Angiotensin II Type 2 Receptor Mediates Vascular Smooth Muscle Cell Apoptosis in Cystic Medial Degeneration Associated With Marfan’s Syndrome

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Background—Cystic medial degeneration (CMD) is a histological abnormality that is common in the aortic diseases associated with Marfan’s syndrome (MFS). Although little known about the mechanism underlying CMD, several recent reports have demonstrated that vascular smooth muscle cell (VSMC) apoptosis could play a substantial role in CMD. On the other hand, angiotensin II (Ang II) has been reported to play an important role in the regulation of VSMC growth and apoptosis via the Ang II type 1 receptor (AT1R) and type 2 receptor (AT2R).

Methods and Results—To elucidate the role of Ang II signaling via the Ang II receptors in CMD, we investigated AT1R and AT2R mRNA expression and tissue concentration of Ang II in MFS aortas (n = 10) and control aortas (n = 12). Furthermore, we examined the effects of an ACE inhibitor, an AT1R blocker, and an AT2R blocker on serum deprivation-induced VSMC apoptosis by organ culture system. AT1R expression was significantly decreased (P < 0.01) and AT2R expression was significantly increased (P < 0.001) in MFS aortas compared with control aortas, and tissue Ang II concentration was significantly higher in CMD than in the control condition (P < 0.01). Both the ACE inhibitor and AT2R blocker significantly inhibited serum deprivation-induced VSMC apoptosis (P < 0.05), although the AT1R blocker did not inhibit apoptosis in cultured aortic media from MFS patients.

Conclusions—Accelerated ACE-dependent Ang II formation and signaling via upregulated AT2R play a pivotal role in VSMC apoptosis in CMD, and the ACE inhibitor could have clinical value in the prevention and treatment of CMD. (Circulation. 2001;104[suppl I]:I-282-I-287.)

Key Words: angiotensin receptors apoptosis muscle, smooth aorta

Cystic medial degeneration (CMD) is a histological abnormality characterized by fragmentation of elastic fibers, fibrosis with collagen production, accumulation of amorphous matrix, and loss of cell nuclei. This histological change is commonly seen in patients with dilatation, aneurysm, or dissection of the ascending aorta associated with hereditary diseases, such as Marfan’s syndrome (MFS), but is not specific to MFS and sometimes also occurs in atherosclerotic aging aortas. Although little is known about the mechanism underlying CMD, several recent reports have suggested that apoptosis of vascular smooth muscle cells (VSMCs) could play an important role not only in atherosclerotic disease but also in aortic disease, including CMD associated with MFS.

Angiotensin II (Ang II) has various effects on blood pressure, hormone secretion, and electrolyte homeostasis. Two major subtypes of the Ang II receptor, the type 1 receptor (AT1R) and the type 2 receptor (AT2R), have been detected by molecular studies. Most of the known effects of Ang II in adult humans are attributable to AT1R, and signals via AT2R have been demonstrated to antagonize AT1R signals. Recent studies have suggested that Ang II may play an important role in the regulation of apoptosis via AT2R. This AT2R expression is at a high level in fetal tissues. Although AT2R expression decreases rapidly after birth, enhanced expression has been reported in adults with cardiovascular diseases, such as cardiac hypertrophy, atherosclerosis, hypertension, and myocardial infarction. Recently, the importance of the renin-angiotensin system (RAS) has been emphasized in patients with cardiac hypertrophy or heart failure. In fact, many clinical studies have proved that ACE inhibitors (ACEIs) and AT1R blockers (AT1RBs) have a beneficial effect on the prognosis in patients with heart failure. On the other hand, Ang II
signaling has been recently reported to play a critical role in the formation of aortic diseases, such as aortic aneurysm or aortic dissection in mice.\textsuperscript{24–26}

In the present study, to investigate the role of the Ang II signaling in CMD, we determined AT1R and AT2R expression, as well as the tissue Ang II concentration. We also examined the effects of an ACEI, an AT1RB, and an AT2R blocker (AT2RB) on apoptosis of VSMCs in CMD associated with MFS.

Methods

Subjects

MFS patients (n=10) and control non-MFS patients (n=12) were examined in the present study. A clinical profile of the MFS patients is shown in Table 1. All patients were not given ACEI or AT1RB therapy before surgery. Aortic wall specimens were obtained from MFS patients undergoing surgery for annuloaortic ectasia (AAE) and aortic regurgitation, and control specimens were collected from non-MFS patients without aortic diseases undergoing surgery for aortic valve disease at Tokyo Women’s Medical University Hospital. These specimens were harvested during surgery and immediately placed into 7.5% buffered formalin and PBS or stored in liquid nitrogen.

Histological Examination

To examine the pathological severity of CMD, we performed staining with Victoria blue and Masson’s trichrome stain. Because variations in the changes were observed within a single specimen, the most severe changes were used for evaluation, and CMD was classified into 4 grades as described previously.\textsuperscript{1,2} Samples displaying histological signs of severe inflammation for any other reason were excluded from further analysis. Immunohistochemical staining for smooth muscle α-actin was carried out with an anti-human smooth muscle α-actin antibody (1A4, Dako) by using an LSAB kit (Dako). Specimens were also observed under a JEM-1200EX (Nikon Denki) electron microscope (EM). The investigators performing histological evaluation were blinded to the clinical data.

Evaluation of VSMC Apoptosis

In Situ Detection of Apoptotic Cells

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) was carried out to detect apoptotic VSMCs in deparaffinized 4-µm-thick sections by use of an ApopTag in situ apoptosis detection kit (Oncor Inc) according to the supplier’s instructions. Sections were lightly counterstained with hematoxylin. Negative controls included omission of terminal deoxynucleotidyl transferase from the labeling mixture.

TABLE 1. Profile of MFS Subjects

<table>
<thead>
<tr>
<th>Subject No.</th>
<th>Age, y</th>
<th>Sex</th>
<th>Maximum Diameter of AAE, cm</th>
<th>AR</th>
<th>Aortic Dissection</th>
<th>HT</th>
<th>Grade of CMD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>36</td>
<td>M</td>
<td>6.0</td>
<td>Moderate</td>
<td>No</td>
<td>No</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>33</td>
<td>M</td>
<td>9.0</td>
<td>Severe</td>
<td>Yes</td>
<td>No</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>26</td>
<td>M</td>
<td>9.2</td>
<td>Moderate</td>
<td>No</td>
<td>No</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>30</td>
<td>M</td>
<td>7.8</td>
<td>Mild</td>
<td>No</td>
<td>No</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>36</td>
<td>F</td>
<td>7.2</td>
<td>Moderate</td>
<td>No</td>
<td>No</td>
<td>4</td>
</tr>
<tr>
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<td>36</td>
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<td>8.5</td>
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<td>Yes</td>
<td>No</td>
<td>4</td>
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<tr>
<td>7</td>
<td>40</td>
<td>F</td>
<td>6.7</td>
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</tr>
<tr>
<td>8</td>
<td>24</td>
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<td>Severe</td>
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<tr>
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<td>M</td>
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<tr>
<td>10</td>
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<td>F</td>
<td>9.0</td>
<td>Mild</td>
<td>Yes</td>
<td>Yes</td>
<td>4</td>
</tr>
</tbody>
</table>

AR indicates aortic regurgitation; HT, hypertension; M, male; and F, female.

Apoptotic Index

Four fields per section within the part of the aorta displaying the most severe dilatation were examined at 400-fold magnification. Two independent investigators counted TUNEL-positive VSMCs, and their observations were averaged. Then the apoptotic index was calculated with the following formula: 100×(number of TUNEL-positive nuclei per field/total number of nuclei per field).

Reverse Transcription–Polymerase Chain Reaction

Total RNA was isolated from arterial medial specimens by using TRIzol reagent (GIBCO-BRL), reverse transcription (RT) of an aliquot (5 µg) of the total RNA sample was performed by use of reverse transcriptase (Superscript II, GIBCO-BRL), and polymerase chain reaction (PCR) of the cDNAs of the human AT1R and AT2R genes was performed by use of a standard protocol. As an internal control, GAPDH was coamplified. The sequences of the oligonucleotide primers used for PCR and the sizes of the predicted PCR products are shown in Table 2. The number of cycles was 35, and the PCR products were analyzed on 1.5% agarose gels.

Linearity of PCR Amplification

To determine whether the PCR conditions were in the linear range, experiments were performed with various amounts of total RNA for each gene. Semiquantification of mRNA expression was achieved by densitometric analysis using NIH Image software. This confirmed linear amplification of RNA concentrations from 0.1 to 5.0 µg (data not shown), indicating that RT-PCR was performed within the linear range in the present study.

Aortic Medial Culture

To investigate the role of Ang II signaling via AT1R and AT2R in CMD, we examined the effects of ACEI, AT1RB, or AT2RB exposure on VSMC apoptosis in MFS aortas. Aortic medial speci-
mens were cut into 6 pieces. Each specimen was washed 3 times with PBS and then placed into culture medium for VSMCs (HuMedia-SG2, Kurabou) with or without 10% FCS in culture dishes. Cultures without serum were treated with either an ACEI (10^{-5} mol/L temocaprilat, a kind gift from Sankyo Pharmaceutical Co, Ltd, or 10^{-5} mol/L perindoprilat, a kind gift from Dai-ichi Pharmaceutical Co, Ltd, Tokyo), an AT1RB (10^{-5} mol/L RNH 6270, a kind gift from Sankyo Pharmaceutical Co, Ltd, Tokyo), an AT2RB (10^{-5} mol/L PD-123319, RBI), or the vehicle and were incubated under 95% O_{2}/5% CO_{2} at 37°C for 48 hours. The medium was changed every 12 hours. After 48 hours, the apoptotic changes of VSMCs were examined.

Counting of Cell Nuclei
Four fields per section from the specimens of cultured aortic media for 48 hours after various stimuli were examined at 200-fold magnification. Two independent investigators counted the number of cell nuclei in the area of 2.5 mm², and their observations were averaged.

Ang II Concentration in MFS Aortas
The tissue Ang II concentration was measured by ELISA using frozen aortic specimens from MFS and control patients and was calculated per wet weight.

Statistical Analysis
Analyses were performed with SAS System 8.1 software (SAS Institute Inc). Results were presented as the mean±SD. The Student t test was used for continuous data. The normality of the distribution of data was evaluated by the Shapiro-Wilks 1-sample test, and the F test was used to assess the homogeneity of variance testing. One-way ANOVA was used to test for statistically significant differences among the groups, and the Dunnett multiple comparison method was applied when appropriate. Two-tailed values of \( P<0.05 \) were considered to be statistically significant.

Results
VSMC Apoptosis in CMD
In a series of Victoria blue– and Masson’s trichrome–stained sections of MFS specimens (data not shown), CMD varied from grade 2 to grade 4. Evidence of VSMC apoptosis was obtained in all CMD lesions by TUNEL staining (Figure 1A) and EM (data not shown). The apoptotic index was significantly higher in CMD than in control samples (\( P<0.01 \), Figure 1B). In the present study, there was no association among CMD grade, apoptosis index, and clinical features,
such as presence of dissection, degree of aortic regurgitation, or AAE diameter.

**AT1R and AT2R Expression in CMD**

We examined AT1R and AT2R expression in the ascending aortas from MFS patients and control patients. Expression of each gene was determined by semiquantitative RT-PCR with primers for GAPDH as an internal control, and the linearity of the PCR was confirmed for each gene as described in Methods. Representative RT-PCR gels are shown in Figure 2A, and the signal intensity data obtained by densitometry are shown in Figure 2B. AT1R mRNA expression was significantly lower in MFS aortas than in control aortas ($P < 0.01$). In contrast, AT2R mRNA expression was significantly higher in MFS aortas than in control aortas ($P < 0.001$).

**Tissue Ang II Concentration**

The Ang II concentration at the tissue level was significantly higher in MFS aortas than in control aortas ($P < 0.01$, Figure 3).

**Effect of RAS Inhibition on VSMC Apoptosis in CMD**

The effects of an ACEI, AT1RB, or AT2RB on serum deprivation–induced VSMC apoptosis were examined in an organ culture system. The results of hematoxylin–eosin staining are shown in Figure 4A, and the number of cell nuclei after various stimuli are shown in the bar graph (Figure 4B). Cell nuclei were dramatically decreased by serum deprivation ($P < 0.05$). Both ACEI and AT2RB treatment significantly inhibited serum deprivation–induced loss of VSMC nuclei ($P < 0.05$), but AT1RB treatment did not (Figure 4B). Serum deprivation–induced decrease in cell nuclei number was revealed to be the result of apoptosis by running TUNEL assays and EM study (data not shown).

**Discussion**

It has become widely accepted that apoptosis is a major mechanism for the control of cell numbers in developing and mature tissues under physiological and pathological conditions. Vascular remodeling in the process of development or in pathological states, such as atherosclerosis, restenosis after angioplasty, and aortic aneurysm, is thought to involve VSMC apoptosis as a basic mechanism. CMD is a common finding that is not specific for MFS and is found in many pathological conditions as well as in aging aortas. Several reports have demonstrated that VSMC apoptosis plays a crucial role in CMD associated with MFS.

Recent studies have also suggested that Ang II plays an important role in the regulation of cell growth and death via AT1R and AT2R. AT2R is ubiquitously expressed in fetal tissues, and its expression decreases rapidly after birth. Although the actions mediated by this receptor in vivo remain unclear, there is increasing evidence that it may activate apoptotic pathways. On the other hand, Ang II has been reported to induce aortic aneurysm or aortic dissection in
hypertensive transgenic mice and atherosclerotic knockout mice. In the present study, we showed the alteration of expression balance between Ang II receptors, the acceleration of Ang II formation, and AT2R-mediated VSMC apoptosis in CMD. AT2R reexpression in adults is not specific for MFS aortas and has been reported to be observed in several pathological vascular remodeling states, such as atherosclerotic aorta and restenotic coronary artery after angioplasty. We also detected a high level of AT2R expression in dilated ascending aortas of non-MFS patients (data not shown), suggesting that AT2R-mediated VSMC apoptosis may play an important role not only in AAE associated with MFS but also in vascular positive remodeling itself. Acceleration of Ang II formation observed in the present study may be the result of positive feedback from downregulated AT1R expression. Furthermore, this Ang II formation might be ACE dependent because ACEI treatment inhibited VSMC apoptosis and because tissue Ang II concentration from 2 patients (given ACEI therapy, 5 mg/d enalapril, before surgery and excluded from the present study) was suppressed to below the detection limit, although in vitro activity of Ang II formation in human aortas has been demonstrated to be mainly chymase dependent. AT2R-mediated VSMC apoptosis demonstrated in the present study is a first in vivo report that is compatible with previous in vitro results obtained with the use of cultured VSMCs.

RAS inhibition is believed to be important for the prevention of cardiovascular remodeling. In fact, many recent studies have proved that ACEI and AT1RB therapy can improve the prognosis and quality of life in patients with heart failure. In the context of RAS inhibition, ACEI and AT1RB treatment might have the same effect when AT1R expression is abundant. However, in disease states with decreased AT1R expression and/or increased AT2R expression, the action of ACEI and AT1RB should be different, as shown in the present study.

Our data suggested that the blockade of AT2R-mediated signaling by ACEI therapy may lead to the inhibition of VSMC apoptosis and, thus, may be useful for the treatment or prevention of CMD in patients with MFS.

Limitations of the Study

Although the results obtained in the present study seem definite, it is important to avoid drawing strong conclusions. Because we studied only part of the diseased aortas, regional differences could not be assessed. RT-PCR used in the present study was only semiquantitative, and protein detection by Western blot analysis should be performed. We could not perform these analyses because of the limitation of human sample quantities. Also, we could show no direct evidence about the clinical importance of the alterations in the expression balance between Ang II receptors and AT2R-mediated VSMC apoptosis. But the large number of uncontrollable variables in patients would make it difficult to determine the relationship between gene expression and clinical features. In failing hearts, Haywood et al showed a decrease of AT1R gene expression by RT-PCR but could not show its clinical significance. However, the survival benefit of the RAS blockade in heart failure has been clinically proven. Thus, modification of the RAS may also be a clinically valuable approach for the treatment of aortic diseases in MFS patients.

Conclusions

VSMC apoptosis in CMD associated with MFS might be caused by signaling of Ang II that is ACE-dependently formed via upregulated AT2R, which could be a novel mechanism underlying the aortic complications that occur in MFS. The inhibition of RAS by ACEI therapy may be of clinical value in the prevention or treatment of CMD and aortic complications, such as AAE, in MFS patients.

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References

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