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Delivery of caveolin-1 gene into arterial walls of rabbits via an infiltrator angioplasty balloon catheter

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Abstract

Gene therapy is a rapidly developing field with great potential for the treatment of atherosclerosis. Choosing the appropriate system for the precise delivery of genes into targeted plaques is still a challenging task for vascular gene therapists. We investigated the site-specific expression of the caveolin-1 gene within the aortic vessel walls in a rabbit model following local delivery of the gene via an infiltrator angioplasty balloon catheter (IABC). The full-length untagged cDNA encoding human caveolin-1 was fused to the myc reporter gene using a pcDNA 3.1 plasmid. The caveolin-1-myc was then infused into the using a pcDNA 3.1 plasmid. The caveolin-1-myc was then infused into the aorta via IABC twice every three days for two weeks. The site-specific expression of caveolin-1 protein was analyzed by immunohistochemisty at two weeks after the last injection. Immunofluorescence analysis and Western blot indicated that caveolin-1-myc was present in the transfected cells. Caveolin-1 protein was detected in the medial layers of rabbit aorta for up to two weeks after IABC infusion and there was no intimal/medial thickening in the infusion area. IABC appears to be an effective gene delivery system for the treatment of cardiovascular diseases.

Key Words：Atherosclerosis, Caveolin-1, Gene therapy, Local delivery system

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Introduction

Gene therapy is a rapidly developing field with great potential for the treatment of atherosclerosis. Successful vascular gene therapy is dependent on the precise delivery of genes into targeted plaques, the efficient transfer of genes into the intimal/medial layers of the target, and the assessment of gene expression over time[1]. Among these criteria, the most challenging task for interventional specialists is choosing the most efficient drug delivery system. Over the last two decades, great advances have been made in percutaneous conronary intervention (PCI) for the treatment of coronary atherosclerotic disease [2]. However, new evidence suggests that the delivery of large quantities of pharmaceutical agents via drug-eluting stents puts patients at risk for sub-acute thrombosis due to delayed or limited re-endovascularization [3-6]. Recently a local delivery device, infiltrator angioplasty balloon catheter (IABC), has been developed to deliver fluid-phase substance directly into the vessel wall after conventional angioplasty[7]. With angioplasty and a specifically designed InjectionPort, the IABC can deliver genes more precisely and efficiently into the vessel wall.

Caveolin-1, the major structural protein of caveolae, is mainly expressed in endothelial cells, macrophages, and smooth muscle cells [8]. This protein has the capacity to bind cholesterol as well as a variety of proteins [9, 10]. Several studies have demonstrated that the over-expression of caveolin-1 enhances the cholesterol efflux in culture systems [11-14]. A decreasing level of caveolin-1 in the arterial wall was reported to be associated with increasing smooth muscle cell proliferation in hypercholesterolemic rabbits [15]. Furthermore, the injection of antennapedia-caveolin-1 (AP-caveolin-1) peptide into animals has been shown to reduce inflammation [16]. Our preliminary study showed that AP-caveolin-1 can also repress the formation of atherogenic plaques in hypercholesterolemic rabbits (unpublished data). In this study, we used IABC to infuse the caveolin-1 gene into rabbit aorta to investigate the site-specific expression of caveolin-1 gene within the vessel walls.

Materials and Methods

Delivery device

A specially designed infiltrator angioplasty balloon catheter (Infiltrator®, Boston Scientific Corporation, Boston, MA) was used in this study (Figure 1). The device consists of a conventional noncompliant angioplasty balloon with three polyurethane injector strips, each with a 0.25-mm high nickel cone (InjectorPort) as an infusate

delivery channel . The balloon is 15 mm long and 3.0–4.0 mm in diameter. The distance covered by the InjectorPort is 10.7 mm. The catheter shaft is a monorail configuration consisting of three adjacent lumens: the first one is used to deliver infusate through the InjectorPort; the second one contains a support wire; the third one is used to inflate and deflate the balloon. The shaft diameter is 4.2 French, and the total length is 132 cm. Before delivery, the balloon is flattened and the injector nipples are recessed. When the infiltrator is inflated in the vessel, the balloon is fully apposed with the vessel wall, and the InjectorPort is radically extended so that it penetrates the intimal/medial layers of the artery.

Construction of caveolin-1-myc plasmids

The full-length untagged cDNA encoding human caveolin-1 (NM001753) was fused, in frame and full-length, to the myc reporter gene using a pcDNA 3.1 plasmid (Invitrogen, San Diego, CA). The myc epitope was attached to the C-terminal end of the caveolin-1gene. The EcoRI restriction site was added to the 3' and 5' ends of human caveolin-1 cDNA by polymerase chain reaction (PCR). After digesting with EcoRI, the PCR product was inserted into the pcDNA 3.1 vector. The proper orientation and sequence of caveolin-1-myc were verified by sequencing.

Transient transfection of plasmids

Transfection was performed as described by Lin et al.[14]. Twenty-four hours before transfection, 3×10^4 cells per well were seeded in 24-well tissue culture plates. On the day of transfection, 0.4 μg of caveolin-1-myc plasmid was diluted in 25 μL of serum-free DMEM. A 1 μL aliquot of lipofectamine (Invitrogen, San Diego, CA) was then diluted in 25 μL of serum-free DMEM. The diluted caveolin-1-myc DNA and the lipofectamine were then gently mixed and incubated at 25°C for 20 min. After incubation, 250 μL of serum-free DMEM was added to the DNA/lipofectamine mixture. The final mixture was added to the cultured cells that had been grown on a 24-well plate. After incubation at 37°C for 5 h, the cells were removed from the 24-well plates, placed into 250 μL of DMEM containing 20% serum, and allowed to grow for an additional 24 h.

Immunofluorescence staining

Endothelial cells were grown on a cover slip in a 24-well plate for 24 h, followed by transfection with caveolin-1-myc plasmid using lipofectamine for 24 h. The cells were then washed with ice-cold PBS-albumin and fixed in 2% paraformaldehyde for 25 min at room temperature. After washing with PBS, the cells were incubated with rabbit anti-human caveolin-1 antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA) for endogenous caveolin-1 or mouse anti-myc antibody (1:200; Zymed, South San Francisco, CA) for exogenous caveolin-1 for 30 min at room temperature. The cells were then washed with PBS, incubated for 30 min with TRITC-conjugated goat anti-rabbit (1:200; Zymed, South San Francisco, CA) or goat anti-mouse IgG (1:200; Zymed, South San Francisco, CA), mounted on slides, and photographed with a Nikon E400 immunofluorescence microscope (Nikon, Tokyo, Japan).

Western blot analysis

Cell lysates were harvested in lysis buffer (tris-HCL, 10 mM, pH 7.4, containing 150 mM NaCl, 1 mM benzamidine, 0.5 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride desolved in dimethyl sulfoxide), homogenized, and centrifuged for 10 min at 800 g at 4°C. The protein concentration was measured with BCA protein assay (Pierce). The proteins (50 μg) was separated by 12% SDS-PAGE and electrotransfered to a polyvinylidene fluoride (PVDF) membrane. Blots were probed with rabbit anti-human caveolin-1 antibody (1:7500; Santa Cruz Biotechnology, Santa Cruz, CA) and mouse anti-myc antibody (1:7500; Santa Cruz Biotechnology, Santa Cruz, CA), followed by HRP-conjugated goat anti-rabbit IgG (1:15000; Zymed, South San Francisco, CA) or goat anti-mouse IgG (1:15000; Zymed, South San Francisco, CA). After washing with PBS, peroxidase activity was visualized using enhanced chemiluminescence (PerkinElmer Life Science, Boston, MA). For an internal control, the same membrane was re-probed with a monoclonal antibody directed against β-actin (1:10000; Sigma, St. Louis, MO). The intensities of the reaction bands were analyzed with the Image Gauge System (Fuji, Tokyo, Japan).

Plasmid or GFP injection

Ten male New Zealand white rabbits, each weighing 3.0~3.5 kg were fed a standard diet (Fu-Shou Co.; Taichung, Taiwan). Animals were anesthetized

intramuscularly with a mixture of ketamine (40 mg/kg) and xylocaine (5 mg/kg). The right femoral artery was exposed and a standard 0.018-inch guidewire (Boston Scientific, Boston, MA) was advanced under ultrasound guidance (Sonos 5500, Philips, equipped with a S12 probe, frequency 5.0–12.0 MHz). The IABC was introduced through the descending aorta and extended to the aortic arch . The balloon was inflated to 6 bar and the three rows of nipples on the longitudinal infiltrator penetrated the vessel wall. Green fluorescent protein (GFP) was used as a marker for the detection of the injection site. Through an independent infiltrator port, caveolin-1-plasmid or GFP was infused directly into the vessel wall. A dose of 0.5 μg/kg caveolin-1-myc plasmid was infused once at three-day intervals for two weeks. The animals were sacrificed at two weeks after the last injection . The aortic arch was collected for fluorescence microscopy and immunohistochemical study.

Fluorescence microscopy and immunohistochemistry

For GFP analysis, the cryostat tissue sections (7 μm thick) of the aortic arches from 3 rabbits were post-fixed in methanol at –20ºC and then washed in PBS. Fluorescence was observed using an E 400 immunofluorescence microscope (Nikon, Tokyo, Japan). For immunohistochemistry, the aortic arches from 7 rabbits were dissected, washed with PBS, and embedded in paraffin. The thick sections were cut (each 6 μm thick) and myc protein was detected via the streptavidin-biotin method. Endogenous peroxidase activity was quenched by treating the tissue sections with 3% hydrogen peroxide for 10 min. Nonspecific background staining was eliminated by incubating the tissue sections with non-immune serum. After washing with PBS, tissue sections were incubated with rabbit anti-human myc-antibody (1:50; Santa Cruz Biotechnology, Santa Cruz, CA) at 37ºC for 1 h, followed by the addition of biotinylated goat anti-rabbit IgG. Tissue sections were then washed and incubated with streptavidin-peroxidase conjugate, followed by the addition of an H_2O_2-DAB substrate-chromogen solution (Zymed, South San Francisco, CA). Immunostaining specificity was checked by treating the control slides as described above, except that the primary antibody was omitted. Tissues were then counterstained with hematoxylin and eosin, dehydrated, mounted, and photographed with a Nikon light microscope (Nikon, Tokyo, Japan).

Results

Transfection of plasmid into endothelial cells in vitro

The immunofluorescence analysis indicated that myc, which represented the exogenous caveolin-1, was present in the transfected cells (Figure 2A), and that endogenous and exogenous caveolin-1 were distributed in the cytoplasm of the transfected cells (Figure 2B). There was no myc-positive staining in the non-transfected cells (Figure 2C). Furthermore, the level of caveolin-1 fluorescent staining was markedly lower in the non-transfected cells than in the transfected cells (Figure 2D).

Western blotting analysis

The expression of caveolin-1 myc protein in transfected cells was analyzed by Western blotting. The results showed that the endogenous caveolin-1 (22 kDa) and exogenous c-myc-tagged caveoiln-1 (29kDa) were present in the caveolin-1-myc plasmid-transfected cells (Figure 3). Actin was used as an internal control.

The expression of caveolin-1-myc protein in the arterial wall

At 48 h after GFP injection via the ICBA balloon catheter, the injection sites were identified as the green fluorescent circles in the medial layer of the aorta (Figure 4A). Two weeks after infusion of caveolin-1-plasmid via IABC, the signals of HRP reaction products representing the caveolin-1-myc protein appeared in the endothelial layer and the medial layer (Figure 4B, 4C). No reaction products were found in the vessel wall of the negative control (Figure 4D). No thrombus or intimal/medial thickening occurred in the infusion area (Figure 4A-D).

Discussion

In this study, we demonstrated that caveolin-1 protein was expressed in the vessel walls of the thoracic aorta for up to two weeks after infusion of the caveolin-1-myc plasmid via IABC. This finding indicates that IABC is an efficacious system for delivering genes in the treatment of cardiovascular diseases. Devices that provide local delivery of drugs or agents are advantageous over systemic delivery because lower concentrations of drug can be administered, thereby causing fewer systemic side effects and hence improving therapeutic efficacy. Over the past few years, a number of devices, such as the Dispatch® balloon (Scimed Inc), the Crescendo® balloon (Cordis Corp)[5] , intrapericardial delivery[17] and stent-based therapy, have became available for delivering agents to the site of the angioplasty. These local delivery devices do not cause marked vascular disruption; however, they are associated with rapid blood washout and a low efficiency of drug delivery after injection. In contrast, the IABC device is suitable for the combination of dilatation and delivery procedures[7]. In the present study, no intimal/medial thickening and no thrombus formation were observed in the treated vessels 48 h or two weeks after IABC infusion of the caveolin-1 gene (Figure 4). The detection of GFP [18] with fluorescence microscopy confirmed the circular distribution of the delivered fluid in the whole vessel after infiltration (Figure 3).

Restenosis is primarily caused by neointimal hyperplasia. Migration and proliferation of smooth muscle cells after mural injury is a principal event that leads to neointimal hyperplasia. A number of genes have been tested in gene therapy to block smooth muscle cell migration and proliferation[19]. Caveolin-1, the main structural protein of caveolae, is involved in the regulation of cellular cholesterol metabolism and lipid uptake, as well as efflux[12, 20]. In smooth muscle cells, caveolin-1 can negatively regulate cell proliferation[14, 16]. In caveolin-1-deficient mice, there is a dramatic enhancement of neointima hyperplasia[21, 22]. Therefore, a change in caveolin-1 could directly impact vascular function and result in the development of atheroma. The regression of atherosclerotic plaques in the hypercholesterolemic rabbits after infusion of caveolin-1 plasmid by IABC is currently under investigation in our laboratory.

Retroviral, adenoviral, and adeno-associated viral vectors can be used for the delivery of genes to the targeted lesions. Although each has its merits, none of these vectors is considered ideal for safe and efficient gene transfer and for stable gene expression[18]. In this study, we demonstrated that an IABC could provide efficacious local application of genes to diseased areas. In addition, we monitored the delivered

caveolin-1 gene for up to two weeks and found no proliferation or migration in the medial layer.

IABC appears to an effective gene delivery device for local treatment of cardiovascular diseases. This device could well be used in preventive therapy for insignificant but vulnerable plaque prior to the development of acute coronary syndrome in order to mitigate adverse reactions caused by stent struts.

Acknowledgements

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Braun-Dullaeus RC, et al. Decreased caveolin-1 in atheroma: Loss of antiproliferative control of vascular smooth muscle cells in atherosclerosis. *Cardiovasc Res* 2005; 68:128-35.

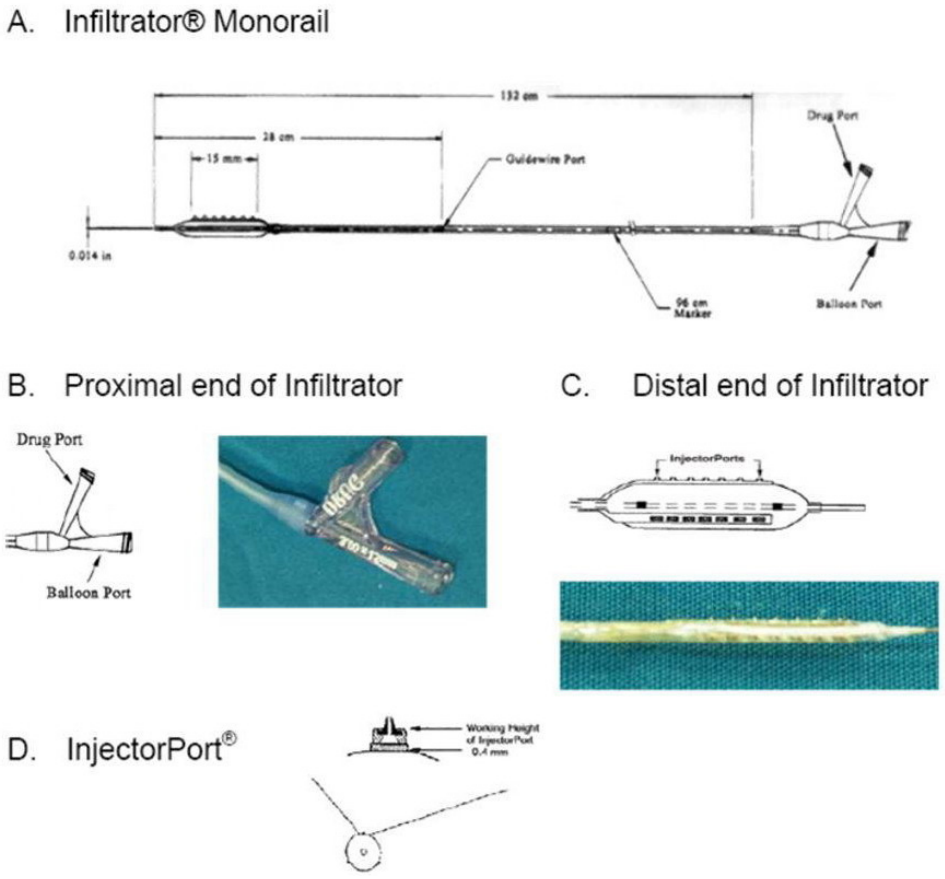

Figure 1. The Infiltrator, the catheter-based delivery system used in this study, consists of a conventional noncompliant angioplasty balloon and a drug delivery system (A)**.** The proximal portion of the Infiltrator includes one port for drug infusion and another port for balloon inflation (B). The distal portion of the Infiltrator includes a 15-mm noncompliant balloon with three polyurethane strips, each with a 0.25-mm high nickel cone (C). The InjectorPort (D) is a vertical hole mounted longitudinally on the surface of the infusate delivery channel .

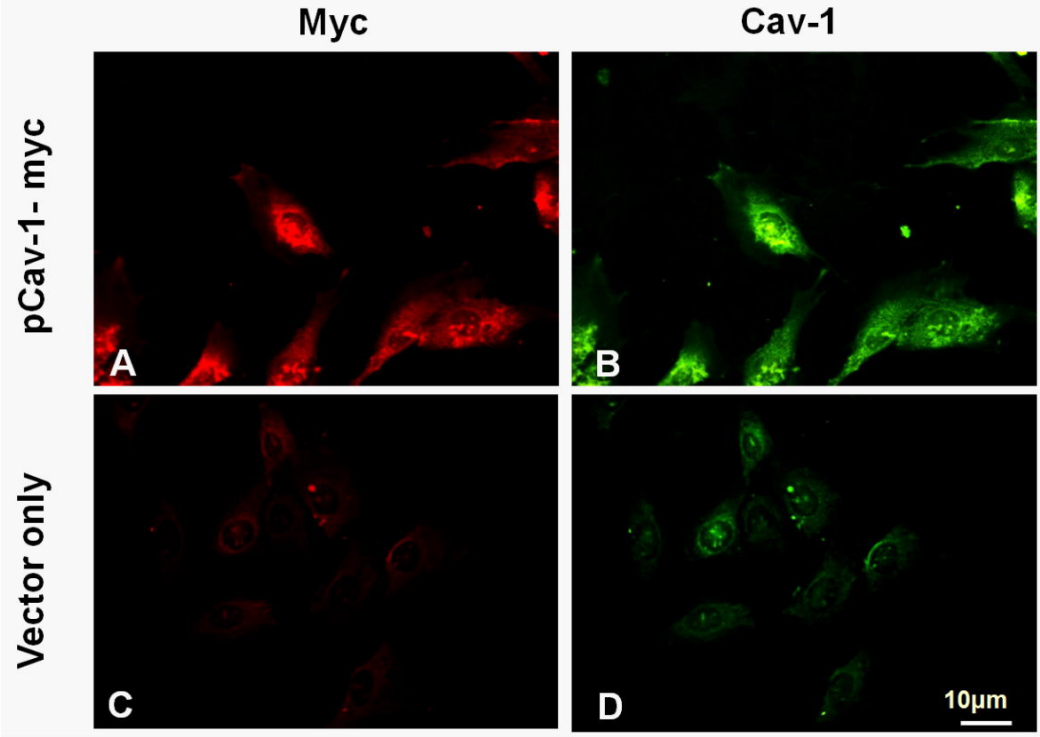

Figure 2. Immunofluorescent micrographs of caveolin-1-myc transfected cells (A, B) and vector-only controls (C, D). The fluorescence of myc protein (red) and caveolin-1 (green) showed high intensity in the transfected cells (A, B) compared to the level of intensity in the vector-only controls (C, D).

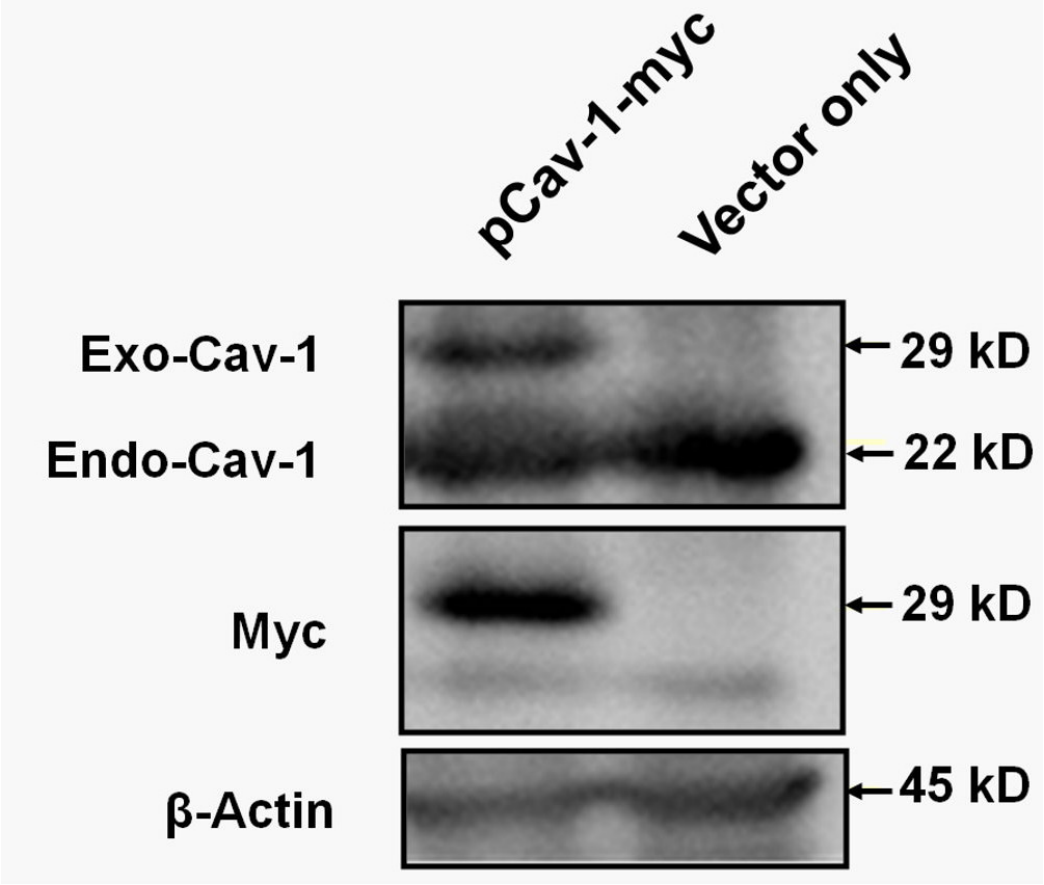

Figure 3. Endothelial cells were transfected with caveolin-1-myc plasmid. Immunoblotting for exo-caveolin-1-myc (Exo-Cav-1), endo-Cav-1, and β-actin in caveolin-1-myc plasmid transfected cells or vector-only controls.

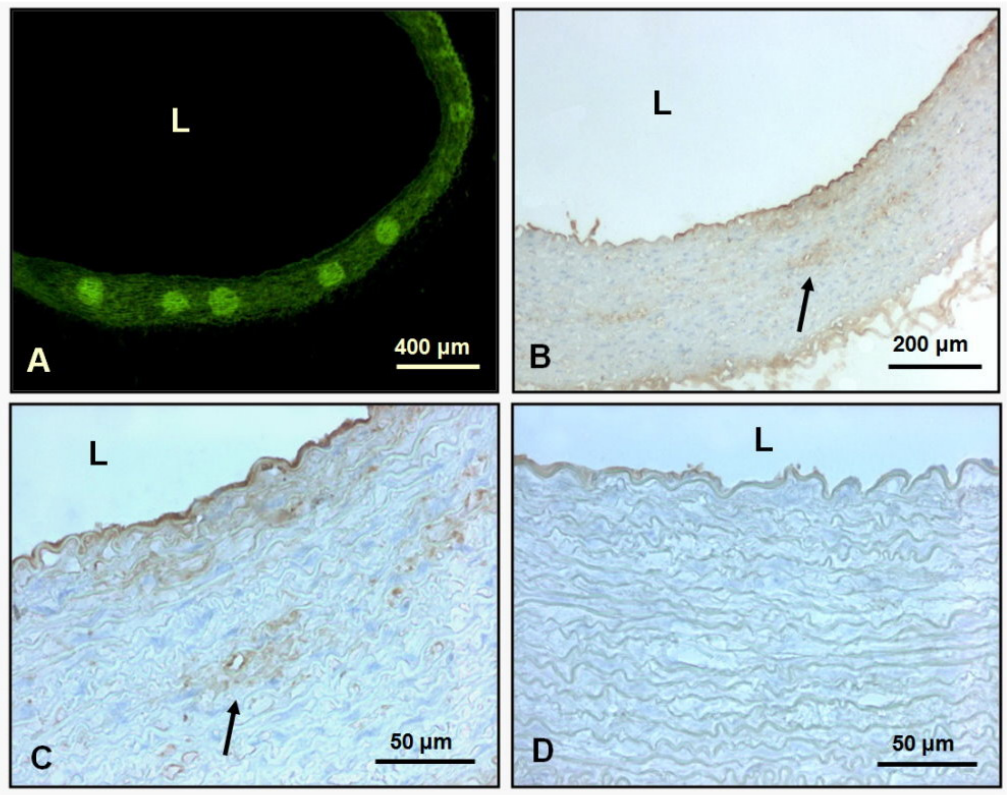

Figure 4. Fluorescent micrograph indicates the location of GFP in the vessel wall after infusion via the IABC (A). The immunohistochemical micrographs reveal expression of caveolin-1-myc (→) in the arterial wall at two weeks after IABC infusion (B). The enlarged image (Fig. 4B) shows the reaction products (→) in the arterial intima and media at the injection site (C). No reaction products were found in the arterial wall in the negative control (D). L: lumen.

利用滲入性血管整型氣球導管將 caveolin-1 基因注入白兔動脈管壁

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摘　　要

基因療法技術在治療動脈粥狀硬化疾病雖然快速的發展，然而將基因準確地送到治療目標，仍是血管基因治療的挑戰。我們以滲入性血管整型氣球導管(infiltrator angioplasty balloon catheter, IABC)將 Caveolin-1 基因注入白兔主動脈管壁後，評估 Caveolin-1 基因在注射部位的表現。首先將人類 caveolin-1 基因及 myc 報導基因利用 pcDNA 3.1 質體合成重組基因 caveolin-1-myc，再利用滲入性血管整型氣球導管將此 caveolin-1-myc 基因注射至白兔主動脈管壁，每三天注射一次，共注射兩週。在最後一次注射的兩週後將血管取下，以免疫組織化學染色法分析 caveolin-1 蛋白質在注射位置的表現。由免疫螢光染色與西方點墨法的結果顯示 caveolin-1-myc 基因成功表現在體外培養的細胞中。經由滲入性血管整型氣球導管注射後的兩週，以免疫組織染色發現 Caveolin-1 蛋白質在白兔主動脈管壁中表現，而在注射部位並沒有上皮細胞或肌肉層之增生。本研究結果顯示滲入性血管整型氣球導管可做爲基因治療心血管疾病的有效注入方式。

關鍵字：動脈粥狀硬化 Caveolin-1, 基因治療, 局部注入方法