A Paracrine Loop Between Adipocytes and Macrophages Aggravates Inflammatory Changes
Role of Free Fatty Acids and Tumor Necrosis Factor α

Takayoshi Suganami, Junko Nishida, Yoshihiro Ogawa

Objective—Weight gain is associated with infiltration of fat by macrophages, suggesting that they are an important source of inflammation in obese adipose tissue. Here we developed an in vitro coculture system composed of adipocytes and macrophages and examined the molecular mechanism whereby these cells communicate.

Methods and Results—Coculture of differentiated 3T3-L1 adipocytes and macrophage cell line RAW264 results in the marked upregulation of proinflammatory cytokines, such as tumor necrosis factor α (TNF-α), and the downregulation of the antiinflammatory cytokine adiponectin. Such inflammatory changes are induced by the coculture without direct contact, suggesting the role of soluble factors. A neutralizing antibody to TNF-α, which occurs mostly in macrophages, inhibits the inflammatory changes in 3T3-L1, suggesting that TNF-α is a major macrophage-derived mediator of inflammation in adipocytes. Conversely, free fatty acids (FFAs) may be important adipocyte-derived mediators of inflammation in macrophages, because the production of TNF-α in RAW264 is markedly increased by palmitate, a major FFA released from 3T3-L1. The inflammatory changes in the coculture are augmented by use of either hypertrophied 3T3-L1 or adipose stromal vascular fraction obtained from obese ob/ob mice.

Conclusions—We postulate that a paracrine loop involving FFAs and TNF-α between adipocytes and macrophages establishes a vicious cycle that aggravates inflammatory changes in the adipose tissue. (Arterioscler Thromb Vasc Biol. 2005;25:0-0.)

Key Words: macrophage ■ adipocyte ■ fatty acids ■ TNF-α ■ obesity

The metabolic syndrome is a constellation of visceral fat obesity, impaired glucose metabolism, atherogenic dyslipidemia, and blood pressure elevation, which all independently increase a risk of atherosclerotic diseases, such as ischemic heart disease and cerebral stroke.1,2 The molecular basis for the clustering of such independent risks of atherosclerosis has not fully been elucidated, with visceral fat obesity considered most important.3,4 Systemic insulin resistance has been implicated as one possible factor that links visceral fat obesity and the adverse metabolic consequences.4,5 Evidence has accumulated indicating that obesity is associated with a state of chronic, low-grade inflammation, suggesting that inflammation may be a potential mechanism whereby obesity leads to insulin resistance.5,6 Indeed, obesity and insulin resistance are strongly associated with systemic markers of inflammation, and, clinically, inflammation has been recognized as a major predictor of atherosclerotic disease.5,7

The adipose tissue is an important endocrine organ that secretes many biologically active substances, such as leptin, adiponectin, tumor necrosis factor α (TNF-α), and monocyte chemoattractant protein 1 (MCP-1), which are collectively termed adipokines.5,8–10 Dysregulated production of proinflammatory and antiinflammatory adipokines seen in visceral fat obesity is associated with the metabolic syndrome,4,6 suggesting that inflammatory changes within the adipose tissue may critically contribute to the development of many aspects of the metabolic syndrome and results in diabetes and atherosclerosis. For example, it has been reported that TNF-α and MCP-1 are upregulated, and adiponectin is downregulated in obese adipose tissue.4,8–11 However, the molecular mechanisms underlying inflammatory changes within the adipose tissue are largely unknown.

The adipose tissue is composed of various cell types: lipid-laden mature adipocytes and the remaining stromal vascular fraction (SVF) that includes blood cells, endothelial cells, and macrophages.12 Recent studies have demonstrated that obese adipose tissue is characterized by increased infiltration of macrophages, suggesting that they are important sources of inflammation in the adipose tissue.13,14 Notably, macrophage infiltration and inflammation-related gene expression in the adipose tissue may precede the development of insulin resistance and atherogenesis.
of insulin resistance in animal models.\textsuperscript{14} It is also important to note that such inflammatory changes are remarkable within visceral fat depots.\textsuperscript{15} The analysis of gene expression profiles of adipocytes and SVF obtained from obese mice and humans revealed that macrophages produces almost all of the TNF-\(\alpha\), whereas mature adipocytes secrete the majority of leptin, and interleukin 6 (IL-6) is expressed roughly equally among adipocytes, macrophages, and nonmacrophage SVF.\textsuperscript{13} These observations suggest that the macrophages infiltrated in the adipose tissue contribute to the elevation in circulating inflammatory markers, including TNF-\(\alpha\) and IL-6, that are common in obesity.\textsuperscript{13} Because local interactions between macrophages and other cell types are well documented under certain pathologic conditions,\textsuperscript{16,17} it is also tempting to speculate the cross-talk between adipocytes and macrophages as a potential mechanism that aggravates inflammatory changes in obse adipose tissue.

In this study, we developed an in vitro coculture system composed of adipocytes and macrophages and examined the molecular basis for the interaction between these cells. The data of this study suggest that a paracrine loop involving free fatty acids (FFAs) and TNF-\(\alpha\) derived from adipocytes and macrophages, respectively, establishes a vicious cycle that aggravates inflammatory changes in obse adipose tissue.

### Materials and Methods

#### Materials and Antibodies

Details are described in online Materials and Methods, available at http://atvb.ahajournals.org.

#### Cell Culture

RAW264 macrophage cell line (RIKEN BioResource Center, Tsukuba, Japan) and 3T3-L1 preadipocytes (American Type Culture Collection, Manassas, VA) were maintained in DMEM (Nacalai Tesque, Kyoto, Japan) containing 10\% FBS (Sanko Junyaku, Tokyo, Japan) and antibiotics, and incubated at 37°C in a humidified 5% \(\text{CO}_2/95\%\) air atmosphere.\textsuperscript{15,18} Differentiation of 3T3-L1 preadipocytes to mature adipocytes was performed using insulin, dexamethasone, and 3-isobutyl-1-methyl-xantihone as described\textsuperscript{14} and used as differentiated 3T3-L1 at day 8 after the induction of differentiation. Hypertrophied 3T3-L1 with larger lipid droplets, when cultured up to day 21, was also used.

#### Coculture of Adipocytes and Macrophages

Coculture of adipocytes and macrophages was performed in 2 different ways as follows (Figure I, available online at http://atvb.ahajournals.org). In the contact system, serum starved differentiated 3T3-L1 (\(\sim 1.5\times10^5\) cells) was cultured in a 6-cm dish, and macrophages (RAW264 or peritoneal macrophages) or SVF (\(1.0\times10^4-10^6\) cells) was plated onto 3T3-L1. The cells were cultured for \(\sim 48\) hours with contact to each other and harvested as a control, adipocytes and macrophages, the numbers of which were equal to those in the contact system, were cultured separately and mixed after harvest. In the transwell system, cells were cocultured by using transwell inserts with a 0.4-\(\mu\)m porous membrane (Corning, Corning, NY) to separate adipocytes from macrophages. After incubation for 24 hours, the cells in the lower well were harvested. In some experiments, the supernatants of differentiated 3T3-L1 and RAW264 were used to replace the medium of each cell type.

#### Quantitative Real-Time Polymerase Chain Reaction

Total RNA was extracted from cultured cells, and quantitative real-time PCR was performed with an ABI Prism 7000 Sequence Detection System using the PCR Master Mix Reagent (Applied Biosystems, Foster City, CA).\textsuperscript{19} Primers used were listed in Table I (available online at http://atvb.ahajournals.org.). Levels of mRNA were normalized to those of 36B4 mRNA.

#### Measurement of MCP-1, TNF-\(\alpha\), and Adiponectin Levels in Culture Media

The MCP-1, TNF-\(\alpha\), and adiponectin levels in culture supernatants were determined by commercially available ELISA kit (MCP-1 and TNF-\(\alpha\), R&D systems, Minneapolis, MN; adiponectin, Otsuka Pharmaceutical, Tokyo, Japan).

#### Western Blotting of Mitogen-Activated Protein Kinases

Whole cell lysates were prepared as described previously.\textsuperscript{17,20} Samples (20 \(\mu\)g protein/lane) were separated by 12.5\% SDS-PAGE, and Western blotting was performed using antibodies against mitogen-activated protein (MAP) kinases.\textsuperscript{17} Immunoblots were developed with horseradish peroxidase-conjugated secondary antibodies and a chemiluminescence kit (ECL Plus, Amersham Biosciences, Piscataway, NJ) and observed with LAS-3000 mini system (Fuji Photo Film, Tokyo, Japan).

#### Lipolysis Assay

Differentiated 3T3-L1 was cocultured with RAW 264 in the medium containing 2\% fatty acid (FA)-free bovine serum albumin for 24 hours in both coculture systems. The concentration of FFAs in the medium was measured using an acyl-coenzyme A oxidase-based colorimetric assay kit (NEFA-C, WAKO Pure Chemicals, Osaka, Japan).

#### Preparation of SVF from the Adipose Tissue

Genetically obese ob/ob mice and wild-type C57BL/6 mice were purchased from Charles River Japan (Yokohama, Japan). The adipose SVF was prepared by collagenase digestion from perigonadal fat tissue of both strains at 12 weeks of age.\textsuperscript{21} The procedure was performed under pentobarbital anesthesia (30 mg/kg). All of the animal experiments were conducted in accordance with the guidelines of Tokyo Medical and Dental University Committee on Animal Research (No. 0050140).

#### Statistical Analysis

Data were expressed as the mean\(\pm\)SE. Statistical analysis was performed using ANOVA followed by Scheffe’s test. A \(P\) value <0.05 was considered to be statistically significant.

#### Results

#### Induction of Inflammatory Changes by Coculture of Adipocytes and Macrophages in the Contact System

Coculture of differentiated 3T3-L1 and RAW264 in the contact system revealed marked upregulation of proinflammatory cytokines MCP-1, IL-6, and TNF-\(\alpha\) (\(P<0.01\)) and significant downregulation of antiinflammatory cytokine adiponectin (\(P<0.05\)) relative to the control culture (Figure 1). The extent of changes in adipocytokine mRNA expression was dependent on the duration of coculture and the number of RAW264 (Figure II, available online at http://atvb.ahajournals.org). To elucidate the specific role of the macrophage, we substituted peritoneal macrophages or undifferentiated 3T3-L1 for RAW264, and examined the effects on MCP-1 mRNA expression (see online Methods). Coculture with peritoneal macrophages, as well as RAW264, exhibited marked upregulation of MCP-1 mRNA expression (Figure III, available online at http://atvb.ahajournals.org). By con-
contrast, coculture with undifferentiated 3T3-L1 showed no apparent induction of MCP-1. These observations indicate that the coculture of differentiated adipocytes and macrophages induces inflammatory changes.

To explore the molecular mechanisms of the interaction between adipocytes and macrophages, we investigated the role of secreted factors in the induction of inflammatory changes in both cells. The conditioned media from RAW264 significantly induced MCP-1 (P<0.01), IL-6 (P<0.05), and TNF-α (P<0.01) mRNA expression in 3T3-L1 (Figure 2a). The media from 3T3-L1 showed no induction of MCP-1 but a significant increase in IL-6 and TNF-α in RAW264 (P<0.05). In this study, TNF-α was mostly derived from RAW264, and only a small amount of TNF-α was secreted from 3T3-L1 (Figure 2b). Moreover, the coculture of 3T3-L1 and RAW264 in the transwell system exhibited marked upregulation of MCP-1 and IL-6 mRNA expression in 3T3-L1 (P<0.01), the extent of which was dependent on the number of RAW264 (Figure 2c). These observations indicate that secreted factors play critical roles in the induction of inflammatory changes in both cells.

Role of Macrophage-Derived TNF-α in Induction of MCP-1 in Adipocytes

We next investigated the role of TNF-α in the induction of inflammatory changes in adipocytes. Blockade of TNF-α using anti-TNF-α neutralizing antibody effectively inhibited the upregulation of MCP-1 mRNA expression in both contact and transwell systems (P<0.01; Figure 3a). Treatment of 3T3-L1 with recombinant TNF-α significantly induced MCP-1 mRNA levels (P<0.01), and the effects were completely abolished by pretreatment of MAP kinase inhibitors (PD98059 and SP600125; P<0.01; Figure 3b). Moreover, the administration of PD98059 and SP600125 in the transwell system dose-dependently prevented the upregulation of MCP-1 mRNA expression in 3T3-L1 (Figure 3c). We also confirmed that the blockade of TNF-α and MAP kinase pathways markedly reduced MCP-1 secretion in the media (P<0.01; Figure 3d). Furthermore, coculture induced the phosphorylation of extracellular signal-regulated kinase (ERK) and C-Jun NH2-terminal protein kinase (JNK) in the contact system (P<0.01; Figure 3e). To elucidate the transcriptional regulation of MCP-1 in adipocytes, we performed a MCP-1 promoter reporter assay using MCP-1/JE-Luc construct (see online Methods). Coculture, as well as recombinant TNF-α markedly increased adipocyte MCP-1 promoter activity, which was significantly inhibited by MAP kinase inhibitors (Figure IV, available online at http://atvb.ahajournals.org). We also confirmed that activator protein 1 was activated in 3T3-L1 by stimulation of TNF-α through the MAP kinase pathways. These observations indicate that macrophage-derived TNF-α plays a critical role in the induction of inflammatory changes in adipocytes, with MAP kinase pathways being activated under the coculture condition.

Role of Adipocyte-Derived FFAs in Induction of TNF-α in Macrophages

We next investigated the role of FFAs in the induction of inflammatory changes in macrophages. Coculture of 3T3-L1 with RAW264 significantly increased the release of FFAs (P<0.01; Figure 4a), which was significantly inhibited by the blockade of TNF-α using an anti-TNF-α neutralizing antibody (P<0.05; Figure 4b). Saturated FAs, such as palmitate and laurate, significantly induced TNF-α mRNA expression in RAW264 (P<0.01; Figure 4c). By contrast, polyunsaturated FAs (PUFAs), such as linoleate and eicosapentaenoic acid (EPA), showed no effects. These observations are consistent with previous reports on FA-induced inflammatory
changes in macrophages and myotubes. Pretreatment with PD98059 or SP600125 significantly inhibited palmitate-induced upregulation of TNF-α in RAW264 (P < 0.05; Figure 4d). These observations, taken together, indicate that macrophage-derived TNF-α increases the release of FFAs from adipocytes, which, in turn, induces inflammatory changes in macrophages at least partly via the MAP kinase pathways.

To elucidate the role of adipocyte-derived endogenous FFAs in the production of TNF-α in macrophages, we examined the effect of insulin in coculture of the contact system, because insulin is well known for the antilipolytic action (Figure V, available online at http://atvb.ahajournals.org). Insulin significantly reduced not only FFA release but also mRNA expression of TNF-α and MCP-1 in coculture, whereas it induced no appreciable inflammatory changes in the control culture. Similar results were obtained using the transwell system (data not shown). These findings suggest that adipocyte-derived endogenous FFAs play a role in the induction of inflammatory changes in coculture.

**Figure 3.** Role of macrophage-derived TNF-α in adipocytes. (a) effects of anti-TNF-α neutralizing antibody on MCP-1 gene expression in both coculture systems. Differentiated 3T3-L1 was cocultured with RAW264 (1 × 10^5 cells/dish) for 24 hours. (b) effects of recombinant TNF-α (10 ng/mL) and MAP kinase inhibitors on MCP-1 gene expression in differentiated 3T3-L1. (c and d) effects of MAP kinase inhibitors on MCP-1 gene expression and secretion in the transwell system. Differentiated 3T3-L1 was cocultured with RAW264 (1 × 10^5 cells/dish) for 24 hours in the presence or absence of MAP kinase inhibitors. (e) enhanced phosphorylation of MAP kinases by coculture (RAW264, 1 × 10^5 cells/dish) in the contact system. Ab indicates anti-TNF-α antibody (1 μg/mL); IgG, nonimmune IgG; PD, PD98059 (20 μmol/L); SP, SP600125 (10 μmol/L). **P < 0.01 vs coculture alone (n = 6).

**Figure 4.** Role of adipocyte-derived FFAs in macrophages. (a) increased release of FFAs by coculture. Differentiated 3T3-L1 was cocultured with RAW264 (1 × 10^5 cells/dish) or cultured with TNF-α (10 ng/mL) for 24 hours. (b) effects of anti-TNF-α neutralizing antibody on FFA release in coculture. Ab indicates anti-TNF-α antibody (1 μg/mL); IgG, nonimmune IgG (1 μg/mL). (c) effects of FFAs on TNF-α gene expression in RAW264. RAW264 was incubated with FFAs (500 μmol/L) for 24 hours in the presence or absence of MAP kinase inhibitors. Pal indicates palmitate (50–500 μmol/L); laurate (La) and linolenate (Li) (500 μmol/L); EPA (E) 50 μmol/L; PD, PD98059 20 μmol/L; SP, SP600125 10 μmol/L. *P < 0.05 vs coculture alone (n = 6).

**Figure 5.** Augmentation of inflammatory changes by coculture with hypertrophied adipocytes or SVF from Obese Mice. We next investigated the impact of adipocyte hypertrophy on the inflammatory changes in adipocytes. We used hypertrophied 3T3-L1 adipocytes with larger lipid droplets, which were cultured for 21 days after the induction of differentiation and showed mild inflammatory changes (the upregulation of MCP-1 and downregulation of adiponectin; P < 0.05; Figure 5a). Coculture of hypertrophied 3T3-L1 and RAW264 in the contact system significantly augmented the inflammatory changes relative to that of differentiated 3T3-L1 with smaller lipid droplets (8 days after differentiation; P < 0.05). The augmentation of MCP-1 mRNA expression was mostly inhibited by treatment with anti-TNF-α neutralizing antibody (P < 0.01; Figure 5b), suggesting the role of TNF-α under this condition.
To elucidate the role of macrophages in obese adipose tissue, we cocultured 3T3-L1 and SVF obtained from wild-type or ob/ob mice in the transwell system. Coculture with SVF from ob/ob mice significantly increased MCP-1 mRNA levels in 3T3-L1 (P<0.01) but that from wild-type mice did not increase (Figure 6a). The SVF from ob/ob mice exhibited more abundant expression of TNF-α, as well as macrophage marker F4/80 relative to wild-type mice (P<0.01; Figure 6b). These observations suggest that SVF from obese mice has the potential to induce inflammatory changes in adipocytes because of an increased macrophage population.

**Discussion**

Obesity is associated with a state of chronic, low-grade inflammation. Recent studies have demonstrated that obese adipose tissue is characterized by increased infiltration of macrophages, suggesting that they are an important source of inflammation in the adipose tissue.\(^{13-15,24}\) It is, therefore, important to elucidate the signals that attract macrophages to the adipose tissue and to dissect the molecular mechanisms whereby adipocytes and macrophages communicate within the adipose tissue. Here we developed an in vitro coculture system composed of differentiated 3T3-L1 adipocytes and the macrophage cell line RAW264. The data herein suggest that our coculture system provides the unique in vitro experimental model system to investigate the functional interaction between adipocytes and macrophages within the adipose tissue.

This study demonstrates for the first time that the coculture of 3T3-L1 and RAW264 results in marked upregulation of proinflammatory cytokines, such as MCP-1, IL-6, and TNF-α, and downregulation of the antiinflammatory cytokine adiponectin, suggesting the functional interactions between adipocytes and macrophages. In this study, MCP-1 is increased in 3T3-L1 rather than RAW264 in the coculture system (Figure 2), which is consistent with a previous study with the laser microdissection technique in which the MCP-1 mRNA expression was not increased in nonadipocytes but in adipocytes in the adipose tissue from obese mice.\(^{10}\) These findings suggest that MCP-1 may serve as a marker of inflammation in adipocytes in the coculture system. Notably, we observed that the inflammatory changes are induced by the coculture of 3T3-L1 and RAW264 without direct contact, indicating that adipocytes and macrophages communicate at least partly via a paracrine mechanism. There appears to be a paracrine interaction between adipocytes and macrophages that controls the inflammatory status in obese adipose tissue in vivo, in which mature adipocytes is enlarged in size, and infiltrated macrophages is increased in number.\(^{13-15,24}\)

Because both adipocytes and macrophages are capable of secreting a number of biologically active substances, it is important to identify the paracrine signals between adipocytes and macrophages. Using an anti-TNF-α neutralizing antibody, we successfully inhibited the upregulation of MCP-1 gene expression in 3T3-L1 cocultured with RAW264. Given that TNF-α is derived mostly from macrophages in the coculture (Figure 2) and is expressed mostly in macrophages in the adipose tissue in vivo,\(^{13,14}\) our data suggest that TNF-α is a major macrophage-derived paracrine mediator of inflammation in the adipose tissue. This discussion does not exclude the possible involvement of other secreted factors than TNF-α in the induction of inflammatory changes in coculture. Additional studies are needed to elucidate the pathophysiologic significance of macrophage-derived secreted factors in obese adipose tissue. Adipose tissue expression of TNF-α is increased in a variety of experimental animal models of obesity and in obese humans,\(^{8,25,26}\) and may represent an important link between obesity and insulin resistance. It is conceivable that increased production of TNF-α by obese adipose tissue not only causes insulin resistance outside the adipose tissue (e.g., the skeletal muscle and liver)\(^{27-29}\) but induces inflammatory changes within the adipose tissue. The direct effects of TNF-α on adipocytes include induction of lipolysis (the release of FFAs)\(^{27,30,31}\) and alterations in expression of adipocytokines such as adiponectin,\(^{32}\) which can aggravate insulin resistance and account, at least partly, for the association between obesity and the metabolic syndrome. This above discussion is supported by a previous study in
which TNF-α-deficient mice rendered obese by a high-fat diet or ob/ob mice with targeted mutations in both p55 and p75 tumor necrosis factor receptors have lower levels of circulating FFAs and are protected from obesity-related insulin resistance relative to obese control mice despite there being no significant difference in body weight and adiposity.29

Recently, Berg et al33 have reported that adipocytes exert a strong inflammatory stimulus on macrophages in vitro and suggested the possibility of a cross-talk between adipocytes and macrophages. Among adipocyte-derived soluble factors (or adipocytokines), FFAs are very unique in that they are rich within the adipose tissue. Elevated circulating FFAs in obesity also potentially contribute to the development of insulin resistance.27 The data of this study suggest that FFAs are an important adipocyte-derived paracrine mediator of inflammation in macrophages because of the following reasons: (1) the release of FFAs from 3T3-L1 is increased when cocultured with RAW264; (2) TNF-α increases the release of FFAs from 3T3-L1; and (3) saturated FAs, such as palmitate, a major FFA released from 3T3-L1, can increase TNF-α production and, thus, induce inflammatory changes in RAW264. The precise mechanisms underlying the FFA-induced TNF-α expression are currently unknown. It is, nevertheless, conceivable that macrophase-derived TNF-α increases the release of FFAs from adipocytes, which, in turn, augments inflammatory changes in macrophages, thereby leading to increased production of TNF-α. We also found that there is no significant induction of inflammatory changes in adipocytes incubated with palmitate (≈500 μmol/L) for 24 hours (unpublished data), suggesting that macrophages are likely to be a target of FFAs in the induction of inflammatory changes in coculture. Interestingly, we observed no induction of inflammatory changes in macrophages by PUFS, suggesting the specificity of FFAs in the induction of inflammatory changes in macrophages. In this regard, it is interesting to note that saturated FAs induce inflammatory markers in macrophages through the activation of toll-like receptors, which is inhibited by unsaturated FAs, such as EPA and docosahexaenoic acid, the major n-3 PUFAs present in fish oil.25

There should be several intracellular signaling pathways involved in the induction of inflammatory changes in adipocytes and macrophages. In this study, we found that the inflammatory changes induced by the coculture of adipocytes and macrophages are significantly abolished by inhibiting MAP kinases. These observations suggest that the activation of MAP kinases is important for the induction of inflammatory changes in adipocytes and macrophages. Using the coculture system, however, it is hard to determine whether MAP kinases are activated in adipocytes, macrophages, or both. In this study, TNF-α-induced and FA-induced inflammatory changes in adipocytes and macrophages, respectively, are also significantly inhibited by MAP kinase inhibitors. In adipocytes, TNF-α has been shown to induce the activation of MAP kinases (ERK, JNK, and p38 MAP kinase),30,34 among which both ERK and JNK are involved in TNF-α-induced lipolysis.30,31 Furthermore, JNK has also mediated the suppressive effect of TNF-α on adiponectin gene expression.34 Conversely, ERK and JNK are involved in agonist-induced TNF-α gene expression in macrophages.35,36 We speculate that activation of MAP kinases in both adipocytes and macrophages within the adipose tissue may contribute to the pathogenesis of obesity itself and the metabolic syndrome.

The concept of adipose tissue endocrinology has been transformed by recognizing infiltrated macrophages as a source of inflammatory signals released from the adipose tissue, as well as a paracrine regulator of adipose tissue metabolism, thereby controlling the metabolic changes associated with obesity. In this study, we found that the inflammatory changes are augmented by use of either hypertrophied adipocytes or SVF obtained from ob/ob mice, suggesting the pathophysiologic implication in obese adipose tissue. A recent study with bone marrow transplantation has suggested that most of the macrophages in the adipose tissue are derived from the bone marrow.13 On the other hand, adipocytes and macrophages may even be interconvertible.37 Once infiltrated, macrophages may be activated in response to FFAs released from hypertrophied adipocytes and produce a larger amount of TNF-α, which, in turn, augments the release of FFAs and inflammatory changes in adipocytes. Previous studies demonstrated that adipose tissue expression of MCP-1 is increased during the course of obesity9,10,14,15 and is significantly correlated with macrophage markers in the adipose tissue,15 suggesting the role of MCP-1 in inflammatory changes of obese adipose tissue. Infiltrated macrophages, when activated, as well as hypertrophied adipocytes, per se, become a substantial source of MCP-1,10,14,15 which may augment the macrophase infiltration in the adipose tissue.38 Collectively, we postulate that a paracrine loop involving adipocyte-derived FFAs and macrophase-derived TNF-α establishes a vicious cycle that aggravates inflammatory changes in obese adipose tissue (Figure VI, available online at http://atvb.ahajournals.org).

In conclusion, this study is the first demonstration that FFAs and TNF-α derived from adipocytes and macrophages, respectively, ORGANIZE A PARACRINE LOOP THAT AGGRAVATES INFLAMMATORY CHANGES in the adipose tissue. The data of this study will also help identify therapeutic targets that may reduce obesity-induced inflammation and, thus, the metabolic syndrome associated with excess adiposity.

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References


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Figure I

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**Contact system**
- co-culture
- control culture

**Transwell system**
- co-culture
- control culture

Adipocytes (3T3-L1)
Macrophages (RAW264, peritoneal macrophages)
Stromal-vascular fraction

Co-culture harvest
Quiescent
24h
~48h
Figure II
Figure III
Figure IV

A  MCP-1/JE-Luc  B  MCP-1/JE-Luc  C  AP-1-Luc

RLA  RLA  RLA

0  5  10  15  0  5  10  15  0  1  2  3  4  5  6
ct  co  (-)  (-)  PD  SP  (-)  (-)  PD  SP  TNF-α  TNF-α

##  ##  **  **  ##  ##  **  **  ##
Figure V
TNF-α fatty acids (saturated) MAPK MCP-1

Infiltrated macrophages

Activation of chemotaxis?

Circulating monocytes

Obese adipose tissue

Hypertrophied adipocytes

MAPK

Figure VI
Online Supplement

Materials and Methods

Materials and Antibodies
Recombinant TNF-α and anti-TNF-α neutralizing antibody were purchased from R&D systems (Minneapolis, MN). Antibodies against mitogen activated protein (MAP) kinases (extracellular signal-regulated kinase (ERK), phospho-ERK, c-Jun NH2-terminal kinase (JNK), and phospho-JNK) and MAP kinase kinase 1 (MEK1) inhibitor PD98059 were obtained from Cell Signaling (Beverly, MA). JNK inhibitor SP600125 was purchased from BIOMOL (Plymouth Meeting, PA). Insulin was obtained from Eli Lilly (Indianapolis, IN). Fatty acids were purchased from Sigma (St. Louis, MO), solubilized in ethanol, and combined with fatty acid- and immunoglobulin-free bovine serum albumin (Sigma) in low serum medium. All other reagents were purchased from Sigma unless otherwise described.

Preparation of Peritoneal Macrophages
Green fluorescence protein (GFP) transgenic mice were kindly provided by Dr. M. Okabe (Osaka University, Suita, Japan). Murine peritoneal macrophages were obtained from GFP transgenic mice at 8-10 weeks of age using 5 mM sodium periodate, and cultured for 24h in Dulbecco’s modified Eagle’s medium (DMEM; Nacalai Tesque, Kyoto, Japan) with 10% fetal bovine serum (FBS; Sanko Junyaku, Tokyo, Japan) to remove non-adherent cells. After serum starvation for 24h in DMEM with 0.5% FBS, the peritoneal macrophages (1 x 10^5 cells/dish) were co-cultured with differentiated 3T3-L1 for 24 h and observed with a phase contrast/fluorescence microscopy (IX71; Olympus, Tokyo, Japan).

Transient transfection and luciferase assay
The luciferase reporter construct (MCP-1/JE-Luc) was kindly provided by Dr. D. Koya (Shiga University of Medical Science, Shiga, Japan). To construct the MCP-1/JE-Luc, the promoter region (2.6 kb) of the murine MCP-1/JE gene in pJECAT2.6 construct (a gift from Dr. J. M. Boss; Emory University, Atlanta, GA) was subcloned into pGL3 vector (Promega, Madison, WI). The 7 x activator protein-1 (AP-1) luciferase reporter construct (AP-1-Luc) was obtained from Stratagene (La Jolla, CA). The luciferase reporter constructs without any cis-acting DNA elements were used as negative controls. A luciferase reporter assay was performed as previously described. In brief, differentiated 3T3-L1 was transiently transfected by electroporation (Nucleofector system; Amaxa, Gaithersburg, MD) with luciferase reporter vectors and pRL-TK (Promega) as an internal control for transfection efficiency. After 24 h of incubation, cells were serum starved for additional 24 h and used for the experiments. The luciferase activity was determined using a Dual-Luciferase Reporter Assay System (Promega).
References


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<td>TNF-α</td>
<td>5’-ACCCTCAGTACAGATCTCCTT-3’</td>
<td>5’-TGGTGTTTGCTACGACGT-3’</td>
</tr>
<tr>
<td>F4/80</td>
<td>5’-CTTTGGCTATGGGCTTCCAGTC-3’</td>
<td>5’-GCAAGAGGACAGAGTTATCGTG-3’</td>
</tr>
<tr>
<td>36B4</td>
<td>5’-GGCCCTGCACCTCGCTTCC-3’</td>
<td>5’-TGCCAGGAGCGCTTGTG-3’.</td>
</tr>
</tbody>
</table>
Figure Legends

Figure I. Protocol of the co-culture system.

Figure II. Time course and number-dependent responses of adipocytokine mRNA expression in the contact system. A, Time course of adipocytokine mRNA expression by co-culture. Differentiated 3T3-L1 and RAW264 (1 x 10⁵ cells/dish) were co-cultured for 6-48 h. Up-regulation of MCP-1 and down-regulation of adiponectin were more marked at the longer time point, whereas macrophage marker F4/80 was not significantly changed by co-culture. B, Number-dependent responses of RAW264 on adipocytokine mRNA expression by co-culture. Differentiated 3T3-L1 and RAW264 (1 x 10³-1 x 10⁵ cells/dish) were co-cultured for 24 h. The extent of increase and decrease in MCP-1 and adiponectin, respectively, by co-culture was dependent on the number of RAW264. There was no appreciable difference in F4/80 between the culture methods. co, co-culture; ct, control culture. *P < 0.05, **P < 0.01 vs. ct at each point. n = 6.

Figure III. Co-culture with differentiated 3T3-L1 and undifferentiated 3T3-L1, RAW264, or peritoneal macrophages in the contact system. A, A representative microscopic view of co-culture of 3T3-L1 and peritoneal macrophages from GFP transgenic mice. x200. B, Changes of MCP-1 mRNA expression by co-culture for 24 h with undifferentiated 3T3-L1, RAW264, or peritoneal macrophages (1 x 10⁵ cells/dish). Co-culture with RAW264 or peritoneal macrophages revealed marked increase in MCP-1 mRNA expression, whereas co-culture with undifferentiated 3T3-L1 showed no significant changes. undiff, undifferentiated 3T3-L1; peri Mφ, peritoneal macrophages; ct, control culture; co, co-culture. ##P < 0.01. n = 6.

Figure IV. Molecular mechanism of MCP-1 mRNA expression in adipocytes. Differentiated 3T3-L1, transiently transfected with AP-1 or MCP-1 promoter
reporter construct, was co-cultured with RAW264 (1 x 10^5 cells/dish) or stimulated with recombinant TNF-α (10 ng/ml) for 8 h in the presence or absence of MAP kinase inhibitors. A, Induction of adipocyte MCP-1 mRNA expression by co-culture in the contact system. B, Induction of MCP-1 mRNA expression by TNF-α and the effects of MAP kinase inhibitors in adipocytes. C, Activation of AP-1 by TNF-α and the effects of MAP kinase inhibitors in adipocytes. MCP-1/JE-Luc, MCP-1 promoter luciferase reporter construct; AP-1-Luc, AP-1-luciferase reporter construct; RLA, relative luciferase activity; ct, control culture; co, co-culture; PD, PD98059 20 μmol/l; SP, SP600125 10 μmol/l. **P < 0.01 vs. (-)/(−). ###P < 0.01. n = 6.

**Figure V. Effects of insulin on inflammatory changes in the contact system.** Differentiated 3T3-L1 was co-cultured with RAW264 (1 x 10^5 cells/dish) with/without insulin (100 nmol/l) for 24 h. A, Decreased release of FFAs by insulin in co-culture. B, Effects of insulin on mRNA expression of TNF-α and MCP-1 in co-culture. co, co-culture; ct, control culture; Ins, insulin 100 nmol/l. **P < 0.01 vs. ct/(−). #P < 0.05, ##P < 0.01. n = 5.

**Figure VI. A paracrine loop involving adipocyte-derived FFAs and macrophage-derived TNF-α may establish a vicious cycle in obese adipose tissue.**