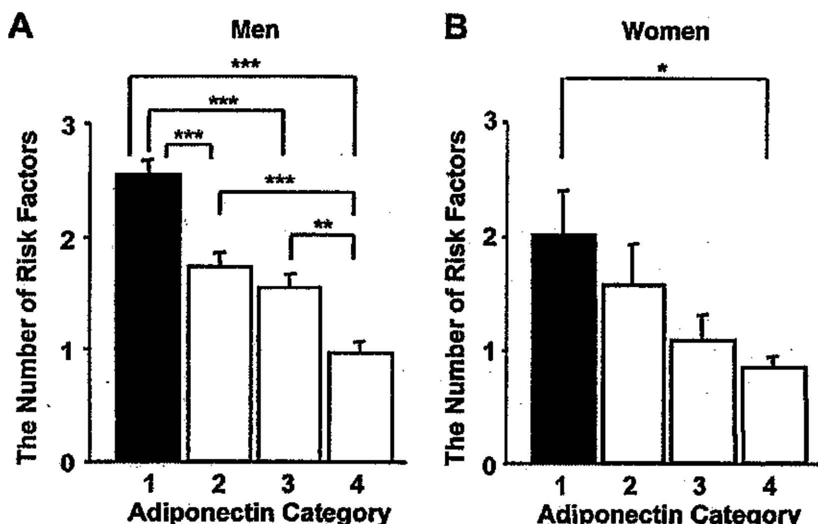

Fig4. Mean number of components of the metabolic syndrome in each of the quartiles based on plasma adiponectin concentration: men (n=479), women (n=182). Risk factors: abdominal obesity, hypertriglyceridemia, low HDL cholesterol, hypertension, high fasting glucose. Data are mean±SEM. *p<0.05, **p<0.01, ***p<0.001 by Kruskal-Wallis test with a Scheffe's test.

relation coefficients between plasma adiponectin and Σplasma glucose, Σplasma insulin were −0.26 and −0.15 in men, −0.29 and −0.31 in women, respectively.).

Hypoadiponectinemia and the Prevalence of Components of the Metabolic Syndrome

We divided the subjects into 2 groups according to their adiponectin concentrations using a cut-off point of 4.0μg/ml and compared the prevalence of each component of the metabolic syndrome in the 2 groups (Fig3). In men, subjects with an adiponectin concentration <4.0μg/ml showed a higher prevalence of abdominal obesity (76.1% vs 52.7%, p<0.001), hypertriglyceridemia (60.6% vs 27.0%, p<0.001), low HDL cholesterol (35.8% vs 16.8%, p<0.001), hypertension (50.5% vs 33.2%, p<0.01) and high fasting glucose (34.0% vs 13.5%, p<0.001) than those with an adiponectin concentration ≥4.0μg/ml. In addition, the female subjects with an adiponectin concentration <4.0μg/ml showed a higher prevalence of hypertriglyceridemia (37.5% vs 13.3%, p<0.05), low HDL cholesterol (18.8% vs 3.6%, p<0.01), hypertension (62.5% vs 36.4%, p<0.05) and high fasting glucose (37.5% vs 13.9%, p<0.05) than those with an adiponectin concentration ≥4.0μg/ml. The prevalence of the metabolic syndrome was higher in subjects with an adiponectin concentration <4.0μg/ml than in those with a concentration ≥4.0μg/ml in both men (52.3% vs 19.6%, p<0.001) and women (37.5% vs 11.4%, p<0.01).

We also categorized the subjects into 4 groups according to their plasma adiponectin concentration: category 1, <4.0μg/ml; category 2, ≥4.0μg/ml, <5.5μg/ml; category 3, ≥5.5μg/ml, <7.0μg/ml; and category 4, ≥7.0μg/ml as described previously.[28] The percentages of subjects in the 4 categories were 22.8%, 28.6%, 23.8% and 24.8%, respectively, in men and 8.8%, 9.9%, 15.9% and 65.4% in women (Table1). The mean number of components of the metabolic syndrome in each adiponectin quartile increased with the decrease in the quartiles of plasma adiponectin concentration in men (category 1: 2.57±1.34; category 2: 1.76±1.16; category 3: 1.54±1.25; category 4: 0.97±1.02) (Fig4A). Comparable data were also obtained in women (category 1: 2.00±1.51; category 2: 1.56±1.54; category 3: 1.07±1.16; category 4: 0.83±1.05) (Fig4B).

Discussion

The metabolic syndrome, representing a cluster of insulin resistance, glucose intolerance, hypertension and dyslipidemia, is a common basis for the development of atherogenic cardiovascular diseases, especially CAD, in industrial countries exposed to overnutrition.[29,30] The molecular basis of the metabolic syndrome has not been elucidated. Adiponectin is an adipose-derived protein with multivalent functions including anti-atherogenic, insulin-sensitizing, lipid-oxidation enhancing, and vasodilatory activities.[12–17,19–22] Therefore, it is possible that decreased plasma concentrations of adiponectin plays a significant role in the development of the metabolic syndrome. In the present study, we demonstrated that the plasma concentration of adiponectin was significantly correlated with each component of the metabolic syndrome. Furthermore, multiplicity of the risk factors was higher in the category of subjects with lower plasma adiponectin concentration. Plasma adiponectin may therefore become a useful biomarker for the metabolic syndrome.

In the present study, we tentatively defined the metabolic syndrome using modified NCEP-ATPIII criteria[25] appropriate for the Japanese population. The cut-off point of WC was 85 cm in men and 90 cm in women according to the criteria for abdominal obesity in the Japanese Society for the Study of Obesity,[26] which corresponded to VFA of 100 cm² determined by CT scan. The cut-off point of HDL-cholesterol level was 40 mg/dl in both men and women, according to the criteria of the Japanese Atherosclerosis Society.[27] Using these criteria, 20.9% of men and 3.6% of women were diagnosed with the metabolic syndrome among a population of Japanese adults.

In the present study, men had significantly lower plasma adiponectin concentrations than BMI-adjusted women (median concentration of plasma adiponectin: 5.4μg/ml vs 8.2μg/ml) as described in previous studies,[18] and the prevalence of hypoadiponectinemia was higher in men (22.8%) than in women (8.8%). The incidence of CAD is lower in women than in men and a higher plasma concentration of the anti-atherogenic protein, adiponectin, may be one of the reasons for the lower risk of CAD in women. It is not appropriate to set a more rigid cut-off value for the simple reason of a higher distribution of plasma adiponectin con-

centrations in women. Subjects with hypoadiponectinemia less than 4.0μg/ml had almost the same number of risk factors whether male (2.57±1.34) or female (2.00±1.51); 52.3% of men and 37.5% of women in this category fulfilled the criteria of the metabolic syndrome. Previously, we reported that subjects with plasma adiponectin concentration less than 4.0μg/ml had a 2-fold increase in the incidence of CAD,[28] and taken together with the present results, we propose setting a plasma adiponectin concentration of less than 4.0μg/ml as the cut-off point for hypoadiponectinemia. However, the number of female subjects diagnosed with the metabolic syndrome in this study was still small and the association of hypoadiponectinemia with the prevalence of CAD in women has not been investigated. Further large-scale surveys are necessary to evaluate the significance of plasma adiponectin in the prevalence of metabolic syndrome in women.

Plasma adiponectin concentrations as an inverse predictor of cardiovascular outcomes has been demonstrated in patients with end-stage renal disease.[31] The relative risk of adverse cardiovascular events was 1.56-fold higher among patients in the lower adiponectin tertile than in those in the higher tertile. Recently, a case–control study of 18,255 participants in the US showed higher plasma adiponectin concentrations were associated with lower risk of myocardial infarction independently of other risk factors in men.[32] Clinical evidence demonstrating that correction of reduced plasma adiponectin can reduce the risk of CAD needs to be accumulated.

The metabolic syndrome is not simply a pathogenic clustering of multiple risk factors by chance. Obesity is frequently accompanied with hypertension, glucose intolerance, and dyslipidemia, but the prevalence of obesity greater than BMI 30 is only 2–3% in eastern Asia and it has been reported that BMI is not associated with CAD in Asians.[33] Establishment of criteria appropriate for eastern Asian populations is needed. Accumulation of fat in the intra-abdominal cavity is associated with hypertension, glucose intolerance, dyslipidemia, and CAD also in the Japanese population and accumulation of visceral fat, even in the non-obese, may play a central role in the metabolic syndrome.[34] In the management of the metabolic syndrome, appropriate biomarkers are needed and inflammatory markers, including C-reactive protein, are candidates in Japanese also.[35,36] Adiponectin will be a novel biomarker for the metabolic syndrome.

Weight reduction increases the plasma adiponectin concentration; 21% reduction in BMI resulted in a 46% increase of plasma adiponectin concentration in obese subjects.[37] Therapeutic agents (ie, adiponectin promoters) can elevate the plasma adiponectin concentration. In mice and humans adiponectin promoters have a nonclassical peroxisome proliferator responsive element, which activates receptor gamma ligands, thiazolidinediones, promoting adiponectin activity and raise its plasma concentration.[38] It has also been reported, still controversially, that blockade of the renin–angiotensin system[39] and the sulfonylurea reagent, glimepiride,[40] increase the plasma adiponectin concentration, but whether these reagents can reduce the incidence of CAD in subjects with the metabolic syndrome, and whether the increase of plasma adiponectin induced by these reagents participates in the reduction of CAD, needs to be clarified in large-scale clinical trials.

In countries exposed to overnutrition, hypoadiponectinemia may become a secondary target for the prevention of atherosclerotic vascular diseases beyond hypercholesterolemia, and the measurement of plasma adiponectin may be useful for management of the metabolic syndrome.

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Adiponectin Stimulates Angiogenesis in Response to Tissue Ischemia through Stimulation of AMP-activated Protein Kinase Signaling*

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Obesity is a risk factor for the development of cardiovascular diseases that are associated with impaired angiogenesis. Adiponectin is an adipocyte-specific adipocytokine with anti-atherogenic and anti-diabetic properties, and its plasma levels are reduced in association with obesity-linked diseases. Here, we investigated whether adiponectin regulates angiogenesis in response to tissue ischemia using adiponectin knock-out (KO) mice. Angiogenic repair of ischemic hind limbs was impaired in adiponectin-KO mice compared with wild-type (WT) mice as evaluated by laser Doppler flow method and capillary density analyses. Adenovirus-mediated supplement of adiponectin accelerated angiogenic repair in both adiponectin-KO and WT mice. Intramuscular injection of an adenovirus encoding dominant-negative AMP-activated kinase diminished the improvement in limb perfusion seen in WT mice and abolished the adiponectin-induced enhancement of perfusion. These data indicate that adiponectin can function to stimulate angiogenesis in response to ischemic stress by promoting AMP-activated kinase signaling. Therefore, adiponectin may be useful in the treatment for obesity-related vascular deficiency diseases.

Obesity contributes to the development of metabolic syndrome and type 2 diabetes (1). Both of these conditions are associated with microvascular rarefaction and reduced collateralization in ischemic tissues (2–5). These circulatory changes can lead to cardiac dysfunction, increased vulnerability to ischemic injury, and impaired wound healing. Presumably obesity-related disorders contribute to the pathogenesis of vascular complications by perturbing the levels of angiogenesis regulatory factors or by decreasing the responsiveness of tissues to proangiogenic agents.

Adiponectin/ACRP30 is a circulating adipocyte-derived cytokine that is down-regulated in association with obesity-linked diseases including coronary artery diseases (6, 7), metabolic syndrome (8), and type 2 diabetes (9). Adiponectin knock-out (KO)[1] mice exhibit diet-induced insulin resistance that is associated with diminished insulin receptor substrate 1-mediated phosphatidylinositol 3-kinase signaling in muscle (10). These mice also display increased intimal hyperplasia in response to acute injury (11) and impaired endothelium-dependent vasodilation (12). Conversely, adiponectin overexpression reduces atherosclerotic lesions in a mouse model of atherosclerosis (13) and has anti-inflammatory effects on the vasculature (6, 14, 15). Collectively, these data suggest that adiponectin acts as a biologically relevant modulator of vascular remodeling with anti-atherogenic and anti-diabetic properties.

In the present study, we investigated whether adiponectin modulates the angiogenic process *in vivo* employing the hind limb model of ischemia-induced angiogenesis in mice. The ability of adiponectin to regulate angiogenesis was investigated with loss- and gain-of-function genetic manipulations using adiponectin-KO mice and an adenoviral vector that expresses adiponectin. Our observations indicate that adiponectin promotes angiogenesis in response to tissue ischemia via the activation of myogenic AMPK-dependent signaling.

EXPERIMENTAL PROCEDURES

Materials—Phospho-AMPK (Thr-172) was purchased from Cell Signaling Technology (Beverly, MA). Tubulin antibody was purchased from Oncogene (Cambridge, MA). Adenovirus vectors containing the gene for β-galactosidase (Ad-βgal), full-length mouse adiponectin (Ad-APN), and dominant-negative AMPKα2 (Ad-dnAMPK) were described previously (11, 16, 17).

Mouse Model of Angiogenesis—Adiponectin-KO (APN-KO) and wild-type (WT) mice in a C57/BL6 background were used for this study (10). Study protocols were approved by the Institutional Animal Care and Use Committee at Boston University. Mice at the ages of 8–11 weeks were subjected to unilateral hind limb surgery under anesthesia with sodium pentobarbital (50 mg/kg intraperitoneally). In this model, the entire left femoral artery and vein were excised surgically (18). Before surgery and on postoperative days 3, 7, 14, and 28, body weight and systolic blood pressure were determined using a tail cuff pressure analysis system in the conscious state.

Measurement of Plasma—Blood samples were collected by heart puncture from mice on postoperative day 7. Total cholesterol, high density lipoprotein cholesterol, and glucose levels were measured with enzymatic kits, and insulin levels were assayed with an enzyme immunoassay kit (Wako Chemicals, Richmond, VA). Mouse adiponectin levels were determined with adiponectin enzyme-linked immunosorbent assay kits (Otsuka Pharmaceutical Co. Ltd., Tokyo, Japan). For this measurement blood was collected from the tail vein at the time of hind

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[1] The abbreviations used are: KO, knock-out; dn, dominant-negative; AMPK, AMP-activated kinase; APN, adiponectin; WT, wild-type; LDBF, laser Doppler blood flow; Ad, adenovirus.

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TABLE I
Characteristics of WT and APN-KO mice

Measurements were made at 7 days postsurgery in mice that were fasted for 6 h (n = 5). Each value is mean ± S.E. BW, body weight (g); sBP, systolic blood pressure (mm Hg); TC, total cholesterol (mg/dl); HDL-C, high density lipoprotein cholesterol (mg/dl); PG, plasma glucose (mg/dl); IRI, immunoreactive insulin (ng/ml); APN, adiponectin (μg/ml).

	BW	sBP	TC	HDL-C	PG	IRI	APN
WT	29.2 ± 1.0	96 ± 3	79.0 ± 9.2	55.7 ± 10.1	137.2 ± 23.4	0.24 ± 0.16	7.53 ± 0.93
APN-KO	28.3 ± 1.3	97 ± 3	82.7 ± 6.1	52.7 ± 12.0	139.5 ± 22.1	0.27 ± 0.20	<0.05

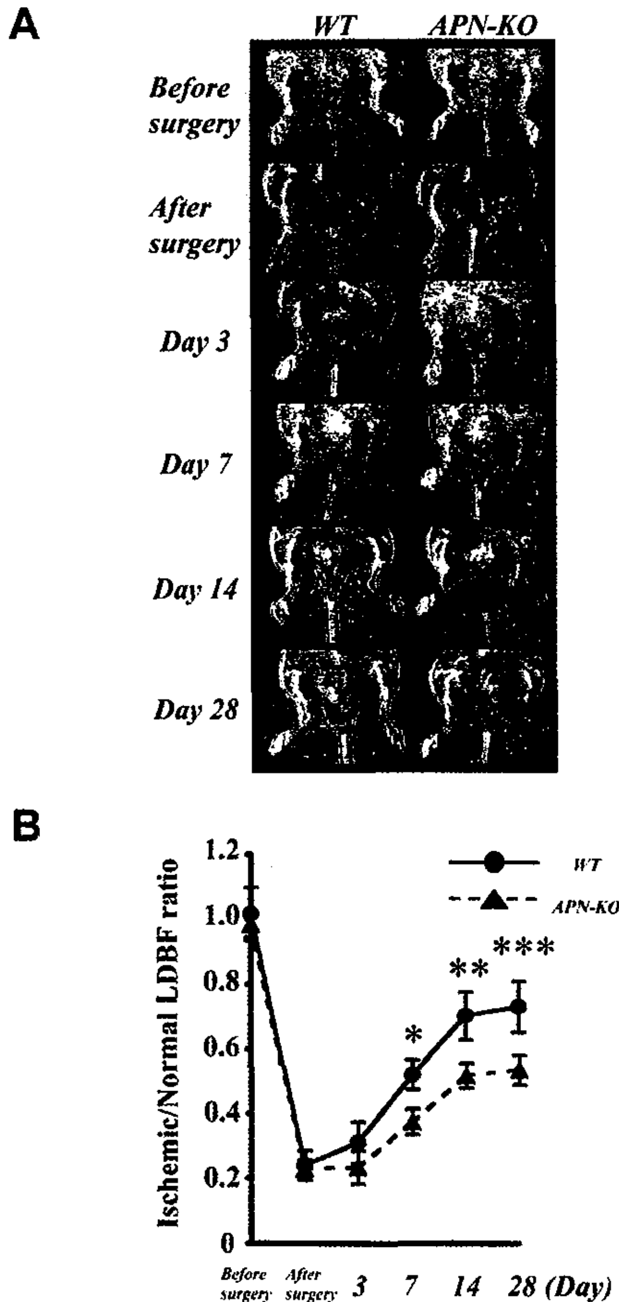

FIG. 1. **Impaired angiogenic response in the ischemic hind limbs of adiponectin-KO mice.** *A*, a low perfusion signal (*dark blue*) was observed in the ischemic hind limbs of APN-KO mice, whereas a high perfusion signal (*white* to *red*) was detected in WT mice on postoperative days 7, 14, and 28. *B*, quantitative analysis of the ischemic/nonischemic LDBF ratio in the WT (*circle*) and APN-KO (*triangle*) mice on postoperative days 7, 14, and 28 (n = 6). *, p < 0.001; **, p < 0.01; ***, p < 0.05 *versus* APN-KO mice.

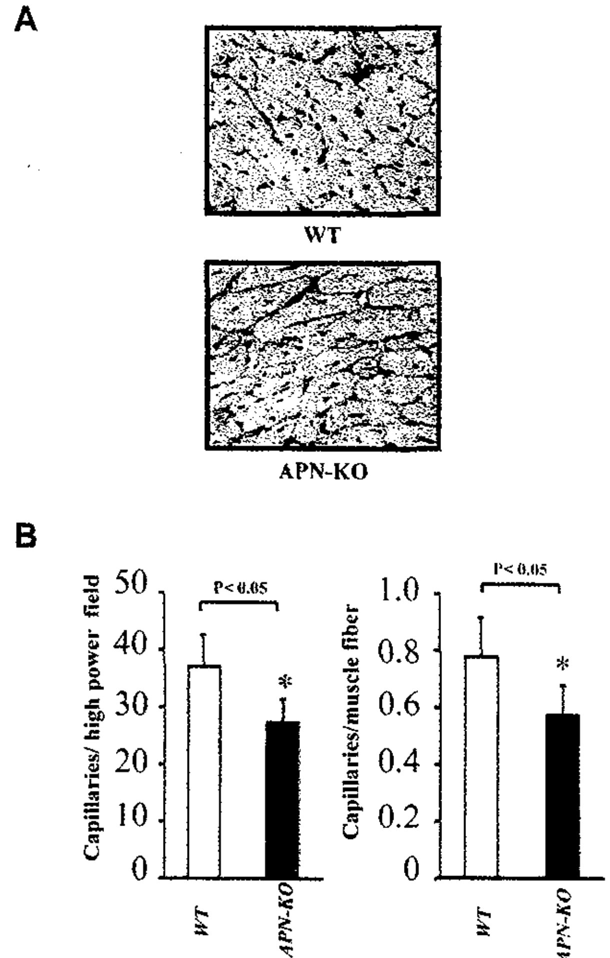

FIG. 2. **Reduced capillary density in ischemic APN-KO mice.** *A*, immunostaining of ischemic tissues with anti-CD31 monoclonal antibody (*brown*) on postoperative day 14. *B*, quantitative analysis of capillary density in WT and APN-KO mice on postoperative day 14 (n = 6 in each group). Capillary density was expressed as the number of capillaries per high power field (×400, *left*) and capillaries per muscle fiber (*right*). *, p < 0.05 *versus* WT mice.

limb surgery, which was 3 and 10 days after the administration of the adenoviral vectors.

Laser Doppler Blood Flow Analysis—Hind limb blood flow was measured using a laser Doppler blood flow (LDBF) analyzer (Moor LDI; Moor Instruments, Devon, UK). Immediately before surgery and on postoperative days 0, 3, 7, 14, and 28, LDBF analysis was performed on legs and feet. Blood flow was displayed as changes in the laser frequency using different color pixels. After scanning, stored images were analyzed to quantify blood flow. To avoid data variations caused by ambient light and temperature, hind limb blood flow was expressed as the ratio of left (ischemic) to right (nonischemic) LDBF.

Capillary Density Analysis—Capillary density within the thigh adductor muscle was quantified by histological analysis. Muscle samples were imbedded in OCT compound (Miles, Elkhart, IN) and snap frozen in liquid nitrogen. Tissue slices (5 μm in thickness) were prepared, and capillary endothelial cells were identified by immunohistostaining for CD31 (PECAM-1; BD Biosciences). Fifteen randomly chosen microscopic fields from three different sections in each tissue block were examined for the presence of capillary endothelial cells for each mouse specimen. Capillary density was expressed as the number of CD31-positive features per high power field (×400) and the number of capillaries per muscle fiber.

TABLE II
Plasma adiponectin levels in each experimental group
All values are adiponectin (μg/ml). Measurements were made at 3 and 10 days postgene transfer in mice that were fasted for 6 h (n = 5).

	WT	WT + β3gal	WT + APN	APN-KO	APN-KO + βgal	APN-KO + APN
Day 3	7.53 ± 0.93	7.23 ± 1.44	13.83 ± 1.55	<0.05	<0.05	9.66 ± 1.72
Day 10	7.31 ± 1.06	7.94 ± 0.24	9.8 ± 0.93	<0.05	<0.05	5.03 ± 0.53

Western Blot Analysis—Tissue samples obtained 3 days after surgery were homogenized in lysis buffer containing 20 mM Tris-HCl (pH 8.0), 1% Nonidet P-40, 150 mM NaCl, 0.5% deoxycholic acid, 1 mM sodium orthovanadate, and protease inhibitor mixture (Sigma). Protein content was then determined by the Bradford method (34). Proteins (50 μg) were separated with denaturing SDS 10% polyacrylamide gels. Following transfer to membranes, immunoblot analysis was performed with the indicated antibodies at a 1:1000 dilution. This was followed by incubation with a secondary antibody conjugated with horseradish peroxidase at a 1:5000 dilution. An ECL Plus Western blotting detection kit (Amersham Biosciences) was used for detection. The relative changes in phosphorylated AMPK were normalized to the tubulin signal and expressed as percent relative to control.

Adenovirus-mediated Gene Transfer—For adenovirus experiments ischemic/nonischemic LDBF ratios were examined 14 days after surgery, which is compatible with the time course of adenovirus-mediated gene expression. In some experiments, 2×10^8 plaque-forming units of Ad-APN or Ad-βgal were injected into the jugular vein of mice 3 days prior to being injected into the ischemic hind limb. Alternatively, 2×10^8 plaque-forming units of Ad-dnAMPK or Ad-βgal were injected into five different sites of adductor muscle in the ischemic limb at the same time that 2×10^8 plaque-forming units of Ad-APN or Ad-βgal were injected into the jugular vein of WT mice 3 days prior to surgery.

Statistical Analysis—Data are presented as mean ± S.E. Statistical analysis was performed by analysis of variance followed by Scheffe's F test. A value of $p < 0.05$ was accepted as statistically significant.

RESULTS

Impaired Ischemia-induced Angiogenesis in Adiponectin-KO Mice—All mice survived after surgical induction of unilateral hind limb ischemia and appeared to be healthy during the follow-up period. Body weight and blood pressure did not differ between the two groups (Table I). No significant differences were observed in the plasma concentrations of glucose, insulin, total cholesterol, or high density lipoprotein cholesterol between APN-KO and WT mice (n = 6). Plasma adiponectin levels were not detected in APN-KO mice. Immediately after left femoral artery and vein resection, the ratio of blood flow between the ischemic and nonischemic hind limbs decreased to 0.24 ± 0.04 in WT and 0.23 ± 0.03 in APN-KO, indicating that the severity of the induced ischemia was comparable in the two groups.

Fig. 1*A* shows representative LDBF images of hind limb blood flow before surgery and at different time points after surgery in the WT and APN-KO mice. In WT mice, hind limb blood flow perfusion fell precipitously after surgery, remained impaired for 3 days, increased to 50–60% of the nonischemic limb by day 7, and ultimately returned to 80% of the nonischemic limb by day 28 (Fig. 1*B*). In contrast to WT mice, flow recovery in the APN-KO mice was impaired. Flow in APN-KO mice was significantly less than WT by the 7th day after surgery, and the flow difference persisted at each of the subsequent time points (14 and 28 days).

To investigate the extent of angiogenesis at the microcirculatory level, capillary density was measured in histologic sections harvested from the ischemic tissues. Fig. 2*A* shows representative photomicrographs of tissue immunostained with CD31. Quantitative analysis revealed that on postoperative day 14 the capillary density was significantly reduced in APN-KO mice compared with WT mice (Fig. 2*B*).

Elevated Adiponectin Levels Promote Angiogenesis in Response to Ischemia—To test whether supplementation of adiponectin could modulate ischemia-induced angiogenesis, an adenoviral vector expressing adiponectin (Ad-APN) was delivered via jugular vein at the time of hind limb surgery in WT and APN-KO mice. Plasma adiponectin levels in Ad-APN-treated WT mice increased to a level 2.0 times higher on the 3rd day and 1.3 times higher on the 10th day compared with the Ad-βgal-treated WT mice (Table II). In APN-KO mice, Ad-APN treatment restored plasma adiponectin to levels similar to those of Ad-βgal-treated WT mice on day 3, but plasma adiponectin levels fell slightly by day 10.

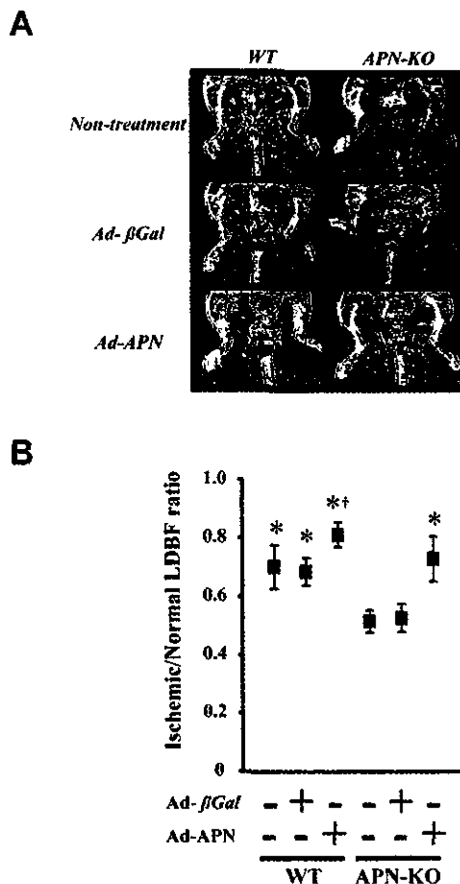

FIG. 3. **Exogenous adiponectin improves perfusion of ischemic limbs in wild-type and adiponectin-KO mice.** *A*, representative LDBF images showing improved perfusion in ischemic limb adenovirus-mediated adiponectin following delivery in WT and APN-KO mice on postoperative day 14. Adenoviral vectors expressing adiponectin or β-galactosidase (control) were delivered intravenously via the jugular vein 3 days prior to surgery. *B*, quantitative analysis of the ischemic/nonischemic LDBF ratio in WT and APN-KO mice with or without Ad-βgal and Ad-APN on postoperative day 14. *, $p < 0.05$ *versus* APN-KO mice without Ad-βgal and Ad-APN; †, $p < 0.01$ *versus* WT mice without Ad-βgal and Ad-APN; n = 5.

Ad-APN-treated WT mice showed a significant increase in limb perfusion 14 days after hind limb surgery compared with control mice that were treated with Ad-βgal (Fig. 3, *A* and *B*) ($p < 0.05$; n = 5). Furthermore, treatment with Ad-APN promoted hind limb perfusion in APN-KO mice to levels similar to those of WT mice (Fig. 3, *A* and *B*) ($p < 0.01$; n = 5). These results demonstrate that the adenovirus-mediated supplementation of adiponectin can rescue the hemodynamic deficit that

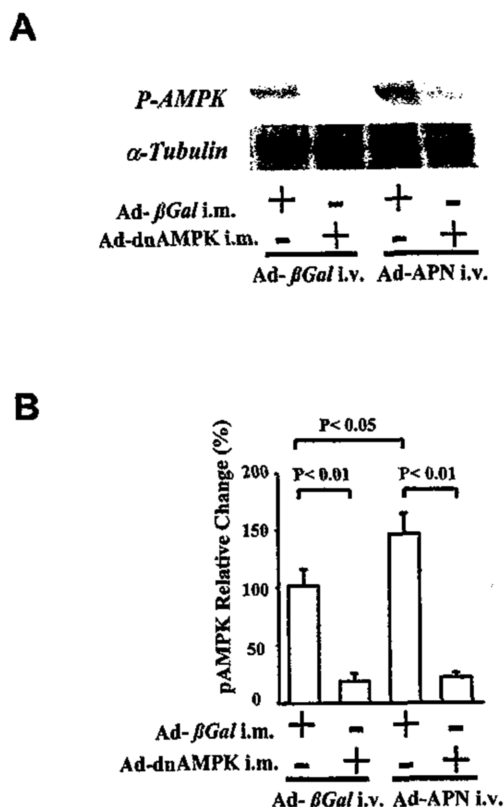

FIG. 4. **Intramuscular injection of Ad-dnAMPK reduces basal and adiponectin-stimulated AMPK signaling in ischemic adductor muscle.** The indicated adenoviral vectors were injected into five sites in the adductor muscle (2 × 10^{8} plaque-forming units total) 3 days prior to ischemic surgery. At the same time the indicated adenoviral vectors were delivered intravenously via the jugular vein. Western immunoblots with the indicated antibodies were performed on the ischemic adductor muscle 3 days postsurgery. *A*, representative immunoblots for phospho-AMPK (*P-AMPK*) in the presence of intravenously (*i.v.*) injected Ad-APN or Ad-βgal along with intramuscular injection (*i.m.*) of Ad-dnAMPK or Ad-βgal on day 3 after induction of limb ischemia. *B*, quantitative analysis of relative changes in phospho-AMPK (*pAMPK*) levels on day 3 after induction of limb ischemia relative to the tubulin signal as a percentage of phospho-AMPK in the ischemic adductor muscle of control mice (*Ad-βgal i.v.* × *Ad-βgal i.m.*). $n = 5$.

is seen in the APN-KO mice and that overexpression of APN can promote angiogenesis in WT mice.

Role of AMPK Signaling on Ischemia-induced Angiogenesis—To examine the role of the AMPK pathway in ischemia-induced angiogenesis, Ad-dnAMPK was injected intramuscularly into the adductor muscle of the ischemic limb at the time of surgery. APN treatment stimulated AMPK phosphorylation in the ischemic adductor muscle (Fig. 4, *A* and *B*). The intramuscular injection of Ad-dnAMPK significantly reduced both basal and adiponectin-induced AMPK phosphorylation.

Intramuscular injection of Ad-dnAMPK also led to a significant reduction in the improvement in limb perfusion in control mice that received an intravenous infusion of Ad-βgal (Fig. 5). Of note, the increase in hind limb blood flow induced by infusion of Ad-APN was abrogated by Ad-dnAMPK. These data indicate that intramuscular AMPK signaling is required for neovascularization following ischemic injury and is essential for the improvement in angiogenesis that occurs in response to exogenous adiponectin.

DISCUSSION

The present study provides *in vivo* evidence that adiponectin plays an important role in the process of ischemia-induced angiogenesis. Angiogenic involvement was demonstrated in a

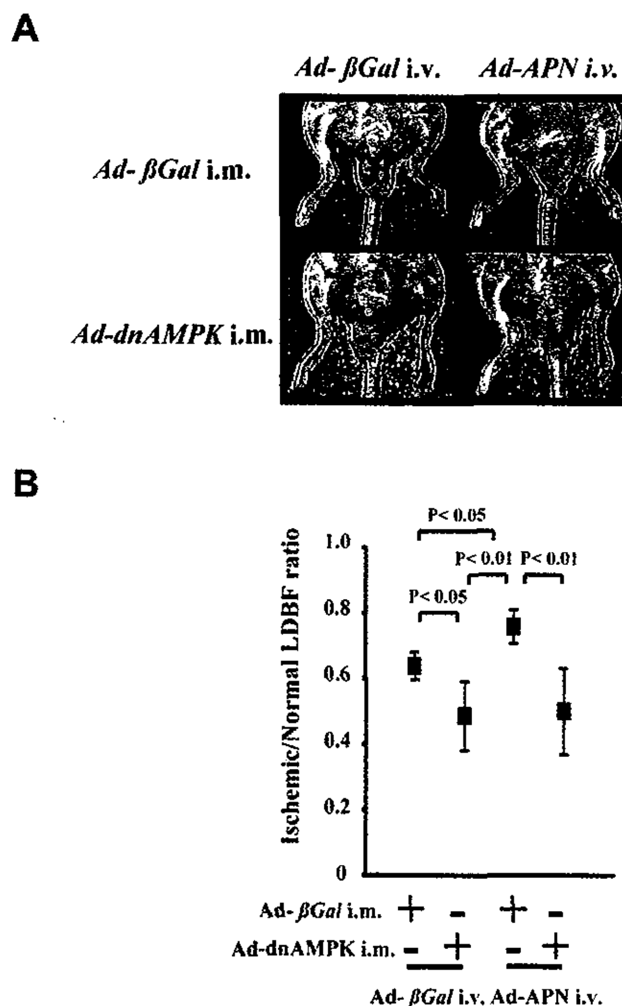

FIG. 5. **AMPK signaling is required for basal and adiponectin-induced angiogenesis in ischemic hind limbs.** *A*, representative LDBF images of the effects of intramuscular delivery of Ad-dnAMPK on hind limb perfusion at 14 days postsurgery. *B*, quantitative analysis of ischemic/nonischemic LDBF ratio hind limb blood perfusion at 14 days postsurgery in WT mice treated with intravenously (*i.v.*) injected Ad-APN or Ad-βgal along with intramuscular (*i.m.*) injection of Ad-dnAMPK or Ad-βgal. All adenoviral vectors were delivered 3 days prior to surgery. The intramuscular injections were performed in the adductor muscle of the ischemic limb. Ad-βgal i.v. × Ad-βgal i.m. group *versus* Ad-APN i.v. × Ad-βgal i.m. group, $p < 0.05$; Ad-βgal i.v. × Ad-βgal i.m. group *versus* Ad-βgal i.v. × Ad-dnAMPK i.m. group, $p < 0.05$; Ad-APN i.v. × Ad-βgal i.m group *versus* Ad-APN i.v. × Ad-dnAMPK i.m. group, $p < 0.01$. $n = 5$.

well established hind limb ischemia model. Adiponectin-KO mice showed impaired recovery of limb perfusion following femoral artery and vein removal, and exogenous adiponectin could rescue the impairment in limb reperfusion. Moreover, wild-type mice supplemented with adiponectin displayed a more rapid recovery of limb perfusion compared with control mice.

The ability of adiponectin to promote angiogenesis is probably due to its ability to stimulate the AMPK-dependent signaling within muscle. Intramuscular injection of an adenoviral vector expressing dominant-negative AMPK reduced both basal and adiponectin-stimulated AMPK signaling in the adductor muscle of the ischemic hind limb. Furthermore, dominant-negative AMPK reduced both basal and adiponectin-stimulated improvements in limb revascularization. AMPK is a stress-activated protein kinase that participates in the regulation of energy and metabolic homeostasis (19, 20). Adiponectin functions as an AMPK activator in multiple cell types including

skeletal muscle, liver, adipocytes, and endothelial cells (21–25). With regard to skeletal muscle, adiponectin-induced AMPK signaling increases glucose metabolism and fatty acid oxidation and promotes insulin sensitivity (24). These conditions promote phosphatidylinositol 3-kinase-Akt signaling within muscle (10, 26), which could lead to angiogenic growth factor synthesis (27).

Adiponectin could also exert a proangiogenic effect in ischemic tissue by acting directly on the vascular endothelium. It has been shown that AMPK signaling mediates adiponectin-induced angiogenic and anti-apoptotic cellular responses in endothelial cells (21, 28). In addition, we have demonstrated that AMPK signaling in endothelial cells is essential for angiogenic cellular responses under conditions of hypoxia *in vitro* (17). In contrast to these findings, it has recently been reported that adiponectin inhibits tumor neovascularization through its ability to induce endothelial apoptosis (29). The reason for this discrepancy is unclear. It is possible that adiponectin differentially affects pathological and physiological angiogenesis as has been described for the effects of statins on vascularization (30). Alternatively, differences in experimental models or endothelial cell types may explain these discrepancies. However, all previous studies evaluating the effects of adiponectin on endothelial cell biology have used recombinant adiponectin proteins that may be subject to preparation-to-preparation variability. Therefore, this study was designed to analyze the role of adiponectin with the exclusive use of loss- and gain-of-function genetic manipulations. Collectively, multiple lines of evidence suggest that adiponectin is a proangiogenic regulator. This hypothesis is further supported by the observation that AMPK signaling is anti-apoptotic in a variety of cell types (31, 32) including endothelial cells (17, 28) and that AMPK-mediated endothelial nitric-oxide synthase phosphorylation (33) will favor a proangiogenic phenotype (18).

Collateral vessel development is impaired in patients and animal models with obesity-related disorders (2–5). The findings reported here suggest that hypoadiponectinemia may contribute the vascular insufficiency that limits blood flow to distal vessels in obese individuals. Taken together, these data suggest that the exogenous supplementation of adiponectin could be beneficial treatment for obesity-related vascular disorders.

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Hypoadiponectinemia Is an Independent Risk Factor for Hypertension

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Abstract—Adiponectin is one of the key molecules in the metabolic syndrome, and its concentration is decreased in obesity, type-2 diabetes, and coronary artery disease. Genetic investigation has revealed that 2 polymorphisms (I164T and G276T) are related to adiponectin concentration and diabetes. To examine whether adiponectin affects hypertension genetically or biologically, we performed a case-control study. A total of 446 diagnosed cases of hypertension (HT) in men and 312 normotensive (NT) men were enrolled in this study. Plasma adiponectin concentration was measured using an enzyme-linked immunosorbent assay system. Single nucleotide polymorphisms were determined by TaqMan polymerase chain reaction method. After adjustment for confounding factors, adiponectin concentration was significantly lower in HT (HT: 5.2±0.2 μg/mL; NT: 6.1±0.2 μg/mL; $P<0.001$). Furthermore, multiple regression analysis indicated that hypoadiponectinemia was an independent risk factor for hypertension ($P<0.001$). Blood pressure was inversely associated with adiponectin concentration in normotensives regardless of insulin resistance. In subjects carrying the TC genotype of the I164T polymorphism, adiponectin concentration was significantly lower (TC: 2.6±0.9 μg/mL; TT: 5.5±0.1 μg/mL; $P<0.01$), and most of them had hypertension. In contrast, the G276T polymorphism was not associated with adiponectin concentration or hypertension. In conclusion, hypoadiponectinemia is a marker for predisposition to hypertension in men. (***Hypertension*. 2004;43:1318-1323.**)

Key Words: blood pressure ■ genetics ■ hypertension, genetic ■ men ■ mutation

Adipose tissue participates in the regulation of a variety of homeostatic processes as an endocrine organ that secretes many biologically active molecules such as leptin, tumor necrosis factor-α, and plasminogen-activator inhibitor type 1, which contribute to the development of cardiovascular disease.[1–5] Furthermore, some of these molecules, such as leptin and plasminogen-activator inhibitor type 1, are known to contribute to the development of hypertension.[6–8] Adiponectin is an adipose tissue-specific collagen-like factor, which is abundant in plasma, and a decrease of adiponectin is associated with obesity[9] and type-2 diabetes.[10] Adiponectin modulates the endothelial inflammatory response in vitro, and its concentration is decreased in patients with coronary artery disease.[10–12] Furthermore, adiponectin has been reported to be associated with lipid metabolism,[13,14] glucose metabolism,[15] and insulin resistance.[13,14,16] It was recently reported that treatment of diabetic animals with adiponectin markedly improved insulin sensitivity via reducing triglyceride accumulation in skeletal muscle.[17] These results suggest that adiponectin is one of the key molecules in the metabolic syndrome.

Hypertension is a common disease that increases the risk for cardiovascular disease, and it is also a component of the metabolic syndrome, which is defined as the combination of obesity, insulin resistance, glucose intolerance, and hyperlipidemia. Hypertensive patients are known to have higher body mass index (BMI), triglyceride level, and insulin resistance compared with normotensive subjects.[18] Even though an association between hypertension and serum adiponectin concentration has been reported by several groups using a small number of subjects,[19–22] the obtained results were not identical. Mallamaci et al[19] reported an increased plasma adiponectin concentration in hypertensive patients with renal dysfunction, but Adamczak et al[20] reported decreased adiponectin in hypertensive subjects. Kazumi et al[21] reported that young Japanese men with high-normal blood pressure had lower adiponectin. Recently, Furuhashi et al[22] reported that only hypertensive patients with insulin resistance showed lower adiponectin concentration. Furthermore, in these studies, the association between plasma adiponectin and hypertension was evaluated without adjusting for confounding factors or without dividing the subjects by sex. It is well known that normal women have a higher adiponectin concentration than men,[23] so sex is a potential confounding factor. Thus, the clinical importance of hypoadiponectinemia in hypertension has not been fully elucidated.

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On the other hand, a genetic investigation revealed that subjects with the I164T polymorphism (T-to-C substitution at nucleotide 517 leading to amino acid substitution from isoleucine to threonine at position 164) more frequently had diabetes and had lower concentrations of adiponectin. It was interesting that all 9 patients with the I164T polymorphism had hypertension.[16] In addition, another report showed that the G276T polymorphism in intron 2 was also associated with type-2 diabetes, partially through affecting plasma adiponectin concentration.[24]

To examine whether adiponectin affects blood pressure genetically or biologically, we performed a case-control study using a large number of subjects. In addition, we confirmed the hypothesis that hypoadiponectinemia is correlated with increased insulin resistance.

Methods

Subjects

A total of 758 male subjects (mean age 58.4±0.4 years, BMI 23.9±0.1 kg/m^2) were selected from people who were admitted and underwent medical investigation at Osaka University Hospital or its affiliated hospitals. The numbers of normotensive subjects and hypertensive subjects were 312 and 446, respectively. Hypertension was defined as a systolic blood pressure of ≥140 mm Hg and/or a diastolic blood pressure of ≥90 mm Hg on repeated measurements, or receiving antihypertensive treatment. Diabetes was defined as fasting plasma glucose of ≥7.0 mmol/L or receiving treatment for diabetes. All subjects enrolled were Japanese, and subjects with ischemic heart disease including myocardial infarction, congestive heart failure, abnormal electrocardiogram results, valvular heart disease, atrial fibrillation, arteriosclerosis obliterans, or renal failure were excluded. The study protocol was approved by the Ethical Committee of Osaka University, and subjects gave informed consent to participate in the present study, including genetic analysis.

Clinical Features

Blood pressure was measured with an appropriate arm cuff and a mercury column sphygmomanometer on the left arm after a resting period of at least 10 minutes in the supine position. Blood pressure was measured by well-trained physicians who were blinded during the study, and 3 measurements at 1 visit were averaged to evaluate the systolic and diastolic blood pressures. After blood pressure measurements, venous blood sampling from all subjects was performed after fasting overnight. Height and body weight were measured, and BMI was calculated. Plasma samples for subsequent assay were stored at −80°C. Insulin sensitivity was estimated using the homeostatic model assessment (HOMA) index (ie, plasma glucose level×[plasma insulin level/22.5]). Insulin resistance was defined as HOMA ≥3. Plasma concentration of adiponectin was determined by a sandwich enzyme-linked immunosorbent assay system (adiponectin ELISA kit; Otsuka Pharmaceutical Co. Ltd.) as previously reported.[9]

The following parameters were also determined: total cholesterol (T-chol), triglyceride (TG), high-density lipoprotein cholesterol (HDL-chol), and serum creatinine (Cr) levels.

Genotype Determination of Adiponectin Polymorphisms

To investigate the association between adiponectin polymorphisms and hypertension, we selected 2 polymorphisms (I164T and G276T) that were previously reported to be related to plasma adiponectin concentration.[16,24] Genomic DNA was prepared from the buffy coat using a QIAmp DNA blood kit (QIAGEN, Valencia, Calif). The genotypes of the I164T and G276T polymorphisms were determined by the TaqMan polymerase chain reaction (PCR) method.[25] The following primers and probes were included in the reactions: I164T, forward primer, 5′-AAC ATT CCT GGG CTG TAC TAC TTT G-3′; reverse primer, 5′-GGC TGA CCT TCA CAT CCT TCA TA-3′; probes, 5′-FAM-CCA CAC CAC AGT CT-3′, 5′-VIC-ACC ACA TCA CAG TCT A-3′; G276T, forward primer, 5′-AGA ATG TTT CTG GCC TCT TTC ATC-3′; reverse primer, 5′-TTC TCC CTG TGT CTA GGC CTT AGT-3′; probes, 5′-FAM-AAA CTA TAT GAA GTC ATT CAT TA-3′, 5′-VIC-CTA TAT GAA GGC ATT CAT TA-3′. The fluorescence level of PCR products was measured using an ABI PRISM 7900 HT Sequence Detector (Applied Biosystems).

TABLE 1. Clinical Characteristics of Study Subjects

Characteristics	HT (n=446)	NT (n=312)
Age, y	59.4±0.5	57.1±0.6*
BMI, kg/m^2	24.4±0.1	23.1±0.2*
Systolic BP, mm Hg	138±1	119±1*
Diastolic BP, mm Hg	83±1	72±1*
Adiponectin, μg/mL	5.2±0.2	6.4±0.2*
T-chol, mmol/L	5.34±0.06	5.17±0.05†
TG, mmol/L	1.77±0.05	1.65±0.07
HDL-chol, mmol/L	1.32±0.02	1.32±0.03
FPG, mmol/L	6.24±0.11	5.98±0.13
HbA1c, %	5.7±0.1	5.7±0.1
HOMA	2.4±0.2	2.1±0.2
Cr, μmol/L	84.6±3.2	90.6±4.2

Values are given as mean±SE. FPG, indicates fasting plasma glucose; other definitions are provided in the text.

*$P<0.01$ and †$P<0.05$ compared with hypertensive subjects for each parameter.

Statistical Analysis

Values are expressed as mean±SE. Associations between hypertension and all other parameters were first analyzed by simple logistic regression and then by multivariate analysis. Differences in genotypes and alleles were examined by χ^2 analysis. The association between polymorphisms and clinical variables was examined by multivariate analysis. The quantitative effects of covariates were assessed by multiple regression analysis. $P<0.05$ was considered statistically significant. All calculations were performed using a standard statistical package (JMP 4.0; SAS Institute Inc).

Results

Plasma Adiponectin Concentration and Hypertension

The average length of time since the first diagnosis of hypertension was 12.5±0.6 years. Furthermore, 342 of 758 hypertensive subjects also had close relatives (parents, brothers, and sisters) who were hypertensive. To assess whether adiponectin was related to hypertension, we compared the clinical characteristics of hypertensive male subjects (HT) and normotensive male subjects (NT) (Table 1). Plasma adiponectin concentration was significantly lower in hypertensive subjects than in normotensive subjects. Age, BMI, and T-chol were also significantly higher in hypertensive men than in normotensive men. Consequently, we selected these parameters as confounding factors. After adjustment for confounding factors (age, BMI, and T-chol), adiponectin concentration was significantly lower in HT (HT: 5.2±0.2 μg/mL; NT: 6.1±0.2 μg/mL; $P<0.001$). Multiple regression analysis revealed that each confounding factor, age, BMI,

TABLE 2. Multiple Logistic Regression Analysis for Hypertension

Term	Estimate	SE	*P*
Age	−0.0497	0.0086	<0.0001
BMI	−0.1144	0.0293	<0.0001
Adiponectin	0.1017	0.0278	0.0003
T-chol	−0.0048	0.0023	0.0374
Intercept	3.7284	1.0341	0.0003

R^2=0.0754 (n=758).

T-chol, and adiponectin concentration, independently affected the risk for hypertension (Table 2).

We examined simple correlations between plasma adiponectin concentration and clinical variables. The hypertensive subjects were divided into 2 groups: with and without antihypertensive medication; the normotensive subjects were divided into 3 subgroups: with diabetes, with insulin resistance (HOMA≥3) but without diabetes, and without insulin resistance or diabetes. Thus, we compared the clinical variables among 5 subgroups (Table 3). Adiponectin concentration significantly increased with age (in hypertensives using medication and normotensives without diabetes or insulin resistance, $P<0.01$, respectively) and HDL-chol (in hypertensives using medication and normotensives without diabetes, $P<0.01$, respectively), and decreased with BMI (in hypertensives using medication and normotensives, $P<0.01$, respectively) and TG (in hypertensives using medication and normotensives with diabetes, $P<0.01$, respectively). Systolic blood pressure was inversely associated with adiponectin concentration in normotensive subjects without diabetes ($P<0.01$). Diastolic blood pressure was inversely associated with adiponectin concentration in normotensive subjects ($P<0.01$). The association between plasma adiponectin concentration and blood pressure in normotensive subjects without diabetes is shown in Figure 1. However, adiponectin concentration was not associated with blood pressure in hypertensives without medication (Table 3).

TABLE 3. Simple Correlations Between Plasma Adiponectin Concentration and Clinical Characteristics

	Hypertensives		Normotensive		
	Medication		Diabetes	Insulin Resistance	
Characteristics	(+) (n=367)	(−) (n=79)	(+) (n=67)	(+) (n=93)	(−) (n=152)
Age	0.21*	0.26†	0.22	0.17	0.44*
BMI	−0.19*	−0.12	−0.36*	−0.36*	−0.37*
T-chol	−0.05	−0.09	−0.20	−0.11	−0.09
TG	−0.21*	−0.18	−0.43*	−0.19	−0.20†
HDL-chol	0.18*	0.29†	0.11	0.27*	0.34*
FPG	−0.06	−0.15	−0.15	−0.32*	−0.10
HbA1C	−0.03	−0.04	−0.18	−0.04	−0.03
HOMA	−0.21†	−0.13	−0.18	−0.25†	−0.25†
Cr	0.15†	0.17	0.48†	0.03	0.07
SBP		−0.02	−0.32†	−0.35*	−0.31*
DBP		−0.05	−0.44*	−0.38*	−0.38*

Data indicates correlation coefficient. FPG indicates fasting plasma glucose; other definitions are defined in the text.

*$P<0.01$ and †$P<0.05$.

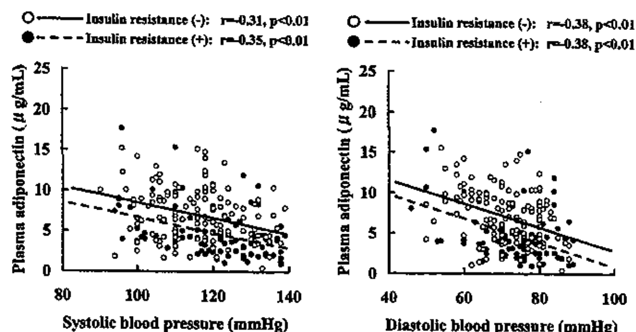

Figure 1. Correlation between plasma adiponectin concentration and blood pressure in normotensives without diabetes. ● indicates subjects with insulin resistance (n=93); ○, subjects without insulin resistance (n=152).

Polymorphisms of Adiponectin and Hypertension

We examined the association between the I164T and G276T polymorphisms and plasma adiponectin concentration. After adjustment for confounding factors (age, BMI, TG, HDL-chol, and HOMA), plasma adiponectin concentration was significantly lower in subjects with the TC genotype of the I164T polymorphism compared with those with the TT genotype (TC: 2.6±0.9 μg/mL; TT: 5.5±0.1 μg/mL; $P<0.01$). No subject with the CC genotype was found in this study. The G276T polymorphism was not significantly related to plasma adiponectin concentration (GG: 5.4±0.2 μg/mL; GT: 5.8±0.2 μg/mL; TT: 4.9±0.4 μg/mL; NS) (Figure 2). We also examined the influence of these polymorphisms on the prevalence of hypertension by case-control study. The G276T polymorphism showed no association with hypertension. Table 4 shows that the TC genotype of the I164T polymorphism was significantly associated with hypertension.

Discussion

The initial finding of the present study was that plasma adiponectin concentration was significantly lower in men

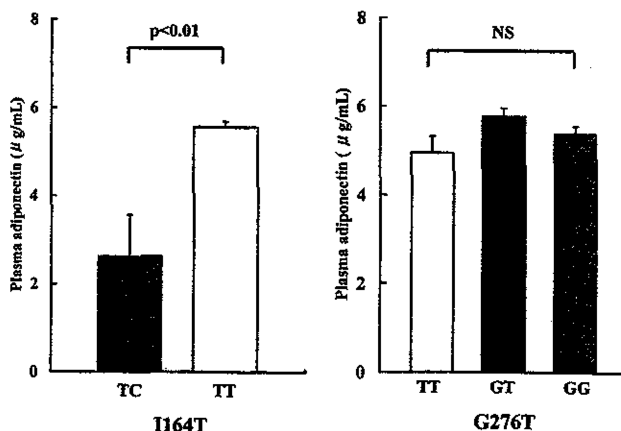

Figure 2. Plasma adiponectin concentration in subjects with I164T and G276T polymorphisms, after adjustment for confounding factors (age, BMI, triglyceride, HDL cholesterol, and homeostatic model assessment index). Data represent mean±SEM.

TABLE 4. Frequencies of Genotypes of Adiponectin Polymorphisms

Polymorphisms		HT	NT	χ^2	P
I164T, n	TT	433	311	6.815	0.009
	TC	13	1		
	GG	225	165		
G276T, n	GT	180	124	0.950	0.622
	TT	41	23		

with hypertension than in normotensive men and was negatively correlated with blood pressure in subjects without hypertension. Furthermore, multiple regression analysis clearly showed that hypoadiponectinemia is an independent risk factor for hypertension. Even though several studies have examined plasma adiponectin level, most of them focused on insulin resistance or diabetes and not on hypertension.

Our results were in accordance with the previous report that HOMA was significantly related to adiponectin concentration.[24] Recently, Furuhashi et al[22] reported that only hypertensive patients with insulin resistance showed a decreased adiponectin concentration. However, the cause–effect relationship among hypoadiponectinemia, insulin resistance, and hypertension has not been clearly elucidated. Even though the consensus has been that insulin resistance is correlated with hypertension,[26,27] the association between insulin and hypertension is controversial.[28–31] In fact, HOMA was not significantly different between hypertensive and normotensive subjects in the present study. As a specific finding of this study, plasma adiponectin level significantly decreased with an increase in blood pressure, even in the normotensives without insulin resistance or diabetes. These results indicate that hypoadiponectinemia may affect the pathogenesis of hypertension at a very early stage without involving insulin resistance. Recently, Lindsay et al[32] reported that there were loci on chromosomes 2, 3, 9, and 10 affecting the circulating adiponectin concentration in the Pima population, suggesting the possibility of an unknown modulator of adiponectin level. However, further investigation is required to examine this hypothesis.

There are 4 possible reasons for the negative correlation between hypertension and plasma adiponectin concentration. First, as Ouchi et al[33] recently reported that plasma adiponectin concentration was independently correlated with the vasodilator response to reactive hyperemia, adiponectin concentration could be an independent parameter of endothelial function. Endothelial dysfunction is an important feature of the early stage of atherosclerosis, which is related to pathogenic conditions including hypertension.[34,35] Furthermore, in adiponectin-knockout mice, hypoadiponectinemia causes diet-induced hypertension. Second, an increase in sympathetic nerve activity, which is common in hypertensives,[36] may inhibit adiponectin gene expression via β-adrenergic stimulation.[37] Third, the reciprocal association of adiponectin and high-sensitive C-reactive protein or increased risk of arteriosclerosis suggests that a low adiponectin concentration might enhance the predisposition to hypertension via vascular injury.[10,11] Fourth, activation of the renin-angiotensin system may be induced in adipose tissue by hypoadiponectinemia, resulting in an increase in fat mass and blood pressure.[38,39] However, further investigation is required to examine these hypotheses.

Another important finding of this study was the positive association between plasma adiponectin concentration and age. There is a supportive report that adiponectin was decreased by sex hormones like androgens, which are suppressed with aging.[23] A reduction in adiponectin clearance in older men is another possible reason for the age-related increase in adiponectin concentration. Furthermore, a previous report also suggested that age is an independent regulating factor for adiponectin concentration.[40] However, it is well known that the prevalence of hypertension, insulin resistance, and diabetes increases with age. There may appear to be a discrepancy, but these results lead to the hypothesis that the implication of hypoadiponectinemia in youth is different from that in old age, and adiponectin may exert an insufficient effect without increasing sufficiently with age. The finding of a lower adiponectin concentration in elderly subjects may indicate the existence of a metabolic disorder like "adiponectin resistance." Further investigation is required to examine these hypotheses.

The final finding of our study was related to adiponectin gene polymorphism. We examined 2 polymorphisms that were previously reported to be related to plasma adiponectin concentration in the Japanese population. Subjects with the TC genotype of the I164T polymorphism showed a significantly lower plasma adiponectin concentration, and most of the C allele carriers had hypertension. Furthermore, we also found a significant association between the TC genotype of the I164T polymorphism and hypertension. It seems to be a novel finding that >80% of C164 carriers were hypertensive in a previous study[16] and in the present study. In contrast, we could not find an association between the G276T polymorphism and adiponectin concentration or hypertension. A previous study has shown an association between theG276T polymorphism and adiponectin concentration only in obese subjects (BMI $\geq$26.7 kg/m^2).[24] Because few obese subjects were included in the present study, we could not conclude a lack of association between the G276T polymorphism and adiponectin.

Study Limitations

This study was designed to be cross-sectional and case-controlled, but not prospective. Several important determinants of plasma adiponectin level, such as body fat content and waist circumference, were not measured in our study. Instead of these measurements, we used HOMA to evaluate insulin resistance. In addition, verification of the cause–effect relationship between hypertension and hypoadiponectinemia would require a study design with a cohort base.

It has been reported that renal function, as indicated by creatinine clearance (Ccr), is an independent regulator of adiponectin concentration in hypertensive subjects.[19] In our study, also, adiponectin concentration was significantly associated with Ccr ($r=-0.38$, $P<0.01$). However, the number of subjects whose Ccr was measured was small (n=102) compared with the total number of study subjects (n=758). The mean Ccr was almost the same in normotensive and hypertensive subjects. Therefore, Ccr was not included in the

discussion of the association between adiponectin and hypertension in this study. However, it was revealed that adiponectin concentration was significantly associated with creatinine in hypertensives using medication and normotensives with diabetes (Table 3), suggesting that hyperadiponectinemia is also involved in the progression of renal damage.

In conclusion, the present findings suggest that a lower plasma adiponectin concentration is significantly associated with hypertension. Interestingly, hypoadiponectinemia is one of the risk factors for hypertension and could be a possible target for antihypertensive treatment.

Acknowledgments

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Adiponectin-induced antiangiogenesis and antitumor activity involve caspase-mediated endothelial cell apoptosis

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Obesity is a risk factor for the development of many severe human diseases such as cardiovascular disorders, diabetes, and cancer, which are tightly linked to angiogenesis. The adipose tissue produces several growth factors/hormones including leptin, tumor necrosis factor α, and adiponectin. It has been found that adiponectin levels are reduced in obesity. Here, we report a unique function of adiponectin as a negative regulator of angiogenesis. *In vitro*, adiponectin potently inhibits endothelial cell proliferation and migration. In the chick chorioallantoic membrane and the mouse corneal angiogenesis assays, adiponectin remarkably prevents new blood vessel growth. Further, we demonstrate that the antiendothelial mechanisms involve activation of caspase-mediated endothelial cell apoptosis. Adiponectin induces a cascade activation of caspases-8, -9, and -3, which leads to cell death. In a mouse tumor model, adiponectin significantly inhibits primary tumor growth. Impaired tumor growth is associated with decreased neovascularization, leading to significantly increased tumor cell apoptosis. These data demonstrate induction of endothelial apoptosis as an unique mechanism of adiponectin-induced antiangiogenesis. Adiponectin, as a direct endogenous angiogenesis inhibitor, may have therapeutic implications in the treatment of angiogenesis-dependent diseases.

neovascularization | cancer | adipocyte | endothelium | Acrp30

Obesity has become a global health problem, and it is linked to the development of many angiogenesis-related diseases such as diabetes, cardiovascular disorders, and cancer (1, 2). Several angiogenic growth factors and hormones are produced by the adipose tissue (3), including vascular endothelial growth factor (VEGF), tumor necrosis factor α, and leptin (4–8). Thus adipocyte-derived factors may play critical roles in regulation of global as well as local tissue and organ function by control of angiogenesis. Like any other tissue growth in the body, the expansion of fat mass depends on angiogenesis (9). Thus, the adipose tissue must be able to switch on an angiogenic phenotype to grow.

Switching on angiogenesis usually requires both up-regulation of angiogenic stimulators and down-regulation of angiogenesis inhibitors (10). It is known that actively growing healthy or pathological tissues express high levels of angiogenic factors. However, overexpression of angiogenic factors may not be sufficient to induce angiogenesis. For example, VEGF is expressed at high levels in several quiescent adult tissues that lack active angiogenesis. This observation indicates that up-regulation of angiogenic factor and down-regulation of angiogenesis inhibitors are both necessary to induce angiogenesis. In the adipose tissue, the balance between angiogenic factors and inhibitors has not been studied. Adiponectin is a unique adipocyte-derived hormone, and its functional targets are not only limited to adipose tissues. Indeed, adiponectin accumulates to very high levels in the circulation. It has recently been found that adiponectin may protect against diabetes and arteriosclerosis (11–14). In obese individuals, adiponectin levels are decreased, while active angiogenesis occurs in the adipose tissue (15). Thus we hypothesize that adiponectin might be a negative regulator of angiogenesis. Here, we provide evidence that adiponectin is a direct angiogenesis inhibitor that induces apoptosis in activated endothelial cells. Further, because angiogenesis is critical for tumor growth and metastasis, we investigate the antitumor activity of adiponectin.

Methods

Reagents, Cells, and Animals. Recombinant full-length human adiponectin or mouse adiponectin (Acrp30) was produced and purified as described (15). Recombinant human fibroblast growth factor 2 (FGF-2) was obtained from Amersham Pharmacia and UpJohn, and the $VEGF_{165}$ was provided by R & D Systems. Primary bovine capillary endothelial (BCE) cells were obtained from Judah Folkman's laboratory; human dermal microvascular endothelial (HDME) cells were obtained from PromoCell (Heidelberg); porcine aortic endothelial (PAE) cells were obtained from Lena Claesson-Welsh's laboratory; and rat vascular smooth muscle (rVSM) cells were provided by Johan Thyberg (Karolinska Institutet). Female or male 5- to 6-wk-old C57BL/6 mice were acclimated and caged in groups of six or fewer. Mice were anaesthetized by injection of a mixture of dormicum/hypnorm (1:1) before all procedures and killed with a lethal dose of CO_2. All animal studies were reviewed and approved by the animal care and use committee of the Stockholm Animal Board.

Proliferation Assay. A standard 72-h BCE cell proliferation assay was performed as described (7). PAE/FGFR-1 cells or rVSM cells were grown in 10% heat-inactivated FCS-F12-Ham's medium. Approximately 10,000 cells per well were seeded in 24-well plates. Samples were assayed in 5% FCS-Ham's medium containing 10–15 ng/ml FGF-2. Murine T241 fibrosarcoma cells were assayed (10,000 cells per well in 24-well plates) in 5% FCS-DMEM containing various concentrations of adiponectin. After 72-h incubation, cells were trypsinized, resuspended in Isoton II solution (Kebo Lab, Stockholm), and counted with a Coulter counter. Results are presented as mean cell number per well (±SEM).

Cell Migration Assay. The motility response of PAE/VEGFR-2 cells to VEGF was assayed by using a modified Boyden chamber technique as described (16). rVSM cells at the density of 25,000 cells per well were seeded in the upper chambers in serum-free F12-Ham's medium containing 0.25% BSA and adiponectin samples.

Abbreviations: VEGF, vascular endothelial growth factor; FGF, fibroblast growth factor; BCE, bovine capillary endothelial; HDME, human dermal microvascular endothelial; PAE, porcine aortic endothelial; rVSM, rat vascular smooth muscle; CAM, chick chorioallantoic membrane; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling; AMC, 7-amino-4-methylcoumarin.

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F12-Ham's medium containing 2% FCS was added to the lower chambers. After a 6- to 9-h incubation, the medium was removed and cells attached to the filter were fixed in 99% methanol and stained with a Giemsa solution. All experiments were performed in six replicates. The number of cells migrating through the filter was counted and plotted as mean number of migrating cells per optic field (×20 magnification) (+SEM).

Chick Chorioallantoic Membrane (CAM) Assay. After 3-d incubation at 37°C, fertilized white Leghorn eggs (OVA Production, Sorgarden, Sweden) were cracked, and chick embryos with intact yolks were carefully placed in 20 × 100-mm plastic Petri dishes. After 6 days of incubation in 4% CO_2 at 37°C, methylcellulose disks containing 5, 10, or 20 μg of adiponectin or PBS were implanted on the CAM of individual embryos. After 2–8 days of incubation, CAMs were examined for the formation of avascular zones around the field of the implanted disks by using a stereoscope (n = 5 embryos per group). Photographs (×20 magnification) were taken on day 8 after implantation.

Mouse Corneal Micropocket Assay. The mouse corneal assay was performed as described (7). Micropellets containing 30 ng of FGF-2, 360 ng of adiponectin, or both were surgically implanted. Eyes were examined on day 5 after pellet implantation. Vessel lengths and clock hours of circumferential neovascularization were measured under a stereoscope (n = 10–11 eyes per group). Areas of maximal neovascularization were calculated.

Morphological Detection of Cellular Apoptosis. BCE or HDME cells grown to 60–70% confluency in 12-well plates were incubated for 6–48 h with various concentrations of human adiponectin in 5% bovine calf serum-DMEM or 1% FCS in endothelial cell growth medium MV (PromoCell), respectively. Cells were harvested and resuspended in PBS containing 30 mM glycerol and 0.1 M NaCl; the cells were dried onto slides and fixed with acetone/methanol (1:1). The cells were stained with Hoecsht dye 33258 (500 ng/ml). Apoptotic cells were counted in random fields under a microscope (×60 magnification, at least 10 fields per sample).

Caspase Activity Assay. BCE cells grown to 70–80% confluency in 12-well plates were incubated for 3–24 h with various concentrations of human adiponectin in 5% BCS-DMEM. The activities of caspase-3-, -8, and -9 were determined fluorometrically by cleavage of substrates: DEVD-7-amino-4-methylcoumarin (AMC), IETD-AMC, or LEHD-AMC (Peptide Institute, Osaka), respectively, according to described methods (17). Approximately 3–5 × 10^5 cells were used for each sample. To inhibit caspase activity, some samples were preincubated for 1 h with z-DEVD-fmk, z-IEDT-fmk, or z-LEHD-fmk (20 μM) (Enzyme Systems Products, Livermore, CA). Cleavage of the fluorogenic peptide substrates was monitored by AMC release in a Fluoroscan II plate reader (Labsystems, Chicago). Fluorescence units were converted to picomole of AMC by using a standard curve generated with free AMC. Data were analyzed by linear regression. Quantification of caspase activity was calculated as fold increase over control samples.

Tumor Experiments. Female 6- to 7-wk-old C57BL/6 mice were used for tumor studies. WT or GFP-expressing murine T241 fibrosarcoma cells growing in log phase were harvested and resuspended in PBS, and 1 × 10^6 cells in 100 μl were implanted s.c. in the middle dorsum of each animal as described (18). Mice were treated with murine adiponectin (Acrp30; 50 μg per mouse) by daily intralesional injections throughout the experiment (n = 6 mice per group). Control animals were injected with PBS. Visible tumors were present after 72 h and measured by using digital calipers on the indicated time points. Tumor volumes were calculated as reported (18). After a 2-wk treatment, the control tumors approached to the size of the Swedish ethical upper limit (1.5 cm^3), and the experiment was terminated at that point. Mice were killed, and the tumor tissues were removed and weighed.

Immunohistochemistry. Growth factor-implanted corneas were snap-frozen in TissueTek (HistoLab, Gothenburg, Sweden) on dry ice and stored at −80°C. Histological sections (12 μm) were stained for CD31 as described (7). Tumors were dissected and fixed in 3% paraformaldehyde, dehydrated, and embedded in paraffin. Thin sections (5 μm) were deparaffinized, blocked, and incubated overnight at 4°C with a biotinylated rat anti-mouse CD31 Ab (1:100; Pharmingen), followed by amplification of the signal by using NEN TSA amplification (Perkin–Elmer) protocol for biotinylated primary Abs. The reaction was developed by addition of diaminobenzidine substrate. Microphotographs were taken under a microscope (×20 magnification) and analyzed by using PHOTOSHOP 7.0 (Adobe Systems, Mountain View, CA). Vessel numbers per field (×10 magnification) were quantified in five to eight random fields per group (four tumors per group, two fields per tumor). The terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) staining was performed according to a standard but modified fluorescein *in situ* death detection protocol (Amersham Biosciences). In brief, deparaffinized thin tumor sections (5 μm) were blocked by using 3% H_2O_2 in methanol for 15 min; antigenic epitopes were retrieved, and the TUNEL reaction mixture was added. The sections were photographed and signals were quantified under fluorescent microscope (×60 magnification) in 5–10 random fields per group.

Whole-Mount Staining. Adiponectin-treated or control GFP-expressing T241 tumors were removed on day 8 after implantation for whole-mount immunohistochemical analysis. Tumors were dissected into thin slices and fixed with 3% paraformaldehyde overnight. Ab epitopes were exposed by proteinase K, and the tissues were permeabilized. Blood vessel endothelial cells were detected by using a rat anti-mouse CD31 (1:300; Pharmingen) primary Ab followed by a secondary goat anti-rat-IgG Ab labeled with Cy5 (Zymed). Apoptotic cells were visualized by TUNEL *in situ* death detection kit as above. Samples were examined by confocal microscopy (×20 magnification; LSM510 confocal microscope, Zeiss). The 3D images were assembled by scanning 10 layers (2–3 μm apart) of each sample by using LSM510 software (Zeiss) and PHOTOSHOP 7.0.

Statistics. Results are presented as mean (+SEM). Statistical evaluation of the results was made by using standard two-tailed Student's t test with EXCEL 5 (Microsoft). Statistical significance was defined as $P < 0.05$.

Results

Inhibition of Endothelial Cell Growth. To study whether adiponectin could inhibit endothelial cell growth, the effect of full-length purified recombinant human and mouse adiponectin (15) were determined by using endothelial cell proliferation and migration assays. Under nonreducing conditions, both human and mouse adiponectin form trimers and large oligomeric complexes (Fig. 1*A*). These large complexes are biologically active (19). Addition of physiologically relevant concentrations of human adiponectin to BCE cells resulted in a dose-dependent inhibition of FGF-2-stimulated endothelial proliferation, with an IC_{50} of 250 ng/ml (Fig. 1*B*). Similarly, mouse adiponectin also inhibited BCE proliferation (Fig. 1*C*). Because endothelial cell growth and angiogenesis can be stimulated by several angiogenic factors, we next determined whether adiponectin could block individual angiogenic factor-induced endothelial cell growth. In FGF receptor-1-overexpressing PAE cells/FGFR, human adiponectin significantly inhibited FGF-2-induced proliferation in a dose-dependent manner, with an IC_{50} of 250 ng/ml (Fig. 1*D*). To study VEGF-induced endothelial activity, we performed endothelial cell migration assay because

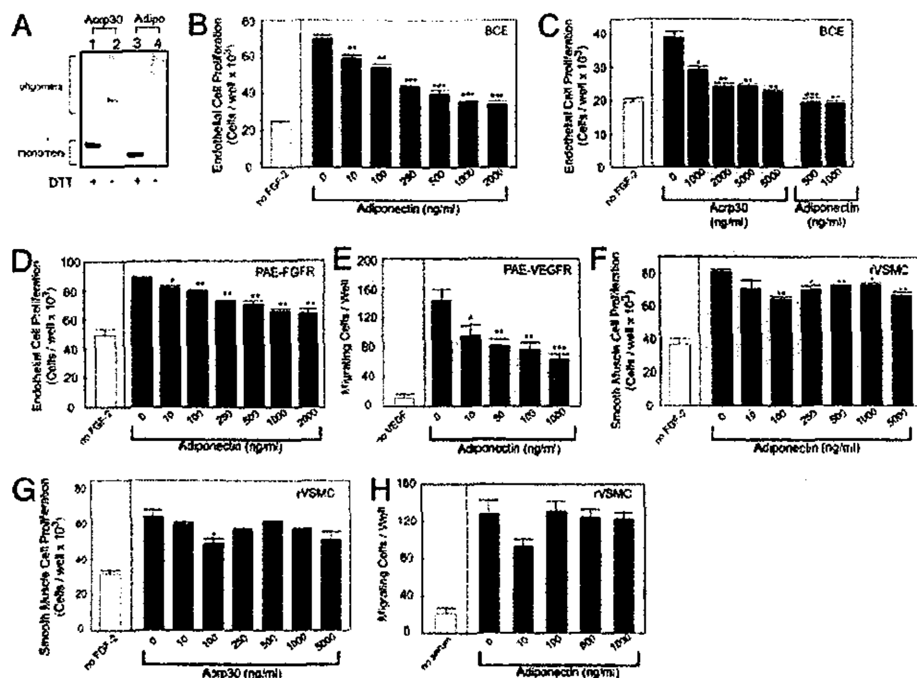

Fig. 1. Adiponectin inhibits endothelial growth. (*A*) Recombinant mouse (Acrp30) or human adiponectin (Adipo) proteins (1 μg) were analyzed on a polyacrylamide gel followed by staining with Coomassie brilliant blue. Trimeric/oligomeric (lanes 2 and 4) and monomeric (lanes 1 and 3) forms of adiponectin were detected under nonreducing and reducing/alkylating conditions, respectively. (*B* and *C*) FGF-2-stimulated BCE cells were incubated with various concentrations of human or mouse adiponectin. (*D*) Human adiponectin was incubated with PAE/FGFR-1 cells stimulated with FGF-2. (*E*) The motility response of PAE/VEGFR-2 cells to VEGF with or without human adiponectin was assayed. FGF-2-stimulated rVSM cells were incubated with human (*F*) or mouse (*G*) adiponectin. (*H*) The motility response of rVSM cells to serum was assayed with or without human adiponectin. Values represent mean number of cells per well (+ SEM). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

VEGF preferentially induced endothelial cell migration rather than proliferation. VEGF receptor-2-overexpressing PAE (PAE/VEGFR-2) cells exhibited dramatically increased cell motility upon stimulation with VEGF. Human adiponectin significantly inhibited VEGF-induced PAE/VEGFR-2 cell migration (Fig. 1*E*). These data suggest that adiponectin blocks a common pathway for endothelial cell proliferation and migration. To study whether adiponectin also inhibited nonendothelial cell growth, we determined its inhibitory activity on rVSM cells. Similar to previous findings (20), adiponectin significantly inhibited rVSM cell proliferation (Fig. 1 *F* and *G*). However, the concentrations required to reach a maximal inhibitory effect on rVSM cells were higher than for endothelial cells. Interestingly, adiponectin showed no inhibitory effect on rVSM cell migration (Fig. 1*H*) or FGF-2-stimulated fibroblast proliferation (data not shown). These findings indicate that adiponectin selectively inhibits endothelial cell growth at low concentrations. Consistent with our data, it has been reported that adiponectin exhibits specific binding to endothelial cells *in vitro* and that it accumulates in the vascular wall *in vivo*, suggesting the existence of a cell surface receptor for adiponectin (21–23). Indeed, adiponectin receptors (AdipoR1 and AdipoR2) have recently been detected on endothelial cells (24).

Inhibition of Angiogenesis *in Vivo*. To investigate its antiangiogenic activity *in vivo*, adiponectin was tested in the CAM and the mouse corneal angiogenesis assays. In the CAM model, adiponectin induced avascular zone formation in the developing embryos. Notably, newly formed microvessels were regressed around the area of adiponectin-implanted disks (Fig. 2*B*, arrows). This antiangiogenic activity appeared to be dose-dependent (Fig. 2*C*). In contrast, control CAMs, implanted with disks without adiponectin, had no avascular zones (Fig. 2*A*). In the mouse corneas, adiponectin significantly inhibited FGF-2-induced neovascularization (Fig. 2*E*) as compared to FGF-2 alone (Fig. 2*D*). Adiponectin alone did not induce angiogenesis in this model (Fig. 2*F*). Quantification analysis revealed the antineovascularization effect of adiponectin to be

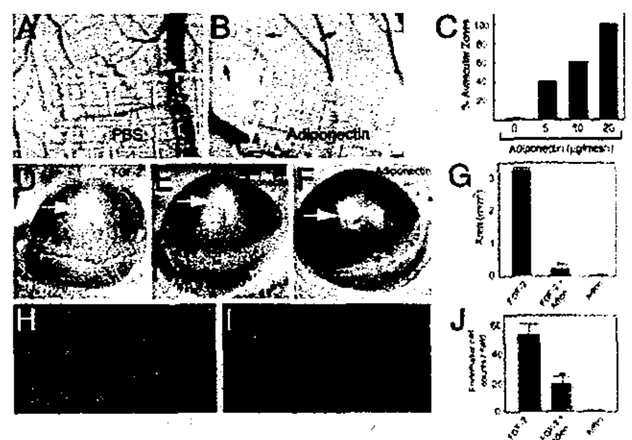

Fig. 2. Antiangiogenic activity of adiponectin. Disks containing human adiponectin were implanted on the developing CAMs. Microphotographs (×20) on day 8 of a control CAM (*A*) and an adiponectin-implanted CAM (*B*; 20 μg per disk) are shown. Arrows point to regressing blood vessels. (*C*) The number of CAMs with avascular zones was quantified at 48 h after implantation. Pellets containing FGF-2 (*D*), FGF-2/adiponectin (*E*), or adiponectin alone (*F*) were implanted into mouse corneal micropockets. Photographs represent ×20 magnification of the mouse eye, and positions of implanted pellets are indicated by arrows. (*G*) Corneal neovascularization was quantified on day 5 as mean maximal area (+ SEM). (*H* and *I*) Immunohistochemical labeling of blood vessels in sections of FGF-2- (*H*) or FGF-2/adiponectin- (*I*) implanted corneas (×20). (*J*) The number of microvessels per microscopic field was quantified. **, $P < 0.01$; ***, $P < 0.001$.

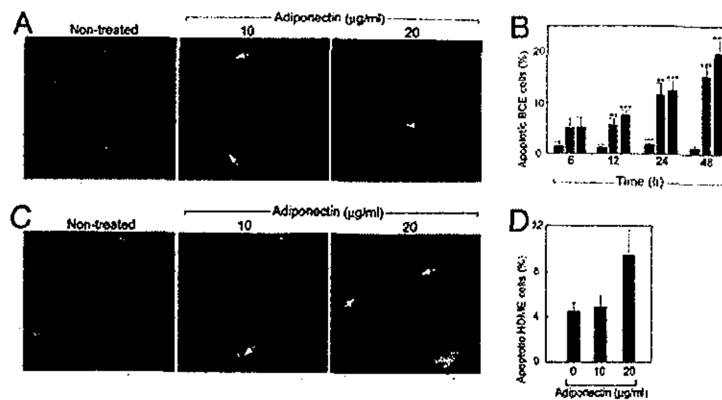

Fig. 3. Adiponectin induces endothelial apoptosis. (*A* and *B*) Apoptotic bodies of adiponectin-treated BCE cells were detected at 24 h by fluorescent microscopy and quantified at different time points. Bars: black, control; red, 10 μg/ml; blue, 20 μg/ml adiponectin. (*C* and *D*) Apoptosis of HDME cells after 24-h incubation with adiponectin. Values represent mean percentage of apoptotic cells per total number of cells per field. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

significant, with 94% reduction of vascularization area ($P < 0.001$) (Fig. 2*G*). Immunohistological examination of the corneas revealed that the number of newly formed microvessels was significantly reduced in the FGF-2/adiponectin-coimplanted corneas as compared with the FGF-2-implanted eyes ($P < 0.01$; Fig. 2 *H–J*). These findings demonstrate that adiponectin is a potent inhibitor of angiogenesis *in vivo*.

Induction of Endothelial Cell Apoptosis and Activation of Caspases. To elucidate the possible mechanisms of adiponectin-mediated antiangiogenesis, we investigated the effect of adiponectin on endothelial cell apoptosis. We found that addition of adiponectin to BCE cells induced a significant proportion of cells to undergo apoptosis, as determined by the appearance of apoptotic bodies (Fig. 3*A*). The induction of endothelial apoptosis became more pronounced after prolonged incubation, with ≈20% of the total cell population showing clear signs of apoptosis by 48 h (Fig. 3*B*). Similar findings were also observed by using HDME cells (Fig. 3 *C* and *D*).

To investigate apoptotic pathways, activation of endothelial caspases was analyzed. First, we determined the involvement of caspase-3, an apoptotic effector, in BCE cells. Activation of procaspase-3 by adiponectin was found to be dose and time dependent. A maximal activation was observed at ≈5 μg/ml adiponectin (Fig. 4*A*). Notably, the physiological levels of adiponectin in both human and mouse serum have been reported to range from 2 to 17 μg/ml (15, 25). Thus, the concentrations of adiponectin used in our system to sufficiently induce apoptosis of activated endothelial cells are physiologically relevant. To standardize our assay systems and compare efficacies of activation of different caspases, we used 1 μg/ml adiponectin in subsequent studies. We found that caspase-3 activity was first elevated at 3–4 h and reached a maximal level (≈6-fold over control) after 18–24 h of incubation (Fig. 4*B*). Activation of pro-caspase-8 was detected already 1 h after addition of adiponectin, and this level of activation persisted during prolonged incubations (Fig. 4*C*). In contrast, activation of procaspase-9 seemed to closely mirror the activation pattern of caspase-3 (Fig. 4*D*). These findings suggest that adiponectin initially activates caspase-8, which subsequently leads to activation of caspases-3 or -9. Pro-caspase-8 is an intracellular component that directly communicates with the death domain of cell membrane receptors, whereas pro-caspase-9 is activated by apoptotic signals released by the mitochondria.

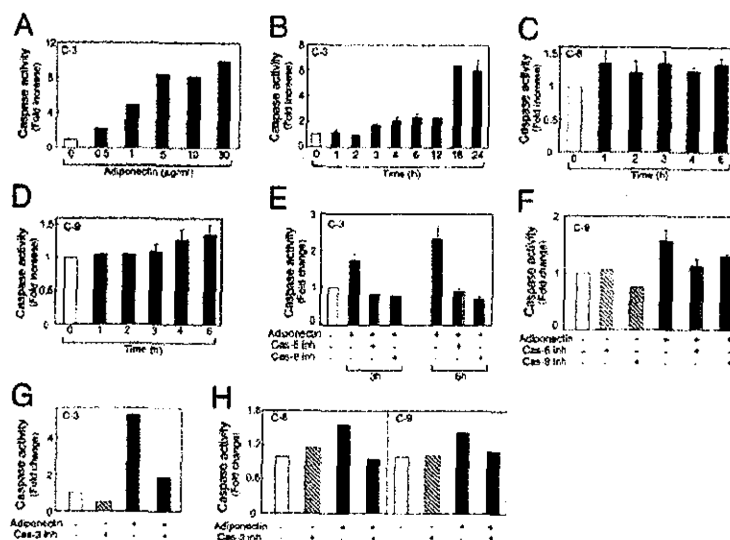

Fig. 4. Activation of endothelial caspase pathways. (*A*) Dose-dependent activation of caspase-3 in adiponectin-treated BCE cells at 24 h. (*B–D*) Time course determination of activation of caspase-3-like (*B*), caspase-8-like (*C*), and caspase-9-like (*D*) enzymes in BCE cells treated with 1 μg/ml human adiponectin. Activation of caspase-3 at 3 or 6 h (*E* and *G*), caspase-9 at 6 h (*F* and *H*), and caspase-8 at 6 h (*H*) was determined in the presence or absence of specific caspase inhibitors.

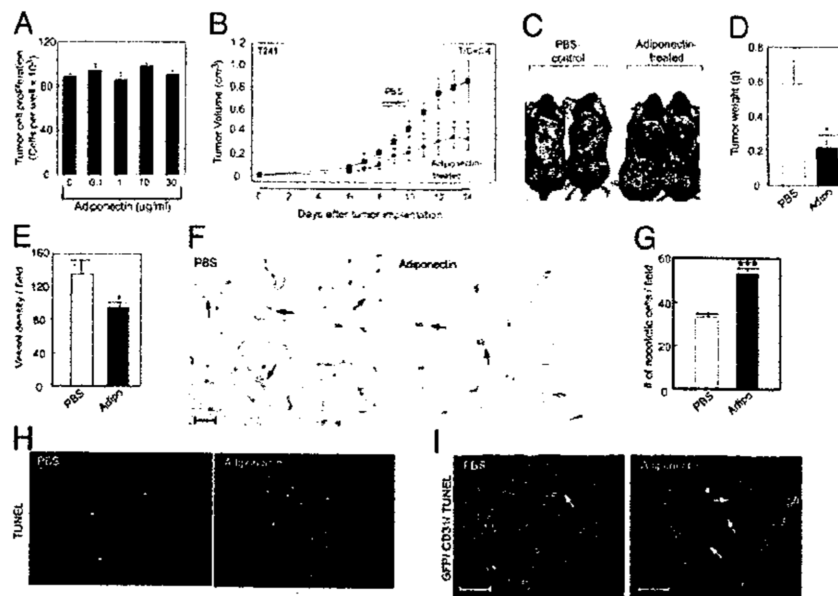

Fig. 5. Suppression of tumor growth and induction of apoptosis. (*A*) T241 tumor cell growth rates *in vitro* in the presence and absence of mouse adiponectin. (*B*) Tumor volumes represent mean determinants of treated and control groups (±SEM). (*C*) Typical examples of tumor-bearing mice of the control or adiponectin-treated groups on day 14. (*D*) Tumor weights at necropsy. (*E*) Tumor vascular density was quantified as numbers of vessels per field (×10). (*F*) Tumor neovascularization was detected by using an anti-CD31 Ab in the adiponectin-treated and control tumors (×20). (Scale bar, 50 μm.) (*G*) Quantification of TUNEL-positive, apoptotic tumor cells. *, $P < 0.05$; ***, $P < 0.001$. (*H*) TUNEL staining (green) for visualization of apoptotic cells in tumor sections. (*I*) Whole-mount staining of tumor blood vessels (×20; green, GFP-T241 tumor cells; blue, CD31-positive tumor vessels; red, TUNEL-positive apoptotic cells (scale bar, 25 μm)). Arrows indicate CD31/TUNEL double-positive structures.

Consistent with these data, addition of cell permeable-specific, irreversible inhibitors of caspase-8 (z-IETD-fmk), caspase-9 (z-LEHD-fmk), or caspase-3-like (z-DEVD-fmk) enzymes to BCE cells revealed that both z-IETD-fmk and z-LEHD-fmk completely prevented adiponectin-induced activation of caspase-3 (Fig. 4*E*). In addition, z-IETD-fmk blocked activation of caspase-9 (Fig. 4*F*). Thus, it is plausible that activation of caspase-8 is the immediate early event of adiponectin-triggered endothelial apoptosis. Furthermore, we found that preincubation of BCE cells with a specific caspase-3 inhibitor repressed not only the activation of caspase-3 (Fig. 4*G*) but also the activation of caspases-8 and -9 after 6 h of incubation (Fig. 4*H*), indicating the existence of an caspase-3-mediated amplification loop in the adiponectin-trigged caspase pathway (26).

Suppression of Tumor Growth and Neovascularization. The antiangiogenic activity of adiponectin encouraged us to determine whether adiponectin could suppress pathological angiogenesis. Tumor growth is angiogenesis-dependent, and suppression of angiogenesis has been shown to inhibit tumor growth (27). To study the possible antitumor activity of adiponectin, we chose a hypervascularized murine T241 fibrosarcoma model. Incubation of T241 tumor cells with adiponectin, even at high concentrations, did not affect tumor cell growth *in vitro* (Fig. 5*A*), suggesting that adiponectin had no direct effect on tumor cells *per se*. *In vivo*, intralesional administration of murine adiponectin resulted in significant suppression of tumor growth. After a 2-wk treatment, ≈60% reduction of tumor volumes and weights was observed (Fig. 5 *B*–*D*). Neovascularization of adiponectin-treated tumors was significantly reduced as compared with control tumors (Fig. 5 *E* and *F*). Interestingly, reduction of tumor neovascularization led to a remarkable increase of tumor apoptosis (Fig. 5 *G* and *H*). The increase of tumor cell apoptosis could be caused by deprivation of survival factors supplied by blood vessels.

To further study whether tumor vessel endothelial cells became apoptotic, we colocalized the CD31-positive signals with TUNEL-positive cells. Interestingly, a remarkable overlap between CD31- and TUNEL-positive signals was found in adiponectin-treated tumors (Fig. 5*I*). In contrast, only a small number of CD31/TUNEL double positive structures was found in the PBS-treated control tumors (Fig. 5*I*). These data demonstrate that adiponection induces tumor vessel apoptosis, which is consistent with our *in vitro* data that adiponectin selectively induces endothelial cell apoptosis.

Discussion

Adiponectin is produced by adipocytes and thought to be an important regulator of lipid and glucose metabolism (13, 14). Our present data demonstrate that adiponectin is a negative regulator of angiogenesis. This finding provides important clues to understanding the biological functions of this protein as a master regulator of many systems. Indeed, the adipose tissue is an important and perhaps the largest endocrine organ in the body. In addition to adiponectin, leptin, resistin, and adipsin are examples of other adipose-derived endocrine hormones or adipokines (3, 28). Although their main targets are located in distal sites, the local functions have not been fully investigated. Recently, leptin has been found to stimulate angiogenesis (6–8). Thus, adiponectin and leptin have opposite effects in regulation of angiogenesis. Like most tissues in the adult body, the quiescent vasculature in the adipose tissue may represent a net balance between the production levels of angiogenic factors and inhibitors. Overproduction of angiogenic factors or decreased levels of inhibitors would result in adipose tissue neovascularization and expansion. Recently, it has been shown that expansion of fat mass in the body is angiogenesis dependent (9). To switch on an angiogenic phenotype, the adipose tissue may have to down-regulate the expression of angiogenesis inhibitors. Our data provide an example of an endogenous adipose-derived angiogenesis inhibitor. Indeed, the levels of adiponectin are decreased in obese animals and humans (13, 15). In contrast, production of leptin is usually elevated in the obese state (29, 30), and it is known that dysregulation of leptin in mice and humans lead to obesity (31, 32). Surprisingly, deletion of adiponectin in mice

does not result in any differences in body weight, suggesting that the adiponectin system might be redundant under physiological conditions (33). Similarly, overexpression of adiponectin in mice did not result in a significant difference of body weight or adiposity (34). Indeed, our present data show that treatment of mice with recombinant adiponectin does not affect adipogenesis (unpublished data), a process that probably depends on angiogenesis. The fact that adiponectin preferentially targets tumor blood vessels reflects that the rate of angiogenesis in the tumor vasculature is higher than that in the slowly expanding adipose tissue. Thus, it appears the antiangiogenic activity of adiponectin is more pronounced under pathological conditions. Another possibility is that tumors produce additional molecules that facilitate the antiangiogenic activity of adiponectin. However, this hypothesis remains to be further investigated.

Very recently, adiponectin has been reported to stimulate endothelial cell growth and angiogenesis (35). Most of these experimental data were obtained by *in vitro* assays using endothelial cells derived from large vessels. The discrepancy of these findings from our present data could be because different systems were used. In those studies, human umbilical vein endothelial cells were used for the *in vitro* studies, whereas we use capillary endothelial cells in our assays. It is believed that capillary endothelial cells are most relevant for studying angiogenic processes *in vitro* because capillaries usually sprout from microvessels but not large vessels. In addition, the effect of adiponectin on endothelial cells *in vitro* cannot be directly translated into its *in vivo* angiogenic activity. For example, transforming growth factor type β is a potent inhibitor for endothelial cell growth *in vitro* but stimulates angiogenesis *in vivo* (36, 37). Our *in vivo* results are consistent with the *in vitro* finding obtained from capillary endothelial cells, suggesting that adiponectin is a potent angiogenesis inhibitor.

There is a correlation between obesity and high incidences of certain cancers, including breast, colon/rectal, and prostate cancer (38, 39). Our findings that adiponectin inhibits tumor neovascularization may imply that obese individuals with low levels of adiponectin are more susceptible to develop various forms of tumors. It would be interesting to investigate whether the reported increased cancer incidences in the obese population (38, 40) correlate with significantly decreased levels of adiponectin. In this study, we also provide molecular mechanisms of the antiangiogenic activity of adiponectin. The finding that adiponectin induces endothelial apoptosis indicates that this protein acts as a direct endogenous inhibitor for blood vessel growth. Indeed, administration of adiponectin to tumor-bearing animals resulted in apoptosis of a significant proportion of tumor blood vessels. Adiponectin may have several therapeutic advantages as compared to angiogenic factor antagonists. As the genome of tumor cells is unstable, the production of multiple angiogenic factors is switched on during tumor progression (41). Thus, antiangiogenic therapy by using, e.g., an anti-VEGF agent may encounter drug resistance problems. In contrast, induction of endothelial apoptosis by adiponectin seems to target a common angiogenic pathway. In this regard, adiponectin may be an effective novel anticancer agent.

In addition to suppression of tumor growth, adiponectin has recently been reported to protect apolipoprotein E-deficient mice from development of atherosclerotic plaques (12). Although the underlying mechanisms need to be further investigated, inhibition of angiogenesis could be involved in the antiatherogenic effect of adiponectin, as suppression of angiogenesis can prevent atherosclerotic plaque growth (42). In type II diabetic individuals, decreased levels of adiponectin may contribute to several severe complications. For example, diabetic retinopathy represents a switch of pathological angiogenesis in the retina. Could the pathological neovascularization in the eye and other tissues be caused by decreased levels of adiponectin in diabetic patients? Our finding that adiponectin acts as a potent angiogenesis inhibitor suggests this possibility.

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