Abstract Endothelin-1 is a potent vasoconstrictor produced by vascular endothelial cells. A recently cloned endothelin-1-selective receptor, the endothelin-A receptor, mediates the vasoconstrictive action of endothelin-1. Because endothelin-1 also possesses mitogenic properties, it may play a role in regulating the proliferation of intimal smooth muscle cells. In this study, we analyzed the expression of endothelin-A receptor gene in the thickened arterial intima of patients with hypertension. Internal mammary artery specimens obtained from 12 patients undergoing cardiovascular surgery were subjected to in situ hybridization using a digoxigenin-labeled cRNA probe. High, homogeneous signals of endothelin-A receptor mRNA were observed in the medial smooth muscle cells of all vessels examined but not in the endothelial cells. Patients with hypertension displayed more severe intimal thickening than those without hypertension. Immunohistochemical analysis suggested that almost all of the intimal proliferative cells originated from smooth muscle cells. In contrast to media, endothelin-A receptor mRNA signals in intimal smooth muscle cells were low and heterogeneous. In the thickened arterial intima of hypertensive patients, the signals were detected just beneath the luminal endothelium but not deep in the intimal smooth muscle cell layer. By contrast, staining with anti-α-smooth muscle actin antibody was more intense in the deep layer than in the subendothelium. These findings suggest that the modulation of endothelin-A receptor gene expression in smooth muscle cells differs between the intima and media. Its regulated expression in intimal smooth muscle cells might affect the proliferative activity of these cells in patients with hypertension. (Hypertension. 1994;23:288-293.)

Key Words • receptors, endothelin • muscle, smooth, vascular • hyperplasia • in situ hybridization

Endothelin (ET) was initially identified in porcine vascular endothelial cells as a potent vasoconstrictor peptide with 21 amino acid residues. Cloning and sequencing of ET genes revealed three isopeptides, ET-1, ET-2, and ET-3, that have subsequently been found in a wide variety of vascular and nonvascular tissues. The discovery of three isopeptides and their different biologic activities raised the possibility that there were multiple ET receptor subtypes. The expression cloning of two ET receptors, the ETA and ETB receptors, has recently been reported. Both receptors contain seven putative transmembrane domains, similar to other G protein–coupled receptors, but these two ET receptors are functionally distinct.

The rank order of binding to the ETA receptor is ET-1 > ET-2 > ET-3, whereas that for the ETB receptor is ET-1 = ET-2 = ET-3. Northern blotting has shown that ET receptor mRNAs are expressed in various organs and tissues including the aorta, heart, lungs, kidneys, and brain. However, the precise distribution of these receptors at the cellular level remains unclear.

Proliferation of smooth muscle cells (SMCs) is a key feature of several pathogenic processes, including atherosclerosis, the vessel wall response to hypertension, and restenosis after coronary angioplasty. Many growth factors, including platelet-derived growth factor, may play a role in regulating the proliferation of SMCs. Growing evidence suggests that in addition to its vasoconstrictor properties, ET-1 possesses mitogenic properties. It stimulates DNA synthesis in cultured vascular SMCs in a dose-dependent manner, and this effect is markedly inhibited by anti-endothelin antibodies. These data suggest that ET-1 plays a role in regulating the proliferation of intimal SMCs as well as in controlling vascular tone. Thus, in this study we analyzed the expression of the ETA receptor gene by means of in situ hybridization in the human vascular system, especially in the thickened arterial intima of patients with hypertension.

Methods

Patients and Tissue Preparation

A total of 39 specimens of internal mammary arteries were obtained from 12 patients undergoing aortocoronary bypass surgery. The characteristics of these patients are listed in the Table. All of these patients gave informed consent. The study was approved by the review committee of Kyoto University. Arterial pressure was measured during cardiac catheterization after medication had been stopped for 24 hours. Eight of these 12 patients had hypertension (blood pressure >140/90 mm Hg). The estimated duration of hypertension ranged from 2 to 15 years. Both hypertensive and normotensive patients received oral calcium antagonists and nitrates because of coronary artery disease and/or hypertension. Two hypertensive patients (patients 6 and 12 in the Table) required additional angiotensin-converting enzyme inhibitors and α-blockers for blood pressure control. Mean arterial pressure, defined as one third of pulse pressure plus diastolic pressure, was 110±6 mm Hg in the hypertensive group and 82±5 mm Hg in the normotensive group.
The normotensive group. Serum creatinine level was 1.1±0.2 mg/dL in the hypertensive group and 1.0±0.1 mg/dL in the normotensive group. There were no significant differences in age or sex distribution between patients with hypertension (59±7 years, male/female=6/2) and those without hypertension (58±2 years, male/female=2/2). Immediately after removal, the specimens were fixed for 2 hours at 4°C in 4% paraformaldehyde in 0.01 mol/L phosphate-buffered saline (PBS). After washing in PBS containing 30% sucrose for 4 hours, specimens were embedded in OCT compound (Miles Laboratories), frozen in dry ice-acetone, and stored at -70°C until use for in situ hybridization.

**Probe Preparation**

An 826-bp EcoRl-EcoRI fragment of human ETa receptor cDNA (nucleotides 256-1081) was subcloned into Bluescript (Stratagene Inc). Using this as a template, anti-sense and sense RNA probes labeled with digoxigenin. (2) Negative control studies were performed. (1) Negative control probe: sections were hybridized with the corresponding control studies. (2) Positive control: sections were hybridized with the corresponding control studies. The unbound conjugate was removed by washing in three changes of buffer 1 for 10 minutes each followed by one wash in buffer 1 for 10 minutes. Then they were postfixed in 4% paraformaldehyde in 0.01 mol/L phosphate-buffered saline (PBS). After washing in PBS containing 30% sucrose for 4 hours, sections were embedded in OCT compound (Miles Laboratories), frozen in dry ice-acetone, and stored at -70°C until use for in situ hybridization.

**In Situ Hybridization Histochemistry**

Sections (4 μm) were cut on a cryostat at -20°C and mounted on glass slides coated with poly-L-lysine (Sigma Chemical Co). After rinsing in PBS, the hydrated sections were placed sequentially in 0.2N HCl for 15 minutes and 3 μg/mL proteinase K (Sigma) in PBS for 15 minutes at 37°C. Then they were postfixed in 4% paraformaldehyde for 5 minutes and quenched twice with 2 mg/mL glycine in PBS for 15 minutes each. The slides were rinsed in PBS between each of these procedures, which were carried out at room temperature unless otherwise stated. After prehybridization for 2 hours at 37°C, 20 μL hybridization buffer containing 20 mg of the digoxigenin-labeled probe was applied to the slides. The hybridization buffer consisted of 3× SSC (pH 7.0), 2.5× Denhardt's solution, 50% deionized formamide, 0.5% sodium dodecyl sulfate, 50 μg/mL sheared salmon sperm DNA, and 10% dextran sulfate. After hybridization for 16 hours at 45°C, slides were washed for 1 hour in 2× SSC at room temperature with shaking. To eliminate nonhybridized strands of cRNA probe and thus decrease the background, sections were treated with RNase A (Sigma) at a final concentration of 20 μg/mL at 37°C for 30 minutes in a solution of 0.5 mol/L NaCl, 10 mmol/L Tris, pH 8.0, and 1 mmol/L EDTA. High-stringency washes then were performed in 0.1× SSC at 50°C for 40 minutes.

**Detection of Digoxigenin**

Before the detection of digoxigenin, sections were incubated for 30 minutes in buffer 1 (0.1 mol/L Tris-HCl, 0.15 mol/L NaCl, pH 7.5) containing 3% normal sheep serum. An alkaline phosphatase-conjugated Fab fragment of a sheep anti-digoxigenin polyclonal antibody (Boehringer Mannheim) was applied at the dilution of 1:500 for 2 hours at room temperature. The unbound conjugate was removed by washing in three changes of buffer 1 for 10 minutes each followed by one wash in buffer 2 (0.1 mol/L Tris-HCl, 0.1 mol/L NaCl, 0.05 mol/L MgCl₂·6H₂O, pH 9.5). The reaction was visualized using nitroblue tetrazolium dye as the substrate. Finally, the slides were rinsed in distilled water and counterstained with methyl green.

**In Situ Hybridization Controls**

To ensure the specificity of the in situ hybridization signals, several control studies were performed. (1) Negative control probe: sections were hybridized with the corresponding concentrations of sense cRNA probe labeled with digoxigenin. (2) RNase or DNase digestion: sections were incubated with RNase A (10 mg/mL) or DNase I (0.5 mg/mL) for 1 hour at 37°C before hybridization.

**Immunohistochemistry**

The cell types in the arterial intima were identified by immunohistochemistry using monoclonal antibodies. Endothelial cells were identified by staining with a monoclonal antibody to human factor VIII-related antigen, SMCs were stained with a monoclonal antibody against human α-smooth muscle-specific actin, and macrophages were identified with a monoclonal antibody to human macrophages (KP1). All of these monoclonal antibodies were purchased from Dako Laboratories. Frozen sections adjacent to those used for in situ hybridization were stained by the indirect immunoperoxidase method, as described previously. Controls included the omission of the primary antibody or the substitution of the primary antibody with nonimmune mouse sera.

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**Table: Characteristics of Normotensive and Hypertensive Patients**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age, y</th>
<th>Sex</th>
<th>SBP/DBP, mm Hg (Mean)</th>
<th>Duration of Hypertension, y</th>
<th>Duration of Antihypertensive Therapy, y</th>
<th>Plasma Cholesterol, mmol/L</th>
<th>History of Diabetes Mellitus</th>
<th>No. of Specimens With ETa Receptor mRNA</th>
<th>Intimal Thickness, %</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>59</td>
<td>M</td>
<td>121/67 (85)</td>
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<td>...</td>
<td>4.28</td>
<td>-</td>
<td>3</td>
<td>0</td>
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<tr>
<td>2</td>
<td>60</td>
<td>F</td>
<td>118/72 (87)</td>
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<td>...</td>
<td>4.18</td>
<td>-</td>
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<td>0</td>
</tr>
<tr>
<td>3</td>
<td>58</td>
<td>F</td>
<td>107/60 (76)</td>
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<td>...</td>
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<td>+</td>
<td>3</td>
<td>0</td>
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<tr>
<td>4</td>
<td>55</td>
<td>M</td>
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<td>...</td>
<td>6.28</td>
<td>-</td>
<td>3</td>
<td>0</td>
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<tr>
<td>5</td>
<td>63</td>
<td>M</td>
<td>148/98 (115)</td>
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<td>1</td>
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<tr>
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<td>65</td>
<td>M</td>
<td>196/78 (117)</td>
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<td>5</td>
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<td>6.01</td>
<td>-</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

SBP indicates systolic blood pressure; DBP, diastolic blood pressure; and ETa, endothelin-A.
Tissue Section Analysis

In situ hybridization staining was performed at the same time for all 39 sections and at least twice on serial sections in each specimen. The presence of the ETα receptor mRNA signals was assessed by light microscopy at ×200 magnification. The staining was judged to be positive when purple hybridization signals were definitely visible at this magnification. Two observers (T.I., S.O.) who were unaware of which group the sections belonged to reviewed the sections and determined whether ETα receptor mRNA was present in the arterial intima. Unanimity in the positivity was acquired among the serial sections for all specimens and between the two observers for all sections.

Quantification of Arterial Intimal Thickening

The luminal narrowing in the internal mammary artery was semiautomatically measured as previously described using an image analyzer (Olympus VIP-21C). The luminal area (L) and the intima within the internal elastic lamella (I) were calculated, and the percent area of luminal narrowing (%LN) was determined as follows: (%LN) = [(L-I)/L] × 100.

Statistical Analysis

Clinicopathological data are expressed as the mean±SD. Statistical comparisons were performed using χ² analysis, Student's t test, or one-way analysis of variance with multiple comparisons when appropriate. Statistical significance was designated at a probability value of less than .05.

Results

Purple hybridization signals of ETα receptor mRNA were observed in the medial SMCs of all the vessels examined (Figure, panels a through f). However, no hybridization signals were detected in the endothelial cells of any of the vessels (panels a through h). Panels g and h are magnifications of panels c and d, respectively. It is difficult to identify the endothelium with negative staining in hybridized sections. Therefore, to identify endothelial cells, we performed immunostainings with anti-factor VIII antigen antibody in the adjacent serial sections (panel i). The signals in medial SMCs were intense and homogeneous within each vessel. Using our method, there was no difference in the signal intensity of medial SMCs between normotensive and hypertensive patients.

In the internal mammary artery specimens, patients without hypertension displayed no or mild intimal thickening (12 specimens; %LN: 0% to 18%, 11±6%) (panel a). However, patients with hypertension displayed significant and severe intimal thickening (27 specimens; %LN: 3% to 52%, 24±13%) (panels b through h) compared with normotensive patients (P<.01). Panels b through f show intimal thickening of various severities taken from different hypertensive patients. The severity of intimal thickening varied among specimens as well as among patients. We could find no correlation between intimal thickness and duration of hypertension or therapy. No hybridization signals were detected in the intima of normotensive patients (panel a). Among 27 specimens from hypertensive patients, ETα receptor gene expression in the intima was observed in 14 (panels c through h) but not in the other 13 (panel b). Intimal thickening in hypertensive patients was more severe in specimens with ETα receptor gene expression (%LN: 12% to 52%, 33±12%) (panels c through h) than those without (%LN: 3% to 25%, 15±8%) (panel b, P<.01). ETα receptor mRNA signals were detected just beneath the luminal endothelium but not deep in the intimal SMC layer (panels c through h). However, the signals in the intima were lower than those in the media. Positive staining was not altered by digestion with DNase (panel i) but was abolished by digestion with RNAse before hybridization (panel j). Parallel in situ hybridization procedures with a sense cRNA probe were performed on all sections. None of the control sections hybridized with the corresponding concentrations of the sense cRNA probe labeled with digoxigenin showed positive staining (panel k).

Immunohistochemistry showed that the luminal surface of the vessels was fully covered by an intact monolayer of endothelial cells stained by anti-factor VIII–related antigen antibody (panel l). No platelet adhesion was noted. Staining with the macrophage antibody showed no positive signals in the intima (panel m). Lipid-containing foam cells or atheromatous plaques were not found. Almost all medial SMCs had high signals of α-smooth muscle actin. In sections adjacent to those used for in situ hybridization, almost all of the intimal proliferative cells were stained by the anti-α-smooth muscle actin antibody. The signals were high in the deep part but low in the subendothelium of the intimal cell layer (panels n through p). Immunohistochemical control sections incubated with nonimmune mouse serum showed no positive staining (panel q).

Discussion

Using the EcoRV–EcoRI fragment of ETα receptor cRNA as a probe for in situ hybridization, we demonstrated ETα receptor gene expression in the medial SMCs of human vessels but not in the endothelial cells. Previous Northern blotting studies using the same fragment showed that ETα receptor mRNA was expressed abundantly in human vessels but not in cultured human umbilical vein endothelial cells. Thus, our in situ hybridization data are compatible with those of the Northern blots. ET-1–induced vasoconstriction is blocked by a specific antagonist for ETα receptor. These findings suggest that ETα receptors on medial SMCs play a part in the vasoconstrictive action of ET-1. However, it recently became apparent that besides ETα receptors, ETβ receptors are also present in vascular SMCs and may significantly contribute to the proliferative and contractile response to ET-1. Thus, in ET-1–treated saphenous vein was not. These findings suggest that the saphenous vein was not. Thus, our in situ hybridization data are compatible with those of the Northern blots. ET-1–induced vasoconstriction is blocked by a specific antagonist for ETα receptor. These findings suggest that ETα receptors on medial SMCs play a part in the vasoconstrictive action of ET-1. However, it recently became apparent that besides ETα receptors, ETβ receptors are also present in vascular SMCs and may significantly contribute to the proliferative and contractile response to ET-1. Thus, in ET-1–treated patients with hypertension than in those without. Thus, intimal SMC proliferation may occur in the internal mammary arteries as a vessel wall response to hypertension. However, all of the patients studied here received aortocoronary bypass surgery, thus having a substantial amount of atherosclerosis in coronary
Photomicrographs show localization of endothelin (ET)\(_A\) receptor mRNA in human arteries. Internal mammary artery specimens obtained during cardiovascular surgery were analyzed by in situ hybridization using a digoxigenin-labeled cRNA probe. The specimen shown in panel a was taken from patient 2 in the Table; b, from patient 10; c, from patient 8; d, from patient 8; e, from patient 12; and f, from patient 6. Panels g and h are magnifications of c and d, respectively. ET\(_A\) receptor mRNA signals were observed in medial smooth muscle cells of all vessels examined but not in endothelial cells (a through h). Patients without hypertension showed no or mild intimal thickening (a); however, patients with hypertension displayed various degrees of intimal thickening (b through h). ET\(_A\) receptor mRNA signals were not detected in mildly thickened intima (b); however, in moderately or severely thickened intima (c through h), signals were detected just beneath the luminal endothelium but not deep in the layer. Positive staining was not altered by pretreatment with DNAse (i) but was abolished by digestion with RNase before hybridization (j). Control sections hybridized with corresponding concentrations of the sense cRNA probe labeled with digoxigenin showed no positive staining (k). An intact monolayer of luminal endothelial cells was immunostained by an antibody to factor VIII-related antigen (l). Anti-human macrophage antibody staining showed no positive signals (m). Almost all intimal cells were transmurally immunostained by the anti-\(\alpha\) smooth muscle actin antibody (n through p); however, subendothelial cells had reduced reactivity to this antibody. Note that panels d and n, e and o, and f and p are serial sections. Immunohistochemical control sections incubated with nonimmune mouse serum were not positively stained (q). Star indicates arterial intima; thick arrow, luminal endothelium; and thin arrow, internal elastic lamina. Original magnification, a through f and i through q: \(\times 200\); g and h: \(\times 400\).

arteries. One normotensive and three hypertensive pa-
tients had abnormal plasma levels of total cholesterol 
(more than 5.20 mmol/L). One normotensive and two 
hypertensive patients had diabetes mellitus. Oxidized 
low-density lipoprotein was reported to stimulate ET-1 
secretion in cultured endothelial cells, macrophages,
and intact blood vessels.\textsuperscript{10,31} Insulin stimulates the gene expression and secretion of ET-1 from bovine aortic endothelial cells.\textsuperscript{22} Therefore, we must consider that not only hypertension but also hypercholesterolemia or diabetes may be involved in the pathogenesis of the arterial intimal thickening observed in this study.

Although we identified ET\textsubscript{A} receptor mRNA signals in the thickened arterial intima of patients with hypertension, ET-1 production at the vessel wall remains unclear. Some studies have shown increased levels of ET-1 in patients with hypertension,\textsuperscript{33,34} whereas others have reported normal circulating levels of ET-1.\textsuperscript{35,36} Lerman et al.\textsuperscript{37} have demonstrated not only the elevation of ET-1 plasma levels in patients with atherosclerosis but also tissue ET-1-like immunoreactivity in atherosclerotic lesions. These findings suggest that local ET-1 plays a role in the progression of atherosclerosis. ET-1 may be released preferentially toward the abluminal side of the endothelium.\textsuperscript{38} Therefore, to clarify the role of ET-1 in the arterial intimal thickening in hypertension, studies on the local production of ET-1 will be required.

Among patients with hypertension, the ET\textsubscript{A} receptor gene was more preferentially expressed in moderately or severely thickened intima than mildly thickened intima. The receptor mRNA signals were detected just beneath the luminal endothelium but not deep in the intimal SMC layer. By contrast, the staining by anti-α-smooth muscle actin antibody was stronger in the deep layer than in the subendothelium. A similar staining gradient by anti-α-smooth muscle actin antibody has also been reported in intimal SMC proliferation after coronary angioplasty\textsuperscript{39} or at sites of primary atherosclerotic lesions.\textsuperscript{40} Gown et al.\textsuperscript{41,42} have previously found that the anti-α-smooth muscle actin antibody has a reduced reactivity with actively proliferating SMCs. Thymidine autoradiography has also shown that SMCs located near the luminal endothelial surface are more actively proliferating than the deeper SMCs in arterial intimal thickening.\textsuperscript{42} Therefore, the staining gradient observed in the thickened intima may mean that the ET\textsubscript{A} receptor gene was expressed in actively proliferating SMCs of the subendothelium but not in quiescent SMCs of the deeper region. These findings suggest that the regulated expression of ET\textsubscript{A} receptor gene affects the proliferative activity of intimal SMCs.

The role of the ET system in hypertension should be investigated not only in terms of receptor expression but also in terms of the vascular response to ET-1. In humans, the sensitivity of vascular SMCs to the effect of ET-1 decreases with advancing age.\textsuperscript{43} In animal models of hypertension, the vasoconstrictive response to ET-1 is still a matter of controversy. Some investigators reported both greater sensitivity and enhanced maximal response in isolated blood vessels of spontaneously hypertensive rats\textsuperscript{44,45} whereas others reported no difference between spontaneously hypertensive rats and Wistar-Kyoto rats in the sensitivity of the aorta to ET-1.\textsuperscript{46,47} In contrast, there is a paucity of information regarding the mitogenic potency of ET-1 in hypertension. It is reported that SMCs derived from spontaneously hypertensive rats proliferate more rapidly when compared with SMCs from normotensive Wistar-Kyoto rats.\textsuperscript{48,49} Our data suggest that the expression of the ET\textsubscript{A} receptor gene in the arterial intima may contribute to the enhanced proliferative activity exhibited by SMCs in hypertension. However, to clarify the pathophysiological importance of the ET system to arterial intimal thickening in hypertension, it is necessary to study the expression of ET\textsubscript{A} and ET\textsubscript{B} receptors and the proliferative response of SMCs to ET-1 in a large number of normotensive and hypertensive patients.

Modulation of ET receptor gene expression also may be involved in other pathological conditions. For example, ET-1 binding to cardiac membranes is reported to be increased by ischemia and reperfusion.\textsuperscript{50} Analysis of ET\textsubscript{A} receptor gene expression in hearts and vessels with various types of pathology should facilitate the elucidation of the pathophysiological significance of the ET system in human cardiovascular disease.

In summary, in situ hybridization data demonstrated the intense expression of the ET\textsubscript{A} receptor gene by medial SMCs of all vessels. However, the ET\textsubscript{A} receptor mRNA signals in intimal SMCs were low and heterogeneous. In the thickened arterial intima of patients with hypertension, the signals were detected in the subendothelium but not deep in the intimal SMC layer. By contrast, almost all of the intimal proliferative cells were stained by the anti-α-smooth muscle actin antibody, and the signals were more intense in the deep layer than in the subendothelium. These findings suggest that modulation of ET\textsubscript{A} receptor gene expression in SMCs differs between the intima and media. Its regulated expression in intimal SMCs might affect their proliferative activity in patients with hypertension.

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