Inhibitory Effect of the Salmosin Gene Transferred by Cationic Liposomes on the Progression of B16BL6 Tumors

Soo In Kim, Keun Sik Kim, Hong Sung Kim, Doo Sik Kim, Yangsoo Jang, Kwang Hoe Chung, and Yong Serk Park

Department of Biomedical Laboratory Science and Institute of Health Science, Yonsei University, Wonju 220-710, South Korea [S. I. K., K. S. K., H. S. K., Y. J.]; Department of Biochemistry, College of Science, Yonsei University, Seoul 120-749, Republic of Korea [D. S. K.]; and Cardiovascular Research Institute and BK 21 Project for Medical Sciences, Yonsei University College of Medicine, Seoul 120-752, Republic of Korea [K. H. C., Y. J.]

ABSTRACT

Salmosin is a novel disintegrin containing the Arg-Gly-Asp sequence that significantly inhibits platelet aggregation, basic fibroblast growth factor-induced endothelial cell proliferation, and tumor progression by antagonizing integrin-mediated cell interactions. Previously, it was shown that daily administration of salmosin was able to inhibit tumor-derived angiogenesis and adherence and proliferation of tumor cells, resulting in suppression of tumor progression. However, it is very difficult to maintain a therapeutic level of salmosin in the blood by systemic administration of the protein. Hence, an alternative strategy for antiangiogenic cancer therapy, based on the in vivo expression of the salmosin gene administered with cationic liposomes, was investigated. The salmosin peptides expressed in vitro inhibited the proliferation of bovine capillary endothelial cells in a dose-dependent manner, presumably as a result of inhibition of cell adhesion mediated via \( \alpha_\beta \) integrin. Subcutaneous administration of the salmosin gene resulted in systemic expression of the gene product and concomitant inhibition of the growth of B16BL6 melanoma cells. Suppression of pulmonary metastases, verified by experimental and spontaneous metastasis models in mice, also resulted from salmosin gene treatment. These results suggest that administration of the salmosin gene complexed to cationic liposomes is effective in maintaining antiangiogenic salmosin at an effective therapeutic level and may be clinically applicable to anticancer gene therapy.

INTRODUCTION

Salmosin is a novel, snake (Gloydius saxatilis) venom-derived disintegrin that is composed of 73 amino acids, including 12 cysteines and an RGD4 sequence (1). In previous studies, it was demonstrated that salmosin inhibited platelet aggregation (1) and tumor growth by suppression of angiogenesis without affecting the normal proliferation of endothelial cells (2). In addition, i.v. administration of the salmosin protein was shown to be able to inhibit proliferation and metastasis of B16 mouse melanoma cells by perturbation of \( \alpha_\beta \) integrin-mediated adherence (3). Besides salmosin, a number of other endogenous angiogenesis inhibitors such as IFN-\( \alpha \) and IFN-\( \gamma \) (4, 5), interleukin 12 (6), angiostatin (7), endostatin (8), and the NH\( _2 \)-terminal fragment of proactin (9) have been identified. Of these antiangiogenic molecules, some such as endostatin and angiostatin have been extensively tested to verify their effectiveness in inhibiting tumor angiogenesis and resultant tumor growth. At present, >20 different antiangiogenic compounds are undergoing evaluation in clinical trials.

It has been well documented that angiogenesis is essential for the growth and metastasis of solid tumors (10). Some angiogenic factors released from tumor cells attract and activate neighboring endothelial cells, which is a pivotal step for tumor progression (11–13). Thus, it is reasonable to presume that inhibition of angiogenesis may provide an important means to control tumor growth and metastasis. In animal models, inhibition of tumor growth by administration of recombinant antiangiogenic proteins has been amply demonstrated (2, 14–17). However, cancer treatment with antiangiogenic molecules, including salmosin, may not be clinically practical because high-dose administration of the functionally active recombinant proteins is required, and it is very difficult to maintain therapeutic levels of these protein drugs in the tissues. Alternatively, it would be reasonable to assume that in vivo transfer of the genes for these antiangiogenic proteins could result in sufficient endogenous expression of the functionally active proteins and provide an effective alternative to exogenous administration.

Nonviral gene delivery using complexes of cationic liposomes and plasmid DNA (lipoplexes) has become popular for in vitro and in vivo research into cancer gene therapy (18). Liposomal vectors provide some distinct advantages over recombinant viral vectors because they are nonpathogenic, are less immunogenic, and are simple to prepare and use. Because cationic liposomes are well tolerated and cause few side effects, they have been used as a safe vehicle for gene transfer in a variety of gene therapy clinical trials (19, 20).

In this study, a possible application of the salmosin gene transferred with the aid of cationic liposomes is proposed as a gene medicine for cancer therapy. Efficient expression vectors containing the salmosin gene were constructed, and cationic liposome-mediated transfer of the gene resulted in expression and active production of biologically functional salmosin. The expressed salmosin was able to inhibit endothelial cell growth in vitro as well as in vivo tumor growth and metastasis. These results strongly supported the premise that antiangiogenic gene therapy with the salmosin gene could be a clinically applicable strategy for cancer treatment. This is the first study of the transfection of a disintegrin gene in an animal model and the demonstration of the in vivo antiangiogenic/anticancer effect of the expressed disintegrin.

MATERIALS AND METHODS

Materials. DOTAP was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Chol and anti-FLAG M2 monoclonal antibody were purchased from Sigma (St. Louis, MO). C57BL/6 and ICR female mice were 8 weeks old and 20–24 g in weight. Transformed human kidney cells (293 cells) were purchased from the American Type Culture Collection (Manassas, VA). The 293 cells were cultured in DMEM supplemented with 10% FBS, 100 mM sodium pyruvate, 500 units/ml penicillin, and 50 \( \mu \)g/ml streptomycin. B16BL6 mouse melanoma cells were provided by Dr. I. J. Fidler at M. D. Anderson Cancer Center (Houston, TX). The melanoma cells were grown in MEM supplemented with 5% FBS, 100 mM vitamin solution, 100 mM sodium pyruvate, 10 mM nonessential amino acid and 500 units/ml penicillin, and 50 \( \mu \)g/ml streptomycin. BCE cells were obtained from bovine adrenal glands as previously described (3) and maintained in 0.25% gelatin-coated culture dishes with DMEM containing 2 ng/ml bFGF supplemented with 10% FCS and sodium pyruvate.

Received 5/1/03; revised 6/25/03; accepted 6/30/03.

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1 This study was financially supported by the Korea Ministry of Commerce, Industry, and Energy and Grant G7 from the Ministry of Science and Technology made in the program year of 2001.

2 These two authors contribute equally to this work.

3 To whom requests for reprints should be addressed, at Department of Biomedical Laboratory Science and Institute of Health Science, Yonsei University, Wonju 220-710, South Korea. E-mail: yspark@dragon.yosei.ac.kr

4 The abbreviations used are: RGD, Arg-Gly-Asp; bFGF, basic fibroblast growth factor; BCE, bovine capillary endothelium; Chol, cholesterol; DOTAP, 1,2-dioleoyl-sn-glycero-3-phospho-L-1(4)-[N,N,N-trimethylammonium chloride; ECM, extracellular matrix.
Preparation of Salmosin Gene Constructs. Salmosin gene cDNA of was synthesized by a standard PCR. The 5’ primer was CCCAAGCTTGGCCACCATGGAACGTGGAAGGGATT and the 3’ primer CCGGAAGCTTCTAGGGAGGCCGGAGAATGT. The PCR product was digested with HindIII and BamHI and then cloned into the pAAV-CMV vector (provided by Dr. M. J. Durig, Jefferson Medical College, Philadelphia, PA) and pFLAG-CMV-1 vector (Sigma) encoding the NH2-terminal FLAG (DYKDDDDK) epitope (Fig. 1). The resulting constructs were designated as pAAV-CMV-Sal and pCMV-FLAG-Sal.

Preparation of Salmosin Gene-containing Lipoplexes. Constructs of pDNA were propagated in Escherichia coli DH5α with ampicillin-induced growth selection and purified using a Qiagen maxiprep kit (Hilden, Germany). Cationic liposomes consisting of DOTAP-Chol (1:1, molar ratio) were prepared by hydration in filtered PBS or 5% dextrose solution followed by extrusion through a polycarbonate membrane filter (100 nm pore size). For in vitro transfection, pDNA and the cationic liposomes (1:10, wt/wt) were mixed in serum-free culture media and incubated for 30 min at room temperature. For in vivo transfection, an equal volume of plasmid DNA solution was added to the liposome solution (in 5% dextrose) by slow dropwise addition with continuous mixing to achieve a 1:10 by weight ratio of DNA/lipid. The resulting lipoplexes were incubated at room temperature for 30 min before administration into mice.

In Vitro and in Vivo Transfection with the Salmosin Gene. The lipoplexes made of DOTAP-based cationic liposomes (100 µg) and pCMV-FLAG-Sal (10 µg) were prepared for in vitro transfection as described earlier and added to 293 cells (~80% confluence) in a 100-mm plate followed by incubation for 4 h at 37°C and 5% CO2. After incubation, the lipoplex containing media was replaced by fresh serum-free media and incubated for an additional 7 days.

In vivo transfection with the salmosin gene was executed in two different ways. The first involved s.c. administration of lipoplexes prepared for in vivo transfection as described above. Lipoplexes (25 µg pDNA/250 µg lipid in 50 µl of saline) were administered s.c. into the dorsal midline of ICR mice. The second method used hydrodynamics-based transfection of naked plasmid DNA (21). Plasmid solutions (10 µg of DNA in 2 ml of saline) were injected i.v. within 5 s into ICR mice via the tail vein. In both experiments, mouse sera were collected at various time points after administration.

Culture media and mouse serum containing the expressed salmosin were run on a 4–20% gradient SDS-PAGE gel. Twenty µl of the culture media were loaded for in vitro expression assay. For in vivo expression assay, 20 µl of 4-fold diluted serum, collected from a transfected mouse at various time points, and as a control, 20 µl of purified salmosin solution (5 mg/ml) were loaded on the gel. Proteins on the gel were transferred to nitrocellulose membranes that were then blocked for 1 h at room temperature with 5% skim milk in Tris-buffered saline. The membranes were treated overnight with anti-FLAG M2 solution at room temperature and then incubated with goat antimouse IgG-horseradish peroxidase conjugate at 37°C for 1 h. FLAG-conjugated salmosin protein bands were visualized by treatment with peroxidase substrate solution containing 3,3’-diaminobenzidine and H2O2.

BCE Cell Proliferation Assay. The assay for endothelial cell growth was performed as described previously (3). Bovine capillary endothelial cells (BCE, 1 × 104 cells/well) were plated onto gelatinized 24-well plates and then incubated at 37°C in 5% CO2 for 24 h. After incubation, the cells were treated for 20 min with various amounts of expressed salmosin in serum-free media. DMEM was supplemented with 5% FCS and 1 ng/ml bFGF. After 72 h of incubation, the cells were dispersed with trypsin and then counted.

Inhibition of Tumor Growth. B16BL6 cells (5 × 104 cells/mouse) were injected s.c. into the dorsal midline of C57BL/6 female mice. When the resulting tumors reached a volume of 50–100 mm³, the mice were randomized into three groups (n = 5). Salmosin DNA (25 µg of pAAV-CMV-Sal or pCMV-FLAG-Sal) was combined with 250 µg of DOTAP/Chol liposomes in a total volume of 50 µl of saline. The lipoplexes were administered s.c. within 5 mm of the tumor site every fourth day. The control group received a comparable injection of pAAV-CMV empty vector. Tumor size measurements were performed twice weekly and were terminated when the control mice began to die. Two biassing diameters of each tumor were measured with calipers, and tumor volume was calculated according to the equation; tumor volume = πr²h/6 (a, long diameter; b, short diameter).

Inhibition of Tumor Metastasis. Two different experimental procedures, experimental metastasis, and spontaneous metastasis were used to examine the effect of salmosin gene administration on tumor metastasis. For experimental metastasis, B16BL6 cells (4 × 10⁴ cells/mouse) were administered via the tail vein into C57BL/6 female mice. DNA (pAAV-CMV-Sal, pCMV-FLAG-Sal, or empty vector) was complexed with cationic liposomes (1:10, wt/wt), and the resulting lipoplexes injected s.c. once daily near the dorsal midline 1 day before tumor inoculation and then on every fifth day. The mice were sacrificed on day 25 and lung colonization determined. The experimental metastasis experiment was repeated three times (n = 8).

For spontaneous metastasis, B16BL6 cells (2 × 10⁴/mouse) were administered s.c. into the foot pad of C57BL/6 female mice. The primary tumors were surgically removed by amputating below knee when they reached a volume of 400–600 mm³. The lipoplex solutions were injected s.c. once into the dorsal midline 1 day before tumor removal and then on every fourth day. Twenty-five days after primary tumor removal, metastatic colonies in the lungs of the mice were counted. The spontaneous metastasis experiment was repeated twice (n = 7).

RESULTS

In Vitro Expression of Recombinant Salmosin. The 293 transformed human kidney cells treated with lipoplexes consisting of DOTAP liposomes and pCMV-FLAG-Sal efficiently released the salmosin gene product into the culture medium. Expression of the salmosin protein was verified by Western blot analysis of the serum-free culture medium (Fig. 2). As expected, immunostaining with anti-FLAG-M2 monoclonal antibody detected the FLAG-salmosin fusion protein band at a position a slightly higher (M₅, ~8000) than that for salmosin.

Systemic Expression of Recombinant Salmosin. To study the systemic expression of salmosin gene in an animal model, two dif-
different methods were adopted. First, lipoplexes containing pCMV-FLAG-Sal were administered s.c. into the dorsal midline of mice. The salmosin gene product was efficiently expressed and released into the circulation. Systemic expression of salmosin was quantitated by immunostaining of sera collected from the transfected mice (Fig. 3A). Gene expression began within a few hours after s.c. administration and reached a maximum in 24 h and was maintained for several days.

The systemic expression of salmosin was also demonstrated by hydrodynamics-based gene transfection. As described above, mice were administered a large volume of pDNA solution (10 μg of DNA in 2.0 ml of saline) i.v. within 5 s. Western blot analysis of sera collected from the hydrodynamically treated mice revealed that the salmosin gene product began to be expressed within a few hours after i.v. administration and was sustained over 4 months (Fig. 3B). Salmosin gene expression, as demonstrated by the relative intensity of the bands on Western blots, reached a peak 8 h after injection and gradually diminished with time. The results of these experiments demonstrated that transfer of the gene by either of these in vivo methods resulted in efficient expression of the salmosin proteins.

**Inhibition of bFGF-induced BCE Cell Proliferation by Expressed Salmosin Gene.** A BCE cell proliferation assay (3) was performed to study the effect of salmosin on endothelial cell growth, which is considered to be a major process in tumor angiogenesis. Measured amounts of salmosin protein in media were applied to BCE cells in culture plates. BCE cell proliferation induced by bFGF was inhibited by the addition of the salmosin-containing media, and the extent of inhibition was found to be dependent on the concentration of salmosin added (Fig. 4). Moreover, a distinct morphological change of the BCE cells into a round shape induced by the salmosin treatment was observed (data not shown). Collectively, these data imply that the expressed salmosin protein is biologically active.

**Inhibition of Tumor Growth by Salmosin Gene Administration.** The effect of s.c. administered salmosin gene on solid tumor growth was examined in a mouse model carrying B16BL6 mouse melanoma tumors. These mice were treated with two different types of lipoplexes containing salmosin genes after their tumors had attained a mass of 50–100 mm³. Subcutaneous administration of the lipoplexes near by the tumors every fourth day remarkably inhibited tumor growth compared with nontreated controls (Fig. 5). The inhibitory effects of both the pAAV-CMV-Sal and pCMV-FLAG-Sal containing lipoplexes were similar.

**Inhibition of Tumor Metastasis by Salmosin Gene Administration.** To examine the inhibitory effect of salmosin gene transfection on tumor metastasis, two different procedures were used to induce metastatic colony growth in mice. The first of these, experimental metastasis, involved direct i.v. administration of B16BL6 melanoma cells via the tail vein. Subcutaneous administration of lipoplexes containing salmosin every fifth day after inoculation with the B16BL6 cells significantly inhibited the formation of pulmonary metastases (Fig. 6). Although nontreated mice had an average of 52 metastatic colonies in their lungs, those treated with pAAV-CMV-Sal or pCMV-FLAG-Sal carried only six or eight colonies, respectively. Reduction of pulmonary metastases by treatment with pAAV-CMV-Sal and pCMV-FLAG-Sal was 89 and 84%, respectively. When colonies ≥2 mm diameter (large colonies), a size known to promote angiogenesis, are considered, the reduction in number caused by treatment with the salmosin gene was similar to that of the total number of colonies.
Although the difference was not statistically significant, treatment with pAAV-CMV-Sal appeared to be slightly more effective than with pCMV-FLAG-Sal. Interestingly, mice treated with lipoplexes containing empty plasmids, pAAV-CMV or pFLAG, each exhibited some reduction in lung colonization when compared with nontreated controls.

The second procedure used to induce tumor metastasis into the lungs involved s.c. administration of B16BL6 cells. The administered tumor cells grew as a primary tumor from which cells were released and developed into metastatic lesions in the lungs of the mice, even after the primary tumors were removed. When the primary tumors grew to ~500 mm³ in size, the tumors were surgically removed. Treatment with the salmosin gene was begun 1 day before the tumor removal. The treatment with lipoplexes containing pAAV-CMV-Sal or pCMV-FLAG-Sal every fourth day after primary tumor removal significantly reduced the spontaneous lung metastasis of B16BL6 cells (Fig. 7). Treatment with pAAV-CMV-Sal or pCMV-FLAG-Sal reduced the average lung colony number from 33 to 7 or 7.8, respectively (76 and 75% inhibition). Mice treated with either, pAAV-CMV, pFLAG, or empty vehicles exhibited a notably smaller number of lung colonies than untreated mice, similar to the results observed with experimental metastasis (Fig. 6). In the spontaneous metastasis experiment, the number of large colonies in the lungs of mice treated with pAAV-CMV-Sal or pCMV-FLAG-Sal was reduced by 92 and 88%, respectively. The two different plasmid constructs coding for salmosin exhibited similar levels of inhibition on B16BL6 pulmonary metastasis in this animal model.

**DISCUSSION**

This study demonstrates that the salmosin gene transferred with the aid of cationic liposomes is able to inhibit tumor progression, presumably because of the antiangiogenic activity of the gene product. In vitro, salmosin peptides released from transfected 293 cells strongly inhibited BCE cell proliferation and, in vivo, expression of the salmosin peptides inhibited growth and pulmonary metastasis of highly metastatic B16BL6 mouse melanoma.

Recently, therapy using antiangiogenic molecules has been recognized as a promising alternative for cancer treatment (22). Among the antiangiogenic drugs, the endogenous antiangiogenic molecules endostatin and angiostatin have been extensively tested against various cancer models in animals (7–9, 16, 17). However, the candidate molecules remain to be proven clinically effective in cancer treatment. From the practical point of view, the clinical application of exog-
We speculate that subdermal smooth muscle cells and epidermal cells may be major target cells transsected and releasing gene products after s.c. administration of pDNA, as reported earlier (26). Surprisingly, a single hydrodynamics-based transfection (21) of the salmosin gene produced sustained systemic release of salmosin peptide from liver cells into the blood stream for several months (Fig. 3B). The collective results of \textit{in vitro} and \textit{in vivo} salmosin expression experiments implied that the pAAV-CMV-Sal and pCMV-FLAG-Sal salmosin gene constructs were functionally active in terms of gene expression and biological activity. The continuous expression of the salmosin peptide induced by hydrodynamics-based transfection or by multiple cationic liposome-aided administrations may indicate that salmosin is not very immunogenic. This low level of immunogenicity may allow salmosin concentrations to be sustained in the blood circulation long enough to have a significant antitumor effect.

The antiangiogenic activity of the gene product was indirectly supported by inhibition of BCE cell growth by the salmosin peptides released from transfected 293 cells. The salmosin in the culture media inhibited BCE cells in a dose-dependent manner (Fig. 4) and also induced a change in the morphology of the cells into a rounded shape (data not shown). It has been well documented that endothelial cells adhere to the ECM via interactions between endothelial integrin molecules and RGD sequences of the ECM during vascular proliferation (27, 28). The adhesive interactions between endothelial cells and ECM molecules are one of pivotal steps regulating the growth, differentiation, and morphogenesis of microvessels during the process of angiogenesis. Therefore, it can be speculated that inhibition of endothelial cell adhesion to the ECM by salmosin expressed \textit{in vivo} may result in the failure of these cells to migrate, proliferate, and form capillary tubes, thus impeding tumor progression (Figs. 5–7). It has also been shown that \( \alpha_3\beta_1 \) integrin mediates the migration and survival of melanoma cells in collagen (29). Thus, the expressed salmosin may directly interfere with the survival of B16BL6 melanoma tumor cells and, consequently, cause a reduction in pulmonary metastases.

Interestingly, the treatment groups that received lipoplexes containing only empty vehicles also exhibited some suppression of lung colonization (Figs. 6 and 7). It has been suggested previously that i.v. administration of cationic lipoplexes elicits a proinflammatory cytokine response that mediates antitumor activity (30). The CpG motifs in the inhibition of tumor growth and metastasis. Oncogene, \textit{19}: 6122–6129, 2000.


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