-α in the acute phase of lung inflammation would lead to attenuation of subsequent lung fibrosis. However, we found that treatment with the anti-MIF antibody did not affect lung fibrosis induced by BLM. The mechanism by which the antibody attenuated acute lung inflammation but did not affect lung fibrosis in our model is not clear from this study. We are not able to exclude the possibility that the suppression of the level of TNF-α in lung tissues by anti-MIF antibody is not sufficient enough to attenuate lung fibrosis in this study. However, not all studies indicate the strong association of acute lung inflammation with subsequent lung fibrosis in the BLM-induced lung injury model. Munger and colleagues (21) have reported that mice with knockout of integrin αvβ6, which regulates transforming growth factor-β activity in vivo, developed lung inflammation but not lung fibrosis after BLM treatment (21). Furthermore, they recently reported that the inflammatory response after BLM in-

Table 4. Lung hydroxyproline content and fibrosis score

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Lung Hydroxyproline Content, μg/lung</th>
<th>Fibrosis Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>153.6 ± 28.4 (6)</td>
<td></td>
</tr>
<tr>
<td>Normal IgG</td>
<td>498.2 ± 58.8* (8)</td>
<td>2.1 ± 0.8 (6)</td>
</tr>
<tr>
<td>MIF Ab</td>
<td>481.5 ± 30.1* (8)</td>
<td>1.9 ± 0.8 (6)</td>
</tr>
</tbody>
</table>

Data are means ± SE; nos. in parentheses are no. of animals. Criteria of grading lung fibrosis are based on the method of Ashcroft (1). *P < 0.05 in statistical comparison with saline controls.
stilization was enhanced, but lung permeability was not, in these α6-deficient mice (28). There have been some other studies in which lung fibrosis independently occurred with BLM treatment in neutrophil-depleted animals, in which acute lung inflammation is thought to be suppressed (11, 35). Although these data indicate the complexity of the relationship between acute lung inflammation and subsequent lung fibrosis, these are consistent with a recent new hypothesis that inflammation is not required for the development of a fibrotic response (14, 31). In our study, anti-MIF antibody attenuated the increase of lung wet weight and total cell number in BAL fluids but not total protein concentration in BAL fluid. These data may indicate that anti-MIF antibody attenuated the accumulation of inflammatory cells (especially neutrophils) to alveolar space but did not attenuate the lung protein leakage. Because excessive proteinaceous exudates within the alveolar space lead to lung fibrosis, this might be another reason why the anti-MIF antibody attenuated acute lung inflammation but did not affect lung fibrosis in this study.

The mechanism by which anti-MIF antibody attenuated neutrophil accumulation in the alveolar space was not clear in this study. We have previously demonstrated that anti-MIF antibody suppresses LPS-induced neutrophil accumulation in rat lungs via its suppressive effect on MIP-2 (20). However, in this study, the level of MIP-2 in lung tissues was not attenuated by anti-MIF antibody. Thus other mechanisms by which anti-MIF antibody attenuates neutrophil accumulation in the alveolar space must exist.

To evaluate fibrosis in the experiment using a high dose of BLM, we examined the lung hydroxyproline content at 10 days post-BLM treatment, which might be too early to evaluate lung fibrosis. However, the lung injury induced by a high dose of BLM was too severe for mice to survive over 10 days, particularly when mice were not treated with the anti-MIF antibody. We thus conducted an additional experiment using a low dose of BLM to examine the effect of the antibody on the lung hydroxyproline content and the lung pathological score for fibrosis at 21 days post-BLM. In both experiments, we could not find any significant effect of anti-MIF antibody on BLM-induced lung fibrosis. Accordingly, we are confident that MIF is not implicated in the fibrosing process of BLM-induced lung injury.

In summary, we demonstrated in this study that an anti-MIF antibody significantly attenuated BLM-induced acute lung inflammation and mortality in mice and that this was, at least in part, mediated by suppression of the level of TNF-α in lung tissues. However, the suppression of acute lung inflammation by the anti-MIF antibody did not affect subsequent lung fibrosis in this model. These data provide further evidence for the proinflammatory role of MIF in acute lung inflammation but do not support the involvement of MIF in lung fibrosis induced by BLM in mice.

We thank Dr. Yoshikazu Kawakami (Konan Hospital, Sapporo, Japan) for constructive comments, Yoko Tani (Hokkaido University School of Medicine) for excellent technical assistance, and Yuka Mizue (Sapporo Immunodiagnostic Laboratory, Sapporo, Japan) for assistance in the measurement of MIF by ELISA.

This work was supported by Scientific Research Grant 2670540 from the Ministry of Education, Science, Sports, and Culture of Japan and a Research Grant for the Intractable Diseases from the Ministry of Health and Welfare of Japan.

REFERENCES


