Macrophage infiltration into adipose tissue may promote angiogenesis for adipose tissue remodeling in obesity

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Pang C, Gao Z, Yin J, Zhang J, Jia W, Ye J. Macrophage infiltration into adipose tissue may promote angiogenesis for adipose tissue remodeling in obesity. Am J Physiol Endocrinol Metab 295: E313–E322, 2008. First published May 20, 2008; doi:10.1152/ajpendo.90296.2008.—The biological role of macrophage infiltration into adipose tissue in obesity remains to be fully understood. We hypothesize that macrophages may act to stimulate angiogenesis in the adipose tissue. This possibility was examined by determining macrophage expression of angiogenic factor PDGF (platelet-derived growth factor) and regulation of tube formation of endothelial cells by PDGF. The data suggest that endothelial cell density was reduced in the adipose tissue of ob/ob mice. Expression of endothelial marker CD31 was decreased in protein and mRNA. The reduction was associated with an increase in macrophage infiltration. In the obese mice, PDGF concentration was elevated in the plasma, and its mRNA expression was increased in adipose tissue. Macrophages were found to be a major source of PDGF in adipose tissue, as deletion of macrophages led to a significant reduction in PDGF mRNA. In cell culture, PDGF expression was induced by hypoxia, and tube formation of endothelial cells was induced by PDGF. The PDGF activity was dependent on S6K, as inhibition of S6K in endothelial cells led to inhibition of the PDGF activity. We conclude that, in response to the reduced vascular density, macrophages may express PDGF in adipose tissue to facilitate capillary formation in obesity. Although the PDGF level is elevated in adipose tissue, its activity in angiogenesis is dependent on the availability of sufficient endothelial cells. The study suggests a new function of macrophages in the adipose tissue in obesity.

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though PDGF expression is elevated locally in tissue remodeling processes such as wound healing and tumor growth, it is not clear whether PDGF expression is increased during adipose tissue growth in obesity. As hypoxia induces PDGF expression (16), we propose that PDGF expression may be increased by adipose tissue hypoxia and may be involved in stimulation of angiogenesis during adipose tissue remodeling in obesity.

The angiogenic activities of PDGF are mediated by signaling pathways of cell membrane receptors of PDGF (47). Ligand engagement leads to PDGF receptor phosphorylation and activation of several signaling pathways, including Src, PI3K, and phospholipase Cγ (PLCγ) (47). The phophatidylinositol 3-kinase (PI3K)/Akt/mTOR (mammalian target of rapamycin) pathway is activated by PDGF (25, 58) and is involved in the recruitment of pericytes (13). It remains to be tested whether the PI3K/Akt/mTOR pathway is involved in tube formation of endothelial cells in response to PDGF. In studies of other angiogenic factors, the PI3K/Akt/mTOR pathway was reported to stimulate proliferation (51) and tube formation of vascular endothelial cells (57). However, there is no direct evidence that this signaling pathway is required by PDGF in the stimulation of tube formation.

In this study, we demonstrated that vascular density was reduced in adipose tissue in ob/ob mice. PDGF expression was elevated in adipose tissue and expressed in macrophages. In cell culture, PDGF stimulated tube formation of endothelial cells, and the activity required ribosomal protein S6 kinase (S6K). These data suggest that macrophage PDGF may play an important role in the stimulation of tube formation in the process of angiogenesis in adipose tissue. The study provides direct evidence for the role of the PI3K/mTOR/S6K signaling pathway in the PDGF-induced tube formation.

**EXPERIMENT PROCEDURES**

**Animals.** Male C57BL/6J-Lepob and C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME) at 6 wk of age and used in the study according to the animal protocol approved by the Institutional Animal Care and Use Committee at the Pennington Biomedical Research Center, Louisiana State university (Baton Rouge, LA). The mice were housed in regular cage with four mice per cage with free access to water and standard chow unless noted otherwise. The serum of the mice was collected from the tail vein. The cage with free access to water and standard chow unless noted otherwise. The mice were housed in regular cage with four mice per cage with free access to water and standard chow unless noted otherwise.

**Cell lines and reagents.** Murine endothelial cell line SVEC4-10 (CRL-2181, ATCC), murine fibroblast cell line 3T3-L1 (CL-173, ATCC), and the RAW 264.7 cell line (TIB-71) were maintained in DMEM with 10% fetal bovine serum in a CO2 (5%) incubator. Mouse fibroblast cell line 3T3-L1 (CRL-2181, ATCC), murine fibroblast cell line 3T3-L1 (CL-173, ATCC), and the RAW 264.7 cell line (TIB-71) were maintained in DMEM with 10% fetal bovine serum in a CO2 (5%) incubator.

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IgG-FITC (sc-2010, Santa Cruz Biotechnology). Microphotographs were taken under a microscope (×20).

Peritoneal macrophages. Primary peritoneal macrophages were isolated from C57BL/6J mice, as described elsewhere (56). The macrophages were transferred to 35-mm tissue culture dishes and treated with hypoxia in serum-free medium 3 days later for collection of supernatant.

Statistical analysis. All of the experiments were conducted at least three times with consistent results. The gel or image from representative experiment is presented. Values are means ± SE of multiple data points. Student's t-test or one-way ANOVA was used in statistical analysis of the data with a significance of P < 0.05.

RESULTS

Decreased density of microvasculature in adipose tissue in ob/ob mice. A decrease in capillary density has been proposed as a mechanism of blood flow reduction in adipose tissue in obesity. However, the reduction in capillary density is controversial (7, 23). One possible reason for the discrepancy is the quantification method for capillary density, which was determined by immunostaining in the studies (7, 23). To resolve the discrepancy, we compared capillary density in adipose tissues of lean mice and ob/ob mice via quantification of endothelial cell marker CD31. The CD31 protein was examined by immunostaining and Western blot. The CD31 mRNA was determined by qRT-PCR. The CD31 protein was detected in the tissue with either colorimetric- or fluorescence-based staining. CD31, distributed in the extracellular matrix of adipocytes, was reduced in obese mice (Fig. 1, A and B). The protein and mRNA of CD31 were both reduced in ob/ob mice (Fig. 1, C and D). These data suggest that neovascularization is reduced in white adipose tissue in obesity.

PDGF expression in adipose tissue of ob/ob mice. To understand the reduction in capillary density, we examined PDGF activity in the adipose tissue of obese mice. As a proangiogenic factor, PDGF is increased in human serum in response to inflammation and is involved in angiogenesis in many tissues (1, 3). However, it is not clear whether blood PDGF is elevated in an obese condition. To address this issue, the PDGF protein was compared in the serum of lean and ob/ob mice. The PDGF protein was increased in obese mice, as indicated by the ELISA data (Fig. 2A). To determine the source of PDGF protein, PDGF mRNA was determined in the adipose tissue by qRT-PCR. The mRNA was increased in the ob/ob mice as well (Fig. 2B). To determine the cell types for the source of increased PDGF, we examined PDGF mRNA in the adipose tissue after deletion of macrophages in the obese mice. The PDGF expression was reduced by macrophage deletion in a dose-dependent manner (Fig. 2C). These data suggest that PDGF is elevated in the blood and that adipose tissue may contribute to the systemic increase in PDGF protein. Macrophages are responsible for the PDGF increase in adipose tissue.

Expression of PDGF by macrophages and adipocytes. The data above suggest that macrophages may be a major producer of PDGF in the adipose tissue of obese mice. To test this question, we compared macrophages and adipocytes for expression of PDGF mRNA. The primary cells and cell lines were used and their activities compared in the basal and hypoxia-treated conditions. In the primary cells, macrophages expressed more PDGF than the adipocytes in the basal condition (Fig. 3A). This pattern of expression was also observed in the cell lines of macrophages (RAW cell) and adipocytes (3T3-L1) (Fig. 3B). These data suggest that PDGF is expressed

![Fig. 1. Vascular density decreased in adipose tissue in ob/ob mice. A and B: sections of epididymal fat pads were stained with CD31 antibody to reveal endothelial cell density. Immunohistostaining was conducted for the endothelial marker CD31. A: CD31 protein is indicated in red from AEC staining. B: CD31 protein is indicated in green fluorescent. Blue stands for nuclei stained by DAPI. Scale bar, 50 μm. C: CD31 protein was determined in whole cell lysate of epididymal fat pads in Western blot. Representative blot is shown with an average signal strength in the bar figure. D: CD31 mRNA was determined in epididymal fat pads by qRT-PCR. In this figure, each data point represents mean ± SE (n = 5). Experiments were repeated 3 times with consistent results.](image-url)
in adipocytes, but the expression level is significantly lower than that of macrophages. The impact of differentiation on PDGF expression was investigated in 3T3-L1 cells. After differentiation, PDGF expression was significantly reduced in 3T3-L1 cells (Fig. 3C). These data suggest that expression of PDGF is in the order of macrophage > preadipocytes > mature adipocytes.

Induction of PDGF expression by hypoxia. Hypoxia in adipose tissue may induce PDGF expression in obese mice, as PDGF-B is a hypoxia response gene (16); this possibility remained to be tested. To test this, PDGF expression was determined in primary macrophages after exposure to ambient hypoxia. As expected, PDGF expression was increased by the hypoxia treatment (Fig. 3D). In the macrophage cell line (RAW), the same effect was observed for hypoxia (Fig. 3D). However, the response of RAW macrophages was stronger than for the primary macrophages. These data further support that macrophages are the major producer of PDGF in the adipose tissue of obese mice. Macrophage infiltration should contribute to the elevated PDGF expression in adipose tissue.

Fig. 2. Platelet-derived growth factor (PDGF) increased in adipose tissue in ob/ob mice. A: serum PDGF was determined by ELISA. B: PDGF mRNA was determined in epididymal fat pads by qRT-PCR. C: PDGF mRNA in adipose tissue after macrophage deletion. Macrophages were reduced in adipose tissue by single ip injection of clodronate liposome in ob/ob mice (6 wk old). PDGF was examined at day 4 after injection. F4/80 is a marker of macrophage. Each data points represents mean ± SE (n = 3).

Fig. 3. PDGF expression in macrophages and adipocytes. A: PDGF mRNA in primary cells. The basal level of PDGF mRNA was determined in primary macrophages and adipocytes of lean mice. B: basal level of PDGF mRNA was determined in macrophage cell lines (RAW cells) and 3T3-L1 adipocytes. C: basal level of PDGF mRNA was determined in 3T3-L1 fibroblasts before and after differentiation into adipocytes. D: induction of PDGF mRNA expression by ambient hypoxia in primary macrophages and RAW macrophages. E: expression of PDGF in adipose tissue of lean mice after macrophage deletion. A single injection of clodronate liposome (150 mg/kg) was used to delete macrophages in lean C57BL/6J mice (8 wk old). Experiments were repeated 3 times with consistent results. Each data point represents mean ± SE (n = 3). *P < 0.05, treated vs. untreated in RAW cells; †P < 0.05, treated vs. untreated in primary macrophages.
In lean mice, preadipocytes or stromal cells may be the primary source of PDGF, as deletion of macrophages did not lead to reduction in PDGF expression (Fig. 3E).

Function of PDGF in regulation of angiogenesis. Given the role of PDGF in angiogenesis, we proposed that macrophage PDGF may be involved in the stimulation of capillary growth in adipose tissue under hypoxia, in which case the supernatant of hypoxia-treated macrophages should be able to stimulate angiogenesis. To test this possibility, the supernatant of RAW macrophages treated by hypoxia was examined in the tube formation assay. Such an assay was used to study PDGF activity elsewhere (2, 12). The results suggest that the macrophage supernatant induced more tube in the assay, and this activity increased in the supernatant of hypoxia-treated macrophages (Fig. 4, A and B, control antibody). In the presence of PDGF antibody, such an increase in tube formation was inhibited (Fig. 4, A and B, PDGF antibody).

**PI3K/Akt pathway in PDGF-stimulated tube formation.** The signaling pathway of PDGF receptor is involved in angiogenesis and is described in several pathological conditions (47). The importance of the PI3K/Akt/mTOR pathway in PDGF-induced angiogenesis was demonstrated in vivo in rodent models (13, 18, 25, 58). PDGF is known to activate the PI3K/Akt pathway in pericytes that normally form part of the capillary wall. It remains to be tested whether the same pathway is involved in tube formation by endothelial cells. To investigate the PI3K/Akt pathway in endothelial cells, we examined PI3K inhibitors on tube formation. In the presence of control insulin was used to induce tube formation (Fig. 5, A and B). In the presence of PI3K inhibitor LY-294002 (100 μM) or wortmannin (1 μM), the insulin-induced tube formation was reduced significantly. Recombinant PDGF was used to stimulate tube formation in the mouse endothelial cells. As expected, the tube formation was induced by the recombinant PDGF, and the effect was blocked by the PI3K inhibitors (Fig. 5, A and B). The data suggest that PI3K activity is required for tube formation induced by PDGF and insulin. The basal level of tube formation was also reduced by the PI3K inhibitors (Fig. 5, A and B), suggesting that PI3K activity is required for tube formation induced by Matrigel, which contains proangiogenic factors.

To verify activation of PI3K/Akt by PDGF, we examined the phosphorylation status of Akt and its downstream molecules in a Western blot. Identical to those observed with insulin in the positive control, the activation markers of Akt, S6K, and GSK3-β were all increased by PDGF (Fig. 5, C and D). These changes were consistently blocked by the PI3K inhibitor LY-294002. We also tested the roles of MAPK kinases in PDGF-induced tube formation with chemical inhibitors to ERK (PD-098095 40 μM), JNK (SP-600125 50 μM), and p38 (SB-203580 2 μM). Although these inhibitors could inhibit the basal level of tube formation, they did not decrease the PDGF-induced tube formation significantly (Supplemental Fig. S1, online only). These data suggest that the PI3K/Akt pathway is required for angiogenesis induced by PDGF.

**mTOR in PDGF-induced tube formation.** mTOR is a major signaling molecule downstream of Akt. Inhibition of angiogenesis by rapamycin in mice suggests that mTOR may mediate PI3K/Akt signaling in the stimulation of angiogenesis in mice (18). In the current study, inhibition of mTOR led to a reduction in tube formation induced by PDGF as well as by insulin (Fig. 6, A and B), suggesting that mTOR is required for the PDGF signaling pathway. Activation of mTOR by PDGF and its inhibition by rapamycin were examined by the phosphorylation status of S6K in Western blot. As was observed for insulin, the phosphorylation was induced by PDGF and blocked by rapamycin (Fig. 6, C and D). As inhibition of protein synthesis by cycloheximide abolished the tube formation induced by PDGF or insulin (Fig. 6A), protein synthesis is required for the tube formation. This observation suggests that mTOR may act through S6K in the PDGF signaling pathway in endothelial cells, as S6K controls gene translation.

**S6K in PDGF-induced tube formation.** S6K is one of the major signaling molecules activated by mTOR and is involved in regulation of protein synthesis. It remains to be tested whether S6K mediates PDGF signal in endothelial cells for tube formation (24, 38, 57). The S6K dominant-negative (S6K-DN) mutant in stable transfection was generated to test S6K activity in PDGF-induced tube formation. The mutant S6K was expressed in the SVEC4-10 cells, as indicated by detection of HA-tag in Western blot (Fig. 7A). Inhibition of S6K function was confirmed, with decreased phosphorylation of its substrate S6 in the transfected cells (Fig. 7B). In response to PDGF, the stable cell line expressing S6K-DN exhibited much fewer tubes.

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**Fig. 4.** Induction of tube formation by PDGF in macrophage supernatant. Tube formation of endothelial cells on Matrigel was used to test proangiogenic activity of PDGF in supernatants of macrophages. Raw cells were treated with hypoxia for 4 h, and its supernatant was collected for induction of tube formation in endothelial cells. PDGF antibody was used to determine activity of PDGF in the supernatant. A: representative images of tube formation of the endothelial cells on Matrigel. B: quantification of tube formation. Experiments were repeated 3 times with consistent results. Each data point represents mean ± SE (n = 3 experiments).
on the Matrigel (Fig. 7, C and D), suggesting that S6K is required by PDGF in the signal transduction.

**Stimulation of tube formation by albumin.** The data above suggest that the signal of tube formation is transduced through PI3K/Akt/mTOR/S6K under the PDGF receptor. It is not clear whether activation of S6K in the absence of activation of upstream signaling molecules including PI3K and Akt is sufficient to induce tube formation. To test this possibility, S6K was activated with albumin (Fig. 8 A). The activation led to an increase in tube formation, and the increase was blocked by rapamycin (Fig. 8, B and C), which suppresses S6K by targeting mTOR. The data suggest that activation of S6K by albumin is able to increase tube formation. Toxicity of rapamycin was examined in an MTT assay. Treatment of the cells with rapamycin for 24 h had no significant effect on cell viability in presence or absence of PDGF (Fig. 8 D).

**DISCUSSION**

Our study suggests that vascular density is decreased in adipose tissue in ob/ob mice. Angiogenesis is required for the development and growth of adipose tissue (7, 41). During development of obesity, angiogenic activity will be increased to compensate for expansion of adipocyte tissue. This compensation ensures quick growth in adipose tissue at the early stage of weight gain. Once the weight gain reaches a level such as BMI >25–30 kg/m², a failure in angiogenic compensation may occur and thus reduce the growth rate of fat tissues. A reduction in adipose tissue blood flow may be a result of such angiogenic failure (53). To explain adipose tissue hypoxia in the obese condition (56), we proposed that angiogenic failure may occur in adipose tissue of obese mice. In the current study, this possibility is supported by the reduction of vascular density in the adipose tissue. The CD31 data from immunohistostaining, Western blot, and qRT-PCR consistently support the lack of endothelial cells in the adipose tissue of ob/ob mice (Fig. 1). A lack of VEGF induction by hypoxia was observed in the adipose tissue in the same condition (56). The VEGF non-response may contribute to the reduced endothelial cells, as VEGF is a primary growth factor for endothelia cells. Macrophages may serve as a stimulator for angiogenesis in adipose tissue in obesity. Adipose tissue contains several types of cells, such as preadipocytes (or stromal cells), adipocytes, macrophages, and endothelial cells. PDGF is expressed in all of these types of cells; however, the expression levels are different. The current study suggests that preadipocytes express more PDGF than mature adipocytes. In obesity, preadipocyte number is reduced, as most of them are differentiated into mature adipocytes. This change may lead to a decrease in local PDGF level, since mature adipocytes produce less PDGF than the preadipocytes (Fig. 3 C). To meet the demand for...
PDGF, macrophage infiltration into adipose tissue is increased to compensate for the loss of preadipocytes for PDGF production (Fig. 2B). Our data support the idea that infiltration and activation of macrophages may contribute to the increased PDGF expression in the adipose tissue of obese mice. This conclusion is supported by the observations that deletion of macrophages led to a significant reduction in PDGF expression in adipose tissue of ob/ob mice. The reduction was observed in obese mice but not in lean mice, which have abundant preadipocytes (Figs. 2C and 3E). Macrophages are second next to platelets in expression of PDGF (32, 40, 43). We believe that, in addition to its roles in chronic inflammation (52, 55), macrophages may serve as an angiogenic stimulator in adipose tissue by expression of PDGF. To our knowledge, this may be the first study to support elevation and function of PDGF in adipose tissue of obesity. Hypoxia in adipose tissue is likely to induce PDGF expression in macrophages (Fig. 3D). In the current study, PDGF expression was determined mainly at the mRNA level. It remains to be tested whether PDGF protein is induced by hypoxia in macrophages.

We did not find literature about PDGF expression in adipose tissue in obesity. Closely related literature includes PDGF regulation of GLUT4 translocation, proliferation, or differentiation of adipocytes in cell culture (19, 31, 36). In vitro, PDGF or its receptor has been shown to promote GLUT4 translocation in adipocytes (31, 36). PDGF has been reported to inhibit adipocyte differentiation (19). In other organs, local PDGF has been suggested to play a role in pathogenesis of proliferative retinopathy and diabetic nephropathy (14, 26, 39). An increase in PDGF has been found in the retinal membranes and vitreous fluid of patients with proliferative diabetic retinopathy (14, 39). A higher PDGF level has also been reported in individuals with additional rubeosis iridis or ischemic nondiabetic retinopathy (14). In the renal biopsy of patients with diabetic nephropathy, expression of PDGF-A and PDGF-B has been reported to be increased in mRNA and protein (26). In mice with type 1 diabetes, the PDGF elevation has been associated with macrophage infiltration into kidney with diabetic nephropathy (9). These observations suggest a role of PDGF in the pathogenesis of diabetic complications.

The current study suggests that the activity of PDGF in angiogenesis is dependent on VEGF activity. Angiogenesis requires endothelial cell proliferation and tube formation. The two events are equally important in the process of formation of capillary. Tube formation requires the presence of endothelial cells; in the absence of endothelial cells, tube formation will not occur. Endothelial cell proliferation is dependent on proangiogenic factor VEGF, which stimulates cell proliferation.
through VEGF receptor 2 in the endothelial cells. The lack of endothelia cells was observed in adipose tissue of ob/ob mice in this study (Fig. 1). The reduction in endothelial cells is supported by quantification of CD31 (endothelial cell marker) in protein and mRNA. This reduction is consistent with our previous observation that VEGF expression was not increased in adipose tissue by obesity in ob/ob mice (56). Lack of leptin in the ob/ob mice may be related to the unresponsiveness of VEGF to obesity or adipose tissue hypoxia. In the wild-type mice, VEGF was increased by diet-induced obesity in the adipose tissue (56). Without sufficient endothelial cells, PDGF will not be able to increase angiogenesis in the adipose tissue by itself.

In addition to stimulation of tube formation, PDGF was reported to play a role in the recruitment of pericytes or the proliferation of vessel smooth muscle cells in angiogenesis. Recruitment of pericytes is required for maintenance of microvascular stability and function (21, 22, 29). This function of PDGF is established in a PDGF knockout study (29). Pericytes are able to transdifferentiate into fibroblasts (stromal cells/preadipocytes) and vessel smooth muscle cells (37). With these activities, it is possible that PDGF is involved in recruitment of preadipocytes into adipose tissue in obesity.

In the current study, we observed that insulin stimulated tube formation, suggesting a role for insulin in the stimulation of angiogenesis. This activity indicates that hyperinsulinemia may serve to stimulate angiogenesis in adipose tissue in the obese condition. Hyperinsulinemia is a prognosis factor for the incidence of microvascular abnormalities in diabetes patients for retinopathy and nephropathy (4). In the present study, insulin exhibited a similar activity to PDGF in the induction of tube formation (Figs. 5 and 6). This observation suggests that hyperinsulinemia may be involved in neovascularization in adipose tissue during the development of obesity.

S6K is required for tube formation induced by PDGF and insulin. S6K is activated by an array of mitogenic or nutrient stimuli, such as insulin, serum, phorbol ester, and PDGF (38, 48, 54). Knockout studies suggest that S6K is an important regulator of cell size and body growth in mice and Drosophila.
(35, 42). S6K1−/− mice are protected against age- and high-fat diet-induced obesity and insulin resistance due to increased energy expenditure (48). In tube formation, S6K may act through induction of FGF-2 expression in endothelial cells (57). In the present study, our data suggest that S6K mediates signals of PDGF and insulin in the induction of tube formation. This is supported by data that inhibition of S6K by dominant-negative mutant or chemical inhibitors (LY, wortmanin, and rapamycin) lead to inhibition of PDGF and insulin activity (Figs. 5–7). These data consistently support the notion that S6K is a critical kinase for endothelial differentiation and tube formation.

In summary, reduction in vascular density is likely a result of a lack of an endothelium growth factor (such as VEGF) in adipose tissue in ob/ob mice. This may contribute to the development of adipose tissue hypoxia and macrophage infiltration. PDGF expression is increased in macrophages in response to the hypoxia. Although PDGF is able to stimulate tube formation, it may not stimulate angiogenesis in the absence of sufficient endothelial cells or VEGF activity. The signaling pathway PI3K/Akt/mTOR/S6K is used by PDGF in the induction of tube formation. With insufficient endothelial cells in the adipose tissue of ob/ob mice, macrophages may fail to stimulate angiogenesis. These possibilities may support a new function for macrophages in adipose tissue. Hyperinsulinemia may also be involved in angiogenesis in adipose tissue in obesity by stimulation of tube formation in endothelial cells.

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