Mast cell deficiency results in the accumulation of preadipocytes in adipose tissue in both obese and non-obese mice

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ABSTRACT

Mast cells have been suggested to play key roles in adipogenesis. We herein show that the expression of preadipocyte, but not adipocyte, marker genes increases in the white adipose tissue of mast cell-deficient (Kit<sup>W-sh/W-sh</sup>) mice under both obese and non-obese conditions. In vitro culturing with adipogenic factors revealed increased adipocytes differentiated from the Kit<sup>W-sh/W-sh</sup> stromal vascular fraction, suggesting the accumulation of preadipocytes. Moreover, the increased expression of preadipocyte genes was restored by mast cell reconstitution in the Kit<sup>W-sh/W-sh</sup> mice. These results suggest positive effects of mast cells on the preadipocyte to adipocyte transition under both physiological and pathological conditions.

1. Introduction

Adipose tissue is important for the maintenance of energy balance due to its ability to store large amounts of triglycerides during periods of energy excess and mobilize these stores during periods of nutritional starvation [1]. Obesity is defined as an increase in adipose tissue mass and is a major risk factor for the development of metabolic disorders, such as type 2 diabetes, atherosclerosis, dyslipidemia and hypertension [2,3]. In the adipose tissue of obese individuals, the stromal vascular fraction (SVF) within white adipose tissue (WAT) is characterized by chronic low-grade inflammation with infiltration of immune cells, such as macrophages and T-cells [4,5]. In particular, macrophages in obese WAT have been shown to play key roles in the progression of inflammation [6,7]. Recent studies have shown that, in addition to macrophages, mast cells are involved in the progression of obesity [8,9]. Mast cell-deficient (Kit<sup>W-sh/W-sh</sup>) mice [10] fed a high-fat diet (HFD) exhibit less body weight gain than wild-type (Kit<sup>+/+</sup>) mice [8]. The production of inflammatory cytokines and proteases in WAT is reduced in Kit<sup>W-sh/W-sh</sup> mice [8]. WAT has recently been defined as an important source of functional mast cell progenitors regardless of the nutritional status [11]. Therefore, we consider that the mast cell functions involved in adipogenesis may not be limited to the progression of obesity. However, the role of mast cells in adipogenesis under physiological conditions has not yet been elucidated.

We herein provide evidence for a novel role of mast cells in adipogenesis under both obese and nonobese conditions. We examined the expression of adipocyte-specific genes in the WAT of Kit<sup>W-sh/W-sh</sup> and Kit<sup>+/+</sup> mice fed either a HFD or ND using quantitative RT-PCR. Consistent with the findings of previous reports, body weight gain induced by HFD was suppressed in the Kit<sup>W-sh/W-sh</sup> mice compared to that observed in the Kit<sup>+/+</sup> mice. However, in contrast to body weight changes, the expression levels of adipocyte markers (Pref-1, AEBP1 and GATA2), but not mature adipocyte markers (aP2, PPARγ, AcSL1 and adipin), were significantly higher in the epididymal WAT and SVF of the Kit<sup>W-sh/W-sh</sup> mice compared to those observed in the Kit<sup>+/+</sup> mice. Notably, an enhanced expression of preadipocyte markers in the Kit<sup>W-sh/W-sh</sup> WAT was observed even in mice fed an ND. The in vitro culture of SVF with adipogenic factors resulted in a greater number of adipocytes differentiated from the Kit<sup>W-sh/W-sh</sup> WAT, suggesting the accumulation of preadipocytes. Finally, the increased expression of preadipocyte markers in the WAT and SVF was restored when the Kit<sup>W-sh/W-sh</sup> mice were reconstituted with bone marrow-derived mast cells (BMMCs). Collectively, these data indicate that mast cell deficiency results in the accumulation of preadipocytes in the WAT of both lean and obese mice, suggesting positive effects of mast cells on the preadipocyte to adipocyte transition in WAT.

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Abbreviations: SVF, stromal vascular fraction; WAT, white adipose tissue; HFD, high-fat diet; ND, normal diet; BMMC, bone marrow-derived mast cell.

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Fig. 1. The body weight and epididymal fat weight of the KitW-sh/W-sh mice fed an HFD were less than those of the Kit+/+ mice. (A) Body weight gain in the Kit+/+ mice (black square) and KitW-sh/W-sh mice (gray circle) fed an ND (left panel) or HFD (right panel) for 16 weeks. The data are presented as the mean ± SD. *P < 0.05, **P < 0.01 versus the Kit+/+ mice. (B and C) Body weight (B) and epididymal fat weight (C) of the Kit+/+ and KitW-sh/W-sh mice that consumed an ND or HFD for 16 weeks. The data are presented as the mean ± SD. *P < 0.05, **P < 0.01 for the comparisons indicated. NS, not significant. n = 12 for each group.
Table 1

| Gene product | Primer | Sequence |
|--------------|--------|----------|
| Pref-1       | 5'     | CTT GCT CCT GCT GGC TTT CG |
| AEBP1        | 3'     | TGG CAC AGA GAC ACC CCT CT |
| GATA2        | 5'     | GCA GAG ACC AGA GGC TGG C |
| α2F          | 3'     | CAG TGG ACA CAC TCC CGG C |
| PPARγ        | 5'     | AAA TCA CGG CAC AGC AGA |
| Acril        | 3'     | TTC ACC AGC TGG TCA CC |
| Adipsin      | 5'     | TGG TAT TAG ACC ATC AGC AG |
| Adiponectin  | 3'     | GTC CAG ACT CCT CGG CTG T |
| MCTP         | 5'     | TCC TGG AGA GAG AGA AAG |
| AdipoC        | 3'    | TCA GCT CCT GCT ATT CCA AGA |
| GAPDH        | 5'     | GCA TGG CGA CTG ACC TCA AC |
| Adiponectin  | 3'     | GCC TGG ATT GAC TGG AGA T |

2. Materials and methods

2.1. Mice

C57BL/6-Kit+/+ and C57BL/6-Kit<sup>W-sh/W-sh</sup> mice were purchased from Japan SLC and RIKEN BRC, respectively. The mice were maintained in the animal facility of Takasaki University of Health and Welfare in accordance with institutional guidelines. The mice were given ad libitum access to food, either ND (CLEA Rodent diet CE-2; 12.6% of calories from fat) or HFD (PMI TestDiet 58Y1; 60.0% of calories from fat) and water. To create a model of diet-induced obesity, male mice were fed an HFD from 6 to 22 weeks of age.

2.2. RT-PCR

Total RNA was isolated from cells and tissues using Isogen (Nippon Gene) and treated with DNase I (Takara). The reverse transcription reactions were performed using the ReverTra Ace qPCR RT Kit (Toyo-o) according to the manufacturer's instructions. Synthesized cDNAs were analyzed using an Mx3000P real-time PCR system (Stratagene) with the GoTaq qPCR Master Mix (Promega), as previously described [12]. The results were normalized to the GAPDH levels. The primers used for real-time PCR are shown in Table 1.

2.3. Isolation of SVF and in vitro adipocyte differentiation

Epididymal WAT (eWAT) was dissected and minced in PBS, followed by digestion with 1 mg/ml of collagenase (Sigma) for 30 min at 37 °C. After digestion, the cell suspension was filtered through a 40-μm nylon mesh (BD Biosciences), followed by centrifugation at 1500 rpm for 5 min to separate floating adipocytes from the SVF pellets. The SVF pellets were treated with a hypotonic buffer to lyse red blood cells and resuspended in DMEM containing 10% fetal bovine serum (FBS), penicillin and streptomycin. The isolated SVF was seeded in 24-well plates and cultured at 37 °C in a 5% CO<sub>2</sub> incubator. As the cells reached confluency, they were cultured with the medium supplemented with 0.5 mM of isobutylmethylxanthine (IBMX), 1 μM of dexamethasone and 10 μg/ml of insulin for 2 days, followed by culturing with 10 μg/ml of insulin for 2 days and without insulin for 3 days. The degree of lipid accumulation in the cells was examined using Oil Red O staining according to a standard procedure [21]. To quantify retention of Oil red O, the lipids were extracted using 150 μl of isopropanol for 30 min, and the absorbance was measured at 540 nm.

2.4. Flow cytometry

The SVF cells were stained with allophycocyanin (APC)-conjugated rat anti-mouse CD117 (BD Pharmingen; clone 28B) and phycoerythrin (PE)-conjugated mouse anti-mouse Fc epsilon receptor I alpha (eBio-science; clone MAR-1) antibodies that recognize c-Kit and the high-affinity IgE receptor alpha subunit (FcεRα), respectively. The staining was performed using PBS containing 2% FBS at 4 °C for 60 min. After washing with PBS, the cell fluorescence was measured using a FACSCanto II flow cytometer (BD Biosciences).

2.5. Reconstitution of mast cells in mast cell-deficient mice

BMMCs were prepared as previously described [13]. In brief, femoral bone marrow cells were harvested from 4- to 8-week-old male Kit<sup>+/+ </sup> mice and cultured with RPMI 1640 supplemented with 10% FBS and 10 ng/ml of recombinant murine interleukin-3 (IL-3; Peprotech). After 2 weeks of culturing, 10 ng/ml of recombinant murine stem cell factor (SCF) (Peprotech) was added. After 4-6 weeks of culturing, 2.0 × 10<sup>8</sup> BMMCs in 200 μl of PBS were injected intraperitoneally (i.p.) into 4-week-old male Kit<sup>W-sh/W-sh</sup> mice, and the mice were used for the experiments after 12–16 weeks of injection. The transferred BMMCs were confirmed using cytospin preparations of peritoneal cells (5 × 10<sup>4</sup> cells) stained with toluidine blue or alcin blue/safranin O.

2.6. Statistics

Comparisons between the two groups were made using Student's t-test. Unless otherwise specified, the data are presented as the mean ± SEM. For all analyses, statistical significance was defined as a P-value of < 0.05.

3. Results

3.1. Mast cell-deficient mice are resistant to diet-induced obesity

Kit<sup>+/+ </sup> and Kit<sup>W-sh/W-sh</sup> mice were fed an HFD or ND, and their body weight was monitored weekly during 16 weeks of feeding (Fig. 1A). In the HFD group, the body weight gain in the Kit<sup>W-sh/W-sh</sup> mice was less than that observed in the Kit<sup>+/+ </sup> mice at 9 weeks and afterwards, whereas the body weights were comparable in the ND group throughout the observation period (Fig. 1A). The Kit<sup>+/+ </sup> mice fed an HFD weighed 39% more than those fed an ND after 16 weeks of feeding (44.04 ± 3.22 and 31.65 ± 1.78 g, respectively, n = 12, Fig. 1B). In contrast, the Kit<sup>W-sh/W-sh</sup> mice fed an HFD weighed only 8% more than those fed an ND (34.78 ± 2.83 and 32.20 ± 1.49 g, respectively, n = 12, Fig. 1B). Consistent with this finding, the eWAT weight of the Kit<sup>W-sh/W-sh</sup> mice was significantly less than that of the Kit<sup>+/+ </sup> mice in the HFD group (1.29 ± 0.53 and 2.15 ± 0.44 g, respectively, n = 12, Fig. 1C), whereas these values were comparable in the Kit<sup>W-sh/W-sh</sup> and Kit<sup>+/+ </sup> mice in the ND group (0.42 ± 0.12 and 0.46 ± 0.17 g, respectively, n = 12, Fig. 1C). These results are consistent with the findings of previous reports [8, 14] and suggest the contribution of mast cells to the development of HFD-induced obesity.

3.2. Mast cell deficiency results in an increased expression of preadipocyte marker genes in WAT

To examine the molecular basis of the resistance to HFD-induced obesity observed in the Kit<sup>W-sh/W-sh</sup> mice, we evaluated the expression of genes involved in adipogenesis using quantitative RT-PCR. In the HFD group, the expression levels of adipocyte marker genes
of and bars) the preadipocyte (AEBP1) and adipocyte Gata2, aP2, PPARY, Acs1, adipin) were examined in the eWAT of the Kit+/+ (black bars) and KitW-sh/W-sh mice (gray bars) fed either an HFD (A) or ND (B) for 16 weeks using quantitative RT-PCR. n = 7 for each group. (C) The mRNA levels of the preadipocyte markers were examined in the eWAT derived from the Kit+/+/ mice (black bars) and KitW-sh/W-sh mice (gray bars) fed an ND using quantitative RT-PCR. n = 6 for each group. The data are presented as the mean ± SEM. *P < 0.05, **P < 0.01 versus the Kit+/+ mice.

3.3. Adipogenic differentiation is more prevalent in the KitW-sh/W-sh SVF than in the Kit+/+ SVF in vitro

We next examined whether preadipocytes obtained from KitW-sh/W-sh SVF are able to differentiate into mature adipocytes in vitro. Since SVF cells are comprised of heterogeneous cell populations, they may contain a considerable amount of hematopoietic progenitor cells positive for the c-Kit expression. Therefore, in order to exclude the possibility that the Kit mutation in non-mast cells intrinsically affects adipocyte differentiation in the SVF of KitW-sh/W-sh mice, we examined the expression of c-Kit and FcεRI in freshly isolated SVF cells of Kit+/+ and KitW-sh/W-sh mice using a flow cytometric analysis (Fig. 3A). While mast cells express both c-Kit and FcεRI, other cell types are generally negative for the FcεRI expression. We found a minor fraction of c-Kit-positive cells at a frequency of 0.43 ± 0.18% in the SVF isolated from the Kit+/+ mice, but not the KitW-sh/W-sh mice. Notably, all c-Kit-positive cells in the Kit+/+ SVF were FcεRI-positive mast cells, suggesting that the effects of the Kit mutation are negligible in non-mast cells (Fig. 3A). Consistent with this finding, a quantitative RT-PCR analysis showed that the expression of the mast cell-specific gene cpa3, which encodes mast cell carboxypeptidase A (MCCPA), was detected in the freshly isolated SVF obtained from the Kit+/+ mice, but not the KitW-sh/W-sh mice. The MCCPA mRNA level in the Kit+/+ SVF was significantly lower (approximately one seventy-fifth) than that observed in the BMMCs prepared from the Kit+/+ mice (data not shown). The low level of the MCCPA mRNA expression was no longer detectable when the cells reached confluency. Given that the BMMCs are isolated and maintained in the presence of IL-3 and SCF, the mast cells in the Kit+/+ SVF were unable to survive in the absence of these cytokines and were eliminated by that time (Fig. 3B). When the SVF cells reached confluency, they were supplemented with adipogenic factors to examine their ability to differentiate into adipocytes. After 7 days of culture with adipogenic factors, the number of cells stained with Oil red O was markedly increased in the SVF obtained from the Kit+/+ mice, and the KitW-sh/W-sh eWAT compared to that observed in the controls (Fig. 3C). A spectrophotometric quantitative analysis also showed greater lipid accumulation in the KitW-sh/W-sh SVF compared to that observed in the controls (Fig. 3D). Consistent with this finding, quantitative RT-PCR revealed that the mRNA levels of adipocyte markers, aP2, PPARY and adiponectin, were significantly higher in the SVF obtained from the KitW-sh/W-sh mice than in the SVF obtained from the controls (Fig. 3E). These results indicate that in vitro adipogenic differentiation is more prevalent in KitW-sh/W-sh SVF than in Kit+/+ SVF. Given that the gain of body weight in response to an HFD was suppressed in the Kit+/+ mice, it is likely that the transition of preadipocytes to adipocytes in vivo is attenuated in KitW-sh/W-sh mice, although KitW-sh/W-sh adipocytes are capable of responding to exogenous adipogenic factors and differentiating into mature adipocytes in vitro.

3.4. The enhanced expression of preadipocyte marker genes is restored by reconstituting mast cells in the KitW-sh/W-sh WAT

To examine whether mast cell deficiency results in impaired maturation of preadipocytes in KitW-sh/W-sh mice, BMMCs prepared from Kit+/+ mice were transferred into the peritoneal cavity of KitW-sh/W-sh mice, and adipogenic differentiation was then examined using the same methods as described above (Fig. 3F). Interestingly, the enhanced expression of preadipocyte markers in the KitW-sh/W-sh eWAT was also observed in the ND group, even though the weight of the eWAT was comparable between the KitW-sh/W-sh and Kit+/+ mice in these groups (Fig. 2B). Consistent results were obtained in the SVF composed of immune cells, fibroblasts, preadipocytes, endothelial cells and mesenchymal stem cells (Fig. 2C).

[15–17] including adipocyte protein 2 (aP2), peroxisome proliferator-activated receptor γ (PPARY), acyl-CoA synthetase long-chain family member 1 (Acs1) and adipin, were comparable in the eWAT prepared from the KitW-sh/W-sh mice compared to those observed in the eWAT prepared from the Kit+/+ mice (Fig. 2A). In contrast, the expression levels of preadipocyte marker genes [18–20], including preadipocyte factor-1 (Pref-1), adipocyte enhancer binding protein 1 (AEBP1) and GATA2, were significantly greater in the eWAT prepared from the KitW-sh/W-sh mice than that observed in the controls (Fig. 2A). Interestingly, the increased expression of preadipocyte markers in the KitW-sh/W-sh eWAT was also observed in the ND group, even though the weight of the eWAT was comparable between the KitW-sh/W-sh and Kit+/+ mice in these groups (Fig. 2B). Consistent results were obtained in the SVF composed of immune cells, fibroblasts, preadipocytes, endothelial cells and mesenchymal stem cells (Fig. 2C).
mice. After 12–16 weeks of transplantation, toluidine blue- and alcin blue/safranin O-positive cells were detected in the peritoneal cavity in the KIt°/°/W-sh mice reconstituted by BMMCs, but not the KIt°/°/W-sh mice (Fig. 4A and B). Furthermore, the expression of MCPPA, a mast cell-specific marker, was detected in the eWAT of the BMMC-transferred, but not PBS-injected, KIt°/°/W-sh mice using quantitative RT-PCR (Fig. 4C). Collectively, these results suggest that the transferred BMMCs were terminally differentiated in the peritoneal cavity and infiltrated into the eWAT of the recipient mice.

We next examined whether the increased expression levels of preadipocyte markers in the KIt°/°/W-sh eWAT were restored by mast cell reconstitution (Fig. 4D). Indeed, the expression levels of Pref-1, AEBP1 and GATA2 were significantly lower in the eWAT and SVF obtained from the mast cell-reconstituted mice than in the controls (Fig. 4D). Taken together, these results suggest that mast cells possibly facilitate the transition of preadipocytes to mature adipocytes in murine WAT.

4. Discussion

Recent studies have demonstrated that a greater number of mast cells reside in the WAT of obese mice receiving an HFD than in the WAT of lean mice and that the body weight gain induced by an HFD is reduced in mast cell-deficient mice [8,14]. Several mechanisms have been proposed to explain how mast cells promote diet-induced obesity. Liu et al. demonstrated that mast cells promote WAT angiogenesis in response to a Western diet by maintaining a high proteolytic activity of cathepsin, which generates angiogenic factors in vascular cells [8]. They showed that mast cells stimulate the cathepsin activity by producing two common cytokines, interleukin-6 and interferon-γ [8]. In a separate study, Tanaka et al. demonstrated that mast cells activate PPARγ, a key regulator of adipogenesis [14]. PPARγ is a ligand-dependent transcription factor that induces the expression of various adipogenic genes and is essential for adipocyte differentiation and fat tissue formation [15]. The authors showed that mast cells activate PPARγ by producing prostaglandin D2 (PGD2), which is metabolized to deoxy-delta-12,14-PGJ2, a natural ligand for PPARγ [14].

The current study provides evidence for a novel role of mast cells in adipogenesis. We demonstrated that the mRNA expression levels of preadipocyte markers were significantly higher in the eWAT and SVF of the KIt°/°/W-sh mice than in the controls and that the increased expression of these genes was restored by mast cell reconstitution. We selected three genes, Pref-1, AEBP1 and GATA2, as representative preadipocyte markers in this study. Notably, these genes are highly expressed in immature preadipocytes and downregulated during adipocyte maturation. Furthermore, the products of these genes are known to repress adipocyte maturation via distinct mechanisms. Pref-1 is a transmembrane protein that contains tandem epidermal
Fig. 4. Mast cell reconstitution in the Kit<sup>W-sh/W-sh</sup> mice restored the enhanced mRNA expression of preadipocyte marker genes in the eWAT. BMMCs prepared from Kit<sup>+/+</sup> mice (2.0 × 10<sup>6</sup> cells) were injected intraperitoneally into Kit<sup>W-sh/W-sh</sup> mice and examined after 12–16 weeks of injection. (A) Photomicrographs of peritoneal cells (× 400) stained with toluidine blue (upper panels) or alcian blue/safranin O (lower panels) on cytopsin preparations. Cells prepared from Kit<sup>+/+</sup> and Kit<sup>W-sh/W-sh</sup> mice with or without reconstitution are shown. (B) Quantification of the mast cells in the cytopsin preparations shown in (A). n = 3 for each group, ND, not detected. (C) The mRNA expression of the mast cell-specific gene Cpa3 (MCCPA) in the eWAT was evaluated using quantitative RT-PCR (n = 5). (D) The mRNA levels of the preadipocyte marker genes were examined in the eWAT (n = 7) and SVF (n = 6) of the Kit<sup>+/+</sup> (black bars) and Kit<sup>W-sh/W-sh</sup> mice with (light gray bars) or without (dark gray bars) reconstitution using quantitative RT-PCR. The data are presented as the mean ± SEM. *P < 0.05, **P < 0.01 for the comparisons indicated. NS, not significant.
growth factor-like repeats [18]. Pref-1 is activated when enzymatically cleaved into a soluble form, which stimulates MAPK kinase ERK signaling [22], thus resulting in the upregulation of SRY (sex determining region Y)-box 9 (Sox9), an inhibitor of adipogenesis. AEBP1 is a unique transcriptional repressor with carboxypeptidase activity. It has been shown that AEBP1 directly binds to the aP2 gene regulatory sequence, repressing its transcription [19]. GATA2 is a zinc finger transcription factor that is known to be essential for hematopoietic and vascular development [23]. The constitutive expression of GATA2 suppresses the preadipocyte–adipocyte transition through, at least in part, direct repression of the PPARγ gene [20]. Collectively, our data suggest that mast cell deficiency results in the sustained expression of a set of genes that maintain preadipocytes in an immature state.

The important point of our findings is that an increased expression of preadipocyte marker genes was observed in the KitW-sh/W-sh eWAT and SVF, even in the mice fed an ND, thus suggesting that mast cells play a novel role in the homeostatic regulation of adipogenesis in nonobese individuals. Interestingly, despite the fact that the body weights were similar between the ND groups, more lipid accumulating cells were differentiated from the SVF of the KitW-sh/W-sh mice than from the controls. Our findings indicate that mast cells in Kit+/−/+/− SVF are unable to survive, likely due to the absence of IL-3 and SCF, and are eliminated by the time of adipogenic differentiation. Therefore, it is unlikely that the increased adipogenic potential in the KitW-sh/W-sh SVF results from the lack of direct effects of mast cells during adipocyte maturation in vitro. Rather, the increased adipogenic potential may result from the expansion of adipocyte progenitor pools or the activation of the adipogenic gene expression program in KitW-sh/W-sh preadipocytes. Further studies are needed to determine the precise roles of mast cells in the homeostatic regulation of adipogenesis.

Taken together with previous results showing that a greater number of mast cells are present in WAT in obese humans and mice than in lean controls [8,9,14], our present findings suggest that mast cells may play a gate-keeper role by facilitating the preadipocyte to adipocyte transition under both physiological and pathological conditions. Thus far, a number of mediators released from mast cells, such as cytokines, chemokines, proteases and proteoglycans, have been suggested to play a role in adipogenesis [24]. As mast cells play diverse roles in adipogenesis, further studies are therefore needed to identify the mediators responsible for each function.

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