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Expression of Endothelin-1 After Endothelial Denudation of Thoracic Aortas in Experimental Hypercholesterolemic Rats

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Abstract

Although endothelin-1 (ET-1) is involved in balloon-induced neointima formation, the role of ET-1 in balloon-induced neointima formation in hypercholesterolemia is unclear. In addition, it remains to be determined whether ET-1 is produced by endothelial cells or vascular smooth muscle cells, or both. We investigated tissue immunoreactive ET-1 levels by immunoblot analysis, localization of ET-1 immunoreactivity by immunohistochemistry, and expression of preproET-1 mRNA by *in situ* hybridization in balloon-induced neointima formation in experimental hypercholesterolemic rats. Serum total cholesterol levels were significantly higher ($p<0.01$) in the 5% cholesterol-diet group (194 ± 17 mg/dl, n=20) than in the normal-diet group (64 ± 2 mg/dl, n=20). Before and after endothelial denudation, plasma ET-1 levels and tissue immunoreactive ET-1 levels were significantly higher in cholesterol-diet rats. The expression of preproET-1 mRNA by *in situ* hybridization was observed in the nuclei of endothelial cells, but not medial smooth muscle cells in normal- or cholesterol diet rats. After endothelial denudation, plasma ET-1 levels and serum total cholesterol levels did not change in either the normal- or the cholesterol-diet rats. Tissue level of ET-1 tended to increase at 3 days after denudation in normal-diet rats (1.0 ± 0.1 vs 2.6 ± 0.2 density ratio, $p<0.05$), although endothelial cells had not yet regenerated. The expression of preproET-1 mRNA by *in situ* hybridization was not observed at 3 days after endothelial denudation in either endothelial or medial smooth muscle cells in normal-diet rats. Four weeks after denudation, regeneration of endothelial cells was almost complete, and an intimal hyperplasia was observed. Tissue ET-1 levels were significantly elevated 4 weeks after endothelial denudation in normal-diet rats (1.0 ± 0.1 vs 7.6 ± 0.2 density ratio, $p<0.05$). The expression of preproET-1 mRNA by *in situ* hybridization was observed in the nuclei of regenerated endothelial cells after endothelial denudation, and in smooth muscle cells migrating into the intima, but was not observed in medial smooth muscle cells in normal-diet rats. A similar pattern was observed in cholesterol-diet rats. We concluded that ET-1 was involved in neointima formation and that ET-1 was produced by both endothelial and neointimal smooth muscle cells, but not medial smooth muscle cells after endothelial denudation in experimental hypercholesterolemic rats. (J Nippon Med Sch 2000; 67: 342—351)

Key words: prepro endothelin-1 mRNA, hypercholesterolemia, neointima formation, angioplasty, restenosis

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Introduction

The introduction of percutaneous transluminal coronary angioplasty (PTCA) has been one of the major advances in the treatment of stenotic human coronary artery lesions[1]. However, neointimal formation and subsequent vascular restenosis are major factors limiting the long-term efficacy of PTCA[2,3], particularly in the presence of hypercholesterolemia. The mechanisms of the neointimal formation and subsequent vascular restenosis are not clear. The cellular and molecular mechanisms underlying intimal hyperplasia involve the interaction of numerous factors and forces, including growth factors, the hemodynamic action of blood flow across surfaces, and endothelins (ETs)[4]. ETs are a family of 21-amino acid peptides, including ET-1, ET-2 and ET-3. ET-1 is the most potent vasoconstrictor that was initially isolated from the supernatant of cultured porcine endothelial cells[5], and exerts various biological effects[6-8]. ET-1 causes a rapid and transient increase in c-fos and c-myc mRNA levels[9] and may stimulate DNA synthesis in vascular smooth muscle cells and fibroblasts[10-12]. Therefore, ET-1 may contribute to the development of atherosclerosis as a growth factor as well as a vasoconstrictor. Recently, it has been demonstrated that plasma ET concentrations are elevated affer PTCA[13-15] as well as advanced human atherosclerosis[16] and hypercholesterolemic pigs[17]. The increase in plasma ET-1 concentrations after PTCA is thought to reflect a rise in vascular production of ET-1 in response to endothelial cell injury. ET-1 production is consistent with the progression of neointimal formation in rats. In addition, exogenously administered ET-1 potentiates neointimal formation, suggesting an important role of ET in restenosis[18,19]. Furthermore, immunohistochemical studies show that endogenous ET-1 levels are elevated within the wall of rabbit carotid arteries after angioplasty[20]. Indeed, blockade of neointimal progression by ET-1 antagonists confirms the role of ET-1 in intimal hyperplasia[21-23]. Thus, ET-1 may be involved in balloon-induced neointima formation. After endothelial denudation, it is expected that ET-1 would be lost and would progressively reappear with re-endothelialization. The mechanism of activation of the ET system after denuding arterial injury is not well known. Azuma et al[20] evaluated the role of ET-1 in neointima formation after endothelial removal in rabbit carotid arteries, and suggested that the increased content of ET-1 in the denuded carotid artery may reflect the increased production/release of ET-1 by the vessel wall. However, in the absence of mRNA analysis, it remains to be determined whether endothelial cell or vascular smooth muscle cell, or both produce ET-1. Although many studies on ET-1 using the rat balloon injury model, or hypercholesterolemic animals have been conducted in recent decades, no report has appeared regarding the ET-1 using the rat balloon injury model in the presence of a hypercholesterolemic state. Accordingly, the primary goal of the present investigation was to determine the significance of local tissue ET-1 in the vascular wall and plasma ET-1 concentrations after endothelial denudation of the thoracic aorta in normal and experimental hypercholesterolemic rats. We also determined simultaneously the localization of ET-1 immunoreactivity by immunohistochemistry, expression of preproET-1 mRNA by *in situ* hybridization, tissue immunoreactive ET-1 levels by immunoblot analysis in the vascular wall, and plasma ET-1 concentrations, at each phase after the balloon injury.

Materials and Methods

All procedures were performed in accordance with the Nippon Medical School Animal Ethics Committee regulations and recommendations.

(1) *Animals and Endothelial denudation*

Fifty male Wistar rats aged 14 weeks and weighing about 400 g were allocated to receive a normal-diet for 4 weeks, and fifty male Wistar rats aged 10 weeks and weighing about 350 g were designed to receive a 5% cholesterol-diet containing 20% beef tallow and 2% cholic acid for 8 weeks.

Endothelial denudation of the thoracic aorta was performed with a vascular balloon catheter (1.8 Fr., Kaneka Medix Corp.) in both diet groups. Baumgartner[24] originally devised the procedure and a modified version has been used by some authors[25,26]. Briefly, under intraperitoneal pentobarbital anesthesia (8 mg/kg) supplemented with ether, a deflated vascular balloon catheter was inserted into the thoracic aorta via the left carotid artery. After inflation of the balloon to

4 mm in diameter with saline solution, the catheter was passed through the aorta three times for a distance of 3 cm. The animals were sacrificed 30 min, 3,7,14 and 28 days after endothelial denudation and their thoracic aortas were dissected out. The control animals in both diet groups were sacrificed without endothelial denudation and their thoracic aortas were dissected out.

(2) *Macroscopic observation*

To confirm the injured area, 1.5 ml Evans blue solution (2.5% in saline) was injected intravenously into randomly selected animals immediately after endothelial denudation. Thirty minutes after the injection, the aortas were dissected out and opened longitudinally to observe the Evans blue uptake macroscopically.

(3) *Histological analysis*

For light microscopic observation, immunohistochemistry, and *in situ* hybridization, the dissected tissues were opened longitudinally and fixed in 4% paraformaldehyde (PFA) solution for 6 h at 4℃, then rinsed in graded sucrose solution (10%, 15% and 20%) in 0.01 M phosphate buffered saline (PBS) containing 0.02% (v/v) diethylpyrocarbonate (Sigma Chemical Corp.) for 6 h at 4℃ each. The tissues were embedded in OCT compound (Miles Lab., IL) and frozen in dry ice-100% ethanol. Frozen sections were cut with a cryostat at a thickness of 5～6 μm, placed on 3-aminopropylmethoxysilane (SILANE)-coated slides and air-dried. The sections were first stained with hematoxylin-eosin for light microscopic observation.

(4) *Immunohistochemistry*

The labeled streptavidin-biotin (LsAB) method was employed using anti-ET-1 monoclonal antibody (Yamasa Inc.), which specifically reacted with the N-terminal of ET-1. Reaction with the primary antibody was performed at a dilution of 1: 500 overnight at 4℃. Biotin-conjugated rabbit anti-mouse IgG (Nichirei Inc.) was diluted to 1: 50 and used as the secondary antibody. The sections were then treated using Histofine SAB-PO kit (Nichirei Inc.), then finally stained with hematoxylin and observed by light microscopy.

(5) *In situ hybridization*

Using the nucleotide sequence of rat ET-1 cDNA[27], a 48-mer oligonucleotide was synthesized as a probe. Synthesis was performed using Cyclone plus DNA synthesizer (Millipore). The oligonucleotide sequence was 5'-GGTGGCAGAAGTAGACACACTCCTTGTC-CATCAGGAGGACAGGAGC-3'. The oligonucleotide was then labeled with digoxigenin (Dig)-dUTP by the 3' tailing method using a DNA tailing kit (Boehringer Mannheim GmbH). The labeling reaction was initiated by combining 100 pmol oligonucleotide, 4 μl tailing buffer, 4 μl of 5 mM $CoCl_2$, 1 μl Dig-dUTP (1 mM stock solution, as supplied by Boehringer Mannheim), 1 μl of 2.5 mM dATP, 50 units terminal transferase, and water up to 20 μl. The mixture was incubated at 37℃ for 15 min and then placed on ice. The reaction was stopped with 2 μl of 200 mM EDTA solution, pH8. Ethanol precipitation was then performed. The sections were rehydrated with PBS and incubated with proteinase K (1 μg/ml, Sigma Chemical Co.) in PBS for 15 min at 37℃. The sections were post-fixed in 4% PFA/PBS for 5 min and quenched twice with glycine (2 mg/ml) in PBS, then kept in 50% (v/v) deionized formamide/2 xSSC. The hybridization buffer contained 0.6 M NaCl, 1 mM EDTA, 10 mM Tris-HCL (pH 7.6), 120 μg/ml herring sperm DNA (Boehringer Mannheim GmbH), 200 μg/ml yeast RNA (Boehringer Mannheim GmbH), 1xDenhardt's solution, 10% (w/v) dextran sulfate (Sigma Chemical Corp.), 40% (v/v) deionized formamide, and 300～500 ng/ml labeled oligonucleotide probe. Eighty microliters of the hybridization buffer were applied to each section followed by incubation in a moist chamber for 18 h at 45℃. The sections were then washed twice with 50% formamide/2xSSC for 1 h at room temperature (RT), then washed twice with 2xSSC for 30 min at RT. For immunological detection, a nucleic acid detection kit (Boehringer Mannheim GmbH) was employed. The sections were washed briefly with buffer 1 solution (100 mM Tris-HCl, 150 mM NaCl, pH 7.6) and incubated with 0.5% (w/v) blocking reagent in buffer 1 solution for 30 min at RT. After washing again briefly with buffer 1 solution, the sections were incubated with a 1: 2000 dilution of polyclonal sheep anti-digoxigenin Fab fragment conjugated to alkaline phosphatase in buffer 1 solution for 30 min at RT. The sections were washed twice with buffer 1 solution for 15 min at RT and equilibrated with the buffer 3 solution containing 100 mM Tris-HCl, 100 mM NaCl and 50 mM $MgCl_2$ (pH 9.5) for 2 min. The sections were then incubated with chromogenic solution containing

NBT and BCIP in a dark box for 60 min. After stopping the reaction with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), the sections were mounted in an aqueous mounting medium (Daido Sangyo Corp.) and observed by light microscope.

(6) *Immunoblot analysis*

The tissues were homogenized and boiled with 1 M acetic acid for 10 min. The homogenate was placed on ice for a while until it was centrifuged at 12000 g for 10 min at 4℃, and the supernatant was used as tissue extracts. Protein concentration in each tissue extract was determined by Bradford's method[28], and 5 μg protein from each tissue extract was blotted onto the nitrocellulose membrane placed in a DOT BLOT MANIFOLD apparatus (Bethesda Research Laboratories) for immunoblot analysis. The membrane was blocked with 5% skim milk, and 0.1% NaN_3 in PBS for 2 h and incubated with the anti-ET-1 primary antibody diluted to 1: 1000 overnight at 4℃. After reaction, the membrane was washed with washing buffer containing 0.01% (v/v) Triton X-100, 25 mM Tris-HCl pH 8.0 and 150 mM NaCl, incubated with biotin-conjugated rabbit anti-mouse IgG 1 (Zymed Laboratories, Inc.) diluted to 1: 2000, and visualized with Histofine Alkaline phosphatase-conjugated streptavidin (Nichirei Inc.). Finally, each dot's density was quantitated by NIH image software.

(7) *Plasma ET-1 and serum total cholesterol levels*

Before each sacrifice, arterial blood (9 ml) was drawn from the lower abdominal aorta into tubes containing 2 mg/ml EDTA-Na_2 and aprotinin and centrifuged at 3000 g for 15 min at 4℃. Plasma was separated and frozen at −20℃ until assay. Plasma ET-1 concentration was measured by [^{125}I]ET-1 assay system. In brief, plasma was added to octadecylsilyl silica (ODS) suspension (60% methanol containing 0.25 mg/ml of C18 particles) and centrifuged at 3000 g for 3 min at 4℃. After precipitates were washed with water, 40% methanol, and acetone, (each 1.5 ml), ET-1 was eluted twice with 1.0 ml of 60% methanol and then dried and reconstituted with 0.25 ml of assay buffer (0.1% human serum albumin, 0.01 M EDTA-Na_2, 0.01% Tween 20, 0.01% NaN_3 and 0.05 M Tris-HCl, pH 8.0 containing 500 kallikrein inhibiting units/ml aprotinin) and subjected to radioimmunoassay (RIA). After 0.2 ml standard ET-1 (Peptide Institute Inc.) or sample was incubated with 0.05 ml diluted to 1: 12500 of rabbit anti-ET-1 serum (Peninsula Laboratories) overnight at 4℃, 0.05 ml [^{125}I]ET-1 (Amersham, Japan) (12000 cpm) was added and the mixture was incubated again overnight at 4℃. The bound from free ligands was separated by adding 0.1 ml goat anti-rabbit serum (Eiken Kagaku Co, Ltd), 0.1 ml normal rabbit serum and 0.1 ml of 25% polyethyleneglycol 6000, and centrifugation at 3000 g for 30 min at 4℃. The radioactivity in the precipitate was counted in a γ-spectrometer. The detection limit of this RIA was 0.82 pg per tube. The cross-reactivity of ET-2 and -3 and big ET-1 in this assay was < 7%, < 7%, and < 17%, respectively. Serum total cholesterol levels were measured by standard laboratory methods.

(8) *Statistical analysis*

Data are expressed as mean ± SEM. All experiments were repeated at least three times. Statistical comparisons were made by repeated-measures ANOVA, and values were considered to be significant at $p<0.05$.

Results

1. Macroscopic observation

Macroscopically, the injured area of the thoracic aorta was stained blue for a distance of 3 cm by Evans blue solution at 30 min after intimal denudation, indicating endothelial removal (**Fig. 1**).

2. Serum total cholesterol levels

Serum total cholesterol levels were significantly higher ($p<0.05$) in the 5% cholesterol-diet group than in the normal-diet group before (246 ± 63 vs 51 ± 2 mg/dl) and after intimal denudation (3 days: 180 ± 27 vs 79 ± 5; 7 days: 190 ± 43 vs 62 ± 2; 14 days: 172 ± 30 vs 66

Fig. 1 Evans blue up-take in the injured area of the thoracic aorta. Macroscopically, the injured area of thoracic aorta was stained blue with Evans blue solution to a length of 2.5 cm.

±3; 28 days: 181±21 vs 64±2 mg/dl).

3. Histological analysis

In both diet groups, the endothelial cells had spindle-like cytoplasm and firmly lined a narrow layer of subendothelial tissue before intimal denudation. At three days after intimal denudation, the endothelial cells were desquamated and the internal elastic lamina was exposed and some smooth muscle cells were observed to be migrating from the media to the luminal surface. At seven days after intimal denudation, the luminal surface of the aortic wall was covered by a monolayer of round or cuboidal cells. The neointima was completely covered with regenerated endothelial cells at 14 days after intimal denudation and the regenerating cells flattened with advancing times. Thickened neointima consisting of proliferative vascular smooth muscle cells and extracellular matrix was observed more markedly in 5% cholesterol-diet group than in normal-diet group, at 28 days after intimal denudation.

4. Immunohistochemistry

In both diet groups, ET-1 immunoreactivity was observed in the cytoplasm of endothelial cells but not in the medial smooth muscle cells before intimal denudation (**Figs. 2 a, f**). Because endothelial cells were desquamated, ET-1 immunoreactivity was not observed at 3 days after intimal denudation in either diet group (**Figs. 2 b, g**). Localization of ET-1 immunoreactivity was observed in the cytoplasm of regenerated endothelial cells at 7, 14 and 28 days after intimal denudation and the intensity of positive staining for ET-1 increased with advancing times after intimal denudation in both diet groups (**Figs. 2 c, h, d, i, e, j**). In the 5% cholesterol-diet group, weak positive staining for ET-1 was observed in proliferative intimal smooth muscle cells directly underlying regenerated endothelial cells at 28 days after intimal denudation (**Fig. 2 j**). Throughout all phases in both diet groups, ET-1 immunoreactivity was absent in medial smooth muscle cells as indicated by the anti-ET-1 monoclonal antibody used in this study.

5. *In situ* hybridization

PreproET-1 mRNA was observed in the nuclei of endothelial cells before intimal denudation in both diet groups (**Figs. 3 a, f**), but was not observed at 3 days after intimal denudation (**Figs. 3 b, g**) in either group. Expression of preproET-1 mRNA was observed in the nuclei of regenerated endothelial cells at 7, 14, and 28 days after intimal denudation in both diet groups (**Figs. 3 c, h, d, i, e, j**). In the 5% cholesterol-diet group, preproET-1 mRNA was weakly present in neointimal smooth muscle cells directly underlying regenerated endothelial cells at 28 days after intimal denudation (**Fig. 3 j**). In the medial smooth muscle cells, the presence of preproET-1 mRNA was not observed throughout any of the phases in either diet groups.

6. Immunoblot analysis

The ET-1 density of each phasic tissue extract in both diet groups was quantitated by NIH image software, and tissue immunoreactive ET-1 levels of each phasic extract were shown as density ratio to baseline density before intimal denudation in the normal-diet group. Tissue immunoreactive ET-1 levels gradually and significantly increased with advancing times after intimal denudation as a consequence of vascular injury in both diet groups and were significantly higher in the 5% cholesterol-diet group than in the normal-diet group, that is, 7.6-fold increase over the baseline at 28 days after intimal denudation in the normal-diet group, 10.2-fold and 17.1-fold increases over the baseline before and at 28 days after intimal denudation, respectively, in 5% cholesterol-diet group (**Fig. 4**).

7. Plasma ET-1 levels

Plasma ET-1 levels did not significantly increase as a consequence of vascular injury in either diet group, but were significantly higher in the 5% cholesterol-diet group than in the normal-diet group before (3.2±0.2 vs 1.8±0.1 pg/ml) and after intimal denudation (3 days: 3.1±0.2 vs 2.0±0.1; 7 days: 3.1±0.2 vs 2.4±0.2; 14 days: 3.1±0.3 vs 2.2±0.1; 28 days: 3.5±0.3 vs 1.8±0.1 pg/ml).

Discussion

Before endothelial denudation, plasma ET-1 levels and tissue immunoreactive ET-1 levels were significantly higher in cholesterol-diet rats than in normal-diet rats. The finding of elevated circulating ET-1 lev-

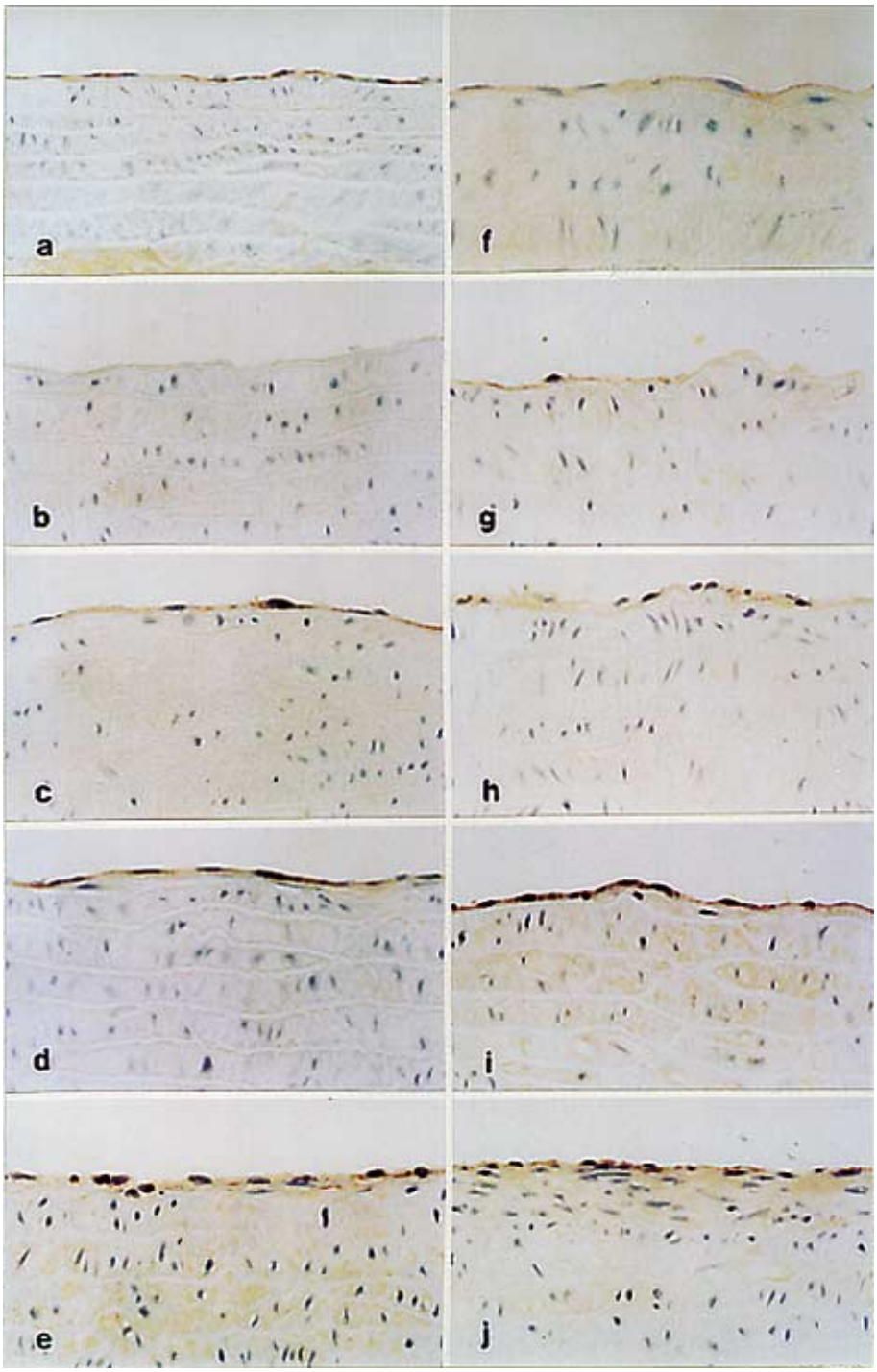

Fig. 2 Immunohistochemical staining for ET-1 in the injured area of thoracic aorta (a-e: normal-diet group; f-j: 5% cholesterol-diet group; a and f: before intimal denudation; b and g; c and h; d and i; e and j: 3, 7, 14, and 28 days after intimal denudation respectively). ET-1 immunoreactivity was observed in the cytoplasm of endothelial cells, but not in the medial smooth muscle cells before intimal denudation in either diet group (Fig. 2 a: ×130, 2 f: ×200). ET-1 immunoreactivity was not observed at 3 days after intimal denudation in either diet group (Fig. 2 b, 2 g: ×130). Localization of ET-1 immunoreactivity was observed in the cytoplasm of regenerated endothelial cells at 7, 14, and 28 days after intimal denudation, and the intensity of positive staining for ET-1 immunoreactivity increased with advancing times after intimal denudation in both diet groups (Fig. 2 c, 2 h; 2 d, 2 i; 2 e, 2 j: ×130).

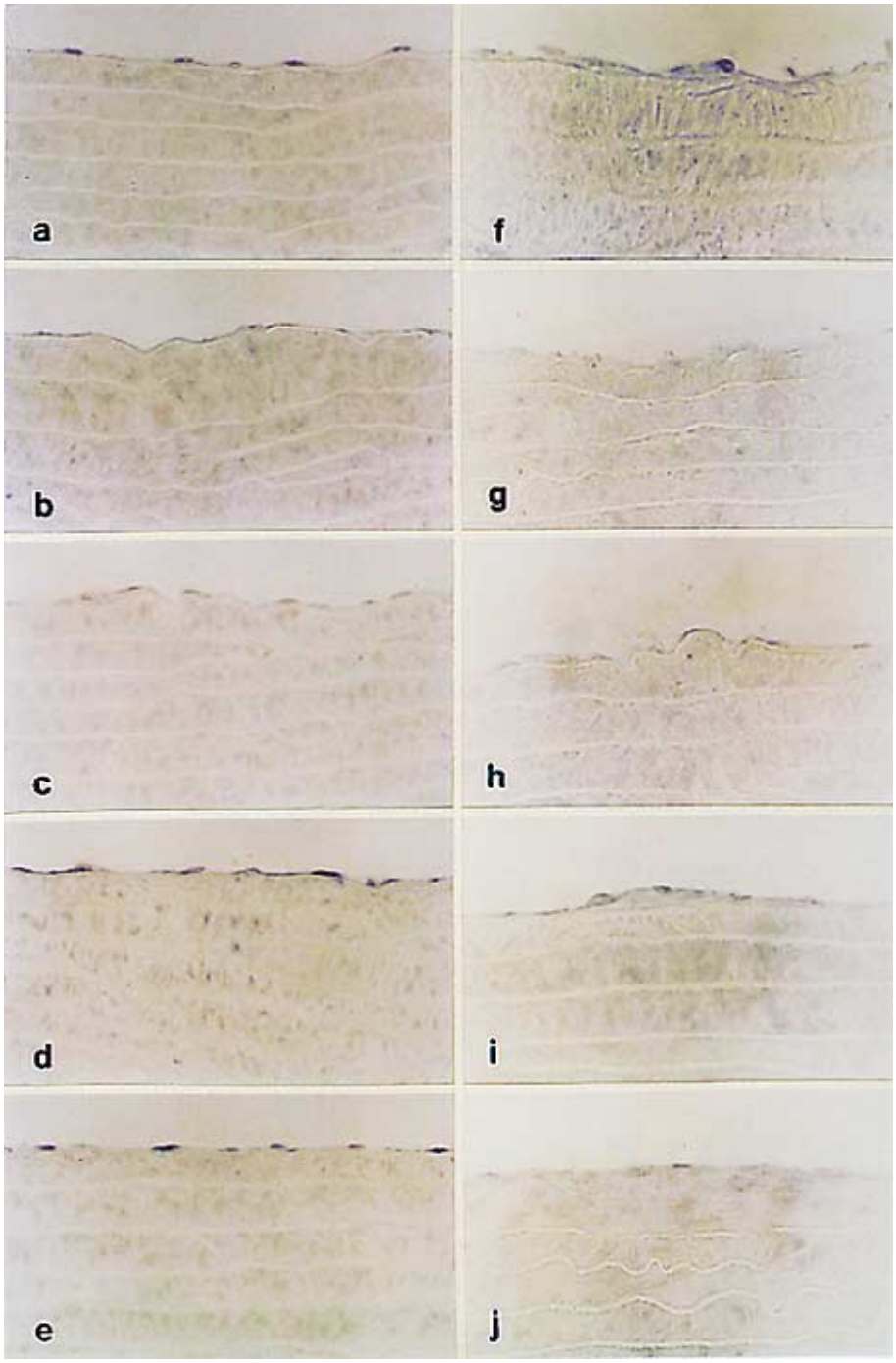

Fig. 3 *In situ* Hybridization for prepro ET-1 mRNA in the injured area of thoracic aorta (a-e: normal-diet group, ×130, f-j: 5% cholesterol-diet group, ×150, a and f: before intimal denudation, b and g; c and h; d and i; e and j: 3, 7, 14, and 28 days after intimal denudation respectively). PreproET-1 mRNA was expressed in the nuclei of endothelial cells before intimal denudation in both diet groups (Fig. 3 a, 3 f), but was not observed at 3 days after intimal denudation (Fig. 3 b, 3 g) in either group. Expression of preproET-1 mRNA was observed in the nuclei of regenerated endothelial cells at 7, 14, and 28 days after intimal denudation in both diet groups (Fig. 3 c, 3 h; 3 d, 3 i; 3 e, 3 j).

els in association with hypercholesterolemia is in accord with a previous investigation, which showed that circulating and tissue ET-1 immunoreactivity are enhanced in advanced atherosclerosis in human and correlate with the severity of the disease[16]. ET-1 mRNA expression is also reportedly elevated in atheroscle-

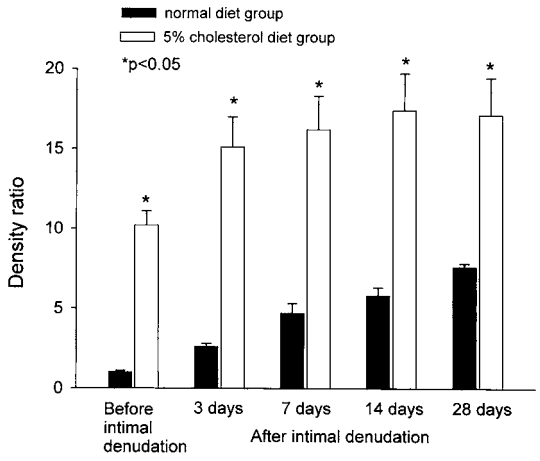

Fig. 4 Tissue immunoreactive ET-1 levels before and after intimal denudation in both normal- (solid bars) and 5% cholesterol-diet groups (open bars). Tissue immunoreactive ET-1 levels were gradually and significantly increased with advancing times after intimal denudation as a consequence of vascular injury in both diet groups and were significantly higher in the 5% cholesterol-diet group than in the normal-diet group.

rotic lesion[29]. However, there is no direct evidence whether ET-1 is produced by endothelial or medial smooth muscle cells. A major finding of the current investigation was that, before endothelial denudation, the expression of preproET-1 mRNA by *in situ* hybridization was observed in the nuclei of endothelial cells, but not in medial smooth muscle cells, in cholesterol-diet rats. Morphologically, neither foam cell formation nor intimal thickening was observed in cholesterol-diet rats. These findings suggest that the endothelial cells in cholesterol-diet rats release more ET-1 than those in the control group. Uyama et al[30] demonstrated that the surface area of each endothelial cell was significantly enlarged in the cholesterol-diet rats compared to the control group, and ET-1 secretion by cultured endothelial cells in diet-induced hypercholesterolemia was increased. Boulanger[31] also documented that oxidized low-density lipoproteins are an endogenous mediator of the augmented release of ET in hyperlipidemia. Furthermore, in diet-induced hypercholesterolemia and atherosclerosis, there is diffuse upregulation of the ET_B receptor, which is localized in endothelial and smooth muscle cells[32,33]. Thus, our results demonstrate that increased ET-1 secretion by endothelial cells in the cholesterol-diet rats is essentially caused by functional alterations in each endothelial cell induced by hypercholesterolemia.

After endothelial denudation, plasma ET-1 and serum total cholesterol levels did not change in either diet group. It is important to note that the tissue level of ET-1 significantly increased at 3 days after denudation both in normal- and in cholesterol-diet rats, although endothelial cells had not yet regenerated. In the present study, the expression of preproET-1 mRNA by *in situ* hybridization was not observed at 3 days after endothelial denudation in both endothelial and medial smooth muscle cells in either diet group, i.e., ET-1 was not yet produced in either endothelial or smooth muscle cells. Clearly, this result indicates that the increased tissue ET-1 is attributable to nonendothelial cell components in the injured artery. The mechanisms underlying increased tissue ET-1 at 3 days after denudation are unknown. It is possible that the increased tissue ET-1 in medial smooth muscle cells may be due to enhanced exposure of the smooth muscle to ET-1, either derived from the circulation or released by tissue adjacent to the lesion. Recently, Wang et al[34] reported that rat balloon injury results in the induction of ET_A receptor mRNA by semiquantitative polymerase chain reaction. They demonstrated that the level of ET_A receptor transcript is increased at 1 day, then reaches a peak at 3 days and 7 days, and maintains an elevated level up to 14 days after balloon angioplasty. Therefore, our results may suggest that binding of ET-1 to the ET_A receptor on medial smooth muscle cells increases at 3 days after endothelial denudation of a whole injured vascular wall. At seven days after endothelial denudation, almost all the injured vascular surface was covered with regenerated endothelial cells, and prepro ET-1 mRNA began to be detectable in regenerated endothelial cells by *in situ* hybridization. Therefore, in addition to increased binding of ET-1 to medial smooth muscle cells such as 3 days after endothelial denudation, ET-1 began to be produced again in regenerated endothelial cells at 7 days after endothelial denudation. Consequently, the amount of ET-1 in a whole-injured vascular wall at 7 days was greater than that at 3 days after endothelial denudation. At four weeks after endothelial denudation, endothelial regeneration was almost complete in both diet groups and a marked intimal hyperplasia was observed in the cholesterol-diet group.

Tissue immunoreactive ET-1 levels were significantly elevated at 2 and 4 weeks after endothelial denudation in both diet groups.

Another major finding of the current investigation was that the expression of preproET-1 mRNA by *in situ* hybridization was observed in the nuclei of regenerated endothelial cells after endothelial denudation in both diet groups, and in smooth muscle cells migrating into the intima at 28 days after endothelial denudation in the cholesterol-diet group, but was not observed in medial smooth muscle cells after endothelial denudation in either diet group. These findings suggest that ET-1 is produced in both endothelial and neointimal smooth muscle cells, but not in medial smooth muscle cells. Although the localization of ET-1 immunoreactivity by immunohistochemistry was not observed in medial smooth muscle cells in any phase in either diet group, ET-1 could still exist in medial smooth muscle cells. Anti-ET-1 monoclonal antibody used in this study specifically reacts with the amino-terminal portion of ET-1. Because the animo-terminal disulfide loop portion of ET-1 binding the ET_B receptor on endothelial cells does not combine with the transmembrane helices IV-VI or with the adjacent loop regions of the ET_B receptor[35], its reactive site seems to be able to bind with the anti-ET-1 monoclonal antibody. On the other hand, the amino-terminal disulfide loop portion of ET-1 binding the ET_A receptor on smooth muscle cells combines with the transmembrane helices IV～VI and the adjacent loop regions of the ET_A receptor[35], and its reactive site does not seem to be able to bind with the anti-ET-1 monoclonal antibody. Therefore, ET-1 immunoreactivity is detectable in endothelial cells but not in medial smooth muscle cells by the anti-ET-1 monoclonal antibody employed in the present study. However, it is plausible to consider that the bindings of ET-1 and the ET_A receptor on medial smooth muscle cells are disrupted when sample arteries are homogenized and boiled with 1 M acetic acid in immunoblot analysis measuring tissue immunoreactive ET-1 levels. Consequently, ET-1 immunoreactivity was detectable not only in endothelial cells but also in medial smooth muscle cells, that is, the tissue immunoreactive ET-1 levels quantitated by immunoblot analysis indicate the summation of ET-1 from both endothelial cells and smooth muscle cells. In such conditions, we obtained the fact that tissue immunoreactive ET-1 levels gradually and significantly increased with advancing times from the baseline when ET-1 was intact (uninjured state), that is, at 3, 7, 14 and 28 days after endothelial denudation.

In summary, ET-1 may be involved in neointima formation. It is produced by both endothelial and neointimal smooth muscle cells, but not medial smooth muscle cells, after endothelial denudation in experimental hypercholesterolemic rats. The present study would purport the idea that ET-1 can be a potential target of therapy for atherosclerosis and angioplasty restenosis.

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