t3.1 Table 3
t3.2 Odds ratio and 95% CI of each risk factor by logistic regression analysis

t3.3	Hypercholesterolemia	4.485* (1.495–12.28)
t3.4	DM	4.167* (1.477–10.81)
t3.5	DM+Hypercholesterolemia	8.652* (2.543–13.68)
t3.6	Hypertension	2.151 (0.845–9.26)
t3.7	Age	2.953**(0.985–10.36)
t3.8	Positive finding in TMTest	17.590*** (6.77–47.02)

t3.9 * $P<.01$.
t3.10 ** $P<.05$.
t3.11 *** $P<.001$.

We therefore divided the patients into four groups (Table 3): Gp HC, hypercholesterolemic patients (n=42; 72.0±0.5 years old; LDL-C, 150.7±10.4 mg/dl; exercise tolerance, 6.4±0.2 METs); Gp DM, diabetic patients (n=30; 72.3±0.9 years old; HbA1C, 7.6±0.5 g/dl; disease duration, 12.0±1.2 years; 6.0±0.5 METs); Gp HC+DM, hypercholesterolemic and diabetic patients (n=36; 71.4±0.8 years old; LDL-C, 149.5±11.5 mg/dl; HbA1C, 7.0±0.3 g/dl; disease duration for diabetes, 12.9±1.1 years; 6.4±0.3 METs); and Gp C, nondiabetic and nonhypercholesterolemic patients (n=39; 71.6±0.9 years old; 6.2±0.4 METs). The mean age and the frequency of other coronary risk factor complications, such as hypertension, smoking, and others, were not significantly different among the four groups. The TMT-positive ratios were 28.6%, 33.3%, 52.7%, and 16.3% in participants from the Gp HC, DM, HC+DM, and C groups, respectively (Fig. 1). Only three participants complained of chest pain during the TMT test (two in Gp HC+DM and one in Gp HC), and all of them became symptom-free within 5 min after exercise; all other positive patients were symptom-free. The ratios of patients receiving CAG per TMT-positive patient within 8 months after TMT were 66.7%, 63.6%, 68.4%, and 62.5% in the Gp HC, DM, HC+DM and C groups, respectively. CAG was not done for the following reasons: (1) patient refusal, lack of understanding of the CAG, and/or coronary intervention due to risk (n=11); (2) a high risk of coronary intervention for other general diseases such as chronic renal failure or cerebral infarction (n=6); and (3) physician refusal due to the risk of coronary intervention or CAG because of cognitive impairment, and others (n=6). In some patients who did not receive CAG but were suspected to have stenotic lesion by other examinations, medication such as anti-platelets and/or NO donors, such as isosorbide dinitrate, was prescribed. More than 75% stenosis was observed in 75.0%, 71.4%, 69.2%, and 60.0% of CAG-receiving patients of the Gp HC, DM, HC+DM, and C groups, respectively, and coronary intervention was performed in all of these cases (Fig. 1). During the 4.1±0.5 years of observation, ischemic coronary diseases such as angina pectoris or acute myocardial infarction occurred in 4.7% (8.3), 3.3% (10.0), 5.5% (5.3), and 0% of patients in the Gp HC, DM, HC+DM, and C groups, respectively (the percentage for TMT-positive patients). Older patients (older than 75 years of age) had more events (7.3% vs. 0%) than did the relatively younger patients (65–74 years; $P<.001$). Cardiac death was significantly more frequent in older

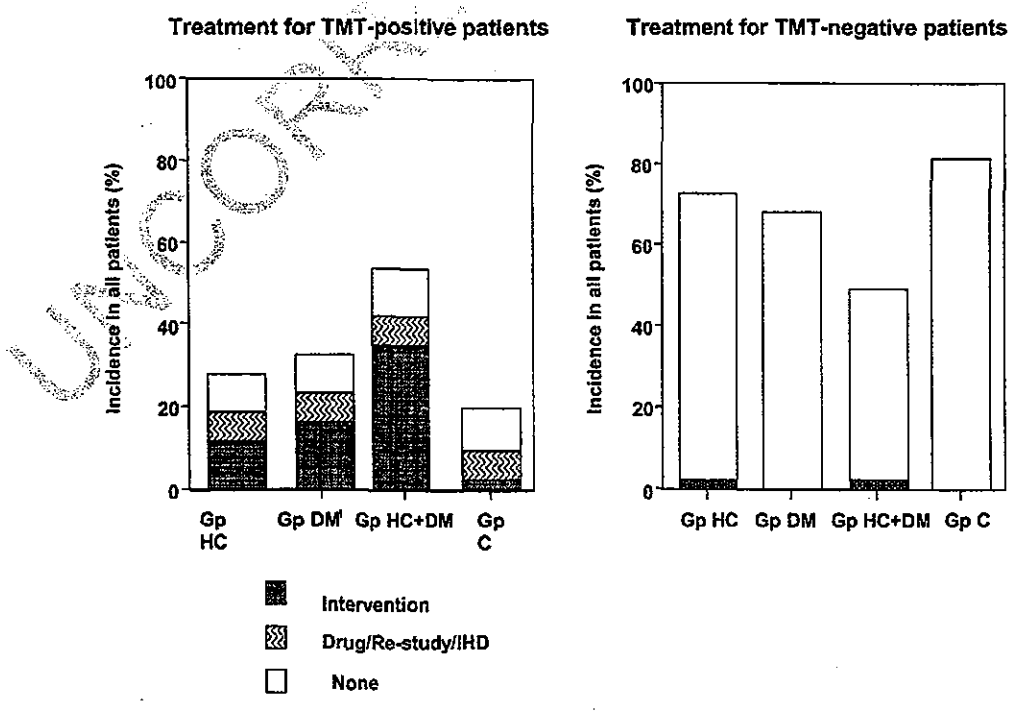

Fig. 1. Left: The frequency of TMT-positive findings and the corresponding treatments chosen for each disease group. Gp HC: hyper-cholesterolemic patients (n=42); Gp DM: diabetic patients (n=30); Gp HC+DM: hypercholesterolemic and diabetic patients (n=36); Gp C: nondiabetic and nonhypercholesterolemic patients (n=39). Right: The frequency of TMT-negative findings and the corresponding treatments chosen for each disease group.

t4.1 Table 4
t4.2 Patients profile who have coronary stenosis by CAG study, acute coronary syndrome, or drug treatment without CAG

t4.3		Percentage (%) of possible IHD				
t4.4		Total	In TMT-positive patients	In TMT-negative patients	Sensitivity of TMT for IHD	Specificity of TMT for IHD
t4.5	Gp HC (42)	23.2	75.0	5.6	72.7≦	96.7
t4.6	Gp DM (30)	26.7	72.7	0	66.7≦	100
t4.7	Gp HC+DM (36)	41.7	73.7	5.9	68.8≦	94.1
t4.8	Gp C (39)	10.3	66.7	0	66.7≦	100

t4.9 Possible IHD means significant stenosis, ACS, and drug treatment during the observation term (4.1±0.5 years).
t4.10 Sensitivity is calculated by (ACS and significant stenosis)/(TMT-positive patients—patients treated by drug without CAG).
Specificity is calculated by (no ACS or no significant stenosis)/(TMT-negative patients).

patients ($P<.001$). Finally, significant stenosis observed by CAG, IHD, or medical intervention during follow-up term was observed in 75.0%, 72.7%, 73.7%, and 66.7% of TMT-positive patients in the Gp HC, DM, HC+DM, and C groups, respectively. Sensitivity and specificity were calculated as shown in Table 4, and they mean the reliability and usefulness of TMT for the diagnosis or speculation of IHD.

4. Discussion

The elderly population is increasing all over the world and Japan is now the world's most aged society. Elderly individuals with IHD have higher rates of physical disability, as defined by a diminished ability to perform the activities of daily living, than do persons without IHD. Older age and clinical manifestations of angina pectoris or chronic heart failure are known to be associated with the highest rates of disability (Morey, Pieper, Crowley, Sullivan, & Puglisi, 2002). The odds ratio for age was also found to be significantly high in the present study (2.953; $P<.05$).

TMT using a protocol for the elderly was shown in the present study to be safe and possibly useful for maintaining independent activities of daily living in the elderly, as the positive ischemic signs evaluated by TMT showed an odds ratio of 17.59 despite the fact that 90% of patients testing positive were asymptomatic. The exercise tolerance (mean=6.1±0.5 METs) determined in the present study indicates that the elderly have the capacity to maintain the activities of daily living, including voidance and using the stairs. The optimal test duration is from 8 to 12 min, and the protocol workloads should be adjusted to permit this duration (Myers & Froelicher, 1993).

The odds ratios for each risk factor, as determined by logistic regression analysis, were the following: DM, 4.167; HC, 4.485; and DM+HC, 8.652 ($P<.01$, respectively). Hypertension, however, was not found to be significant (2.151; $P=.053$). Although the importance of diabetes as a coronary risk factor is well known, almost all patients with a positive TMT test were asymptomatic and showed a relatively high percentage of coronary stenosis. TMT is useful in screening for diabetic coronary macroangiopathy. The frequency of the TMT-positive ratio was found to be relatively high in the present study; we speculate that this finding was due to the fact that the study participants had suffered from diabetes for long periods and to our adoption of the standards of the AHA exercise-tolerance test (Gibbons, Balady, Basley, et al., 1997). We also examined 166 patients younger than 65 years as young control participants; these patients underwent TMT using a symptom-limited modified Bruce protocol and were followed for 4.0±0.8 years (data not shown). Their positive ratios were less than 15%, even in the patients with diabetes complicated with hyperlipidemia (data not shown). Despite a paucity of data on the predictive value of stress tests in older populations, current stress-testing guidelines extend the following recommendations to all adults aged 65 and older (Gibbons et al., 1997). The value of exercise training in patients older than 65 years is supported by a recent study involving 772 men with coronary heart disease, in which physical activity (walking, in particular) for a total of at least 4 hours per week was associated with a significant reduction in overall mortality. Thus, TMT should be useful in cardiac-rehabilitation programs for the elderly. Regarding the interpretation of these findings, a number of limitations should be mentioned. Goyara, Jacobsen, Pellikka, et al. (2000) found that exercise capacity, but not ST-segment changes, was predictive of mortality and cardiovascular events, but they did not distinguish patients who were older than 75 years of age from those who were younger. These findings do not agree with those of the present study, although we cannot identify the reason for this discrepancy. In our study, some patients did not undergo CAG due to patient or physician refusal, and others. Although the risk associated with CAG is small, some physicians cannot justify it fully to patients. This study was also confined to those patients who were referred for exercise testing and thus were able to walk on a treadmill. Despite these facts, our results demonstrated conclusively that TMT was useful for the prevention and management of ischemic coronary artery disease in elderly patients, especially in those with diabetes.

Especially, the high value of specificity of TMT means that TMT-negative finding means the less possibility of IHD and that TMT can be used as screening test of IHD for independent elderly.

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A new HMG-CoA reductase inhibitor, pitavastatin remarkably retards the progression of high cholesterol induced atherosclerosis in rabbits

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Abstract

Background: The remarkable anti-atherosclerotic effects of 3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitor have not been demonstrated in diet induced severe hyperlipidemia in rabbit model. *Objective:* We have investigated the effect of pitavastatin, a newly developed statin, on atherosclerosis in rabbits. *Methods and results:* Oophorectomized female NZW rabbits were fed 0.3% cholesterol chow for 12 weeks with or without pitavastatin (0.1 mg/kg per day) (Gp.NK and HCD). The level of serum cholesterol was decreased in Gp.NK compared with Gp.HCD (772.8 ± 70.2 versus 1056.9 ± 108.3 mg/dl), whereas no significant alterations were observed in triglyceride and HDL-cholesterol. NO dependent response stimulated by acetylcholine and calcium ionophore A23187 and tone related basal NO response induced by N^G-monomethyl-L-arginine acetate were all improved by pitavastatin treatment. Pitavastatin treatment increased the level of cyclic GMP in the aorta of cholesterol fed rabbits. In the aorta, the expression of eNOS mRNA was significantly up regulated and O_2^- production was slightly reduced in Gp.NK animals. Atherosclerotic area was significantly decreased in aortic arch and thoracic aorta from Gp.NK compared with those from Gp.HCD (15.1 ± 5.3 versus 41.9 ± 10.2%, 3.1 ± 1.1 versus 7.9 ± 1.2% in Gp.NK and Gp.HCD aortic arch and thoracic aorta). Anti-macrophage staining area, the MMP1 or 2 and the nitrotyrosine positive area were decreased in Gp.NK. *Conclusion:* Pitavastatin retards the progression of atherosclerosis formation and it improves NO bioavailability by eNOS up-regulation and decrease of O_2^-.

Keywords: Nitric oxide; Endothelial nitric oxide synthase; Superoxide anion; HMG-CoA reductase inhibitor; Atherosclerosis

1. Introduction

3-Hydroxymethyl-3-glutaryl-CoA (HMG-CoA) reductase inhibitors (statins) are potent inhibitors of cholesterol biosynthesis in the liver by blocking the conversion of HMG-CoA to mevalonate [1]. They were widely used for the treatment of hyperlipidemia and used for the prevention of coronary artery disease. Landmark clinical trials with pravastatin (WOSCOPS) and simvastatin (4S) demonstrate that these statins decrease the serum cholesterol level and reduce the incidence of myocardial infarction and also cardiovascular mortality [2,3]. Additionally, several large statin trials such as AFCAPS/TEXCAPS and LIPID showed the beneficial effect of other statins [4,5].

Pitavastatin is a newly developed statin whose cholesterol reducing effect is stronger than the other new statins such as atorvastatin or lovastatin and its side effects such as liver dysfunction, were reported less when compared to the other statins [6]. However, the anti-atherosclerotic effect of pitavastatin on high cholesterol diet induced atherosclerosis was unknown in the rabbit model. High cholesterol diet itself inhibits HMG-CoA reductase activity of cells throughout the body, especially in the liver. In addition, application of statin inhibits HMG-CoA reductase absolutely, and the complete suppression of HMG-CoA reductase inhibitor may result in it being impossible of produce cell membrane composed of cholesterol, a life-threatening condition. It is thus very important to evaluate the anti-atherosclerotic effect of

Abbreviations: NO, nitric oxide; NOS, nitric oxide synthase; e-NOS, endothelial nitric oxide synthase; $ONOO^-$, peroxynitrite

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strong statin administration using high cholesterol diet induced atherosclerosis animal models.

HMG-CoA reductase inhibitors were shown to improve the endothelial function in a short time period [7]. Superoxide anion (O_2^-) production was increased in vessels of hyperlipidemic rabbits, and the release of peroxynitrite; $ONOO^-$ (formed from the reaction of NO and O_2^-) release was also increased in atherosclerosis [8]. These studies demonstrated that atherosclerosis was closely related to the level of NO production and reactive oxygen species (ROS). Hence, the present study was decided to determine whether the anti-atherosclerotic effects of pitavastatin is observed, and whether it is mediated by its lipid lowering effect and/or nitric oxide or superoxide mediated system in high cholesterol diet induced atherosclerosis in oophorectomized female rabbits. We used rabbits because they are herbivorous and easy to make atherosclerotic and it is also easy to damage liver function by high cholesterol diet or statins. Further, as sex steroids are known to affect on atherosclerosis formation via NO and antioxidant action, we used oophorectomized female rabbits in this study [9].

2. Materials and methods

2.1. Chemicals and solutions

Acetylcholine chloride (ACh), prostaglandin F2α (PGF2α), indomethacin and L-monomethyl-arginine (L-NMMA) were purchased from Sigma Chemical Co. (St. Louis, MO). Nitroglycerin (NTG) was from Nihon Kayaku Co. (Tokyo, Japan). Krebs'–Henseleit solution (118 mM NaCl, 4.7 mM KCl, 1.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 11 mM glucose, and 0.002 mM EDTA; disodium ethylendiamine-tetraacetic acid, pH7.4) was saturated with 95% O_2/5% CO_2. All concentrations are those in the final bath.

2.2. Animals

A total of 28 female New Zealand white rabbits, 3–4 months aged, weighing about 2.0 to 2.4 kg were obtained from Kitayama Rabbis (Ina, Japan). The rabbits were housed individually at 20 ± 3 °C with free access to water. Twenty rabbits were bilaterally oophorectomized and 8 were left non-oophorectomized. Four weeks after oophorectomy, the rabbits were divided into two groups (n—each = 10) and treated for 12 weeks. Gp.HCD was fed HCD (regular diet plus 0.3% cholesterol; Gp.NK was fed HCD with pitavastatin (0.1 mg/kg per day). Separately, 10 oophorectomized female rabbits were fed with regular diet with or without pitavastatin (0.1 mg/kg per day)(Gp.R, Gp.R-NK). Feeding was restricted to 120 g per day. Blood was withdrawn 24 h after the last feeding. All the rabbits were appeared to be healthy throughout the course of study. All the experiments were conducted in accordance with institutional guidelines for animal research.

2.3. Determination of plasma lipids

Plasma lipids levels were measured by enzymatic assays as described previously [10].

2.4. Isometric tension measurements

After twelve weeks of treatment, the rabbits were sacrificed by exanguination after being anesthetized with pentobarbital (50 mg/kg i.v.). The thoracic aorta was carefully taken from the portion of the orifice of left first costal artery down to the portion enclosed by the diaphragm, and cut into 2-mm wide transverse rings. Isometric tension measurement was performed as described before [11]. The rings were stretched to their optimal force, which was predetermined as the contractile response to 122 mM KCl, mounted in organ chambers and bathed in Krebs' Henseleit solution at 37 °C. Prostaglandin F2α induced sub-maximal force (2.6×10^{-6} M). Endothelium-dependent relaxation induced by ACh and endothelium-independent relaxation by NTG were determined. To investigate tone-related basal NO release assessed by responses to L-NMMA from aortic rings, moderate vascular tone (35–50% of the contraction obtained with 122 mM KCl) was induced by low prostaglandin F2α concentrations (0.8×10^{-6} M). In some experiments, indomethacin (5×10^{-6} M) was added for 60 min before the experiment to rule out contribution of prostanoids.

2.5. Histological evaluation of atherosclerosis and assays for tissue cholesterol content

Cross-sections of the aorta adjacent to segments of vascular responses were examined [12]. Briefly, the contours of the lumen and the internal elastic lamina (IEL) were traced. The mean surface involvement by atherosclerotic lesion per vessel (extent) was calculated after dividing the lesion circumference by the circumference of the internal elastic lamina. The circumferences of the lesion and the healthy region were defined as the circumferences of the respective parts of the internal elastic lamina. The area occupied by atherosclerotic lesions (total lesion burden: size/thickness) was defined as the % area bounded by the lumen and internal elastic lamina for luminal area (n = 6 for one vessel). The intima/media ratio was also measured. A 0.8 cm-long segment was homogenized and lipids were extracted and resuspended, then cholesterol levels were measured [13].

2.6. Immunocytochemical analyses

Cross-sections of the thoracic aorta were analyzed as described previously [14]. They were incubated with primary monoclonal antibody [for anti-macrophages (RAM11), smooth muscle cells (HHF35), MMP-1 and -2, nitrotyrosine or iNOS] for 60 min at room temperature. Negative controls

included substitution of primary antiserum for either PBS or irrelevant antibodies. Each field was scored for number of each antibody positive cells on slides and analyzed statistically as described by previous report [14]. Five samples were prepared from each rabbit.

2.7. Determination of cyclic GMP (cGMP)

The aortic cGMP concentration was determined by a specific radioimmunoassay (RPN226, Amersham, Buckinghamshire, England) [15]. Four aortic rings (each wet weight is 10 ± 1 mg) per rabbit were investigated.

2.8. Measurement of endothelial NO synthase (eNOS) mRNA

The expression of eNOS mRNA in the arterial wall was measured by RT-PCR methods [16]. Briefly, to make a DNA competitor, we designed and synthesized two primers [5′-ATTTAGGTGAC-ACTATAGAATACCAGTGTCCAA-CATGCTGCTGGAAATTGGTACGGTCATCATC-TGAC-AC-3′ (sense primer), 5′-TAAAGGTCTTCTTCCTGGTGA-TGCCAATACATC-AAACGCCGCGAC-3′ (anti-sense primer)] based on the sequences of human eNOS cDNA. We used a competitive RNA transcription kit (Takara Shuzo, Otsu, Japan). eNOS cDNA primers amplify a product with a predicted length of 486 bp, and the competitor was produced at a length of 558 bp. The same amount of mRNA was corrected using a β-actin.

2.9. Detection of aortic superoxide anion (O_2^-) generation

Formation of O_2^- from vessel was assayed by measuring the intensity of chemiluminescence probes in the presence of one of the Cypridina luciferin analogs, 2-methyl-6-(p-methoxyphenyl)-3,7-dihydroimidazo[1,2-a] pyrazine-3-one (MCLA) [17]. In brief, the O_2^- generation signal from the 2 mm length of vessel with or without endothelium was detected by a luminescence reader (BLR-201, Aloka Co., Tokyo). To ensure the specificity of MCLA to detect O_2^- increasing concentrations of SOD (1–50 U/ml) were added to the tissues.

2.10. Data analysis

Results were expressed as mean±S.E.M. Data were compared by analysis of variance with repeated measurements. A level of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Plasma lipid concentration

Plasma lipid levels were measured before oophorectomy and after 0, 4, 8 and 12 weeks of oophorectomy. The addition of 0.3% cholesterol to the diet increased the total cholesterol level significantly compared with the baseline value. Plasma cholesterol levels were decreased in the pitavastatin group at 4, 8 and 12 weeks after oophorectomy compared with that of the HCD group. There were no significant differences in other lipid components such as HDL-C observed between the control and treated group animals (Table 1). Pitavastatin treatment in the regular diet group did not show any change of lipid profile (data not shown).

3.2. Histological examination of atherosclerosis

Histological examination of the thoracic aortae revealed more atheromatous lesions, as indicated by the mean percentage of luminal encroachment and the mean lesion area, in the hypercholesterolemic (Gp.HCD) than in NK104 treated groups (Gp.NK) (Fig. 1). Regular diet group showed no atherosclerotic lesion w/wo pitavastatin treatment.

3.3. Endothelium-dependent and -independent relaxation

In all groups, ACh produced endothelium dependent relaxations (EDRs) of the aortic rings with an intact en-

Table 1
Profile of plasma biochemistry

	BeO	0 week	4 weeks	8 weeks	12 weeks
T.Chol. (mg/dl)					
HCD	58.2±4.9	62.2±5.5	884.2±110.5	1082.2±123.3	1056.9±110.4
NK104	56.2±5.5	59.1±4.9	582.2*±95.8	752.4*±91.7	772.8*±79.1
Control	59.2±5.9	58.1±5.9	54.2±4.9	60.4±6.3	56.2±5.3
T.G. (mg/dl)					
HCD	48.5±6.3	51.2±8.3	54.2±10.1	57.4±13.7	52.9±24.0
NK104	46.2±6.5	49.1±5.1	51.2±10.3	52.1±11.7	40.8±6.8
Control	49.6±5.1	48.6±7.1	47.4±5.9	50.8±8.9	53.1±15.3
HDL-C (mg/dl)					
HCD	34.4±4.8	31.2±4.3	34.1±2.1	32.2±3.1	34.4±4.3
NK104	33.2±5.1	31.4±3.4	37.2±4.6	36.2±4.6	34.4±3.2
Control	34.6±5.1	31.5±2.1	31.4±5.2	30.8±4.9	30.1±5.1

* $P < 0.05$ vs. control.

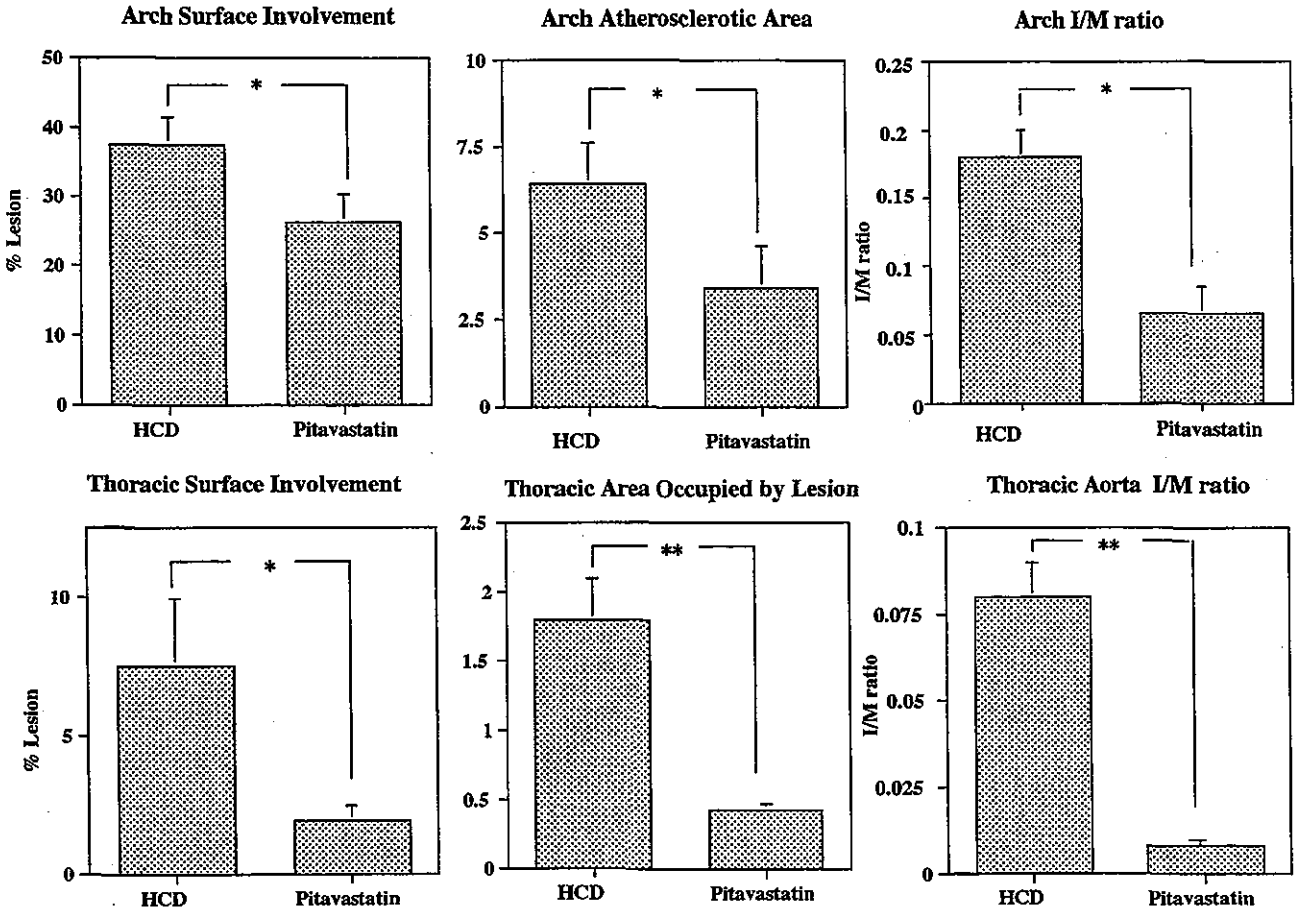

Fig. 1. Histological evaluation of atherosclerotic area (upper) of the thoracic aortae (lower). Left: The surface involvement of atherosclerotic area in the aortic arch and the thoracic aorta from of rabbits (Gp.HCD: high cholesterol diet [HCD, standard diet plus 0.3% cholesterol], Gp.NK, pitavastatin: HCD plus pitavastatin (1 mg/kg per day) $*P < 0.05$, $**P < 0.01$. Center: The area occupied by atherosclerotic areas of the aortic arch and the thoracic aorta from four groups of rabbits. Right: The Intima/Media ratio of the aortic arch and the thoracic aorta from four groups of rabbits.

dothelium (Fig. 2, left). The magnitude of the relaxation of aorta from the hypercholesterolemic animals (Gp.HCD) was diminished compared to those from regular diet group w/wo pitavastatin. However, EDRs in arteries from Gp.NK was remarkably larger than that from Gp.HCD. There was no significant difference in the response of vessels from regular diet group (Gp.R) and pitavastatin treated hypercholesterolemic animals (Gp.R-NK, data not shown). The non-receptor mediated vasorelaxation by calcium ionophore, A23187 showed the same tendency as ACh induced relaxation (data not shown). The endothelium-independent vasodilator, NTG, produced concentration-dependent relaxation in the thoracic aortic rings. No significant difference in relaxation was observed in aortic rings of all groups (Fig. 2, center). The inhibition of NOS by L-NMMA led to a contractile response in the aortic rings. The L-NMMA contractile response was higher in pitavastatin treatment (Gp.NK) (Fig. 2, right). Preincubation of indomethacin did not affect EDRs (data not shown).

3.4. Tissue cyclic GMP concentration

NO activates soluble guanylate cyclase in smooth muscle cell and led to produce cGMP. We examined cGMP concentration in homogenate samples of rabbit aorta. Pitavastatin treatment showed a significant increase of cGMP level Gp.NK as compared with HCD group ($3.11 + 0.42$ versus $2.24 + 0.34$ pmol/wet g in Gp.NK versus Gp.HCD, $P < 0.05$).

3.5. Detection of mRNA for endothelial NO synthase

The ethidium bromide-stained bands were quantified by densitometry from a photograph of the gel. The signal for eNOS increased about 50% in samples from aortae of hypercholesterolemic rabbits (Gp.HCD), as compared to those from control (Gp.R)(data not shown). The amount of eNOS mRNA was increased in Gp.NK compared with that in Gp.HCD.

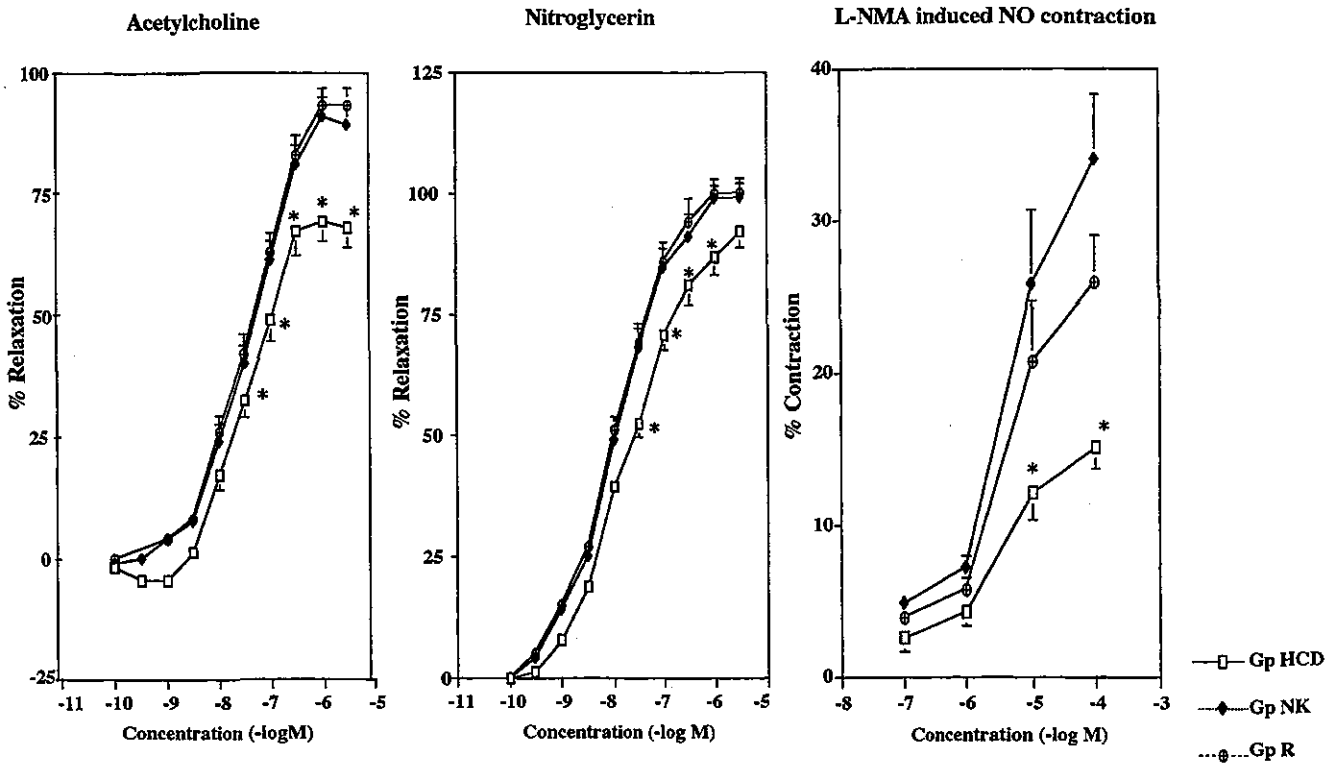

Fig. 2. Left: Cumulative concentration-response curves to acetylcholine (ACh) during contraction evoked by prostaglandin F2α (2.6×10^{-6} M) in the thoracic aortas of rabbits fed with a high-cholesterol diet (HCD), HCD plus pitavastatin (NK), or a regular rabbit chow (R). Significant difference ($^*P < 0.05$) vs. HCD. Data are shown as means ± S.E.M. Center: Cumulative concentration-response curves to L-NMMA during contraction evoked by prostaglandin F2α (0.8×10^{-6} M). Right: Cumulative concentration-response curves to nitroglycerin (NTG) during contraction evoked by prostaglandin F2α (2.6×10^{-6} M) in the thoracic aortas. There is no significant difference between three groups.

3.6. An aortic superoxide anion production

We measured superoxide anion production from arterial wall with lucigenin analogue (MCLA). The chemiluminescence signals (CL signals) as superoxide anion production increased in aorta from cholesterol fed rabbits (Gp.HCD) as compared with regular diet group of rabbits (Gp.R) (Fig. 3 right). CL signals from vascular tissue with endothelium showed a decrease in Gp.NK as compared with HCD group. It means that the amount of O_2^- released is greater in aorta from HCD group than in those from pitavastatin group. In aorta without endothelium, CL signals were decreased in pitavastatin treated rabbits as compared to cholesterol fed rabbits (Fig. 3 right). The endothelium dependent chemiluminescence was drastically decreased in pitavastatin treated group. In other words, the relative difference of aortic O_2^- generation between HCD group and pitavastatin group was higher in the part of endothelium, and pitavastatin treatment decreased O_2^- generation more in endothelium than that in components of vessels other than endothelium. The amount of O_2^- released decreased in Gp.NK compared with in Gp.HCD.

3.7. Immunohistochemical study

Immunohistochemical analyses demonstrated a significant decrease in the number of macrophage derived cells in the atherosclerotic lesions in pitavastatin treated rabbits as compared to those from HCD group (Fig. 4). At the same time, the number of smooth muscle derived cells in atherosclerotic lesions of pitavastatin treated rabbit aortae tended to be decreased without statistical significance (data not shown). Pitavastatin treatment not only reduced the area of atherosclerosis, but also decreased the area stained by the macrophage antibody, the area stained by the iNOS antibody, and the areas positive for $ONOO^-$ established by nitrotyrosine staining. MMP-1 (interstitial collagenase), a matrix metalloproteinase that initiates collagen degradation, was localized predominantly in macrophages. The expression of MMP-1 and MMP-2 decreased in the pitavastatin treated group compared with that of Gp.HCD.

4. Discussion

Epidemiological studies have shown that lipid lowering therapy with statins such as simvastatin leads to a significant reduction in cardiac mortality and morbidity [2–5]. Atorvastatin was also shown to reduce the progression of coronary atherosclerosis through its strong lipid lowering action. In this experiment, we tried to investigate the anti-atherosclerotic effect of pitavastatin, a newly developed

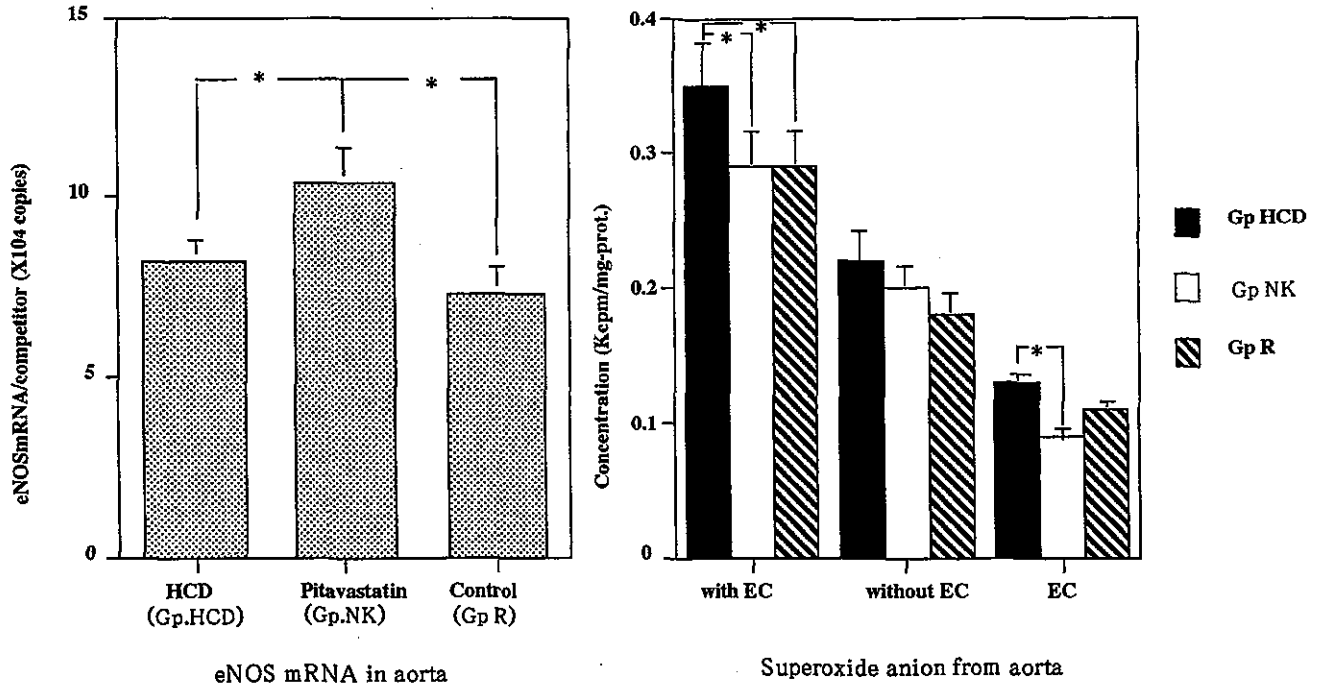

Fig. 3. *Left: Quantification* of eNOS mRNA in aortic arch using competitive RT-PCR. Ethidium bromide-staining gel after electrophoretic resolution of the competitor (558 bp) and eNOS target bands (486 bp) were determined. Relative amounts of eNOS mRNA to competitors using densitometry was shown. Data are shown as means ± S.E.M. Significant difference ($*P < 0.05$). Right: Effects of Pitavastatin on superoxide production from rabbit aortae. 'Endothelium+' means the data of vessel with endothelium. 'Endothelium−' means the data of vessel without endothelium.

statin, on aorta by selecting dose of pitavastatin which was reported to be comparable to the dose used in humans [6]. Attention has recently been focused on the molecular mechanisms responsible for these effects of statins, as well as their lipid lowering action. The present study therefore focuses on the status of endothelial functions, especially NO related, as determined by vascular responses. We measured nitric oxide metabolites, cGMP concentration, and eNOS mRNA expression in oophorectomized rabbits with or without pitavastatin treatment. In addition, we examined the O_2^- generation in the vessels with or without endothelium, immunohistochemistry related to peoxynitrite, matrix metalloprotease and apoptosis, and atherosclerotic lesions of hypercholesterolemic rabbits with or without pitavastatin treatment.

The HMG-CoA reductase inhibitors are potent inhibitors of cholesterol biosynthesis [1], decreasing serum cholesterol level by blocking the hepatic conversion of HMG-CoA to L-mevalonate in cholesterol biosynthetic pathway [1]. In the present study, serum cholesterol level was significantly decreased whereas no difference was observed in TG and HDL cholesterol (Table 1). This lipid lowering effect of pitavastatin in high cholesterol induced atherosclerotic rabbit model may be stronger than that of fluvastatin or simvastatin which we did previously, and the data is compatible with clinical data reported in Japan [18–20]. On the other hand, there are few reports concerning the effect of atorvastatin or lovastatin where the lipid lowering effects in rabbits were reported to be stronger than simvastatin or fluvastatin in hyperlipidemic patients. As these statins have inhibited HMG CoA reductase strongly, it may cause liver damage in rabbit. Pitavastatin was reported to have stronger LDL receptor induction in liver, however weaker HMG-CoA reductase inhibition than atorvastatin or simvastatin [21].

The EDRs were impaired in animals with experimentally induced atherosclerosis, which has been correlated to the decreased biological activity of endothelium derived NO [9,13]. The present investigation shows that endothelium dependent nitric oxide mediated relaxation in response to acetylcholine and calcium ionophore. A23187 and tone-related basal NO release evaluated by L-NMMA contraction were improved significantly by pitavastatin treatment (Fig. 2). The improvement of endothelial function by statin is often attributed to the reduction in serum cholesterol concentration. Indeed, a study demonstrated that a single treatment of LDL apheresis is sufficient to significantly improve EDRs in hypercholesterolemic humans [22]. Further, tissue cGMP concentration in aorta was also increased by pitavastatin treatment (Fig. 3). NO activates vascular smooth muscle soluble guanylate cyclase, thereby increasing cGMP in turn responsible for decreased intracellular Ca^{2+} concentration. The increased cGMP concentration clearly indicates that the increased production and bioavailabity of NO. In other words, increase of cGMP and greater contraction of aorta in response to L-NMMA shows increase of the basal

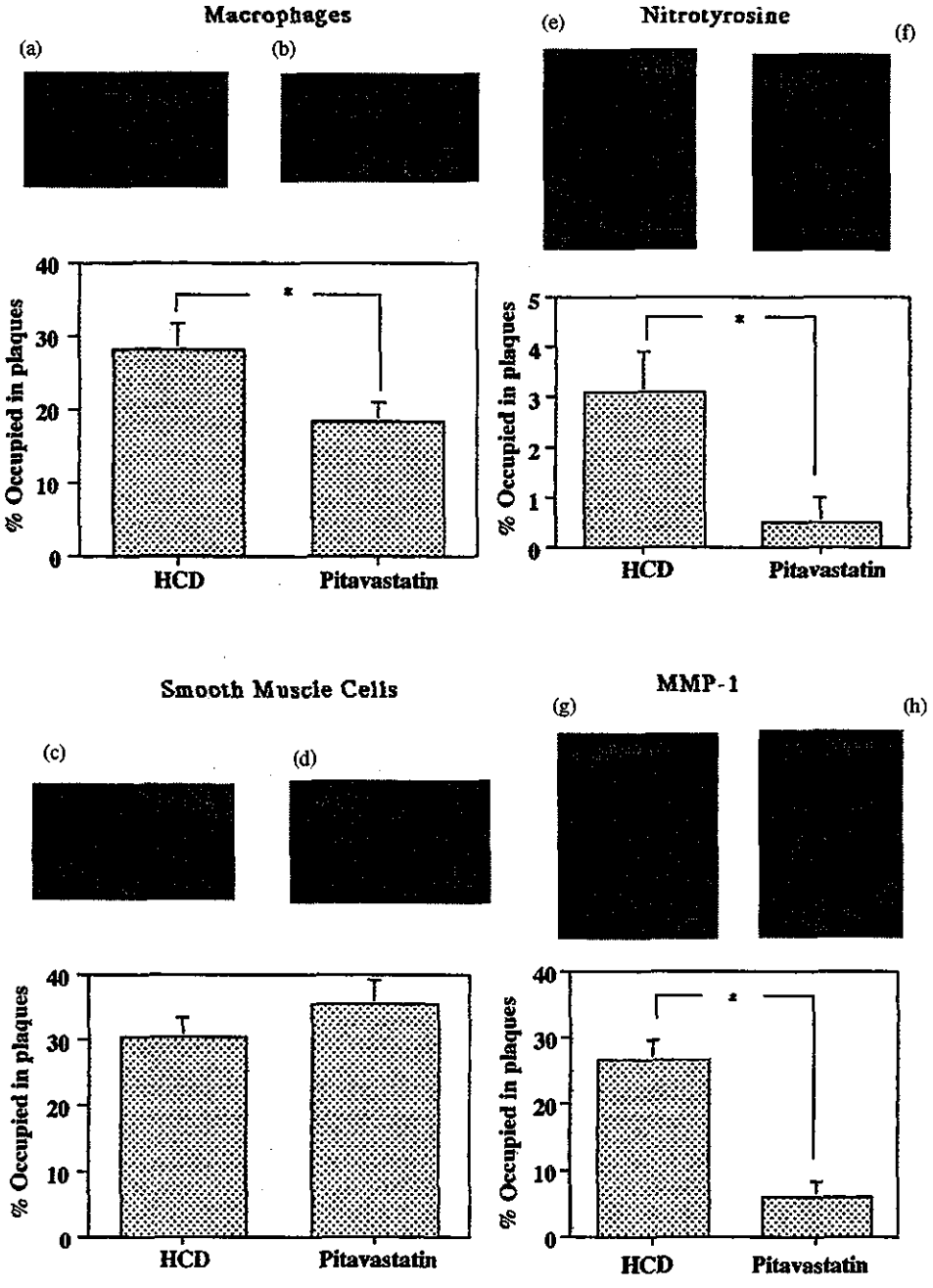

Fig. 4. Photograph: Immunohistochemical analysis of the thoracic aortae of rabbits from the HCD group and NK group (pitavastatin) using macrophages (a, b), smooth muscle cells (c, d), nitrotyrosine, a marker of peroxynitrite (e, f) and MMP-1 (g, h). Original magnification: 100× (a, b, c, d); 200× (e, f, g, h). Bar is 50 μM. Graph: % occupied area in atherosclerotic plaque by macrophages, smooth muscle cells, nitrotyrosine and MMP-1 evaluated by immuno-histochemistry.

release of NO. We hypothesized two mechanism of this improved NO bioavailability.

The eNOS mRNA expression was increased significantly in the aorta of pitavastatin treated rabbits (Fig. 3). This result is compatible with the observation that eNOS mRNA expression was increased in simvastatin treated cultured endothelial cells without changing lipid sub-fraction in the medium [23], and that the eNOS mRNA expression was increased by the stabilization of mRNA, not by the stimulation of transcription [23]. eNOS upregulation and inhibition of iNOS induction by statin were also reported [24]. We have also observed the increased expression of eNOS mRNA and protein in pitavastatin treated cultured bovine aortic endothelial cells and that it was also mediated by the stabilization of eNOS mRNA (data not shown). The increased expression of eNOS mRNA attributes increased NO synthesis. In endothelial cells, eNOS protein is translocated to the caveolae for myristoylation and palmitoylation. Our preliminary experi-

ment based on immunohistochemical study showed that the majority of eNOS protein exists in cytoplasm of endothelial cells in atherosclerotic lesions of cholesterol diet fed rabbits whereas almost all of eNOS exist in membranous part of aortic endothelial cells of regular diet fed rabbits (data not shown). The eNOS mRNA was increased by cholesterol diet in this study and recent other studies [16]. Taken together, we speculated the possibility that eNOS activity was regulated by both mRNA level and location of protein in cells.

The other mechanism of improved NO bioavailability is the decrease of O_2^- production. The oxidative inactivation of NO is regarded as an important cause of its decreased biological activity. O_2^- reacts with NO faster than SOD and forms peroxynitrite anion [25]. The peroxynitrite anion oxidizes sulfhydryls groups and yields products indicative for hydroxyl radical reaction with deoxyribose and dimethyl sulfoxide. These reactions induce membrane lipid peroxidation, to stimulate progression of vascular atherosclerosis. The presence of peroxynitrite-derived nitrotyrosines in atherosclerotic lesions has been demonstrated in our previous study in rabbit models [26]. The vascular release of superoxide was increased significantly in hypercholesterolemia and atherosclerosis [9]. This study shows that O_2^- production was decreased in arteries by pitavastatin treatment, especially in endothelial cells. Among several oxidases, as O_2^- producing enzymes, three are possible candidates in the release of O_2^- from endothelial cells. In the hypercholesterolemic rabbit, increasing serum activity of xanthine oxidase release increased amounts of O_2^- [27]. Recently, NO was reported to inhibit, in vitro [28], xanthine oxidase and xanthine dehydrogenase, which are present in endothelial cells. NADPH oxidase exists in culture endothelial cells and smooth muscle cells activated by TNF-α, and its activity is increased in hypercholesterolemia [29]. eNOS was also one of the candidates, because it was reported to release O_2^- in diabetic vessels [30]. Preliminarily, our data have shown that pitavastatin decreases O_2^- from NADPH oxidase in endothelial cells, and eNOS did not release O_2^- in high-cholesterol diet induced atherosclerosis (data not shown). However, we have to consider that statins may have a potential effect on superoxide production by mitochondria, considering the potential effects of statins on the metabolism of CoQ10 [31]. Coenzyme Q can undergo oxidation/reduction reactions in other cell membranes such as lysosomes, plasma membranes; deficiency of coenzyme Q has been described based on failure of biosynthesis by statins [31].

Nitroglycerin mediated endothelium independent relaxation is also improved by pitavastatin treatment. We speculated that it was due to the retardation of atherosclerosis formation by pitavastatin. However it is possible that pitavastatin has some effect on smooth muscle cell sensitivity to NO: It maybe necessary to elucidate more to understand the underlying mechanism.

Accumulation of macrophages in the vascular wall might be responsible for a variety of pathological events, such as generation of superoxide radicals, oxidation of LDL, subsequent foam cell formation, and release of cytokines, resulting in smooth muscle cell proliferation, and migration. The present investigation depicts the decreased number of macrophages in the intima following pitavastatin treatment. It may be due to the prevention of macrophages adhesions and migration by increasing NO bioavailability.

To determine other mechanisms of the anti-atherosclerotic effect of pitavastatin, we investigated the proportion of MMP-1 and 2 positive areas. The interstitial collagenase (matrix metalloproteinase-1, MMP-1) and MMP-2 expression in the lesion were measured by quantitative image analysis [32]. MMP-1 is localized predominantly in the macrophages and that plays a key role in initiating collagen degradation. The baseline lesions in the HCD group expressed high levels of MMP-1. Macrophage-related proteolysis might contribute to weakness of the protective fibrous cap of the plaque (Fig. 4). A reduction of both the macrophage content and the expression of immunoreactive MMP-1 were observed in aortae from pitavastatin treated rabbits. Immunoreactive MMP-2 showed the same tendency. This suggests that pitavastatin treatment plays a major role in plaque stabilization. Conclusively, the present study demonstrates that pitavastatin safely reduces plasma cholesterol level in high-cholesterol diet induced atherosclerosis, and that the anti-atherosclerotic effect of pitavastatin is mediated at least partly by increasing endothelium dependent vascular responses, eNOS mRNA expression, cGMP level and decreasing superoxide anion production. The antiatherosclerotic property of pitavastatin is due to two major pathways: one is due to its pleiotropic effect, such as improvement of endothelial function; the second stems from its lipid lowering effect. Although suggest the experiment was carried out in rabbits, the results suggest new possibilities of the usefulness of pitavastatin in cases of atherosclerosis, due to its NO bioavailability.

Acknowledgements

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Gene transfer of endothelial NO synthase, but not eNOS plus inducible NOS, regressed atherosclerosis in rabbits

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Abstract

The effects of in vivo gene transfer of endothelial nitric oxide synthase (eNOS) and inducible NOS (iNOS) genes on severe atherosclerosis were investigated in rabbits. The recombinant adenoviruses, Ad.eNOS and Ad.iNOS, which respectively express eNOS and iNOS, were constructed. Atherosclerosis was induced by a balloon injury followed by a high cholesterol diet for 12 weeks. The rabbits were divided into six groups: Gp cont (no treatment); Gp null (adenovirus sham-infected); Gp eNOS (Ad.eNOS); Gp iNOS (Ad.iNOS); Gp e+i (Ad.eNOS plus Ad.iNOS); and Gp heNOS (a high dose of Ad.eNOS). Examinations were carried out 7 days after gene transfer. Plasma lipid levels were not significantly changed, but transfection with Ad.eNOS (Gp eNOS and Gp heNOS) decreased the tissue cholesterol concentration and regressed atherosclerotic lesions. Vessels treated with Ad.iNOS (Gp iNOS and Gp e+i) showed iNOS staining in the atheroma, and slight staining at other parts of the vessels; those treated with Ad.eNOS showed eNOS staining in the endothelium and subintima, and slight staining at other parts. Ad.eNOS transfection, but not Ad.iNOS or Ad.eNOS+Ad.iNOS transfection, improved the impaired aortic endothelium-dependent relaxation (EDR) and basal NO-dependent response, increased tissue cyclic GMP (cGMP), and decreased the release of O_2^- from vessels. eNOS treatment showed a decreasing tendency in regions with peroxynitrite staining, MMP1 staining, and suspected apoptosis. In conclusion, in vivo gene transfer of eNOS, but not iNOS or eNOS plus iNOS, regressed atherosclerosis. The relations among NO, O_2^-, and peroxynitrite may be critical, and lipid resorption from the lesions may be responsible for the regression.

Keywords: Atherosclerosis; Nitric oxide; Gene theraphy

1. Introduction

The atherogenic process is characterized by an early deficit in nitric oxide (NO) and related biomolecules [1,2]. Chronic inhibition of NO in addition to a high cholesterol diet has been shown to induce severe atherosclerosis [3]. These data suggest that NO has direct effects on the progression of atherosclerosis. In fact, NO has anti-atherosclerotic effects, because it can inhibit monocyte adhesion to endothelium, smooth muscle cell chemotaxis, and proliferation [4]. It has been speculated that improving NO bioavailability, which in turn helps to restore the impaired endothelium-dependent relaxation (EDR) in atherosclerotic vessels, is important to stabilize atheroma and regress atherosclerotic lesions—two important goals in the treatment of atherosclerosis. On the other hand, removing dietary cholesterol has not been consistently shown to restore EDR [5,6]. In addition, clinical and experimental trials have failed to show sufficient regression of atheroma by dietary cholesterol reduction [5,7]. Even if such a regression occurs, it is

Abbreviations: NO, Nitric oxide; NOS, Nitric oxide synthase; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; NTG, nitroglycerin; EDR, endothelium-dependent relaxation; L-NMA, N^G-monomethyl-L-arginine acetate, inhibitor of NO synthase; ACh, acetylcholine chloride; SOD, superoxide dismutase; PGF2α, prostaglandin F2α.

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expected to take years to obtain a partial regression of atherosclerosis in humans, although such a regression occurs quickly in animals [5,7]. We speculated that vascular gene transfer of nitric oxide synthase (NOS) isoforms via adenoviruses might be effective to hasten the process of NO-induced regression of the atheroma and lesions associated with atherosclerosis. Nabel et al. [8] successfully transfected genes in vivo by using a double-balloon catheter in a pig ileofemoral artery. Ooboshi et al. [9] showed that ex vivo endothelial nitric oxide synthase (eNOS) gene transfer improved the impaired EDR, and Leyen et al. [10] reported that in vivo eNOS gene transfer inhibited neointimal vascular lesions in rabbits. However, the effects of inducible NOS (iNOS) on the vascular function and the regression of atherosclerosis remain a subject of controversy [11,12]. In this study, we first ensured the successful regression of severe atherosclerosis by eNOS gene transfer. To speculate on the dose-dependency of the transferred NOS, we prepared two doses of Ad.eNOS vectors. To determine whether or not iNOS gene transfer is beneficial, we compared the effects of in vivo gene transfer of eNOS and iNOS in a model of advanced atherosclerosis with vascular dysfunction. We investigated the exact role played by iNOS when combined with eNOS gene transfer to confirm its synergistic effect with eNOS gene transfer.

2. Materials and methods

2.1. Chemicals

Acetylcholine chloride (ACh), prostaglandinF2α (PGF2α), indomethacin, and N^G-monomethyl-L-arginine acetate (L-NMA) were purchased from Sigma (St. Louis, MO). Nitroglycerin (NTG) was obtained from Nihon Kayaku (Tokyo, Japan). Monoclonal antibodies against eNOS and iNOS (Transduction Laboratories, Lexington, KY), rabbit macrophages (RAM11), muscle actin-specific one (HHF35) (DAKO, Carpinteria, CA), nitrotyrosine (Upstate Biotechnology, Lake Placid, NY), MMP-1 (Fuji Yakuhin, Takaoka, Japan) and β galactosidase (Chemicon International, Lexington, NY) were also used [6,13].

2.2. Construction and purification of recombinant adenovirus

Recombinant adenoviruses containing eNOS cDNA and iNOS cDNA were constructed using an ADENOQUEST Kit (Quantum, Quebec, Canada) [14]. Briefly, bovine eNOS cDNA (provided by Dr. T. Michel, Harvard University) and rat iNOS cDNA (provided by Drs. H. Esumi and Y. Ogura, National Cancer Center, Tokyo) were cloned into the AdBM5pAG vector. The resulting plasmid was co-transfected with viral DNA into 293 cells. For all of the studies, the viral titers were adjusted to 2×10^9 pfu/ml. Adenoviruses carrying an *Escherichia coli* Lac Z gene encoding a nucleus-localized variant of β galactosidase (Ad. β gal) or no cDNA (Ad.null) were also used. To investigate the expression of eNOS and iNOS in normal and atherosclerotic aorta, we separately prepared four rabbits (two animals were fed with a regular diet, and other two animals were fed with 0.5% cholesterol and regular diet for 12 weeks). Gene transfer of eNOS and iNOS was performed using dispatch catheter as described in Section 2.3 into abdominal aorta in regular diet and high cholesterol diet groups of rabbits. Animals were sacrificed at day 4 for the detection of the transgene [14].

2.3. Animals

Male New Zealand white rabbits (age, 3–4 months; weight, 2.0–2.4 kg) were obtained from Kitayama Rabbis (Ina, Japan) and housed with free access to water. The abdominal aortae were injured by means of a balloon catheter as described previously [15]. In brief, a 3Fr Fogarty catheter was inserted from right femoral artery and advanced as far as the just below diaphragm. The balloon was inflated with 0.6 ml of saline and the catheter pulled three times until reaching a portion of the bifurcation of iliac arteries. Finally, the balloon was deflated and the catheter withdrawn. After aortic injury, atherosclerosis was induced by feeding regular chow plus 0.5% cholesterol for 12 weeks. The animals were divided into six groups ($n=8$ each): Gp cont (no treatment); Gp null (treated with Ad.null); Gp eNOS (Ad.eNOS); Gp iNOS (Ad.iNOS); Gp e+i (Ad.eNOS plus Ad.iNOS); and Gp heNOS (high dose of Ad.eNOS). In an additional experiment, the rabbits were treated with Ad. β gal, Ad.eNOS plus Ad.null, or Ad.iNOS plus Ad.null after aortic injury and feeding with regular chow plus 0.5% cholesterol for 12 weeks ($n=6$, each). As an additional control, six rabbits were fed regular chow for 12 weeks, with neither aortic injury nor cholesterol supplementation (Gp regular control). In all experimental trials, transfection of genes into the arteries was done using a 3.2 Fr. dispatch catheter as follows (Boston Scientific, Boston, MA). Under anesthesia with pentobarbital (20 mg/kg intravenously), a catheter was advanced into the abdominal aorta to facilitate intramural drug delivery via longitudinal strips (about 3.5 cm) of six raw-profile 0.254-mm injector plots capable of penetrating delivery. Inflation of the balloon at 6 atm was followed by injection of 0.3 ml (Gp cont, Gp null, Gp eNOS and Gp iNOS), 0.6 ml (Gp e+i) or 3 ml (Gp heNOS) of viral solution over a period of 3 min. In an additional experiment, the viral injection volume was 0.3 ml (β gal treatment group) or 0.6 ml (Ad.null plus Ad.eNOS treatment group or Ad.null plus Ad.iNOS treatment group). All experiments were conducted in accordance with the institutional guidelines for animal research, and conforms with the Guide for the Care and Use of

Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996).

2.4. Western blot analysis for eNOS and iNOS

The levels of eNOS and iNOS expression in vessels were determined by Western blot analysis [16]. Band intensities were analyzed densitometrically by the National Institute of Health IMAGE program.

2.5. Vascular response

Seven days after gene transfer, the rabbits ($n=6$ each group) were anesthetized with pentobarbital (50 mg/kg

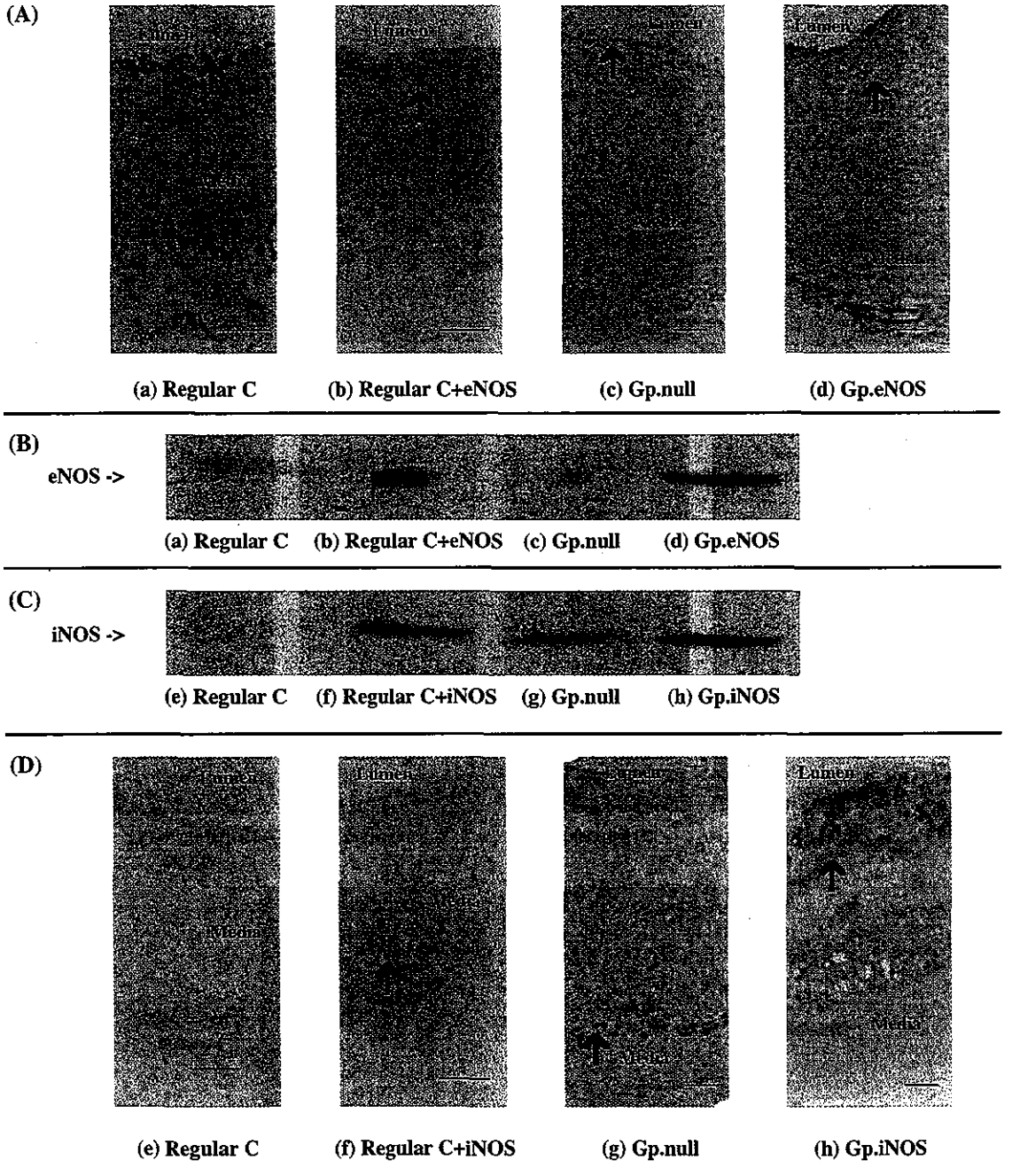


Fig. 1. Upper: localization of eNOS expression by immunostaining (arrows). Arteries from Gp regular c (a), gene transfer of eNOS to Gp regular c (b), Gp null (c), and Gp eNOS (d) were stained. Middle: Western blot analysis for eNOS. Lane 1: control (aorta from Gp regular c); Lane 2: aorta from Gp regular c transfected with eNOS; Lane 3: atherosclerotic control aorta from Gp null; Lane 4: aorta from Gp eNOS (transfected with eNOS to atherosclerotic artery). Western blot analysis for iNOS (f). Lane 1: control (aorta from Gp regular c); Lane 2: aorta from Gp regular c transfected with iNOS. Lane 3: atherosclerotic control (aorta from Gp null), Lane 4: aorta from Gp iNOS (Ad.iNOS). Lower: localization of iNOS expression by immunostaining (arrows). The artery from Gp regular c (e), gene transfer of iNOS to Gp regular c (f), Gp null (g), and Gp iNOS (h) were stained. The scale bar represents 50 μm.

intravenously) and sacrificed by exsanguination. Vascular responses were investigated as described previously [15]. Briefly, aortae were cut into 2-mm-wide transverse rings. The rings were stretched to their optimal force, which was predetermined by a force of 122 mM KCl, and mounted in chambers filled with Krebs'–Henseleit solution at 37 °C. The response of endothelium-intact rings to ACh and that of endothelium-denuded rings to NTG were determined under submaximal contraction induced by PGF2α (2.6×10^{-6} M) [17]. Tone-related basal NO-dependent contractile responses to L-NMA were assessed under moderate tone (about 40% contraction by KCl) induced by PGF2α (0.8×10^{-6} M) [17].

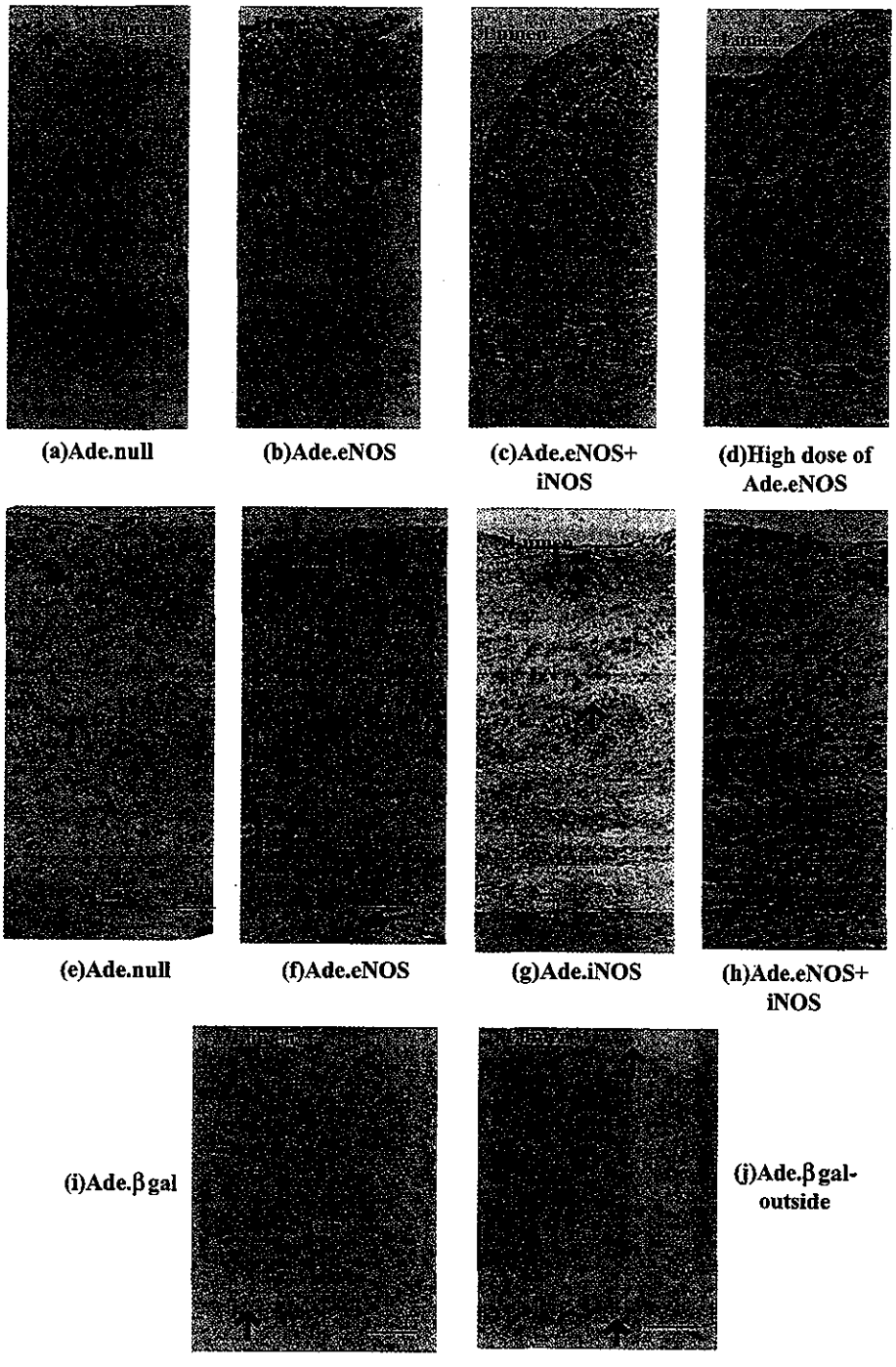


Fig. 2. Upper: localization of eNOS expression by immunostaining. Arteries infected with: (a) Ad.eNOS (Gp eNOS); (b) Ad.eNOS (Gp eNOS); (c) Ad.eNOS plus Ad.iNOS (Gp e+i); and (d) a high dose of Ad.eNOS (Gp heNOS) were stained. Original magnification, × 150. Middle: localization of iNOS (e, f, g and h) expression by immunostaining. Arteries infected with: (e) Ad.null; (f) Ad.eNOS; (g) Ad.iNOS; (h) Ad.eNOS+iNOS. Original magnification, × 100. Lower: localization of β gal expression by immunostaining. The section from the gene transfer site with Ad. β-gal (Gp β-gal) (i). The section from the site outside the gene transfer site of Ad. β-gal (Gp β-gal) (j). Original magnification, × 150. The scale bar represents 50 μm.

Table 1
Lipid and NO related profile

	Tissue			Tissue		Tissue		
	T. Chol. (mg/dl)	T.G. (mg/dl)	HDL-C (mg/dl)	T. Chol. (mg/wet g)	E. Chol. (mg/wet g)	cGMP (pmol/wet g)	NOx (nM/wet g/24 h)	$O2^-$ release (μM)
Gp cont	1771.0 ± 210.3	66.9 ± 8.9	67.4 ± 7.3	5.62 ± 0.26	3.18 ± 0.42	3.04 ± 0.28	4.6 ± 0.7	0.31 ± 0.06
Gp null	1826.1 ± 171.3	61.8 ± 8.2	62.0 ± 4.0	5.45 ± 0.16	3.05 ± 0.31	2.63 ± 0.21	4.4 ± 0.6	0.34 ± 0.04
Gp eNOS	1690.5 ± 170.4	62.4 ± 8.5	63.1 ± 5.0	4.78* ± 0.41	2.49* ± 0.41	5.95* ± 0.77	8.1* ± 2.5	0.25* ± 0.04
Gp iNOS	1804.7 ± 183.3	56.2 ± 4.9	59.5 ± 5.7	6.09 ± 0.38	3.31 ± 0.58	3.03 ± 0.45	5.1 ± 1.8	0.43* ± 0.05
Gp e+i	1725.0 ± 110.7	52.9 ± 3.7	52.0 ± 2.7	6.67 ± 0.57	3.47 ± 1.35	3.53 ± 0.17	5.6 ± 1.2	0.45* ± 0.06
Gp heNOS	1712.5 ± 191.4	59.4 ± 9.8	51.1 ± 7.2	4.69* ± 0.51	2.53* ± 0.42	5.81* ± 0.71	9.1* ± 2.1	0.27* ± 0.04
Gp regular c	84.1 ± 12.3	39.1 ± 6.9	49.6 ± 7.7	1.02 ± 0.18	N.D.	3.33 ± 0.75	4.9 ± 1.1	0.19 ± 0.04

T. Chol.: total cholesterol, T.G.: triglyceride, T.P.: total protein, g.t.: gene transfer, N.D.: not detected.
*$P<0.05$ vs. Gp null.

In some studies, indomethacin (5×10^{-6} M) was added to the chambers to rule out the contribution of prostanoids.

2.6. Measurement of lipids

Serum lipids were measured by enzymatic assays [18].

2.7. Measurement of cyclic GMP (cGMP)

The basal concentrations of cGMP in aortae were determined by an enzyme-linked immunoassay (Amersham, Buckinghamshire, UK) [19]. Four rings were used from each rabbit.

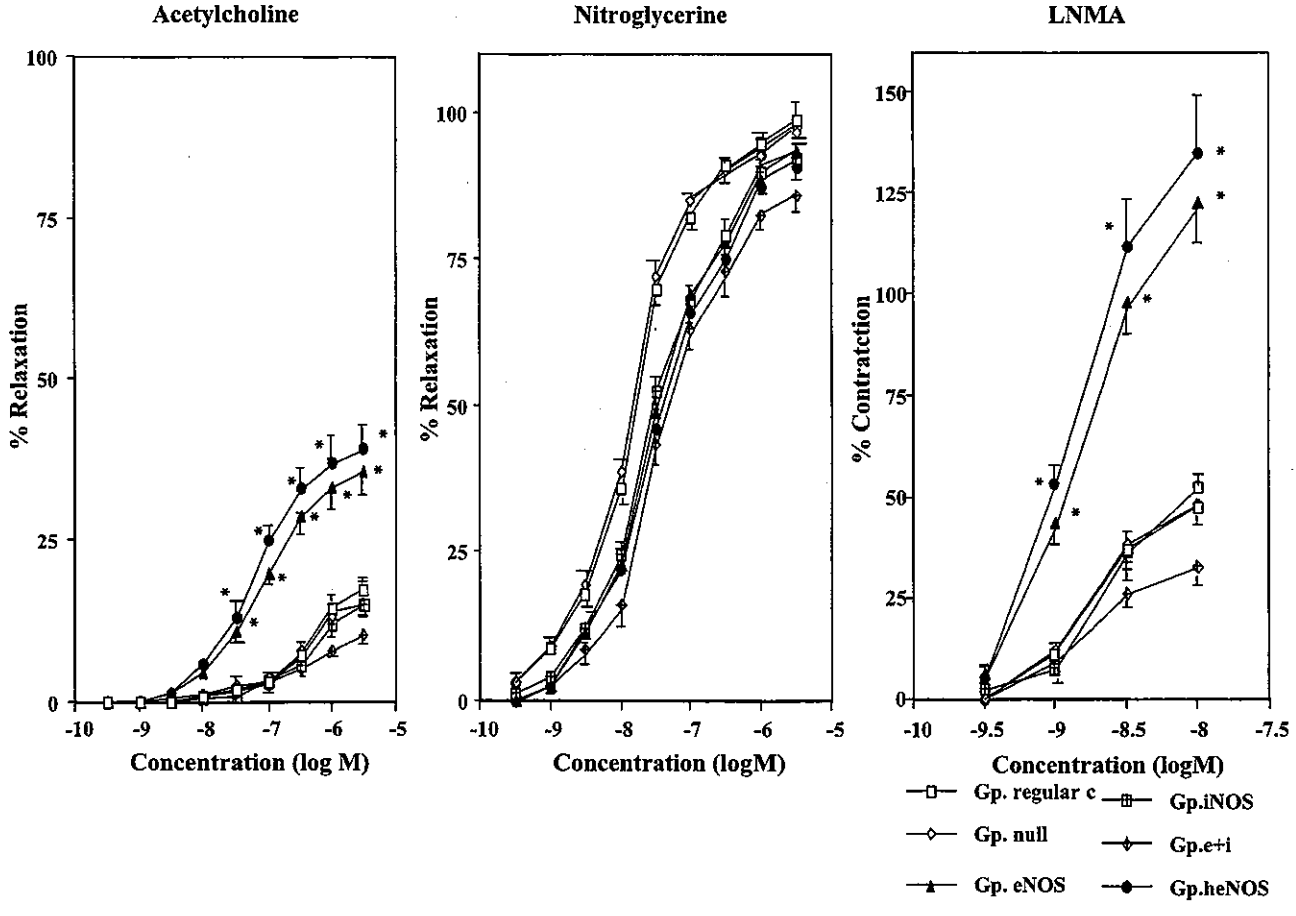


Fig. 3. Cumulative concentration–response curves to each of the agonists in the abdominal aortae of seven groups, which were precontracted by prostaglandin F2α: Gp cont: no treatment; Gp null: Ad.null; Gp eNOS: Ad.eNOS; Gp iNOS: Ad.iNOS; Gp e+i: Ad.eNOS plus Ad.iNOS; Gp heNOS: high amount of Ad.eNOS. Left: cumulative concentration–response curves to acetylcholine. Middle: cumulative concentration–response curves to nitroglycerin. Right: cumulative concentration–response curves to L-NMA; NOS inhibitor. % indicates the percentage vs. the magnitude of the contraction level by PGF2α. *$P<0.05$ vs. Gp null.

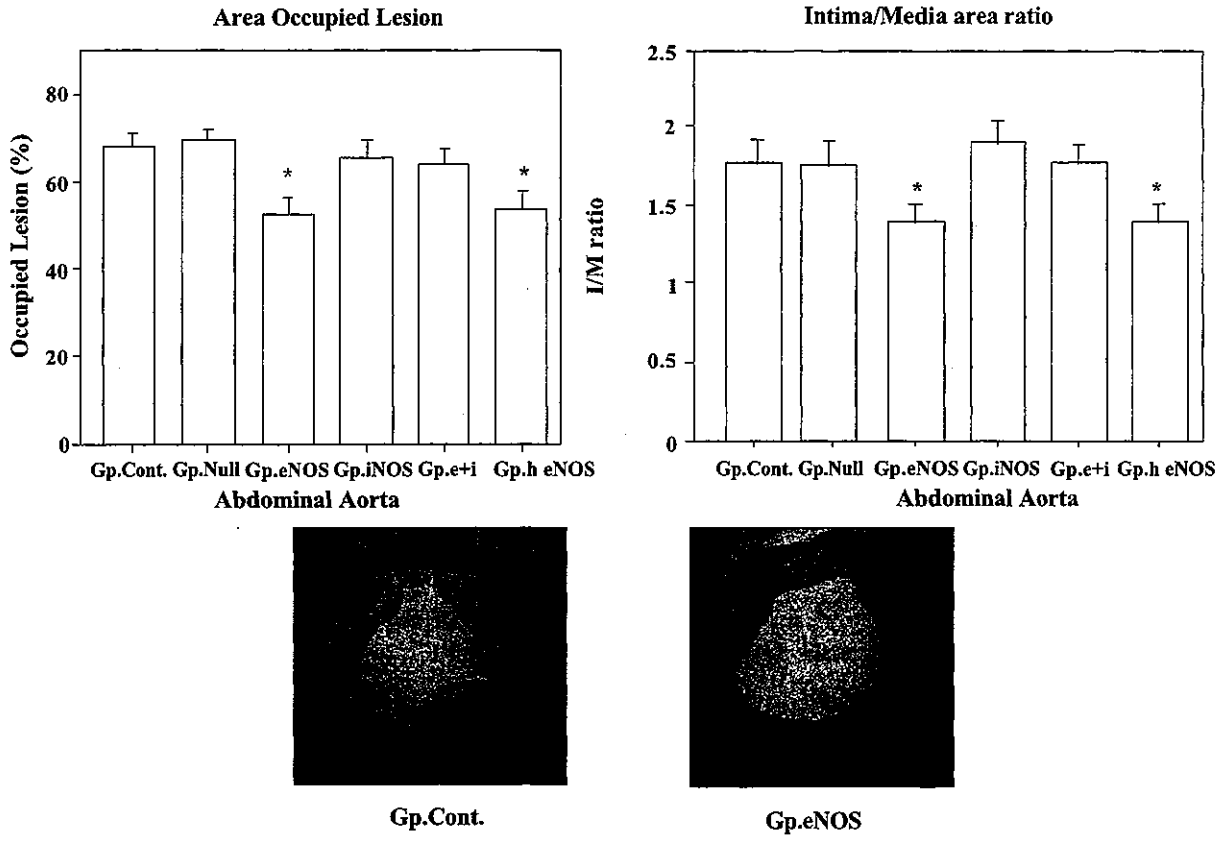


Fig. 4. Histological evaluation of the atherosclerotic area of the aorta as indicated by the mean lesion area (% occupied lesion, left), and the intima/medial ratio (I/M ratio, Right). Gp cont: no treatment; Gp null: Ad.null; Gp eNOS: Ad.eNOS; Gp iNOS: Ad.iNOS; Gp e+i: Ad.eNOS plus Ad.iNOS; Gp heNOS: high amount of Ad.eNOS. Lower: a section stained with H.E. from Gp cont and Gp eNOS. Original magnification, × 40. The scale bar represents 200 μm.

2.8. Measurement of nitrite and nitrate (NO_2^-/NO_3^-) and detection of aortic superoxide generation

NOx (sum of nitrite and nitrate, NO_2^-/NO_3^-) was measured in medium containing 2 mm-wide of abdominal aorta from each group in 24 well culture dishes (medium: 100 μl of phenol red free DMEM + 10% fetal calf serum, cultured for 24 h). An NO detector-HPLC system (ENO10; Eicom, Kyoto, Japan) was used as previously reported [20]. Formation of O_2^- was assayed by measuring the intensity of chemiluminescence probes at physiological pH (7.4). Its signal was detected by a luminescence reader (BLR-201; Aloka, Tokyo). The O_2^- generation signal was defined as the inhibitory signal by superoxide dismutase (SOD, 100 U/ml).

2.9. Histological evaluation of atherosclerosis and assays for tissue

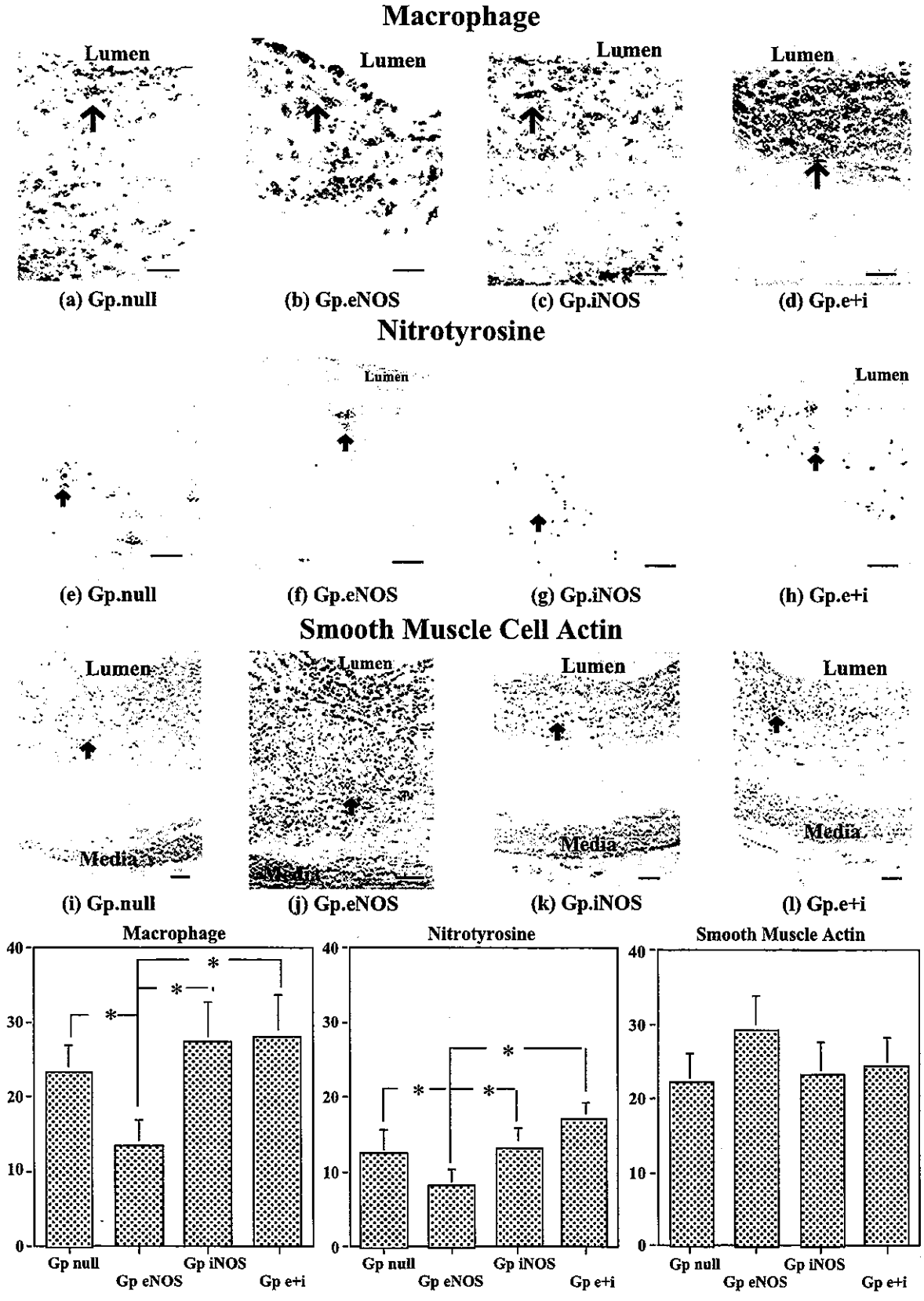
Cross sections of the aorta adjacent to the segments of vascular responses were examined [21]. The contours of the lumen and the internal elastic lamina (IEL) were traced. The area occupied by lesions was defined as the ratio of the area bounded by the lumen and IEL (atherosclerotic area) to the area bounded by the IEL (mean of six sections for one vessel). The intima/media ratio was also measured. A 0.8-cm-long segment of the gene-transferred portion was homogenized, and lipids were extracted to measure the cholesterol level [18].

2.10. Immunohistochemical study

This study was performed as described previously [22]. Tissues were sectioned into 5-mm-thick slices. Primary monoclonal antibodies (macrophages, smooth muscle cells, nitrotyrosine, MMP-1, or β galactosidase, each one × 250) were applied. Sections were incubated with biotinylated immunoglobulin and incubated with horseradish peroxidase-labeled avidin solution. DNA nick-end labeling of tissue sections (TUNEL staining) was performed as described previously [22]. Negative

Fig. 5. The representative immunohistochemical staining of abdominal aortae. Upper: a section stained with a monoclonal antibody against macrophages (arrow) from (a) Gp null: Ad.null; (b) Gp eNOS: Ad.eNOS; (c) Gp iNOS: Ad.iNOS; and (d) Gp e+i: Ad.eNOS plus Ad.iNOS. Original magnification, × 150. Middle upper: a section stained with a monoclonal antibody against nitrotyrosine (arrow): the marker of $ONOO^-$ from (e) Gp null, (f) Gp eNOS, (g) Gp iNOS, and (h) Gp e+i. Original magnification, × 150. Middle lower: a section stained with a monoclonal antibody against the marker of smooth muscle cell actin (arrow) from (i) Gp null, (j) Gp eNOS, (k) Gp iNOS, and (l) Gp e+i. Original magnification, × 100. Lumen: luminal area; media: media. The scale bar represents 50 μm. Lower: % area occupied by macrophages, nitrotyrosine and smooth muscle cells in atherosclerotic plaque was evaluated by immunohistochemistry (each group, $n = 8$).

controls included substitution of primary antibody with irrelevant antibodies. Each slide was scored for the presence of cells that were positive for each type of antibody, and was statistically analyzed as described in our previous report [6]. Five pieces of aortae were prepared from each rabbit.



2.11. Statistical analysis

Data are presented as the means ± S.E.M. Student's *t*-test was used for determination of statistical significance, and Kruskal–Wallis one-way ANOVA was used for multiple comparisons. Values of $p < 0.05$ were considered to indicate statistical significance.

3. Results

All rabbits appeared to be healthy throughout the study period, and body weight and serum total protein were not significantly different among the groups.

3.1. Transgene expression in severe atherosclerotic aortae

Immunohistochemical staining of each NOS gene showed that the gene transfers were successful. Staining of intrinsic eNOS and iNOS was apparent in vessels from the Gp cont. However, only eNOS was observed in vessels from the Gp regular cont (Fig. 1). In the case of vessels treated with Ad.eNOS (Gp eNOS, Gp e+i and Gp heNOS), positive staining for eNOS was observed in a region of the endothelium, throughout the entire subintimal area, and slightly in the media and adventitial area (Fig. 2). In vessels treated with Ad.iNOS (Gp iNOS and Gp e+i), positive staining for iNOS was observed in part of the atheromatic area, especially around the necrotic core, and slightly in the endothelium and adventitial area (Fig. 2). The levels of eNOS and iNOS in the Gp e+i seemed to be almost the same as those in Gp eNOS and Gp iNOS. Even transfection of cells with a large amount of Ad.eNOS resulted in only a slight increase of eNOS expression (Fig. 2). In an additional experiment, the level of eNOS in the Ad.eNOS plus Ad.null treatment group and that of iNOS in the Ad.iNOS plus Ad.null treatment group were almost identical to those in the Gp eNOS and Gp iNOS, respectively (data not shown). Vessels treated with Ad.β galactosidase showed pronounced β galactosidase staining in the atheromatic regions, and slight staining in the adventitia, media, and endothelium (Fig. 2). However, the part of vessels outside of Ad. β galactosidase infection showed little β galactosidase staining (Fig. 2).

3.2. Western blot analysis for eNOS and iNOS

Expression of eNOS was confirmed in aortae of all groups, whether atherosclerosis was induced or not. Pronounced expression of eNOS was confirmed in aortae of the Ad.eNOS transfection groups (5.7 ± 1.6 times staining compared to that of the regular control group). Slight expression of iNOS was detected in aortae from groups in which advanced atherosclerosis was induced. Pronounced iNOS expression was detected in arteries treated with Ad.iNOS (Fig. 1). The amount of iNOS in Ad.iNOS-transduced arteries (Gp iNOS) was also comparable with that of eNOS in Gp eNOS. Even transfection of cells with a large amount of Ad.eNOS produced only a slight increase of eNOS expression (2.1 ± 0.4 times staining compared to that of Gp eNOS).

3.3. Blood chemistry

There were no significant differences in serum lipid levels (Table 1).

3.4. Vascular responses

Contraction by PGF2α in the Ad.eNOS-transfected groups (Gp eNOS, 1.9 ± 0.2 g; Gp heNOS, 1.8 ± 0.2 g) was slightly smaller than that of the other groups (2.2 ± 0.2, 2.2 ± 0.1, 2.0 ± 0.3 and 2.3 ± 0.3 g in the Gps cont, null, iNO, and e+i). Balloon injury and atherogenic diet diminished the ACh-induced relaxation in the aortae (Fig. 3). Relaxation was almost eliminated in the aorta of Gp cont, Gp null, Gp iNOS, and Gp e+i animals. Ad.eNOS transfection remarkably improved EDR (Fig. 3). The EDR levels in vessels from Gp e+n and Gp i+n were almost the same as those in vessels from Gp eNOS and Gp iNOS (data not shown). No significant difference was observed in NTG-induced relaxation among the aortae of all groups (Fig. 3). Tone-related basal NO-dependent contraction induced by L-NMA was highest in the aortae from Gp eNOS and Gp heNOS (Fig. 3). In an additional experiment, the contraction was also increased in the aortae from the Ad.eNOS plus Ad.null group, but was not increased in the group treated with Ad.iNOS plus Ad.null (data not shown). This finding indicates that the aortae from Gp eNOS, Gp heNOS, and the Ad.eNOS plus Ad.null group released larger amounts of tone-related basal NO than the vessels of the other groups. Preincubation with indomethacin did not affect EDR (data not shown).

3.5. Histological evaluation of atherosclerosis and aortic cholesterol content

Ad.eNOS regressed the atherosclerotic lesions in the abdominal aorta; however, transfection with a large amount of Ad.eNOS (Gp heNOS) resulted in only a slight increase in regression relative to that in Gp eNOS (Fig. 4). In an additional experiment, the total areas of lesions in the aortae of the Ad.eNOS plus Ad.null group or Ad.iNOS plus Ad.null group were almost identical to that of Gp eNOS or Gp iNOS, respectively. Total and esterified cholesterol content in vessels exhibited the same tendency as the atherosclerotic areas (Table 1).

3.6. Measurement of cyclic GMP

The concentrations of cGMP in aortic tissues from Gp eNOS and Gp heNOS increased significantly compared to other groups (Table 1).