Downregulation of tumstatin expression by overexpression of ornithine decarboxylase

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Abstract. Tumor angiogenesis, a pivotal process for cancer growth and metastasis, requires both upregulation of pro-angiogenic molecules and downregulation of anti-angiogenic molecules. Anti-angiogenesis therapy represents a promising way for cancer treatment. Tumstatin, a novel endogenous angiogenesis inhibitor, inhibits endothelial cell proliferation, pathological angiogenesis and tumor growth. Ornithine decarboxylase (ODC), overexpressed in various cancers, is associated with cell transformation, tumor invasion and angiogenesis. We found that the expression of tumstatin was suppressed in ODC-overexpressing human cancer cells and renal carcinoma tissues. We presumed that ODC overexpression may downregulate the expression of tumstatin. To be able to test this hypothesis, we generated HEK293 cells that overexpress ODC (ODC transfectants) and characterized the following experimental groups: PBS-treated group, mock transfectants, ODC transfectants, ODC transfectants transfected with pcDNA-ODCr (an antisense ODC-expressing plasmid) group and putrescine-treated group. The effect of ODC overexpression on tumstatin expression was examined by reverse transcriptase-polymerase chain reaction (RT-PCR), western blot analysis and dual luciferase reporter assay. ODC-overexpressing cells and putrescine-treated cells showed suppressed tumstatin mRNA and protein expression, and decreased tumstatin gene promoter activity. Thus, ODC overexpression suppresses the expression of tumstatin, which may provide fundamental evidence for the combination of anti-angiogenic therapy and conventional therapy for cancer treatment.

Introduction

Tumstatin has been found to be a target of autoantibodies in patients with Goodpasture syndrome (1). This novel endogenous anti-angiogenic molecule is the bioactive NC1 domain (28 kDa) of colIVα3 chain, liberated from the basement membrane through cleavage by matrix metalloproteinase (2-4). Tumstatin can specifically inhibit proliferation of endothelial cells, cause G1 arrest of vascular endothelial growth factor (VEGF)- and basic fibroblast growth factor (bFGF)-stimulated endothelial cells, induce apoptosis of proliferating endothelial cells and consequently inhibit pathological angiogenesis (5-7). Tumor growth in many mouse xenograft models treated with tumstatin is suppressed because tumstatin can induce endothelial cell-specific apoptosis (8-10). It is currently considered to be a promising anti-angiogenic and antitumor agent for its unique property of causing ‘tumor stasis’ (11).

Polyamine, comprised of putrescine, spermine and spermidine, not only plays an important role in the maintenance of normal cell function, but also is involved in the formation of multiple malignant phenotypes including tumor angiogenesis (12-14). Ornithine decarboxylase (ODC), the first rate-limiting enzyme of polyamine biosynthesis, catalyzes the decarboxylation of ornithine to produce putrescine (15,16). ODC, associated with cell growth, proliferation, transformation and angiogenesis, has been shown to be overexpressed in various cancers (15,17-19). Nude mice inoculated with ODC-overproducing NIH3T3 cells developed well-vascularized tumors which were vascularized abundantly (18). This tumor neovascularization was elicited not by VEGF and bFGF, but by a novel angiogenesis factor which promotes endothelial cell proliferation and migration. Concomitant to this is the production of thrombospondins, an inhibitor of angiogenesis, which appear to be decreased in ODC-transformed cells. On the other hand, DL-α-difluoromethylornithine (DFMO), an irreversible inhibitor of ODC, can inhibit tumor angiogenesis and subsequent tumor growth (20,21). The inhibitory effect of DFMO on B16 melanoma cells was much less than that on bovine pulmonary artery endothelial cells. Therefore, Takahashi et al presumed that the antitumor effect of DFMO...
is mostly attributed to tumor angiogenesis inhibition by poly-
amine depletion (20).

Nemoto et al found that ODC overexpression facilitates
angiogenesis by suppressing the expression of endostatin,
which is also an endogenous angiogenesis inhibitor (22). We
presumed that overexpression of ODC may downregulate the
expression of tumstatin. To overexpress ODC, we generated
the plasmid pcDNA-ODC and transfected it into HEK293
cells to establish ODC transfectants. Subsequently, the effect
of ODC overexpression on tumstatin expression was examined
in the following cell lines: PBS-treated cells, mock transfec-
tants, ODC transfectants, ODC transfectants transfected
with pcDNA-ODCr, and putrescine-treated cells. The data
presented here show that ODC overexpression downregulates
the tumstatin level.

Materials and methods

Sample collection. Thirty-eight cancerous samples paired with
noncancerous tissues adjacent to the cancer tissue of kidney
were obtained from Shandong Provincial Hospital, Shandong,
China. Informed consent was obtained from all patients before
surgery.

Plasmid construction. Full-length human ODC gene sequence
was amplified using the following primers: 5'-CCGTCCTAG
ATGACCACTTTGGTAAATGA-3' and 5'-AGAAAGCTT
ACACATTATACTAGCCG-3' (enzyme recognition sites are
underlined). An ODC-overexpressing plasmid pcDNA-ODC
was constructed by inserting the ODC cDNA into an expres-
sion vector pcDNA3.1/Myc-His(-)A, and the correct plasmid
was identified by restriction enzyme digestion and DNA
sequencing. The antisense ODC expressing vector pcDNA-
ODCr was constructed as described previously (23).

Cells, stable transfection and treatment. HEK293 cells
(human embryonic kidney cell line), ACHN cells (human
renal carcinoma cell line), HELF6 cells (human embry-
onic lung fibroblast cell line) and A549 cells (human lung
carcinoma cell line) were cultured in Dulbecco's modified
Eagle's medium (DMEM), DMEM, RPMI-1640 medium and
Ham's/F-12 medium, respectively, supplemented with 10%
fetal bovine serum, 100 U/ml of penicillin and 100 µg/ml
streptomycin.

HEK293 cells transfected with pcDNA3.1/Myc-His(-)A and
pcDNA-ODC were selected using G418. At 3-4 weeks
later, the positive clone was picked, digested with trypsinase
and further cultured to establish mock and ODC transfectants.

Untreated HEK293 cells, mock transfectants and ODC
transfectants were plated in 6-well plates. At 24 h later,
untreated HEK293 cells were treated with PBS or 100 µM
putrescine, while the ODC transfectants were transfected
with pcDNA-ODCr. All five groups of cells (PBS-treated,
mock transfectants, ODC transfectants, pcDNA-ODC and
pcDNA-ODCr transfectants, and putrescine-treated group)
were harvested 72 h post-treatment for further analysis.

Semi-quantitative reverse transcriptase-polymerase chain
reaction (RT-PCR). Total RNA was isolated from kidney tissues
and cells were treated as described above and then used for
reverse transcription according to the manufacturer's protocol
(Fermentas). PCR analysis was performed using the following
primers: β-actin (5'-CCACTGGCATGATGACGAC-3' and
5'-GGGGATCGAGTCAGTGGTC-3'), ODC (5'-CCGTCCTAG
AAGCCAGATGACGAC-3' and 5'-AGAAAGCTT
ACACATTATACTAGCCG-3'), tumstatin (5'-GACCC
ACCATGCGAGTGCGTGAAGA-3' and 5'-GGCTCGAG
TCAGTGTCTTTTCTTATGC-3'), endostatin (5'-AGCA
CTCTCTTTTACGTC-3' and 5'-TGGCTACCTGGAGGC
GACA-3'), and VEGF (5'-GATCCCGAAGCGGGCG
CATGC-3' and 5'-GCTTCACCCGACTGCGGCCG
TGC-3'). Band intensities for ODC, tumstatin, endostatin and VEGF fragment were quanti-
fied and normalized to the intensity of the β-actin signal.

Western blot analysis. Western blot analysis was performed
using cell lysates. ODC was observed using the specific mouse anti-human monoclonal antibody (Sigma). Tumstatin was
detected with the antibody prepared by our laboratory (24).
Bands were visualized by the electrochemiluminescence
protein detection system (Millipore). An immunoblot with
antibody against β-actin was used as a control.

Dual luciferase reporter assay of tumstatin gene promoter.
The tumstatin gene promoter was amplified from total DNA
extracted from human peripheral blood, using the following
primers: 5'-GGTACCAGCAACATCTGCGATATGGTC
GGCTCTCTGACGTC-3' and 5'-AAGCTTTCAGAGCCTGGGCGAGTC
-3'). The amplified
products were digested, purified and inserted into the
pGL3-basic null vector to form the reporter construct
pGL-tumstatin (2.2 kb). HEK293 cells were plated in 24-well
plates at a density of 1x10^5 cells/well prior to transfection.
Using Lipofectamine™ 2000, the cells were co-transfected
with pRL-TK and the following constructs: pGL3-primary
(2.2 kb). The cells were collected 48 h later for the promoter
activity assay.

Using the same procedure, pGL-tumstatin (2.2 kb) and
pRL-TK were co-transfected into HEK293 cells treated as
described above (categorized into five groups). The cells were
harvested 48 h later for the promoter activity assay using the
dual-luciferase reporter system (Promega) according to the
manufacturer's protocol.

Statistical analysis. Data were expressed as the means ± SD.
Correlation analysis and ANOVA were performed by
SPSS 13.0 statistical software package and P<0.05 was consid-
ered statistically significant.

Results

The expression of ODC and tumstatin in renal tissues and
cells. We detected the expression of ODC and tumstatin in
various tumor cells. In the ACHN and A549 cells, ODC
was overexpressed, while the expression of tumstatin and
endostatin were markedly suppressed in comparison with the
corresponding normal cells (HEK293 and HELF6, respec-
tively), as determined by RT-PCR and western blot analysis
(Table 1).

Subsequently, RT-PCR was performed to detect the
expression levels of ODC and tumstatin in human renal
cancer and adjacent normal tissues. ODC mRNA overexpression was detected in 32 of 38 cancerous tissues, and not in any of the corresponding normal tissues (Fig. 1A). In these 32 ODC-overexpressing kidney cancerous samples, 24 had downregulated tumstatin mRNA expression when compared with the adjacent normal tissues (Fig. 1B). Statistical analysis revealed a correlation between ODC gene expression and tumstatin expression (P<0.05).

Establishment of mock transfectants and ODC transfectants. Full-length human ODC cDNA (1,386 bp) was cloned into pcDNA3.1/Myc-His(-)A expression vector to generate the eukaryotic expression plasmid pcDNA-ODC, which was identified and confirmed by restriction enzyme digestion and DNA sequencing. Subsequently, RT-PCR and western blot analysis were performed to detect whether the recombinant plasmid could be expressed in eukaryotic cells (HEK293) (data not shown).

HEK293 cells transfected with pcDNA3.1/Myc-His(-)A and pcDNA-ODC were selected in the presence of G418 for 3–4 weeks, and the surviving cells were established as mock transfectants and ODC transfectants, respectively. Compared with mock transfectants, the expression level of ODC mRNA (Fig. 2A) and protein (Fig. 2B) in ODC transfectants was increased by 200 and 196%, respectively. However, the ODC mRNA and protein expression level in ODC transfectants transfected with pcDNA-ODCr recovered to the same level as that in mock transfectants (Fig. 2), which indicates that pcDNA3.1-mediated antisense ODC could inhibit the expression of ODC in ODC transfectants.

ODC overexpression suppressed tumstatin expression. In order to examine the effect of ODC overexpression on tumstatin expression, we generated transfectants overexpressing ODC and mock transfectants containing vector alone. Then HEK293 cells were subjected to different conditions: PBS treatment, mock transfection, ODC transfection, pcDNA-ODC + pcDNA-ODCr (ODC transfectants transfected with pcDNA-ODCr) and putrescine treatment. The expression levels of ODC and tumstatin in HEK293 cells treated as described above were detected by semi-quantitative RT-PCR and western blot analysis. ODC mRNA and protein expression level in ODC transfectants transfected with pcDNA-ODCr recovered to the same level as that in mock transfectants (Fig. 2), which indicates that pcDNA3.1-mediated antisense ODC could inhibit the expression of ODC in ODC transfectants.
The expression of endostatin in ODC transfectants and putrescine-treated group was significantly inhibited, but the downregulation of endostatin expression in ODC transfectants was restored upon transfection of pcDNA-ODCr (Fig. 3C), while VEGF mRNA expression level remained unchanged (Fig. 3D) (Table II).

After demonstrating that ODC overexpression results in the downregulation of tumstatin mRNA and protein levels, we then examined the effect of ODC overexpression on the tumstatin gene promoter. A luciferase reporter plasmid pGL-tumstatin 2.2 kb containing the full-length promoter region (2,149 bp) was constructed and identified by restriction enzyme digestion and DNA sequencing. Subsequently, the tumstatin gene promoter luciferase reporter plasmid was transfected into HEK293 cells and luciferase activity assay was performed. As shown in Fig. 5A, wherein M1/M2 represents the relative luciferase activity, the 2,149 bp fragment exhibited promoter activity. pGL-tumstatin (2.2 kb) showed no promoter activity.

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Tumstatin is regarded as a promising anticancer therapeutic agent because of its high potency and distinct anti-angiogenic activity and a C-terminal (amino acids 185-203) having antitumor cell activity. Tumstatin exerts its antitumor properties with an N-terminal (amino acids 54-132) targeting the αvβ3 integrin in endothelial cells. Tumstatin exerts an antitumor effect by binding to the αvβ3 integrin in endothelial cells and melanoma cells (5,7,10). Endostatin, another endogenous inhibitor of angiogenesis, originates from the α1 chain of type XVIII collagen (32). Tumstatin and endostatin share a 14% amino acid homology and exhibit distinct anti-angiogenic and anti-tumor activities. Tumstatin inhibits tumor progression by paracrine secretion and facilitates the downregulation of endogenous angiogenic inhibitors such as VEGF, bFGF, angiogenin and angiogenic stimulators such as tumor angiogenic stimulators have been investigated as potential angiogenic inhibitors and synthetic inhibitors or antibodies to angiogenic stimulators have been investigated as potential therapeutic agents against tumors because of their promising toxicities. DFMO by irreversibly inactivating ODC, and these inhibitions are reversed by exogenous putrescine and spermidine (20,38). Due to this, DFMO has been used as chemotherapeutic agent in clinical trials for cancers, although it exhibits dose-limiting toxicity.

In the present study, we found that ODC-overexpressing human cancer cells (ACHN and A549) and renal cancer tissues have reduced expression of tumstatin. ODC overexpression can downregulate the expression of type XVIII collagen and endostatin (20,22). Therefore, we hypothesized that ODC overexpression can inhibit the expression of tumstatin. To examine the effect of ODC overexpression on tumstatin expression, we generated an ODC overexpressing plasmid pcDNA-ODC and established ODC-overexpressing HEK293 cells. In this study, cells subjected to five different conditions were examined: PBS treatment (control group), mock transfection, ODC transfection, ODC transfectants transfected with pcDNA-ODCr, and putrescine treatment. The overexpression of ODC in ODC transfectants was suppressed by the antisense plasmid pcDNA-ODCr. This experimental setup allowed us to examine the expression level of tumstatin relative to the level of ODC. The effect of the antisense plasmid pcDNA-ODCr on ODC was similar to that of DFMO. RT-PCR and western blot results showed that ODC overexpression and putrescine matched that in mock transfectants (38.96±3.22) upon transfection with the antisense plasmid pcDNA-ODCr (39.04±3.68) (Fig. 5B). These results indicate that ODC overexpression and putrescine suppressed the expression of tumstatin by inhibiting promoter activity.

**Discussion**

Angiogenesis, characterized by generation of new capillaries, plays an important role in physiological processes such as wound healing and in pathological disorders such as cancer (25,26). Tumor angiogenesis is indispensable for solid tumor growth and metastasis (27,28). Neovascularization in tumors supplies tumor with oxygen and nutrition, stimulates tumor progression by paracrine secretion and facilitates the hematogenous metastasis of tumor cells (29). Targeting the blood vessels feeding tumors could result in tumor starvation and tumor regression. Therefore, tumor angiogenesis presents an essential target of therapeutic intervention for cancer.

Tumor angiogenesis requires both upregulation of angiogenic stimulators such as VEGF, bFGF, angiogenin and downregulation of endogenous angiogenic inhibitors such as tumstatin, endostatin and thrombospondin (30). Endogenous angiogenic inhibitors and synthetic inhibitors or antibodies to angiogenic stimulators have been investigated as potential therapeutic agents against tumors because of their promising antitumor activity (27,28).

Tumstatin, a novel endogenous angiogenic inhibitor, specifically suppresses proliferation of endothelial cells, induces apoptosis of endothelial cells, and inhibits pathological angiogenesis and tumor growth (31). It has distinct antitumor properties with an N-terminal (amino acids 54-132) possessing anti-angiogenic activity and a C-terminal (amino acids 185-203) having antitumor cell activity. Tumstatin exerts an antitumor effect by binding to the αvβ3 integrin in endothelial cells and melanoma cells (5,7,10). Endostatin, another endogenous inhibitor of angiogenesis, originates from the α1 chain of type XVIII collagen (32). Tumstatin and endostatin share a 14% amino acid homology and exhibit distinct anti-angiogenic activities (6). Anti-angiogenesis effect of tumstatin is ten times more potent than that of endostatin, therefore tumstatin is regarded as a promising anticancer therapeutic candidate (6,33). However, tumstatin alone failed to achieve tumor regression. It might have an adjuvant role in tumor treatment and is effective against tumors when administered in combination with conventional therapy (33). Many studies have demonstrated that the addition of tumstatin or endostatin increases the antitumor efficacy of conventional therapies (34-36). Thus the combined treatment of these agents could be used for targeting cancer in the future.

The role of ODC and polyamine in cancer has been a focus of many research studies. Elevated ODC activity has been detected in many cancers and thought to be associated with cell transformation, tumor invasion and angiogenesis (14,16-19). The ODC gene is considered to be an oncogene because overexpression of ODC results in malignant transformation of NIH3T3 cells (18,37). B16 melanoma-induced angiogenesis, rapid neovascularization and tumor growth are inhibited by DFMO by irreversibly inactivating ODC, and these inhibitions are reversed by exogenous putrescine and spermidine (20,38). Due to this, DFMO has been used as chemotherapeutic agent in clinical trials for cancers, although it exhibits dose-limiting toxicity.

In this study, cells subjected to five different conditions were examined: PBS treatment (control group), mock transfection, ODC transfection, ODC transfectants transfected with pcDNA-ODCr, and putrescine treatment. The overexpression of ODC in ODC transfectants was suppressed by the antisense plasmid pcDNA-ODCr. This experimental setup allowed us to examine the expression level of tumstatin relative to the level of ODC. The effect of the antisense plasmid pcDNA-ODCr on ODC was similar to that of DFMO. RT-PCR and western blot results showed that ODC overexpression and putrescine matched that in mock transfectants (38.96±3.22) upon transfection with the antisense plasmid pcDNA-ODCr (39.04±3.68) (Fig. 5B). These results indicate that ODC overexpression and putrescine suppressed the expression of tumstatin by inhibiting promoter activity.
inhibited the expression of tumstatin mRNA and protein, while the suppression of tumstatin expression in ODC transfectants was rescued after transfection of pcDNA-ODC. The expression level of VEGF mRNA remained unchanged, demonstrating that the effect of ODC overexpression and putrescine on promoting angiogenesis was not associated with VEGF. This is consistent with a previous report by Nemoto et al (22). In order to better understand the negative effect of ODC on tumstatin expression, we examined the effect of ODC on tumstatin gene promoter activity. The results from the dual luciferase reporter assay indicate that ODC overexpression and putrescine suppressed the expression of tumstatin by inhibiting tumstatin promoter activity. Taken together, these results support that ODC may promote tumor angiogenesis by suppressing tumstatin expression in many cancers. This finding provides novel evidence for the efficacy of combining anti-angiogenic therapy with conventional therapy for cancer treatment.

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