Involvement of mast cells in adipose tissue fibrosis

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Submitted 30 January 2013; accepted in final form 3 December 2013

Fibrosis can occur in various aged or chronic inflammatory tissues; liver (2, 24), lung (5), and pancreatic (33) fibrotic diseases are common worldwide. Fibroblast proliferation is important for tissue regeneration and repair, but excessive formation of fibrous connective tissue impairs tissue architecture and function, eventually resulting in organ failure. Currently, there is no specific treatment for fibrosis; therefore, the prevention of pathogenesis of fibrosis is likely the only strategy against fibrosis.

Interestingly, some researchers have reported that obese adipose tissue also exhibits fibrosis (4, 13). Adipose tissue fibrosis is associated with insulin resistance (13, 25), which is a characteristic feature of obesity and type 2 diabetes and plays a major role in the pathogenesis of the disease (19). Therefore, suppression of adipose tissue fibrosis is considered to be of great significance for the prevention of metabolic syndrome. However, the mechanism of adipose tissue fibrosis has not been clarified.

In other tissues, such as liver (2, 24) and lung (5), mast cells are observed in the fibrous region, which promotes fibroblast growth and collagen synthesis by secreting various mediators including tryptase. Recently, mast cells have been found to be accumulated in obese adipose tissue (1, 15), preferentially in fibrosis bundles (4). These facts suggest that mast cells in obese adipose tissue may participate in the development of fibrosis in adipose tissue.

The objective of the present study was to clarify the relationship between fibrosis and mast cells in obese adipose tissue, as well as to determine the origin of infiltrating mast cells. In the present study, fibrosis and the accumulation of mature mast cells were observed in obese adipose tissue. We indicate that immature mast cells that have infiltrated into nonobese adipose tissue gradually mature with the progression of obesity and diabetes. We further demonstrate that mouse tryptase, mast cell protease-6 (MCP-6), secreted from mature mast cells in obese adipose tissue, enhances collagen mRNA expression in fibroblasts, which inhibits preadipocyte differentiation. Our findings suggest that mast cells are involved in the process of fibrosis of obese adipose tissue, which might accelerate insulin resistance.

MATERIALS AND METHODS

Animals. Twenty male 5-wk-old db/db mice and 6-wk-old C57BL/6Jc1 mice were purchased from Nippon CLEA (Tokyo, Japan). All the mice were individually housed in plastic cages in a room with controlled temperature (24 ± 1°C) and lighting (alternating 12 h periods of light and dark). All db/db mice had free access to 60 kcal % high-fat diet (D12492; Research Diet) and water for 0, 5, 7, or 10 wk. We measured the body weights of mice every week. At the end of the experimental period, anesthetized mice were killed by cervical dislocation after overnight fasting, and blood samples were collected from the palpebral veins for the determination of blood glucose by Glucose Clinitest Wako (Wako Pure Chemical Industries, Osaka, Japan). Epididymal adipose tissue samples were collected, from which stromal vascular (SV) cells were obtained for flow cytometric analysis (FACS). Mesenteric adipose tissue samples were collected for the tissue culture and for the determination of mRNA expression levels. The animal care procedures and methods were approved by the Animal Care Committee of Kyoto University. Mice were treated in accordance with the “Guideline for the Care and Use of Laboratory Animals”, Kyoto University.

FACS. SV cells were isolated from epididymal adipose tissue of db/db mice as described previously (11), with slight modifications. Briefly, the obtained adipose tissue samples of 5–10 mice each were pooled and cut into small pieces and digested with 1 mg/ml collagenase type I (Sigma-Aldrich, Saint Louis, MO) at 37°C for 1 h. The
3T3 fibroblasts were purchased from American Type Culture Collection. The resulting digest was filtered through a 100-μm nylon mesh, and SV cells were centrifuged at 1,500 rpm for 5 min. SV cells were incubated with Fe blocking solution (eBioscience, San Diego, CA) for 10 min on ice and then triply stained with a phycoerythrin (PE)-Cy5-conjugated anti-mouse CD45 antibody (eBioscience), a PE-conjugated anti-mouse CD117 (c-kit) antibody (eBioscience), and a fluorescein isothiocyanate (FITC)-conjugated anti-mouse FcεRIα antibody (eBioscience). After incubation with the antibodies, the cells were washed twice with FACS buffer and analyzed on a FACSCalibur (BD Biosciences, San Jose, CA) with CellQuest software (BD Biosciences).

Adipocyte histology: immunohistochemistry and Masson's trichrome staining. Mesenteric adipose tissue samples were fixed in 4% paraformaldehyde at 4°C overnight, embedded in paraffin, and cut into 8-μm-thick sections. For immunohistochemical staining, deparaffinized sections were treated with citrate buffer (pH 6.0) at 121°C for 15 min to retrieve antigens and with 0.3% hydrogen peroxide for 10 min at room temperature to quench endogenous peroxidase activity. After blocking with normal donkey serum (Millipore, Billerica, MA) for 30 min, the sections were incubated with an anti-mast cell tryptase antibody (FL-275) (sc-32889; Santa Cruz Biotechnology, Dallas, TX), which is recommended for the detection of mast cell tryptase of mouse, rat, or human origin, at 4°C overnight, followed by a biotinylated secondary antibody (anti-rabbit IgG; GE Healthcare, Buckinghamshire, UK) at room temperature for 1 h and then streptavidin-horseradish peroxidase (GE Healthcare) at room temperature for 30 min. The reaction was developed with diaminobenzidine reagent for 10 min. For Masson's trichrome staining, deparaffinized sections were stained with Weigert's iron hematoxylin solution (Sigma-Aldrich) for 5 min, Biebrich scarlet-acid fuchsin solution (Sigma-Aldrich) for 3 min, 10% sodium phosphotungstate-10% phosphomolybdic acid (Sigma-Aldrich) for 5 min, and aniline blue solution (Sigma-Aldrich) for 5 min.

Preparation of adipose tissue-conditioned medium. Mesenteric adipose tissue samples (300 mg) from db/db mice were minced and incubated in 10 ml of M199 medium (Nacalai Tesque, Kyoto, Japan) supplemented with 50 μg/ml gentamicin (Invitrogen, Carlsbad, CA) at room temperature for 10 min. For Masson's trichrome staining, aspirates were coated with fibronectin (10 μg/ml, Biomedical Technology) and stored at −20°C until use.

Preparation of bone marrow-derived mast cells. Bone marrow cells were harvested from the femoral bone marrow of 6-wk-old male C57BL/6Jcrl mice. The cells were cultured in α-MEM (Nacalai Tesque) supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin mixed solution (Invitrogen), 5 ng/ml stem cell factor (PeproTech, Rocky Hill, NJ), 5 ng/ml interleukin (IL)-3 (PeproTech), 2 ng/ml IL-4 (PeproTech), and 50 μM β-mercaptoethanol at 37°C in a humidified 5% CO₂ atmosphere. After 72 h, the aliquots of the culture medium were collected as adipose tissue-conditioned medium (ATCM) and stored at −20°C until use.

Table 1. Sequences of primer sets used for PCR

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gies, Stoughton, MA) at room temperature overnight and then air-dried. After labeling of BMMCs with 10 μg/ml DiIC12 (3) fluorescent dye (BD Biosciences) at 1 × 10⁷ cells/ml for 1 h, the cells were suspended at 3 × 10⁶ cells/ml in α-MEM supplemented with 1% P/S and placed in the upper wells. The bottom wells were filled with culture medium (control) or ATCM from db/db mice at various obese stages as described above. After 72 h of incubation at 37°C in a humidified 5% CO₂ atmosphere, BMMCs that had migrated to the lower side of the filter were measured on the basis of fluorescence intensity at 549/565 nm, using Infinit F200, a bottom-reading fluorescence plate reader (Tecan Group, Männedorf, Switzerland).

**Statistical analysis.** The data are presented as mean ± SE. Student’s t-test was used to assess statistically significant differences between groups of two. Comparisons between multiple groups were carried out using one-way ANOVA and Dunnet’s multiple comparison tests. Differences were considered significant at *P* < 0.05.

**RESULTS**

**Body weight and plasma glucose in db/db mice.** Body weights in 5-, 10-, 12-, and 15-wk-old mice were 25.2 ± 0.2, 47.6 ± 0.2, 56.2 ± 0.8, and 57.3 ± 2.9 g, respectively. Fasting

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Fig. 1. A: mast cells increased in obese adipose tissue. Mast cell protease-6 (MCP-6)-positive cells (arrows) were detected by immunohistochemical staining of mesenteric adipose tissue from 15-wk-old db/db mice. B: the stromal vascular (SV) fraction was isolated from the epididymal adipose tissue of various obese diabetic stages of db/db mice, and the percentages of CD45⁺/c-kit⁺/FcεRI⁺ triple-positive mast cells among total SV cells were measured by FACS.
plasma glucose levels in 5-, 10-, 12-, and 15-wk-old mice were 200 ± 35, 400 ± 58, 517 ± 45, and 641 ± 37 mg/dl, respectively. These results indicated that the degree of hyperglycemia increased with the progression of obesity and still continued to increase after body weight gain had reached a plateau.

Mast cells accumulated in visceral adipose tissue with the progression of obesity and diabetes. To characterize mast cell accumulation in visceral adipose tissue with the progression of obesity and diabetes, we performed immunohistochemical analysis and FACS. In immunohistochemical analysis, we used an antibody against MCP-6, one of the major proteins stored in the granules of mature mast cells. Indeed, the antibody we used in this study was raised against human mast cell tryptase but could cross-react with mouse tryptase (mMCP-6), as described in this study was raised against human mast cell tryptase but could cross-react with mouse tryptase (mMCP-6), as described in the data sheet. As shown in Fig. 1A, MCP-6-positive cells increased in the mesenteric adipose tissue of 15-wk-old mice. In the FACS analysis, we first gated the SV population on CD45-positive status and showed the FACS histogram with c-kit and FcεRI. The percentages of CD45+/c-kit+/FcεRI+ triple-positive cells among total SV cells were very low (~0.18%) in the epididymal adipose tissue of 5-wk-old mice; however, they increased with the progression of obesity and diabetes (Fig. 1B).

Fibrosis and collagen expressions were enhanced in obese adipose tissues. As mast cells are observed in many fibrous regions and are considered to promote fibroblast growth and collagen synthesis, we next performed Masson’s trichrome staining, which stains the collagen-rich fibrotic regions blue, in obese adipose tissue. The mesenteric adipose tissue of 15-wk-old mice was more deeply stained than that of 5-wk-old mice (Fig. 2A). Collagen types I, IV, V, and VI are reported to be abundantly expressed in obese adipose tissue in mice, whereas collagen types II and III show relatively lower expression levels (13). Therefore, we measured the mRNA expression levels of the former four types of collagens in adipose tissue of various obese stages. Collagen 4α1 mRNA expression level in adipose tissue significantly increased in 10- and 12-wk-old mice but not in 15-wk-old mice compared with 5-wk-old mice (Fig. 2B). The mRNA expression levels of other types of collagen in adipose tissue significantly increased in 15-wk-old mice compared with 5-wk-old mice (Fig. 2B).

MCP-6 derived from obese adipose tissue induced collagen 5 expression in fibroblasts and adipose tissues. Next, we investigated whether MCP-6 enhanced collagen expression in fibroblasts in obese adipose tissues. Treatment with normal rabbit IgG (species/isotype-matched control IgG) did not affect the expression levels of any collagen types (data not shown). Collagen 1α1 mRNA expression in NIH-3T3 fibroblasts was not affected by ATCM from 15-wk-old mice compared with that from 5-wk-old mice (Fig. 3A). On the other hand, collagen 4α1 mRNA expression in the cells treated with ATCM from 15-wk-old mice was significantly suppressed by anti-MCP-6 antibody (Fig. 3B). Collagen 5α1 mRNA expression was significantly enhanced by ATCM from 15-wk-old mice, and this enhancement was significantly suppressed by anti-MCP-6...
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Collagen V inhibited 3T3-L1 preadipocyte differentiation. To identify the pathophysiological significance of the enhanced expression of collagen V in obese adipose tissue, we investigated the effect of collagen V on preadipocyte differentiation. The mRNA expression levels of the adipocyte markers, aP2 and LPL in 3T3-L1 cells that differentiated on collagen V-coated plates significantly decreased compared with those in cells that differentiated on non-collagen-coated plates, whereas other types of collagen did not affect the mRNA expression of adipocyte markers (Fig. 5A). Similar results were obtained for the accumulation of lipid droplets (Fig. 5B).

BMMCs migrated to nonobese adipose tissues. To determine the obese stage at which immature mast cells migrate to adipose tissue with the progression of obesity and diabetes, we examined BMMC migration activities to ATCM from various stages of diabetes. ATCM from 5-wk-old mice significantly induced the migration of a larger number BMMCs than control or that from 10-, 12-, and 15-wk-old mice (Fig. 6).

Mast cell maturation in adipose tissue with progression of obesity and diabetes. To determine whether immature mast cells that infiltrated into nonobese-stage adipose tissue undergo maturation with the progression of obesity and diabetes, we measured the mRNA expression level of MCP-6 in BMMCs treated with ATCM from various stages of diabetes. MCP-6 mRNA expression level significantly increased in BMMCs treated with ATCM from 15-wk-old mice compared with the control (Fig. 7). As candidate factors that induce the maturation of mast cells in adipose tissue, we focused on fatty acids, whose level of secretion from adipose tissue increases with the progression of obesity and diabetes (5). Palmitic acid and stearic acid significantly increased MCP-6 mRNA expression level; however, unsaturated linolenic acid did not affect the expression level of MCP-6 mRNA (Fig. 8).

DISCUSSION

Fibrosis is observed in various organs such as liver (2, 24), lung (5), and pancreas (33), which severely impairs tissue architecture and function, resulting in organ failure. Recently, fibrosis has also been observed in obese adipose tissue (4, 13), which is related to insulin resistance (13, 25). However, the mechanism of adipose tissue fibrosis has not been fully clarified.

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Numerous mast cells are observed in obese adipose tissue (1, 15), preferentially in fibrosis bundles (4). Mast cells in liver are
reported to secrete various mediators in granules, including tryptase, that promote fibroblast growth and collagen synthesis (7, 16, 23). Therefore, we hypothesized that mast cells in obese adipose tissue also secrete tryptase, involving in the adipose tissue fibrosis.

In the present study, we observed the Masson’s trichrome-positive fibrosis and accumulation of mast cells in the adipose tissues derived from severely obese diabetic mice (15-wk-old mice). Furthermore, we observed that the enhanced expressions of collagen 5 mRNA by ATCM from 15-wk-old mice were suppressed by the cotreatment with the neutralizing anti-MCP-6 antibody. MCP-6, one of the mast cell-derived granule components, is the counterpart of human tryptase because of its predicted tryptic-like substrate specificity (18, 22). Collagen V consists of about 1–5% of the total collagens in healthy tissue; however, it increases during fibrogenesis in liver (31) and lung (20), playing an anchoring role between collagen fibrils (31, 32). Collagen V is also reported to increase

Fig. 4. MCP-6 enhanced collagen 5 mRNA expression in fibroblasts and adipose tissue. NIH-3T3 cells (A) and epididymal adipose tissue from C57Bl/6J mice (B) were treated with 1 or 10 ng/ml recombinant mouse MCP-6 for 6 h. Expression levels of collagen mRNAs were quantified as described in Fig. 2 legend. Values are means ± SE of 4 replicates. *P < 0.05 vs. culture treated without MCP-6.

Fig. 5. Collagen V inhibited preadipocyte differentiation. 3T3-L1 preadipocytes were induced to differentiate on plates coated with various types of collagens. A: expression levels of adipocyte markers aP2 and LPL mRNAs were quantified as described in Fig. 2 legend. B: lipid droplet was stained with Oil red O and photographed. The quantity of Oil red O was determined by measuring absorbance at 550 nm. Values are means ± SE of 4 replicates. *P < 0.05 vs. culture on noncoated plates.

Fig. 6. ATCM from nonobese stage enhanced bone marrow-derived mast cell (BMMC) migration. Migration of fluorescence-labeled BMMCs to ATCM from various obese stages of db/db mice during 72 h was quantified on the basis of fluorescent intensity and represented as a relative value. Values are means ± SE of 4 replicates. **P < 0.01 vs. control.

Fig. 7. Expression of the maturation marker of mast cells was enhanced by ATCM from severely obese diabetic stage. BMMCs were treated with ATCM from 5- or 15-wk-old db/db mice for 24 h. Expression level of MCP-6 mRNA was determined by semiquantitative RT-PCR. The relative amount of MCP-6 transcript was normalized to the amount of 36B4 transcript. Values are means ± SE of 4 replicates. *P < 0.05 vs. control.
These results suggest that collagen V may not be involved in adipose tissue fibrosis.

To investigate the pathophysiological significance of adipose tissue fibrosis, we examined the effect of collagen 6 on preadipocyte differentiation. Preadipocyte differentiation is considered to be important in mediating insulin sensitivity in adipose tissue (6). In fact, preadipocyte differentiation is impaired in patients with insulin resistance (21, 30). The present study demonstrated that only collagen V among the various types of collagens inhibited preadipocyte differentiation, suggesting that collagen V expression enhanced by the mast cell-derived mediator MCP-6 may cause the aggravation of insulin resistance. On the other hand, Khan et al. (13) reported that the increased expression of collagen VI in obese adipose tissue induces insulin resistance probably via the enhancement of shear stress of plasma membrane of adipocytes. Therefore, adipose tissue fibrosis may be associated with insulin resistance via multiple mechanisms.

To further investigate the pathogenesis of adipose tissue fibrosis associated with mast cells, we next examined the origin of mature mast cells accumulated in obese adipose tissue. In general, mast cells are considered to infiltrate into peripheral tissues in an immature form and complete their maturation in the tissues they infiltrate (9, 16). In the present study, the migration of BMMCs, an immature model of mast cells, was enhanced only by ATCM from severely obese mice (15-wk-old mice). These findings suggest that immature mast cells that infiltrate into nonobese-stage adipose tissue may be gradually stimulated to mature with the progression of obesity and diabetes.

As candidate factors that promote the maturation of mast cells in adipose tissue, we focused on saturated fatty acids (10, 25), especially in fibrotic areas (26). These facts and our findings suggest that collagen V induced by MCP-6 is associated with adipose tissue fibrosis.

On the other hand, myofibroblasts that specifically express α-smooth muscle actin (α-SMA) under the control of TGF-β plays the central role in many fibrotic tissues (8, 14, 29). Recently, α-SMA was also reported to be expressed in fibrotic regions in adipose tissue (4, 17). Furthermore, Spencer and colleagues (25, 26) reported that M2 macrophages are associated with adipose tissue fibrosis by secreting TGF-β. In the present study, we also measured α-SMA expression in MCP-6-treated NIH-3T3 cells and adipose tissue; however, MCP-6 did not induce α-SMA mRNA expression in these cells and tissue (data not shown). From these facts and results, it was considered that mast cell-derived MCP-6 is involved in the process of adipose tissue fibrosis by specifically upregulating collagen V expression.

In obese adipose tissue, collagen VI is considered to be the main extracellular matrix component associated with fibrosis (13). In the present study, ATCM treatment with NIH-3T3 cells also induced collagen 6 mRNA expression; however, enhanced expression of collagen 6 was not fully suppressed by the cotreatment with anti-MCP-6 antibody. Moreover, recombinant MCP-6 did not induce collagen 6 mRNA expression in NIH-3T3 cells and adipose tissue. These results suggest that collagen 6 may be mainly induced by other factors than MCP-6 in ATCM. On the other hand, collagen 4 mRNA expression was significantly suppressed by anti-MCP-6 antibody; however, it was neither induced by recombinant MCP-6 in NIH-3T3 cells and adipose tissue nor increased in 15-wk-old obese adipose tissue. Collagen IV is a basement membrane protein that is observed in the surroundings of adipocytes, not in fibrotic areas (26). These results suggest that collagen IV may not be involved in adipose tissue fibrosis.

In summary, collagen 6 secreted from mature mast cells may induce collagen V expression in fibroblasts, which may be involved in the process of fibrosis in obese adipose tissue.

**Fig. 8.** Saturated fatty acids enhanced maturation of BMMCs. BMMCs were treated with palmitic, stearic, or linolenic acid (1, 10, 20 μM) for 24 h. Expression level of MCP-6 mRNA was quantified as described in Fig. 7 legend. Values are means ± SE of 4 replicates. *P < 0.05 and **P < 0.01 vs. control (cont).

**Fig. 9.** Proposed model for the development and function of mast cells in obese adipose tissues. Immature mast cells that infiltrated into adipose tissues in nonobese stages are gradually stimulated to mature with the progression of obesity and diabetes. In the adipose tissues in severely obese stage, MCP-6 secreted from mature mast cells may induce collagen V expression in fibroblasts, which may be involved in the process of fibrosis in obese adipose tissue.
secreted from hypertrophied adipocytes and involved in insulin resistance (12). As a result, saturated fatty acids were found to dose-dependently enhance the expression of maturation markers in BMMCs; however, an unsaturated fatty acid did not enhance the maturation. These results indicate that saturated fatty acids are some of the factors that stimulate mast cell maturation in obese adipose tissue. Saturated fatty acids induce chronic inflammation through macrophage activation, which leads to insulin resistance (27). The results presented here suggest that saturated fatty acids may induce insulin resistance by enhancing the maturation of the resident immature mast cells involved in fibrosis as well as by enhancing chronic inflammation provoked by the activation of macrophages in obese adipose tissue.

In conclusion, we found that immature mast cells that infiltrate into adipose tissue at the nonobese stage gradually mature with the progression of obesity and diabetes, and that MCP-6 secreted from mature mast cells induces collagen V expression in obese adipose tissue, which may contribute to the process of adipose tissue fibrosis (Fig. 9). Furthermore, we suggested that induction of collagen V by MCP-6 might accelerate insulin resistance via the suppression of preadipocyte differentiation. However, it remains to be noted that the current study is limited to mRNA expressions in vitro experiments. Further studies on protein levels and/or in vivo studies will strengthen the contribution of mast cells to adipose tissue fibrosis. Moreover, elucidation of the detailed mechanism of the infiltration of mast cells into adipose tissue will help in preventing the fibrosis involved in insulin resistance.

ACKNOWLEDGMENTS

We gratefully appreciate Dr. Yoshii Nishino (Kyoto Sangyo University) and Dr. Masayuki Funaba (Kyoto University) for providing technical advice about preparation of BMMCs.

GRANTS

This work was supported by grants-in-aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (22228001, 22780111) and by a grant from Career-Support Program for Woman Scientists at Chiba University. R. Yu was supported by the National Research Foundation of Korea (NRF) with a grant funded by the Korean government (MEST) (2012R1A2A4A01002702).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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22. Reynolds DS, Gurley DS, Austen KF, Serafin WE. Cloning of the cDNA and gene of mouse mast cell protease-6. Transcription by progen-


