Growth inhibitory effect of triple anti-tumor gene transfer using Semliki Forest virus vector in glioblastoma cells

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Abstract. The gene delivery of multiple tumor suppressors can provide an efficient tumor therapy in the case of malignant human glioblastomas containing multiple genetic alteration and inactivation. As such, the current study presents a new delivery system that can simultaneously express three anti-tumor genes using a Semliki Forest virus (SFV) vector in the expectation of combined or synergistic effects of angiogenesis inhibition by angiostatin and apoptosis induction by p53, PTEN and the rSFV particle itself. Recombinant SFV (rSFV) containing three anti-tumor genes (rSFV-Agt/p53/PTEN) were found to efficiently transduce and express each anti-tumor gene in glioblastoma cells. In addition, rSFV-Agt/p53/PTEN also resulted in a more effective induction of apoptosis *in vitro* and inhibition of tumor growth in nude mice when compared with other rSFVs containing only one or two anti-tumor genes. Accordingly, the current results demonstrate that a triple anti-tumor gene transfer using an rSFV vector would be a powerful strategy for regional cancer gene therapy.

Introduction

Malignant glioblastoma is the most common primary tumor occurring in the central nerve system and is characterized by rapid cell proliferation, high invasiveness, genetic alterations and increased vascularity (1-3). A number of new therapies are being developed in glioblastoma treatment, among which is the delivery of DNA encoding tumor suppressor genes to the tumor site by gene delivery systems.

Semliki Forest virus (SFV)-based expression as a useful vector for gene therapy is an ideal delivery system because it is suicidal, has a broad host range, replicate in the cytoplasm of host cells, has an inserted gene capacity of at least 5 kb, and can express high levels of recombinant proteins (4,5). This system has already been shown to be effective in recombinant vaccine studies, suggesting that it could be used for *in vivo* transfer of the prospective genes for inducing anti-tumor response.

The tumor suppressor gene transferred to a variety of tumor tissues was *p53*, which plays a central role in regulating cell division and apoptosis (6,7). As such, the introduction of the *p53* gene into a *p53*-deficient tumor or even *p53*-positive tumor cells inhibited the tumor growth (8,9). Another tumor suppressor gene, *PTEN*, mapped to 10q23, has also been observed in a mutated form in primary glioblastoma and other tumor cells (10). Various functional studies of PTEN, including experiments using animals as a model system, have demonstrated that it plays a significant role in inducing G1 cell-cycle arrest and apoptosis, along with regulating cell adhesion, migration, and differentiation (11,12). The introduction of wild-type *PTEN* into glioblastoma cells containing endogenous mutant alleles has been found to cause growth suppression (13,14).

Angiogenesis is also known to be an essential requirement for progressive growth of brain tumors. Therefore, the inhibition of angiogenesis may represent a potentially powerful approach to tumor therapy. Angiostatin, an internal peptide fragment of plasminogen, has been shown to potently inhibit the migration and proliferation of endothelial cells *in vitro* and the growth of tumor cells by inducing apoptosis *in vivo* (15-17). The results of anti-angiogenic gene therapy utilizing an angiostatin gene (*agt*) have demonstrated this to be an effective anti-tumor method to suppress tumor-induced angiogenesis and tumor growth (18-20). As mentioned above, the gene transfer of *p53, PTEN*, or *agt* can be effective in suppressing tumor growth (21). For synergistic anti-tumor effects, the cotransfection of more than one vector containing different anti-tumor genes into tumor cells has also been attempted (22,23). However, the maximal synergy of any anti-tumor effect can not be expected because two or three separate viruses can not be coinfected into identical cells with a high efficiency following *in vivo* administration.

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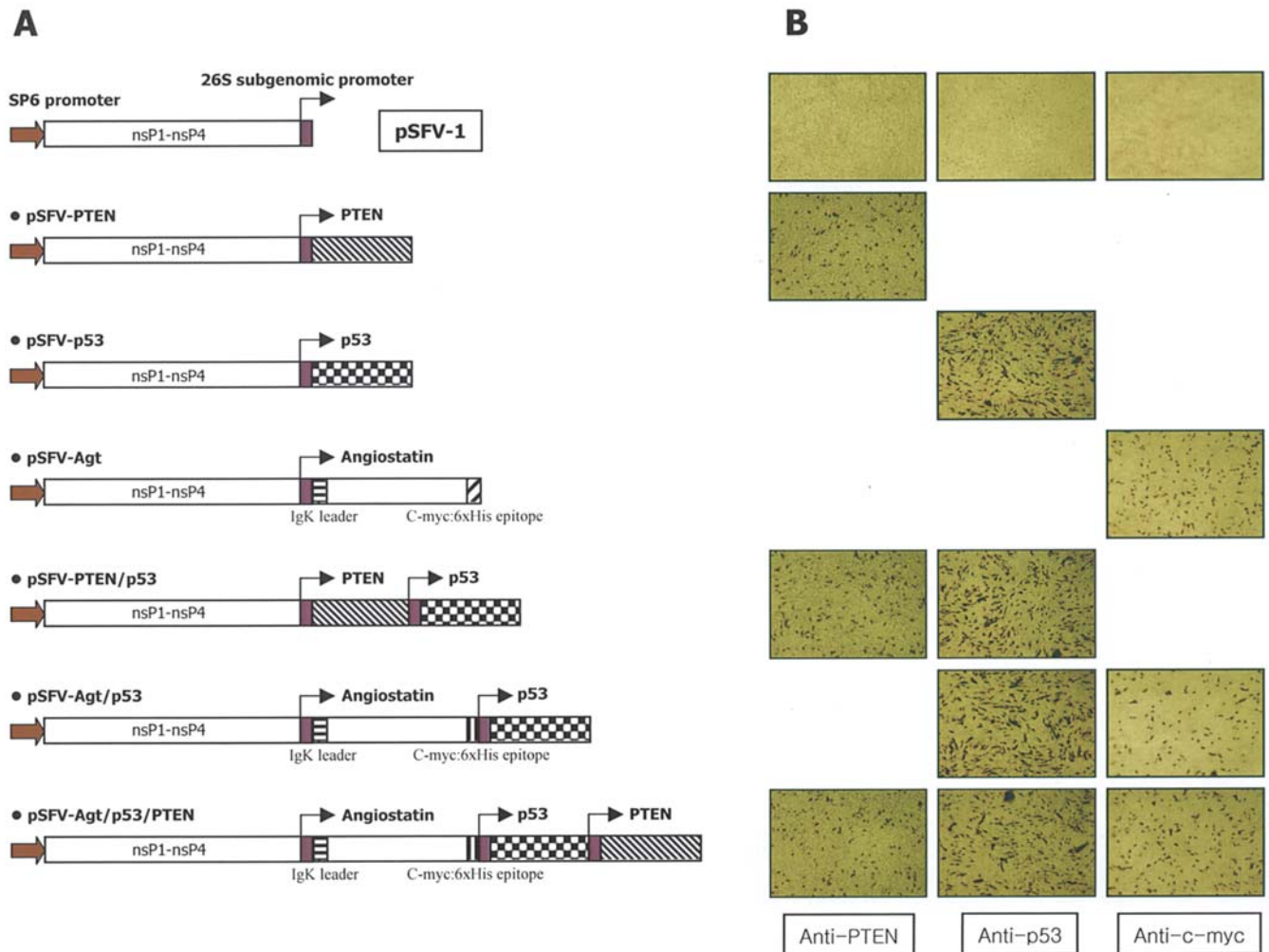E-mail: cjkim@cnu.ac.kr

Figure 1. Schematic diagram of each plasmid (A) and expression of anti-tumor proteins, as shown by immunocytochemistry in BHK-21 cells with anti-PTEN antibody (B, first row), anti-p53 antibody (B, second row) and anti-c-myc antibody (B, third row). The cells were transfected with *in vitro* transcribed RNAs from the plasmids of pSFV1, pSFV-PTEN, pSFV-p53, pSFV-Agt, pSFV-PTEN/p53, pSFV-Agt/p53, and pSFV-Agt/p53/PTEN, respectively.

In this study, we have used SFV vectors carrying a single anti-tumor gene (pSFV-p53, pSFV-PTEN, and pSFV-Agt) or two or three genes (pSFV-PTEN/p53, pSFV-Agt/p53, and pSFV-Agt/p53/PTEN), all under the control of each subgenomic 26S promoter, and we demonstrate the local overexpression of anti-tumor genes in malignant glioblastoma. The rSFV expressing three anti-tumor genes, *p53, PTEN*, and *agt*, simultaneously was found to be more efficient in inducing the apoptosis-mediated regression of brain tumors than any of the other rSFVs expressing one or two genes. Consequently, these results indicate that an SFV-based vector offers a very effective system for delivering and expressing functional protein genes concurrently in *in vivo* gene therapy.

Materials and methods

Cell culture. The human glioblastoma cell lines, U87MG and U251MG, were prepared and maintained in Dulbecco's modified essential medium (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (Gibco BRL), 2 mM L-glutamine (Sigma, St. Louis, MO), penicillin (0.1 U/ml), and streptomycin (0.1 μl/ml). Baby hamster kidney cells (BHK-21) were obtained from the Korean Type Culture

Collection (KCTC, Daejon, Korea) and maintained in Dulbecco's minimal Eagle's medium (DMEM) supplemented with 10% FBS (Gibco BRL).

Construction of recombinant plasmids and rSFVs carrying anti-tumor genes. Human *p53, PTEN*, and *angiostatin* cDNAs were obtained from the plasmids, pCEP4-p53, pSecTaq2A-Agt, and pcDNA3-PTEN, respectively. The SFV vectors, pSFV1 and pSFV-helper plasmids, were kindly provided by Dr Marc Girard at the Pasteur Institute, France. Each anti-tumor gene amplified by PCR was inserted independently into the *Bam*HI site of pSFV1 to generate pSFV-p53, pSFV-PTEN, and pSFV-Agt. The amplified 1.4-kb angiostatin gene product included a murine Igκ chain leader sequence upstream of the angiostatin and c-*myc* epitope and six tendem histidine residue sequences downstream of angiostatin. The expression cassette for p53, including a subgenomic 26S promoter, was amplified using the pSFV-p53 plasmid as the template. This fragment was then subsequently cloned into pSFV-PTEN and pSFV-Agt to generate pSFV-PTEN/p53 and pSFV-Agt/p53, respectively. The PCR fragment of PTEN, including the 26S promoter sequence, was amplified using pSFV-PTEN as the template and then cloned into pSFV-Agt/

p53 to generate pSFV-Agt/p53/PTEN (Fig. 1). To generate the rSFVs, *in vitro*-transcribed RNA from each recombinant plasmid (pSFV-lacZ, pSFV-p53, pSFV-PTEN, pSFV-Agt, pcSFV-Agt/p53, pSFV-PTEN/p53 and pSFV-Agt/p53/PTEN) along with RNA transcribed from the pSFV-helper were cotransfected into the BHK-21 cells by electroporation. The rSFVs were then harvested from the cell culture fluid after 48 h of incubation by ultracentrifugation at 100,000 x g for 3 h, resuspended in a TNE buffer (100 mM NaCl, 0.5 mM EDTA, 20 mM Tris-HCl, pH 7.4), and stored at -70˚C until use. The transfected cells were fixed using 4% paraformaldehyde and blocked for 30 min in 1% gelatin. The blocking solution was then removed and the cells reacted with a primary antibody for 3 h at room temperature. Next, the cells were rinsed in PBS and reacted with a biotinylated secondary antibody (Vector Laboratories) for 1 h at room temperature. After reacting with the avidin-biotin reaction solution (Vector Laboratories) for 30 min, a DAB solution containing 3,3'-diaminobenzidine tetrahydrochloride and 0.2% hydrogen peroxide was added. The expression of the anti-tumor genes was finally detected using inverted microscopy.

Infection of rSFVs and Western blot analysis. Each rSFV produced with the helper-packing system was activated using α-chymotrypsin (Boehringer Mannheim, Mannheim, Germany). The cells were infected with rSFVs at a multiplicity of infection (MOI) of 10. After 48 h of incubation, the cells were washed and scraped into PBS, pelleted, and then lysed with 200 μl of an RIPA buffer (10 mM Tris-HCl, pH 7.6, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40 and 1 μl/ml PMSF). To detect the secreted angiostatin, the culture supernatant was harvested and concentrated to 1/20 of the original volume. Thereafter, 30 μl of each lysate or cell supernatant was resolved using a 12% discontinuous SDS-PAGE and transferred onto PVDF membranes (Bio-Rad, Hercules, CA). The membranes were probed with an anti-p53, anti-PTEN, or anti-c-myc anti-body, and then the target proteins were detected by chemiluminescence (SuperSignal Substrate, Pierce, Rockford, IL).

Cell viability and tumorigenicity assay. Each line of glioblastoma cells was seeded at $1x10^4$ cells/well in 6-well plates for 20 h before viral infection. The cells were infected in triplicate with rSFVs produced from each plasmid containing the anti-tumor genes and the ß-galactosidase gene as the control at an MOI of 5. After 48 h of incubation, triplicate dishes of each treatment were counted according to the trypan blue exclusion method using a hemocytometer. For tumor formation in the nude mice assay, U251MG cells were plated at $1x10^6$ cells/flask. After overnight incubation, the cells were infected with rSFVs, as mentioned above, for 3 h. The infected or mock cells were trypsinized and resuspended in PBS, and then $5x10^6$ cells were injected into athymic nu/nu mice (KRIBB). Four weeks after injection, calipers were used to measure the tumor diameters in three dimensions and the tumor volumes were calculated.

Apoptotic cell detection assay. The apoptosis of the infected cells by the rSFVs was assessed using an apoptosis detection system (Promega, Madison, WI) which measured the fragmented DNA of the apoptotic cells using a TUNEL assay. The apoptotic index was analyzed based on the percentage of positive cells using flow cytometry (FACScalibur, Becton-Dickinson, Franklin Lakes, NJ).

Inhibition of tumor growth in vivo. To determine the inhibition of tumor growth *in vivo*, nude mice were injected subcutaneously with $1x10^6$ U251MG cells. After developing visible tumors (=14 days post-inoculation), the mice were injected twice at 3-day intervals with $1x10^9$ rSFVs in PBS intratumorally. The tumor volumes were measured using calipers and calculated prior to the virus injection and subsequently at 5-day intervals up to 40 days post-injection.

Results and Discussion

Construction of SFV vector carrying anti-tumor genes. The alphavirus vector based on SFV has been demonstrated as a favorably comparable gene transfer vector (24). Since this vector is a transient RNA-based expression system, this obviates certain nuclear complications, such as random RNA splicing or host chromosomal integration. The replicon contains plus and minus strand promoters, the genes for the replicase complex, and a cloning site for foreign gene insertion.

The replicon contains the packaging signal and is packaged into virions when helper functions are provided *in trans*. The defective helper construct only provides genes encoding structural proteins. When these two RNA molecules transcribed *in vitro* are introduced into host cells, both RNAs are replicated and recombinant SFV (rSFV) particles containing the replicon and a foreign gene are produced. rSFV particles are very stable and can be concentrated to titers exceeding 10^{10} infectious units per milliliter. Furthermore, they also have the ability to infect both dividing and non-dividing cells, adsorb rapidly to cells at the injection site, and are capable of one round of multiplication only. In the current study, SFV replicons were constructed carrying either the *p53, PTEN*, or *agt* gene under the control of the SFV 26S promoter, resulting in pSFV-p53, pSFV-PTEN, and pSFV-Agt, respectively. To achieve an efficient secretion of the expressed agt, an Igκ leader sequence was inserted in front of the *agt* gene.

In vitro expression of three anti-tumor genes. The tumor suppression activity of the three anti-tumor genes was demonstrated using other viral systems in glioblastoma cells. Previously, the combined gene transfer of two anti-tumor genes based on the co-injection of two separate viral vectors containing different anti-tumor genes was investigated to determine the synergistic effects. Although the co-injection of multiple viral vectors expressing different anti-tumor genes into a tumor was found to be more effective than the injection of a single viral vector including only one anti-tumor gene, it was suggested that treatment with a single vector coexpressing multiple anti-tumor genes might be even more effective. Accordingly, for the simultaneous expression of multiple anti-tumor genes using a single vector, the second and third expression cassettes each containing the 26S promoter and functional gene at the end of the first gene were connected, resulting in pSFV-PTEN/p53, pSFV-Agt/p53, and pSFV-Agt/p53/PTEN (Fig. 1A). rSFVs containing one, two, or
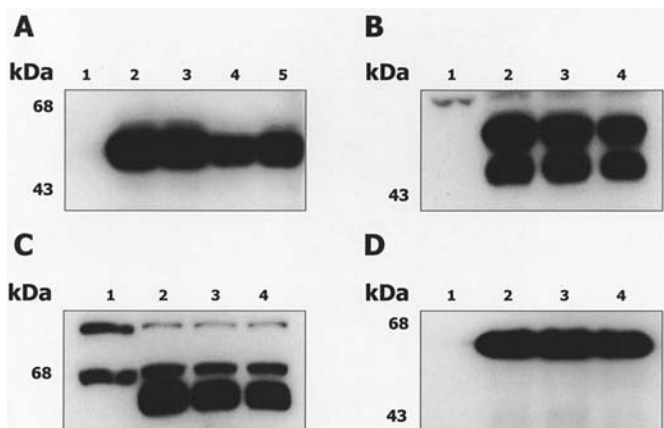
Figure 2. Expression of anti-tumor proteins, p53 (A), PTEN (B), and angio-statin (C) in U251MG cells and secreted angiostatin in supernatant of infected cells (D), as shown by Western blotting. The cells were infected with either rSFV-lacZ (lane 1 of A-D), rSFV-p53 (A, lane 2), rSFV-PTEN (B, lane 2), rSFV-Agt (lane 2 of C and D), rSFV-PTEN/p53 (lane 3 of A and B), rSFV-Agt/p53 (lane 4 of A and lane 3 of C and D), or rSFV-Agt/p53/PTEN (lane 5 of A and lane 4 of B-D).
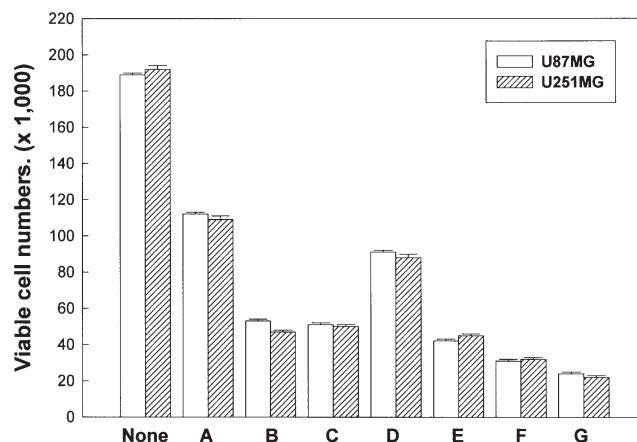

Figure 3. Viable cell count assay of U87MG and U251MG cells infected with rSFVs (none, no infection; A, rSFV-lacZ; B, rSFV-p53; C, rSFV-PTEN; D, rSFV-Agt; E, rSFV-Agt/p53; F, rSFV-PTEN/p53; and G, rSFV-Agt/p53/ PTEN). Mean cell numbers 48 h after infection; bars, SD (three replicates).

three different anti-tumor genes were then generated by the cotransfection of BHK-21 cells with RNA transcripts from each expression replicon and helper plasmid. The transient expression of each SFV replicon containing anti-tumor genes in BHK-21 cells was examined 48-h post-transfection by immunocytochemistry using monoclonal antibodies (Fig. 1B). The expression efficiency of each anti-tumor gene, whether cloned alone or with other genes in the SFV vector, seemed to be consistent. In addition, the expression level of each anti-tumor gene was also examined 48-h post-infection with rSFV using Western immunoblotting (Fig. 2). This experiment verified the highly efficient expression of the anti-tumor proteins [p53 (53 kDa), Fig. 2A; PTEN (55 kDa), Fig. 2B; and angiostatin (64 kDa), Fig. 2C] in U251MG cells. Angio-statin was also detected in the culture supernatant of the rSFV-Agt infected U251MG cells (Fig. 2D). The expression level of the anti-tumor proteins and infectivity of the rSFV were unaffected by the presence of a second or third gene connected in cis with their own 26S promoter. Accordingly, these results suggest that the SFV polymerase produced a similar amount of subgenomic RNAs to encode the three different anti-tumor proteins, each under the control of a separate 26S promoter, thereby enabling the rSFV to package a replicon RNA containing three different foreign genes with a total size >4 kb. Consequently, this would appear to be the first report demonstrating the overproduction of three different proteins based on the transfer of a viral vector carrying three different genes into mammalian cells.

In vitro study of synergistic effect of anti-tumor proteins on tumor growth. Glioblastoma cells (U87MG and U251MG) were infected with rSFV-lacZ, rSFV-p53, rSFV-PTEN, rSFV-Agt, rSFV-PTEN/p53, rSFV-Agt/p53, or rSFV-Agt/p53/PTEN [multiplicity of infection (MOI) =5]. Because both cell lines express a mutant type of p53 and PTEN, we could examine the unambiguous effect of wild-type p53 and PTEN expressed in infected cells. At 48 h post infection with rSFV-lacZ, approximately 35% of the cells had died, which

coincided with previous results where SFV induced apoptosis in a p53-independent manner (25). Infection with rSFV-p53, rSFV-PTEN, or rSFV-Agt in glioblastoma cells induced approximately 75%, 75%, and 50% cell death, respectively. Approximately 75-80% cells died after infection with rSFV-PTEN/p53 or rSFV-Agt/p53. In particular, after infection with rSFV-Agt/p53/PTEN, most cells (>85%) died (Fig. 3). To check whether the marked increase in the cell death of U251MG, mutated by both the p53 and PTEN genes, was due to apoptosis induced by infection with rSFV, a terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay was performed and the results were analyzed by flow cytometry.

To assess the apoptosis rate due to the anti-tumor proteins, the infected cells were examined 24-h post-infection. Fig. 4 shows the frequency of apoptosis in U251MG cells infected with rSFVs, as measured by TUNEL assay. After the glio-blastoma cells were infected with rSFV-lacZ, an increased number of apoptotic cells were observed compared to the mock control. However, a higher apoptotic frequency was observed in cells infected with rSFVs containing anti-tumor gene(s) than in rSFV-lacZ-infected cells. In particular, cells infected with rSFV-Agt/p53/PTEN showed the highest frequency of apoptosis (~82%) compared to any other rSFV-infected cells (~19-74%). This result suggests that the three different anti-tumor proteins coexpressed in the glioblastoma cells synergistically induced the apoptosis of U251MG cells.

The cytotoxicity induced by rSFV-lacZ-infected cells suggests that regional rSFV-mediated delivery of anti-tumor proteins may be useful for local tumor control.

In vivo study of synergistic effect of anti-tumor proteins on tumor growth. To check the effect of the rSFVs on tumor growth, U251MG cells infected with rSFVs were injected into nude mice (Table I). For this purpose, U251MG cells were infected with rSFV-lacZ, rSFV-p53, rSFV-PTEN, rSFV-Agt, rSFV-Agt/p53, rSFV-PTEN/p53, or rSFV-Agt/p53/PTEN (MOI=10), and then the transduced U251MG cells were injected subcutaneously into nude mice. The tumor size was
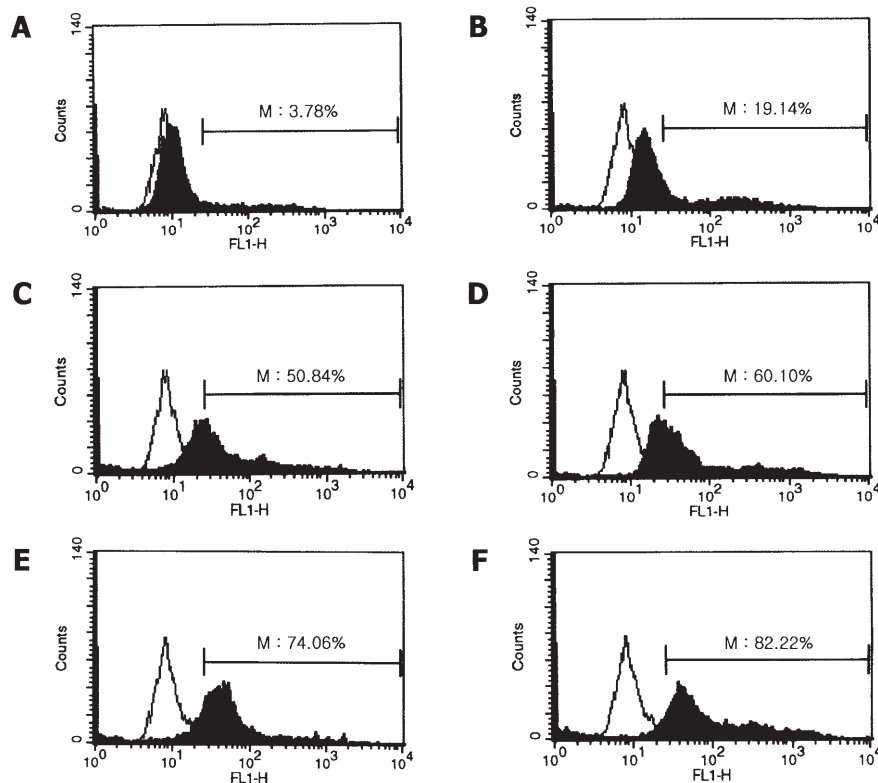
Figure 4. Analysis of apoptosis in U251MG cells based on TUNEL assay. The cells were infected with the rSFVs, then the apoptotic index was analyzed based on the percentage using flow cytometry (A, control; B, rSFV-lacZ; C, rSFV-p53; D, rSFV-Agt/p53; E, rSFV-PTEN/p53; and F, rSFV-Agt/p53/PTEN).

Table I. Tumor growth in nude mice injected with U251MG cells infected *ex vivo* with rSFVs.

Treatment	No. of mice with tumors/no. of mice injected	Mean tumor volume ($mm^3 \pm SD$)
Mock	6/6	3124±801
rSFV-lacZ	5/6	1634±754
rSFV-p53	5/6	726±194
rSFV-PTEN	5/6	751±367
rSFV-Agt	6/6	692±259
rSFV-Agt/p53	6/6	312±103
rSFV-PTEN/p53	5/6	479±68
rSFV-Agt/p53/PTEN	5/6	156±72

For the effect of rSFVs on tumor growth *ex vivo*, the transduction of rSFV-infected U251MG cells was performed. U251MG cells (5×10^6) were infected with rSFVs (MOI=10) and then washed 4-5 h later. These infected cells were introduced subcutaneously into athymic nu/nu mice (n=6). Tumor volumes were measured after 4 weeks. The average sizes of the tumors and standard deviation are given.

measured 4 weeks post inoculation. The size of the tumors formed by U251MG cells infected with rSFV containing one or two tumor-suppressor genes was 10-25% of that of the control tumors developed based on the inoculation of uninfected U251MG. The rSFV-Agt/p53/PTEN-infected cells formed tumors that were only 6% of the size of the control tumors. As such, the growth of the glioblastoma tumor cells was more suppressed by rSFV particles containing a combination of *agt, p53*, and *PTEN* than by rSFV particles containing only one or two these genes.

To assess the potential therapeutic efficiency, an *in vivo* experiment was also conducted using pre-established tumors developed from U251MG glioblastoma in nude nice and the direct injection of rSFVs. Whereas the rSFVs carrying only a single gene exhibited a marginal growth-retarding effect, the coexpression of two genes led to an almost complete inhibition of tumor growth for at least 20 days. In addition, the rSFV carrying the three genes (*agt, p53*, and *PTEN*) significantly extended the growth inhibition of tumors to 40 days compared to the other rSFVs carrying one or two genes (Fig. 5). Accordingly, these results provide further support for the use of the SFV vector in cancer gene therapy, particularly for the delivery of three synergistic anti-tumor genes using a single vector. The combined high-level expression of p53, PTEN, and angiostatin substantially increased the regression of the established tumors, compared with the vectors expressing only one or two anti-tumor proteins. The direct intratumoral injection of rSFVs expressing p53, PTEN, and angiostatin simultaneously was demonstrated to be very effective in suppressing tumors by reducing tumor growth or angiogenesis. Therefore, rSFVs expressing p53, PTEN, and angiostatin in combination would appear to represent a highly effective means of *in vivo* therapy for patients.

With intratumoral injection of rSFVs, the treated mice remained healthy and no lesions were detected in the tissue surrounding the tumor (25). This may be because rSFVs
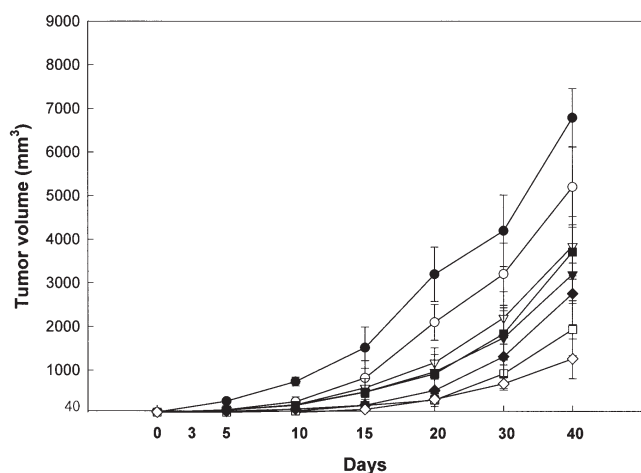
Figure 5. Inhibition of tumor growth following intratumoral injection with rSFVs. U251MG cells (1×10^6) were inoculated into nude mice subcutaneously. At 14 days post inoculation, each rSFV (1×10^9) was injected into a visible tumor (●, no injection; ○, rSFV-lacZ; ■, rSFV-p53; inverted empty triangle, rSFV-PTEN; ▼, rSFV-Agt; ◆, rSFV-PTEN/p53; □, rSFV-Agt/p53; and ◇, rSFV-Agt/p53/PTEN). Injection was then repeated 3 days later.

adsorb rapidly to cells at the injection sites and only one round of multiplication is possible. When rSFVs are injected into the brain tumor directly, it is necessary to be cautious because of apoptosis in the normal tissue surrounding the tumor.

As regards the gene transfer of tumor-suppressor genes, several types of viral vectors have been developed. However, most previous experiments have been performed using an adenoviral or retroviral vector system for the delivery of a single tumor-suppressor gene [*p53* (8,9,26,27), *PTEN* (13,14), and *p16INK4* (23,28)] or anti-angiogenic gene (*agt*) (18-20,29). It was reported that the transfer of a recombinant adenovirus encoding a p27-p16 fusion gene (30) and the combined transfer of two recombinant adenovirus encoding the *p16INK4* and *p53* genes (23), respectively, led to a more efficient inhibition of tumor growth than the transfer of a recombinant virus carrying only a single tumor-suppressor gene. The current results also suggest that rSFV particles containing three anti-tumor genes, such as *p53, PTEN*, and *agt*, would be a convenient and efficient method for delivering three genes into each tumor cell, thereby synergistically inducing the most effective suppression of tumor cell growth.

Acknowledgements

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