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Tumor Necrosis Factor-α Regulates the Gene Expression of Macrophage Migration Inhibitory Factor through Tyrosine Kinase-Dependent Pathway in 3T3-L1 Adipocytes[1]

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Macrophage migration inhibitory factor (MIF) has been rediscovered as a proinflammatory cytokine, pituitary hormone, and glucocorticoid-induced immunoregulator. We have recently identified the expression of MIF in adipocytes and found that tumor necrosis factor (TNF)-α stimulates its secretion from 3T3-L1 adipocytes. Since adipocytes are regarded as a potential source of various biologically active substances, we examined in more detail the effect of TNF-α on MIF expression in 3T3-L1 adipocytes in the present study. We found that TNF-α induced MIF mRNA in dose- and time-dependent manners. After stimulation with TNF-α, the amount of intracellular MIF protein was unchanged or slightly decreased, concomitant with increased release of this protein into the extracellular space. This observation indicates that TNF-α stimulates MIF secretion from the constitutively expressed intracellular pool of 3T3-L1 adipocytes and promotes *de novo* synthesis of MIF. From evaluation of the mechanism of MIF gene expression, we found that tyrosine kinase inhibitors, either genistein or herbimycin A, suppressed the MIF mRNA induction by TNF-α. The results suggest the possibility that upregulation of MIF mRNA expression by TNF-α is mediated by a tyrosine kinase-dependent pathway. Taken together, the present observations shed light on the role of MIF in the metabolism of obesity and diabetes.

Key words: adipocyte, diabetes, macrophage migration inhibitory factor, tumor necrosis factor α, tyrosine kinase.

From several lines of recent evidence, adipocytes are recognized as not merely lipid-laden cells serving as the major repository of energy stores, but also as endocrine cells regulating systemic energy balance through secretion of bioactive molecules. In brief, adipocytes have the potential to secrete leptin, adipsin, type 1 plasminogen activator inhibitor (PAI-1), and tumor necrosis factor-α (TNF-α). Leptin, the *ob* gene product, is able to modulate food intake and energy expenditure (*1*). Adipsin, the rodent homologue of human complement factor D, is a serine protease that can activate the alternative complement pathway (*2, 3*). PAI-1 contributes to the regulation of fibrinolysis through inactivation of both tissue-type and urokinase-type plasminogen activators (*4*), and it is reported that adipose tissue is the major source of the elevated plasma PAI-1 often observed in obesity (*5*). TNF-α is overexpressed in adipose tissues of obese rodents and humans (*6-9*), and exogenous administration of TNF-α has the potential to affect lipid and glucose metabolisms (*7*). Moreover, TNF-α has been demonstrated to affect the expression of leptin (*10*) and PAI-1 (*11*) in adipose tissue. From these findings, the adipocyte-derived secretory substances appear to constitute a local regulatory network of the metabolic pathway in adipose tissue.

Macrophage migration inhibitory factor (MIF) was identified for the first time as a T cell-derived cytokine that inhibits the migration of macrophages and concentrates them at inflammatory loci (*12, 13*). We cloned rat MIF cDNA and reported its physicochemical properties, including the tertiary structures of both human and rat MIF (*14-19*). Although MIF had long been considered to be produced only by activated T cells, recent reports have demonstrated its expression in many cells of various organs (*20-26*). At present, MIF has been identified as a proinflammatory cytokine, a pituitary hormone, and a glucocorticoid-induced immunoregulator involved in various immunological and inflammatory events (*27*). Recently, we found high expression of MIF mRNA and MIF protein in the rat epididymal fat pad and murine 3T3-L1 adipocytes (*28*). Accordingly, MIF is considered to be a previously unrecognized novel "adipocytokine." This protein is synthesized and secreted by adipocytes and is likely to have broad-spectrum pathophysiological roles beyond the immunological system in an autocrine/paracrine or endocrine fashion.

During the course of our MIF study, we also found that

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the MIF secretion from 3T3-L1 adipocytes was augmented by TNF-α. TNF-α is profoundly involved in the lipid and glucose metabolisms of adipocytes. For example, TNF-α is elevated in the state of obesity, which may cause insulin resistance (*7*). Thus, in this study, we focused on the role of TNF-α, particularly in the kinetics of MIF mRNA expression in adipocytes. We here report evidence that TNF-α may upregulate MIF mRNA expression *via* a tyrosine kinase-dependent pathway.

MATERIALS AND METHODS

Reagents—The following materials were obtained from commercial sources: nitrocellulose membrane filters from Millipore (Bedford, MA, USA); Isogen RNA extraction kit from Nippon Gene (Tokyo); Genistein and Staurosporine from Calbiochem (La Jolla, CA, USA); Herbimycin A and Okadaic acid from Wako (Osaka); heat-inactivated fetal calf serum (FCS) from Hyclone Labs (Logan, UT, USA); dexamethasone, d-biotin, penicillin, streptomycin, insulin, and isobutylmethylxanthine from Sigma (St. Louis, MO, USA); horseradish peroxidase-conjugated goat anti-rabbit antibody from Pierce (Rockford, IL, USA); Histofine SAB-PO kit from Nichirei (Tokyo); Protein A Sepharose from Pharmacia (Uppsala, Sweden); random primer labeling kit from Takara (Kyoto); [α-^{32}P]dCTP from Du Pont-NEN (Boston, MA, USA), and ECL kit from Amersham (Buckinghamshire, UK). All other chemicals used were of analytical grade. Human recombinant TNF-α was a generous gift from Dainippon Pharmaceutical (Tokyo).

A polyclonal anti-rat MIF antibody was generated by immunizing New Zealand white rabbits with purified recombinant rat MIF as described (*25, 26*). The IgG fractions (4 mg/ml) were prepared using Protein A Sepharose according to the manufacturer's protocol.

Cells—3T3-L1 murine adipose cells were purchased from the American Type Culture Collection (Rockville, MD, USA). These cells were designated preadipocytes. They were grown in Dulbecco's MEM-25 mM HEPES containing 10% FCS, 100,000 U/liter penicillin, 100 mg/liter streptomycin, and 8 mg/liter *d*-biotin (medium A) in a humid atmosphere of 5% CO_2 at 37°C. Post-confluent cells were exposed to medium A supplemented with 0.5 mM isobutylmethylxanthine, 1 μM dexamethasone, and 10 μg/ml insulin to induce differentiation. After 72 h, the medium was replaced with medium A supplemented with 5 μg/ml insulin, and the thereafter cells were fed every 3 days. The cells were used as adipocytes 12-15 days after induction.

Northern Blot Analysis—Northern blot analysis was carried out as previously described (*14*). In brief, total RNA from murine 3T3-L1 adipocytes was extracted and separated by electrophoresis on agarose gels containing 0.6 M formaldehyde, and blotted onto nylon membrane filters. Hybridization was carried out with a rat MIF cDNA probe, radiolabeled with [α-^{32}P]dCTP by use of a random primer labeling kit. The hybridization was performed in a solution containing the radiolabeled rat MIF cDNA probe, 50% formamide, 0.75 M NaCl, 1% sodium dodecyl sulfate (SDS), 20 mM Tris-HCl (pH 7.5), 2.5 mM EDTA, 0.5× Denhardt's solution (1×Denhardt's: 0.2% bovine serum albumin, 0.2% polyvinylpyrolidone, 0.2% Ficoll), and 10% dextran sulfate at 42°C overnight. After hybridization, the filters were washed with 0.2×standard saline citrate (SSC) (1×SSC: 0.15 M NaCl, 0.015 M sodium citrate, 0.1% SDS) at 65°C and subjected to autoradiographic analysis. As a control, the filters were probed with radiolabeled rat β-actin prepared essentially as described above. Hybridization signals were digitized and quantified with an MCID Image Analyzer (Fuji Film, Tokyo). Background values were determined from equivalent surface areas near each hybridization signal and were subtracted from each value before normalization.

Western Blot Analysis—Western blot analysis was carried out by the method of Towbin *et al.* (*29*). In brief, samples were dissolved in 20 μl of Tris-HCl (50 mM, pH 6.8) containing β-mercaptoethanol (1%), SDS (2%), glycerol (20%), and bromophenol blue (BPB) (0.04%), and heated at 100°C for 5 min. They were then subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described (*30*). The electrophoresed proteins were transferred onto a nitrocellulose membrane at 50 mA for 1 h using a semi-dry blot transfer apparatus (Bio-Rad). The membrane was intensively washed with phosphate-buffered saline (PBS), then incubated with the anti-rat MIF polyclonal antibody (1 : 1,000 in dilution of 4 mg/ml IgG) for 1 h at room temperature, and with peroxidase-conjugated anti-rabbit IgG for 1 h at room temperature. Proteins were then visualized with an ECL kit as recommended by the manufacturer. Protein concentration was determined with a Micro BCA protein assay reagent kit.

Immunohistochemistry—Immunohistochemistry for adipocytes on a culture slide glass was performed using a Histofine SAB-PO kit according to the manufacturer's protocol. Cells were first immersed in methanol containing 0.3% H_2O_2 for 30 min to block endogenous peroxide activity. Following three washes in cold sucrose-PBS (PBS containing 10% sucrose), non-specific staining was blocked by incubation with 10% normal goat serum in PBS for 10 min. The sample was then incubated with an anti-rat MIF antibody (1 : 500 dilution of 4 mg/ml) for 30 min at room temperature. Following three washes in cold sucrose-PBS, it was incubated with a biotinylated goat anti-rabbit IgG and avidin-biotin-peroxidase complex at room temperature for 30 min. After three additional washes, the reaction was developed in 3,3′-diaminobenzidine tetrahydrochloride containing 0.01% H_2O_2. The sections were counterstained with hematoxylin. Lastly, the sample was washed in distilled water, and the tissue sections were rinsed well with distilled water and mounted in alkylacrylates.

Enzyme-Linked Immunosorbent Assay (ELISA) of MIF—The anti-rat MIF antibody dissolved in PBS (50 μl) (4 μg/ml) was added to each well of a 96-well microtiter 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% BSA for blocking and left for 20 min at room temperature. The samples of culture supernatants of adipocyte cell line 3T3-L1 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 h at room temperature. After the plate was washed three times with PBS containing 0.05% Tween 20 (washing buffer), 50 μl of biotin-conjugated anti-MIF antibody was added to each well. Following incubation for 1 h at room temperature, the plate was again washed three times with the washing buffer. Avidin-conjugated horse-

radish peroxidase was added to each well, and the microtiter plate was incubated for 15 min at room temperature. After washing three times, the substrate solution (10 ml) containing 8 mg of *o*-phenylenediamine and 4 μl of 30% H_2O_2 in citrate phosphate buffer (pH 5.0) was prepared and the substrate solution (50 μl) was added to each well. After incubation for 20 min at room temperature, the reaction was terminated with 25 μl of 4 N sulfuric acid. The absorbance was measured at 492 nm using an ELISA plate reader (Bio-rad, Model 3550).

RESULTS

Effects of TNF-α on MIF Expression in 3T3-L1 Adipocytes—After treatment of 3T3-L1 adipocytes with human recombinant TNF-α for 24 h, the level of MIF mRNA increased in a dose-dependent manner within the concentration range from 0.5 to 50 nM as examined by Northern blot analysis (Fig. 1A). We next performed a time-course analysis of MIF mRNA expression. The MIF mRNA was induced as early as 6 h after the addition of 50 nM TNF-α, and increased by approximately 5-fold after 24 h (Fig. 1B). This increase persisted for at least 48 h after the stimulation (data not shown). On the other hand, Western blot analysis demonstrated only a slight decrease of intracellular MIF protein during the TNF-α treatment, suggesting a stimulatory effect of TNF-α on MIF excretion from the intracellular MIF pool of adipocytes (Fig. 2).

In parallel with these changes, MIF concentrations in the culture media of these cells were significantly increased by TNF-α stimulation. As shown in Fig. 3, an approximately 6-fold increase of MIF concentration (20.9±1.8 ng/ml) was observed 24 h after TNF-α stimulation compared with that in the absence of the stimulation (3.4±0.4 ng/ml). This supports the result of Western blot analysis, showing that

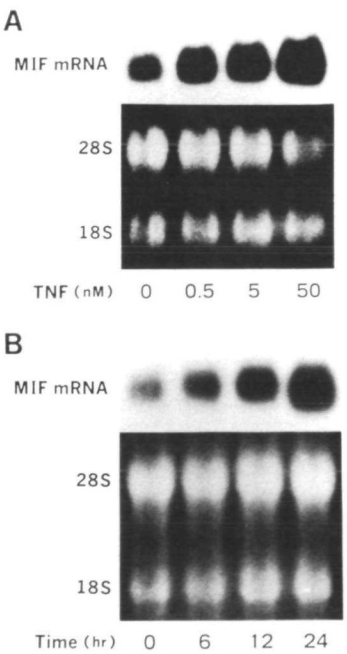

Fig. 1. **Effects of TNF-α on MIF mRNA expression in 3T3-L1 adipocytes.** Northern blot analysis was carried out as described in "MATERIALS AND METHODS." (A) 3T3-L1 adipocytes were incubated in 60-mm culture dishes with 0, 0.5, 5, and 50 nM TNF-α for 24 h. (B) The cells were incubated with 50 nM TNF-α for the indicated times. Results are representative of three independent experiments.

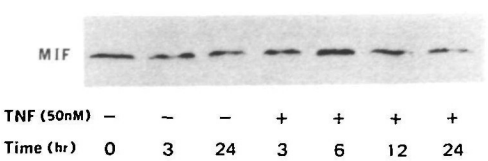

Fig. 2. **Effect of TNF-α on the amount of intracellular MIF protein in 3T3-L1 adipocytes.** Western blot analysis was performed as described in "MATERIALS AND METHODS." The cells were incubated with 50 nM TNF-α for the indicated times. Results are representative of three independent experiments.

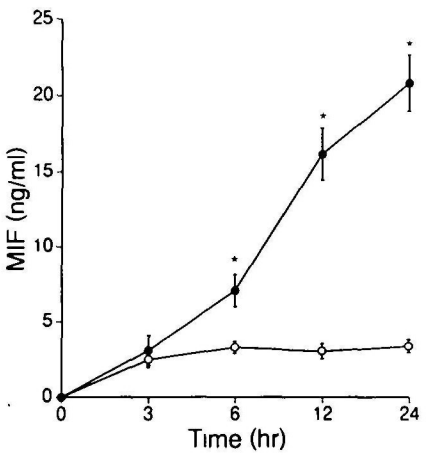

Fig. 3. **Time course analysis of TNF-α-induced MIF release by 3T3-L1 adipocytes.** MIF content in the culture medium was measured at the indicated times using ELISA as described in "MATERIALS AND METHODS." The post-confluent preadipocytes in 60-mm dishes were induced to differentiate. The cells were incubated in 2 ml of medium in the presence (●) or absence (○) of 50 nM TNF-α. $^*p<0.001$ ($n=6$).

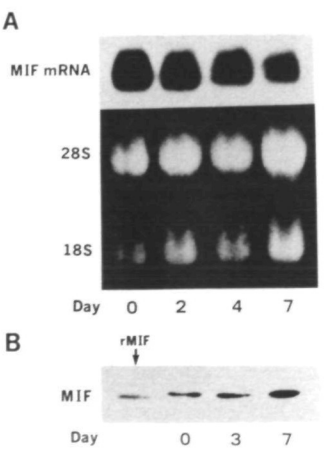

Fig. 4. **MIF expression in association with adipose differentiation.** 3T3-L1 cell differentiation was carried out from Day 0 to Day 7 after treatment with isobutylmethylxanthine, dexamethasone and insulin as described in "MATERIALS AND METHODS." (A) MIF mRNA and (B) intracellular MIF protein were quantified at the indicated time after induction of differentiation. rMIF: recombinant MIF.

TNF-α had the potential to stimulate the release of the intracellular MIF protein into the culture media.

MIF Expression in Association with Cell Differentiation—Several reports indicate that TNF-α causes "de-differentiation" of adipocytes (*31-33*). So we examined MIF expression of 3T3-L1 cells before induction of adipose differentiation, and compared it with TNF-α-induced changes in the MIF expression level in mature adipocytes. MIF mRNA was more abundant in confluent preadipocytes than in adipocytes (Fig. 4A). In contrast, a marked increase of intracellular MIF protein was observed in parallel with adipose differentiation (Fig. 4B). This observation was further confirmed by immunohistochemical analysis where MIF protein was clearly detected in mature adipocytes but not in preadipocytes (Fig. 5). MIF protein in mature adipocytes was mostly localized in the cytoplasm, which was in agreement with our previous reports using the rat epididymal fat pad and other tissues (*23-25, 28*).

This apparent discrepancy between the levels of mRNA and intracellular protein would be explained if preadipocytes secrete more MIF protein than adipocytes. However, MIF concentrations in the culture media of preadipocytes were unexpectedly lower than those of mature adipocytes (data not shown). This suggested accelerated turnover of MIF protein in preadipocytes compared to mature adipocytes, though the precise mechanism remains to be elucidated.

Effects of Tyrosine Kinase Inhibitors on MIF mRNA Expression Induced by TNF-α—Regulation of MIF gene expression is still poorly understood. In this study, we tried

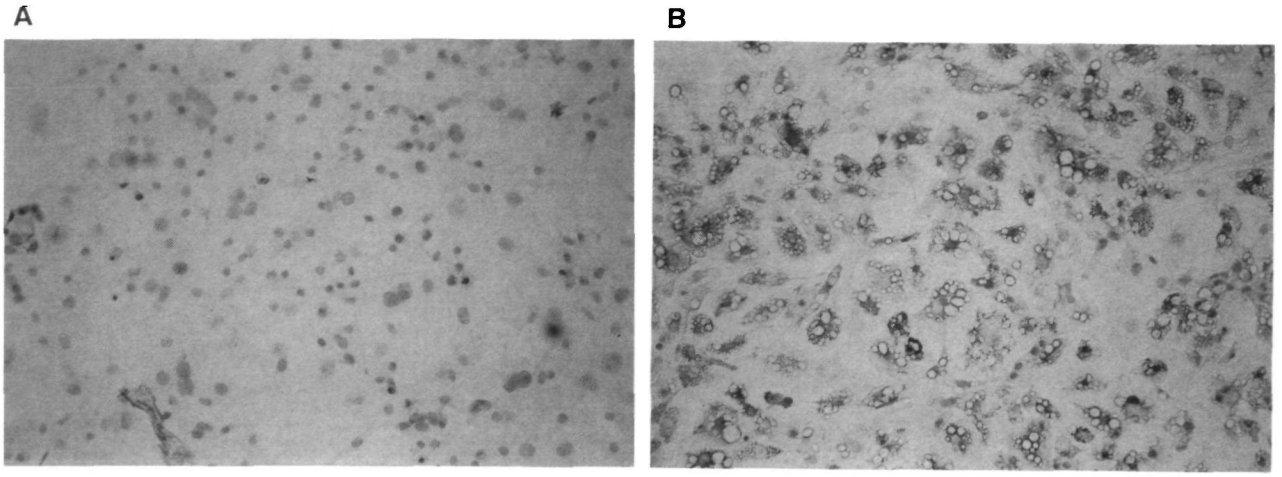

Fig. 5. **Immunohistochemistry of 3T3-L1 cells for MIF.** Confluent preadipocytes (A) and differentiated adipocytes (B) were prepared on the same glass slide, and fixed and stained as described in "MATERIALS AND METHODS" (×400). The control sample prepared by addition of excess rat MIF (1,000-fold) to the primary antibody showed no positive staining (data not shown).

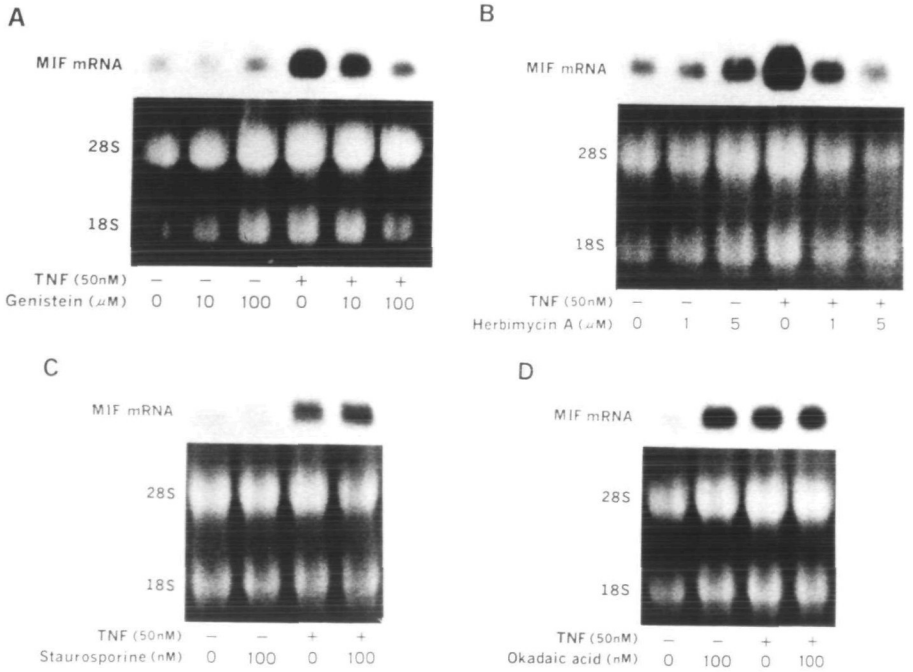

Fig. 6. **Effects of kinase/phosphatase inhibitors on TNF-α-induced MIF mRNA expression.** Northern blot analysis was carried out as described in "MATERIALS AND METHODS." 3T3-L1 adipocytes were preincubated for 30 min in the presence of genistein (A), herbimycin A (B), staurosporine (C), or okadaic acid (D). Then they were incubated for 20 h with or without 50 nM TNF-α.

to find a clue within the signal transduction pathway responsible for the effect of TNF-α on MIF mRNA in this adipocyte culture system. TNF-α is known to stimulate several intracellular signaling pathways, including activation of protein kinases (*34*). We here examined whether genistein, a widely used tyrosine kinase inhibitor (*35*), modulated the effect of TNF-α on MIF mRNA expression. When genistein was coincubated with TNF-α for 24 h, the tyrosine kinase inhibitor suppressed the TNF-α-induced MIF mRNA in a dose-dependent manner (Fig. 6A). A similar result was obtained by the use of herbimycin A, a structurally-distinct tyrosine kinase inhibitor from genistein (*36*). The upregulation of MIF mRNA by TNF-α was almost completely blocked by 5 μM herbimycin A (Fig. 6B). This effect was specific for tyrosine kinase inhibitors, because neither the serine/threonine kinase inhibitor staurosporine nor the serine/threonine phosphatase inhibitor okadaic acid affected TNF-α-induced MIF mRNA expression (Fig. 6, C and D). In contrast, okadaic acid induced MIF mRNA in unstimulated cells (Fig. 6D), but this needs further evaluation. Nonetheless, these results indicated that activation of tyrosine kinase might be essential for the induction of MIF gene expression by TNF-α.

DISCUSSION

TNF-α has been found to be a critical component in the mechanism of insulin resistance (*7*). We have recently reported the constitutive expression of MIF mRNA and MIF protein in 3T3-L1 adipocytes and shown that TNF-α augmented the excretion of MIF protein into the extracellular space (*28*). However, the mechanism of the TNF-α effect on MIF mRNA expression has not been fully investigated. In the present study, we showed that TNF-α markedly upregulated MIF mRNA of 3T3-L1 adipocytes in dose- and time-dependent manners. The amount of intracellular MIF protein was unchanged or slightly decreased by the TNF-α stimulation, whereas MIF contents in the culture media were significantly increased. This observation suggests a possible mechanism by which TNF-α stimulates the release of preserved intracellular MIF concomitant with MIF mRNA expression and *de novo* protein synthesis. Indeed, preadipocytes secrete less MIF than mature adipocytes, even though preadipocytes express more MIF mRNA but store much less the MIF protein.

The remarkable change in the MIF expression level in the distinct stages of adipose differentiation indicates the possibility that MIF may be profoundly involved in the process of cell differentiation and dedifferentiation. This possibility is supported by the fact that elevation of MIF mRNA expression was observed in parallel with differentiating cells of the lens (*22*). On the other hand, TNF-α is reported to cause "dedifferentiation" of mature adipocytes (*31-33*). TNF-α suppresses several adipocyte-specific genes such as PPARγ (*33*), C/EBPα (*37*), GLUT4 (*6, 37*), and adipsin (*6*). From the morphological features of mature adipocytes, TNF-α appears to be able to reduce intracellular lipid accumulation (*33*). Accordingly, it may be hypothesized that the MIF protein turnover rate in preadipocytes is much more accelerated by TNF-α, that is, MIF may be rapidly metabolized for use in cell differentiation, resulting in an apparent decrease of intracellular MIF protein in spite of a high expression level of MIF mRNA. More precise experiments, *e.g.*, pulse-chase biosynthetic studies using [^{35}S]methionine, are required to assess the metabolic processing of intracellular MIF protein.

TNF-α exerts its biological functions by binding to its specific receptors (TNFRs), p55 (type 1), and p75 (type 2) (*38*), which have been identified in adipocytes (*39*). Because the human recombinant TNF-α that we used in this study is reported to bind only to murine p55 TNFR, TNF-α-induced synthesis and release of MIF is considered to be mediated by this type of receptor. TNF-α is known to activate various intracellular signal transduction pathways (*34*). A number of studies have demonstrated that TNF-α activates tyrosine kinases in various cell types, and several genes are reported to be regulated by this protein through a tyrosine kinase-dependent process. For example, TNF-α-induced expression of some adhesion molecules is suppressed by tyrosine kinase inhibitors such as genistein (*40-42*). Genistein suppresses TNF-α-induced PAI-1 upregulation (*43*). In this study, we found that genistein and herbimycin A almost completely blocked TNF-α-induced MIF gene expression. Thus, it is strongly suggested that the activation of tyrosine kinase is essential for TNF-α-induced MIF expression. To the best of our knowledge, there has been no report on MIF mRNA expression in association with the signal transduction pathways. This is the first evidence to indicate the involvement of a particular signaling pathway in the regulation of MIF gene expression.

Nuclear factor kappa B (NFκB) is an essential component for regulation of gene expressions by TNF-α (*34*). Since the potential enhancer/promoter motifs of the mouse MIF gene contain one NFκB site (*44*) and TNF-α-induced activation of NFκB is inhibited by a tyrosine kinase inhibitor (*45*), it is likely that this potent transcription factor may regulate the tyrosine kinase-dependent upregulation of MIF mRNA initiated by TNF-α. Indeed, NFκB is activated by LPS through a tyrosine kinase-dependent pathway (*46*) as in the case in which MIF mRNA is induced by this endotoxin (*21*). In addition, it is of further interest that administration of an anti-MIF antibody decreases lethality in LPS-injected animals (*21*), and a similar inhibitory effect is also produced by a tyrosine kinase inhibitor (*47*). Hence, the reduction of LPS-induced lethality by the tyrosine kinase inhibitor may be due to the suppression of LPS-induced MIF expression, though this needs further evaluation.

Adipose tissue, occupying sometimes more than 30-40% of total body weight in the state of obesity, is regarded as a huge endocrine organ to produce a variety of cytokines such as TNF-α and MIF (*7, 28*). A number of studies indicate that TNF-α overexpressed in adipose tissue may play an important role in the pathogenesis of insulin resistance in obesity and diabetes (*6-9*). It should be noted that the stimulation of p55 TNFR by human recombinant TNF-α, which we used in this study, was sufficient to cause insulin resistance in 3T3-L1 adipocytes (*39*). Therefore, induction of MIF expression by TNF-α may be mediated by the identical TNFR in adipocytes. We demonstrated that TNF-α enhances MIF mRNA expression of adipocytes and secretion of intracellular MIF into extracellular space. In a similar manner, TNF-α facilitates MIF secretion in macrophages, and MIF in turn induces the secretion of

TNF-α (*20*). Thus, MIF might mediate, at least in part, the pathophysiological effect of TNF-α in adipose tissue. At present, we consider that TNF-α-induced MIF may further facilitate the adipose expression of TNF-α *via* a positive feedback loop, which boosts the effect of TNF-α.

Finally, we showed for the first time evidence that TNF-α-induced MIF gene expression might be mediated by a tyrosine kinase-dependent pathway. Recently, an interesting finding was reported that MIF overrides glucocorticoid-induced inhibition of secretion of cytokines, including TNF-α (*48*). Since glucocorticoids elicit profound effects on glucose and the fatty acid metabolisms in adipocytes, it is likely that glucocorticoids, MIF and TNF-α may functionally interact in a complex cytokine network for the regulation of the metabolism of adipose tissue. Very recently, it was found that MIF was secreted by pancreatic β cells in a glucose-dependent manner, indicating that MIF might be an autocrine regulator for insulin secretion (*49*). Taken together, these findings, including our present results, indicate that adipocyte-derived MIF is likely to play an important role in lipid and carbohydrate metabolisms, and provide further understanding of the pathophysiological role of MIF beyond the immunological system.

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REFERENCES

1. Caro, J.F., Sinha, M.K., Kolaczynski, J.W., Zhang, P.L., and Considine, R.V. (1996) Leptin: the tale of an obesity gene. *Diabetes* **45**, 1455-1462
2. Cook, K.S., Min, H.Y., Johnson, D., Chaplinsky, R.J., Flier, J.S., Hunt, C.R., and Spiegelman, B.M. (1987) Adipsin: a circulating serine protease homologue secreted by adipose tissue and sciatic nerve. *Science* **237**, 402-405
3. White, R.T., Damm, D., Hancock, N., Rosen, B.S., Lowell, B.B., Usher, P., Flier, J.S., and Spiegelman, B.M. (1992) Human adipsin is identical to complement factor D and is expressed at high levels in adipose tissue. *J. Biol. Chem.* **267**, 9210-9213
4. Vassalli, J.D., Sappino, A.P., and Belin, D. (1991) The plasminogen activator/plasmin system. *J. Clin. Invest.* **88**, 1067-1072
5. Shimomura, I., Funahashi, T., Takahashi, M., Maeda, K., Kotani, K., Nakamura, T., Yamashita, S., Miura, M., Fukuda, Y., Takemura, K., Tokunaga, K., and Matsuzawa, Y. (1996) Enhanced expression of PAI-1 in visceral fat: Possible contributor to vascular disease in obesity. *Nature Med.* **2**, 800-803
6. Hotamisligil, G.S., Shargill, N.S., and Spiegelman, B.M. (1993) Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. *Science* **259**, 87-91
7. Hotamisligil, G.S. and Spiegelman, B.M. (1994) TNF-α: a key component of obesity-diabetes link. *Diabetes* **43**, 1271-1278
8. Hotamisligil, G.S., Armer, P., Caro, J.F., Atkinson, R.L., and Spiegelman, B.M. (1995) Increased adipose tissue expression of tumor necrosis factor-α in human obesity and insulin resistance. *J. Clin. Invest.* **95**, 2409-2415
9. Kern, P.A., Saghizadeh, M., Ong, J.M., Bosch, R.J., Deem, R., and Simsolo, R.B. (1995) The expression of tumor necrosis factor in human adipose tissue. Regulation by obesity, weight loss, and relationship to lipoprotein lipase. *J. Clin. Invest.* **95**, 2111-2119
10. Grunfeld, C., Zhao, C., Fuller, J., Pollock, A., Moser, A., Friedman, J., and Feingold, K.R. (1996) Endotoxin and cytokines induce expression of leptin, the *ob* gene product, in hamsters. A role for leptin in the anorexia of infection. *J. Clin. Invest.* **97**, 2152-2157
11. Samad, F., Yamamoto, K., and Loskutoff, D.J. (1996) Distribution and regulation of plasminogen activator inhibitor-1 in murine adipose tissue in vivo. *J. Clin. Invest.* **97**, 37-46
12. Bloom, B.R. and Bennett, B. (1996) Mechanism of a reaction in vitro associated with delayed-type hypersensitivity. *Science* **153**, 80-82
13. David, J.R. (1996) Delayed hypersensitivity in vitro: its mediation by cell-free substances formed by lymphoid cell-antigen interaction. *Proc. Natl. Acad. Sci. USA*, **56**, 72-77
14. Sakai, M., Nishihira, J., Hibiya, Y., Koyama, Y., and Nishi, S. (1994) Glutathione binding rat liver 13K protein is the homologue of the macrophage migration inhibitory factor. *Biochem. Mol. Biol. Int.* **33**, 439-446
15. Nishihira, J., Kuriyama, T., Sakai, M., Nishi, S., Ohki, S., and Hikichi, K. (1995) The structure and physicochemical properties of rat liver macrophage migration inhibitory factor. *Biochim. Biophys. Acta* **1247**, 159-162
16. Nishihira, J., Kuriyama, T., Nishino, H., Ishibashi, T., Sakai, M., and Nishi, S. (1993) Purification and characterization of human macrophage migration inhibitory factor: Evidence for specific binding to glutathione and formation of subunit structure. *Biochem. Mol. Biol. Int.* **31**, 841-850
17. Suzuki, M., Murata, E., Tanaka, I., Nishihira, J., and Sakai, M. (1994) Crystallization and a preliminary X-ray diffraction study of macrophage migration inhibitory factor from human lymphocytes. *J. Mol. Biol.* **235**, 1141-1143
18. Sugimoto, H., Suzuki, M., Nakagawa, A., Tanaka, I., Fujinaga, M., and Nishihira, J. (1995) Crystallization of rat liver macrophage migration inhibitory factor (MIF) for MAD analysis. *J. Struct. Biol.* **115**, 331-334
19. Suzuki, M., Sugimoto, H., Nakagawa, A., Tanaka, I., Nishihira, J., and Sakai, M. (1996) Crystal structure of the macrophage migration inhibitory factor from rat liver. *Nature Struct. Biol.* **3**, 259-266
20. Calandra, T., Bernhagen, J., Mitchell, R.A., and Bucala, R. (1994) The macrophage is an important and previously unrecognized source of macrophage migration inhibitory factor. *J. Exp. Med.* **179**, 1895-1902
21. Bernhagen, J., Calandra, T., Mitchell, R.A., Martin, S.B., Tracey, K.J., Voelter, W., Manogue, K.R., Cerami, A., and Bucala, R. (1993) MIF is a pituitary-derived cytokine that potentiates lethal endotoxaemia. *Nature* **365**, 756-759
22. Wistow, G.J., Shaughnessy, M.P., Lee, D.C., Hodin, J., and Zelenka, P.S. (1993) A macrophage migration inhibitory factor is expressed in the differentiating cells of the eye lens. *Proc. Natl. Acad. Sci. USA* **90**, 1272-1275
23. Shimizu, T., Ohkawara, A., Nishihira, J., and Sakamoto, W. (1996) Identification of macrophage migration inhibitory factor (MIF) in human skin and its immunohistochemical localization. *FEBS Lett.* **381**, 199-202
24. Matsuda, A., Tagawa, Y., Matsuda, H., and Nishihira, J. (1996) Identification and immunohistochemical localization of macrophage migration inhibitory factor in human cornea. *FEBS Lett.* **385**, 225-228
25. Onodera, S., Suzuki, K., Matsuno, T., Kaneda, K., Kuriyama, T., and Nishihira, J. (1996) Identification of macrophage migration inhibitory factor in murine neonatal calvariae and osteoblasts. *Immunology* **89**, 430-435
26. Suzuki, H., Kanagawa, H., and Nishihira, J. (1996) Evidence for the presence of macrophage migration inhibitory factor in murine reproductive organs and early embryos. *Immunol. Lett.* **51**, 141-147
27. Bucala, R. (1996) MIF rediscovered: Cytokine, pituitary hormone, and glucocorticoid-induced regulator of the immune response. *FASEB J.* **10**, 1607-1613
28. Hirokawa, J., Sakaue, S., Tagami, S., Kawakami, Y., Sakai, M., Nishi, S., and Nishihira, J. (1997) Identification of macrophage migration inhibitory factor in adipose tissue and its induction by tumor necrosis factor-α. *Biochem. Biophys. Res. Commun.* **235**, 94-98
29. Towbin, H., Staehelin, T., and Gordon, J. (1979) Electrophoretic transfer of protein from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**, 4350-4354

30. Schagger, H. and Von Jagow, G. (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* **166**, 368-379
31. Torti, F.M., Dieckmann, B., Beutler, B., Cerami, A., and Ringold, G.M. (1985) A macrophage factor inhibits adipocyte gene expression: an in vitro model of cachexia. *Science* **229**, 867-869
32. Torti, F.M., Torti, S.V., Larrick, J.W., and Ringold, G.M. (1989) Modulation of adipocyte differentiation by tumor necrosis factor and transforming growth factor beta. *J. Cell. Biol.* **108**, 1105-1113
33. Zhang, B., Berger, J., Hu, E.I., Szalkowski, D., Whitecarrington, S., Spiegelman, B.M., and Moller, D.E. (1996) Negative regulation of peroxisome proliferator-activated receptor-gamma gene expression contributes to the antiadipogenic effects of tumor necrosis factor-alpha. *Mol. Endocrinol.* **10**, 1457-1466
34. Vilcek, J. and Lee, T.H. (1991) Tumor necrosis factor, new insights into the molecular mechanisms of its multiple actions. *J. Biol. Chem.* **266**, 7313-7316
35. Akiyama, T.J., Ishida, J., Nakagawa, S., Ogawara, H., Watanabe, S., Itoh, N., Shibuya, M., and Fukami, Y. (1987) Genistein, a specific inhibitor of tyrosine-specific kinase. *J. Biol. Chem.* **262**, 5592-5595
36. Uehara, Y., Fukasawa, H., Murakami, Y., and Mizuno, S. (1989) Irreversible inhibition of *v-src* tyrosine kinase activity by herbimycin A and its abrogation by sulfhydryl compounds. *Biochem. Biophys. Res. Commun.* **163**, 803-809
37. Stephens, J.M. and Pekala, P.H. (1991) Transcriptional repression of the GLUT4 and C/EBP genes in 3T3-L1 adipocytes by tumor necrosis factor-α. *J. Biol. Chem.* **266**, 21839-21845
38. Lewis, M., Tartaglia, L.A., Lee, A., Bennet, G.L., Rice, G.C., Wong, G.H., Chen, E.Y., and Goeddel, D.V. (1991) Cloning and expression of cDNAs for two distinct murine tumor necrosis factor receptors demonstrate one receptor is species specific. *Proc. Natl. Acad. Sci. USA* **88**, 2830-2834
39. Peraldi, P., Hotamisligil, G.S., Buurman, W.A., White, M.F., and Spiegelman, B.M. (1996) Tumor necrosis factor (TNF)-α inhibits insulin signaling through stimulation of the p55 TNF receptor and activation of sphingomyelinase. *J. Biol. Chem.* **271**, 13018-13022
40. Weber, C., Negrescu, E., Erl, W., Pietsh, A., Frankenberger, M., Zeigler-Heitbrock, H.W., Seiss, W., Weber, P.C. (1995) Inhibitors of protein tyrosine kinase suppress TNF-stimulated induction of endothelial cell adhesion molecules. *J. Immunol.* **155**, 445-451
41. Burke-Gaffney, A. and Hellewell, P.G. (1996) Tumor necrosis factor-α-induced ICAM-1 expression in human vascular endothelial and lung epithelial cells: modulation by tyrosine kinase inhibitors. *Brit. J. Pharmacol.* **119**, 1149-1158
42. May, M.J., Wheeler-Jones, C.P.D., and Pearson, J.D. (1996) Effects of protein tyrosine kinase inhibitors on cytokine-induced adhesion molecule expression by human umbilical vein endothelial cells. *Brit. J. Pharmacol.* **118**, 1761-1771
43. van Hinsbergh, V.W.M., Vermeer, M., Koolwijk, P., Grimbergen, J., and Kooistra, T. (1994) Genistein reduces tumor necrosis factor-α-induced plasminogen activator inhibitor-1 transcription but not urokinase expression in human endothelial cells. *Blood* **84**, 2984-2991
44. Mitchell, R., Bacher, M., Bernhagen, J., Pushkarskaya, T., Seldin, M., and Bucala, R. (1995) Cloning and characterization of the gene for mouse macrophage migration inhibitory factor (MIF). *J. Immunol.* **154**, 3863-3870
45. Reddy, S.A.G., Chaturvedi, M.M., Darnay, B.G., Chan, H., Higuchi, M., and Aggarwal, B.B. (1994) Reconstitution of nuclear factor κB-activation induced by tumor necrosis factor requires membrane-associated components. *J. Biol. Chem.* **269**, 25369-25372
46. Geng, Y., Zhang, B., and Lotz, M. (1993) Protein tyrosine kinase activation is required for lipopolysaccharide induction of cytokines in human blood monocytes. *J. Immunol.* **151**, 6692-6700
47. Novogrodsky, A., Vanichkin, A., Patya, M., Gazit, A., Osherov, N., and Levitzki, A. (1994) Prevention of lipopolysaccharide-induced lethal toxicity by tyrosine kinase inhibitors. *Science* **264**, 1319-1322
48. Calandra, T., Bernhagen, J., Metz, C.N., Spiegel, L.A., Bacher, M., Donnely, T., Cerami, A., and Bucala, R. (1995) MIF as a glucocorticoid-induced modulator of cytokine production. *Nature* **377**, 68-71
49. Waeber, G., Calandra, T., Roduit, R., Haefliger, J.A., Bonny, C., Thompson, N., Thorens, B., Temler, E., Meinhardt, A., Bacher, M., Metz, C., Nicod, P., and Bucala, R. (1997) Insulin secretion is regulated by the glucose-dependent production of islet β cell macrophage migration inhibitory factor. *Proc. Natl. Acad. Sci. USA* **94**, 4782-4787