Early growth response gene-1 decoy oligonucleotides inhibit vascular smooth muscle cell proliferation and neointimal hyperplasia of autogenous vein graft in rabbits

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Received 21 October 2014; received in revised form 13 January 2015; accepted 17 February 2015

Abstract

OBJECTIVES: The excess proliferation of vascular smooth muscle cells (VSMCs) and the development of intimal hyperplasia is a hallmark of vein graft failure. This study aimed to verify that a single intraoperative transfection of early growth response gene-1 (Egr-1) decoy oligonucleotide (ODN) can suppress vein graft proliferation of VSMCs and intimal hyperplasia.

METHODS: In a rabbit model, jugular veins were treated with Egr-1 decoy ODN, scrambled decoy ODN, Fugene6, or were left untreated, then grafted to the carotid artery. The vein graft samples were obtained 48 h, 1, 2 or 3 weeks after surgery. The thickness of the intima and intima/media ratio in the grafts was analysed by haematoxylin-eosin (HE) staining. The expression of the Egr-1 decoy ODN transfected in the vein was analysed using fluorescent microscopy. Egr-1 mRNA was measured using reverse transcription-polymerase chain reaction. The expression of Egr-1 protein was analysed by Western blot and immunohistochemistry.

RESULTS: Transfection efficiency of the ODN was confirmed by 4′, 6-diamidino-2-phenylindole staining. In the grafts treated with Egr-1 decoy ODN, our study achieved statistically significant inhibition of intimal hyperplasia by ~58% at 3 weeks. Transfection of Egr-1 decoy ODNs decreased the protein expression of Egr-1 and Egr-1 mRNA.

CONCLUSIONS: We confirmed that gene therapy using in vivo transfection of an Egr-1 decoy ODN significantly inhibits proliferation of VSMC and intimal hyperplasia of vein grafts in a rabbit model.

Keywords: Early growth response gene-1 • Decoy • Gene therapy • Intimal hyperplasia

INTRODUCTION

The excess proliferation of vascular smooth muscle cells (VSMCs) and the development of intimal hyperplasia is a hallmark of vein graft failure and a method to decrease it in order to restrain the restenosis of vein grafts is the key aim in coronary artery bypass grafting (CABG) [1]. Although several methods using medication, external sheaths and stents have been attempted to reduce the rate of restenosis, they have not achieved satisfactory results [2].

In current studies, gene therapy provides a promising new method to address this problem. Previous studies have demonstrated that Egr-1 can activate the intimal hyperplasia and inhibit VSMC apoptosis in vein grafts; inhibition of Egr-1 expression might therefore prevent excessive VSMC proliferation [3] and help to prevent vein graft failure.

As a transcription factor (TF), Egr-1 may contribute to vascular proliferation and inflammation. We believed that Egr-1 might be a therapeutic target against vein graft failure. The policy of decoy oligonucleotide (ODN) includes double-stranded ODN transfection, whose sequence corresponds to the sequence of the TF in investigated binding sites. These ODNs may compete with native TF binding sites, resulting in downstream target gene regulation by inhibiting TF [4]. Egr-1 has been shown to have an important role in neointimal hyperplasia after balloon injury in rabbits; in the study, the inhibitory effects of the Egr-1 decoy were explained by the inhibition of vascular inflammation and proliferation through decreased expression and activity of Egr-1-dependent genes [5]. Here, we have designed an Egr-1 decoy ODN, transfected it into rabbit autologous vein grafts, and then evaluated the neointimal hyperplasia, proliferation of VSMCs and the expression of Egr-1.

METHODS

Early growth response gene-1 decoy ODN design and synthesis

A double-stranded phosphorothioate Egr-1 decoy ODN (TaKaRa Biotech Co. Dalian, China) was synthesized. The Egr-1 decoy ODN sequence was 5′-CCGAGAGCGGGGGCGAGCGTG-3′; the sequence
of the scrambled ODN (SCR) used as a negative control, which included unrelated oligonucleotides, was 5’-GAGGCGGGCGAG TCGGAGGG-3’. Egr-1 ODN labelled with fluorescein isothiocyanate (FITC) at the 5’end of one strand was used for analysis of ODN distribution using fluorescent microscopy following transfection.

Experimental animal model and transfection of ODNs

This study was approved by the ethics committee of our institution. It was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animals were supplied by the experimental Animal Center of Tongji University. Sixty-four male New Zealand white rabbits 12–15 weeks old, weighing 3–3.5 kg were used in this experiment. They were randomly divided into five groups (n = 16/group; Groups A and B used same group rabbits but a different lateral jugular vein).

The groups were as follows:

Group A: normal group (contralateral normal external jugular vein of Group B).
Group B: untreated group (jugular vein to carotid artery interposition grafts in rabbits without other treatment).
Group C: control group (vein grafts received treatment with Fugene6 solution [30 µl Fugene6 (transfection reagent for the transient and stable transfection of animal cells)]).
Group D: SCR group [vein grafts received treatment with SCR decoy solution (200 µl mixture containing 500 µg ODNs and 30 µl Fugene6)].
Group E: decoy group [vein grafts received treatment with Egr-1 decoy solution (200 µl mixture containing 500 µg ODNs and 30 µl Fugene6)].

During surgery, a 3 cm segment of the right external jugular vein was harvested under anaesthesia. A catheter was cannulated into the distal side of the vein graft and ligated, the other side being kept open. The cannulated vein graft was placed into a small plastic container. The catheter was then ligated in the container’s mouth, so that the vein graft was sealed in the container and was only connected to the outside through the cannula. The ODN solution (decoy and scrambled decoy) or Fugene6 alone was individually injected into the container through the cannula, which ensured that both the vein lumen and the ectorrhoea were in the fluid. Using a pressure device, the fluid was maintained at 300 mmHg for 20 min. After heparin (500 U/kg) was administered, 7-0 polypropylene sutures were used to suture the two sides of the vein graft individually to the carotid artery in an end-to-side fashion. The carotid artery was then ligated between the two anastomotic stoma and the incision was closed.

Forty-eight hours, 1, 2 or 3 weeks after surgery, animals were sacrificed and the graft vein specimens were harvested at each time point (n = 4/group/time point). One segment of the harvested vein in the decoy group was frozen in liquid nitrogen 48 h after surgery, and prepared for measurement of transfection efficiency.

Histopathology and immunohistochemistry

Compared with the normal jugular vein, the harvested vein grafts of all groups were thick and swollen. Vein grafts and the contralateral external jugular vein were fixed in 10% neutral formalin for 24 h. Dehydration was performed, then the grafts were wax-dipped. The wax block was embedded and the vein graft was cut into 5 µm thick cross-sections. After HE staining, six sections were selected around the circumference of the vein to measure the thickness of the intima and intima/media ratio. For each vein graft, three different levels of cross-section from the same paraffin blocks were required to calculate the average values.

Streptavidin-biotin complex staining was performed. The nucleus or cytoplasm had positive brown-yellow [DAB (Diaminobenzidine)] or red [AEC (3-amin-9-ethylcarbazole)] particles, respectively, at 400 times magnification using a light microscope. The percentage of positive cells was determined.

Reverse transcriptase polymerase chain reaction analysis

The cDNA was prepared by reverse transcription of 1 mg total RNA according to the manufacturer’s instructions (Cat. no. DRR037A, TaKaRa, Dalian, China), followed by polymerase chain reaction (PCR) amplification. The Egr-1 primers were as follows: forward: 5’-GCGGAGAAAGGACAAGAAA-3’, reverse: 5’-GGAGGAATAGGA GGTGGG-3’. The primers for β-actin, which is a housekeeping gene and used for normalization, were as follows: forward: 5’-CATTCCGCGATTGAGAG-3’, reverse: 5’-GGGAGGAGGATG ATTTCT-3’. PCR products were analysed by 1.5% agarose gel electrophoresis and the SYBR Green PCR core reagent according to established protocols. DNA bands were photographed and the intensities were quantified using the 2 ΔΔct method on the Gel Imaging System.

Western blot analysis

The specimens were lysed, then total protein was extracted according to the manufacturer’s instructions (Cat. no. KG1050, TaKaRa, Dalian, China). Protein extracts (50 µg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and then transferred to nitrocellulose membrane. The membrane was incubated with a primary antibody, and visualized using a horseradish peroxidase conjugated secondary goat anti-mouse IgG antibody at a 1: 300 dilution. The nitrocellulose membrane was photographed, and analysed on the Gel Imaging System.

Statistical analysis

All values are expressed as mean ± SD. Statistical analysis was performed using Student’s t-test and one-way analysis of variance. The SPSS software version 13.0 for Windows was used in data analysis. When P < 0.05, the result was considered to be statistically significant.

RESULTS

Transfection efficiency

After transfection with FITC-ODNs, frozen sections of vein grafts were analysed by the fluorescence microscopy. Transfection of FITC-ODNs resulted in widespread fluorescence in vascular intima and media of vein grafts in the decoy group, which was confirmed.
by 4′, 6-diamidino-2-phenylindole (DAPI) staining (Fig. 1). The fluorescence expression value of total cells was 69 ± 2.36%.

Inhibition of intimal hyperplasia

The neointimal hyperplasia was significantly reduced in the Egr-1 decoy ODN-treated vein, but not in the untreated, Fugene6 alone and SCR ODN-treated groups (Fig. 2). Three weeks after surgery, intimal thickness in the grafts of the Egr-1 decoy ODN group (41.33 ± 3.78 µm) was 56% lower than that in the SCR group (93.50 ± 3.73 µm, P < 0.05), 55% lower than that in the Fugene6 group (92.83 ± 3.06 µm, P < 0.05) and 58% lower than that in the untreated group (97.17 ± 2.04 µm, P < 0.05) (Fig. 3). Regarding intimal thickness, no significant difference was found between the SCR group, the Fugene6 group or the untreated group. The rate of intimal/media in the Egr-1 decoy ODN group was 47, 48 and 49% lower compared with the SCR, Fugene6 group and untreated group at 3 weeks (P < 0.05), respectively (Fig. 4).

Inhibition of vascular smooth muscle cell proliferation

No expression of proliferating cell nuclear antigen (PCNA) protein was detected in the normal vein. There were slightly disordered VSMCs in the media 48 h after surgery compared with the control and SCR group. There was a slightly positive expression of PCNA protein by immunohistochemistry at 48 h; a positive cell rate of 3.6 ± 0.5% in the Egr-1 decoy ODN group, and 5.1 ± 0.6% (P < 0.05) in the SCR group. The expression of PCNA protein reached a peak at 3 weeks 23.4 ± 5.2% in the Egr-1 decoy ODN group, and 23.4 ± 5.2% in the SCR group. There was a slightly positive expression of PCNA and SCR group. The expression of Egr-1 mRNA reached a peak at 3 weeks (1.89 ± 0.01) in the Egr-1 decoy ODN group, which was significantly lower than that observed in the SCR group (2.91 ± 0.09, P < 0.05) (Fig. 6). The levels of Egr-1 protein detected by immunohistochemistry supported the RT-PCR results, the tendency of positive cells was consistent with the RT-PCR results in each group. This showed that the Egr-1 decoy ODN could inhibit the expression of Egr-1 mRNA in VSMCs.

Western blot

Western blot analysis showed that the Egr-1 protein levels reached a peak at 48 h, decreased after 1 week, increased again at 2 weeks and decreased again at 3 weeks. The expression of Egr-1 protein in the grafts treated with Egr-1 decoy ODN was lower at each time point compared with SCR, Fugene6 alone and untreated groups (Fig. 7). This showed that the Egr-1 decoy ODN could inhibit the expression of Egr-1 in VSMCs, thus supporting the observations obtained using RT-PCR and immunohistochemistry.

DISCUSSION

Proliferation and migration of VSMCs can lead to neointimal hyperplasia and vascular remodelling. Vein graft intimal hyperplasia can be induced by a variety of factors, such as vascular injury, haemodynamic changes in pressure, vascular spasm, inflammation and ischaemia; intracellular pathway responses to these stimuli are more complex [6]. Egr-1 is a Cys2-His2-type zinc-finger TF, whose expression is triggered by physical or chemical stimulation, and plays an important role in cell growth, differentiation and injury repair [4, 7–8]. More than 300 genes are regulated by Egr-1, including genes encoding transcriptional regulators, signalling proteins, cell cycle regulatory proteins, growth factors and cytokines, suggesting that Egr-1 is a key mediator of inflammation and apoptosis in vascular cells [9]. Cao et al. [10] indicated that differential regulation of Egr-1 expression by glucose and TNF-α in endothelial cells may be an important consideration in the mechanisms linking these factors to the development of vascular dysfunction in metabolic disorders. Zhang et al. [11] demonstrated that Egr-1 may promote endothelial cell (EC) proliferation and result in vein graft restenosis; as a key factor in vein graft restenosis, it could be a target for the prevention of restenosis after CABG surgery.

As double-stranded DNA, decoy ODNs have a stable duplex structure, small molecular weight, simple synthesis and sequence specificity, which can inhibit multiple genes regulated by the same cis-element. Previous investigations have confirmed that decoy ODNs could cure disease [4]. In one study, where vein grafts were treated with Nuclear factor xB (NFκB) decoy ODN in a rabbit model, the accumulation of VSMCs in the neointima was significantly suppressed, and intimal hyperplasia was suppressed by ~50%; the NFκB decoy ODN had also been tested in 17 patients, significant coronary artery restenosis was found in only 1 patient after 6 months [6]. In another rabbit model, neointimal hyperplasia was inhibited in jugular vein grafts treated with E2F decoy ODN; in humans, E2F-decoy-treated infrainguinal vein grafts had fewer occlusions at 12 months [6].

There have only been a few studies on the use of Egr-1 decoy ODN in vein grafts, but it appears to have potential. This study aimed to prove the efficacy of Egr-1 decoy ODN transfection in

![Figure 1: Forty-eight hours after grafting, FITC-ODNs are located in the vein graft of a rabbit model (×400). Widespread green fluorescence (FITC-ODNs) was observed in vascular areas using fluorescence microscopy, with punctiform fluorescence staining, suggesting nuclear fluorescence in the transfected cells. This experiment was performed thrice. The fluorescence expression value of total cells was 69 ± 2.36%. Scale bar: 50 µm. FITC: fluorescein isothiocyanate.](image-url)
vivo for restenosis therapy in vein grafts. Successful transfection of FITC-ODNs resulted in widespread fluorescence in vascular intima and media of vein grafts in the decoy group, which was confirmed by DAPI staining. We found that blockade of Egr-1 activity by transfection of Egr-1 decoy ODN inhibited neointimal hyperplasia (by \( \sim 58\% \)) in vein grafts in rabbits for up to 3 weeks.

Common gene transfer methods include viral or non-viral methods, and they all have advantages and disadvantages [6]. The local application of short hairpin RNA using organ-targeted gene delivery may be a promising gene therapeutic tool [12]. In our study, satisfactory inhibition of Egr-1 mRNA expression showed that gene therapy using transfection of Egr-1 decoy ODN could obtain good results. The expression of Egr-1 mRNA reached a peak at 3 weeks, \((1.89 \pm 0.01)\) in the Egr-1 decoy ODN group, \((2.91 \pm 0.09, P < 0.05)\) in the SCR ODN group. We demonstrated in vivo evidence for effective suppression of Egr-1 activation in the vein graft by transfection of synthetic Egr-1 decoy ODN.

Egr-1 protein expression in vein grafts is biphasic; Egr-1 protein reached a peak at 48 h, then the expression of Egr-1 protein declined at 1 week, increased again at 2 weeks and decreased again at 3 weeks. The expression of Egr-1 protein in the grafts treated with Egr-1 decoy ODN was lower at each time point compared with the grafts that were treated with SCR decoy ODN, Fugene6 alone and untreated grafts. The results showed that Egr-1 decoy ODNs could effectively inhibit proliferation of VSMCs.

PCNA is a cofactor of DNA polymerase, which can promote cell proliferation and be regulated by Egr-1 [13]. Egr-1 decoy ODNs decreased the protein expression of PCNA, Egr-1 and Egr-1mRNA. The use of Egr-1 ODNs to prevent vascular proliferation diseases appears feasible.
CONCLUSION

Our present data suggest that Egr-1 is important in neointimal hyperplasia and that an Egr-1 decoy ODN can decrease expression of Egr-1 and Egr-1-dependent genes, thereby inhibiting neointimal hyperplasia and proliferation of VSMCs in vein grafts. These results suggest that gene therapy using in vivo transfection of an Egr-1 decoy ODN might be a new therapeutic option. It will have practical significance in the prevention of restenosis of vein grafts, and hopefully be used in clinical cardiac surgery.

Funding

This study was supported by the Shanghai Municipal Natural Science Foundation (no. 11ZR1433600).

Conflict of interest: none declared.

REFERENCES


