Production and Release of Macrophage Migration Inhibitory Factor from Human Adipocytes

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Background and aim of the study: Macrophage migration inhibitory factor (MIF) has been identified as a critical mediator of inflammatory responses. Because of its potent migration inhibition activity, it regulates macrophage accumulation in tissues. We therefore analyzed whether human adipocytes produce MIF, in the search of candidate mediators of macrophage infiltration of obese adipose tissue. Methods: Human adipose tissue samples were obtained from various depots. The precursor cells were allowed to differentiate under defined adipogenic culture conditions. MIF expression was analyzed by RT-PCR, ELISA, and immunocytochemistry. Results: Human preadipocytes secreted MIF in a differentiation-dependent fashion with maximum concentrations at d 12, whereas MIF mRNA was detected in both undifferentiated and differentiated cells at relatively constant levels. Immunocytochemical analysis showed that MIF protein was present in preadipocytes and more pronounced in differentiated adipocytes. Freshly isolated mature adipocytes from sc, omental, and mammary depots released MIF at rates of up to 10,000 pg/ml/24 h. Most importantly, MIF production was positively correlated with donor body mass index. Secretion of MIF was not influenced by lipopolysaccharide, interferon-γ, or IL-4. The rates of MIF release from sc and omental adipocytes were similar but approximately 10 times higher compared with mammary adipocytes. Conclusions: Human preadipocytes and mature adipocytes from different depots spontaneously release substantial amounts of MIF. Expression levels were positively associated with donor body mass index. Hence, MIF may be an obesity-dependent mediator of macrophage infiltration of adipose tissue. (Endocrinology 146: 1006–1011, 2005)

Here is growing evidence that adipose tissue is not an inert lipid-storing organ but instead represents a multifunctional tissue that secretes a variety of factors that may directly contribute to obesity-related diseases such as atherosclerosis and type 2 diabetes mellitus (1, 2). Recent studies showed that obesity is associated with elevated serum levels of a wide range of inflammatory markers including C-reactive protein (CRP), IL-6, IL-18, and TNF-α and that elevated serum levels of these proteins are reduced after weight loss (3–7). Because it has been hypothesized that low-grade chronic inflammation may be etiologically related to the development of atherosclerosis and type 2 diabetes, obesity and adipose tissue-derived inflammatory mediators may play a crucial role in the pathogenesis of these diseases. In vitro studies established that both adipocytes and stromal cells of adipose tissue express and secrete cytokines and chemokines such as IL-6 and TNF-α (8–12). MIF is a pleiotropic cytokine that participates in the immune and stress response. It is a key mediator for the septic shock syndrome and also exerts proinflammatory activities (13). MIF was found to prolong the lifespan of macrophages by protecting them from apoptosis, thus maintaining inflammatory processes. Additional proinflammatory activity is generated by counteracting glucocorticoid-induced suppression of the immune system (14, 15) and by up-regulating TNF-α expression in peripheral immune cells (16).

MIF is expressed in both immune and nonimmune cells including various peripheral tissues (17–20). Recently, MIF was also identified as a product of mouse 3T3-L1 adipocytes (21, 22). In addition, MIF serum levels are elevated in patients with type 2 diabetes (23), and MIF expression is regulated by insulin and may be associated with insulin resistance (24–26). Recently, obese adipose tissue has been reported to exhibit severe macrophage infiltration (27, 28). Therefore, MIF may serve as a link between obesity and insulin resistance and may promote the development of type 2 diabetes.

The aim of this study, therefore, was to test the hypothesis that MIF is secreted by human adipose tissue and to investigate which factors regulate its expression.

Materials and Methods

Subjects

Adipose tissue samples (5–10 g) were obtained from young, healthy, normal-weight females [age < 40 yr, body mass index (BMI) < 27 kg/m²] undergoing elective mammary reduction or from the visceral and sc depot of patients undergoing abdominal surgery [age 49 ± 18 yr (range 22–91 yr), BMI 37 ± 15 kg/m² (range 21–70 kg/m²)]. All subjects were free of metabolic or endocrine diseases as assessed by routine laboratory investigations. The procedure for obtaining human adipose tissue was approved by the Ethical Committee of the Heinrich-Heine-Universität Düsseldorf.

Cell culture

Adipose tissue samples were prepared as described previously (29). Briefly, connective tissue and visible blood vessels were removed with
scissors, and fat lobules were cut into small pieces. The minced tissue was then digested in PBS (10 mmol/liter) containing 250 U/ml collagenase (Biochrom, Berlin, Germany) and 2% BSA (Sigma, Munich, Germany) for 90 min at 37 C in a shaking water bath. Stromal cells were collected by a 10-min centrifugation at 200 × g. The supernatants with the adipocytes were discarded. The sedimented cells were incubated in an erythrocyte lysis buffer (154 mmol/liter NH₄Cl, 5.7 mmol/liter KH₂PO₄, and 0.1 mmol/liter EDTA) for 10 min to remove red blood cells. Then, cells were filtered through a nylon mesh with a pore size of 150 μm to separate undigested material. To obtain a pure stromal cell fraction the cell suspension was filtered again through a cell strainer with a pore size of 70 μm. Finally, cells were resuspended in DMEM/Ham’s F-12 medium (vol/vol, 1:1) supplemented with 10% fetal bovine serum (Biochrom, Berlin, Germany) and seeded into 12-well culture plates (Biochrom) at a density of approximately 3 × 10⁴/cm². The serum-containing medium was replaced after 16 h by serum-free DMEM/F-12 medium supplemented with 10 mg/ml human transferrin (Sigma), 66 nmol/liter human insulin (Sigma), 100 nmol/liter cortisol (Aventis Pharma, Frankfurt, Germany), 0.2 nmol/liter T3 (Sigma) and 0.05 g/liter gentamycin (Invitrogen Life Technologies, Gaithersburg, MD). To induce adipocyte differentiation, cells were exposed to 1 μg/ml triptolide (Sankyo Europe, Düsseldorf, Germany) and 0.5 mM isobutyl-methylxanthine (Serva, Heidelberg, Germany) for the first 4 d. Medium was changed every other day. On d 16, approximately 50–70% of the cells had acquired an adipocyte phenotype detectable by multiple cytoplasmic lipid inclusions. Such cultures were incubated for 24 h with either 1 μg/ml lipopolysaccharide (Sigma) or 1 ng/ml interferon (IFN)-γ or 50 ng/ml IL-4 (R&D Systems, Minneapolis, MN), respectively.

**Suspension culture of human adipocytes**

In parallel experiments, mature adipocytes were isolated. In contrast to the before mentioned protocol a shorter collagenase digestion of only 60 min in Krebs-Ringer buffer (pH 7.4) containing 4% BSA was performed. Cells were carefully centrifuged and washed twice with Krebs-Ringer buffer, supplemented with 0.1% BSA. After a filtration step through a 250-μm nylon mesh (VWR International, Darmstadt, Germany) cells were allowed to recover from preparation for 24 h in DMEM/F12 supplemented with 2% BSA. After this period, 400 μl packed adipocytes in 4 ml DMEM/F12 + 0.25% BSA were incubated for 24 h either with IFN-γ (0.1 ng/ml) or IL-4 (5 ng/ml). Cells and the buffer medium were separately stored at −20 C for later analyses.

**Real-time RT-PCR**

All PCR reagents were purchased either from Applied Biosystems (ABI, Foster City, CA) and used according to the instructions of the manufacturer. Total RNA from 0.5 × 10⁶ cells was isolated with the NucleoSpin RNA II kit from Machery-Nagel (Düren, Germany) and transcribed with the iScript Synthesis kit (Bio-Rad, München, Germany). Quantitative PCR was performed in 20-μl reactions using an amount of 7.5 ng mRNA equivalent. 18S RNA was used to normalize for the starting amount of cDNA. Amplification was performed with the ABI 7000 sequence detection system using the following protocol: amplification was performed during 40 cycles (PCR initial activation step: 15 min, 95 C; denaturation: 30 sec, 95 C; annealing: 30 sec, 55 C; extension: 45 sec, 72 C). FAM (6-carboxylfluorescein)-labeled probes were used in the Assay on Demand platform from ABI. The following reference sequences were provided by the company: AGCCCGGAAGGTCTACATCAACT for MIF (accession no: NM_002415) (catalog no. Hs00236988_g1) and TG GAGGCGAAGTGGCTGGCCAGCAG for 18S RNA (accession no. x03205) (catalog no. Hs99999901_s1).

**Measurement of MIF protein**

Concentrations of MIF protein in cell culture supernatants were measured by using a highly sensitive sandwich ELISA (R&D Systems, Wiesbaden, Germany) with a detection limit of 20 pg/ml. All supernatants were analyzed in duplicates. Mean intra- and interassay variations were less than 5% and 20%, respectively.

**Immunohistochemistry**

For immunohistochemical detection a monoclonal mouse antihuman MIF antibody (R&D Systems, Minneapolis, MN) was used. Positive signals were detected using the horseradish peroxidase-diaminobenzidine (DAB) system (R&D Systems) according to the instructions of the manufacturer. Stromal cells isolated from adipose tissue were cultured under the conditions mentioned before. For immunohistochemistry, cells were washed with PBS and fixed with pure methanol for 2 h at −20 C. For isotype control, cells were treated in the same manner and incubated with a monoclonal IgG₁ antibody (R&D Systems).

**Measurement of glycerol-3-phosphate dehydrogenase (GPDH) activity**

GPDH activity was determined as a marker of adipose differentiation according to an established procedure (31). Briefly, cells were washed twice in PBS and harvested in a Tris-buffer (0.05 mol/liter Tris/HCl, 1 mmol/liter EDTA, 1 mmol/liter mercaptoethanol, pH at 7.4). After sonication of cells for complete cell lysis and centrifugation GPDH activity was assessed spectrophotometrically by addition of reduced nicotinamide adenine dinucleotide and dihydroxyacetone phosphate (Sigma). Specific activity was calculated and expressed as milliunits per milligrams of protein. For protein determination an established method including protein precipitation with trichloroacetic acid was used (31).

**Statistical analysis**

Results were expressed as mean ± s.d of at least three experiments in triplicate. For comparisons ANOVA was used. P < 0.05 was considered to be statistically significant. Possible associations between BMI and sc and omental MIF expression was described by Spearman’s rank correlation test.

**Results**

**MIF production and mRNA expression in cultured preadipocytes**

In the first set of experiments, MIF secretion into the culture medium was measured in cultured stromal cells from human adipose tissue undergoing in vitro adipose differentiation. To relate the course of MIF secretion with the differentiation process, GPDH activity was measured at defined time points. GPDH activity as a marker of adipose differentiation was undetectable on d 0 but increased continuously throughout the culture time until d 16 (Fig. 1A). During this time period, the original fibroblast-like cells acquired an adipocyte phenotype as assessed by multiple large lipid droplets.

After inoculation, human preadipocytes secreted substantial amounts of MIF into the culture medium. During the course of adipose differentiation, there was no significant increase in the release of MIF into the culture medium, although there was a trend toward higher MIF levels at d 12 and 16 (400–600 pg/ml) (Fig. 1B).

Additional experiments were performed to assess the expression of MIF mRNA in fat cells differentiating in vitro. During the differentiation process (between d 4 and 24), we detected substantial MIF mRNA expression in both undifferentiated and differentiated cells (Fig. 1C). 18S RNA was used for normalization.

**MIF release from freshly isolated human adipocytes**

In addition, MIF release from freshly isolated adipocytes from different fat depots was assessed. As shown in Fig. 2, mature fat cells secreted substantial amounts of MIF over a
24-h incubation period. Interestingly, sc abdominal adipocytes exhibited a much greater potency of MIF release compared with mammary fat cells (Fig. 2). On a per cell basis, freshly isolated fat cells from the abdominal region produced approximately 10 times more MIF than in vitro differentiated preadipocytes (data not shown).

When sc and omental adipocytes from single donors were compared, there was no difference between the two depots. There was a close positive correlation of MIF release into the culture medium (r = 0.7273, P = 0.0074; n = 12) (Fig. 3A). The secretion of MIF from adipocytes depended on the donor BMI. As shown in Fig. 3B, MIF release from adipocytes and BMI were positively correlated in sc (r = 0.80; P = 0.002) and omental (r = 0.56; P = 0.057) fat cells.

**Immunocytochemistry**

To further demonstrate MIF expression by adipocytes, immunohistochemistry was performed. These experiments revealed a specific cytoplasmatic staining for MIF in preadipocytes and, even more pronounced, in in vitro differentiated adipocytes (Fig. 4, C and F). The finding that adipocytes dem-

![Fig. 1](image1.png)  
**Fig. 1.** MIF secretion into the culture medium of in vitro-differentiating human adipocytes compared with control cells without adipogenic factors. A, Time-course of adipose differentiation assessed by the development of GPDH activity in human preadipocytes in primary culture (*, P < 0.05; **, P < 0.01). B, MIF concentrations in culture supernatants 24 h after addition of fresh medium. Data are given as mean ± SD of three experiments performed in duplicate. C, MIF mRNA expression during the course of differentiation compared with undifferentiated control cultures. Data are given as mean ± SD of four independent experiments in triplicates.

![Fig. 2](image2.png)  
**Fig. 2.** Comparison of MIF release into the culture medium from mature human adipocytes maintained in DMEM/F12 medium supplemented with 2% BSA. Cells were kept in suspension culture for 24 h in a 1:10 dilution. MIF concentrations in supernatants from sc abdominal and mammary adipocytes after 24 h.

![Fig. 3](image3.png)  
**Fig. 3.** A, Comparison of MIF release from omental and sc adipocytes after 24 h of suspension culture from 12 individual donors. Results reveal a high correlation among both depots (Spearman’s rank correlation: r = 0.73, P = 0.007). B, Effect of BMI on MIF release from omental (om) and sc adipocytes after 24 h of culture (Pearson correlation, om: r = 0.56, P = 0.057 (n = 12); sc: r = 0.80, P = 0.002 (n = 12).
onstrate a positive staining for MIF protein is arguing against the possibility that contaminating cells of the stromal fraction
are responsible for MIF production in the supernatants.

**Effect of lipopolysaccharide (LPS), IFN-γ, IL-4, and insulin on MIF release**

The possible immunoregulation of MIF secretion from fat cells was analyzed by exposing *in vitro* differentiated human adipocytes to either LPS, or to the antagonistic cytokines IFN-γ and IL-4. As demonstrated in Fig. 5, MIF secretion was not affected upon exposure to 1 μg/ml LPS. MIF production was also not modified by the addition of 1 ng/ml IFN-γ, an inducer of a Th-1 response, or of 50 ng/ml IL-4, a stimulator of a Th-2 response. This was true for *in vitro* differentiated adipocytes from the sc, the omental, and the mammary adipose tissue depot. Similar data were obtained when freshly isolated mature adipocytes were exposed to these stimuli for 24 h (data not shown).

In addition, because insulin has been shown to be a possible regulator of MIF secretion, administration to the cells was tested at a supraphysiological concentration of 66 nM. Incubation with insulin for 24 h neither increased MIF mRNA expression (mean increase 1.42-fold, range 0.2- to 4.4-fold, n = 8, P > 0.05) nor MIF production by mature adipocytes (mean ratio of MIF levels in insulin vs. control cultures 1.0, range 0.6–1.4, n = 12, P > 0.05 for sc adipocytes; mean ratio 0.9, range 0.7–1.0, n = 4, P > 0.05 for omental adipocytes.

**Discussion**

In this study, we investigated the capacity of primary human adipocytes to produce and secrete MIF by using ELISA, quantitative RT-PCR and immunohistochemical staining. Our experiments clearly indicate that cultures of preadipocytes from human adipose tissue express MIF spontaneously. The adipose differentiation process of the cells was paralleled by an increase of MIF secretion. Analyses by quantitative RT-PCR revealed the presence of MIF mRNA in cultures of differentiating human preadipocytes. Cultures of preadipocytes may contain a small fraction of contaminating monocytes/macrophages that are known to be an important production site for MIF (13). We therefore conducted immunocytochemical studies using MIF-specific antibodies and isotype controls. MIF-specific staining was detectable and was more prominent in cells from differentiated vs. control cultures. Most importantly, the strongest staining for MIF was observed in mature adipocytes, recognizable by the accumulation of lipid droplets.

Freshly isolated mature adipocytes also spontaneously secreted substantial amounts of MIF during a 24-h culture period. Interestingly, comparative experiments also demon-
also analyzed the effects of fat depot and donor BMI on MIF secretion, which have not been investigated before.

Another novel and important finding of this study is that MIF secretion from both sc and omental adipocytes was closely correlated with the BMI of the individual donor. This could be of particular significance in view of the recently demonstrated association of obesity with macrophage infiltration in adipose tissue (27, 28). Therefore, MIF may qualify as a candidate adipocyte product contributing to macrophage infiltration of fat tissue in obesity. Monocyte chemoattractant protein-1 (MCP-1) is another mediator that has been discussed in the context of macrophage infiltration in obesity (27, 28). Interestingly, MIF has been reported to counteract MCP-1-mediated chemotaxis of human peripheral blood monocytes (34). Because adipocytes cosecrete MIF and MCP-1 (our unpublished data), MIF is a potential candidate that may contribute to macrophage accumulation in obese fat tissue. This aspect merits further attention.

Another interesting finding of our study was the lack of responsiveness of mature adipocytes from sc, omental, and mammary to LPS, IFN-γ, or IL-4. Although sufficiently high concentrations of these immunostimulatory compounds were used in the culture models that potently induced or modulated cytokine secretion in monocytes/macrophages, dendritic cells and endothelial cells (35, 36) (and unpublished data), the release of MIF into the culture medium remained unaffected. This observation is important from a technical point of view because it confirms the absence of monocytes/macrophages in our culture system. The latter cells are well known to secrete increased amounts of MIF upon stimulation with LPS or IFN-γ (37–39). In addition, these data also suggest that the immunological properties of adipocytes may be different from those of innate immune cells with respect to the regulation of MIF expression. To date, little is known about the functional role of MIF in metabolic disorders such as type 2 diabetes and in atherosclerosis. As recent findings in murine cells suggest, insulin seems to be a potential regulator of MIF expression and secretion (24). In contrast to these findings insulin didn’t seem to have an intrinsic effect on neither mRNA nor protein level in the human model arguing for the constitutive character of MIF secretion from fat cells. However, recent studies indicated that MIF serum concentrations are elevated in subjects with type 2 diabetes mellitus (23) or insulin resistance (26). Whether MIF is directly involved in the pathophysiological processes leading to insulin resistance and atherosclerosis or simply an innocent bystander remains to be determined.

Taken together, our experiments show that adipocytes spontaneously express and secrete MIF protein. Differences in the amounts of MIF secretion were observed between adipocytes from different fat depots. An intriguing observation was that constitutive MIF secretion from adipocytes increased with increasing BMI of the donor. From this observation and the known physiological function of MIF, it is tempting to speculate that this cytokine contributes to macrophage infiltration into adipose tissue in obesity.

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