POTENTIAL ROLE OF VACUOLAR H⁺-ADENOSINE TRIPHOSPHATASE IN NEOINTIMAL FORMATION IN CULTURED HUMAN SAPHENOUS VEIN

Hajime Otani, MD^a
Hideyasu Ohmiya, MD^a
Reiji Hattori, MD^a
Hirofumi Fujii, MD^a
Hideki Ninomiya, MD^a
Masakuni Kido, MD^a
Hideki Kawaguchi, MD^a
Motohiko Osako, MD^a
Hiroji Imamura, MD^a
Tetsuo Ohta, MD^b
Shoji Ohkuma, PhD^c

Objective: Vacuolar H⁺-adenosine triphosphatase plays a pivotal role in pH regulation and molecular transport across the vacuolar membranes and is involved in cell proliferation and transformation. In the present study, possible involvement of vacuolar H⁺-adenosine triphosphatase in neointimal formation was investigated in an organ culture model of human saphenous vein.

Methods and results: Cultured saphenous vein segments developed neointimal formation and marked thickening of the media within 14 days. Neointimal formation and medial thickening were completely inhibited by 10 nmol/L bafilomycin A₁, a selective inhibitor of vacuolar H⁺-adenosine triphosphatase, although structurally related macrolide antibiotics FK-506 and erythromycin were without an effect. The neointimal cells were positive for α -smooth muscle actin and vimentin but negative for desmin, indicative of myofibroblasts. The emergence of myofibroblasts was inhibited, and endothelial cells were preserved in the saphenous vein segments treated with bafilomycin A₁. Uptake of bromodeoxyuridine, a proliferation marker, by myofibroblasts was abrogated in the saphenous vein segments treated with 10 nmol/L bafilomycin A₁. Detection of apoptotic cells by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling concomitant with identification of desmin-expressing smooth muscle cells demonstrated that neointimal myofibroblasts, but not medial smooth muscle cells, that expressed desmin underwent apoptosis by treatment with bafilomycin A₁.

Conclusions: These results suggest that vacuolar H⁺-adenosine triphosphatase may be involved in myofibroblast growth that contributes to neointimal formation and medial thickening in cultured human saphenous vein. Increased sensitivity of myofibroblasts, but not endothelial cells, and differentiated smooth muscle cells to bafilomycin A₁ may have potential therapeutic implications in the treatment for vein graft disease. (*J Thorac Cardiovasc Surg* 2000;119:998-1007)

Neointimal formation is known to develop in saphenous vein (SV) grafts after coronary artery bypass surgery. The SV grafts are prone to have a number of

noxious stimuli during the perioperative period (ie, dissection, overdistention to secure side branch ligation, ischemia, chemical cardioplegia, and enhanced mechanical stress in the arterial circulation). Long-term patency of the SV grafts has been compromised by neointimal formation and subsequent progression of atherosclerosis.¹⁻³ Thus improvement of the outcome of surgical revascularization depends on the suppression of such undesirable vascular remodeling. However, the exact molecular mechanism for neointimal formation in SV grafts remains unknown.

There is a growing body of evidence that vacuolar-type H⁺-adenosine triphosphatase (V-ATPase) plays a crucial role in regulating cell growth.⁴⁻⁶ V-ATPases reside on the membranes of acidic organelles such as synaptic vesicles, chromaffin granules, platelet dense granules, secretory granules, lysosomes, and the trans-Golgi network and maintain an acidic environment by

From the Department of Thoracic and Cardiovascular Surgery,^a Kansai Medical University, Department of Surgery (II),^b School of Medicine, Kanazawa University, and the Laboratory of Biochemistry,^c Department of Molecular and Cell Biology, Faculty of Pharmaceutical Science, Kanazawa University, Kanazawa, Japan.

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Address for reprints: Hajime Otani, MD, Department of Thoracic and Cardiovascular Surgery, Kansai Medical University, 10-15 Fumizono-cho, Moriguchi City, Osaka 570, Japan (E-mail: otanih@takii.kmu.ac.jp).

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pumping protons with the energy of adenosine triphosphate (ATP) hydrolysis.⁷⁻⁹ The acidic pH within such organelles is proposed to be necessary for cell growth and differentiation.¹⁰⁻¹²

V-ATPases are highly sensitive to bafilomycin A₁ (BA₁), a macrolide antibiotic with a 16-membered lactone ring isolated from *Streptomyces griseus*.¹⁴ In vitro sensitivity assay of different ATPases to BA₁ revealed that V-ATPases were inhibited by nanomolar concentrations of BA₁, whereas mitochondrial H⁺-ATPase and gastric H⁺-ATPase activities were not affected by BA₁ at concentrations up to 1 mmol/L.¹⁴ This highly selective nature of BA₁ to inhibit V-ATPases has been taken advantage of as a probe in clarifying the role of V-ATPases in various experimental models. Ohta and colleagues¹⁵ have demonstrated that BA₁ inhibits the growth of the human cancer cell line Capan-1 in vivo in the rat. Our recent study has proved that BA₁ is capable of inhibiting neointimal hyperplasia in the cryopreserved rat aortic allograft.¹⁶ The potential role of V-ATPase in tumorigenesis prompted us to investigate the inhibitory effect of BA₁ on neointimal formation in cultured human SV. The results of the present study suggest that neointimal myofibroblasts, but not endothelial cells, and differentiated smooth muscle cells are susceptible to inhibition of V-ATPase.

Methods

Preparation of SV segments. Segments of freshly prepared SV were obtained from 40 patients (37 male patients; age, 64.2 ± 7.4 years [range, 51-80 years]) who underwent coronary artery bypass operations. Informed consent was obtained from those patients according to the ethical permission issued by the Ethical Committee of Kansai Medical University. A segment (approximately 2 cm) of SV was obtained immediately after dissection and collected in tissue culture medium (RPMI 1640 [Roswell Park Memorial Institute, Buffalo, NY] with HEPES buffer, 20 mmol/L, pH 7.4) containing sodium heparin, 4 IU/mL.

Tissue culture procedures. The SV segment was freed of excess fat and connective tissue under sterile conditions, opened with the luminal surface uppermost, and cut approximately into 1-cm segments. The SV segments were then incubated in RPMI 1640 medium containing sodium bicarbonate supplemented with glutamate (2 mmol/L), 30% fetal bovine serum and penicillin: streptomycin, 100 µg/mL.

Medium pH was adjusted to 7.35 to 7.45 by adding sodium hydroxide, if necessary, during incubation in a 5% carbon dioxide–air incubator at 37°C. The tissue culture medium was changed every 48 to 72 hours. BA₁, concanamycin A, erythromycin (obtained from Sigma Chemical Co, St Louis, Mo), and FK-506 (generous gift from Fujisawa Pharmaceutical Co, Tokyo, Japan) were dissolved in dimethylsulfoxide and diluted with tissue culture medium to yield desired concentrations (5 nmol/L–1 µmol/L) and 0.1%

dimethylsulfoxide. Control cultures were performed with medium containing 0.1% dimethylsulfoxide. The effect of BA₁ on cell proliferation was examined in cultured SV segments, which were supplemented with 50 µmol/L of 5-bromo-2'-deoxyuridine (BrdU) in the presence or absence of BA₁ between days 11 and 12 in culture. At the end of the cultivation, the SV segments were fixed with 10% formaldehyde in 0.1 mol/L phosphate buffer, pH 7.2, for light microscopy, or with 2.5% glutaraldehyde in 0.1 mol/L sucrose/sodium cacodylate HCl buffer, pH 7.2, for electron microscopy. The glutaraldehyde-fixed SV segments were postfixed in 1% osmium tetroxide, dehydrated, critical point dried in liquid carbon dioxide, and viewed with a scanning electron microscope (Hitachi S-700; Hitachi, Tokyo, Japan). The dehydrated sections were embedded also in Epon 812 fixative, cut with a diamond knife, double stained with uranyl acetate and lead citrate, and examined on a transmission electron microscope (Hitachi H-7000; Hitachi).

Immunohistochemistry. The formaldehyde-fixed and paraffin-embedded segments were cut into 4 µm-thick sections and deparaffinized. The sections were incubated with proteinase K (Roche Molecular Biochemicals [formerly Boehringer-Mannheim Biochemica, Mannheim, Germany]) at a concentration of 40 µg/mL for 10 minutes at 37°C. Immunostaining for BrdU and cell marker proteins (ie, von Willebrand factor, α-smooth muscle actin [α-SMA], desmin, and vimentin) was performed as described previously.^{17,18} The primary antibodies used in the present study were mouse monoclonal anti-BrdU antibody (50:1 dilution), mouse monoclonal F8/86 antibody recognizing von Willebrand factor (50:1 dilution), mouse monoclonal 1A4 antibody recognizing α-SMA (50:1 dilution), mouse monoclonal D33 antibody recognizing desmin (100:1 dilution), and mouse monoclonal V9 antibody recognizing vimentin (5:1 dilution; all obtained from Dako JAPAN, Tokyo, Japan).

Immunohistochemical detection of apoptotic cells was performed by a terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method¹⁹ using a kit (Apop Tag Plus; Oncor Inc, Gaithersburg, Md). Double immunofluorescence experiments to identify TUNEL-positive cells concomitant with desmin-expressing cells were performed by the TUNEL method with fluorescein isothiocyanate-conjugated rabbit anti-sheep immunoglobulin G (400:1 dilution; Dako) as a secondary antibody. The sections were then incubated with mouse monoclonal D33 antibody as described earlier, followed by staining with tetra-rhodamine isothiocyanate-conjugated rabbit anti-mouse immunoglobulin G (200:1 dilution; Dako). The fluorescence staining was viewed with confocal laser microscope (Olympus Co, Tokyo, Japan).

Quantitative measurements. Neointimal areas were delineated by the inner edge of the internal elastic laminae. Medial areas were traced between the internal elastic laminae and the inner border of the external elastic laminae. Adventitial areas were defined between the inner border of the external elastic laminae outward to the free edge of the connective tissue. Intimal and medial thickness was measured in sections stained with elastica van Gieson stain with

Fig 1. Transverse sections of human SV segments stained with elastica van Gieson. **A**, A freshly isolated vein segment before culture. **B**, A vein segment after 14 days in culture. **C**, A vein segment after 14 days in culture in the presence of 10 nmol/L of bafilomycin A₁. Arrowheads and arrows point to the internal elastic laminae and the external elastic laminae, respectively. (Original magnification, $\times 40$.)

the use of a calibrated grid at 5 to 10 points of the middle portion on 2 sections from each SV graft; the mean value was calculated to obtain the data.

BrdU-labeled cells were counted on 3 sections from each segment. TUNEL-positive cells with positive or negative expression of desmin were identified by double immunofluorescence confocal laser microscopy as described earlier. The number of TUNEL-positive cells with positive immunofluorescence staining for desmin in the media and those with negative immunofluorescence staining for desmin in the neointima were counted on 3 sections from each segment at magnification $\times 600$. The percentage of TUNEL-positive cells was calculated by dividing the number of TUNEL-positive cells with positive immunofluorescence staining for desmin in the media or the number of those with negative immunofluorescence staining for desmin in the neointima by the total number of cells with a respective immunoreactivity pattern for desmin in each layer.

Statistics. All numeric data are presented as mean $\pm$ SD. One-way analysis of variance and the Scheffé multiple comparison test were used to compare the multigroup variables.

Results

Effects of BA₁ on neointimal formation and medial thickening. Elastica van Gieson stain showed no visible intimal layer above the inner border of internal elastic laminae in the freshly isolated SV segments after preparation of culture (Fig 1, A). After 14 days in culture, neointimal formation occurred above the inner

border of internal elastic laminae, associated with marked thickening of the media (Fig 1, B). Treatment of cultured SV segments with 10 nmol/L of BA₁ for 14 days abolished neointimal formation and medial thickening (Fig 1, C).

Quantitative evaluation of the effect of BA₁ on the vessel wall hyperplasia showed that significant inhibition of neointimal formation and medial thickening was evident by 5 nmol/L of BA₁ (Fig 2). At 10 nmol/L, BA₁ completely inhibited neointimal formation and medial thickening of the SV segments. A similar inhibitory effect was observed with 10 nmol/L of concanamycin A, another macrolide antibiotic known as a selective and potent inhibitor of V-ATPase.¹³ In contrast, FK-506 and erythromycin, which are the structurally related macrolide antibiotics, failed to inhibit neointimal formation and medial thickening at a concentration up to 1 μ mol/L.

Effects of BA₁ on endothelial cell coverage. The freshly isolated SV segments were entirely covered with a layer of endothelial cells in scanning electron microscopy and immunostaining for von Willebrand factor (Fig 3, A and D). All of the SV segments after 14 days in culture were led to complete uncoverage of endothelial cells, which were replaced by neointimal cells with smooth muscle cell-like morphologic features (Fig 3, B and E). Cultivation of the SV segments in the presence of 10 nmol/L of BA₁ preserved endothe-

Fig 2. Effects of BA₁ and the structurally related macrolide antibiotics on thickness of the neointima (A) and the media (B). Human SV segments were cultured for 14 days in the absence or the presence of indicated concentrations of BA₁, concanamycin A, FK-506, and erythromycin. The *filled bars* indicate neointimal thickness; the *open bars* indicate medial thickness. Preculture (n = 12), vehicle (n = 10), 5 nmol/L BA₁ (n = 10), 10 nmol/L BA₁ (n = 10), 10 nmol/L concanamycin A (n = 8), FK-506 (n = 10), erythromycin (n = 10). Results are expressed as mean ± SD. ND, Not detected. *P < .001; **P < .0001 vs vehicle.

Fig 3. Intimal surface structure of human SV segments. Scanning electron micrographs (A-C; original magnification, ×1000) and immunohistochemical staining for von Willebrand factor (D-F; original magnification, ×400) are representative of 10 vein segments examined under each of the following conditions: A and D, a freshly isolated vein segment before culture; B and E, a vein segment after 14 days in culture; and C and F, a vein segment after 14 days in culture in the presence of 10 nmol/L of BA₁.

lial cell coverage on the intimal surface (Fig 3, C and F).

Effects of BA₁ on expression of cytoskeletal markers. It has been proposed that neointimal cells proliferated after animal models of endoluminal injury of coronary arteries and animal models of vein graft surgery are myofibroblasts.^{17,18,20,21} This possibility was tested in our organ culture model of human SV segments by the determination of the expression of cytoskeletal protein markers (ie, α -SMA, desmin, and vimentin). The freshly isolated SV segments showed bundles of smooth muscle cell layers in the media, which were positive for α -SMA and desmin (Fig 4, A and B). These SV segments also contained non-muscle cells with negative immunostaining for α -SMA and desmin but positive immunostaining for vimentin, which were distributed between the medial smooth muscle cell layers and in the adventitia (Fig 4, C). Immunohistochemically detectable myofibroblasts, which have been characterized by positive immunostaining for α -SMA and vimentin but negative immunostaining for desmin,²² were not found in the freshly isolated SV segments. The organ culture of the SV segments for 14 days markedly altered the distribution of the cells. Although α -SMA-positive cells were distributed uniformly in the neointima (Fig 4, D), the neointimal cells were exclusively negative for desmin but were positive for vimentin (Fig 4, E and F). Thus the neointimal cells expressed a phenotypic feature consistent with myofibroblasts. In the media, α -SMA-positive cells were distributed inhomogeneously, and most of the α -SMA-positive cells were also positive for desmin or vimentin. Because desmin is an intermediate filament protein usually found only in muscle cells but not in fibroblastic populations,²³ this finding indicates that the medial cells were composed of differentiated smooth muscle cells and myofibroblasts. It should also be noted that the number of smooth muscle cells with positive immunostaining for desmin was reduced in comparison to that in the freshly isolated SV segments. A considerable number of myofibroblasts were localized around the free edge of the adventitia (not shown). The SV segments that were cultured in the presence of 10 nmol/L of BA₁ for 14 days showed bundles of cell layers with immunoreactivity patterns for α -SMA and desmin, similar to those observed in the freshly isolated SV segments and suggestive of differentiated smooth muscle cells. In serial sections, α -SMA appeared to colocalize with desmin in layers of the medial cells (Fig 4, G and H). Only vimentin-positive non-muscle cells were distributed between the medial smooth muscle cell layers and in the adventitia as observed in the freshly isolated SV segments (Fig 4, I). Immunohistochemically detectable myofibroblasts were not found in the BA₁-treated SV segments.

Effects of BA₁ on BrdU uptake. BrdU was used to identify proliferating cells in the cultured SV segments. When BrdU was administered to the SV segments between days 11 and 12 in culture, the intimal cells incorporated BrdU in their nuclei (Fig 5, A). Quantitative analysis of the number of BrdU-labeled cells in the cultured SV segments without BA₁ treatment showed that $45.6\% \pm 20.1\%$ ($n = 8$ segments) of the neointimal cells expressed positive immunostaining for BrdU. In contrast, incubation with BrdU in the presence of 10 nmol/L of BA₁ abrogated BrdU uptake ($0.8\% \pm 0.5\%$; $n = 8$) by the neointimal cells (Fig 5, B).

Effects of BA₁ on apoptosis. These observations suggest that BA₁ induces the inhibition of neointimal formation and that medial thickening may be caused by growth arrest of myofibroblasts. However, it is also possible that BA₁ could induce apoptotic cell death,¹⁵ which might be responsible for reducing the number of myofibroblasts. This possibility was tested by the treatment of SV segments with 50 nmol/L of BA₁ between days 11 and 12 in culture. Transmission electron microscopy showed the characteristic morphologic features of apoptosis in the neointimal cells within 24-hour treatment with BA₁. The neointimal cells showed chromatin condensation at the periphery of the nucleus associated with cellular shrinkage and the formation of vesicles in the presence of the intact plasma membrane (Fig 6, A). The neointimal cells underwent nuclear fragmentation and disintegration of intracellular organelles after the BA₁ treatment for 48 hours (Fig 6, B). Most of the neointimal cells were found to undergo apoptosis as detected by TUNEL immunostaining after 48-hour treatment with 50 nmol/L of BA₁, although a smaller number of TUNEL-positive cells were found in the media (Fig 6, C). Under double immunofluorescence confocal laser microscopy, TUNEL-positive cells in the neointima were devoid of immunofluorescence staining for desmin, whereas most of the medial cells expressing desmin were negative for TUNEL (Fig 6, D).

The dose response effects of BA₁ on the number of TUNEL-positive cells with positive immunofluorescence staining for desmin and the number of those with negative immunofluorescence staining for desmin were tested by incubating 10-day cultured SV segments with 3 different concentrations of BA₁ for 48 hours (Fig 7). BA₁ at a concentration of 5 nmol/L did not significantly increase the number of TUNEL-positive cells, with either immunoreactivity pattern for desmin. However, 10 nmol/L of BA₁ significantly increased the number of TUNEL-positive cells, with negative immunofluorescence staining for desmin. BA₁ at a concentration of 50 nmol/L induced apoptosis on more than two thirds of cells with negative immunofluorescence staining for desmin in the neointima, without a significant effect on

Fig 4. Immunohistochemical staining for α -SMA, desmin, and vimentin. Serial sections obtained from the freshly isolated SV segment were immunostained for α -SMA (A), desmin (B), and vimentin (C). Note that vimentin-positive cells are distributed between the medial smooth muscle cell layers (arrows) and in the adventitia (arrowhead). Serial sections obtained from the SV segment after 14 days in culture were immunostained for α -SMA (D), desmin (E), and vimentin (F). Serial sections obtained from the SV segment after 14 days in culture in the presence of 10 nmol/L of BA₁ were immunostained for α -SMA (G), desmin (H), and vimentin (I). The arrow points to the vimentin-positive cell. *m*, Media; *a*, adventitia; *i*, intima. (Original magnification, $\times 100$.)

Fig 5. Immunohistochemical staining for BrdU-labeled cells. The SV segment after 10 days in culture was incubated with 50 $\mu\text{mol/L}$ of BrdU for 48 hours in the absence or presence of 10 nmol/L of BA_1 . **A**, Incubation with BrdU in the absence of BA_1 . **B**, Incubation with BrdU in the presence of BA_1 . *i*, Intima; *m*, media. (Original magnification, $\times 400$.)

the number of those cells with positive immunofluorescence staining for desmin in the media. These observations indicate that BA_1 induces apoptosis on the neointimal myofibroblasts and that those cells are more susceptible to apoptosis by inhibition of V-ATPase compared with the medial smooth muscle cells expressing desmin.

Discussion

The present study is the first to propose that V-ATPase may play a crucial role in vessel-wall hyperplasia. Neointimal formation and medial thickening occurred in cultured human SV segments that had been freshly isolated from the patients who had undergone coronary artery bypass operations. Endothelial cells were lost from the intimal surface and replaced by the neointimal cells by 10 days in culture. Neointimal formation, medial thickening, and the loss of endothelial cells were prevented by nanomolar concentrations of BA_1 and concanamycin A but not by the structurally related macrolide antibiotics FK-506 and erythromycin. The highly selective nature of BA_1 and concanamycin A to inhibit V-ATPase activity strongly suggests that V-ATPase is involved in the vessel-wall hyperplasia in cultured human SV segments.

The results of the present study argue for the potential role of myofibroblasts in vessel-wall hyperplasia and suggest further that V-ATPase is crucial for myofibroblast growth. The freshly isolated SV segments did not contain myofibroblasts, which have been characterized by positive immunostaining for $\alpha\text{-SMA}$ and vimentin but negative immunostaining for desmin.²² However, myofibroblasts were grown in the neointima during 14 days in culture. Cultivation of the SV seg-

ments in the presence of 10 nmol/L of BA_1 inhibited the emergence of myofibroblasts. The same concentration of BA_1 abrogated BrdU uptake by the neointimal cells in 10-day cultured SV segments. These results indicate that BA_1 -induced suppression of neointimal hyperplasia is not a consequence of the inhibition of myofibroblast migration to the intima but is due to the growth arrest of myofibroblasts and that BA_1 -induced attenuation of medial thickening is also attributable to reduced synthesis of extracellular matrix by myofibroblasts. We have previously demonstrated that both BA_1 and FK-506 are capable of inhibiting neointimal hyperplasia in the cryopreserved rat aortic allograft.¹⁶ The evidence shown here substantiates the assumption that BA_1 and FK-506 inhibit neointimal hyperplasia through a distinct mechanism (ie, V-ATPase inhibition and immunosuppression, respectively).

The ability of BA_1 to inhibit the emergence of myofibroblasts in cultured SV segments may not only result from growth arrest but may also be ascribed to cell death through apoptosis. The present study demonstrated that the treatment of 10-day cultured SV segments with 50 nmol/L of BA_1 for 48 hours produced morphologic changes characteristic for apoptosis. Even lower concentrations of BA_1 significantly increased the number of TUNEL-positive cells. On the other hand, endothelial cells and desmin-expressing smooth muscle cells appear to have survived under the BA_1 treatment. The relative insensitivity of differentiated smooth muscle cells to BA_1 was documented by the fact that desmin-positive smooth muscle cells in the media withstood the 48-hour challenge of 50 nmol/L BA_1 , which caused apoptosis on most desmin-negative cells in the neointima.

Fig 6. The apoptotic neointimal cells detected with transmission electron microscopy (**A** and **B**) and with TUNEL with the use of light microscopy (**C**) or confocal laser microscopy (**D**). The neointimal cell of the vein segment treated with 50 nmol/L of BA₁ for 24 hours after 10 days in culture shows chromatin condensation at the periphery of the nucleus associated with cellular shrinkage and formation of vesicles in the presence of intact plasma membrane (**A**). The neointimal cell shows nuclear fragmentation and disintegration of intracellular organelles after 48 hours of treatment with BA₁ (**B**). (Original magnifications, **A** and **B**, $\times 8000$.) **C**, The vein segment treated with 50 nmol/L of BA₁ for 48 hours after 10 days in culture shows that most of the neointimal cells are positive for TUNEL, although few medial cells are positive for these indices of apoptosis. *i*, Intima; *m*, media. (Original magnification, $\times 200$.) **D**, A serial section of the vein segment was doubly immunostained for TUNEL and desmin with fluorescein isothiocyanate-conjugated and tetra-rhodamine isothiocyanate-conjugated secondary antibodies, respectively. The immunofluorescence staining was visualized with confocal laser microscopy. Note that the neointimal cells with positive immunofluorescence for TUNEL (arrowhead) are negative for desmin, although medial cells with negative immunofluorescence for TUNEL are positive for desmin (arrow). Bar, 10 μ m.

The molecular mechanisms underlying V-ATPase involvement in myofibroblast growth remain elusive. However, some pathophysiologic roles of V-ATPase in regulating myofibroblast growth are anticipated from the viewpoint that the stimulation of V-ATPase activity could provide a desirable milieu for cell proliferation and survival. First, acidification of vacuolar compartments in eukaryotic cells by V-ATPase serves a number of important functions for mitogenic activity, such as insulin and epidermal growth factor receptor recycling and reuse.^{24,25} Moreover, full mitogenic activity of bFGF has been shown to require internalization of the

growth factor or the growth factor-receptor complex by V-ATPase-dependent endocytosis followed by translocation to the nucleus.²⁶ Second, V-ATPase may be involved in facilitating molecular transport across organelle membranes. Unlike mitochondrial F-type H⁺-ATPases which synthesize ATP at the expense of proton motive force, the vacuolar systems of eukaryotes are energized primarily by V-ATPase that functions as an ATP-dependent proton pump to generate proton motive force.⁹ The electrochemical proton gradient is used in driving the coupled transport of essential ions and macromolecules, which are usually impermeant

Fig 7. The dose response effect of BA₁ on apoptosis. The SV segments after 10 days in culture were treated with 50 nmol/L of BA₁ for 48 hours. Double immunofluorescence confocal laser microscopy for identifying TUNEL-positive cells with or without expression of desmin was performed as described. The percentage of TUNEL-positive cells was calculated by dividing the number of TUNEL-positive cells with positive immunofluorescence staining for desmin in the media or the number of those cells with negative immunofluorescence staining for desmin in the neointima by the total number of cells with a respective immunoreactivity pattern for desmin in each layer. *Open bars* indicate medial cells expressing desmin; *filled bars* indicate neointimal cells not expressing desmin. Results are expressed as mean $\pm$ SD of 7 experiments. * $P < .05$; ** $P < .0001$, compared with the vehicle (0.1% dimethylsulfoxide) group; † $P < .05$; †† $P < .001$, compared with the desmin-positive medial cell group.

through vacuolar membranes. Therefore, the inhibition of V-ATPase would result in the retardation of molecular trafficking, leading to growth arrest and even cell death. Finally, the activation of V-ATPase may be involved in the regulation of cytosolic pH. The maintenance of cytosolic pH is crucial for cell growth and survival. It has long been known that cytosolic alkalization provokes cell proliferation,²⁷ whereas intracellular acidosis is an important trigger for hypoxia-, ischemia-, and irradiation-induced cell death.²⁸⁻³⁰ To elucidate the exact role of V-ATPase in myofibroblast growth, the function and localization of V-ATPase remain to be investigated.

The present study was designed to address the possible involvement of V-ATPase in the vessel wall hyperplasia with the use of an organ culture model of human SV. This model allowed the resolution of spatial and temporal distributions of vascular cells within the vascular wall. However, this organ culture model may be difficult to extrapolate directly to in vivo situations, and the results should be interpreted with caution. During 14 days of culture of the SV seg-

ments, it is likely that there are significant zones of hypoxia, especially in the medial core, that may increase susceptibility of the vascular cells to BA₁, leading to the overestimation of the proapoptotic effect of BA₁. However, our present study showed that there was no increase in the number of TUNEL-positive cells in the media compared with the neointima, irrespective of the absence or presence of BA₁. In addition, the preservation of endothelial cells by BA₁ treatment is unlikely to be due to re-endothelialization but is attributed to the inhibition of myofibroblast growth. Nevertheless, preservation of endothelial cells and differentiated smooth muscle cells in BA₁-treated SV segments provides significant clinical implications, because these cells play an important role in normal vascular physiologic and morphologic features. Maintaining the integrity of vascular structure after vein graft surgery would prevent subsequent progression of vein graft disease.

In conclusion, the present study provides a new insight into the mechanism of neointimal formation. The results suggest that V-ATPase may be involved in myofibroblast growth that contributes to neointimal formation and medial thickening in cultured human SV. The inhibition of myofibroblast growth and preservation of endothelial cells and differentiated smooth muscle cells by BA₁ may have potential therapeutic implications in the treatment for vein graft disease.

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