Original Article

Tumstatin peptide 19 can induce Hep-2 cell apoptosis through the mitochondrial apoptotic pathway

Li-Na Wang1*, Qiu-Ying Li2*, Jin-Hua Zhao3, De-Jun Jin2, Chao Wang2

1Department of Pathophysiology, Harbin Medical University, Harbin 150081, P. R. China; 2Department of Otolaryngology-Head and Neck Surgery, The Second Affiliated Hospital of Harbin Medical University, Harbin 150081, P. R. China; 3Department of Otolaryngology, Heilongjiang Provincial Hospital, Harbin 150036, P. R. China. *Equal contributors.

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Abstract: Tumstatin is an endogenous vascular endothelial cell growth inhibitory factor. In addition, it is a strong angiogenesis inhibitor and selectively inhibits endothelial cell proliferation. Therefore, tumstatin is currently considered to be a potential antiangiogenic and antitumor drug with excellent prospects. In this study, we performed the MTT assay, trypan blue staining, and the TUNEL assay to investigate the effects of tumstatin peptide 19 on the proliferation and apoptosis of Hep-2 cells. We found that tumstatin significantly inhibited the survival rate and obviously induced the apoptosis of Hep-2 cells. Western blot analysis of Hep-2 cells exhibited that the Bax protein expression increased and the Bcl-2 protein expression decreased in the cytoplasm and mitochondria in the tumstatin group, whereas the cytochrome C expression was significantly suppressed in the mitochondria, and the caspase-3 p20 expression was upregulated in the cytoplasm, compared to the control group. Therefore, tumstatin can inhibit cell proliferation through regulating Bax/Bcl-2/cytochrome C/caspase-3 pathway-induced apoptosis in laryngeal cancer Hep-2 cells.

Keywords: Tumstatin, Hep-2 cells, apoptosis, cytochrome C, caspase-3

Introduction

Laryngeal carcinoma is one of the most common malignant tumors of the head and neck. More than 95% of laryngeal tumors are squamous cell carcinomas (SCC). There is still no satisfactory treatment for laryngeal carcinoma, especially for advanced-stage tumors. Surgical treatment usually leads to the removal of laryngeal tissues, which severely affects the patients’ life, whereas the use of chemotherapeutics to treat laryngeal carcinoma results in side effects and possible drug resistance. Increasing evidence indicates that angiogenesis is essential not only for tumorigenesis and proliferation, but also for tumor invasion and metastasis [1].

Tumstatin is a 28-kDa protein fragment cleaved from collagen type IV that serves as both an antiangiogenic and antitumorigenic agent [2]. There are two activation domains on the tumstatin fragment, one is at the N-terminus (74-98 amino acids) and is responsible for inhibiting angiogenesis, and the other is a peptide of 19 amino acids (185-203 amino acids) near the C terminus and is primarily responsible for inhibiting tumorigenesis [3, 4]. Its antitumor characteristics can significantly inhibit cell proliferation and migration in melanoma cells, fibrosarcoma, and some epithelial tumor cells [5-7]. However, the functions of tumstatin peptide 19 in laryngeal carcinoma are still unclear.

The aim of this study was to determine whether tumstatin peptide 19 can inhibit cell proliferation through inducing apoptosis in laryngeal cancer Hep-2 cells. We measured the expression levels of Bax, Bcl-2, cytochrome C, and caspase-3 P20, which are associated with the mitochondrial apoptotic pathway, after tumstatin peptide 19 treatment in Hep-2 cells. The results of this study will reveal whether tumstatin peptide 19 might be a potential antitumor clinical therapeutic strategy for patients with laryngeal carcinoma.
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Material and methods

Cell culture

Hep-2 cells were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in a 5% CO₂ humidified atmosphere at 37°C and grown in Dulbecco’s modified Eagle’s medium (HyClone, Thermo Fisher Scientific), containing 10% fetal bovine serum (HyClone, Thermo Fisher Scientific) and 1:100 Penicillin/Streptomycin (Invitrogen, Thermo Fisher Scientific).

MTT assay

Cells were inoculated into a flat-bottomed 96-well plate at a density of 1 × 10³ cells/well. After 24 h of culture, the cells were adherent to the plate. Then, tumstatin peptide 19 (a gift from the Department of Biochemistry, Harbin Medical University. It was synthesized and purified as described by Liu Y et al. [8]) at various final concentrations (0, 44, 88, 132, 176, and 220 μg/ml) was added to the cells. After 72 h, the culture medium was discarded, 100 μl of MTT (5 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) was added, the cells were incubated for 4 h, and then the supernatant was removed. Thereafter, insoluble formazan was dissolved by the addition of 100 μl DMSO. The absorbance of the solution in each well was measured at a wavelength of 490 nm using a Perkin-Elmer ELISA reader (HTS7000 plus). The growth inhibition rate was calculated according to the following formula: survival rate = experimental well reading/control well reading × 100%. The experiment was repeated five times, and the mean value was obtained.

Growth curves

Cells (5 × 10³ cells/well) were inoculated into a 24-well plate. After the cells were cultured for 24 h and adhered to the plate, tumstatin peptide 19 (final concentration of 44 μg/ml; tumstatin group) or phosphate-buffered saline (PBS; control group) was added to the medium. Three wells were selected daily and stained with trypan blue (Sigma-Aldrich, St. Louis, MO, USA) for cell counts for six successive days, and the mean value was calculated. The experiment was repeated five times, and the mean value was obtained.

The TUNEL enzymatic labeling assay

TUNEL assay kit for apoptosis detection was purchased from Roche Company. The experiment was performed according to the standard procedure. Briefly, cells were inoculated into the coverslip-treated 6-well plate at 2 × 10⁴ cells/well, and after the cells were cultured for 24 h and adhered to the coverslip, the cells were treated with or without tumstatin peptide 19 (final concentration of 44 μg/ml) for 24 h. The coverslips were dried and fixed with 4% paraformaldehyde for 30 min. Pre-treatment with 0.03% hydrogen peroxide resulted in the irreversible inactivation of endogenous peroxidase. The cells were then washed with PBS three times. The cells were permeabilized by immersing the coverslips in 0.1% Triton X-100 solution in PBS for 2 min on ice. After washing with PBS, 50 μl of TUNEL reaction solution was added, and the cells were incubated at 37°C for 60 min. Next, 50 μl of phorbol 12, 13 dibutyrate (PDB) was then added into the medium, and the cells were incubated at 37°C for 30 min. After washing with PBS, 50 μl of diamino-benzidine (DAB) was added, and the cells were incubated at room temperature for 10 min. The coverslips were then washed with PBS three times, mounted in mounting medium (Vector Laboratories, CA, USA), and sealed with nailpolish. The prepared samples were observed and photographed under an Olympus microscope.

Western blotting

Western blot analysis was used to detect the expression levels of Bax, Bcl-2, cytochrome C, and caspase-3 active fragment P20. Hep-2 cells were treated with 44 μg/ml tumstatin peptide 19 for 12, 24, and 48 h. All the cells were collected by centrifugation at 600g for 5 min before cells were washed twice with cold PBS and digested with trypsin-EDTA solution (Beyotime, Nantong, China). Then, the cells were resuspended in ice-cold lysis buffer (320 mM sucrose, 1 mM K-EDTA and 10 mM Tris-HCl, pH 7.4) and homogenized by a homogenizer in ice/water about 30 times. After removing the nuclei and cell debris by centrifugation at 800 g for 10 min at 4°C, the supernatants were further centrifuged at 10000 g for 10 min at 4°C, and the resulting mitochondrial pellets (mitochondrial proteins) were resuspended in lysis buffer. The supernatants from the 10000
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Figure 1. Survival rate of Hep-2 cells in the present of different concentration (0, 44, 88, 132, 176, and 220 μg/ml) of tumstatin peptide 19 was assessed by MTT assay.

Figure 2. Trypan blue staining to measure the concentration of Hep-2 cells with or without tumstatin peptide 19 treatment for six successive days.

g centrifugation were centrifuged once more at 14000 g for 10 min at 4°C and then sediments were collected (cytoplasmic proteins). The protein concentrations of all samples were measured with the Bradford method, and equal amounts of protein were analyzed by western blot analysis. All of the samples, including the cytoplasmic and mitochondrial proteins, were mixed with 6 × sodium dodecyl sulfate (SDS) sample buffer, boiled for 10 min, and electrophoresed at 80 V through a 10% SDS-polyacrylamide denaturing gel. Separated proteins were electrotransferred to polyvinylidene fluoride film (Bio-Rad), and the expression levels of Bax, Bcl-2, cytochrome C, caspase-3 active fragment P20, and actin proteins were detected with the desired antibodies, including monoclonal anti-Bax antibody (1:2000; Santa Cruz Biotechnology, Inc, Dallas, TX, USA), polyclonal anti-Bcl-2 antibody (1:2000; Santa Cruz Biotechnology, Inc, Dallas, TX, USA), monoclonal anti-cytochrome C antibody (1:2000; Santa Cruz Biotechnology, Inc, Dallas, TX, USA), polyclonal anti-P20 antibody (1:2000; Santa Cruz Biotechnology, Inc, Dallas, TX, USA), and monoclonal anti-actin antibody (1:2000; Santa Cruz Biotechnology, Inc, Dallas, TX, USA), respectively. The expression levels were calculated using ImageJ software (NIH, Bethesda, MD, USA).

Statistical analysis

All results were analyzed by PrismDemo 5 statistical software and presented as the arithmetic means ± standard deviation. The Student’s t test was performed for statistical analysis. The difference was considered to be significant if P < 0.05.

Results

The toxicity of tumstatin peptide 19 on the survival rate of Hep-2 cells

Based on previous studies showing that tumstatin can inhibit tumorigenesis and angiogenesis in various tumors, we evaluated the potential antitumor effects of tumstatin peptide 19 on Hep-2 cells. The cells were incubated with different concentrations (44, 88, 132, 176, and 220 μg/ml) of tumstatin peptide 19 in cell culture medium. After 48 h, we found that the Hep-2 cells survived in a dose-dependent manner (Figure 1). A higher concentration of tumstatin peptide 19 led to a lower survival rate. For example, a 50% mortality rate was observed with 44 μg/ml tumstatin peptide 19 treatment, whereas a mortality rate of 90% was observed with 220 μg/ml tumstatin peptide 19 treatment, suggesting that a dose of 44 μg/ml tumstatin peptide 19 should be suitable for the following experiments.

Tumstatin peptide 19 significantly inhibits the proliferation of Hep-2 cells

To investigate the effects of tumstatin peptide 19 on the proliferation of Hep-2 cells, we performed trypan blue staining to measure the concentration of Hep-2 cells with or without tumstatin peptide 19 treatment. As shown in Figure 2, in the tumstatin peptide 19-treated group, we found that the cell proliferation was
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Remarkably suppressed since 2 days post-treatment, compared with the control group. Meanwhile, in the control group, the cell number was only decreased slightly since 4 days post-treatment, which probably is a result of cell contact inhibition. Our results demonstrated that 44 μg/ml tumstatin peptide 19 treatment significantly inhibited the proliferation of Hep-2 cells.

Tumstatin peptide 19 suppresses cell proliferation through apoptosis of Hep-2 cells

To further investigate the mechanism of the suppression of cell proliferation in tumstatin peptide 19-treated Hep-2 cells, we next performed a TUNEL assay to examine the apoptosis of Hep-2 cells with or without tumstatin peptide 19 treatment. The TUNEL assay revealed that most of the tumstatin peptide 19-treated Hep-2 cells were apoptotic cells, which displayed brown staining, and even spherical or fragmented cells were observed, whereas apoptotic cells were not observed in the control group (Figure 3). These results suggested that tumstatin peptide 19 suppressed cell proliferation through inducing the apoptosis of Hep-2 cells.

The mitochondrial apoptotic pathway is associated with the inhibition of Hep-2 cell proliferation

Since mitochondria play important roles in the apoptotic network and are essential for many apoptotic pathways, we next investigated whether the mitochondrial apoptotic pathway is associated with the inhibition of Hep-2 cell proliferation. We measured the expression levels Bax, Bcl-2, cytochrome C, and caspase-3 P20, which are involved in the mitochondrial apoptotic pathway, at various time points (12, 24, and 72 h) with or without tumstatin peptide 19 treatment. As shown in Figure 4, we found that the expression levels of Bax and Bcl-2 proteins were regulated after tumstatin peptide 19 treatment in the cytoplasmic samples. After tumstatin peptide 19 treatment, the Bax expression was remarkably upregulated, whereas the Bcl-2 expression was gradually downregulated in the cytoplasmic samples (Figure 4). In addition, similar results of the alteration of the expression levels of these two proteins were found in the mitochondrial samples (Figure 5). Notably, the alteration of the expression levels of Bax and Bcl-2 proteins in the mitochondrial samples was more significant than that in the cytoplasmic samples.

Similarly, the expression level of cytochrome C protein was upregulated in the cytoplasm and downregulated in the mitochondria, gradually, after tumstatin peptide 19 treatment (Figure 6). Moreover, in the cytoplasm, the expression level of caspase-3 active fragment P20 was significantly increased (Figure 7). Together, these results suggest that the expression levels of all these proteins, including Bax, Bcl-2, cytochrome C, and caspase-3 active fragment P20, which are mitochondrial apoptotic pathway-related factors, were regulated by the tumstatin peptide 19 treatment.

Discussion

Angiogenesis has been emerging as a hotspot in cancer research. At the early stage of tumor progression, due to the limited blood supply, angiogenesis occurs as the tumor microenvironment changes. Previous studies have demonstrated that tumstatin is a novel, effective angiogenic inhibitor that originates from collagen IV, which is found in the vascular basement...
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Figure 4. Determination of the expression levels of Bax and Bcl-2 proteins in the cytoplasm of Hep-2 cells with or without tumstatin peptide 19 treatment at various time points (12, 24, 48, and 72 h). (A) Bax and Bcl-2 proteins in the cytoplasm were examined by western blot analysis. Quantitative analysis of the expression levels of Bax (B) and Bcl-2 (C) in the cytoplasm of Hep-2 cells. The intensity of each band was quantified using densitometry, and the data were normalized to the β-actin protein band intensity. *P < 0.05 versus the Control group.

Figure 5. Determination of the expression levels of Bax and Bcl-2 proteins in the mitochondria of Hep-2 cells with or without tumstatin peptide 19 treatment at various time points (12, 24, 48, and 72 h). (A) Bax and Bcl-2 proteins in the mitochondria were examined by western blot analysis. Quantitative analysis of the expression levels of Bax (B) and Bcl-2 (C) in the mitochondria of Hep-2 cells. The intensity of each band was quantified using densitometry, and the data were normalized to the β-actin protein band intensity. *P < 0.05 versus the Control group.
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Figure 6. Determination of the expression level of cytochrome C protein in the cytoplasm and mitochondria of Hep-2 cells with or without tumstatin peptide 19 treatment at various time points (12, 24, 48, and 72 h). A. Cytochrome C protein in the cytoplasm was examined by western blot analysis. B. Quantitative analysis of cytochrome C protein expression in the cytoplasm of Hep-2 cells. C. Cytochrome C protein in the mitochondria was examined by western blot analysis. D. Quantitative analysis of cytochrome C protein expression in the mitochondria of Hep-2 cells. The intensity of each band was quantified using densitometry, and the data were normalized to the β-actin protein band intensity. *P < 0.05 versus the Control group.

Figure 7. Determination of the expression level of caspase-3 P20 in the cytoplasm of Hep-2 cells with or without tumstatin peptide 19 treatment at various time points (12, 24, 48, and 72 h). A. Caspase-3 P20 in the cytoplasm was examined by western blot analysis. B. Quantitative analysis of caspase-3 P20 protein expression in the cytoplasm of Hep-2 cells. The intensity of each band was quantified using densitometry, and the data were normalized to the β-actin protein band intensity. *P < 0.05 versus the Control group.

membrane. It is a polypeptide fragment of the α3 chain noncollagen domain 1 of type IV collagen [9]. It was discovered as an antigen of Goodpasture syndrome [2]. Monboisse et al. have found that the alpha 3 chain of type IV collagen can prevent activation of human polymorphonuclear leukocytes [10]. In 2000, Maeshima et al. demonstrated that it can induce apoptosis through inhibiting the proliferation of endothelial cells in capillaries [11]. Tumstatin has two different active antitumor domains. One is a peptide of 19 amino acids (185-203 amino acids) near the C-terminus that has the direct antitumor effect of suppressing tumor cell proliferation and inducing tumor cell apoptosis. The other is a peptide of 25 amino acids (74-98 amino acids) near the N-terminus that has the indirect antitumor effect of suppressing tumor angiogenesis [12]. Recent studies have indicated that tumstatin can inhibit the proliferation of melanoma cells, glioma cells, fibrosarcoma and gastric...
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carcinoma cells, etc. [6, 13-15]. However, its functions in laryngeal carcinoma are still unknown.

In our study, after treatment with various concentrations of tumstatin peptide 19, we found that the survival rates of Hep-2 cells were reduced in a dose-dependent manner. In addition, tumstatin peptide 19 treatment (a final concentration of 44 µg/ml) exhibited significant inhibitory effects on the proliferation of Hep-2 cells, and the inhibition of the proliferation in the tumstatin peptide 19-treated group was much more than that in the nontreated group at 24 h post-treatment (Figure 2).

Moreover, the TUNEL assay showed that tumstatin peptide 19 treatment inhibited the proliferation through inducing apoptosis (Figure 3). As is known, Bcl-2 is the key factor in the apoptotic process. It is transported to the mitochondria and induces the degradation of mitochondria through upregulating the expression of certain apoptotic factors [16]. Therefore, the apoptosis is regulated by apoptotic protein (Bax) or antiapoptotic protein (Bcl-2). The expression levels of these two proteins result in the induction or inhibition of the apoptotic process [17]. Our results demonstrated that Bax expression was gradually increased, whereas Bcl-2 expression was significantly decreased in both the cytoplasm and mitochondria after tumstatin peptide 19 treatment, indicating that tumstatin peptide 19 could effectively regulate the expression levels of Bax and Bcl-2 proteins to induce apoptosis.

It is believed that there are three apoptosis pathways. One is a membrane signaling pathway, the second is a mitochondrial signaling pathway, and the third is an endoplasmic reticulum signaling pathway. In the mitochondrial pathway of apoptosis, Bax can be transported from the cytoplasm to the mitochondria after apoptotic signaling stimulation. Most often, Bax activation requires a direct interaction with a member of the BH3-only protein family [18]. This causes Bax conformational changes, leading to its oligomerization within the mitochondrial outer membrane, which probably results in the release of cytochrome C to induce the mitochondrial apoptotic pathway [19]. Our results also demonstrated the upregulation of Bax protein and the downregulation of Bcl-2 protein in the cytoplasm, especially in the mitochondria, indicating that tumstatin peptide 19 induced the mitochondrial apoptotic pathway through regulating the expression levels of Bax and Bcl-2 proteins (Figures 4, 5).

Under normal conditions, cytochrome C settles among phospholipids along the mitochondrial inner membrane. In the apoptotic process, cytochrome C is released from the mitochondria to the cytoplasm and forms the apoptosisosome together with Apaf-1, pro-caspase-9, and ATP/dATP. The activated caspase-9 in the apoptosisosome can active caspase-3, which is a 32-kDa protein that cleaves two active fragments (P20 and P10) that can interact with cytoskeletal protein or deoxyribonuclease (DNase) to start the caspase cascade and induce cell apoptosis [20]. Our results demonstrated that most of the cytochrome C was localized in the cytoplasm in tumstatin peptide 19-treated Hep-2 cells, whereas most of the cytochrome C was expressed in the mitochondria in nontreated cells, indicating that tumstatin peptide 19 can also regulate the translocation of cytochrome C in the cytoplasm and mitochondria (Figure 6). Additionally, we found that the expression level of caspase-3 P20 was significantly upregulated, indicating that the caspase-3-induced apoptotic pathway can be activated by tumstatin peptide 19 treatment.

In summary, our study demonstrated that tumstatin peptide 19 can significantly inhibit the proliferation of Hep-2 cells in a dose-dependent manner. Further investigation revealed that the tumstatin peptide 19-mediated regulation of Bax/Bcl-2 expression promotes the release of cytochrome C from the mitochondria to the cytoplasm and subsequently activates the caspase-3 pathway to induce the mitochondrial apoptotic pathway in Hep-2 cells. Our findings showed that tumstatin peptide 19 might be a potential antitumor clinical therapeutic strategy for patients with laryngeal carcinoma.

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Disclosure of conflict of interest

None.
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Address correspondence to: Dr. Chao Wang, Department of Otolaryngology-Head and Neck Surgery, The Second Affiliated Hospital of Harbin Medical University, No. 246, Xuefu Road, Harbin 150081, Heilongjiang, P. R. China. Tel: +86 451 86296837; Fax: +86 451 86296837; E-mail: hjwangchao@163.com

References


