J. Vet. Sci. (2002), 3(1), 41-45

JOURNAL OF
Veterinary
Science

Expression of Angiostatin Using DNA-Based Semliki Forest Virus Replicon

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ABSTRACT

Angiogenesis is recognized as a critical factor in the growth of tumor cells and plays a key role in the tumor metastasis. Recent studies for antiangiogenic substances are getting popular. The angiostatin, one of the antiangiogenic substances, leads to the increased apoptosis of the tumor cells by inhibiting the neovascularization of the tumor. The angiostatin was identified as the internal fragments of the plasminogen which has no antiangiogenic activity. By hydrolysis of the plasminogen, the angiostatin can be produced. In this study, we constructed the SFV-derived DNA vector by employing the cytomegalovirus immediate early enhancer/ promoter (CMV). This vector makes it possible to transfect the cells with DNA without the *in vitro* transcription process. The C-*myc* epitope and polyhistidine residue sequences were placed in downstream of the angiostatin gene to make it eligible to detect the expressed protein. The murine Ig κ-chain V-J2-C signal sequence was placed in upstream to secrete the expressed protein from the cells. We confirmed the expression of angiostatin in the BHK-21 cells using DNA-based SFV replicon.

INTRODUCTION

Angiogenesis, the formation of new vessels from the preexisting microcapillaries, is recognized increasingly as a critical factor in a broad spectrum of diseases. The potential therapeutic benefits for the treatment of tumors, with antiangiogenic substances therefore are very high (5). Angiostatin was initially isolated from mice bearing a Lewis lung carcinoma, and was identified as a 38 kDa internal fragment of plasminogen that encompasses the first four kringles of the molecule (3, 9, 7). The kringles are the conserved domains in a number of plasma coagulation-related proteins. A kringles is approximately 80 amino acid residues in a double loop conformation held together by three disulfide bonds (18). The kringle domains were named because of the appearance being reminiscent of the Danish pastry of the same name. Angiostatin has been shown to efficiently inhibit the growth of a broad spectrum of murine and human tumor models in mice (11, 13). By inhibiting the neovascularization of the tumor, the angiostatin treatment leads to the increased apoptosis of the tumor cells (2, 15, 21).

Prokaryotic expression systems have been well established to produce large amounts of angiostatin, but these proteins are not post-translationally modified and may not be folded correctly. Furthermore, the insolubility of prokaryotic recombinant proteins often decreases the yield of the soluble and active proteins (22). It is of a great importance to establish a eukaryotic expression system. To overcome these limitations, we produced the recombinant angiostatin using pCI-neo (Promega) and Semliki Forest virus (SFV) expression vector.

SFV, a member of the *Alphavirus* genus of the family Togaviridae, is an enveloped virus with a single-stranded RNA genome of positive polarity (20). Many properties of alphavirus vectors make them a desirable alternative to other virus-derived vector systems being developed, including the potential of a high-level expression of up to 10^8 molecules of the heterologous protein per cell, a broad host range, and the ability to infect the nondividing cells (4, 14, 16, 19). In addition, replication occurs entirely in the cytoplasm of the infected cells as an RNA molecule without the DNA intermediate.

Upon the infection, the RNA genome functions as mRNA for the translation of nonstructural proteins. This subsequently replicates the virus by copying the plus-strand RNA genome into minus-strand RNA and *vice versa*. The minus-strand RNA also serves as a template for the synthesis of a short subgenomic RNA which encodes the structural proteins (1, 17). Transcription starting at the internal subgenomic promoter in the minus-strand results in the production of large amounts of subgenomic mRNA (8, 10, 12). SFV-derived vectors are based on the insertion of a genomic SFV cDNA into an SP6 promoter plasmid, and subsequent modification by deletion of the SFV structural genes to allow for the insertion of heterologous DNA as part of the SFV replicon. Since the *in vitro* transcripts from such constructs also encode the SFV replicase, high levels of expression of the heterologous gene can be achieved by

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This work was supported by National Research Laboratory (NRL) Program Grant (2000-N-NL-01-C-171) from the Ministry of Science and Technology, Korea.

directly transfecting the recombinant RNA into cells (6, 12).

Although this system can be used, the preparation of capped RNA vectors by the *in vitro* transcription is necessary before the transfection and the RNA molecules are unstable in general. Therefore, it is of great use to construct a DNA vector based on self-amplifying system of SFV. In this study, we investigated the possibility of using DNA-based plasmid expression vector to directly initiate the alphavirus RNA replication cascade in the transfected mammalian cells.

MATERIALS AND METHODS

Construction of plasmids

Polymerase chain reaction (PCR) with the cytomegalovirus (CMV) immediate-early (IE) enhancer/promoter sequence was performed using the pCI-neo (promega) as a template with the sense primer (5'-ACATGCATGCGTCCGTTACATA ACTTAC-3') and the antisense primer (5'-ACATGCATG CGTCCGGAGGCTGGATCGG-3'). The amplified CMV-IE was digested with and inserted into the pSFV-1 digested with *Sph* I, generating pcSFV. PCR amplification of human angiostatin gene was performed using the pSecTaq2A/Agt kindly provided by Dr. S. H. Lee (National Cancer Center) as a template with the sense primer (5'-CCCAGATCTATGG AGACAGACACACTC-3') and the antisense primer (5'-CCC AGATCTTAGAAGGCACAGTCGAGGC-3'). The amplified 1.4 kb, angiostatin gene containing the murine Ig κ-chain V-J2-C signal sequence in upstream of angiostatin and C-*myc* epitope and polyhistidine residue sequences in downstream, was inserted into the pGEM-T (Promega), generating pGEM/Agt. The angiostain gene from pGEM/Agt digested with *Bgl* II, was ligated to pcSFV vector resulting in pcSFV/Agt. PCR amplification of human angiostatin gene was performed using the pcSFV/Agt as the template with the same primers. Amplified DNA fragments were filled in by using Klenow fragment (TaKaRa). pCI-neo (Promega) was digested with *Sma* I and dephosphorylated. The angiostatin gene treated with Klenow fragment was introduced into *Sma* I site of the dephosphorylated pCI-neo.

Cell culture

BHK-21 (Baby Hamster Kidney-21) cell was grown in MEM (Minimum Essential Media, GIBCO BRL), supplemented with 5 % fetal bovine serum (GIBCO BRL), 20 mM HEPES (USB), 2 mM L-gluatmine (Sigma), and 0.1 U/ml penicillin and 0.1 μg/ml streptomycin (Sigma). CHO-K1 (Chinese Hamster Ovary-K1) cell was grown in Ham's F-12 medium (GIBCO BRL), supplemented with 10 % fetal bovine serum. Cells were washed in PBS (phosphate buffered saline, Sigma), trypsinized with 1x trypsin-EDTA (GIBCO BRL) and subcultured in 1 : 3. Cells were incubated at 37℃ in a humidified atmosphere of 5 % CO_2.

RNA and DNA Transfection into Mammalian Cells

To transfect with RNA, the recombinant pSFV plasmid DNA was digested with *Spe* I restriction enzyme (TakaRa) to linearize the plasmid. This linearized plasmid were used as templates for *in vitro* transcription. Briefly, 50 $\mu\ell$ transcription reactions contained 40 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 5 mM DTT (TaKaRa), 1 mM each of ATP, CTP and UTP, 0.5 mM GTP (Roche), 1 mM CAP analogue M7G(5')ppp(5')G (Roche), 50 units SP6 RNA polymerase (TaKaRa). This mixture was incubated at 37℃ for 1.5 hr. The BHK-21 cells were washed twice and resuspended in PBS at 10^7cells/ml. The resuspended cells were mixed with the RNA transcripts. The mixture was electroporated with two consecutive pulses at 0.83 kv and 25 μF (Bio-Rad Gene pulser) and transferred to 100 mm tissue culture dishes (Nunc). To transfect with DNA, cells at the concentration of 1×10^5 cells/well were plated in the 4-well plates. One μg of plasmid DNA was added to the predilluted mixtures of FuGENE 6 (Roche). After the mixtures of FuGENE 6 reagent and plasmid DNA were incubated for 15 min, the mixtures were added to the wells.

Immunocytochemistry

The cells were washed twice in PBS and fixed on the slide glass by ice-cold methanol at 4℃ for 15 min. After washing the fixed cells twice in PBS, the blocking solution (1 % gelatin in PBS) was added and incubated at room temperature for 1 hr. The blocking solution was removed and the cells were reacted with the primary antibody for 3 hr at room temperature. The cells were washed in PBS three times and the biotinylated secondary antibody (Vector) was added and incubated further 1 hr at room temperature. The cells were washed in PBS and reacted with HRP-avidin-biotin reaction solution (Vector) for 30 min. The cells were finally washed in PBS and visualized by adding DAB (3,3'-diaminobenzidine, Vector) solution.

SDS-PAGE and Western Blot Analysis

The cell pellets were lyzed with 1 % NP40 (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 2 mM EDTA and 1 $\mu\ell$/mℓ PMSF) for 30 min on ice and centrifuged at 12,000 x g for 10 min. Sodium dodecylsulfate-polyacrylamide gel electrophoresis was performed following the method of Laemmli.

RESULTS

In vitro RNA transcription

To transfect the RNA to the BHK-21 cells, RNA was synthesized *in vitro* as described in materials and methods. The RNA production monitoring was carried out by the electrophoresis of 2 $\mu\ell$ aliquot in 1 % agarose gel (Fig. 1). As shown in the lane 2 of Fig. 1, a clear band was observed.

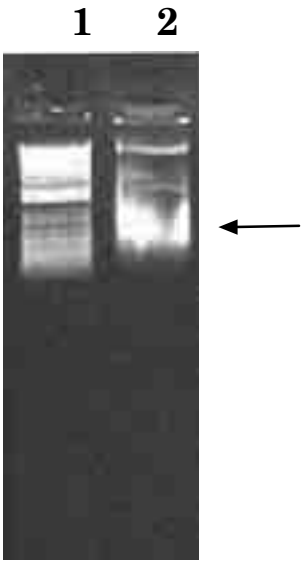

Fig. 1. *In vitro* RNA transcripts from the linearized DNA (lane 1: 1 kb DNA ladder, lane 2: pcSFV/Agt). The arrow indicates the RNA transcripts.

Expression of Angiostatin Gene in Mammalian Cells

We investigated whether the angiostatin could be produced in the cytoplasm of the BHK-21 cells using an SFV-based expression system. For the control, the pCI-neo expression system was employed.. Immunocytochemistry was performed as described in materials and methods. The angiostatin gene contained the C-*myc* epitope and polyhistidine residues in downstream of it. The expression of the angiostatin gene was confirmed with the mouse anti-His and the anti-C-*myc* monoclonal antibody. The dark brown staining in the cytoplasm and nucleus of the transfected cells indicates the expression of the angiostatin protein (Fig. 2 and 3).

Expression of angiostatin was detected in most cells transfected with RNA from pcSFV/Agt(Fig. 2, B). When the plasmid DNA was directly transfected as described in materials and methods, the expression levels of angiostatin were slightly decreased (Fig. 2, D). These results indicated that angiostatin was successfully expressed by RNA and DNA based SFV replicon.

On the other hand, the BHK-21 cells transfected with pCI-neo/Agt showed a significantly decreased expression level of angiostatin (Fig. 3, B). It can be inferred that SFV expression system is more efficient than pCI-neo expression system. The expression level of the CHO-K1 cells transfected with pCI-neo/Agt was similar to that of the BHK-21 cells (Fig. 3, D). At 48 hr post-transfection, the expression of angiostatin were analyzed by the immunoblotting. As shown in Fig. 4, the expression of angiostatin was confirmed.

DISCUSSION

In this study, we constructed DNA- and RNA-based Semliki Forest virus replicons by inserting the cytomegalovirus immediate early enhancer/promoter (CMV) in upstream of the SP6 promoter in the SFV vector. It is desirable to apply the DNA vector based on the self-amplifying system of SFV, because RNA molecules are unstable in general. The current drawbacks of the DNA-based expression system is the poor transfection efficiency, and the low expression level. One approach to overcome these disadvantages may be using the vectors such as the SFV-derived DNA vector described here which expresses the foreign gene efficiently.

A major advantage of the SFV-derived plasmid DNA vector is a high-level expression of exogenous gene using the self-amplifying systems of SFV. In addition, this vector is

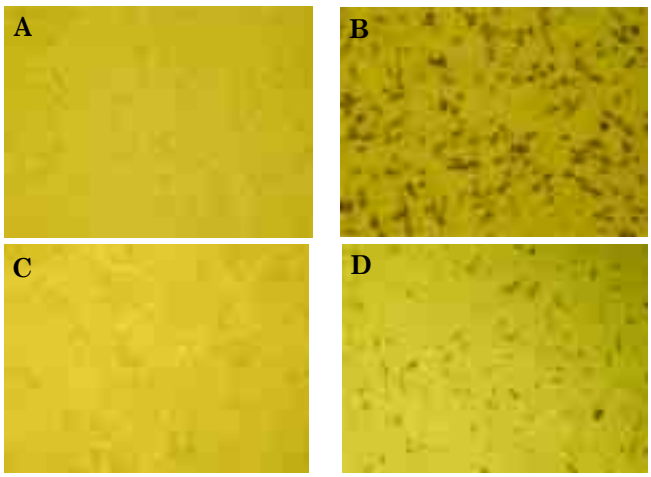

Fig. 2. Immunocytochemistry of angiostatin expressed in BHK-21 cells. Cells were transfected with control (A), pcSFVAgt *in vitro* transcribed RNA (B), control (C), pcSFV/Agt plasmid DNA (D).

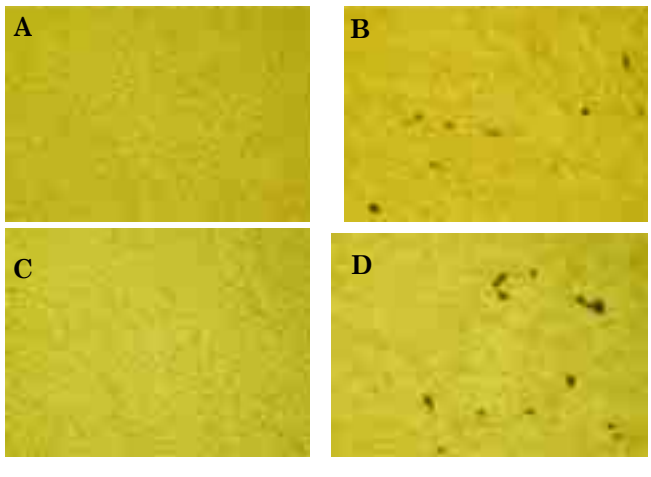

Fig. 3. Immunocytochemistry of angiostatin expressed in BHK-21 cells (A and B), and CHO-K1 (C and D). Cells were transfected with pCI-neo/Agt plasmid DNA (A and C: control, B and D: cells transfected with pCI-neo/Agt).

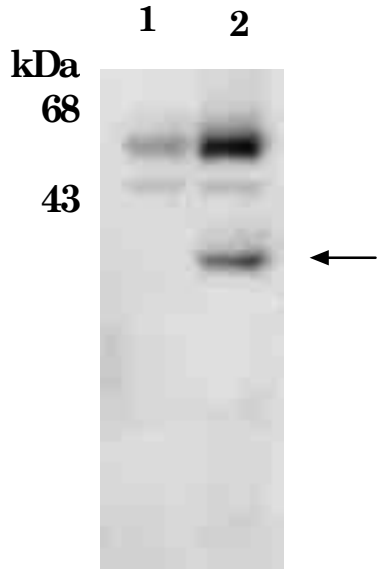

Fig. 4. Western-blot analysis of angiostatin in BHK-21 cell lysate. Cells were transfected with control (lane 1), or pcSFV/Agt (lane 2) and detected with monoclonal anti-histidine antibody. The arrow indicates the expressed angiostatin.

transfected into cells as double-stranded DNA. There is no need to perform the *in vitro* transcription and mRNA capping that are required for the transfection of the RNA-based SFV vectors. The conversion of alphavirus-derived replicon into a plasmid DNA-based expression system is the primary requisite step toward the development of the alphavirus-based gene transfer systems which parallel the classic retrovirus-based producer cell configurations (9).

The angiostatin has been shown to be a physiopathological inhibitor of the angiogenesis, driving the metastasis into a dormant state. Though the basic scientific backgrounds of the action mechanism of the angiostatin is very attractive for the further researches, the ultimate importance in this field is the potential to use this understanding for the treatment of cancer and other angiogenesis-related diseases. The most direct approach is the large-scale preparation of this recombinant angiostatin protein.

Several approaches are under development to apply this angiostatin to human use. A prolonged administration of the purified angiostatin at the high dosage was indeed required to maintain the cytostatic intra-tumoral concentrations of angiostatin. Accordingly it is of a great importance to produce the angiostatin efficiently in the eukaryotic expression system. In this study, we produced the recombinant angiostatin using pCI-neo (Promega) and SFV expression vector.

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