Angiogenic Role of LYVE-1-positive Macrophages in Adipose Tissue

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Abstract—Here we report the discovery of a characteristic dense vascular network (DVN) in the tip portion of epididymal adipose tissue in adult mice. The DVN is formed by angiogenesis rather than by vasculogenesis, and has functional blood circulation. This DVN and its subsequent branching may provide a new functional route for adipogenesis. The recruitment, infiltration, and accumulation of bone marrow-derived LYVE-1⁺ macrophages in the tip region are crucial for the formation of the DVN. Matrix metalloproteinases (MMPs) and the VEGF-VEGFR2 system are responsible not only for the formation of the DVN, but also for the recruitment and infiltration of LYVE-1⁺ macrophages into the epididymal adipose tissue tip region. SDF-1, but not the MCP-1-CCR2 system, is a critical factor in recruitment and ongoing retention of macrophages in this area. We also demonstrate that the tip region of epididymal adipose tissue is highly hypoxic, and thus provides a microenvironment conducive to the high expression and enhanced activities of VEGF, VEGFR2, MMPs, and SDF-1 in autocrine and paracrine manners, to create an ideal niche for the recruitment, retention, and angiogenic action of macrophages. These findings shed light on the complex interplay between macrophage infiltration, angiogenesis, and adipogenesis in the tip region of adult epididymal adipose tissue, and provide novel insight into the regulation of alternative outgrowth of adipose tissue. (Circ Res. 2007;100:0-0.)

Key Words: adipogenesis ■ angiogenesis ■ endothelial cell differentiation ■ endothelial cell growth ■ lymphatic vessel hyaluronan receptor 1 ■ macrophages ■ matrix metalloproteinases ■ monocyte chemoattractant protein-1 ■ vascular endothelial growth factor ■ vascular endothelial growth factor receptors

Adipose tissue is a unique organ that has reversible growth depending on the balance of fat metabolism. It is mainly composed of adipocytes supported by stromal-vascular tissue, which contains vascular endothelial cells, macrophages, and poorly characterized stem cells. Developmental growth of adipocytes through adipogenesis (defined as development of adipoblasts into differentiated adipocytes) is accompanied by the growth of vasculature in adipose tissue. Recent studies using pharmacological agents or cell implantation have proposed that the growth of adipose tissue is angiogenesis-dependent. However, little is known about the nature of how angiogenesis governs the growth of adipose tissue and, inversely, how the growth of adipose tissue affects the growth of vasculature.

Macrophages are released from the bone marrow as immature monocytes and circulate in the blood before extravasation into their target tissues, where they differentiate into resident macrophages. Thus, macrophages are found in every tissue of the body and, depending on the local microenvironment, acquire specialized functions including phagocytosis, antigen presentation, tissue remodeling, and the secretion of a wide range of growth factors and cytokines. The distribution and accumulation of macrophages in certain tissue are mediated by several CC chemokines and growth factors. Macrophages, particularly in obese subjects, can constitute up to 40% of the cell population within an adipose tissue depot. The increased number of macrophages in adipose tissue is a likely source of secreted proinflammatory factors, which are responsible for inducing features of metabolic syndrome. However, under normal circumstances, macrophages are also widely distributed among adipocytes, and constitute 5% to 10% of the cell population in several adipose...
tissues depots. Besides their inflammatory role, it is unknown whether macrophages may have other roles in adipose tissue. Here, we demonstrate an angiogenic role for macrophages in the tip region of epidymal adipose tissue (EAT), which may be involved in the outgrowth of adipose tissue. Furthermore, we clarify the main mediators and underlying mechanisms responsible for the recruitment and retention of macrophages in this tissue.

Materials and Methods

Animals and Treatment
Specific pathogen-free C57BL/6J and CCR2(−/−) (C57BL/6J genetic background) mice were purchased from Jackson Laboratory (Jackson Labs, Bar Harbor, Maine). GFP⁺ mice (C57BL/6J genetic background) were a gift from Dr Masaru Okabe (Osaka University, Japan). Mice were bred in our pathogen-free animal facility and 8- to 9-week-old male mice were used for this study otherwise specifically indicated. Animal care and experimental procedures were performed under approval from the Animal Care Committees of KAIST and Dankook University. For systemic depletion of macrophages, mice were treated with intraperitoneal injections of clodronate liposome (CDL, 12 or 40 mg/kg) as previously described.ETO. To block endogenous VEGF, mice were treated with intraperitoneal injection of sFlt-1-Fc (5 μg/d for 2 weeks, R&D Systems) as previously described. As a control, dimeric-Fc protein was applied in the same manner. To block VEGFR2, mice were treated with intraperitoneal injections of SU11248 (60 mg/d for 8 days, ImageCo, Seoul, Korea) as previously described. As a control, vehicle containing 0.1% DMSO was applied in the same manner. To block matrix metalloproteinases (MMPs), mice were treated with intraperitoneal injections of zoleudronic acid (ZA, Zometra, 100 μg/kg/d for 2 weeks, Novartis Pharma Stein AG, Stein, Switzerland). As a control, vehicle containing 0.1% DMSO was applied in the same manner. To block SDF-1, mice were treated with intraperitoneal injections of anti-SDF-1 blocking antibody (50 μg thrice per week, R&D systems). As a control, nonspecific anti-rat IgG antibody was applied in the same manner.

Bone Marrow Transplantation
Bone marrow cells (2×10⁶) were harvested from the femurs and tibias of GFP⁺ mice by flushing with ice-cold Dulbecco’s phosphate buffered saline (DPBS, Sigma-Aldrich). The recipient mice (C57BL/6J), 8 to 10 weeks old, were sublethally irradiated at a dose of 4.5 Gy with a γ irradiator (Gammacell 3000, MDS Nordion Inc, Canada). Bone marrow cells were then injected intravenously into the recipient mice 16 hours after irradiation. The bone marrow transplanted mice were examined at the indicated times.

Isolation of Adipocytes and LYVE-1⁺ Macrophages, and RT-PCR
Mice were anesthetized by intramuscular injection of a combination of anesthetics (80 mg/kg ketamine and 12 mg/kg xylazine). The bone marrow transplanted mice 16 hours after irradiation. The bone marrow transplanted mice were sacrificed. Mice were then injected intravenously with the appropriate primers (see supplementary Table I). The bone marrow transplanted mice were examined at the indicated times.

Histological and Morphometric Analysis
Mice were anesthetized by intramuscular injection of a combination of anesthetics (80 mg/kg ketamine and 12 mg/kg xylazine). EAT were fixed by vascular perfusion of 1% paraformaldehyde in PBS, removed, and whole-mounted. For detection of functional blood circulation, 400 μg of fluorescein-conjugated Baudhina Purpurea lectin (Vector Laboratories, Inc, Burlingame, Calif) was injected intravenously 10 minutes before perfusion-fixation. For detection of hypoxia, Huyoxprobe-1 (pimonidazole hydrochloride, 60 mg/kg, Chemicon International) was injected 90 minutes before perfusion-fixation. After blocking with 5% goat serum in PBST (0.3% Triton X-100 in PBS) for 1 hour at room temperature, the whole-mounted EAT were incubated overnight at 4 with one or more of the following primary antibodies: (a) for blood vessels, hamster anti-PECAM-1 antibody, clone 2H8, 1:1,000 (Chemicon International, Temecula, Calif); (b) for macrophages and leukocytes, rat monoclonal anti-mouse LYVE-1 antibody, clone 1:1,000; TH rat-mouse F4/80 antibody, clone CEAM-1, 1:1,000 (Serotec, Oxford, UK), rat anti-mouse CD11b antibody, clone M1/70, 1:1,000 (BD Pharmingen) and rat anti-mouse CD45, clone 30-F11, 1:1,000 (BD Pharmingen); (c) for adipocytes, guinea pig polyclonal anti-perilipin antibody, 1:1,000 (Research Diagnostics); (d) for apoptotic cells, rabbit polyclonal anti-caspase-3 antibody, 1:500 (Cell Signaling Technology); (e) for hypoxic cells, FITC-conjugated mouse monoclonal anti-Huyoxprobe-1 antibody, clone 4,3.11.3, 1:500 (Chemicon International); and (f) for VEGFR-2, rabbit polyclonal anti-VEGFR-2 antibody, 1:100 (TQ14). After several washes in PBST, whole-mounted EAT were incubated for 1 hour at room temperature with 1 or more secondary antibodies: (a) Cy3- or Cy5-conjugated anti-hamster IgG antibody, 1:500 (Jackson ImmunoResearch Laboratories); (b) FITC- or Cy5-conjugated anti-rat antibody or anti-rabbit antibody, 1:500 (Jackson ImmunoResearch Laboratories); (c) Cy3-conjugated anti-guinea pig antibody (Jackson ImmunoResearch Laboratories). For control experiments, the primary antibody was omitted or substituted with preimmune serum. Signals were visualized and digital images were obtained using a Zeiss Apotome microscope and a Zeiss LSM 510 confocal microscope equipped with argon and helium-neon lasers (Carl Zeiss). For determining the extent of macrophage infiltration, triple-stained color images with anti-LYVE-1, anti-PECAM-1, and anti-perilipin antibodies were captured with a Zeiss LSM 510 confocal microscope. Using ImageJ software (http://rsb.info.nih.gov/ij), the DNV area was selected as a region-of-interest from PECAM-1/perilipin-stained images, and LYVE-1-stained images were converted to 8-bit gray scale. Area densities of the LYVE-1-stained images were measured from the pixels in the region-of-interest; only pixels over a certain level (>50 intensity value) were taken to exclude background fluorescence. For determining the hypoxic region, anti-Huyoxprobe-1 antibody-stained images of Huyoxprobe-1 injected and uninjected EATs were captured from a confocal microscope. After conversion to 8-bit grayscale by ImageJ software, the mean area densities of DNV and body portions of EAT, and section of implanted LLC tumor were measured from the pixels without background fluorescence. To avoid nonspecific fluorescence of anti-Huyoxprobe-1 antibody, the mean area densities were subtracted from the values of Huyoxprobe-1-un.injected EAT.

Statistics
Values presented are means ± standard deviation (SD). Significant differences between means were determined by analysis of variance followed by the Student-Newman-Keuls test. Statistical significance was set at P<0.05 or P<0.01.

Results
Presence of a Characteristic Dense Vascular Network in the Tip Region of EAT
To gain insight into the reciprocal interaction between the growth of adipose tissue and the growth of vasculature, we...
chose to examine whole-mounted EAT of male mice (C57BL/6) during postnatal development. As EAT lacks adjacent tissues, it is relatively easy to visualize blood vessels and adipocytes at the 3-dimensional level by coimmunostaining with antibodies against PECAM-1 (a component of vascular endothelial cells) and perilipin (a membrane protein that surrounds lipid droplets). In 2-week-old mice, these characteristics are detected mainly in the EAT tip region. The DVNs (black arrowheads) are detected mainly in the EAT tip region of 6- and 8-week-old mice. Occasionally, a few variably-sized protrusions of adipose tissue (blue arrows) are observed near the tip region. C, Where a characteristic DVN is present, there are no lipid-containing adipocytes (blue). D, Number of DVN exceeding 250 μm in diameter in the EAT tip region and whole EAT mass (g) at the indicated age. Bars represent means ± SD from 4 to 8 mice. *, P<0.01 vs 2-weeks of age. Scale bar, 100 μm.

Figure 1. Presence of a characteristic dense vascular network (DVN) in the tip region of EAT in adult mouse. Vasculature in whole-mounted EAT in mice ranging from 2 to 12 weeks are visualized with PECAM-1 immunostaining (red or reddish brown) (A) vascular endothelial cells having sprouting (white arrowheads) and filopodia (green arrowheads) are detected in most of the vasculature of 2-week-old mice, whereas, in 8 week-old mice, these characteristics are detected mainly in the EAT tip region. B, The DVNs (black arrowheads) are detected mainly in the EAT tip region of 6- and 8-week-old mice. Occasionally, a few variably-sized protrusions of adipose tissue (blue arrows) are observed near the tip region. C, Where a characteristic DVN is present, there are no lipid-containing adipocytes (blue). D, Number of DVN exceeding 250 μm in diameter in the EAT tip region and whole EAT mass (g) at the indicated age. Bars represent means ± SD from 4 to 8 mice. *, P<0.01 vs 2-weeks of age. Scale bar, 100 μm.

Approximately 75% to 85% (n=4) of the DVN is fluorescein-labeled lectin, indicating that most DVNs have blood circulation. D, Large numbers of macrophages are localized in the upper portion of and around a characteristic DVN. Scale bar, 100 μm.

Figure 2. Relationships between adipocytes, vasculature, functional circulation, and macrophage distribution in the tip region of EAT. Whole-mounted EAT from 8-week-old mice are visualized by immunostaining of perilipin (for adipocytes, blue), PECAM-1 (for vasculature, red) and LYVE-1 (for macrophages, green), and merged. A, A characteristic DVN consisting of a short segment and large diameter is present in the tip region, where very few lipid-containing adipocytes and large numbers of macrophages are present. B, Between arrows and arrowheads, blood vessels having large diameters and a large vascular network, lipid-containing adipocytes, and occasional macrophages are present, whereas between arrowheads and bars, a characteristic DVN, very few lipid-containing adipocytes, and large numbers of macrophages are present. C, Fluorescein-labeled lectin was injected intravenously into mice, and the image of fluorescein-lectin (green) in the DVN (red) was obtained. From these observations, we sought to address the question of whether there is a relationship between the growth of adipose tissue and the formation of the DVN in the adult EAT tip region. Additionally, we sought the mechanism and reason behind the formation of the DVN in this region.

Relationships Between Adipocytes, Vasculature, Functional Circulation, and Macrophage Distribution in the Tip Region of EAT

To define the DVN, the tip region of EAT was examined in more detail with immunostaining experiments. This analysis revealed that the DVN is composed of a short segment with a large diameter (Figure 2A), resembling a primitive vascular plexus formed by vasculogenesis during early embryogenesis. To examine distribution of lymphatic vessels in this region, we also performed immunostaining of LYVE-1 (lymph vessel endothelial hyaluronan receptor-1), which is a marker of lymphatic endothelial cells in the whole-mounted EAT. However, there was no LYVE-1+ lymphatic vessel. Instead, this region was infiltrated by macrophages having variable shapes and positive staining for LYVE-1 (Figure 2A), suggesting that the formation of the DVN in the tip region could be related to the infiltration of macrophages. Higher magnifications of these images clearly showed frequent vascular sproutings in the outer margins of the DVN and many infiltrated macrophages of varied shapes in the DVN area (supplementary Figure IA in the online data.
supplement at http://circres.ahajournals.org). In the tip region, our triple immunostained images revealed that approximately 90% of these LYVE-1− cells were CD11b+, which is well-known marker for macrophages, indicating that LYVE-1 staining was an accurate marker for macrophages in this tissue (supplementary Figure IIA). In comparison, approximately 30% to 40% or 40% to 50% of the LYVE-1− cells were F4/80+ (other macrophage marker) or CD45+ (common leukocyte marker), respectively (supplementary Figure IIB and IIC). Moreover, isolated LYVE-1− macrophages from the SVCs of EAT displayed active phagocytic activity (supplementary Figure III). These results suggest that the LYVE-1− cells are different subset of macrophages. In fact, recent report33 has described that LYVE-1 is expressed not only in lymphatic endothelial cells, but also in a subset of macrophages in the wound healing tissues and implanted malignant tumor tissues. Other triple immunostained images suggested close interactions between LYVE-1− macrophage infiltration, formation of the DVN, and adipogenesis in the EAT tip region (Figure 2B). Compared with the lower portion of the tissue, the middle area of the tip region has smaller and nonuniformly-sized lipid-containing adipocytes with less organized blood vessels having wider diameters and an enlarged vascular network (Figure 2B). In the top portion of this newly-formed adipose tissue, the DVN was of characteristic shape without mature lipid-containing adipocytes (Figure 2B). Again, in this DVN, there was a massive infiltration of macrophages, suggesting that macrophages may play a role in the formation of a DVN in the tip portion. Higher magnifications of these images clearly showed such interactions between macrophage infiltration, formation of DVN, and adipogenesis in the EAT tip region (supplementary Figure IB). From these observations, we speculated that longitudinal outgrowth of EAT into the upper part of the abdominal cavity might result from the preceding macrophage-induced formation of the DVN and subsequent adipogenesis in the EAT tip region. For lipid transport from circulation to immature preadipocytes to occur, blood vessels would need to be functional to systemic blood circulation. To examine whether the DVN has blood circulation, fluorescein-labeled lectin was injected intravenously, and the image of fluorescein-lectin was obtained by fluorescence microscopy. Approximately 75% to 85% of the DVN was fluorescein-lectin-positive (Figure 2C and supplementary Figure IV in the online data supplement at http://circres.ahajournals.org), indicating that the DVN in the EAT tip region has blood circulation. Furthermore, red blood cells were often observed in the DVN of the nonperfused adipose tissue (data not shown). Thus, the DVN in the EAT tip region has blood circulation and is able to transport lipids from the blood into immature preadipocytes in order for them to become lipid-accumulating mature adipocytes.

Role of LYVE-1+ Macrophages in the Formation of DVN

We then analyzed the regional interaction between the DVN and LYVE-1+ macrophages. Notably, the location of accumulated LYVE-1+ macrophages was always proximal to and around the DVN (Figure 2D and 2E), implying that LYVE-1+ macrophages may affect the formation of the DVN. To examine the role of macrophages in the formation of the DVN, macrophages were depleted by single intraperitoneal treatment of clodronate liposome (CDL), 12 or 40 mg/kg as previously described15,16 in the 8- to 9-week-old mice. This treatment efficiently depletes macrophages in a dose-dependent manner by inducing selective apoptosis of macrophages in all organs, including EAT, for 1 to 2 weeks (Figure 3A). In this situation, the number of the DVN exceeding 250 μm in diameter was dramatically reduced in a dose-dependent manner (Figure 3B). Moreover, vascular sprouting and filopodia were rarely detected in the outer margins of the DVN. However, 3 weeks after this treatment, massive infiltration of macrophages and the characteristic shape of the DVN were similarly observed in the EAT tip region (data not shown), suggesting that circulating macrophages may dynamically replenish this region with fresh macrophages, which then participate to form the DVN.

Origin of Macrophages and Angiogenic Role in the Formation of DVN

To clarify the origin of the macrophages in the EAT tip region, 8-week-old mice transplanted with GFP+ bone marrow cells were examined. At 12 weeks after bone marrow
transplantation (BMT) with GFP⁺ cells, approximately 90% to 95% of macrophages in the tip region were GFP⁺ (Figure 3C), indicating that most of the macrophages were derived from bone marrow through circulation. To evaluate whether bone marrow-derived cells act as endothelial progenitor cells (EPCs) to form endothelial cells in the DVN, the colocalization of PECAM-1 immunostaining and GFP⁺ (EPCs) to form, endothelial cells in the DVN, the colocalization of PECAM-1 immunostaining and GFP⁺ was carefully examined. However, based on our vigorous observations, we found no GFP⁺ cells in the endothelial cells of any characteristic DVNs (Figure 3D), clearly indicating that the formation of the DVN is induced by angiogenesis through active sprouting, not by vasculo genesis.24,25

**Role of MMPs in Macrophage Infiltration and Formation of DVN in the Tip Region of EAT**

There is ample evidence that macrophage-derived growth factors and cytokines play a major role in angiogenesis in certain microenvironments.9,10,26 Therefore, we compared the expression patterns of several angiogenic growth factors and cytokines in enriched LYVE-1⁺ macrophages, adipocytes, and LYVE-1⁺ macrophage-depleted stromal-vascular cells (SVCs) (Figure 3E) of the EAT tip region by semi-quantitative RT-PCR. Selective expression of adiponectin in the enriched adipocytes and PECAM-1 in the LYVE-1⁺ macrophage-depleted SVCs reflected the high efficiency of cell separation and enrichment for each cell type from the EAT (Figure 3E). Interestingly, the enriched LYVE-1⁺ macrophages more abundantly expressed MMP-9, MMP-12, MMP-7, chemokine receptor-4 (CXCR4) and TNF-α, whereas the adipocytes more abundantly expressed VEGF-A, VEGF-D, angiopoietin-1 (Ang1) and MMP-8, and the LYVE-1⁺ macrophage-depleted SVCs more abundantly expressed VEGF-C and MMP-2 (Figure 3F and supplementary Figure V). Angiopoietin-2 (Ang2) was expressed in all 3 cell types, while macrophage chemoattractant protein-1 (MCP-1) was expressed in the enriched LYVE-1⁺ macrophages and LYVE-1⁺ macrophage-depleted SVCs, and stromal cell-derived factor-1 (SDF-1) was in the adipocytes and in the enriched LYVE-1⁺ macrophage-depleted SVCs (Figure 3F and supplementary Figure V). Thus, the RT-PCR analysis strongly suggested that MMPs secreted from the macrophages, adipocytes, and SVCs could play a certain role in the formation of the DVN in the adult EAT tip region. All members of the MMP family contain Zn²⁺ at the catalytic site.27 ZA is a nitrogen-containing (amino-) bisphosphonate that inhibits proteolytic activities of MMPs possibly through Zn²⁺ chelation.28,29 ZA has been used for reducing complications of bone metastasis in patients with multiple myeloma and several solid tumors with minimal side effects,30,31 and for targeting MMP-9-expressing macrophages and reducing angiogenesis in experimental mice.32 Therefore, to test the role of MMPs in the formation of the DVN, we inhibited MMP activity in mice by treatment with ZA (100 μg/kg/d for 2 weeks).32 The treatment with ZA exerted significant reductions not only in the formation of the DVN and vascular sprouting and filopodia in the outer margins of the DVN, but also in macrophage infiltration in the tip region (Figure 4A and 4B). These results indicate that the action of MMPs is critically involved in macrophage infiltration and subsequent formation of vascular sprouting, filopodia, and DVN in the EAT tip region. To further clarify the role of MMPs in macrophage infiltration from circulating macrophages into the tip region, mice were treated with intraperitoneal injections of ZA (100 μg/kg/d for 2 weeks) 1 week after macrophage depletion by CDL (40 mg/kg). Two weeks after the treatment with ZA, the treated mice showed not only markedly-reduced macrophage accumulation in the tip region, but also a severely-reduced number of DVN (Figure 4A and 4B), indicating that the MMPs secreted by the macrophages, adipocytes, and SVCs play a critical role in macrophage infiltration into the EAT tip region.

**Role of VEGF-VEGFR System in Macrophage Infiltration and Formation of DVN in the Tip Region of EAT**

Because angiogenesis is critically dependent on VEGF action,33,34 and all 3 cell types in EAT express the 2 main forms of VEGF (VEGF₁₆₄ and VEGF₁₂₅), we examined the role of...
VEGF in the formation of the DVN in the EAT tip region by blocking VEGF by intraperitoneal treatment with sFlt1-Fc (5 µg/d for 2 weeks). As a control, dimeric-Fc protein was given in the same manner. Two weeks after treatment, mice treated with sFlt1-Fc showed not only a reduced number and size of DVN and attenuated angiogenic sprouting and filopodia in the DVN, but also a moderately reduced number of macrophages in the tip region (Figure 4A and 4B), whereas mice treated with the control dimeric-Fc protein showed no difference from untreated mice. Moreover, variably-shaped protruding bumps, having a small amount of macrophages without lipid-containing adipocytes, were observed in the tip region (Figure 4A and 4B). These data indicate that the action of VEGF in the tip region is important in the formation of the DVN, and that VEGF itself might act on macrophage recruitment and infiltration, possibly through the VEGF-VEGFR1 system. To test whether VEGF affects the recruitment and infiltration of bone marrow-derived macrophages into the tip region, the mice were treated intraperitoneally with sFlt1-Fc (5 µg/d for 2 weeks) 1 week after macrophages depletion by CDL. Two weeks after treatment with sFlt1-Fc, the treated mice showed not only a markedly-reduced number of macrophages in the tip region, but also severely inhibited sprouting and filopodia in the outer margins of the DVN (Figure 4A), indicating that VEGF itself in the tip region could act on macrophage recruitment and infiltration. The role of VEGFR2 is critical in angiogenesis and angiogenic vasculature expresses VEGFR2 abundantly. Therefore, levels of VEGFR2 in the vasculature of EAT were examined. Indeed, VEGFR2 was more highly expressed in the vasculatures of the DVN than in the mature vasculature in the body region of EAT (supplementary Figure VI). To examine role of VEGFR2 in the formation of the DVN, VEGFR2 was blocked by intraperitoneal treatment with SU11248 (60 mg/d for 8 days) as previously described. This treatment markedly inhibited the formation of the DVN and, to a lesser extent, macrophage infiltration, but the effects were less than those in response to VEGF neutralization with sFlt1-Fc (Figure 4B). In addition, lipid-containing adipocytes were not detected in the protruding bump. These results indicate that VEGFR2 plays a key role in the formation of the DVN, and that VEGFR2-dependent formation of the DVN could facilitate subsequent adipogenesis in the adult EAT tip region.

The Tip Region of EAT is Highly Hypoxic

Hypoxia is well known to induce angiogenesis, linking vascular oxygen supply to metabolic demand. Massive infiltrations of macrophages have been found in hypoxic areas, including avascular areas of tumors. Furthermore, considering that the expression of VEGF and VEGFR2 are mainly dependent on hypoxia and that the actions of the MMPs, VEGF, and VEGFR2 are enhanced in hypoxia, we have reasoned to determine the extent of hypoxia in EAT using immuno-detection of Hypoxyprobe-1. Consistent with previous predictions, mature adipocytes in the body region of the adipose tissue were highly hypoxic (Figure 5A and 5K), showing a strong Hypoxyprobe-1 signal in the cytoplasm compared with other cells and tissues of other organs, including the lung (Figure 5C and 5K). Noticeably, the tip region of EAT was more hypoxic compared with the body region (Figure 5D-5K). This difference could be because of insufficient blood supply through the immature blood vessels to this locale. In fact, the extent of hypoxia in the tip region of EAT was higher than that of hypoxic areas in solid cancer tissues (Figure 5J and 5K). Thus, the extreme hypoxia in this locale may contribute to high expression levels of VEGF and VEGFR2 and may enhance the actions of VEGF/VEGFR2/MMPs in the formation of the DVN.

Role of SDF-1 in Macrophage Infiltration and Formation of DVN in the Tip Region of EAT

Our RT-PCR analysis (Figure 3F) also suggested that it might have a reciprocal interaction for the recruitment and retention of macrophages between CXCR4-expressing macrophages and SDF-1-expressing SVCs. To examine whether SDF-1 is necessary for the ongoing retention of macrophages in the EAT tip region, the function of SDF-1 was blocked by intraperitoneal treatment with an anti-SDF-1 blocking anti-
body (50 μg/thrice per week). As a control, nonspecific anti-rat IgG antibody was as administered in the same manner. Two weeks after treatment, the mice treated with anti-SDF-1 showed moderately reduced numbers of macrophages and a small number of immature DVN (Figure 4A and 4B), whereas the mice treated with control antibody showed no difference compared with untreated mice. To examine whether SDF-1 is necessary for macrophage mobilization and recruitment to occur in the EAT tip region, mice were treated with an intraperitoneal injections of anti-SDF-1 blocking antibody, as described above, one week after macrophages depletion by CDL. Two weeks after treatment with the anti-SDF-1 blocking antibody, the treated mice showed markedly reduced numbers of macrophages and showed no DVN in the tip region (Figure 4A and 4B). These data indicate that the action of SDF-1 is critically involved not only in macrophage retention, but also in macrophage mobilization and infiltration, and subsequent DVN formation in the EAT tip region.

**Role of MCP-1/CCR2 System in Macrophage Infiltration and Formation of DVN in the Tip Region of EAT**

These data raise the question of how macrophages are infiltrated into the EAT tip region. We hypothesized that MCP-1 (also known as C-C motif chemokine ligand 2, CCL2) might be involved in macrophage infiltration. MCP-1 is known to be a major chemokine for macrophage infiltration in adipose tissue, particularly during obesity, and its receptor, CCR2, plays a role in macrophage infiltration in adipose tissue. Our immunostaining showed that a relatively abundant amount of MCP-1 was detectable not only in the vascular area but also in the avascular area of the EAT tip region (Figure 6A). To examine the role of MCP-1/CCR2 in macrophage infiltration in the EAT tip region, we compared the pattern of macrophage accumulation between CCR2 mice and wild-type (WT) littermates. Our data indicated no difference in the pattern of macrophage accumulation or in characteristics of the DVN between CCR2 mice and WT littermates (Figure 6B and 6C). To clarify the role of the MCP-1/CCR2 system in the recruitment, infiltration, and ongoing retention of macrophages in EAT, macrophages in both CCR2 mice and WT littermates were depleted by CDL, and the pattern of macrophage accumulation was examined. One week after the treatment, all macrophages had completely disappeared from the adipose tissue of both CCR2 mice and WT littermates (Figure 6B). Three weeks after the treatment, the accumulation of macrophages and development of DVN in the EAT tip region of CCR2 mice were indistinguishable from those of WT littermates (Figure 6B), whereas fewer infiltrated macrophages were observed in the body region of EAT in CCR2 mice compared with WT littermates (Figure 6B). The latter finding is consistent with a previous report. These data suggest that the MCP-1/CCR2 system is not a critical factor in macrophage infiltration into the EAT tip region.

**Discussion**

Little is known about how angiogenesis governs the growth of adipose tissue. Here we show the angiogenic role of macrophages that accumulate largely in the tip region of adult EAT, which is extremely hypoxic (Figure 7). These macrophages are LYVE-1, originate from the bone marrow, and induce active angiogenesis through the secretion of MMP-9, MMP-12, and MMP-7, and activation of the VEGF-VEGFR2 system. This macrophage-induced angiogenesis is essential for subsequent adipogenesis, which may prove to be important for longitudinal outgrowth of adipose tissue. SDF-1 and VEGF, but not the MCP-1/CCR2 system, are critical factors for the mobilization, recruitment and retention of macrophages to this region (Figure 7). Thus, we have elucidated the angiogenic role of LYVE-1 macrophages that accumulate largely in the EAT tip region and show that they could be involved in regulating the outgrowth of adipose tissue. Furthermore, we unveil the main mediators and underlying

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**Figure 6.** Role of MCP-1/CCR2 system in macrophage infiltration and formation of DVN in the tip region of EAT. A, MCP-1 was immunostained (green). B, Macrophages and DVN are visualized by immunostaining of LYVE-1 (blue) and PECAM-1 (red) in the 8–9-week-old wild type (WT) and CCR2 mice during the control period (0 w), and 1 and 3 weeks after CDL (40 mg/kg) treatment. Scale bar, 100 μm. C, The density of macrophages per given density of blood vessels in DVN (Mac/BV in DVN) is expressed as a ratio of the amount of blue signal to the amount of red signal in the EAT tip region in the control period (0 w) and 3 weeks after CDL (40 mg/kg) treatment in WT and CCR2 mice. Bars represent means ±SD from 4 to 6 mice. NS, not significant vs WT.
mechanisms responsible for the recruitment and retention of macrophages in this tissue (Figure 7).

We had initially been interested in examining the distribution of lymphatic vessels in EAT by immunostaining of LYVE-1, which is a marker of lymphatic endothelial cells.22 By chance, we discovered from the coimmunostaining results that most LYVE-1 cells in EAT were macrophages rather than lymphatic endothelial cells. Indeed, our results reveal that the LYVE-1 cells are variably-shaped single cells rather than continuous tube-like lymphatic endothelial cells. Moreover, many of the LYVE-1 cells expressed well-known markers for macrophages: 90% were CD11b, and 30% to 40% were F4/80. Importantly, LYVE-1+ cells undergo apoptosis in all organs including EAT by active phagocytosis during 1 to 2 weeks of treatment with clodronate liposome.15,16 Moreover, isolated LYVE-1+ cells from the EAT display active phagocytic activity. These ample pieces of evidence indicate that the LYVE-1+ cells are variably-shaped single cells rather than continuous tube-like lymphatic endothelial cells. Moreover, of these LYVE-1+ cells, only lymphatic endothelial cells have the homeobox domain related transcriptional factor PROX-1, distinguishing LYVE-1+ macrophages from lymphatic endothelial cells. The mechanisms of LYVE-1 expression and the functional significance of LYVE-1 beyond hyaluronan transport have not been defined in detail. However, recent reports showed that LYVE-1 knockout mice displayed an apparently normal phenotype, with no visible alterations in hyaluronan metabolism, lymphatic vessel development and maintenance, dendritic cell trafficking, or tumor growth. In comparison, it is known that the expression and function of LYVE-1 in macrophages is upregulated in hypoxic and inflammatory conditions, and by a variety of growth factors and cytokines.44 These conditions were observed in the microenvironment of the EAT tip region. In turn, LYVE-1+ macrophages, as activated macrophages, could produce a vast number of factors that promote angiogenesis; as their bone marrow-derived monocytic precursors, they secrete factors that mediate their own migration and infiltration into hypoxic areas.9,10,26 Thus, the large number of infiltrated LYVE-1+ macrophages could actively participate in the formation of DVN in the hypoxic EAT tip region (Figure 7).
the DVN. Therefore, we sought to determine whether the MMPs are essential for macrophage recruitment and infiltration into the locale and for formation of the DVN. Our data obtained from using MMP inhibitor ZA indicate that the MMPs play a critical role in macrophage infiltration and macrophage-induced angiogenesis for the formation of DVN in the EAT tip region (Figure 7).

VEGF is one of the adipokines that are secreted from adipose tissues; such factors could be beneficial in regenerative cell therapy using adipose stromal cells to treat ischemic diseases in a paracrine manner. Our study reveals that VEGF plays critical roles not only in DVN formation, but also in macrophage recruitment and infiltration to the EAT tip region in a paracrine manner. Moreover, VEGF, in concert with Ang1, may act to help maintain of vascular integrity in adipose tissue in a paracrine manner. Thus, VEGF in adipose tissues could have multiple functions as one of the adipokines. Our data also indicate that the action of SDF-1 is critically involved not only in the ongoing retention of macrophages, but also in macrophage mobilization and infiltration into the EAT tip region. The importance of chemokine SDF-1 in the recruitment and retention of bone marrow-derived CXCR4 (SDF-1 receptor)-positive circulating progenitor cells into hypoxic ischemic and regenerative tissues for subsequent angiogenesis has been well established. Likewise, in our study, hypoxia in the EAT tip region could induce expression of SDF-1 and thus spurs its role in macrophage retention; bone-marrow-derived macrophage recruitment, mobilization, and infiltration; and subsequent angiogenesis. CXCR4 is the sole SDF-1 receptor and its expression in macrophages is enhanced by hypoxia. Likewise, LYVE-1–macrophages in the EAT tip region express CXCR4, and its expression can be enhanced by hypoxia in EAT. Thus, the recruitment and ongoing retention of macrophages is the result of a reciprocal interrelationship between CXCR4-expressing LYVE-1–macrophages and SDF-1-expressing vascular-stromal cells, and this relationship is eventually involved in macrophage-induced angiogenesis and the subsequent angiogenesis–dependent outgrowth of the EAT tip region (Figure 7).

Previous studies indicate that the growth of adipose tissue is angiogenesis–dependent. However, these observations are obtained from situations of active growth of adipose tissues, such as in obese mice or implantation of preadipocytes. In this study, we have focused on studying the role of locally accumulated LYVE-1–macrophages and locally active angiogenesis in adipogenesis only at the EAT tip region. It is envisaged that macrophages and blood vessels in the rest of EAT of normal are relatively quiescent and that the process of adipogenesis in this tissue is relatively slow. Our systemic treatments that targeted MMPs, the VEGF-VEGFR2 system, and SDF-1 could mainly have their effects on the tip region of adipose tissues, but not on the main portion of the adipose tissues. This may explain why we did not observe a marked reduction in total EAT fat mass and body weight during the 2 weeks exposure to several different treatments. However, in the future, the role of tip growth in EAT on total growth of EAT remains to be elucidated with long-term treatments with several specific agents that affect the formation of DVN or angiogenesis.

In conclusion, our findings shed light on the close interactions between macrophage infiltration, angiogenesis, and adipogenesis in the tip region of adult EAT.

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Disclosures
None.

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Adipokines: inflammation and the pleiotropic role


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Supplementary Table, Figures and Their Legends

Angiogenic Role of LYVE-1-positive Macrophages for Adipogenesis in Adipose Tissue

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(Angiogenic Role of Macrophage in Adipogenesis)

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Supplementary Table 1

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Supplementary Figure Legends

Supplementary Figure 1. Higher magnification views revealing the relationships between adipocytes, vasculature, and macrophages in the tip region of EAT. Whole-mounted EAT from 8-week-old mice are visualized by immunostaining for perilipin (for adipocytes, blue), PECAM-1 (for vasculature, red), and LYVE-1 (for macrophages, green); merged view is shown. (A) A characteristic DVN consisting of a short segment and large diameter is present in the tip region, where very few lipid-containing adipocytes and large numbers of macrophages are present. White arrowheads indicate vascular sprouting, which is common in the outer margins of the DVN. (B) Between arrowheads and bars, a characteristic DVN, with very few lipid-containing adipocytes and many macrophages present. Scale bar, 100 μm.

Supplementary Figure 2. Characterization of LYVE-1+ macrophages in the tip region of EAT. Whole-mounted EAT from 8-week-old mice are visualized by immunostaining for LYVE-1 (green), (A) CD11b (macrophage marker), (B) F4/80 (macrophage marker) or (C) CD45 (common leukocyte marker, red), PECAM-1 (for vasculature, red), and; merged views are shown. Upper portions of white dotted lines in A and C regard as the tip regions of EAT. Approximately 90%, 30-40% or 40-50% of variable shapes of LYVE-1+ cells are double stained with CD11b, F4/80 or CD45. Scale bar, 100 μm.

Supplementary Figure 3. Phagocytic activity of isolated LYVE-1+ cells from the EAT. Isolated LYVE-1+ cells from the EAT of 8-week-old mice are incubated for 4 hrs
in the presence of 0.026% (w/v) of FITC-labeled latex beads (2.0 μm in diameter) that have been opsonized with mouse IgG. Then the cells are washed, fixed, and phase contrast and fluorescent microscopic images of the cells are obtained. The merged view reveals that isolated LYVE-1+ cells contain the FITC-labeled latex beads in their cytoplasm (arrows) by phagocytosis. Scale bar, 20 μm.

**Supplementary Figure 4. Relationships between adipocytes, vasculature, functional circulation, and macrophage distribution in the tip region of EAT.** FITC-labeled lectin was injected intravenously into 8-week-old mice, and the image of FITC-lectin (green) in the DVN (PECAM-1 immunostaining, blue) of the tip region was obtained, and merged. Macrophages are visualized by immunostaining of LYVE-1 (red), and merged. Approximately 75-85% (n=4) of the DVN is FITC-lectin+, indicating that most DVNs have blood circulation. Scale bar, 100 μm.

**Supplementary Figure 5: Densitomeric quantitative analyses of RT-PCR profiles of various angiogenic factors in LYVE-1+-enriched SVC (a), adipocytes (b) and LYVE-1+-depleted SVC (c) in the EAT tip region as depicted in Figure 3.** The densitometric analyses are presented as the relative ratio of (a) or (b) to (c). (c) is arbitrarily presented as 1. Bars represent the mean ± SD from 3 experiments. *, P<0.05 versus c.

**Supplementary Figure 6. VEGFR2 is highly expressed in DVNs of various sizes in the tip region of EAT.** Whole-mounted EAT from 8-week-old mice are visualized with immunostaining of PECAM-1 (for vasculature, red), VEGFR2 (green), LYVE-1 (for
macrophages, blue), and merged views. Various types of DVN express VEGFR2 abundantly, and many accumulated macrophages are located ahead of and around the DVN. Scale bar, 100 μm.