Critical Role of Bone Marrow Angiotensin II Type 1 Receptor in the Pathogenesis of Atherosclerosis in Apolipoprotein E–Deficient Mice

Daiju Fukuda, Masataka Sata, Nobukazu Ishizaka, Ryozo Nagai

Objective—It is suggested that the angiotensin II (Ang II)–Ang II type 1 receptor (AT1R) pathway plays a pivotal role in the pathogenesis of atherosclerosis. Recently, bone marrow (BM) cells were reported to express AT1R. Here, we investigated the role of AT1R in BM in the pathogenesis of atherosclerosis.

Methods and Results—Genetic ablation or pharmacological blockade of AT1R led to a significant reduction and stabilization of atherosclerotic lesions in ApoE−/− mice. To elucidate the role of AT1R in BM, we generated several BM chimeric mice. Ang II promoted atherosclerosis progression in the BM chimeric mice that had AT1aR in BM, regardless of the absence of AT1aR in the recipient vasculature (P<0.05). BM chimeric mice whose BM AT1aR was disrupted showed significantly less atherosclerotic lesions in aorta (P<0.05) and more stable plaque with reduced accumulation of BM-derived cells compared with BM chimeric mice that had AT1aR-positive BM. Most of the BM-derived cells in atheroma were positive for a macrophage marker and expressed matrix metalloproteinase (MMP)-9 and monocyte chemotactic protein-1.

Conclusions—Our findings suggest that AT1R in BM plays an important role in the pathogenesis of atherosclerosis.

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Key Words: angiotensin II type I receptor • bone marrow • atherosclerosis • MMP-9 • MCP-1

It is a widely accepted view that atherosclerosis is a chronic inflammatory disease. Although multifactorial in etiology, vascular inflammation produces atherosclerosis through continuous recruitment of circulating leukocytes into the vessel wall and by contributing to an oxidant-rich inflammatory milieu. Recent advances in immunology have dissected several molecular pathways that induce and promote inflammatory responses in atherosclerotic lesions.

The renin–angiotensin system (RAS) has been suggested to play a role in the pathogenesis of atherosclerosis by simulating a series of coordinated cellular and molecular events observed in atherosclerotic lesions. Angiotensin II (Ang II) induces the production of reactive oxygen species and stimulates the expression of adhesion molecules and chemokines, leading to endothelial dysfunction, adhesion and invasion of leukocytes, lipid deposition, and smooth muscle cell proliferation. These observations suggest that local effects of an activated RAS in the vessel wall play a central role in the pathogenesis of chronic vascular inflammation by directly acting on resident vascular cells.

The RAS is reported to be involved in the maintenance of cell proliferation and organ remodeling under physiological or pathophysiological conditions in many tissues other than the cardiovascular system. It was suggested that an activated RAS has local effects in bone marrow (BM), which contributes to the regulation of both normal and malignant hematopoietic processes. It was demonstrated that Ang II increases hematopoietic progenitor cell proliferation. Recently, Cassis et al reported that Ang II type 1a receptor (AT1aR) and that AT1aR expressed on infiltrating cells exerted modest regulation of Ang II–induced atherosclerosis in LDL receptor–deficient mice. It was suggested that the presence of AT1aR in resident tissue was required for the initiation of Ang II–induced atherosclerosis and aneurysm formation.

Although ApoE-deficient and LDL receptor–deficient mice are the most widely used mouse models for atherosclerosis, they differ markedly in lesion type and in their susceptibility to different atherogenic stimuli. Here, we tested the hypothesis that local effects of an activated RAS, especially AT1aR, in BM may play a role in the pathogenesis of atherosclerosis in ApoE-deficient mice. Analyses of BM chimeric mice revealed that AT1aR in BM plays an important role in progression and destabilization of atherosclerotic plaques. We performed a detailed analysis of cellular components of plaque composition and investigated the molecular
mechanism by which BM AT1aR contributes to progression and destabilization of atherosclerotic plaque.

Methods

Animals
ApoE-deficient (ApoE−/−) mice were originally purchased from Jackson Laboratory. Mice deficient in AT1aR, the type 1a receptor of Ang II, (AT1aR−/−) were described previously.6 GFP mice were described previously.7 Double knockout mice deficient in ApoE and AT1aR were generated by cross-breeding ApoE−/− mice and AT1aR−/− mice. Furthermore, we also generated GFP-positive ApoE−/−AT1aR+/+ mice (ApoE−/−AT1aR+/+ GFP−/− mice) and GFP-positive ApoE−/−AT1aR−/− mice (ApoE−/−AT1aR−/− GFP+/+ mice). We administered 10 mg/kg/d olmesartan, an AT1R blocker, or 30 mg/kg/d hydralazine to 6-week-old male ApoE−/− mice by gavage every day for 24 weeks. An osmotic mini-pump (Alzet) was used to infuse Ang II (5 mg/kg/d).

Bone Marrow Transplantation
Bone marrow transplantation (BMT) was performed as described previously.7 At 4 weeks after BMT, all animals were started on a Western type diet. We used only BM chimeric mice, in which more than 80% of BM had been replaced by donor BM. All experimental procedures and protocols were approved by the Animal Care and Use Committee of the University of Tokyo.

Preparation of Aortas and Atherosclerotic Lesions
Lipid deposition was quantified by en face analysis of the aorta as previously described.8 The atherosclerotic lesions in aortic root were analyzed by Oil red-O staining, Sirius-red staining, and immunohistochemistry as previously described.9

Laser Microdissection and Quantitative Real-Time Polymerase Chain Reaction
Total RNA was isolated from the atherosclerotic lesions of the aortic root with a Laser Microdissection System (AS LMD, Leica) according to the manufacturer’s instructions. First strand cDNA was synthesized from the obtained total RNA using a Quantitect Reverse Transcription Kit (QIAGEN) for quantitative real-time polymerase chain reaction (PCR).

Statistical Analysis
Numerical values are expressed as mean±SEM. Comparison of parameters between 2 groups was performed by unpaired Student t test. A value of P<0.05 was considered significant.

For further details, please refer to the supplemental materials (available online at http://atvb.ahajournals.org).

Results
Effects of Genetic Ablation or Pharmacological Blockade of AT1R on Atherosclerotic Plaque Formation
We generated ApoE−/−AT1aR−/− double knockout mice by cross-breeding ApoE−/−AT1aR+/+ mice and ApoE+/+ AT1aR−/− mice. We compared atherosclerotic lesion progression between male ApoE−/−AT1aR−/− mice (n=9) and ApoE−/−AT1aR−/− mice (n=7) fed normal chow. As previously reported, systolic blood pressure was significantly lower in ApoE−/−AT1aR−/− mice (90.1±2.9 mm Hg) than in ApoE−/−AT1aR+/− mice (101.1±3.4 mm Hg; P=0.03). Plasma total cholesterol level was significantly higher in ApoE−/−AT1aR−/− mice (911±102 mg/dL) than in ApoE−/−AT1aR+/− mice (513±41 mg/dL; P=0.006). At 32 weeks of age, en face Sudan IV staining of the aortic arch revealed a significant reduction in atherosclerotic lesion formation in ApoE−/−AT1aR+/+ mice (1.4±0.3 versus 4.2±0.7 mm²; P=0.003; Figure 1A). Furthermore, Oil red O staining and Sirius-red staining of atherosclerotic lesions in the aortic root revealed significantly decreased lipid deposition (8.4±2.4 versus 18.1±2.8%, P=0.04) and increased collagen content (27.4±5.3 versus 7.9±4.0%, P=0.03) in the plaques of ApoE−/−AT1aR−/− mice compared with those of ApoE−/−AT1aR+/+ mice (Figure 1B and 1C). We infused Ang II (5 mg/kg/d) or vehicle into the 20-week-old mice for 2 weeks. In ApoE−/−AT1aR+/+ mice, Ang II markedly accelerated atherosclerotic lesion formation in aortic arch (9.3±1.4 versus 2.1±0.4 mm²; P=0.0001) associated with significant elevation in systolic blood pressure (143.0±3.2 versus 95.5±2.6 mm Hg; P<0.0001). On the other hand, in ApoE−/−AT1aR−/− mice, there was no significant difference in atherosclerotic lesion area (0.8±0.3 versus 1.5±0.5 mm², P=0.20) or in blood pressure (92.1±4.2 versus 80.5±4.5 mm Hg, P=0.09) between the Ang II–treated and vehicle-treated groups.

Next, we administered 10 mg/kg/d olmesartan (n=6), an AT1R blocker, or 30 mg/kg/d hydralazine (n=6) to 6-week-old male ApoE−/−AT1aR+/+ mice fed a Western-type diet every day by gavage for 24 weeks. There was no significant difference between the 2 groups in systolic blood pressure.
(hydralazine, 64.7±2.6 versus olmesartan, 61.7±1.4 mm Hg; \( P=0.52 \)) or plasma total cholesterol level (hydralazine, 527±33 versus olmesartan, 523±27 mg/dL, \( P=0.93 \)). Consistent with the effects of genetic ablation of AT1aR, en face Sudan IV staining of the aortic arch revealed significant suppression of atherosclerotic lesion progression by olmesartan (2.8±0.6 versus 5.1±0.5 mm\(^2\), \( P=0.01 \)) (Figure 1D). Furthermore, Oil red O staining of the plaques in the aortic root revealed that olmesartan decreased lipid content (7.3±1.3 versus 14.5±2.9\%, \( P=0.048 \)) with increased collagen content (38.7±4.3 versus 23.1±4.5\%, \( P=0.03 \)) as detected by Sirius red staining.

**Effects of Restoration of BM AT1aR on Atherosclerosis in ApoE\(^{-/-}\)AT1aR\(^{-/-}\) Mice**

To evaluate the potential contribution of AT1aR in BM to the pathogenesis of atherosclerosis, we generated several combinations of BM chimeric mice. We performed BMT from ApoE\(^{-/-}\)AT1aR\(^{-/-}\) mice to ApoE\(^{-/-}\)AT1aR\(^{-/-}\) mice at 10 to 14 weeks of age. We also performed BMT from ApoE\(^{-/-}\)AT1aR\(^{+/+}\) mice to ApoE\(^{-/-}\)AT1aR\(^{-/-}\) mice at the same age. These BM chimeric mice had AT1aR in BM, but not in their innate vascular cells. At 12 weeks after BMT, white blood cell count was similar between the AT1aR\(^{-/-}\) recipients repopulated with AT1aR\(^{+/+}\) BM and AT1aR\(^{-/-}\) BM (5.6±0.4 versus 4.9±0.5 \times 10\(^{3}\) /μL, \( P=0.28 \)). From 12 weeks after BMT, we infused 5 mg/kg/d Ang II or vehicle into these BM chimeric mice for 8 weeks using an osmotic mini-pump. Ang II infusion into these BM chimeric mice elevated blood pressure significantly compared with vehicle infusion, though these mice had no AT1aR in their vasculature. There was no significant difference in blood pressure or in plasma cholesterol level between Ang II-treated AT1aR\(^{-/-}\) recipient mice repopulated with AT1aR\(^{+/+}\) BM and Ang II-treated AT1aR\(^{-/-}\) recipient mice repopulated with AT1aR\(^{-/-}\) BM (Systolic blood pressure; 97.0±6.2 versus 107.4±3.6 mm Hg, \( P=0.16 \); Total cholesterol level; 728±50 versus 650±54 mg/dL, \( P=0.32 \)). After 8 weeks of infusion, en face Sudan IV staining of the aortic arch revealed that atherosclerotic lesions in AT1aR\(^{-/-}\) recipients with AT1aR\(^{+/+}\) BM (\( n=8 \)) were significantly larger than those in AT1aR\(^{-/-}\) recipients with AT1aR\(^{-/-}\) BM (\( n=7 \); 1.9±0.5 versus 0.6±0.2 mm\(^2\), \( P=0.03 \)) (Figure 2). Histological analysis of atherosclerotic lesions in the aortic root revealed that lipid deposition detected by Oil red O staining was accelerated in AT1aR\(^{-/-}\) recipients with AT1aR\(^{+/+}\) BM compared with those in AT1aR\(^{-/-}\) recipients with AT1aR\(^{-/-}\) BM (12.1±2.2 versus 5.5±1.4\%, \( P=0.03 \)). Collagen content demonstrated by Sirius-red staining was decreased in AT1aR\(^{-/-}\) recipients with AT1aR\(^{+/+}\) BM compared with that in AT1aR\(^{-/-}\) recipients with AT1aR\(^{-/-}\) BM (9.7±1.5 versus 18.2±2.6\%, \( P=0.01 \)). We measured mRNA expression of MMP-9, MCP-1, and vascular cell adhesion molecule (VCAM-1) in the plaques by means of a laser microdissection system and quantitative RT-PCR. MMP-9 expression in AT1aR\(^{-/-}\) recipients with AT1aR\(^{-/-}\) BM tended to be greater compared with that in AT1aR\(^{-/-}\) recipients with AT1aR\(^{-/-}\) BM (3.5±1.5 versus 0.7±0.2 [arbitrary unit], \( P=0.11 \)). There was no statistical difference in MCP-1 (7.6±2.4 versus 3.6±0.8 [arbitrary unit], \( P=0.15 \)) or VCAM-1 (5.6±0.4 versus 6.0±0.3 [arbitrary unit], \( P=0.58 \)) expression. Among the vehicle treated mice (\( n=7 \) for each group), collagen content in atheroma in AT1aR\(^{-/-}\) recipients with AT1aR\(^{-/-}\) BM was significantly decreased compared with that in AT1aR\(^{-/-}\) recipients with AT1aR\(^{-/-}\) BM, although atherosclerotic lesion area in aorta (Figure 2), lipid content in atheroma and RNA expression in the lesion were similar (supplemental Table I). Taken together, these results suggest that BM transplantation from AT1aR\(^{+/+}\) donors to AT1aR\(^{-/-}\) recipients could restore Ang II–induced acceleration of atherosclerosis and plaque destabilization.

**Effects of Targeted Disruption of BM AT1aR on Atherosclerosis in ApoE\(^{-/-}\)AT1aR\(^{-/-}\) Mice**

Next, to keep track of BM-derived cells in the process of atherosclerotic lesion progression, we replaced BM of ApoE\(^{-/-}\)AT1aR\(^{-/-}\) mice with that of ApoE\(^{-/-}\)AT1aR\(^{+/+}\)GFP\(^{+/+}\) mice or ApoE\(^{-/-}\)AT1aR\(^{-/-}\)GFP\(^{+/+}\) mice at 10 weeks of age. The former BM chimeric mice lacked AT1aR only in BM, and the latter chimeric mice had AT1aR in both BM and the vasculature. At 12 weeks after BMT, white blood cell count was similar between the AT1aR\(^{-/-}\) recipient mice repopulated with AT1aR\(^{-/-}\) BM and the AT1aR\(^{-/-}\) recipient mice repopulated with AT1aR\(^{-/-}\) BM (6.9±0.6 versus 6.3±0.8 \times 10\(^{3}\) /μL, \( P=0.52 \)). In these BM chimeric mice, we compared the effects of Ang II on atherosclerotic lesion formation. We infused Ang II from 12 weeks after BMT. After 8 weeks infusion, en face Sudan IV staining of the aortic arch revealed that acceleration of atherosclerotic lesion was significantly attenuated in the AT1aR\(^{-/-}\) recipients repopulated with AT1aR\(^{-/-}\) BM (\( n=7 \)) compared with that in the AT1aR\(^{-/-}\) recipients repopulated with AT1aR\(^{-/-}\) BM (\( n=9 \); 5.8±0.9 versus 9.8±1.1 mm\(^2\), \( P=0.02 \); Figures 2 and 3A), with reduced accumulation of GFP-positive cells (5.8±0.3 versus 9.3±1.3 mm\(^2\), \( P=0.03 \); Figure 3A). In these
2 types of BM chimeric mice, there was no significant difference in blood pressure (136.9 ± 4.7 versus 141.7 ± 3.1 mm Hg, \( P = 0.40 \)) or in total cholesterol level (1019 ± 87 versus 912 ± 76 mg/dL, \( P = 0.37 \)). In atherosclerotic plaques in the aortic root in the AT1aR−/− mice, there was no significant difference in blood pressure between the AT1aR+/+ recipients with AT1aR+/+ BM and those with AT1aR−/− BM. Similar to Ang II–treated mice, atherosclerotic lesion of aorta was significantly attenuated in the AT1aR+/+ recipients with AT1aR−/− BM (n=5) compared with that in the AT1aR−/− recipients with AT1aR+/+ BM (n=6; Figure 2). However, lipid deposition, collagen content, and RNA expression in atheroma were similar in the AT1aR+/+ recipients repopulated with AT1aR+/+ BM or AT1aR−/− BM (supplemental Table I).

To investigate how AT1aR positive BM cells contribute to the pathogenesis of atherosclerosis, we examined gene expression in the plaques by means of a laser microdissection system and quantitative RT-PCR (n=4 in each group) at 4 weeks after Ang II infusion (Figure 4A). Expressions of MMP-9 (3.0-fold, \( P = 0.04 \)) and MCP-1 (7.1-fold, \( P = 0.02 \)) in the AT1aR+/+ recipients repopulated with AT1aR−/− BM was significantly greater than those in the AT1aR+/+ recipients repopulated with AT1aR+/+ BM. On the other hand, there was no significant difference in VCAM-1 expression between the 2 BM chimeric mice. Accumulation of BM-derived GFP-positive cells was significantly accelerated in AT1aR+/+ recipients with AT1aR−/− BM (n=8) compared with that in AT1aR+/+ recipients with AT1aR+/− BM (n=7; 60.3 ± 3.8 versus 38.4 ± 1.9%, \( P = 0.0003 \)). Most of the BM-derived cells were positive for a macrophage marker (Figure 4B). Furthermore, the percentage of BM-derived GFP-positive cells among the MMP-9−positive cells (72.7 ± 6.3 versus 38.2 ± 1.9%, \( P = 0.0003 \)) or the MCP-1−positive cells (55.1 ± 2.8 versus 42.5 ± 4.8%, \( P = 0.10 \)) was greater in the AT1aR+/+ recipient with AT1aR+/− BM than in the AT1aR+/+ recipient with AT1aR−/− BM (Figure 4C and 4D).

**Discussion**

In this study, we demonstrated that genetic ablation or pharmacological blockade of AT1R effectively suppressed atherosclerotic lesion formation with more stabilized morphological characteristics of the plaque. We found that AT1aR-positive BM cells accelerated atherosclerotic lesion progression and plaque destabilization, even if the recipient vasculature cells did not express AT1aR. On the other hand, lack of AT1aR in BM cells decreased atherosclerotic lesion progression and stabilized plaques with or without Ang II infusion despite the existence of AT1aR in vascular cells. Histological studies revealed that accumulation of BM-derived cells in atherosclerotic lesions was enhanced when AT1aR was expressed in BM cells. Moreover, the existence of AT1aR in BM significantly increased the expression of MMP-9 and MCP-1 in atherosclerotic plaques. The percentage of BM-derived cells among the MCP-1− or MMP-9−expressing cells in the lesions was decreased by the disruption of AT1aR in BM. Most of the BM-derived cells accumulated in the lesions were positive for a macrophage marker. Taken together, our present study demonstrated...
functional contribution of the AT1aR in BM to the pathogenesis of atherosclerosis in vivo.

The RAS has been considered to be a circulating hormonal system that regulates blood pressure and flow. Recent studies have provided evidence for local effects of an activated RAS, particularly in the cardiac, vascular, and renal systems. It is now well established that Ang II has significant proinflammatory actions on the vessel wall, leading to progression of atherosclerosis. It is well known that there are 2 different types of Ang II receptors, AT1R and AT2R, in mammals. Both AT1R and AT2R have been identified in the vessel wall. In rodents, 2 AT1R subtypes, AT1aR and AT1bR, have been identified. In the vasculature, AT1aR is predominant and mediates most of the physiological and pathophysiological responses to Ang II. It is well known that there are 2 different types of Ang II receptors, AT1R and AT2R, in mammals. Both AT1R and AT2R have been identified in the vessel wall. In rodents, 2 AT1R subtypes, AT1aR and AT1bR, have been identified. In the vasculature, AT1aR is predominant and mediates most of the physiological and pathophysiological responses to Ang II. There is increasing evidence of cross-talk between RAS and dyslipidemia in atherogenesis. It was demonstrated that hypercholesterolemia stimulates angiotensin peptide synthesis and increased the density of AT1R, suggesting that Ang II–AT1R pathway may mediate, at least in part, the atherogenic effects of hypercholesterolemia. Consistently, previous reports demonstrated that inhibition of AT1R-signaling reduces atherosclerosis. The greatest AT1R density has been found on vascular smooth muscle cells and endothelial cells. Thus, the antiatherogenic effects of AT1R blockade are thought to result from inhibition of AT1R-mediated signaling in resident vascular cells.

Recent reports suggest that local effects of an activated RAS exist in BM and functions to promote differentiation and proliferation of BM cells. Our preliminary study revealed that AT1aR was abundantly expressed in BM, whereas other receptors were hardly detected (supplemental Figure IA). We also found that AT1aR could be detected in atherosclerotic lesions in the AT1aR/H11001/H11001 recipients repopulated with AT1aR/H11001/H11001 BM (supplemental Figure IB). Thus, we here focused on AT1aR in BM, although it is plausible that other receptors of Ang II may participate in the atherogenic effects of Ang II.

Previous reports have demonstrated that AT1R in the vasculature mediates upregulation of adhesion molecules and chemokines, thus promoting infiltration of inflammatory cells into the vessel wall. MCP-1 in vascular cells is one of the essential inflammatory mediators in Ang II–induced progression of atherosclerosis. Several reports have demonstrated that monocytes/macrophages release MCP-1 through activation of the Ang II–AT1R pathway in vitro. Our results showed the expression of MCP-1 from BM-derived cells in plaques. Selective disruption of AT1aR in BM significantly decreased MCP-1 expression in plaques in ApoE−/−/AT1aR−/− mice that received Ang II infusion for 4 weeks. It was suggested that AT1aR-positive BM-derived cells themselves could be a source of MCP-1 in plaques. As well as MCP-1,
MMPs are demonstrated to be expressed in atherosclerotic lesions.\textsuperscript{18} Especially, MMP-9 is important for the resorption of extracellular matrix and contributes to progression and destabilization of atherosclerosis. An AT1R antagonist is reported to inhibit MMP-9 expression in a mouse model of atherosclerosis.\textsuperscript{19} Our present results showed that AT1aR-positive BM-derived cells are an important source of MMP-9.

Recently, when this study was being conducted, Cassis et al reported that Ang II (1.0 μg/kg/min) promotes vascular pathology via AT1aR in LDL receptor-deficient mice.\textsuperscript{20} Consistent with our findings, repopulation of AT1aR\textsuperscript{+/+} mice with AT1aR\textsuperscript{−/−} BM resulted in modest reductions in Ang II–induced atherosclerosis.\textsuperscript{20} Here, we confirm the importance of AT1aR in BM. In addition, we demonstrate that AT1aR-positive BM cells not only accelerate accumulation of BM-derived cells in the lesions through MCP-1 expression, but also contribute to plaque progression and destabilization by secretion of MMP-9, at least in part. Thus, our study significantly extends the findings of Cassis et al and provides novel insights into the mechanism by which Ang II promotes atherosclerosis progression and instability.

Unexpectedly, Cassis et al found that AT1aR\textsuperscript{−/−} recipient mice were dramatically protected from Ang II (1.44 mg/kg/d for 4 to 6 weeks after BMT).\textsuperscript{21} Assays of immune function numbers of donor cells can be observed in peripheral blood at a longer period (5.0 mg/kg/d for 8 weeks) could promote AT1aR in BM. However, Ang II infusion at a higher dose for Ang II–induced atherosclerosis.\textsuperscript{20} Here, we confirm the importance of AT1aR in BM. In addition, we demonstrate that AT1aR-positive BM cells not only accelerate accumulation of BM-derived cells in the lesions through MCP-1 expression, but also contribute to plaque progression and destabilization by secretion of MMP-9, at least in part. Thus, our study significantly extends the findings of Cassis et al and provides novel insights into the mechanism by which Ang II promotes atherosclerosis progression and instability.

Unexpectedly, Cassis et al found that AT1aR\textsuperscript{−/−} recipient mice were dramatically protected from Ang II (1.44 mg/kg/d for 4 weeks)-induced vascular pathologies irrespective of BM donor genotype, suggesting that the presence of AT1aR in resident tissue is required for the initiation of Ang II–induced atherosclerosis.\textsuperscript{20} In this study, we also found that atherosclerosis development was notably retarded in AT1aR\textsuperscript{−/−} mice with vehicle infusion regardless of the existence of AT1aR in BM. However, Ang II infusion at a higher dose for a longer period (5.0 mg/kg/d for 8 weeks) could promote atherosclerosis significantly even in AT1aR\textsuperscript{−/−} recipients when the BM cells were repopulated with AT1aR\textsuperscript{+/+} BM. Others reported that there are several differences in the pathogenesis of dyslipidemia and atherosclerosis between ApoE\textsuperscript{−/−} and LDL-R\textsuperscript{−/−} mice.\textsuperscript{3} Moreover, we infused Ang II into BM chimeric mice at 12 weeks after BMT, whereas Cassis et al started Ang II treatment at 7 weeks after BMT. In our study, blood cell count at 12 weeks after BMT was similar among 4 BMT groups. At 40 weeks after BMT, the blood cell count and the BM cell composition were also similar in the AT1aR\textsuperscript{+/+} recipients repopulated with AT1aR\textsuperscript{+/+} BM and AT1aR\textsuperscript{−/−} BM (supplemental Table II). However, many reports documented that only the limited number of engrafted cells are matured with skewed population frequencies at 4 to 6 weeks after BMT, although high numbers of donor cells can be observed in peripheral blood at 4 to 6 weeks after BMT.\textsuperscript{21} Assays of immune function indicate deficient functions at earlier time points after BMT. Thus, the differences in the type of hypercholesterolemic mice, the protocol of Ang II infusion, and the timing of Ang II infusion relative to the BMT might lead to the different results by us and by Cassis et al.

The dose of Ang II used in our study might be high compared with those used in previous studies. However, 5 mg/kg/d Ang II did not cause apparent toxic effects, such as increased mortality (supplemental Table III), changes in plasma lipid profile, or body weight loss, in the BM chimeric mice. Moreover, in our preliminary study, 3.0 mg/kg/d of Ang II infusion was not sufficient for blood pressure elevation and acceleration of atherosclerosis even in AT1aR\textsuperscript{+/+} recipients repopulated with AT1aR\textsuperscript{+/+} BM (Fukuda and Sata, unpublished data), suggesting that higher dose of Ang II would be required to evaluate the effects of Ang II in BM chimeric ApoE\textsuperscript{−/−} mice repopulated with exogenous BM after lethal irradiation. Therefore, we chose the dose of 5 mg/kg/d of Ang II in this study.

Unexpectedly, in our study, ApoE\textsuperscript{−/−}AT1aR\textsuperscript{−/−} mice showed significantly higher total cholesterol level than that in ApoE\textsuperscript{−/−}AT1aR\textsuperscript{+/+} mice. This was inconsistent with previous reports.\textsuperscript{22} Analysis of lipid profile showed similar pattern in ApoE\textsuperscript{−/−}AT1aR\textsuperscript{+/+} mice and ApoE\textsuperscript{−/−}AT1aR\textsuperscript{−/−} mice (data not shown). ApoE\textsuperscript{−/−}AT1aR\textsuperscript{−/−} mice showed significantly lower blood pressure compared with ApoE\textsuperscript{−/−}AT1aR\textsuperscript{+/+} mice, consistent with a previous report.\textsuperscript{22} It could be possible that alteration of blood pressure and lipid level may affect the development of atherosclerosis in ApoE\textsuperscript{−/−}AT1aR\textsuperscript{−/−} mice. However, in our experiment on pharmacological blockade of AT1R, there were no significant difference in blood pressure and cholesterol level between olmesartan-treated mice and hydralazine-treated mice. In our studies with BM chimeric mice, there was no significant difference in blood pressure or plasma cholesterol level in AT1aR\textsuperscript{−/−} recipients or AT1aR\textsuperscript{+/+} recipients regardless of BM AT1aR genotype. Therefore, our data obtained with the BM chimeric mice appear to be independent of blood pressure and plasma cholesterol level.

In summary, our results suggest that AT1aR expressed not only on vascular cells but also on BM-derived cells plays a role in the pathogenesis of atherosclerosis, at least in part. Therefore, blockade of AT1R not only in vascular cells but also in BM could be an important strategy to prevent the progression and destabilization of atherosclerotic plaques.

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Disclosures
None.

References


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Supplementary Methods

Animals

ApoE-deficient (ApoE-/-) mice (B6.129P2-Apoe<sup>tm1Unc</sup>/J, Stock # 002052) were originally purchased from Jackson Laboratory. Mice deficient in AT1aR, the type 1a receptor of Ang II, (AT1aR-/-) were generated from a germline chimera derived from TT2 embryonic stem cells with a targeted mutation of the AT1a gene as described previously. GFP mice, which are transgenic mice (C57BL/6 background) that ubiquitously express enhanced green fluorescent protein (GFP), were donated by Dr Masaru Okabe (Osaka University, Osaka, Japan). All mice were backcrossed 10 times into a C57BL/6 background. To investigate the participation of the Ang II-AT1R pathway in atherosclerosis, we generated double knockout mice deficient in ApoE and AT1aR by cross breeding ApoE-/- mice and AT1aR-/- mice. Genotypes were determined by PCR amplification of tail DNA. Heterozygous animals were crossed until homozygous double knockout mice were obtained. Furthermore, to elucidate the contribution of BM cells to atherosclerosis, we also generated GFP-positive ApoE-/-AT1aR+/+ mice (ApoE-/-AT1aR+/+GFP+/+ mice) and GFP-positive ApoE-/-AT1aR-/− mice (ApoE-/-AT1aR-/−GFP+/+ mice).

Bone marrow transplantation

Bone marrow transplantation (BMT) from either male ApoE-/-AT1aR+/+ mice or male ApoE-/-AT1aR-/− mice to female ApoE-/-AT1aR-/− mice and BMT from either male...
ApoE-/-AT1aR+/+GFP+/- mice or male ApoE-/-AT1aR-/-GFP+/- mice to female ApoE-/-AT1aR+/+ mice were performed as described previously. At 4 weeks after BMT, all animals were started on a western type diet. We used only BM chimeric mice, in which more than 80% of BM had been replaced by donor BM as determined by in-situ hybridization for the Y chromosome in female recipient mice repopulated with male BM. Flow cytometry was also used in recipient mice repopulated with AT1aR+/+GFP+/- or AT1aR-/-GFP+/- BM. All experimental procedures and protocols were approved by the Animal Care and Use Committee of the University of Tokyo and complied with the “Guide for the Care and Use of Laboratory Animals” (NIH publication No. 86-23, revised 1985).

**Blood pressure and plasma cholesterol measurement**

Blood pressure of each mouse was measured with a tail-cuff system (BP-98A, Softron) in conscious animals. In each animal, the mean value of three measurements was used for comparison. At the time of sacrifice, blood was collected from the heart into an EDTA-containing tube. After blood samples were centrifuged, plasma was removed and stored at –80°C until measurement of total cholesterol level.

**Preparation of aortas and atherosclerotic lesions**

Lipid deposition was quantified by en face analysis of the aorta as previously described. Mice were sacrificed with an overdose of pentobarbital, and perfused with 0.9% sodium chloride solution at a constant pressure via the left ventricle. Both their heart and whole aorta
were immediately removed. The whole aorta was excised and opened longitudinally and fixed with 4% paraformaldehyde. To quantify the atherosclerotic lesions in the thoracic aorta, we analyzed the aorta by en face Sudan IV staining. Sudan IV-positive area in the aortic arch was measured. Sirius red polarization microscopy was performed to visualize interstitial collagen, as previously described.

**Histology and immunohistochemical analysis**

Each heart was cut in the plane between the lower tips of the right and left atria. The upper portion was snap-frozen in OCT compound (TissueTek, Tokyo). Then, the aortic root was sectioned serially (5 μm intervals) from the appearance of the aortic valve to the ascending aorta until the valve cusps were no longer visible. Frozen sections were used for Oil red O staining and Sirius red staining for the detection of lipid and collagen, respectively. Some sections were incubated with anti-MOMA-2 antibody (Serotec) followed by the avidin-biotin complex technique and stained with Vector Red substrate kit (Vector). Each section was counterstained with hematoxylin. To investigate the characteristics of BM-derived cells, the remaining sections were incubated with anti-matrix metalloproteinase-9 antibody (MMP-9) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-MCP-1 antibody (R&D Systems, Minneapolis, MN) and anti-MOMA-2 antibody (Serotec) followed by Cy3-conjugated secondary antibody. We enhanced the GFP signal with an anti-GFP antibody (Molecular Probes, Eugene, OR) followed by Alexa Fluor 488-conjugated secondary antibody (Molecular
Nuclei were counterstained with Hoechst 33258. The sections were mounted with a Prolong Antifade Kit (Molecular Probes) and observed under a confocal laser scanning system (FLUOVIEW FV300, Olympus, Tokyo).

**Sirius red polarization method to detect collagen**

Sirius red polarization microscopy was performed to visualize interstitial collagen. Fresh-frozen sections (5 μm) were rinsed with distilled water and incubated with 0.1% Sirius red (Sigma-Aldrich, St. Louis, MO) in saturated picric acid for 90 minutes. Sections were rinsed twice with 0.03 N HCl for one minute each time and then immersed in distilled water. After dehydration with 70% ethanol for 30 seconds, the sections were coverslipped. The stained sections were examined under a polarization microscope (Eclipse LV100POL, Nikon, Tokyo). Images were digitized with a CCD camera (Digital Sight DS-2Mv, Nikon). As the fiber thickness increases, the color changes from green to red.

**Detection of Ang-II receptors in BM and atherosclerotic plaque**

Total RNA was extracted using total RNA extraction kit (Agilent Inc.) from BM cells of C57BL/6 mice, ApoE-/-AT1aR+/+ mice, ApoE-/-AT1aR+/+ mice, BMT AT1aR+/+ --> AT1aR-/- mice, BMT AT1aR+/+ --> AT1aR-/- mice, BMT AT1aR+/+ --> AT1aR-/+ mice and BMT AT1aR+/+ --> AT1aR+/+ mice. Reverse transcription was performed with 1μg of RNA, random primers and MMLV reverse transcriptase (ReverTraAce-α, TOYOBO). PCR primers were as follows: AT1aR (201bp), sense 5’-TCACCTGCATCATCATCTCTGG-3’, and antisense
5’-AGCTGGTAAGAATGATTAGG-3’; AT1bR (488bp), sense
5’-GCAGCATTTAGCTAGACAGTTC-3’, and antisense
5’-GCCTACGAAATCTTAACACAC-3’; AT2R (160bp), sense
5’-CCTTTTGATAATCTCAACGCAA3’; and antisense
5’-GACAAACAAACAGTGAGACCACA-3’; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (452bp), 5’-ACCACAGTCCATGCCATCAC-3’ and 5’TCCACCACCCCTGGTGTGTA-3’. PCR reactions were carried out for 30 cycles of 30 seconds of denaturation at 94°C, 30 seconds of annealing at 60°C, and 1 minute of extension at 72°C followed by 10 minutes of final extension. After agarose-gel (3%) electrophoresis in the presence of ethidium bromide, the PCR products were revealed by UV irradiation. To investigate expression of Ang II receptors in atherosclerotic lesions, we performed laser microdissection and quantitative real-time PCR. Probes for AT1aR, AT1bR, AT2R and GAPDH were purchased from Applied Biosystems. GAPDH was used as an internal control.

**Measurement of peripheral blood cell count**

Measurement of peripheral blood cell count was performed with LC-152 (HORIBA) at 12 weeks after BMT.

**Cell sorting analysis**

BM cells were harvested from femur and tibia of BMT AT1aR-/- --> AT1aR+/+ mice (n=3) and BMT AT1aR+/+ --> AT1aR+/+ mice (n=3). Peripheral blood was also collected from these mice in EDTA
containing tube, and then red blood cells were removed using PharM Lyse (BD Biosciences). BM cells and peripheral white blood cells were stained with biotin conjugated primary antibody for each lineage marker (Gr-1, Mac-1, CD3e, B220 and TER-119) followed by streptavidin-phycoerythrin (BD Pharmingen). Dead cells were excluded with propidium iodide staining. Percentage of each marker positive population was analyzed by flow cytometer (EPICS XL, Coulter).

**Laser microdissection and RNA extraction**

To investigate the characteristics of atherosclerotic lesions, we collected atherosclerotic plaques from snap-frozen sections (5 μm) of the aortic root of each BM chimeric mice. Sections were fixed in cold ethanol /acetic acid (19:1) for 3 min. After fixation, sections were washed in cold DEPEC water for 1 min, then stained with 0.05% toluidine blue for 3 min. After drying, laser capture was performed with a Laser Microdissection System (AS LMD, Leica) according to the manufacturer’s instructions. For each mouse, we collected 18 lesions and extracted total RNA with an RNeasy Micro Kit (QIAGEN). First strand cDNA was synthesized from the total RNA using a Quantitect Reverse Transcription Kit (QIAGEN) for quantitative real-time PCR.

**Quantitative real-time PCR**

To quantify the differential expression of RNA, real-time PCR was performed on Mx3000P (STRATAGENE) using TaqMan Universal PCR Master Mix (Applied Biosystems).
Expression of the MMP-9, MCP-1 and vascular cell adhesion molecule-1 (VCAM-1) genes was measured by TaqMan Probe method. GAPDH was used as an internal control. These probes were purchased from Applied Biosystems. All measurements were duplicated and the mean value was used.

References for supplementary Methods


M. Statins alter smooth muscle cell accumulation and collagen content in established atheroma of watanabe heritable hyperlipidemic rabbits. *Circulation.* 2001;103:993-999.
Supplementary Figure I

A. BM cells were harvested from femur and tibia of C57BL/6 mice, ApoE-/-AT1aR+/+ mice, ApoE-/-AT1aR+/+ mice, BMT AT1aR-/- --> AT1aR-/- mice, BMT AT1aR+/+ --> AT1aR-/- mice, BMT AT1aR+/+ --> AT1aR+/+ mice and BMT AT1aR-/- --> AT1aR+/+ mice. RNA was extracted from BM cells and then real-time PCR performed to analyze Ang II receptor expression. AT1aR was expressed in wild type mice, ApoE-/-AT1aR+/+ mice and BM chimeric mice whose BM had been replaced with that of AT1aR+/+ mice. Neither AT1bR nor AT2R was detected in BM.

B. Expression of Ang II receptors in atherosclerotic lesions. RNA was extracted from atheroma dissected from aortic root using laser microdissection system. Expression of Ang II receptor expression was analyzed by real-time PCR. GAPDH was used as an internal control. Data were presented as mean ± SEM (arbitrary units). All donors and recipients were ApoE-/- background.
**Supplementary Table I**

**Comparison of effect of vehicle infusion in BMT mice**

<table>
<thead>
<tr>
<th>(donor)</th>
<th>AT1aR+/+</th>
<th>AT1aR/-</th>
<th>p</th>
<th>AT1aR+/+</th>
<th>AT1aR/-</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>(recipient)</td>
<td>AT1aR+/+</td>
<td></td>
<td></td>
<td>AT1aR/-</td>
<td></td>
<td></td>
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<tr>
<td>systolic blood pressure, mmHg</td>
<td>100.4±13.7</td>
<td>95.1±4.5</td>
<td>0.68</td>
<td>75.2±4.0</td>
<td>81.3±3.1</td>
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<td>total cholesterol level, mg/dL</td>
<td>586±20</td>
<td>568±40</td>
<td>0.67</td>
<td>569±84</td>
<td>717±35</td>
<td>0.13</td>
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<td>plaque characteristics</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Oil red O staining, %</td>
<td>9.0±1.4</td>
<td>7.0±1.5</td>
<td>0.37</td>
<td>7.8±1.9</td>
<td>6.5±1.7</td>
<td>0.65</td>
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<tr>
<td>Sirius red staining, %</td>
<td>16.6±1.4</td>
<td>16.6±1.80</td>
<td>1</td>
<td>10.9±1.6</td>
<td>21.4±4.3</td>
<td>0.03</td>
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<tr>
<td>RNA expression in plaques</td>
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<td></td>
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<tr>
<td>MMP-9/GAPDH (arbitrary unit)</td>
<td>1.1±1.0</td>
<td>0.006±0.005</td>
<td>0.41</td>
<td>0.6±0.5</td>
<td>0.03±0.02</td>
<td>0.29</td>
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<td>MCP-1/GAPDH (arbitrary unit)</td>
<td>5.6±0.6</td>
<td>4.4±1.1</td>
<td>0.37</td>
<td>4.5±0.7</td>
<td>9.5±2.8</td>
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<td>VCAM-1/GAPDH (arbitrary unit)</td>
<td>6.5±0.7</td>
<td>6.9±0.2</td>
<td>0.73</td>
<td>10.3±0.4</td>
<td>11.4±0.7</td>
<td>0.2</td>
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</table>

Data were presented as mean±SEM. All donors and recipients were ApoE-/- background.
## Supplementary Table II

### Comparison of peripheral blood cell count and BM cell composition

<table>
<thead>
<tr>
<th></th>
<th>(donor) AT1aR+/+</th>
<th>AT1aR-/- AT1aR+/+</th>
<th>p</th>
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</thead>
<tbody>
<tr>
<td><strong>blood cell count</strong></td>
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<tr>
<td>white blood cell (x10^3/mL)</td>
<td>2.8±0.9</td>
<td>2.6±0.8</td>
<td>0.9</td>
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<tr>
<td>red blood cell (x10^6/mL)</td>
<td>7.6±0.2</td>
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<tr>
<td>platelet (x10^3/mL)</td>
<td>594.3±48.8</td>
<td>662.7±196.8</td>
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<td><strong>peripheral white blood cell composition</strong></td>
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<tr>
<td>Gr-1, %</td>
<td>15.7±5.4</td>
<td>16.8±3.4</td>
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<td>Mac-1, %</td>
<td>14.7±3.1</td>
<td>17.0±1.3</td>
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<tr>
<td>CD3e, %</td>
<td>11.1±1.4</td>
<td>14.0±3.2</td>
<td>0.46</td>
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<tr>
<td>B220, %</td>
<td>23.3±6.8</td>
<td>20.5±1.4</td>
<td>0.71</td>
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<tr>
<td><strong>bone marrow cell composition</strong></td>
<td></td>
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<tr>
<td>Gr-1, %</td>
<td>19.4±6.7</td>
<td>7.7±3.2</td>
<td>0.19</td>
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<tr>
<td>Mac-1, %</td>
<td>19.9±6.3</td>
<td>11.1±2.6</td>
<td>0.26</td>
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<tr>
<td>CD3e, %</td>
<td>2.4±0.9</td>
<td>1.9±0.5</td>
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<tr>
<td>B220, %</td>
<td>6.3±1.5</td>
<td>3.6±0.7</td>
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<td>TER-119, %</td>
<td>6.1±1.2</td>
<td>4.4±0.4</td>
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Data were presented as mean±SEM (arbitrary units).
All donors and recipients were ApoE-/- background.
**Supplementary Table III**

**Mortality of the ApoE-/- mice with BMT during infusion of AngII or vehicle**

<table>
<thead>
<tr>
<th>BMT</th>
<th>Treatment</th>
<th>Number of animals</th>
<th>Premature death</th>
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<tr>
<td>AT1R+/+→AT1R+/+</td>
<td>Ang II</td>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>vehicle</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>AT1R-/→AT1R+/+</td>
<td>Ang II</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>vehicle</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>AT1R+/+→AT1R-/</td>
<td>Ang II</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>vehicle</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>AT1R-/→AT1R-/</td>
<td>Ang II</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>vehicle</td>
<td>8</td>
<td>1</td>
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