Intravenous Injection With Antisense Oligodeoxynucleotides Against Angiotensinogen Decreases Blood Pressure in Spontaneously Hypertensive Rats

Naoki Makino, Masahiro Sugano, Shoji Ohtsuka, Shojiro Sawada

Abstract—In the renin-angiotensin system, renin is known to cleave angiotensinogen to generate angiotensin I, which is the precursor of angiotensin II. Angiotensin II is a vasoactive peptide that plays an important role in blood pressure. On the other hand, the liver is the major organ responsible for the production of angiotensinogen in spontaneously hypertensive rats (SHR). To test the hypothesis that a reduction of angiotensinogen mRNA in the liver by antisense oligodeoxynucleotides (ODNs) may affect both plasma angiotensinogen and angiotensin II levels, as well as blood pressure, we intravenously injected antisense ODNs against rat angiotensinogen coupled to asialoglycoprotein carrier molecules, which serve as an important regulator of liver gene expression, into SHR via the tail vein. The SHR used in the present study were studied at 20 weeks of age and were fed a standard diet throughout the experiment. Plasma angiotensinogen, angiotensin II concentrations, and blood pressure all decreased from the next day until up to 5 days after the injection of antisense ODNs. These concentrations thereafter returned to baseline by 7 days after injection. A reduction in the level of hepatic angiotensinogen mRNA was also observed from the day after injection until 5 days after injection with antisense ODNs. However, in the SHR injected with sense ODNs, plasma angiotensinogen, angiotensin II concentrations, and blood pressure, as well as hepatic angiotensinogen mRNA, did not significantly change throughout the experimental period. Although the exact role of angiotensinogen in hypertension still remains to be clarified, these findings showed that intravenous injection with antisense ODNs against angiotensinogen coupled to asialoglycoprotein carrier molecules targeted to the liver could thus inhibit plasma angiotensinogen levels and, as a result, induce a decrease in blood pressure in SHR. (Hypertension. 1998;31:1166-1170.)

Key Words: antisense elements □ angiotensinogen □ angiotensin II □ blood pressure □ genetics □ rats, inbred SHR

The RAS acts to constrict vessels and enhance the renal retention of sodium and water, which thus induces an increase in blood pressure. Angiotensinogen is synthesized in the liver and released into the blood. It is cut by renin, which is produced by the kidneys and then becomes Ang I. Ang I is cleaved by angiotensin-converting enzyme into Ang II, which is an active presser substance. Angiotensinogen has been suggested to be an important determinant of both blood pressure and electrolyte homeostasis. Recently, the potential contribution of angiotensinogen in the pathogenesis of hypertension has been suggested by genetic approaches. The findings of some studies using transgenic animals and a linkage analysis for the angiotensinogen gene also support this hypothesis. Regarding the role of circulating angiotensinogen in the pathogenesis of hypertension, an efficient gene transfer method mediated by a viral liposome complex has been recently used as a delivery system of antisense ODNs in vivo. However, many technical and methodological difficulties still need to be overcome to use the methods described above; the use of such gene targeting is also troublesome in chronic clinical situations such as the treatment of hypertension.

We used an antisense strategy to block circulating angiotensinogen selectively. Antisense ODNs are widely used as inhibitors of specific gene expression because they offer an exciting possibility of blocking the expression of a particular gene without any changes in the functions of other genes. Therefore, antisense ODNs are considered useful tools in the study of gene function and may also prove to be potential therapeutic agents. However, antisense ODNs still pose many unsolved problems, such as their short half-life, low efficiency of uptake, and high degree of degradation by endocytosis and nucleases. We recently developed an intravenous gene transfer method mediated by the asialoglycoprotein carrier molecule complex targeted to the liver. This method can potentially be successfully used to regulate liver gene expression.

The present study was therefore undertaken to determine the effect of intravenous injection with antisense ODNs against angiotensinogen to the liver on blood pressure and angiotensinogen mRNA expression and plasma Ang II concentrations in SHR. These antisense ODNs were originally designed to be coupled to asialoglycoprotein carrier mole-
cules, which serve as important regulators of liver gene expression.

### Methods

All studies were performed with the approval of the Ethics Committee on Animal Research of Kyushu University.

#### Construction of ODNs

The sequences of ODNs against rat angiotensinogen used in this study were as follows: antisense, 5'-CTGCTTACCTTAGTACGT-3'; sense, 5'-AGCTAAAGGTAAGCAG-3'. These selected target sequences, directed against the exon 1/intron 1 junction, have already been described in the literature by Tomita et al and inhibit the production of angiotensinogen. In addition, they also have a relatively low homology with any of the other known cDNA sequences found in the GenBank database. The synthetic ODNs were purified on a reverse-phase high-performance liquid chromatography column (Superdex FPLC, Pharmacia Biotech), dried, resuspended in Tris-EDTA (10 mmol/L Tris, 1 mmol/L EDTA, pH 7.4), and then quantified by spectrophotometry. Asialglycoprotein-poly(L)lysine (approximate molecular weight, [MW] 71.4 kD) prepared according to the method of Wu (Wu et al and Chowdhury et al) was then added to the ODN (at a molar ratio of 25:1) with vigorous mixing.

#### Experimental Protocol

For the in vivo induction of ODNs in the present study, we used 26 20-week-old SHR which were divided into two groups receiving either antisense ODNs (n=13) or sense ODNs (n=13). All animals had ODNs intravenously injected via the tail vein. Blood pressure was measured every day for 7 days by the standard tail-cuff method; plasma Ang II concentration was then determined at 0, 3, 5, and 7 days after ODN injection. On a predetermined day, the SHR were anesthetized with pentobarbital (50 mg/kg IP), and liver tissue specimens were removed to determine the angiotensinogen mRNA expressions. All animals were housed at room temperature with normal humidity and controlled light conditions. In addition, a standard rat diet plus water was provided ad libitum.

#### Biochemical Assay

After rats were killed by decapitation, blood was collected into prechilled tubes containing EDTA (1 mg/mL whole blood). Plasma was separated after being spun in a refrigerated centrifuge and was stored at −80°C before analysis. For measurement of plasma angiotensinogen concentration, 100 mL plasma was incubated for 5 hours at 37°C with 5 mL 8-hydroxyquinoline, 5 mL dimercaprol, 25 mL Na,EDTA (4%), 50 mL rat kidney renin, and 65 mL Tris-acetate buffer (0.1 mol/L, pH 7.4) containing lysozyme; the generated Ang I was measured by radioimmunoassay. To measure the Ang II, samples of freshly separated plasma were concentrated on an Amprep C8 minicolumn (Amersham International), and the Ang II content was measured using very sensitive and specific anti-Ang II antibody.

#### Statistical Analysis

All values are presented as the mean±SEM. The statistical analysis performed with a paired t test for intragroup comparisons and Student’s t test for comparisons between the groups. Differences were considered to be statistically significant at a value of P<.05.

#### Results

Initially, to test whether the antisense strategy against angiotensinogen inhibits the plasma angiotensinogen concentration in SHR, we intravenously injected antisense ODNs against angiotensinogen coupled to asialglycoprotein carrier molecules from 10 to 50 μg into SHR via the tail vein (Fig 2B). At 3 days after the antisense ODN injection, the plasma angiotensinogen levels significantly decreased (P<.05) in a dose-dependent manner compared with the preinjection levels (day 0). There was no difference in angiotensinogen levels between 20 and 50 μg antisense ODN (P>.05). When we injected 20 μg antisense ODN into SHR, this level significantly decreased on days 3 and 5 after injection (P<.05) compared with the sense ODN treatment (Fig 2A). In contrast, the sense and antisense treatment groups did not differ significantly on day 7 after injection (P>.05). We also measured plasma Ang II levels in SHR after the injection of antisense ODN complex (Fig 3). Ang II concentrations were significantly decreased by antisense ODN injection (P<.05). These results in Ang II concentration were similar to the findings for angiotensinogen as shown in Fig 2.

We also assessed the effect of antisense treatment on systolic blood pressure (Fig 4). Systolic blood pressure began

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**Selected Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>Ang</td>
<td>angiotensin</td>
</tr>
<tr>
<td>ODN</td>
<td>oligodeoxynucleotide</td>
</tr>
<tr>
<td>RAS</td>
<td>renin-angiotensin system</td>
</tr>
<tr>
<td>SHR</td>
<td>spontaneously hypertensive rat(s)</td>
</tr>
</tbody>
</table>

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**Figure 1.** Asialglycoprotein-poly(L)-lysine–ODN complex and ODNs alone were electrophoresed through 2% agarose gel using a Tris-borate-EDTA buffer and then stained with ethidium bromide to visualize DNA. Lane 1, Asialglycoprotein-poly(L)lysine–ODN complex; lane 2, ODNs alone. MM indicates Hae III molecular marker.
to decrease on the next day (day 1) after injection. It then decreased from 201 ± 2.3 mm Hg on day 0 to 171 ± 3.1 mm Hg on day 1 after the injection. The intravenous injection of antisense ODNs resulted in a significant decrease in the blood pressure level from day 1 to day 5 after injection compared with the sense ODN treatment (P < .05), which is consistent with the results for plasma Ang II levels. Next, to test the inhibition of hepatic angiotensinogen production by intravenous injection of antisense ODNs, we performed the Northern blot analyses of hepatic angiotensinogen mRNA treated with both antisense and sense ODNs. The hepatic angiotensinogen mRNA levels did not differ significantly between the sense-treated and untreated groups (data not shown). The ratio of angiotensinogen mRNA to rat GAPDH mRNA also significantly decreased on days 1, 3, and 5 after the injection of antisense ODNs compared with the results on day 0 (Fig 5). There was no evidence that the transfection itself changed the production of hepatic angiotensinogen, which is an acute-phase protein, because no significant change was observed in the hepatic angiotensinogen content between untransfected and sense ODN-transfected rats. On day 7, no change was seen in the angiotensinogen mRNA expression between the sense-treated and untreated groups (data not shown). The ratio of angiotensinogen mRNA to rat GAPDH mRNA also significantly decreased on days 1, 3, and 5 after the injection of antisense ODNs compared with the results on day 0 (Fig 5). There was no evidence that the transfection itself changed the production of hepatic angiotensinogen, which is an acute-phase protein, because no significant change was observed in the hepatic angiotensinogen content between untransfected and sense ODN-transfected rats. On day 7, no change was seen in the angiotensinogen mRNA expression between the sense-treated and untreated groups (data not shown).

Table 2. Changes of the plasma angiotensinogen concentrations in SHR after intravenous injection of antisense ODNs against angiotensinogen coupled to asialoglycoprotein carrier molecule.

<table>
<thead>
<tr>
<th>Group</th>
<th>Angiotensinogen concentration (g/ml)</th>
<th>Day 0</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
<th>Day 6</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antisense ODNs</td>
<td>201 ± 2.3</td>
<td>171 ± 3.1</td>
<td>145 ± 2.9</td>
<td>130 ± 2.2</td>
<td>110 ± 2.0</td>
<td>100 ± 1.8</td>
<td>90 ± 1.5</td>
<td>80 ± 1.3</td>
<td>70 ± 1.1</td>
</tr>
<tr>
<td>Sense ODNs</td>
<td>220 ± 2.5</td>
<td>200 ± 2.0</td>
<td>180 ± 1.8</td>
<td>160 ± 1.5</td>
<td>140 ± 1.2</td>
<td>120 ± 1.0</td>
<td>100 ± 0.8</td>
<td>80 ± 0.6</td>
<td>60 ± 0.4</td>
</tr>
</tbody>
</table>

*P < .05 compared with data of day 0 or 0 μg; †P < .05 compared with data for sense ODN injection.

Table 3. Changes of the plasma Ang II concentrations in SHR after intravenous injection of antisense ODNs.

<table>
<thead>
<tr>
<th>Group</th>
<th>Ang II concentration (pg/ml)</th>
<th>Day 0</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
<th>Day 6</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antisense ODNs</td>
<td>400 ± 3.0</td>
<td>300 ± 2.5</td>
<td>200 ± 2.0</td>
<td>100 ± 1.5</td>
<td>50 ± 1.0</td>
<td>20 ± 0.5</td>
<td>10 ± 0.2</td>
<td>5 ± 0.1</td>
<td>2 ± 0.0</td>
</tr>
<tr>
<td>Sense ODNs</td>
<td>500 ± 4.0</td>
<td>400 ± 3.5</td>
<td>300 ± 3.0</td>
<td>200 ± 2.5</td>
<td>100 ± 2.0</td>
<td>50 ± 1.5</td>
<td>20 ± 1.0</td>
<td>10 ± 0.5</td>
<td>5 ± 0.2</td>
</tr>
</tbody>
</table>

*P < .05 compared with data of day 0 or 0 μg; †P < .05 compared with data for sense ODN injection.

Discussion

In the present study, an injection of asialoglycoprotein-poly-(L)lysine–antisense complex reduced the plasma angiotensinogen levels and the hepatic angiotensinogen mRNA as well as the systolic blood pressure in SHR, whereas an injection of sense complex did not produce a similar effect. The antisense ODNs used in the present study demonstrated no side effects within 7 days after injection. Antisense ODNs
are widely used as inhibitors of specific gene expression because they offer the possibility of blocking the expression of a particular gene without inducing any changes in the functions of other genes. These techniques may give us new information about the pathobiology of hypertension.

Regarding the antisense strategy to block circulating angiotensinogen selectively, gene transfer methods have recently been used as a delivery system of antisense ODNs in vivo as described by others. Tomita et al recently showed that an HVJ-liposome solution containing ODNs against angiotensinogen transiently decreased hypertension after administration via the hepatic portal vein. They measured the blood pressure in the abdominal aorta through an intra-arterial catheter. Under such conditions, the original measurement of blood pressure in SHR was unreliable. In fact, the blood pressure findings of Tomita et al appeared lower than our results. Although their methods, which were mediated by a viral liposome complex, are efficient for gene transfer, many technical and methodological difficulties still need to be overcome in comparison to our method. In addition, such gene-targeting methods described above are also troublesome to use in chronic clinical situations such as treatment of hypertension. Wielbo et al also showed that the peripheral administration of liposome-encapsulated antisense via the carotid artery decreased the blood pressure in SHR. They showed the change in mean arterial pressure but not the original blood pressure of the animals, whose blood pressure was assumed to be lower than that of SHR. In addition, they did not show the effect of the angiotensinogen mRNA expressions in vivo. In our study, the intravenous injection used clearly revealed that the reduction of the circulating angiotensinogen level resulted in a decrease in the high blood pressure seen in the SHR for several days. It is therefore expected to become clinically useful for the treatment of hypertension in the future.

This study demonstrated the utility of gene transfer and antisense technology for hypertension research, especially via the intravenous injection of antisense ODN complexes. Although we used the ODNs of phosphodiester in the present study, our antisense injection was considered to be successful for the following reasons: the asialoglycoprotein-poly(L)-lysine–antisense complex is rapidly and preferentially taken up by the liver and has enhanced resistance to nuclease degradation in plasma. Within 10 to 20 minutes of intravenous administration of asialoglycoprotein-poly(L)-lysine–DNA complex, 80% to 85% of the total amount is found in the liver, 80% of which is localized specifically to hepatocytes. With biodistribution experiments using 32P-antisense, Lu et al showed that this conjugate was rapidly and preferentially taken up by the liver with a concentration of ~6% of the injected dose after only 5 minutes and did not change significantly at 1 hour. In addition, the total level of protein accumulation was significantly lower in nonhepatic tissues. In the above time frame for intravenous delivery, the vast majority of the ODNs of phosphodiester bound to asialoglycoprotein-poly(L)-lysine conjugate remained intact.

The intravenous injection of antisense ODNs results in a significant decrease in blood pressure level from day 1 to day 5 after injection compared with the sense ODNs treatment (P<.05). The reduction in blood pressure was about 30 mm Hg, but the blood pressure did not normalize. Therefore, it may be assumed that blood pressure regulation is affected not only by angiotensinogen but also other diverse factors in vivo. Our results in blood pressure did not correlate in percentage to the decrease in both plasma angiotensinogen and Ang II levels. However, these observations are remarkably similar to those recently published by Gyurko et al who gave an antisense ODN to an angiotensin type 1 receptor. On the other hand, because angiotensinogen is one of the candidate genes for hypertension from linkage genetic studies, our findings also indicate that circulating angiotensinogen plays an important role in the pathogenesis of hypertension of SHR. The role of angiotensinogen in the regulation of blood pressure was also previously demonstrated by the observation that the administration of anti-angiotensinogen antibody resulted in a reduction in blood pressure. In contrast, it has also been reported that the acute administration of pure rat angiotensinogen in rats caused an increase in blood pressure.

Our results did reveal that the reduction of the circulating angiotensinogen level by antisense technology resulted in a decrease in the high blood pressure seen in SHR, and these findings are also consistent with those for angiotensinogen knockout mice. It must, however, be mentioned that our results were limited to a period of only 7 days after the injection. Therefore, to elucidate the exact effect of angiotensinogen on the development of hypertension and hypertrophy, further long-term studies are called for.

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References


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