CXCL3 positively regulates adipogenic differentiation

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Abstract Chemokines are a family of cytokines inducing cell migration and inflammation. Recent reports have implicated the roles of chemokines in cell differentiation. However, little is known about the functional roles of chemokines in adipocytes. Here, we explored gene expression levels of chemokines and chemokine receptors during adipogenic differentiation. We have found that two chemokines, chemokine (C-X-C motif) ligand 3 (CXCL3) and CXCL13, as well as CXC chemokine receptor 2 (CXCR2), are highly expressed in mature adipocytes. When 3T3-L1 cells and ST2 cells were induced to differentiate, both the number of lipid droplets and the expression levels of adipogenic markers were significantly promoted by the addition of CXCL3, but not CXCL13. Conversely, gene knockdown of either CXCL3 or CXCR2 by specific siRNA effectively inhibited the course of adipogenic differentiation. CXCL3 treatment of 3T3-L1 cells significantly induced the phosphorylation of ERK and c-jun N-terminal kinase (JNK). Furthermore, CXCL3-induced CCAAT-enhancer binding protein (C/EBP)β and δ expression was suppressed by both ERK and JNK-specific inhibitors. Furthermore, chromatin immunoprecipitation assay revealed functional binding of PPARγ2 within the cxcl3 promoter region. Taken together, these results have indicated that CXCL3 is a novel adipokine that facilitates adipogenesis in an autocrine and/or a paracrine manner through induction of c/ebpβ and c/ebpδ.

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Chemokines are a family of structurally related small proteins that play important roles in the chemotaxis of various cell types. Chemokines are classified into four groups, C, CC, CXC, and CX3C chemokines, based on the position of four conserved cysteine residues that form two disulfide bonds paring the first with the third and the second with the fourth cysteines (1). Approximately 50 chemokines have been identified so far in humans and mice, respectively (2). Their chemotactic properties are profoundly involved in the pathogenesis of inflammation, tumors, and autoimmune diseases (3). On the other hand, constitutive expression of some chemokines is essential for the physiological processes of migration and homing of various types of cells in developmental homeostasis (4). Notably, some recent studies have shown regulatory roles of the chemokine/chemokine receptor signaling axis in the differentiation of mesenchymal stem cells (MSCs) (5), osteoblasts, and osteoclasts (6). These reports have suggested that some types of cellular differentiation are modulated by the chemokine expression levels in the tissue microenvironments.

It has recently been revealed that adipose tissues are highly active endocrine organs. Adipocytes produce and release a variety of physiologically active peptides and proteins, known as adipokines (7). About 50 adipocyte-derived substances have been isolated and characterized. Among them, leptin, adiponectin, interleukin-6 (IL-6), TNF-α, and plasminogen activator inhibitor-1 are well-known adipokines that are involved in inflammation, type 2 diabetes, cancers, hypertension, and metabolic disorders (8, 9). Monocyte chemotactic protein-1 (MCP-1), which is also referred to as CCL2, is accepted as an adipocyte-produced chemokine (10, 11). MCP-1 plays an important role in pro-inflammatory states and insulin resistance induced by increased adipose tissues (12). However, little is known about the secretion and functional roles of other chemokines in adipocytes.

The differentiation state of adipocytes is an important factor affecting adipokine production in vitro and in vivo (7). Overexpression of two inflammatory cytokines, TNF-α...
and IL-6, was observed with the expansion of adipose tissues. On the other hand, the expression of adiponectin, a cytokine with anti-inflammatory properties, is decreased in mature adipocytes.

Adipokine receptors are expressed in a variety of cell types, explaining why adipokines affect systemic lipid and glucose homeostasis (1). Importantly, previous studies have reported that several adipokine receptors such as leptin (13), IL-6 (14), and TNF-α receptors (15) are expressed in adipocytes, raising the possibility that adipokines function as local modulators of adipocyte physiology in autocrine/paracrine manners. However, detailed functional effects of each adipokine on adipocytes are still poorly understood.

In this study, we explored how adipocyte-derived chemokines affect the adipogenic differentiation of two cell lines, 3T3-L1 and ST-2. It has been found that several chemokines including CXC chemokine ligand (CXCL) 2, CXCL3, and CXCL13 are abundantly expressed in both cell lines during their adipogenic differentiation. Among these chemokines, CXCL3 distinctly facilitates adipogenic differentiation through ERK and c-jun N-terminal kinase (JNK)-mediated induction of CCAAT-enhancer binding protein (c/ebp)β and c/ebpδ in autocrine/paracrine manners.

**MATERIALS AND METHODS**

**Reagents and antibodies**

Recombinant mouse CXCL2 and CXCL3 proteins were purchased from Prospec (Rehovot, Israel). Recombinant mouse CXCL13 protein was purchased from Peprotech (Rocky Hill, NJ). Lipopolysaccharide (LPS), purified from *Escherichia coli* O55:B5 by phenol extraction and ion exchange chromatography (catalog number L5418), and recombinant mouse TNF-α was purchased from Sigma (St. Louis, MO). U0126, a specific mitogen-activated protein kinase kinase (MEK) inhibitor; SP600125, a specific JNK inhibitor; and SB203580, a specific p38 kinase inhibitor, were purchased from Funakoshi (Tokyo, Japan). Antibodies recognizing phosphorylated forms of ERKs, p38 kinases, and JNKs were purchased from Cell Signaling Technology (Danvers, MA). Antibodies against ERK1/2, p38 kinases, JNK1/2, and PPARγ were also from Cell Signaling Technology. Antibody against β-actin was from Santa Cruz Biotechnology (Santa Cruz, CA).

**Cell culture**

3T3-L1, a mouse preadipocytic cell line, was obtained from DS Pharma Biomedical Co. Ltd. (Osaka, Japan), and maintained in DMEM (Wako, Osaka, Japan) containing 10% FBS, 50 units/ml penicillin, and 50 mg/ml streptomycin. ST2, a mouse MSC line, was obtained from RIKEN Cell Bank (Tsukuba, Japan) and maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (Wako) containing 10% FBS, 50 U/ml penicillin, and 50 mg/ml streptomycin. Adipogenic differentiation of 3T3-L1 and ST2 were induced by the addition of 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 1.7 μM insulin, and 1 μM dexamethasone (DEX) in the culture medium. Osteogenic differentiation of ST2 was induced by the addition of 280 μM 1-l-ascorbic acid 2-phosphate trisodium and 5 mM β-glycerophosphate in the culture medium. Adipogenic progenitor cells were isolated from the subcutaneous adipose tissue of C57BL/6J mice. Briefly, the adipose tissue was minced and digested with 2 mg/ml type I collagenase at 37°C for 45 min. After filtration through a 100 μm filter (BD Falcon, Bedford, MA) to remove cellular debris, the tissue was centrifuged at 1,300 rpm for 5 min. Collected cells were suspended and cultured in DMEM containing 10% FBS. Primary osteoblasts were isolated from newborn mouse calvariae as previously described (16).

**Oil Red O staining**

Lipid droplet appearance was determined by Oil Red O staining. Briefly, cells were washed with Ca²⁺-free PBS twice and fixed in 10% formaldehyde in PBS for 1 h at 4°C. After three washes with distilled water and one wash with 60% isopropanol in distilled water, the cells were stained in 0.5% (w/v) Oil Red O in isopropanol for 15 min at room temperature. The remaining dye was washed out by three washes with distilled water. After the staining, Oil Red O was eluted with isopropanol and measured by OD540 using a microplate reader (Thermo Fisher Scientific, Waltham, MA). Blank wells were stained with dye and rinsed in the same manner. These values were subtracted from the experimental data points to control for stain retention by the walls of the wells. Each staining assay with three biological replicates was performed three times.

**RNA interference**

3T3-L1 cells were transfected with siRNA duplexes specific for murine CXCL1: r(GACAGUGUGGGAGGCUGU)dTdT and r(ACAGCCUCCCACACAUGC)dTdT, CXCL2: r(GUGAGUUGGGAAUACGU)dTdT and Ur(ACGUUUGCCACCUAC)dTdT, CXCL3: r(CCUGAGAGUCAUACCUAU)dTdT and r(AUAGUAUAGACUACCCAG)dTdT, CXC chemokine receptor 2 (CXCR2): r(CUUCUAGAAGAUCGCAG)dTdT and r(AGCCAUAUUGAGAAD)dTdT obtained from Sigma Aldrich, or nontargeting control siRNA duplexes (Control siRNA-A; Santa Cruz Biotechnology) using Hilymax (Dojindo, Kumamoto, Japan) according to the manufacturer’s instructions.

**Quantitative PCR analysis**

Total RNA was isolated with Isogen II (Wako) and reverse-transcribed with ReverTra Ace® (Toyobo, Osaka, Japan) in the presence of an oligo(dT) primer and RNase inhibitor (Takara Bio Inc., Otsu, Japan) at 37°C for 1 h. Real-time PCR was conducted using Step-One Plus (Life Technologies, Carlsbad, CA). The cDNA synthesized from 1 μg of total RNA was amplified in a 10 μl volume with 0.11 X SYBR Green I (Cambrex, Rockland, ME), 0.1 mM deoxynucleoside triphosphates, 0.2 μM each primer, 0.1 μM ROX™ reference dye (Life Technologies), and 1 unit Blend Taq DNA polymerase (Toyobo) under the following conditions: 94°C for 2 min, and then 55 PCR cycles at 94°C for 30 s, 60°C for 20 s, and 72°C for 20 s. Fluorescent signals were measured in real time, and then each sample was quantified. To determine the absolute number of copies of the target transcript, PCR product dilutions ranging from 10⁴ to 10⁸ copies were used to generate standard curves. The mRNA expression of chemokines, osteogenic markers, and adipogenic markers was analyzed using primers previously described (17) or shown in Table 1.

**ELISA**

Conditioned medium was collected and centrifuged at 200 g for 10 min. These supernatants were analyzed for CXCL3 contents using growth-regulated oncogene-γ (GRO-γ) Mouse SimpleStep ELISA Kit (Abcam, Cambridge, UK) according to the manufacturer’s instructions.

**Western blot analysis**

Cells were lysed in RIPA lysis buffer [150 mM NaCl, 1.0% Nonident-P40, 0.5% deoxycholic acid, 0.1% SDS, 50 mM Tris (pH
8.0), 0.1% Na$_2$VO$_4$, and protease inhibitor cocktail (Funakoshi, Tokyo, Japan). Immunoblotting was performed as previously described (18).

**Table 1. Real-time PCR primers used in this study**

| Gene Symbol | Primers [5′-3′] |
|-------------|----------------|
| ccr1        | ATTCACCCCTGCCCTACAGA |
| ccr2        | TTCCGCGGTCGAGATTCA |
| ccr3        | AGTTCGCGTCGCAAGAAG |
| ccr4        | GCCTGTGGAGAGAGGT |
| ccr5        | GCTAGCGCACTGAGGAGCCGGCAG |
| ccr6        | All PCR products were linked to pGEM-easy vector (Promega, Madison, WI), digested with KpnI and NotI, and the inserts were subcloned into a luciferase reporter vector pGL4.19 (Promega). To generate site-directed mutagenesis of the putative PPARG binding site of ccr3 gene, mutated DNA fragments constructed by PCR using two primer pairs, GAGTGTGAGGAGAGTGAAGCAGAC and GGGTTGCTGTGAGAGACAGTCA, were stably cotransfected with G418-resistant plasmid into 3T3-L1 cells by HiHmax according to the manufacturer’s instructions, followed by selection with 1.0 mg/ml G418. As a negative control cell line, pGL4.19 vector was stably transfected into 3T3-L1 cells. After G418 selection, three individual cell lines were isolated for each plasmid and induced to differentiate in adipogenic differentiation medium for 4 days. Relative luciferase activities were measured by Dual-Luciferase reporter assay kit (Promega) according to the manufacturer’s instructions.

**Chromatin immunoprecipitation assay**

Chromatin immunoprecipitation assay (ChIP) assay was performed as described previously (19). PCR amplifications were carried out using Blend Taq DNA polymerase (Toyobo) under the following conditions: 94°C for 2 min, and then 30 PCR cycles at 94°C for 30 s, 60°C for 20 s, and 72°C for 20 s. The ccr3 primer-specific primers were as follows: site 1 (+900 to +428) sense, TACCCCTGACTCACTGACATAC and antisense, AAAACACCGGAGCAGAGGA; and site 2 (+427 to +227), GGAGTCTCTGACCAAGGGGCAGTC and antisense, AAATCCCCCAGGACGCTTCAGC. The PCR products were analyzed by electrophoresis on a 1.5% agarose gel and quantitated by ImageJ (NIH). For each reaction, 1% of cross-link released chromatin was saved and reversed at 55°C for 6 h followed by proteinase K digestion and DNA extraction, and recovered DNA was used as input control.

**RESULTS**

**Gene expression of ccr3 is strongly induced during adipogenic differentiation**

For the purpose of exploring the roles of chemokines in adipogenic differentiation, we induced 3T3-L1, a mouse preadipocyte cell line, to differentiate into adipocytes for 4 or 8 days and analyzed the mRNA expression levels of various chemokine and chemokine receptor genes. The degree of adipogenic differentiation were confirmed by mRNA levels of fatty acid binding protein 4 (fabp4) (Fig 1A). Consistent with previous reports (12), cel2 (mcp-1), which belongs to the adipokine family, was highly expressed in mature adipocytes. Other than cel2, mRNA expression of ccl6, ccl11, ccl12, ccl24, ccl27, ccr3, ccr9, ccr10, and ccr13 was also significantly increased during the adipogenic differentiation. Among the examined chemokines, ccr3 and ccr13 mRNAs were most highly increased (Fig. 1A). As for chemokine receptors, cxr1, cxr1, cxr3, cxr10, cxr1, cxr2, cxr4, cxr5, and cxr3r1 mRNA were significantly increased in differentiated adipocytes (Fig. 1B). However, the absolute numbers of copies of ccl6, ccl11, ccl12, ccl27, ccr3, ccr10, ccr1, and ccr3r1 were only modest in undifferentiated 3T3-L1 cells.

Subsequently, we analyzed the chemokine and chemokine receptor expression of a mouse MSC line, ST2, cultured under adipogenic and osteogenic differentiation conditions. The degrees of adipogenic and osteogenic differentiation were confirmed by the expression levels of fabp4 and bone γ-carboxyglutamate protein (bglap), respectively (Fig. 1C). Similarly to 3T3-L1 cells, the mRNA levels of ccr3 and ccr13 were significantly increased with the induction of adipogenic differentiation (Fig. 1C). The mRNA level of ccr12 was also highly increased in the inducing condition of adipogenic differentiation. Among chemokine receptors, the
Fig. 1. mRNA inductions of chemokine and chemokine receptor genes during adipogenic or osteogenic differentiation of 3T3-L1 and ST2 cells. A, B: 3T3-L1 cells were induced to differentiate by a combination of DEX, insulin, and IBMX for 4 or 8 days. Total RNAs were isolated and reverse transcribed. The gene expression of fabp4, chemokine (A), and chemokine receptor (B) genes was analyzed by real-time PCR. Each experiment was performed at least three times producing consistent results. Relative mRNA expression levels in comparison with ribosomal protein L13a are shown. Error bars represent SD. C, D: ST2 cells were cultured in adipogenic differentiation media as in (A) or osteogenic differentiation media (280 μM L-ascorbic acid 2-phosphate trisodium and 5 mM β-glycerophosphate) for 10 days. Gene expression levels of adipogenic (fabp4) and osteogenic (bglap) markers, and chemokines and chemokine receptors were analyzed as in A. E: The culture supernatants were collected from undifferentiated (day 0) and differentiated (days 4 and 8) 3T3-L1 cells. CXCL3 ELISA was performed at least three times in three different cells.
mRNA expression of cer1, cer3, cer9, csrc2, and csrc5 was facilitated during the course of adipogenic differentiation (Fig. 1D). Furthermore, the amount of CXCL3 protein secretion was significantly increased during adipogenic differentiation (Fig. 1E). Notably, in contrast to adipogenesis, none of the chemokine or chemokine receptor expression was significantly altered with the induction of osteogenic differentiation.

**CXCL3 is a promoting factor for adipogenic differentiation**

We next examined the functional roles of CXCL3 and CXCL13, whose mRNA levels were highly induced during adipogenic differentiation of both 3T3-L1 and ST2 cells. Notably, the mRNA levels of csrc2 and csrc5, which are receptors for CXCL3 and CXCL13, respectively, were also increased during adipogenic differentiation in both cell lines (Fig. 1B, D). This result raised the possibility that CXCL3 and CXCL13 acted locally on adipocytes to directly regulate their cellular functions. Thus, we induced adipogenic differentiation of ST3-L1 cells with the addition of recombinant CXCL3 or CXCL13. Adipogenic differentiation was evaluated by the appearance of lipid droplets in the cytoplasm visualized by Oil Red O staining. As a result, lipid droplet appearance and absorbance value (OD540) were significantly increased by the addition of CXCL3, but not CXCL13 (Fig. 2A). Interestingly, treatment with CXCL2, another ligand of CXCR2, did not affect the differentiation. We also examined the effect of CXCL3 treatment on the gene expression of adipogenic markers. Being consistent with the observation of Oil Red O staining, mRNA induction of adipogenic marker genes, fabp4, pparg2, and cebp4, were significantly facilitated with the treatment by CXCL3. Moreover, the protein expression level of PPARγ2 was promoted with CXCL3 treatment (Fig. 2C). It was also found that the expression of cebp and cebp4, which are crucial transcription factors to initiate the differentiation cascade of preadipocytes, was also significantly promoted by CXCL3 at day 3.

We also analyzed the effects of CXCL3 treatment on adipogenic and osteogenic differentiation of ST2 cells. ST2 cells were cultured in either adipogenic or osteogenic differentiation medium with the addition of recombinant CXCL3 protein. Being consistent with the results of 3T3-L1 cells, the mRNA expression levels of pparg2 and fabp4 were significantly promoted by CXCL3 treatment in the adipogenic condition (Fig. 3A). In contrast, neither CXCL2 nor CXCL13 treatment affected adipogenic differentiation. When cultured in the osteogenic condition, the mRNA expression of bglap and runt-related transcription factor 2 (runx2), an essential transcription factor of osteogenic differentiation, was not altered by the addition of CXCL3 (Fig. 3B).

Furthermore, we examined the effects of CXCL3 on adipogenic differentiation of primary adipo-progenitor cells isolated from mouse subcutaneous adipose tissue. Being consistent with the results of 3T3-L1 cells, the gene expression of the analyzed adipogenic markers, fabp4, pparg2, cebp, and cebp4, was significantly increased with the treatment by CXCL3 (Fig. 3C). We also used primary osteoblasts derived from newborn mouse calvariae for the osteogenesis experiments and found that CXCL3 treatment did not significantly affect the expression levels of osteogenic markers (data not shown). Collectively, these results indicated that CXCL3 has promotive effect on adipogenic differentiation, but not osteogenic differentiation.

**Adipocyte-derived CXCL3 affects adipogenic differentiation by autocrine/paracrine manners**

Mature adipocytes expressed CXCL1, CXCL2, and CXCL3 (Fig. 1A), all of which share the CXCR2 as their specific signaling receptor. Hence, in order to elucidate the physiological roles of CXCL3/CXCR2 axis in adipogenesis, we performed siRNA-mediated gene knockdown experiments. 3T3-L1 cells were transiently transfected with CXCL1, CXCL2, CXCL3, or CXCR2-specific siRNA, followed by cell culture in adipogenic differentiation medium. Being consistent with the aforementioned result (Fig. 1), the fold induction ratio of cxcl2 mRNA expression was more significant than those of cxcl1, cxcl2, and cxcl2 mRNAs under the adipogenic culture condition (Fig. 4C). The suppressive effects of the siRNA transfections were confirmed by decreased protein and mRNA expression levels of the target chemokines (Fig. 4A, C). The results of Oil Red O staining revealed that CXCL3 and CXCR2 siRNA transfection significantly suppressed the appearance of lipid droplet (Fig. 4B). The induction of fabp4 and pparg2 mRNAs, as well as PPARγ2 proteins, was also inhibited by the transfection of CXCL3 and CXCR2 siRNAs (Fig. 4D). On the other hand, knockdown of cxcl1 or cxcl2 mRNA failed to significantly inhibit the lipid formation. Although knockdown of cxcl2 had a modest but significant inhibitory effect on fabp4 and pparg2 mRNA expression, it did not attenuate the protein expression level of PPARγ2 (Fig. 4C). These findings indicated that the CXCL3/CXCR2 signaling axis specifically promotes adipogenic differentiation of 3T3-L1 cells.

We then introduced CXCL3 siRNA to the adipogenesis experiment by recombinant CXCL3 in order to eliminate the background effects of endogenous CXCL3. 3T3-L1 cells were treated with either CXCL3 or control siRNA and incubated in adipogenic differentiation media with or without CXCL3 recombinant protein. As shown by PPARγ2 protein expression (Fig. 4E), the enhancing effect of adipogenesis by recombinant CXCL3 was more evident with the knockdown of endogenous CXCL3, further confirming the promotive effect of CXCL3 on adipogenesis.

**CXCL3 promotes cebp and cebp4 expression through ERK and JNK activation**

We next explored the intracellular signaling pathway induced by CXCL3 stimulation. 3T3-L1 cells were treated with recombinant CXCL3 protein and analyzed for the activated intracellular signaling molecules. CXCL3 rapidly induced significant phosphorylation of ERK (Fig. 5A). JNK was modestly phosphorylated, whereas phosphorylation of p38 was not induced by CXCL3 treatment. On the contrary, CXCL2 and CXCL13 did not induce the phosphorylation...
Notably, U-0126, a specific MEK inhibitor, significantly inhibited the CXCL3-induced expression of c/ebpβ and c/ebpδ. SP600125, a specific JNK inhibitor, suppressed the CXCL3-induced expression of c/ebpδ but not c/ebpβ. In contrast, the treatment by SB203580, a specific p38 inhibitor, did not influence CXCL3-induced expression of either c/ebpβ or c/ebpδ. These results have indicated that ERK activation is essential for the increased mRNA expression of c/ebpβ and c/ebpδ, while JNK activation is essential for that of any of the three protein kinases. In addition, we confirmed that CXCR2 knockdown using siRNA significantly inhibited CXCL3-induced ERK/JNK phosphorylation (Fig. 5B).

Subsequently, we examined whether short-term treatment by CXCL3 induced the expression of transcriptional factors that are crucial for adipogenic differentiation. It was found that c/ebpβ and c/ebpδ mRNA expression was increased by CXCL3 within 3 h in 3T3-L1 cells (Fig. 5C).
CXCL3 is an accessorial factor for adipogenic differentiation

We found that CXCL3 treatment rapidly induced the mRNA expression of c/ebp and c/ebp (Fig. 5B), which encode transcription factors initiating the adipogenic differentiation processes (20). It has been reported that these transcription factors increase the mRNA expression levels of pparg2 and c/ebp (21). To evaluate whether CXCL3 can be the initiating factor for adipogenesis, 3T3-L1 cells were cultured in normal cell culture media with the addition of recombinant CXCL3 protein. The mRNA levels of c/ebp and c/ebp were increased by CXCL3 treatment for 3 and 6 days. As for the late adipogenic differentiation marker genes, pparg2 and fabp4 mRNA levels were slightly elevated by CXCL3 treatments at day 6. However, these mRNA expression levels induced by CXCL3 were lower than those induced by the addition of general differentiation cocktail (insulin, IBMX, and DEX) (Fig. 6A). Moreover, higher-dose CXCL3 treatment (100 ng/ml) failed to increase the mRNAs of these late differentiation markers (data not shown).

To clarify the detailed effects of CXCL3 on adipogenesis, we treated the undifferentiated adipocytes by the combination of adipogenic cocktail including DEX, IBMX, insulin, and CXCL3 for 5 days (Fig. 6B). The addition of CXCL3 promoted fabp4, c/ebp, and c/ebp expression in all combination of cytokine cocktails. The induction levels of c/ebp and c/ebp were higher in the treatment of CXCL3/DEX/IBMX, CXCL3/DEX/insulin, and CXCL3/IBMX/insulin than in general differentiation inducers (DEX/IBMX/insulin). The expression of pparg2 was only promoted in CXCL3/IBMX/insulin combination. However, fabp4 induction was modestly increased in IBMX/insulin-treated cells. These results have indicated that CXCL3 alone have accessorial roles of c/ebp and c/ebp inductions in the presence of adipogenic inducers and insufficient to induce the full differentiation process of adipo-progenitor cells.

CXCL3 expression is highly induced in the inflammation states of adipocytes

Recent lines of evidence have revealed that obesity involves chronic low-grade inflammation (22, 23), indicating that adipocytes are exposed to inflammatory cytokines. We then examined the effects of inflammatory signals on the expression of cxcl3 and cxcl13 in undifferentiated or differentiated adipocytes. cxcl3 mRNA expression was remarkably increased by both LPS and TNF-α stimulations in premature adipocytes (Fig. 7). Interestingly, the response was higher in mature adipocyte than in adipo-progenitor cells. In contrast, these inflammatory factors did not affect cxcl13 expression. Thus, CXCL3 secretion may be induced from adipocytes in mature adipose tissue of obese subjects.

Cxcl3 promoter activity is regulated by the binding of PPARγ2

In order to analyze the transcriptional activation mechanism of cxcl3 during adipogenic differentiation, we searched the 1,000 bp upstream region of the mouse cxcl3 gene

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Fig. 3. CXCL3 treatment facilitates adipogenesis, but not osteogenesis, in ST2 cells. A: ST2 cells were cultured in adipogenic differentiation medium with the treatment of 10 ng/ml CXCL2, CXCL3, or CXCL13 for 4 or 10 days. Total RNAs were isolated and reverse transcribed. Adipogenic marker gene expression was analyzed by real-time PCR. Each experiment was performed at least three times producing consistent results. Relative mRNA expression levels compared with RPL13a are shown. Error bars represent SD. Statistical significance was determined by Student’s t-test (* P < 0.01). B: ST2 cells were cultured in osteogenic differentiation medium with 10 ng/ml of CXCL2 or CXCL3. Osteogenic marker gene expression was examined as in A. C: ST2 cells were cultured in adipogenic differentiation medium with the treatment of 10 ng/ml CXCL3 for the indicated days. Adipogenic marker gene expression was analyzed as in A.

c/ebp in CXCL3-treated 3T3-L1 cells. Moreover, CXCL3-induced c/ebp expression was significantly suppressed with the treatment by CXCR2 siRNA (Fig. 5D). These results have suggested that the promotive effects of CXCL3 on adipogenesis are mainly mediated by CXCR2.
Fig. 4. Transfection with CXCL3- or CXCR2-specific siRNA inhibits adipogenic differentiation of 3T3-L1 cells. A: 3T3-L1 cells were incubated in adipogenic differentiation with CXCL3-specific siRNA. The supernatants of culture medium were collected from undifferentiated (day 0) and differentiated (day 8) cells. CXCL3 ELISA was performed at least three times in three different cells. B: 3T3-L1 cells were cultured in adipogenic differentiation medium with the transient transfection of CXCL1-, CXCL2-, CXCL3-, or CXCR2-specific siRNA for 8 days. Cells were stained with Oil Red O to determine lipid droplet appearances. After the staining, extracted dye was monitored spectrophotometrically at 540 nm. Each staining assay with three biological replicates was performed three times. Error bars represent SD. C: After the treatment as in B, total RNAs were isolated and reverse transcribed. The indicated gene expression was analyzed by real-time PCR. Each experiment was performed at least three times producing consistent results. Relative mRNA expression levels compared with RPL13a are shown. Error bars represent SD. Statistical significance was determined by Student’s t-test (* P < 0.01). D: After the treatment as in B, cells...
for the putative binding sites of adipogenesis-related transcriptional factors using JASPAR (http://jaspar.genereg.net/). We found five, two, and two potential binding sites for PPARγ2, C/EBPa, and C/EBPB, respectively (Fig. 8A). We then established 3T3-L1 cells stably transfected with each of the six reporter plasmids containing various lengths of the 5′-upstream regions of the mouse cxcl3 gene (pGL-1000, -885, -491, -448, -420, and -336) (Fig. 8A). At least three independent clones for each construct were isolated. These cells were induced to differentiate into adipocytes for 4 days, and the luciferase reporter activities were measured. It was found that the promoter activities were significantly increased during adipogenic differentiation in cells stably transfected with pGL4-1000, pGL4-885, pGL4-491, and pGL4-448 constructs (Fig. 8B). The subsequent deletion (pGL4-420) resulted in a partially reduced promoter activity. The reporter activity was completely abrogated by the further deletion (pGL4-336).

The region between −448 and −336 seemed important for the activation of cxcl3 promoter during adipogenic differentiation, which contains two putative binding sites for PPARγ2 and none for C/EBPa nor C/EBPB. Each of the two putative PPARγ2 response element (PPRE) sites within this region (−436 to −421 and −352 to −337) was then mutated by site-directed mutagenesis in the pGL4-1000 wild-type cxcl3 promoter construct (Fig. 8C). In stably integrated 3T3-L1 cells, each of the two PPRE mutations (mD and mE) significantly reduced the reporter activity in adipogenesis (Fig. 8D). Furthermore, the promoter activity was completely abrogated by the double mutation (mD + mE). Therefore, CXCL3 induction is presumably regulated by these two PPREs during adipogenic differentiation.

In order to confirm that the interaction between PPARγ2 and the predicted PPRE sequences actually occurs in the endogenous cxcl3 gene, we performed the ChIP assay using an antibody specific to PPARγ2. The presence of the PPRE sequences in the immunoprecipitated chromatin was analyzed by PCR using two pairs of primers (−900/−428 and −427/−223) to target the PPARγ2 binding regions (Fig. 8C). The ChIP assay results showed PPARγ2 binding to −427/−223 but not −900/−428 of the cxcl3 5′-upstream region only after the induction of adipogenic differentiation (Fig. 8E). These results have indicated that the two PPREs in 5′ upstream region between −448 and −336 are functionally involved in the activation of cxcl3 promoter during adipogenic differentiation.

**DISCUSSION**

CXCL3, which is also referred to as GRO-γ, macrophage inflammatory protein 2β (MIP-2β), or neutrophil chemoattractant-2 (CINC-2), is a small cytokine consisting of 69 amino acids with 7.7 kDa molecular mass and belonging to CXC chemokine subfamily (24, 25). Previous studies have reported that CXCL3 is produced by macrophages (24, 26), osteoblasts (17), airway epithelium (27), dendritic cells (28), synovial fibroblasts (29), and cancers (30). Our present study is the first report showing significant production of CXCL3 by differentiated adipocytes (Fig. 1). CXCL3 is known to be involved in inflammation (24), cell proliferation (25), cell migration (31, 32), angiogenesis (33), and metastasis (33, 34). One of the significant novel findings of our study is the promotive effect of

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were lysed in RIPA lysis buffer at the indicated day. Cell lysates were separated by SDS-PAGE, and Western blotting was performed with the indicated antibodies. E: 3T3-L1 cells were cultured in adipogenic differentiation medium with the transient transfection of CXCL3-specific siRNA and CXCL3 recombinant proteins for 8 days. Cells were lysed in RIPA lysis buffer at the indicated day. Cell lysates were separated by SDS-PAGE, and Western blotting was performed with the indicated antibodies.
CXCL3 on adipogenic differentiation. When adipogenic differentiation was induced, both the lipid droplet appearance and the adipogenic marker gene expression were significantly promoted by the addition of recombinant CXCL3 protein in both 3T3-L1 (Fig. 2) and ST2 cells (Fig. 3). It should be noted, however, the treatment with CXCL3 alone is insufficient to induce complete differentiation of adipocytes (Fig. 6), indicating that the role of CXCL3 in adipogenesis is supportive. Previous studies also showed that the chemokine-induced signaling pathways are functionally involved in differentiation of several cell types. For example, stromal-derived factor-1, which is also referred to as CXCL12, was reported to be required for bone morphogenetic protein 2-induced osteogenic differentiation of C2C12 and ST2 cell (5). Fractalkine, which is also called CX3CL1, plays roles in an early stage of osteoblastic
CXCL3 expression is highly induced in the inflammation states of adipocytes. 3T3-L1 cells were cultured with or without addition of adipogenesis-inducing factors for 8 days. Undifferentiated or differentiated cells were stimulated by 10 ng/ml of LPS or 10 ng/ml of TNF-α for 3 h. Total RNAs were isolated and reverse transcribed. The expression of cxcl3 and cxcl13 genes was analyzed by real-time PCR. Each experiment was performed at least three times producing consistent results. Relative mRNA expression levels compared with RPL13a are shown. Error bars represent SD. Statistical significance was determined by Student’s t test (* P < 0.01).

Moreover, we found that CXCL3 expression was significantly induced by LPS and TNF-α in differentiated adipocytes (Fig. 7), suggesting that the inflammatory response to produce CXCL3 is higher in differentiated adipocyte than in adipo-progenitor cells. Some previous studies have shown that inflammatory stimuli including LPS show inhibitory effects on adipogenic differentiation (44, 45). Notably, LPS suppressed adipogenesis mainly by downregulating the expression of late adipogenic markers such as PPARγ2 and Fabp4 and inhibiting C/EBPα transcriptional activity (46). Being similar to LPS, TNF-α has been reported to down-regulate the expression of C/EBPα and PPARγ2 in adipocytes (47, 48). On the other hand, we have found that CXCL3 treatment promotes the initial adipogenic inducers, C/EBPβ and CEBPβ, in preadipocytes (Fig. 5C). We presume that the CXCL3 secretion from adipocytes induced by LPS or TNF-α partially compensate for the lack of the expression of late adipogenic inducers by promoting the expression of C/EBPβ and C/EBPβ. Being consistent with this hypothesis, a previous report demonstrated that TNF-α significantly induced C/EBPβ and C/EBPβ expression in adipocytes (49). Meanwhile, our findings also suggested that adipogenic differentiation might be regulated by other types of cells, such as macrophages, in adipose tissue. It is widely accepted that the infiltration of macrophages into adipose tissue is a crucial physiological event during the expansion and hyperplasia of adipocytes (50, 51). Previous studies have shown that CXCL3 expression in macrophages is significantly induced after the exposure to TNF-α and LPS (26, 27). The coordinated mechanism for CXCL3 production from both macrophages and differentiated adipocytes may be physiologically important for the induction of mature adipocytes.

Additionally, CXCL3 production by adipocytes might have a possible role to induce dynamic functional changes in the infiltrating immune cell populations during the development of adipocyte hyperplasia. Adipose tissue macrophages are known to be distinguished based on the expression of immune markers. Interestingly, chemokine receptors, CCR2 and CX3CR1, are reported to be candidate antigens for subdividing both the diversity of macrophages and their function (52, 53). Other studies showed that TNF-α derived from adipocytes and macrophages up-regulates inflammatory cytokines and downregulates anti-inflammatory adiponectin (54). Taken together, CXCL3 might be one of the candidate molecules involved in a paracrine loop between adipocytes and macrophages to define their phenotypes and functions in adipose tissue.

CXCL3 gene is located on chromosome 5 in a cluster with other CXC chemokines, CXCL1 (GRO-α), CXCL2 (GRO-β, MIP-2α), CXCL5 (epithelial cell-derived neutrophil

differentiation and the maintenance of osteoclastogenic precursor cells (6). Thus, taken together with our present finding, at least some types of chemokines seem to be important regulatory factors in cell differentiation.

Recent studies have shown that gene deletions of chemokine and chemokine receptors have phenotypic effects on adipose tissues. Genetic deficiency in CCR2 (35) and CX3CR1 (36) reduced food intake and attenuated the development of obesity in mice fed a high-fat diet. These phenotypes were explained by decreased macrophage infiltration into adipose tissue and central nervous system dysregulating the feeding behavior. In adipocyte-specific CXCR4 knockout mice, uncoupling protein 1 expression was significantly increased to support thermogenic activity of brown adipose tissue (37). Moreover, a conditional deletion of CXCR7, the alternative receptor of CXCL12, in adipocytes has revealed that CXCR7 regulates blood cholesterol by promoting its uptake in adipose tissue (38).

We have found that CXCL3 expression is highly induced during adipogenic differentiation (Fig. 1A, C). CXCR2, which is the receptor for CXCL3, was also expressed in adipocyte progenitor cell lines, 3T3-L1 and ST2 (Fig. 1B, D). These results raised the possibility that CXCL3 physiologically influences adipogenesis in autocrine/paracrine manners in adipocytes. Our subsequent data using siRNA transfection have demonstrated that both CXCL3 and CXCR2 knockdowns suppress adipogenic differentiation (Fig. 4). Several studies have also reported that adipocyte-derived cytokines and growth factors are important differentiation regulators for adipocytes themselves. Midkine (39), angiotensin 1-7 (40), and prostacyclin (41) promote adipogenic differentiation by autocrine mechanisms, whereas interleukin-11 (42) and activin A (43) have been reported to inhibit the differentiation of adipocytes. Collectively, it seems reasonable to presume that some of the adipocyte-derived secretory factors control the degree of their own differentiation.

In addition, CXCL3 expression was induced in late stage of adipogenic differentiation. We have shown that two PPREs in the 5′ upstream region are involved in the activation of cxcl3 promoter during adipogenic differentiation (Fig. 8). Therefore, CXCL3 seems to be secreted from mature adipocytes rather than undifferentiated adipo-progenitor cells. We presume that the functional role of CXCL3 on adipogenesis is mainly mediated in a paracrine manner promoting the early stage differentiation of surrounding cells.
Fig. 8. *Cxcl3* promoter activity is regulated by the binding of PPARγ2. A: Schematic diagram of putative PPARγ2 binding sites in the 5′ upstream region of the mouse *cxcl3* gene. Six reporter plasmids containing variable lengths of *cxcl3* gene upstream region are shown. A: pGL-1000. B: pGL4-885. C: pGL4-491. D: pGL4-480. E: pGL4-336. B: 3T3-L1 cells were stably transfected with one of the six pGL4.17-cxcl3 reporter plasmids described in A. The cells were induced to differentiate into adipocytes for 4 days. Cytoplasmic lysates were analyzed for luciferase activities in triplicate. Error bars represent SD. Three independent cell clones were isolated and analyzed for each reporter plasmid producing similar results. The results from a typical cell clone are shown for each construct. C: The upstream nucleotide sequence of the transcriptional starting point in the mouse *cxcl3* gene. Two putative PPRE sites were mutated by site-directed mutagenesis (mD: 5436 to 5421; mE: 5352 to 5337) in the pGL4-1000 wild-type *cxcl3* promoter construct to analyze their functional significance. The mutated bases are shown in italic lower cases. D: The mutated (mD, mE, and mD + mE) and the wild-type control constructs were stably transfected into 3T3-L1 cells. Luciferase reporter assay was performed as in B. E: 3T3-L1 cells were induced to differentiate for 4 days. Chromatins were extracted and immunoprecipitated with an antibody against PPARγ2. PCR analyses of the immunoprecipitated DNA were carried out. The positions of the PCR primer pairs are shown in C.
activating peptide-78), and CXCL7 (neutrophil-activating peptide-2) in mouse (55). Interestingly, although CXCL2 and CXCL3 share CXCR2 and CXCR1 as their specific receptors, CXCL2 failed to significantly promote lipid droplet appearance or adipogenesis-related gene expression during adipogenesis (Fig. 2A, B). These results were consistent with the finding that CXCL3-induced ERK and JNK phosphorylation levels were significantly higher than those induced by CXCL2 in 3T3-L1 cells (Fig. 5A). Furthermore, mRNA levels of Fadb4 and PPARγ2 were more significantly attenuated by CXCL3 siRNA than by CXCL2 siRNA (Fig. 4C). We presumed that the cause of these results was the different affinities of these two chemokines for the common receptors. Previous studies have investigated the selectivity and affinity of rat (36) and human (57) CXCR1 and CXCR2 ligands using the competition binding experiments, demonstrating that the biological activities and signal transduction pathways are different among chemokines that share the same receptor. In addition to the lower affinity level, it should also be noted that the expression level of cxcl2 was only slightly induced during adipogenic differentiation (Fig. 1A). Therefore, CXCL2 may only have an additional role in the physiological condition of adipocytes. As for the other CXCR2 ligands, the mRNA expression levels of CXCL1, CXCL5, and CXCL7 were much lower than that of CXCL3 (Fig. 1A, C).

Adipocytes moderately express both CXCR1 and CXCR2 mRNAs during adipogenic differentiation (Fig. 1B, D). We presume, however, that CXCR2 is a more dominant mediator of CXCL3 signals during adipogenesis due to the following two reasons. First, the fold induction of CXCR2 mRNA expression during adipogenic differentiation was more significant than that of CXCR1 (Fig. 1B, D). Second, the phosphorylation levels of ERK and JNK by CXCL3 treatment was almost completely abolished by CXCR2 siRNA transfection (data not shown).

We found that CXCL3 treatment induces significant phosphorylation of ERK in 3T3-L1 cells (Fig. 5A). ERK phosphorylation is a crucial event controlling the differentiation of various cell types including adipocytes (58). Some previous studies have shown that ERK phosphorylation has a promotive effect on adipogenesis (59, 60). In contrast, we and other groups previously reported that ERK activation has inhibitory effects on adipogenic differentiation (18, 61). We speculate that the apparent discrepancies among these studies including ours might be caused by the different roles of ERK depending on the adipogenic differentiation stages. Activation of ERK seems to be a fundamental trigger in the initial step of adipogenesis. Treatment with the combination of insulin, IBMX, and DEX, which are universally used as the basal initiators of adipogenic differentiation, has been reported to induce ERK phosphorylation in adipogenic progenitor cells (59). The normal adipocyte differentiation process is tightly orchestrated by a set of interdependently acting transcriptional factors, including PPARγ2, C/EBPα, C/EBPβ, and C/EBPδ (62). Among them, PPARγ2 is considered as the master transcriptional factor of adipogenesis. The promoters of adipocyte-phenotypic genes such as Fabp4 and lipoprotein lipase contain the binding sites for PPARγ2 (63). C/EBPα plays a crucial role in the later stages of differentiation to maintain high-level expression of PPARγ2 and adipocytic phenotypes (64). In contrast to PPARγ2 and C/EBPα, C/EBPβ and C/EBPδ are expressed at early stages during adipogenesis. While their expression is not specific for the adipocytic lineage, transient expression of C/EBPβ and C/EBPδ induces the burst expression of PPARγ2. We showed that CXCL3 treatment induced the mRNA expression of c/ebpβ and c/ebpδ in an ERK-dependent manner promoting the adipogenic differentiation induced by the combination of insulin, IBMX, and DEX (Fig. 5B). On the other hand, moderate induction of C/EBPβ and C/EBPδ mRNAs by CXCL3 treatment alone was insufficient to promote the expression of Pparγ2, c/ebpa, and fabp4 (Fig. 6). Thus, CXCL3 appears to play a supportive role in the induction of C/EBPβ and C/EBPδ through the activation of ERK in the early stage of adipogenic differentiation.

JNK is also an important signaling molecule regulating various types of cell differentiation (16, 65, 66). Lee et al. (67) have reported that JNK partly has an inhibitory effect on adipogenesis. JNK activation was involved in UV A-induced expression of migration inhibitory factor, which reduces the protein level of PPARγ2 in human adipose tissue-derived MSCs (hAMSCs) and 3T3-L1 cells (67). Our present study has demonstrated that CXCL3 treatment induces the phosphorylation of JNK (Fig. 5A) and JNK activation is involved in the CXCL3-induced c/ebpδ expression in 3T3-L1 cells (Fig. 5B). Being consistent with the functional role of JNK in adipogenic differentiation, it was found that the JNK phosphorylation level was increased at the early stage (day 1 to day 3) of adipogenic differentiation (data not shown). Moreover, treatment with a JNK-specific inhibitor suppressed the lipid droplet appearance and the gene expression of adipogenic markers (data not shown). These observations have suggested that JNK signaling...
activity is physiologically indispensable for adipogenic differentia-
tion. Being consistent with our findings, Lee et al. (68) previously reported that treatment by artemisinc acid induced the reduction of C/EBPβ expression through the 
inhibition of JNK in hAMSCs. Therefore, we suppose that 
JNK activation induced by CXCL3/CXCR2 signaling axis plays a significant role in the initial phase of adipocytic differentiation.

In summary, our study has demonstrated that CXCL3 is a novel adipokine that facilitates adipogenic differentia-
tion through ERK- and JNK-induced expression of c/ebpβ and
and possibly suggests new clinical approaches to chronic metabolic diseases and adiposity by controlling adipocyte-derived chemokine production.  

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