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Macrophage migration inhibitory factor induces phagocytosis of foreign particles by macrophages in autocrine and paracrine fashion

S. ONODERA,* K. SUZUKI,* T. MATSUNO,* K. KANEDA,* M. TAKAGI‡ & J. NISHIHIRA† *Department of Orthopaedic Surgery and †Central Research Institute, Hokkaido University School of Medicine, Sapporo, and ‡Department of Orthopaedic Surgery, Tohoku University School of Medicine, Sendai, Japan*

SUMMARY

Exposure to foreign particles sometimes causes inflammatory reactions through production of cytokines and chemoattractants by phagocytic cells. In this study, we focused on macrophage migration inhibitory factor (MIF) to evaluate its pathophysiological role in the phagocytic process. Immunohistochemical analysis of human pseudosynovial tissues retrieved at revision of total hip arthroplasty showed that infiltrating mononuclear and multinuclear cells were positively stained by both an anti-CD68 antibody and anti-human MIF antibody. For *in vitro* study, MIF was released from murine macrophage-like cells (RAW 264.7) in response to phagocytosis of fluorescent-latex beads in a particle dose-dependent manner. Northern blot analysis showed marked elevation of the MIF mRNA level in the phagocytic macrophage-like cells. Moreover, pretreatment of RAW 264.7 cells with rat recombinant MIF increased the extent of phagocytosis by 1·6-fold compared with the control. Taken together, these results suggest that MIF plays an important role by activating macrophages in autocrine and paracrine fashion to phagocytose foreign particles.

INTRODUCTION

The human body is often exposed to numerous inert and pathogenic particles. Once these particles are taken into the body, macrophages primarily respond to them for self-defence. Inhaled particles in the lung and inert particles around artificial joints are readily ingested by macrophages. On artificial joint tissue, particulate wear debris generated from the artificial materials after joint replacement surgery is phagocytosed by infiltrating macrophages. Histologically, macrophages and multinuclear giant cells phagocytosing wear particles can be seen within the joint tissues around loosened prostheses.[1–4] As for the pathological state subsequent to phagocytosis, various inflammatory mediators are released within these tissues, which causes bone resorption via osteoclast activation and results in implant loosening.[5–7]

In the study of macrophage recruitment, macrophage migration inhibitory factor (MIF) in the guinea-pig was the first lymphokine reported to prevent the random migration of macrophages out of capillary tubes.[8,9] However, the underlying mechanism of macrophage recruitment remains to be elucidated. MIF was at first considered to be exclusively secreted by activated T lymphocytes; however, recent reports indicate that a variety of cells and tissues other than T lymphocytes have the potential to produce the protein.[10–14] To date, a variety of biological functions of MIF have been identified, including its ability to enhance the capacity of macrophages to kill intracellular parasites and tumour cells, increases in tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) production, counter-regulation of glucocorticoid suppression of cytokine production, and an increase in nitric oxide synthesis.[15–18] It is known that IL-1β, TNF-α and nitric oxide are released from activated macrophages in the process of tissue damage caused by particle-induced inflammation accompanied by phagocytosis. Thus, it is speculated that the co-operative action of MIF with other cytokines may be involved in the mechanism of phagocytosis.

In 1989, human MIF cDNA was cloned, which revealed that MIF consists of 114 amino acid residues.[19] Recently, we cloned rat MIF cDNA and reported its physicochemical properties, and revealed the tertiary structure of rat MIF at 2·2 Å resolution.[20–24] The structural analysis showed that MIF forms a unique homotrimeric form with a unique parallel βαβ motif. We here report the presence of MIF within the tissues around loosened artificial joints observed by immunohistochemistry. Furthermore, we present evidence for involvement of MIF in the foreign body reaction by *in vitro* studies. The present results suggest that MIF produced by macrophages is profoundly involved in the mechanism of phagocytosis in both an autocrine and a paracrine fashion.

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Correspondence: Dr J. Nishihira, Central Research Institute, Hokkaido University School of Medicine, Kita-15, Nishi-7, Kita-ku, Sapporo 060, Japan.

MATERIALS AND METHODS

Materials

The following materials were obtained from commercial sources. Nitrocellulose membrane filters were from Millipore (Bedford, MA); Isogen RNA extraction kit from Nippon Gene (Tokyo, Japan); heat-inactivated fetal calf serum (FCS) from Hyclone Labs (Logan, UT); horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody from Pierce (Rockford, IL); Histofine SAB-PO kit from Nichirei (Tokyo, Japan); 3,3′-diaminobenzidine tetrahydrochloride and *o*-phenylethylenediamine from Wako (Osaka, Japan); protein A–Sepharose from Pharmacia (Uppsala, Sweden); HRP-conjugated goat anti-mouse IgG antibody from Bio-Rad (Hercules, CA); anti-human CD68 antibody from DAKO (Tokyo, Japan), and Fluorebrite carboxylate microspheres (2·5% solid latex, mean diameter 0·75 μm) from Polyscience (CA, USA). All other chemicals used were of analytical grade.

Polyclonal anti-human and anti-rat MIF antibodies were generated by immunizing New Zealand White rabbits with purified recombinant human and rat MIF as described previously.[11,13] The IgG fractions were prepared using protein A–Sepharose according to the manufacturer's protocol.

Immunohistochemistry

Ten samples of loosening tissues were retrieved at revision of total hip replacement (THR) surgery. These included the bone–cement interface and pseudocapsule synovium around the loosened prostheses. These samples were embedded and frozen in Tissue-Tek OCT compound, and stained with a Histofine SAB-PO kit. In brief, 6-μm cryostat sections were fixed in acetone and sequentially treated with 0·6% H_2O_2, normal goat serum, anti-human MIF polyclonal antibody, anti-rabbit IgG, and streptavidin–biotin peroxidase complex (ABC). ABC-binding regions were visualized with 3,3′-diaminobenzidine tetrahydrochloride. They were further counterstained with Meyer-Haematoxylin. Serial sections of each slide were also stained for CD68, a surface antigen of human macrophages, using an anti-CD68 IgG monoclonal antibody and HRP-conjugated goat anti-mouse IgG antibody.

Cells

RAW 264.7, a murine macrophage-like cell line, was purchased from the American Type Culture Collection (Rockville, MD). Cells were grown in Dulbecco's modified Eagle's minimal essential medium (DMEM) containing 2 nM glutamine, 10% FCS, and 50 μg/ml penicillin and streptomycin, and incubated at 37°.

Phagocytosis assay

Cells of murine macrophage cell line RAW 264.7 were dispensed into wells of a 48-well tissue culture plate (1×10^5 cells in 0·3 ml of DMEM plus 10% FCS). After 3 hr of incubation, the supernatants were aspirated and these cells were incubated for a further 12 hr in media containing various concentrations of fluorescent latex beads. Then each supernatant was collected by centrifugation at 5000 ***g*** to remove the latex beads, and stored at −80° until use. At the same time, the cells were harvested by treatment with trypsin (0·2%), ethylenediaminetetraacetic acid (EDTA; 0·02%) and gentle scraping, washed three times with phosphate-buffered saline (PBS), and subjected to flow cytometric analysis of particle uptake. This uptake was morphologically confirmed by fluorescence microscopy. For the time–course study of MIF secretion, macrophages were challenged with latex beads at the concentration of 2500 μg/ml. The supernatants were collected at designated times (0·5, 3, 6, 9, 12 and 18 hr after stimulation). These samples were also examined for quantification of MIF.

Enzyme-linked immunosorbent assay (ELISA) of MIF

The rat MIF IgG polyclonal antibody dissolved in PBS (50 μl) was added to each well of a 96-well microtitre plate, which was then left for 30 min at room temperature. The plate was washed three times with distilled water. All wells were filled with PBS containing 0·5% bovine serum albumin (BSA) for blocking and left for 20 min at room temperature. The samples of supernatants were diluted 10-fold with PBS containing 0·5% BSA. After removal of the blocking solution, these diluted samples were added in duplicate to individual wells and incubated for 1 hr at room temperature. After the plate was washed three times with PBS containing 0·05% Tween-20 (washing buffer), 50 μl of biotin-conjugated MIF IgG was added to each well. Following incubation for 1 hr at room temperature, the plate was again washed three times with the washing buffer. Then, an avidin-conjugated goat anti-rabbit IgG antibody was added to each well, after which the microtitre plate was incubated for 15 min at room temperature and washed three times with the washing buffer. The substrate solution (10 ml) contained 8 mg of *o*-phenylethylenediamine and 4 μl of 30% H_2O_2 in citrate phosphate buffer adjusted to pH 5·0. The substrate solution (50 μl) was added to each well. After incubation for 20 min at room temperature, the reaction was stopped with 25 μl of 8 M sulphuric acid. The absorbance was measured at 492 nm by an ELISA plate reader (Bio-rad, Model 3550).

Northern blot analysis

RAW 264.7 cells were dispensed into 100-mm dishes (3×10^6 cells in 2 ml DMEM containing 10% FCS). After they reached confluence, the media were changed to those containing latex beads in the concentrations described above, and the cells were cultured for a further 12 hr. Then they were harvested by gentle scraping, washed twice in PBS to remove free particles and used for Northern blot analysis. Total RNA was extracted from the cell pellets with an Isogen total RNA extraction kit. Pelleted RNA was resuspended in TE (10 mM Tris–HCl, 1 mM EDTA, pH 7·4). Total RNA (20 μg) was denatured and dissolved in 1% agarose formaldehyde gels. The RNA was then transferred to nylon membranes, and cross-linked by ultraviolet irradiation. Prehybridization and hybridization were carried out in 0·5 M $NaPO_4$/1 mM EDTA/7% sodium dodecyl sulphate (SDS)/150 mg/ml RNA at 65°. The membranes were washed with 0·1% SSC containing 1% SDS at 22° and 52° before autoradiography and quantitative densitometric analysis by MCID Image Analyser (Fuji Film, Tokyo, Japan).

Flow cytometric analysis

RAW 264.7 cells were dispensed into six 35-mm dishes (3×10^6 cells in 2 ml of DMEM containing 10% FCS). After a 3 hr incubation, non-adherent cells were washed out and the medium was changed to those containing various amounts of rat recombinant MIF (0, 1 and 10 μg/ml), and the cells were

pretreated with or without MIF for a further 48 hr. Then the culture media were changed to those containing fluorescence-coated latex beads (500 μg/ml, 2 ml per dish) and the cells were further incubated. After 20 min and 2 hr, the cells were collected by treatment with trypsin (0·2%) and EDTA (0·02%) and gentle scraping, washed three times with PBS to remove free particles, fixed in 50 μl of 2·5% formaldehyde, and quantified for phagocytosis of particles by flow cytometry. Uptake of fluorescent particles by RAW 264.7 cells was measured using a FACScan (Becton-Dickinson, Mountain View, CA). Phagocytosis was analysed in 10 000 cells/sample to determine the overall uptake of particles by the population.

All data are shown as mean values and standard deviations. The concentration of MIF measured by ELISA was analysed by unpaired *t*-test. For assessment of the effects of time and MIF concentrations on phagocytosis, two-way analysis of variance (ANOVA) was performed, followed by Fisher protected least-significant difference (PLSD) analysis for multiple comparison.

RESULTS

Immunohistochemistry

Immunohistochemistry using the anti-human MIF antibody showed the existence of MIF in all the samples tested. Positive MIF staining was observed within the cytoplasm of mononuclear cells and multinuclear giant cells (Fig. 1 a1 and a2). This reaction was observed both within the pseudosynovial tissue and bone–cement interface membrane. In the absence of the primary anti-MIF antibody or with the addition of an excess amount of MIF, the positive reaction was not observed (Fig. 1 b1 and b2). Normal hip joint synovium, which was retrieved at hemipelvectomy operation, showed no positive reaction in the synovial cells against anti-human MIF antibody and a slight positive reaction in the capillary endothelial cells (Fig. 1c). Polarized light microscopic study of the same tissue specimens showed numerous intracellular and extracellular foreign bodies of amorphous shape, that were consistent with polyethylene fragments (×100) (Fig. 1d). The staining of serial sections for CD68 showed positive reactions in identical cells which were positive for the antibody against MIF (Fig. 1 e1 and e2). This indicated that macrophages were the main source of MIF in the tissues around the loosened implants.

Phagocytosis and MIF release

RAW 264.7 cells were incubated with varying amounts of particles *in vitro* in a medium containing 10% FCS. Figure 2 shows the amounts of MIF released into the media. High particle uptake resulted in a marked increase of MIF release by macrophages. The maximum increase was obtained at the concentrations of 1500 and 2500 μg/ml of latex beads (unpaired *t*-test, $P<0·01$ versus control). Addition of particles in serum-free medium did not result in the elevation of MIF release (data not shown). Flow cytometric analysis of the particle uptake showed that the average fluorescence intensity of latex beads normalized by the cell number was increased in proportion to the concentration of the beads added to the medium (Fig. 3a). It was confirmed by fluorescence microscopy that most of the particles were internalized by the macrophages (Fig. 3b). Endotoxin levels in the particle samples were low (<0·1 ng/ml). Next, we investigated the time–course of particle-induced MIF release by the macrophages. MIF was detected in culture supernatants of macrophages without stimulation by latex beads. This indicated that macrophages in the steady-state constitutively produced MIF (Fig. 4).

Northern blot analysis of MIF mRNA

Changes of the MIF mRNA expression by phagocytosis were examined. After phagocytosis, as described above, RAW 264.7 cells were harvested and the total RNA was extracted for Northern blot analysis. The expression level of MIF mRNA was normalized by that of β-actin mRNA used as a control. The values presented in the figure were normalized by β-actin signal value. Uptake of latex particles at the dose of more than 1000 μg/ml caused a marked increase in the expression of MIF mRNA (Fig. 5). Densitometric analysis showed increased expression of MIF mRNA (8-fold) after particle uptake at the dose of 1000 μg/ml. The result indicated that the expression of MIF mRNA in monocyte/macrophage lineages could be induced by foreign-particle stimulation, which might led to excessive inflammatory and immunological reactions.

Effect of MIF on phagocytosis

As shown in Table 1, increasing concentrations of rat recombinant MIF produced a progressive promotion in the mean particle uptake per cell. At 20 min after administration of particles, no difference of particle uptake was seen between the control and cells pretreated with the two different doses of MIF. At 120 min, an increase of particle uptake could be demonstrated in cells pretreated with MIF at both doses ($P<0·01$ and $P<0·0001$, respectively). The increase of particle uptake was promoted 1·6-fold by treatment with the high dose of MIF compared to the control group.

DISCUSSION

In this study, we demonstrated by immunohistochemistry *in vivo* that the majority of MIF-positive cells were macrophages within tissues exposed to inert particles. We also showed for the first time that MIF could be released by murine macrophages in the process of phagocytosis of foreign particles. ELISA showed that a large amount of MIF was released to extracellular space in a particle-dose-dependent manner. Northern blot analysis showed that MIF mRNA expression was concomitantly elevated by foreign-particle stimulation. These facts suggested that macrophages readily produced MIF in response to foreign-particle stimulation. MIF is known to have the potential to recruit macrophages at infection loci; however, the role of MIF in the mechanism of phagocytosis still remains to be elucidated. To date, there have been several lines of *in vitro* studies regarding release of proinflammatory substances, TNF-α, IL-1β and proteolytic enzymes by inert and toxic particles.[25–30] In association with these cytokines, it is reported that MIF stimulates monocytes/macrophages to secrete TNF-α and IL-1β.[10,16] Considering these facts, it is considered that MIF may be profoundly involved in the mechanisms of not only the inflammatory response but also phagocytosis in concert with these proinflammatory cytokines.

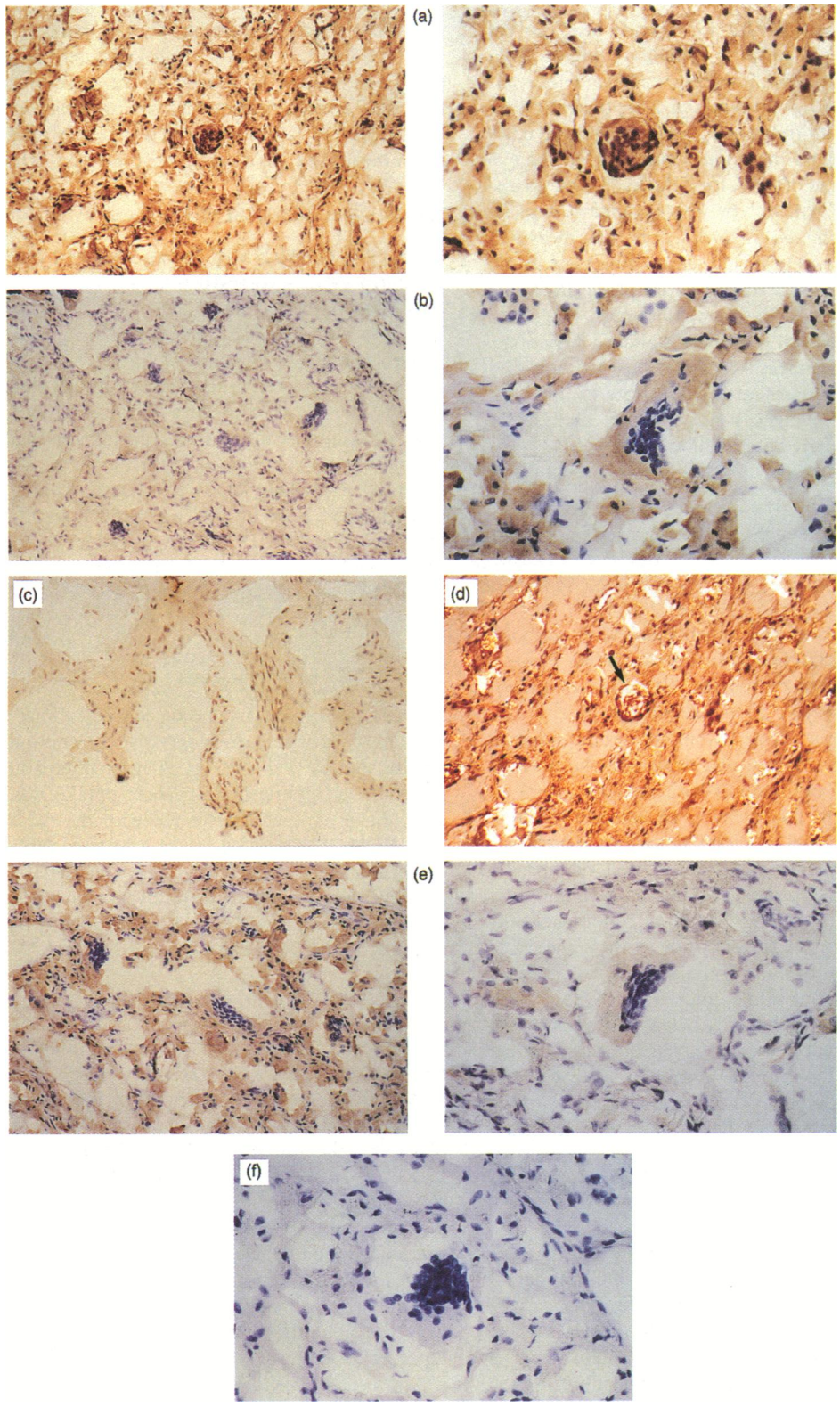

Figure 1. Immunohistochemistry of the pseudocapsule synovium around a loosened hip prosthesis by an anti-human MIF antibody and anti-CD68 antibody. The tissue specimens were stained with a Histofine SAB-PO kit as described in the Materials and Methods. (a1) The pseudocapsule synovium around a loosened hip prosthesis stained for the anti-MIF antibody (×100). (a2) The high magnification of (a1) (×400). (b1) The pseudocapsule synovium reacted with non-immune rabbit IgG as a control (×100). (b2) The high magnification of (b1) (×400). (c) A normal hip joint synovium stained with the anti-human MIF antibody (×100). (d) The pseudocapsule synovium observed by polarized light microscopy showing numerous intracellular briefringent foreign bodies (indicated by an arrow) appearing in white colour (×100). (e1) The pseudocapsule synovium around a loosened hip prosthesis stained for the anti-CD68 antibody (×100). (e2) The high magnification of (e1) (×400). (f) The pseudocapsule synovium reacted with non-immune mouse IgG as a control (×400).

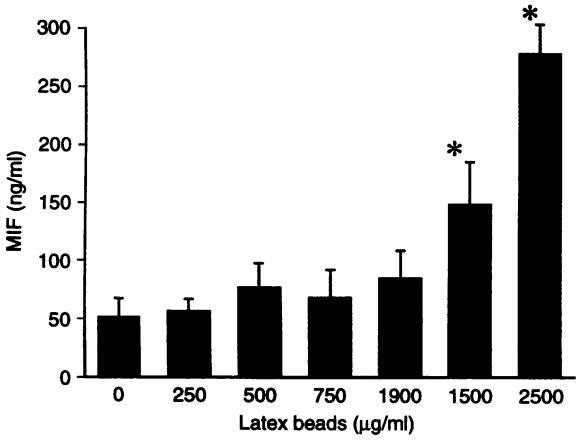

Figure 2. MIF released by RAW 264.7 macrophages after particle stimulation. The amount of MIF was determined by ELISA as described in the Materials and Methods. Each point is the mean ± SD of at least six experiments. $*P < 0{\cdot}01$, unpaired *t*-test.

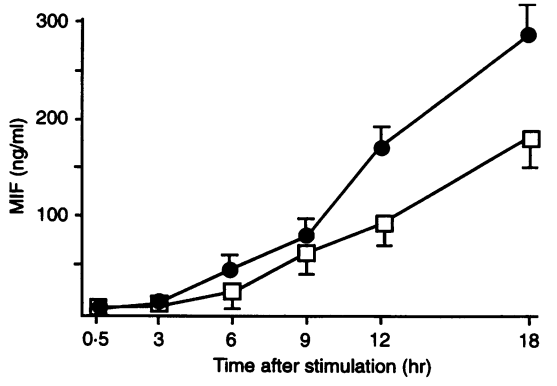

Figure 4. Time–course of particle-induced MIF release from RAW 264.7 cells. The cell cultures were incubated with medium (DMEM + 10% FCS) containing 2500 μg/ml of latex beads or medium as a control. Supernatants were collected at intervals as indicated. Each point is the mean ± SD of at least five experiments. (●), latex beads added; (□), control.

A complex immune system has evolved in vertebrates; however, phagocytosis still remains the principal effector mechanism for the ultimate disposal of invading foreign particles or unwanted cells.[31] Macrophages, the major phagocytes, can ingest particles by extending pseudopodia over the particle surface until the particle is totally enveloped. This process requires specific interaction between particle surface ligands and ligand-receptors located at the membranes of the pseudopodia.[32] From the data available to date, it is clear that at least two ligand–ligand receptor mechanisms mediate phagocytosis. The first mechanism, called opsonin-dependent phagocytosis, is a process via which ingestion of foreign particles coated with IgG or fragments of the third complement component can facilitate phagocytosis. The second mechanism, called opsonin-independent phagocytosis, mediates particle ingestion in the absence of immune opsonins. This phagocytic mechanism is important as a primary defence because the human body is often exposed to immunologically inert particles. In this context, it is of interest to determine in which mechanism MIF is involved.

We have often observed phagocytosis in the pseudosynovial tissues around loosened artificial joints where particulate wear debris of polyethylene is continuously generated. Synovial tissue is known to consist mainly of three different cell types:

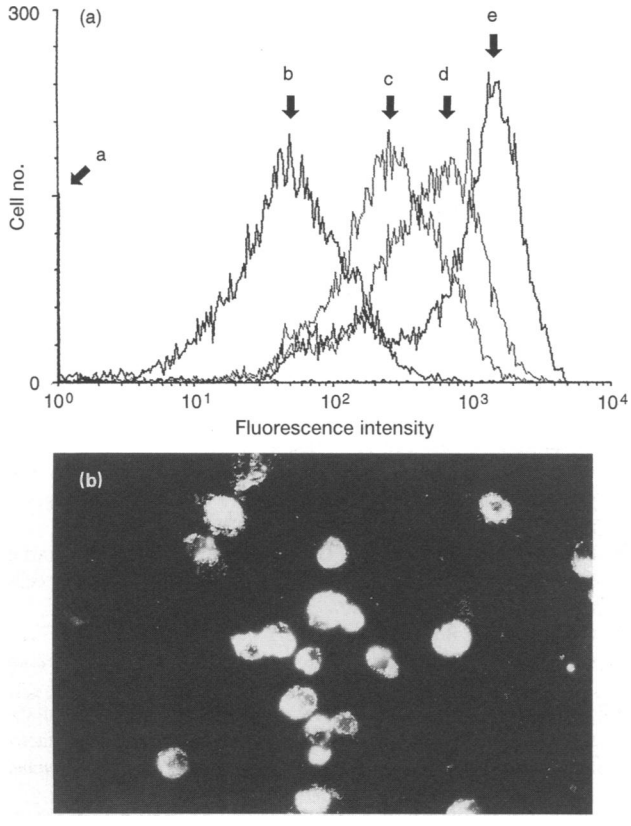

Figure 3. Flow cytometric analysis of RAW 264.7 macrophages after exposure to various concentrations of fluorescent latex beads. (a) The concentrations of latex beads added were 0, 100, 500, 1000 and 2500 μg/ml, indicated by arrows of a, b, c, d, and e, respectively. The result is typical of five studies on different samples. (b) Photomicrograph of fluorescence microscopic study of particle-challenged macrophages by the dose of 2500 μg/ml latex beads (×400).

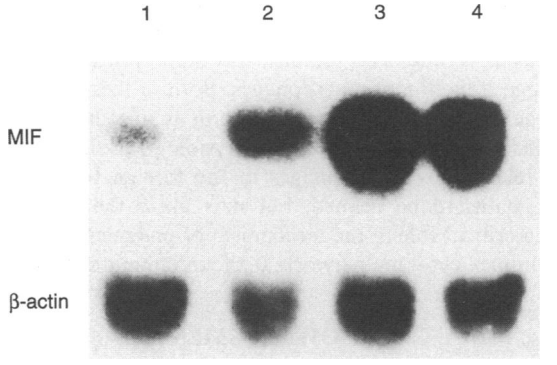

Figure 5. Northern blot analysis on the changes of MIF mRNA in RAW 264.7 cells. The cells were harvested at 12 hr after incubation with various concentrations of latex beads. Total RNA was extracted from the cells, and radiolabelled MIF and β-actin nucleotide probes as described in the Materials and Methods. Lanes 1, 2, 3 and 4 show MIF mRNA levels at the doses of 0, 500, 1000 and 2500 μg/ml, respectively. The relative MIF mRNA levels normalized by those of β-actin are shown at the bottom of each lane. The result is typical of three studies on different samples.

Table 1. Effect of MIF on phagocytosis of latex beads by RAW 264.7 cells

Concentration of MIF for pretreatment† (μg/ml)	20 min	120 min
0	27·07 ± 0·78‡	261·14 ± 4·59
1	23·15 ± 0·94	299·21 ± 12·42*
10	31·01 ± 6·23	413·25 ± 11·23**

*Significantly different from control ($P < 0·01$).

**Significantly different from control ($P < 0·0001$).

†Two-way analysis of variance demonstrated a significant difference with respect to MIF concentration ($P < 0·0001$) and time ($P < 0·0001$). There was significant interaction between the MIF concentration and time ($P < 0·0001$).

‡Fluorescent intensity (arbitrary unit) was expressed as the mean ± SD of three experiments.

macrophage-like cells, fibroblast-like cells and dendritic cells. A large proportion of the synovial cells consists of macrophage-like cells.[33] So far there has been no report regarding whether particulate wear debris generated from the surface of artificial joints is opsonized before ingestion. One possible mechanism may be non-specific adsorption of IgG onto such particles followed by binding and ingestion via FC receptors (FcR). In association with this process, our present *in vitro* data showed the elevation of MIF at both protein and mRNA levels in response to particle phagocytosis. This phenomenon was seen in the presence of FCS, but not in the absence of FCS. So far, it is known that fluorescent latex beads are readily phagocytosed both in the presence and absence of serum.[31] Thus, the phagocytosis mediated, at least in part, by MIF demonstrated in this study appeared to be an opsonin-dependent and FcR-mediated mechanism, though it needs further evaluation.

In conclusion, MIF is considered to play an important role in foreign body reaction. Apart from the classical effect known as migration inhibition and concentration of macrophages at the inflammatory loci, the present results showed two novel biological aspects of MIF. One was induction or promotion of tissue damage by regulating other inflammatory cytokines such as IL-1 and TNF-α, and the other promotion of the scavenger function of macrophages. Both of these phenomena are results of macrophage activation in an autocrine or paracrine fashion. The precise pathophysiological significance of MIF released by macrophages in the foreign body reaction still remains to be defined, but it is likely that the protein plays a critical role in the mechanism of phagocytosis through the complex cytokine network at the inflammatory locus.

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