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• LIVER CANCER •

Inhibitory effect of endostatin expressed by human liver carcinoma SMMC7721 on endothelial cell proliferation *in vitro*

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Abstract

AIM: **To construct a stable transfectant of human liver carcinoma cell line SMMC7721 that could secret human endostatin and to explore the effect of human endostatin expressed by the transfectant on endothelial cell proliferation.**

METHODS: **Recombinant retroviral plasmid pLncx-Endo containing the cDNA for human endostain gene together with rat albumin signal peptide was engineered and transferred into SMMC7721 cell by lipofectamine. After selection with G418, endostatin-transfected SMMC7721 cells were chosen and expanded. Immunohistochemical staining and Western blot were used to detect the expression of human endostatin in transfected SMMC7721 cells and its medium. The conditioned medium of endostatin-transfected and control SMMC7721 cells were collected to cultivate with human umbilical vein endothelial cells for 72 hours. The inhibitory effect of endostatin, expressed by transfected SMMC7721 cells, on endothelial proliferation *in vitro* was observed by using MTT assay.**

RESULTS: **A 550 bp specific fragment of endostatin gene was detected from the PCR product of endostatin-transfected SMMC7721 cells. Immunohistochemistry and Western blot analysis confirmed the expression and secretion of foreigh human endostatin protein by endostatin-transfected SMMC7721 cells. *in vitro* endothelial proliferation assay showed that 72 hours after cultivation with human umbilical vein endothelial cells, the optical density (OD) in group using the medium from endostatin-transfected SMMC7721 cells was 0.51±0.06, lower than that from RPMI 1640 group (0.98±0.09) or that from control plasmid pLncx-transfected SMMC7721 cells (0.88±0.11). The inhibitory rate for medium from endostatin-transfected SMMC7721 cells was 48%, significantly higher than that from empty plasmid pLncx-transfected SMMC7721 cells (10.2%, $P<0.01$).**

CONCLUSION: **Human endostatin can be stably expressed by SMMC7721 cell transferred with human endostain gene and its product can significantly inhibit the proliferation of human umbilical vein endothelial cell *in vitro*.**

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INTRODUCTION

Recent studies have shown that angiogenesis is essential for tumor growth and metastases[1-9]. Hanahen *et al*[10,11]noted that angiogenesis is regulated by a balance between factors of proangiogenesis and antiangiogenesis. The pathological formation of new blood vessel could be generated if the balance was undermined[12]. This provides the rationale for antiangiogenic therapy for cancer. Endostatin, a specific inhibitor of endothelial cell proliferation, first isolated as a M_r22000 protein from the conditioned medium of a murine hemangioendothelioma cell line (EOMA), is a C-terminal fragment of collagen 18a consisting of 184 amino acids. Its potent antiangiogenic effect can specifically inhibit the proliferation and migration of endothelial cell and subsequently promote the development of apoptosis and atrophy of tumor without direct influence on tumor cell or nonneoplastic cell growth[13-21]. To explore the inhibitory effect of human endostain expressed by SMMC7721 on endothelial cell proliferation, retroviral pLncx carrying human endostatin gene was used to transfect human liver carcinoma cell line SMMC7721, and the supernatant of transfected SMMC7721 was collected to incubate with human umbilical vein endothelial cell (HUVEC) *in vitro*.

MATERIALS AND METHODS

Plasmids

The plasmid pUC19-Endo containing the cDNA for human endostain was generously provided by Professor Genxin Xu (Nanjing Military Medical College, Nanjing, China). In this plasmid, human endostain cDNA was put downstream from rat serum albumin signal peptide and influenza virus HA tag. Plasmid pUC19-Endo and retroviral pLncx were digested with HindIII and Cla Ⅰ respectively, and resulting products were ligated. The recombinant plasmid was designated as pLncx-Endo. Correct in-frame insertion of human endostatin cDNA was confirmed by electrophresis showing the pattern of pLncx-Endo digested with restriction enzyme.

Cell lines

Virus packaging cell PA317 and NIH3T3 cell lines were provided by Dr. Qian (Second Military Medical University, Shanghai, China). PA317 and NIH3T3 cells were maintained in DMEM supplemented with 100mL·L^{-1}fetal bovine serum, 100units/ml penicillin and 100ug/ml streptomycin. HUVEC was purchased from Shanghai Cellular Research Institute. SMMC7721 and HUVEC were maintained in 1640 medium.

Tranfection of PA317 cells and determination of viral titre

Plasmids of pLncx-Endo and pLncx were transferred into PA317 cells respectively by lipofectamine (Gibco) following the manufacturer's instructions. G418 selection at 500mg·L^{-1} was added at the same time. Two weeks after transfection, G418-resistant colonies emerged and were expanded. The supernatant of G418-resistant PA317 colony was collected and diluted to infect NIH3T3 cells with a final

concentration of polybrene at 2mg·L^{-1}. After transfection, NIH3T3 cells were also placed under G418 selection. Two weeks later, G418-resistent NIH3T3 colonies were counted for determination of viral titre.

Generation of stable transfectant

Total of 5×10^5 SMMC7721 cells were plated on 6-well plate and incubated for 24h. The cells were rinsed with serum-free 1640 medium twice, and 100μL supernatant of endostatin-transfected PA317 colony was added and incubated for 3h. Another 3mL 1640 medium was added with the final concentration of polybrene at 2mg·L^{-1} and G418 at 500mg·L^{-1}. Four weeks after transfection, G418-resistent cells were expanded for preservation and detected for endostatin-HA fusion protein by immunohistochemistry and Western blot analysis. The G418-resistent colony was designated as SMMC-Endo. Control transfectant (SMMC-pLncx) was generated in a similar way except that the parent plasmid pLncx-Endo was replaced by pLncx.

PCR amplification of endostatin gene

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

Immunohistochemical staining

Immunohistochemical staining was accomplished utilizing an avidin-biotin technique. Anti-HA monoclonal antibody was purchased from Jing Mei Biotechnology Co. Ltd. SMMC-Endo and SMMC-pLncx cells were grown on six-well glass slides and fixed in acetone. After washing in PBS, the cells were incubated with a 10m·L^{-1} H_2O_2 solution at room temperature for ten minutes to quench endogenous peroxidases. Nonspecific binding was blocked with 50mL·L^{-1} normal horse serum at room temperature for five minutes. The cells then were incubated with anti-HA at a 1:300 dilution at 4℃ overnight. Following washing in PBS, the secondary antibody, biotinylated anti-rat Ig G, was added and the cells were incubated at room temperature for an 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 ×400.

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 24h. The medium was replaced with 1ml serum-free RPMI 1640 and collected after 48h. One mL of conditioned medium was concentrated in a microconcentrator (Amicon, Berverly, MA) to 20μL and subjected to a 120g·L^{-1} reducing SDS/PAGE gel. Protein were transferred to a nitrocellulose membrane and incubated overnight in 50mL·L^{-1} nonfat milk in PBS at 4℃. After briefly washing in 10mL·L^{-1} nonfat milk, the membrane was incubated with anti-HA mouse monoantibody diluted 1:500. After three 10min washes in 10mL·L^{-1} nonfat milk, the membrane was incubated in horseradish peroxidate-conjudated antimouse immunoglobulin diluted 1:1000. After three 10min washes in TBS, the proteins were detected using the Amersham ECL kit.

Endothelial cell proliferation assay

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

RESULTS

Identification of a recombinant retroviral pLncx-Endo and determination of the recombinant virus titre

The plasmid pLncx and the recombinant retroviral pLncx-Endo were digested by Hind III and Cla Ⅰ respectively. Only in recombinant retroviral pLncx-Endo contained a 640-bp endostatin gene fragment separated by electrophoresis in 10g·L^{-1} agarose gel (Figure 1). It proved that the foreign endostatin gene together with signal peptide and HA-tag was correctly inserted in retroviral pLncx. After transfection of NIH3T3 cells with supernatant of endostatin-transfected PA317, NIH3T3 cells were maintained in DMEM supplemented with G418 500mg·L^{-1}. Two weeks later, total 34 colonies were detected under microscopy and the titre of the recombinant virus (pLncx-Endo) was 1.36×10^{8}cfu·L^{-1}.

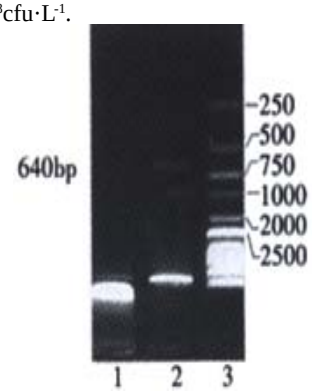

Figure 1 Identification of recombinant plasmids digested with restriction enzymes (Hind III and Cla Ⅰ)
1: pLncx plasmid digested with Hind III and Cla I;2: pLncx-Endo plasmid digested with Hind III and Cla I;3: DNA Marker.

Generation of stable transfectants

The PCR products amplified from DNA of SMMC-Endo and SMMC-pLncx cells were analyzed under ultraviolet light after 10g·L^{-1} agarose gel electrophoresis. A 550-bp fragment was seen in the PCR product from DNA of SMMC-Endo cells, but not from the control (Figure 2). It indicated that endostatin was successfully transferred into SMMC7721 cells by way of retroviral pLncx-Endo. The expression of endostatin in the transfected SMMC-Endo cells was also detected by immunohistochemical staining. A lot of brown granules were seen in endostatin-transfected SMMC-Endo cell cytoplasms while control SMMC-pLncx cells showing negative. Thus, it proved that endostatin

gene can be expressed stably in SMMC7721 cells (Figure3). Transgene expression was also tested by Western blot for the expressed protein. On a reducing 120g·L^{-1} SDS/PAGE gel, a distinct band at around M_r22000,corresponding to the size of endostatin, was visualized in the supernatant of SMMC-Endo cells but not in the supernatant of SMMC-pLncx cells. Monoclonal mouse anti-HA antibody reacted positively in a Western blot with the M_r22000 protein only. It was confirmed that endostatin could be efficiently secreted into the supernatant of cells transduced by retroviral pLncx-Endo (Figure 4).

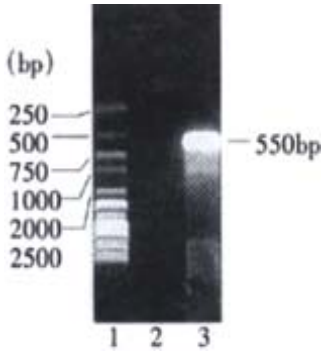

Figure 2 Analysis of PCR product of SMMC7721 transferred with pLncx-endo by 1% agarose gel electrophoresis.1: DNA Marker;2: PCR product of SMMC7721 cell DNA transferred with pLncx;3: PCR product of SMMC7721 cell DNA transferred with pLncx-endo

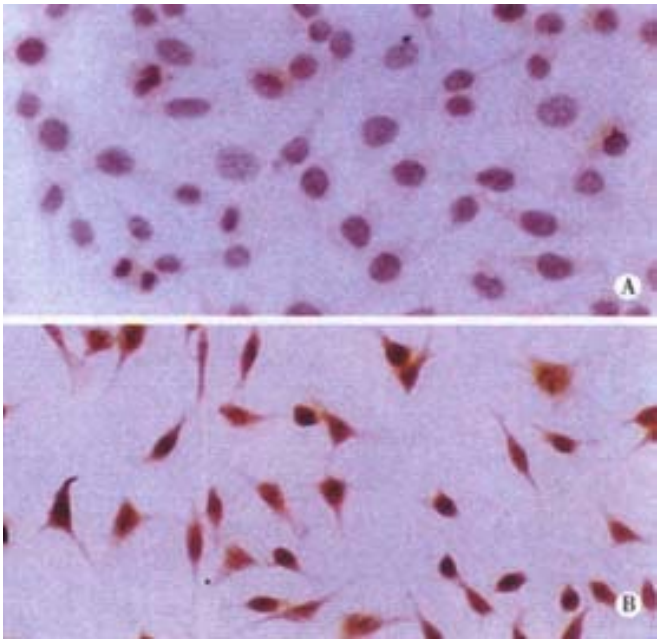

Figure 3 Expression of human endostatin-HA fusion protein in endostatin-transfected cells. Anti-HA monocolonal antibody was applied to SMMC7721 transferred with pLncx (A) and SMMC7721 transferred with pLncx-endo (B), followed by a HRP-conjugated secondary antibody. Hematoxylin counterstain. ×400.

Human endostatin inhibits endothelial cell proliferation

Three days after incubation with conditioned medium, cell number, as measured by absorance (OD), was quantified by using a colorimetric MTT assay. The results showed that the optical density in groups using concentrated conditioned medium from SMMC7721 cells, RPMI1640 and SMMC-pLncx cells were 1.01±0.09,0.98±0.09 and 0.88±0.1 respectively. It revealed that conditioned medium both from SMMC7721 cells and empty plasmid pLncx-transfected SMMC7721 cells did not have inhibitory effect on the growth of HUVEC, compared with 1640 medium (P>0.01). While the optical density in group using conditioned medium from endostatin-transfected SMMC-Endo cells was 0.51±0.06, significantly lower than that from SMMC-pLncx group (0.88±0.1). It meant that inhibitory rate on endothelial proliferation for conditioned medium from endostatin-transfected SMMC7721 group was 48%, significantly higher than that from control pLncx-transfected SMMC7721 group (10.2%, P<0.01), (Figure5).

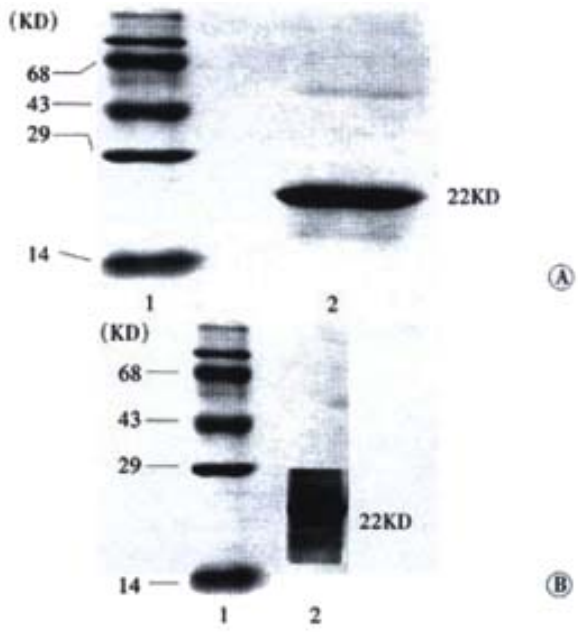

Figure 4 SDS-PAGE analysis and Western blot of endostatin expressed in supernatant of viral transduced SMMC7721cells(A) SDS-PAGE analysis; 1, protein marker; 2, supernatant of SMMC7721 cells transfected with pLNCX-Endo;(B) Western blot analysis; 1, protein marker; 2, supernatant of SMMC7721 cells transfected with pLNCX-Endo

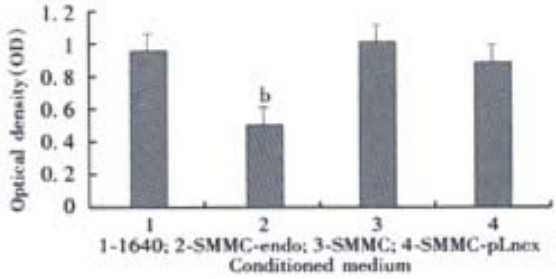

Figure 5 Inhibition of endothelial cell proliferation by conditioned medium from transfected and untransfected cells. Conditioned medium from endostatin-transfected SMMC-Endo cells (2), conditioned medium from SMMC7721 cells (3), and conditioned medium from SMMC-pLncx cells (4) were concentrated and applied to cultivate with 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. ^bP<0.01, compared with conditioned medium from control SMMC-pLncx cells.

DISCUSSION

It is well known that the growth and metastases of tumor is dependent on the formation of new blood vessel. The new blood vessel provides not only nutrient for tumor, but also the ways for excretion and metastases. Numerous studies have proven that tumor cells will stop growing or die when it exceeds 2mm to 3mm in diameter if new blood vessel for tumor is not formed[5,6]. So, anti-angiogenesis is one of the effective ways to inhibit and control the development of tumor by inducing tumor dormancy or apoptosis.

Endostatin is a new kind of potent antiangiogenic factor

consisting of 184 amino acids in C- terminal fragment of endogenous collagen 18a. It was isolated as a M_r22000 protein from conditioned medium of the EOMA murine hemangioendothelioma cell line by Professor O'Reilly in 1997[22-27]. *In vivo* and *in vitro* experiments have demonstrated that endostatin have specific inhibitory effect on tumor metastases and primary tumor with no observed sign of toxicity[6,28-33]. Furthermore, the genome of endothelial cell, targeted by endostatin, has a stable inherent property and rare mutation. So, there is no acquired resistance to endostatin during endostatin therapy. But the production of functional polypeptide has proven difficult because of its unstable physical property. In addition, antiangiogenic therapy with endostatin in cancer requires prolonged administration and high doses of the recombinant protein. It will result in heavy economic burden and inconvenience to recipients by repeated administrations. Therefore, transfer of foreign endostatin gene into host cells represents an alternative method to treat tumor by generating high efficient endostatin in areas around tumor[34-36]. The aims of generating a high efficient protein with no toxin and keeping a long time and relatively high expression of endostatin can be achieved by single administration[37]. A few groups have demonstrated that antiangiogenic gene therapy with viral vectors is a potentially useful approach for inhibiting tumor growth in mouse model[35,38-44]. By the way, gene transfer mediated by retroviral vectors is most commonly used among the various ways of transducing methods[45]. As retroviral vectors can be integrated into chromosome of host cells, gene transferred by retrovirus can be inherited to next generation and stably expressed in host cells. In this experiment, in order to explore the effect of endostatin on endothelial cell proliferation expressed by SMMC7721, endostatin gene was inserted into retroviral vector pLncx by recombinant technology and subsequently used to infect human liver carcinoma cell line SMMC7721. After transfection, PCR products and immunohistochemical staining showed that endostatin gene had been successfully transferred into and expressed in endostatin-transfected SMMC7721 cells. For the purpose of the protein expressed by SMMC7721 cells being excreted outside the cell, rat albumin signal peptide, which can lead to the expressed protein being secreted outside cell while without any effect on the activity of the protein, was put into the upstream of endostatin gene during the construction of recombinant plasmid[6,28,46]. The effect of signal peptide was also demonstrated by Western blot analysis, which revealed that endostatin protein did exist in the concentrated supernatant of endostatin-transfected SMMC7721 cells. The endothelial cell proliferation assay indicated that conditioned medium from endostatin-transfected SMMC7721 cells significantly inhibited the proliferation of endothelial cell by 48%, compared to conditioned medium from control SMMC7721 cells transferred with empty plasmid pLncx. In another word, endostatin expressed by SMMC7721 cells can remarkably inhibit the proliferation of endothelial cell. In conclusion, gene therapy with endostatin mediated by retrovirus is effective *in vitro*,and perhaps it might also have a significant inhibitory effect on tumor growth *in vivo*, but that remains to be confirmed by further experiments.

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