



CLINICAL RESEARCH

Expression of angiostatin cDNA in human gallbladder carcinoma cell line GBC-SD and its effect on endothelial proliferation and growth

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Abstract

AIM: To explore the influence of angiostatin up-regulation on the biologic behavior of gallbladder carcinoma cells *in vitro* and *in vivo*, and the potential value of angiostatin gene therapy for gallbladder carcinoma.

METHODS: A eukaryotic expression vector of pcDNA3.1(+) containing murine angiostatin was constructed and identified by restriction endonuclease digestion and sequencing. The recombinant vector pcDNA3.1-angiostatin was transfected into human gallbladder carcinoma cell line GBC-SD with Lipofectamine 2000, and paralleled with the vector and mock control. The resistant clone was screened by G418 filtration. Angiostatin transcription and protein expression were examined by RT-PCR, immunofluorescence and Western-blot. The supernatant was collected to treat endothelial cells. Cell proliferation and growth *in vitro* were observed under microscope.

RESULTS: Murine angiostatin cDNA was successfully cloned into the eukaryotic expression vector pcDNA3.1(+). After 14 d of transfection and selection with G418, macroscopic resistant cell cloning was formed in the experimental group transfected with pcDNA 3.1(+)-angiostatin and vector control. But untreated cells died in the mock control. Angiostatin was detected by RT-PCR and protein expression was detected in the experimental group by immunofluorescence and Western-blot. Cell proliferation and growth *in vitro* in the three groups were observed respectively under microscope. No significant difference was observed in the growth speed of GBC-SD cells between groups that were transfected with and without angiostatin. After treatment with supernatant,

significant differences were observed in endothelial cell (ECV-304) growth *in vitro*. The cell proliferation and growth were inhibited.

CONCLUSION: Angiostatin does not directly inhibit human gallbladder carcinoma cell proliferation and growth *in vitro*, but the secretion of angiostatin inhibits endothelial cell proliferation and growth.

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Key words: Angiostatin; Gallbladder carcinoma; Endothelial cell

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INTRODUCTION

Gallbladder carcinoma is the most common malignant tumor of the biliary tract and a particularly high incidence is observed in Chile, Japan, and northern India. After common treatment, the prognosis of gallbladder carcinoma patients is poor^[1].

A large body of work by a number of laboratories in the past 3 decades has provided both direct and indirect evidence that tumor growth and metastasis are accompanied with the growth of new blood vessels^[2].

As previously reported, angiostatin is a potent antiangiogenic endogenous protein^[3]. In the present study, we constructed a mammalian expression vector that was cloned as an angiostatin gene. Following stable transfection of this vector into human gallbladder carcinoma cell line GBC-SD, we observed the expression of angiostatin and its antiangiogenic effect by evaluating the influence of cultured human umbilical vein endothelial cells (ECV-304).

MATERIALS AND METHODS

Cell lines and reagents

The human gallbladder carcinoma cell line GBC-SD and the endothelial cell line ECV-304 were purchased from

China Center for Type Culture Collection. EcoRI, XbaI, PmeI, HindIII, XbaI and PvuI were purchased from Bio-son Corporation. LipofectamineTM 2000 and G418 were purchased from Gibco Company. Recombinant eukaryotic expression vector pcDNA3.1(+)-angiostatin and rabbit anti-HA tag monoclonal antibody were presented by Dr. Ji-cheng Zhang. Trizol reagent, reverse transcriptase, and Taq DNA polymerase were purchased from Life Technologies, Inc. FITC was purchased from Boster Biological Technology Co.

Cell culture

Gallbladder carcinoma cells and endothelial cells were cultured in RPMI1640 (Gibco) supplemented with 10% fetal bovine serum (FBS) (Sijiqing, Hangzhou, China), penicillin (100 units/mL) and streptomycin (100 mg/mL) in a humidified atmosphere containing 5% CO₂ at 37 °C.

Recombinant eukaryotic expression vector pcDNA3.1(+)-angiostatin construction

Mouse angiostatin cDNA fragment encoding for the NH₂-terminal secretory signal sequence(SS) and kringle1-4(K1-4) regions of mouse plasminogen, fused with an antigenic epitope tag HA(HA tag) to the COOH terminus of kringle 4, was inserted into eukaryotic expression vector pcDNA3.1(+). The structure of the recombinant vector pcDNA3.1(+)-angiostatin was confirmed by restriction endonuclease digestion and sequencing. Recombinant clones were identified by HindIII, XbaI and PmeI digestion. Positive clones were further confirmed by sequencing.

Transfection of pcDNA3.1-angiostatin into GBC-SD

Gallbladder carcinoma cells (GBC-SD) in logarithmic growth phase were plated on 24-well plates at 2×10^5 cells/well, and approximately 80% confluence was obtained after overnight incubation. Cultured GBC-SD cells were divided into three groups: transfected with recombinant pcDNA3.1-angiostatin (group A), pcDNA3.1 (+) vector (group B) and without pcDNA3.1 (+) vector (group C). Transfection was performed according to the instructions of LipofectamineTM 2000 reagent kit (Gibco). The cells were then cultured in complete medium containing G418 (50 mg/L) for 14 d. G418-resistant pooled cells were subjected to further studies. Then the isolated resistant cell clones were selected and amplified. Cell growth curve was also plotted.

Analysis of angiostatin transcript by RT-PCR

Total RNA was isolated from GBC-SD cells using Trizol reagent. Reverse transcription was performed with 1 µg of total RNA in a total volume of 20 µL containing reverse transcriptase. The PCR reagents, including 0.5 units of Taq DNA polymerase, were added to a final volume of 25 µL. A 35-cycle amplification profile consisted of denaturation at 94 °C for 1 min, annealing at 56 °C for 1 min, and extension at 72 °C for 1 min. Primers used in PCR were designed according to the reported angiostatin cDNA sequence. The primer sequences were as follows: 5'end: 5'-CCCAACATGGACCATAAGGAAGT-3' and 3'end: 5'-TGTGGGCAATTCCACAACACTC-3'. Human-actin primers were used as positive controls. Negative controls

without RNA and reverse transcriptase were also assessed. The PCR products were identified by 1% agarose gel electrophoresis.

Analysis of angiostatin protein by Western blot and immunofluorescence cytochemistry

GBC-SD cells with pcDNA3.1(+)-angiostatin in logarithmic growth phase were implanted into 6-well plates. After 24 h, the glass flake was taken out and immunofluorescence cytochemistry was carried out. For immunofluorescence, cells on glass flake were fixed for 15 min in 3.7% formaldehyde in PBS containing 1 mM EGTA at room temperature. After fixation, the cells were made permeable by incubating with 0.2% Triton X-100 in PBS for 15 min, and then washed with PBS. After being blocked with PBS including 1% BSA, antibodies were applied and incubated for 1 h at 37 °C. The cells were then stained with secondary antibodies. After being washed with PBS, the expression was detected under fluorescent microscope.

The cells in the three groups were cultured in conditioned media for 5 d. Cell supernatant was mixed with lysine-sepharose and incubated at 4 °C overnight. The resin was washed with Tris-HCl (pH8.0) and protein was eluted. Protein concentration was determined by bicinchoninic acid assay (BCA) with bovine serum albumin as standard. Equal aliquots (40 µg) of protein from cell supernatants were subjected to electrophoresis on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel. Proteins were transferred onto PVDF membranes (mMILLIPORE) using the transfer buffer for 2 h. The membranes were then blocked for 1.5 h using 5% nonfat dried milk in phosphate-buffered saline containing 0.1% Tween 20 (PBS-T), washed with PBS-T, and incubated at 4 °C overnight in the presence of anti-rabbit HA-tagged antibody. The membranes were washed with PBS-T and incubated with secondary peroxidase-conjugated anti-rabbit immunoglobulin G for 1 h. Following washing with PBS-T, immunoreactive bands were visualized by the enhanced chemiluminescence system (Amersham Life Science).

Viable cell number counting and growth curve

In logarithmic growth phase of the three groups of gallbladder carcinoma cells (GBC-SD), the supernatant was collected. Endothelial cells (ECV-304) were plated onto 24-well plates at 1×10^4 cells/well and ECV-304 cells were treated with supernatant separately for 7 d (supernatant/medium=1/9). Viable cells were counted under microscope everyday and growth curves were plotted.

Statistical analysis

Data were analyzed by statistical software of SPSS 10.0. Differences between groups were examined by Student's *t* test. *P* < 0.01 was considered statistically significant.

RESULTS

Identification of recombinant plasmid pcDNA3.1(+)-angiostatin

The recombinant plasmid was released by restrictive digestion with HindIII, XbaI and PmeI. As shown in electro-

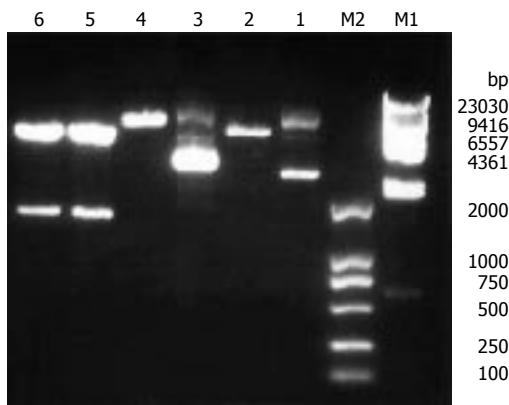


Figure 1 Identification of angiotensin cDNA insertion in pcDNA3.1(+)-angiotensin. M1: λ DNA/Hind III marker; M2: DNA marker DL2000; lane 1: pcDNA3.1(+)-plasmid; lane 2: pcDNA3.1(+)-EcoRI; lane 3: pcDNA3.1(+)-angiotensin; lane 4: pcDNA3.1(+)-angiotensin/XbaI; lane 5: pcDNA3.1(+)-angiotensin/PmeI; lane 6: pcDNA3.1(+)-angiotensin/ Hind III +XbaI.

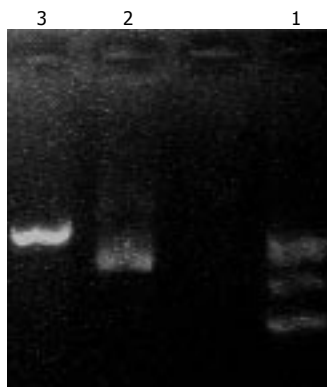


Figure 2 pcDNA3.1(+)-angiotensin linearization. Lane 1: λ DNA/Hind III marker; lane 2: pcDNA3.1(+)-angiotensin; lane 3: pcDNA3.1(+)-angiotensin /PvuI.

phoresis, the linear recombinant plasmid was about 7.0kb, being a fragment of 1.4kb, suggesting that angiotensin fragment was inserted into the pcDNA3.1 (+) vector, named recombinant plasmid pcDNA3.1- angio (Figure1). The sequence obtained was the same as the reported sequence of angiotensin cDNA, indicating that the murine gene was successfully cloned into the eukaryotic expression vector pcDNA3.1 (+).The recombinant plasmid was linearized by *pvu*I(Figure 2).

Vector-mediated expression of angiotensin in vitro

Gallbladder carcinoma cells (GBC-SD) transfected with the corresponding vectors were selected by G418 for 14 d. The transfected pcDNA3.1-angiotensin and vector control groups formed macroscopic cell clones (Figure 3), but the mock group of cells was completely dead after 8 d of selection.

Angiotensin expression in transfected cells

GBC-SD cells transfected with pcDNA3.1- angiotensin were prepared. Total RNA was extracted and used as the template. A band was detected at 1.4 kb with PCR using angiotensin primers, indicating the presence of angiotensin cDNA in the GBC-SD cancer cells (Figure 4).

Angiotensin protein expression in vitro

Immunofluorescence showed signals of angiotensin in the

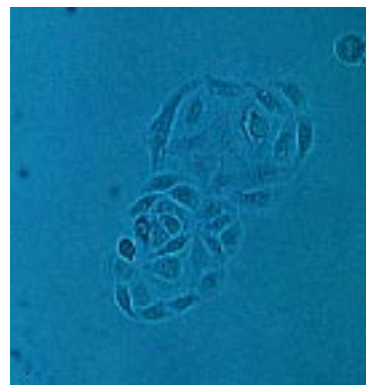


Figure 3 Gallbladder carcinoma cell clones transfected with pcDNA3.1(+)-angiotensin. $\times 400$

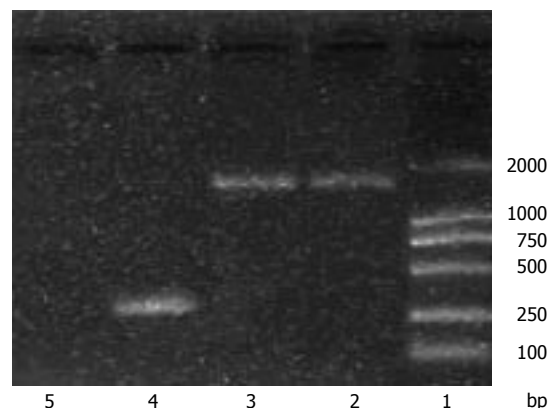


Figure 4 Analysis of angiotensin transcript by RT-PCR. Lane1: DNA marker DL2000; lanes 2, 3: angiotensin; lane 4: positive control; lane 5: negative control.

experimental group of cell clones genetically engineered but not in the controls (Figure 5A). Supernatants of cultured cells in three groups were collected and analyzed by Western blotting. A band in a molecular mass of 58000 kb was detected with rabbit anti-HA-tagged antibody from the cells transfected with pcDNA3.1-angiotensin, but no band was detected in mock control group or vector control group (Figure 5B).

Biological activity of angiotensin protein expression in vitro

To detect the biological activity of encoded angiotensin *in vitro*, tumor cells transfected with or without the corresponding vectors were cultured for 7 d to plot cell growth curve (Figure 6A). Under microscope, no obvious difference was observed in the cell morphology of the three groups of cells. Cell growth curves indicated no change in cell growth speed and doubling time in the three groups. These results indicate that up-regulated angiotensin expression could neither directly inhibit cell growth and proliferation nor affect cell cycle *in vitro*. But the biological activity of cells treated with supernatant of GBC-SD/ pcDNA3.1(+)-angiotensin and cell growth were obviously inhibited. The cell growth curve of the vector and mock control groups was similar (Figure 6B). These results indicate that angiotensin expression could directly inhibit endothelial cell growth and proliferation *in vitro*.

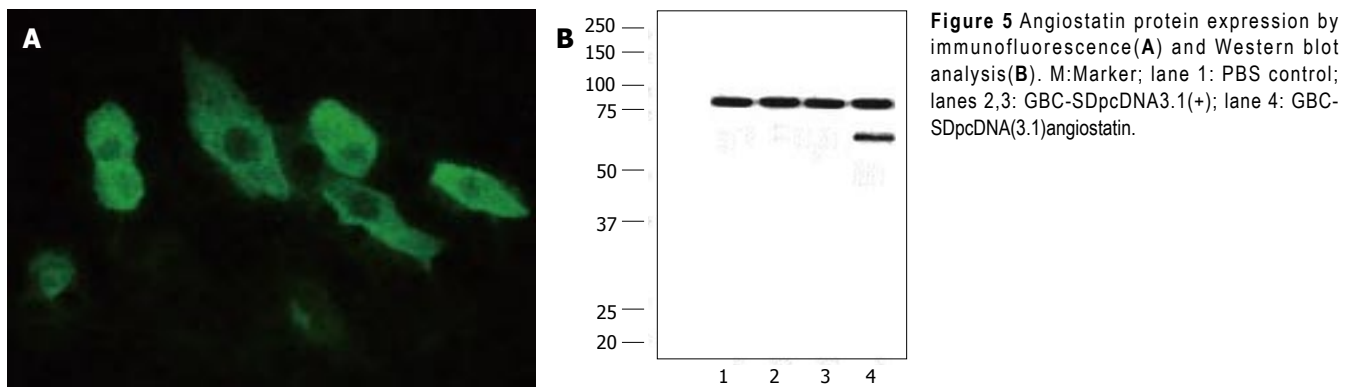


Figure 5 Angiostatin protein expression by immunofluorescence(A) and Western blot analysis(B). M:Marker; lane 1: PBS control; lanes 2,3: GBC-SDpcDNA3.1(+); lane 4: GBC-SDpcDNA(3.1)angiostatin.

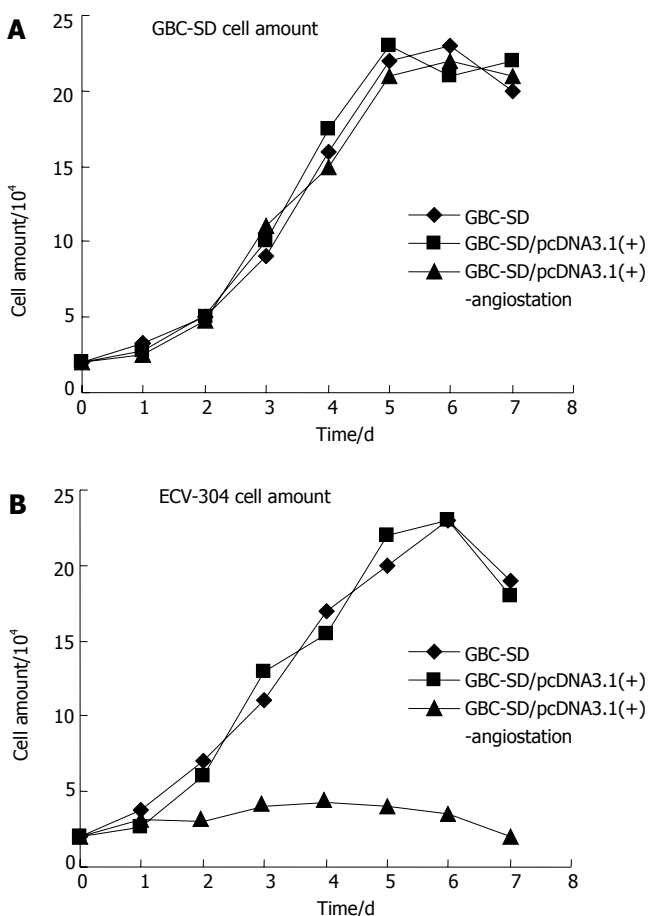


Figure 6 Cell growth curves of GBC-SD(A) and ECV-304(B).

DISCUSSION

Angiogenesis refers to the formation of new blood vessels. Since Dr. Folkman^[15] raised the hypothesis of tumor angiogenic dependence, experimental evidence has validated that tumor growth companies new blood vessel growth. At the prevascular stage, tumor is unable to grow to a size beyond 2-3 mm³ and remains in its dormant state. However, once the angiogenic phenotype of tumor is switched on, tumor growth rate changes from linear to exponential^[4-6]. Infiltration of new blood vessels in tumors not only supplies nutrients and oxygen for tumor cells, but also removes the waste products produced by tumor cells. In addition, endothelial cells can communicate directly with tumor cells by producing tumor growth promoting factors.

Angiogenesis begins when a fibrin clot forms on the adventitial surface of an existing blood vessel^[7], which is followed by sprouting of new capillaries. The initial phase begins with increased vascular permeability and local degradation of the vessel wall. Endothelial cells enter the tumor stroma and proliferate. At this time, the cells may be most vulnerable to agents that interfere with their proliferation, since they lack protection from other cell types^[8]. The next step in vessel formation is recruitment of pericytes, followed by smooth muscle cells.

It appears that a switch to the angiogenic phenotype requires a local change in the balance between angiogenic factors and inhibitors^[9]. Among the angiogenic factors, fibroblast growth factor (FGF)^[10] and vascular endothelial growth factor (VEGF)/vascular permeability factor (VPF)^[11] are most commonly expressed in tumors. Others are inhibitors such as angiostatin^[12], thrombospondin-1^[13], 16-kd prolactin fragment^[14], interferon- α and β ^[15], and endostatin^[16]. Because cancer cannot grow or spread without the formation of new blood vessels^[17], scientists are trying to find ways to stop angiogenesis.

Tumor angiogenesis is often the consequence of an angiogenic imbalance in which pro-angiogenic factors predominate over anti-angiogenic factors^[5]. Furthermore, angiogenesis is essential for growth and metastasis of most solid cancers^[18]. Gallbladder carcinoma is not a grossly vascular tumor, but is related to angiogenesis^[19]. Antiangiogenic treatment may be necessary and potential for gallbladder carcinoma.

Angiostatin is an internal fragment of plasminogen and may contain either the first three (K1-3) or four (K1-4) kringle domains^[20]. A similar activity has been reported for kringle 5 (K-5) of plasminogen^[21]. Three receptors of angiostatin on endothelial cells have been reported to date. Adenosine triphosphate (ATP) synthase is one of the receptors. The presence of this typically mitochondrial enzyme on the endothelial cell surface is somewhat surprising^[22]. Two other potential target receptors of angiostatin are angiomotin^[23] and integrin $\alpha(v)\beta(3)$ ^[24]. These receptors on the surface of endothelial cells may be located at sites so proximal to one another that angiostatin may be able to interact with more than one simultaneously^[25]. Alternatively, multiple targets could be implicated in binding to different kringles of angiostatin as a function of receptor and peptide presentation^[26].

Although angiostatin is a potent inhibitor of angiogenesis and tumor growth^[27], the need for high doses, repeated injections and long-term administration of this protein

have made it less attractive for clinical trials^[28]. In order to develop alternative strategies for therapy, we have investigated the possibility of angiostatin gene therapy. When mouse angiostatin cDNA was transfected into human gallbladder carcinoma cell line GBC-SD and endothelial cell line (ECV-304), angiostatin cDNA was present in cancer cells, and a secreted form of angiostatin was established by immunofluorescence and Western-blot. Although angiostatin had no direct effect on gallbladder carcinoma cell growth *in vitro*, the supernatant of ECV-304 cells inhibited the growth of endothelial cells *in vitro*. Xu *et al*^[29] engineered a recombinant adeno-associated virus (AAV) vector encoding mouse angiostatin and found that it suppresses metastatic liver cancer in mice. In our study, similar results were achieved using Lipofectamine 2000 for transfection. These results support that angiostatin-gene therapy is a potential strategy in the treatment of gallbladder carcinoma.

In conclusion, angiostatin has no direct effect on gallbladder carcinoma cell growth *in vitro*, but inhibits the growth of ECV-304 cells *in vitro*.

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