Inhibition of intimal hyperplasia via local delivery of vascular endothelial growth factor cDNA nanoparticles in a rabbit model of restenosis induced by abdominal aorta balloon injury

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Abstract. In-stent restenosis (ISR) is one of the major factors affecting long-term outcomes of percutaneous coronary interventions. Vascular endothelial growth factor (VEGF) has been hypothesized to have a positive role in preventing ISR, however, this remains controversial. The aim of the present study was to assess whether nanoparticles can be used to deliver VEGF to injured arteries and whether this is beneficial in preventing restenosis. New Zealand White rabbits were randomly divided into a control group, an empty nanoparticles group and a VEGF nanoparticles group (n=6 in each group). Polylactic-polyglycolic acid VEGF nanoparticles were prepared using a phacoemulsification method. A rabbit model of restenosis was established following abdominal aorta balloon injury, and VEGF gene nanoparticles, empty nanoparticles or normal saline were delivered locally at the site of injury via a GENIE Catheter™ perfusion balloon. Intimal proliferation determination and immunohistochemistry analysis were performed at day 28 following arterial injury. Compared with the control and empty nanoparticle groups, the neointima area (0.49±0.09, 0.48±0.08 and 0.19±0.11 mm^2, respectively; P<0.001) and proliferation index (0.32±0.03, 0.32±0.05 and 0.13±0.06, respectively; P<0.001) were significantly lower in the VEGF nanoparticles group. In addition, in the VEGF nanoparticles group, the immunoreactivity of α-actin and proliferating cell nuclear antigen were significantly lower (P≤0.001), while the immunoreactivity of VEGF was higher (P=0.01). Therefore, the results revealed that local delivery of VEGF gene nanoparticles reduced intimal thickening and cell proliferation following abdominal aorta balloon injury in a rabbit model, demonstrating the efficacy of this therapy against restenosis.

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Introduction

Percutaneous coronary intervention (PCI) is known to effectively improve the prognosis of patients with coronary heart diseases, particularly those with acute coronary syndrome (1). However, in-stent restenosis (ISR) is a major concern that can compromise the long-term outcome of PCI (2). The mechanisms of restenosis secondary to PCI injury are very complex, and include local reendothelialization and vascular remodeling mediated by a variety of inflammatory cells, cytokines and growth factors. Poor reendothelialization, and excessive migration and proliferation of vascular smooth muscle cells in the tunica media, can result in obstructive neointimal hyperplasia, and are considered to be the major mechanisms involved in restenosis following PCI (3).

Vascular endothelial growth factor (VEGF) is a homodimer glycoprotein (molecular weight, 45 kDa) composed of two identical peptide chains connected by disulfide bonds. There are five isoforms of the VEGF gene resulting from alternate splicing: VEGF121, VEGF145, VEGF165, VEGF189 and VEGF206; among these, VEGF165 is the biologically active form (4). The human VEGF gene is ~14 kb in length and consists of eight exons. VEGF is primarily secreted by endothelial cells, macrophages and fibroblasts. VEGF can stimulate mitosis and angiogenesis by binding to VEGF receptors on the surface of vascular endothelial cells (5).

The majority of animal studies indicate that local delivery of the VEGF gene is able to promote vascular reendothelialization and prevent restenosis (6-8), although this remains controversial. Asahara *et al* demonstrated that VEGF protein stimulated vascular reendothelialization after local delivery into rat carotid arteries following balloon injury (9). The authors concluded that VEGF reduced the intimal thickening resulting from the proliferation of smooth muscle cells. In addition, the authors succeeded in treating balloon injuries in rabbit iliac arteries using a locally delivered plasmid DNA construct,

phVEGF165, which supported the concept of using VEGF gene therapy against restenosis (7). However, Dulak *et al* (7) demonstrated that a different plasmid DNA construct (pSG5VEGF165) was unable to inhibit intimal hyperplasia in a rabbit model of hypercholesterolemia. Normocholesterolemic rabbits were found to benefit from VEGF following an arterial injury; however, since hypercholesterolemia *per se* appeared to increase plasma VEGF levels in the model, hypercholesterolemic rabbits did not receive any benefit from exogenous VEGF, which may be due to the already increased levels of VEGF and the decreased availability of nitric oxide (7,10). This is consistent with the observations of a previous study that performed adenoviral transfer of VEGF in rabbits, and demonstrated that the therapeutic effect of VEGF was nitric oxide-dependent (11). Two studies using a pig model revealed that liposome-mediated VEGF gene transfer prevented the regression of microvessels, enhanced the accumulation of elastin in the adventitia, reduced the amount of myofibroblasts in the adventitia and induced a healing inflammatory response. These mechanisms indicated a potential role for VEGF transfer in the prevention of restenosis (12,13). Furthermore, a mouse model of adenovirus-mediated VEGF transfer showed that VEGF accelerated endothelial repair and inhibited neointima formation following an arterial injury (14). An additional study investigating adenoviral transfer of VEGF in rabbits revealed that VEGF accelerated the restoration of endothelium integrity and decreased intimal hyperplasia following an arterial injury (15). In addition, rabbits implanted with VEGF-eluting stents were found to undergo accelerated reendothelialization in the injured artery (16). However, results from randomized controlled studies indicate that local delivery of the VEGF gene into an injured coronary artery, using an adenovirus or liposome as a vector, is not effective at preventing restenosis and improving the long-term outcomes of patients (17,18). This may be due to inadequate VEGF concentrations or the short period of time that effective concentrations of VEGF are available for action on local blood vessels.

Nanoparticles are an emerging vector for delivering gene therapy, with excellent tissue penetration ability, good absorption and sustained release (19). In addition, the use of nanoparticles bypasses the requirement for conventional vectors to carry the gene. Viral vectors are known to be associated with certain limitations, including the induction of a host immune response, random insertional mutagenesis, the eventual presence of a wild-type vector in the administered preparation and unsuitable tissue tropism (20,21). Similarly, a low efficiency and transient gene expression have been reported with the use of liposomal vectors (22). By contrast, polylactic-polyglycolic acid (PLGA) is safe and has an excellent biocompatibility, and is extensively used in medicine with US Food and Drug Administration approval (23,24). Nanoparticles can further enhance local drug concentrations and thereby yield the desired therapeutic effects with excellent tissue penetration and high cellular absorption rates. A previous study demonstrated that nano- and microparticles can maintain measurable drug concentrations for days after the injection (25). Nanoparticles are becoming valued as a potential method for the treatment of restenosis (26). Guzman *et al* investigated PLGA nanoparticles incorporated with dexamethasone for local delivery in a rat carotid model of restenosis. The authors used immunofluorescence to show that nanoparticles were present in each of the arteries' three layers at 3 h and 24 h post-treatment, and were present in the adventitial layer from days three to seven (27). Furthermore, the arterial vasa vasorum provided a path for nanoparticles to reach the adventitial layer (8). The adventitial layer has been hypothesized to function as a storage pool for nanoparticles, facilitating their release. A previous study in mice revealed that VEGF delivery using PLGA nanoparticles enhanced vascular growth and connectivity (23). An additional study in dogs demonstrated that stents coated with VEGF nanoparticles enhanced the reendothelialization of injured arteries (28). Furthermore, a pig model that coeluted VEGF and paclitaxel from a nanoparticle-coated stent was shown to have similarly favorable results (29).

Since PLGA particles exhibit a promising efficacy, VEGF nanoparticles were hypothesized to effectively induce the expression of VEGF in a rabbit model of vascular restenosis induced by aorta balloon injury. In the present study, a rabbit model of vascular restenosis was established by abdominal aorta balloon injury. The VEGF gene nanoparticles were prepared using nanoscale particle technology and were locally delivered to determine their beneficial effects on the restenosis of injured arteries. The results of the present study may lead to novel therapeutic options to limit restenosis following percutaneous coronary interventions in patients with myocardial infarction.

Materials and methods

Ethical statement. Experimental protocols of the study were approved by the Ethics Committee of Peking Union Medical College Hospital (Beijing, China; approval ID, XJYYLL-2012107). Animal testing was performed in accordance with the international guiding principles for biomedical research (30), and the animals used in the experiments were cared for according to these guidelines.

Preparation and characterization of the VEGF gene nanoparticles. A phacoemulsification method was used to prepare the nanoparticles (31). Briefly, 200 mg PLGA (Birmingham Polymers, Inc., Pelham, AL, USA) was dissolved in a solution of indichloromethane containing the VEGF165 cDNA (4 ml; obtained from the Department of Cardiology of the Peking Union Medical College Hospital). Next, a 0.5% polyvinyl alcohol solution (Sigma-Aldrich, St. Louis, MO, USA) was added and the samples were placed in sonicating, ice-bath conditions. The solution was centrifuged at 64,000 x g for 30 min until complete volatilization was achieved. The VEGF gene nanoparticles were subsequently freeze-dried into pellets, and stored in a dry environment at low temperatures. Prior to use, the VEGF gene nanoparticles (6.6 mg/ml) were dissolved in 0.9% NaCl to create a final VEGF gene concentration of ~0.4 mg/ml. The nanoparticles' range of diameters and ζ-potential were detected using a laser particle sizer (Brookhaven Instruments Corporation, Holtsville, NY, USA). Particle morphology was observed using scanning electron microscopy (Hitachi, Ltd., Tokyo Japan), and the microstructure was observed using transmission electron microscopy (Hitachi, Ltd.). VEGF gene encapsulation rates

were calculated using the following formula: Encapsulation rate (%) = (total amount of loaded gene - amount of gene in the supernatant)/total amount of loaded gene x 100%.

VEGF nanoparticle bioactivity assay. A bioactivity assay was used to demonstrate that the VEGF nanoparticles were biologically active; thus, if a VEGF gene product was detectable in the cells. Briefly, the media layer of a rabbit coronary artery was treated with 1.0 mg/ml collagenase I (Gibco®, Invitrogen Life Technologies, Hong Kong, China) and 10 U/ml elastase (Gibco®, Invitrogen Life Technologies) at 37˚C for 10 h. The resulting mixture was centrifuged at 120 x g for 7 min and the supernatant was discarded. Primary cells were prepared for culture using the collagen gel embedded method (32), with a collagen gel matrix and Media 199 supplemented with 10% fetal bovine serum and antibiotics (all from Gibco®, Invitrogen Life Technologies). Primary cells were subcultured in a culture dish, and maintained in culture medium at 37˚C with 5% carbon dioxide. Subsequently, 2 μg VEGF nanoparticle solution (20 μg/ml) or 0.9% NaCl was added to the cells. VEGF expression levels in the medium were measured using a commercial ELISA kit (CytImmune Sciences, Rockville, MD, USA). Experiments were performed in triplicate.

Establishment of an animal model of restenosis and drug delivery through a perfusion balloon catheter. A total of 18 New Zealand male rabbits (aged, 4 months; purchased from The institute of Laboratory Animal Science, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing) were randomly divided into the control (n=6; receiving normal saline), empty nanoparticles (n=6; receiving empty nanoparticles) and VEGF nanoparticles (n=6; receiving VEGF gene nanoparticles) groups. A further two rabbits did not receive any treatment and were not allocated into a group. A rabbit model of vascular restenosis was established in all rabbits by abdominal aorta balloon injury (33). Briefly, each rabbit received aspirin (12.5 mg/day) intragastrically from the day prior to surgery until euthanasia. Rabbits were anesthetized via an ear vein injection of sodium pentobarbital (30 mg/kg). A 30-mm incision was created over the right femoral artery and the femoral artery was dissected. The distal end of the femoral artery was ligated, and the proximal end was occluded. The femoral artery was subsequently removed using ophthalmic scissors, and a sheath guide wire was implanted into the artery, followed by the insertion of a 5-French sheath (Cordis Corporation, Fremont, CA, USA). A 40-mm incision was made along the ventral (right) side of the midline and the abdominal aorta was dissected. The aortas obtained from the two untreated rabbits were termed 'normal aortas'; the aortas in control group were from the restenosis rabbits receiving normal saline. A site 10 mm below the renal artery was selected as the nanoparticle delivery site, and a suture was placed at the proximal end. The size of the balloon catheter (Cordis Corporation) was selected according to the outer diameter of the abdominal aorta. The balloon catheter was located at the labeled site (~250 mm) in the abdominal aorta along the guide wire. The balloon injury model was created by inflating the balloon to a pressure of 10 atm, and moving the balloon retrograde by 100 mm three times for 15 sec each. The balloon catheter was then removed.

The perfusion balloon, GENIE Catheter™ (Acrostak Corporation, Geneva, Switzerland), is a new local drug delivery catheter designed to deliver various liquid therapeutic agents into arteries. A GENIE Catheter™ with an outer diameter matched to the aorta's lumen size was located at the injury site using a guide wire via a 5-French sheath. VEGF gene nanoparticles, empty nanoparticles or normal saline (0.9%) were delivered to the injury site using the GENIE Catheter™, with a perfusion pressure of 2-3 atm over 5 min. The GENIE Catheter™, guide wire and 5-French sheath were subsequently removed. The proximal end of the artery was ligated and each wound layer was individually sutured. Penicillin (80 MU) was intramuscularly administrated daily for three days after surgery.

Intimal hyperplasia of the injured artery. All rabbits were euthanized through air embolization at day 28 after surgery. The injured abdominal aorta was removed from the site where the drug had been delivered. The removed aorta was washed with 10% phosphate-buffered saline, fixed in formaldehyde, and embedded in paraffin for hematoxylin and eosin staining. The neointima area (NIA), media area (MA) and proliferation index (PI) of the aorta were calculated following Weigert's staining. The PI was calculated using the following formula: PI = NIA/MA. Picro-sirius red staining was used to detect collagen expression, while immunohistochemistry was used to determine the expression of α-actin, proliferating cell nuclear antigen (PCNA), matrix metalloproteinase-2 (MMP-2), tissue inhibitor of MMP-2 (TIMP-2), VEGF and C-reactive protein (CRP). The numbers of positively stained cells were counted in five randomly selected fields from each section. The positive expression index (PEI) was calculated using the following formula: PEI (%) = number of positively-stained cells/total number of cells in five fields x 100%.

Statistical analysis. Statistical analyses were performed using SPSS 10.0 software (SPSS, Inc., Chicago, USA). The results are expressed as the mean ± standard deviation for continuous data. The Kolmogorov-Smirnov test was used for normalized tests. Differences among three groups were assessed by analysis of variance, while intergroup differences were further evaluated using the Bonferroni method. The χ^2 and Fisher's exact tests were used to analyze categorical data, where $P<0.05$ was considered to indicate a statistically significant difference.

Results

Nanoparticle characterization and biological activity. The average diameter of the nanoparticles was 78.82 nm (range, 58.28-105.7 nm). The average ζ-potential was -12.2. The VEGF gene encapsulation efficiency was 98% and the amount of loaded gene was 4.67%.

The bioactivity assay results revealed that the cells treated with VEGF nanoparticles expressed VEGF at 243.5±111.5 ng/l, while the saline-treated cells did not express VEGF.

Histological examination of the intima following injury and treatment. All the rabbits survived following the aorta balloon injury and the local delivery of VEGF nanoparticles. In the control and empty nanoparticle groups, histological examina-

Table I. Indices of intimal proliferation at day 28 after balloon injury and treatment.

Parameters	Control group (n=6)	Empty nanoparticles group (n=6)	VEGF nanoparticles group (n=6)	P-value
Neointima area, mm^2	0.49±0.09	0.48±0.08	0.19±0.11[a]	<0.001
Media area, mm^2	1.53±0.26	1.55±0.39	1.75±1.43	0.889
Proliferation index	0.32±0.03	0.32±0.05	0.13±0.06[a]	<0.001

[a]P<0.001, vs. control and empty nanoparticle groups. VEGF, vascular endothelial growth factor.

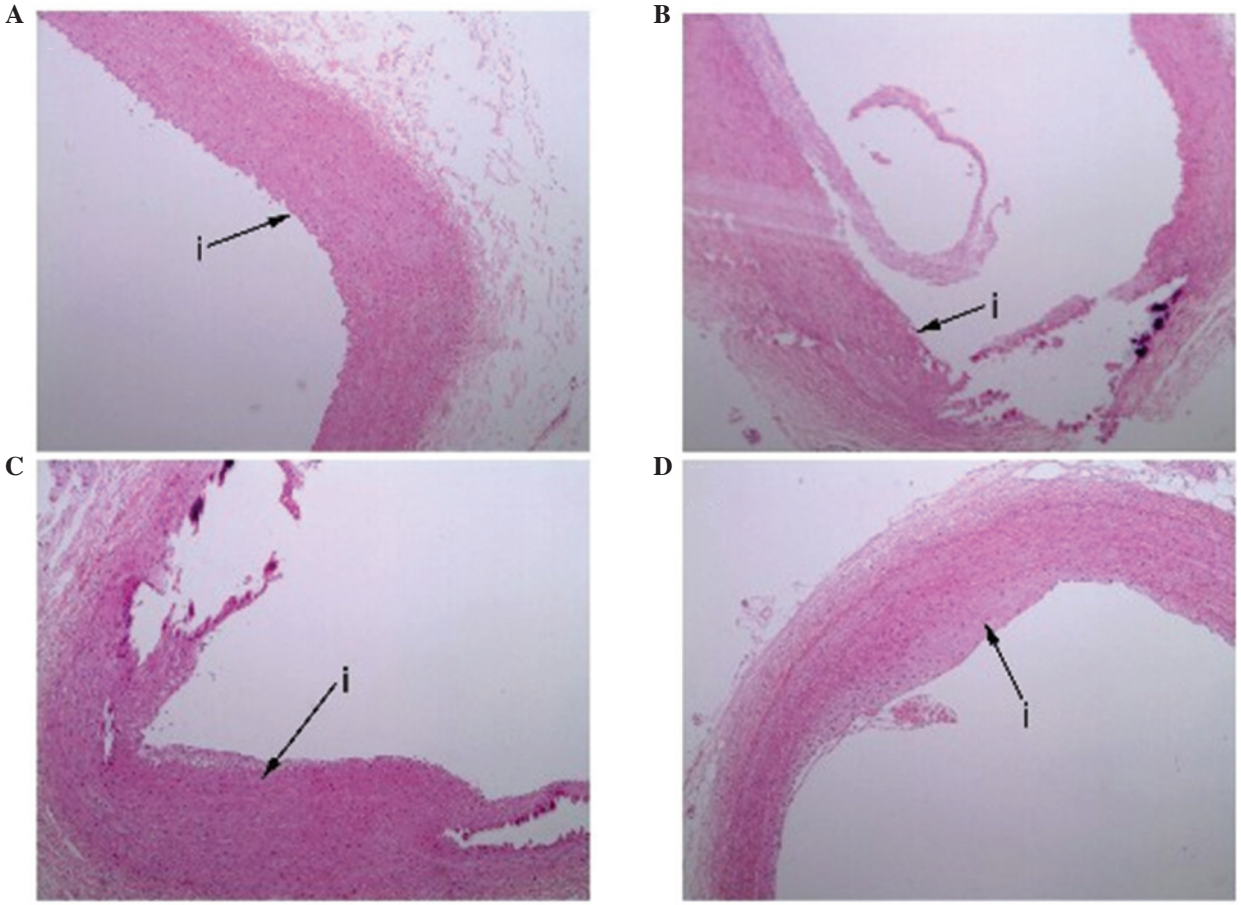

Figure 1. Hematoxylin and eosin staining of a rabbit aorta at day 28 after balloon injury (magnification, x40) in the (A) normal, (B) control, (C) empty nanoparticle and (D) vascular endothelial growth factor nanoparticle groups.'i' indicates the tunica intima.

tion revealed partially denuded endothelial cells with intimal thickening, and hyperplasia of foam cells, smooth muscle cells and fibrous tissue, as well as the rupture of the internal elastic lamina (Fig. 1). By contrast, these pathological changes were rarely observed in the VEGF nanoparticles group at day 28 after balloon injury. Based on Weigert's staining (Fig. 2), the VEGF nanoparticles group exhibited a decreased neointima area (VEGF nanoparticles, 0.19±0.11 mm^2 vs. empty nanoparticles, 0.48±0.08 mm^2 and controls, 0.49±0.09 mm^2; P<0.001) and a decreased proliferation index (VEGF nanoparticles, 0.13±0.06 vs. empty nanoparticles, 0.32±0.05 and controls, 0.32±0.03; P<0.001) when compared with the two other groups (Table I). Small amounts of type III and type II collagen were observed in the media and adventitia of the vessel walls from the three groups (Fig. 3).

Immunohistochemical examination of the intima following injury and treatment. α-actin was used to identify smooth muscle cells, while PCNA was used to determine the extent of cell proliferation. MMP-2 plays an important role in extracellular matrix degradation and cell migration, while TIMP-2 is the inhibitor of MMP-2. Thus, the VEGF nanoparticles group showed decreases in the PEI of α-actin (VEGF nanoparticles, 34.7±9.6% vs. empty nanoparticles, 65.7±16.2% and controls, 65.0±21.3%; P=0.001) and PCNA (VEGF nanoparticles, 21.0±8.6% vs. empty nanoparticles, 69.5±13.7% and controls, 63.0±17.3%; P<0.001), and an increase in the PEI of VEGF (VEGF nanoparticles, 45.8±10.5% vs. empty nanoparticles, 27.5±12.5% and controls, 25.7±10.2%; P=0.01). The PEIs of MMP-2, TIMP-2 and CRP were similar between the three groups (Table II).

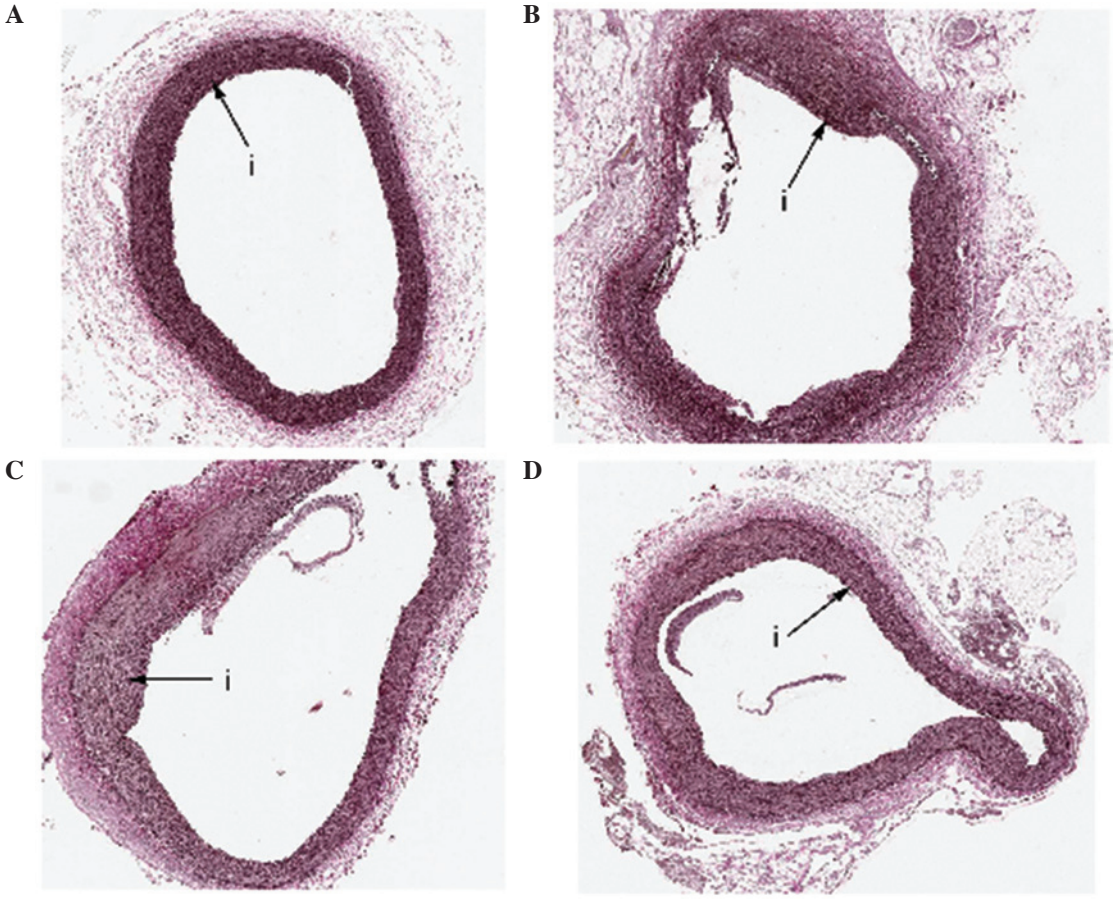

Figure 2. Weigert's staining of a rabbit aorta at day 28 after balloon injury (magnification, x40) in the (A) normal, (B) control, (C) empty nanoparticle and (D) vascular endothelial growth factor nanoparticle groups. 'i' indicates the tunica intima.

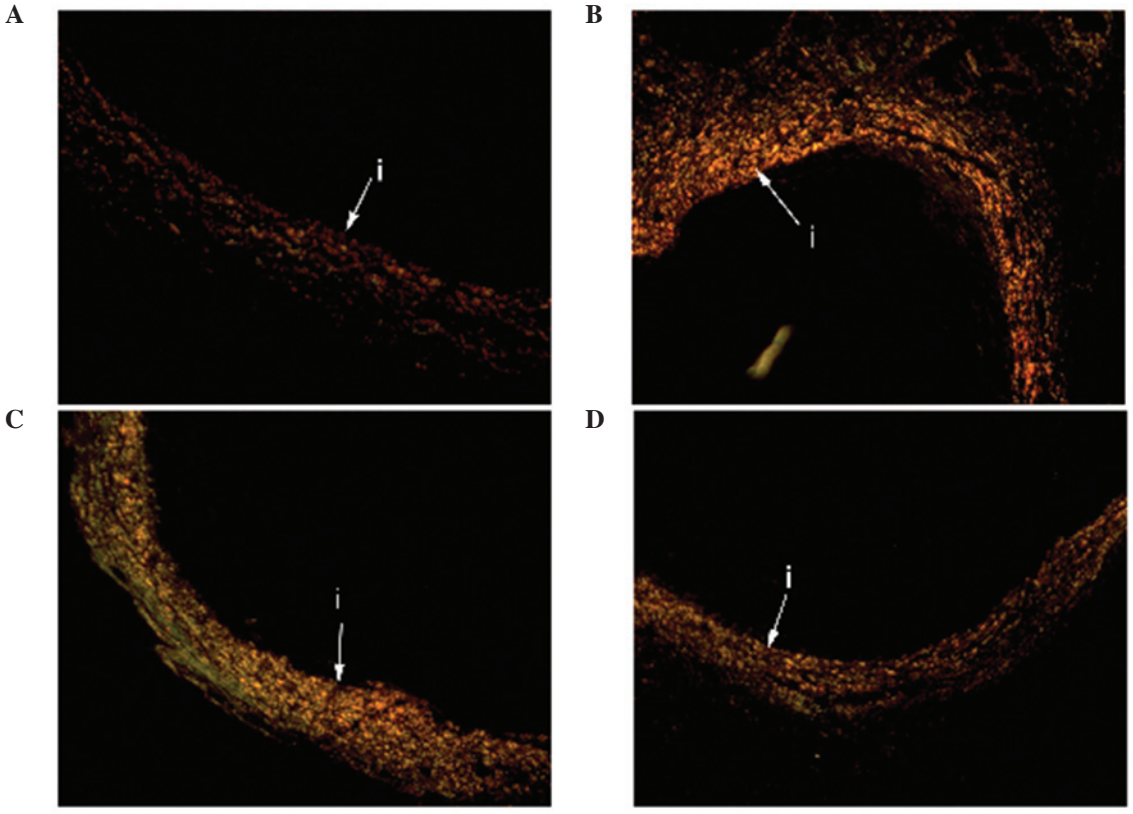

Figure 3. Picro-sirius red staining of a rabbit aorta at day 28 after balloon injury (magnification, x40) in the (A) normal, (B) control, (C) empty nanoparticle and (D) vascular endothelial growth factor nanoparticle groups. 'i' indicates the tunica intima.

Table II. Positive expression indexes (%) at day 28 after balloon injury and treatment.

Parameters	Control group (n=6)	Empty nanoparticles group (n=6)	VEGF nanoparticles group (n=6)	P-value
α-actin	65.0±21.3	65.7±16.2	34.7±9.6[a]	0.001
PCNA	63.0±17.3	69.5±13.7	21.0±8.6[b]	<0.001
MMP-2	61.7±14.4	56.8±8.7	57.2±11.6	0.735
TIMP-2	56.8±8.7	61.5±15.0	49.8±9.0	0.229
VEGF	25.7±10.2	27.5±12.5	45.8±10.5[c]	0.012
CRP	61.7±11.5	60.5±10.3	57.8±12.1	0.836

[a]P=0.003, vs. control and empty nanoparticle groups; [b]P<0.001, vs. control and empty nanoparticle groups; [c]P=0.036 and P=0.020, vs. control group and empty nanoparticles group, respectively. VEGF, vascular endothelial growth factor; PCNA, proliferating cell nuclear antigen; MMP, matrix metalloproteinase; TIMP-2, tissue inhibitor of MMP; CRP, C-reactive protein.

Discussion

The aim of the present study was to investigate whether VEGF nanoparticles can effectively induce the expression of VEGF in a rabbit model of vascular restenosis. Immunohistochemical analyses of the injured abdominal aortas from the experimental rabbits demonstrated that treatment with VEGF nanoparticles significantly increased the number of cells that were positive for VEGF expression when compared with the control cells or those that had been treated with empty nanoparticles, indicating that the VEGF nanoparticles were able to induce VEGF expression.

The present study also investigated whether local delivery of VEGF was able to effectively improve intimal hyperplasia in the rabbit restenosis model. This issue is important since previous studies investigating the use of VEGF for the treatment of restenosis have produced conflicting results (6-14). In general, the results indicate that when high cholesterol is involved in model establishment, the beneficial effects of VEGF are mitigated (7,12); however, a number of studies using liposome- and virus-mediated VEGF transfer to injured arteries in a variety of animal models have demonstrated similar beneficial results (13,15). The present study showed that characteristics observed in the rabbit model of vascular restenosis, including intimal thickening, proliferation of foam cells and smooth muscle cells and an increase in fibrous tissues, were all decreased following VEGF nanoparticle treatment. The lower rate of proliferation in the muscle cells was confirmed by the decreased numbers of α-actin-positive cells, while the lower overall rate of cell proliferation was demonstrated by the lower number of PCNA-positive cells following VEGF nanoparticle treatment. Therefore, the results from the present study concur with the observations from previous studies, despite using a completely different delivery method.

The two major differences between the present study and previous studies were the use of a perfusion balloon catheter as a delivery system and nanoparticles as gene vectors. The GENIE Catheter™ has been approved for clinical use in the local delivery of medication for ISR, branch lesions and small vessel diseases. A key feature of the GENIE Catheter™ is that it can maintain drug concentrations and perfusion pressure at the site of injury with small doses and without any damage to the vessel walls. Herdeg *et al* demonstrated the safety and efficacy of locally administering paclitaxel through the GENIE Catheter™ as a PCI strategy for treating patients with coronary heart disease (34).

An additional potential benefit of the method used in the present study is that unlike certain methods, such as adenovirus delivery, nanoparticle delivery is unlikely to trigger an immune response. The results of the current study indicated that nanoparticles themselves do not trigger an immune response, since the results observed with empty nanoparticles were similar to those obtained in the control group. The uptake of nanoparticles by cells has been shown to be dependent on particle size (35). The average and range of particle sizes used in the present study were in accordance with those that have been shown to have the most success at transferring into arterial walls (36). Results from the present study and from previous studies performed in different animal models strongly suggest that the use of nanoparticles for the sustained delivery of VEGF is an appropriate and efficient method of promoting reendothelialization following an arterial injury (23,28,29).

A small number of studies have assessed the effects of VEGF transfer to arterial injuries in humans. Three studies used adenoviruses and liposomes to transfer VEGF in patients undergoing PCI for a coronary event (17,18,37). These three studies demonstrated that the short- and long-term safety was adequate. In addition, the results showed an increase in vascularity, but without any effect on the clinical restenosis rate. As previously discussed, the efficacy of adenovirus- and liposome-mediated VEGF transfer is lower compared with the efficacy achieved using VEGF-containing PLGA nanoparticles. Therefore, future clinical trials using VEGF nanoparticles in humans may result in a higher efficacy (8,23,24).

The present study has several limitations. Firstly, the efficacy of VEGF gene nanoparticles was demonstrated without comparing with other VEGF gene vectors. However, the previously used vectors, viruses and liposomes, have been shown to have a low efficacy and a number of issues associated with safety (17,38,39). Nevertheless, a future study should compare all three modalities. Secondly, the positive expression rate was calculated using an immunohistochemistry assay, rather than a more direct semi-quantitative polymerase chain reaction (PCR) or real-time PCR method, to assess the expression of

the proteins in the vessel wall. Thirdly, only one concentration of VEGF was applied in the nanoparticles; thus, future studies should investigate the effects of different concentrations. Finally, the biodistribution, bioavailability and biodegradation of the nanoparticles were not analyzed in the model used in the present study. Assessment of these parameters is planned for a future study.

In conclusion, the present study demonstrated the efficacy of VEGF gene nanoparticles for the treatment of restenosis following vascular injury, using an animal model. The results provide a new direction for the clinical application of VEGF gene therapy. However, this conclusion requires further confirmation by future studies.

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