Expression of the Angiotensinogen Gene and Localization of Its Protein in the Human Heart

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Background. There have been no reports on the presence of the tissue renin–angiotensin system in the human heart, although the presence of angiotensinogen has been described in the animal heart.

Methods and Results. To determine whether angiotensinogen is synthesized in the human heart, we examined angiotensinogen messenger RNA (mRNA) synthesis in autopsy hearts by using ribonuclease protection assay. As a result, angiotensinogen mRNA was detected in the atrial muscle, muscles of the conduction system, and the left ventricular wall. In the left ventricular wall, mRNA expression was more prominent in the subendocardial muscles than in the midventricular or epicardial muscles. Using a monoclonal antibody to human angiotensinogen in immunoblotting experiments, we detected two closely spaced bands at approximately 70 kd in the heart, which was quite consistent with the human angiotensinogen molecule. Immunohistochemical studies with this monoclonal antibody demonstrated intense immunoreactivity in the atrial muscles, the muscles of the conduction system, and those of the subendocardial layers.

Conclusions. We conclude that angiotensinogen was synthesized in the human heart. It was evident that the localization of angiotensinogen was not ubiquitous in the cardiac muscles, showing its predilection for the atrial muscles, muscles of the conduction system, and subendocardial layer of the left ventricle. (Circulation 1992;86:138–146)

Key Words • angiotensinogen • immunohistochemistry • monoclonal antibody • ribonuclease protection assay • human heart

The renin–angiotensin (RA) system plays a role in the regulation of blood pressure and of fluid and electrolyte homeostasis. Renin cleaves angiotensinogen to produce a decapeptide, angiotensin I (Ang I), which is then converted by angiotensin converting enzyme (ACE) to angiotensin II (Ang II), the most active pressor substance known. Angiotensinogen is synthesized in the liver, renin is produced by the kidneys, and ACE is released from the lungs. Together, they form the major circulating RA system. Recently, molecular cloning and sequence analysis of complementary DNAs (cDNAs) coding for rat angiotensinogen and human angiotensinogen have provided an opportunity to study angiotensinogen at the messenger RNA (mRNA) level. In addition, renin cDNAs have been cloned for analysis of the mRNA of renin in the mouse and in humans. Advances made in the development of molecular tools have enabled investigators to analyze the tissue RA system. In rats, it has been reported that angiotensinogen is synthesized in the kidney, brain, and heart. At the cellular level, in studies on cultured cells, angiotensinogen has been shown to be produced by endothelial cells and smooth muscle cells. Moreover, renin mRNA has been detected in extrarenal tissues. This suggests that the tissue RA system plays an important role independently of the circulating RA system in both the physiological and pathological states.

Ang II appears to play an important role in cardiac hypertrophy. The authors of a recent clinical study reported that ACE inhibitor (ACEI), which inhibits the conversion of Ang I to Ang II, caused regression of cardiac hypertrophy. Thus, the tissue RA system may be involved in the development of cardiac hypertrophy and other cardiac disorders in humans. However, few studies have focused on the possibility of local synthesis of angiotensinogen in human organs, especially in the heart. This report describes experiments designed to show whether a tissue RA system exists in the human heart. First, we looked for angiotensinogen mRNA in the human heart and liver by performing a ribonuclease protection assay in which we used angiotensinogen complementary RNA (cRNA). We also examined the localization of angiotensinogen in the liver and heart by immunohistochemistry and Western blotting, using a monoclonal antibody to angiotensinogen. We were able to demonstrate that angiotensinogen mRNA was transcribed in the human heart as well as in the liver.

Methods

Tissues

Human hearts (eight nondiseased and seven diseased) were obtained from 15 patients at autopsy, which
TABLE I. Data for 15 Human Hearts at Autopsy

<table>
<thead>
<tr>
<th>Case</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Clinical diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>29</td>
<td>F</td>
<td>AML, VF</td>
</tr>
<tr>
<td>2</td>
<td>56</td>
<td>M</td>
<td>HCM, AML, OMI</td>
</tr>
<tr>
<td>3</td>
<td>68</td>
<td>M</td>
<td>HCM changing into DCM</td>
</tr>
<tr>
<td>4</td>
<td>39</td>
<td>F</td>
<td>DCM</td>
</tr>
<tr>
<td>5</td>
<td>40</td>
<td>F</td>
<td>HCM</td>
</tr>
<tr>
<td>6</td>
<td>77</td>
<td>M</td>
<td>Renal cancer</td>
</tr>
<tr>
<td>7</td>
<td>61</td>
<td>M</td>
<td>Esophageal cancer</td>
</tr>
<tr>
<td>8</td>
<td>58</td>
<td>M</td>
<td>ASR, MSR</td>
</tr>
<tr>
<td>9</td>
<td>14</td>
<td>F</td>
<td>Anaphylactic shock</td>
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<tr>
<td>10</td>
<td>23</td>
<td>M</td>
<td>DCM</td>
</tr>
<tr>
<td>11</td>
<td>62</td>
<td>M</td>
<td>Malignant lymphoma</td>
</tr>
<tr>
<td>12</td>
<td>68</td>
<td>F</td>
<td>Encephalitis</td>
</tr>
<tr>
<td>13</td>
<td>65</td>
<td>M</td>
<td>Colon cancer</td>
</tr>
<tr>
<td>14</td>
<td>70</td>
<td>M</td>
<td>Gastric cancer, hepatic failure</td>
</tr>
<tr>
<td>15</td>
<td>45</td>
<td>F</td>
<td>Acute myocarditis</td>
</tr>
</tbody>
</table>

Cases 1 to 10 were used for immunohistochemical studies, cases 11 and 12 for protein extraction, and cases 13, 14, and 15 for RNA assay. AML, acute myelocytic leukemia; VF, ventricular fibrillation; HCM, hypertrophic cardiomyopathy; AML, acute myelocytic infiltration; OMI, old myocardial infarction; DCM, dilated cardiomyopathy; ASR, aortic stenosis and regurgitation; MSR, mitral stenosis and regurgitation.

was performed within 3 hours after death (Table 1). Cases 1–10 were used for immunohistochemical analysis, cases 11 and 12 for protein analysis, and cases 13, 14, and 15 for the ribonuclease protection assay. For immunohistochemical study, organs were fixed with freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer (PB) overnight at room temperature and embedded in paraffin. For extraction of RNA and protein, disease-free organs were snap-frozen in liquid nitrogen immediately after pathomorphological examination and stored at −80°C until used. During extraction of RNA from the conduction systems of the hearts, tissues were carefully dissected from both atrium and ventricle under low-magnification microscopy as described previously. The left ventricular wall was divided into four parts: papillary muscle, endocardium (inner one third of the cross section of the ventricular wall), middle layer (middle one third), and epicardium (outer one third).

**Extraction of Total RNA**

Tissues from the patients were homogenized individually with a modified single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction with the RNaseql (Cinna/Biotex Laboratories International, Inc., Friendswood, Tex.). To the homogenate, 0.2 ml chloroform per 2 ml homogenate was added, and after shaking, the specimen was stored on ice for 15 minutes. The suspension was centrifuged at 12,000g for 15 minutes, an equal volume of isopropanol was added to the upper aqueous phase, and the specimen was stored for 45 minutes at −20°C. The sample was centrifuged for 15 minutes at 12,000g, and the precipitated RNA formed a white pellet. The pellet was washed twice with 75% ethanol/Tris/EDTA/natrium (0.1 M NaCl, 10 mM Tris-Cl, 1 mM EDTA). The precipitated RNA was dissolved in Tris/EDTA (10 mM Tris-Cl, 1 mM EDTA, pH 8.0) and quantified by measurement of absorbance at 260 nm.

**Preparation of Radiolabeled Complementary Angiotensinogen RNA Probe**

Human angiotensinogen cDNA3 cloned into plasmid pHag3 was excised with both SpI (sequence No. 395) and EcoRI (sequence No. 1805), which includes the coding region of the synthetic peptide we used for immunization. The 1,411–base-pair fragment was inserted into the paired-promoter vector pAM 19 (Amerham) by directional cloning. The DNA template was linearized with BstE II restriction endonuclease, which cut a single site, 1,004 base pairs downstream from the SP6 promoter and 407 base pairs upstream from the T7 promoter. cRNAs were labeled with α-32P-CTP. The antisense RNA probe was prepared by the use of T7 RNA polymerase, and the sense RNA probe with the use of SP6 RNA polymerase, in vitro. The length of the antisense RNA probe was 407 base pairs, and of the sense RNA probe was 1,004 base pairs. The specific activity of these RNA probes was 0.7 to 1.0 × 106 cpm/μg.

**Ribonuclease Protection Assay**

The 32P-labeled sense and antisense RNAs were mixed with total RNAs from human tissues and precipitated with ethanol. The pellets were resuspended in hybridization buffer (Ambion, Inc.) and heated at 80°C for 3 minutes, then hybridized at 43°C overnight. All the RNAs except control samples were then treated with RNase A and T1 before treatment with SDS and protease K. Samples were extracted with phenol/chloroform, precipitated with ethanol, resuspended in a loading buffer containing 80% formamide, heated for 3 minutes at 80°C, and electrophoresed on 5% polyacrylamide gel containing 8 M urea. The gel was transferred to chromatography paper, covered with plastic wrap, and exposed to x-ray film (Kodak XAR) at −80°C for 3 hours.

**Tissue Extracts**

Protein was extracted from the human tissues as follows. The tissues samples, each ~1 g wet weight, were homogenized in 50 mM PB–7 mM EDTA (pH 7.0) and centrifuged at 6,000g for 20 minutes at 4°C. The supernatant was adjusted to 2.3 M ammonium sulfate and stirred overnight. The precipitates obtained by centrifugation at 7,000g for 1 hour were dissolved and dialyzed for 24 hours at 4°C against the same buffer. The dialysate was then snap-frozen and stored at −20°C until it was used.

**Production of Monoclonal Antibodies to Human Angiotensinogen**

For immunization of human angiotensinogen, two cultures of splenic lymphocytes from female Balb/c mice (CLEA, 6–8 weeks old) were added separately to 0.5 μg and 5.0 μg of synthetic peptide (human angiotensinogen 1–13, composed of 13 amino acids, molecular weight: 1645.86; Peninsula Laboratories, Inc.) together with 20 μg of N-acetylmuramyl-L-ararayl-d-isoglutamine (Calbiochem). The cultures were incubated at 37°C in 5% CO₂ for 4 days before fusion. A mixture of two cell cultures (with 0.5 and 5.0 μg of antigen previously added) was fused with myeloma cells (PAI) generously provided by Dr. Shigeo Ohta (Department of Biochemistry, Jichi Medical College, Japan). The fusion products were maintained in
**A**

Ribonuclease Protection Assay

![Image of ribonuclease protection assay result](FIGURE1B.png)

RNA applied in each lane: 15μg

**B**

Ribonuclease Protection Assay of Human Heart

![Image of ribonuclease protection assay result](FIGURE1B.png)

RNA applied in each lane: 15μg

**C**

Ribonuclease Protection Assay of Human Heart

![Image of ribonuclease protection assay result](FIGURE1B.png)

RNA applied in each lane: 20μg

**Figure 1.** mRNA analysis for angiotensinogen cRNA probe by ribonuclease protection assay. Panel A: A \(^{32}\)P-labeled RNA probe was hybridized to 10μg of total RNA from human liver and 50μg of total RNA from human kidney, left ventricle (LV), and right atrium (RA) and incubated with RNase A and T1. For control samples, RNA probe was hybridized to 10μg of total RNA from yeast with and without digestion by RNase A and T1. The protected fragments were analyzed by electrophoresis and autoradiography. The autoradiogram was performed for 3 hours. The protected band (lane 1-4, 407 nucleotides) is smaller than the unprotected band (lane 6, 423 nucleotides). Panels B and C: The same RNA probe was hybridized to 15μg of total RNA from the conduction system, papillary muscle, endocardial layer, middle layer, epicardial layer, atrium and liver of case 14 (panel B). In case 15, 20μg of total RNA from various parts of the heart and liver was hybridized (panel C).

hypoxanthine/aminopterin/thymidine medium (Boehringer-Mannheim) and after 10 days were placed in hypoxanthine/thymidine medium (Boehringer-Mannheim).

Supernatant media from wells containing clones were tested for antibodies by ELISA. After one of the positive tissue culture supernatants was selected, cloning was performed by limiting dilution (0.3 hybridoma per well), and 26 immunopositive clones (ATN 1–26) were obtained. From these 26 clones, one clone, which recognized only human angiotensinogen, was selected.
This monoclonal antibody (ATN-3) did not cross-react with Ang I, Ang II, or bovine serum albumin on ELISA and immunospot analysis. The immunospot test was performed according to the methods of Miyazaki. One microgram of antigen was spotted on a gelatin-coated nitrocellulose membrane, and the membrane was baked with 5 g of paraformaldehyde at 80°C for 1 hour, after which it was incubated with the newly generated monoclonal antibody at room temperature for 3 hours. After incubation with biotinylated goat anti-mouse immunoglobulin; Biogenex, San Ramon, Calif), the sections were reacted with avidin-biotin complex (ABC) at 37°C for 30 minutes, the membrane was visualized with 3,3'-diaminobenzidine. The immunoglobulin subtype of the antibody was IgM k.

Analysis of Proteins Extracted From Various Organs

Proteins extracted from the atrium and ventricle were dissolved in 100 μl of 50 mM PB, and that from the liver was dissolved in 500 μl of 50 mM PB. Ten microliters of each solution added was applied to each lane. The proteins separated by SDS-PAGE were transferred to nitrocellulose membranes by the semi–dry blot method. The immunoreaction was performed by the ABC method with the newly generated monoclonal antibody.

Immunohistochemical Techniques

The ABC method was applied to the paraffin-embedded tissues after fixation with 10% formalin and 4% paraformaldehyde/0.1 M phosphate buffer. Briefly, after deparaffinization with xylene and hydration with ethanol, the sections were pretreated with 1% normal goat serum to avoid nonspecific binding and with 1% H₂O₂ methanol for quenching of endogenous peroxidase activity. The sections were then incubated with the primary antibody at a dilution of 1:800 at 4°C overnight. After washing with PBS, the sections were incubated with a second antibody (biotinylated goat anti-mouse immunoglobulin; Biogenex, San Ramon, Calif.). The sections were then reacted with ABC (Elite ABC kit, Vector Laboratories Inc., Vectastain) and visualized with 3,3'-diaminobenzidine tetrahydrochloride.

In control sections, the primary antisera were substituted by the corresponding preimmune serum or preabsorbed antiserum. Preabsorption was carried out by incubation of 1 μl of primary antibody with 100 μg of synthetic peptide in 400 μl of PBS at 4°C overnight. As positive controls, liver sections were reacted with anti-CEA (Dako, Japan), which always stains bile canaliculi of the liver, and sections from the atrium were reacted with anti-ANP, which always gives positive results.

Results

Ribonuclease protection analysis of RNAs from various tissues in human organs showed that the antisense RNA probe detected a single protected band of 407 nucleotides in length in liver, kidney, left ventricle (LV), and right atrium (RA), indicating that angiotensinogen mRNA was present in these organs (Figure 1A). However, there was no positive signal in yeast with RNase digestion. The sense RNA probe used as a negative control did not hybridize with RNAs from liver, kidney, LV, and RA, except for yeast RNA without digestion.

To examine the distributional difference of angiotensinogen mRNA in parts of the heart, we excised the specimens from the atrium, the conduction system that contains the His bundle and the major branching bundles and the four separated ventricular tissues consisting of papillary muscle and subendocardial, midcardial, and epicardial layers. Two autopsy cases (cases 14 and 15) were used for this analysis. In both cases, angiotensinogen mRNA was detected in the atrium, conduction system, and all three layers of the LV (Figures 1B and 1C). The amount of angiotensinogen mRNA from the epicardium was less than that from the endocardial and midcardial layers. There were two protected bands (400 and 340 nucleotides), as shown in Figure 1B. A 340-nucleotide band was thought to be a product of alternatively spliced angiotensinogen mRNA. The smaller amount of angiotensinogen mRNA from the liver of case 14 compared with that from case 15 may reflect the terminal hepatic failure that occurred in case 14.

Western blotting with the newly generated antibody showed two closely spaced bands of angiotensinogen
=70 kD in size in extracts from both liver and heart. An additional high-molecular-weight form of angiotensinogen (150 kD) was found only in the liver (Figure 2).

For immunohistochemistry, the most suitable method for the present study (among the various methods tried) was fixation with 4% paraformaldehyde in 0.1 M PB and embedding in paraffin. As negative control, culture medium containing 10% fetal calf serum and preabsorbed antiserum was applied in place of the primary antibody, and goat serum was used as the second antibody.

In the heart, immunoreactivity was found in the myocardial cells of the atrium (Figure 3A). The negative control staining did not yield any positive results (Figure 3B). The myocardial cells of the right atrial auricle were intensely stained. The cells adjacent to the endocardium exhibited a stronger reaction than did those near the epicardium (Figure 4A). In the ventricle, an immunopositive reaction was recognized in the papillary muscle and subendocardial layer, but the intensity was weaker than that in the atrium. Immunoreactive cells were present in a mottled pattern (Figure 4B). In contrast, intense immunoreactivity was found in the conduction system, i.e., in the sinoatrial node (Figure 5A), atrioventricular node (Figure 5B), and major branching bundle (Figure 5C). There was no immunoreactive signal in the connective tissue of the endocardium or in the cardiac valve, fat cells, endothelial cells, or the smooth muscle cells of the large coronary arteries. There were some variations in staining intensity among 10 cases examined, probably because of postmortem fixation time, but the
staining pattern described above was similar to both diseased and nondiseased hearts.

In the liver, positive immunostaining of angiotensinogen was observed in only a few hepatocytes in one of the 10 samples tested. The other nine cases showed no immunoreaction. Although we tested a liver sample that gave an intense angiotensinogen mRNA signal (case 15), the staining was again faint. The validity of the staining procedure was confirmed by the positive results of CEA staining for bile canaliculi applied to parallel sections.19

Discussion
In this study, we showed that both angiotensinogen mRNA and its protein were present not only in the liver

FIGURE 4. Immunohistochemical staining of the human heart. Panel A: Diffuse and intense immunoreactivity was seen in the myocardial cells adjacent to the endocardium (end) of the right atrial auricle, whereas those near the epicardium (epi) remained unstained. Original magnification, ×40. Panel B: Angiotensinogen-immunoreactive muscle cells were found in the myocardial cells in the papillary muscles and subendocardial layers. Original magnification, ×40.
FIGURE 5. Angiotensinogen-immunoreactivity of the conduction system. Panel A: Myocytes constituting the sinoatrial node were stained positively. Original magnification, ×200. Panel B: Most cells of the atrioventricular node were positive. Original magnification, ×40. Panel C: Muscle fibers of the major branching bundle gave positive result. Original magnification, ×40.
but also in the heart. The presence of mRNA of angiotensinogen in the heart suggests that angiotensinogen is synthesized in extrahepatic tissues in humans. Angiotensinogen mRNA has been detected previously in rat hearts, but there have been no reports on associated immunohistochemical findings.

The immunogen that we used to make monoclonal antibodies was a synthetic peptide of human angiotensinogen, which is composed of 13 amino acids. Ang I consists of the sequence of the first 10 of these amino acids, and Ang II is composed of the first eight amino acids (Figure 6). The newly generated monoclonal antibody recognized only the angiotensinogen-specific domain; it did not cross-react with Ang I or Ang II. Tewksbury reported that, when a purified human angiotensinogen from plasma was subjected to SDS-PAGE, they detected two closely spaced major bands. In our study, the monoclonal antibody recognized two such bands in both the liver and the heart on Western blotting, in agreement with Tewksbury’s data. In addition, we found a high-molecular-weight band (150 kd) in the liver. Eggens and colleagues reported that the presence of a high-molecular-weight protein could indicate that, in human plasma, multiple forms of angiotensinogen exist in the presence of conditions such as pregnancy, a high-estrogen state, and uremia. The 150-kd molecule could be one of the high-molecular-weight forms of human angiotensinogen, but its exact molecular structure remains unknown.

We observed overt and intense immunohistochemical staining of angiotensinogen in hearts, indicating the presence of angiotensinogen in cardiac muscle. This was the most prominent finding in the present study. Moreover, the immunoreactive myocytes were confined mostly to the atrium, the subendocardial layer of the ventricle, and the conduction system, and fewer immunoreactive cells were present in the epicardial layer. The results were consistent with the presence of angiotensinogen mRNA on ribonuclease protection assay. The pattern of distribution of the angiotensinogen-positive cells is similar to that of the human atrial natriuretic peptide (hANP), which functions as a smooth muscle relaxant. Mochizuki and Wharton found hANP in atrial muscle as well as in the conduction system and in the subendocardial layer of the LV of the human heart, with a distribution identical to that of angiotensinogen. Matsubara et al reported that ANP, a hormone with natriuretic and vasodilatory effects, was synthesized mainly in the atrium; however, ventricular production of ANP increased during cardiac hypertrophy.

Previous reports have shown that there are specific receptors for Ang II in the myocytes and the conduction system of the rat heart. Dzau et al suggested that Ang II can be internalized by cardiac muscle and vascular cells, with subsequent localization in the mitochondria and nuclei. Moreover, Ang II was found to be capable of increasing angiotensinogen mRNA levels. In the present experiments, we have provided evidence that angiotensinogen is synthesized in the human heart, with a distribution identical to that of hANP. However, the physiological role of a similar localization of angiotensinogen and hANP in the heart remains unknown. Our immunohistochemical study showed positive immunostaining in very few hepatocytes, with a less intense level than that in the heart. An explanation for this phenomenon might be that, in the normal physiological state, angiotensinogen is quickly released into the blood stream after synthesis. Another explanation is that alternative RNA splicing could be operational in the heart and liver, and that our monoclonal antibody detects an alternative isoform of angiotensinogen. For confirmation of this hypothesis, an in situ hybridization technique is needed, and such studies are currently in progress.

References

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