
Yukio Arita, MD, PhD; Shinji Kihara, MD, PhD; Noriyuki Ouchi, MD, PhD; Kazuhiisa Maeda, MD, PhD; Hiroshi Kuriyama, MD, PhD; Yoshihisa Okamoto, MD; Masahiro Kumada, MD; Kikuko Hotta, MD, PhD; Hiroshi Kuriyama, MD, PhD; Yoshihisa Okamoto, MD; Masahiko Takahashi, MD, PhD; Tadashi Nakamura, MD, PhD; Ichiro Shimomura, MD, PhD; Masahiro Muraguchi, PhD; Yasukazu Ohmoto, PhD; Tohru Funahashi, MD, PhD; Yuji Matsuzawa, MD, PhD

Background—Vascular smooth muscle cell proliferation plays an important role in the development of atherosclerosis. We previously reported that adiponectin, an adipocyte-specific plasma protein, accumulated in the human injured artery and suppressed endothelial inflammatory response as well as macrophage-to-foam cell transformation. The present study investigated the effects of adiponectin on proliferation and migration of human aortic smooth muscle cells (HASMCs).

Methods and Results—HASMC proliferation was estimated by [3H] thymidine uptake and cell number. Cell migration assay was performed using a Boyden chamber. Physiological concentrations of adiponectin significantly suppressed both proliferation and migration of HASMCs stimulated with platelet-derived growth factor (PDGF)-BB. Adiponectin specifically bound to 125I-PDGF-BB and significantly inhibited the association of 125I-PDGF-BB with HASMCs, but no effects were observed on the binding of 125I-PDGF-AA or 125I-heparin–binding epidermal growth factor (EGF)–like growth factor (HB-EGF) to HASMCs. Adiponectin strongly and dose-dependently suppressed PDGF-BB–induced p42/44 extracellular signal–related kinase (ERK) phosphorylation and PDGF receptor autophosphorylation analyzed by immunoblot. Adiponectin also reduced PDGF-AA–stimulated or HB-EGF–stimulated ERK phosphorylation in a dose-dependent manner without affecting autophosphorylation of PDGF receptor or EGF receptor.

Conclusions—The adipocyte-derived plasma protein adiponectin strongly suppressed HASMC proliferation and migration through direct binding with PDGF-BB and generally inhibited growth factor–stimulated ERK signal in HASMCs, suggesting that adiponectin acts as a modulator for vascular remodeling. (Circulation. 2002;105:2893-2898.)

Key Words: atherosclerosis ■ muscle smooth ■ growth substances

Obesity, the most common nutritional disorder in the industrial countries, is associated with increased cardiovascular mortality and morbidity.1–3 Adipose tissue is not simply an energy storage organ but also a secretory organ, producing a variety of bioactive molecules, including leptin, tumor necrosis factor-α, plasminogen activator inhibitor type 1, and adiponectin, that may directly contribute to the development of cardiovascular diseases.4–13 Adiponectin is an adipose-specific plasma protein belonging to the collectin family.14,15 Plasma concentrations of adiponectin ranged from 3 to 30 μg/mL16 and decreased in patients with coronary artery disease (CAD) and patients with type 2 diabetes with macroangiopathy.10,17–19 Screening of mutations in adiponectin gene revealed that a patient carrying missense mutation showed markedly decreased plasma level of adiponectin and had CAD.20 These data suggest that hypoadiponectinemia can be considered a risk factor of CAD.

Vascular smooth muscle cell proliferation induced by platelet-derived growth factor (PDGF) or heparin-binding epidermal growth factor (EGF)–like growth factor (HB-EGF) is considered crucial for the development of vascular diseases.21 Adiponectin specifically bound to subendothelial collagens22 and abundantly accumulated into subendothelial space of acute injured lesion of human artery,12 suggesting that the adipocyte-derived plasma protein may play a physiological role in the vascular wall when the endothelial barrier is broken.
injured. We hypothesized that adiponectin might act as an endogenous modulator for vascular remodeling. In this study, we investigate the inhibitory mechanism of adiponectin on the responsiveness of the human aortic smooth muscle cell (HASMC) to various growth factors.

**Methods**

**Proteins and Antibodies**

Recombinant human platelet-derived growth factor (PDGF)-BB, PDGF-AA, and heparin-binding EGF-like growth factor (HB-EGF) were purchased from R&D systems. [methyl-1H] thymidine and 125I-PDGF-BB were obtained from Amersham. Recombinant PDGF-AA or HB-EGF was labeled by Na-125I (Amersham) and Iodo-Gen (Pierce). The iodinated protein was purified by passage over Sephadex G-25 (Pharmacia Biotech). Anti-PDGF β-receptor, anti-PDGF α-receptor, and anti-EGF receptor polyclonal antibodies were purchased from Upstate Biotechnology. Antiphosphotyrosine monoclonal antibody (PY99) was obtained from Santa Cruz Biotechnology. Anti-p42/44 extracellular signal–related kinase (ERK) antibody and antiphosphorylated p42/44 ERK antibody were purchased from New England Biolabs.

**DNA Synthesis and Cell Number of HASMCs**

HASMCs were maintained as described.23 Cells from passage 4 to 5 were used for experiments. HASMCs were treated for 18 hours in DMEM (Nacalai tesque, Japan) containing 2% FCS with 10 ng/mL of PDGF-BB, PDGF-AA, or HB-EGF in the presence or absence of the indicated amounts of adiponectin and then exposed to [methyl-1H] thymidine (Amersham) at 20 μCi/mL for 6 hours. The cells were trypsinized and retrieved onto glass fiber filters using an automatic cell harvester, and [methyl-1H] thymidine uptake was measured in a direct β counter.

Cell number was determined with the hemocytometer measurement method described earlier.24 Human recombinant adiponectin was prepared as described.16

**Migration Assay of HASMCs**

Migration assays were performed using a Boyden chamber. HASMCs were trypsinized and washed with serum-free DMEM, and aliquots (200 μL) of HASMCs suspension (5×10^5 cells/mL) were added to the transwell inserts precoated with collagen type I. Migration was induced by PDGF-BB (10 ng/mL) with or without adiponectin (30 μg/mL) added to the lower chamber beneath the insert membrane. The transwell chambers were then incubated for 6 hours under culture condition. Nonmigrating HASMCs on the top of the membrane were removed by gentle scraping. HASMCs at the lower surface of the membrane were fixed with ethanol and stained with hematoxylin. The filters were removed, and the number of cells migrating to the lower surface of the filter was evaluated microscopically by counting the number of stained nuclei per high-power field (×400). All assays were performed in triplicate, and each sample was counted randomly in 3 different areas in the center of the membrane. The HASMC migration activity is reported as cells per high-power field.

**Immunoblot**

HASMCs were treated with PDGF-BB, PDGF-AA, or HB-EGF at a final concentration of 10 ng/mL with or without the indicated amount of adiponectin for 5 minutes at room temperature. Cells were placed immediately on ice, washed with ice-cold PBS, and solubilized with solubilization buffer (1% Triton X-100, 50 mmol/L HEPES, pH 7.5, 150 mmol/L NaCl, 10% glycerol, 1.5 mmol/L MgCl2, 10 mmol/L NaF, 10 mmol/L sodium diphosphate decahydrate 1% aprotinin, 5 μg/mL leupeptin, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L sodium orthovanadate, and 1 mmol/L dithiothreitol). The cell lysates were incubated with 2 μg/mL of anti-PDGF β-receptor, anti-PDGF α-receptor, or anti-EGF receptor polyclonal antibody for 2 hours at 4°C, followed by incubation for 1 hour at 4°C with protein A Sepharose beads prewashed with ice-cold solubilization buffer. Beads were washed twice with ice-cold solubilization buffer. Samples were resuspended in 1×Laemml buffer, and the proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. The membranes were incubated for 2 hours at room temperature with

![Figure 1. Adiponectin inhibits PDGF-BB-induced HASMC proliferation and migration. A, Effect of adiponectin on PDGF-BB-induced, PDGF-AA–induced, or HB-EGF–induced DNA synthesis in HASMCs. HASMCs were treated with the indicated concentrations of adiponectin either in the presence or absence of PDGF-BB, PDGF-AA, or HB-EGF (10 ng/mL each) for 24 hours. B, Effect of adiponectin on cell number of HASMCs stimulated by PDGF-BB. HASMCs were treated with PDGF-BB (10 ng/mL) either in the presence or absence of adiponectin (30 μg/mL) for 5 days. Medium was changed every 24 hours. C, Effect of adiponectin on migration of HASMCs stimulated by PDGF-BB. HASMCs were trypsinized and incubated in the Boyden chamber for 6 hours at 37°C with 10 ng/mL of PDGF-BB either in the presence or absence of 30 μg/mL of adiponectin. These experiments were repeated 3 times, and the data (mean±SD) from a representative experiment were shown.](http://circ.ahajournals.org/issue/6/11/2894/Circulation_2894_Fig1.jpg)
blocking solution (PBS containing 0.2% Tween 20 and 5% BSA), followed by incubation for 2 hours at room temperature with PBS containing 0.2% Tween 20 and the indicated antibody. Then the membranes were rinsed 4 times with wash buffer (PBS containing 0.2% Tween 20) and incubated for 1 hour at room temperature with horseradish peroxidase conjugated anti-mouse or anti-rabbit antibody. The membranes were rinsed 4 times. The bands were visualized by ECL detection system (Amersham) and quantified by using NIH Image. Band volume was determined as band intensity per area according to the manufacturer’s instructions.

125 I-Growth Factor Binding to HASMCs
Specific binding of 125I-PDGF-BB, 125I-PDGF-AA, or 125I-HB-EGF was studied in HASMC-seeded monolayers at 1×10⁴ cells/well in 24-well plates. HASMCs were rinsed once with binding buffer (DMEM containing 0.5% BSA) and incubated for 3 hours at 4°C with binding buffer containing the indicated amount of 125I-PDGF-BB, 125I-PDGF-AA, or 125I-HB-EGF in the presence or absence of adiponectin. After rinsing 3 times, cells were solubilized for 3 hours at room temperature with 1 N NaOH, and the total binding was determined by measuring the radioactivity by a γ-scintillation counter. Nonspecific binding was determined by the addition of a 100-fold excess of unlabeled PDGF-BB, PDGF-AA, or HB-EGF. The counts were subtracted from the total counts. Binding assays were performed in triplicate in 3 independent experiments.

Immunoprecipitation of 125I-PDGF-Adiponectin Complexes
125I-PDGF-BB or 125I-PDGF-AA (5 ng each) was incubated with adiponectin (5 μg) or BSA (5 μg) in the presence or absence of unlabeled PDGF-BB or PDGF-AA for 3 hours at room temperature. Samples were incubated with anti-adiponectin polyclonal antibody or nonimmune rabbit serum for 1 hour, followed by incubation with protein A Sepharose for 1 hour. The immune complex-bound Sepharose beads were washed 5 times and dissociated in 1×Laemmli buffer at 95°C for 3 minutes. The radioactivities of the samples were measured by a γ-scintillation counter. Binding assays were performed in 3 independent experiments.

Results
Adiponectin Inhibits Proliferation and Migration of HASMCs Stimulated by Growth Factors
Treatment of HASMCs with PDGF-BB, PDGF-AA, or HB-EGF (10 ng/mL each) increased DNA synthesis 3-fold compared with untreated control. Physiological concentrations of adiponectin inhibited DNA synthesis stimulated with PDGF-BB in a dose-dependent manner. The increased DNA synthesis induced by 10 ng/mL of PDGF-BB, PDGF-AA, or
HB-EGF was inhibited by 30 μg/mL of adiponectin by 90%, 30%, or 50%, respectively (Figure 1A). We confirmed the suppressive effect of adiponectin on PDGF-BB–induced HASMC proliferation by the cell count. Cell number was increased 2-fold of control by PDGF-BB (10 ng/mL) treatment for 5 days. Adiponectin (30 μg/mL) treatment totally abolished PDGF-BB–induced HASMC proliferation (Figure 1B). No significant apoptotic cells were observed during this incubation period (data not shown).

Next we investigated the effect of adiponectin on PDGF-BB–induced migration of HASMCs. The migration was increased to 1.8-fold of control by 10 ng/mL of PDGF-BB. Physiological concentrations of adiponectin dose-dependently suppressed PDGF-BB–induced migration of HASMCs. Adiponectin (30 μg/mL) interfered the specific binding of 125I-PDGF-BB to HASMCs (Figure 3A). The inhibitory effects were observed in a dose-dependent manner (Figure 3B). Binding of 125I-PDGF-AA or 125I-HB-EGF was significantly suppressed by physiological concentrations of adiponectin (Figures 3C and 3D, bottom).

Adiponectin Inhibits Binding of 125I-PDGF-BB to HASMCs

Because adiponectin inhibited PDGF-β receptor autophosphorylation of HASMCs, we examined the effect of adiponectin on the binding of 125I-PDGF-BB to HASMCs. HASMCs were incubated with 125I-PDGF-BB in the presence or absence of 10 μg/mL of adiponectin. Adiponectin (10 μg/mL) interfered the specific binding of 125I-PDGF-BB to HASMCs (Figure 3A). The inhibitory effects were observed in a dose-dependent manner (Figure 3B). Binding of 125I-

Effects of Adiponectin on Growth Factor–Induced p42/44 ERK Phosphorylation and Receptor Autophosphorylation

To investigate the mechanism by which adiponectin inhibits the PDGF-BB–induced mitogenic effect on HASMCs, we examined the effect of adiponectin on PDGF-BB–stimulated ERK phosphorylation. ERK phosphorylation was increased ∼10-fold of control by stimulation with 10 ng/mL of PDGF-BB. Physiological concentrations of adiponectin dose-dependently suppressed PDGF-BB–induced ERK phosphorylation. The increased ERK phosphorylation by PDGF-BB was inhibited by 30 μg/mL of adiponectin by 70% (Figure 2A). Next, we investigated PDGF β-receptor autophosphorylation in PDGF-BB–stimulated HASMCs. PDGF β-receptor autophosphorylation was increased 14-fold of control by 10 ng/mL of PDGF-BB. Physiological concentrations of adiponectin inhibited PDGF β-receptor autophosphorylation in a dose-dependent manner. The increased PDGF β-receptor autophosphorylation was suppressed by 30 μg/mL of adiponectin by 80% (Figure 2B).

In contrast to PDGF-BB, adiponectin had no effects on PDGF-AA–stimulated or HB-EGF–stimulated autophosphorylation of each receptor (Figures 2C and 2D, top). However, the increased ERK phosphorylation with PDGF-AA or HB-EGF was significantly suppressed by physiological concentrations of adiponectin (Figures 2C and 2D, bottom).

Figure 3. Effect of adiponectin on the binding of 125I-PDGF or 125I-HB-EGF to HASMCs. A, Effect of adiponectin on 125I-PDGF-BB binding to HASMCs. HASMCs were incubated with 125I-PDGF-BB in the presence (○) or absence (●) of 10 μg/mL adiponectin. B, Dose-response effect of adiponectin on 125I-PDGF-BB binding to HASMCs. HASMCs were incubated with 25 ng/mL of 125I-PDGF-BB in the presence or absence of indicated concentrations of adiponectin. C, Dose-response effect of adiponectin on 125I-PDGF-AA binding to HASMCs. HASMCs were incubated with 25 ng/mL of 125I-PDGF-AA in the presence or absence of indicated concentrations of adiponectin. Nonspecific binding was defined as binding in the presence of a 100-fold excess of unlabeled ligand. The experiments were repeated 3 times, and the data (mean±SD) were shown.
PDGF-BB to HASMCs was inhibited by 30 μg/mL of adiponectin by 80%. We also investigated the effect of adiponectin on the binding of 125I-PDGF-AA or 125I-HB-EGF to HASMCs. No inhibitory effect of adiponectin was observed on the binding of 125I-PDGF-AA or 125I-HB-EGF to HASMCs (Figures 3C and 3D).

**Binding of 125I-PDGF and Adiponectin**

To investigate the direct association of adiponectin with PDGF, 125I-PDGF-BB or 125I-PDGF-AA (5 ng each) was incubated with adiponectin or BSA and then immunoprecipitated with the anti-adiponectin polyclonal antibody. PDGF-BB was significantly immunoprecipitated with adiponectin and anti-adiponectin polyclonal antibody, but no specific association was observed between adiponectin and PDGF-AA (Figure 4A). The relative competition of unlabeled PDGF-BB revealed that PDGF-binding capacity of adiponectin was 30% of PDGF-BB/μg adiponectin (Figure 4B).

**Discussion**

In the present study, we demonstrated that physiological concentrations of adiponectin (3 to 30 μg/mL) had significant inhibitory effects on PDGF-BB–induced proliferation and migration of vascular smooth muscle cells. The increased DNA synthesis induced by 10 ng/mL of PDGF-BB was inhibited by 30 μg/mL of adiponectin by 90%. Adiponectin directly associated with PDGF-BB, and its binding capacity was 2 ng PDGF-BB/μg adiponectin. Adiponectin inhibited PDGF-BB binding to HASMCs and blocked PDGF β-receptor–mediated mitogenic signaling. We next examined the effect of adiponectin on PDGF-AA and HB-EGF. Adiponectin had no inhibitory effect on the binding of PDGF-AA or HB-EGF to HASMCs. However, adiponectin suppressed PDGF-AA–induced or HB-EGF–induced DNA synthesis and ERK phosphorylation. In addition, adiponectin treatment 1 hour after addition of PDGF-BB inhibited PDGF-BB–induced DNA synthesis by 30% (data not shown). These data suggest that adiponectin may partially inhibit the postreceptor ERK-mediated signaling pathway of these growth factors, although additional investigation is required to elucidate the precise cross-talk mechanism. PDGF-BB is secreted from activated platelets and vascular foam cells and plays an important role in the development of atherosclerosis. Excess migration and proliferation of HASMCs results in thickening of the vascular wall and contributes to the eventual occlusion of the artery. The biological activities of PDGF have been reported to be modulated by binding extracellular matrices and secreted protein acidic and rich in cysteine (SPARC) and thrombospondin-1 and α2-macroglobulin. Interstitial collagens can interact with PDGF, suggesting that they act as a storage depot for immobilized PDGF. SPARC, thrombospondin-1, and α2-macroglobulin interact with PDGF and modulate the biological activity of PDGF. Our findings clearly demonstrated that adiponectin specifically bound to PDGF-BB, not with PDGF-AA. Adiponectin is an important molecule in the regulation of PDGF-BB that is released from platelets or secreted by activated macrophages like SPARC and α2-macroglobulin. Different from these matrix-like proteins produced from vascular cells, adiponectin is a plasma protein specifically secreted from adipocytes. Adiponectin strongly binds to interstitial collagen type I, III, and V in vitro and was immunohistochemically localized in the balloon-injured rat carotid artery and the acute injured lesion of human aorta in vivo. Adiponectin accumulates to the subendothelial space of injured vascular wall containing smooth muscle cells from blood circulation and may inhibit proliferation and migration of smooth muscle cells through modulating the PDGF-BB activity.

We have reported that adiponectin was eluted in the fractions with molecular weight from 290 to 900 kDa by gel filtration of human plasma, suggesting that adiponectin exists as a large multimeric molecule like other soluble defense collagens. Raines et al reported the presence of plasma...
constituents, which have the capacity to bind to PDGF and inhibit the binding of PDGF to its cell-surface receptor. They defined 3 fractional classes with molecular weights of 40, 150, and >500 kDa by gel filtration. One of these molecules had been identified as a 725-kDa glycoprotein, α2-macroglobulin.29–31 The fraction >500 kDa might contain adiponectin.

Adiponectin is abundantly present in human plasma, accounting for 0.01% of the total plasma protein. Plasma adiponectin levels are reduced in patients with coronary artery disease, including myocardial infarction. Recently we have demonstrated that adiponectin acts as a modulator of monocyte adhesion to endothelial cells and macrophage-to-cell transformation.10–12 In addition to these functions, adiponectin could modulate excessive proliferation and migration of HASMCs by attenuating PDGF-BB signal, indicating that this naturally circulating substance may have a potential preventive role against the atherogenic process.

In summary, we demonstrated that adiponectin strongly suppressed PDGF-BB–induced proliferation and migration of HASMCs through direct association with PDGF-BB. Our findings suggest that the adipocyte-specific plasma protein adiponectin acts not only as a negative regulator of the endothelial adhesion molecule expression and macrophage-to-cell transformation but also as a modulator of smooth muscle cell proliferation, providing a pivotal fundamental mechanism for the link between overnutrition and atherosclerosis.

Acknowledgments

This work was supported by grants from the Japanese Ministry of Education and Japan Society for Promotion of Science-Research for the Future Program.

References


Yuji Arita, Shinji Kihara, Noriyuki Ouchi, Kazuhisa Maeda, Hiroshi Kuriyama, Yoshihisa Okamoto, Masahiro Kumada, Kikuko Hotta, Makoto Nishida, Masahiko Takahashi, Tadashi Nakamura, Ichiro Shimomura, Masahiro Muraguchi, Yasukazu Ohmoto, Tohru Funahashi and Yuji Matsuzawa

_Circulation_. 2002;105:2893-2898; originally published online May 20, 2002; doi: 10.1161/01.CIR.000018622.84402.FF

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2002 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/105/24/2893

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ 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 _Circulation_ is online at:
http://circ.ahajournals.org//subscriptions/