Angiotensin II Type 1a Receptor Is Involved in Cell Infiltration, Cytokine Production, and Neovascularization in Infarcted Myocardium

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Objective—Angiotensin II is critically involved in left ventricular remodeling after myocardial infarction. Neovascularization has been thought to prevent the development of left ventricular remodeling and deterioration to heart failure. To elucidate the role of angiotensin II in neovascularization during cardiac remodeling, we induced myocardial infarction in angiotensin II type 1a receptor (AT1) knockout (KO) mice.

Methods and Results—There were more vessels in the border zone of infarcted hearts of wild-type (WT) mice and AT1KO mice at 14 days after operation, compared with in the left ventricle of sham-operated mice, and the number was larger in WT mice than in AT1KO mice. Consistent with these observations, the infarcted heart of AT1KO mice expressed lower levels of matrix metalloproteinase and endothelial nitric oxide synthase activity. More inflammatory cells such as granulocytes and macrophages were infiltrated in the infarcted hearts of WT mice than AT1KO mice at 4 days. A variety of cytokines and chemokines were increased in infarcted hearts of WT and AT1KO mice, and many of them were more remarkable in WT mice than in AT1KO mice at 14 days.

Conclusions—AT1 plays a critical role in inflammatory cell infiltration, cytokine production, and neovascularization in infarcted hearts.

Key Words: angiotensin II ■ AT1 receptor ■ neovascularization ■ myocardial infarction ■ cardiac remodeling

Left ventricular remodeling after myocardial infarction (MI) causes progression of heart failure and death. The remodeling process is characterized by progressive expansion of the initial infarct area and dilation of the left ventricular lumen, with cardiomyocyte replacement by fibrous tissue deposition in the ventricular wall. Once these processes develop, the infarcted heart accelerates the deterioration of ventricular dysfunction, leading to heart failure.

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Accumulating evidence has suggested that the renin-angiotensin system (RAS) plays an important role in left ventricular remodeling after MI, and that inhibition of RAS with angiotensin-converting enzyme (ACE) inhibitors and angiotensin II (AngII) type 1a receptor (AT1) blockers suppresses the cardiac remodeling and reduces the mortality after MI in clinical studies and experimental models.1-3 We also reported that AngII plays a critical role in cardiac remodeling and mortality after MI using AT1 knockout (KO) mice.4 Although cardiac dysfunction was more prominent and mortality was higher in wild-type (WT) mice than AT1KO mice after MI,4 the precise mechanism of how AngII induces left ventricular remodeling remains unknown.

It has been reported that neovascularization within the infarcted tissue is an integral component of the remodeling process and that induction of neovascularization reduces infarcted area and mortality.5 There are several controversial reports regarding the effects of AngII on vascularization. Some reports have shown that AngII induces neovascularization in tumors, ischemic legs, and retina,6-8 but others have reported that inhibition of RAS stimulates neovascularization.9 It has also been reported that an ACE inhibitor does not inhibit vascular growth during the early phase of post-infarcted cardiac remodeling and scar formation.10 To elucidate the role of AngII in neovascularization in the heart, we induced MI and examined the number of vessels in AT1KO mice.

Methods

Animals

Eight-week-old male WT mice and AT1KO mice11 from the same genetic background were used in the present study (SLC, Shizuoka,
Japan. Mice were housed under climate-controlled conditions with a 12-hour light/dark cycle and were provided with standard food and water ad libitum as described previously. All protocols were approved by the Institutional Animal Care and Use Committee of Chiba University.

MI Model
MI was produced in male WT and AT1KO mice by left coronary artery ligation as described previously. Mice were sacrificed at 1, 4, 7, and 14 days after the operation. Sham-operated control mice did not receive coronary artery ligation. Before procedure, systolic blood pressure (SBP) and heart rate were measured by using a tail-cuff method.

Hydralazine Treatment
It has been reported that SBP was lower in AT1KO mice than WT mice. To examine the effect of BP on angiogenesis, we administered hydralazine (Norvartis Pharmaceuticals, Tokyo, Japan) to WT mice. WT mice were treated with hydralazine (3 mg/kg per day) through osmotic mini pump (Alzet, Palo Alto, Calif) from 1 week before procedure to euthanization. Our preliminary experiments showed that SBP in WT mice treated with this dose of hydralazine was similar to that in AT1KO mice.

Number of Capillaries and Arterioles
We examined neovascularization by measuring the number of capillaries and arterioles in light microscopic sections taken from the border zone of the infarcted heart. Capillary endothelial cells and smooth muscle α-actin (SMA) were identified by immunohistochemical staining with anti-platelet/endothelial cell adhesion molecule (PECAM) antibody and anti-SMA antibody (PROGEN Biotechnik GmbH, Heidelberg, Germany), respectively. Ten random microscopic fields were examined and the number of capillaries and arterioles were expressed as the number of PECAM-positive capillaries and SMA-positive arteriole/high-power field (HPF) (×400).

Histological Analysis for Inflammatory Response
Immunohistochemical analysis was performed with anti-Ly6G antibody, anti-Mac3 antibody, and anti-CD3 antibody (BD Pharmingen, San Diego, Calif) to detect granulocytes, macrophages, and T lymphocytes, respectively, according to the supplier’s instructions. We counted positive cells in 10 random microscopic HPFs.

Western Blot Analysis
Protein extracts were obtained after homogenization of infarcted myocardium; 100 μg of protein was separated on a polyacrylamide gel and electroblotted onto nitrocellulose transfer membrane (Schleicher & Schuell, Dassel, Germany). The membrane was blocked with 5% skim milk and 0.5% BSA in PBS with 0.1% Tween-20 (T-PBS) and then probed with anti-matrix metalloproteinase (MMP)-2 antibody, anti-MMP-9 antibody, anti-Akt antibody, anti-endothelial nitric oxide synthase (eNOS) antibody, and anti-phospho-Akt antibody (Cell Signaling, Beverly, Mass) for 1 hour at room temperature. After incubation with the primary antibody, the membrane was washed in T-PBS and was incubated for 1 hour with peroxidase-conjugated secondary antibody. The reaction was detected using ECL detection reagent kit (Amersham Pharmacia, Buckinghamshire, UK).

NOS Activity Assay and NOS Inhibition
The NOS activity (calcium-dependent) in infarcted myocardium was examined using NOS assay kit (Calbiochem, San Diego, Calif) according to the manufacturer’s instruction. To examine the role of eNOS in angiogenesis after MI, we administered a NOS inhibitor, N²-nitro-L-arginine methyl ester (L-NAME) (4 mg/kg per day) via drinking water to WT mice from 1 week before procedure to euthanization.

Ribonuclease Protection Assay
Total RNA was extracted from left ventricles with RNAzol B (Biotec Laboratories) and analyzed by ribonuclease protection assay. Multi-probes template sets, mCK-3b and mCK-5, were available with reagents of in vitro transcription and ribonuclease protection assay (RiboQuant; Pharmingen). For all hybridization assays, we used 2 μg total RNA from the sham-operated mice hearts and the MI hearts.

Implantation of Sarcoma Cells
S180 sarcoma was inoculated subcutaneously into the right axilla of male WT and AT1KO mice at a dose of 2×10⁵ cells in 0.2 mL PBS/mouse. The mice were euthanized on day 14, and the tumor was removed and weighted. The number of capillaries in the tumor was counted as described.

Statistical Analysis
Data were shown as mean±SEM. Multiple group comparison was performed by one-way ANOVA followed by the Bonferroni procedure for comparison of means. Comparison between 2 groups was analyzed by the two-tailed Student t test or two-way ANOVA. Values of P<0.05 were considered statistically significant.

Results
AT1 Plays a Critical Role in Neovascularization After MI
We have previously reported that an infarcted size was significantly reduced in AT1KO mice compared with WT mice. We therefore examined whether AT1 was involved in angiogenesis during the remodeling process. Immunohistochemical studies using anti-PECAM antibody revealed that the number of capillaries was increased in the border zone of the infarcted hearts of WT and AT1KO mice 14 days after MI as compared with hearts of sham-operated mice (Figure 1A), and this increase was more prominent in WT mice than in AT1KO mice (Figure 1A and 1B). Immunohistochemical analysis using anti-SMA antibody showed that the number of arteriole was also increased in the border zone of the infarcted hearts of WT mice on day 14 after MI, but not AT1KO mice (Figure 1C and 1D). These results suggest that AT1 has stimulating effects on neovascularization after MI.

There was no significant difference in the heart rate between WT mice and AT1KO mice, but SBP in AT1KO mice was lower than that in WT mice (Figure 1E), which might affect neovascularization. To examine the effect of BP on angiogenesis after MI, we administered hydralazine to WT mice. SBP in WT mice treated with hydralazine was as low as that in AT1KO mice (Figure 1E). There was no significant difference in the capillary density between WT mice with hydralazine and WT mice without the treatment (Figure 1F). These results indicate that lower BP of AT1KO did not account for decreased angiogenesis in ischemic myocardium.

AT1 Mediates Cell Infiltration After MI
Because it has been reported that inflammation is an important trigger for ischemia-induced neovascularization and that the RAS plays an important role in inflammatory responses, we examined infiltration of inflammatory cells in the heart after MI. The numbers of granulocytes, macrophages, and T lymphocytes were examined in the myocardium on day 4 after MI using anti-Ly6G, anti-Mac3, and anti-CD3 antibodies, respectively. Many granulocytes (Figure 2A and 2D), macrophages (Figure
and T lymphocytes (Figure 2C and 2F) were observed in the heart of WT and AT1KO mice after MI. The numbers of infiltrative cells such as granulocytes and macrophages were much larger in WT mice than those in AT1KO mice (Figure 2G), suggesting that AT1 is critically involved in cell infiltration in the myocardium after MI.

AT1 Induces MMPs and Activates Akt-1

Because it has been reported that MMPs are important for cell invasion into extravascular space and that MMPs play a critical role in vascularization, we next examined protein levels of MMP-2 and MMP-9 in the heart after MI. MMP-2 was increased on day 1 after MI in both WT mice and AT1KO mice (Figure 3A and 3B). MMP-9 was increased from day 4 in both WT mice and AT1KO mice (Figure 3A and 3C). The increases of MMP-2 and MMP-9 were more remarkable in WT mice compared with AT1KO mice (Figure 3B and 3C).

We examined another angiogenic factor, Akt-1. The protein level of Akt-1 was increased in WT mice and AT1KO mice after MI (Figure 3D), and the increase was more prominent in WT mice compared with AT1KO mice (Figure 3B and 3C).

To further elucidate the role of eNOS activity in infarcted myocardium, we administered L-NAME to WT mice to reduce NO production. Treatment with L-NAME partially but significantly reduced the capillary density in the infarcted heart of WT mice (Figure 4D). In contrast, inhibition of NO production had no effect on neovascularization after MI in AT1KO mice. These results suggest that AngII-induced eNOS activation partly regulates angiogenesis after MI.
AT1 Is Involved in Induction of Cytokines and Chemokines in Infarcted Myocardium

Various cytokines and chemokines have been reported to play a critical role in left ventricular remodeling after MI.24 We examined various cytokines and chemokines using ribonuclease protection assay. The expression levels of tumor necrosis factor-α, interleukin (IL)-6, transforming growth factor (TGF)-β1, TGF-β2, TGF-β3, interferon-inducible protein-10 (IP-10), monocyte chemoattractant protein-1 (MCP-1), exotoxin, RANTES, macrophage inflammatory protein (MIP)-α, MIP-1β, and MIP-2 were increased in infarcted myocardium in WT and AT1KO mice (Figure 5A and data not shown). Most of these cytokines and chemokines such as TGF-β1, TGF-β2, TGF-β3, IP-10, MCP-1, MIP-1α, MIP-1β, and MIP-2 were more strongly upregulated in WT mice compared with AT1KO mice (Figure 5B and data not shown).

Tumor Progression Is Inhibited in AT1KO Mice

Because tumor growth depends on angiogenesis,25 we also investigated the role of AT1 in tumor angiogenesis. The tumor size in WT mice was larger than that in AT1KO mice 14 days after implantation of sarcoma cells (Figure 6A). The much more capillaries were observed in the tumor in WT mice compared with AT1KO mice (Figure 6B and 6C). These results indicate that AngII plays an important role in angiogenesis of tumors as well as ischemic hearts.
Discussion

AT1 Is Involved in Neovascularization After MI
It has been reported that an increase of neovascularization improves cardiac function and mortality, and that inhibition of RAS is effective to prevent post-infarction cardiac remodeling. It is unknown, however, whether activation of AngII/AT1 signaling induces or prevents vascularization in the infarcted heart. Some reports have demonstrated that AngII induces neovascularization in various experimental models including tumors, ischemic limb, retina, and choroidal membrane. To the contrary, there are some reports showing that inhibition of RAS induces neovascularization. We thus examined in this study the role of AngII/AT1 in neovascularization during left ventricular remodeling and tumor progression using AT1KO mice. AT1KO mice exhibited less capillaries and arterioles than WT mice, suggesting that AT1 plays a pivotal role in neovascularization of the heart after MI and tumors.

AT1 Signal Plays an Important Role in Induction of MMPs and Cell Infiltration
To elucidate the molecular mechanism of how AngII induces neovascularization, we examined several molecules that have been reported to play an important role in angiogenesis. MMP-2 and MMP-9, gelatinases that digest basement membrane and play a critical role in cell invasion, have been reported to be necessary for vascularization. It has been reported that the treatment with ACE inhibitors decreases MMP-2 at mRNA and protein levels in vitro and inhibits endothelial-cell migration by blocking the activity of MMP-2 and MMP-9. In this study, protein levels of MMP-2 and MMP-9 were increased after MI and the increase was attenuated in AT1KO mice compared with WT mice. Histological examination revealed that infiltration of inflammatory cells such as granulocytes and macrophages was more remarkable in WT mice than AT1KO mice. These results and observations suggest that AngII induces transendothelial migration of inflammatory cells at least in part through enhanced production of MMP-2 and MMP-9. MMPs have been also demonstrated to contribute to tissue remodeling in a number of disease states, and inhibition of MMPs prevents ventricular remodeling after MI. Mice with targeted disruption of MMP-9 have attenuated ventricular remodeling and decreased cardiac rupture after infarction. Our previous report also demonstrated that left ventricular dimension was
smaller in AT1KO mice than WT mice 4 weeks after MI. These results suggest that MMPs activation via the AT1 signaling pathway is involved in neovascularization as well as in post-infarcted cardiac remodeling.

**Activation of Akt-1 and eNOS by AngII Induces Angiogenesis in the Infarcted Myocardium**

Akt plays an important role in cell survival, cell migration and angiogenesis. Akt phosphorylates, and eNOS thereby promoting angiogenesis. In this study, protein levels of Akt-1 were more increased in the heart of WT mice than AT1KO mice and Akt-1 was activated only in the heart of WT mice. Although there was no significant difference in protein levels of eNOS between WT mice and AT1KO mice, eNOS activity was significantly increased in WT mice compared with AT1KO mice. Moreover, the increase of capillaries in WT mice was partially inhibited by L-NAME treatment. These results suggest that AngII-induced activation of eNOS, mediated possibly by Akt, enhances angiogenesis in ischemic myocardium.

**AngII Is Involved in the Production of Various Cytokines**

The inflammation in cardiovascular diseases is associated with the activation of a variety of cells including lymphocytes, monocytes/macrophage, endothelial cells, smooth muscle cells, and cardiac myocytes, which express and secrete proinflammatory cytokines and chemokines. These cytokines can modulate cardiac function and cardiovascular remodeling. Various cytokines were increased after MI, and the increases of TGF-β, MIP-1, IP-10, and MCP-1 were more prominent in WT mice compared with AT1KO mice. These results suggest that AngII is involved in production of various cytokines after MI, which induce post-infarcted cardiac remodeling including impaired cardiac function and increased fibrosis.

Chemokines represent a family of inflammatory cytokines that induce chemotaxis of leukocyte subsets into inflammatory tissues. CC-chemokines are potent chemoattractants and activators for monocytes and lymphocytes, whereas most CXC-chemokines attract neutrophils. MCP-1 recruits monocytes, which produce proteolytic enzymes, reactive oxygen species, and inflammatory cytokines. Recent studies have shown that neovascularization in response to tissue ischemia depends on macrophage infiltration and that local infusion of MCP-1 markedly increases collateral and peripheral conductance in hindlimb ischemia model. Moreover, inflammatory cytokines such as IL-1, IL-6–related cytokines, and MCP-1 have been reported to induce myocardial dysfunction and cardiac remodeling through promotion of cardiomyocyte hypertrophy and apoptosis as well as alteration in extracellular matrix in the myocardium. The MCP-1 overexpression mice showed hypertrophied left ventricular wall, dilated left ventricular dimension, and decreased cardiac function. In this study, the MCP-1 expression was suppressed and the number of macrophage infiltrated into myocardium was less in AT1KO mice after MI compared with WT mice. These results and observations suggest that reduction of MCP-1 expression and macrophage infiltration might be related to less left ventricular remodeling despite less neovascularization in the heart of AT1KO mice.

AngII/AT1 signaling has 2 roles in left ventricular remodeling after MI. Activation of AT1 induces expression of chemokines and infiltration of inflammatory cells, which cause neovascularization possibly through enhanced expression of MMPs and activation of Akt. The enhanced neovascularization may prevent left ventricular remodeling by inhibition of cardiomyocyte apoptosis. However, AngII/AT1-induced cardiomyocyte hypertrophy, increased fibrosis, enhanced cytokines, and MMPs expressions induce left ventricular remodeling. Taken together with the previous reports, inhibition of AngII/AT1 signal is important for preventing cardiac remodeling after MI, although it may suppress neovascularization.

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