Restenosis is one of clinical limitations for vein graft in coronary bypass graft. It has been proved that signal pathway IGF-1 and its receptor (IGF-1R) activated by hemodynamic mechanical stretch are responsible for the vascular smooth muscle cells proliferation in vein graft neointima formation. Unfortunately, there is no routinely successful method to resolve this problem. Gene delivering to vein graft possesses great therapeutic potential to prevent neointima formation. Polymer is one kind of nanoparticles, which can activate the process of endocytosis of cells. In this study, we evaluated the transfection efficiency and therapeutic potential of polymer-based transfection of plasmids expressing GFP and shRNAs targeting IGF-1R (pGFPshIGF-1Rs) to smooth muscle cells and rabbit external jugular vein graft. Results showed that polymer-based transfection provided high efficiency of transgene expression in smooth muscle cells in vitro. In vitro, IGF-1R-specific shRNA transfected by polymer inhibited IGF-1R protein expression by 52 ± 3.6%, when compared with mock transfected cells. In vivo delivering efficiency of pGFPshIGF-1R plasmid into the rabbit external jugular vein graft was significantly high in the polymer-based transfection group, when compared with negative control group. In vivo, polymer-based transfection IGF-1R-specific shRNA efficiently inhibited the expression of IGF-1R protein by 77 ± 3.6%, 65.6 ± 4.9%, and 76.7 ± 4.3% at 24, 48, and 72 h, respectively, when compared with negative control group. Our findings indicated that polymer-based transfection may be a promising technique that allows the targeting of gene therapy for vein graft restenosis.

Keywords vein graft restenosis; gene therapy; polymers; igf-1r

Original Article

Polymer-based delivering of shRNA to rabbit aortic smooth muscle cells suppressed the expression of IGF-1R in vitro and in vivo

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Introduction

Vein grafts used in coronary artery bypass graft often developed into stenosis due to the proliferation of vascular smooth muscle cells (VSMCs). A vital factor is the hemodynamic mechanical stress induced by blood pressure [1,2], which subsequently activates the IGF-1/insulin-like growth factor-1 receptor (IGF-1R) signaling pathway [3]. It has been demonstrated that IGF-1/insulin-like growth factor-1 receptor (IGF-1R) signaling pathway plays an essential role in VSMC proliferation, inducing neointima proliferation in vein grafts [4]. Proliferation of VSMCs can also be regulated by other growth factors including PDGF, bFGF, thrombin, and angiotensin II [5,6], but IGF-1 has a special role in this process because it regulates the cell cycle of VSMCs and functions both as an endocrine and an autocrine/paracrine growth factor [7,8]. Growth factors, such as bFGF and PDGF, activate IGF-1R transcription and up-regulate IGF-1R expression in VSMCs. Accordingly, the stimulation of an IGF-1/IGF-1R autocrine loop can be pivotal in mediating the growth factor-induced activation of VSMCs. In this regard, IGF-1R may be a new target for preventing vein graft stenosis.

Gene therapy for vein graft disease is effective; however, relatively low efficacy and safety remain to be problems in gene transfer technologies. Viral vectors can achieve efficient arterial gene transfer in vivo [9–11], but there is a potential of immunologic and cytotoxic complications [12]. Non-viral methods for in vivo gene transfer, such as hydrogel balloon-mediated gene delivery [13], cationic lipid [14], and electroporation-mediated gene transfection [15] have been widely used to introduce DNA into the arterial wall, but the efficiency is relatively low. Synthetic delivery systems such as non-lipid cationic polymers, e.g. polyethylenimine (PEI), polyamidoamine (PAMAM), and poly-γ-lysine have been investigated as alternatives to viral vectors [16–18], because they are easier to produce, raise fewer safety issues, and pose fewer problems for repeated administration than viral gene transduction. However, the feasibility of polymer-based gene transfection to smooth muscle cells has never been systematically evaluated. In this study, plasmid DNA conjugated with polymer was applied to assess the efficiency of polymer-based gene transfection to smooth muscle cells in vitro and in vivo.
Materials and Methods

Cell culture
Under sterile conditions, aortic arteries were excised from the New Zealand rabbits (Zhejiang Academy of Medical Science, Hangzhou, China) as previously described [19]. The vessels were rinsed three times in phosphate buffered saline (PBS) containing antibiotics, then cut longitudinally and dissected into small pieces. Explants were placed in 100-mm culture plates. Five to six hours later, 5 ml of Dulbecco-modified eagle medium (Gibco, Carlsbad, USA) supplemented with 10% fetal bovine serum (Gibco) and antibiotics/antimycotic was carefully added to the culture plates in order not to disturb the adhered explants. Explants were cultured at 37°C in an incubator with 5% CO₂. Medium was removed and replaced every 3 days. Inverted light microscope was used to examine the morphology of culture growth every day. Cells started to grow from explants within 1 week. Two weeks later, cell cultures became ~75% confluent. Aortic smooth cells were used between passages 3 and 5.

Animals
The New Zealand rabbits were raised in clean environment and surgery procedure was performed according to Zhejiang University’s institutional guidelines established for animal care and use.

Preparation of plasmid expressing GFP and shRNA targeting IGF-1R
Plasmids expressing GFP and shRNA targeting IGF-1R (pGFPshIGF-1R) were purchased from Genepharmaceutical (Shanghai, China) and the complementary sequences of ds-oligos encoding shRNAs and the negative control were as follows: IGF1R-shRNA1, 5'-GGAGGGTCTCTTTCTACTA CATTCAAGAGATGTAGTAGAAGGAGACCTCGCTT-3'; IGF1R-shRNA2, 5'-GCGAGGTCTCCTTCTACTAGCACAACTACTGCTCCAAAGATTC AAGAGATTTTGGAGACGAGGACTCTCGTT-3'; negative control shRNA (shNC), 5'-CTTCTCCGAACGTGGTACCG TCAAGAGATTACGTGACACGTTCGGAGAATT-3'.

Polymer-based delivering of sh-RNA into smooth muscle cell in vitro and in vivo
In vitro transfection was performed according to manufacturer’s instruction in a 6-well plate. Briefly, the transfection mixture contained 6 μl of MegaTran 1.0 (Origene, Rockville, USA) diluted in antibiotics and serum-free DMEM to a volume of 500 μl. A total of 2 μg of pGFPshIGF-1R was diluted in antibiotics and serum-free DMEM to 500 μl, added to the diluted polymer solution to a total volume of 1 ml, and mixed by pipetting. For the control, 500 μl of antibodies and serum-free DMEM containing 6 μl of polymer were added instead of diluted pGFPshIGF-1R solution. Mixtures were incubated at room temperature for 15 min. Prior to transfection, the medium was removed and cells were gently washed twice with PBS. Transfection mixtures were added directly to the cells and the cells were subsequently incubated at 37°C in an incubator with 5% CO₂ for 48 h. Lipo2000 (Invitrogen, California, USA) was used to deliver pGFPshIGF-1R into smooth muscle cells according to manufacturer’s instruction.

To avoid possible nonspecific effects of the transfection reagents or the transfection methods, mock transfected cells were applied to provide a more severe control.

In vivo transfection was performed as previously described [20]. Briefly, animals were sedated with ketamine HCl (10 mg/kg IM) and anesthetized with pentobarbital (25–30 mg/kg IV). A segment of 1- to 2-cm external jugular vein graft was surgically exposed through the placement of vascular clamps on the distal external jugular vein and proximal common jugular vein, and the internal jugular vein was ligated. The control group received 0.2 ml of PBS per rabbit, and the lipofection group received 0.2 ml of Lipo2000/DNA mixture. In polymer-based delivering group, a total of 0.2–0.3 ml of MegaTran1.0/DNA mixture was instilled into the isolated external jugular veins segment through internal jugular vein. After 20 min of incubation, the vector-containing medium was withdrawn, and the blood flow through the common and external jugular vein was re-established.

Flow cytometry and fluorescence microscopy to analyze efficiency of transfection
Flow cytometry was performed as described previously [21]. Briefly, cultured smooth muscle cells in well plates were washed with PBS, and then trypsinized for 5 min to detach the cells from the well plates. The cell suspension was diluted with PBS to a volume of 500 μl. The expression of GFP was analyzed by using a FACSCalibur dual laser flow cytometer (Becton-Dickinson, Arlington, USA). For each cell sample, 1 × 10⁵ events were collected. Transfection efficiency was expressed as the percentage of GFP-positive vital cells minus the mock transfected control cells. Cells were also visually examined for the presence of green fluorescence by using Olympus IX71 fluorescence microscope (Olympus, Tokyo, Japan).

Reverse transcription polymerase chain reaction
Total RNA was extracted with TRIzol reagent (Invitrogen) according to manufacturer’s instruction. The polymerase chain reaction (PCR) product (10 μl) was electrophoresed on 2% agarose, stained with ethidium bromide and analyzed by UV absorption using Quantity One Software (Quantity One 1-D; Bio-Rad, Hercules, USA).

Western blot analysis for IGF-1R expression in vitro and in vivo
Western blot assay was performed as described previously [21]. Briefly, 30 μg of total proteins from smooth muscle...
cells and 60 μg of total tissue proteins from rabbit jugular vein graft were separated on 10% sodium dodecyl sulphate-polyacrylamide gels, then analyzed by using anti-IGF-1R and anti-β-actin (Bios, Beijing, China) antibodies.

Immunofluorescence staining
The smooth muscle cells were identified with the presence of α-smooth muscle actin by using monoclonal antibodies (Abcam, Cambridge, Britain). As described previously [22], a cover glass was placed in the culture dish before the cells were seeded. When 50% confluence was reached, the cover glass was fixed with 10% paraformaldehyde in PBS for 10 min, permeabilized with 0.1% Triton X-100 in PBS for 5 min, washed three times with PBST, and blocked with 5% BSA for 1 h. Then, mouse monoclonal anti-α-smooth muscle actin antibody (1 : 500 in PBST; Abcam) was applied after the cells were washed three times with PBST, then incubated at 4°C for 12 h. The cells were incubated with goat anti-rabbit IgG/Alexa Fluor 555 at 37°C for 2 h, washed with PBST for three times (5 min per wash), further incubated with DAPI (Roche, Basel, Switzerland) at 37°C for 30 min, and then visualized by using an Olympus IX71 fluorescence microscope. For vein graft section staining, the same procedure was performed.

Statistical analysis
Data were expressed as mean ± SD from at least three parallel experiments. Student’s two-tailed t test and one-way analysis of variance were used to determine the statistical significance of differences in two and multiple groups, respectively. P < 0.05 was considered statistically significant. SPSS 17.0 software (SPSS inc., Chicago, USA) was used to analyze data.

Results
IGF-1R-specific shRNA (pGFPshIGF-1R) efficiently inhibits IGF-1R expression
The growth of the cells was observed from vessel explants within 1 week (Fig. 1). Primary culture of rabbit aortic smooth muscle cells grew to 70% confluence in ~2.5 weeks. At confluence, cultured VSMCs had uniform morphology that was characterized by elongated cells in parallel rows and by the overlapping layers forming ridges, which is the characteristic of smooth muscle cell [22]. Figure 1 showed that VSMCs morphology was retained after several passages and the cultured smooth muscle cells showed uniform immunostaining for α-smooth muscle actin. The purity of cultured smooth muscle cell reached almost 100%.

To assess the interference effect, reverse transcription (RT) PCR and western blot assays were performed to evaluate the interference effects of shIGF-1R on IGF-1R expression in smooth muscle cells. Results showed that IGF-1R mRNA level in smooth muscle cells 48 h after transfection with the pGFPshIGF-1R-1 and pGFPshIGF-1R-2 was 58.8 ± 4.7% and 50.7 ± 2.0%, respectively, when compared with shNC.

![Figure 1. Growth of VSMCs cells from arterial explants](image-url)
Compared with shIGF-1R-1 group, the shIGF-1R-2 group showed a more potent inhibition for IGF-1R mRNA expression (Fig. 2A,C, 49 ± 1.0% of shNC group). Moreover, compared with shNC group, shIGF-1R-2 inhibited IGF-1R expression by 45% ± 1.5% (Fig. 2B,D), while the interference effect of shIGF-1R-1 only reached 25.5 ± 0.7%, suggesting that the shIGF-1R-2 plasmid possessed a more potent inhibition for IGF-1R expression.

Polymer-based gene transfection has higher efficiency in cultured smooth muscle cells

In order to obtain higher transfection efficiency, we assessed transfection condition in 24-well plates by modulating the cell density per well and the ratio between shIGF-1R and polymer. When the cell confluence was >80%, the ratio of shIGF-1R between polymers was 1 : 3 (w/v) and the transfection efficiency reached the peak. Under fluorescence microscope, the polymer-based transfection group had higher fluorescence intensity than the lipofection group (Fig. 3A,B). Flow cytometry analysis indicated that the lipofection efficiency of pGFPshIGF-1R was 11.0 ± 4.1%, when compared with the control (mock transfected cells), while polymer-based transfection efficiency of shIGF-1R was 28.6 ± 1.2%, when compared with the control after 48 h (Fig. 3D,E).

Polymer-based transfection allows site-specific gene delivery in vivo

To evaluate whether polymer-based transfection can be a feasible strategy for target-specific gene therapy in vivo, local gene transfection was performed with MegaTran 1.0/DNA mixture to rabbit jugular vein graft. The DNA mixture without MegaTran 1.0 was used as control. High expression level of GFP was observed in the jugular vein graft after polymer-based transfection at 24, 48, and 72 h (Fig. 4). However, only scattered GFP expression was found in vein graft with lipofection, suggesting that polymer-based transfection could cause specific targeting and the uptake of plasmid DNA into specific target sites (data not shown).

Polymer-based transfection specifically silences IGF-1R protein expression in vitro and in vivo.

To evaluate the effect of IGF-1R gene silencing by delivering specific shRNA into smooth muscle cells using polymer, western blot assay was performed. Result showed that IGF-1R expression was down-regulated by 49 ± 2.0% in polymer group, when compared with control (Fig. 5A,C), however, in lipofection group, IGF-1R expression was down-regulated by 10.4 ± 0.5%, suggesting that polymer-based transfection was an effective way to deliver the target gene.

To further confirm whether the plasmid which expressed GFP and shRNA specific for IGF-1R is effective in the silencing of IGF-1R expression in vein graft, MegaTran 1.0/DNA mixture was locally applied into rabbit external jugular vein graft. Moreover, to determine the difference of GFP and shRNA expression in vein graft after transfection, fluorescence microscopy, and western blot assays were performed to analyze the efficiency of shRNAs delivered by polymer. The expression of GFP remained about 1 week in vein grafts. Western blot analysis showed that shRNAs delivered by both lipofection and polymer effectively inhibited IGF-1R expression in vivo. shRNAs delivered by polymer silenced IGF-1R expression more significantly than by lipofection (**P < 0.05). The difference within polymer group was not significant (P > 0.05). The efficiency of shRNAs delivered by polymer reached 77.0 ± 3.6%, 65.6 ± 4.9%, and 78.0 ± 4.3% at 24, 48, and 72 h, respectively, after pGFPshIGF-1R was instilled (Fig. 5B,D), while averagely...
These results confirmed that the efficiency of gene transfection delivered by polymer was more effective than by lipo2000.

**Discussion**

Gene therapy can be effective for the treatment of restenosis after angioplasty. Although reports have suggested successful...
gene therapy strategies for vascular disease in pre-clinical animal models with available non-viral gene transfer methods [23–26], efficacy and safety are major limitations of gene transfer techniques for humans [27]. The majority of the non-viral vectors used for gene therapy, such as PEI, PAMAM, poly-L-lysine, were developed for favorable transfection efficiency in gene transfer [16, 28–30]. However, there are very few reports on introducing foreign DNA into smooth muscle cells using polymer. MegaTran 1.0 is an efficient and versatile agent for gene delivery, which can be used for in vitro transfection and possesses advantages including high efficiency, low toxicity, simple application, and extreme affordability [31–33].

Our results confirmed that polymer-based gene transfection can effectively deliver plasmid DNA into smooth muscle cells and vein graft in rabbit. In smooth muscle cells, the polymer-based transfection efficiency of pGFPshIGF-1R was 28.6 ± 1.2%, when compared with control (Fig. 3), in contrast, lipofection efficiency with Lipo2000 was only 11.0 ± 4.1% (Fig. 3D). We also found that the efficiency was affected by the ratio between shIGF-1R and polymer (data not shown). As other transfection methods, it is an endocytosis-dependent process [34], activating cellular uptake mechanisms rather than promoting uptake. This mechanism of the cellular uptake of transfected genes has been elucidated by transmission electron microscopy. Our results showed that polymer contributed to targeting delivery of therapeutic genes in vivo, indicating that the targeting of vessel with polymer increased the site specificity and thus the selectivity of the therapy, and contributed to the reduction of side effects and therapy cost. Furthermore, IGF-1R-specific shRNA of polymer into aortic smooth muscle cells silenced expression of IGF-1R with high suppression efficacy of 49 ± 2.0% (Fig. 5A,C), while IGF-1R-specific shRNA of lipofection with Lipo2000 silenced the expression of IGF-1R with a lower suppression efficacy of 10.4 ± 0.5% (Fig. 5A,C). This variability might be caused by different amounts of IGF-1R-specific shRNA delivered into aortic smooth muscle cells by polymer and lipofection. Furthermore, Fig. 5(B,D) showed that shRNA delivered by polymer down-regulated IGF-1R expression by 77.0 ± 3.6%, 65.6 ± 4.9%, and 78.0 ± 4.3% at 24, 48, and 72 h, respectively. Moreover, the inhibitory effect of shRNA became obvious when the time of pGFPshIGF-1R in the body was extended. And the silencing efficiency by lipofection reached only 52.8 ± 2.5% averagely at 24, 48, and 72 h.

Synthetic delivery systems have been investigated as an alternative to viral vectors because they are easier to produce, raise fewer safety issues, and pose fewer problems for repeated administration than viral gene transduction [35]. Depending on the type of transfected cell, delivery system, the size of the transfection system, or the ratio of DNA to reagent [36], the efficiency was acceptable though less efficient than viral vectors for gene transfection into smooth muscle cells.

Figure 5. Down-regulation of IGF-1R in smooth muscle cells by plasmids expressing shRNA specific for IGF-1R delivered using polymer in vitro (A,C) and in vivo (B,D). In vitro IGF-1R gene expression was down-regulated by 49 ± 2.0%, when compared with the control, *P < 0.05. The in vivo silencing efficiency of shRNAs delivered by polymers reached 77.0 ± 3.6%, 65.6 ± 4.9%, and 78.0 ± 4.3% at 24, 48, and 72 h, respectively, **P < 0.01. The statistical differences between 24, 48, and 72 h were not significant (P > 0.05).
In summary, we successfully applied commercially available polymer-mediated gene delivery in primarily cultured aortic smooth muscle cells in vitro and further achieved targeting transfection in vivo, which provided a novel method of targeting therapeutic gene delivery for gene therapy of vein graft disease. Based on this therapeutic potential, we hypothesized that the inhibition of IGF1R activity in vein grafts using polymer would be an effective approach to suppress graft neointimal hyperplasia in a rabbit model.

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References

17. Chunna Wu, Chao D, Minjian K, Aiqiang D, Jianfang Q and Daming J. Polymer-based delivering of shRNA suppressed the expression of IGF-1R in vivo and further achieved tar-