Periadventitial Adipose Tissue Plays a Critical Role in Vascular Remodeling

Minoru Takaoka, Daisuke Nagata, Shinji Kihara, Ichiro Shimomura, Yu Kimura, Yasuhiko Tabata, Yoshihiko Saito, Ryozo Nagai, Masataka Sata

Rationale: Obesity is associated with a high incidence of cardiovascular complications. However, the molecular link between obesity and vascular disease is not fully understood. Most previous studies have focused on the association between cardiovascular disease and accumulation of visceral fat. Periadventitial fat is distributed ubiquitously around arteries throughout the body.

Objective: Here, we investigated the impact of obesity on inflammation in the periadventitial adipose tissue and on lesion formation after vascular injury.

Methods and Results: High-fat, high-sucrose feeding induced inflammatory changes and decreased adiponectin expression in the periadventitial adipose tissue, which was associated with enhanced neointima formation after endovascular injury. Removal of periadventitial fat markedly enhanced neointima formation after injury, which was attenuated by transplantation of subcutaneous adipose tissue from mice fed on regular chow. Adiponectin-deficient mice showed markedly enhanced lesion formation, which was reversed by local delivery, but not systemic administration, of recombinant adiponectin to the periadventitial area. The conditioned medium from subcutaneous fat attenuated increased cell number of smooth muscle cells in response to platelet derived growth factor-BB.

Conclusions: Our findings suggest that periadventitial fat may protect against neointimal formation after angioplasty under physiological conditions and that inflammatory changes in the periadventitial fat may have a direct role in the pathogenesis of vascular disease accelerated by obesity. (Circ Res. 2009;105:906-911.)

Key Words: neointima ■ adipocyte ■ periadventitial tissue ■ smooth muscle cell ■ adipocytokine

Obesity is a fast growing public health problem in industrialized countries and is associated with cardiovascular complications. Abdominal obesity is independently associated with cardiovascular disease, such as stroke, heart failure, and myocardial infarction. Furthermore, recent reports suggest that obesity is an important risk factor for restenosis after percutaneous coronary intervention.

It was reported that serum levels of circulating proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin-6, plasminogen activator inhibitor-1, and monocyte chemoattractant protein-1, are increased in overweight people with enhanced accumulation of visceral fat, whereas levels of antiinflammatory adipocytokines such as adiponectin (APN) are decreased. In contrast to visceral fat, little attention has been paid to the role of periadventitial fat in vascular remodeling under physiological and pathological conditions. Periadventitial fat has been considered to mainly act a structural support for blood vessels.

Here, we investigated the effect of obesity on periadventitial adipose tissue and on lesion formation after vascular injury. Our findings support the idea that obesity induces changes in periadventitial tissue, which potentially affect lesion formation after vascular injury.

Methods

Animals

C57BL/6 mice were purchased from Japan SLC Inc (Shizuoka, Japan). APN-deficient mice (C57BL/6 background) have been described previously. Green fluorescent protein (GFP) mice, which are transgenic mice (C57BL/6 background) that ubiquitously express enhanced GFP, were donated by Dr Masaru Okabe (Osaka University, Osaka, Japan). All mice were backcrossed 10 times into a C57BL/6 background. To examine the effects of perivascular adipose tissue on injured arteries, neointimal hyperplasia in injured arteries was examined at 4 weeks after the vascular injury.

Mice received standard chow diet (STD) or a high-fat high-sucrose diet (HF/HS) (45 kcal% fat, 20 wt% sucrose, D12451, Research Diets).
All experimental procedures were approved by the University of Tokyo Ethics Committee for Animal Experiments and strictly adhered to the guidelines for animal experiments of the University of Tokyo.

**Mouse Vascular Wire Injury**

Vascular injury was induced as described previously. To deliver APN into the perivascular area, recombinant mouse APN (5 μg per mouse; Wako Pure Chemical Industries Ltd) or PBS was administered using a gelatin hydrogel for 4 weeks after vascular injury. Gelatin hydrogel was implanted on both the left and right femoral arteries at the same time as vascular injury. One artery received APN (APN gel group, n=6) and the other received PBS (PBS gel group, n=6). Other mice received recombinant mouse APN (5 μg per mouse) at the subcutaneous space (APN SC group, n=6) after wire-induced injury.

**Transplantation of Adipose Tissue to Perivascular Area**

Wire injury was performed with or without periadventitial adipose tissue. Then, 10 mg of visceral and subcutaneous fat tissues was harvested from the epididymis and flank, respectively, of lean or obese WT mice. Then, 400 mg of each sample was incubated at 37°C in 1 mL of DMEM medium containing 50 U/mL penicillin and 50 μg/mL streptomycin with 0.5% FBS for 4 hours.

**Quantification of Neointimal Hyperplasia**

For morphometric studies, the femoral arteries were harvested 4 weeks after injury. Digitalized images were analyzed using Image analysis software (Image J, NIH). The lumen, internal elastic lamina, and external elastic lamina were defined. The intimal (tissue between lumen and internal elastic lamina) and medial (tissue between internal elastic lamina and external elastic lamina) areas were recorded. Neointima/media area ratio was calculated.

**Immunohistochemical Staining and Confocal Microscopic Observation**

Paraffin-embedded sections (4 μm thick) were deparaffinized and blocked using 2% rabbit or horse serum. The sections were then incubated with anti-Mac3 antibody (BD Pharmingen), followed by incubation with avidin–biotin complex and Vector Red substrate (Vector Laboratories). Sections were counterstained with hematoxylin. To observe the GFP signal for histological analysis, the sections were then incubated with rabbit anti-GFP antibody (Molecular Probes) followed by Alexa Fluor 488–conjugated donkey anti-rabbit immunoglobulin antibody for immunohistochemical staining. The sections were observed under a confocal microscope (FLUOVIEW FV300, Olympus).

**Measurement of Proinflammatory Cytokines**

Total RNA was isolated from pooled perivascular adipose tissue (n=3 to 4 for each group) using a Qiagen RNeasy Mini kit, after which cDNA was generated using a reverse transcription kit (Qiagen). Real-time PCR was then carried out in an MxPro Mx3000P (Stratagene) using SYBR GREEN PCR Master Mix (TOYOBO). The following primers were used: 5'-GATG-GCACAGATGGCACCCTC-3' and 5'-CTTGATGTCCTGTGACTTTGTTAT-3' for mouse APN; 5'-CCTCTGACCACTGCTACATCT-3' and 5'-TGCGAACCACTGCTACATCT-3' for mouse TNF-α; 5'-ACAAACACACAGCCCTTCCATG-3' and 5'-CAGTTTCCCAAGAGATAG-3' for mouse PDGF. The number of cell in proliferation was determined by MTS assay using CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega).

**Adipose Tissue Conditioned Medium**

Visceral and subcutaneous fat tissues were harvested from the epididymis and flank, respectively, of lean or obese WT mice. Then, 400 mg of each sample was incubated at 37°C in 1 mL of DMEM medium containing 50 U/mL penicillin and 50 μg/mL streptomycin with 0.5% FBS for 4 hours.

**Measurements of APN Protein Levels**

APN concentration in the conditioned medium was measured using a mouse APN ELISA kit (Otsuka Pharmaceutical Co Ltd) according to the instructions of the manufacturer. The minimum detectable concentration of APN was 15.6 pg/mL.

**Cell Culture**

Rat vascular smooth muscle cells (SMCs) were seeded in 96-well plates at an initial density of 2×10^3 cells per well and allowed to attach for 24 hours. The cells were serum starved for 24 hours and then incubated with or without recombinant platelet-derived growth factor (PDGF)-BB (R&D Systems Inc) in the presence or absence of 100 μL/mL conditioned medium for 48 hours. Adenovirus vectors containing the gene for dominant negative AMP-activated protein kinase (dn-AMPK), constitutively active AMPK (ca-AMPK), and GFP were described previously. SMCs were transduced with the indicated adenoviral vectors at a multiplicity of infection of 50 plaque-forming units for 24 hours before treatments. The number of cell in proliferation was determined by MTS assay using CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega).

**Statistical Analysis**

All results are expressed as means±SEM. Differences between groups were evaluated for statistical significance using Student t test. Values of P<0.05 were considered significant.

**Results**

To investigate the effect of obesity on lesion formation after vascular injury, 6-week-old C57BL6 mice were fed STD or HF/HS diet for 16 weeks. The HF/HS mice increased body weight by 54% (STD, 25.4±0.1 g; HF/HS, 39.0±2.2 g). Histological studies demonstrated abundant macrophage infiltration in adventitial adipose tissue as determined by anti-Mac3 immunostaining (Figure 1A). Consistent with these changes, quantitative RT-PCR analysis revealed that APN expression was downregulated with upregulation of proinflammatory cytokines within the perivascular adipostat of HF/HS mice (Figure 1B). We inserted a wire into the femoral artery of STD and HF/HS mice. At 4 weeks after the

**Non-standard Abbreviations and Acronyms**

<table>
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<tr>
<th>Abbreviation</th>
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<tr>
<td>APN</td>
<td>adiponectin</td>
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<tr>
<td>ca-AMPK</td>
<td>constitutively active AMPK</td>
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<tr>
<td>dn-AMPK</td>
<td>dominant negative AMP-activated protein kinase</td>
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<td>GFP</td>
<td>green fluorescent protein</td>
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<td>HF/HS</td>
<td>high-fat, high-sucrose</td>
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<td>PDGF</td>
<td>platelet-derived growth factor</td>
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<td>SMC</td>
<td>smooth muscle cell</td>
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<td>STD</td>
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<td>TGFβ</td>
<td>transforming growth factor B</td>
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<td>TNF-α</td>
<td>tumor necrosis factor</td>
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injury, intimal hyperplasia was enhanced in HF/HS mice compared to that in STD mice (P<0.01) (Figure 1C).

To investigate the impact of the phenotypic changes in periadventitial adipose tissue on vascular remodeling after injury, we replaced the periadventitial adipose tissue with exogenous fat after insertion of the wire. First, the periadventitial fat tissue around the femoral artery was carefully removed and the artery was injured. Then, subcutaneous fat from a GFP mouse was transplanted around the injured artery. Histological analysis showed the presence of GFP-positive adipocytes in close proximity to the injured artery at 1 week and 4 weeks after transplantation (Figure 2A), indicating that the transplanted fat attached and survived around the artery. There was no statistical difference between subcutaneous fat and visceral fat in the efficiency of grafting, as determined by immunofluorescence staining of the cross-section at 4 weeks after the grafting. Fat transplantation induced neovascularization around the artery (Online Figure I, available in the Online Data Supplement at http://circres.ahajournals.org). Morphometric analysis revealed that neointimal hyperplasia was markedly enhanced by removal of periadventitial fat in STD mice (Figure 2B). Transplantation of subcutaneous fat from STD mice markedly attenuated the neointima (Figure 2C), suggesting that perivascular adipose tissue may have a protective role in neointimal hyperplasia in STD mice. Atheroprotective effect of exogenous fat tissue was not observed when subcutaneous fat from HF/HS mice or visceral fat was transplanted (Figure 2C, Online Figure II).

To analyze which molecule in periadventitial fat contributes to the attenuation of lesion formation in STD mice, we focused on APN. Consistent with a previous report,22 APN-deficient mice showed severe neointimal thickening and increased proliferation of vascular SMCs in response to vascular injury, compared with C57BL6 WT mice (P<0.05) (Figure 3A and 3B). To investigate the local effects of periadventitial fat APN on vascular remodeling, recombinant APN was delivered locally to the adventitial space in APN-deficient mice, using gelatin hydrogel.19 Gelatin hydrogel filled with APN or PBS was transplanted into the periadventitial area. Four weeks after endovascular injury, perivascular delivery of APN to the femoral artery reduced endovascular injury-induced neointimal hyperplasia compared with that in the PBS-delivered artery in APN-deficient mice (P<0.001) (Figure 3C). These findings indicate that APN secreted from periadventitial fat has a protective role in lesion formation in response to endovascular injury.

Next, we investigated the effects of humoral factors secreted from fat tissue on SMCs proliferation in vitro. The conditioned medium from the subcutaneous fat of STD mice, but not of HF/HS mice, attenuated increased SMC numbers induced by PDGF-BB (P=0.05) (Figure 4A). The conditioned medium of HF/HS fat increased SMC numbers (P=0.001). RT-PCR analysis revealed that proinflammatory cytokine expression was upregulated in the HF/HS fat than STD fat (Online Figure III). Pretreatment with a neutralizing anti–TNF-α antibody suppressed the increased SMC numbers (P=0.05). Furthermore, the conditioned medium from the subcutaneous fat of APN-deficient mice increased SMC numbers compared with conditioned medium from STD subcutaneous fat (P=0.05) (Figure 4A). We measured APN level in the conditioned medium from the subcutaneous fat of STD and HF/HS mice using an ELISA kit. APN protein levels were significantly higher in the conditioned medium from STD mice than in that from HF/HS mice (P=0.01) (Figure 4B). These results showed that inflammatory cytokines such as TNF-α secreted from fat increased SMCs proliferation and inflammatory cell infiltration.
numbers and that APN secreted from fat suppressed SMC numbers in response to PDGF-BB stimulation. These findings indicate that humoral factors secreted from adipose tissues play a crucial role in regulation of SMCs proliferation.

To investigate the mechanism by which APN modified SMCs number cultured with PDGF-BB, we stimulated cultured SMCs with PDGF-BB in the presence or absence of APN. APN suppressed the increased SMC number induced by PDGF-BB ($P<0.01$) but had no effect on SMCs number in the absence of PDGF-BB (Figure 5). To test whether AMPK signaling was involved in the action of APN, we pretreated SMCs with adenoviral vector expressing dn-AMPK or ca-AMPK. Transduction with dn-AMPK abrogated the suppressive effects of APN on the increased SMCs number induced by PDGF-BB. On the other hand, ca-AMPK suppressed increased SMCs number induced by PDGF-BB ($P<0.05$) (Figure 5). These results indicated that AMPK mediates suppressive effect of APN on the PDGF-BB-induced increased SMCs number.

### Discussion

In this study, we investigated the effect of obesity on inflammation in periadventitial adipose tissue and on lesion formation after vascular injury. Obesity-induced inflammation in periadventitial adipose tissue, with upregulation of inflammatory adipocytokines and downregulation of the anti-inflammatory adipocytokine APN. These changes were associated with enhanced neointima formation after endovascular injury. Obesity-induced enhancement of lesion formation was attenuated by replacement of periadventitial adipose tissue with exogenous subcutaneous fat. Our findings suggest that obesity causes inflammatory changes and downregulation of APN in the periadventitial adipose tissue, which potentially influence lesion formation after vascular injury.

Endothelial injury induces adhesion and migration of leukocytes, macrophages, and bone marrow–derived progenitor cells into the artery wall. It is well known that proinflammatory cytokines have a fundamental role in medi-
ating the initiation and progression of vascular lesion formation.\textsuperscript{23–25} In contrast to these well-characterized processes within the vessel wall, changes in the adventitia during vascular remodeling have been largely neglected.

The adventitial and periadventitial tissues are composed of various cell types including adipocytes, vascular cells, macrophages, T cells, mast cells, and fibroblasts.\textsuperscript{26–30} It was demonstrated that perivascular adventitial adipose tissue releases a transferable adventitia-derived relaxing factor that acts by tyrosine kinase–dependent activation of K\textsuperscript{+} channels in vascular SMCs.\textsuperscript{31} Recent reports showed that periadventitial fat secretes various kinds of chemokines and might contribute to the progression of obesity-associated atherosclerosis.\textsuperscript{32} Epicardial adipose tissue has been reported to be a source of inflammatory mediators\textsuperscript{33} and is an indicator of cardiovascular risk.\textsuperscript{34} Quite recently, Chatterjee et al reported that perivascular adipocytes exhibit reduced differentiation and proinflammatory state, suggesting that dysfunction of perivascular adipose tissues induced by fat feeding may link metabolic signals to inflammation in the blood vessel wall.\textsuperscript{35} Patients with obesity\textsuperscript{14} and coronary artery disease\textsuperscript{36} have low plasma APN levels. It was suggested that perivascular adipocytes loose activities to secrete APN in obesity, leading to hypoxia, inflammation, and oxidative stress.\textsuperscript{37} These results suggested that perivascular fat may play a role in increased risk of cardiovascular disease in obese individuals. However, the physiological and/or pathological role of perivascular adipose tissue in the maintenance of vascular homeostasis and in pathological vascular remodeling remains to be elucidated.\textsuperscript{31}

In this study, we provide direct evidence that perivascular fat may protect against neointimal formation after angioplasty under physiological conditions and that inflammatory changes in the periadventitial fat may have a direct role in the pathogenesis of vascular disease accelerated by obesity. It was suggested that APN secreted from perivascular fat may play a protective role in neointima formation of the adjacent artery in situ after vascular injury in lean mice.

We made efforts to transplant periadventitial fat from one blood vessel to the other. However, it was impossible to obtain enough amount of periadventitial fat for the transplantation to coat the artery of another mouse. Alternatively, in this study, we implanted subcutaneous or visceral fat from other mice around the wire-injured femoral artery after removal of endogenous perivascular fat. Our experiments using subcutaneous or visceral fat transplantation from the mice fed on STD or HF/HS diet successfully demonstrated the importance of adipose tissues in perivascular area in regulating neointima formation in the injured vessel via paracrine effects of adipocytokines. Consist with notion, local delivery, but not systemic administration, of APN to perivascular area effectively inhibited neointima formation.

In summary, we provide direct evidence that obesity increases periadventitial adipose tissue inflammation and may contribute to pathological vascular remodeling in response to injury. Our findings support the notion that changes in periadventitial adipose tissue may have a role in obesity-associated cardiovascular complications. The results described here suggest the previously unappreciated role of periadventitial fat in the regulation of vascular remodeling.

**Sources of Funding**

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Disclosures

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Material and Methods

*Immunohistochemical staining*

Paraffin-embedded sections (4-µm thick) were deparaffinized and blocked using 2% rabbit or horse serum. The sections were then incubated with anti-CD31 antibody (BD Pharmingen), followed by incubation with avidin-biotin complex and Vector Red substrate (Vector Laboratories). Sections were counterstained with hematoxylin.
Online supplement Figure I

Fat transplantation induced neovascularisation around the artery

Fat transplantation induced accumulation of vascular endothelial cells around the artery. Immunohistochemical analysis showed accumulation of CD31-positive vascular endothelial cells (arrows) within periadventitia in fat transplantation mice at one week. Scale bar, 50 μm. Results are expressed as mean ± S.E.M. ***P<0.01. n=6.
Online supplement Figure II

Transplantation of subcutaneous or visceral fat from standard chow diet (STD) or high fat high sucrose diet (HF/HS) mice after removal of perivascular adipose tissue

Morphometric analysis of injured femoral arteries at 4 weeks. Subcutaneous or visceral fat from STD (n=6 each) or HF/HS mice (n=6 each) was transplanted after removal of endogenous periadventitial adipose tissue. Results are expressed as mean ± S.E.M. *P<0.05, **P<0.01, NS, not statistically significant.
Online supplement Figure III

Obese and visceral fat induced inflammatory changes in adipose tissue

Expression of mRNA in subcutaneous and visceral fat from STD (n=3 each) and HF/HS (n=3 each) wild-type C57BL6 mice. Expression level was assessed by real-time PCR normalized to GAPDH level. Results are expressed as mean ± S.E.M. *P<0.05. STD, standard chow diet. HF/HS, high fat high sucrose diet.