Inhibition effect of pcDNA-tum-5 on the growth of S180 tumor

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Abstract Tumor growth and metastasis depend on vessel formation, and inhibition of angiogenesis of tumor by production of anti-angiogenic drugs should be a promising approach for cancer therapy. Tumstatin is an angiogenesis inhibitor. The anti-angiogenic activity of tumstatin is localized to the 54–132 amino acids. The gene fragment encoding amino acids 45–132 of tumstatin (tum-5) was subcloned into pcDNA3.1 (pcDNA-tum5). Tum-5 protein could be expressed and secreted in CHO cells after transfection. The conditioned medium (containing tum-5 protein) from the transfectant has an anti-angiogenic effect on HUVEC cells in vitro. The anti-tumor effect of pcDNA-tum5 on mice bearing S180 tumors was evaluated. The results showed that pcDNA-tum-5 has a significant inhibition activity in the growth of the

tumors. This study suggests that the gene delivery of tum-5 may be an effective strategy for cancer therapy.

Keywords Tumstatin · Gene therapy · Anti-tumor effect · Anti-angiogenic effect

Introduction

Angiogenesis is required for a variety of physiological processes such as development, wound repair, and tissue regeneration. Moreover, it is also one major pathogenic step in the process of tumor growth and metastasis (Folkman et al. 1995). Because nonmalignant endothelial cells (EC) are genetically stable and unlikely to mutate into drug-resistant variants, tumor angiogenesis has become an important area in cancer treatment (Denekamp et al. 1993). Several anti-angiogenic proteins have been identified that suppress the growth of cancer (Cameliet et al. 2000). Tumstatin, the NC1 domain of $\alpha 3$ chain of type IV collagen, was identified as possessing anti-angiogenic activity (Maeshima et al. 2000a, Petitclerc et al. 2000). The anti-angiogenic activity of tumstatin was localized to the 54–132 amino acid region (tum-5) by using deletion mutagenesis (Maeshima et al. 2000a). Subsequently, the anti-angiogenic activity was localized to a 25 amino acid region encompassing amino acids 74–98, which contains the entire anti-angiogenic activity associated with tumstatin. Tum-5 domain

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binds and functions via $\alpha v\beta 3$ in an RGD independent manner (Maeshima et al. 2001). The full-length tumstatin and fragments containing regions 54–132 and 74–98 amino acids exhibit significant anti-angiogenesis activity in Matrigel plug assays. They suppress tumor growth associated human renal carcinoma (786-O) and prostate carcinoma (PC-3) in mouse xenograft models and induced endothelial cell specific apoptosis (Maeshima et al. 2000a; Maeshima et al. 2000b; Maeshima et al. 2001).

To construct a novel anti-tumor agent, the gene fragment encoding the amino acids 45–132 of tumstatin was subcloned into pcDNA3.1 expression vector. We investigated the anti-angiogenic effect and the anti-tumor activity of pcDNA-tum-5 on mice bearing S180 sarcoma tumor (Ma et al. 2007). The results showed that pcDNA-tum-5 has an inhibition effect on the formation of vessels and tumor growth.

Materials and methods

Making pcDNA-tum-5 constructs

The pBluescript-tum-5(containing the region of tum-5) was used as a template. PCR amplification was performed by using a standard protocol with the primers: 5'-CCCAAGCTTACCATGAAACATCTGTG-3'(upstream); 5'-CGCGGATCCCTAGGCGATCGCAGGACCTTCA C-3'(downstream). *Bam*H I and *Hind*III restriction sites were introduced to the primers respectively. The resulting PCR products were digested with *Hind*III and *Bam*HI, purified, and inserted into pcDNA3.1 vector. The recombinant plasmid (pcDNA-tum-5) was confirmed by sequencing.

Animal, cell lines and polyclonal rabbit anti-tum-5 antibody

BALB/c mice (4–6 weeks) were fed under specific pathogen-free conditions (The animal experiment was approved by an Institutional Animal Care and Use Committee in China). CHO and S180 cells were maintained in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 37 °C in 5% CO₂. All cell lines were purchased from the National Rodent Laboratory Animal Resource

(Shanghai, China). Polyclonal rabbit anti-tum-5 antibody was a kind gift from the Immunology Department of the Fourth Military Medical University, China.

Generation of stable transfectants

Tum-5 transfectants were generated by transfection of pcDNA-tum-5 into CHO cells using Lipofect-AMINE 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Two days later, the cells were placed under G418 (Gibco, Paisley, UK) selection at 800 µg/mL with replacement of the medium every 2 days. Two weeks after transfection, G418-resistant colonies were expanded. The expression and secretion of tum-5 were identified by RT-PCR and Western blot. Control transfectants were generated in a similar manner.

RT-PCR analysis

Total cellular RNA was extracted with Trizol Reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions, and cDNA was prepared from 1 µg of total RNA by reverse transcription using RevertAid First Strand cDNA Syntheses kit (MBI Fermentas, Fermentas AB, Lithuania) according to the user manual and amplified by PCR using the primers as described above.

Western blot analysis

Stable transfectants were incubated in DMEM supplemented with 10% FBS and 800 µg/mL G418. The cells were rinsed with phosphate-buffered saline (PBS) at 80% confluence and incubated in serum-free medium for 2 days. The conditioned medium was harvested, cleared of cell debris, and concentrated 20-fold with Milipore membranes (Millipore, Billerica, MA) with a Mr 5,000 cut-off. Total protein from the conditioned media was subjected to electrophoresis under reducing conditions on an 18% polyacrylamide gel, transferred onto a polyvinylidene difluoride membrane, and incubated overnight in PBS containing 5% bovine serum albumin (BSA) at 4 °C. After briefly washing with 0.1% Tween 20 (Sigma, St. Louis, MO)

in PBS, the membrane was incubated with polyclonal rabbit anti-tum-5 antibody diluted 1:5,000. After washing with 0.1% Tween 20 in PBS, the membrane was incubated in goat anti-rabbit horseradish peroxidase-conjugated IgG (Amersham, Arlington Heights, IL) at a dilution of 1:5,000. The expression of tum-5 protein was detected using the enhanced ECL kit (Amersham, Arlington Heights, IL).

Proliferation of transfected CHO cells (stable transfectants) in vitro

CHO cells were trypsinized and plated onto 96-well plates at a density of 2,000 cells per well in triplicate, and then cultured at 37 °C in 5% CO₂. At designed time point, cells were placed in DMEM without phenol red containing 5 mg/mL MTT (Sigma, St. Louis, MO) for 4 h. After removal of the media, formazan crystals were dissolved with 150 µL by vigorous shaking. Optical density of each well was determined using an automatic plate reader at 490 nm wave length. All experiments were conducted in triplicate.

Assay of tum-5 protein concentration

Assay of protein concentration was performed using indirect competitive ELISA method as described previously (Hurley et al. 2004). Briefly, ELISA plates were coated overnight at 4 °C with 100 µL/well tum-5 protein (Meng et al. 2006) at optimized concentration of 0.2 µg/mL in PBS. The plates were blocked with 250 µL/well PBS containing 3% BSA. After washing with 0.1% Tween 20 in PBS, the plate was incubated with 50 µL/well of polyclonal rabbit anti-tum-5 antibody optimally diluted 1:5,000 and 50 µL/well conditioned medium containing tum-5 protein as competitor. After washing 3 times, the plate was incubated with 100 µL/well goat anti-rabbit horseradish peroxidase-conjugated IgG at an optimal dilution of 1:4,000 in PBS. After washed another 3 times, the plate was then incubated for 15 min with 100 µL/well of the O-phenylenediamine (0.4% W/V) and 2 mM H₂O₂ as substrate. The reaction was stopped by addition of 50 µL/well of 2M sulfuric acid, and the absorbance was measured at 490 nm on an automatic plate reader.

Endothelial cell proliferation assay

HUVEC cells were trypsinized and plated onto 96-well plates at a density of 5,000 cells per well for 24 h, and then placed in 200 µL conditioned medium from the stable transfectants per well. The plates were incubated for 72 h at 37 °C in 5% CO₂. The numbers of cells were quantified using MTT assay described above. The test was performed in quadruplicate.

Endothelial tube assay

Matrigel (Collaborative Biomedical, Bedford, MA) was added (100 µL) to each well of a 96-well plate and allowed to be polymerized. HUVEC cells, were seeded into the plate at a density of 5,000 cells per well and treated with the conditioned medium containing tum-5 at 37 °C for 24 h., and viewed using a CK2 Olympus microscope. Tests were performed in triplicate.

Migration assay

The effect of conditioned medium containing tum-5 on the migration of endothelial Cells was tested. HUVEC cells were trypsinized, resuspended and seeded onto the upper wells of Millicell Insert (8-mm pore size, 6.5-mm diameter; Millipore, Billerica, MA, US) at a density of 100,000 per well in 200 µL conditioned medium from stable pcDNA-tum-5 or pcDNA3.1 transfectants. Lower wells of the Millicell Insert contained 600 µL DMEM with 10% FBS. After 12 h, membranes were fixed with methanol and stained with crystal violet before counting under phase-contrast microscopy. The result of the inhibition of EC migration was measured by counting the number of migrated cell in five random non-overlapped 100 × view fields. The experiment was performed in triplicate.

Expression of tum-5 in muscle tissues

Mice were randomly divided into 2 groups (pcDNA-tum-5 and pcDNA 3.1, *n* = 3 for each). Each mouse received one muscle injection in leg (100 µg plasmid). After 3 days, muscle tissues injected with

pcDNA-tum-5 or pcDNA 3.1 were excised from animals. The expression of tum-5 protein was analyzed by RT-PCR as described above.

Animal studies

Tumors were generated by injection of 5×10^5 S180 cells in 100 μ L PBS into right flank subcutaneously of 4–6 weeks old healthy BALB/c mice. When the tumors grew an average volume of about 4 mm³, the mice were divided randomly into 4 groups ($n = 8$ for each group): 0.9% sodium chloride only (control), pcDNA 3.1, pcDNA-tum-5 and Cyclophosphamide (CTX). Mice were treated with pcDNA 3.1 (100 μ g/day), pcDNA-tum-5 (100 μ g/day), or CTX (20 mg/kg). Each mouse received 6 muscle injections in legs every 3 days. Tumor width (W) and length (L) were measured every 3 days with calipers. The tumor volume (TV) was determined by the following formula: $TV (mm^3) = (L \times W^2)/2$. Mice were sacrificed after 18 days and the tumor was excised from each mouse and weighed. The inhibition rates of growth of S180 xenografted tumors were calculated according to the formula: $\text{inhibition rate}(\%) = (1 - \text{tumor weight in test group} / \text{tumor weight in control}) \times 100\%$. This experiment was performed in triplicate.

Statistics

Data were analyzed by Student's test. Results were expressed as the means $\pm$ standard deviations (SD). Differences were considered significant when the p value was less than 0.05.

Results

Generation of stable transfectants secreting tum-5

Tum-5 sequence was inserted into a mammalian expression plasmid pcDNA 3.1, and the resulting plasmids were designated pcDNA-tum-5 (Fig. 1a). Two plasmids, pcDNA-tum-5 and pcDNA 3.1(control), was transfected into CHO cells using LipofectAMINE 2000 reagent. All the vectors contain aminoglycoside phosphotransferase II gene, which confers resistance to aminoglycosides such as

Fig. 1 Construction and expression of tum-5. (a) Construction of pcDNA-Tum-5 (b) Total RNA was isolated from clones selected by G418 resistance and analyzed by RT-PCR using specific primers to detect the expression of tum-5. RNA from CHO cells transfected with pcDNA3.1 (Lane 1). CHO cells transfected with pcDNA-tum-5 (Lane 2). (c) The expression of tum-5 protein in the conditioned medium from CHO cells transfected with pcDNA-Tum-5 (Lane 2) was detected by Western blot. The conditioned medium from CHO cells transfected with pcDNA 3.1 (Lane 1) was used as control

Geneticin (G418), allowing the selection of stable transfectants. The expression and secretion of tum-5 were identified by RT-PCR (Fig. 1b) and Western blot analysis (Fig. 1c).

Proliferation ability of stable transfectants secreting tum-5

In order to test the proliferation ability of the stable expression tum-5 transfectants in vitro, MTT assay was performed. The result showed that tum-5 did not influence the growth of the host cells in vitro (Fig. 2) after transfection.

Assay of tum-5 protein in conditioned medium

Indirect competitive ELISA was used to determine the concentration of tum-5 protein secreted in conditioned medium. From the established standard curve (Fig. 3) and regression equation ($y = -15.05 \ln(x) + 113.07$,

$R^2 = 0.97$. $y(\text{inhibition rate}) = B(\text{absorption value with competitor tum-5 addition}) \cdot B_0(\text{absorption value without competitor addition})^{-1}$; $x = \rho[\text{tum-5}]/(\text{ng} \cdot \text{mL}^{-1})$. The concentration of tum-5 protein secreted in conditioned medium was 547 ng/mL.

Fig. 2 Proliferation of the stable transfectants in vitro. The pcDNA-tum-5 transfected CHO cells were plated onto 96-well plates. Three days later, cell numbers were quantified by using a colorimetric MTT assay. The pcDNA 3.1 transfected CHO cells and untransfected CHO cells were used as control

Fig. 3 Standard curve for the optimized indirect competitive ELISA for tum-5 protein. Plates were coated with 0.2 μg/mL tum-5 protein. The primary antibody-polyclonal rabbit anti-tum-5 antibody optimally diluted 1:5,000; secondary antibody-goat anti-rabbit horseradish peroxidase-conjugated IgG at an optimal dilution of 1:4,000

Tum-5 inhibiting endothelial cell proliferation

The biological activity of secreted tum-5 was tested against HUVEC cells. The conditioned medium from the pcDNA 3.1 transfectant did not inhibit HUVEC cell growth as compared with HUVEC cells with DMEM alone. The conditioned medium from pcDNA-tum-5 transfectants significantly inhibited HUVEC cell proliferation by 39% ($p < 0.05$), compared with conditioned medium from the pcDNA 3.1 transfectants (Fig. 4).

Effect of tum-5 on the tube formation of endothelial cells

When HUVEC cells are cultured on Matrigel matrix, they rapidly align and form hollow tube-like structures. The conditioned medium containing tum-5 significantly inhibited endothelial tube formation compared with control (Fig. 5).

Tum-5 inhibiting migration of endothelial cells

Angiogenesis is highly dependent on endothelial cell motility and invasion. Therefore, we examined the effects of tum-5 on migration of HUVEC cells. After 12 h incubation, migration of HUVEC cells induced by conditioned medium containing tum-5 was reduced by about 34% compared to that by conditioned medium from the control pcDNA3.1 transfectant (Fig. 6).

Fig. 4 Inhibition of endothelial cell proliferation by the conditioned medium from the transfected cells. HUVEC cells were incubated with the conditioned medium from CHO cells transfected with pcDNA-tum-5 or pcDNA3.1. Three days later, cell numbers were quantified by using a MTT assay

Fig. 5 Tum-5 inhibiting endothelial tube formation. HUVEC cells were allowed to form tubes on Matrigel-coated plates incubated with the conditioned medium and viewed at 100 $\times$ original magnification

Fig. 6 Tum-5 inhibiting migration of endothelial cells. Cells migrated through the membrane of Millicell Inserts were counted in five random non-overlapped 100 $\times$ view fields

Fig. 7 Expression of tum-5 protein after muscle injection. *in vivo*. The muscle tissues injected pcDNA-tum-5 or pcDNA3.1 were removed 3 days after injection. Total RNA was extracted and the expression of tum-5 was detected by RT-PCR using specific tum-5 primers. Muscle samples injected with pcDNA3.1 (Lane1–3). Muscle samples injected with pcDNA-tum-5 (Lane 4–6)

Expression of tum-5 in muscle tissues injected with pcDNA-tum-5

We examined the expression of tum-5 in muscle tissues injected with pcDNA-tum-5. The RT-PCR results showed that tum-5 expression was detectable in muscle tissues in pcDNA-tum-5 group, whereas no expression was detected in control (Fig. 7).

Anti-tumor effect of pcDNA-tum-5

To investigate the effect of pcDNA tum-5 on tumor growth, the plasmid was injected into BALB/c mice

Fig. 8 Inhibition effect of pcDNA-tum-5 on tumor growth. Mice were separated into four groups: control, pcDNA-tum-5, pcDNA3.1 and CTX. Tumor volume was measured every 3 days. At the end of the treatment, all mice were sacrificed, tumor weight was determined (a) and the inhibition rates (b) were calculated. There is a significant difference between pcDNA-tum-5 treated group and pcDNA3.1 control group ($p < 0.05$)

bearing S180 tumors. A significant anti-tumor effect of tum-5 was observed ($p < 0.05$). After 18 days of treatment, the weight of tumor from pcDNA-tum-5 group was significantly lower than that from pcDNA 3.1 control group ($p < 0.05$) (Fig. 8a). The inhibition rate was 29.4% (Fig. 8b).

Discussion

Prolonged administration of recombinant protein such as angiostatin and endostatin *in vivo* is required in anti-angiogenic therapy in cancer. However, production of these functional polypeptides may be difficult. In addition, many of these biological agents are unstable *in vitro* and *in vivo* (Putney and Burke 1998). Direct *in vivo* gene delivery of these polypeptides could represent an alternative approach to purified protein administration and allow reaching continuous high level of drugs which is needed for optimal clinical benefits (Folkman et al. 1998; Tanaka et al. 1998; Goldman et al. 1998). Although viral vectors are commonly used for gene transfer due to their high transfection efficiency, safety issues and the toxicity of viral vectors may preclude their use in humans in the near future (Chen et al. 1999). Nonviral vectors have been shown to be effective in gene transfer (Lesson-Wood et al. 1995; Nabel et al. 1992; Stewart et al. 1992; Xu et al. 1997).

In the recent years, a number of angiogenesis inhibitors that target ECs and inhibit tumor growth have been discovered. Tumstatin has showed the genetic evidence for a physiological role in the negative regulation of tumor growth progression. The 54–132 amino acid region of tumstatin is so called tum-5. We choose tum-5 for tumor treatment by gene delivery because it contains the entire anti-angiogenic activity of tumstatin. In this study, pcDNA-tum-5 was generated and transfected into CHO cells. After G418 selection, we found that tum-5 protein could be expressed and secreted into the medium in a stable transfectant. In order to observe whether tum-5 has toxicity to the transfectant, the proliferation of the stable transfectant was detected. The result showed that the expression and secretion of tum-5 protein did not disturb the normal growth of the transfectant compared with control, suggesting that tum-5 is safe for the transfectant.

Angiogenesis is essential for the progression of solid tumors (Maeshima et al. 2000a; Meng et al. 2006). The progressive growth and metastasis of malignant neoplasms depend on new blood vessel formation from host vessels. Tumstatin inhibits the proliferation of endothelial cells, causes G1 arrest of VEGF- and bFGF-stimulated EC and induces apoptosis of proliferating EC through upregulation of caspase 3 (Maeshima et al. 2000a; Maeshima et al.

2000b; Maeshima et al. 2001). To detect whether the secreted tum-5 protein has anti-angiogenic activity, the effect of the protein on HUVECs was investigated *in vitro*. The results indicated that tum-5 protein could markedly restrain the tube formation and the migration of HUVECs, suggesting that tum-5 secreted by CHO cells has an effective anti-angiogenesis effect on HUVECs.

Since tum-5 expressed and secreted in CHO cells has a marked inhibitory action on HUVECs, we tested the anti-tumor activity of pcDNA-tum-5 in mice bearing S180 tumors. The results showed that pcDNA-tum-5 had a significant tumor growth inhibition effect on the tumors. After 18 days of treatment, the weight of tumors from pcDNA-tum-5 group was significantly lower than control. The tumor inhibition rate could reach 29.4%, suggesting that pcDNA-tum-5 is a good tumor inhibition agent. The full-length tumstatin and fragments containing regions 54–132(tum-5) have significant anti-tumor activity (Maeshima et al. 2000a). Our previous study also showed that tum-5 protein could suppress tumor growth and the tumor inhibition rate was 27% (Meng et al. 2006). Compared with tum-5 protein, the antitumor effect of pcDNA-tum-5 appeared to be similar. But preparation of pcDNA-tum-5 is much easier than that of tum-5 protein.

Gene delivery represents an efficient method to achieve intratumoral constant concentrations of biological polypeptide agents, for their short half life *in vivo*, which is necessary to acquire clinical benefits, although the amount of protein delivered by this approach can not be quantified (O'Reilly et al. 1994). This study also supports that gene delivery is an attractive strategy that can effectively bring the anti-angiogenic or anti-tumor polypeptide inhibitor to tumor. Our data from this study suggests that pcDNA-tum-5 may be a useful candidate for tumor treatment.

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