Original Article

Construction of an adenovirus vector carrying the human tissue inhibitor of metalloproteinase 2 gene

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Summary **This study sought to construct an adenoviral vector carrying the human tissue inhibitor of the metalloproteinase 2 (TIMP-2) gene for use in gene therapy. A recombinant adenovirus (AdTIMP-2) containing a human TIMP-2 cDNA fragment was generated by homologous recombination in BJ5183 bacteria. Recombinant plasmids were screened by antibiotic selection. The adenovirus vector was then packaged and amplified in HEK293 cells. A recombinant adenoviral vector carrying human TIMP-2 was constructed. The titer was 4×10^{11} pfu/mL after purification. The expression of the TIMP-2 gene in HEK293 cells was detected by PCR. A recombinant adenoviral vector carrying human TIMP-2 was successfully constructed and is available for further use in gene therapy for vascular disease.**

Keywords: Tissue inhibitor of metalloproteinase, Adenoviral vector, Gene therapy

1. Introduction

Abdominal aortic aneurysm (AAA) is a complex multifactorial disease, and inflammation appears to play a fundamental role in AAA development and progression (*1*,*2*). In a previous study, the current authors found that expression of matrix metalloproteinases (MMPs) increased in experimental abdominal aortic aneurysms in rats and that tetracycline inhibited the development of experimental abdominal aortic aneurysms *in vivo* through the inhibition of MMP-2 and MMP-9 expression (*3*,*4*). Many investigators have described the involvement of various members of MMP family in the degradation of extracellular matrix, and MMP-2 and MMP-9 in particular seem to play a pivotal role in this process (*5*,*6*). In addition, MMPs activity is regulated by the binding of endogenous inhibitors known as tissue inhibitors of metalloproteinases (TIMPs), 4 of which are now known. TIMP-2 selectively binds to pro-MMP-2, an interaction that can prepare the enzyme for activation by membrane type MMP-1 (MT1-MMP) (*7*). Therefore, the current study investigated the role of TIMP-2 in the formation of abdominal aneurysms using a recombinant adenovirus gene transfer system. An adenovirus vector carrying human TIMP-2 gene was first constructed and then the vector was propagated in human embryo kidney 293 (HEK 293) cells.

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2. Materials and Methods

2.1. *Construction of a recombinant adenovirus vector containing the human TIMP-2 gene*

A first generation (E1-, E3-) recombinant adenoviral vector was used to construct an expression vector for human TIMP-2 using the AdEasy™ system (Agilent Technologies, Inc., Santa Clara, CA, USA). This simplified system was first introduced by He TC, *et al.* (*8*). Briefly, the human TIMP-2 gene was released by *Eco*RI and *Xba* I digestion from the pGEM-4 vector

and inserted into the multicloning site (MCS) of the pBluescript vector, generating plasmid pBluescript-TIMP-2. The resultant plasmid was linearized by digestion with *Sal* I and *Xba* I. Then, the human TIMP-2 cDNA was inserted into the *Sal* I and *Xba* I restriction sites of the shuttle vector pAdTrack-CMV (cytomegalovirus). The resultant plasmid was designated the pAdTrack-CMV-TIMP-2 vector.

The resultant plasmid was linearized by digestion with *Pme* I and subsequently co-transformed into electrocompetent *E. coli* BJ5183 with an adenoviral backbone plasmid (pAdEasy-1; Agilent Technologies, Inc.). Electroporation was performed in 2.0 mm cuvettes at 2,500 V, 200 ohms, and 25 μF in a Gene Pulser electroporator (Bio-Rad Laboratories, Hercules, CA, USA). The cells were immediately placed in 500 μL of L-Broth and grown at 37°C for 20 min. One hundred twenty-five microliters of the cell suspension were then inoculated into four 10-cm Petri dishes containing L-agar plus 25 μg/mL of kanamycin. After 16-20 h of growth at 37°C, about 10-25 colonies per dish were obtained. Recombinants were selected for kanamycin resistance and were confirmed by restriction digestion with *Pac* I. Once recombinants were confirmed, supercoiled plasmid DNA was transformed into DH10B cells for large-scale amplification by electroporation.

Finally, the recombinant adenoviral plasmid was digested with *Pac* I and transfected into HEK 293 cells using the Lipofectamine (Invitrogen, Carlsbad, CA, USA) method for adenovirus packaging. Transfected cells were monitored for enhanced green fluorescence protein (GFP) expression and collected 7-10 days after transfection. The cells were lysed by three cycles of freezing and thawing. The viral lysates were then collected after centrifugation. The primary recombinant adenovirus with the human TIMP-2 (AdTIMP-2) was propagated by re-infecting HEK 293 cells and was purified by CsCl density gradient ultracentrifugation. Purified viruses were stored in phosphate-buffered saline (PBS) containing 10% glycerol at −80°C at a concentration of 2×10^{10} plaque-forming units (pfu)/mL. The control recombinant adenovirus AdCMV-GFP was similarly constructed.

2.2. *Identification of recombinant adenoviral particles by PCR*

On day 10, HEK 293 cells were collected and pelleted together along with any cells floating in the culture. After three cycles of freezing/thawing, 5 μL of viral lysate were used for detection of the TIMP-2 gene in adenoviral particles with PCR. Primers used in PCR reactions were as follows: TIMP-2, sense primer 5'-CCG AAT TCT GCA GCT GCT CCC CGG TGC ACC CG-3', and antisense primer 5'-GGA AGC TTT TAT GGG TCC TCG ATG TCG AG-3'. PCR was performed in a 50 μL reaction system with 25 cycles (94°C for 20 sec, 65°C for 30 sec, and 72°C for 60 sec). PCR products were subjected to electrophoresis in a 1.0% agarose gel.

3. Results

3.1. *Generation of the shuttle vector pAdTrack-CMV-TIMP-2*

The full length of the TIMP-2 gene was released from the recombinant shuttle vector pAdTrack-CMV-TIMP-2 by *Sal* I/*Xba* I and *Bgl* II/*Xba* I. Sequencing of the TIMP-2 gene yielded 791 bp and was in accordance with what had been previously published in Genbank (Figure 1).

3.2. *Construction of adenoviral vectors pAdTIMP-2 and pAdCMV-GFP for homologous recombination in bacteria*

Zero point five to 1.0 mg of pAdTrack-CMV-TIMP-2 was linearized with *Pme* I and mixed with 0.1 mg of supercoiled pAdEasy-1. Then, electrocompetent *E. coli* BJ5183 cells were added, and electroporation was performed. The cell suspension was then inoculated into 10-cm Petri dishes containing L-agar plus 25 μg/mL of kanamycin. Thirty clones that were kanamycin-resistant were obtained. Smaller colonies (usually representing recombinants) were selected and grown in 2 mL of L-Broth containing 25 μg/mL of kanamycin. Clones were first screened by analyzing their supercoiled sizes on agarose gels and comparing them to pAdEasy-1 controls. The clones that had inserts were further tested by restriction endonuclease digestion, typically with Pac I. As shown in Figure 2, the TIMP-2 gene was detected as a 791-bp diagnostic fragment. Typical digestion of pAdTIMP-2 with *Pac* I yielded the diagnostic fragments

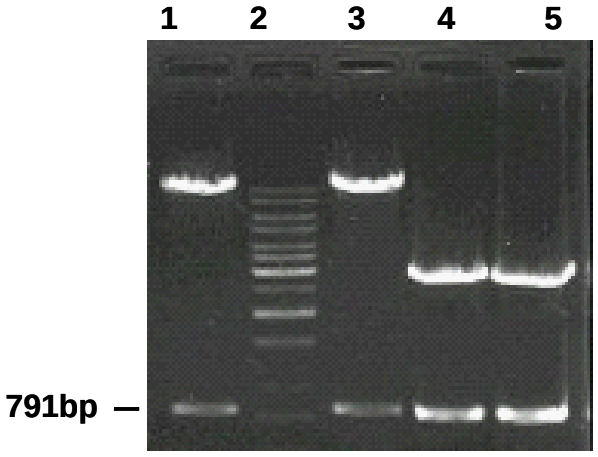

Figure 1. Identification of pAdTrackCMV-TIMP-2. Lane 1, pAdTrackCMV-TIMP-2 (linearized with *Sal* I and *Xba* I); lane 2, DNA ladder; lane 3, pAdTrackCMV-TIMP-2 (linearized with *Bgl* II and *Xba* I); lane 4, pBS-TIMP-2 (linearized with *Sal* I and *Xba* I); lane 5, pGEM-TIMP-2 (linearized with *Eco*RI and *Xba* I).

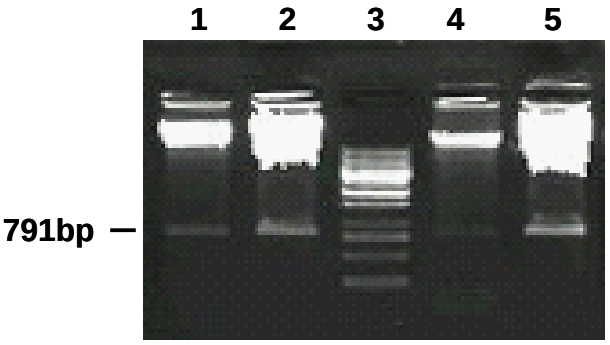

Figure 2. Different clones of recombinant pAdTIMP-2 constructs identified by *Eco*RI and *Xba* I. Some clones in lanes 1, 2, and 5 yielded 791-bp TIMP-2 diagnostic fragments; lane 3, DNA ladder.

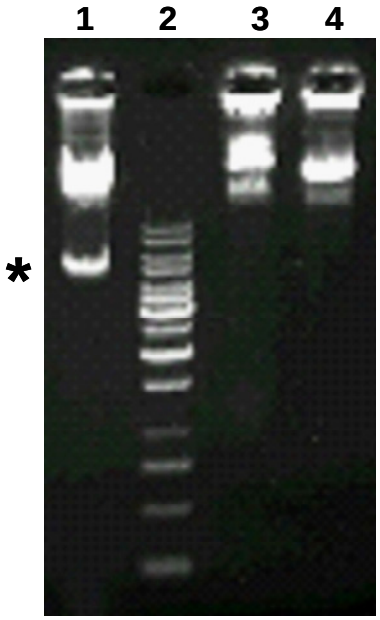

Figure 3. Digestion of pAdTIMP-2 with *Pac* I. Lane 1, pAdTIMP-2 (linearized with *Pac* I); lane 2, DNA ladder; lane 3, pAdMP-2; lane 4, pAdEasy-1.

indicated with an asterisk (Figure 3, lane 1). Figure 4 shows that both the final viral vector pAdTIMP-2 and shuttle vector pAdTrackCMV-TIMP-2 yielded the target gene by restriction endonuclease digestion. The control adenoviral vector pAdCMV-GFP was constructed using the same procedure.

3.3. *Generation of recombinants AdTIMP-2 and AdCMV-GFP*

Transfection of recombinant adenoviral particles in HEK 293 cells was evaluated by tracing the expression of GFP proteins under fluorescence microscopy. As shown in Figure 5, on day 2 after transfection GFP can be seen in about 20% of HEK 293 cells. On day 10, HEK 293 cells were harvested by scraping cells off flasks and pelleted them along with any cells floating in the culture. Finally, the recombinant adenovirus was prepared and purified by CsCl density gradient

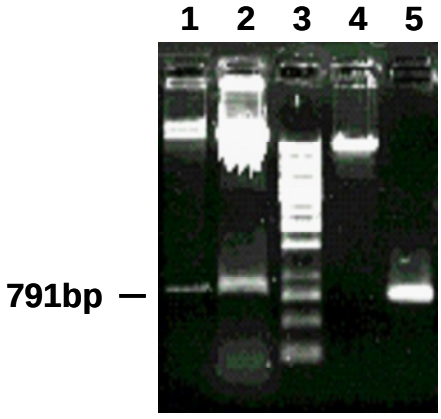

Figure 4. Identification of pAdTIMP-2. Lane 1, pAdTIMP-2 (linearized with *Eco*RI and *Xba* I); lane 2, pAdTrackCMV-TIMP-2 (linearized with *Sal* I and *Xba* I); lane 3, DNA ladder; lane 4, pAdTrack CMV (linearized with *Sal* I and *Xba* I); lane 5, target gene TIMP-2 DNA.

ultracentrifugation as described in Materials and Methods, and its titer was 4×10^{11} pfu/mL. The viral solution was diluted and stored in PBS containing 10% glycerol at −80°C. The control adenoviral vector AdCMV-GFP was constructed *via* a similar method.

3.4. *Identification of TIMP-2 in adenoviral particles by PCR*

After three cycles of freezing and thawing, 5 μL of viral lysate were used for detection of the TIMP-2 gene in adenoviral particles with PCR. At the same time, the shuttle vector AdTrackCMV-TIMP-2 was selected as a positive control. A fragment of 590 bp was obtained, indicating the correct generation of recombinant adenovirus (Figure 6).

4. Discussion

Gene therapy is the transfer of genetic material into somatic cells to effect changes in the pathogenetic processes that contribute to a disease (*9*). Vascular gene therapy has been at the forefront of this field since the first human attempt at gene transfer focused on patients with severe peripheral vascular disease (*10*). Currently, gene therapy techniques are mostly used to treat malignant tumors, and cases of cardiovascular diseases account for about 3 to 17% of all cases of gene therapy, including neointimal hyperplasia or restenosis in vein grafts, arteriosclerosis, peripheral ischaemic vascular disease, and coronary heart disease (*11*).

Gene therapy for vascular disease has particular advantages both in theory and in practice. For example, it can provide long-term expression of a desired protein in tissues and also allows the therapy to target a specific process involved in the disease of interest. In addition, ease of access to the vascular system is another advantage for this type of therapy (*11*).

Replication-defective recombinant adenovirus is an

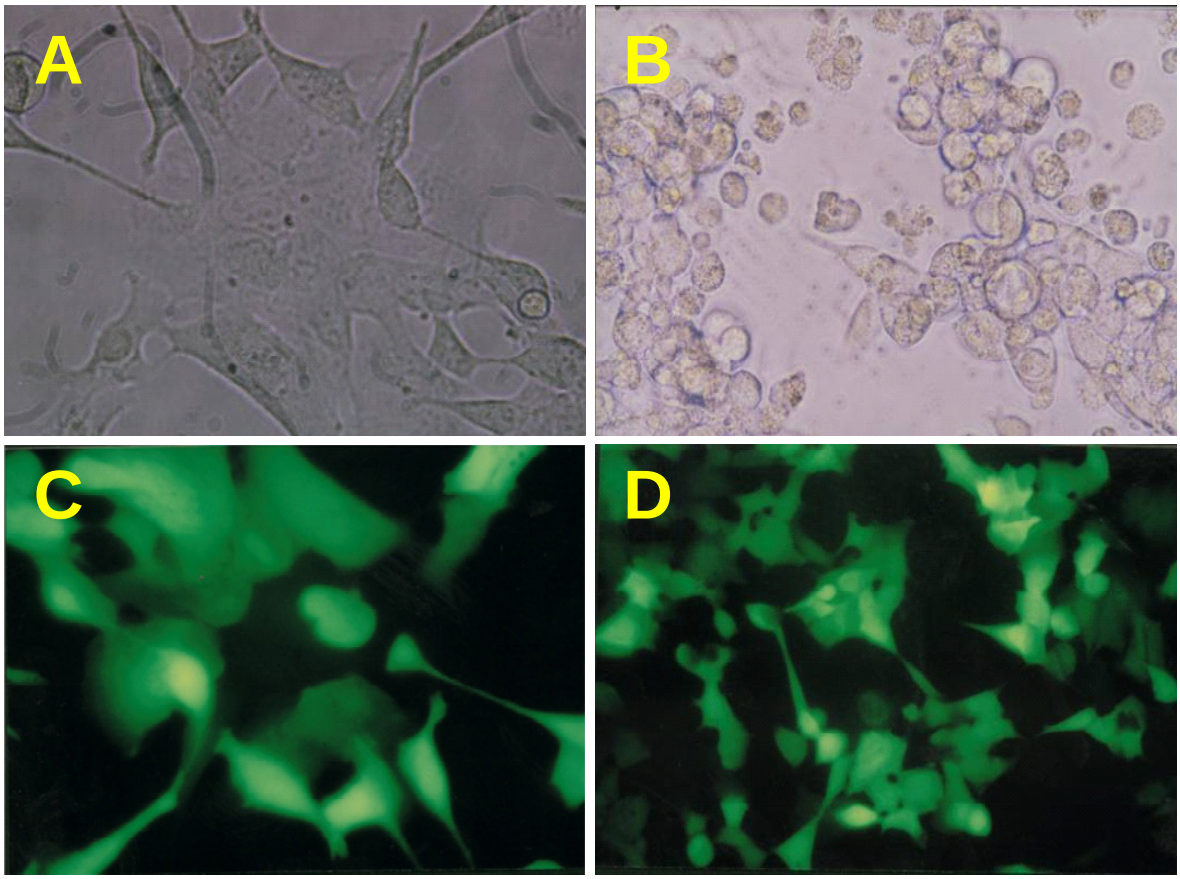

Figure 5. Packaging of recombinant adenovirus AdTIMP-2 in HEK 293 cells. A, normal HEK 293 cells, which were flat and spindly in shape; B, 10 days after transfection of pAdTIMP-2 linearized with *Pac* I into HEK 293 cells, typical comet-like adenovirus-producing foci were observed along with central lysis of cells; C, 2 days after transfection of pAdTIMP-2 linearized with *Pac* I into HEK 293 cells, fluorescence microscopy revealed GFP in about 10~20% of cells; D, 7 days after transfection, GFP was noted in about 50~60% of HEK 293 cells. Over time, the expression of GFP became more marked. A and B, phase contrast microscopy; C and D, fluorescent microscopy. Original magnification, A, B, and D, ×200; C, ×400.

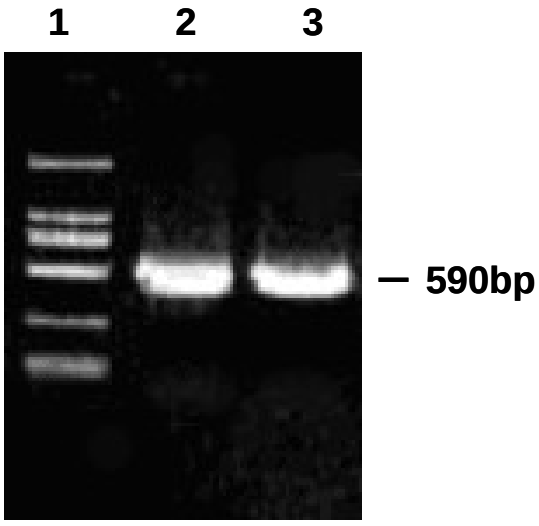

Figure 6. Identification of recombinant adenoviral particles using PCR. Lane 1, DNA ladder; lane 2, AdTIMP-2; lane 4, AdTrackCMV-TIMP-2; A fragment of 590 bp was observed in lanes 2 and 3, indicating the correct generation of recombinant adenovirus.

effective *in vivo* vector system for vascular cell types. It has the advantages of relative ease of penetration into non-dividing cells and allowing preparation of high titer viral stocks (*12*). The conventional method of generating recombinant adenovirus involves homologous recombination in mammalian cells that have the ability to complement defective adenoviruses. Screening individual plaques formed in HEK 293 cells allows the identification of desired recombinants. However, the conventional method has drawbacks in terms of low efficiency of homologous recombination and the time needed for the completion of virus production. Repeated rounds of plaque purification are also needed. In contrast, the AdEasy system of homogenous recombination in bacteria as used in the current study has the advantages of speed and ease. The ability to recover reasonable quantities of homogeneous viruses, without plaque purification, represents another major practical advantage. Furthermore, a GFP tracer is incorporated into the adenoviral backbone, allowing direct observation of all stages of virus production.

Increased proteolysis by MMPs has been reported to be associated with cancer cell invasion, rheumatoid arthritis, and more recently with the neointima formation that characterizes vascular disease (*13,14*). In a previous study of adenovirus-mediated TIMP gene transfer in a cultured human saphenous vein model, TIMP-2 was shown to inhibit neointimal thickening

primarily by inhibiting MMP activity and hence smooth muscle cell migration (*15*). Recently, Xiong *et al*. also found that TIMP-2 promotes aortic enlargement *in vivo*, presumably through its role as a cofactor in the activation of MMP-2 (*16*). The current study successfully constructed a recombinant adenovirus vector carrying the TIMP-2 gene and obtained high titers of virus solution. The study confirmed that the recombinant AdTIMP-2 can be used for further transfection for other cell types.

Replication-defective adenoviruses containing TIMP-2 can now be constructed more easily by homogenous recombination in bacteria than with conventional techniques. The current study has laid the groundwork for further study of gene therapy for vascular disease.

Acknowledgement

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