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• LIVER CANCER •

Retrovirus-mediated gene transfer of human endostatin inhibits growth of human liver carcinoma cells SMMC7721 in nude mice

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Abstract

AIM: To study the effect of human endostatin mediated by retroviral gene transfer on the growth of human hepatocarcinoma cell line SMMC7721 in nude mice.

METHODS: Human endostatin gene together with rat serum albumin signal peptide was transferred into human liver carcinoma SMMC7721 cells by retroviral vector pLncx to build a stable transfectant (SMMC-endo). PCR and Western blot analysis were used to verify the transfection and secretion of human endostatin gene in SMMC7721 cells. The endothelial cell proliferation assay *in vitro* was conducted to test the biological activity of the expressed human endostatin. The inhibitory effect of endostatin expressed by transfected SMMC7721 on the growth rates of tumor cells *in vivo* was observed. The mean microvessel density in the specimen was also counted.

RESULTS: PCR amplification proved that the genome of SMMC-endo cells contained a 550bp specific fragment of endostatin gene. Western blot analysis confirmed the secretion of human endostatin gene in the conditioned medium of transfected SMMC-endo cells. The endothelial proliferation assay showed that the conditioned medium of SMMC-endo cells significantly inhibited the proliferation of human umbilical vein endothelial cells by 48 %, significantly higher than that of SMMC-pLncx (10.2 %, $P<0.01$). *In vivo* experiments revealed that only in 3 out of 5 mice tumors were formed and the mean size of flank tumors from SMMC-endo cells was 94.5 % smaller than that from the control SMMC-pLncx cells 22 days after tumor inoculation ($P<0.001$). The mean microvessel density in tumor samples from SMMC-endo cells was only 8.6±1.1, much fewer than that of 22.6 ±4.5 from SMMC-pLncx cells ($P<0.01$).

CONCLUSION: Human endostatin mediated by retroviral gene transfer can inhibit human liver carcinoma cell SMMC7721 growth in nude mice.

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INTRODUCTION

It has become clear that angiogenesis not only is essential for a number of physiological processes such as embryonic development, organ and tissue regeneration, but also plays a pivotal role in tumor growth and metastases[1-4]. Folkman[2] demonstrated that suppression of tumor angiogenesis leads to tumor starvation and tumor regression. Thus, the tumor vascular system has become an important target for cancer therapy, and methods to inhibit angiogenic process provide an unique opportunity to inhibit tumor growth[4]. An increasing number of antiangiogenic agents have been discovered recently. These included angiostatin,endostatin and interleukin-12[5-7]. Among them, endostatin is one of the most hopeful antiangiogenic proteins. Its antiangiogenic effect, most potent discovered so far, can specifically inhibit the proliferation and migration of endothelial cell, and subsequently promote the development of apoptosis and regression of tumor[8-14]. However, antiangiogenic therapy with endostatin requires multiple and prolonged administrations, and the problems of such as bioactive protein production in large quantities, high costs and the cumbersome daily administration can often be met during cancer therapy[15]. Gene transfer therapy could provide an alternative approach to continuous local delivery of this antiangiogenic factor *in vivo*[16]. So, in order to explore the effect of human endostatin expressed by human liver carcinoma cell on tumor growth, stable transfectant from human liver carcinoma cell line SMMC7721 transferred with human endostatin gene was built and the inhibitory effect of endostatin on tumor growth *in vitro* and *in vivo* were observed.

MATERIALS AND METHODS

Cell culture

Human liver carcinoma cell line SMMC7721 was kept by our laboratory. Human umbilical vein endothelial cell line (HUVEC) was purchased from Institute of Cell Biology, Chinese Academy of Sciences. HUVEC and human liver carcinoma cell line SMMC7721 were maintained in RPMI 1640 medium (Gibco) supplemented with 100 mL· L^{-1} FBS, 100 units/mL penicillin and 100 ug/mL streptomycin. Retrovirus packaging cell line PA317 was kindly provided by Dr.Qian Qijun (Second Military Medical University, Shanghai, China), and maintained in DMEM (Gibco) supplemented with 100 mL· L^{-1} FBS,100 units/ml penicillin and 100 μg/mL streptomycin.

Plasmid

The recombinant retroviral plasmid pLncx-endo containing the cDNA for human endostain gene with a HA tag attached to the C-terminus as a fusion protein was engineered by our laboratory[17]. In this plasmid, human endostain cDNA was put downstream of rat serum albumin signal peptide.

Generation of stable transfectants

Recombinant plasmid pLncx-endo was transferred into PA317

cells by Lipofectamine (Gibco) following the manufacturer' s instructions. G418 selection at 500 μg/mL was added at the same time. Two weeks after transfection, G418-resistant colonies emerged and were expanded respectively. The supernatants of G418-resistant PA317 colony were collected and stored at -80 ℃ for usage. 5×10^5 SMMC7721 cells were plated on 6-well plate and incubated for 24 h. The cells were rinsed with serum-free RPMI 1640 medium twice, and 100 μL supernatant of endostatin- transfected PA317 colony was added and incubated for 3 h. Another 3 mL 1640 medium was added with the final concentration of polybrene at 2 mg· L^{-1} and G418 at 500 mg· L^{-1}. Four weeks after transfection, G418-resistent cells were expanded for preservation and tested for endostatin-HA fusion protein by immunohistochemistry and Western blot analysis. The endostatin-transfected colony was designated as SMMC-endo. Control transfectant (SMMC-pLncx) was generated in a similar way except the parent plasmid pLncx-endo was replaced by empty plasmid pLncx.

PCR amplification of endostatin from transfected SMMC7721 cells

According to human endostain sequence, two primers were devised. The primers used were E1: 5' CCG GAA TTC ATG CAC AGC CAC CGC GAC TTC CAG CCG and E2: 5' GCC GGA TCC CTA CTT GGA GGC AGT CAT GAA GCT. SMMC-endo and SMMC-pLncx cells were harvested and DNA extracted. PCR was performed in 50 μL reactive volume containing 2 μL cDNA, 2 μL 10×PCR buffer, 2 μL 4×dNTP (2 mmol· L^{-1}), 50 pmol· L^{-1} primer, and 1 μL *Tag* DNA polymerase. The samples were subjected to 30 thermal cycles of 5 min at 94 ℃ for predenaturation, 1 min at 94 ℃ for denaturing, 1 min at 60 ℃ for annealing, 1 min at 72 ℃ for extension, and 10 min at 72 ℃ for final extension after the last cycle. PCR products were checked on 10 g· L^{-1} agarose gels (containing 0.5 mg· L ethidium bromide).

Western blot analysis

SMMC-endo and SMMC-pLncx cells were plated in six-well plates at 2.5×10^5 cells/well respectively and incubated for 24 h. The medium was replaced with 1 mL serum-free RPMI 1640 and collected after 48 h. One mL of conditioned medium was concentrated in a microcon 10 microconcentrator (Amicon, Berverly, MA) to 20 μL and subjected to a 120 g· L^{-1} reducing SDS/PAGE gel. Protein was transferred to a nitrocellulose membrane and incubated overnight in 50 mL· L^{-1} nonfat milk in PBS at 4 ℃. After briefly washing in 10 mL· L^{-1} fat-free milk, the membrane was incubated with anti-HA mouse monoantibody diluted 1:500. After three 10-min washes in 1 mL· L^{-1} fat-free milk, membrane was incubated in horseradish peroxidate-conjudated antimouse immunoglobulin diluted 1:1000. After three 10-min washes in TBS, proteins were detected using 3,3-diaminobenzidine as the chromagen.

Endothelial cell proliferation assay

SMMC-endo, SMMC-pLncx and SMMC7721 cells were plated onto six-well culture plates at a density of 2.5×10^5 cells/well and incubated for 24 h. The cells were washed with PBS, and 1.5 mL of serum-free RPMI1640 were added and incubated for another 48 h. The total of 9 mL serum-free RPMI 1640 were collected and concentrated to 1.8 ml using Centriplus 10 concentrator (Amicon), and stored at -80 ℃ for usage. HUVEC cells were seeded at a density of 4000 cells/well into gelatinized 40-well culture plates and incubated (37 ℃, 50 mL· $L^{-1}CO_2$) for 24 h in 100 μL RPMI 1640 medium. The medium was replaced with 20 μL of above concentrated conditioned medium and incubated for 30 min. 80 μL of RPMI 1640 supplemented with 100 mL· L^{-1} fetal bovine serum and 1 μg· L^{-1} bFGF (Sigma) was then added for 72 h. The numbers of cells were determined using a colorimetric MTT assay. Tests were conducted in quadruplicate.

In vivo evaluation of tumor growth

To test whether endostatin expressed by host cells could inhibit tumor growth *in vivo*, we used the human liver carcinoma cell line SMMC7721. This model has been widely used in experiment. 4-week-old female BALB/c (nu/nu) mice (purchased from Shanghai Institute of Cancer Research) were chosen. SMMC7721 cells were inoculated s.c. into the right flank of nude mice and changes in tumor growth were monitored. In this experiment, flank tumors were generated by s.c. injection of 5×10^5 cells in 100 μL of HBSS into the right flank of athymic BALB/c (nu/nu) mice for SMMC-endo and control SMMC-pLncx cells (n=5). Tumors were measured by calipers in two dimensions every 2 days, and the volume was calculated as length×width^2×0.52.

Histological analysis

To determine the effect of endostatin on vascular growth *in vivo*, microvessel density counting was conducted on frozen sections of tumors from SMMC-endo and SMMC-pLncx cells as described previously[18] by immunostaining with anti CD_{34} (Gibco). Anti-CD_{34} antibody was used at a dilution of 1:500. Immunohistochemistry was accomplished utilizing an avidin-biotin technique. SMMC-endo and control SMMC-pLncx were grown on six-well glass slides and fixed in acetone at room temperature. After washing in PBS, the cells were incubated with a 10 mL· L^{-1} H_2O_2 solution at room temperature for ten minutes to quench endogenous peroxidases. Nonspecific binding was blocked with 50 mL· L^{-1}normal horse serum at room temperature for five minutes. The cells were then incubated with anti-HA at a 1:300 dilution at 4 ℃ overnight. Following washing in PBS, the secondary antibody, biotinylated antimouse, was added and the cells were incubated at room temperature for one hour. After washes in PBS, Vectastain reagent (a solution containing strepavidin-horseradish peroxidase) was added and then incubated at room temperature for ten minutes. 3,3-diaminobenzidine was used as the chromagen. After ten minutes, the brown color signifying the presence of antigen bound to antibodies was detected by light microscopy and photographed at ×200. The blood vessels were counted from five areas in each tumor section.

RESULTS

Generation of stable transfectants

A 550-bp fragment was seen in the PCR product from DNA of SMMC-endo cells,but not from the control (Figure 1). On a reducing 120 g· L SDS/PAGE gel, a distinct band at around M_r22 000, corresponding to the size of endostatin, was only visualized in the supernatant of SMMC-endo cells. Monoclonal mouse anti-HA antibody reacted positively in a Western blot with the M_r22 000 protein only. It confirmed that endostatin protein expressed by transfected SMM-endo cells could be secreted into the supernatant of cells (Figure 2).

Antiangiogenic effect of human endostatin expressed by endostatin-transfected cells

The effect of expressed endostatin on HUVEC proliferation was tested. The result showed that there were no significant differences among the concentrated conditioned media from SMMC7721, SMMC-pLncx cells and RPMI1640 medium in inhibiting the growth of HUVEC. Compared to RPMI 1640 medium, the inhibitory rate on HUVEC proliferation for conditioned medium from SMMC-endo cells was 48 %,

significantly higher than that of 10.2 % for control SMMC-pLncx. There was a significant difference on the antiangiogenic effect between conditioned medium from SMMC-endo and SMMC-pLncx (P<0.01, Figure3).

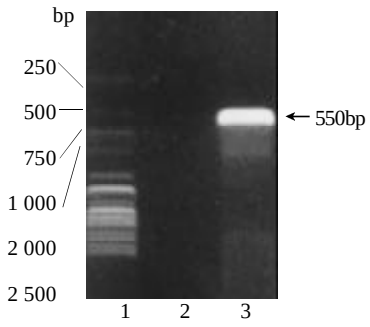


Figure 1 Analysis of PCR product of endostatin-transfected SMMC7721 cells by 1 % agarose gel electrophoresis. 1: DNA Marker. 2: PCR product of SMMC-pLncx. 3: PCR product of SMMC-endo.

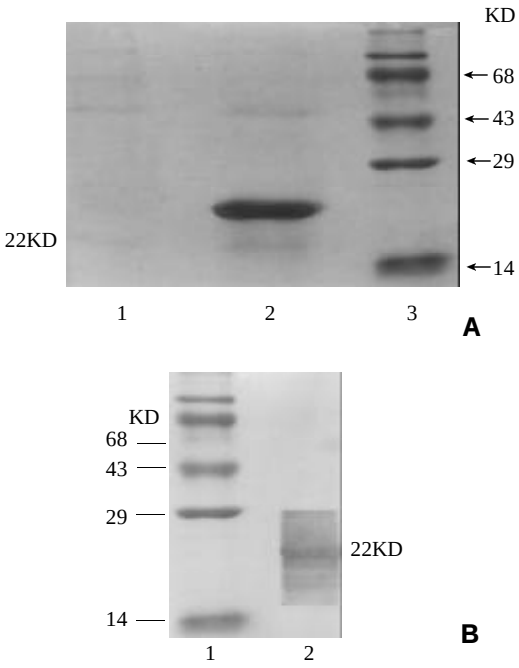


Figure 2 SDS-PAGE analysis and Western blot of endostatin expressed in supernatant of virally transduced SMMC7721cells (A) SDS-PAGE analysis; 1, supernatant of control SMMC-pLncx cells; 2, supernatant of endostatin-transfected SMMC-endo cells; 3, protein marker; (B) Western blot analysis; 1 protein marker; 2, supernatant of endostatin-transfected SMMC-endo cells.

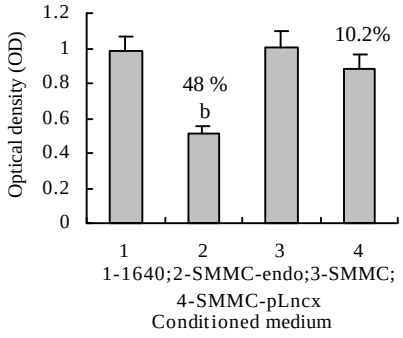
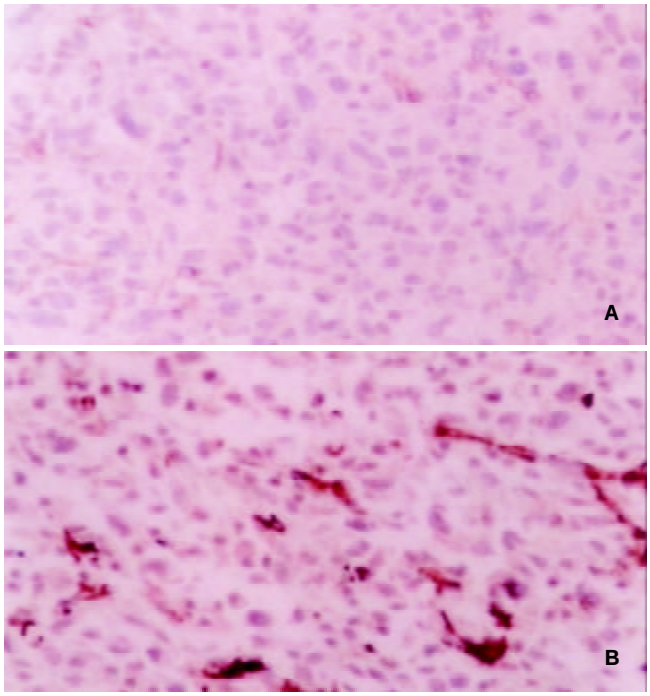


Figure 3 Inhibition of endothelial cell proliferation by conditioned medium from SMMC-endo cells. Conditioned medium from SMMC-endo (2), SMMC7721 (3) and SMMC-pLncx (4) were concentrated and applied to HUVEC cells grown in 40-well plate. Three days later, cell number, as measured by absorbance(OD), was then quantified by using a colorimetric MTT assay. Bars, SD. ^{b}P<0.01, compared with conditioned medium from control SMMC-pLncx.

Human endostatin inhibits tumor growth in nude mice

To determine the anti-tumor effect of endostatin expressed by transfected SMMC7721 cell, 5×10^5 cells were injected s.c. into right flanks of nude BALB/c mice. SMMC7721 cells and SMMC-pLncx cells formed tumor rapidly within 14 days. While flank tumors from SMMC-endo cells grew very slowly in nude mice. The first palpable tumor from SMMC-endo cells appeared 16 days after injection and only in 3 out of 5 nude mice formed tumors. The mean size of flank tumors from SMMC-endo cells was 94.5 % smaller than that from SMMC-pLncx cells 22 days after tumor inoculation, a significant difference between SMMC-endo and SMMC-pLncx groups (P<0.001, Figure 4). The mean microvessel density(MVD) in tumors was determined by utilizing anti- CD_{34}. The number of vessels was counted and the results showed that the MVD in tumor samples from SMMC-endo cells was only 8.6±1.1, much fewer than that of 22.6±4.5 from SMMC-pLncx cells (P<0.01, Figure 5). It meant that endostatin expressed by SMMC-endo cells could decrease tumor vascularization.



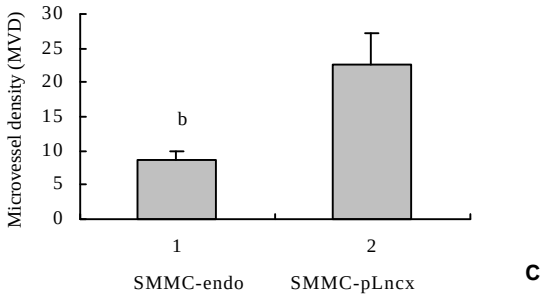


Figure 5 Tumor sections were stained with an antibody reactive to CD_{34}. A: Tumor section from endostatin-transfected

g1roup showed only a few positively stained vascular endothelial cells. B: Similar section of the control group showed highly vasuclarized tumor tissue. C: Microvessel density (MVD) was quantified by counting of positively stained endothelial cells from 5 fields in each tumor section. Bars, SD. $^bP<0.01$, compared with control SMMC-pLncx . ×200.

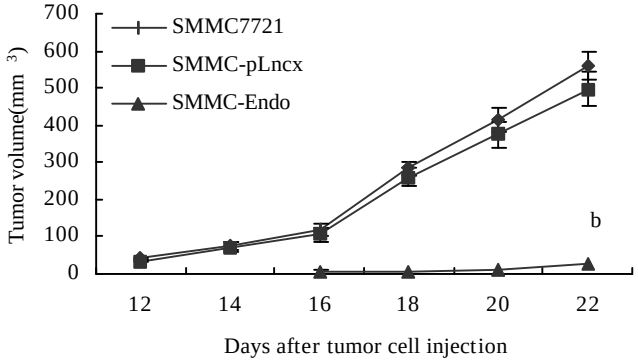


Figure 4 Inhibition on the growth of human liver carcinoma implanted in nude mice by human endostatin. $^bP<0.001$, SMMC-endo compared with control SMMC-pLncx.

DISCUSSION

It is known to all that blood supply is necessary for tumor progression and metastasis[1-4]. Folkman[2] noted that tumor will stop growing or die when it exceeds 2 mm to 3 mm in diameter if new blood vessel for tumor is not formed. Numerous studies have also shown that inhibition of tumor growth and metastases could be reached by administration of recombinant antiangiogenic proteins[15,19-21]. Moreover, the genome of endothelial cells, targeted by antiangiogenic proteins has a stable inheritance property and rare mutation rate. So, unlike tumor cells in chemotherapy, acquired resistance to recombinant antiangiogenic protein is rarely developed during antiangiogenic therapy. Therefore, antiangiogenic therapy is probably one of the most effective and promising approaches to cancer treatment[14].

Antiangiogenic therapy will require sustained maintenance of therapeutic levels *in vivo*[22,23]. Continuous delivery and high doses of recombinant antiangiogenic protein in circulation by repeated administrations seem expensive and impossible. Gene therapy transfer of foreign antiangiogenic gene into host cells represents an alternative method to tumor therapy. The aim of generating high efficient protein in areas around tumor with no toxin and keeping long time relatively high expression of antiangiogenic protein can be achieved by a single administration of gene transfer. There have been successful reports of antiangiogenic gene therapy with viral vectors being used to treat tumor[24-27]. Endostatin is a new kind of potent antiangiogenic factor consisting of 184 amino acids in C-terminal fragment of endogenous collagen18 a. It was first isolated as a M_r20 000 protein from conditioned medium of the EOMA murine hemangioendothelioma cell line by Professor O' Reilly in 1997[28-33]. *In vivo* and *in vitro* experiments had demonstrated that endostatin has specific inhibitory effect on the formation of new blood vessel. It could inhibit tumor growth with reduction of virtually all tumor neovascularization and without detectable systemic sign of toxicity[34-37]. In the same way, antiangiogenic therapy with endostatin in cancer requires prolonged administration and high doses of recombinant protein. Furthermore, the production of soluble functional polypeptide endostatin has proven difficult and nearly 95 % of the recombinant protein used will be excreted out of the body because of its insoluble and instable property[38]. However, all of the above problems can be overcome by gene transfer of endostatin gene. As gene transfer mediated by retroviral is most commonly used among the various ways of transducing methods and retroviral can be integrated into chromosome of host cells. So, gene transfer mediated by retrovirus can be inherited to next generation and stably expressed in host cells for a long duration[27]. In this experiment, recombinant retroviral plasmid pLncx-endo was transferred into human liver carcinoma cell SMMC7721 by using lipofectamine. The following PCR examination and Western blot analysis confirmed the transfection and stable expression of human endostatin by SMMC-endo cells. The expressed protein was secreted outside of cells under the influence of rat albumin signal peptide[10,39]. It revealed that a stable transfectant that could secrete human endostatin was built. *In vitro* endothelial cell proliferation assay verified the biological activity of endostatin, which showed that conditioned medium from SMMC-endo could significantly inhibit the proliferation of endothelial cells by 48 %, while the inhibitory rate for conditioned medium from control SMMC-pLncx was only 10.2 %. It suggested that the secreted protein expressed by the stable transfectant had a potent antiangiogenic effect[40,41]. In vivo experiments showed that tumor formations from SMMC-endo cells were dramatically inhibited. Compared to control group, only in 3 out of 5 mice inoculated with SMMC-endo cells tumors were formed and a remarkable reduction in tumor size was also exhibited 22 days after tumor injection. The mean size of tumors from SMMC-endo cells was about 94.5 % smaller than that from control SMMC-pLncx. Meanwhile, histological analysis showed that the MVD in tumors from SMMC-endo cells was also remarkably decreased compared to control SMMC-pLncx. It meant that human endostatin expressed by SMMC7721 could indeed inhibit the formation of tumor *in vivo*. In another word, endostatin arrests tumor growth by inhibiting the formation of microvessels *in vivo*, and gene transfer therapy mediated by retrovirus could meet the requirements for tumor treatment. But we also noted that the expressed endostatin did not completely inhibit the formation of tumor in nude mice. Some proangiogenic factors such as VEGF and bFGF, produced by SMMC7721 cell itself may be responsible for this phenomenon[42,43]. Another probable reason is the low amount of endostatin expressed by retroviral plasmid pLncx. So, in a word, gene transfer of endostatin mediated by retroviral pLncx could significantly inhibit the growth of SMMC7721 cells in nude mice by affecting angiogenesis and is probable one of the effective ways to deal with tumor. But the application of new efficient expression plasmid and combined therapy with multiple genes may further improve the therapeutic effect of endostatin in the future.

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