Endothelin-1 and Its mRNA in the Wall Layers of Human Arteries Ex Vivo

Gian Paolo Rossi, MD; Stefania Colonna, MD; Edoardo Pavan, MD; Giovanna Albertin, BSc; Foscarina Della Rocca, MD; Gino Gerosa, MD; Dino Casarotto, MD; Saverio Sartore, BSc, PhD; Paolo Pauletto, MD; Achille C. Pessina, MD, PhD

Background—The participation of endothelin-1 (ET-1) in the control of vascular tone in humans has been questioned, on the basis of the finding of subthreshold immunoreactive (ir) ET-1 plasma levels. However, because most ET-1 is secreted abluminally, it might attain a higher concentration in the tunica media than in plasma. Furthermore, evidence indicates that vascular smooth muscle cells (VSMCs) can synthesize ET-1 on stimulation in vitro. We therefore looked for irET-1 in the different layers of the wall of human arteries, including renal, gastric, and internal thoracic artery wall, obtained ex vivo from consenting patients with coronary artery disease and/or high blood pressure undergoing surgery, as well as from young organ donors.

Methods and Results—We performed immunohistochemistry with specific anti–ET-1 and anti-vWF antibodies followed by detection with an avidin-biotin complex ultrasensitive kit. The presence of preproET-1 and human endothelin-converting enzyme-1 (hECE-1) mRNA was also investigated by reverse transcription–polymerase chain reaction in homogenates of vessel wall, including preparations deprived of both endothelium and adventitia, and in isolated VSMCs. We detected irET-1 in the endothelium of all arteries and in the tunica media of internal thoracic artery from most patients with coronary artery disease. PreproET-1 and hECE-1 mRNA was also detected in VSMCs isolated from these vessels. irET-1 and irvWF staining in endothelium and tunica media was measured by use of microscope-coupled computer-assisted technology. Significant correlations between the amount of irET-1 in the tunica media and mean blood pressure (P<0.05), total serum cholesterol (P<0.05), and number of atherosclerotic sites (P<0.001) were found. Thus, in organ donors, irET-1 was detectable almost exclusively in endothelial cells, whereas in patients with coronary artery disease and/or arterial hypertension, sizable amounts of irET-1 were detectable in the tunica media of different types of arteries. In addition, VSMCs isolated from these vessels coexpressed the preproET-1 and hECE-1 genes.

Conclusions—Collectively, these findings are consistent with the contention that endothelial damage occurs in most patients with atherosclerosis and/or hypertension and that ET-1 is synthesized in VSMCs of these patients. (Circulation. 1999;99:1147-1155.)

Key Words: arteries ■ endothelin ■ antibodies ■ RNA

Endothelins are a family of isopeptides with multiple biological actions that may be relevant for the regulation of vascular tone, blood pressure, and sodium and water homeostasis.1-3 Endothelin-1 (ET-1), originally isolated from the supernatant of porcine aortic endothelial cells, is the predominant isopeptide in the vascular endothelium.4 Because of its pleiotropic effects on the vasculature, it was hypothesized to intervene in the regulation of vascular tone under pathophysiological conditions and to play a role in the pathogenesis of hypertension, atherosclerosis, and related cardiovascular disease.4-7 However, this contention was challenged on the basis of the observation that although plasma levels of the peptide were increased in atherosclerotic patients and in hypertensives with cardiovascular disease,8-11 they were either only slightly increased or normal in most hypertensives.6,12 Furthermore, they are generally subthreshold, ie, lower than the 30 to 300 pmol/L that elicits a vasoconstrictor response in various smooth muscle (SM) preparations in vitro.5,7 In vitro studies with endothelial cell monolayers suggest that ≥80% of ET-1 would be secreted abluminally through the basolateral portion of endothelial cells toward the tunica media, where it could act in a paracrine fashion.13,14 but this polarization was not found in the coronary vascular bed of isolated rat hearts.15 In addition, it was reported that vascular smooth muscle cells (VSMCs) could express the preproET-1 gene in vitro under several experimental conditi...
tions,16 thereby raising the possibility that this might also occur in vivo. We therefore hypothesized that the peptide could be detected in the tunica media of human vessels in vivo, where it was synthesized locally.

Thus, our purposes were to investigate (1) the presence and distribution of immunoreactive (ir) ET-1 in the different layers of the wall of these vessels, (2) the expression of preproET-1 gene in the tunica media and in VSMCs of human arteries obtained ex vivo from young organ donors and from patients with coronary artery disease and/or high blood pressure, and (3) the clinical correlates of irET-1 in the arterial wall.

Methods

Selection of Subjects

Consecutive otherwise healthy organ donors (n = 5) and consenting patients undergoing coronary artery bypass graft surgery (n = 19; age, 42 to 77 years), unilateral nephrectomy for kidney cancer (n = 1), or surgery for lung (n = 2) and gastric (n = 1) cancer (age, 69 to 83 years) were investigated. The latter patients were selected so as to obtain different types of artery. Most arterial specimens were histologically normal, but 50% of those from coronary artery disease patients showed some intimal thickening.

In all patients, the presence and extension of atherosclerotic involvement was determined as described by Lerman et al. The study followed the local ethical guidelines for human investigations.

Preparation of Vascular Tissues

After excision, tissues were immediately frozen in isopentane, cooled at −70°C over dry ice in the operating room, and stored at −195°C in liquid nitrogen until they were used for analyses. Frozen 7- to 10-μm sections were cut with a cryostat (Leitz 1720 Digital) at −20°C and used for the immunohistochemistry studies. They were examined for localization of the mature ET-1 peptide and vWF as a marker of the endothelium.

Immunohistochemistry for ET-1

To visualize the distribution of irET-1 peptide, the following primary antibodies were used: rabbit anti–ET-1 antiserum (Peninsula Laboratories Inc) that shows a 7% cross-reactivity for ET-2 and ET-3 and antibodies were used: rabbit anti–ET-1 antiserum (Peninsula Laboratories Inc) that shows a 7% cross-reactivity for ET-2 and ET-3 and no cross-reactivity to nonrelated peptides. The reaction was stopped with tap water. The sections were mounted with polyvinyl alcohol (Sigma).

Quantification of irET-1

For each artery section, ≥6 high-quality images were examined with the microscope (magnification ×20) and acquired on a personal computer coupled to the microscope by use of specially developed software (Studio Casti Imaging) under identical conditions of light in black and white using a gray scale, white being set to a maximum brightness value of 250 and black to 0. On each image, measurements on endothelium, tunica media, and background, the latter being defined as a tissue-free portion of the image in the vessel lumen, were performed. The endothelium was identified by comparison of each section with an adjacent one stained for vWF, the tunica media being defined as the portion of wall between the internal and external elastic laminae. The area of interest was outlined with a cursor, and the average gray was measured. To exclude the confounding effect of different backgrounds, the background value of each section was subtracted from each reading. The average within-section coefficients of variation of the measurement of irET-1 in the endothelium and tunica media were 20% and 17%, respectively.

Immunofluorescence Studies

The monoclonal SM-E7 anti-SM myosin antibody, which binds selectively to SM-type myosin heavy chains (MHC) 1 and 2 (SM-1 and -2) expressed in VSMCs,20,21 was used. Cryosections 7 μm thick from arteries and veins were fixed in 1.5% p-formaldehyde, rinsed in PBS for 10 minutes at room temperature, and then incubated at 37°C for 30 minutes with the appropriate dilution of SM-E7 (IgG, 2.5 μg/mL) antibody. After being rinsed with PBS, the sections were treated with a rabbit-anti-mouse IgG coupled with tetramethylrhodamine isothiocyanate (Dako) and mounted in polyvinyl alcohol. The specimens were observed with an Olympus BX 50 fluorescence microscope.

SM Cell Cultures

Arteries collected in the operating room were immediately placed in sterile Ringer’s solution with penicillin and streptomycin at 4°C, cleaned of fat and connective tissue, and cut under a stereoscopic microscope. The intima was scraped away and the adventitia stripped away from the media in DMEM. The media was chopped fine and stored away from the media in DMEM. The media was chopped fine and stored away from the media in DMEM. The media was chopped fine and stored away from the media in DMEM. The media was chopped fine and stored away from the media in DMEM.
trypsinized and seeded into 25-cm² Petri dishes. Subconfluent cell cultures at the first passage were used after being growth-arrested. Viability of cells was evaluated by dye exclusion test with trypan blue. An aliquot of acetone-fixed cells was used for immunofluorescence studies, and another aliquot of 250,000 cells was processed for RNA extraction with RNAzol B (Duotech).

## Gene Expression Studies

Samples of internal thoracic artery (ITA) (n=6) maintained on ice were carefully deprived of both endothelium and adventitia under a microscope. Reverse transcription–polymerase chain reaction (RT-PCR) was performed from total RNA isolated by the guanidinium isothiocyanate method by use of specific primers as reported. Amplification was carried out for 38 cycles at 94°C for 1 minute; 54°C and 60°C, as annealing temperature, for preproET-1 and human ECE-1 (hECE-1), respectively, for 1 minute; and extension at 72°C for 1 minute. To rule out the possibility of amplifying genomic DNA, in some experiments the PCR was carried out with no prior RT of the RNA. Detection of the PCR amplification products was carried out by size-fractionation on 2% agarose gel electrophoresis and ethidium bromide staining.

## Statistical Analysis

Results are expressed as mean±SD or SEM. Comparisons were carried out by 1-way ANOVA followed by Bonferroni’s test or with Kruskal-Wallis test for variables not normally distributed. To investigate the relationship between demographic features, mean blood pressure values, number of atherosclerotic sites, and irET-1, a stepwise forward regression analysis (inclusion cutoff value of 0.05) was used. Pearson coefficient was also estimated to assess the relationship of individual variables; a value of P<0.05 was considered statistically significant. For analyses, we used the SPSS for Windows statistical package (version 8.0, SPSS Inc).

## Results

### Clinical Features of the Subjects

The demographic and clinical features of the subjects studied are summarized in Table 1. In 16 cases (67%), the indication for surgery was coronary artery disease, and in 4 (17%) it was cancer. Arterial hypertension was present in 58%, diabetes mellitus in 21%, and hypercholesterolemia in 12% of the subjects.

### Immunohistochemistry

Staining of the endothelium for irET-1 was seen in all types of arteries, including hypogastric, mesenteric (Figure 1), ITA, and femoral artery. However, in all arteries from patients with coronary artery or other diseases, irET-1 was detected not only in the endothelium (Figures 2 and 3, arrows) but also in...
the tunica media. The staining of the latter was homogeneous and involved almost exclusively the muscle bundles, as confirmed by further experiments carried out with combined immunocytochemistry for ET-1 and immunofluorescence analysis for VSMC-specific antibodies (see below). Similar results were obtained with the monoclonal anti–ET-1 antibody (not shown). No staining was observed in any negative controls carried out. The correlation matrix and a regression

Figure 1. Sections of superior mesenteric artery obtained from a 12-year-old organ donor. A, Exclusive staining of endothelium with anti–ET-1 antibody (magnification ×30). Details of staining of endothelial cells with anti–ET-1 antibody are shown at higher magnification in B (×75) and C (×300). Negative controls on serial cryosections incubated with preimmune rabbit antiserum, shown in D (magnification ×300) and E (magnification ×75), demonstrate no staining. Preservation of endothelium is demonstrated on adjacent cryosections by immunostaining with an anti-vWF antibody in F (magnification ×300) and G (magnification ×75). iel indicates internal elastic lamina.

Figure 2. Examples of immunohistochemistry of cryostatic sections of human arteries incubated with a specific rabbit anti–ET-1 antibody (A and C); adjacent sections were incubated with preimmune rabbit IgG (B and D) as negative controls. Substrate for peroxidase reaction was 3-amino-9-ethylcarbazole; therefore, positive cells are stained in red. Staining of endothelium (arrows), tunica media (m, *) and adventitia (a) is evident. A and B, Renal artery sections from a 68-year-old man with arterial hypertension and coronary artery disease; C and D, gastric artery sections from a 74-year-old man with arterial hypertension and left ventricular hypertrophy. Magnification ×150.
analysis carried out with irET-1 in the tunica media as dependent variable (Table 2) revealed significant direct relationships between irET-1 in the media and mean blood pressure levels, total serum cholesterol, irET-1, and irvWF in the endothelium; an inverse relationship between irET-1 in the media and number of sites involved by atherosclerosis was also found.

**Immunofluorescence**

As expected, all medial cells of cryosections were recognized by the SM-E7. Almost all intimal cells also reacted to this antibody (Figure 3D), indicating that the thin intimal layer was composed primarily of VSMCs. A few cells in the adventitia were stained with this antibody, and in some cases, the anti–ET-1 antibody (not shown) also recognized them. At the immunofluorescence analysis, cultured cells were all positive to both the anti–α-actin and anti–SM-MHC antibodies, and with both antibodies, the immunofluorescence pattern typical of VSMCs was evident (not shown).

Double immunofluorescence experiments with the monoclonal anti–ET-1 antibody and the anti–hECE-1 antisera VP-16 showed the colocalization of irET-1 and hECE-1 in VSMCs isolated from human aorta (Figure 4). No immunofluorescence was seen in the control experiments (not shown).

**Table 2. Correlation Matrix of Immunoreactive ET-1 of the Tunica Media With the Other Variables**

<table>
<thead>
<tr>
<th></th>
<th>irET1med</th>
<th>Age</th>
<th>EF</th>
<th>MBP</th>
<th>Cholesterol</th>
<th>No. of Atherosclerotic Sites</th>
<th>IrvWFend</th>
<th>irET1end</th>
</tr>
</thead>
<tbody>
<tr>
<td>irET1med</td>
<td>1.000</td>
<td>0.080</td>
<td>-0.013</td>
<td>0.223*</td>
<td>-0.387‡</td>
<td>-0.387‡</td>
<td>0.239*</td>
<td>0.669‡</td>
</tr>
<tr>
<td>Age</td>
<td>1.000</td>
<td>-0.557‡</td>
<td>-0.038</td>
<td>0.388‡</td>
<td>-0.029</td>
<td>-0.090</td>
<td>-0.101</td>
<td></td>
</tr>
<tr>
<td>EF</td>
<td>1.000</td>
<td>-0.307†</td>
<td>-0.486‡</td>
<td>-0.187</td>
<td>0.134</td>
<td>0.191</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MBP</td>
<td>1.000</td>
<td>0.383‡</td>
<td>-0.056</td>
<td>-0.087</td>
<td>0.029</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol</td>
<td>1.000</td>
<td>-0.175</td>
<td>0.346‡</td>
<td>0.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of atherosclerotic sites</td>
<td>1.000</td>
<td>-0.100</td>
<td>-0.008</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IrvWFend</td>
<td>1.000</td>
<td>0.122</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>irET1end</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

irET1med indicates irET-1 in the tunica media; EF, ejection fraction; MBP, mean blood pressure; irvWFend, irWF in the endothelium; and irET1end, irET-1 in the endothelium.

*P<0.05, †P<0.01, ‡P<0.001.
Gene Expression
The RT-PCR allowed detection of the preproET-1 and hECE-1 mRNA in all vessel specimens examined (Figure 5). Amplified cDNA fragments of the expected size for both the preproET-1 (314 bp) and hECE-1 (567 bp) and for the control genes were easily detected in samples of the normal arterial wall (Figure 5, top) and in preparations in which both endothelium and adventitia were carefully removed (Figure 5, top). No amplification was seen in the control PCR containing either no cDNA (water) or total RNA without any prior RT, thereby ruling out the possibility of false-positive results and of amplification of genomic DNA, respectively. Hybridization with capture probes specific for each cDNA investigated confirmed the specificity of the amplification products for each mRNA (not shown). Of interest, similar results for both the preproET-1 and the hECE-1 in VSMCs isolated from ITA were found (Figure 5, bottom).

Discussion
Our immunohistochemistry results show that ET-1 is found mainly on endothelium of different types of arteries from young, healthy normotensive individuals (Figure 1). However, they also show that irET-1 is detectable in the tunica media, and more precisely on VSMCs, of human arteries obtained ex vivo from patients with coronary artery disease and/or hypertension (Figures 2 and 3). This finding is in keeping with earlier reports on atherosclerotic coronary arteries, transplant coronary arteries, atherosclerotic plaques, VSMCs isolated from atherosclerotic coronary arteries, or angioplasty-induced neointima formation. Several lines of evidence support the contention that irET-1 is localized in VSMCs. First, the distribution of the immunostaining within the tunica media of these arteries is consistent with a predominant expression of irET-1 on SM bundles, ie, on VSMCs (Figures 2 and 3), and it is well established that VSMCs are the only cell type present in the normal tunica media. Second, the irET-1–stained cells were conclusively...
identified as VSMCs by use of SM-E7, an antibody that recognizes only VSMCs, regardless of their differentiative state, and therefore stained all cells of the tunica media (Figure 3). Rare VSMCs expressing ET-1 were also recognized by the anti-myosin antibody in the adventitia close to the external elastic lamina (Figure 3), but a few adventitial cells expressing irET-1 (not shown) were not, thereby suggesting that adventitial myofibroblasts can also express ET-1. Thus, collectively, our ex vivo findings accord well not only with the in vitro demonstration of a predominant abluminal secretion of ET-1 but also with the contention that in patients with coronary artery disease and/or hypertension, the peptide is found in the tunica media, at variance with findings in otherwise healthy young organ donors, in whom the peptide was detectable primarily in the endothelium (Figure 1). Because both ET_{A} and ET_{B} receptors were found on VSMCs of the media, it might be that the irET-1 detected in this layer is the endothelium-derived peptide bound to its receptors. However, the secretion of ET-1 is constitutive and the half-life of secreted ET-1 in vivo is short (between 1.5 and 7 minutes), because it is quickly inactivated in vascular cells, and the intracellular half-life of ET-1 mRNA is only \( \approx 15 \) minutes. Thus, we propose that ET-1 is synthesized in the tunica media, a hypothesis further suggested by 2 sets of evidence: (1) the demonstration that ET-1 mRNA can be induced in vitro in human VSMCs by growth factors and vasoactive peptides and (2) the identification of consensus sequences for a number of regulators, including shear stress, the acute-phase reactant regulatory elements, transforming growth factor-\( \beta \), insulin, thrombin, epinephrine, interleukin-1, and angiotensin II, in the 5' flanking region of the preproET-1 gene and of the hECE-1 gene. Therefore, to investigate this question further, we sought the expression of the preproET-1 and hECE-1 genes in homogenates of the vessel wall of ITA. We found that both genes are expressed not only in homogenates of the wall of these vessels but also, although less intensely, in preparations of ITA that were carefully denuded in vitro of both the endothelium and the adventitia (Figure 5, top). Because endothelial cells on capillaries of the media might account for these results, to prove that the preproET-1 and ECE-1 mRNA come from VSMCs, we isolated cells from ITA media and could show that cultured VSMCs coexpress the preproET-1 and hECE-1 mRNAs (Figure 5, bottom) at an early passage in culture. Thus, although we cannot rule out the possibility that the preproET-1 gene is activated once cells are placed in culture, our present findings, as well as the demonstration that irET-1 is released by cultured human VSMCs, suggest that ET-1 can be made by VSMCs of the tunica media in vivo. This is further supported by the demonstration of a colocalization of both immunoreactive ET-1 and hECE-1 in VSMCs isolated and cultured from the human aorta (Figure 4).

Our arteries were obtained from a population with a heterogeneous cardiovascular risk profile. Most were from patients in their fifth decade and older who underwent major surgical procedures for coronary artery disease or cancer, and some were from younger organ donors. Thus, to gain insight into the mechanisms linked to ET-1 biosynthesis in the arterial wall layers, we carried out a regression analysis of the number of sites involved by atherosclerosis and immunostaining for vWF (top) and ET-1 (bottom) in endothelium of human arteries. (Table 2), which unveiled several interesting significant relationships. First, a direct correlation between irvWF and irET-1 in the endothelium was found, and an inverse relationship between irvWF in the endothelium and the number of sites involved by atherosclerosis was seen (Figure 6). This finding indicates that more extensive atherosclerotic involvement is associated with progressive loss of endothelial expression of irvWF. This inverse relationship, although statistically significant (Table 2), was less evident when irET-1 in the endothelium was taken into consideration, suggesting that the loss of irvWF, ie, of endothelial integrity, is not closely paralleled by a decrease of ET-1 synthesis. We also noticed a significant correlation between irET-1 in the media was directly related (\( P<0.05 \)) to the level of mean blood pressure and total serum cholesterol, 2 well-known cardiovascular risk factors, whereas irET-1 in the endothelium was not. We therefore suggest that although endothelial irET-1 is subjected to short-term regulation and/or to loss of endothelial lining integrity, the ET-1 in the media constitutes a more stable pool. A contribution of aging cannot be conclusively ruled out at this stage, but our analysis did not provide support to the contention of a relationship of irET-1 with age either in the endothelium or in the tunica media. The fact that irET-1 in the media was inversely related to the...
extension of atherosclerosis, ie, to the number of atherosclerotic sites, is intriguing in view of the previous finding of a direct relationship between plasma levels of irET-1 and sites of atherosclerosis. However, it may indicate either a more important role of ET-1 in the initiation rather than in advanced stages of atherosclerosis and/or an increased turnover of ET-1 at the vascular wall leading to increased plasma levels of the peptide.

Our findings, along with the detection of preproET-1 and ECE-1 mRNA in the tunica media and in isolated VSMCs, suggest that ET-1 synthesis can be activated in vivo in the VSMCs of ITA and other types of arteries from patients with and without coronary artery disease and/or hypertension. Nonetheless, they speak against an exclusive activation of ET-1 biosynthesis in endothelial cells under most pathophysiological conditions, a conclusion that accords well with the results of other investigators.

It has recently been shown that blockade of both endothelin receptors with the nonselective agent TAK-044 and inhibition of hECE-1 with an intravenous infusion of phosphoramidon cause peripheral vasodilatation in humans. It was therefore proposed that in vivo generation of ET-1 plays a pivotal role in the maintenance of vascular tone. By showing an ET-1 biosynthesis in endothelial cells under most pathophysiological conditions, a conclusion that accords well with the results of other investigators, but not all investigators.

Acknowledgments
We gratefully acknowledge the valuable collaboration of all our colleagues of the Departments of Cardiac Surgery (directed by Prof Dino Casarotto) and of Drs Umberto Tedeschi, Umberto Cillo, Giacomo Zanus, and Enrico Gringeri of the P.G. Cevese Institute of Clinical Surgery 1 (directed by Prof Davide D’Amico) of the University of Padua.

References


Endothelin-1 and Its mRNA in the Wall Layers of Human Arteries Ex Vivo
Gian Paolo Rossi, Stefania Colonna, Edoardo Pavan, Giovanna Albertin, Foscarina Della Rocca, Gino Gerosa, Dino Casarotto, Saverio Sartore, Paolo Pauletto and Achille C. Pessina

Circulation. 1999;99:1147-1155
doi: 10.1161/01.CIR.99.9.1147

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1999 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/99/9/1147

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org/subscriptions/