Original Research

Construction of a recombinant vector based on AAV carrying human endothelial nitric-oxide synthase gene[1]

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KEY WORDS nitric oxide; nitric-oxide synthase; plasmids; gene therapy; adeno-associated virus

ABSTRACT

AIM: To construct an AAV based vector carrying human endothelial nitric-oxide synthase (eNOS) cDNA and study its expression *in vitro* for future gene therapy. **METHODS:** eNOS cDNA was inserted into the *Eco*R I site of pSNAV-1 containing the cytomegalovirus (CMV) promoter and inverted terminal repeat sequences of adeno-associated virus. The constructed vector was transfected into BHK and C2C12 cells. eNOS cDNA and mRNA were detected by polymerase chain reaction (PCR) and reverse transcription-PCR (RT-PCR), respectively. **RESULTS:** By restriction enzyme digestion analysis, it was proved that eNOS cDNA was inserted into pSNAV-1 in a proper direction. PCR detection demonstrated that pSNAV-eNOS was transferred into both BHK and C2C12 cells. RT-PCR analysis showed that these pSNAV-eNOS transfected cells could express eNOS mRNA. **CONCLUSION:** pSNAV-eNOS was successfully constructed with the ability to express human eNOS mRNA in cultured mammalian cells.

INTRODUCTION

Nitric oxide (NO) produced in the endothelial cells exerts critical and diverse functions, such as regulating blood pressure and regional blood flow through vasodilatation, inhibiting platelet aggregation, leukocyte adhesion, and smooth muscle cell proliferation, and promoting endothelial survival and proliferation[1]. Endothelial dysfunction is a common denominator for a variety of cardiovascular diseases, including atherosclerosis, hypertension, stroke, postangioplasty restenosis, transplant vasculopathy, diabetic vasculopathy, and coronary heart diseases[1-3]. It has recently been appreciated that altered function of endothelial nitric oxide synthase (eNOS) and/or decreased availability of NO can account for a broad array of clinical manifestations in patients with endothelial dysfunction[1-3]. Thus, eNOS is an attractive target for treatment of cardiovascular diseases, and endogenous NO production through eNOS gene transfer can be a novel strategy to restore the impaired NO system.

In the present study, we constructed a new recombinant plasmid of pSNAV-eNOS and studied its expression in two kinds of cultured mammalian cells. This is to make arrangements for future eNOS gene therapy in animal models. Because the constructed pSNAV-eNOS contains inverted terminal repeat (ITR)

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of adeno-associated virus (AAV), it is also easy to develop a recombinant AAV vector for eNOS gene transfer as a perfect gene transfer vector[4,5].

MATERIALS AND METHODS

Construction of pSNAV-eNOS pUC13-eNOS was kindly provided by Dr Kenneth D BLOCH[6]. With *Eco*R I digestion, the cDNA encoding human eNOS was excised as a 4.0 kb fragment. The eNOS cDNA was then re-cloned into the *Eco*R I site of pSNAV-1[7], between the strong enhancer/promoter of the cytomegalovirus (CMV) immediate early genes and the simian virus (SV) 40 polyadenylation signals (polyA). The plasmid also contains two packaging signal ITR of AAV and a neomycin resistant gene (NEO). To test the correct cloning, pSNAV-eNOS was identified with *Eco*R I and *Xho* I restricton enzyme digestion. The plasmid map of the constructed pSNAV-eNOS was illustrated in Fig 1.

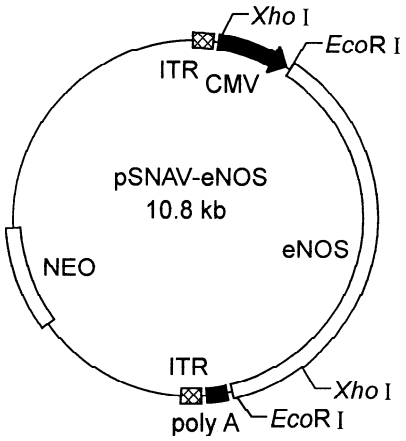

Fig 1. The plasmid map of the constructed pSNAV-eNOS. ITR: the inverted terminal repeat of the adeno-associated virus; CMV: the enhancer/promoter of the cytomegalovirus immediate early genes; eNOS: human endothelial nitric-oxide synthase cDNA; poly A: the simian virus 40 polyadenylation signals; NEO: the neomycin resistant gene.

Amplification and purification of plasmid DNA The plasmid DNA used for transfection of cultured cells was purified with a plasmid purification kit (Qiagen) according to the instruction. In brief, 100 mL cultured bacterial cells were harvested. After the procedure of resuspension, lysis, and neutralization, the supernatant containing plasmid DNA was applied to the Qiagen-tip. The DNA was redissolved in a suitable volume of Tris edetic acid (TE) buffer. DNA concentration was determined by UV spectrophotometry.

Cell culture and transfection The baby hamster kidney BHK cells and murine C2C12 myoblasts were respectively maintained in 1640 and DMEM medium supplemented with 10 % fetal calf serum, benzylpenicillin 100 kU/L, and streptomycin 0.1 g/L, at 37 °C in a humidified atmosphere containing 5 % CO_2. The plasmid DNA (pSNAV-eNOS and pSNAV-1) was transfected into BHK and C2C12 cells by lipofectamine™ (Bethesda Research Labs). Twenty-four hours posttransfection, the transfected BHK and C2C12 cells were selected in the antibiotic G418 (1 g/L) for 2 weeks. The resistant clones containing eNOS cDNA and mRNA were identified by polymerase chain reaction (PCR) and reverse transcription-polymerase chain reaction (RT-PCR), respectively.

Identification of eNOS cDNA in transfected cells Genomic DNA was prepared from BHK and C2C12 cells and used for PCR with primers specific for eNOS (5'-TCA GGC CCG GGA CTT CAT CAA CC-3' positioned in exon 3 of eNOS genomic sequence and 5'-GGA AGG CCG GGG ACA GGA AAT AGT-3' positioned in exon 10). The reaction mixture for PCR amplification was subjected to 35 cycles of denaturation (95 °C, 45 s), annealing (61 °C, 45 s), and extension (72 °C, 90 s). The PCR products were 1062 bp in length and identified by agarose-gel electrophoresis.

Identification of eNOS mRNA in transfected cells Total RNA were isolated from BHK and C2C12 cells using the Fast-Track kit (Invitrogen, San Diego, CA). The transcript was first subjected to reverse transcriptation using an oligo (dT) primer and reverse transcriptase (Life Technologies) to generate first-strand cDNA. The resulting pool was amplified by PCR using the primers specific for eNOS and β-actin. For eNOS, the PCR primers, conditions, and products were the same as described above. For actin, the PCR primers were 5'-CCTTCCTGTGCATGGAGTCCT-3' and 5'-GGAGCAATGATCTTGATCTTC-3', and the PCR conditions involved denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s for 30 cycles, and the PCR products were 202 bp in length. Here, β-actin was used as an internal control.

RESULTS

Identification of the constructed pSNAV-eNOS The cDNA encoding human eNOS was excised as a 4.0 kb *Eco*R I fragment from the plasmid pUC13-eNOS (Fig 2). Only one fragment of 6.8 kb was obtained

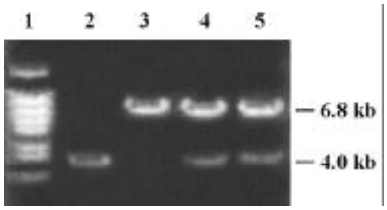

Fig 2. Identification of the constructed pSNAV-eNOS. Lane 1: λ/*Bst* II DNA marker; Lane 2: pUC13-eNOS digested by *Eco*R I; Lane 3: pSNAV-1 digested by *Eco*R I; Lane 4: pSNAV-eNOS digested by *Eco*R I; Lane 5: pSNAV-eNOS digested by *Xho* I.

from pSNAV-1 with *Eco*R I digestion. The successfully constructed plasmid pSNAV-eNOS could be cut into two *Eco*R I fragments of 6.8 kb and 4.0 kb, and the former was stronger than the latter on agarose-gel electrophoresis, indicating that the inserted fragment was monocopy rather than multicopys. After digestion with *Xho* I, the recombinant plasmid in which eNOS was inserted with the same direction as CMV promoter was cut into two fragments of 6.9 kb and 3.9 kb, and the former was stronger than the latter on agarose-gel electrophoresis.

Detection of eNOS cDNA in transfected cells

Total DNA in BHK and C2C12 cells was extracted to detect by PCR if pSNAV-eNOS DNA was transfected into the cultured cells. The PCR products of 1062 bp fragment for eNOS were amplified from BHK and C2C12 cells transfected with pSNAV-eNOS but not from control cells transfected with pSNAV-1 (Fig 3).

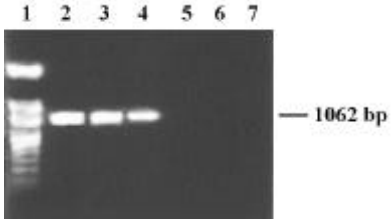

Fig 3. PCR detection of eNOS cDNA in transfected cells. Lane 1: Go3S 100 bp plus DNA marker; Lane 2: positive control; Lane 3: BHK cells transfected with pSNAV-eNOS; Lane 4: C2C12 cells transfected with pSNAV-eNOS; Lane 5: BHK cells transfected with pSNAV-1; Lane 6: C2C12 cells transfected with pSNAV-1; Lane 7: negative control.

eNOS mRNA expression in transfected cells

Total RNA extracted from BHK and C2C12 cells was reversely transcribed to check if eNOS gene transfected cells could express eNOS mRNA. In RT-PCR detection with β-actin primers, 202 bp fragment was found in both pSNAV-eNOS and pSNAV-1 transfected cells. However, in RT-PCR detection with eNOS primers, 1062 bp fragment was found only in pSNAV-eNOS transfected cells, not in pSNAV-1 transfected cells (Fig 4).

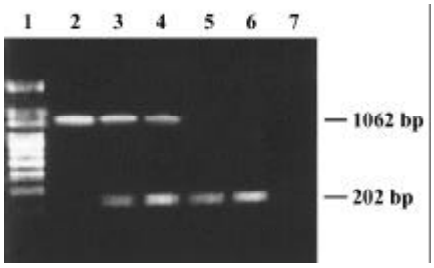

Fig 4. RT-PCR detection of eNOS and β-actin mRNA in transfected cells. Lane 1: Go3S 100 bp plus DNA marker; Lane 2: positive control (β-actin RT-PCR detection had no positive control); Lane 3: BHK cells transfected with pSNAV-eNOS; Lane 4: C2C12 cells transfected with pSNAV-eNOS; Lane 5: BHK cells transfected with pSNAV-1; Lane 6: C2C12 cells transfected with pSNAV-1; Lane 7: negative control.

DICUSSION

The present study developed a new plasmid vector containing human eNOS cDNA, ie, pSNAV-eNOS, and investigated its expression in two kinds of mammalian cells *in vitro*. The inserted human eNOS cDNA was proved to be monocopy and in the same orientation as the CMV promoter using *Eco*R I and *Xho* I restriction enzyme digestion. In transfected BHK and C2C12 cells, eNOS DNA and RNA were identified by PCR and RT-PCR, respectively. eNOS fragments were obtained in both kinds of cultured cells transfected with pSNAV-eNOS, but not with pSNAV-1. These results indicated that human eNOS mRNA was successfully transcribed from pSNAV-eNOS in cultured cells.

A variety of gene transfer vectors, such as naked plasmid, liposome, and virus, have been experimentally applied in eNOS gene transfer to cardiovascular system[8-10]. The advantages and disadvantages have been reported for these vectors. AAV was discovered to be a promising vector, because it can efficiently transduce wide spectrum of cells including vascular cells, renal cells, cardiac myocytes, and others, integrate into genomes of mammalian cells with long-term transgene expression, and induce very low immunogenicity[4,5]. The only *cis*-elements required for AAV genome replication, encapsidation, and integration are the two sequences of ITR at each end of the genome. The

optimal DNA fragment for AAV packaging is between 4.1 kb and 4.8 kb, and AAV can also package a vector larger than its genome size, up to 5.2 kb[4]. Our constructed pSNAV-eNOS plasmid contained a packaging fragment (CMV promoter-eNOS-SV40 poly A) between two ITR that is about 4.8 kb and appropriate for AAV packaging. Therefore, pSNAV-eNOS can be used as the eNOS AAV transfer plasmid for further preparation of recombinant AAV vector carrying human eNOS cDNA. This is also a good reason why we chose pSNAV-1 to construct pSNAV-eNOS.

eNOS gene transfer to cardiovascular system is not only a powerful research tool, but also a potential therapy for certain cardiovascular diseases[2,11]. As a practical tool, eNOS gene transfer can be used to elucidate the mechanisms involved in the physiological and pathological conditions. For example, endothelial dysfunction produced by elevated oxidant stress can be effectively ameliorated by more NO generation through eNOS gene transfer[10,12], clearly indicating that the balance between NO and O_2^- plays an important role in the maintenance of vascular function and structure. As a potential therapy, the treatment effects of eNOS gene transfer have begun to be explored in animal models of cardiovascular diseases, such as hypertension, cerebral vasospasm, atherosclerosis, and postoperative complications of restenosis and graft vasculopathy[2], in which endogenous NO production, as a result of eNOS gene transfer, may provide a cardiovascular protection. We will use the constructed pSNAV-eNOS for further *in vivo* studies on the gene therapy of cardiovascular diseases and for future development of the perfect eNOS gene transfer vector.

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