Endovascular Injury Induces Rapid Phenotypic Changes in Perivascular Adipose Tissue

Minoru Takaoka, Hiroshi Suzuki, Seiji Shioda, Kenji Sekikawa, Yoshihiko Saito, Ryozo Nagai, Masataka Sata

Objective—Accumulating evidence suggests that adipose tissue not only stores energy but also secretes various bioactive substances called adipocytokines. Periadventitial fat is distributed ubiquitously around arteries throughout the body. It was reported that inflammatory changes in the periadventitial fat may have a direct role in the pathogenesis of vascular diseases accelerated by obesity. We investigated the effect of endovascular injury on the phenotype of perivascular fat.

Methods and Results—Endovascular injury significantly upregulated proinflammatory adipocytokines and downregulated adiponectin within periadventitial fat tissue in models of mouse femoral artery wire injury and rat iliac artery balloon injury. Genetic disruption of tumor necrosis factor (TNF)-α attenuated upregulation of proinflammatory adipocytokine expression, with reduced neointimal hyperplasia after vascular injury. Local delivery of TNF-α to the periadventitial area enhanced inflammatory adipocytokine expression, which was associated with augmented neointimal hyperplasia in TNF-α-deficient mice. Conditioned medium from a coculture of 3T3-L1 and RAW264 cells stimulated vascular smooth muscle cell proliferation. An anti-TNF-α neutralizing antibody in the coculture abrogated the stimulating effect of the conditioned medium.

Conclusion—Our findings indicate that endovascular injury induces rapid and marked changes in perivascular adipose tissue, mainly mediated by TNF-α. It is suggested that the phenotypic changes in perivascular adipose tissue may have a role in the pathogenesis of neointimal hyperplasia after angioplasty. (Arterioscler Thromb Vasc Biol. 2010;30:1576-1582.)

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

It is well known from both experimental and clinical observations that proinflammatory cytokines have a fundamental role in the initiation and progression of atherosclerosis.1–4 In the setting of angioplasty, it is generally accepted view that the first changes that precede neointimal formation take place in the endothelium and the luminal side of the vessel wall.5 Endothelial denudation induces adhesion and migration of leukocytes, macrophages, and bone marrow-derived cells into the artery wall, which contribute to smooth muscle cell (SMC) proliferation.6,7 On the other hand, little attention has been paid to the changes in the adventitia in response to injuries. It was reported that the adventitia is highly populated by various cell types, including leukocytes, macrophages, T cells, and mast cells, together with increased differentiation of resident fibroblasts into myofibroblasts after vascular injury.7–11

Accumulating evidence suggests that adipose tissue not only stores energy but also secretes various bioactive substances called adipocytokines. Obesity induces chronic inflammation in adipose tissue, which is associated with upregulation of inflammatory cytokines and downregulation of adiponectin.12–14 Previous studies suggested that excess accumulation of visceral fat is responsible for these changes. Perivascular fat is widely distributed throughout the arterial tree. Recently, we reported 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.15

This study was designed to investigate the effect of endovascular injury on perivascular adipose tissue. Our results demonstrate that endovascular injury induces rapid and marked changes in periadventitial adipose tissue, which potentially influence neointimal hyperplasia. Our findings also provide insights into the mechanism by which vascular injury leads to phenotypic changes in perivascular adipose tissue.

Received on: October 27, 2009; final version accepted on: May 6, 2010.

From the Department of Cardiovascular Medicine, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan (M.T., R.N.); Division of Cardiology, Department of Internal Medicine, Showa University Fujigaoka Hospital, Yokohama, Kanagawa, Japan (H.S.); First Department of Anatomy, Showa University School of Medicine, Tokyo, Japan (S.S.); PrevenTec, Tsukuba, Japan (K.S.); First Department of Internal Medicine, Nara Medical University, Nara, Japan (Y.S.); Department of Cardiovascular Medicine, Institute of Health Biosciences, The University of Tokushima Graduate School, Tokushima, Japan (M.S.).

This manuscript was sent to Kenneth Walsh, Consulting Editor, for review by expert referees, editorial decision, and final disposition.

Correspondence to Masataka Sata, MD, PhD, Professor and Chairman, Department of Cardiovascular Medicine, Institute of Health Biosciences, The University of Tokushima Graduate School, 3-18-15 Karamoto-cho, Tokushima 770-8503, Japan. E-mail sata@clin.med.tokushima-u.ac.jp © 2010 American Heart Association, Inc.

Arterioscler Thromb Vasc Biol is available at http://atvb.ahajournals.org DOI: 10.1161/ATVBAHA.110.207175
Materials and Methods

Animals

C57BL/6 mice were purchased from Japan SLC, Inc. Tumor necrosis factor (TNF)-α-deficient mice (C57BL/6 background) were previously described. Monocyte chemotactic protein (MCP)-1-deficient and interleukin (IL)-6-deficient mice (C57BL/6 background) were purchased from Clea Japan, Inc. 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 Model of Vascular Injury

Vascular injury was induced in mice as described previously. A straight spring wire (0.38 mm in diameter, No.C-SF-15-15, COOK) was inserted into the femoral artery and left in place for 1 minute to denude and dilate the artery. Blood flow was restored. Perivascular adipose tissue was collected at day 1, 7, and 28 after the injury. The injured artery was harvested at day 1 and 28. Osmotic minipumps (Alzet) filled with either recombinant TNF-α (R&D Systems) or PBS were implanted beside the injured arteries at the time of vascular injury in TNF-α-deficient mice. Either TNF-α (0.5 or 5 ng/day) or PBS was delivered locally for 4 weeks.

Rat Model of Vascular Injury

In the rat model, a 1.5-mm percutaneous transluminal coronary angioplasty catheter (Apollo 1.5×20 mm; Avantec Vascular Corp.) was introduced through the carotid artery to the left iliac artery using a guide wire and a C-arm digital fluoroscopy system (Sirus Power/C; Hitachi Medico). The balloon was dilated at 8 atmospheres for 1 minute. At 24 hours after the vascular injury, the perivascular adipose tissue was collected for gene expression analysis.

Histological Analysis

For morphometric analysis, the femoral arteries were harvested at 4 weeks after the injury. Digitalized images of these vessels were obtained and analyzed using image analysis software (Image J, National Institutes of Health). Paraffin-embedded sections (4 μm thick) were deparaffinized and blocked with 0.5% horse serum. The sections were then incubated with primary antibody against F4/80 (Serotec), CD3 (Pharmingen), MCP-1 (R&D Systems), or IL-6 (Santa Cruz Biotechnology, Inc.), followed by incubation with anti-rat immunoglobulin secondary antibody, avidin-biotin complex, and Vector Red substrate (Vector Laboratories). Sections were counterstained with hematoxylin. For immunofluorescent staining, the sections were incubated with rabbit anti-TNF-α antibody (Rockland) and rat anti-F4/80 antibody (Serotec) followed by incubation with secondary antibodies [Alexa Fluor 488-conjugated anti-rabbit immunoglobulin (Molecular Probes) and Alexa Fluor 647-conjugated anti-rat immunoglobulin (Molecular Probes)]. The sections were observed under a confocal microscope (FLUOVIEW FV300; Olympus). Electron microscopic study was performed (Hitachi H-7600) as described previously.

Evaluation of Adipocyte Expression

RNA was isolated from pooled perivascular adipose tissue (n = 3 to 6 for each group) using a QIAGEN RNasy Minikit (QIAGEN, Inc.), after which cDNA was synthesized using a Quantitect Reverse Transcription kit (QIAGEN, Inc.). 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′-GATGGCGAGATGGGCACCCTC-3′ and 5′-CTTGGCAGTGTGGCCTGTCAT-3′ for mouse IL-6; 5′-TTCTACACTTCCCAAGAAGATGGT-3′ for mouse IL-6; 5′-GATGGGCTGTGTGTTACGCCC-3′ and 5′-TCTTGGCAGATGGCCCTGTCAT-3′ for mouse IL-6; 5′-ATGACAACTTTGTCGACTCTTT-3′ and 5′-GGTCCGGACCCCTGTCAT-3′ for GAPDH.

Western Blot Analysis

Perivascular adipose tissue was harvested from the rat iliac artery at 24 hours after balloon injury and homogenized. Proteins were separated in denaturing sodium dodecyl sulfate 12.5% polyacrylamide gels and transferred onto a membrane. The membrane was incubated with an anti-adiponectin antibody (R&D Systems) at a dilution of 1:1000, followed by incubation with a secondary antibody conjugated with horseradish peroxidase. The signal was detected with an ECL-PLUS Western Blotting Detection System (GE Healthcare). The β-actin signal was used to quantify the relative content of adiponectin.

Cell Culture

Murine 3T3-L1 preadipocytes, RAW264 macrophages, and rat vascular SMCs were grown in DMEM containing 50 U/mL penicillin/50 μg/mL streptomycin with 10% FBS at 37°C in 5% CO2. 3T3-L1 preadipocytes were seeded in 6-well plates at an initial density of 1×105 cells per well and incubated for an additional 2 days to allow the cells to reach confluence. 3T3-L1 cells were then exposed to induction medium containing 10% FBS/10 μg/mL insulin/1 μmol/L dexamethasone/500 μmol/L 3-isobutyl-1-methylxanthine (IBMX) for up to 48 hours. The differentiated 3T3-L1 cells were fed a maintenance medium containing 10% FBS/5 μg/mL insulin in DMEM and left undisturbed for an additional 9 days with a change of medium every 3 days (maturation period).

Coculture of Adipocytes and Macrophages

Coculture of adipocytes and macrophages was performed as described previously. RAW264 macrophages at an initial density of 1×106 cells per well were cocultured with serum-starved differentiated 3T3-L1 adipocytes in the presence or absence of anti-TNF-α-neutralizing antibody (1 μg/mL; R&D Systems). As a control, adipocytes and macrophages were cultured separately. TNF-α concentration was measured using a mouse TNF-α ELISA kit (eBioscience) according to the manufacturer’s instructions.

SMC Proliferation Assay

Rat vascular SMCs were seeded in 96-well plates at an initial density of 2×105 cells per well and allowed to attach for 24 hours. The cells were starved for 24 hours and then incubated with conditioned medium for 48 hours. Cell proliferation was evaluated by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay using CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega).

Statistical Analysis

All results are expressed as mean±SEM. Differences between the 2 groups were assessed by Student’s t tests. Data among more than 2 groups were assessed by ANOVA followed by Bonferroni multiple comparisons test. Values of P < 0.05 were considered significant.

Results

Endovascular Injury Induced Rapid and Marked Changes in Adipocyte Profile in Perivascular Adipose Tissue

To determine whether endovascular injury influences adipocyte expression within periadventitial fat, we injured the femoral artery of wild-type C57BL/6 mice. A large wire was inserted into the femoral artery. This wire injury led to complete endothelial denudation and marked dilatation of the
lumen. Real-time PCR analysis revealed that endovascular wire injury induced marked downregulation of adiponectin expression (P<0.05) and upregulation of proinflammatory cytokines, such as MCP-1, TNF-α, IL-6, and plasminogen activator inhibitor type-1 (P<0.05), in periadventitial fat at 1 day after the injury (Figure 1A). These results indicate that endovascular wire injury affects not only the injured artery itself but also periadventitial adipose tissue.

Next, we used a rat iliac artery transluminal balloon injury model to avoid potential inflammation in the adventitia induced by the surgical procedure. A percutaneous transluminal coronary angioplasty balloon was inserted into iliac artery and inflated for 1 minute. After 1 day, there was upregulation of proinflammatory cytokines and downregulation of adiponectin in the periadventitial fat (P<0.05) (Figure 1B). Adiponectin downregulation was also confirmed at protein level. These results indicate that endovascular balloon injury affects expression of adipocytokines in perivascular fat in the rat.

**Inflammatory Cells Infiltrated Into Perivascular Fat in Response to Endovascular Injury**

To investigate whether endovascular injury induces histological changes in periadventitial fat, we performed immunohistochemical staining and electron microscopic observation. Immunohistochemical analysis detected accumulation of F4/80-positive macrophages and CD3-positive T cells in periadventitial fat after endovascular injury in mice (Figure 2A through 2C). Electron microscopic observation revealed accumulation of inflammatory cells, including foam cells (Figure 2D), macrophages (Figure 2E), and mast cells (Figure 2F) within periadventitial adipose tissues in rats.

**TNF-α Mediated Adipocytokine Changes Induced by Endovascular Injury**

To obtain insights into the mechanism by which endovascular injury induced phenotypic modification of periadventitial fat, we induced wire-mediated endovascular injury in C57BL/6 wild-type, TNF-α-deficient, MCP-1-deficient, and IL-6-deficient mice. Downregulation of adiponectin induced by...
vascular injury was attenuated in MCP-1-deficient mice (Figure 3A). Upregulation of proinflammatory cytokines was markedly attenuated in TNF-α-deficient mice compared with that in wild-type mice at day 1. Downregulation of adiponectin and upregulation of proinflammatory cytokines were most prominent at day 1 compared with those at day 7 or day 28 in wild-type mice (Figure 3B). Immunohistochemical analysis showed that MCP-1 (Figure 3C) and IL-6 (Figure 3D) were upregulated at protein level in perivascular fat at 1 day after wire-mediated endovascular injury in wild-type mice compared with that in TNF-α-deficient mice. E, Immunofluorescent staining showed that infiltrating F4/80-positive macrophages (red) strongly and adipocytes moderately expressed TNF-α (green).

Figure 3. TNF-α mediates phenotypic modification of perivascular adipose tissue in response to endovascular injury. A, Expression of adipocytokines mRNA in perivascular fat around the femoral artery of wild-type C57BL/6 (n=4), TNF-α-deficient (TNF-α KO) (n=3), MCP-1 KO (n=6), and IL-6 KO (n=6) mice at 1 day after endovascular wire injury. Expression of mRNA was analyzed by quantitative real-time PCR. Data were normalized to that of GAPDH and expressed as mean±SEM, *P<0.05, **P<0.01, NS, not significant. B, Expression of adipocytokines mRNA in perivascular fat around the femoral artery of wild-type C57BL/6 (open columns) and TNF-α KO mice with that in wild-type C57BL/6 mice using wire injury model. Osmotic minipumps filled with either TNF-α or PBS were implanted beside the injured arteries. Local delivery of TNF-α increased proinflammatory adipocytokines in perivascular adipose tissue (Figure 4B). However, adiponectin expression was not affected. Four weeks after endovascular injury, perivascular delivery of TNF-α to the femoral artery (5 ng/day) enhanced neointimal hyperplasia compared with the contralateral femoral artery onto which PBS was delivered in TNF-α-deficient mice (Figure 4C). These findings indicate the central role of TNF-α in mediating vascular injury signaling for the change in adipocytokine expression in adjacent perivascular adipose tissue.

**Conditioned Medium From Coculture of Adipocytes and Macrophages Stimulated SMC Proliferation**

It was reported that coculture of differentiated 3T3-L1 adipocytes and the macrophage cell line RAW264 resulted in marked upregulation of proinflammatory adipocytokines and downregulation of the antiinflammatory cytokine adiponec- 
tin.19 To examine the effect of macrophage infiltration into adipose tissue on vascular remodeling, SMCs were cultured with conditioned medium from coculture of differentiated 3T3-L1 adipocytes and RAW264 macrophages. Conditioned medium from coculture stimulated rat vascular SMC proliferation more than that from the control culture (Figure 5A). TNF-α concentration increased in the conditioned medium from the coculture compared with that from the control culture (Figure 5B). A neutralizing anti-TNF-α antibody abrogated the stimulating effect of the conditioned medium.
from the coculture on vascular SMC proliferation. The anti-TNF-α antibody had no significant effect on SMC proliferation in the presence of medium from the control culture of 3T3-L1 or RAW alone (Figure 5A). A neutralizing anti-MCP-1 or anti-IL-6 antibody also abrogated the stimulating effect of the conditioned medium from the coculture on vascular SMC proliferation (please see supplemental data, available online at http://atvb.ahajournals.org).

Discussion
In the present study, we found that endovascular injury induces rapid phenotypic modification of perivascular adipose tissue. Proinflammatory adipocytokines were upregulated, and adiponectin was downregulated. These changes were associated with infiltration of inflammatory cells. It was suggested that TNF-α plays a central role in the changes in periadventitial adipose tissue, which potentially influence neointimal formation following vascular injury.

It has been considered that endovascular injury causes inflammatory and proliferative responses in the injured vessels on the luminal side. Numerous studies have focused on the key molecules that transmit endothelial injury signals to bring about infiltration of inflammatory cells into the vessel wall from the luminal side. On the other hand, accumulating evidence suggests that inflammatory response in the adventitia may also play a crucial role in the pathogenesis of vascular diseases. Roles of the vasa vasorum, mast cells, fibroblasts, leukocytes, macrophages, and adipose tissue in the adventitia have been reported. Cellular components of adipose tissue include mature adipocytes, preadipocytes, microvasculature, nerve fibers, monocytes, macrophages, and other blood cells. Here, we detected foam cells, which are usually found in atherosclerotic lesions and within the periadventitia following angioplasty. Moreover, we also observed activated macrophages and mast cells with granules within periadventitial adipose tissue. It has

Figure 4. TNF-α plays an atherogenic role in adipocytokines changes and in neointima formation after endovascular injury. A, Morphometric analysis of injured femoral arteries from wild-type C57BL/6 mice and TNF-α KO mice at 4 weeks after wire-induced injury (n=7 each). B, PBS or TNF-α was delivered locally to the perivascular area in TNF-α-deficient mice. Expression of adipocytokines mRNA in periadventitial fat around femoral artery in TNF-α KO mice with or without TNF-α administration at 4 weeks after endovascular wire injury (n=3 each). Expression of mRNA was analyzed by quantitative real-time PCR. Expression was normalized to that of GAPDH. Bars denote mean±SEM, *P<0.05. NS, not significant. C, Effect of perivascular TNF-α administration on neointimal formation in TNF-α KO mice. Morphometric analysis of injured femoral arteries in TNF-α KO mice with or without TNF-α administration at 4 weeks after wire-induced injury (n=3 each).
been reported that a vicious cycle between adipocytes and macrophages aggravates inflammatory changes in adipose tissue. Our results indicate that endovascular injury may contribute to an augmented interaction between local adipocytes and inflammatory cells within periadventitial adipose tissue.

Dysregulated secretion of adipocytokines, ie, upregulated secretion of inflammatory cytokines, such as TNF-α, IL-6, MCP-1, and plasminogen activator inhibitor type-1, and downregulated secretion of antiinflammatory cytokines, such as adiponectin, has been shown to be involved in the pathogenesis of the metabolic syndrome. Clinical studies have shown that visceral obesity is a more important risk factor for cardiovascular disease than is sc obesity. On the other hand, periadventitial adipose tissue was considered as an organ mainly acting as a structural support for blood vessels. Recent studies suggested that perivascular adipose tissue, particularly epicardial fat, plays an important role in vessel remodeling under physiological and pathological conditions. It has been shown that periadventitial fat is an important source of adipocytokines, which might contribute to the progression of obesity-associated atherosclerosis. Greenstein et al suggested that adiponectin secreted from local perivascular adipose tissue physiologically modulates local vascular tone by increasing nitric oxide bioavailability, which is lost in obesity. Although some clinical studies have suggested that the characteristics of epicardial fat are altered in patients with coronary artery disease, it remains to be determined whether vascular injury induces phenotypic changes in adjacent perivascular fat. In this study, we investigated the changes in periadventitial adipose tissues in response to endovascular injury in detail. Our results indicate that endovascular injury induces periadventitial fat inflammation.

Previous studies have reported that adiponectin-deficient mice showed severe neointimal thickening and increased proliferation of vascular SMCs in injured arteries. Recently, we showed that adiponectin secreted from perivascular fat may play a protective role in neointima formation after vascular injury in lean mice and that obesity causes inflammatory changes and downregulation of adiponectin in the periadventitial adipose tissue, which potentially influence enhanced lesion formation after vascular injury. In the present study, we showed the role of TNF-α secreted from perivascular fat. It is likely that changes in both adiponectin and inflammatory adipocytokine expression in perivascular adipose tissue potentially play a role in the pathogenesis of neointimal formation after angioplasty.

We investigated phenotypic modification of periadventitial fat induced by endovascular injury in wild-type, TNF-α-deficient, MCP-1-deficient, and IL-6-deficient mice. Interestingly, downregulation of adiponectin was attenuated in MCP-1-deficient mice, suggesting a crucial role of MCP-1 in regulation of adiponectin expression in periadventitial fat. Upregulation of TNF-α and IL-6 was attenuated in MCP-1-deficient mice. On the other hand, there was no significant difference between wild-type and IL-6-deficient mice in expression of adiponectin, MCP-1, and TNF-α in perivascular fat at 1 day after injury. Taken together, these results suggest that TNF-α plays a central role in phenotypic modulation of periadventitial fat induced by endovascular injury.

We demonstrated that TNF-α-deficient mice have decreased periadventitial fat inflammation and attenuated neointimal hyperplasia in response to endovascular injury. Continuous periadventitial administration of recombinant TNF-α protein augmented periadventitial fat inflammation and exacerbation of neointimal progression in response to endovascular injury in TNF-α-deficient mice. Clinical studies have shown an increased risk of recurrent coronary events in myocardial infarction patients with elevated TNF-α and an association between carotid atherosclerosis and plasma TNF-α level. Our previous study demonstrated that elevated TNF-α contributed to lesion formation after angioplasty in a mouse myocardial infarction model. It was shown that TNF-α-deficient mice have attenuated atherosclerosis and that TNF-α inhibition improved neointimal hyperplasia after angioplasty in animal models. Clinical studies also reported that obese patients have an elevated TNF-α level. It was reported that a vicious cycle between adipocytes and macrophages via TNF-α and free fatty acids aggravates inflammatory changes in adipose tissue in obesity. Although these clinical and experimental studies suggest that TNF-α is one of the key molecules in both cardiovascular disease and obesity, only the role of systemically circulating TNF-α, but not local distribution of TNF-α, has been focused in the pathogenesis of vascular diseases. Our present study suggests that periadventitial TNF-α may directly influence the pathogenesis of vascular remodeling.

Our in vitro study showed that the conditioned medium from coculture of differentiated 3T3-L1 adipocytes and RAW264 macrophages induced marked SMC proliferation compared with individual culture of adipocytes or macrophages. Blockade of TNF-α with a neutralizing antibody in coculture effectively inhibited the stimulating effect of the conditioned medium on SMC proliferation. These results suggest that TNF-α mediates the phenotypic change of adipocyte in response to vascular injury. It was reported that periadventitial inflammatory cells contribute to vascular remodeling after angioplasty. Our findings suggest that periadventitial adipose tissue and infiltrating inflammatory cells may have synergistic deleterious effects on lesion formation in response to vascular injury.

In summary, our findings suggest that endovascular injury induces rapid and marked changes in perivascular adipose tissue. It is likely that TNF-α may mediate these changes. Phenotypic change of perivascular fat may have a potential role in neointimal hyperplasia after vascular injury.

Sources of Funding
This work was supported in part by the Program for Promotion of Basic and Applied Researches for Innovations in Bio-oriented Industry and by grants from the Ministry of Education, Culture, Sports, Science, and Technology (Knowledge Cluster and New Research Area) and the Ministry of Health, Labor, and Welfare of Japan.

Disclosures
None.
References


Endovascular Injury Induces Rapid Phenotypic Changes in Perivascular Adipose Tissue
Minoru Takaoka, Hiroshi Suzuki, Seiji Shioda, Kenji Sekikawa, Yoshihiko Saito, Ryozo Nagai
and Masataka Sata

Arterioscler Thromb Vasc Biol. 2010;30:1576-1582; originally published online May 20, 2010;
doi: 10.1161/ATVBAHA.110.207175
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272
Greenville Avenue, Dallas, TX 75231
Copyright © 2010 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://atvb.ahajournals.org/content/30/8/1576

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2010/05/20/ATVBAHA.110.207175.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published
in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the
Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for
which permission is being requested is located, click Request Permissions in the middle column of the Web
page under Services. Further information about this process is available in the Permissions and Rights
Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online
at:
http://atvb.ahajournals.org//subscriptions/
Online Data Supplement

Stimulatory effect of conditioned medium from co-culture of adipocytes and macrophages on SMC proliferation

RAW264 macrophages at an initial density of $1 \times 10^5$ cells per well were co-cultured with serum-starved differentiated 3T3L1 adipocytes in the presence or absence of anti-MCP-1 neutralizing antibody (R&D Systems, 10 $\mu$g/ml) or anti-IL-6 neutralizing antibody (R&D Systems, 1 $\mu$g/ml) ($n=3$). As a control, adipocytes and macrophages were cultured separately. Stimulatory effect of the conditioned mediums on SMC proliferation was evaluated. Rat vascular SMCs were seeded in 96-well plates at an initial density of $2 \times 10^3$ cells per well and allowed to attach for 24 hours. The cells were starved for 24 hours and then incubated with conditioned medium for 48 hours. Cell proliferation was evaluated by MTS assay using CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega). Bar, mean ± S.E.M. *, $p < 0.05$. **, $p < 0.05$. NS, not significant. Ab, antibody.