Transplanted Perivascular Adipose Tissue Accelerates Injury-Induced Neointimal Hyperplasia
Role of Monocyte Chemoattractant Protein-1


Objective—Perivascular adipose tissue (PVAT) expands during obesity, is highly inflamed, and correlates with coronary plaque burden and increased cardiovascular risk. We tested the hypothesis that PVAT contributes to the vascular response to wire injury and investigated the underlying mechanisms.

Approach and Results—We transplanted thoracic aortic PVAT from donor mice fed a high-fat diet to the carotid arteries of recipient high-fat diet–fed low-density lipoprotein receptor knockout mice. Two weeks after transplantation, wire injury was performed, and animals were euthanized 2 weeks later. Immunohistochemistry was performed to quantify adventitial macrophage infiltration and neovascularization and neointimal lesion composition and size. Transplanted PVAT accelerated neointimal hyperplasia, adventitial macrophage infiltration, and adventitial angiogenesis. The majority of neointimal cells in PVAT-transplanted animals expressed α-smooth muscle actin, consistent with smooth muscle phenotype. Deletion of monocyte chemoattractant protein-1 in PVAT substantially attenuated the effects of fat transplantation on neointimal hyperplasia and adventitial angiogenesis, but not adventitial macrophage infiltration. Conditioned medium from perivascular adipocytes induced potent monocyte chemotaxis in vitro and angiogenic responses in cultured endothelial cells.

Conclusions—These findings indicate that PVAT contributes to the vascular response to wire injury, in part through monocyte chemoattractant protein-1–dependent mechanisms. (Arterioscler Thromb Vasc Biol. 2014;34:1723-1730.)

Key Words: adipose tissue | hyperplasia

Obesity is associated with expansion of metabolically active adipose tissues leading to local and systemic inflammation, insulin resistance, and dyslipidemia, all of which can contribute to cardiovascular disease.1 In addition to these systemic effects, obesity is associated with expansion of perivascular adipose tissue (PVAT) immediately adjacent to the adventitia of large arteries. Emerging evidence suggests that PVAT may contribute to the pathogenesis of vascular disease.2

PVAT is anatomically colocalized with atherosclerotic lesions in humans, correlating with plaque burden and vascular calcification.3,4 Also, inflammatory cell infiltration was reported to be markedly increased in PVAT surrounding atherosclerotic human aorta as compared with nondiseased aorta.5 Moreover, inflammatory gene expression was shown to be upregulated,5,6 and expression of adiponectin, an anti-inflammatory adipokine, was downregulated,7,8 in PVAT surrounding diseased human coronary arteries. Thus, both the amount of PVAT and the degree of PVAT inflammation correlate with cardiovascular disease in humans.

Inflammation of human PVAT may in part relate to the unique properties of human perivascular adipocytes, which express higher levels of chemokines compared with adipocytes from other depots. In particular, expression of monocyte chemoattractant protein-1 (MCP-1) is ≈10- to 40-fold higher in perivascular adipocytes (coronary artery) compared with corresponding subcutaneous and perirenal adipocytes derived from the same subjects.9 Likewise, human perivascular adipocytes surrounding the radial artery secrete more MCP-1 than visceral or subcutaneous adipocytes derived from the same patients.10 MCP-1 is best known for its role in recruiting monocytes/macrophages to the arterial wall, but it may...
Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

To study the local effect of transplanted adipose tissue in vivo, PVAT was collected from the thoracic aorta of donor C57Bl/6J (wild type [wt]) male animals fed an HFD for 2 weeks. Ingual SQAT was harvested from the same group of donor mice and used as a control. PVAT from donor MCP-1−/− male mice on a C57Bl/6J background fed an HFD was also harvested for a second treatment group. We transplanted 2 to 3 mg of PVAT (wt or MCP-1−/−) or SQAT (wt) per mouse and executed wire injury of the left common carotid arteries of HFD-fed low-density lipoprotein receptor knockout mice 14 days after transplantation.22–25 To control for surgical manipulations, sham transplantation was performed on the contralateral right common carotid arteries and the left common carotid arteries of separate mice.

At the time of wire injury, sham-transplanted segments were devoid of fat and showed no evidence of fibrosis or adhesions (Figure 1A and 1B), whereas PVAT- (Figure 1C and 1D) and SQAT- (not shown) transplanted segments demonstrated glistening fat that was incorporated into the carotid adventitia and exhibited grossly visible neovessels. Histology of PVAT-transplanted arteries demonstrated a mixed population of white and brown adipocytes abutting the adventitia, with inflammatory cells dispersed throughout the adipose tissue (Figure 2). Assessment of gene expression demonstrated that in transplanted PVAT and SQAT, mRNA expression of adiponectin and leptin was similar to endogenous fat harvested from the corresponding depots of recipient mice, suggesting that adipose phenotype was unaffected by the transplantation procedure (Figure II in the online-only Data Supplement). Comparing PVAT-transplanted animals with sham-transplanted animals, total cholesterol (1085±187 versus 1368±457 mg/dL, respectively; P=0.38) and triglyceride levels (440±175 versus 484±134 mg/dL, respectively; P=0.75) were unchanged. These findings indicate that the transplanted adipose tissue acquired sufficient nutritive blood flow within 2 weeks to maintain viability and that the phenotype of adipose tissue and systemic lipid levels were not significantly affected by the transplantation procedure.

Fourteen days after wire injury, the mice were euthanized, carotid arteries were harvested, and neointimal formation was

Figure 1. Surgical images taken during wire injury procedure. Two weeks after either sham transplantation (A and B) or transplantation of 2 to 3 mg of perivascular adipose tissue (C and D), wire injury was performed (B and D). The carotid artery (black arrows) was ligated with silk sutures proximally and distally, relative to the carotid bifurcation. Note that transplanted perivascular adipose tissue is healthy appearing, with incorporated vessels, at the time of wire injury (blue arrows, C and D).
examined in PVAT-transplanted versus sham-transplanted and SQAT-transplanted arteries. Histological analysis demonstrated that, compared with sham controls (Figure 2A), transplanted PVAT caused an increase in injury-induced neointimal area in low-density lipoprotein receptor knockout mice in the setting of HFD (neointimal formation, Figure 2B). In contrast, transplanted PVAT from MCP-1−/− mice failed to induce a robust neointimal formation (Figure 2C). Quantification analysis revealed that, at 14 days after injury, wt PVAT resulted in ≈3-fold increase in neointimal formation compared with sham transplant control (Figure 2D, Figure III in the online-only Data Supplement). Transplanted SQAT was not statistically different in influencing neointimal hyperplasia compared with sham transplantation. Taken together, these data suggest that transplanted PVAT augments neointimal hyperplasia through a mechanism that is dependent on locally produced MCP-1.

To determine the composition of the neointimal formation in PVAT-transplanted mice, we performed immunostaining for α-smooth muscle actin (Figure 3). We found that compared with sham-transplanted controls (Figure 3A), mice transplanted with wt PVAT exhibited a smooth muscle actin–rich neointimal formation, suggesting a predominant VSMC composition of the neointima (Figure 3B and Figure IV in the online-only Data Supplement). We also detected rare cells expressing the macrophage marker F4/80 dispersed throughout the neointima (Figure 4B, black arrow). Notably, mice transplanted with PVAT from MCP-1−/− mice exhibited marked reduction in smooth muscle actin–positive neointimal staining (Figure 3C and Figure IV in the online-only Data Supplement), suggesting reduced accumulation of VSMC in the neointima.

In view of previous reports that HFD induces inflammation of adipose tissues, especially visceral adipose tissues, we surmised that inflammatory cell infiltration in the adjacent vascular adventitia would be enhanced in PVAT-transplanted arteries subjected to wire injury. Indeed, staining for F4/80 demonstrated increased infiltration of macrophages into

**Figure 2.** Carotid arteries from low-density lipoprotein receptor knockout (LDLR−/−) mice after wire injury. Compared with sham-transplanted control (A), transplanted wild-type (wt) perivascular adipose tissue (PVAT) resulted in a robust neointimal response (NI, B). Note that the transplanted adipocytes are healthy appearing and in intimate contact with the lamina adventitia. Transplanted monocyte chemoattractant protein-1–deficient (MCP-1−/−) PVAT was not associated with robust neointimal (NI) formation (C). Scale bar=200 μm. D, Transplanted PVAT increases cross-sectional area of neointimal lesion after wire injury. *P<0.05 PVAT-injury vs all other groups. Data were analyzed by 1-way ANOVA followed by pairwise multiple comparison procedures (Holm–Sidak method). SQAT indicates subcutaneous adipose tissue.
the adventitia after PVAT transplantation (Figure 4B, red arrows) as compared with sham transplantation (Figure 4A) or SQAT transplantation (Figure 4F). Staining for a second macrophage marker, Mac3, showed similar results, as well as clustering of macrophages near the border of PVAT with the adventitia, as has been demonstrated in atherosclerotic human aorta (Figure V in the online-only Data Supplement). HFD upregulates MCP-1 expression in visceral adipose tissues, and this inflammatory mediator can contribute to recruitment of leukocytes and adipose inflammation.26,27 Transplantation of MCP-1−/− PVAT, however, produced similar numbers of infiltrating adventitial macrophages compared with the wt PVAT (Figure 4C and 4D, Figure V in the online-only Data Supplement), suggesting that other chemokines from PVAT could compensate for MCP-1 gene deletion to promote macrophage recruitment to the adventitia. Consistent with

Figure 4. Inflammation after perivascular adipose tissue (PVAT) transplantation and wire injury. F4/80 staining revealed few adventitial macrophages after wire injury surrounding sham-transplanted arteries (A, red arrow). In contrast, transplanted wild-type (wt) PVAT markedly increased the presence of adventitial macrophages surrounding injured arteries (B, red arrows); transplanted monocyte chemoattractant protein-1−/− (MCP-1−/−) PVAT was associated with a similar increase in the infiltration of adventitial macrophages (C, red arrows). In all panels, the blue line demarcates the external elastic lamina of the injured vessel; black arrow in B points to neointimal F4/80 staining. Scale bar=50 μm. Quantification of adventitial macrophages in all 3 groups is shown in D: sham transplant, 81±12 cells/mm²; MCP-1−/− PVAT, 174±48 cells/mm²; wt PVAT, 220±108 cells/mm² (all data expressed as mean±SD). *P<0.01 vs sham transplant. E, mRNA levels of inflammatory cytokines tumor necrosis factor-α (TNF-α), MCP-1, and macrophage inflammatory protein-1α (MIP-1α) in intact perivascular adipose tissue of C57Bl/6J mice fed a chow or high-fat diet (HFD) for 2 weeks. mRNA levels of selected genes were quantified by quantitative reverse transcription polymerase chain reaction after normalizing to the house-keeping gene RPLPO (ribosomal protein large O) according to previously described methods.9 Results are normalized to the expression in chow-fed animals (red line). *P<0.001 vs chow from 3 independent experiments. Data in D and E were evaluated by 1-way ANOVA followed by Student–Newman–Keuls testing. F, Monocyte migration through endothelial monolayer toward conditioned medium from cultured subcutaneous (SQ) or perivascular (PV) murine adipocytes. Monocytes were stained with DAPI (4',6-diamidino-2-phenylindole), captured in 3 random view fields (x40), and quantified using ImageJ. *P<0.001 vs SQ. Data were compared by 1-way ANOVA followed by a 2-tailed Student t test to evaluate levels of significance at 95% confidence interval.
Data were compared by 1-way ANOVA followed by a 2-tailed Student’s *t* test to evaluate levels of significance at 95% confidence interval.

In a separate series of experiments, we conditioned medium for 4 hours at 37°C by incubating with 10 μg of intact, freshly harvested PVAT from either MCP-1−/− or wt mice fed a Western diet for 4 weeks. We found that MCP-1 deletion did not diminish monocyte migration using the same in vitro assay (Figure VIII in the online-only Data Supplement). Taken together, these results suggest that perivascular adipocytes are more potent than subcutaneous adipocytes at eliciting monocyte chemotaxis and that production of other chemokines in PVAT compensates for loss of MCP-1 to promote leukocyte recruitment.

The grossly visible blood vessels coursing through PVAT (Figure 1) suggested enhanced neovascularization induced by the transplanted fat. This was confirmed by staining for the endothelial marker factor VIIIa–related antigen, which showed markedly increased adventitial neovessel formation after PVAT transplantation as compared with sham transplantation (Figure 5B, red arrows). Specifically, injured sham-transplanted adventitia was characterized by small vascular structures with poorly defined lumens, whereas PVAT transplantation was associated with an extensive adventitial vasculature between the transplanted PVAT and external elastic lamina of the artery. Interestingly, transplanted PVAT from MCP-1−/− mice failed to elicit a robust angiogenic response in the adventitia, and the change in neovessel density was not statistically significant compared with sham transplantation (Figure 5C).

Adipocytes are powerful inducers of angiogenesis. To compare the angiogenic potential of perivascular adipocytes with subcutaneous and visceral adipocytes, we performed an in vitro bioassay. Human perivascular preadipocytes were differentiated into mature adipocytes, as described previously, and conditioned medium from the cultured cells was collected and applied to quiescent, subconfluent human coronary artery endothelial cell. Medium from identically processed subcutaneous and perirenal adipocytes derived from the same subjects was used for comparison purposes. We found that conditioned medium from differentiated cultures of human perivascular adipocytes strongly induced human coronary artery endothelial cell to elongate and form branching structures indicative of angiogenesis (Figure 6). The angiogenic effects of conditioned medium from perivascular adipocytes far exceeded those elicited by subcutaneous or perirenal adipocytes.

Vascular endothelial growth factor (VEGF) is a powerful angiogenic factor that has been implicated in adventitial neovascularization after endothelial injury and is produced by adipocytes. Therefore, we examined release of VEGF into the medium by perivascular adipocytes. As compared with subcutaneous adipocytes, perivascular adipocytes released approximately twice as much VEGF into the medium during incubation (94±35 versus 49±5 pg) for an equal number of cells.
in this blood vessel. Moreover, data in the SMPG knockout mouse suggest an atheroprotective effect of PVAT when animals are housed at a reduced temperature, presumably attributable to increased systemic metabolic activity from adaptive thermogenesis.18

Several studies have examined the impact of transplanted adipose tissue on local vascular biology. In 2 studies, endogenous PVAT was removed and replaced with subcutaneous or visceral fat before performing wire injury of the femoral artery.19,21 In these studies, subcutaneous fat transplanted from mice fed a normal diet inhibited neointimal formation after injury, whereas subcutaneous fat transplanted from high-fat, high-sucrose diet–fed mice had no effect.19 Transplantation of visceral epididymal adipose tissue also diminished neointimal hyperplasia, and this protective effect was lost when the proinflammatory angiopoietin-like protein 2 gene was overexpressed in the transplanted adipose tissue.21 In a third study, atherosclerosis was quantified after transplantation of visceral or subcutaneous fat to the carotid artery.20 In this model, epididymal fat transplantation augmented atherosclerosis, whereas subcutaneous fat had minimal effect.29 In the latter study, the investigators transplanted 60 mg of fat, which is disproportionate to the amount of PVAT that spontaneously forms around conduit arteries. Nevertheless, the augmentation of atherosclerosis observed in that study was clearly a local phenomenon because vessels devoid of transplanted fat were unaffected. Therefore, studies to date have yielded conflicting results, suggesting that the influence of PVAT on vascular pathology is complex and dependent on the particular blood vessel and the experimental model used.

The results obtained in the current study are not strictly comparable with any of the prior publications, because it is the only study in which authentic PVAT was transplanted. PVAT exhibits a distinct phenotype as compared with subcutaneous and epididymal adipose tissues, which must be taken into account when interpreting results from this study in the context of prior publications. To add to the complexity, PVAT in rodents and perhaps humans comprised both brown and white adipose tissue, depending on the anatomic location.34,35 The divergent metabolic and inflammatory state of brown versus white adipose tissues may be an important factor in determining how PVAT locally influences vascular pathophysiology in rodent models and in humans.

PVAT differs from both subcutaneous and epididymal adipose tissues, both under basal conditions and after dietary manipulations, which may relate in part to the unique biochemical and molecular properties of perivascular adipocytes.8,36 One of the most striking differences between perivascular adipocytes and adipocytes derived from other depots is increased expression of chemokines, especially MCP-1 (10–40-fold increased as compared with subcutaneous and perirenal adipocytes). Our results with PVAT transplanted from MCP-1−/− mice clearly implicate MCP-1 secretion by PVAT in the vascular response to wire injury. Based on these findings, we expected that neointimal proliferation associated with PVAT transplantation would consist largely of inflammatory cells. Rather, we observed that most of the cells stained positively for α-smooth muscle actin, with relatively few inflammatory cells scattered
throughout the neointima. Moreover, although PVAT transplantation triggered pronounced adventitial macrophage infiltration, it was not significantly diminished by ablation of MCP-1 expression in the PVAT. It is possible that polarization of the macrophages was affected by MCP-1 gene deletion in PVAT, based on the limited data with inducible nitric oxide synthase and Ym1 gene expression. Thus, phenotypic changes in macrophages could have indirectly contributed to the reduction in neointimal remodeling observed in the mice transplanted with MCP-1−/− PVAT. Given the small amount of available tissue, however, we were not able to specifically isolate macrophages from the PVAT tissue, nor were we able to assess expression of other genes related to macrophage polarization. Nevertheless, taken together with the immunostaining data for α-smooth muscle actin, these findings suggest that the effects of MCP-1 expression by PVAT on the vascular response to wire injury are largely independent of monocyte/macrophage recruitment.

Besides functioning as a chemokine to recruit inflammatory cells, MCP-1 has been demonstrated to elicit diverse effects on the vascular wall, including stimulation of VSMC proliferation and migration.37,38 For example, incubation of human VSMC with MCP-1 stimulated an increase in proliferating nuclear cell antigen and cyclin A expression and a 2-fold increase in cell numbers.38 Mechanistically, MCP-1–induced VSMC proliferation was found to be independent of nuclear factor κB activation and mediated via phosphorylidyinositol 3-kinase activation.37 Whether MCP-1 expressed in PVAT acts on VSMC to enhance the neointimal response to injury remains to be determined. Also, it is conceivable that the MCP-1 might act on progenitor cells in the PVAT depot or the vascular adventitia, triggering their migration to the neointima and differentiation into a VSMC phenotype.39 Interestingly, progenitor cells isolated from the aortic adventitia and transferred to the adventitial side of vein grafts implanted into hyperlipidemic mice were demonstrated to migrate to the intima, providing support for such a mechanism.39

We also observed that PVAT transplantation markedly stimulated adventitial angiogenesis, a finding that may shed light into the mechanisms of adventitial vasa vasorum proliferation after short-term high-fat feeding.32 In vitro studies demonstrated that perivascular adipocytes, as compared with their subcutaneous and perirenal counterparts, release higher quantities of soluble factors, such as VEGF, that induce proangiogenic responses in endothelial cells. Interestingly, the adventitial angiogenesis was significantly attenuated by transplanting PVAT from MCP-1−/− mice. A previous report suggested that MCP-1 can induce angiogenesis via upregulation of VEGF gene expression.40 Further studies will be required to determine whether this mechanism underlies the potent angiogenic effects of PVAT.

In summary, we report here that transplantation of 2 to 3 mg of PVAT to the carotid artery is sufficient to enhance vascular responses to wire injury in HFD-fed, hyperlipidemic mice, leading to accumulation of a VSMC-rich neointima and prominent adventitial inflammation and angiogenesis. The pathogenic effects of PVAT transplantation in this model are mediated in part by expression of MCP-1.

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Disclosures
None.

References


Significance

Perivascular adipose tissue (PVAT) surrounds most conduit arteries, and the amount and inflammatory state of PVAT correlates with the presence of vascular disease in humans. However, the mechanisms whereby PVAT interacts with the blood vessel wall to regulate vascular disease are poorly understood. We devised a novel model of adipose tissue transplantation to the common carotid artery, which is normally devoid of PVAT. We found that transplanted PVAT augmented injury-induced neointimal hyperplasia in the setting of high-fat diet, whereas subcutaneous adipose tissue had no effect. The effects of PVAT transplantation were in part dependent on monocyte chemotactrant protein-1 released locally by the adipose tissue, contributing to both accumulation of neointimal smooth muscle cells and adventitial angiogenesis. These findings provide new insight into the mechanisms whereby PVAT and monocyte chemotactrant protein-1 regulate vascular disease.
Transplanted Perivascular Adipose Tissue Accelerates Injury-Induced Neointimal Hyperplasia: Role of Monocyte Chemoattractant Protein-1


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Supplemental Material

**Transplanted perivascular adipose tissue accelerates injury-induced neointimal hyperplasia: role of MCP-1**


**Materials and Methods**

**Transplantation and wire injury.** To study the local effect of transplanted PVAT in vivo, 2-3 mg of PVAT was collected from the inferior margin of the lesser curvature of the thoracic aorta of donor C57Bl/6J (Stock #664 from the Jackson Laboratory) animals fed a HFD (Harlan-Teklad TD.88137, 42% calories from fat) for 4 weeks. The PVAT was carefully inspected using a dissection microscope, which enabled us to collect white adipose tissue accumulating at the margin of the more extensive underlying brown adipose tissue depot adjacent to the aortic wall (Supplemental Figure 1). The collected PVAT, or subcutaneous adipose tissue (SQAT) as a control, was transplanted to HFD-fed LDL receptor knockout mice, also in a C57Bl/6J background (Stock #2207, the Jackson Laboratory). Two weeks after transplantation, we executed wire injury of carotid arteries as described. Using a midline neck incision, the left external carotid artery (LECA) was looped with 6-0 silk suture for temporary vascular control. A transverse arteriotomy was made in the LECA and a resin probe introduced and advanced approximately 5 mm to the aortic arch. Two weeks after injury, the mice were sacrificed, and blood was withdrawn from the ventricles and assayed for cholesterol and triglyceride levels using commercially available kits (Infinity kits, Thermo Scientific), as described previously. Both carotid arteries were processed for paraffin embedding, as previously described. Serial 5-μm sections were cut from the paraffin-embedded blocks, prepared for histological analyses, and examined as described previously. In some experiments, tissue was processed for extraction of mRNA and subjected to real-time PCR as described below.

**Immunocytochemistry.** Sections were stained for macrophages/foam cells with an anti-mouse macrophage monoclonal antibody (mAb) to F4/80 or Mac3 (Accurate Chemical and Scientific Corp), or for smooth muscle actin (SMA)-positive VSMC cells with mAb 1A4 (Dako Corp). Rabbit anti-factor VIII-related antigen (18-0018, Zymed. Laboratories, Invitrogen, Carlsbad,
CA) was used to stain for endothelial cells to identify adventitial neovessels\textsuperscript{1-4}. Adventitial neovessel density and macrophage density were calculated by counting the number of factor VIII-related antigen positive microvessels or F4/80 positive macrophages, respectively, within 150 \( \mu m \) of the luminal surface and normalizing to the corresponding cross-sectional area. Neointimal area and SMA immunostaining in the neointima were quantified using ImageJ software (version 1.42; National Institutes of Health)\textsuperscript{7}. The intima was traced with a freehand tool in a macro field and then processed in a binary fashion with the stain hue as the threshold color\textsuperscript{7}. Individual areas were added to determine the total area of positive immunostaining in the intima per image (40x).

**Adipocyte cell culture.** Human SQ, PV and perirenal preadipocytes were collected from enzymatically digested adipose tissues from candidates for organ donation as described\textsuperscript{8}. Human preadipocytes were cultured and differentiated into adipocytes as described previously\textsuperscript{8}. Conditioned medium from these adipocyte cultures was assayed for VEGF concentration by ELISA, using previously reported methodology\textsuperscript{9}. Murine SQ and PV tissues from C57Bl/6J mice fed a Western diet or chow diet for 2 weeks were finely minced and digested for 90 minutes at 37 °C in 0.3% by mass solution of collagenase type I (Worthington Biochemical Corporation, Cat. #4196). Preadipocytes were maintained in DMEM/F12/10% FBS until differentiated when confluent. Adipocyte differentiation was induced by replacing the maintenance medium with a commercially available differentiation medium (Cell Applications), which was replaced every 4 days. Differentiated adipocytes were examined by DIC microscopy to visualize cytoplasmic lipid droplet accumulation. All experiments were performed on cells at passage 2 or less.

**Assay for pro-inflammatory cytokines by real-time PCR.** Total RNA was extracted from tissues and cells and relative gene expression calculated as described\textsuperscript{8}. Primer sequences are listed in the supplementary Table.

**Endothelial cell culture.** Human coronary endothelial cells (HCAEC) were cultured as previously described\textsuperscript{9}. Cells were grown to approximately 50% confluence prior to exposure to conditioned medium from adipocytes. Twenty four hours after exposure to equivalent aliquots of conditioned medium from human perivascular, subcutaneous or perirenal adipocytes, the HCAEC cultures were examined under phase contrast microscope by three blinded observers, and the number of branching, tube-like structures per 10 high power fields was counted and averaged to derive an index of angiogenesis.
**Monocyte/macrophage transendothelial migration assay.** bEnd.3 cells (a murine endothelial cell line, ATCC, Manassas, VA) were seeded at 20,000 cells/well in 3μm pore-size transwell inserts (BD Falcon, cat. # 353492) and grown to a confluent monolayer. Aliquots of conditioned media from isolated murine subcutaneous and perivascular adipocytes were placed in 24-well plates, and transwell inserts were then placed in the wells. 3x10^5 J774.1 cells (murine monocyte/macrophages, ATCC) in serum-free media were added onto the luminal side of the inserts and left to adhere for 1hr, after which non-adherent cells were aspirated; fresh serum free media was added, and the adherent J774.1 cells were allowed to migrate overnight. The following day, J774.1 cells remaining on the luminal side of the inserts were removed using a cotton swab, the inserts were excised, and the migrated J774.1 cells present on the abluminal side of the inserts were fixed with methanol, stained with DAPI, and counted in three random view fields (40x) excluding the edges.

**Statistical Analyses.** Data are reported as the number of animals in each group, and expressed as mean±SD. Treatment groups were compared by 1-way analysis of variance (ANOVA) followed by a 2-tailed Student’s t test to evaluate levels of significance at 95% confidence. Differences were deemed significant at p<0.05.

**References**


Supplemental Materials

Transplanted perivascular adipose tissue accelerates injury-induced neointimal hyperplasia: role of MCP-1


Supplemental

Table.

Primers sequences used in this study.

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Supplemental Figure I. Representative anatomy and histology of thoracic aortic PVAT. C57Bl/6J mice were fed a chow diet (upper panels) or high fat diet (lower panels) for 4 weeks. Mice were euthanized, and thoracotomy was performed to expose PVAT at the lesser curvature of the thoracic aorta (black arrows) using a dissecting microscope. A strip of white adipose tissue was visually identified at the inferior margin of the lesser curvature (white arrows, right panels) and marked with a black tissue marking pen. Aortic arch with PVAT was harvested en bloc and processed for H&E staining (middle panels). White arrows in middle panels correspond to the regions marked in the right panels, identifying the site where PVAT was harvested for transplantation. Left panel shows 20x magnification of the adipose tissue histology contained within the insert (note the black marking).
Supplemental Figure II. Comparison of expression of the adipocyte genes adiponectin and leptin in transplanted SQ or PV adipose tissue versus the corresponding endogenous adipose tissues from the same animals. Expression levels were normalized to the SQ data and expressed as % SQ values. Because of the small volume of transplanted adipose tissues, tissues were pooled from 3-4 animals and run in duplicate to obtain these results.
Supplemental Figure III. Scatter plot of individual neointimal area data points from experiments shown in Figure 2. Please see legend for Figure 2 for additional experimental details.
**Supplemental Figure IV.** Quantification of α-smooth muscle actin expression in the neointima. Images (40x) from each animal were analyzed using ImageJ software, and the total area (μm²) of positively stained intima was calculated and expressed relative to sham PVAT transplantation + wire injury (column A). Column B represents wt PVAT transplantation + wire injury, and column C represents MCP-1/- PVAT transplantation + wire injury.
Supplemental Figure V. Representative staining for Mac3 (brown staining) in carotid artery sections (all photographed at 10x). Panel A: sham PVAT transplantation + wire injury, Panel B: wt PVAT transplantation + sham injury, Panel C: wt PVAT transplantation + wire injury, Panel D: MCP-1/- PVAT transplantation + wire injury. Black arrows denote location of external elastic lamina, and red arrows in B-D show clustering of macrophages near transplanted PVAT.
Supplemental Figure VI. Representative immunostaining for T cells in carotid artery sections. Left panels show trichrome staining, middle panels CD3 staining, and right panels nuclear staining (DAPI). A: wt PVAT transplantation + wire injury. B: MCP-1/- PVAT transplantation + wire injury. Images (all photographed at 20x) are representative of histology from 3 separate mice in each group.
Supplemental Figure VII. Expression (mRNA) of iNOS and Ym1 in transplanted PVAT. Mice underwent transplantation of PVAT or SQAT and two weeks later were subjected to wire injury. After 2 more weeks, tissues were harvested and RNA isolated to quantify mRNA expression of iNOS and Ym1 by real-time PCR. Data were normalized to wt values and expressed as fold change. *, p<0.05 compared to corresponding WT value, n=3-4.
Supplemental Figure VIII. Comparison of potency of conditioned medium from wt PVAT and MCP-1-/- PVAT to elicit monocyte chemotaxis. After feeding the mice a Western diet for 4 weeks, PVAT (10 mg) was harvested and incubated in vitro in culture medium for 4 hours. Media were collected and aliquots placed in 24-well plates fitted with inserts to elicit monocyte/macrophage transendothelial migration as described in Materials and methods. N=3, p=n.s.