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Lentiviral vector mediated expression of Bax and hepatocyte growth factor inhibits vein graft thickening in a rabbit vein graft model

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Vein graft failure caused by vein graft thickening of the arterialized vein after bypass surgery is a main problem in clinical vascular surgery. Gene therapy is increasingly being recognized as a relevant treatment option for vein graft failure. In this study, we aimed to develop a novel recombinant lentivirus for the delivery of hepatocyte growth factor (HGF) and Bax in a rabbit vein graft model of bypass grafting. A bypass model was made in rabbits using the right jugular vein interposed end-to-end to the ipsilateral carotid artery. A lentivirus vector harboring HGF and Bax cDNAs (Lenti-HGF-Bax) was constructed and transduced into the venous grafts. Vein grafts were stained with hematoxilyn and eosin, and Masson. HGF and Bax expression in vein grafts was detected by immunohistochemical and Western blot analysis. Our results showed that vein graft thickening was reduced by 47.2 ± 7.4 % in lenti-HGF-Bax treated rabbits, compared to controls. Meanwhile, the ratio of intima/media area was reduced in lentil-HGF-Bax treated rabbits, compared to controls. The number of HGF and Bax positive cells was increased in vein grafts from rabbits treated by lenti-HGF-Bax, compared to those from controls. Furthermore, protein levels of HGF and Bax were both significantly increased in grafts derived from rabbits treated by lenti-HGF-Bax, compared to those from control. In conclusion, Lenti-HGF-Bax inhibits vein graft thickening in vein grafts and is a promising agent for preventing vein graft failure.

1. Introduction

Currently, surgical revascularization (bypass surgery) with autologous vein graft is the main approach for the therapy of both coronary and peripheral artery diseases. However, the long-term outcome of bypass surgery is limited due to bypass failure caused by vein graft thickening of the arterialized vein after bypass surgery (Harskamp et al. 2013; Prevost et al. 2013). Vein graft failure results from endothelial dysfunction and inflammation, and involves the proliferation and migration of vascular smooth muscle cells (VSMCs) (Schwartz 1997). Gene therapy is increasingly being recognized as a relevant treatment option for vein graft failure (Wang et al. 2014; Southerland et al. 2013). Lentiviral vectors are reported to be superior to other vectors with regard to the safety and efficacy of gene delivery, especially for terminally differentiated target cells (Schambach and Baum 2008). Lentiviral vectors efficiently transduce cells and stably integrate into the host genome to allow long-term expression of the transgene, and therefore are good vehicles for long-term gene delivery (Schambach and Baum 2008). Up to now, few studies have utilized lentiviral vectors for gene therapy of vein graft failure (Robertson et al. 2012; Eefting et al. 2009; Dedieu et al. 2000).
Hepatocyte growth factor (HGF) acts as the angiogenic factor that induces the migration and proliferation and inhibits the apoptosis of endothelial cells, contributing to angiogenesis (Morishita et al. 1999). Bax is a well-known pro-apoptotic factor and a recent study showed that Bax is involved in the regulation of the proliferation, migration and apoptosis of VSMCs (Wang et al. 2014). Therefore, we attempted to employ both HGF and Bax for gene therapy of vein graft disease. In this study, we developed a novel recombinant lentivirus harboring HGF and Bax cDNAs, and prepared a bypass model in rabbits using the right jugular vein interposed end-to-end to the ipsilateral carotid artery. We transduced the lentiviral vector into the venous grafts and observed the efficacy.

2. Investigations and results

2.1. *Lenti-HGF-Bax inhibits vein graft thickening in vein grafts*

To demonstrate the inhibitory effect of lenti-HGF-Bax on neointima formation in vein grafts, vein segments were treated with GFP/HGF/Bax lentivirus or control GFP lentivirus and cultured for 4 weeks. We found that green fluorescence present in intima in Group III was thinner than that in Group II, whereas no green fluorescence could be detected in Group I (Fig. 1).
Next, we performed hematoxilyn and eosin and Masson staining of the vein grafts. As shown in Figs. 2 and 3, the intima was thinner in Group III than in Group I and II. Furthermore, quantitative morphometric analysis of vein grafts showed that vein graft thickening was reduced by $47.2 \pm 7.4\%$ in lenti-HGF-Bax treated rabbits, compared to controls in Group I and II (Fig. 4A). Meanwhile, the ratio of intima/media area was reduced in

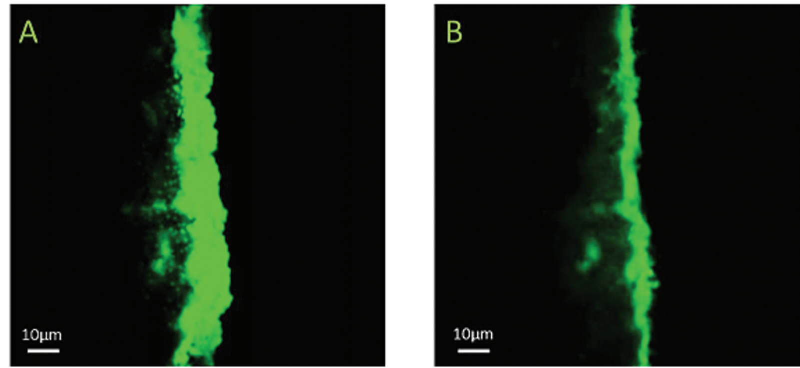

Fig. 1: Lenti-HGF-Bax inhibits vein graft thickening in vein grafts. After 28 days of culturing, vein grafts were assessed under fluorescent microscope. Green fluorescence was detected in Group II (A) and Group III (B).

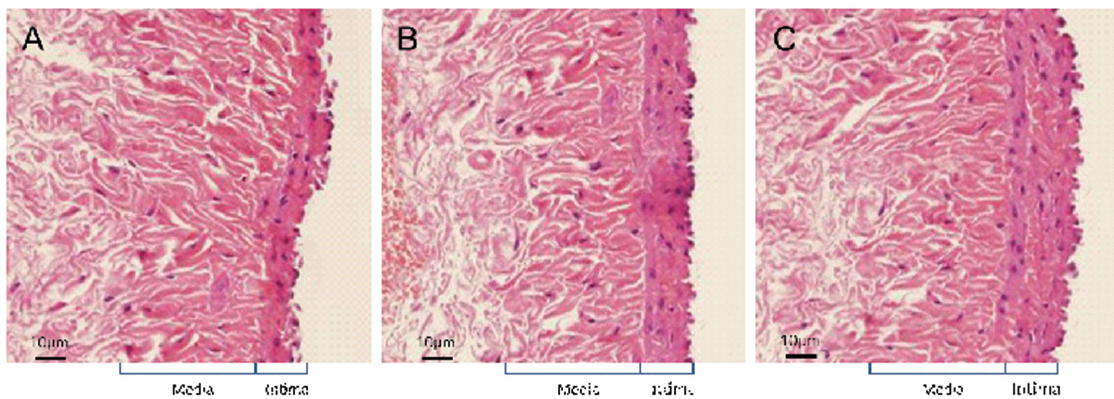

Fig. 2: Histopathologic examination of vein grafts by hematoxylin and eosin staining 28 days after operation (original magnification 200 ×). **A**. Group III (recombinant lentivirus HGF-Bax), **B**. Group II (empty vehicle), **C**. Group I (DMEM).

lentil-HGF-Bax treated rabbits, compared to controls in Group I and II (Fig. 4B). Taken together, these data demonstrate that Lenti-HGF-Bax inhibits vein graft thickening in vein grafts.

2.2. *Lenti-HGF-Bax increases HGF and Bax expression in vein grafts*

To confirm that the effects of Lenti-HGF-Bax on vein graft thickening are due to the overexpression of HGF and Bax, we performed immunohistochemistry analysis of HGF and Bax in vein grafts. The results showed that the number of HGF positive cells was increased in vein grafts from rabbits treated by lenti-HGF-Bax, compared to those from controls (Fig. 5A). Furthermore, the number of Bax positive cells was increased in vein graft from rabbits treated by lenti-HGF-Bax, compared to those from controls (Fig. 5B).

In addition, we performed Western blot analysis of HGF and Bax expression in vein grafts. The results showed that the protein levels of HGF and Bax were both significantly increased in grafts derived from rabbits treated by lenti-HGF-Bax, compared to those from control (Fig. 5C, D). Taken together, these data indicate that Lenti-HGF-Bax could increase HGF and Bax expression in vein grafts.

3. Discussion

In the present study, we evaluated the effects of lentiviral gene delivery of both Bax and HGF on vein graft thickening in a rabbit

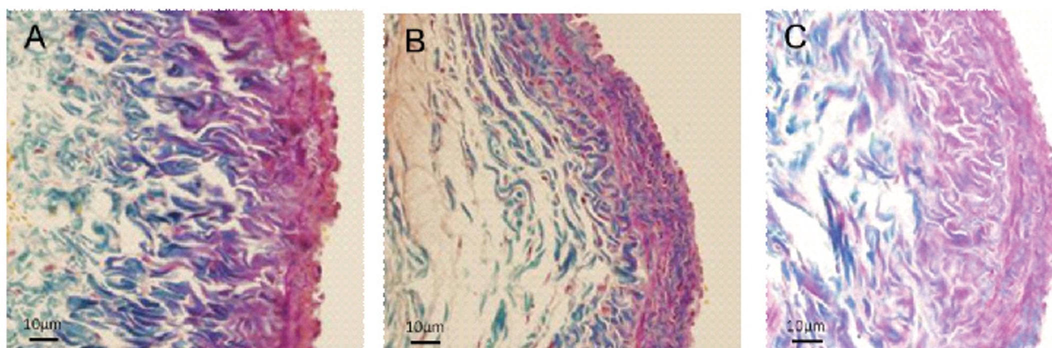

Fig. 3: Pathologic examination of vein grafts by Masson staining (original magnification 200 ×). **A**. Group III (recombinant lentivirus HGF-Bax), **B**. Group II (empty vehicle), **C**. Group I (DMEM).

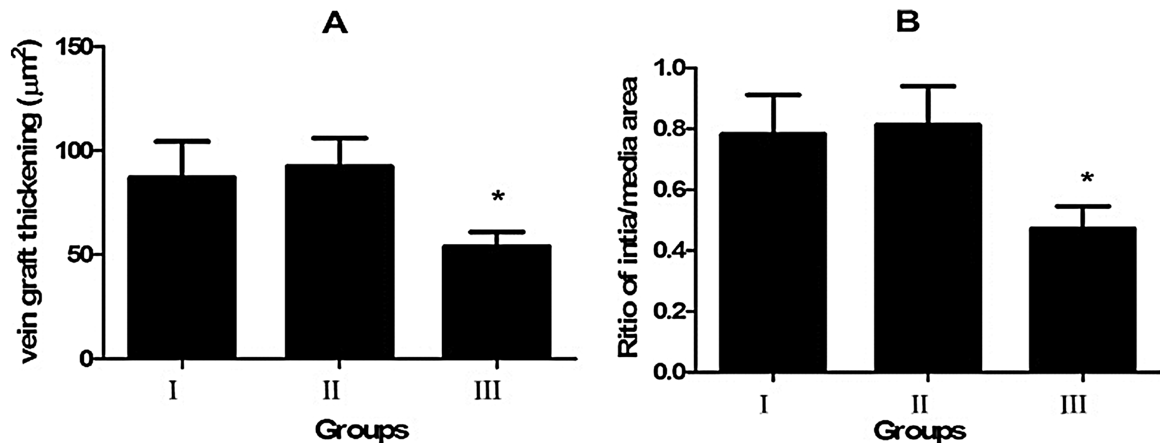

Fig. 4: Quantitative morphometric analysis of vein grafts. Four weeks after surgery, vein graft thickening (A) and the ratio of intima/media area (B) were quantified using six random selected sections per segment. Areas were expressed in square micronmetres (mean + SD). *P < 0.05 compared with Group I. Group I (DMEM), Group II (empty vehicle), Group III (recombinant lentivirus HGF-Bax).

in vivo vein graft model. Lenti-HGF-Bax vector was designed in order to promote the apoptosis of EC cells and inhibit the proliferation and migration of VSMCs, which are crucially involved in vein graft thickening.

HGF has been shown to play key role in the regulation of the proliferation and migration of VSMCs and neointima formation (McKinnon et al. 2006). Therefore, we postulated that the dual modulation of the apoptosis of ECs and the proliferation and migration of VSMCs by overexpression of Bax and HGF may provide a new approach to reduce neointima formation and vein graft remodeling. By fluorescent macroscopic analysis we showed that recombinant lentiviral vector facilitated an effective gene delivery of Bax and HGF in an autologous rabbit venous bypass grafts *in vivo* model. By morphometric analysis we demonstrated that Lenti-HGF-Bax effectively inhibited vein graft thickening in vein grafts. In addition, by immunohistochemistry and Western blot analysis we confirmed that Lenti-HGF-Bax could increase HGF and Bax expression in vein grafts. Taken together, these results suggest that overexpression of Bax and HGF could inhibit vein graft thickening.

Interestingly, we found that immunohistochemical staining of HFG was detected mainly in VSMCs related to the medial layer, while immunohistochemical staining of Bax was detected mainly in ECs related to the intimal layer. Cells from the media and intima are known to be the origin of neointimal hyperplasia in bypass grafts. Further studies are needed to confirm that Bax induces the apoptosis of ECs and HGF modulates the proliferation and migration of VSMCs.

Adenoviral vectors have shown high efficiency for gene delivery and expression in vein grafts but could induce a strong immuno/inflammatory response to exacerbate neointimal formation in vein grafts, thus limiting their clinical application

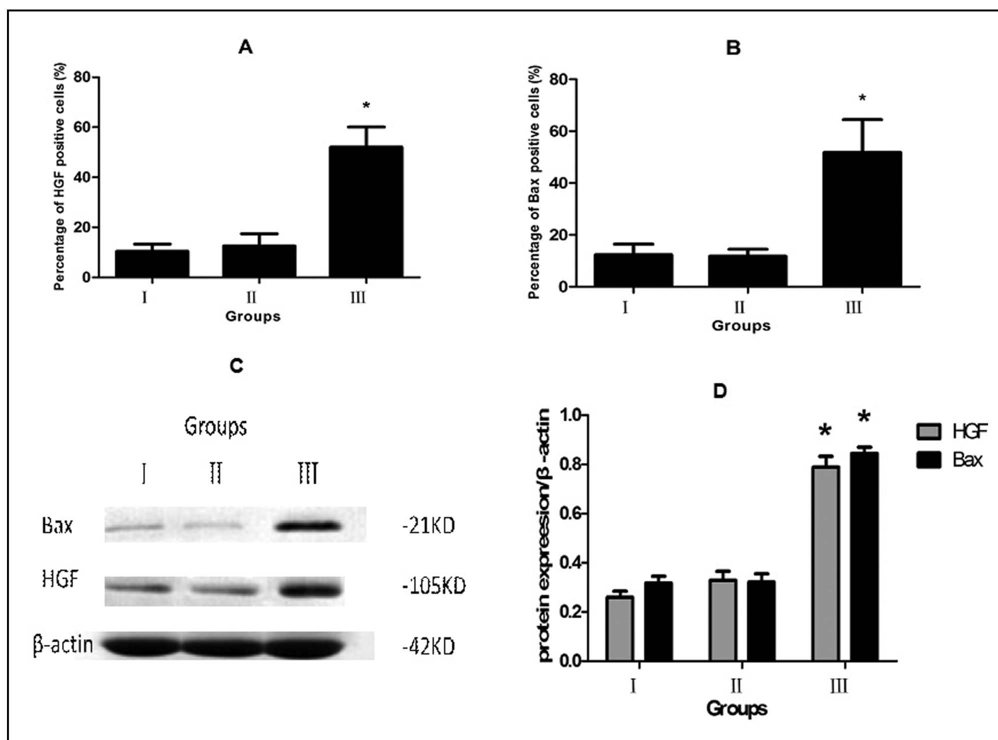

Fig. 5: Expression of HGF and Bax in vein grafts. A. HGF immunopositive cells as a percentage of the total cells in cross-sections (mean + SEM). B. Bax immunopositive cells as a percentage of the total cells in cross-sections (mean + SEM). C. Representative blots showing the protein levels of HGF and Bax in vein grafts. β-actin was loading control. D. Densitometry analysis of HGF and Bax protein levels in vein grafts. *P < 0.05 compared with Group I. Group I (DMEM), Group II (empty vehicle), Group III (recombinant lentivirus HGF-Bax).

(Newman et al. 1995; Yla-Herttuala and Martin 2000). Several non-viral vectors have been developed as an alternative to adenoviral vectors, they induce no immuno/inflammatory response they exhibit poor efficiency of gene delivery compared to adenoviral vectors. In this study, we employed lentivirus vector and found that lentivirus mediated expression of Bax and HGF was detectable beyond 4 weeks after the operation, indicating the durable expression of the transduced factors. Thus lentivirus vector is a efficient and suitable vector for gene therapy of vein graft failure.

In summary, we developed a novel lentiviral vector Lenti-HGF-Bax that achieves efficient transduction of vein grafts and high expression of HGF and Bax in vein grafts. Lenti-HGF-Bax inhibits vein graft thickening in vein grafts and is a promising agent for preventing vein graft failure.

4. Experimental

4.1. Lentiviral vector construction

The hybrid cDNAs attB1-K-hBAX/T2A/eGFP/P2A/hHGF-attB2 were made using PCR and inserted into pLV.EX2d.null-EF1A > eGFP vector to construct HGF-Bax-GFP vector, the empty vector was used as a negative control. Lentiviruses were generated by transfecting 293FT cells with auxiliary plasmid (Invitrogen) and 4 μg pLV vector. The transfection mix was replaced with fresh culture medium (without antibiotics) 6-12 h after transfection. Supernatant containing infectious lentiviruses was harvested 72 h after transfection, centrifuged at 3,000 rpm for 15 min at 4 °C, and filtered through a 0.45-μm filter (Millipore). Lentivirus aliquots were prepared and stored at −80 °C for further use.

Lentivirus was titrated using 293FT cells seeded in a 6-well plate (Corning, Corning, NY). Frozen aliquots of vectors were thawed. Virus was added at 10^{-3}–10^{-7} dilution in volumes of 30 μl, and a control was included which was devoid of virus. After 24 h, medium was replaced with 2 ml DMEM. Lentiviral vectors were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 72 h. Finally, the cells were observed under a fluorescent microscope and the percentage of transduction was calculated.

4.2. Rabbit vein graft model

White New Zealand rabbits (female, weight 3–3.5 kg) were obtained from the Experimental Animal Center of Qingdao Institute for Drug Control. Animals were maintained under standardized, sterilized conditions (25 ± 2 °C, 60-70% humidity, 12 h dark/light cycle) in a specific pathogen free (SPF) laboratory. The rabbits were acclimatized to the housing condition 1 week before the experiment. All the experiments were conducted under the guidelines of the Care and Use of Laboratory Animals by National Institutes of Health (NIH Publication No. 85-23, revised 1996). Rabbits were anesthetized with ketamine (100 mg/kg bodyweight) and xylacin (50 mg/kg bodyweight) intramuscularly.

Eighteen rabbits were assigned randomly into three different groups (n = 6). End to side anastomosis was made to the right common carotid artery using the right external jugular vein. The vein was first dissected free from the surrounding tissues and all branches were ligated. Heparin sodium 125 U/kg was injected intravenously after the vessels were freed. The vein segments were gently flushed to remove residual blood placed in the DMEM buffer solution for 30 min at room temperature. Group I was treated with DMEM medium. Group II was treated with DMEM medium containing GFP lentivirus (9×10^7 TU/ml). Group III was treated with DMEM medium containing GFP/HGF/Bax lentivirus (7.8×10^7 TU/ml).

The vein segments were interposed into ipsilateral carotid arteries in an end-to-end fashion with 7-0 polypropylene monofilament sutures. Intravenous penicillin G procaine (0.5 million units) was administered during surgery and then daily for three days postoperatively. The rabbits were fed aspirin 2 mg/kg/day for anticoagulation from the second postoperative day until the end of the study. 28 days after the procedure, all animals from each group were euthanized by an overdose of sodium pentobarbital. Each graft was divided into three parts at the center. Grafted segments were harvested for biochemical, immunohistochemical, and morphometric analysis.

4.3. Morphometric and immunohistochemical analysis

Each vein graft was sliced into 5 μm from the proximal, middle, and distal segments. Crosssections of three tissues from each vein graft were prepared and stained with hematoxilyn and eosin, and Masson. The extent of intimal hyperplasia was expressed as an intima/media index, intima thickness divided by media thickness. The intima area and media area were measured using the NIH Image computer assisted analyzer (National Institutes of Health, Bethesda, MD, USA). The average of the six values was considered to represent the intimal hyperplasia of the section, and the average of the three sections was considered to represent those of the graft.

For immunohistochemical staining, the vein graft tissues were stained by HGF and Bax antibodies with the use of the streptavidin–biotin–complex peroxidase kit (Boster, Wuhan, China). Finally, the slides were washed, dehydrated and mounted for microscopic examination on the Olympus microscope. The images were analyzed by Media Cybernetics Image-Pro Plus analysis system. To calculate the positive index, eight high-power fields (×400) per section were selected randomly and positive cells and total cells were counted in the neointima in a blinded manner. The number of positive cells divided by the total number of cells of the vein graft was defined as the HGF and Bax index.

4.4. Western blot analysis

The snap-frozen parts of each vein graft were ground into powder and lysed in radioimmunoprecipitation assay (RIPA) buffer supplemented with protease inhibitors. The protein concentrations of the lysate were quantified using the bicinchoninic acid (BCA) protein assay kit (Beyotime Institune of Biotechnology, China). Equal amounts of protein were separated by 10% sodium dodecyl sulfate/polyacrylamide gel electrophoresis and transferred to polyvinyli-dene fluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA). The membranes were blocked with 5% non-fat dry milk in PBST (PBS with 0.05% Tween-20) for 1 h, and then incubated at 4 °C overnight with HGF, Bax or β-actin antibody (Sigma, St. Louis, MO, USA) in blocking buffer. Next the membranes were washed with PBST and incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h, and developed with the ECL Western blotting system. Protein levels were normalized to β-actin.

4.5. Statistical analysis

Values were presented as the mean ± standard deviation (SD) and analyzed by SPSS 17.0 software. The results were evaluated by using one-way analysis of variance (ANOVA) followed by LSD test. Significant differences were defined as $P < 0.05$.

Competing interests: The authors declare that they have no competing interests.

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