Obesity-associated mouse adipose stem cell secretion of monocyte chemotactic protein-1

Hui Ren Zhou,1 Eun-Kyoung Kim,1,2 Hyojung Kim,1 and Kate J. Claycombe1

Departments of 1Food Science and Human Nutrition and 2Neurology and Ophthalmology, Michigan State University, East Lansing, Michigan

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STUDIES HAVE SHOWN that obesity-associated increases in circulating proinflammatory cytokine levels decrease with a reduction in body weight, particularly with decreased body adipose tissue mass (4, 10, 34), suggesting adipose tissue is an important source of obesity-associated increases in inflammatory responses. A number of cytokines and chemokines, including monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor-α (TNF-α), interleukin (IL)-1, IL-6, IL-8, and IL-18, have been shown to be regulated according to the changes in body adiposity (45). However, MCP-1 can potentially exert a higher degree of adverse effects compared with other adipokines. For example, an obesity-associated increase in MCP-1 expression (31, 37, 44) results in infiltration and accumulation of macrophages in adipose tissue (9, 44, 47), and these macrophages subsequently secrete other inflammatory cytokines and chemokines that further accentuate obesity-induced adipose tissue inflammation (9, 44, 47). By activating this secondary inflammatory response in already inflammanogenic adipose tissue, MCP-1 may have a more profound effect on obesity-associated risks than other adipokines.

MCP-1 is a member of the C-C motif chemokine ligand-2 (CCL2) chemokines family of proteins that are known to induce leukocyte migration to the inflammatory organs and tissues (14, 30). It has been shown that the action of MCP-1 is mediated by MCP-1 binding to its seven-transmembrane-domain G protein-coupled CCL2 receptor (CCL2R). CCL2R activation has been shown to cause activation of downstream activators such as phosphatidylinositol 3-kinase and inositol trisphosphate, which leads to increased intracellular calcium. A rise in intracellular calcium concentration has been shown to activate Rho protein and mitogen-activated protein kinases (39, 48). Although the major function of MCP-1 is chemotaxis, recent studies suggest that MCP-1 also exert nonchemotaxis effects, which include the induction of adhesion molecules expression (17), tissue factor secretion (32), and smooth muscle cell proliferation (39, 42).

Studies have shown that a variety of cell and tissue types, including adipose tissue (3, 5, 12, 18), adipocytes (7, 13, 21, 36, 40, 41), preadipocyte (15, 38), cardiomyocytes (29), hepatic stellate cells (2, 26, 27), epithelial cells (19, 49), endothelial cells (20), and monocyte (23), secrete MCP-1. Interestingly, obese rodents have increased adipose tissue mass and increased levels of MCP-1 in plasma (31, 37, 44). In addition, adipose tissue stromal vascular fraction (SVF) cells have been shown to secrete higher levels of MCP-1 compared with adipocytes (11).

The SVF cells of adipose tissue are comprised of heterogeneous cell types, including macrophages, preadipocytes, fibroblasts, and nondifferentiated mesenchymal stem cells (1). To date, macrophages within mouse adipose tissue are thought to be the predominant source of proinflammatory cytokines (25). The resident macrophages in mouse adipose tissue, which are positively correlated with obesity, are the primary sources of TNF-α and IL-6 (44). Mice resident macrophages content is correlated with MCP-1 mRNA level (3, 6). In addition, a few studies have demonstrated that undifferentiated stem cells produce MCP-1 (22, 35). However, no studies have determined if other adipose tissue SVF cells play a role in MCP-1 secretion. Moreover, specific adipose tissue cell types that play a major role in obesity-associated increases in plasma MCP-1 have not been determined. Our studies show that adipose stem cells make up a majority of MCP-1-secreting cell types in the
adipose SVF of leptin-deficient obese (ob/ob) mice. Furthermore, we demonstrate that adipose stem cells have the potent secretory MCP-1 secretory potential in basal and lipopolysaccharide (LPS)-stimulated conditions.

Thus we hypothesized that leptin-deficient ob/ob mice have increased adipose tissue MCP-1 mRNA and MCP-1 secretion levels compared with lean mice because of increased MCP-1 secretion and an increased number of MCP-1-secreting adipose stem cells. To address our hypothesis, we isolated SVF cells, adipose stem cells (CD34⁺), macrophages (F4/80⁺), and adipocytes from adipose tissues of ob/ob mice and compared these cells’ potential to secrete MCP-1. In addition, to determine which specific adipose tissue depot contributes more significantly to circulating MCP-1 levels, we investigated MCP-1 secreting potential of isolated CD34⁺ cells from visceral and subcutaneous adipose tissue depots of ob/ob mice.

**MATERIALS AND METHODS**

Materials. LPS derived from *Escherichia coli* serotype O111:B4 with an activity of 1.5 × 10⁶ EU/ml was purchased from Sigma (St. Louis, MO). All chemicals were obtained from Sigma unless otherwise noted.

Mice. Adipose tissue and plasma were collected from 4-mo-old male C57BL/6J lean control and leptin-deficient obese C57BL/6J-ob/ob mice purchased from Jackson Laboratory (Bar Harbor, ME). Mice consumed a nonpurified diet (Teklad 22/5 Rodent Diet; Harlan, Indianapolis, IN) and water ad libitum. Mice were killed by CO₂ asphyxiation before excising adipose tissue. All conditions and handling of animals in this study were conducted with approved protocols by the Michigan State University Committee on Animal Use and Care.

Isolation of primary adipose cells. White adipose tissue was excised from lean and ob/ob mice, minced, and digested using 0.25% collagenase containing 2 mg/ml of collagenase type I (Worthington Biochemical, Lakewood, NJ) in Hanks’ balanced salt solution (HBSS) supplemented with sodium bicarbonate (0.35 g/l) at 37°C in a shaking water bath for 1 h. Adipose tissues were collected from subcutaneous inguinal (SI), subcutaneous dorsal bronchial region (SB), visceral perirenal (VP), and visceral epidydimal (VE) regions. The digested adipose tissue cells were filtered through 100-μm nylon cell strainers (BD Biosciences, Bedford, MA). The floating mature adipocytes were isolated by centrifugation (450 g for 1 min), washed twice with DMEM (Cellgro Mediatech, Herndon, VA), and resuspended with fresh DMEM supplemented with 10% heat-inactivated fetal bovine serum (HIFBS; GibCO, Grand Island, New York), 100 IU penicillin (P), and 100 μg/ml streptomycin (S). The resulting SVF pellet was treated with red blood cell lysis buffer for 5 min at room temperature. After addition of 10 ml of PBS and centrifugation, the pellet was resuspended in DMEM supplemented with 10% HIFBS and P/S and incubated in a 5% CO₂ humidified incubator 3–5 days until confluent CD34⁺ cells were isolated from SVF cells using magnetic cell sorting (MACS) according to the manufacturer’s instructions. Briefly, CD34⁺ cells were stained with a R-phycocerythrin (PE)-conjugated anti-CD34 antibody (eBioscience, San Diego, CA), and macrophages were stained with a PE-conjugated anti-F4/80 antibody (Invitrogen, Carlsbad, CA). Subsequently, the cells were magnetically labeled with anti-PE microbeads. The cell suspension was then loaded on a column that was placed in the magnetic field of a MACS separator. The magnetically labeled cells were retained in the column while the unlabeled cells ran through. After removal of the column from the magnetic field, the cells were eluted with MACS buffer and resuspended in 10% HIFBS DMEM P/S medium. Cells were plated in 24-well cell culture plates with 1 × 10⁶ cells·ml⁻¹·well⁻¹ for MCP-1 protein level measured by ELISA or in six-well cell culture plates with 5 × 10⁶ cells·5 ml⁻¹·well⁻¹ for real-time RT-PCR assays. For peritoneal macrophage isolation, peritoneal exudate cells from thioglycollate (1 ml of 9% broth for 3 days)-injected mice were obtained by peritoneal lavage with cold Ca²⁺- and Mg²⁺-free HBSS (Invitrogen). Peritoneal macrophages were collected by centrifugation at 200 g at 4°C for 10 min followed by resuspension and plated on cell culture dishes or plates (Falcon Labware, Lincoln Park, NJ). Cells were then allowed to adhere for 2 h at 37°C in 5% CO₂, at which time nonadherent cells were removed by vigorous washing. To minimize the potential proinflammatory effects resulting from use of collagenase and thioglycollate on the isolated cells, we washed, cultured, and passed cells (from passages 2–6) as indicated in the legends for Figs. 1–4.

**Enzyme-linked immunosorbent assay.** MCP-1 enzyme-linked immunosorbent assay (ELISA) was performed using a mouse CCL2/JE Duoset ELISA Development Kit (R & D Systems, Minneapolis, MN) with Immulon 4 HBX microtiter plates (Franklin, MN) according to the manufacturer’s procedure. Absorbances were measured at 450 nm with a Vmax Kinetic Microplate Reader ( Molecular Devices, Menlo Park, CA) using Softmax software.

**Real-time RT-PCR.** Total RNA was extracted from CD34⁺ macrophages and SVF cells using a RNeasy Mini Kit (Qiagen, Valencia, CA). The total RNA of adipocytes was extracted with a RNeasy Lipid
Tissue Mini Kit (Qiagen). Total RNA (10 ng/ml) was used to measure MCP-1 mRNA by real-time RT-PCR. The primers, probe, and endogenous control (18S-rRNA) were purchased as Taqman assay reagents (Applied Biosystems, Foster City, CA). Taqman One Step PCR Master Mix (Applied Biosystems) was used to quantify MCP-1 and 18S-rRNA following the manufacturer’s instructions on an ABI Prism 7900 (Applied Biosystems). 18S-rRNA was used to normalize target gene expression. Target gene expression levels were calculated relative to the control group.

Statistical analysis. Data were analyzed using Sigma Stat for Windows (Jandel Scientific). Data were subjected to one-way ANOVA, and pairwise comparisons were made by Bonferroni or Student-Newman-Keul’s methods. Differences were considered significant at P < 0.05.

RESULTS

Obesity-associated increases in serum and adipose tissue MCP-1 levels. We first tested if plasma MCP-1 is increased with increased adiposity. Our results showed that plasma MCP-1 is significantly higher in the ob/ob mice compared with lean mice (Fig. 1A). Furthermore, basal levels of adipose tissue MCP-1 mRNA are significantly higher in the adipose tissues of ob/ob mice compared with lean mice (Fig. 1B).

MCP-1 expression in CD34+, F4/80+, mixed SVF cells, and adipocytes. To compare the degree to which stem cells contribute to total adipose tissue expression of MCP-1, we isolated adipose tissue primary stem cells, macrophages, SVF cells, and adipocytes. These cells were then cultured and stimulated in the presence and absence of LPS to determine MCP-1 levels. As shown in Fig. 2, CD34+, F4/80+, and SVF cells express significantly higher levels of MCP-1 when compared with adipocytes in both basal and LPS-stimulated conditions. The basal and LPS-stimulated level of MCP-1 mRNA of CD34+, F4/80+, and SVF cells was not significantly different.

MCP-1 secretion by adipose CD34+ stem cells. To confirm whether adipose CD34+ stem cells secrete MCP-1, we isolated adipose CD34+ stem cells using a magnetic column with PE-conjugated mouse CD34+ antibodies and anti-PE microbeads. The average purity of the isolated CD34+ cell that was determined using flow cytometry analysis was 88.2% (n = 4). As shown in Fig. 3A, LPS stimulation increased MCP-1 secretion compared with control with significant accumulation during the incubation time period. Furthermore, LPS stimulation resulted in significant increases in MCP-1 mRNA expression in adipose CD34+ stem cells. This induction was time dependent, with the peak expression at 6 h of LPS stimulation as shown in Fig. 3B.

MCP-1 expression comparison of adipose tissue depots. We isolated CD34+ cells from four different adipose tissue depots, and cultured and tested levels of MCP-1 secretion ex vivo in the presence and absence of LPS stimulation. As shown in Fig. 4, in VE and VP depots, the secretion of MCP-1 was slightly higher than that of SI and SB. However, in response of LPS treatment, MCP-1 secretion was maximally induced, and no depot-specific difference was observed.

DISCUSSION

Data presented in our current study indicate that adipose tissue may play a major role in increasing plasma MCP-1.
levels in the leptin-deficient obese mice. As shown in Fig. 1, increases in the plasma MCP-1 levels of ob/ob mice compared with lean mice (Fig. 1A) nearly parallels that of adipose tissue MCP-1 mRNA levels (Fig. 1B). Other immune cell-containing tissues and their contribution to the obesity-associated increases in MCP-1 levels were not addressed in our current study. However, we previously showed that the obesity-associated increases in other proinflammatory cytokines such as IL-6 were mostly accounted for by the adipose tissue when compared with liver and spleen of ob/ob mice (16). The MCP-1 expression pattern is also similar to that of IL-6 in the ob/ob mice compared with lean mice has not been addressed in our current study and thus needs to be addressed in the future studies.

To further characterize which of the adipose tissue cell type(s) contribute most significantly to the obesity-associated increases in plasma MCP-1 levels, we isolated several candidate cell types from the epididymal adipose tissues of ob/ob mice, including adipocytes, macrophages, stem cells, and mixed SVF fraction cells, and compared their MCP-1-secreting potentials for both basal and under LPS-stimulated conditions. In agreement with data shown in a recent study (12), results from our study also showed that SVF cells express significantly higher levels of MCP-1 compared with adipocytes (Fig. 2). We have measured fresh undigested adipose tissue MCP-1 mRNA levels and compared these values with the threshold cycle (Ct) values of isolated adipocytes and CD34+ cells, as shown in the Table 1. Our data showed that there is more MCP-1 mRNA expression in the isolated CD34+ cells compared with the undigested fresh adipose tissue when adipocyte MCP-1 expression (Ct-FAM) signals were normalized to 18S signals (Ct-VIC). We further characterized the major cell type source of MCP1 within SVF and showed that adipose CD34+ stem cell and F4/80+ cells express significantly higher levels of MCP-1 mRNA compared with adipocytes.

In addition, in our current study, we showed that adipose CD34+ stem cell and F4/80+ cells also express significantly higher levels of MCP-1 mRNA compared with adipocytes. Interestingly, basal MCP-1 mRNA levels of F4/80+ cells were moderately higher than MCP-1 mRNA levels of CD34+ cells, whereas LPS-induced MCP-1 mRNA levels of SVF, CD34+, and F4/80+ cells showed no differences (Fig. 2). Using FACS analysis, we observed that 30 and 15% of adipose tissue SVF cells were comprised of CD34+ and F4/80+ cells, respectively (unpublished data). Thus we suggest that the twofold higher cell numbers of adipose CD34+ stem cells compared with F4/80+ macrophages point to a potentially predominant role of adipose CD34+ stem cells in obesity-associated increases in plasma MCP-1. In addition to adipose resident cells, we also tested a potential contribution from the nonadipose tissue resident macrophages. For example, using thioglycollate-elicited peritoneal macrophages from ob/ob mice, we showed that LPS-stimulated MCP-1 mRNA expression levels of F4/80+ nonadipose tissue resident macrophages are significantly less (1.73-fold increase compared with control) than the MCP-1 mRNA expression levels of adipose tissue resident macrophages (32.58-fold increase compared with control). The reason(s) for the differences in MCP-1 expression between the nonadipose tissue and adipose tissue resident macrophages is unknown and merits further investigation. However, recent studies showed that locally acting paracrine factors that are secreted by adipocytes and preadipocytes may contribute to the

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**Table 1. Tissue- and cell type-dependent MCP-1 real-time RT-PCR cycle numbers**

<table>
<thead>
<tr>
<th>Tissue Types</th>
<th>Cell Types (Ct-FAM)</th>
<th>Treatments (Ct-VIC)</th>
<th>MCP-1 [(Ct-FAM) − (Ct-VIC)]</th>
<th>18S-rRNA</th>
<th>MCP-1/18S-rRNA</th>
</tr>
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<tbody>
<tr>
<td>Fresh undigested adipose tissue</td>
<td>NA</td>
<td>None</td>
<td>27.050</td>
<td>22.240</td>
<td>4.810⁺</td>
</tr>
<tr>
<td>Adipose tissue</td>
<td>Adipocytes</td>
<td>Control</td>
<td>39.000</td>
<td>27.475</td>
<td>11.525⁺</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LPS</td>
<td>39.000</td>
<td>27.832</td>
<td>11.168⁺</td>
</tr>
<tr>
<td></td>
<td>SVF cells</td>
<td>Control</td>
<td>32.767</td>
<td>28.772</td>
<td>3.996⁺</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LPS</td>
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<td>28.479</td>
<td>−0.777⁻</td>
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<tr>
<td></td>
<td>CD34⁺ cells</td>
<td>Control</td>
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<td>28.649</td>
<td>4.134⁺</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LPS</td>
<td>27.581</td>
<td>28.314</td>
<td>−0.733⁻</td>
</tr>
<tr>
<td></td>
<td>F4/80⁺ cells</td>
<td>Control</td>
<td>32.859</td>
<td>28.784</td>
<td>4.075⁺</td>
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<td></td>
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<td>LPS</td>
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<td>28.244</td>
<td>−0.721⁻</td>
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<td></td>
<td></td>
<td>LPS</td>
<td>23.053</td>
<td>21.687</td>
<td>1.366⁺</td>
</tr>
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</table>

Values are expressed as means ± SE; n = 3 experiments. LPS, lipopolysaccharide. Cycle nos. (Ct) are expressed as monocyte chemotactic protein (MCP)-1 (Ct-FAM) and 18S-rRNA (Ct-VIC). Normalized MCP-1 values to 18S-rRNA [(Ct-FAM) − (Ct-VIC)] without the same letter differ statistically at P < 0.05. NA, not appreciable.
secondary increases in adipose resident cell activation and subsequent macrophage chemotaxis from circulation into adipose tissue (46).

In addition, a very recent work that characterized the different phenotypes of macrophages in lean and high-fat-induced obese mice suggests that diet-induced obesity leads to a shift from an alternatively protective state of adipose tissue macrophages to an activated state (M1) that exhibits proinflammatory events; these events are associated with selectively increased gene expression of TNF-α and inducible nitric oxide synthase, and several M1 markers (24). This study also showed a higher population of F4/80+CD11b+CD11c+ macrophage cells in high-fat-diet-fed animals compared with lean mice. Although not demonstrated in our current study, it is possible that there is a difference in the subpopulation of F4/80+ cells in ob/ob mice that contributes to changes in proinflammatory cytokine expression/secretion. However, according to our results, it is unlikely that F4/80+ cells contribute at higher levels than that of the CD34+ cells in increasing MCP-1 levels. This may be due to the fact that F4/80+ cells showed similar levels of MCP-1 expression compared with the CD34+ cells and have approximately half as many cells in the SVF compared with the CD34+ cells.

In our study, we used CD34 as an adipose stem cell marker to characterize the percentage of these cells in the adipose tissue SVF and their MCP-1-secreting potential. Interestingly, recent studies have shown that the rodent adipose tissues contain CD34+ cells (8), and human adipose tissue resident CD34+ cells do not exhibit hematopoietic stem cell-like ability (28, 33). Furthermore, these human adipose tissue CD34+ stem cells have been suggested not to be originating from the local adipose tissue vasculature but rather preadipocyte lineage cells that can be differentiated into adipocytes (33). Accordingly, potent MCP-1-secreting adipose tissue CD34+ cells in the adult ob/ob mouse may also posses adipocyte lineage potential.

To demonstrate that we used relatively pure CD34+ cells, we conducted FACS analysis of CD34+ and F4/80+ cells from the preadipocyte fraction (negatively selected from the SVF cells). According to our data, ~55% of adipose tissue SVF cells are comprised of preadipocytes in the ob/ob mouse; 2.3 and 1.7% of this preadipocyte fraction were made up of CD34+ and F4/80+, respectively. Furthermore, we have measured purity of CD34+ and F4/80+ cells that were isolated from adipose tissues. According to our FACS data, F4/80+ cells were 81.74% and CD34+ cells were 83.88% of SVF cells (data not shown).

In agreement with recent studies (3, 12), data from our current study also showed significantly higher basal MCP-1 secretion from the visceral adipose tissue compared with subcutaneous adipose tissue depots (Fig. 4). However, LPS-stimulated MCP-1 secretion showed no significant differences (Fig. 4). Although mice adipose stem cell expression of MCP-1 increased with LPS stimulation in our current study, we acknowledge that use of LPS and the consequent changes in MCP-1 gene expression do not necessarily reflect the physiological change seen in human obesity. Whether obesity-associated circulating factors such as leptin and insulin induce adipose CD34+ stem cell production of MCP-1 in an adipose tissue depot-specific manner needs to be investigated further.

Taken together, the data presented in this study demonstrated that obesity-associated increases in plasma MCP-1 levels in ob/ob mice may be primarily due to adipose CD34+ stem cell production of MCP-1. Our data also demonstrate that CD34+ cells isolated from the visceral adipose tissue secrete significantly higher levels of MCP-1 compared with the CD34+ cells from the subcutaneous adipose tissues under basal conditions.

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REFERENCES


