Role of Endogenous Endothelin in the Development of Graft Arteriosclerosis in Rat Cardiac Allografts

Antiproliferative Effects of Bosentan, a Nonselective Endothelin Receptor Antagonist

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Background—The purpose of this study was to determine whether endothelin-1 (ET-1) contributes to the development of graft arteriosclerosis and whether the orally active nonpeptide endothelin receptor antagonist bosentan, which blocks both ET_A and ET_B receptors, can protect against this pathologic damage.

Methods and Results—Recipient male Lewis rats were divided into three groups; group 1 received heterotopic heart transplantations from Lewis donors and groups 2 and 3 received transplantations from Brown-Norway donors; group 3 recipients also received bosentan orally at the dose of 20 mg/kg per day for 120 days. All recipients were given cyclosporine and were euthanized at examination 120 days after transplantation. Plasma ET-1 levels were significantly higher in group 2 than in group 1 (6.99±0.91 and 4.15±0.83 pg/mL, respectively). Strong ET-1 immunoreactivity was seen in both the thickened neointima and the media of the coronary arteries in group 2 but not in group 1. The mean ratio of the coronary luminal area to the total vascular area in group 2 (19.0±11.7%) was significantly lower than that in group 1 (34.2±9.9%) and was significantly increased in group 3 (33.2±9.2%).

Conclusions—These results show that local upregulation of ET-1, mainly in the thickened neointima and the media of the coronary arteries, may play an important role in the pathogenesis of graft arteriosclerosis by stimulating ET_A receptors, ET_B receptors, or both. Orally active bosentan might be a useful agent for the clinical prevention of graft arteriosclerosis. (Circulation. 1998;97:2346-2351.)

Key Words: endothelin • receptors • coronary disease • arteriosclerosis • transplantation

Endothelin-1, in addition to its vasoconstrictor effects,1 is known to act as a strong mitogen.2–4 In vitro studies have demonstrated that ET-1 induces the expression and release of several protooncogenes that can promote smooth muscle cell proliferation.5,6 Furthermore, the ET_A receptor subtype—selective antagonist BQ-123 prevents ET-1—induced mitogenesis in rat smooth muscle cells.7 These results are supported by those of an in vivo study in which ET-1 promoted neointimal formation after rat carotid artery balloon angioplasty.8 However, the role of endothelin in the development of graft arteriosclerosis has not been elucidated.

Plasma ET-1 levels have been shown to be elevated after solid organ transplantation,11,12 and ET-1 immunostaining has also been observed at sites of occlusive and subocclusive intimal proliferation in both experimental heart transplantation models13,14 and human graft coronary artery disease,15 which suggests an important role of ET-1 in the development of graft coronary artery disease. However, it remains unclear whether ET-1 expression actually promotes the disease or is merely a result of increased ET synthesis induced by various cytokines or other growth factors.

Endothelin receptor antagonists are crucial in the unraveling of the physiologic role of ET-1 in the development of graft arteriosclerosis. Endothelin receptor antagonists that block both ET_A and ET_B receptors have been recently developed.16,17 One of these, bosentan (Roche Co, Ltd), an orally active nonpeptide endothelin antagonist (Ro 47 to 0203, 4-tert-butyl-N-[6-(2-hydroxyl-ethoxy)-5-(2-methoxy-phenoxy)-2,2′-bipyridimin-4-y]benzenesulfonamide), exhibits affinity for both ET_A and ET_B receptors (K_i 4.7 and 95 nmol/L, respectively) and competitively inhibits the receptors. Clozel et al17 have reported that a single oral dose of 100 mg/kg body wt of bosentan blocks the action of pressor doses of intravenously injected big ET-1 for more than 24 hours. The present study was designed to use bosentan to assess the contribution of endogenous ET-1 to the pathogenesis of graft arteriosclerosis.
Methods

Animals

Adult male Lewis rats (LEW:RT1<sup>+</sup>) and Brown-Norway rats (BN:RT1<sup>+</sup>), weighing 200 to 250 g, were purchased from Charles River Japan, Inc (Yokohama, Japan), and were housed under conventional conditions and fed a standard diet, following the Principles of Laboratory Animal Care, formulated by the Institute of Laboratory Animal Resources, and the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health.

Operation

Rats were anesthetized with ether, and heterotopic heart transplantation was performed by the modified technique of Ono-Lindsey. The ischemic time was no more than 30 minutes, and there was no significant difference between groups. An external gastrostomy tube was inserted into the stomach and exteriorized through the back of the neck. Cardiac graft survival was determined by daily abdominal palpation.

Experimental Design

Three groups of recipient LEW rats were studied. Those in group 1 (n=7) received heterotopic heart transplantsations from LEW donors to assess the contribution of surgical manipulation and cyclosporine administration; those in groups 2 (n=7) and 3 (n=7) received heterotopic heart transplants from Brown-Norway donors; in addition, group 3 recipients received oral bosentan (20 mg/kg per day) through the gastrostomy tube for 120 consecutive days after transplantation. This oral dose has been demonstrated to block the action of pressor doses of intravenously injected big ET-1. The blocking effect of the oral dose (20 mg/kg per day) on exogenously administered ET-1 (1 nmol/kg) was examined in a separate series of experiments described below, which showed that this dose produced significant block of both ET<sub>1</sub> (P=0.0321) and ET<sub>2</sub> (P=0.011) receptors.

Immunosuppression

From the day of transplantation, all recipients were treated intramuscularly three times per week with 5 mg/kg of cyclosporine.

Euthanasia

After 120 days, all rats were euthanized by deep pentobarbital anesthesia. The donor and recipient hearts and the recipient’s lung, liver, and kidneys were removed and frozen in OCT compound (Miles Scientific) in cryomold in liquid nitrogen for histopathologic and immunohistochemical examination.

Measurement of Plasma ET-1

At the time of euthanasia, a PE50 tube, attached to a syringe, was inserted through the right carotid artery, and 6 mL of blood was collected to measure plasma ET-1 levels. Each blood sample was immediately placed in a chilled tube containing aprotinin (300 kallikrein inhibiting units/mL) and EDTA (2 mg/mL). After centrifugation, the plasma was decanted and stored at −20°C until analysis. After extraction of ET-1 on C18 Sep-Pac cartridges (Waters Associates), the concentration was measured by radioimmunoassay with an anti–ET-1 antibody (Peninsula Laboratory Inc) and <sup>125</sup>I-labeled ET-1 (Amersham Japan Co). The level of cross-reactivity with ET-2, ET-3, or big ET-1 is very low (<0.1%).

Immunohistochemistry

Frozen sections, 5 μm thick, were cut from the frozen recipient and transplanted donor hearts and recipient lung, liver, and kidney and immersion-fixed in aceton for 10 minutes. Mouse monoclonal anti–ET-1 antibody (American Research Products Inc) or anti–smooth muscle–specific α-actin antibody (1A4 DAKO), diluted 1:10 and 1:50, respectively, were used as primary antibody, with binding detected by biotin-labeled anti-mouse immunoglobulins, followed by an avidin-biotin-peroxidase complex (Vectastain ABC kit, Vector). The bound peroxidase was reacted with the substrate 3,3′-diaminobenzidine tetrahydrochloride and NiSO<sub>4</sub>·6H<sub>2</sub>O in Tris (hydroxyl methyl) aminomethane · HCl buffer (pH 7.6) and the sections dehydrated and covered with a coverslip. In the negative controls, prepared identically except for the primary antibody, no immunoreactivity was seen.

Histopathology and Grading of Graft Coronary Disease

The frozen transplantation hearts, in OCT compound, were sliced into 5-μm sections, which were subjected to EVG staining. The Billingham classification was used to evaluate graft vessel disease, with grading by histologic appearance on a scale of 0 to 4, with grade 0 being an unaffected vessel, grade 1 accumulation of inflammatory cells, grade 2 more advanced changes, including definite intimal proliferation and thickening but <50% occlusion of the lumen, grade 3 high-grade occlusion of the vessel, with >50% occlusion of the lumen, and grade 4 100% occlusion of the lumen. The percentage of diseased vessels (number of diseased vessels ×[grade 1]/number of vessels investigated) was calculated for each group. Between 176 to 211 arteries from each group were scored.

We also analyzed between 76 to 94 coronary artery sections from each group to measure luminal, intimal, and medial cross-sectional areas, using an image analysis computer system (Adobe Photoshop 3.0 J, NIH Image 1.61/pc), then calculated the ratio of the luminal area to the total vascular area (percent luminal area, percent lumen) according to Tanaka et al. In brief, the percent lumen was determined as the luminal area/(medial + intimal + luminal) areas. An external caliper of >50 μm was used for the calculation of the percent lumen because it was difficult to trace in small arteries. As a result, between 76 to 94 arteries from each group were scored.

Blocking Effect of Orally Administered Bosentan on Exogenously Administered ET-1

In a separate series of experiments, the blocking effect of orally administered bosentan on exogenously administered ET-1 was tested. Fourteen LEW rats were anesthetized with ether, and an external gastrostomy tube was inserted into the stomach and exteriorized through the back of the neck. The rats were divided into two groups, with those in the bosentan-treated group (n=7) receiving 20 mg/kg per day of bosentan (4 mg/mL in water) and those in the vehicle-treated group (n=7) an equivalent volume of water for 14 days through the external gastrostomy tube. On day 13, the rats were again anesthetized with ether, and the right femoral artery and vein were cannulated with polyethylene tubes (PE10, connected to PE50). On the following day, the MAP (mm Hg) and HR (bpm) were measured with the rats in the conscious state and recorded with the use of a polygraph system (Nihon Koden) and MacLab data acquisition system (model 8s, AD Instruments Inc). ET-1 (Peptide Institute, 2.5 μg/kg =1 nmol/kg, 100 μL) was then injected through the venous catheter, and the hemodynamic parameters (MAP and HR) were monitored and recorded over the next 40 minutes.

Statistics

The data were expressed as mean±SD. All statistical comparisons were performed with a commercially available statistical package for the Macintosh personal computer (STAT VIEW-J 4.11, Abacus Concepts). Differences between plasma ET-1 concentrations in groups 1 and 2 and between the hemodynamic parameters after intravenous injection of ET-1 in the vehicle- and bosentan-treated groups were assessed by the unpaired t test. A one-way factorial
ANOVA, followed by Scheffe’s multiple comparison test, was used to compare the mean percent diseased vessels and mean percent lumen in the three groups. Differences were considered significant at the level of $P < 0.05$.

Results

Plasma Concentration of ET-1

ET-1 levels in both the syngeneic and allogeneic graft recipients were higher than normal control levels ($< 2.30$ pg/mL), being $4.15 \pm 0.83$ and $6.99 \pm 0.91$ pg/mL in groups 1 and 2, respectively. The levels in the allograft recipients (group 2) were significantly higher than those in syngeneic grafts recipients ($\times 1.7$, $P < 0.05$).

Immunohistochemical Staining for ET-1 and $\alpha$-Actin

EVG staining and immunohistochemical staining of ET-1 and $\alpha$-actin were performed on three serial sections of the hearts. In group 2 (allograft recipients), discrete ET-1 cellular immunoreactivity was seen in both the thickened neointima and the media of the coronary arteries (Figure 1A to F) and slight staining of ET-1 was seen in parenchyma (Figure 1F). No ET-1 immunoreactivity was seen in the coronary arteries of the transplanted heart in group 1 (syngeneic graft recipients; data not shown) or in those of the native heart in group 2 (G). Chronic administration of bosentan (20 mg/kg per day) for 120 days ameliorates graft arteriosclerosis. H, EVG findings graded 1 in group 3, accompanied with only a little accumulation of inflammatory cells. Scale bars, 50 $\mu$m.

Figure 1. EVG and immunohistochemical staining of $\alpha$-actin and ET-1. A (EVG), B ($\alpha$-actin staining), and C (ET-1 staining), Serial sections from group 2 in the Billingham classification grade 2. A and B, Coronary arterial lesions with definite intimal thickening that stained positive for $\alpha$-smooth muscle actin; C, Discrete ET-1 cellular immunoreactivity in both the thickened neointima and the media. D (EVG), E ($\alpha$-actin staining), and F (ET-1 staining), Serial sections from group 2 in the Billingham classification grade 3. D and E, Coronary arterial lesions with high-grade occlusive and a proliferative cellular component staining positive for $\alpha$-smooth muscle actin. This coronary artery showed severe intimal thickening and stretched internal and partially destroyed elastic laminae (arrow). F, ET-1–positive immunoreactivity in both the thickened neointima and the media. No ET-1 immunoreactivity was seen in the coronary arteries of the native heart in group 2 (G). Chronic administration of bosentan (20 mg/kg per day) for 120 days ameliorates graft arteriosclerosis. H, EVG findings graded 1 in group 3, accompanied with only a little accumulation of inflammatory cells. Scale bars, 50 $\mu$m.

EVG Staining and Evaluation of Graft Coronary Arteriosclerosis

The Table shows the morphometric analysis of coronary artery disease in the three groups. EVG staining in group 1 (syngeneic grafts) showed the coronary arteries to be almost normal, with, at most, cellular accumulation within Billingham classification grade 1 and only occasionally grade 2. Staining in group 2 (allografts) demonstrated the presence of all grades (0 to 4) of diseased coronary arteries, many...
showing severe intimal thickening, with stretched internal and partially destroyed elastic laminae (Figure 1A and ID). In contrast, the grading in group 3 was less severe (0 to 2) and the luminal area greater (Figure 1H). In group 2, the percentage of diseased vessels was significantly increased and the mean percent lumen significantly decreased compared with group 1. Again, these morphometric parameters were significantly improved in group 3. These results strongly suggest that ET-1 may contribute to the progressive graft coronary artery disease by stimulating ETA and/or ETB receptors.

Blockading Effect of Bosentan on Exogenous ET-1
In a separate series of experiments, the blockading effect of orally administered bosentan (20 mg/kg per day) or vehicle for 14 days on exogenously administered ET-1 was tested. An intravenous bolus injection of ET-1 (1 nmol/kg) induced a biphasic MAP response, consisting of a transient fall (ETB receptor stimulation), followed by a sustained increase (ETA receptor stimulation) (Figure 2A). According to the arterial baroreflex, transient tachycardia was followed by sustained bradycardia (Figure 2B). In the bosentan-treated group, the Δ decrease (minimum preinjection; mm Hg) in the MAP after ET-1 injection was significantly attenuated compared with that in the vehicle-treated group (−25.1 ± 8.1 and −36.0 ± 3.4, respectively; \( P = .011 \)), as was the Δ increase (maximum preinjection; mm Hg) (37.5 ± 11.2 and 52.0 ± 5.5, respectively; \( P = .0321 \)). These data show that 20 mg/kg per day of bosentan has a blockading effect on both ETA and ETB receptors stimulated by ET-1.

Discussion
The major findings of the present study are as follows: (1) 120 days after transplantation, plasma ET-1 levels were slightly but significantly increased and ET-1 production markedly increased in both the thickened neointima and the media of the coronary arteries in heterotopic rat cardiac allografts compared with syngeneic grafts. (2) Oral administration of a nonselective endothelin antagonist, bosentan (20 mg/kg per day for 120 days), resulted in suppression of graft arteriosclerosis development. These results strongly suggest that endogenous ET-1 contributes to the development of graft coronary arteriosclerosis through the ET receptor–mediated signal transduction system in rat cardiac allografts and that oral administration of bosentan might be a useful means of preventing graft arteriosclerosis.

In the current study, after 120 days, the plasma ET-1 concentration in allograft recipients was slightly but significantly higher than that in syngeneic graft recipients. A sustained increase in plasma ET-1 levels has also been seen in organ transplantation patients. However, the mechanism involved in the increase in plasma ET-1 levels is not completely understood. Various factors can induce endothelial activation, which stimulates the release of ET-1 by endothelial cells. Russell et al demonstrated a marked

![Figure 2. Effect of chronic administration of bosentan on changes in MAP (A) and HR (B) induced by exogenous ET-1 in conscious rats. Arrows indicate intravenous injection of ET-1 (1 nmol/kg). Values are mean ± SD for seven animals.](http://circ.ahajournals.org/content/109/11/2398/F2.large.jpg)
increase in the expression of interferon-γ and tumor necrosis factor-α in the LEW to F344 rat cardiac allograft undergoing chronic rejection. In vivo, those cytokines are known to induce ET-1 production in a variety of cells. Thus an immunologic response–based mechanism might be one explanation for this sustained increase in plasma ET-1 levels. In vitro, cyclosporine is known to induce endothelial cell injury, resulting in cell lysis and detachment and increased endothelin secretion. However, some groups have reported that cyclosporine has no effect on plasma ET-1 levels. When we administered cyclosporine to both syngeneic and allograft recipients to study the effect of the different transplants under the same condition of endothelialitis, plasma ET-1 levels were found to be increased compared with normal levels even in syngeneic graft recipients, in which no immunologic response is expected (allograft recipients, 6.99±0.91; syngeneic graft recipients, 4.15±0.83; normal level <2.30 pg/mL). Although it is still controversial whether cyclosporine induces ET-1 secretion, our data suggest that cyclosporine administration contributes to the increased plasma ET-1 levels seen in our experimental model. Thus the effect of cyclosporine may explain a slight low percent lumen for isograft (34%) compared with the normal value of 40% shown in Reference 20.

Our immunohistochemical studies revealed that the discrete ET-1 cellular immunoreactivity, in both the thickened neointima and the media of the coronary arteries, was distinctly higher in cardiac allografts than in the recipients’ own hearts or in syngeneic grafts. The lung and kidney are known to express more ET-1 than other organs. Strong ET-1 immunoreactivity of the bronchial smooth muscle cells was also seen in both syngeneic and allograft recipients. However, in the current study, no strong immunoreactivity was seen in the kidney, an endothelial cell–rich tissue, in either group 1 or group 2. Those data suggest that plasma ET-1 mainly originates from the lung (groups 1 and 2) or coronary arteries (group 2 only). In the current study, the plasma ET-1 concentration in allograft recipients was significantly higher than that in syngeneic graft recipients, which suggests that the difference in plasma ET-1 levels between these groups mainly depends on local upregulation of ET-1 production in both the thickened neointima and the media of the coronary arteries. Forbes et al have reported ET-1 staining of intimal myocytes at sites of occlusive and subocclusive intimal proliferation in allografts, whereas Watschinger et al have reported local upregulation of ET-1 in the cardiac allograft rejection model without cyclosporine administration and showed the major ET-1–expressing cell type to be mononuclear inflammatory cells in the neointima. In a clinical study with double-label immunohistochemistry for ET-1 and α-actin, Ravalli et al have clearly shown the most common cell types immunostaining for ET-1 to be neointimal smooth muscle cells and endothelial cells. Thus regardless of the cell type expressing ET-1, locally upregulated ET-1 in the thickened neointima and the media of the coronary arteries might act on coronary smooth muscle in a paracrine/autocrine fashion.

It remains unclear whether the ET-1 expression is an actual promoter of disease during the development of graft arteriosclerosis. However, ET receptor antagonists are crucial in unraveling the pathogenic role of ET-1 in the progression of the disease. Bosentan given for 120 days (20 mg/kg body wt per dose) significantly inhibited the development of coronary arteriosclerosis in cardiac allografts. It is suggested that the ET receptor–mediated signal transduction system stimulates the mitogen-activated protein kinase cascade and several protooncogenes. The current study demonstrates that interruption of the ET-1–induced potential mitogenic pathway is antiarteriosclerotic and implicates that development of new endothelin antagonists or endothelin-converting enzyme inhibitors could lead to new therapeutic approaches in prevention of graft arteriosclerosis. Previous studies of ET-1–induced mitogenesis have mainly focused on the role of the ETα receptor. Respective roles of ETα and ETβ receptors in the development of graft arteriosclerosis are still uncertain. Recently, Carratu et al have clearly demonstrated that in addition to ETα receptors, ETβ receptors are involved in ET-1–induced proliferation of ovine airway smooth muscle and that a nonselective antagonist, bosentan, also inhibited the mitogenesis. Those studies suggest that the development of graft arteriosclerosis might be suppressed by the inhibition of both ETα- and ETβ-mediated signal transduction systems. Future studies are needed to determine the respective contribution of the receptors to the progression of graft arteriosclerosis in this rat cardiac allograft model.

In conclusion, the present results indicate that increased levels of ET-1, mainly expressed in the coronary arteries, play an important role in the pathogenesis of graft arteriosclerosis by stimulating ETα receptors, ETβ receptors, or both. The current study is the first to demonstrate the efficacy of an ET receptor antagonist in graft arteriosclerosis in rat cardiac allografts. Orally active bosentan might be a potentially useful agent in the clinical prevention of graft arteriosclerosis.

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