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<th>Tum-1, a tumstatin fragment, gene delivery into hepatocellular carcinoma suppresses tumor growth through inhibiting angiogenesis.</th>
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Abstract. Since hepatocellular carcinoma (HCC) is a hypervascular cancer, anti-angiogenic therapy is a promising approach to treat HCC. In the present study, we investigated the antiangiogenic and antitumor effects of tum-1, a fragment of tumstatin, gene transduction into HCC in vitro and in vivo. Tum-1 gene was cloned into a pSecTag2B mammalian expression vehicle to construct pSecTag2B-tum-1. pSecTag2B-tum-1 or vehicle were transfected into human HCC cells, PLC/PRF/5 cells stably and Huh-7 cells transiently. pSecTag2B-tum-1 transfection slightly repressed the proliferation of both PLC/PRF/5 and Huh-7 cells in vitro. Addition of conditioned media (CM) from tum-1 expressing PLC/PRF/5 cells significantly inhibited the spontaneous and vascular endothelial growth factor (VEGF)-induced proliferation and migration of human umbilical vein endothelial cells (HUVEC) in vitro with diminishing the VEGF-induced phosphorylation of both Akt and extracellular signal-regulated kinase (ERK) that are known to mediate VEGF-induced proliferation and migration of endothelial cells. In vivo experiments, intratumoral injection of pSecTag2B-tum-1 significantly repressed the growth of pre-established Huh-7 tumors in athymic mouse models accompanying the decreased density of CD34 positive vessels in tumors. In conclusion, our results suggest that antiangiogenic gene therapy using tum-1 gene may be an efficient strategy for the treatment of HCC.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common fatal malignancies worldwide, and is especially common in several parts of Asia and Africa (1). Although advances in medical technology have permitted the early recognition and treatment of HCC (1,2), the annual death rate from HCC exceeds 30,000 in Japan (3). Therefore, there is a need to develop new strategies to treat HCC.

Recently, it has been reported that antiangiogenic agents sufficiently inhibit tumor growth in vivo (4). Since HCC is a hypervascular cancer, antiangiogenic therapy might be particularly effective in the treatment of patients with HCC. However, systemic administration of antiangiogenic agents, such as thalidomide (5) or TNP-470 (6), might not be the most efficient method for locally aggressive tumors. Therefore, it is conceivable that antiangiogenic gene delivery to cancer cells could be suitable for the treatment of HCC, where it can increase the local concentration of therapeutic endogenous agents. In fact, we have reported that the introduction of antiangiogenic genes such as angiostatin and pigment epithelium-derived factor (PEDF) genes into HCC cells exhibited significant antitumor effect in xenograft models (7,8).

The noncollagenous domain of α3 chain of type IV collagen, namely tumstatin, has the antiangiogenic property by inhibiting endothelial cell proliferation and inducing their apoptosis via an interaction with αvβ3 integrin (9-15). Antiangiogenic effect of tumstatin has been studied in xenograft models, where tumstatin repressed the growth of several cancer types including renal cell carcinoma, prostate cancer, melanoma and lung carcinoma (9-11,13). In the present study, we constructed a mammalian expression vector expressing tum-1 which consists of 54-244 amino acids of tumstatin and has antiangiogenic activity (9). Following stable transfection of this vector into HCC cells, we examined the antiangiogenic activity of tum-1 using cultured human umbilical vein endothelial cells (HUVEC) in the presence or
absence of vascular endothelial growth factor (VEGF). In addition, we investigated the angiogenic and antivirus effects of tum-1 in vivo by intratumoral injection of tum-1 expression vector into HCC tumor implanted subcutaneously in athymic nude mice.

Materials and methods

Cell culture. Human HCC cell lines, PLC/PRF/5 and Huh-7 cells, were maintained in RPMI supplemented with 10% bovine calf serum. Human umbilical vascular endothelial cells (HUVEC) were purchased from Sankyo Junyaku (Tokyo, Japan) and were grown in endothelial cell growth medium 2. HUVEC were grown to <6 passages for all experiments.

Plasmid construction and transfection. The human tum-1 cDNA was kindly provided by Dr Y. Maeshima (Department of Medicine and Clinical Science, Okayama University, Japan) and cloned into pSecTag2B mammalian expression vector containing hexahistidine tag (Invitrogen, Carlsbad, CA) to construct pSecTag2B-tum-1. To establish the PLC/PRF/5 cells stably expressing tum-1/hexahistidine chimera protein, 10 μg of pSecTag2B-tum-1 was transfected into the cells by the lipofection method (Life Technologies, Inc., Gaithersburg, MD). After transfection, the cells were cultured in fresh medium containing Zeocin (100 μg/ml) for 2 weeks. Zeocin-resistant pooled populations were subjected to further studies. As a control, pSecTag2B vehicle was also stably transfected into PLC/PRF/5 cells. In addition, pSecTag2B vehicle or pSecTag2B-tum-1 was transiently transfected into Huh-7 cells.

Preparation of conditioned media. PLC/PRF/5 cells stably transfected with pSecTag2B-tum-1 or vehicle were plated on 100-mm dishes. After 24 h, the medium was replaced with 5 ml serum-free RPMI and incubated for 48 h. Then, conditioned media (CM) from pSecTag2B-tum-1-transfected PLC/PRF/5 cells (CM-tum-1), vehicle-transfected PLC/PRF/5 cells (CM-Mock) and non-transfected PLC/PRF/5 cells (CM-N) were collected and used in the assay. In some experiments, CM-tum-1 was passed through the His trap kit (Amersham) and used for the His pull-down assay. As a control, pSecTag2B vehicle or pSecTag2B-tum-1 or vehicle were transfected into PLC/PRF/5 cells and stable clones were selected.

Proliferation of hepatoma cells. PLC/PRF/5 cells stably transfected with vehicle (Mock) or pSecTag2B-tum-1 (tum-1) were seeded onto 96-well culture plates at ~5x10³ cells/well and were incubated for 72 h. Cell proliferation was evaluated using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI). Similarly, non-transfected PLC/PRF/5 cells were seeded onto 96-well culture plates. After 24 h, the medium was replaced with 100 μl of CM-N, CM-Mock, CM-tum-1 and CM-tum-1ΔHis. After 48 h, cell proliferation was determined. Whereas, Huh-7 cells seeded onto 96-well culture plates were transiently transfected with vehicle (Mock) or pSecTag2B-tum-1 (tum-1) and cultured for 48 h, cell proliferation was determined.

Proliferation and migration of HUVEC. HUVECs were plated onto 96-well culture plates at approximately 5x10³ cells/well and incubated for 24 h. Medium was replaced with 100 μl of CM-Mock or CM-tum-1 with or without 10 ng/ml of recombinant human vascular endothelial growth factor (VEGF, R&D systems, Minneapolis, MN, USA). After 48 h, cell proliferation was determined by the CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit. Migration of HUVEC was analyzed using 8.0-μm 24-well Transwells (Corning, Acton, MA) as described previously (7,8). Briefly, 600 μl of CM-Mock or CM-tum-1 with or without 10 ng/ml of VEGF was placed in the lower chamber. HUVEC (~2x10⁴) suspended in 200 μl of serum-free RPMI were added to the upper chamber. After 24-h incubation, non-migrating cells were removed from the upper surface of the membrane with a cotton swab. Cells migrating to the lower surface were fixed with methanol and stained with Giemsa. Cell number was counted with a light microscope under a high power field (magnification x200).

Western blotting. HUVEC were incubated with CM-Mock or CM-tum-1 in the presence or absence of 10 ng/ml of VEGF for 30 min. Then, the cells were washed twice with phosphate-buffered saline (PBS), lysed by addition of lysis buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 100 μg/ml PMSF, 1 μg/ml of aprotinin, 1% NP40, 0.5% sodium deoxycholate and 1 mM sodium o-ovanadate] for 10 min at 4°C, and insoluble materials were removed by centrifugation at 14,000 rpm for 30 min at 4°C. The supernatant was collected, and the protein concentration was determined using a Bio-Rad protein assay kit (Melville, NY, USA). The same amount of protein from each lysate or conditioned medium (10 μg/well) was analyzed by electrophoresis on 8-12% SDS polyacrylamide gel and transblotted onto nitrocellulose membrane. Blots were blocked with a solution of 5% non-fat dry milk/Tris-buffered saline containing 0.1% Tween-20 (TBS-T) for 1 h and then incubated overnight at 4°C in the presence of rabbit anti-hexahistidine (ICN, Costa Mesa, CA), rabbit anti-human phosphor-Akt, rabbit anti-human Akt, rabbit anti-human phosphor-ERK1/2 and rabbit anti-human ERK1/2 (Cell Signaling, Beverly, MA, USA). The membranes were washed with TBS-T and were incubated with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G. After washing with TBS-T, immunoreactive bands were visualized using the ECL chemiluminescence system (Amersham).

In vivo study. Four-week-old male BALB/c nu/nu athymic mice were purchased from Charles River (Yokohama, Japan). Animal experiments were performed in accordance with institutional guidelines, and the study was approved by the Ethics Committee of Nagasaki University. Huh-7 cells (3x10⁶) were implanted subcutaneously into the left thigh. Tumor volume was calculated as follows; tumor volume = length (mm) x width² (mm) x 1/2. When the tumor volume reached 100 mm³, pSecTag2B-tum-1 [75 μg plasmid/100 μl of TE (Tris ethylenediamine tetra acetic acid) buffer] with 20 μl of lipofectin was injected into the tumor at day 1, 8, 15 and 22. As a control, pSecTag2B vehicle (75 μg plasmid/100 μl of TE) with lipofectin was injected similarly. Each group consisted
of five mice. Tumor volume was measured every 2-3 days until 25 days after first injection. Tumors were removed and analyzed by reverse-transcriptase polymerase chain reaction (RT-PCR) and immunohistochemistry.

**RT-PCR.** Total RNA was extracted from tumors and PLC/PRF/5 cells stably transfected pSecTag2B-tum-1. RNA was used after contaminating DNA was completely removed by DNase I treatment. RT-PCR was performed according to the instructions provided by the supplier of the OneStep RT-PCR Kit (Qiagen, Valencia, CA), using primers specific for tum-1 derived from pSecTag2B-tum-1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a control. Reaction mixtures (10 μl) were loaded on 1.2% agarose gels and visualized by ethidium bromide staining.

**Immunohistochemistry.** Immunohistochemistry was performed using anti-mouse CD34 antibody (Serotec Ltd., Oxford, UK) and anti-mouse αSMA (Actin, Smooth Muscle, Shandon Immunon™, USA). Tissue samples of the tumor extracted from each mouse were cut into 4-μm-thick sections and mounted on aminopropyltriethoxysilane-coated glass slides. Sections were immunostained with anti-CD34 at a dilution of 1:100 for 60 min using the Streptavidin Peroxidase technique (SAB) (Histomouse™ Plus Kits, Zymed Laboratories Inc., South San Francisco, USA). The second staining with anti-αSMA was conducted by making the section reactive primary antibody, anti-αSMA (dilution 1:100), at 4˚C overnight, and by using the SAB technique. The sections were stained with the mixture of a commercial chromogen (VIP) and hydrogen peroxide and hematoxylin for counterstaining.

**Statistical analysis.** All data were expressed as mean ± SD. Differences between groups were examined for statistical significance using Student’s t-test. All reported p-values are two-tailed, and those <0.05 were considered statistically significant.

**Results**

**Tum-1 gene introduction slightly represses the proliferation of HCC cells.** Tum-1 expression plasmid (pSecTag2B-tum-1) was stably transfected into PLC/PRF/5 cells, and the secretion of tum-1 protein from the cells was analyzed by Western blotting. As shown in Fig. 1A, conditioned medium (CM) from PLC/PRF/5 cells stably transfected with pSecTag2B-
tum-1 (CM-tum-1) contained the tum-1/hexahistidine chimera protein detected by anti-hexahistidine antibody, and this chimera protein was effectively removed by the His trap kit (CM-tum-1ΔHis) (Fig. 1A). Cell proliferation assay showed that the growth of PLC/PRF/5 cells stably transfected with pSecTag2B-tum-1 was slightly slower than that of PLC/PRF/5 cells from non-transfected cells (CM-Mock), CM from vehicle transfected cells (CM-tum-1), CM from pSecTag2B-tum-1 transfected cells. Similarly, addition of CM-tum-1 repressed the spontaneous and VEGF-induced migration of HUVEC (Fig. 3B). Since phosphatidylinositol 3-kinase (PI3K)-protein kinase B (PKB/Akt) and extracellular signal-regulated kinase (ERK) signaling pathways are involved in the VEGF-induced proliferation and migration of HUVEC (16-18), we analyzed the effects of tum-1 on the VEGF-induced phosphorylation of Akt and ERK by Western blotting (Fig. 4). Phosphorylation of both Akt and ERK was constitutively detected in HUVEC, which was further upregulated by VEGF. However, addition of CM-tum-1 repressed the VEGF-induced phosphorylation of both Akt and ERK to the basal level.

**Antitumor effect of tum-1 gene introduction in vivo.** Huh-7 cells were subcutaneously implanted and tumors were established in athymic nude mice because Huh-7 cells were more efficiently transplantable than other cell lines. After reaching an adequate size, the tumor was directly injected with pSecTag2B-tum-1, and the effect of treatment on tumor size was determined. Injection of pSecTag2B-tum-1 resulted in a significant reduction of tumor volume compared with vehicle-injected tumors at day 24 (p<0.01; Fig. 5). The expression of tum-1 mRNA in the pSecTag2B-tum-1-injected tumors (at day 24) was confirmed by RT-PCR (Fig. 6A), where the density of CD34 and αSMA positive vessels/fields was significantly lower than those in the vehicle-injected tumors (Fig. 6B and C). These results indicate that the tumor suppressive effect of tum-1 gene injection was closely related to the vascularity of tumors.

**Discussion**

Tumstatin, a non-collagenous domain of α3 chain of type IV collagen, consists of 244 amino acids (9,14), which is cleaved from type IV collagen by matrix metalloproteinase-9 (14,19). Tumstatin inhibits proliferation and causes apoptosis of endothelial cells through αvß3 integrin interaction in an RGD-independent manner (9-14). Tumstatin also inhibits tube formation of endothelial cells on Matrigel and induces G1 endothelial cell cycle arrest (14). Deletion mutants of tumstatin including tum-1 (54-244 amino acids), tum-2 (1-132 amino acids) and tum-5 (54-132 amino acids) retain the anti-angiogenic activity (9-11).

In the present study, tum-1 expression plasmid vector (pSecTag2B-tum-1) was introduced into HCC cells. HCC cells transfected with pSecTag2B-tum-1 produced the detectable tum-1/hexahistidine chimera protein in its CM. Surprisingly, stable and transient tum-1 gene transfection into PLC/PRF/5 and Huh-7 cells, respectively, slightly repressed
the proliferation of these cells. Recent reports revealed that αvß3 integrin, the receptor of tumstatin, is expressed in several HCC cell lines including Huh-7 cells (20) and also in clinical samples of HCC (21). Therefore, it is possible that tum-1 could inhibit the proliferation of HCC cells through interacting with αvß3 integrin. Similar observation was reported that the gene introduction of tumstatin or its C-terminal residues 185-203 into melanoma cells directly inhibited the proliferation and invasiveness of these cells in vitro (22).

A potential mechanism of antiangiogenic function of tumstatin has been reported that tumstatin inhibits activation of focal adhesion kinase (FAK), phosphatidylinositol 3-kinase (PI3K), protein kinase B (PKB/Akt), and mammalian target of rapamycin (mTOR), and it prevents the dissociation of eukaryotic initiation factor 4E protein (eIF4E) from 4E-binding protein 1, resulted in the inhibition of cap-dependent protein synthesis in endothelial cells (23,24). Since PI3K-PKB/Akt signaling plays a key role in cell growth and survival in a variety of cells (25), it is conceivable that inhibition of this
signaling leads to the growth arrest and apoptosis of endothelial cells. Furthermore, recent studies revealed that VEGF promoted angiogenesis through activating PI3K-PKB/Akt signaling (16,17), and that luteolin, an antiangiogenic compound, repressed the VEGF-induced angiogenesis by inhibiting PI3K-PKB/Akt signaling (26). In contrast to tumstatin, endostatin, another antiangiogenic fragment released from \( \alpha_1 \) chain of type XVIII collagen, interacts with \( \alpha_5\beta_1 \) integrin and inhibits activation of FAK, and ras-raf-ERK signaling, but not PI3K-PKB/Akt signaling, and it prevents endothelial cell migration with no effect on proliferation and survival (24), indicating that tumstatin has a distinct antiangiogenic mechanism from endostatin. In this study, addition of CM containing tum-1 repressed not only spontaneous and VEGF-induced proliferation of Akt and ERK. Huh-7 cells were incubated with indicated CM with or without 10 ng/ml of VEGF for 30 min, and the levels of phosphorylation of Akt (A) or ERK (B) were analyzed by Western blotting. Results shown are from one representative experiment from a total of four performed.

Non-viral gene delivery systems are less efficient at inducing transgene expression and have shorter-term expression compared with viral delivery systems. Despite the expected low efficiency of gene induction, intratumoral injection of tum-1 expression plasmid vector (pSecTag2B-tum-1) significantly repressed the Huh-7 tumor growth accompanying the decreased density of CD34 and \( \alpha\)SMA positive vessels compared with vehicle injection. These results suggest that a sufficient bystander effect was achieved by this strategy, and if the transgene is expressed intratumorally, highly efficient therapeutic gene induction may not be necessarily required. We have reported that PEDF gene transduction into Huh-7 tumor significantly repressed its growth in athymic mouse models as well as tum-1 shown in this study, but PEDF did not directly inhibit the proliferation of Huh-7 cells \textit{in vitro} (8). Therefore, it is likely that antitumor effect of tum-1 \textit{in vivo} could be attributable to its anti-
angiogenic activity rather than direct antiproliferative activity against Huh-7 cells. In conclusion, it is possible that intratumoral gene injection of tumstatin or its active derivatives including tum-1 is a promising strategy for the treatment of HCC.

References


Figure 6. Tum-1 mRNA expression and CD34-positive vessel density in the tumors. (A) Exogenous tum-1 mRNA expression in the tumor was analyzed by RT-PCR. Total RNA was extracted from tumor tissues. RT-PCR was performed with primers specific for tum-1 and GAPDH (internal control). Total RNA from PLC/PRF/5 cells stably transfected with pSecTag2B-tum-1 was used as a positive control (right column); N, negative control; Mock, vehicle injected tumor; tum-1, pSecTag2B-tum-1 injected tumor. (B) Representative photographs of a harvested tumor stained immunohistochemically with anti-CD34 and anti-αSMA antibody. Arrows indicate CD34 (brown) and αSMA (violet) positive vessels (original magnification x200); a, Mock (vehicle) injected tumor; b, pSecTag2B-tum-1 injected tumor. (C) Number of CD34 (brown) and αSMA (violet) positive vessels/field. Data represent a number of CD34 and αSMA positive vessels counted from the five different fields of each sample. Data are mean ± SD (n=5); *p<0.01 vs Mock.