

# Endothelin-1(1-31) Levels are Increased in Atherosclerotic Lesions of the Thoracic Aorta of Hypercholesterolemic Hamsters

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## Abstract

**Objective:** The novel vaso-constricting 31-amino acid-length endothelin-1 [ET-1(1-31)] is selectively produced by human mast cell chymase via its action on big ET-1. However, the pathological role of ET-1(1-31) in atherosclerosis remains unclear. The aim of this study was to clarify vasoconstrictive response and expression of ET-1(1-31) in atherosclerotic aorta. **Methods and Results:** Syrian golden hamster, was used for preparing the atherosclerotic models by the administration of a high cholesterol diet (HC), treatment with the nitric oxide synthase inhibitor (*N*<sup>ω</sup>-nitro-L-arginine methylester, L-NAME) alone, or both (HC and L-NAME) for 40 weeks. Early atherosclerosis was observed in the case of HC or L-NAME alone treatments respectively and severe atherosclerosis was observed in the case of combined HC and L-NAME treatment. Vasoconstriction induced by ET-1(1-31) was not altered by the atherosclerotic changes, but the expression pattern of ET-1(1-31) were different at each stage of the atherosclerotic aorta. ET-1(1-31) was observed rarely in normal aortas or in early atherosclerotic lesions, but ET-1(1-31) expression was dramatically increased in aortic neointima and adventitia in a state of atherosclerosis with severe inflammation. **Conclusion:** ET-1(1-31) might play in a role of promoting atherosclerosis, and especially be involved in inflammatory mediation during the progression of atherosclerosis.

## Key words

endothelin, atherosclerosis, nitric oxide, hypercholesterolemia, hamster.

## Introductions

Endothelin (ET)-1(1-21), a 21 amino acid peptide produced from big ET-1 hydrolysis by the ET converting enzyme,<sup>1)</sup> was isolated from the supernatant of cultured porcine coronary artery endothelial cells as a bioactive peptide that has constrictive activity with respect to smooth muscle.<sup>2)</sup> ET-1(1-21) plays an important role in the maintenance of basal vascular tone<sup>3)</sup> and has been implicated in the pathophysiology of vasospastic reactions.<sup>4)</sup> In addition, ET-1(1-21) has mitogenic properties on vascular smooth muscle cells (VSMCs),<sup>5)</sup> and ET-1(1-21)-like immuno-reactivity had been observed in atherosclerotic coronary plaques.<sup>5)6)</sup> Moreover, increased ET-1 mRNA expressions has been reported in atherosclerotic carotid lesions.<sup>7)</sup> Thus, the possibility that ET-1(1-21) might play an important role in the development of atherosclerosis, cannot be excluded.

We previously reported that 31-amino acid endothelins [ETs(1-31)] is formed via cleavage of big ETs at the Tyr<sub>31</sub>-Gly<sub>32</sub> bond by human mast cell chymase or other

chymotrypsin-type proteases.<sup>8)9)</sup> ET-1(1-31) exerts a vasoconstricting action, the activity of which is about 10 times weaker than that of ET-1(1-21) in the porcine coronary artery.<sup>10)11)</sup> Our recent investigations of porcine coronary artery demonstrated that ET-1(1-31) is involved in VSMC proliferation similarly to that of ET-1(1-21).<sup>12)</sup> These findings suggest that the ET-1(1-31) may be associated with atherosclerosis. However, the pathophysiological role of ET-1(1-31) in atherosclerosis remains unclear.

A hamster model was used to investigate the role of chymase-induced ET-1(1-31) in atherosclerosis, since the specificity and activity of hamster chymase is similar with that of human.<sup>13)</sup> The hypercholesterolemic hamster is a useful model for an early pro-atherogenic event, namely subendothelial monocyte-macrophage foam cell formation.<sup>14)</sup> Histological changes for atherosclerosis usually remain in the early stage in the hamster model induced by high cholesterol/fat diet alone for the periods of up to 12 months.<sup>15)</sup> Hypercholesterolemia, smoking, hypertension, and diabetes mellitus are important risk factors for atherosclerosis, which are associated with reduced nitric oxide (NO)-dependent vasodilation, even before the development of clinically or morphologically apparent atherosclerosis.<sup>16)</sup> In addition, endogenously formed NO may also protect against the formation of foam cells and media hypertrophy, i.e. against the structural component of atherosclerosis.<sup>17)</sup> We have been successful in developing a severe atherosclerosis model via the long-term inhibition of NO production in the hypercholesterolemic hamster model.

In the present study, we functionally and pathophysiologically correlated ET-1(1-31) with atherosclerosis by measuring vasoconstriction in atherosclerotic arteries with immunohistochemical changes in ET-1(1-31) with the progression of atherosclerosis, using several stages of atherosclerosis in the hamster model.

## Materials and Methods

### Animals, Diet and Experimental Groups

Twenty-four Syrian golden, specific pyrogen-free male hamsters, aged 5 weeks, were obtained from Japan SLC (Shizuoka, Japan). After 1 week of adaptation, the hamsters were randomly divided into 4 subgroups as follows: SD; standard diet with normal tap water, SD/NAME; standard diet with 1 mg/mL *N*<sup>ω</sup>-nitro-L-arginine methylester (L-NAME) in the drinking water, HC; diet supplemented with 0.3% cholesterol (w/w) and 10% coconut oil (w/w) (Oriental Yeast, Osaka, Japan) as high cholesterol diet with normal water, and HC/NAME; high cholesterol diet with L-NAME containing water. The diets and water were provided ad libitum. The hamsters were housed individually in temperature- (23±2°C) and humidity- (50–60%) controlled rooms, with 12 h of light and 12 h of darkness daily. The protocol was in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals prepared by Institute of Laboratory Animal Resources (ILAR).

### **Measuring Blood Pressure and Heart Rate**

The systolic blood pressure and heart rate of the hamsters were measured by means of a tail manometer using an oscillometric method (TK-370C, UNICOM, Chiba, Japan).

### **Aortic Tissue Preparation and Blood sample**

Animals were fasted overnight and anesthetized with an intraperitoneal injection of sodium pentobarbital (10 mg/100 g body weight), and blood sample (3 mL) was collected from the abdominal aorta. Aortic arches and thoracic aortas were removed and placed in cold Krebs-Ringer bicarbonate solution (KRB). Residual blood and perivascular tissue were removed from the aortas in cold KRB. Thoracic aortas were cut into 3 mm rings for measurement of vascular reactivity. Aortic arch rings were fixed by immersion with 4% buffered p-formaldehyde solution for more than 24 hours at 4°C for measurement of fatty streak accumulation and histological analysis. After fixation, the aortic arches were cut into 2 rings. One was used for histological and immuno-histochemical analysis, after dehydration in ethanol and chloroform, and embedded in paraffin. The other sample was used for measurement of fatty streak accumulation.

### **Quantification of Aortic Fatty Streak**

After fixation, the inner surface of aortic arch was stained with Oil Red O (ORO) for 25 min at room temperature. The aortic arches were cut longitudinally and mounted on a glass slide cover slip with aqueous mounting medium (AQUATEX, MERCK, Darmstadt, Germany). The images of the aortic arches were converted into a gray-scaled picture and the ORO-stained fatty streak area measured by the planimetric analysis software (AtImage, programed by Mr. Y. Endo).

### **Histological and Morphological Analysis**

Aortic arches embedded in paraffin were sectioned to a thickness of 3  $\mu$ m. Serial cross sections were routinely stained with hematoxylin-eosin (HE) or used for immunohistochemistry. The image of the aortic arch was captured and the thickness of aortic intima and adjoining media measured by image analysis software (Photoshop, Adobe Systems, San Jose, CA, U.S.A).

### **Vascular Reactivity**

Each aortic ring was placed in a 5 mL organ chamber (Micro East Magnus, Kishimoto Medical, Kyoto, Japan) and mounted on two stainless steel wires in order to measure the isometric tension. The aortic rings were suspended in KRB solution at 37°C and a mixture of 95% O<sub>2</sub>-5% CO<sub>2</sub> was continuously bubbled into the solution. The rings were equilibrated for 1 hour under a resting tension of 0.7 g, and the solution was exchanged at 20 minutes intervals. Vasoconstrictive responses induced by the cumulative concentrations of ET-1(1-21) or ET-1(1-31) were expressed as relative constriction induced by a 50 mM KCl

solution.

### **Immunization for ET-1(1-31)**

A female rabbit was used for immunization. The immunogen was a synthetic peptide composed of the C-terminal hexapeptide, ET-1(26-31). The immunogen was injected into the rabbit 5 times over a 2-weekly interval and blood was collected as rabbit anti-ET-1(1-31) anti-serum. The collected serum was then eluted from a protein A column (PIERCE, Rockford, IL, USA) with Immuno pure IgG binding buffer (PIERCE), and was fractionated by affinity chromatography on a column of immunogen peptide-coupled CEHVVP peptide. The cross-reactivities of the antibody to ETs, big ETs, vasopressin, and bradykinin (respectively in 5 µg/ml) were measured by enzyme immunoassay. A small amount of cross-reactivity of the ET-1(1-31) antibody to big ET-1 (5.2% relative reactivity of ET-1(1-31)) was observed but not to the other peptides (<0.01% relative reactivity of ET-1(1-31)).

### **Immunohistochemistry**

Immunohistochemical staining with the rabbit monoclonal antibody against ET-1(1-21) (Peptide Institute, Osaka, Japan) and ET-1(1-31) (laboratory made) was performed in serial sections of the aortic arches. The IC<sub>50</sub> (median inhibitory concentration) values of both diluted antibodies were determined by enzyme immunoassay and these immunoreactivities were made equal. Aortic sections were rehydrated in phosphate-buffered saline (PBS), and nonspecific immunoglobulin-binding sites were then blocked by diluted goat normal serum. The sections were incubated with a monoclonal primary antibody for overnight at 4°C. Monoclonal antibodies were diluted by PBS containing 0.1% bovine albumin (w/v). Instead of the primary antibodies, the dilution buffer was used as a negative control. The presence of antigen was detected with biotinylated goat anti-rabbit antibody followed by incubation with biotin avidin-alkaline phosphatase complex (VECTASTAIN ABC-AP kit, VECTOR Laboratories, Burlingame, CA, USA). Alkaline phosphatase was visualized by incubation with an alkaline phosphatase substrate (VECTOR blue AP-substrate kit, VECTOR Laboratories). The stained area for ET-1(1-21) and ET-1(1-31) were quantified by means of a planimetric analyzer. To clarify the localization of ET-1(1-21) and ET-1(1-31) in more detail, we used avidin-peroxidase complex instead of ABC-AP after the second biotinylated antibody. Diaminobenzadine was used as the peroxidase substrate and hematoxylin as the nuclear counterstain.

### **Reagent**

Cholesterol, L-NAME, and p-formaldehyde were purchased from WAKO chemicals (Tokyo, Japan). ET-1(1-21), ET-1(1-31), bradykinin, and vasopressin were purchased from the Peptide Institute (Osaka, Japan). Oil Red O and avidin-peroxidase were purchased from Sigma (St. Louis, MO, U.S.A.). Diaminobenzadine was purchased from Dojindo (Kumamoto, Japan).

### Statistical Analysis

The statistical analysis of differences was estimated by the Student's t-test. A value of  $p < 0.05$  or  $p < 0.01$  was considered statistically significant.

### Results

#### Biochemical and Physiological Measurements

Table 1 shows the body weight, heart weight, serum lipid values, systolic blood pressure, and heart rate in each experimental. There was no difference in the above parameters among the groups at the beginning of the experiments. Increases in body weight were not significantly different between each group. The intake of cholesterol during the experiments was the same for the HC and HC/NAME groups, and the intake of L-NAME was the same for the SD/NAME and HC/NAME groups. Serum levels of total cholesterol and systolic blood pressure tended to increase during the long-term oral administration of L-NAME ( $p < 0.05$ ). Serum levels of triglyceride decreased and total cholesterol increased as the result of administration of L-NAME ( $p < 0.05$ ). Heart weights were significantly increased by the oral administration of L-NAME ( $p < 0.05$ ).

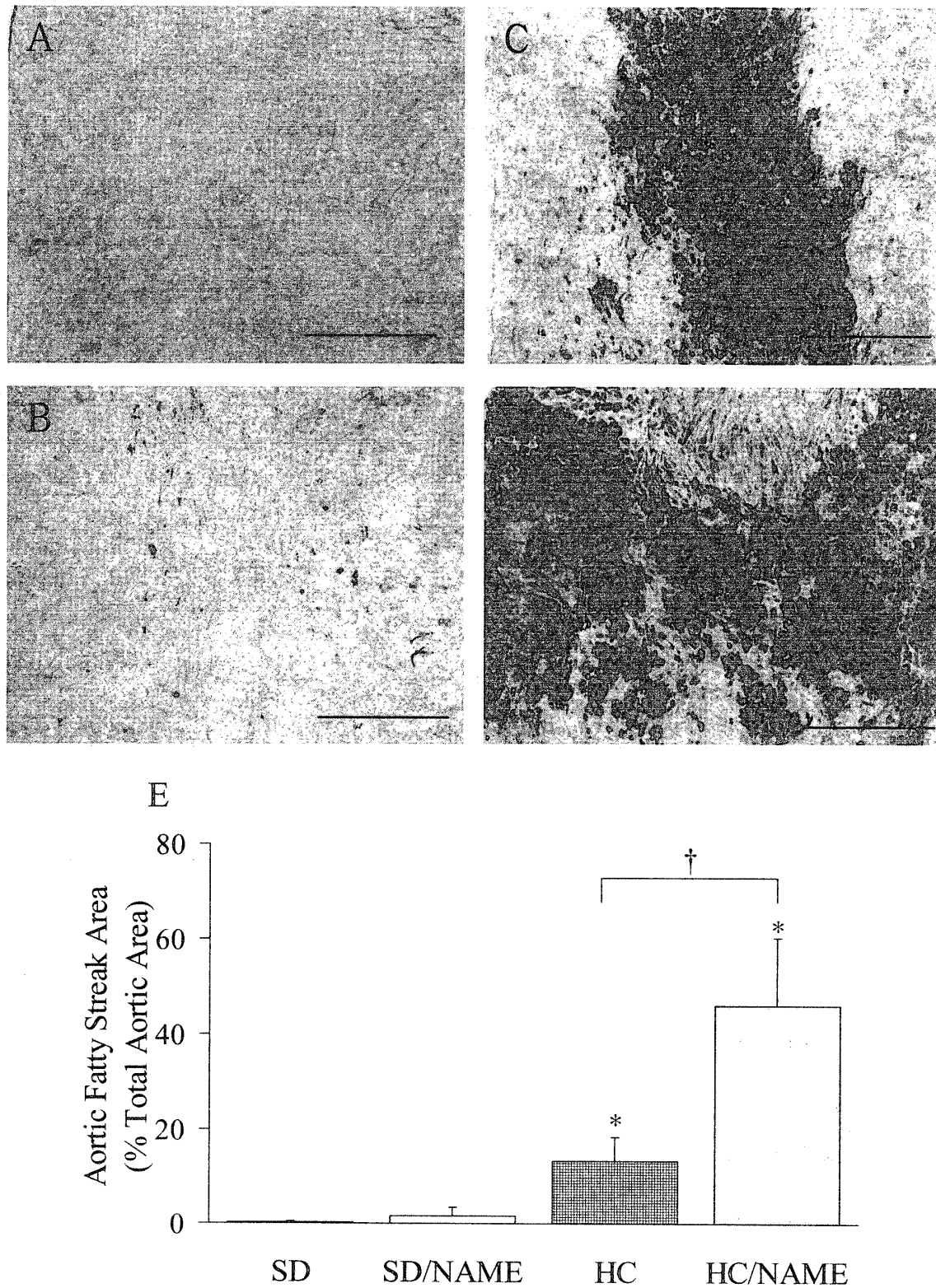
#### Fatty Streak Accumulation of Aortic Arch

Fatty streak accumulation on the inner surface of the aortic arch was observed by examination of Oil Red O-positive droplets under light microscopy (Fig. 1). Fatty streaks were barely detectable in aortic arches in the SD group (Fig. 1A). A few small droplets were observed in aortic arches in the SD/NAME group (Fig. 1B). Large and broad fatty droplets were observed in the aortic arches from HC- and HC/NAME-treated hypercholesterolemic hamsters (Figs. 1C and 1D). The fatty streak areas in aortic arches in the HC/NAME group were significantly larger than those in the HC group (Fig. 1E).

**Table 1. Body Weights and Lipid Value in Each Hamster Group**

	SD	SD/NAME	HC	HC/NAME
Baseline Body Weight, g	91.3±5.5	91.3±5.5	88.3±3.8	91.5±6.6
Increase in Body Weight, g	55.4±7.1	55.4±7.1	56.2±11.5	46.7±19.5
Cholesterol intake, mg/day	—	—	18.9±1.41	18±1.27
L-NAME intake, mg/day	—	7.25±0.31	—	6.59±1.27
Heart weight, g	0.44±0.06	0.44±0.06	0.52±0.06**	0.52±0.06**
Total cholesterol, mg/dL	151±31	151±31	196±21*	472±73 <sup>#</sup>
HDL-cholesterol, mg/dL	87±22	87±22	129±25*	151±13
Triglyceride, mg/dL	102±44	102±44	41±33*	197±159
Systolic Blood Pressure, mmHg	98±12	110±17	100±12	122±26
Heart Rate, beat/min	289±24	279±16	293±19	283±21

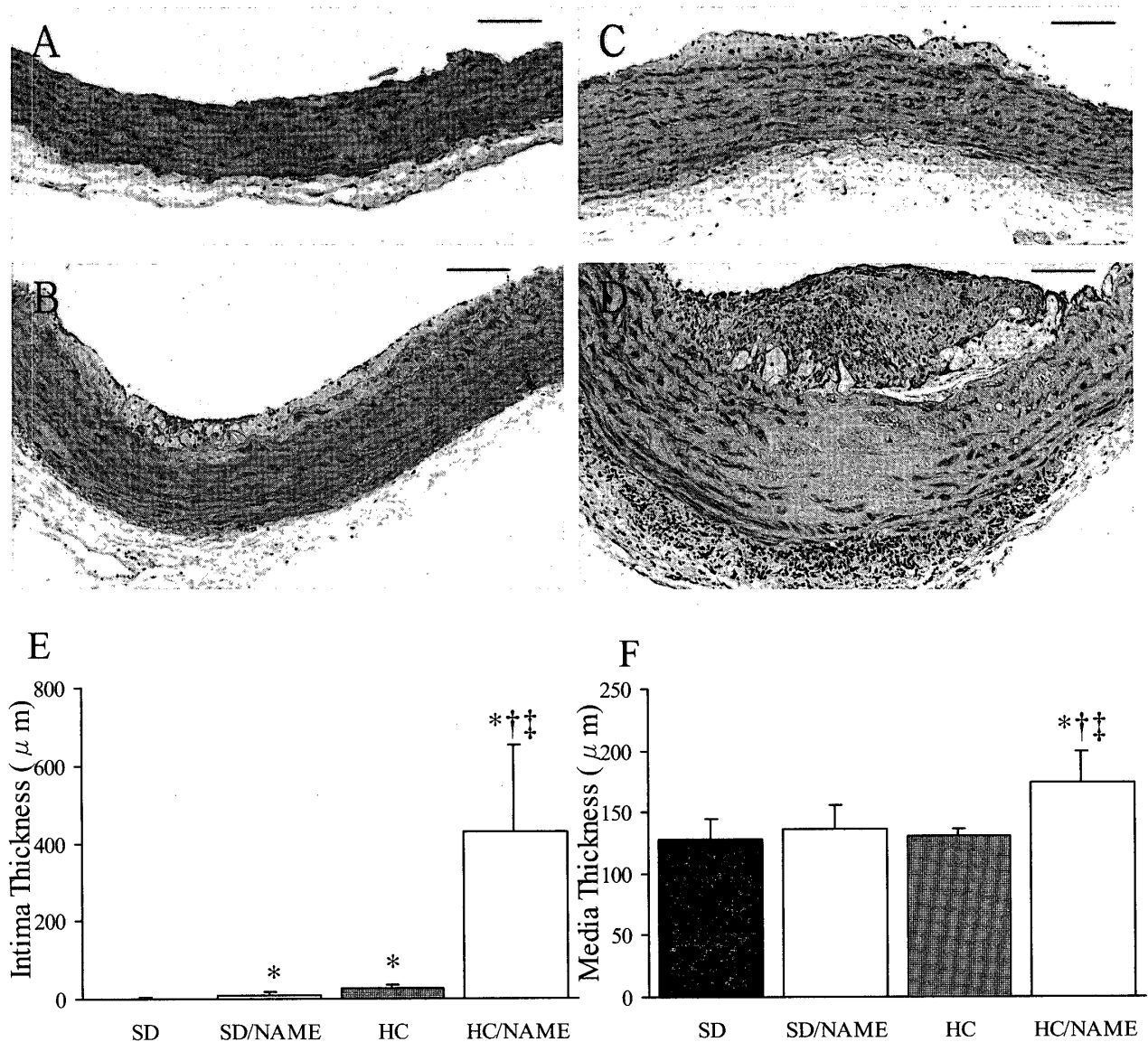
HDL-cholesterol indicates high-density cholesterol; L-NAME, N<sup>ω</sup>-nitro-L-arginine methylester. Values are shown as mean±STDEV. \*Significant difference,  $p < 0.05$  versus SD-treatment; <sup>#</sup>Significant difference,  $p < 0.05$  versus HC-treatment.



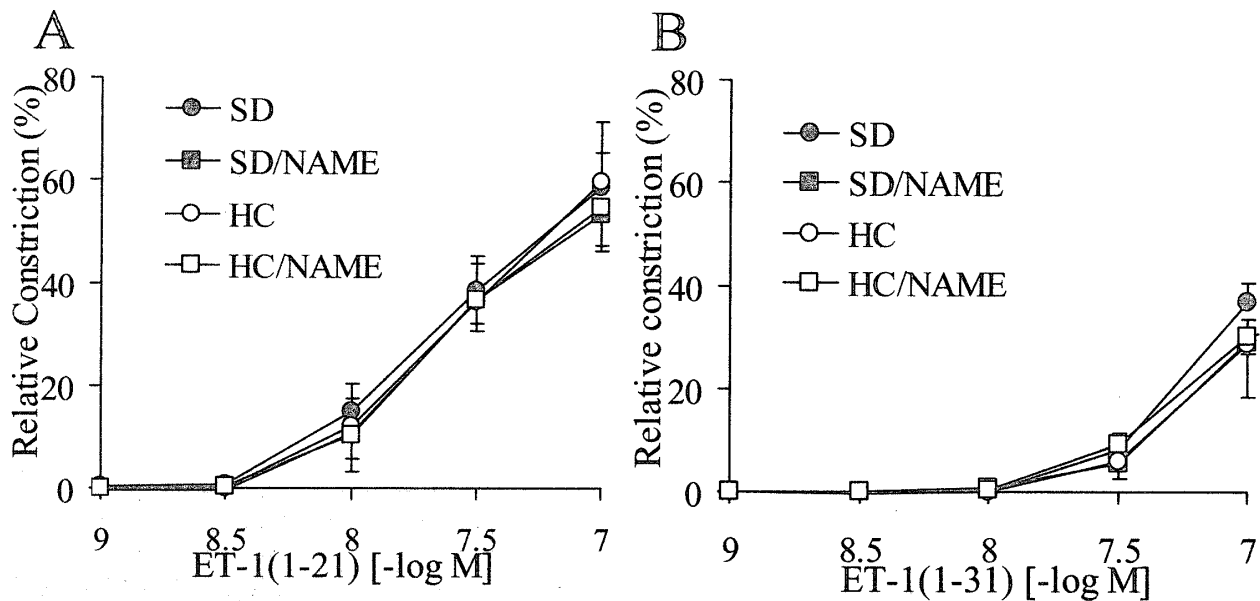
**Figure 1.** Histological photomicrographs of the inner surface of hamster aortic arches stained with Oil Red O (ORO). (A) SD. (B) SD/NAME. (C) HC. (D) HC/NAME. Red spots indicate an ORO-stained fatty streak. Blood flow was from upper side to lower side. Bar=0.5 mm. (E) ORO-stained fatty streak area was measured as the relative total aortic inner surface area. \*Significant difference,  $p < 0.01$  versus SD. †Significant difference,  $p < 0.01$  versus HC. Values are shown as mean ( $n=6$ ) and STDEV.

### Histological Analysis

Cross sections of the aortic arch in each group were stained with hematoxylin and eosin, and the thickness of intima and media measured, in order to estimate the atheromatous lesions. Obvious atheromatous lesions were not detectable in aortic arches in the SD group (Fig. 2A). Aortic arches in the SD/NAME and HC groups had slightly thickened neointima (Figs. 2B and 2C), presumably an indicator of early atherosclerosis.<sup>20)21)</sup> Furthermore, aortic arches in the HC/NAME group had significantly thickened neointima and media (Figs. 2D, 2E, and 2F). In the high power fields of the aortic arch in the HC/NAME group, cell-rich necrotic areas were observed in many parts of the media (Fig. 5D).



**Figure 2.** Photomicrographs of cross sections of hamster aortic arches in each group stained with Hematoxylin and Eosin (HE) and the thickness of intima and media on hamster aortic arch. (A) SD. (B) SD/NAME. (C) HC. (D) HC/NAME. Upper sides of the photographs indicate the intimal sides and lower sides indicate the adventitial sides. Bar=0.1 mm. (E) Thickness of intima. (F) Thickness of media. \*Significant difference,  $p < 0.05$  versus SD. †Significant difference,  $p < 0.05$  versus SD/NAME. ‡Significant difference,  $p < 0.05$  versus HC. Values are shown as mean ( $n=6$ )  $\pm$  STDEV.



**Figure 3.** Relative constrictive response induced by ET-1(1-21) or ET-1(1-31) in hamster aortic rings in each group. (A) ET-1(1-21). (B) ET-1(1-31). ●, SD; ○, SD/L-NAME; ■, HC; □, HC/L-NAME. Data indicates the relative constriction compared with constriction induced by 50 mM KCl. Values are shown as mean (n=6) and STDEV.

Moreover infiltration of fibrocytes, histocytes and neutrophils in the neointima were observed (Fig. 5A), while fibrocytes, histocytes, neutrophils, eosinophils and lymphocytes in the adventitia were observed (Fig. 5G).

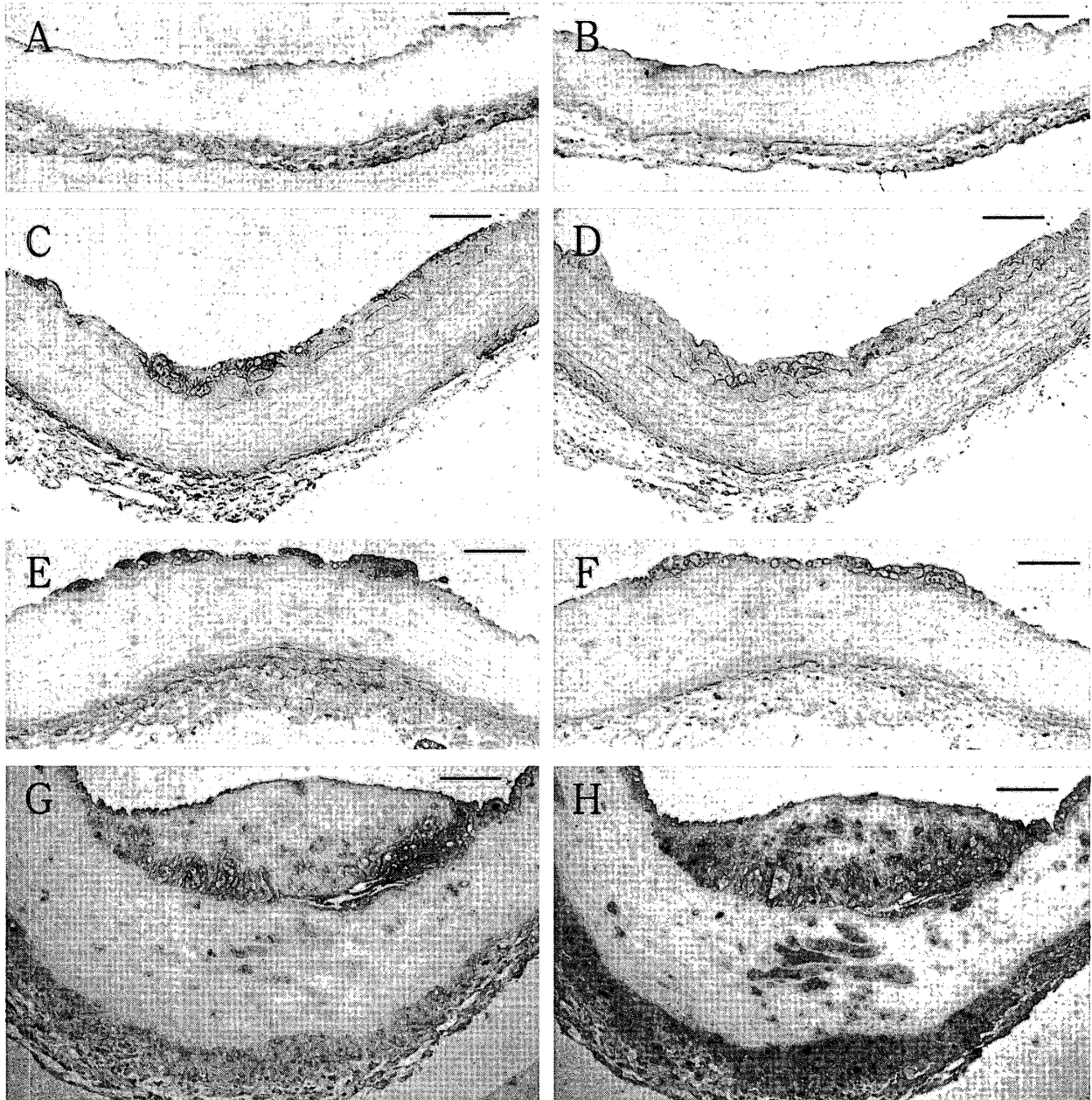
#### Vasoconstrictive Response Induced by ET-1(1-21) and ET-1(1-31)

Figure 3 shows the constrictive response induced by ET-1(1-21) and ET-1(1-31) for each group. ET-1(1-21) and ET-1(1-31) evoked a constriction of the aortic rings in a concentration-dependent manner. ET-1(1-21) induced a greater constriction of the aorta than ET-1(1-31) (Figs. 3A and 3B). However the constrictive response induced by ET-1(1-21) and ET-1(1-31) were not statistically different among the groups (Figs. 3A and 3B).

#### Immunohistochemical Analysis for ET-1(1-21) and ET-1(1-31)

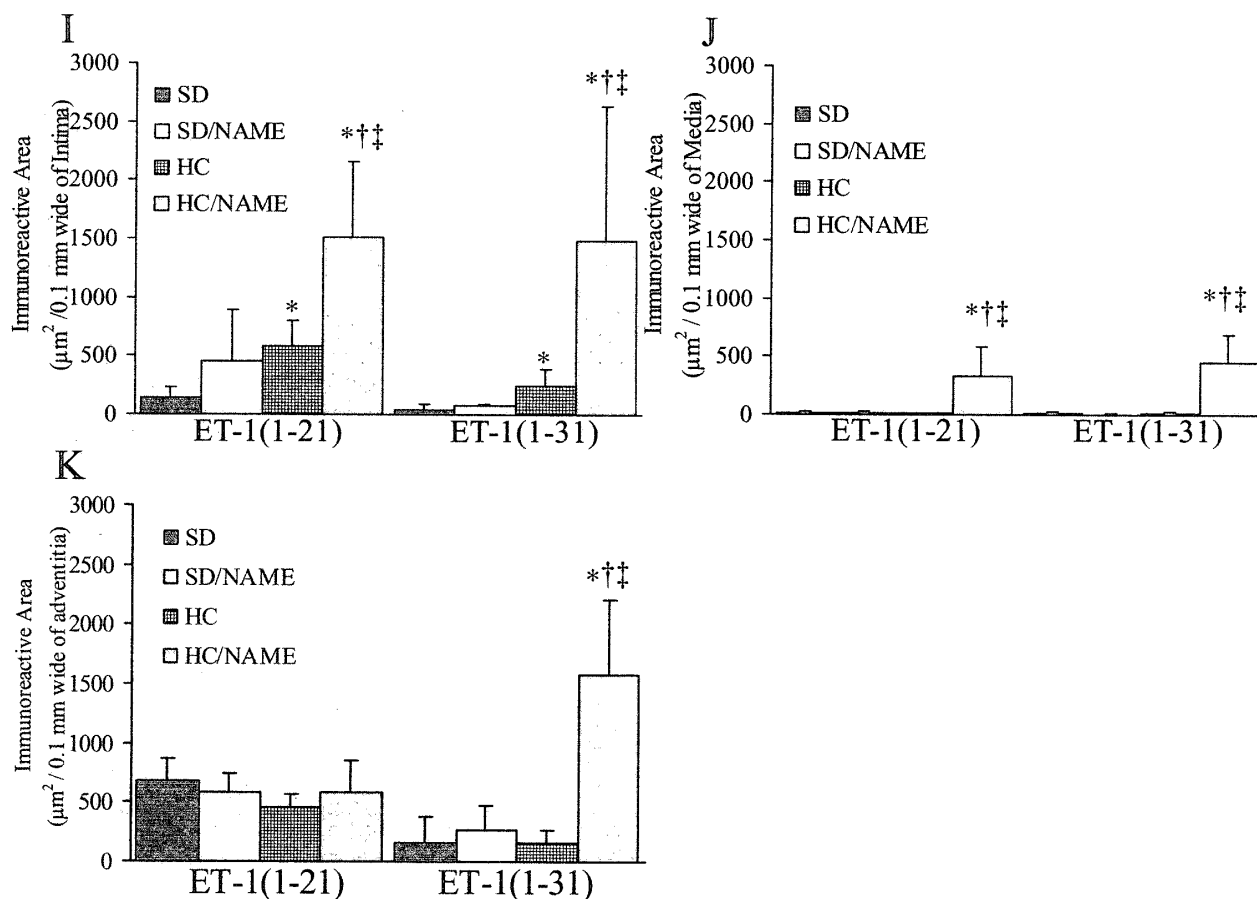
ET-1(1-21)- and ET-1(1-31)-like immuno-reactivity in aortic arches was estimated by immunohistochemistry and semiquantitative morphometric analysis. Aortic arches of the SD group were used as a normal model (Figs. 4A and 4B), the SD/NAME and HC group as an early atherosclerosis model (Figs. 4C, 4D, 4E, and 4F), and the HC/NAME group as a severe atherosclerosis model with severe inflammation (Figs. 4G and 4H). ET-1(1-21)-like immuno-reactivity was low in the intima and adventitia of aortic arches in the normal model (Figs. 4A) and ET-1(1-31)-like immuno-reactivity was much less (Figs. 4B). In early and severe atherosclerosis models, ET-1(1-21)-like immuno-reactivity was increased in the neointima compared with normal models (Figs. 4C, 4E, and 4G), whereas ET-1(1-31)-like immuno-reactivity was observed, only to a slight extent around the neointima,





**Figure 4-1.** ET-1(1-21)- and ET-1(1-31)-like immuno-reactivity on cross sections of hamster aortic arches in each group. Left side photographs; ET-1(1-21). Right side photographs; ET-1(1-31). (A and B) SD. (C and D) SD/NAME. (E and F) HC. (G and H) HC/NAME. Serial sections were stained with anti-ET-1(1-21) or anti-ET-1(1-31) antibody. Blue reaction products indicated immunoreactive area. Upper side of the photograph indicates the intimal side and lower side indicates the adventitial side. Bar=0.1 mm.

suggesting that inflammation was in the early stage (Figs. 4D and 4F). However, in severe atherosclerosis, ET-1(1-31)-like immuno-reactivity was significantly increased in both neointima and adventitia (Fig. 4H). In a semiquantitative analysis, ET-1(1-21)-like immuno-reactivity was observed to a slight extent in the intima and media from normal aortas, and gradually increased only in the neointima as atherosclerotic lesions became histologically advanced but not in adventitia (Figs. 4A and C). ET-1(1-31) was expressed

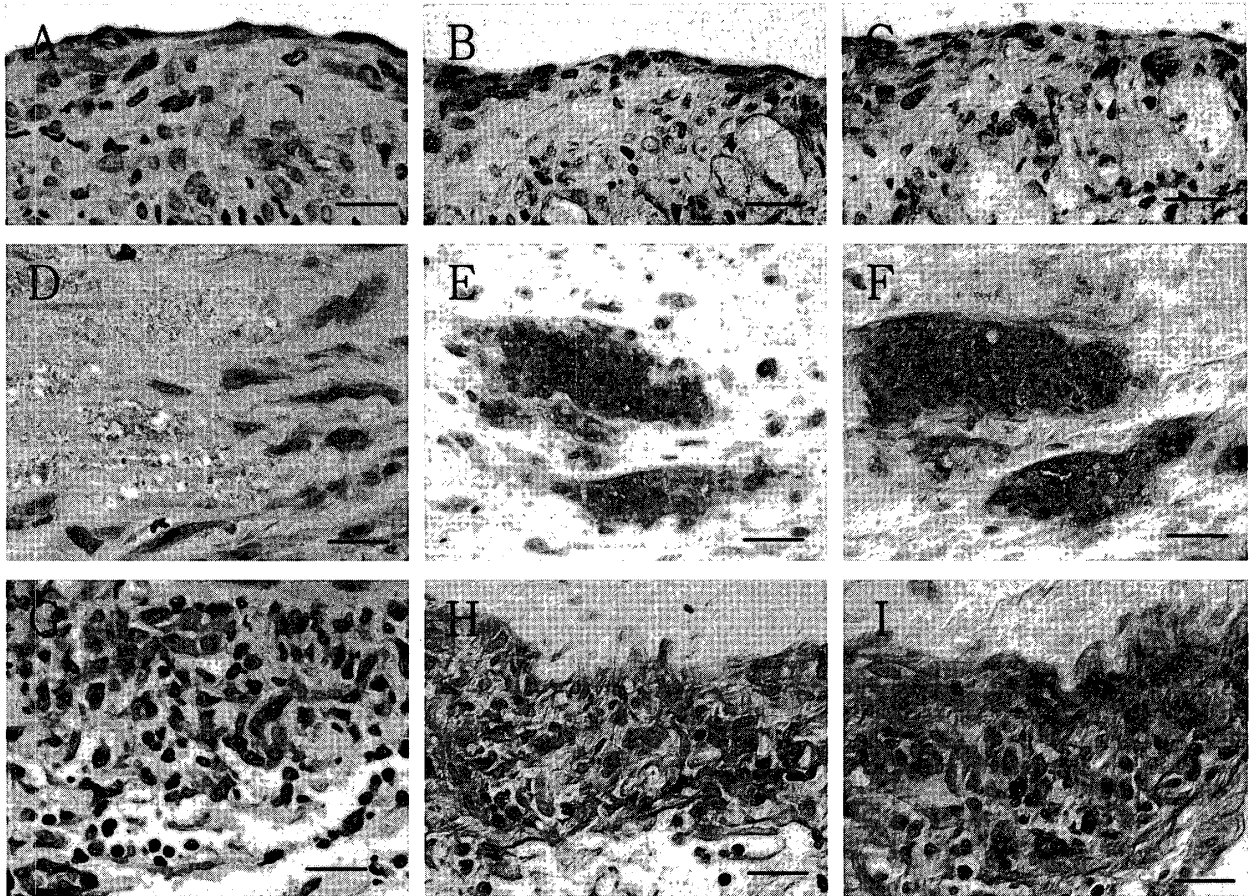


**Figure 4-2.** (I, J, and K); Semiquantitative analysis of immunoreactive area. (I) Intima. (J) Media. (K) Adventitia. \*Significant difference,  $p < 0.05$  versus SD. †Significant difference,  $p < 0.05$  versus SD/NAME. ‡Significant difference,  $p < 0.05$  versus HC. Values are shown as mean ( $n=6$ )  $\pm$ STDEV.

only slightly in normal aorta, and observed minor extent in the neointima from early atherosclerosis (Figs. 4A), but was significantly increased in not only the neointima but also the adventitia from severe atherosclerosis (Figs. 4A and 4C). At high power fields of aortic arches from the HC group with a nuclear counterstain, both ET-1(1-21)- and ET-1(1-31)-like immuno-reactivity were observed within the necrotic area of media (Figs. 5E and 5F), and within or around inflammatory hematocytes, mainly histocytes (macrophages), in the neointima and the adventitia (Figs. 5B, 5C, 5H, and 5I). However the localization between ET-1(1-21)- vis-a-vis ET-1(1-31)-like immuno-reactivity were similar.

## Discussion

We have shown for the first time that changes in ET-1(1-31) expression are depend on the stages of atherosclerosis, to a greater extent than that of ET-1(1-21). A marked increase in ET-1(1-31) in severe atherosclerosis suggests that not only ET-1(1-21) but also ET-1(1-31) is an important mediator of the progression of atherosclerosis.



**Figure 5.** High power fields of cross sections of hamster aortic arches in the HC/NAME group stained with Hematoxylin and Eosin (HE), and immunohistochemistry for ET-1(1-21) and ET-1(1-31). Left side photographs; HE stain. Middle photographs; ET-1(1-21). Right side photographs; ET-1(1-31). (A, B, and C) Intima. (D, E, and F) Media. (G, H, and I) Adventitia. Serial sections were stained with anti-ET-1(1-21) or anti-ET-1(1-31) antibody. Brown reaction products indicated immunoreactive area. Hematoxylin was used as nuclear counterstain in immunohistochemistry. Bar=20  $\mu$ m.

### Atherosclerosis Model

Early atherosclerosis,<sup>18)19)</sup> including thickening of the intima and/or macrophage-foam cell formation was detected in aortic arches in SD/NAME- and HC-treated hamsters. Severe atherosclerosis,<sup>18)19)</sup> including fibrous plaques, medial thickening, and serious inflammation was observed in aortic arches in HC/NAME-treated hamsters. In the present study, mechanical injury to the vascular wall by experimental angioplasty or transgenic animals were not used, but, rather, a 'biochemical treatment' was employed, i.e., the long-term administration of high cholesterol/fat diet and a NO synthase inhibitor, which caused severe atheromatous lesions in the hamster. Since, using this biochemical approach, it was relatively easy to differentiate between stages in the atherosclerotic model compared with other methods, this may be a useful approach for evaluating atherosclerosis.

### **Effect of Long-term Oral Administration of L-NAME to Atherosclerotic Progression in Hypercholesterolemic Hamster**

The effect of a combination of L-NAME and a high cholesterol/fat diet on atherosclerosis is not well established in the literature. It has been reported that the L-NAME group, a potent inhibitor of NO production, increases serum total cholesterol<sup>22)</sup> and promotes the atherosclerotic involvement of the thoracic aorta in hypercholesterolemic rabbit.<sup>20)21)</sup> On the other hand, Nakamura et al and Böger et al suggested that L-NAME failed to promote the atherosclerotic involvement of the thoracic aorta or coronary artery in a hypercholesterolemic rabbit.<sup>22)23)</sup> In the present study, we demonstrated that the long-term oral administration of L-NAME promoted the development of atherosclerotic lesions in the aortic arch and impaired endothelial function in hypercholesterolemic hamsters. The mechanisms for how L-NAME promotes atherosclerosis remain still unclear. NO is not only a potent vasodilator but also has an inhibitory effect in platelet adhesion and aggregation.<sup>24)25)</sup> NO also has been reported to inhibit monocyte adherence to cultured endothelial cells<sup>26)</sup> and to have antiproliferative activity in on vascular cells.<sup>27)38)</sup> A recent study indicated that the long-term oral administration of L-arginine, a metabolic precursor for NO, reverses the attenuated endothelium-dependent relaxation of aortic rings and prevents atherosclerotic development in hypercholesterolemic rabbits.<sup>30)</sup> We also observed that systolic blood pressure was increased as the result of the oral administration of L-NAME. Reports have appeared suggest that high doses of NO synthase inhibitors given to rats acutely<sup>30)</sup> or chronically<sup>31)</sup> cause an increase in systolic blood pressure. Thus, reducing the production of endothelium-derived NO is atherogenic and L-NAME-induced hypertension might have more than a few involvements in progression of atherosclerosis.

### **Constrictive Response induced by ET-1(1-31) on Normal and Atherosclerotic Aorta**

Constriction induced by ET-1 in atherosclerosis is not well established in the literature. ET-1(1-31) had a vasoconstrictive activity in hamster thoracic aorta that was about 10 times less than that of ET-1(1-21) in this study. The sensitivities of both ET-1(1-21) and ET-1(1-31) to vasoconstrictive responses were not altered at different stages of atherosclerosis (Fig. 4). Maguire et al and Pernow et al also reported that vasoconstrictive activity, induced by ET-1(1-21), was not different between histologically atherosclerotic coronary arteries and normal coronary arteries.<sup>32)33)</sup> These findings suggested that ET-1(1-31) might have a minimal effect on maintaining the homeostasis of vascular tone, and that the sensitivities of ET-1(1-31) to vasoconstrictive responses might not be related to the progression of atherosclerosis.

### **ET-1(1-31)-like Immuno-reactivity in Different Histological Stages of Atherosclerosis**

ET-1(1-21)-like immuno-reactivity was observed to only a slight extent in the normal aorta, and gradually increased in neointima as atherosclerotic lesions became histologically advanced (Fig. 4). ET-1(1-21) plays an important role in the maintenance of basal

vascular tone<sup>3)</sup> and a number of reports have suggested that the expressions of ET-1(1-21) gene levels and ET-1(1-21)-like immuno-reactivity are increased in atherosclerotic lesions.<sup>5-7)</sup> ET-1(1-31)-like immuno-reactivity was barely observed in normal aortas and to only a slight extent in early atherosclerotic lesions, especially around the neointima as inflammation was beginning (Fig. 4). Moreover ET-1(1-31)-like immuno-reactivity levels were dramatically increased in, not only the neointima but also the adventitia in the case of severe atherosclerosis with serious inflammation (Fig. 4). It has recently been reported that ET-1(1-31) was the predominant bioactive peptide among the ET-derivatives in human neutrophils.<sup>34)</sup> In addition, ET-1(1-31) exhibited chemotactic activities toward human neutrophils and monocytes<sup>35)</sup> and stimulated eosinophil recruitment.<sup>36)</sup> These findings suggest that ET-1(1-31) exerts a role in inflammation, but presumably at different stages of atherosclerosis from that of ET-1(1-21). Atherosclerosis is generally to be an inflammatory disease.<sup>37)</sup> ET-1(1-31)-like immuno-reactivity was observed around macrophages as ET-1(1-21)-like immuno-reactivity was observed in this study (Fig. 5). This suggests that ET-1(1-31) had a minimal effect on vascular tone but may be involved in the inflammatory mediation during the progression of atherosclerosis, which might have a different pathophysiological significance from conventional ET-1(1-21).

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