Original Article

Essential Role of Angiotensin II Type 1a Receptors in the Host Vascular Wall, but Not the Bone Marrow, in the Pathogenesis of Angiotensin II–Induced Atherosclerosis

Jun-ichiro KOGA[1)], Kensuke EGASHIRA[1)], Tetsuya MATOBA[1)], Mitsuki KUBO[1)], Yoshiko IHARA[1)], Masaru IWAI[2)], Masatsugu HORIUCHI[2)], and Kenji SUNAGAWA[1)]

The angiotensin II (Ang II) type 1a (AT1a) receptor is expressed on multiple cell types in atherosclerotic lesions, including bone marrow–derived cells and vascular wall cells, and mediates inflammatory and proliferative responses. Indeed, Ang II infusion accelerates atherogenesis in hyperlipidemic mice by recruiting monocytes and by activating vascular wall cells. Here, we investigated the relative roles of AT1a receptors in the bone marrow *vs.* the vascular wall in Ang II–induced atherogenesis. Apolipoprotein E–knockout ($ApoE^{-/-}$) mice with or without bone marrow AT1a receptor were generated by experimental bone marrow transplantation using $AT1a^{+/+}$ or $AT1a^{-/-}$ recipients. In these mice, 28-d Ang II infusion induced significant atherosclerosis in the aorta, and the severity of plaque formation was not affected by the absence of bone marrow AT1a receptor. We then generated $AT1a^{-/-}ApoE^{-/-}$ mice with or without bone marrow AT1a receptor. Ang II–induced plaque formation was blunted irrespective of the presence of bone marrow AT1a receptor. Host AT1a receptor deficiency was found to suppress Ang II–induced reactive oxygen species production. In addition, AT1a receptor deficiency also impaired monocyte chemoattractant protein-1 (MCP-1) and vascular cell adhesion molecule-1 (VCAM-1) expression in the arterial wall 7 d after Ang II initiation. These molecules normally initiate later macrophage-mediated inflammation in the vascular wall. By contrast, AT1a receptor deficiency in the bone marrow did not affect MCP-1–induced monocyte chemotaxis *in vitro*. In conclusion, AT1a receptors in the host vascular wall, but not in the bone marrow, are essential in Ang II–induced atherogenesis. (*Hypertens Res* 2008; 31: 1791–1800)

***Key Words*: atherosclerosis, angiotensin II, oxidative stress, inflammation**

Introduction

It is now recognized that the renin-angiotensin system plays an important role in the pathogenesis of atherosclerotic and hypertensive cardiovascular diseases. Inhibiting the renin-angiotensin system by angiotensin-converting enzyme inhibitors or angiotensin II (Ang II) type 1 (AT1) receptor blockers is a practical target for therapy in patients with cardiovascular disease (*1–4*). The central importance of the renin-angio-

From the [1)]Department of Cardiovascular Medicine, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan; and [2)]Department of Molecular and Cellular Biology, Division of Medical Biochemistry and Cardiovascular Biology, Ehime University School of Medicine, Toon, Japan.

This study was supported by Grants-in-Aid for Scientific Research (14657172, 14207036) from the Ministry of Education, Culture, Sports, Science and Technology, Japan, by Health Science Research Grants (Research on Translational Research and Research on Nano-Medicine) from the Ministry of Health, Labour and Welfare, Japan, Uehara Life Science Foundation, Tokyo, and Takeda Life Science Foundation.

Address for Reprints: Kensuke Egashira, M.D., Ph.D., Department of Cardiovascular Medicine, Graduate School of Medical Sciences, Kyushu University, 3–1–1, Maidashi, Higashi-ku, Fukuoka 812–8582, Japan. E-mail: egashira@cardiol.med.kyushu-u.ac.jp

Received March 19, 2008; Accepted in revised form July 2, 2008.

tensin system in atherogenesis has been proven in AT1a receptor deficient mice when atherosclerosis was induced by hyperlipidemia (*5*) and Ang II infusion (*6*). We also reported that diabetes-induced acceleration of atherosclerosis was blunted in AT1a$^{-/-}$ apolipoprotein E–knockout (ApoE$^{-/-}$) mice (authors' unpublished observation).

Infusion of Ang II into hypercholesterolemic mice dramatically accelerates the atherosclerotic process, leading to the development of extensive atherosclerotic plaque formation (*7*). We have recently demonstrated that blockade of the monocyte chemoattractant protein-1 (MCP-1) pathway limits Ang II–induced progression and destabilization of atherosclerotic lesions in hyperlipidemic ApoE$^{-/-}$ mice (*8*). Therefore, Ang II–mediated acceleration of atherogenesis is characterized by the recruitment and activation of monocytes/macrophages and the degradation of elastin and collagen layers, suggesting that Ang II changes the lesion composition into a more destabilized phenotype. Thus, identification of the critical inflammatory pathway involved in Ang II–induced atherosclerosis might aid in the development of novel therapeutic strategies to reduce atherothrombotic events.

The role of Ang II and/or AT1 receptors in atherogenesis may be more complex. Ang II is thought to accelerate atherogenesis by stimulating AT1 receptors in the multiple cell types of atherosclerotic lesions, such as endothelial cells, smooth muscle cells, and leukocytes. Activation of the Ang II–AT1 receptor pathway mediates recruitment and activation of monocytes (*9*), endothelial migration and angiogenesis (*10*), and migration/proliferation of vascular smooth muscle cells (*11*, *12*). It is impossible, however, to dissect the relative pathobiologic role of leukocytes *vs*. non-leukocyte cells in the arterial wall using the systemic absence of AT1a receptors or AT1 receptor blockers. We previously reported that 1) Ang II upregulates the MCP-1 receptor (C-C chemokine receptor 2 [CCR2]) in circulating monocytes (*13*) and 2) Ang II–induced vascular remodeling and atherosclerosis were blunted in donor CCR2 deficient ApoE$^{-/-}$ mice created by bone marrow transplantation (BMT) (*13*, *14*). Recently, Cassis *et al*. (*15*) and Fukuda *et al*. (*16*) independently reported that 1) Ang II–induced atherosclerosis was partly reduced in mice with bone marrow AT1a receptor deficiency and 2) host AT1a receptors are also important for the formation of Ang II–induced atherosclerosis. In these studies, their analysis was focused on the degree of atherosclerotic plaque formation. In contrast, the underlying mechanisms by which AT1a receptors on the bone marrow cells *vs*. those on host vascular wall cells mediate atherosclerotic lesion formation remained unclarified.

The aim of this study was to address the relative roles of AT1a receptors in the bone marrow *vs*. the host arterial wall in Ang II–induced atherogenesis of hyperlipidemic ApoE$^{-/-}$ mice. In addition, we investigated the underlying mechanisms by analyzing phenotypic changes in bone marrow cells and vascular wall cells. We used a BMT technique to generate ApoE$^{-/-}$ mice with AT1a receptor deficiency in the bone marrow or the host arterial wall and demonstrated an essential role of the host AT1a receptors in Ang II–induced atherogenesis.

Methods

Experimental Animals

Male ApoE$^{-/-}$ mice (B6.129P2-Apoe<tm1Unc>/J, stock No.002052, C57Bl/6J background) were purchased from Jackson Laboratory (Bar Harbor, USA). AT1a$^{-/-}$ApoE$^{-/-}$ mice were generated by crossing ApoE$^{-/-}$ mice with AT1a$^{-/-}$ mice (*17*). Littermate AT1a$^{+/+}$ApoE$^{-/-}$ mice were used as control mice. Male CCR2$^{-/-}$ and CCR2$^{+/+}$ mice were used only for monocyte chemotaxis experiments (*14*). All mice were fed a normal diet and given water ad libitum.

Bone Marrow Transplantation

The study protocol was reviewed and approved by the Committee on the Ethics of Animal Experiments, Kyushu University Graduate School of Medical Sciences.

BMT was performed as described previously (*13*, *14*). In brief, bone marrow cells were harvested from the femurs and tibias of either AT1a$^{+/+}$ApoE$^{-/-}$ or AT1a$^{-/-}$ApoE$^{-/-}$ mice. The 8-week-old AT1a$^{+/+}$ApoE$^{-/-}$ recipient mice received 1×10^7 bone marrow cells (0.3 mL) 4 h after whole-body irradiation with 9 Gy of γ rays at 1 Gy/min *via* a Gamma Cell 40. The AT1a$^{-/-}$ApoE$^{-/-}$ recipient mice also received 1×10^7 bone marrow cells from AT1a$^{+/+}$ApoE$^{-/-}$ or AT1a$^{-/-}$ApoE$^{-/-}$ mice after whole-body irradiation.

Four weeks after BMT, the four groups of mice were infused with phosphate-buffered saline (PBS) or angiotensin II (Sigma Aldrich, St. Louis, USA) at 1.9 mg/kg/d by an osmotic pump (Alzet, Cupertino, USA). On day 7 or 28 of treatment, mice were euthanized for morphometric, immunohistochemical, and biochemical analysis. Peripheral blood samples were collected from the inferior vena cava to measure serum lipid content. The aortas were isolated and fixed in 10% buffered formalin for histological and immunohistochemical analyses. Systolic blood pressure and heart rate were measured with a tail cuff system before and 28 d after treatment.

Bone Marrow Chimerism Analysis by the Fluorescence *In Situ* Hybridization

Bone marrow chimerism was examined by the fluorescence *in situ* hybridization (FISH) method. In brief, female ApoE$^{-/-}$ recipient mice were transplanted with total bone marrow cells from male donor AT1a$^{+/+}$ApoE$^{-/-}$ or AT1a$^{-/-}$ApoE$^{-/-}$ mice after γ irradiation, as described above. Bone marrow cells were harvested 28 d after BMT and stained with total mouse Y chromosome probes (Qbiogene Inc., Morgan Irvine, USA). Quantitative analysis was performed by counting Y-chromosome–positive cells and displayed as a Y-chromosome–posi-

Table 1. Flow Cytometric Analysis of the Bone Marrow Leukocyte Composition

	Marker positive ratio (%)			
	Gr-1 (granulocyte)	Mac-1 (monocyte/macrophage)	CD3ε (T cell)	B220 (B cell)
$AT1a^{+/+}ApoE^{-/-}$	63.0±3.7	64.0±6.0	0.8±0.1	29.3±5.3
$AT1a^{-/-}ApoE^{-/-}$	68.4±7.7	74.6±9.6	1.0±0.3	30.7±0.9

Data are mean±SEM. After collection of total bone marrow cells from tibia, cells were incubated with phycoerythrin conjugated anti-lineage marker antibody. The marker positive ratio was determined by flow cytometry ($n=3$ each). AT1a, angiotensin II type 1a; ApoE, apolipoprotein E.

Table 2. Blood Pressure, Lipid Profile, and Body Weight

AT1a receptor genotype				
Donor	+/+	–/–	+/+	–/–
Host	+/+	+/+	–/–	–/–
Systolic blood pressure (mmHg)				
PBS	90±13	102±3	82±4	94±5
Ang II	133±5*	144±5*	85±3	86±4
Serum total cholesterol (mg/dL)				
PBS	677±47	714±54	625±48	614±41
Ang II	599±72	770±82	539±36	578±21
Serum triglyceride (mg/dL)				
PBS	47±3	45±5	47±7	47±3
Ang II	54±11	54±5	35±7	30±4
Body weight (g)				
PBS	23±1	22±1	17±2	18±3
Ang II	20±2	22±1	20±2	17±2

AT1a, angiotensin II type 1a; PBS, phosphate-buffered saline; Ang II, angiotensin II. Data are mean±SEM. *$p<0.01$ *vs*. PBS.

tive ratio. Over 100 cells per mouse were counted.

Bone Marrow Leukocyte Composition Analysis

Bone marrow leukocyte composition was analyzed by flow cytometry (EPICS ALTRA; Beckman Coulter, Fullerton, USA). BM cells were harvested from the tibia and femur of $AT1a^{+/+}ApoE^{-/-}$ or $AT1a^{-/-}ApoE^{-/-}$ mice and incubated with phycoerythrin-conjugated primary antibodies specific for markers for each lineage (granulocyte, Gr-1; monocyte, Mac-1; T cells, CD3ε; B cells, B220; BD Pharmingen, San Diego, USA). The marker-positive ratio for each marker was analyzed.

Staining of Atherosclerotic Lesions and Morphometric Analysis

To evaluate atherosclerotic lesions, the aorta was stained with oil red O solution for 10 min at 37°C, as previously described (*13*, *18*, *19*). The whole aorta from aortic arch to iliac bifurcation was excised and opened longitudinally. Color images were taken with a digital microscope and atherosclerotic lesion area was quantified using Scion Image software (Scion Corporation, Frederick, USA). Lesion area was determined by calculating the percentage of oil red O staining area relative to the total area of the aortic endothelial surface.

Histopathology and Immunohistochemistry

For immunohistochemical analysis, serial paraffin or frozen sections of the aortic root were prepared as described previously (*13*, *14*). Briefly, the aortic root was harvested from the heart and fixed overnight in 10% buffered-formalin. After fixation, the tissue was embedded in paraffin or frozen with OCT compound. Serial cross sections (6 μm thick) throughout the entire length of the aortic root were used for analysis. The sections were subjected to immunostaining using rat anti-mouse macrophage monoclonal antibodies (Mac-3; BD Pharmingen, San Diego, USA), goat anti-mouse MCP-1 antibodies (R&D Systems Inc., Minneapolis, USA), and anti-human vascular cell adhesion molecule 1 (VCAM-1) antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, USA). The respective non-immune IgGs were used as negative controls. The sections were incubated with biotinylated goat anti-rat IgG or rabbit anti-goat IgG (Nichirei, Tokyo, Japan), followed by diaminobenzidine (DAB) for visualization. The sections were then counterstained with Mayer's hematoxylin. The number of macrophages accumulated in the aortic root

lesion and the MCP-1 staining area was estimated. Analysis was performed using a microscope with a computerized, digital image analysis system and Scion Image Software.

Abdominal Macrophage Chemotaxis Assay

Abdominal fluid that contains macrophages was harvested 4 d after intra-peritoneal injection of thioglycolate. Macrophage migration was measured in 96-well chemotaxis chambers (Neuro Probe Inc., Gaithersburg, USA). MCP-1 (50 ng/mL) in RPMI 1640 was added to the lower wells and the isolated macrophages (1×10^7 cells/mL) were placed in the upper wells. After incubation for 90 min at 37°C, the upper surface of the membrane was washed with PBS and the migrated cells on the lower surface were counted after staining with trypan blue. The number of cells per field was counted. All assays were performed in triplicate.

Detection of Superoxide Production

The thoracic aortas were excised and snap frozen with OCT compound. The intact sections (20 μm thick) from the arterial segments were enzymatically unfixed and simultaneously incubated with dihydroethidium (DHE, 20 μm/L) in PBS for 30 min at 37°C in a chamber protected from the light, as previously described (*20*). DHE is oxidized to ethidium bromide in the presence of superoxide, which intercalates into nuclear DNA and causes red fluorescence. The fluorescence was visualized by fluorescence microscopy using an excitation wavelength of 543-nm and a 585-nm long-pass filter.

Statistics

All data are reported as the mean±SEM. Statistical analysis of differences was performed by ANOVA followed by Bonferroni's correction for multiple comparisons. p values <0.05 were considered to be statistically significant.

Results

Generation of the Four Groups of Chimeric Mice

We examined the bone marrow from donor $AT1a^{+/+}ApoE^{-/-}$ and $AT1a^{-/-}ApoE^{-/-}$ mice by flow cytometry and found that there were no significant differences between these mice with regard to leukocyte composition (granulocytes, monocytes/macrophages, T cells, and B cells) (Table 1). We then performed BMT using bone marrow from $AT1a^{+/+}ApoE^{-/-}$ and $AT1a^{-/-}ApoE^{-/-}$ donor mice into $AT1a^{+/+}ApoE^{-/-}$ and $AT1a^{-/-}ApoE^{-/-}$ recipients. Complete replacement of bone marrow cells by donor-derived cells was confirmed by FISH analysis. The bone marrow chimeric ratio was 99.4±0.4% in $AT1a^{+/+}ApoE^{-/-}$ mice transplanted with $AT1a^{+/+}ApoE^{-/-}$ bone marrow cells and 99.4±0.4% in $AT1a^{-/-}ApoE^{-/-}$ mice transplanted with $AT1a^{-/-}ApoE^{-/-}$ bone marrow cells. These results suggested a complete replacement of bone marrow cells and no influence of bone marrow AT1a receptors on graft survival. Each group of mice was fed a normal chow diet for 4 weeks after BMT, followed by chronic saline or Ang II infusion for an additional 4 weeks. Ang II infusion caused arterial hypertension to a similar extent in $AT1a^{+/+}$ recipient mice with or without bone marrow AT1a receptor, but did not cause hypertension in $AT1a^{-/-}$ recipient mice (Table 2). There were no significant differences in serum cholesterol, triglyceride, or body weight among the groups infused with saline or Ang II (Table 2).

Role of AT1a Receptors in the Host Arterial Wall *vs.* the Bone Marrow in Ang II–Induced Atherogenesis

As we have reported previously (*13*, *14*), chronic Ang II infusion induces atherosclerotic lesions in $AT1a^{+/+}$ recipients whose bone marrow was reconstituted with donor $AT1a^{+/+}$ bone marrow. The percentages of plaque areas in the mice infused with PBS or Ang II were 1.2±0.2 and 5.3±0.8%, respectively. Ang II infusion also induced atherosclerotic lesions in $AT1a^{+/+}$ recipient mice whose bone marrow was reconstituted with donor $AT1a^{-/-}$ bone marrow. The degrees of Ang II–induced atherosclerosis were similar between these two groups (Fig. 1A). By contrast, Ang II did not induce significant atherosclerotic lesion formation in $AT1a^{-/-}$ mice that received donor $AT1a^{+/+}$ or $AT1a^{-/-}$ bone marrow (Fig. 1B).

Macrophage infiltration is a hallmark of dyslipidemia, hypertension-induced atherogenesis, and atherosclerosis in Ang II infusion model animals. We examined the degree of macrophage infiltration into aortic atherosclerotic lesions by immunohistochemistry for a macrophage marker (Mac-3). Ang II infusion induced remarkable macrophage infiltration in the aortic neointima in $AT1a^{+/+}$ mice that received donor $AT1a^{+/+}$ or $AT1a^{-/-}$ bone marrow. As summarized in Fig. 2B, the severity of macrophage infiltration was equivalent, irrespective of the presence of bone marrow AT1a receptor. By contrast, Ang II–induced macrophage infiltration was blunted in $AT1a^{-/-}$ recipient mice, again irrespective of the presence of bone marrow AT1a receptor (Fig. 2).

Ang II–Induced Superoxide Production and MCP-1 Induction in Chimeric Mice

Ang II in known to cause superoxide production in vascular smooth muscle cells that may activate redox-dependent signals that regulate inflammatory molecules including MCP-1 and VCAM-1 (*14*, *21*, *22*). To verify the possible mechanisms by which host AT1a receptor accelerates Ang II–induced macrophage infiltration, we examined reactive oxygen species (ROS) generation as well as the expression of MCP-1 and VCAM-1 1 week after Ang II infusion was started (when atherosclerotic changes were hardly observed). DHE fluorescence was observed in the media of host $AT1a^{+/+}$ mice,

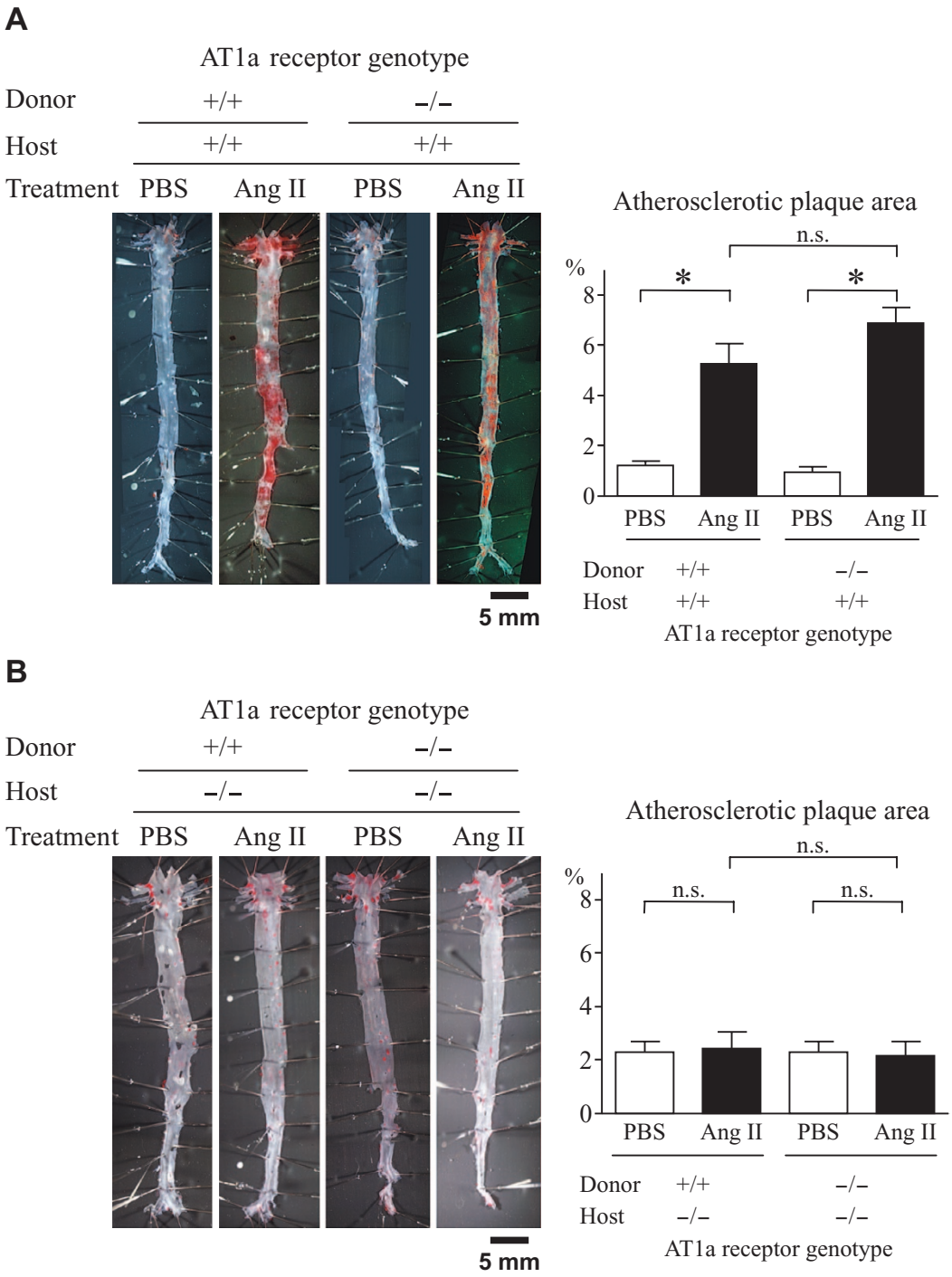
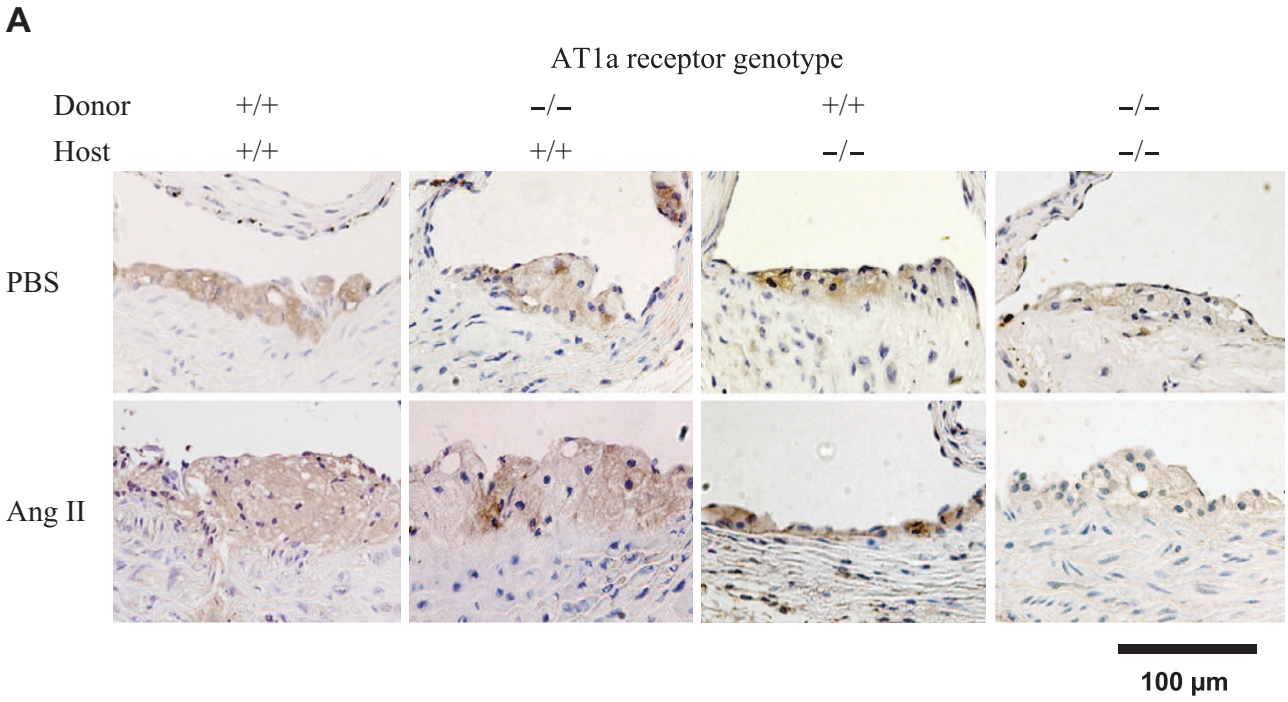


Fig. 1. *Role of AT1a receptors in host-derived cells and in bone marrow–derived cells in Ang II–induced atherosclerosis. A: Photomicrographs of the intraluminal surface of the whole aorta from the aortic arch to the iliac bifurcation stained with oil red O in AT1a$^{+/+}$ recipient mice repopulated with AT1a$^{+/+}$ or AT1a$^{-/-}$ bone marrow and infused with PBS or Ang II for 4 weeks. Lipids containing atherosclerotic plaques are stained red. Bar indicates 5 mm. Bar graph shows quantitative analysis of plaque area stained with oil red O staining. Data are expressed as the percentage of oil red O stained area to the total area of aortic endothelial surface. Data are reported as mean±SEM (n=6 to 8 each). *p<0.01 vs. PBS. n.s., not significant. B: Photomicrographs of the intraluminal surface of the whole aorta from the aortic arch to the iliac bifurcation stained with oil red O in AT1a$^{-/-}$ recipient mice repopulated with AT1a$^{+/+}$ or AT1a$^{-/-}$ bone marrow and infused with PBS or Ang II for 4 weeks. Bar graph shows quantitative analysis of plaque area stained with oil red O staining. Data are reported as mean±SEM (n=6 to 8 each). n.s., not significant.*



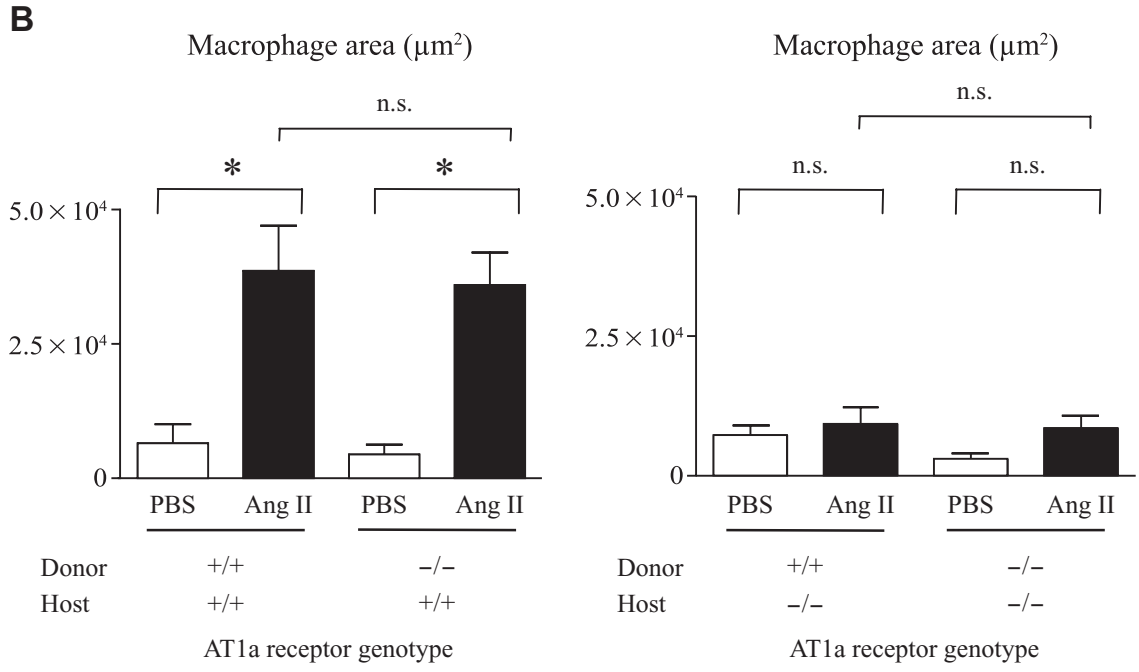


Fig. 2. *Mac-3 positive macrophage infiltration in aortic atherosclerotic plaques. A: Photomicrographs of cross-sections of the aortic root stained immunohistochemically for Mac-3 in AT1a$^{+/+}$ or AT1a$^{-/-}$ recipient mice repopulated with AT1a$^{+/+}$ or AT1a$^{-/-}$ bone marrow and infused with PBS or Ang II for 4 weeks. Bar indicates 100 μm. B: Quantitative analysis of Mac-3 positive area (μm²) per section in each group of chimeric mice. Data are reported as mean ± SEM (n = 5 to 7 each). *p < 0.01 vs. PBS.*

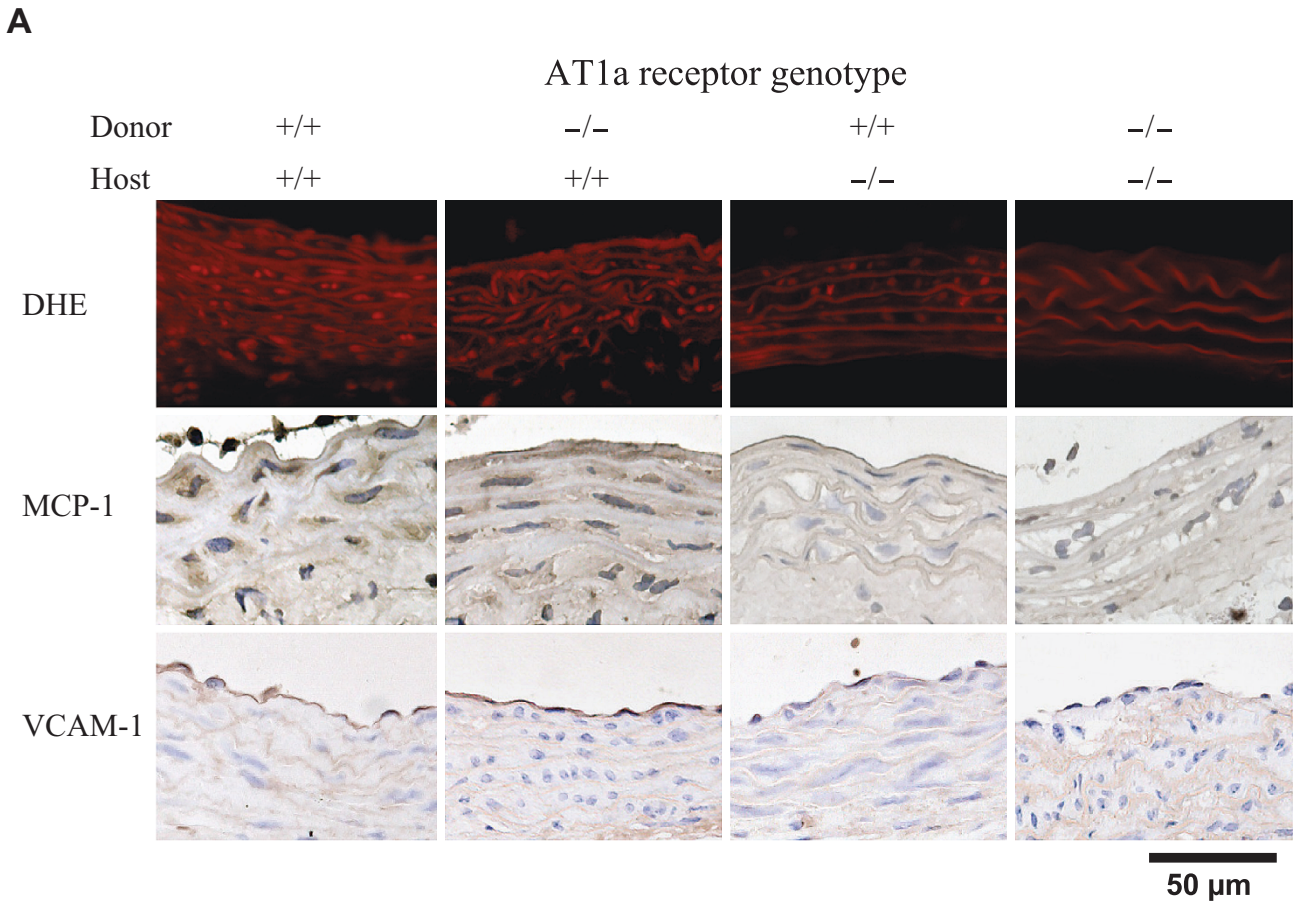


Fig. 3. *Angiotensin II–induced production of superoxide, MCP-1, and VCAM-1 in chimeric mice. A: Photomicrographs of cross-sections of the aortic root stained with dihydroethidium (DHE), MCP-1, or VCAM-1 antibody in $AT1a^{+/+}$ or $AT1a^{-/-}$ recipient mice repopulated with $AT1a^{+/+}$ or $AT1a^{-/-}$ bone marrow and infused with Ang II for 1 week. B: Quantitative analysis of superoxide (DHE staining), MCP-1, and VCAM-1 production. Superoxide production is expressed as the relative fluorescence intensity using $AT1a^{+/+}$ recipient with $AT1a^{+/+}$ bone marrow as a control. MCP-1 production was evaluated based on MCP-1 staining area (μm^2) per section. VCAM-1 production was evaluated as the ratio of the number of VCAM-1-expressing endothelial cells to total endothelial cells. Data are reported as mean±SEM ($n=4$ to 5 each). * $p<0.05$ vs. $AT1a^{+/+}$ recipient with $AT1a^{+/+}$ bone marrow.*

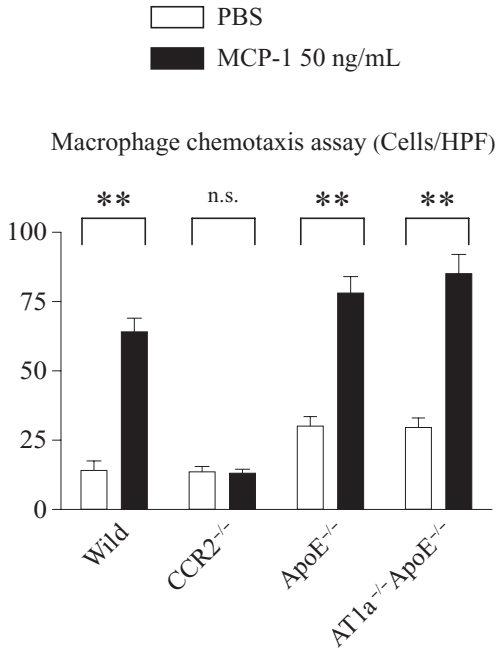


Fig. 4. *Preserved MCP-1-induced chemotaxis in AT1a$^{-/-}$ ApoE$^{-/-}$ macrophages. Macrophage chemotaxis in response to MCP-1 (50 ng/mL) in wild type, CCR2$^{-/-}$, AT1a$^{+/+}$ApoE$^{-/-}$, and AT1a$^{-/-}$ApoE$^{-/-}$ mice. Data are reported as mean±SEM (n=8 each). **p<0.01 vs. PBS. n.s., not significant.*

irrespective of the presence of donor AT1a receptor, which was blunted in host AT1a$^{-/-}$ mice (Fig. 3). At this time point, the majority of ROS-producing cells are considered to be smooth muscle cells, because few macrophages infiltrate into the vascular wall and vascular remodeling has not occurred. The presence/absence of bone marrow AT1a receptor did not affect ROS generation (Fig. 3B). No apparent DHE fluorescence was detected in the aorta from PBS-infused animals (data not shown). As expected, Ang II caused MCP-1 induction in the medial smooth muscle cells and VCAM-1 expression in the intimal monolayer of host AT1a$^{+/+}$ mice. The induction of these molecules was blunted in host AT1a$^{-/-}$ mice (Fig. 3), whereas no MCP-1 or VCAM-1 staining was detected in the media of aorta from PBS-infused animals (data not shown).

Preserved MCP-1–Mediated Macrophage Chemotaxis in AT1a$^{-/-}$ApoE$^{-/-}$ Mice *In Vitro*

To examine effect of AT1a receptor deficiency on macrophage function, the chemotactic response of abdominal cavity macrophages to MCP-1 was evaluated in wild-type, CCR2$^{-/-}$, AT1a$^{+/+}$ApoE$^{-/-}$, and AT1a$^{-/-}$ApoE$^{-/-}$ mice. As expected, the chemotactic response was blunted in macrophages from CCR2$^{-/-}$ mice. In contrast, the chemotaxis capacity was preserved in AT1a$^{+/+}$ApoE$^{-/-}$ and AT1a$^{-/-}$ApoE$^{-/-}$ mice (Fig. 4).

Discussion

BMT experiments provide a useful approach to dissect the role of bone marrow–derived cells and host arterial wall cells in the pathogenesis of vascular diseases. In our present study, we found that Ang II–induced atherogenesis was not affected by the presence or absence of AT1a receptors in donor-derived bone marrow. By contrast, recent studies using BMT (*15*, *16*) have shown that AT1a receptor deficiency in donor bone marrow partially but significantly suppressed Ang II–induced atherosclerosis in host AT1a$^{+/+}$ mice. Although the precise mechanism remains unclarified, it is possible that bone marrow AT1a receptor regulates monocyte chemotactic function. We have previously reported that CCR2 expression and activity were upregulated in circulatory monocytes in Ang II–infused mice. However, CCR2 expression and activity were blunted by treatment with an AT1 receptor blocker or in AT1a receptor deficient mice (*14*). Other studies have shown that CCR2 on monocytes is essential in mediating monocyte infiltration and subsequent atherosclerosis induced by a high-fat diet (*23*) or by Ang II infusion (*13*). Thus, AT1a receptor deficiency in donor bone marrow may suppress monocyte recruitment through MCP-1/CCR2 signaling. However, in our present study, AT1a receptor deficiency in the bone marrow did not affect Ang II–induced macrophage infiltration or atherosclerotic plaque development. This result suggests a minor role of AT1a receptors in bone marrow–derived cells in Ang II–induced atherogenesis in this model. In consensus with these results, we showed that AT1a deficiency did not affect monocyte chemotaxis in response to MCP-1, which was not examined in the previous studies (*15*, *16*). One explanation for the difference between studies is that we used less atherogenic stimuli (normal diet, Ang II 1.9 mg/kg/d for 4 weeks) in this study in comparison with the studies by Cassis *et al*. (high fat diet, Ang II 1.4 mg/kg/d for 4 weeks) (*15*) and Fukuda *et al*. (high fat diet, Ang II 5 mg/kg/d for 8 weeks) (*16*). Stronger stimuli may reveal a role of bone marrow AT1a receptor through yet unclarified mechanisms. Another possible explanation may be the bone marrow maturity after BMT, because we analyzed histology 8 weeks after BMT; Cassis *et al*. analyzed histology 11 weeks after BMT (*15*) and Fukuda *et al*. at 20 weeks after BMT (*16*). We confirmed complete repopulation of leukocytes 4 weeks after BMT by FISH experiments. The extent of atherosclerotic lesions in control BMT mice was equivalent to that of ApoE$^{-/-}$ mice that were administered Ang II (*7*). We have also previously shown that repopulated bone marrow–derived monocytes with or without CCR2 determined Ang II–induced atherosclerosis (*13*), suggesting that repopulated monocytes are sufficiently functional to form Ang II–induced atherosclerosis after BMT using our protocol. However, it is still possible that different leukocyte maturity involving the renin-angiotensin system may affect the results.

The most remarkable finding of our present study is that

Ang II–induced acceleration of atherosclerosis was abolished in host AT1a$^{-/-}$ mice, irrespective of the presence of bone marrow AT1a receptor. This indicates an essential role of the AT1a receptor in host arterial wall cells in Ang II–induced atherogenesis in ApoE$^{-/-}$ mice. These findings are in consensus with recent studies (*15*, *16*); however, we further extended the knowledge of the underlying mechanisms by examining the effect of host AT1a deficiency at 7 d after the initiation of Ang II infusion. We found that host AT1a receptor contributes to superoxide production and MCP-1 expression in the media and VCAM-1 expression in the endothelial cells during the initial phase of Ang II–induced atherogenesis. Vascular smooth muscle cells are considered the main producers of MCP-1 because few macrophages infiltrate into the vascular wall at this time point. It is notable that these changes, including the changes in MCP-1 expression, took place before the infiltration of monocytes/macrophages that were observed later time point, when bone marrow–derived macrophages act as a major source of MCP-1 (*14*, *16*). Ang II is known to activate NAD(P)H oxidase in vascular smooth muscle cells, which increases oxidative stress and subsequent redox-dependent signaling, thus leading to inflammation and proliferation of vascular cells (*21*, *24–26*). These mechanisms are proven to be critical in several forms of vascular pathogenesis (*8*, *18*, *19*, *27*, *28*), and the present study suggests that the AT1a receptor in the vascular wall cells is essential for initiating this process during Ang II–induced atherogenesis.

Host AT1a$^{-/-}$ mice did not show a pressor response to Ang II, which may contribute to the attenuation of atherogenesis. It is unlikely that the lack of a pressor response to Ang II contributed primarily to the observed effect of AT1a deficiency in the host artery during atherogenesis because it is well established that Ang II accelerates atherosclerosis independent of the pressor response in hyperlipidemic mice (*5*). There remains a possibility that AT1a receptors existing in cell types other than those that are bone marrow–derived may affect the results because we used systemic AT1a receptor deficient mice as recipients. Vascular smooth muscle or endothelium selective AT1a receptor deficient mice are necessary as recipients to elucidate the role of each cell type.

In conclusion, the present study demonstrated that AT1a receptors in the host artery, rather than bone marrow–derived cells, are essential for the initiation of Ang II–induced atherogenesis. AT1a receptors in host arterial wall cells regulate the expression of MCP-1 and VCAM-1, possibly *via* increased oxidative stress, and provide an inflammatory condition that in turn leads to monocyte/macrophage recruitment to the vascular wall and atherosclerotic plaque formation. These findings provide another explanation for the pleiotropy of angiotensin-converting enzyme inhibitors and/or AT1 receptor blockers beyond their effects on blood pressure.

References

1. Fox KM: Efficacy of perindopril in reduction of cardiovascular events among patients with stable coronary artery disease: randomised, double-blind, placebo-controlled, multicentre trial (the EUROPA study). *Lancet* 2003; **362**: 782–788.
2. Yusuf S, Sleight P, Pogue J, Bosch J, Davies R, Dagenais G: Effects of an angiotensin-converting–enzyme inhibitor, ramipril, on cardiovascular events in high-risk patients. The Heart Outcomes Prevention Evaluation Study Investigators. *N Engl J Med* 2000; **342**: 145–153.
3. Brasier AR, Recinos A 3rd, Eledrisi MS: Vascular inflammation and the renin-angiotensin system. *Arterioscler Thromb Vasc Biol* 2002; **22**: 1257–1266.
4. Dzau VJ: Theodore Cooper Lecture: tissue angiotensin and pathobiology of vascular disease: a unifying hypothesis. *Hypertension* 2001; **37**: 1047–1052.
5. Wassmann S, Czech T, van Eickels M, Fleming I, Böhm M, Nickenig G: Inhibition of diet-induced atherosclerosis and endothelial dysfunction in apolipoprotein E/angiotensin II type 1A receptor double-knockout mice. *Circulation* 2004; **110**: 3062–3067.
6. Daugherty A, Manning MW, Cassis LA: Angiotensin II promotes atherosclerotic lesions and aneurysms in apolipoprotein E–deficient mice. *J Clin Invest* 2000; **105**: 1605–1612.
7. Weiss D, Kools JJ, Taylor WR: Angiotensin II–induced hypertension accelerates the development of atherosclerosis in apoE-deficient mice. *Circulation* 2001; **103**: 448–454.
8. Ni W, Kitamoto S, Ishibashi M, *et al*: Monocyte chemoattractant protein-1 is an essential inflammatory mediator in angiotensin II–induced progression of established atherosclerosis in hypercholesterolemic mice. *Arterioscler Thromb Vasc Biol* 2004; **24**: 534–539.
9. Kranzhofer R, Browatzki M, Schmidt J, Kubler W: Angiotensin II activates the proinflammatory transcription factor nuclear factor-kappaB in human monocytes. *Biochem Biophys Res Commun* 1999; **257**: 826–828.
10. Tamarat R, Silvestre JS, Durie M, Levy BI: Angiotensin II angiogenic effect *in vivo* involves vascular endothelial growth factor- and inflammation-related pathways. *Lab Invest* 2002; **82**: 747–756.
11. Weber H, Taylor DS, Molloy CJ: Angiotensin II induces delayed mitogenesis and cellular proliferation in rat aortic smooth muscle cells. Correlation with the expression of specific endogenous growth factors and reversal by suramin. *J Clin Invest* 1994; **93**: 788–798.
12. Liu G, Espinosa E, Oemar BS, Lüscher TF: Bimodal effects of angiotensin II on migration of human and rat smooth muscle cells. Direct stimulation and indirect inhibition *via* transforming growth factor-beta 1. *Arterioscler Thromb Vasc Biol* 1997; **17**: 1251–1257.
13. Ishibashi M, Egashira K, Zhao Q, *et al*: Bone marrow–derived monocyte chemoattractant protein-1 receptor CCR2 is critical in angiotensin II–induced acceleration of atherosclerosis and aneurysm formation in hypercholesterolemic mice. *Arterioscler Thromb Vasc Biol* 2004; **24**: e174–e178.
14. Ishibashi M, Hiasa K, Zhao Q, *et al*: Critical role of monocyte chemoattractant protein-1 receptor CCR2 on monocytes in hypertension-induced vascular inflammation and remodeling. *Circ Res* 2004; **94**: 1203–1210.
15. Cassis LA, Rateri DL, Lu H, Daugherty A: Bone marrow

transplantation reveals that recipient AT1a receptors are required to initiate angiotensin II–induced atherosclerosis and aneurysms. *Arterioscler Thromb Vasc Biol* 2007; **27**: 380–386.
16. Fukuda D, Sata M, Ishizaka N, Nagai R: Critical role of bone marrow angiotensin II type 1 receptor in the pathogenesis of atherosclerosis in apolipoprotein E deficient mice. *Arterioscler Thromb Vasc Biol* 2008; **28**: 90–96.
17. Sugaya T, Nishimatsu S, Tanimoto K, *et al*: Angiotensin II type 1a receptor–deficient mice with hypotension and hyperreninemia. *J Biol Chem* 1995; **270**: 18719–18722.
18. Inoue S, Egashira K, Ni W, *et al*: Anti-monocyte chemoattractant protein-1 gene therapy limits progression and destabilization of established atherosclerosis in apolipoprotein E–knockout mice. *Circulation* 2002; **106**: 2700–2706.
19. Ni W, Egashira K, Kitamoto S, *et al*: New anti–monocyte chemoattractant protein-1 gene therapy attenuates atherosclerosis in apolipoprotein E–knockout mice. *Circulation* 2001; **103**: 2096–2101.
20. Nakano K, Egashira K, Ohtani K, *et al*: Catheter-based adenovirus–mediated anti–monocyte chemoattractant gene therapy attenuates in-stent neointima formation in cynomolgus monkeys. *Atherosclerosis* 2007; **194**: 309–316.
21. Griendling KK, Minieri CA, Ollerenshaw JD, Alexander RW: Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. *Circ Res* 1994; **74**: 1141–1148.
22. Tummala PE, Chen XL, Sundell CL, *et al*: Angiotensin II induces vascular cell adhesion molecule-1 expression in rat vasculature: a potential link between the renin-angiotensin system and atherosclerosis. *Circulation* 1999; **100**: 1223–1229.
23. Guo J, Van Eck M, Twisk J, *et al*: Transplantation of monocyte CC-chemokine receptor 2–deficient bone marrow into ApoE3-Leiden mice inhibits atherogenesis. *Arterioscler Thromb Vasc Biol* 2003; **23**: 447–453.
24. Touyz RM, Yao G, Viel E, Amiri F, Schiffrin EL: Angiotensin II and endothelin-1 regulate MAP kinases through different redox-dependent mechanisms in human vascular smooth muscle cells. *J Hypertens* 2004; **22**: 1141–1149.
25. Ni W, Zhan Y, He H, Maynard E, Balschi JA, Oettgen P: Ets-1 is a critical transcriptional regulator of reactive oxygen species and p47(phox) gene expression in response to angiotensin II. *Circ Res* 2007; **101**: 985–994.
26. Mollnau H, Wendt M, Szocs K, *et al*: Effects of angiotensin II infusion on the expression and function of NAD(P)H oxidase and components of nitric oxide/cGMP signaling. *Circ Res* 2002; **90**: E58–E65.
27. Egashira K: Molecular mechanisms mediating inflammation in vascular disease: special reference to monocyte chemoattractant protein-1. *Hypertension* 2003; **41**: 834–841.
28. Kitamoto S, Nakano K, Hirouchi Y, *et al*: Cholesterol-lowering independent regression and stabilization of atherosclerotic lesions by pravastatin and by antimonocyte chemoattractant protein-1 therapy in nonhuman primates. *Arterioscler Thromb Vasc Biol* 2004; **24**: 1522–1528.