Application of Monoclonal Antibodies for Endothelin to Hypertensive Research

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We developed six kinds of monoclonal antibodies against endothelin (ET)-1 recognizing different epitopes with high affinities ($5 \times 10^{-10}$ M$^{-1}$ to $5 \times 10^{-11}$ M$^{-1}$). Using these monoclonal antibodies, we developed radioimmunoassays for ET-1 with different specificities. Cross-reactivities with ET-2 ranged from 80% to 100%, and those with ET-3 ranged from 3% to 60%. Patients with essential hypertension ($n=20$) showed a significant elevation in the plasma ET-1-LI level compared with age-matched control subjects ($n=12$) ($30.1 \pm 1.4$ pg/ml versus $18.5 \pm 0.9$ pg/ml, $p<0.01$). The plasma ET-1-LI level in hypertensive patients in stages II and III (World Health Organization classification) was significantly higher than that in those patients in stage I. There was no significant correlation between the plasma ET-1-LI level and systolic blood pressure ($r=0.11$), diastolic blood pressure ($r=-0.13$), or age ($r=0.24$) in all patients studied who had essential hypertension. In the neutralization experiment, monoclonal antibodies attenuated ET-1-induced contraction of rat aortic rings and the pressor action of ET-1 in pithed rats in vivo. The present study demonstrates the elevated plasma ET-1-LI level in patients with essential hypertension. Monoclonal antibodies developed in this study can become powerful tools to investigate the pathophysiological significance of ET in essential hypertension. (Hypertension 1990;15:734-738)

Endothelin (ET) is a novel 21 amino acid peptide purified from culture medium of porcine aortic endothelial cells. The intravenous administration of ET induces the sustained elevation of arterial pressure with initial hypotensive response in experimental animals. ET produces a vasoconstriction in isolated aortic, renal, and coronary arteries in vitro. These observations raise the possibility that ET plays important roles in the development or maintenance of hypertension.

In the present study, to investigate the implication of ET in essential hypertension, we prepared six kinds of monoclonal antibodies against ET-1 and set up radioimmunoasays (RIAs) with different specificities. Using the RIA, we measured the plasma ET-1-LI level in patients with essential hypertension. In addition, we report here the application of these antibodies to blockade of vasoconstricting responses to exogenously administered ET-1 in vitro and in vivo.

Methods

Peptide

ET-1, ET-2, and ET-3 were purchased from Peptide Institute Inc., Minoh, Japan. Human big ET (human ET[53–90]) and a common carboxy terminal fragment of ET (ET[17–21]) were synthesized by the solid phase method.

Immunization

Adult female BALB/c mice were immunized with the conjugate containing 10 µg ET-1 emulsified in complete Freund's adjuvant as previously described. They were given a booster injection every 3 weeks and bled 10–14 days after each booster injection. Five of 20 mice gave a positive antibody response.

Preparation of Monoclonal Antibody

Fusion of spleen cells with a nonproducing mouse myeloma cell line, X63-Ag8.653, was carried out with...
Measurement of Plasma Endothelin-1-Level

50% polyethylene glycol (PEG 4000, Merck, Darmstadt, FRG) according to the method described previously.7,8 Hybridoma selection, cloning, and ascitic preparation were performed as reported elsewhere.7,8

Characterization of Monoclonal Antibody

Isotyping of the monoclonal antibody was performed by the Ouchterlony technique.7,8 Binding affinity was examined by the Scatchard plot analysis.

Purification of Monoclonal Antibody

The monoclonal antibody was purified using a Protein A affinity column (Affi-Gel Protein A MAPS II Kit, Bio-Rad Laboratories, Richmond, Calif.).

Subjects and Blood Sampling

We studied 20 patients with essential hypertension before drug treatment (age, mean±SD, 51.7±2.8 years) and 12 age-matched control subjects (age 50.0±0.6 years). According to the World Health Organization classification, 13 of the hypertensive patients were in stage I, 5 in stage II, and 2 in stage III. All patients had a serum creatinine level less than 1.5 mg/dl. Their systolic and diastolic blood pressure and heart rate were 160±5 mm Hg, 98±2 mm Hg, and 71±3 beats/min, respectively. Their daily uptake of sodium chloride was 10.6±0.7 g. Blood was collected from an antecubital vein in the recumbent position after an overnight fast.

Measurement of Plasma Endothelin-1-LI Level

The plasma ET-1-LI level was measured by RIA with the monoclonal antibody (KY-ET-1-I) as previously reported.6 The extraction of ET from plasma was carried out using polystyrene beads coated with the purified monoclonal antibody (KY-ET-1-I). Recoveries of 25 pg and 50 pg ET-1 and 50 pg big ET added to 1 ml plasma were 52%, 55%, and 48%, respectively.6 Interassay and intra-assay variations in the RIA were 6.8±2.8% and 7.3±2.2%, respectively.6

Measurements of Other Plasma Hormone Levels

We also measured plasma levels of various hormones, such as atrial natriuretic peptide, arginine vasopressin, norepinephrine, epinephrine and aldosterone, and plasma renin activity, as previously reported.9-11

Neutralization Experiments

Contractile responses of rat aortic strips were performed as previously reported.12 Aortic rings were incubated in an organ bath perfused at 37°C with balanced salt solution containing (meq) Na+ 139.7, K+ 5.4, Ca2+ 2.2, Mg2+ 1.0, Cl− 131.5, and HCO3− 20. Purified monoclonal antibody (0.05–5 µg/ml) was added 3 minutes before the ET-1 administration.

Effects of monoclonal antibody on ET-1-induced pressor action was examined in pithed rats in which the evaluation of the vascular smooth muscle contraction or relaxation was directly assayed because of the lack of the autonomic nervous reflex. Pithed rats were made as previously reported.13 Arterial pressure was monitored directly via the catheter inserted into the femoral artery. Purified antibody or the same dose of mouse immunoglobulin G1 (400 µg/kg) was injected 60 minutes before the bolus injection of ET-1 (0.3 nmol/kg).

Statistical Analysis

Values are expressed as mean±SEM. Univariate analysis of mean data between groups was performed by the Student’s t test. Univariate analysis of proportions between groups was performed by the χ² test. Linear regression analysis was used to determine correlations between results.

Results

Monoclonal Antibodies and Radioimmunoassays for Endothelin-1

Six kinds of monoclonal antibodies (KY-ET-1-I–VI) were obtained. Characterization of monoclonal antibodies are summarized in Table 1. Analysis by the Scatchard plot revealed that all antibodies have high affinities for ET-1 (Kd 5.3×10^10 M⁻¹ to 4.8×10^11 M⁻¹). Although all antibodies recognized ET-2 (cross-reactivity from 80% to 100%), cross-reactivities with ET-3 were from 3% to 60% on a molar basis. KY-ET-1-I exhibited the highest cross-reactivity with ET-3 among six antibodies. All antibodies also recognized human big ET. As shown in Table 1, the value of 50% inhibitory concentration of each RIA with each one of six antibodies ranged from 2 pg/tube to 10 pg/tube. The RIA with KY-ET-1-I was the most sensitive for ET-1.

Table 1. Characterization of Monoclonal Antibodies Against Endothelin-1

<table>
<thead>
<tr>
<th>Antibody</th>
<th>IgG subclass</th>
<th>Kd (M⁻¹)</th>
<th>IC₅₀ (pg/tube)</th>
<th>ET-2</th>
<th>ET-3</th>
<th>Human big ET</th>
<th>Cross-reactivities (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KY-ET-1-I</td>
<td>IgG1</td>
<td>7.0×10¹⁰</td>
<td>6</td>
<td>100</td>
<td>60</td>
<td>100</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>KY-ET-1-II</td>
<td>IgG1</td>
<td>2.1×10¹¹</td>
<td>4</td>
<td>90</td>
<td>40</td>
<td>100</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>KY-ET-1-III</td>
<td>IgG2a</td>
<td>2.4×10¹¹</td>
<td>4</td>
<td>90</td>
<td>35</td>
<td>100</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>KY-ET-1-IV</td>
<td>IgG1</td>
<td>4.8×10¹¹</td>
<td>2</td>
<td>80</td>
<td>20</td>
<td>80</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>KY-ET-1-V</td>
<td>IgG1</td>
<td>5.3×10¹⁰</td>
<td>10</td>
<td>90</td>
<td>15</td>
<td>50</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>KY-ET-1-VI</td>
<td>IgG2a</td>
<td>6.0×10¹⁰</td>
<td>8</td>
<td>80</td>
<td>3</td>
<td>50</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

IgG, immunoglobulin G; Kd, association constant; IC₅₀, value of 50% inhibitory concentration; ET, endothelin.
Plasma Endothelin-1–LI Level in Patients With Essential Hypertension

The plasma ET-1–LI level in patients with essential hypertension was 30.1 ± 1.4 pg/ml and was significantly elevated compared with that in age-matched control subjects (18.5 ± 0.9 pg/ml) (Figure 1). Patients in stages II and III showed higher plasma ET-1–LI level than patients in stage I (33.9 ± 1.8 versus 28.1 ± 1.7 pg/ml). Patients were divided according to their plasma ET-1–LI levels into those with levels below (group A) and above (group B) the average value (30 pg/ml) of the total patients. Five of eight patients in group B were in stage II or III, whereas two of 12 patients in group A were in stage II or III. Thus, the incidence of organ complications in group B was significantly higher than that in group A (Figure 2).

The correlation coefficients between the plasma ET-1–LI level and clinical and laboratory parameters are summarized in Table 2. There was no significant correlation between the plasma ET-1–LI level and systolic or diastolic blood pressure in patients with essential hypertension. The plasma ET-1–LI level was not correlated with any plasma hormone levels, which were involved in the control of blood pressure and water balance.

Neutralization Experiments

Synthetic ET-1 induced a vasoconstriction in isolated rat aortic rings in a dose-dependent manner. The administration of purified monoclonal antibody (KY-ET-1-I) dose-dependently shifted the dose–response curve to the right (Figure 3, upper panel). In pithed rats, the elevation in mean arterial pressure induced by ET-1 was significantly suppressed by pretreatment with the purified monoclonal antibody 60 minutes before the ET-1 administration (Figure 3, lower panel).

Discussion

In the present study, we prepared six kinds of monoclonal antibodies against ET-1 and applied them to RIAs and neutralization experiments. Association constants of these antibodies ranged from 5.3 × 10^10 M^-1 to 4.8 × 10^11 M^-1. All antibodies have higher affinities than the ET-1 monoclonal antibody reported recently. The value of 50% inhibitory concentration in each RIA with each one of six antibodies ranged from 2 pg/tube to 10 pg/tube. Using the RIA with the most sensitive antibody (KY-ET-1-IV), we directly detected plasma ET-1–LI in human and rat plasma (our unpublished observation). In the present study, we used monoclonal antibody–coated beads for extraction of ET from

**TABLE 2. Correlation Between Plasma Endothelin-1–LI Immunoreactivity Level and Clinical Parameters**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Correlation coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemodynamics</td>
<td></td>
</tr>
<tr>
<td>SBP</td>
<td>r = 0.11</td>
</tr>
<tr>
<td>DBP</td>
<td>r = -0.13</td>
</tr>
<tr>
<td>HR</td>
<td>r = 0.13</td>
</tr>
<tr>
<td>Hormone</td>
<td></td>
</tr>
<tr>
<td>ANP</td>
<td>r = -0.08</td>
</tr>
<tr>
<td>PRA</td>
<td>r = -0.002</td>
</tr>
<tr>
<td>PAC</td>
<td>r = 0.40</td>
</tr>
<tr>
<td>NE</td>
<td>r = 0.41</td>
</tr>
<tr>
<td>E</td>
<td>r = 0.04</td>
</tr>
<tr>
<td>AVP</td>
<td>r = 0.19</td>
</tr>
<tr>
<td>Renal function</td>
<td></td>
</tr>
<tr>
<td>CRE</td>
<td>r = -0.07</td>
</tr>
<tr>
<td>BUN</td>
<td>r = 0.29</td>
</tr>
<tr>
<td>Age</td>
<td>r = 0.24</td>
</tr>
</tbody>
</table>

SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; ANP, plasma atrial natriuretic peptide level; PRA, plasma renin activity; PAC, plasma aldosterone concentration; NE, plasma norepinephrine concentration; E, plasma epinephrine concentration; CRE, serum creatinine level; BUN, serum blood urea nitrogen level.

**FIGURE 1.** Bar graph showing plasma endothelin-1–LI immunoreactivity in patients with essential hypertension. **p < 0.01 versus control.

**FIGURE 2.** Pie chart showing relation between plasma endothelin level and incidence of organ complications.
The role of the increased plasma ET-1-LI level in essential hypertension is not clear at present. Recently, Komuro et al\textsuperscript{16} reported that ET-1 possesses a proliferative effect on vascular smooth muscle cells. It may be possible, therefore, that the increased secretion of ET from endothelial cells plays an obligatory role in the development and aggravation of atherosclerotic changes in underlying intima and media and thereby is related to organ complications. On the other hand, a low dose of ET-1 is reported to cause vasodilation instead of vasoconstriction via the production of prostaglandin I\textsubscript{2}, or endothelium-derived relaxing factor.\textsuperscript{17-19} Elucidation of the precise role of ET in essential hypertension must await the development of a sensitive and specific antagonist for ET.

Koyama et al\textsuperscript{20} recently reported a pronounced elevation in the plasma ET-1-LI level in patients with uremia. In the present study, there was no significant correlation between the plasma ET-1-LI level and serum creatinine level in hypertensive patients with normal renal functions. However, we observed a positive correlation between the plasma ET-1-LI level and the serum creatinine level in a larger population of hypertensive patients whose serum creatinine level ranged from 0.5 to 9.0 mg/dl (data not shown).

Recently, Suzuki et al\textsuperscript{14} reported, using the enzyme immunoassay, that the plasma ET-1 level in normal subjects is 1.5±0.32 pg/ml, and this value appears to be lower than that in the present study.\textsuperscript{8} The difference depends on the specificity of the assay system used in each laboratory. As we mentioned above, ET-1-LI in plasma consists of ET-1, big ET, and another peptide (6,000 d), and the ratio of ET-1 to total ET-1-LI in plasma is about 1:5.\textsuperscript{6} KY-ET-1-I equally recognizes ET-1 and big ET, and it also recognizes the 6,000 d peptide,\textsuperscript{6} although the enzyme immunoassay developed by Suzuki et al\textsuperscript{14} recognizes only ET-1. Therefore, the plasma ET-1 level in normal subjects in our assay system is comparable with that reported by Suzuki et al.\textsuperscript{14} However, Ando et al\textsuperscript{21} reported a much lower normal value of plasma ET-LI level by the RIA. The reason responsible for the difference is not clear at present.

For the neutralization experiment, high affinity antibodies are required. Dissociation constant of ET receptors in vascular smooth muscle cells was reported to be 4×10^{-10} M (K\textsubscript{s} = 2.5×10^{-10} M\textsuperscript{-1}).\textsuperscript{22} Association constants of antibodies developed in the present study are more than 5.3×10^{10} M\textsuperscript{-1}. In fact, the purified monoclonal antibody (KY-ET-1-I) attenuated the ET-1-induced vasoconstriction in vitro and pressor action in vivo in this study. These observations raise the possibility that these monoclonal antibodies are useful to investigate roles of endogenous ET in essential hypertension. Neutralization experiments are now in progress in our laboratories.

In conclusion, we have developed six kinds of monoclonal antibodies against ET-1 and set up sensitive RIAs with different specificities. Using the RIA, we have demonstrated the elevated plasma ET-1-LI level in hypertensive patients whose serum creatinine level ranged from 0.5 to 9.0 mg/dl. In these antibodies, each antibody recognizes different epitopes. Therefore, these antibodies will be used for the two-antibody sandwich immunoassay in the near future in our laboratory.

In this study, we also demonstrated an elevated plasma ET-1-LI level in patients with essential hypertension. This observation clearly indicates the possible pathophysiological significance of ET in essential hypertension. The plasma ET-1-LI levels of patients in stages II and III were significantly higher than those of patients in stage I. In addition, patients with higher plasma ET-1-LI levels tended to have organ complications more frequently. These observations raise the possibility that the elevation of the plasma ET-1-LI level is related to organ complications, including atherosclerosis, associated with hypertension. Recently, we reported, using gel permeation chromatography, that ET-1-LI in normal plasma consists of ET-1, big ET, and another precursor form of ET (6,000 d).\textsuperscript{6} It remains to be clarified which components are preferentially increased in the patients with essential hypertension. Parallel increases of ET-1 and big ET were also reported in patients with acute myocardial infarction.\textsuperscript{15} Therefore, it is possible that the elevation of the plasma ET-1-LI level was due to parallel increases of ET-1, big ET, and another component. Further studies with a large number of patients are now taking place in our laboratory to elucidate the clinical implication of ET in essential hypertension.
level in patients with essential hypertension. Monoclonal antibodies and RIAs developed in this study can become powerful tools to investigate a pathophysiological significance of ET in essential hypertension.

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