Targeted Deletion of Angiotensin II Type 2 Receptor Caused Cardiac Rupture After Acute Myocardial Infarction

Sahoko Ichihara, MD, PhD; Takaaki Senbonmatsu, MD, PhD; Edward Price, Jr, MS; Toshihiro Ichiki, MD, PhD; F. Andrew Gaffney, MD; Tadashi Inagami, PhD

Background—Accumulating evidence has suggested that the cardiac renin-angiotensin system is activated during the remodeling process after myocardial infarction (MI). Although 2 types of angiotensin II receptors (AT₁ and AT₂) are upregulated in the infarcted tissue, the contribution of AT₂ to the subsequent fibrogenetic phase of wound healing is less certain. This study was conducted to evaluate the role of AT₂ in wound healing after MI using an in vivo intervention study in mice with MI.

Methods and Results—We examined myocardial hypertrophy, cardiac fibrosis, and morphological evidence of fibrillar collagen accumulation at the infarcted and noninfarcted regions in male mice lacking the AT₂ receptor (Agtr2⁻/⁻) and age-matched wild-type (WT) animals. Of the Agtr2⁻/⁻ mice, 63.6% died of cardiac rupture, whereas 23.5% of the WT mice died of the same cause within 1 week. The extent of fibrosis and that of collagen gene expression in Agtr2⁻/⁻ mice were significantly reduced compared with WT mice at 1 week after coronary ligation. Furthermore, MI resulted in a marked increase in the prostaglandin E₂ (PGE₂) level at 4 days after surgery in Agtr2⁻/⁻ mice. In WT mice, the PGE₂ level was also elevated after MI but to a significantly lesser extent than in Agtr2⁻/⁻ mice.

Conclusions—A chronic loss of AT₂ by gene targeting prevented the collagen deposition and caused cardiac rupture. The markedly elevated PGE₂ may be a mechanism that inhibits collagen synthesis in the infarcted region of Agtr2⁻/⁻ mice.

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Key Words: angiotensin II ▪ collagen ▪ myocardial infarction ▪ prostaglandins ▪ remodeling

Wound healing after myocardial infarction (MI) is associated with migration and proliferation of various cell types, such as fibroblasts, endothelial cells, and inflammatory cells, and with the accumulation of several components of the extracellular matrix (ECM), composed principally of collagen. The amount and distribution of collagen in the myocardium can potentially contribute to altered ventricular function after MI. The cardiac renin-angiotensin system is activated during the remodeling process. Local concentration and generation of angiotensin II (Ang II) and the number of Ang II receptors were increased in infarcted hearts. Of the 2 major Ang II receptor isoforms, AT₁ and AT₂, it is generally accepted that most of the traditional Ang II functions in the cardiovascular system are attributable to AT₁. Although both AT₁ and AT₂ are upregulated in the infarcted as well as noninfarcted tissue, whether AT₂ contributes to the subsequent fibrogenetic phase of wound healing is less certain. In our recent studies, pressure overload by aortic banding or hypertension by Ang II infusion failed to induce myocardial hypertrophy and cardiac fibrosis in mice lacking the AT₂ receptor gene (Agtr2⁻/⁻), suggesting that AT₂ may play an essential role for myocardial hypertrophy and cardiac fibrosis in various conditions of the heart.

See p 2167

Ang II induces synthesis of ECM proteins, both directly and indirectly, by stimulating expression of the profibrotic factor transforming growth factor-β₁ (TGF-β₁). Ang II also has several paracrine functions that serve to stimulate endothelial cells of the neovasculature to elaborate nitric oxide, bradykinin, prostaglandins (PGs), and various steroids. The cardiac fibrosis seen in association with hypertension or MI is mediated by the release of bradykinin and PGs. Evers et al have shown that bradykinin stimulates a marked release of cyclooxygenase products from the infarcted rabbit myocardium in association with inflammatory cell invasion during the first week after MI. Furthermore, isolated canine cardiac fibroblast-like cells were found to have greater cyclooxygenase activity and PGE₂ production in the infarcted tissue compared with fibroblasts of the noninfarcted tissue. Recently, Siragy et al demonstrated that Agtr2⁻/⁻ mice, compared with wild-type (WT) mice, had higher basal levels of PGE₂ and lower prostaglandin F₂α (PGF₂α) levels in renal...
interstitial fluid under basal or dietary sodium restriction. Given the appearance of PGs in inflammatory cells in healing wound, these observations suggest possible differences in the response of heart to infarction in AT2 gene-deleted mice. The present study was conducted to evaluate the role of AT2 in wound healing after MI. Furthermore, we investigated the regulation of myocardial collagen synthesis involving TGF-β or PGs in wound healing after MI by an in vivo intervention study in mice with MI.

Methods

Animal Model
Male Agtr2−/−Y (n=97) and WT (n=98) mice 12 to 16 weeks old (Jackson Laboratory, Bar Harbor, Me) were used after 8 backcrosses to C57BL/6. In a mouse anesthetized with pentobarbital (10 mg/kg IP), MI was produced by permanent occlusion of the left coronary artery with a 10-0 nylon surgical suture under artificial ventilation as previously described. For the sham experiments, the same procedure was performed except for coronary ligation. One week or 6 weeks after surgery, these mice were killed after determination of physiological properties. Hearts were removed and cut into 6 sections. Infarct size was calculated and expressed as a percentage of MI size. LVDD and LVDS were increased 4- to 6-fold in both wild-type (WT) and Agtr2−/−Y mice at the same points as previously reported. Relative thickness, percent fractional shortening, and LV mass were calculated by the following formulas: relative thickness= thickness/LVDD/LVDD, percent fractional shortening= (LVDD−LVDD/LVDD)×100, and LV mass=1.055×(LVDD+PW+LVDD)−LVDD, respectively.

Physiological Properties
Mouse tail-cuff systolic blood pressure and body weight were measured before and 1 and 6 weeks after surgery. Transthoracic echocardiography was performed for measurements of LV internal diameter at end diastole (LVDD) and end systole (LVDS), interventricular septal wall thickness (IVS), and posterior wall thickness (PW) at the same points as previously reported. Relative thickness, percent fractional shortening, and LV mass were calculated by the following formulas: relative thickness= thickness/LVDD/LVDD, percent fractional shortening= (LVDD−LVDD/LVDD)×100, and LV mass=1.055×(IVS+PW+LVDD)−LVDD, respectively.

Morphometric Measurements
Fixed tissues were dehydrated, embedded in paraffin, sectioned at 4-μm thickness, and stained with hematoxylin-eosin and the van Gieson solution. Infarct area was calculated as the percentage of MI relative to the entire ventricular tissue area as shown by the following formula as previously described: Infarct area (Ca+Ir)=(Ca+Ir)/Ca+Nir, where Ca is connective tissue area, Ma is intact and necrotic muscle fiber area, Ir is infarcted region, and Nir is noninfarcted region. The collagen fraction was calculated as the ratio of the sum of the total area of interstitial fibrosis to the sum of the total connective tissue area plus the myocyte area in the entire visual fields of the section as previously reported.

Northern Blot Analysis
Total RNA was extracted from hearts of mice at 1 week after surgery, separated on a 1.2% agarose/formaldehyde gel, and blotted onto a Hybond-N membrane (Amersham Pharmacia Biotech). Northern blot analysis of collagen I, collagen III, fibronectin, and TGF-β was performed as previously reported.

PG Enzyme Immunoassay
PGs were extracted from tissue samples by use of ODS-silica reverse-phase columns (Sep-Pak C18, Waters) as previously described. A [3H]-labeled PG was added as a tracer for calculation of the recovery factor. The myocardial content of PGE2 and PGF2α was determined by use of enzyme immunoassay kits (Cayman Chemical Co) as previously described and expressed as picograms of PGE2 or PGF2α per milligram of protein.

Results

Cardiac Rupture and Survival after MI
The survival rate after MI was compared between Agtr2−/−Y and WT mice. The number of the mice that died within 1 week after ligation was signifi cantly greater in Agtr2−/−Y mice than in WT mice (Figure 1). The early death within 1 week after ligation was 46.4% in Agtr2−/−Y mice and 23.6% in WT mice. The most prevalent cause of the early death was cardiac rupture. Of the Agtr2−/−Y mice, 63.6% (n=21) died of cardiac rupture after MI, whereas 23.5% (n=4) of WT mice died of the same cause within 1 week. In this study, there was no significant difference in survival rate between WT and Agtr2−/−Y mice in 6 weeks after MI.

LV Remodeling After MI
The systolic arterial pressure in both strains remained at basal levels throughout the experiments with coronary ligation as examined at 1 and 6 weeks (data not shown). The echocardiographic parameters of surviving mice are shown in Table 1. Both LVDD and LVDS were increased significantly over their respective baseline values in WT mice after ligation. The percentage changes of these parameters were significantly greater in WT mice than in Agtr2−/−Y mice at 6 weeks after ligation (Table 2). Relative wall thickness and LV mass/body weight ratio in WT mice were significantly greater than those in Agtr2−/−Y mice at 1 and 6 weeks

Statistical Analysis
Data are expressed as mean±SEM. Analyses of survival after MI were carried out by the Kaplan-Meier method with the log-rank (Cox-Mantel) method. Differences between values obtained at each time point were evaluated by repeated-measures ANOVA, followed, if ANOVA revealed significant differences, by Tukey’s test for multiple comparisons. For other data, statistical significance was determined by the Mann-Whitney U test. A level of P<0.05 was considered statistically significant.
after ligation. However, there were no differences in percentage changes from baseline values of these parameters between the 2 strains. LV percent fractional shortening decreased after ligation in both strains.

**Infarct Size, Infarct Area, and Collagen Volume Fraction**

Data for the infarct size, infarct area, and the extent of fibrosis in surviving mice at 1 week after ligation are presented in Table 3. The infarct size was similar in the 2 strains after ligation. Infarct area in the infarcted region of *Agtr2−/−Y* mice was reduced significantly compared with WT mice. MI scar thinning at 1 week after ligation in both strains is shown in Figure 2. The extent of fibrosis at the site of MI was significantly different at 1 week after ligation between the 2 strains. In the noninfarcted region, the collagen volume fraction in *Agtr2−/−Y* mice was also reduced compared with WT mice at 1 week after ligation. To test whether coronary ligation results in the stimulation of the cascade of fibrotic signals, we determined the levels of mRNAs of collagen I, collagen III, and fibronectin. These mRNAs were significantly elevated at 1 week in WT mice (Figure 3). In *Agtr2−/−Y* mice, however, there were no significant changes after MI. The level of TGF-β1 mRNA was increased in both strains, but we could not find significant differences in this value between the 2 strains.

**TABLE 2. Percentage Change in Echocardiographic Measurement From Baseline**

<table>
<thead>
<tr>
<th></th>
<th><em>Agtr2−/−Y</em></th>
<th>WT</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>One week after ligation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔLVDD, %</td>
<td>8.22±1.38</td>
<td>11.42±3.30</td>
<td>0.42</td>
</tr>
<tr>
<td>ΔLVDS, %</td>
<td>13.08±1.84</td>
<td>18.13±3.78</td>
<td>0.27</td>
</tr>
<tr>
<td>ΔRelative wall thickness, %</td>
<td>-0.43±1.87</td>
<td>-2.28±2.88</td>
<td>0.61</td>
</tr>
<tr>
<td>ΔLVM/BW, %</td>
<td>29.70±4.74</td>
<td>30.71±7.71</td>
<td>0.92</td>
</tr>
<tr>
<td>ΔM-mode FS, %</td>
<td>-10.23±4.29</td>
<td>-12.27±2.40</td>
<td>0.62</td>
</tr>
<tr>
<td>Six weeks after ligation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔLVDD, %</td>
<td>15.08±2.25</td>
<td>24.59±3.64</td>
<td>0.03</td>
</tr>
<tr>
<td>ΔLVDS, %</td>
<td>20.43±2.64</td>
<td>32.94±5.20</td>
<td>0.04</td>
</tr>
<tr>
<td>ΔRelative wall thickness, %</td>
<td>-5.33±2.61</td>
<td>-9.37±2.85</td>
<td>0.31</td>
</tr>
<tr>
<td>ΔLVM/BW, %</td>
<td>29.35±6.51</td>
<td>48.15±7.79</td>
<td>0.07</td>
</tr>
<tr>
<td>ΔM-mode FS, %</td>
<td>-13.19±3.13</td>
<td>-14.72±3.61</td>
<td>0.75</td>
</tr>
</tbody>
</table>

Abbreviations as in Table 1.

**TABLE 3. Remodeling of Myocardium in 1 Week After Coronary Ligation**

<table>
<thead>
<tr>
<th></th>
<th><em>Agtr2−/−Y</em></th>
<th>WT</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infarct size, %</td>
<td>41.3±4.2</td>
<td>39.1±3.6</td>
<td></td>
</tr>
<tr>
<td>Area, %</td>
<td>26.1±1.1*</td>
<td>33.3±1.3</td>
<td></td>
</tr>
<tr>
<td>Collagen volume fraction</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infarcted region, %</td>
<td>47±3*</td>
<td>53±4</td>
<td></td>
</tr>
<tr>
<td>Noninfarcted region, %</td>
<td>2.2±0.6*</td>
<td>3.0±0.5</td>
<td></td>
</tr>
</tbody>
</table>

*P<0.05 vs shamt-operated *Agtr2−/−Y* mice.
Analysis of PGs
To determine whether there were any differences in the myocardial content of the arachidonic acid metabolites that are induced after MI, we measured the myocardial content of the major arachidonic acid metabolites (PGE₂ and PGF₂α). MI resulted in a marked increase in PGE₂ levels at 4 days after ligation in Agtr₂−/Y mice (Figure 4). In WT mice, PGE₂ levels were also elevated, but to a much lesser extent. PGF₂α levels were also elevated after ligation in both Agtr₂−/Y and WT mice. There was no significant difference between the 2 strains.

Discussion
We found that MI resulted in LV free wall rupture more frequently in Agtr₂−/Y mice than in WT mice. The fragility of ventricular wall in Agtr₂−/Y mice may be associated with markedly elevated PGE₂ and deficiency in the formation of the collagen matrix in the infarcted region of Agtr₂−/Y mice.

Factors that promote LV structural remodeling after MI include increased LV preload and afterload, increased wall stress, and increased angiotensin II levels. It was described that both the AT₁ and AT₂ subtypes of Ang II receptors were upregulated in the infarcted as well as in the noninfarcted tissue. Several investigators suggested that AT₂ plays some vital roles in myocytes or fibroblasts after MI. Kuizinga et al demonstrated that the increase in DNA synthesis in cardiac endothelial cells and interstitial fibroblasts after MI was mediated through AT₂ in rat MI models. In the present study, we also found that chronic loss of AT₂ by gene targeting prevented the collagen deposition in the infarcted region. These results suggest that AT₂ mediates the interstitial DNA synthesis and collagen deposition after MI.

Cardiac rupture is an acute fatal complication in the first few days after MI. Hypertension, cardiac hypertrophy, infarct expansion, and delayed thrombolysis have been related to cardiac rupture, but the exact relevance of these risk factors has remained controversial. The ECM proteins play an important role in the healing process after MI. The strength and stiffness of the scar collagen provide mechanical stability to the injured tissue. This stability is important, because the level of accumulation of the original collagen matrix of the heart in the first few days after MI is associated with expansion of the infarcted tissue and, in extreme cases, may result in the rupture of the infarcted ventricular wall. Furthermore, agents that inhibit collagen synthesis were shown to be associated with the increase of the risk of cardiac rupture in patients. In the present study, we found that collagen deposition and collagen gene expression were inhibited and caused cardiac rupture in Agtr₂−/Y mice.

We demonstrated that coronary ligation in the mice resulted in transmural anterior infarction that is followed by marked hemodynamic alteration similar to that seen in patients with anterior MI, as previously reported. Early LV
dilation may be caused by the Frank-Starling effect, which results in an increase in the length of the noninfarcted region in response to a reduction in contractile muscle mass. In contrast to this adaptive physiological mechanism, infarct expansion is caused by the pathological processes that stretch and thin the infarcted myocardial segment. In later periods, early infarct expansion and regional ventricular dilation may be accompanied or followed by a phase of global ventricular dilation in the noninfarcted region. In the present study, within 1 week after ligation, Agtr2−/−/Y mice died of cardiac rupture more frequently, and infarct area of the infarcted region in Agtr2−/−/Y mice was significantly less at 1 week after ligation than in WT mice. However, there were no differences in geometric and functional remodeling between the 2 strains at 1 week after ligation. At 6 weeks after ligation, WT mice consistently exhibited more marked remodeling, such as further progression of LV enlargement and fibrosis. In contrast, Agtr2−/−/Y mice exhibited mild LV dilation at 6 weeks after ligation. These results suggest that early LV dilation after MI may be inadequate to account for the generalized cardiac dilation. The ventricular enlargement of the late phase would be influenced primarily by infarct healing, which was associated with extensive fibroblast proliferation and collagen deposition.

In the present study, we found that PGE₂ was increased by a significantly greater extent at 4 days after ligation in Agtr2−/−/Y mice than in WT mice. The ligature also increased PGF₂α levels in Agtr2−/−/Y mice, but to a lesser extent than in WT mice. A previous study demonstrated that during MI synthesis of thromboxane, PGE₂, and prostacyclin were elevated, and the production was significantly increased at 3 days after infarction. PGE₂ inhibits collagen production by several mechanisms, including reduction in the uptake of proline, inhibition of collagen gene expression, and a decrease in collagen types I and III mRNA levels. Furthermore, PGF₂α has been shown to be released from cells after experimental infarction and to stimulate hypertrophic growth. It was recently reported that the pharmacological blockade of AT₂ potentiated the Ang II–induced production of PGE₂. Moreover, Siragy et al demonstrated that Agtr2−/−/Y mice had higher PGE₂ and lower PGF₂α levels in renal interstitial fluid than WT mice. We also demonstrated that the formation of PGE₂ and PGF₂α was associated with the action of AT₂ in this study. The markedly elevated levels of PGE₂ in Agtr2−/−/Y mice may attenuate collagen accumulation after MI. The lesser levels of PGF₂α may also account for the reduced LV wall thickness in Agtr2−/−/Y mice.

Cardiac fibrosis is characterized by inflammatory cell infiltration, fibroblast proliferation, and excess deposition of ECM proteins, including collagen. Fibroblast proliferation and collagen synthesis are known to be regulated, at least in part, by a complex interaction between stimulatory and inhibitory mediators. Several stimulatory mediators, including TGF-β₁, platelet-derived growth factor, insulin-like growth factor-1, and thrombin, as well as inhibitory mediators, such as interferon, glucocorticoids, epidermal growth factor, and PGE₂, have been suggested to play a role in the pathogenesis of cardiac fibrosis. In this study, there was no significant difference in the level of TGF-β₁ mRNA. The cardiac collagen synthesis stimulated after MI would be inhibited by markedly elevated myocardial levels of PGE₂ in Agtr2−/−/Y mice. Cardiac fibrosis is also the result of both exaggerated collagen synthesis and insufficient collagen degradation. Collagen degradation involves matrix metalloproteinases (MMPs). It has been shown that PGE₂ has a role in the elevation of MMP-9 in cultures of human fetal membranes. Recently, Heymans et al demonstrated that deficiency of MMP-9 prevented cardiac rupture after MI. It is possible that the destruction of ECM by MMP-9 induced by the markedly elevated PGE₂ results in cardiac rupture of the ventricular wall after MI. The degradation of ECM plays an important role in the healing process after MI, and AT₂ may play a vital role in these processes.

**Study Limitations**

Physiological measurements and histological and biological analysis were performed only in surviving mice. The reason why there were no differences in echocardiographic measurements between the 2 strains at 1 week after ligation might be subject to survival bias. Furthermore, mean infarct size was comparable in the surviving population of both strains. It has been shown that the cardioprotective effects of acute AT₂ blockade with PD was associated with AT₂ upregulation. Therefore, infarct size of nonsurviving Agtr2−/−/Y mice might...
be more extensive than that of WT mice. We cannot exclude the possibility that in nonsurviving Agtr2−/Y mice, expansion might be more extensive and cause cardiac rupture.

In summary, we found that the excessive interstitial fibrosis and collagen accumulation after MI were prevented in the infarcted and the noninfarcted regions in Agtr2−/Y mice. The number of mice that died of cardiac rupture within 1 week after MI was significantly greater in Agtr2−/Y mice than in WT mice. We also demonstrated that the levels of PGE2 were significantly increased after MI in Agtr2−/Y mice. The marked elevation of PGE2 may inhibit collagen synthesis in the infarcted region of Agtr2−/Y mice and cause cardiac rupture. Although it remains uncertain whether these effects are directly mediated by AT2 or through PGE2, these findings indicate that AT2 plays a vital role in inducing cardiac fibrosis and collagen accumulation after MI. The regulation of AT2 on collagen degradation should also be targeted for future investigation.

Acknowledgments
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