Inhibition of Endothelin (ET)-1- and ET-2-Induced Vasoconstriction by Anti-ET-1 Monoclonal Antibody

Tomoyuki Koshi, Takahiro Torii, Koichi Arai, Toshiyuki Edano, Mitsuteru Hirata, Masao Oikuchi, and Tetsuo Okabe
Tokyo Research Laboratories, Kowa Co., Ltd., 2-17-43 Noguchi-cho, Higashinakayamacho, Tokyo 189, Japan and The Third Department of Internal Medicine, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, Japan. Received August 9, 1990

We produced a monoclonal antibody to endothelin (ET)-1, tested cross-reactivities with the related peptides by enzyme immunoassay, and investigated the effects of the antibody on ET-1- or ET-2-induced vasoconstriction of rat isolated thoracic aorta. The antibody recognized ET-1, ET-2 and ET-3, and the immunoreactive site proved to be the N-terminal region but not the C-terminal region of ET-1. Moreover, at an approximate molar-equivalent concentration, the antibody absorbed ET-1 and ET-2, and significantly inhibited ET-1- and ET-2-induced vasoconstriction notwithstanding the presence of the endothelin receptor.

Keywords: endothelin-1; endothelin-2; anti endothelin-1 cross-reactivity

Introduction
Endothelin-1 (ET-1) is the most potent vasoconstrictor peptide in hitherto known constrictors, is produced by vascular endothelial cells, and consists of 21-amino acid residues containing two intramolecular disulfide linkages.1) Recently, endothelin-related genes were found in human genomes, and three correspondent peptides designated as ET-1, ET-2 and ET-3. ET-2 and ET-3 are also potent constrictors and exhibit distinct pharmacological activities. Their structures are different from ET-1 in the second to the seventh amino acid positions of the N-terminal fragment.2) In addition, it has been demonstrated that immunoreactive substances like endothelin exist in human plasma, and the plasma level is increased in patients with various disorders.3) This paper describes the production of anti ET monoclonal antibody and the possibility of immunological therapy with the antibody.

Materials and Methods
Chemicals
Materials were obtained from the following commercial sources and used according to the procedures given by the respective suppliers: Sodium pentobarbital from Abbott Laboratories (North Chicago); Formylcellulofine and Protein A-cellulofine from Seikagaku Kogyo Co., Ltd. (Tokyo); Sepharose 6B from Pharmacia LKB Biotechnology (Uppsala); Freund’s complete adjuvant (CFA) and incomplete adjuvant (IFA) from Difco Laboratories (Detroit); ET-1, ET-2 and big ET from Peptide Institute Inc. (Osaka); Porcine thryoglobulin (PTG) from Servo Feinbiochemica GMBH & Co. (New York); β-α-galactosidase from Boehringer Mannheim GMBH (Mannheim). The ET-1 isomer, as described by S. Kumagaye et al., was synthesized by biological peptide synthesizer (applied biosystem model 430). Lysylendopeptidase-digested ET-1 was obtained as described by S. Kimura et al.5)

Animals
Wistar rats and BALB/c mice from Japan SLC, Inc. (Shizuoka).

Preparation of Murine Monoclonal Anti ET-1 Immunoglobulin G (IgG) and Murine Normal IgG
ET-1 (1.1 mg) was dissolved in 4.4 ml of 50 mM sodium phosphate buffer (pH 7.4) containing 4.4 mg of PTG, and 110 mg of 1-(3-dimethylaminopropl)-3-ethylcarboxidimide was added. The reaction mixture was stirred magnetically overnight at room temperature (r.t.). The reaction solution was dialyzed for 48 h against 3 1 of 10 mM sodium phosphate buffer (pH 7.4) containing 4.4 mg of PTG, and then 150 mM NaCl at 4°C, and then 8.5 ml of ET-1-PTG conjugate solution (0.52 mg protein/ml) was obtained. The conjugated antigen (50 μg protein), emulsified with an equal volume of CFA, was administrated intraperitoneally in BALB/c mice. Two weeks later, the antigen (50 μg protein) emulsified with an equal volume of IFA was injected intraperitoneally in mice. Immunizations by this method were repeated 3 times at 3 week intervals. Four days after the last i.p. injection, splenocytes of immunized mice were taken out, and were fused with mouse myeloma cell line P3-X63 Ag8U.1.9) The mixture of cells was plated in 768 wells of 96 well plates. Hybridomas were screened for production of the monoclonal anti ET-1 antibodies by using the competitive enzyme immunoassay with ET-1-β-galactosidase conjugate. Selected hybridomas were cloned at least twice by limiting dilution. A monoclonal anti ET-1 (IgG) antibody, ET-MA51 (IgG), was selected. The antibody was purified from ascites fluids produced in BALB/c mice by precipitation with ammonium sulfate, followed by Protein A-cellulose column chromatography and dialization, and then was lyophilized.

Murine normal IgG was purified from sera in a similar manner.

Enzyme Immunoassay
The enzyme immunoassay was performed by the competitive binding procedure. β-α-Galactosidase-conjugated ET-1 was synthesized in a similar way to the preparation of β-α-galactosidase-conjugated bradykinin by Ueno et al.7) ET-MA51 solution (100 μl, 2 μg/ml) in 50 mM sodium carbonate buffer, pH 9.6) was added to each well of microtiter plates. After incubation at 25°C for 2h, followed by washing 3 times with 250 μl of phosphate buffered saline (PBS) containing 0.05% (w/v) Tween 20 (PBS-T), and standard ET-1 (or sample, 100 μl) in PBS-T and 100 μl of β-α-galactosidase-conjugated ET-1 diluted 2000 times in PBS-T were added to each well. After incubation at 4°C for 16h, followed by washing 3 times with 250 μl of PBS-T, and 100 μl of 0.2% 4-methylumbelliferyl-β-galactoside, in 20 mM sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl, 0.1% (w/v) NaNO3, 0.1% (w/v) bovine serum albumin and 2 mM MgCl2 (Buffer A), was added to each well. After incubation at 25°C for 2h, the reaction was terminated by the addition of 0.2 M sodium phosphate buffer (100 μl, pH 10.3). The amount of 4-methylumbellifereone was measured with a spectrophotometer (FCA, Pandex) at 450nm (emission wave length) and at 365 nm (excitation wave length) in duplicate.

Characterization of ET-MA51
Isotyping of ET-MA51 was performed by the Ouchterlony technique. Crossreactivity experiments were performed by the enzyme immunoassay using ET-1 related peptides, ET-2, ET-3, porcine big ET, ET-1 (16-21), ET-1 (4-10), porcine big ET (22-39), synthesized ET-1 isomer linked with Cys1-Cys15 and Cys4-Cys15 bonds (type B ET-1), and lysylendopeptidase-digested ET-like peptide containing 21 amino acid residue, of which Lys6-Glu10 bond was cleaved (Nicked ET-1).

Absorption of ET-1 and ET-2 by ET-MA51
ET-MA51 was dissolved in PBS-T, and an absorption of ET-1 or ET-2 was examined by the enzyme immunoassay. Namely, 150 μl of ET-1 (8 pmol/ml) or ET-2 (8 pmol/ml) was added into 150 μl of the diluent of ET-MA51, and the mixture was incubated at 37°C for 1h, and then the amount of ET-1 or ET-2 was measured.

Inhibition of ET-1 and ET-2-Induced Vasoconstriction by ET-MA51
In Rat Thoracic Aorta
Wistar strain male rats, weighing 200 to 300 g, were stunned and killed by bleeding, and then the thoracic aortae were removed rapidly. Ring segments (3 mm width) were prepared and were mounted between hooks in a plastic organ bath soaked with 3 ml of Krebs–Henseleit solution aerated with a gas mixture of 95% O2 and 5% CO2, and maintained at 37°C. The composition of Krebs-Henseleit solution was as follows (in mM): NaCl, 118.4; KCl, 4.7; CaCl2, 2.5; MgSO4, 1.2; NaHCO3, 25.0; glucose, 10.0. The magnitude of vasoconstriction was measured isometrically with a force-displaced transducer (Orinert T7-30-240) and recorded on a thermal rectorder (NEC San-e Instruments, Rectigraph SK) through an amplifier (Nihon-kohden AR-621G). The resting tension

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Table 1. Inhibition of ET-1- and ET-2-Induced Vasoconstriction by ET-MA51 in Rat Thoracic Aorta

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>ET-1-Induced Vasoconstriction</th>
<th>ET-2-Induced Vasoconstriction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean</td>
<td>S.E. (%)</td>
</tr>
<tr>
<td>Non-treated</td>
<td>6</td>
<td>53.8</td>
<td>8.7</td>
</tr>
<tr>
<td>Normal IgG</td>
<td>6</td>
<td>49.5</td>
<td>6.4</td>
</tr>
<tr>
<td>Antibody 0.3 μg/ml</td>
<td>6</td>
<td>34.5</td>
<td>5.5</td>
</tr>
<tr>
<td>Antibody 1 μg/ml</td>
<td>6</td>
<td>2.7</td>
<td>0.8</td>
</tr>
</tbody>
</table>

The greatest tension developed by endothelin (10 ng/ml) was expressed as a percent of the maximum response 80 nm K⁺. Data express the mean ± S.E. of constriction in six experiments. N.S.: not significant. a) and b) were statistically significant from the control at p < 0.05 and p < 0.01, respectively.

Fig. 1. Crossreactivity in the Enzyme Immunoassay Using ET-MA51 for ET-1 and Other ET-1 Related Peptides

Data show the dose–response curve for ET-1 (●), ET-2 (○), ET-3 (▲), porcine big ET (△), ET (16–21) (□), ET (4–10) (□), ET-1 type B (◇), porcine big ET (22–39) (◇) and Nicked ET-1 (●) in enzyme immunoassay using ET-MA51 and β-galactosidase-labeled ET-1. B/Bo enzyme activity bound to the solid phase without ET-1 or ET-1 related peptides (B) enzyme activity bound to the solid phase with added ET-1 or ET-1 related peptides.

Fig. 2. Absorption of ET-1 and ET-2 by EM-MA51

150 μl of ET-1 (8 pmol/ml) or ET-2 was added to 150 μl of diluent ET-MA51, and the mixture was incubated at 37°C for 1 h. The absorption percent of ET-1 (●) and ET-2 (○) with ET-MA51 were examined by the enzyme immunoassay.

Results and Discussion

Characterization of ET-MA51. The obtained ET-MA51 belonged to the IgG1 subclass. The standard curves and crossreactivity experiments in the enzyme immunoassay, using ET-MA51, for ET-1 and the other ET-1 related peptides are shown in Fig. 1. In the assays, the minimal detectable concentration (at 90% B/Bo) was 40 pg/ml, and the 50% B/Bo value of ET-1 was 230 pg/ml. The ET-MA51 showed 150% crossreactivity with ET-2; 60% with ET-3; 40% with porcine big ET; 10% with type B ET-1, respectively. On the other hand, ET-MA51 showed no crossreactivity with ET-1 (16–21), ET-1 (4–10), porcine big ET (22–39) and Nicked ET-1.

These facts, that not only type B ET-1, Nicked ET-1 but also ET (16–21) show a significant fall or a lack in immunoactivity, implied that the steric restricted N-terminal region (ET(1–15)) possessing two disulfide bonds (Cys¹–Cys¹⁵, Cys³–Cys¹¹) but not the C-terminal region (ET(16–21)) plays an important role in the ET-1-related peptide being recognized by ET-MA51.

Absorption of ET-1 and ET-2 by ET-MA51. ET-MA51 absorbed ET-1 and ET-2 dose-dependently as shown in Fig. 2. Namely, 0.7 and 7.0 pmol of ET-MA51 absorbed, on one hand, 42% and 95% of ET-1 (1.2 pmol), and on the other hand, 55% and 96% of ET-2 (1.2 pmol), respectively. Besides, about 50% of 1.2 pmol of ET-1 and ET-2 were neutralized with 0.9 and 0.6 pmol of ET-MA51, respectively.
These results show not only correspondence with the fact that ET-2 exhibited a 1.5 fold potent cross-reactivity to ET-1, but also a high affinity of ET-MA51 for ET-1 and ET-2 at the equivalent molar concentrations. 

**Inhibition of ET-1 and ET-2-Induced Vasoconstriction by ET-MA51 in Rat Thoracic Aorta** ET-1 induced a slow-developing and long-lasting constriction of the rat aorta at a concentration of 10 ng/ml. ET-2 was more potent (about 150%) than ET-1. The ET-1- or ET-2 (each 10 ng/ml)-induced constriction of the aorta was significantly ($p<0.01$) inhibited with 1 μg/ml of ET-MA51 as shown in Table 1. Table 1, while the ET-MA51 pretreatment (10 μg/ml) did not affect K+ ($2.5 \times 10^{-3}$ M)- and/or a dl-norepinephrine ($2 \times 10^{-7}$ M)-induced constriction comparable to that induced by ET-1 (10 ng/ml) (data not shown).

These results also show the high affinity of ET-MA51 for ET-1 and ET-2.

In addition, an antibody to toxins has been clinically used since the discovery of an antiserum therapy for tetanus. It has recently been found that the immunoreactive levels of ET related peptides rise in the human plasma of patients with acute myocardial infarction, uremia, subarachnoidal hemorrhage, and in bronchial exudate during acute asthmatic episodes. Moreover, ET-1 can also stimulate phospholipase A$_2$ and oxygen radical formation. And so, endogenous ET-1 can be a candidate for the attack of various disorders caused by the constriction of smooth muscle cells, or by an increase of an oxygen radical, thromboxan A$_2$ and leukotrien. Immunological prophylactic and therapeutic applications of this antibody might be possible, since it has high cross-reactivities and high affinities, as it were, high neutralizing abilities, with ET-1 related peptides.

**References**