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Pressurization facilitates adenovirus-mediated gene transfer into vein graft

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Abstract We investigated whether application of non-distending hydrostatic pressure facilitates gene transfer into vein grafts. An external jugular vein was placed in a chamber with 100 µl adenovirus solution at a titer of 10^{10} pfu/ml and was pressurized to up to 8 atm above ambient pressure for 10 min. Histochemical analysis demonstrated a positive transgene expression in all layers of the vessel wall. Gene transfer with 8 atm pressurization resulted in an approximately 50 times higher transgene expression than that without pressurization. Under 8 atm pressurization, the efficiency of gene transfer reached a plateau at 7.5 min. The application of hydrostatic pressure may improve the effectiveness of intraoperative genetic engineering of vein grafts.
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Key words: Vein graft; Adenovirus; Pressurization; β-Galactosidase; Hydrostatic pressure

1. Introduction

Vein grafts are utilized as a conduit for aortocoronary bypass grafting to improve myocardial flow and for vein graft-covered stents to manage perforated bypass-grafts [1] or native vessels [2]. However, there would seem to be potential problems in vein grafts, such as thrombosis and neointimal hyperplasia [3]. Replication-deficient adenovirus is a useful tool to introduce exogenous genes to control vascular responses in pathophysiological states [4,5]. It was recently reported that the application of hydrostatic pressure facilitates the delivery of oligodeoxynucleotide into cardiovascular tissues [6]. In the present study, we characterize the effect of pressurization during ex vivo adenovirus gene transfer to vein grafts. In vivo transgene expression in infected autologous vein grafts will also be discussed.

2. Materials and methods

All experiments followed a protocol approved by the Animal Research Committee of the University of Tokyo.

2.1. Experimental animal preparation and pressure-supported gene transfer

Male Japanese white rabbits (2000–3000 g) were used for the present study. After the induction of anesthesia (pentobarbital, 50 mg/kg, i.v., and ketamine, 50 mg/kg, i.m.), the left external jugular vein was carefully dissected out. Each vein graft was then mounted onto a Palmaz–Schatz stent (Johnson and Johnson). Vein grafts were then placed in a custom-made small chamber (Medikit) with 100 µl adenovirus solution dissolved in Dulbecco's modified Eagle's medium (DMEM, Gibco) for up to 10 min under up to 8 atm above ambient pressure by using a standard angioplasty inflation device (Encore, Scimed). The vein segment was surrounded by pressurized viral solution both luminally and abluminally, thereby avoiding distension or stretch of the vessel wall. Vein grafts were subsequently incubated in Medium 199 (M199, Gibco) supplemented with 10% fetal calf serum (FCS) for a further 48 h to allow the expression of transferred genes at the protein level. In some experiments, three sutures (Prolene 8-0, Ethicon) were applied at each side of the stent to stabilize the vein graft on the stent strut before gene transfer. An autologous vein graft-covered stent was implanted into the abdominal aorta immediately after the gene delivery using a standard angioplasty balloon catheter/guide wire (0.014 inch) system. Animals were killed 48 h after the stent implantation and expression of the transferred gene was examined.

2.2. Recombinant adenoviruses

Two replication-deficient, second generation recombinant adenovirus constructs were made and purified as described previously [7]. Ad-HO-1 and Ad-LacZ encode a rat heme oxygenase-1 (HO-1, a kind gift from Dr. S. Shibahara, Tohoku University School of Medicine, Japan), and an *Escherichia coli* lacZ, respectively. The cDNAs in these vectors were driven under the control of cytomegalovirus immediate early gene enhancer and promoter.

2.3. Histochemical analysis

For the histochemical analysis of β-galactosidase expression, gross vein specimens or fresh frozen sections (5 µm thick) of the vein segments mounted on silanized slides were fixed in 0.2% glutaraldehyde for 10 min and stained for 16 h in X-Gal chromogen as described previously [8].

2.4. Protein purification and immunoblot analysis

Purification of the protein from the vein samples was carried out following the protocol described elsewhere [9]. Anti-HO-1 monoclonal antibody (OSA 111, StressGen) and horseradish peroxidase-conjugated anti-mouse antibody (Jackson ImmunoResearch) was used at 1/1000 and 1/2000 dilutions, respectively. The ECL Western blotting system (Amersham Life Sciences) and lumino-analyzer (Fuji Photo Film) were used for detection of bands. The intensity of bands was semi-quantified using computer software, NIH Image.

2.5. Cell culture and pressure-supported gene transfer to cultured cells

Rabbit aortic vascular smooth muscle cells (VSMCs) were isolated from the thoracic aorta of male Japanese white rabbits using the methods described previously [10]. Cells were grown in DMEM supplemented with 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin. After trypsinization of the cells, cells (1×10^5) were placed in the same chamber with 100 µl adenovirus solution (1×10^6–3×10^9 pfu/ml) dissolved in DMEM for 10 min under 8 atm above ambient pressure by using the standard angioplasty inflation device as described above. The cells were then seeded on to the collagen type 1 cover glass with a diameter of 12 mm (Iwaki), which was placed in a

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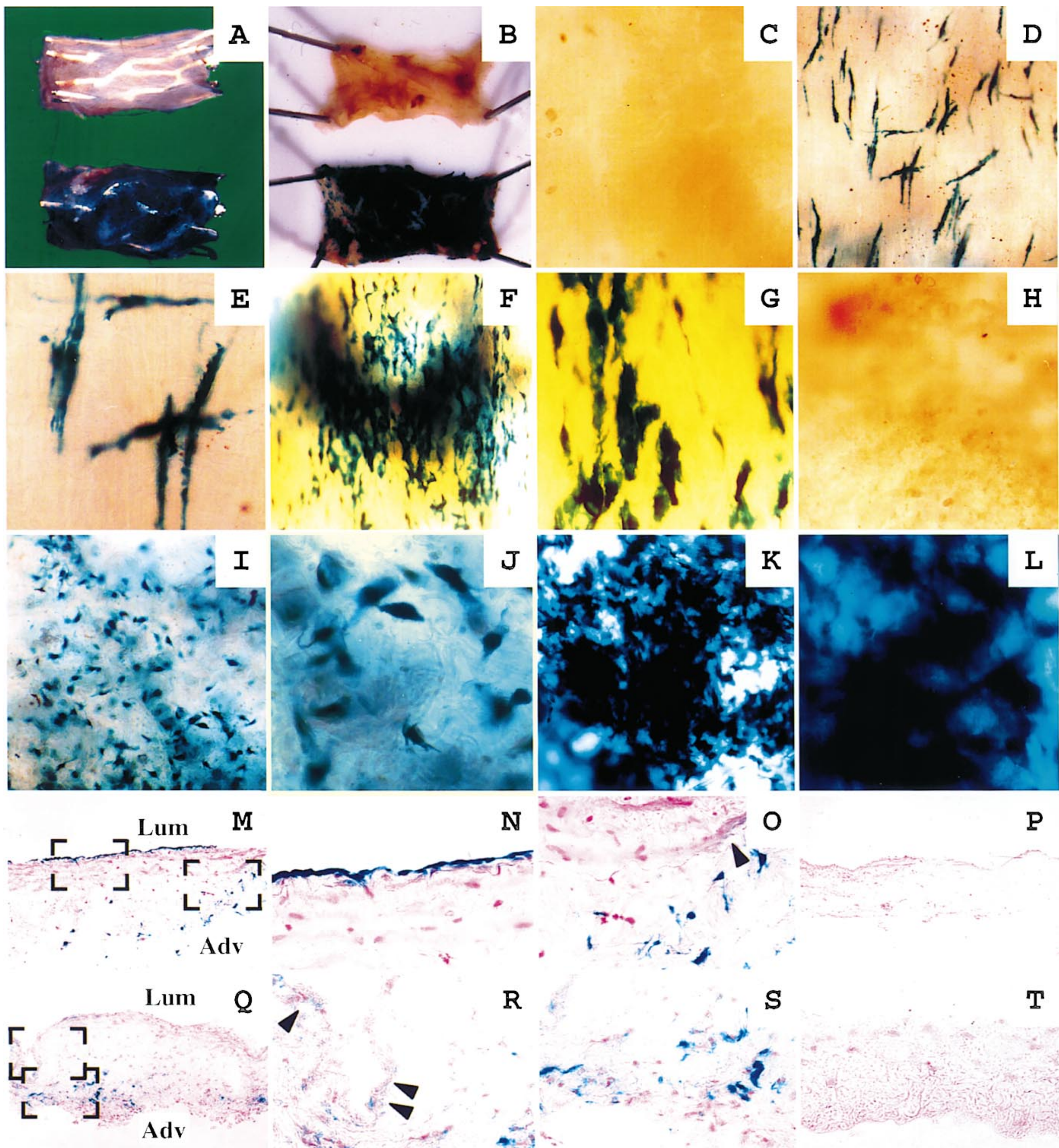

Fig. 1. Macroscopic and light microscopic views of vein segments that had been subjected to pressure-supported ex vivo adenovirus-mediated gene transfer followed by en face X-Gal staining. Vein segments were infected with either Ad-LacZ (A and B, lower panels, D–G, I–O, Q, R and S) or Ad-HO-1 (A and B, upper panels, C, H, P and T). X-Gal staining of vein segments was performed 48 h after incubation with adenoviruses at a titer of 10^{10} pfu/ml under 8 atm for 5 min (D, E, I and J) and 10 min (A–C, F–H, K–T). A and B: En face macroscopic images of gross vein specimen (A) and the adventitial surface of longitudinally opened vein grafts (B). C–L: En face light microscopic views using longitudinally opened vein segments. Views from the luminal (C–G) and from the adventitial side (H–L) are shown. M–T: X-Gal staining of the frozen sections of vein grafts. After gene transfer, vein grafts were organ cultured in M199 or implanted into abdominal aorta (Q–T). Vein grafts were harvested 48 h after the gene transfer. N and O are magnified views of left and right bracketed regions in M, respectively, and R and S are magnified views of upper and lower bracketed regions in Q, respectively. Lum and Adv indicate the luminal surface and the adventitia, respectively. Original magnifications ×250 (C, D, F, H, I, K, M, P and Q) and ×800 (E, G, J, L, N, O, R, S and T).

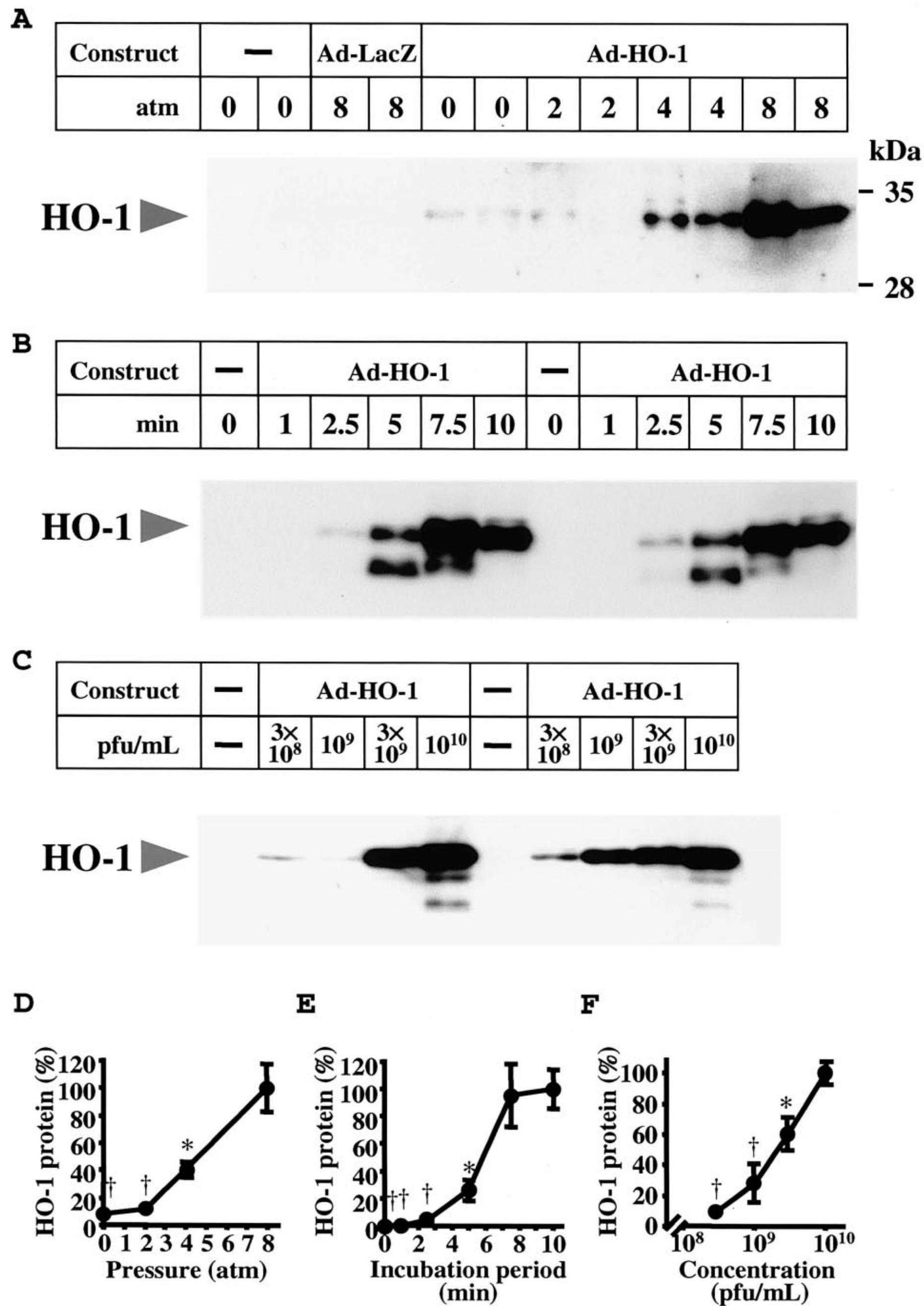

Fig. 2. Efficiency of pressure-supported adenovirus-mediated gene transfer. Rabbit jugular vein segments were subjected to adenovirus-mediated gene transfer of the indicated gene, at the indicated concentrations for the indicated length of time. Pressure dose–response (A, D), time course (B, E) and viral titer–response (C, F) data are shown. Expression of rat HO-1 was examined using anti-HO-1 mAb (OSA 111). A–C: representative immunoblots. D–F: relative intensity of HO-1 bands in comparison with the intensity of the HO-1 bands in the samples infected with Ad-HO-1 at a titer of 10^{10} pfu/ml for 10 min at 8 atm was calculated. Data from 4–6 samples in each point are summarized in line graphs. $^{*}P<0.01$ and $^{\dagger}P<0.001$ versus samples from vein segments infected with 10^{10} pfu/ml Ad-HO-1 at 8 atm for 10 min.

24 well plate, together with the adenovirus solution. Five minutes after seeding the cells onto the cover glass, cells were washed three times with DMEM to remove adenovirus in the culture medium. Then the cells were incubated in DMEM supplemented with 10% FCS for a further 48 h to allow expression of the transferred genes at the protein level. After fixing the cells in 0.2% glutaraldehyde for 10 min, the expression of β-galactosidase was examined.

2.6. Statistical analysis

Data are expressed as mean ± S.E.M. ANOVA followed by a multiple comparison test was used for comparisons on the initial data before expression as a percentage of the control using the statistical analysis software, Statistica ver. 5.1J (StatSoft Inc.). A value of $P<0.05$ was considered to be statistically significant.

3. Results and discussion

3.1. Adenovirus-mediated transfer of lacZ genes

β-Galactosidase expression was examined histochemically 48 h after ex vivo adenovirus-mediated gene transfer at a titer of 10^{10} pfu/ml under 8 atm for 10 min. Marked β-galactosidase expression was observed in the vein grafts infected with

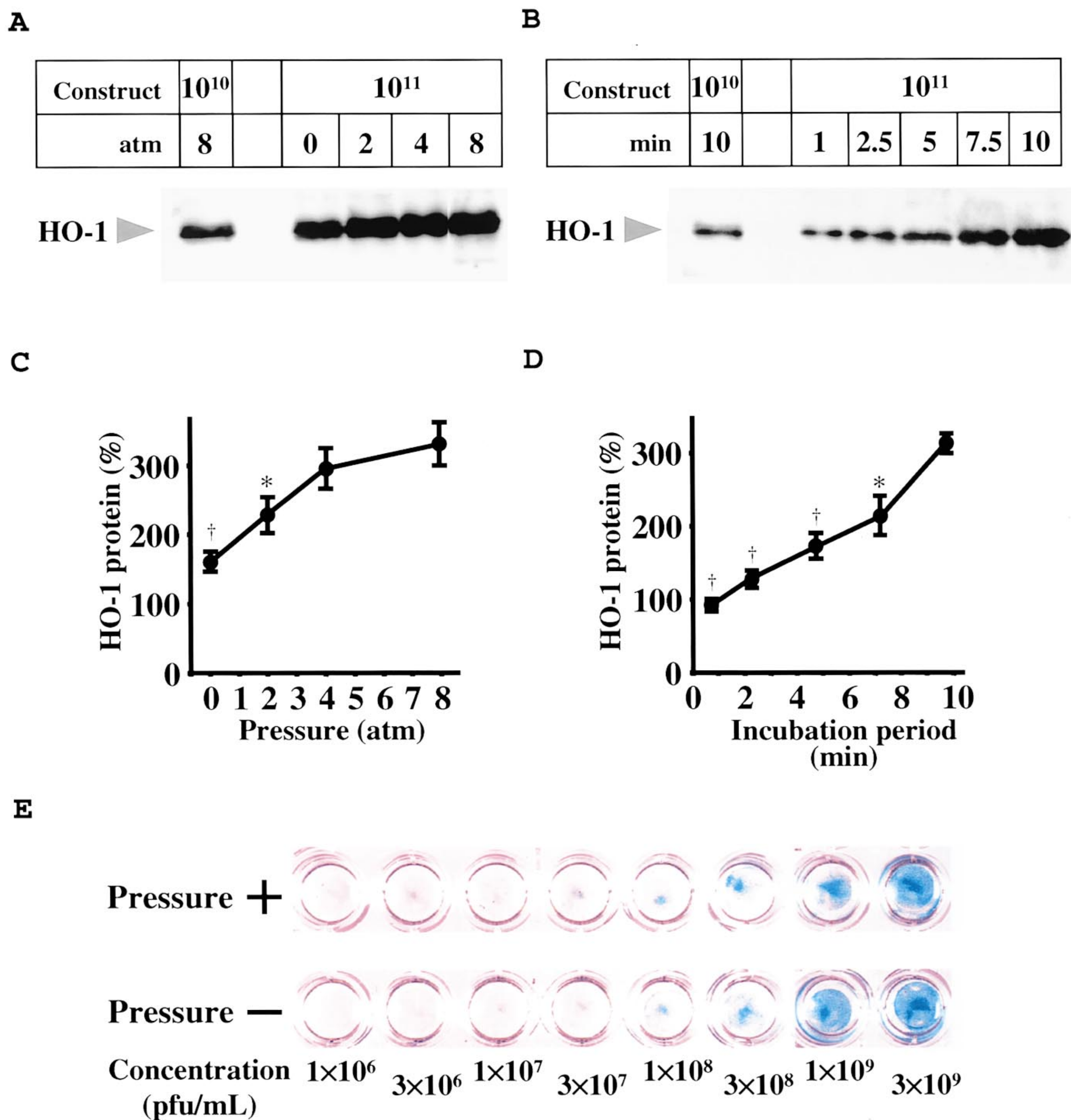

Fig. 3. Efficiency of pressure-supported gene transfer at a higher concentration and efficiency of gene pressure-supported gene transfer to cultured smooth muscle cells. A–D: Efficiency of pressure-supported gene transfer at a titer of 10^{11} pfu/ml. Rabbit jugular vein segments were subjected to gene transfer of Ad-HO-1, at the indicated concentrations for the indicated length of time. Pressure dose–response (A, C) and time course (B, D) data are shown. Expression of rat HO-1 was examined using anti-HO-1 mAb (OSA 111). A and B: Representative immunoblots. C and D: Relative intensity of HO-1 bands in comparison with the intensity of the HO-1 bands in the samples infected with Ad-HO-1 at a titer of 10^{10} pfu/ml for 10 min at 8 atm was calculated. Data from 4–6 samples in each point are summarized in line graphs. $^{*}P<0.05$ and $^{\dagger}P<0.001$ versus samples from vein segments infected with 10^{11} pfu/ml Ad-HO-1 at 8 atm for 10 min. E: effect of pressurization on gene transfer to cultured VSMCs. VSMCs were incubated with Ad-LacZ at the indicated concentrations with or without pressurization under 8 atm for 10 min. X-Gal staining was performed 48 h after gene transfer. Representative photograph of two independent experiments with similar results is shown.

Ad-LacZ (Fig. 1A,B, lower panels), but not in those infected with Ad-HO-1 (Fig. 1A,B, upper panels). A 10 min incubation with Ad-LacZ solution seemed to result in more β-galactosidase expression (Fig. 1F,G,K,L) than a 5 min incubation with the viral solution (Fig. 1D,E,I,J) under 8 atm.

X-Gal staining of the frozen sections of vein segments subjected to ex vivo Ad-LacZ gene transfer showed β-galactosidase expression in cells of all layers of the venous wall (Fig. 1M–O). Positive signals were detected in the medial smooth muscle cells in a dotted fashion (Fig. 1O, arrowhead). No

staining was observed in the vein graft infected with Ad-HO-1. A relatively higher transgene expression in the adventitial cells in the vessel wall was compatible to the previous finding [11]. We also investigated in vivo gene expression of vein graft-covered stents after implantation into abdominal aortas. In the in vivo situation, positively stained cells lining the luminal surface had been lost (Fig. 1Q), which may have been caused by balloon inflation during stent delivery or by exposure to the arterial circulation. On the other hand, positively stained cells in the medial (Fig. 1R, arrowheads) and adventitial (Fig. 1S) layers could be observed. Hematoxylin eosin staining of the specimens showed no apparent injury in pressure-subjected vessel wall (Ishizaka, unpublished observation).

3.2. *Semi-quantitative analysis of the efficiency of gene transfer of Ad-HO-1*

To evaluate the factors influencing the efficiency of pressure-supported gene transfer, the expression of rat HO-1 was investigated by immunoblot analysis. In a preliminary study, we tested two monoclonal anti-HO-1 antibodies, OSA111 and clone 23 (Transduction Laboratory), and found that the former detects rat HO-1 but not rabbit HO-1 and the latter detects rabbit HO-1 but not rat HO-1 (data not shown). Therefore, we used OSA111 to assess the efficiency of gene transfer of Ad-HO-1, which carries the rat HO-1 sequence, in a semi-quantitative manner.

HO-1 expression increased with increasing pressure and did not reach a plateau even at 8 atm (Fig. 2A,D). In the vein segments subjected to Ad-HO-1 infection (10^{10} pfu/ml, 8 atm, 10 min), the expression of endogenous (rabbit) HO-1 was markedly downregulated compared to non-infected controls (data not shown). Longer durations from 1 to 10 min yielded greater HO-1 expression, but these efficiencies were not further increased at 7.5 min (Fig. 2B,E). Increasing viral titers from 3×10^8 to 10^{10} pfu/ml yielded greater transfection efficiencies under 8 atm (Fig. 2C,F).

We then examined the efficiency of Ad-HO-1 infection at a titer of 10^{11} pfu/ml. As shown in Fig. 3, HO-1 expression was approximately three times higher in the veins subjected to Ad-HO-1 infection at a titer of 10^{11} pfu/ml than in those subjected to Ad-HO-1 infection at a titer of 10^{10} pfu/ml. HO-1 expression increased with increasing pressure, but these efficiencies were not further increased at 4 atm (Fig. 3A,C). Longer durations from 1 to 10 min yielded greater HO-1 expression, but these efficiencies did not reach a plateau even at 10 min (Fig. 3B,D).

3.3. *Adenovirus-mediated transfer of lacZ genes to cultured VSMCs*

Finally, in order to gain insight into the mechanisms of pressure-induced facilitation of adenovirus gene transfer, we performed pressure-supported Ad-LacZ infection to cultured VSMCs ??using the same pressurization system. There were no obvious differences in the X-Gal staining between the cells with pressurization and without pressurization during gene transfer (Fig. 3E). This finding suggests that pressure-induced facilitation of gene transfer to vein segments was not due to the change of the physical properties of membrane of vascular cells, but due to the increased infiltration of the virus particle toward the target cells.

Two findings may be of note in the present study in consideration of their clinical utility. First, the efficiency of adenovirus-mediated gene delivery to vein grafts did not reach a plateau even at 8 atm. Previous work demonstrated that the application of pressures of several atmospheres at the time of in vivo gene transfer causes extensive vessel wall damage [12]. In the present study, however, hydrostatic and non-distending pressure was applied evenly along the entire vessel, thereby minimizing damage to the vascular cells, since this range of hydrostatic pressure is applied in hyperbaric treatment or in diving and is, therefore, considered 'physiological' [13]. Second, the efficiency of adenovirus transfer (10^{10} pfu/ml) reached a plateau at 7.5 min under 8 atm hydrostatic pressure. This relatively short duration of incubation period compared to previous studies [14,15] may increase the feasibility of exploiting intraoperative adenovirus-mediated gene transfer as a potential therapeutic strategy at the time of bypass grafting, for example by using vein graft as a conduit and implantation of vein graft-covered stents.

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