BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **235,** 94–98 (1997)
ARTICLE NO. RC976745

Identification of Macrophage Migration Inhibitory Factor in Adipose Tissue and Its Induction by Tumor Necrosis Factor-α

Junichi Hirokawa,* Shinji Sakaue,* Seiichi Tagami,* Yoshikazu Kawakami,* Masaharu Sakai,† Shinzo Nishi,† and Jun Nishihira‡,[1]

**First Department of Medicine, †Department of Biochemistry, and ‡Central Research Institute, Hokkaido University School of Medicine, Sapporo 060, Japan*

Received April 10, 1997

Macrophage migration inhibitory factor (MIF) has been rediscovered as a proinflammatory cytokine, pituitary hormone, and glucocorticoid-induced immunoregulator. A survey of tissue distribution revealed that MIF expression is not limited to T lymphocytes, but exists in several other tissues; however, its presence in adipose tissue has never been investigated. In this study, we examined the expression of MIF in adipose tissue using the rat epididymal fat pad and murine 3T3-L1 adipocytes. Northern and Western blot analyses revealed the expression of MIF mRNA and MIF protein, respectively, in both the fat pad and the adipocyte cell line. In immunohistochemistry, a positive staining reaction with an anti-rat MIF antibody was detected largely in the cytosol of adipocytes of the epididymal fat pad. To examine the production and release of MIF by adipocytes, we examined its content in the culture medium of the 3T3-L1 adipocytes. The results showed that MIF content was 1.6 ± 0.48 ng/ml (mean ± SD) after 24 hr culture, and the content was increased up to 9.7 ± 2.8 ng/ml by stimulation with TNF-α (50 nM). Since TNF-α produced in adipocytes is known to induce insulin resistance, the results suggest the possibility that MIF plays an important role in the mechanism of insulin resistance often observed in obesity and diabetes via regulation of TNF-α expression.

Obesity is pathological condition of the adipose tissues, characterized by an increase in the number and the size of the adipocytes themselves, and is associated with several disorders, including non-insulin dependent diabetes mellitus, hypertension, and coronary heart disease. Recent studies have shown that adipose tissue is not only an energy storage organ, but also secretes biologically active molecules, such as tumor necrosis factor (TNF)-α, ob protein (leptin), and type-1 plasminogen activator inhibitor (PAI-1) (1-3). Obesity is frequently associated with insulin resistance and abnormal glucose homeostasis, and the local cytokine network appears to be dysregulated in several models of obesity.

Among these cytokines, pluripotent TNF-α has been thought to play an important role in the mechanism of insulin resistance in the states of obesity and diabetes. Studies in animal models showed that TNF-α mediates insulin resistance through its overexpression in fat tissues (1, 4, 5). In humans, obese individuals express 2.5-fold more TNF-α mRNA in fat tissues relative to those of lean controls in accordance with production of TNF-α protein (6). However, the precise mechanism linking obesity to insulin resistance remains largely unknown.

Macrophage migration inhibitory factor (MIF) was identified in activated T lymphocytes as a cytokine that inhibited the migration of macrophages out of capillary tubes in vitro (7). MIF had long been considered to be produced only by activated T lymphocytes, and believed to contribute to cell-mediated immunity (8). However, recent reports identified MIF in tissues other than T lymphocytes, such as macrophages, anterior pituitary cells, and lens (9-11). We cloned rat MIF cDNA, reported its physicochemical properties, and demonstrated the tertiary structures of both human and rat MIF (12-16). During the course of our MIF study on the tissue distribution, we found that MIF was expressed in human skin, cornea, reproductive organs and osteoblasts as well (17-20). The immunohistochemical findings on the MIF protein suggested the possibility that MIF might be profoundly involved not only in inflammatory and immunological responses but also in cell growth and wound repair. From the available data, it appears that MIF may function as an inflammatory

[1] To whom correspondence should be addressed. Fax: +81-11-706-7864. E-mail: j_nisihi@med.hokudai.ac.jp.

0006-291X/97 $25.00

cytokine, pituitary hormone, and glucocorticoid-induced immunoregulator (21).

It was reported that MIF expression could be exceedingly induced by TNF-α (9). Since the overexpression of TNF-α in adipose tissue can induce insulin resistance, it is essential to investigate whether MIF present in adipose tissues is involved in the mechanism of insulin resistance. In this study, we examined expression of MIF in adipocytes and the effect of TNF-α on MIF expression. We here show that MIF was expressed in the rat epididymal fat pad and murine 3T3-L1 adipocytes, and this protein was secreted into the extracellular space by TNF-α stimulation. This finding suggests the possibility that MIF affects insulin resistance via the regulation of TNF-α expression in adipose tissues.

MATERIALS AND METHODS

Reagents. The following materials were obtained from commercial sources. Nitrocellulose membrane filters were from Millipore (Bedford, MA, USA); Isogen RNA extraction kit from Nippon Gene (Tokyo, Japan); heat-inactivated fetal calf serum (FCS) from Hyclone Labs (Logan, UT, USA); horseradish peroxidase-conjugated goat anti-rabbit antibody from Pierce (Rockford, IL, USA); DAKO CSA system from DAKO (Carpinteria, CA, USA); 3,3′-diaminobenzidine tetrahydrochloride and o-phenylenediamine from Wako (Osaka, Japan); Protein A Sepharose from Pharmacia (Uppsala, Sweden); DNA labeling kit from Takara (Kyoto, Japan); [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 Co. (Tokyo, Japan).

A polyclonal anti-rat MIF antibody was generated by immunizing New Zealand white rabbits with purified recombinant rat MIF as described (19, 20). The IgG fractions (4 mg/ml) were prepared using Protein A Sepharose according to the manufacturer's protocol.

Animals and cells. All experiments were carried out on male 6-week-old SD rats. The rats were sacrificed by ketamine and xylazine injection. The animal experiments conformed to the Regulations for Animal Experiments of the Institute for Animal Experimentation, Institute, Hokkaido University School of Medicine. The 3T3-L1 cells, a murine adipose cell line, were purchased from the American Type Culture Collection (Rockville, MD, USA). Cells were grown in Dulbecco's MEM containing 10% FCS, in a humid atmosphere containing 5% CO_2 at 37°C. Post-confluent cells were exposed to a medium containing 10% FCS, 0.5 mM isobutylmethylxanthine, 100 nM dexamethasone and 10 μg/ml insulin to induce differentiation. After 72hr this medium was replaced with DMEM supplemented with 10% FCS and 5 μg/ml insulin, and the cells were then fed every 3 days. The cells 9 days after induction were used.

Northern blot analysis. Northern blot analysis was carried out as previously described (22). In brief, total RNAs from the rat epididymal fat pad and murine 3T3-L1 adipocytes were extracted and separated by electrophoresis on agarose gels containing 0.6 M formaldehyde, and blotted onto nylon membrane filters. Hybridization was carried out with the rat MIF cDNA probe, radiolabeled by [α-^{32}P]-dCTP using a random primer labeling kit. The hybridization was performed in a solution containing the radiolabeled 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 for 16 h. 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 cDNA prepared as described above.

Western blot analysis. Western blot analysis was carried out by the method of Towbin et al. (23). In brief, the samples were dissolved in 20 ml of Tris-HCl (50 mM, pH 6.8) containing 2-mercaptoethanol (1%), SDS (2%), glycerol (20%) and bromophenol blue (BPB) (0.04%), and heated at 100°C for 5 min. The samples were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described (24). The electrophoresed proteins were transferred onto a nitrocellulose membrane at 50 mA for 1 hr using a semi-dry blot transfer apparatus (Bio-Rad). Then the membrane was intensively washed with PBS, and incubated with the anti-rat MIF polyclonal antibody (1000 in dilution) for 1 hr at room temperature, and reacted with peroxidase-conjugated anti-rabbit IgG (1:1000 in dilution) for 1 hr at room temperature. After the reaction, proteins were visualized with an ECL kit as recommended in the manufacturer's protocol. Protein concentration was determined with a Micro BCA protein assay reagent kit.

Immunohistochemistry. Paraffin-embedded, paraformaldehyde-fixed sections of the epididymal fat pad on a glass slide were deparaffinized with xylene, rehydrated by immersion in a graded series of ethanol washes, and stained with DAKO CSA system according to the manufacture's protocol. In brief, the sections were treated with 3% hydrogen peroxide for 5 min, and rinsed with the washing buffer (0.05 M Tris-HCl, pH 7.6) containing 0.3 M NaCl and 0.1% Tween 20. Non-specific staining was blocked by PBS with 0.015 M sodium azide. Incubation with the primary antibody (5000-fold dilution of 4 mg/ml IgG) was carried out in a humid chamber at 4°C overnight. The control tissue sample was prepared by the addition of 1000-fold excess rat MIF to the primary antibody before the reaction. The slides were then washed and treated sequencially with biotinylated goat anti-rabbit IgG, streptavidin-biotin complex, biotiny tyramide and 0.02% hydrogen peroxide in PBS containing streptavidin-peroxidase, and 3,3′-diaminobenzidine tetrahydrochloride. The sections were counterstained with hematoxylin. Lastly, the samples were 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 IgG polyclonal antibody dissolved in PBS (50 μl) 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 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 anti-MIF antibody was added to each well. Following incubation for 1 hr at room temperature, the plate was again washed three times with the washing buffer. Avidin-conjugated horseradish 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) contained 8 mg of o-phenylenediamine and 4 μl of 30% H_2O_2 in citrate phosphate buffer (pH 5.0). Finally, 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 by an ELISA plate reader (Bio-rad, Model 3550).

RESULTS

Expression of MIF mRNA in epididymal fat pad and 3T3-L1 adipocytes. Northern blot analysis of the epididymal fat pad adipose tissue revealed the expression

of MIF mRNA (Fig. 1, lane 1). Because the epididymal fat pad contained stromal-vascular cells, including capillary endothelial cells, fibroblasts, mast cells and macrophages other than mature adipocytes, we performed collagenase-digestion of the adipose tissues to exclude these non-adipose cells. Furthermore, we confirmed the expression of the MIF mRNA in the adipocytes using 3T3-L1 adipocytes (Fig.1, lane 2). The results revealed that MIF was constitutively expressed in adipocytes.

Identification of MIF protein in epididymal fat pad and 3T3-L1 adipocytes. To confirm the presence of MIF protein in adipose tissues, Western blot analysis was performed on the epididymal fat pad and 3T3-L1 adipocytes. The samples were electrophoresed on SDS-polyacrylamide gel and were transferred electrophoretically to a nitrocellulose membrane. The transferred proteins were reacted with the anti-rat MIF antibody and visualized using an ECL kit (Fig. 2). A single positive-stained band was observed at a relative molecular mass of 12.5 kDa on both samples. As a positive control, recombinant rat MIF (10 ng) was also shown. No specific band was visualized when a non-immune rabbit IgG was used (data not shown). Immunoblot analysis clearly demonstrated the intracellular presence of MIF in adipocytes.

Immunohistochemical localization of MIF. To determine the intracellular localization of the MIF protein in adipocytes, we carried out immunohistochemical analysis on the adipose tissue of the rat epididymal fat pad. The immunohistochemical analysis revealed that MIF protein was mostly present in the cytosols of mature adipocytes (Fig. 3A). The control tissue sample prepared by the addition of excess rat MIF (1000-fold) to the primary antibody did not show any positive staining (Fig. 3B). This immunohistochemical result together with those of Northern and Western blot analyses indicated that MIF was synthesized de novo by adipocytes and that the protein was localized in the cytoplasm.

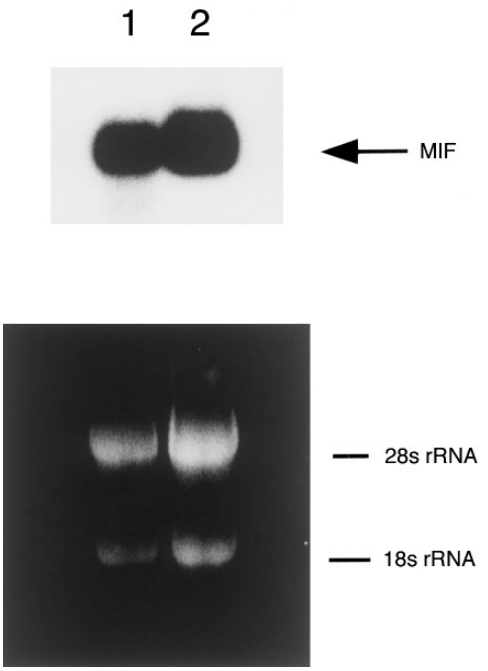

FIG. 1. Northern blot analysis of MIF mRNA on adipocytes. Northern blot analysis was carried out as described in Materials and Methods. Lane 1, the epididymal fat pad; lane 2, 3T3-L1 adipocytes. The profile of total RNA is shown at the bottom of each lane.

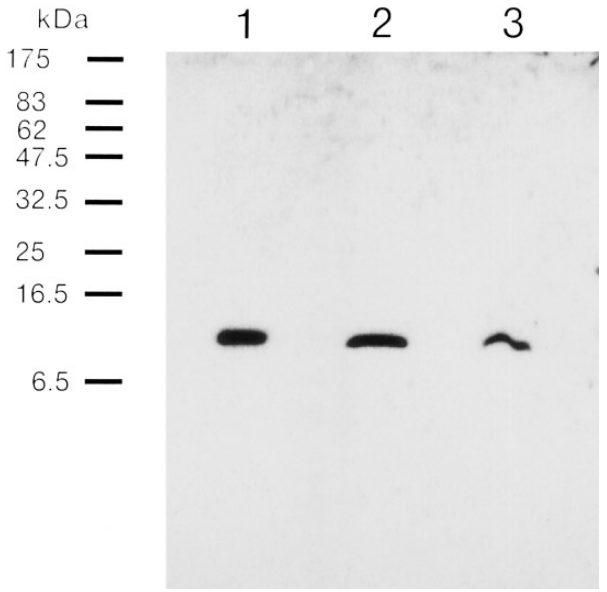

FIG. 2. Western blot analysis of adipocytes for MIF. The cells were collected, electrophoresed, transferred to a nitrocellulose membrane, and visualized by chemiluminescence using an ECL kit as described in Materials and Methods. Lane 1, rat recombinant MIF (10 μg); lane 2, the epididymal fat pad; lane 3, 3T3-L1 adipocytes. The prestained molecular marker (New England Biolabs) is also shown.

MIF content in 3T3-L1 adipocyte culture medium. To examine whether MIF identified in the cytoplasm of adipocytes could be secreted into the extracellular space, we measured MIF content in the medium of 3T3-L1 adipocytes using ELISA. The MIF concentration of the medium of 5×10^6 cells was 1.6 ± 0.48 ng/ml (mean $\pm$ SD) after 24-hr culture (5×10^6 cells in 5 ml medium). This indicated that MIF was biosynthesized by adipocytes and that part of the protein was readily secreted into extracellular space. We further examined the effect of TNF-α on MIF secretion by the adipocytes. Twenty-four hr treatment of 3T3-L1 adipocytes with human recombinant TNF-α (50 nM) significantly increased secretion of the MIF protein into the culture medium (9.7 ± 2.8 ng/ml). This suggested the possibility that TNF-α present in adipocytes regulated the MIF production, which might cause the insulin resistance.

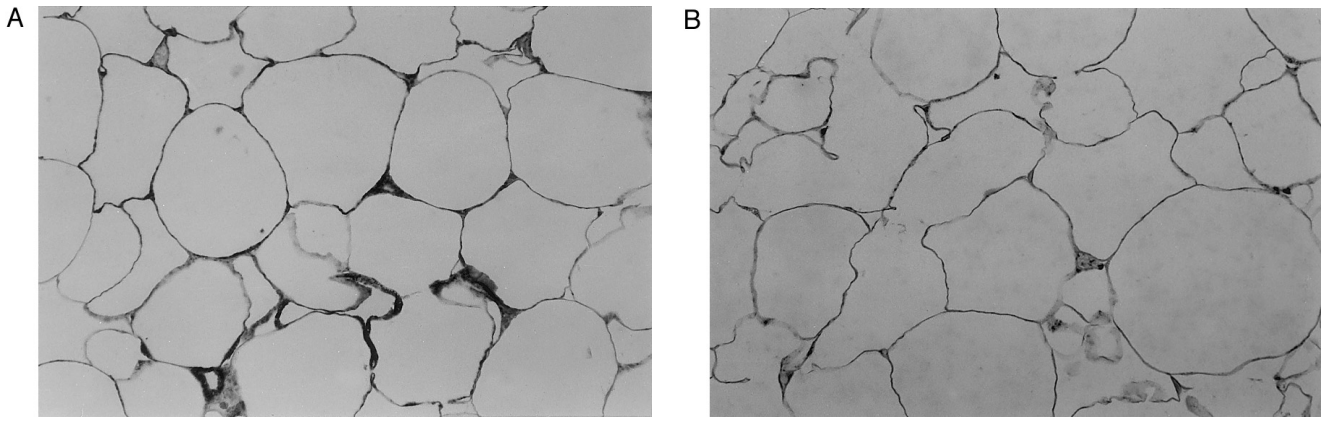

FIG. 3. Immunohistochemistry of the epididymal fat pad for MIF. The adipose tissues prepared from the epididymal fat pad were fixed and stained using a DAKO CSA as described in Materials and Methods. A, adipose tissue reacted with anti-rat MIF antibody (×400). B, a control tissue specimen reacted in the anti-rat MIF antibody pretreated with a 1000-fold excess of rat recombinant MIF (×400).

DISCUSSION

This is the first report demonstrating the expression of MIF mRNA and the identification of the MIF protein in adipocytes. The immunohistochemical study revealed that the MIF protein was localized largely in the cytosol. This cellular distribution is consistent with those of other tissues (17-19). MIF is a multifunctional cytokine possessing a variety of biological functions, e. g., potentiation of endotoxin shock, enhancement of macrophage adherence, phagocytosis, and tumoricidal activity (25, 26). These biological actions are likely to be exerted through both autocrine-paracrine and endocrine routes (9, 10, 21).

It is of note that TNF-α increased the secretion of the MIF protein in the murine 3T3-L1 adipocytes (Fig. 4). It has been previously reported that MIF can induce TNF-α production (9). Thus, the two proteins may function in concert in various pathophysiological states. From the data available to date, it is known that adipocytes have the potential to release several biologically active molecules, such as TNF-α, leptin and PAI-1 (1-3). In particular, TNF-α appears to play an important role in inducing insulin resistance in obesity and diabetes. It was reported that chronic, low-level administration of TNF-α to rodents could induce systemic insulin resistance (27). Moreover, several reports have suggested an association of TNF-α overexpression in adipose tissue with the state of peripheral insulin resistance (4-6). Considering these facts together, it is possible that MIF might regulate the insulin effect in adipose tissues of obesity and diabetes in concert with TNF-α and vice versa, and the dysregulation might cause insulin resistance.

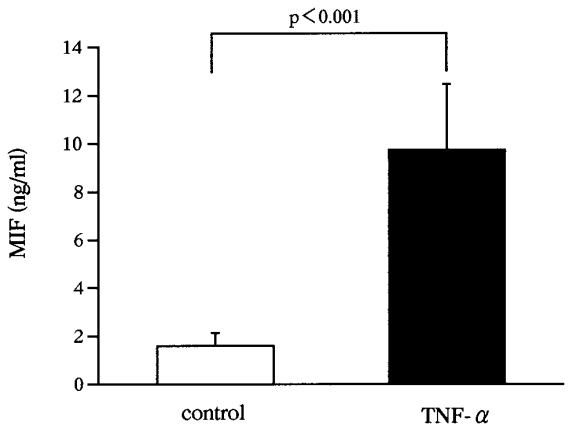

FIG. 4. Effect of TNF-α on MIF release on 3T3-L1 adipocytes. We measured MIF content in the medium of 3T3-L1 adipocytes using ELISA as described in Materials and Methods. 5×10^6 cells were cultured in 5 ml of medium for 24 hr in the presence or absence of human recombinant TNF-α.

Very recently, abundant quantities of MIF were demonstrated in the endocrine pancreas by immunohistochemical analysis, suggesting a role for MIF in glucose metabolism (28). This showed that the differentiated insulin-secreting β-cell line INS-1 had the potential to express MIF, and its production was regulated by glucose. Therefore, it was considered that the islet MIF might regulate insulin secretion by β-cells in an autocrine fashion. That is, MIF might regulate insulin secretion in a positive manner and play an important role in carbohydrate metabolism. Accordingly, it should be considered that MIF might be important in the pathogenesis of obesity and diabetes in association with insulin resistance; though the precise mechanism remains to be elucidated.

Insulin resistance is also seen in other pathophysiological states, including dyslipidemias and atherosclerosis, often complicated with thrombosis (29-31). Elevation of PAI-1, a member of the serpin family, constitutively expressed in adipose tissues, is associated with an increased risk for thrombotic disease, and the

mRNA levels are often elevated in severely atherosclerotic human arteries (32-34). Recently, it was reported that MIF was purified from the bovine brain by using an affinity column with a synthetic peptide corresponding to the C-terminal region of the serpin family including PAI-1 (35). This finding suggested that MIF could also be involved in the regulation of thrombosis and fibrinolysis by the formation of a complex with PAI-1. Thus, it is possible that MIF might also be involved in the regulation of thrombosis and fibrinolysis; however, this needs further evaluation.

Finally, obesity and diabetes are among the most common human health problems in industrialized societies. It is of importance that obesity is an independent risk factor for the development of atherosclerosis and cardiovascular diseases. The mechanistic link between obesity/diabetes and insulin resistance is still poorly understood. Treatment with an anti-MIF antibody reduced the production of TNF-α (36). Accordingly, the regulation of MIF production may improve the state of insulin resistance, and it would be of interest to test whether anti-MIF agents could improve insulin resistance. In this context, the present results may help, at least in part, to elucidate the mechanism of insulin resistance. Currently we are studying the adipose expression of MIF in association with insulin resistance in diabetic animals.

ACKNOWLEDGMENTS

We thank Dr. Jun Ohsumi and Ms. Sachiko Sakakibara for their technical advice on cell culture. This research was supported by a Grant-in-Aid for Research (07670162) from the Ministry of Education, Science, and Culture of Japan and grants from the Akiyama Foundation and Ohyama Foundation.

REFERENCES

1. Hotamisligil, G. S., Shargill, N. S., and Spiegelman, B. M. (1993) *Science* **259,** 87–91.
2. Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L., and Friedman, J. M. (1994) *Nature* **372,** 425–432.
3. Samad, F., Yamamoto, K., and Loskutoff, D. J. (1996) *J. Clin. Invest.* **97,** 37–46.
4. Hotamisligil, G. S., and Spiegelman, B. M. (1994) *Diabetes* **43,** 1271–1278.
5. Hofmann, C., Lorenz, K., Braithwaite, S. S., Palazuk, B. J., Hotamisligil, G. S., and Spiegelman, B. M. (1994) *Endocrinology* **134,** 264–270.
6. Hostamisligil, G. S., Armer, P., Caro, J. F., Atkinson, R. L., and Spiegelman, B. M. (1995) *J. Clin. Invest.* **95,** 2409–2415.
7. Bloom, B. R., and B. (1996) *Science* **153,** 80–82.
8. David, J. R. (1996) *Proc. Natl. Acad. Sci., USA* **56,** 72–77.
9. Calandra, T., Bernhagen, J., Mitchell, R. A., and Bucala, R. (1994) *J. Exp. Med.* **179,** 1895–1902.
10. Bernhagen, J., Calandra, T., Mitchell, R. A., Martin, S. B., Tracey, K. J., Voelter, W., Manogue, K. R., Cerami, A., and Bucala, R. (1993) *Nature* **365,** 756–759.
11. Wistow, G. J., Shaughnessy, M. P., Lee, D. C., Hodin, J., and Zelenka, P. S. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 1272–1275.
12. Nishihira, J., Kuriyama, T., Sakai, M., Nishi, S., Ohki, S., and Hikichi, K. (1995) *Biochim. Biophys. Acta.* **1247,** 159–162.
13. Nishihira, J., Kuriyama, T., Nishino, H., Ishibashi, T., Sakai, M., and Nishi, S. (1993) *Biochem. Mol. Biol. Int.* **31,** 841–850.
14. Suzuki, M., Murata, E., Tanaka, I., Nishihira, J., and Sakai, M. (1994) *J. Mol. Biol.* **235,** 1141–1143.
15. Sugimoto, H., Suzuki, M., Nakagawa, A., Tanaka, I., Fujinaga, M., and Nishihira, J. (1995) *J. Struct. Biol.* **115,** 331–334.
16. Suzuki, M., Sugimoto, H., Nakagawa, A., Tanaka, I., Nishihira, J., and Sakai, M. (1996) *Nature Struct. Biol.* **3,** 259–266.
17. Shimizu, T., Ohkawara, A., Nishihira, J., and Sakamoto, W. (1996) *FEBS Lett.* **381,** 199–202.
18. Matsuda, A., Tagawa, Y., Matsuda, H., and Nishihira, J. (1996) *FEBS Lett.* **385,** 225–228.
19. Onodera, S., Suzuki, K., Matsuno, T., Kaneda, K., Kuriyama, T., and Nishihira, J. (1996) *Immunology* **89,** 430–435.
20. Suzuki, H., Kanagawa, H., and Nishihira, J. (1996) *Immunol. Lett.* **51,** 141–147.
21. Bucala, R. (1996) *FASEB J.* **10,** 1607–1613.
22. Sakai, M., Nishihira, J., Hibiya, Y., Koyama, Y., and Nishi, S. (1993) *Biochem. Mol. Biol. Int.* **33,** 439–346.
23. Towbin, H., Staehelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci, USA* **76,** 4350–4354.
24. Schagger, H., and Von Jagow, G. (1987) *Anal. Biochem.* **166,** 368–379.
25. Weiser, W. Y., Pozzi, L. M., and David, J. R. (1991) *J. Immunol.* **147,** 2006–2011.
26. Pozzi, L. M., and Weiser, W. Y. (1991) *Cellular Immunol.* **145,** 372–379.
27. Lang, C. H., Dobrescu, C., and Bagby, G. J. (1992) *Endocrinology* **130,** 43–52.
28. Waeber, G., Calandra, T., Roduit, R., Haefliger, J. A., Thompson, N., Thorens, B., Temler, E., Meinhardt, A., Bacher, M., Metz, C., Nicod, P., and Bucala, R. (1996) *Diabetes* **45** (Suppl. 2), 161A.
29. Vague, P., Juhan Vague, I., Aillaud, M. F., Badier, C., Viard, R., Alessi, M. C., and Collen, D. (1986) *Metabolism* **35,** 250–253.
30. Auwerx, J., Bouillon, R., Collen, D., and Geboers, J. (1988) *Arteriosclerosis* **8,** 68–72.
31. 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) *Nature Med.* **2,** 800–803.
32. Wiman, B., and Hamsten, A. (1984) *J. Biol. Chem.* **259,** 3644–3647.
33. Hamsten, A., Wilman, B., Faire, U. D., and Blomback, M. (1985) *N. Engl. J. Med.* **313,** 1557–1563.
34. Wiman, B., and Hamsten, A. (1990) *Semin. Thromb. Haemost.* **26,** 207–216.
35. Nishibori, M., Nakaya, N., Mori, S., Kawabata, M., Tahara, A., and Saeki, K. (1996) *Jpn. J. Pharmacol.* **71,** 259–262.
36. Calandra, T., Bernhagen, J., Metz, C. N., Spiegel, L. A., Bacher, M., Donnely, T., Cerami, A., and Bucala, R. (1995) *Nature* **377,** 68–71.