Human circulating eosinophils secrete macrophage migration inhibitory factor (MIF). Potential role in asthma.

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Research Article

Macrophage migration inhibitory factor (MIF) is a potent proinflammatory mediator that has been shown to potentiate lethal endotoxemia and to play a potentially important regulatory role in human acute respiratory distress syndrome (ARDS). We have investigated whether eosinophils are an important source of MIF and whether MIF may be involved in the pathophysiology of asthma. Unstimulated human circulating eosinophils were found to contain preformed MIF. Stimulation of human eosinophils with phorbol myristate acetate in vitro yielded significant release of MIF protein. For example, eosinophils stimulated with phorbol myristate acetate (100 nM, 8 h, 37 degreesC) released 1,539+/-435 pg/10(6) cells of MIF, whereas unstimulated cells released barely detectable levels (< 142 pg/10(6) cells, mean+/-SEM, n = 8). This stimulated release was shown to be (a) concentration- and time-dependent, (b) partially blocked by the protein synthesis inhibitor cycloheximide, and (c) significantly inhibited by the protein kinase C inhibitor Ro-31,8220. In addition, we show that the physiological stimuli C5a and IL-5 also cause significant MIF release. Furthermore, bronchoalveolar lavage fluid obtained from asthmatic patients contains significantly elevated levels of MIF as compared to nonatopic normal volunteers (asthmatic, 797.5+/-92 pg/ml; controls, 274+/-91 pg/ml). These results highlight the potential importance of MIF in asthma and other eosinophil-dependent inflammatory disorders.

Human Circulating Eosinophils Secrete Macrophage Migration Inhibitory Factor (MIF)

Potential Role in Asthma

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Abstract

Macrophage migration inhibitory factor (MIF) is a potent proinflammatory mediator that has been shown to potentiate lethal endotoxemia and to play a potentially important regulatory role in human acute respiratory distress syndrome (ARDS). We have investigated whether eosinophils are an important source of MIF and whether MIF may be involved in the pathophysiology of asthma. Unstimulated human circulating eosinophils were found to contain preformed MIF. Stimulation of human eosinophils with phorbol myristate acetate in vitro yielded significant release of MIF protein. For example, eosinophils stimulated with phorbol myristate acetate (100 nM, 8 h, 37°C) released 1,539±435 pg/10^6 cells of MIF, whereas unstimulated cells released barely detectable levels (< 142 pg/10^6 cells, mean±SEM, $n = 8$). This stimulated release was shown to be (*a*) concentration- and time-dependent, (*b*) partially blocked by the protein synthesis inhibitor cycloheximide, and (*c*) significantly inhibited by the protein kinase C inhibitor Ro-31,8220. In addition, we show that the physiological stimuli C5a and IL-5 also cause significant MIF release. Furthermore, bronchoalveolar lavage fluid obtained from asthmatic patients contains significantly elevated levels of MIF as compared to nonatopic normal volunteers (asthmatic, 797.5±92 pg/ml; controls, 274±91 pg/ml). These results highlight the potential importance of MIF in asthma and other eosinophil-dependent inflammatory disorders. (*J. Clin. Invest.* 1998. 101:2869–2874.) Key words: granulocyte • inflammation • lung • protein kinase C • protein synthesis

Introduction

Macrophage migration inhibitory factor (MIF)[1] has been shown to be an important modulator of the inflammatory response (for review, see reference 1). This mediator was originally described as a T lymphocyte–derived protein that inhibited the random migration of guinea pig peritoneal macrophages (2–4). MIF has subsequently been shown to be a key modulator of both inflammatory and immune responses and has been implicated in macrophage activation and in antigen-driven T cell responses (5, 6). MIF is released from immune cells upon glucocorticoid stimulation, and once released MIF has the unique ability to override glucocorticoid-mediated inhibition of cytokine secretion by both LPS-stimulated monocytes and antigen-activated T cells (7, 8), and human alveolar cells obtained from patients with the acute respiratory distress syndrome (ARDS) (9). Animal studies have shown that MIF has the ability to overcome glucocorticoid protection against lethal endotoxemia (8). It is a mediator in septic shock and this has been highlighted by the ability of neutralizing anti-MIF antibodies to protect animals from lethal endotoxemia (10). Thus, MIF not only exerts proinflammatory effects, but also counteracts the antiinflammatory action of the glucocorticoids. MIF also has recently been shown to be secreted by the corticotrophic/thyrotrophic cells of the anterior pituitary after physiological stress or LPS stimulation (10, 11).

In this paper we report that eosinophils, after stimulation with phorbol myristate acetate (PMA), recombinant human (rh) C5a, and rhIL-5, are also an important cellular source of MIF. In addition we show that (*a*) MIF is stored preformed in significant quantities in eosinophils, (*b*) eosinophils are capable of synthesizing MIF, and (*c*) protein kinase C (PKC) is implicated in the regulation of MIF secretion. Given the involvement of eosinophils in allergic inflammatory disease and the role MIF plays in counteracting the antiinflammatory action of the glucocorticoids, we analyzed alveolar fluid derived from the lungs of stable asthmatic patients and found significantly elevated MIF levels compared to normal control alveolar fluid. In this paper we describe the eosinophil as a cellular source for MIF and highlight the potential importance of MIF as a modulator of the inflammatory response in asthma and other allergic inflammatory diseases.

Methods

Materials. Sodium citrate was purchased from Phoenix Pharmaceuticals Ltd. (Gloucester, UK). Percoll and dextran were obtained from Pharmacia Biotechnologies (St. Albans, UK). Iscove's DME, PBS, HBSS, and supplements (penicillin and streptomycin) were from Life Technologies (Paisley, UK). Sterile tissue culture plasticware was

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1. *Abbreviations used in this paper:* ARDS, acute respiratory distress syndrome; BAL, bronchoalveolar lavage; MIF, macrophage migration inhibitory factor; PKC, protein kinase C; PMA, phorbol myristate acetate; rh, recombinant human.

purchased from CorningCostar (High Wycombe, UK) and Falcon (Baker Scientific, Runcorn, UK). PMA, rhC5a, and cycloheximide were purchased from Sigma Chemical Co. Ltd. (Poole, UK). Dynabeads (M-450) were purchased from Dynal (Merseyside, UK). Ro-31,8220 was purchased from Calbiochem–Novabiochem Ltd. (Nottingham, UK) and rhIL-5 from R&D (Abingdon, UK).

Eosinophil isolation and culture. Eosinophils were isolated by a modification of a previously described method (12, 13). Briefly, eosinophils derived from the peripheral blood of normal donors were isolated by dextran sedimentation followed by centrifugation through discontinuous plasma–Percoll gradients. Granulocytes were taken from the 70/81% layer and the eosinophils were purified from contaminating neutrophils by an immunomagnetic separation step, using sheep anti–mouse IgG Dynabeads coated with the murine antineutrophil antibody 3G8 (anti-CD16; a gift from Dr. J. Unkeless, Mount Sinai Medical School, New York). Cells were mixed with washed 3G8 Dynabeads at a bead/neutrophil ratio of 3:1 on a rotary mixer at 4°C for 20 min, and beads with bound neutrophils were removed magnetically by stationary contact (3 min) with a magnet (Magnetic Particle Concentrator MPC-1; Dynal). This procedure was repeated once to yield an eosinophil population of $> 98\%$ purity and $> 99\%$ viability. After purification, cells were washed sequentially in HBSS without calcium or magnesium and HBSS with calcium and magnesium before resuspending in Iscove's DME with 2% autologous serum. Eosinophils (10^6/ml) were cultured in Iscove's DME with 2% autologous serum at 37°C in a 5% CO_2 atmosphere.

MIF ELISA. Immunoreactive MIF in collected cell supernatants, lysates, and patient samples were quantitated via standard ELISA as published previously (9). Plates were coated with an anti–human MIF mAb and were blocked and incubated using standard methods. Rabbit polyclonal anti-MIF sera were used as secondary antibodies, followed by goat anti–rabbit IgG conjugated to alkaline phosphatase substrate (para-NPP). MIF levels were determined by extrapolating from a quadratic standard curve using purified, human rMIF (range 0–12 ng/ml) (14). The sensitivity of this ELISA was ≥ 150 pg/ml.

Patient details. Asthmatic patients attending the Respiratory Outpatients Department at the Western General Hospital, (Edinburgh, UK) were considered eligible for study enrollment. Our normal nonsmoking control group was paramedical staff within the Western General Hospital. Informed written consent was obtained from volunteers or asthmatic patients before bronchoscopy. Asthmatic patients were eligible for enrollment if they (*a*) were > 18 yr of age, (*b*) had not been prescribed oral corticosteroid medication for at least 3 mo before enrollment, (*c*) had a forced expiratory volume-1 (FEV1) > 1 liter and (*d*) had oxygen saturation values on air of $> 90\%$ before the procedure. This study was approved by the Lothian Health Board Ethics Committee.

Bronchoalveolar lavage (BAL) procedure. BAL sampling was performed as described previously (15). Briefly, asthmatic patients were prescribed nebulized salbutamol (2.5 mg) before bronchoscopy. Premedication consisted of phenoperidine (up to 1 mg) and midazolam (up to 3 mg) intravenously. A fiber-optic bronchoscope was introduced intranasally and passed through the vocal cords into the lungs. The distal end of the bronchoscope was wedged into either the lingula or right middle lobe. Eight 30-ml aliquots of 0.9% NaCl solution were instilled and gently aspirated immediately. All bronchoscopy procedures were performed by the same bronchoscopist (A.P. Greening). On average, 76% of instilled NaCl solution was recovered. Recovered fluid and cells were stored at 4°C until processed. All samples were processed within 1 h of collection.

For collected samples, processing initially entailed straining the lavage fluid through sterile gauze to remove mucous. The strained fluid was then centrifuged at 400 *g* at 4°C for 10 min to recover cells. Total cell counts were performed using a hemocytometer. Aliquots of cells were pelleted onto glass slides using a cytospin 2 (Shandon Scientific, Cheshire, UK) and then stained with Diff-Quick (Merz–Dade AG, Dudingen, Switzerland), a modified Wright–Giemsa stain. Differential counts were determined by counting 500 cells under oil im-

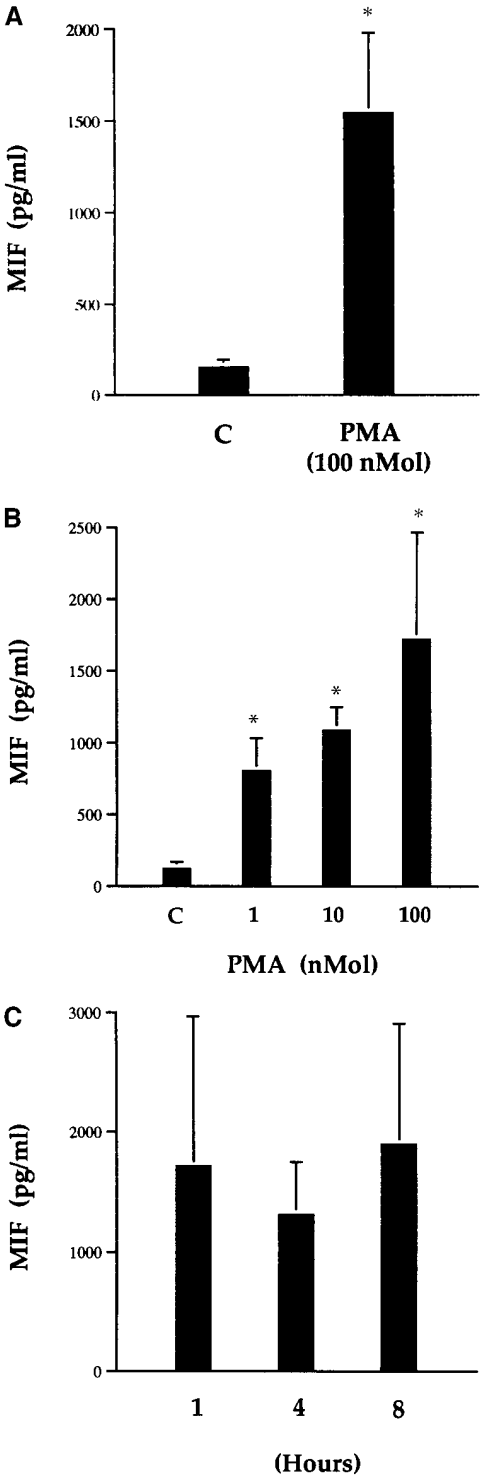

Figure 1. PMA stimulates eosinophils to secrete MIF. Human eosinophils (10^6/ml in Iscove's DME containing 2% autologous serum) were cultured with (*A*) PMA (100 nM) for 8 h ($n = 8$); (*B*) PMA (1, 10, and 100 nM PMA for 8 h) ($n = 4$); and (*C*) PMA (100 nM) for 1, 4, and 8 h ($n = 3$). The cells were centrifuged (800 *g*, 2 min) and the supernatant assessed for the presence of MIF by specific ELISA. Results are expressed as the amount of MIF (pg/ml) released mean± SEM where significant difference above control values is represented by $*P < 0.05$.

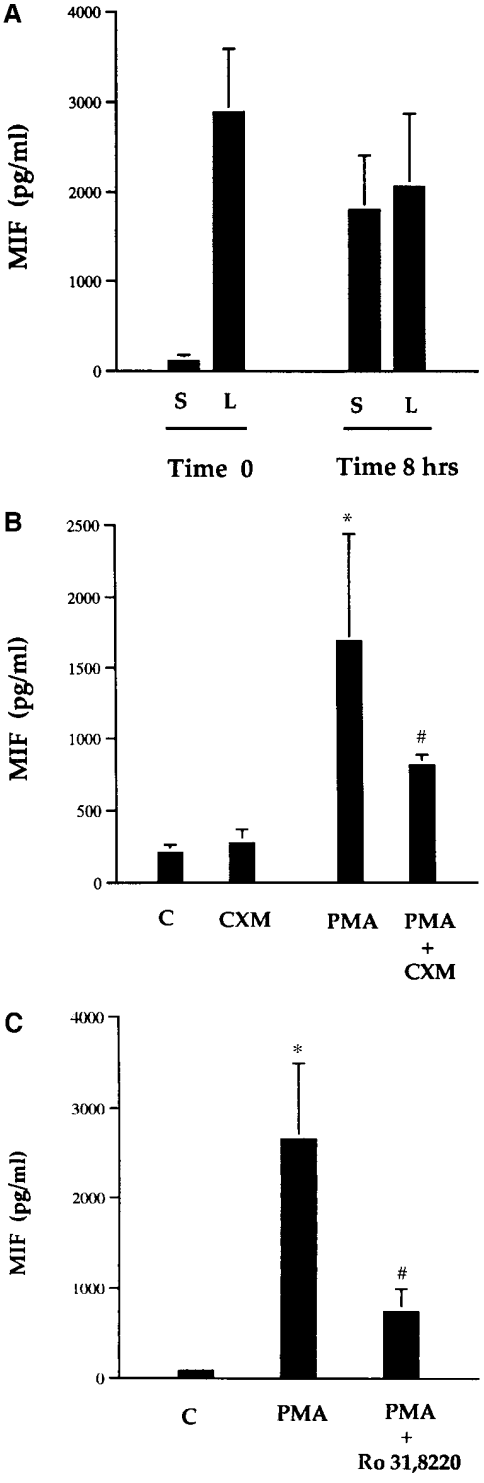

Figure 2. MIF is both stored and newly synthesized and its secretion is controlled by PKC. Eosinophils (10^6/ml in Iscove's DME containing 2% autologous serum) were cultured with (*A*) buffer or PMA (100 nM) for 8 h and MIF levels in cell lysates (*L*) and supernatants (*S*) were determined ($n = 3$); (*B*) PMA (100 nM) for 8 h in the absence and presence of cycloheximide (*CXM*) (1 μM; $n = 4$; *C*, control) and (*C*) PMA (100 nM) for 8 h in the absence or presence of Ro-31, 8220 ($n = 3$). The cells were centrifuged (800 *g*, 2 min) and the supernatant and/or cell lysate assessed for the presence of MIF by specific ELISA. Results are expressed as the amount of MIF (pg/ml) mean±SEM where significant difference above control values are represented by $^*P < 0.05$ and differences between PMA-stimulated cells and CXH- or Ro-31,8220-treated cells are represented by $^{\#}P < 0.05$.

mersion (×100). The lavage fluid supernatant was respun at 1,000 *g* for 10 min at 4°C to remove cellular debris and stored at −70°C until analyzed at a later date. MIF was assayed in unconcentrated, neat BAL samples and MIF results are expressed per milliliter of collected BAL fluid.

Statistical analysis. The results are expressed as mean±SEM. Statistical analysis was performed by the Student's *t* test or by one-way ANOVA followed by the Newmans–Keuls post-test. Analysis of MIF levels in BAL fluid was performed using the Spearman rank correlation. Differences were considered significant when $P < 0.05$. Calculations were performed using the statistical software package Statview (Abacus Concepts, Berkeley, CA).

Results

MIF secretion from PMA-stimulated eosinophils. Eosinophils were found to be efficient secretors of MIF after PMA stimulation. In 8-h cultures, human blood–derived eosinophils released a mean value of 1539±435 pg/ml MIF per million cells after PMA stimulation (100 nM) (Fig. 1 *A*) ($P = 0.0008$). This secretion was concentration-dependent with 1, 10 and 100 nM PMA inducing 803±230, 1,084±161, and 1,718±48 pg/ml MIF, respectively, in collected 8-h supernatants (Fig. 1 *B*). The time course of MIF secretion after stimulation (Fig. 1 *C*) revealed mean MIF release at 1, 4, and 8 h of 1,706±1,259, 1,309±430, and 1,886±1,013 pg/ml, respectively.

MIF is stored preformed in eosinophils. The observation that MIF was found in eosinophil supernatants 1 h after PMA stimulation suggested that significant MIF resides preformed in eosinophils. Analysis of unstimulated eosinophil lysates and supernatants revealed a mean level of MIF of 111±66.2 and 2,883±706 pg/ml in control supernatant and lysate samples, respectively. 8 h after PMA stimulation, supernatant and lysate values were 1,793±610 and 2057±807 pg/ml, respectively (Fig. 2 *A*). Pretreatment with cycloheximide (1 μM) resulted in a reduction in MIF secretion after PMA stimulation by 53% compared to untreated stimulated cells (Fig. 2 *B*).

Influence of PKC inhibitors on MIF secretion. Preincubation of eosinophils with Ro-31,8220 (1 μM), a PKC inhibitor, resulted in an attenuation of PMA-induced MIF secretion by 72% in 8-h supernatants. PMA secretion was reduced from a mean of 2,663±831 to 744±254 pg/ml after preincubation with the PKC inhibitor (control, 86±46 pg/ml) (Fig 3).

IL-5 and C5a induce eosinophil MIF secretion. In relation to the pathophysiology of inflammatory lung disease and asthma, we investigated whether mediators known to be implicated in these diseases (e.g., IL-5 and C5a) could induce MIF secretion from eosinophils. Incubation of human eosinophils with rhIL-5 (100 ng/ml) resulted in a mean MIF level of 1,169 pg/ml in collected supernatants. Preincubation with rhC5a (100 nM) resulted in a mean level of 1,167 pg/ml (Table I).

MIF alveolar levels in asthma. Nonatopic normal volunteers and stable asthmatic patients were bronchoscoped and BAL samples obtained. Significantly elevated alveolar MIF levels were found in asthmatic patients compared to controls ($P < 0.005$) (asthmatic, 797.5±92 pg/ml; controls, 274±91 pg/ml) (Fig. 3). The mean number of total BAL cells obtained

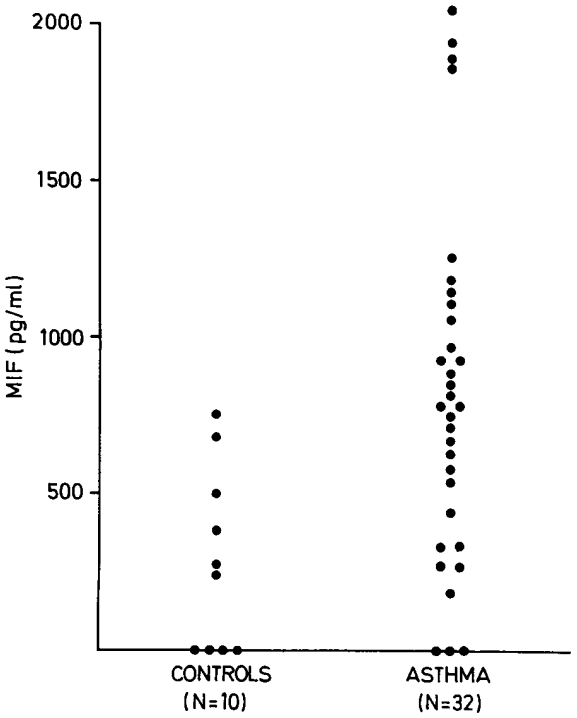

Figure 3. MIF levels are elevated in BAL fluid obtained from stable asthmatic patients compared to levels in alveolar fluid obtained from normal controls. BAL samples were obtained from normal controls ($n = 10$) and asthmatic patients ($n = 32$) as described in Methods and MIF was determined by specific ELISA. Results are expressed as the amount of MIF (pg/ml) obtained in each individual BAL sample. The mean±SEM values obtained from asthmatic patients were 797.5±92 and 274±91 pg/ml from normal controls ($P < 0.005$).

was 11.37×10^6. No significant correlation was obtained between alveolar MIF levels and total BAL cell number ($P = 0.42$), total number of eosinophils ($P = 0.79$), macrophages ($P = 0.60$), lymphocytes ($P = 0.54$), or neutrophils ($P = 0.42$).

Discussion

MIF was originally described over 30 yr ago as a soluble activity produced by activated T cells that inhibited the random migration of cultured guinea pig macrophages (3, 4). Recently, MIF has been rediscovered to be a critical regulatory mediator whose secretion is induced by glucocorticoids and that has the capacity, in its own right, to counterregulate the inhibitory effects of glucocorticoids in the immune system (4). This dual role of maximizing inflammatory activity by being directly proinflammatory, and by indirectly inhibiting maximal antiinflammatory glucocorticoid activity, led us to investigate the potential role of MIF in pulmonary inflammatory disease. ARDS represents a catastrophic form of acute lung injury that arises as a consequence of overwhelming inflammation. We have recently described a potential regulatory role for MIF in ARDS (9). In this study, we showed evidence within lung tissue and within the alveolar airspace of excessive MIF production. In addition MIF was shown to both augment proinflammatory cytokine production and override the antiinflammatory effects of glucocorticoids on ex vivo ARDS alveolar cells.

Table I. Physiological Stimuli C5a and IL-5 Induce MIF Secretion

	Exp. 1	Exp. 2
Control (unstimulated)	439	544
IL-5 (100 ng/ml)	843	1495
C5a (100 nM)	913	1420

Human eosinophils (10^6/ml) in Iscove's DME (containing 2% autologous serum) were cultured alone (control), with rhC5a (100 nM), or with rhIL-5 (100 ng/ml) for 8 h. The cells were centrifuged (800 *g*, 2 min) and the supernatant assessed for the presence of MIF by specific ELISA (each treatment assayed in triplicate).

This work led us to consider the potential role of MIF in an allergic inflammatory disease, namely asthma. The eosinophil has been implicated as a key cell type in the pathogenesis of allergic inflammatory diseases such as bronchial asthma, allergic rhinitis, and atopic dermatitis (16, 17). Eosinophils are recruited to inflammatory foci by the concerted effects of specific chemoattractants, adhesion molecules, and extracellular matrix proteins. Once recruited, eosinophils, if further exposed to inflammatory stimuli, become activated and can undergo a variety of functional responses including degranulation, liberation of reactive oxygen intermediates (ROI), and synthesis and release of inflammatory mediators and cytokines. Secreted products such as eosinophil cationic protein, major basic protein, eosinophil protein X, and eosinophil peroxidase, as well as reactive oxygen intermediates, assist in destruction of invading organisms and contribute to tissue damage associated with allergic diseases and asthma. For example, in asthma, elevated levels of eosinophil-derived products are found in the BAL fluid and correlate positively with the severity of the disease. Furthermore, when certain eosinophil-derived products are administered into the lungs of animals, bronchial hyperresponsiveness and lung damage can occur, further implicating the eosinophil as detrimental and contributing to the pathogenesis of asthma (for review, see references 16 and 18). Clinically, administration of glucocorticoids has been used successfully as a way of reducing inflammatory cell–induced tissue damage and amelioration of eosinophilic diseases. Although the mechanism of action of glucocorticoids in such diseases is unknown, in patients with hypereosinophilia there is a marked reduction in the number of circulating eosinophils, an inhibition of eosinophil recruitment to inflammatory sites, a dampening of eosinophil responsiveness, and an increase in eosinophil apoptosis with subsequent removal by macrophages (13, 19–22). Thus, identifying and characterizing eosinophil-derived mediators and defining their biological effects will help to elucidate the cellular mechanisms involved in eosinophilic diseases and have far-reaching implications in developing therapeutic targets.

In this study we report that eosinophils are an important source of MIF, a cytokine capable of counteracting the effects of glucocorticoids. We show that PMA-induced MIF secretion occurs rapidly, with significant extracellular MIF being found within 1 h after stimulation. Even at 8 h, despite pretreatment with cycloheximide, MIF levels attained 47% of untreated

PMA positive control. This suggests a possible role for MIF in both early and later eosinophil-induced inflammation in asthma and other allergic inflammatory diseases.

PMA is known to directly interact with PKC and therefore bypass classical receptor/G protein effects. We investigated whether the concentration- and time-dependent PMA-induced MIF release was also mediated by this pathway. Using the specific PKC inhibitor Ro-31,8220 we show that the PMA-stimulated response was almost totally blocked by this inhibitor implying that PKC regulates PMA-stimulated release. We were also interested in determining whether other significant eosinophil activators that have been implicated in asthma disease pathogenesis (23, 24) stimulated MIF secretion from human eosinophils. Both IL-5 and C5a enhanced MIF secretion from these cells. The levels of MIF secretion after stimulation by rhC5a and rhIL-5 were $\sim$ 70% of levels obtained by stimulation with PMA.

With our identification of the eosinophil as an additional circulating cellular source for MIF, and the well recognized role MIF plays in counteracting the antiinflammatory actions of glucocorticoids, we wished to investigate whether elevated intraalveolar MIF levels exist in asthmatic patients. In BAL samples obtained from stable asthmatic patients significantly elevated intraalveolar MIF levels were detected compared to nonsmoking controls ($P < 0.005$). These findings support the role for MIF in contributing to sustaining the pulmonary inflammatory response in asthma. There was no direct correlation between MIF levels and total eosinophil numbers in BAL samples. This does not necessarily mean that the MIF we detected was not derived from eosinophils, since a direct correlation between these parameters would occur only if the whole eosinophil population uniformly secreted MIF throughout their tissue life-span in asthmatic lungs, and many other factors are likely to impinge on the kinetics of eosinophil MIF secretion in situ. Nevertheless it is important to recognize that other cells, including macrophages and lymphocytes (3–5), may also contribute to the levels of MIF that we detected in BAL fluid from asthmatics.

To date MIF has been implicated in immune diseases of the kidney and in ARDS (9, 25, 26). In ARDS we have found the median intraalveolar MIF levels in a severe form of acute inflammatory lung injury, namely ARDS, to be 1,190 pg/ml (9). The median level found in our stable asthmatic population was 772.5 pg/ml. One could envisage that in acute inflammatory asthmatic exacerbations requiring hospital admission, intraalveolar MIF levels would be elevated still further. This raises the possibilities of MIF as a significant contributor to sustaining the heightened inflammatory response, and of anti-MIF as a novel therapeutic strategy in asthma and other allergic inflammatory diseases. Further work is required to address these important questions.

In asthma, it is possible that in addition to MIF's potent proinflammatory activity, its ability to override the antiinflammatory activity of glucocorticoids may also significantly contribute towards an exaggerated pulmonary inflammatory response in asthma. Glucocorticoid therapy represents the gold standard antiinflammatory treatment in asthma. However, it is well recognized that a proportion of patients fail to respond to prescribed glucocorticoids and these patients have been termed steroid-resistant asthmatics (27, 28). Today, the problem of a blunted clinical response or resistance to prescribed glucocorticoids is well recognized, particularly in hospital-based asthma practice. MIF could potentially also play a role in this blunted response to steroids. Our findings of significant intrapulmonary MIF evident within the alveolar airspaces of stable asthmatics combined with our knowledge of the ability of MIF to override, in a concentration-dependent manner, glucocorticoid antiinflammatory function on ex vivo inflammatory cells suggest that MIF may play a role in inhibiting maximal endogenous or exogenous steroid activity in inflammatory diseases such as asthma. This finding is potentially of therapeutic importance, as an anti-MIF strategy would not only be antiinflammatory in its own right, but would also act by removing the potential inhibitory role of MIF on glucocorticoid function. This could serve to restore maximal antiinflammatory steroid activity, and reset the balance within the pulmonary inflammatory response away from excessive inflammation, airway constriction, and tissue damage.

Acknowledgments

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