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

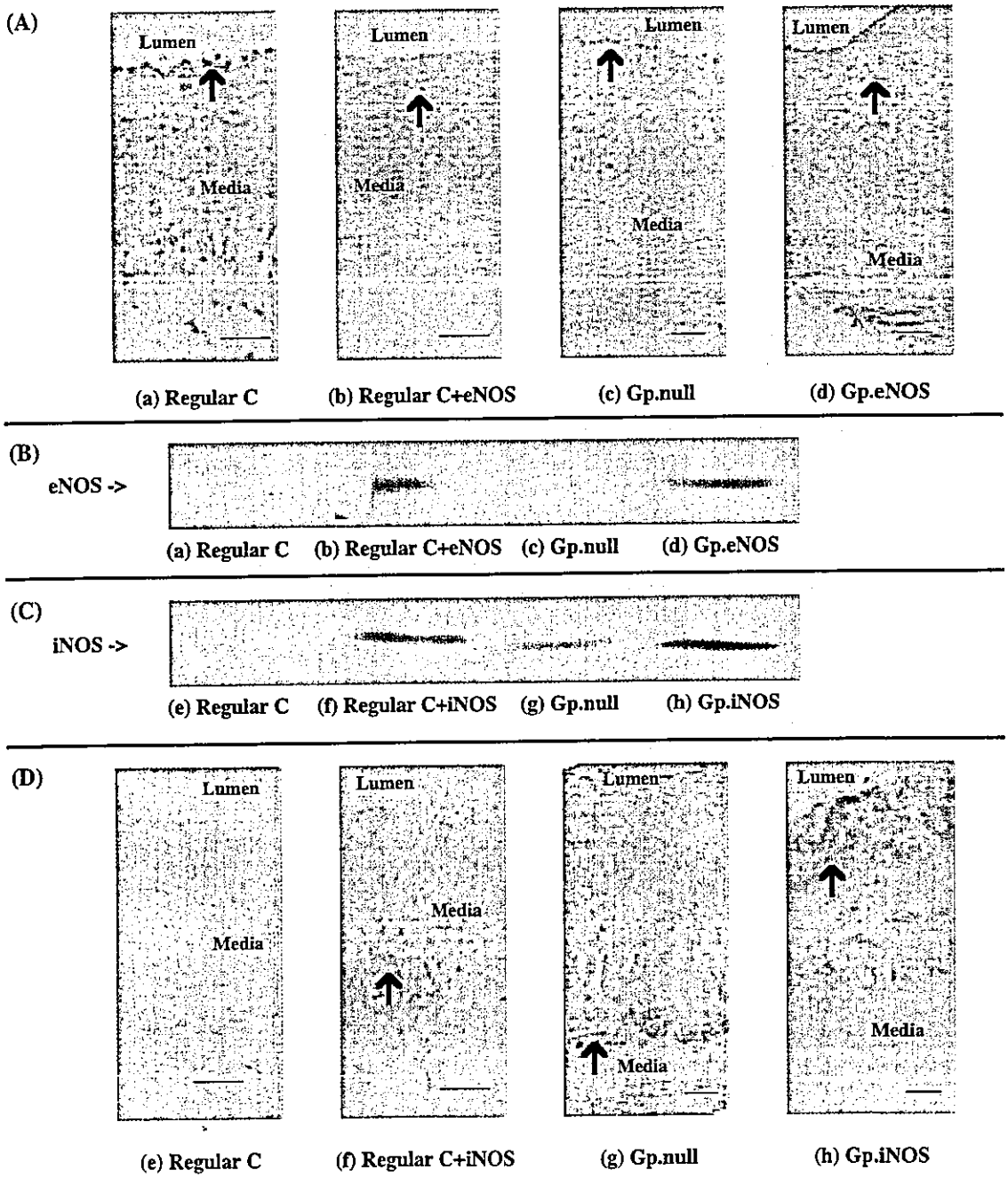


Fig. 1. Upper: localization of eNOS expression by immunostaining (arrows). Arteries from Gp regular c (a), gene transfer of eNOS to Gp regular c (b), Gp null (c), and Gp eNOS (d) were stained. Middle: Western blot analysis for eNOS. Lane 1: control (aorta from Gp regular c); Lane 2: aorta from Gp regular 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].

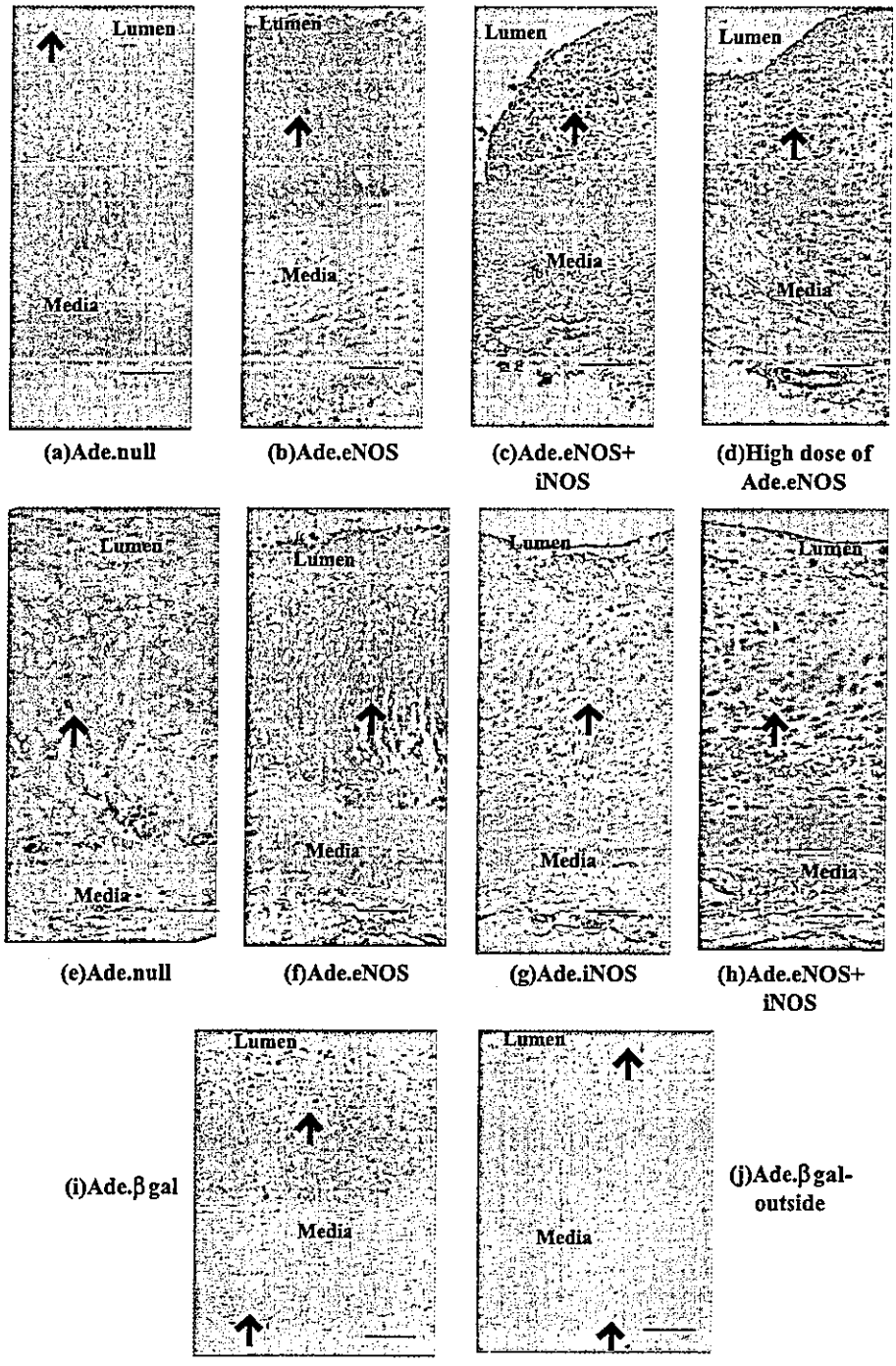


Fig. 2. Upper: localization of eNOS expression by immunostaining. Arteries infected with: (a) Ad.eNOS (Gp eNOS); (b) Ad.eNOS (Gp eNOS); (c) Ad.eNOS plus Ad.iNOS (Gp e+i); and (d) a high dose of Ad.eNOS (Gp heNOS) were stained. Original magnification, × 150. Middle: localization of iNOS (e, f, g and h) 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.

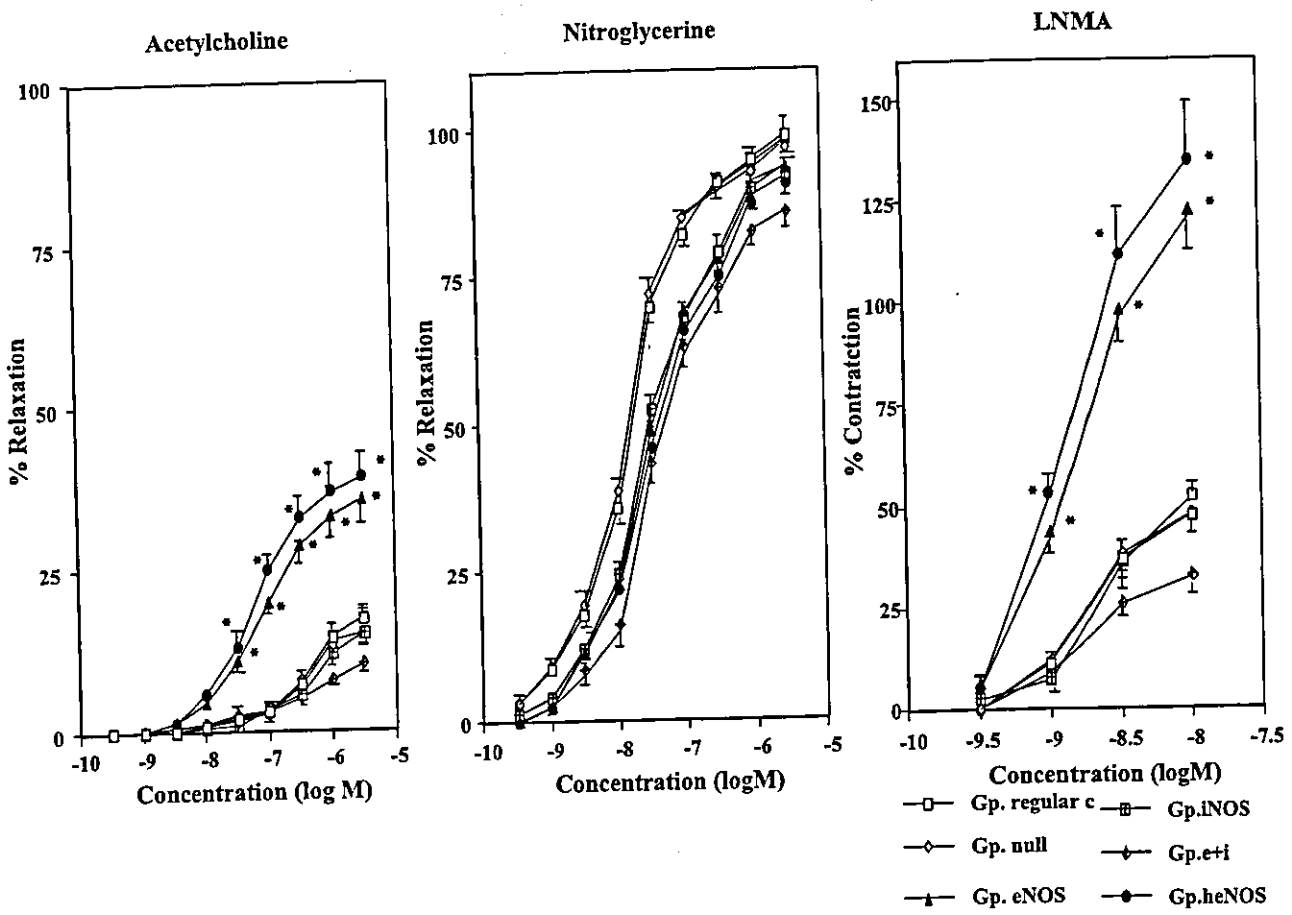


Fig. 3. Cumulative concentration–response curves to each of the agonists in the abdominal aortae of seven groups, which were precontracted by prostaglandin F2α: Gp cont: no treatment; Gp null: Ad.null; Gp eNOS: Ad.eNOS; Gp iNOS: Ad.iNOS; Gp e+i: Ad.eNOS plus Ad.iNOS; Gp heNOS: high amount of Ad.eNOS. 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.

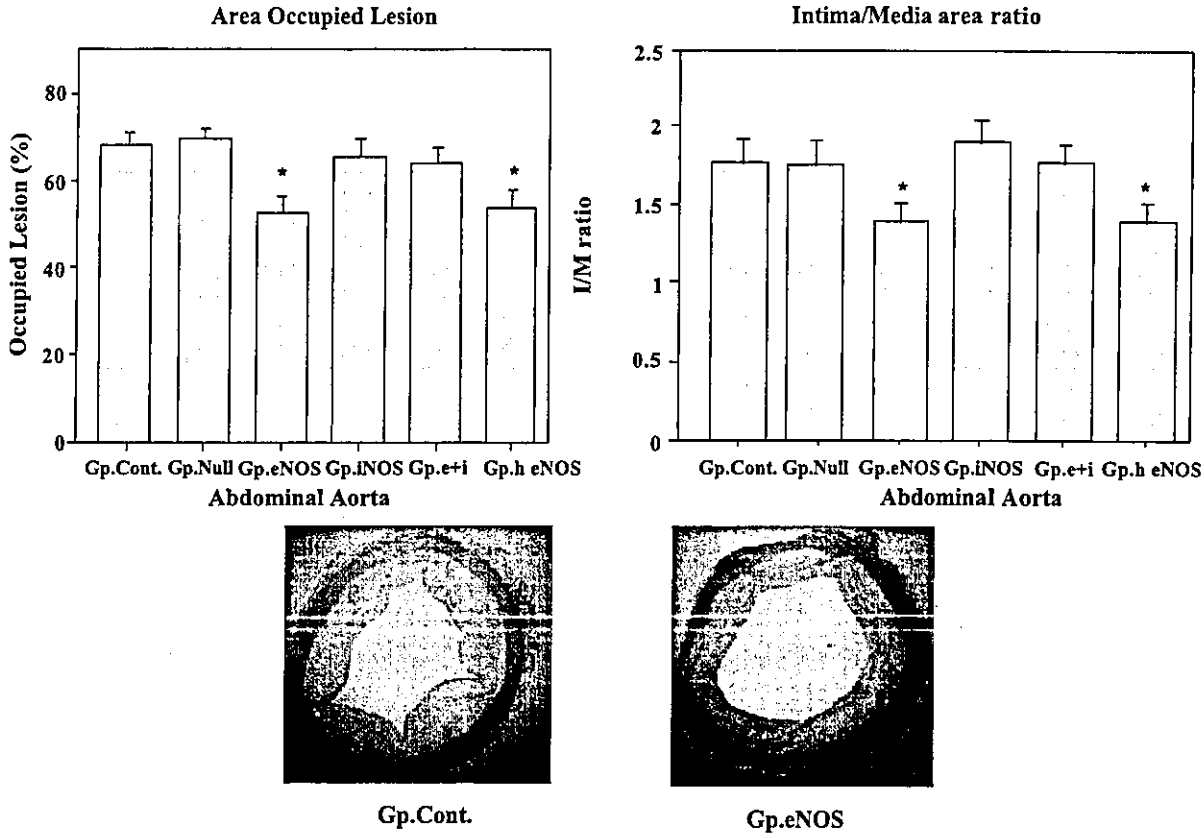


Fig. 4. Histological evaluation of the atherosclerotic area of the aorta as indicated by the mean lesion area (% occupied lesion, left), and the intima/medial ratio (I/M ratio, Right). Gp cont: no treatment; Gp null: Ad.null; Gp eNOS: Ad.eNOS; Gp iNOS: Ad.iNOS; Gp e+i: Ad.eNOS plus Ad.iNOS; Gp heNOS: 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

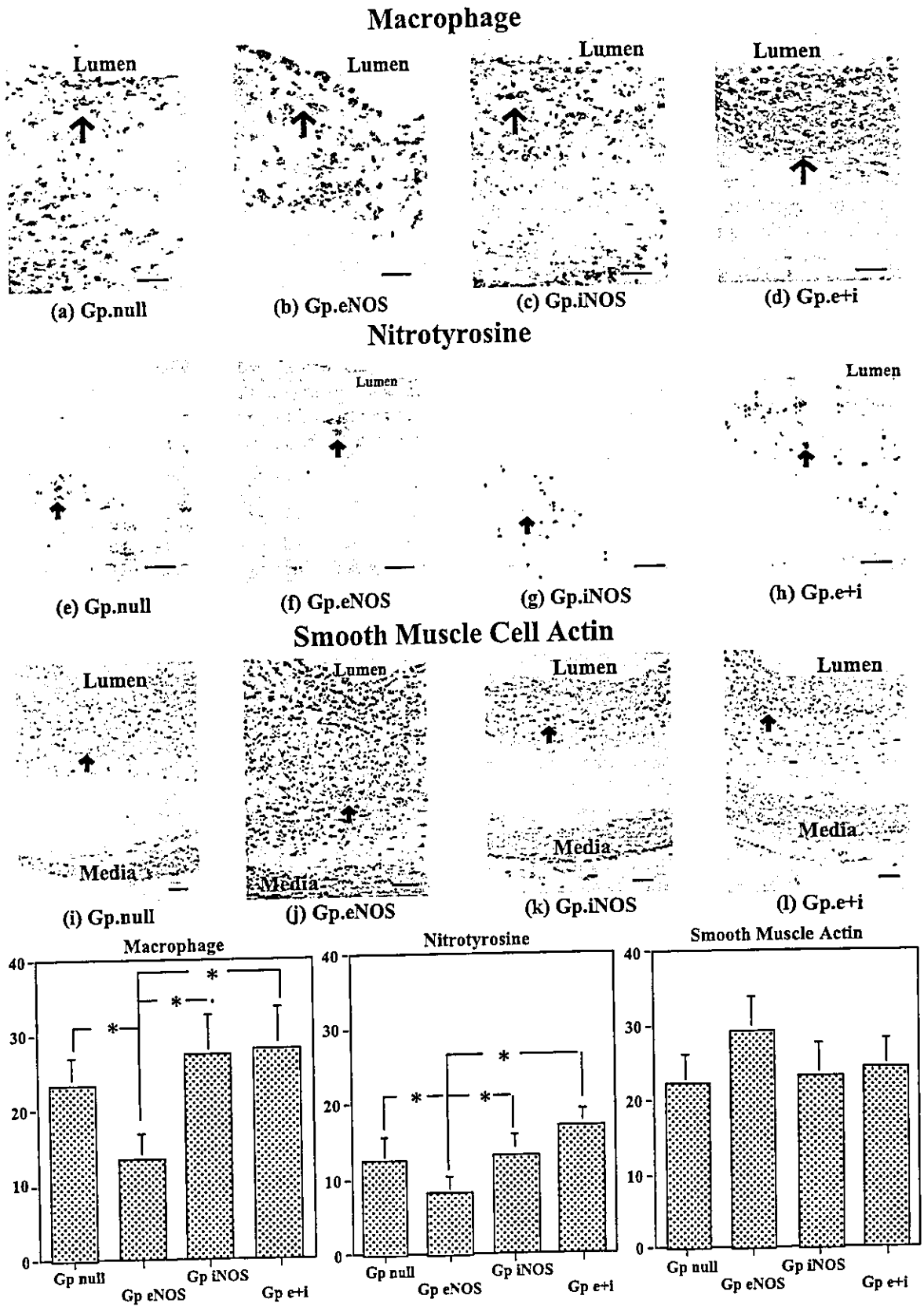
Cross sections of the aorta adjacent to the segments of vascular responses were examined [21]. The contours of the lumen and the internal elastic lamina (IEL) were traced. The area occupied by lesions was defined as the ratio of the area bounded by the lumen and IEL (atherosclerotic area) to the area 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).

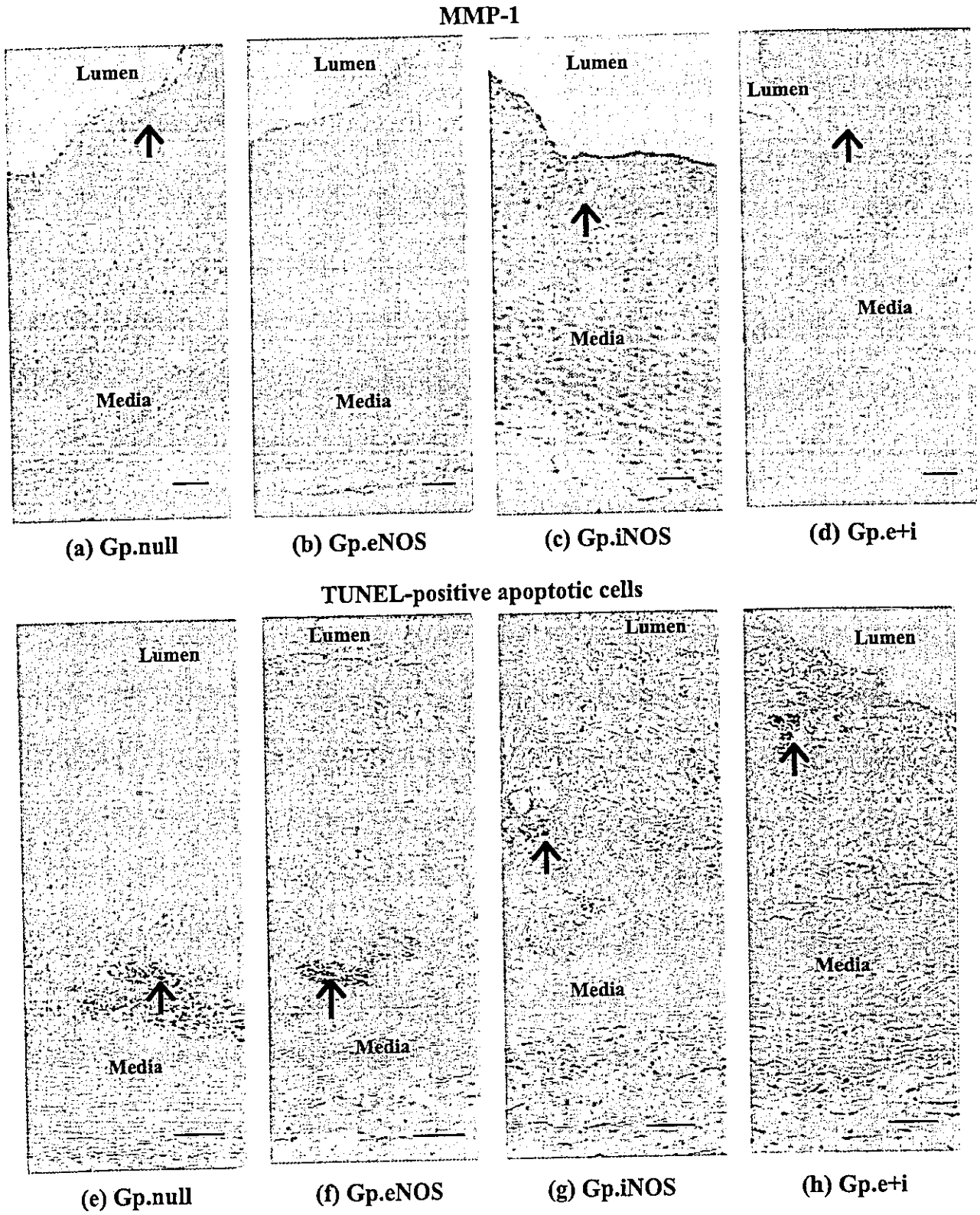


Fig. 6. Upper: expression of MMP-1-positive cells (arrow). (a) Gp null, Ad.null. (b) Gp eNOS, Ad.eNOS. (c) Gp iNOS, Ad.iNOS. (d) Gp e+i, Ad.eNOS plus Ad.iNOS. Strong expression of MMP-1 was observed in the lesions of Gps null, iNOS, and e+i. The expression was decreased in Gp eNOS. Original magnification, × 100. Lower: expression of TUNEL-positive apoptotic cells (arrow). (e) Gp null. (f) Gp eNOS. (g) Gp iNOS. (h) Gp e+i. Apoptotic cells were detected in Gps null, iNOS and e+i. However, apoptotic cells were rare in Gp eNOS. The scale bar represents 50 μm.

3.7. Nitrite and nitrate (NO_2^-/NO_3^-) and detection of aortic superoxide generation

Levels of NOx in medium (sum of NO_2^- and NO_3^-) in each group are shown in Table 1. iNOS treatment increased NOx in the regular control group of rabbits, but not in atherosclerotic rabbits (data not shown). The chemiluminescence signals from O_2^- production were greater in the aortae of iNOS gene-transfected groups, and the eNOS gene-transfected groups showed a slight decrease (Table 1). As an additional experiment described in Section 2.2, we separately prepared rabbits fed with a regular diet, and gene transfer of eNOS or iNOS was performed using dispatch catheter. NOx was increased, and O_2^- was not increased in the aortae from eNOS gene transfer rabbit as well as those from iNOS gene transfer rabbits (data not shown).

3.8. Immunocytochemical analysis

Atheroma in the abdominal aorta was composed of many macrophage-derived foam cells and intimal smooth muscle cells (Fig. 5). Gene transfer of Ad.eNOS not only reduced the area of atherosclerosis, but also decreased the area stained by the macrophage antibody and the areas positive for $ONOO^-$ established by nitrotyrosine staining (Fig. 5). The area stained by the smooth muscle cell actin was not different among the six groups, although it tended to be higher in the eNOS groups. MMP-1 (interstitial collagenase), a matrix metalloproteinase that initiates collagen degradation, was localized predominantly in macrophages. Although the difference did not reach the level of statistical significance, the expression of MMP-1 tended to be decreased in vessels of Gp eNOS and Gp heNOS (Fig. 6). A TUNEL-positive area was observed around a necrotic core in the atheroma as an apoptosis-susceptible area. The apoptosis-susceptible area seemed to be decreased in Gp eNOS and Gp heNOS; however, these differences were not statistically significant (Fig. 6).

4. Discussion

In the present study, in vivo gene transfer of eNOS, but not that of iNOS, resulted in a regression of advanced atherosclerosis and an improvement of EDR. This study has four major findings. First, NOS expression was increased throughout the vessels after transfection, demonstrating the highly efficient adenovirus-mediated gene transfer into severely atherosclerotic arteries. Second, adenovirus-mediated transfer of the eNOS gene alone restored EDR in atherosclerotic vessels. Third, eNOS gene transfer regressed atherosclerosis, and this effect appeared to have been due to a decrease of tissue lipids, although apoptosis or a decrease of extracellular matrix components may also have played a role. Finally, transfection with the iNOS gene failed to improve EDR and did not regress atherosclerosis. Further, simultaneous transfection of both the eNOS and iNOS genes also failed to improve EDR and did not regress atherosclerosis. In other words, transfection of iNOS blocked the beneficial effect of eNOS transfection.

4.1. Highly efficient adenovirus-mediated gene transfer in severe atherosclerosis

Gene transfers of NOS isoforms have been reported previously [9–12,23–27]. However, there have been only a few reports of in vivo gene transfer in advanced atherosclerotic vessels [9]. These studies used a high-cholesterol diet to induce atherosclerosis, resulting in patchy lesions that complicated the evaluation of gene transfer. In this study, we induced severe atherosclerosis by balloon injury and a high-cholesterol diet, resulting in severe atherosclerosis around the whole luminal area of vessels. The successful expression of the transgene (eNOS or iNOS) was confirmed by immunohistochemistry and Western blotting. We employed a 3-min perfusion at 6 atm. This choice was based on a previous study in which the efficacy of gene transfer was enhanced at a pressure greater than 4 atm, and in which no change in efficacy occurred between 2 and 40 min of perfusion [24]. Thahlil et al. [25] reported that the transduction rate is lower in medial smooth muscle cells than in the neointima or endothelium. Our data are almost identical to theirs. We used the same volumes of Ad.eNOS and Ad.iNOS, and separately used a 10-times higher titer of Ad.eNOS to evaluate whether or not the anti-atherosclerotic effect was dependent on the amount of NO.

4.2. Restoration of NO function of advanced atherosclerosis by Ad.eNOS

Gene transfer of eNOS remarkably improved the severely impaired EDR in atherosclerosis. The amount of transgene used in our experiment cannot be directly compared to that in the report by Ooboshi et al. [9]; however, the improvement of EDR by in vivo eNOS gene transfer in the present study is comparable with their report. In the present study, the animals receiving a high eNOS transfection (Gp heNOS) did not show any remarkable change in EDR or in the area of atherosclerosis compared to those receiving a smaller amount of the transgene (Gp eNOS). This finding suggest that the efficiency of the transgene might not be dose-dependent, and that a high dose of gene transfer may be insufficient to achieve a restoration of impaired EDR and complete regression. Regarding the mechanism of EDR improvement, we first hypothesized that regression of atherosclerosis could improve EDR, as the severity of atherosclerosis is inversely correlated with the impairment of EDR [15]. However, in the present study, the EDR improvement was much larger than that of the atherosclerosis regression. We speculated that EDR improvement occurs when NO bioavailability improves as a result of eNOS gene transfer, in turn related to the regression of

atherosclerosis. In fact, basal NO (as evaluated by tone-related basal NO release, NOx from vessels, and tissue cGMP concentration) was increased by eNOS gene transfer, but not by iNOS gene transfer. In the present study, the decrease level of O_2^- due to eNOS gene transfer also increased NO bioavailability.

4.3. The mechanisms of regression of atherosclerosis in response to eNOS

The pathological findings in the abdominal aortae of the present study were similar to previous findings in coronary arteries; i.e., in both cases the vessels were rich in macrophage-derived foam cells and lipids [15]. In the present study, eNOS gene transfer facilitated a significant regression of lesions, as well as a decrease in macrophages, indicating that the atheroma was stabilized. We previously established that there is a significant inverse relation between basal NO and the severity of atherosclerosis [13,15], and our present data suggest that this relation is involved in the NO release after eNOS gene transfer. Although previous studies have investigated the possibility of the regression of atherosclerosis by in vivo gene transfer [9,26–28], this might be the first demonstration of regression in a model of severe atherosclerosis induced by balloon injury and a high-cholesterol diet. The regression may be caused by the absorption of tissue lipid, while a cell decrease due to apoptosis or a decrease of the extracellular matrix such as collagen fibers may be a concern (Table 1 and Fig. 6). Absorption of tissue lipid can be caused by NO released from transferred eNOS, and we can speculate that HDL may be partially related to this absorption through increased NO release [29]. The apoptosis-suspected area by Tunnel staining tended to be small in Gp eNOS and Gp heNOS. Because $ONOO^-$ has cytotoxicity and the decreased level of O_2^- by eNOS gene transfer may contribute to a reduction in the substrate of $ONOO^-$, it could contribute to apoptosis. We cannot fully rule out the possibility that cell death occurred before our observation, because the effect of adenovirus gene transfer has been reported to peak at 3 days after transfection [27]. The role of apoptosis in regression should be elucidated further. Quian et al. [28] reported that monocyte recruitment occurred fairly quickly—i.e., within 24 h—after Ad.nNOS gene transfer. We speculate that a similar mechanism might occur by Ad.eNOS treatment for advanced atherosclerosis. We cannot fully rule out the possibility that other mechanism is responsible for regression, since our examination was made 7 days, rather than 24 h, after gene transfer. The tendency of a decrease in the MMP1-positive area supports the hypothesis that eNOS gene transfer stabilized the atheroma. The ratio of SMCs in atheroma did not differ significantly among the different groups of rabbits, although it tended to be relatively high in the Ad.eNOS transferred groups. We speculated that this occurs due to more decrease in macrophages in eNOS gene transfer group. Preliminarily, we did immunohistochemistry using anti-proliferating cellul nuclear antigen (PCNA), and TUNEL positive area looks like larger than anti-PCNA positive area in atheroma of each group on 7 days after gene transfer. We speculate the decrement in monocyte adhesion and the lipid in atheroma may be one of the possible mechanisms of regression, although other mechanisms may play a role. Further studies will be needed to establish the mechanism by which eNOS gene transfer regresses atherosclerosis.

4.4. Failure to improve impaired EDR and regression of severe atherosclerosis in response to Ad.iNOS, or Ad.eNOS plus iNOS

Neither gene transfer of iNOS nor that of eNOS plus iNOS improved EDR (Fig. 3). Transfection of empty vector with or without Ad.eNOS or Ad.iNOS did not affect EDR or the area of atherosclerosis compared to that of Gp eNOS, Gp iNOS, or Gp cont, indicating that the iNOS transgene not only impairs the improvement of EDR but also impairs the regression effect of eNOS. These data are somewhat contrary that iNOS is effective for improving vascular function, such as in the spastic arteries of various animal models [11,26,30]. As for the cause of the discrepant effects between previous and present studies, we speculate that the difference of enzyme activity of transferred gene between eNOS and iNOS, and the presence of intrinsic iNOS and O_2^- releasing enzymes in advanced atherosclerosis are important, although iNOS was transferred into iNOS-poor areas in previous studies [11,26,30]. In fact, the release of O_2^- was not increased, and that of NO was increased after iNOS gene transfer into normal aorta (3.5). The NO release from the Ad.eNOS-transfected atherosclerotic vessel was different from the Ad.iNOS-transfected vessel (Table 1). Both genes have the component of L-arginine (as substrate) binding site and BH4 (as cofactor) binding site. Ca^{2+}/calmodulin binding site is located in eNOS gene; however, it is already incorporated in iNOS gene. It is speculated that intracellular Ca^{2+} concentrations, even with transferred status, regulate NO release from eNOS and that iNOS is characterized by a greater specific activity, producing much larger quantities of NO in a calcium- and agonist-independent fashion [31]. eNOS releases O_2^- under conditions of depleted substrates or cofactors [32]. Large amount of transferred eNOS gene (Gp heNOS) may not be able to release NO by relative depletion of L-arginine or BH4. Intrinsic iNOS in atheroma is distributed in deep areas of atherosclerotic plaque (Fig. 2). These areas tend to be hypoxic with a relatively limited supply of arginine from the vasa vasorum or lumen [33,34]. The transferred iNOS was also distributed in all other components of blood vessels as well as in deep areas of the atherosclerosis. We suggest that iNOS releases O_2^- as well as NO, since iNOS is known to release O_2^- under conditions that deplete substrates, such as hypoxia, or that deplete arginine [35]. The report by Gunnett [12] may support our data. Macrophages or other inflammatory cells release O_2^- from NADPH oxidase or xanthine/xanthine

oxidase when stimulated by cytokines such as interferon γ, which also induce iNOS and co-localize in atheroma (data not shown). Nitrotyrosine, a marker of $ONOO^-$, was observed in the atherosclerotic regions, and its density was much greater in vessels transfected with iNOS and eNOS plus iNOS (Fig. 5). The response of O_2^- to a large amount of NO from transferred iNOS may have caused greater quantities of $ONOO^-$ and blocked the beneficial effect of NO from transferred eNOS. These differential effects between transgenes are interesting and important in gene therapy. As there are many O_2^- releasing enzymes such as NADPH oxidase and xanthine/xanthine oxidase as well as eNOS or iNOS, further studies like the experiment of iNOS gene transfer with or without antioxidant supplementation is necessary to elucidate the mechanisms.

4.5. Clinical significance of the present study

These findings may be relevant for improving blood flow and preventing thrombosis in advanced atherosclerotic arteries. The results of this study indicate that eNOS gene transfer should be clinically applied not only for prevention of atherogenesis, but also for the regression of advanced atherosclerosis. Because human atherosclerotic lesions, including intimal thickening, begin as early as childhood and by middle age are often quite prominent in the aorta and coronary arteries, hence regression is an important treatment tool. Regression and stabilization of atheroma is the main objective in the management of atherosclerotic lesions in humans. In the future, we expect that such clinical regression will be applicable for unstable angina or acute coronary syndrome. Although we must continue to work for a means of complete regression, the present results showed that eNOS gene transfer clearly improved the endothelial function and induced a regression of advanced atherosclerosis.

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References

[1] Lefer AM, Ma XL. Decreased basal nitric oxide release in hypercholesterolemia increases neutrophil adherence to rabbit coronary artery endothelium. Arterioscler Thromb 1993;13:771–6.
[2] Cooke JP. Role of nitric oxide in progression and regression of atherosclerosis. West J Med 1996;164:419–24.
[3] Cayatte AJ, Palacino JJ, Horten K, Cohen RA. Chronic inhibition of nitric oxide production accelerates neointima formation and impairs endothelial function in hypercholesterolemic rabbits. Arterioscler Thromb 1994;14:746–52.
[4] Ignarro LJ. Biological actions and properties of endothelium-derived nitric oxide formed and released from artery and vein. Circ Res 1989;65:1–21.
[5] Harrison DG, Armstrong ML, Freiman PC, Heistad DD. Restoration of endothelium-dependent relaxation by dietary treatment of atherosclerosis. J Clin Invest 1987;80:1808–11.
[6] Hayashi T, Esaki T, Muto E, Kano H, Iguchi A. Endothelium-dependent relaxation of rabbit atherosclerotic aorta was not restored by control of hyperlipidemia—the possible role of peroxynitrite. Atherosclerosis 1999;147:349–67.
[7] Aikawa M, Voglic SJ, Libby P, et al. Dietary lipid lowering reduces tissue factor expression in rabbit atheroma. Circulation 1999;100: 1215–22.
[8] Nabel EG, Nabel GJ. Complex models for the study of gene function in cardiovascular-biology. Annu Rev Physiol 1994;56:741–61.
[9] Ooboshi H, Toyoda K, Faraci FM, Lang MG, Heistad DD. Improvement of relaxation in an atherosclerotic artery by gene transfer of endothelial nitric oxide synthase. Arterioscler Thromb Vasc Biol 1998;18:1752–8.
[10] Von der Leyen HE, Gibbons GH, Dzau VJ, et al. Gene therapy inhibiting neointimal vascular lesion-in vivo transfer of endothelial nitric oxide synthase gene. Proc Natl Acad Sci U S A 1995;92: 1137–41.
[11] Shears II LL, Kibbe MR, Murdock AD, et al. Efficient inhibition of intimal hyper-plasia by adenovirus-mediated inducible nitric oxide synthase gene transfer to rats and pigs in vivo. J Am Coll Surg 1998;187:295–306.
[12] Gunnett CA, Lund DD, Heistad DD, et al. NO-dependent vasorelaxation is impaired after gene transfer of inducible NO-synthase. Arterioscler Thromb Vasc Biol 2001;21:1281–7.
[13] Esaki T, Hayashi T, Muto E, Kuzuya M, Iguchi A. Expression of inducible nitric oxide synthase in T lymphocytes and macrophages of cholesterol-fed rabbits. Atherosclerosis 1997;128:39–47.
[14] Chen AF, O'Brien T, Katusic ZS. Transfer and expression of recombinant nitric oxide synthase genes in the cardiovascular system. Trends Pharmacol Sci 1998;19:276–86.
[15] Hayashi T, Kano H, Thakur NK, Sumi D, Iguchi A. Physiological concentration of 17 β estradiol retards the progression of severe atherosclerosis induced by cholesterol diet plus balloon injury via NO. Arterioscler Thromb Vasc Biol 2000;20:1613–21.
[16] Sumi D, Hayashi T, Jayachandran M, Iguchi A. Estrogen prevents destabilization of endothelial nitric oxide synthase mRNA induced by tumor necrosis factor α. Life Sci 2001;69:1651–60.
[17] Hayashi T, Ignarro LJ, Fukuto JM, Chaudhuri G. Basal release of nitric oxide from aortic rings is greater in female rabbits than in male rabbits. Proc Natl Acad Sci U S A 1992;89:11259–63.
[18] Lipid Research Clinics Program Manual of Laboratory Operations. vol. 1, ed. 2. US Dept. of Health, Education and Welfare, Publ. No. (NIH). Washington, DC, US. Govt. Printing Office, pp. 1–76, 1982.
[19] Gaskell SJ, Finlay EM, Pike AW. Analyses of steroids in saliva using highly selective mass spectrometric techniques. Biomed Mass Spectrom 1980;7:500–4.
[20] Hayashi T, Muto E, Iguchi A, et al. Dehydroepiandrosterone retards atherosclerosis formation through the conversion to estrogen—the possible role of nitric oxide. Arterioscler Thromb Vasc Biol 2000; 20:782–92.
[21] Weiner BH, Ockene IS, Hoogasian JJ. Inhibition of atherosclerosis by cod-liver oil in a hyperlipidemic swine model. N Engl J Med 1986;315:841–5.
[22] Esaki T, Hayashi T, Muto E, Kano H, Thakur NK, Iguchi A. Expression of inducible nitric oxide synthase correlates with the incidence of apoptotic cell death in atheromatous plaques of human coronary arteries. Nitric Oxide: Biol Chem 2000;4:561–71.
[23] Lund DD, Faraci FM , Miller FJ Jr., Heistad DD. Gene transfer of endothelial nitric oxide synthase improves relaxation of carotid arteries from diabetic rabbits. Circulation 2000;101:1027–33.
[24] Chapman GD, Lim CS, Gammon RS, et al. Gene transfer into coro-

nary arteries of intact animals with a percutaneous balloon catheter. Circ Res 1992;71:27–33.
[25] Thahlil O, Brami M, Feldman LJ, Branelec D, Steg PG. The dispatch catheter as a delivery tool for arterial gene transfer. Cardiovasc Res 1997;33:181–7.
[26] Kibbe MR, Tzeng E, Gleixner SL, et al. Adenovirus-mediated gene transfer of human inducible nitric oxide synthase in porcine vein grafts inhibits intimal hyperplasia. J Vasc Surg 2001;34:156–65.
[27] Channon KM, Qian H, Nepliouева V, et al. In vivo gene transfer of nitric oxide synthase enhances vasomotor function in carotid arteries from normal and cholesterol-fed rabbits. Circulation 1998;98: 1905–11.
[28] Qian H, Nepliouева V, Shetty GA, Channon KM, George SE. Nitric oxide synthase gene therapy rapidly reduces adhesion molecule expression and inflammatory cell infiltration in carotid arteries of cholesterol-fed rabbits. Circulation 1999;99:2979–82.
[29] Tretjakovs P, Kalnins U, Dabina I, et al. Plasma HDL-cholesterol has an effect on nitric oxide production in coronary heart disease patients without LDL hypercholesterolemia. Med Sci Monit 2000;6:507–11.
[30] Fukumoto Y, Shimokawa H, Takeshita A, et al. Vasculoprotective role of inducible nitric oxide synthase at inflammatory coronary lesions induced by chronic treatment with interleukin-1 beta in pigs in vivo. Circulation 1997;96:3104–11.
[31] Morris SM Jr., Billiar TR. New insights into the regulation of inducible nitric oxide synthases. Am J Physiol 1994;266:E829–39.
[32] Vasquez-Vivar J, Martasek P, Whitsett J, Joseph J, Kalyanaraman B. The ratio between tetrahydrobiopterin and oxidized tetrahydrobiopterin analogues controls superoxide release from endothelial nitric oxide synthase: an EPR spin trapping study. Biochem J 2002;362:733–9.
[33] Kai H, Kuwahara F, Imaizumi T, et al. Coexistence of hypercholesterolemia and hypertension impairs adventitial vascularization. Hypertension 2002;39:455–9.
[34] Barker SG, Tilling LC, Martin JF, et al. The adventitia and atherogenesis: removal initiates intimal proliferation in the rabbit which regresses on generation of a neoadventitia. Atherosclerosis 1994;105: 131–44.
[35] Xia Y, Zweuer JL. Superoxide and peroxynitrite generation from inducible nitric oxide synthase in macrophages. Proc Natl Acad Sci U S A 1997;94:6954–8.