Acta Pharmacologica Sinica (2009) 30: 1307–1315
 1671-4083/09 $32.00
www.nature.com/aps

Original Article

The anti-tumor properties of two tumstatin peptide fragments in human gastric carcinoma

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Aim: The aim was to study the anti-tumor activities and mechanisms of two synthetic peptide fragments of tumstatin (alpha3 (IV) NC1 domain) in human gastric carcinoma cells *in vitro* and *in vivo*.
Methods: MTT assay and cell cycle assay were used to study the anti-tumor and anti-angiogenic activities of two peptide fragments *in vitro*. Apoptosis induced by the two peptide fragments was demonstrated by TUNEL assay and morphological observation. The orthotopic tumor model was established to investigate the activities of two peptide fragments *in vivo*. Intratumor vascularization and the expressions of VEGF, bFGF, Fas, FasL, Bax, Bcl-2, and caspase 3 were determined using immunohistochemistry and Western blot analysis.
Results: Peptide 19 inhibited SGC-7901 proliferation and induced apoptosis both *in vitro* and *in vivo*. Notably, peptide 21 suppressed the proliferation of HUVEC-12 cells *in vitro*. Each peptide arrested both cell lines at the G_0/G_1 phase of the cell cycle, and they also synergistically suppressed *in vitro* and *in vivo* tumor growth. Immunohistochemistry and Western blot analysis revealed the strong expression of Fas, FasL and caspase 3 in orthotopic tumor tissues treated with peptide 19 alone or in combination with peptide 21. Decreased expressions of VEGF and bFGF and decreased microvessel density (MVD) in orthotopic tumor tissues were seen in mice treated with peptide 21 alone or in combination with peptide 19.
Conclusion: Two tumstatin peptide fragments facilitate two unique antitumor activities. Thus, they are drug candidates in the treatment of gastric carcinoma.

Keywords: tumstatin peptide; anti-cancer drug; apoptosis; angiogenesis

Acta Pharmacologica Sinica (2009) 30: 1307–1315; doi: 10.1038/aps.2009.111; published online 24 August 2009

Introduction

Angiogenesis, the formation of new capillaries from pre-existing blood vessels, is generally suppressed in healthy adult organisms and is turned on temporarily in such settings as the female reproductive cycle or during tissue repair processes. However, uncontrolled angiogenesis is associated with a number of pathological disorders, including diabetic retinopathy, rheumatoid arthritis, and tumor growth and metastasis[1, 2]. Optimal tumor growth beyond 1 mm of volume is not possible without neovascularization[1, 3–5]. Moreover, tumors metastasize into other organs via newly formed blood vessels[3, 6]. It is thought that angiogenesis is maintained through a delicate balance between growth factors and inhibition factors.

Tumstatin is an endogenous angiogenesis inhibitor that is derived from type IV collagen. Tumstatin is the noncollagenous domain of type α3 (IV) collagen, a basement membrane collagen found in kidney, lung, testis, and other vascular basement membranes[7]. Tumstatin inhibits angiogenesis by inducing apoptosis and inhibits endothelial cell proliferation through its binding to αvβ3 integrin, leading to suppression of cap-dependent protein translation[8–10]. Maeshima *et al*[11] used deletion mutagenesis to demonstrate that the anti-angiogenic activity of tumstatin is localized to amino acids 54–132. Subsequently, the anti-angiogenic activity was localized to a 25 amino acid region encompassing amino acids 74–98 (T7-peptide), which contains the entire anti-angiogenic activity associated with tumstatin[12]. The region is distinct from the 185 to 203 region that is responsible for the anti-tumor activity of tumstatin[9, 13]. A synthetic peptide encompassing residues 183−205 of the NC1 domain of the α3 [IV] chain specifically inhibited activation of polymorphonuclear leukocytes[14]. This peptide binds to an integrin complex, promotes adhesion and chemotaxis, and inhibits proliferation of various human cancer

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Received 2009-02-15 Accepted 2009-06-22

cell lines[13, 15].

Surprisingly, there has been a lack of research on the use of tumstatin for the treatment of gastric tumors, considering its potential for inhibiting angiogenesis and tumor growth in gastric tumor models. This potential is suggested by studies on experimental tumor models of malignant melanoma, bronchopulmonary carcinoma, and malignant glioma. The present experiments were designed to demonstrate the *in vitro* and *in vivo* antitumor properties of two synthetic tumstatin peptides: peptide 19, which corresponds to residues 185−203 of the NC1 domain of the α3 [IV] chain, and peptide 21, a T7 mutant, in human gastric carcinoma cells. We also explored the different mechanisms of antitumor activities for these two synthetic tumstatin peptides. The combination of peptide 19 with peptide 21 was also explored for improvement in antitumor efficacy.

Materials and methods

Synthetic peptides

The NC1α3 (IV) (185−203) peptide 19, CNYYSNSYSFWLASLNPER; the corresponding scrambled peptide, YAPLWNRSSFENSLNYSCY; and peptide 21, MPFLFCNVNDVCNFASRNDYS, were purchased from Multiple Peptide Synthesis (San Diego, CA) and Syn Pep Corp (Dublin, CA).

Cell lines and cell culture

Human gastric cancer SGC-7901 cells (Shanghai Cell-biological Institute, Chinese Academy of Sciences) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (10% FBS-DMEM) and 2 U/mL of penicillin-streptomycin mixture and incubated in 5% CO_2-95% air at 37 °C. Human umbilical vein endothelial cells HUVEC-12 (Shanghai Cell-biological Institute, Chinese Academy of Sciences) were grown in Endothelial Cell Medium-2 supplemented with 2% fetal bovine serum, R^3-IGF-1, hydrocortisone, ascorbic acid, hFGF, VEGF, hEGF, GA-1000, and heparin as recommended by the manufacturer. All cells were maintained at 37 °C in 5% CO_2.

MTT assay

SGC-7901 cells and HUVEC-12 cells were trypsinized, seeded at 1×10^3 cells/well in 96-well plates, and treated with peptide 19, peptide 21, and the two peptides together at various concentrations (0, 15, 30, 45, and 60 μg/mL). Twenty-four hours later, the effects on cell growth were examined by MTT assay: 20 μL of MTT (Sigma Co) solution (5 mg/L in PBS) was added to each well, and the cells were incubated for 4 h at 37 °C. The adherent cells were subsequently solubilized with 150 μL dimethyl sulfoxide (DMSO). The absorbance (*OD*) at 570 nm was recorded using an ELISA reader (Bio-Rad). The inhibition rate of cell proliferation was calculated by the following formula: Inhibition rate (%)=$(OD_{control} - OD_{treated})/OD_{control}$.

TUNEL assay

In brief, SGC-7901 cells were treated with control peptide (34 μg/mL), peptide 19 (34 μg/mL), peptide 21 (34 μg/mL) or peptide 19 (17 μg/mL) and peptide 21 (17 μg/mL) together. After 48 h the number of apoptotic cells was determined using the in situ cell Death Detection kit from Roche Diagnostics (Mannheim, Germany) following the manufacturer's instructions. The apoptotic cells (fluorescent green staining) were counted under a fluorescence microscope. The apoptotic index was defined by the percentage of fluorescent green cells among the total number of cells in each sample. Three fields with 100 cells per field were randomly counted for each sample.

Transmission electron microscopy

For electron microscope analysis of apoptosis, pretreated SGC-7901 cells and HUVEC-12 cells were fixed in 1% glutaraldehyde and 4% paraformaldehyde in PBS, postfixed in 1% osmium tetroxide in PBS, dehydrated and subsequently embedded in epoxy resin. Ultrathin sections (80 nm) were stained with uranyl and lead acetates and examined under a Hitachi H-600 electron microscope at 80 kV (Hitachi, Tokyo, Japan).

Cell cycle assay

SGC-7901 cells and HUVEC-12 cells were treated with peptide 19 (34 μg/mL), peptide 21 (34 μg/mL) or peptide 19 (17 μg/mL) and peptide 21 (17 μg/mL) together. After 24 h of incubation, the cells were washed in PBS and fixed in 70% ethanol overnight at 4 °C. Propidium iodine (10 g/mL) supplemented with RNaseA (200 g/mL) was added to the cells for 30 min (at 37 °C) prior to FACS analysis.

Animals

Five- to six-week-old female nude athymic BALB/c nu/nu mice were purchased from Beijing Wei Tong Li Hua Laboratory Animal Centre (qualified certificate No SCXK (Jing) 2002-2003). They were kept under specific pathogen-free conditions and fed autoclaved pellets and water *ad libitum*. The general health status of the animals was monitored daily. All experiments were carried out with the approval of the Institutional Ethical Committee for Animal Experiments of Heilongjiang Cancer Center Research Institute.

Orthotopic human gastric cancer xenografts

After ip injection of 0.4 mL SGC-7901 cells at a concentration of 5×10^7/mL, nude mice developed peritoneal carcinomatosis similar to that of advanced gastric cancer. When nude mice developed massive ascites production, the malignant ascite cells were taken to culture *in vitro*. Following their culture, 0.2 mL of the malignant ascite cells at a concentration of 5×10^7/mL was injected hypodermically into another mouse. The tumors were measured using Vernier calipers, and the volume was calculated using the standard formula (length×width$^2\times$ 0.52)[16]. The tumors were allowed to grow to about 100 mm^3. Mice were anesthetized with 2.5% avertin and the tumors were resected aseptically; the tumor tissue was subsequently cut into smaller pieces of about 1−2 mm^3. One piece of this tumor was implanted on the back of another anes-

thetized nude mouse. After being passaged hypodermically in 5 nude mice, the tumor tissue was cut into smaller pieces of about 1−2 mm^3. Mice were anesthetized and an incision was made through the left upper abdominal pararectal line and peritoneum. Two to three pieces of tumor were implanted with biological albumin gel on the top of the nude mouse stomach where the serosa had been injured (Shanghai Li Kang Rui Biological Product Co, Ltd) and the abdominal wall and the skin were subsequently closed. Animals were kept in a sterile environment[17].

Drug treatment

Five weeks later, mice were randomly divided into groups of 5 mice. Control peptide (4.4, 6.6, 8.8 mg/kg), peptide 19 (4.4, 6.6, 8.8 mg/kg), peptide 21 (4.4, 6.6, 8.8 mg/kg), or a combination of peptide 19 and peptide 21 (2.2, 3.3, 4.4 mg/kg) was iv injected every other day for 30 days in sterile PBS. Body weight was monitored every 10 days. Thirty days later, mice were sacrificed and tumor sections were obtained from control and drug-treated groups and examined by light microscope. The tumor volume was calculated using the standard formula (length×width^2×0.52) and tumor tissue was placed in 10% buffered formalin for paraffin fixation.

Immunohistochemical analyses

Sections were deparaffinized and rehydrated. They were then heated in citrate buffer (0.01 mol/L, pH 8.0) in an 800-W microwave oven for 12 min for antigen retrieval. Endogenous peroxidase in sections was inactivated in 2% H_2O_2 for 10 min. The sections were then blocked in 3% normal horse serum in 0.2 mol/L PBS (pH 7.4), followed by incubation with a rabbit anti-Fas monoclonal antibody (diluted at 1:100) or a rabbit anti-FasL antibody (diluted at 1:50, Sigma Co). Sections were incubated in primary antibody for 2 h at room temperature and then processed following standard ABC immunostaining (Vector Laboratory, Burl-ingame, CA). Immunoreactive products were visualized using 3,3′-diaminobenzidine/H_2O_2. To verify the specificity of the immunoreactions, some sections were incubated with either PBS or normal mouse IgG as a replacement for the Fas antibody. Additionally, all sections were immunolabeled with a rabbit monoclonal Bcl-2 antibody (diluted at 1:200) or with Bax, bFGF, caspase 3, or VEGF (diluted at 1:200; 1:100; 1:100; 1:200, respectively; Sigma Co). Fas, FasL, caspase 3, Bcl-2, Bax, bFGF, and VEGF immunostaining in the cancer was evaluated microscopically and recorded as a percentage of positive cells (labeling frequency %). Positive expression was defined as positive staining cells ≥10%. All sections were coded and observed by an investigator who was blinded for study protocols.

Immunohistochemistry of intratumor vascularization

Intratumor vascularization was examined by immunohistochemical analysis as described previously[18]. The sections were stained with anti-CD34 Ab (at 1:100 dilution, Sigma Co) and a second peroxidase-conjugated goat anti-rat IgG Ab (1:100 dilution, Santa Cruz biotechnology). Immunoperoxidase staining was carried out using a Simplestain mouse MAX-PO Kit (Nichirei, Tokyo, Japan). The density of microvessels was quantified by first scanning the tumor at low power and identifying five areas at the tumor periphery that contained the maximum number of discrete microvessels and then counting the individual microvessels.

Western blot analysis

Tumor tissues were lysed with a hand-held homogenizer using lysis buffer (10 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L DTT/1 mmol/L NaF/0.5% NP-40/0.5 mmol/L PMSF/0.2 mmol/L sodium orthovanadate/2 μg/mL of aprotinin, leupeptin and pepstatin). Lysates were incubated at 4 °C for 20 min with rotation. After centrifugation at 14 000 r/min (20 800×*g*) for 12 min, the supernatants were collected and boiled in loading buffer, separated by SDS-PAGE, and transferred onto a nitrocellulose membrane. Membranes were blocked with 5% (*w/v*) milk/PBS/0.1% Tween 20. Immunodetection was performed as described in the ECL kit protocol (Amersham Pharmacia): blots were incubated for 2 h at room temperature with specific antibody, washed with PBS, and incubated for another 30 min at room temperature with the peroxidase-conjugated antibodies. Western-blot analysis was performed with Fas, FasL, caspase3, Bcl-2, Bax, bFGF, VEGF, and β-actin antibodies (Santa Cruz Biotechnology Inc). All experiments were performed in triplicate.

Statistical analysis

SPSS10.0 for Windows (SPSS Inc) was used to analyze the data and plot curves. Pearson's chi square test and *t*-test were used to compare the statistical significance of the differences in data from the two groups. A level of $P<0.05$ was considered statistically significant.

Results

Effects of tumstatin peptides on proliferation of HUVEC-12 and SGC-7901 cells

In order to assess the selective inhibitory effect of tumstatin peptides on proliferation of endothelial cells and human gastric cancer cells, the MTT assay was used to measure the viabilities of HUVEC-12 and SGC-7901cell lines after a 24-h treatment of tumstatin peptide 19 or peptide 21. The results show that peptide 19 significantly inhibited the proliferation of SGC-7901 cells. However, peptide 21 had little effect on the proliferation of SGC-7901 cells (Figure 1A). Peptide 21 potently suppressed proliferation of HUVEC-12 cells, whereas peptide 19 did not affect the proliferation of vascular endothelial cells (Figure 1B). Moreover, treatment with both peptides together resulted in a synergistic decrease of proliferation compared with the inhibitory effect of each peptide alone in both HUVEC-12 and SGC-7901 cell lines ($P<0.05$).

Effects of tumstatin peptides on cell apoptosis

After 48 h of treatment with peptides we observed an induction of apoptosis in SGC-7901 cells. We found that 13.3%±1.5% of the cells treated with peptide 19 alone and 17.7%±2.5%

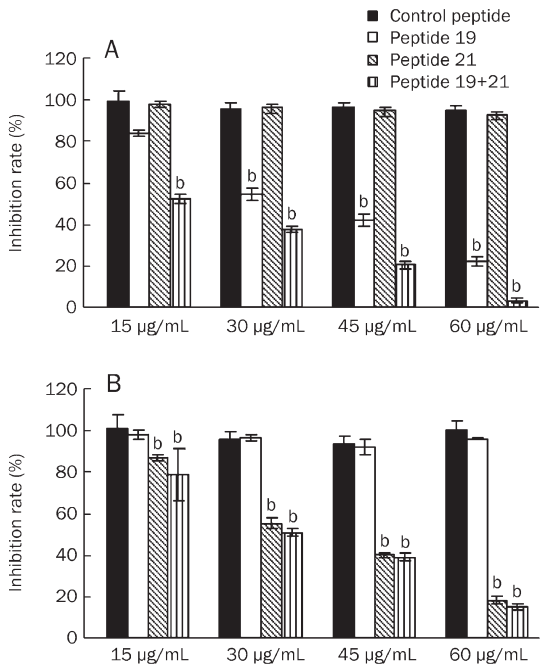

Figure 1. Effects of tumstatin peptides on SGC-7901 and HUVEC-12 proliferation by MTT assay *in vitro*. (A) SGC-7901 cells; (B) HUVEC-12 cells. $^{b}P<0.05$: compared with each control peptide.

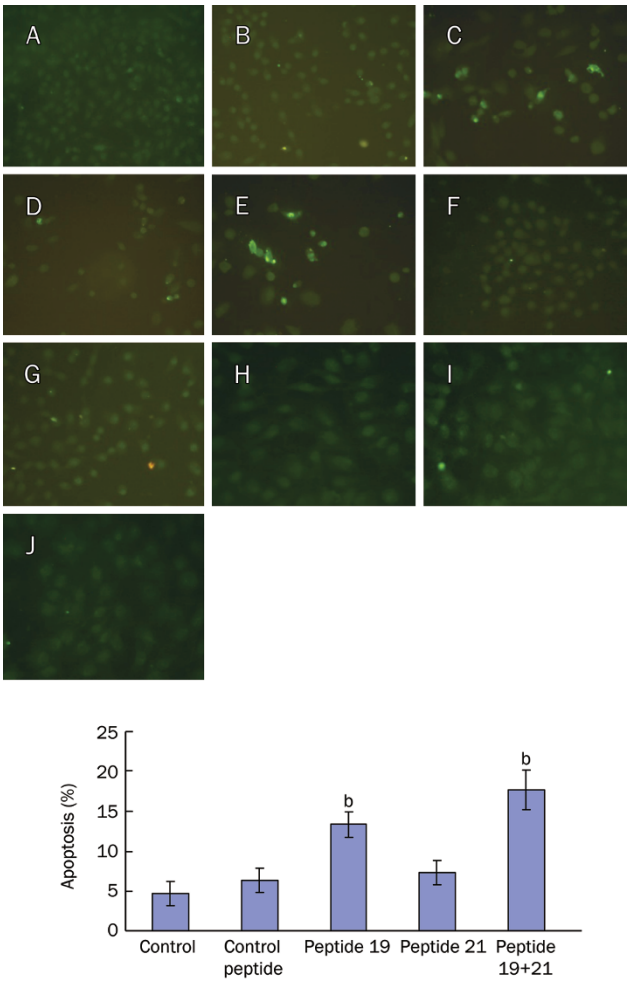

Figure 2. Tumstatin peptides induce apoptosis in SGC-7901 and HUVEC-12 cells by TUNEL assay *in vitro*. A–E ×200: SGC-7901 cells. (A) SGC-7901 cell; (B) Control peptide; (C) peptide 19; (D) peptide 21; (E) peptide 19 and peptide 21; Apoptotic index: $^{b}P<0.05$ compared with control). F–J ×200: HUVEC-12 cells (F: HUVEC-12 cells; G: Control peptide; H: peptide 19; I: peptide 21; J: peptide 19 and peptide 21).

of the cells treated with peptide 19 and peptide 21 together underwent apoptosis, whereas only 4.7%±1.5% did so in the control peptide group. These differences were statistically significant ($P<0.05$). SGC-7901 control cells had the lowest rate of spontaneous apoptosis, 4.7%±1.5%, which rose to 7.3%±1.5% ($P>0.05$) after peptide 21 treatment as shown in Figure 2A–2E. We also noted a concurrent additive effect in the induction of apoptosis by treatment with peptide 19 and peptide 21 together in SGC-7901 cells. The rate of induced apoptosis increased by about 3.5-fold from 4.7%±1.5% (controls) to 17.7%±2.5%, whereas the use of each peptide alone was not as effective, as shown in Figure 2. We did not observe significant apoptosis in HUVEC-12 cells treated with tumstatin peptides (Figure 2F–2J).

Effects of tumstatin peptides on morphology of SGC-7901 cells and HUVEC-12 cells

Electron microscopy of SGC-7901 cells treated with peptide 19 alone or the combination of the two peptides showed typical apoptosis characterized by volume reduction, chromatin condensation, nuclear fragmentation, and the presence of apoptotic bodies (Figure 3B, 3D) when compared with the control cells (Figure 3A). These changes were not observed in SGC-7901 or HUVEC-12 cells after treatment with peptide 21 (Figure 3C, 3F, 3G, 3H).

Cell cycle assay

To study the effect of tumstatin peptide treatment on proliferation at different phases of the cell cycle, we treated exponentially growing cells with tumstatin peptides for 24 h. Conventional DNA FCM showed that 56.23% of the HUVEC-12 cells treated with control peptides were found in G_0/G_1 phase, 31.38% in S phase, and the remaining cells in G_2/M phase. However, treatment with peptide 19, peptide 21, or the two peptides together resulted in an accumulation of cells in the G_0/G_1 phase. The fraction of G_0/G_1 DNA content accounted for 49.45% and 54.49% in the untreated SGC-7901 cells and control SGC-7901 cells. After treatment with peptide 19, peptide 21, or the two peptides together, the fraction of G_0/G_1 DNA content increased to 69.72%, 68.10%, and 69.78%, respectively, indicating that tumstatin peptides could arrest the cell cycle at the G_0/G_1 phase in both HUVEC-12 and SGC-7901 cells (Table 1).

Inhibition of tumor growth by tumstatin peptides in tumor-bearing mice

Treatment with peptide 19 or the combination of both pep-

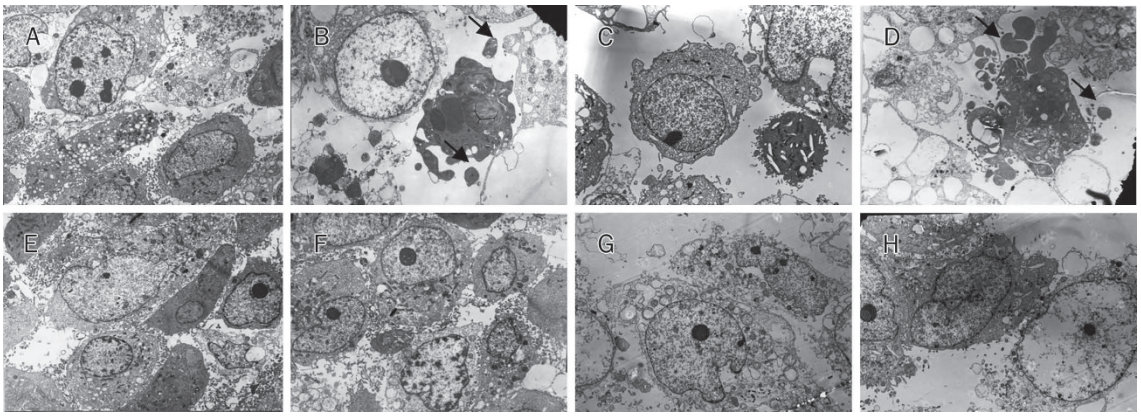

Figure 3. SGC-7901 cells and HUVEC-12 cells treated with tumstatin peptides under electron microscope. A–D EM ×2500: SGC-7901 cell (A: control peptide; B: peptide 19; C: peptide 21; D: peptide 19 and peptide 21). E–H EM ×2500: HUVEC-12 cell (E: control peptide; F: peptide 19; G: peptide 21; H: peptide 19 and peptide 21). Arrows represent apoptotic bodies.

Table 1. Effects of tumstatin peptides 19 and peptide 21 on the cell cycle distribution of SGC-7901 cells and HUVEC-12 cells by flow cytometry assay.

	Groups	G_1 (%)	G_2 (%)	S (%)
HUVEC-12	Control	56.77	12.02	31.21
	Control peptide	56.23	12.39	31.38
	Peptide 19	65.43	10.33	24.24
	Peptide 21	74.65	11.22	14.13
	Peptide 19+21	74.84	10.75	14.41
SGC-7901	Control	49.45	16.19	34.37
	Control peptide	54.49	15.08	30.43
	Peptide 19	69.72	15.46	14.82
	Peptide 21	68.10	14.32	17.58
	Peptide 19+21	69.78	15.07	15.15

Table 2. Tumor volumes after treatment with peptide 19, peptide 21, or peptide 19 and peptide 21 together in orthotopic model (Tumor volumes: mean±deviation)

Group	Doses (mg/kg)	Tumor volums (mm^3)	*t*	*P*
Controls		375.17±99.57		
Control peptide	4.4 mg/kg	365.98±81.88	0.362	0.736
	6.6 mg/kg	343.91±89.17	0.789	0.474
	8.8 mg/kg	350.49±95.78	0.344	0.748
Peptide 19	4.4 mg/kg	257.09±45.44	3.829	0.019[b]
	6.6 mg/kg	183.72±26.28	4.002	0.016[b]
	8.8 mg/kg	69.96±14.93	7.278	0.002[b]
Peptide 21	4.4 mg/kg	342.65±52.87	0.469	0.664
	6.6 mg/kg	277.96±56.23	1.928	0.126
	8.8 mg/kg	152.29±37.69	5.250	0.006[b]
Peptide 19+21	2.2 mg/kg+2.2 mg/kg	166.70±26.55	4.424	0.011[b]
	3.3 mg/kg+3.3 mg/kg	113.93±24.52	5.381	0.006[b]
	4.4 mg/kg+4.4 mg/kg	33.20±11.26	7.253	0.002[b]

[b]$P<0.05$: compared with each control peptide.

tides yielded considerable inhibition of tumor growth (Figure 4). Significant tumor regression was observed at day 30 in animals treated with peptide 21 in the concentrations of 8.8 mg/kg ($P<0.05$). Treatment with the two peptides together had a synergistic inhibitory effect on tumor growth compared with treatment with either single peptide ($P<0.05$) (Table 2). All animals were alive until sacrifice, but 16% body weight loss occurred by day 20 after the peptide combination (4.4 mg/kg of each peptide) treatment. A 30% loss in body weight

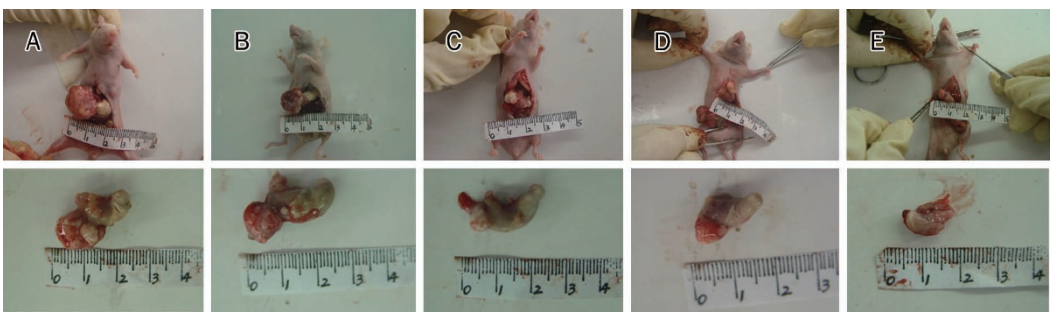

Figure 4. Tumstatin peptides inhibited gastric tumor growth in orthotopic model. (A) No treatment; (B) Treated with control peptide; (C) Treated with peptide 19 (8.8 mg/kg); (D) Treated with peptide 21 (8.8 mg/kg); (E) Treated with peptide 19 (4.4 mg/kg) and peptide 21 (4.4 mg/kg).

was observed with this treatment by day 30. In other groups, mice experienced <11% body weight loss.

Inhibition of angiogenesis by tumstatin peptides in tumor-bearing mice

We then examined the effect of tumstatin peptides on *in vivo* angiogenesis in tumors by immunostaining with CD34. Invasive growth of local cancer cells into lymph ducts was observed in control groups (Figure 5A). Tumors from animals receiving PBS (Figure 5B), control peptide, or peptide 19 (Figure 5C, 5D) showed intense CD34 staining, indicating the presence of extensive angiogenesis in the tumors. However, tumors from animals treated with peptide 21 alone or the combination of the two peptides together showed a significant reduction in microvessel density (Figure 5E, 5F) (P=0.006 and 0.001, respectively) (Table 3).

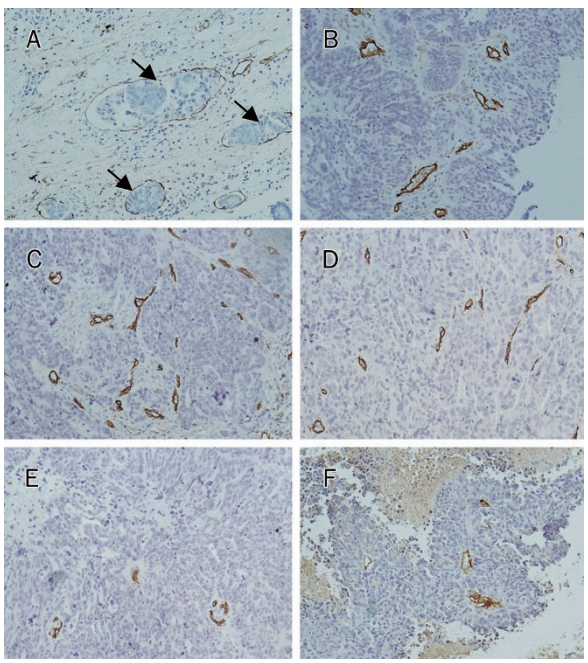

Figure 5. Intratumor vascularization examined by immunohistochemical expression of CD34 ×200. (A) invasive growth of local cancer cells into lymph ducts (arrows) in untreated mice; (B) Treated with PBS; (C) Treated with control peptide (8.8 mg/kg); (D) Treated with peptide 19 (8.8 mg/kg); (E) Treated with peptide 21 (8.8 mg/kg); (F) Treated with peptide 19 (4.4 mg/kg) and peptide 21 (4.4 mg/kg).

Expression of Fas, FasL, caspase 3, Bcl-2, Bax, VEGF, and bFGF in tumor tissues

26.67%, 13.33%, and 6.67% tumors were positive for Fas, FasL, and caspase 3 in the mouse gastric cancer tissues treated with control peptide. However, 30 days after treatment with peptide 19 or the two peptides together, the expression levels of all three proteins increased in the gastric cancer tissues of mice (Figure 6A–6C). The expression of Fas, FasL, and caspase 3 remained low in animals treated with peptide 21. The expression of Bcl-2 and Bax was similar in the three treated groups and the control peptide group (Figure 6D, 6E). The expression of VEGF and bFGF was high in the control peptide group and the peptide 19 group (Figure 6H, 6I). Treatment with peptide 21 (Figure 6F, 6G) or with the two peptides together decreased the expression of VEGF and bFGF in gastric cancer tissues (Table 4). We further confirmed these findings by Western blot analysis. The data showed that the expression levels of Fas, FasL, and caspase3 were increased in mouse gastric cancer tissues treated with peptide 19 or peptide 19 and peptide 21, whereas the expression of Bcl-2 and Bax was similar in the three treated groups and the control peptide group (Figure 7).

Table 3. Density of microvessels in the tissues of orthotopic model (MVD: mean±deviation).

	Mean MVD	*P*
Control	22.2±3.6	
Control peptide	20.0±2.6	0.448
Peptide 19	19.0±3.9	0.352
Peptide 21	8.8±2.4	0.006[b]
Peptide 19+21	5.0±2.9	0.001[b]

[b]P<0.05: compared with control.

Discussion

Angiogenesis, the process by which new blood vessels are derived from preexisting capillaries, is considered essential for tumor growth[1, 19]. The tumor microenvironment influences the induction of tumor angiogenesis[3, 19, 20]. The angiogenic switch is turned "on" when levels of endogenous angiogenesis stimulators, such as VEGF and bFGF, exceed those of endogenous angiogenesis inhibitors[1, 5, 20, 21]. Tumstatin is one such endogenous angiogenesis inhibitor.

In the present study, we demonstrate the anti-tumor properties of two tumstatin synthetic peptides: peptide 19, which corresponds to residues 185–203 of the NC1 domain of the α3 [IV] chain, and peptide 21, a T7 mutant, in human gastric carcinoma cells *in vitro* and *in vivo*. We chose these two peptide fragments for tumor treatment because peptide 19 contains anti-tumor cell activity and we believed that peptide 21 would contain the anti-angiogenic property of tumstatin. Therefore, in combination with peptide 19, peptide 21 may have an adjuvant role in the treatment of gastric cancer. Peptide 19 inhibited proliferation and induced apoptosis in human gastric carcinoma cells. In contrast, peptide 21 specifically suppressed proliferation in endothelial cells, causing them to accumulate in G_0/G_1. Peptide 21 did not induce apoptosis in HUVEC-12 or SGC-7901 cells. In addition, peptide 19 and peptide 21 exhibited synergistic anti-tumor effects *in vitro* and *in vivo*. Our results confirmed that tumstatin synthetic peptide 19 selectively inhibited the proliferation of tumor cells, and tumstatin synthetic peptide 21 did not influence the growth or proliferation of tumor cells. None of the whole NC1 domains inhibited proliferation of cancer cell lines, as observed with

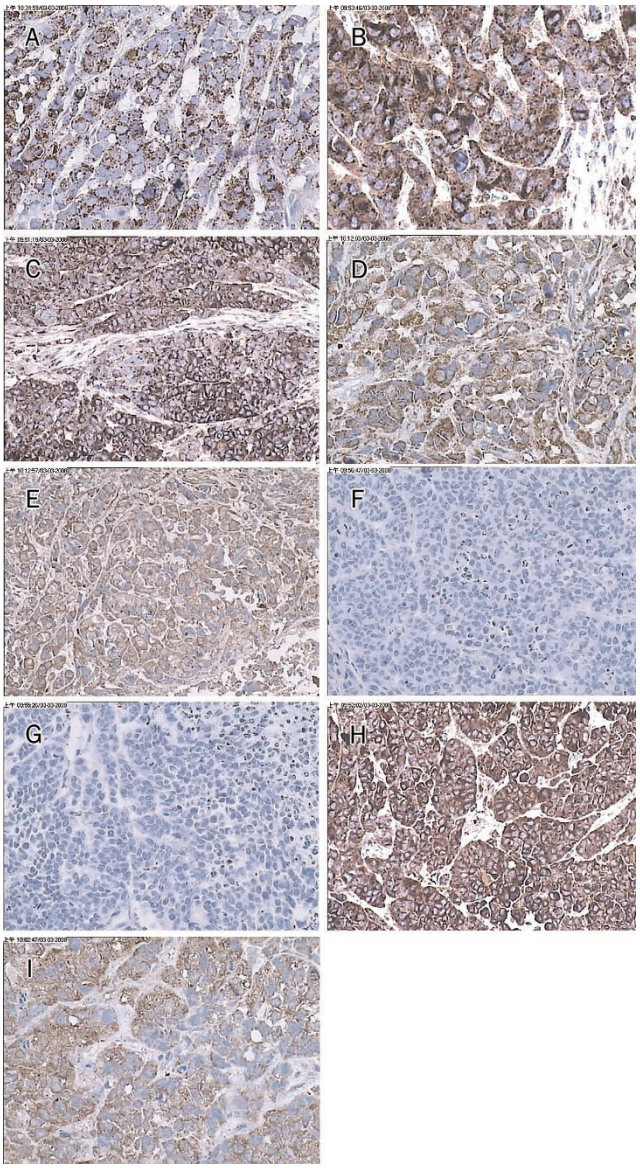

Figure 6. Immunohistochemical expression of Fas; FasL; caspase3; Bcl-2; Bax; VEGF and bFGF in orthotopic gastric carcinoma samples or orthotopic gastric carcinoma treated with peptide 19 and peptide 21. Plasma or membrane staining was frequently observed in tumour cells treated with peptide 19 (A, B, C, D, and E; Fas; FasL; caspase 3; Bcl-2 and Bax, respectively A×400, B×400, C×200, D×400, and E×200). Absent expression of bFGF (F×200) or VEGF (G×200) was observed in tumour cells treated with peptide 21. Expression of bFGF (H×200) and VEGF (I×400) were high in orthotopic gastric carcinoma samples without treatment.

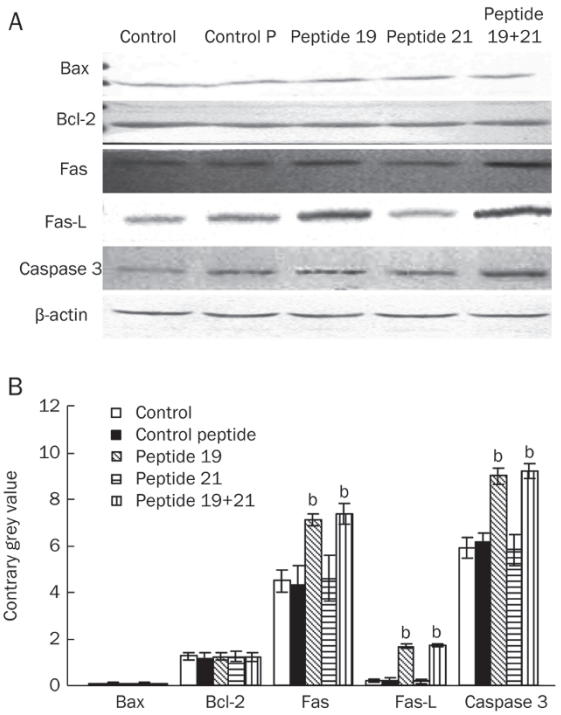

Figure 7. (A) Western blot analysis showed that the expression levels of Fas, FasL, caspase 3 were upregulated in orthotopic gastric carcinoma tissues treated with peptide 19 or peptide 19 and peptide 21. (B) The histogram shows the results from applying Glyco Band-Scan software. (Control P: Control peptide; $^{b}P<0.05$ *vs* each control).

the 185-205(α3(IV)NC1) peptide (peptide 19), indicating that this effect is dependent on partial degradation of the NC1 domain[22].

This research also investigated the effect of these two peptide fragments on the growth of human gastric cancer xenografts in a mouse model. The animal model used in these gastric cancer experiments had the characteristics of orthotopic syngenic tumors (SGC-7901cells) in an immunocompetent host (nude mice). Thus, the results from this study are clinically relevant. Our results show that peptide 19 suppressed the growth of tumor xenografts in a dose-dependent manner. However, peptide 21 suppressed the growth of tumor xenografts only at higher concentrations. These findings are consistent with previously reported results. Previous data showed that tumstatin delayed primary tumor growth and metastasis but failed to achieve tumor regression in animal models. Treatment with both peptide fragments caused some weight loss in the experimental animals, which was tolerable when less than 8.8 mg/kg peptide was used. Higher concentrations (over 8.8 mg/kg) of tumstatin were not used in this study because of high toxicity.

We observed *in vivo* that mice treated with peptide 21 had a lower number of CD34-positive vessels along with an impairment in angiogenesis. *In vitro*, we did not detect apoptotic endotheliocytes in cells treated with peptide 21. These results indicate that peptide 21 selectively inhibits endotheliocytes and induces apoptosis of new vessels in tumors, but does not affect normal endotheliocytes.

CAO *et al* reported that a fusion peptide made of tumstatin-derived peptides a.a. 74–98 and a.a. 197–215 connected by the human IgG3 upper hinge region possesses antiangiogenic and antitumor cell proliferation properties. They showed that this peptide potently inhibited the proliferation of human endothelial (HUVEC-12) cells and human colon cancer (SW480) cells

Table 4. Expressions of Fas, FasL, Bcl-2, Bax, VEGF, bFGF, and caspase 3 in orthotopic gastric carcinoma tissues.

Antibody	Control	Control peptide		Peptide 19		Peptide 21		Peptide 19 and 21	
	Positive (%)	Positive (%)	*P*	Positive (%)	*P*	Positive (%)	*P*	Positive (%)	*P*
Fas	20 (1/5)	26.67 (4/15)	1.000	80.0 (12/15)	0.031[b]	27.8 (4/15)	1.000	86.7 (13/15)	0.014[b]
FasL	20 (1/5)	13.33 (2/15)	1.000	86.7 (13/15)	0.014[b]	20.0 (3/15)	1.000	93.3 (14/15)	0.005[b]
Bcl-2	80 (4/5)	93.33 (14/15)	0.447	86.7 (13/15)	1.000	93.3 (14/15)	0.447	100.0 (15/15)	0.250
Bax	40 (2/5)	27.77 (4/15)	0.613	40 (6/15)	1.000	33.33 (5/15)	1.000	33.33 (5/15)	1.000
VEGF	80 (4/5)	80 (12/15)	1.000	86.7 (13/15)	1.000	20.0 (3/15)	0.031[b]	13.3 (2/15)	0.014[b]
bFGF	80 (4/5)	73.33 (11/15)	1.000	73.3 (11/15)	1.000	6.7 (1/15)	0.005[b]	6.7 (1/15)	0.005[b]
Caspase 3	0 (0/5)	6.67 (1/15)	1.000	60.0 (9/15)	0.038[b]	13.3 (2/15)	1.000	66.7 (10/15)	0.033[b]

[b]*P*<0.05: compared with each control peptide.

in vitro, with no inhibition of proliferation in Chinese hamster ovary (CHO-K1) cells. The peptide also significantly inhibited human endothelial cell tube formation and suppressed tumor growth of SW480 cells in a mouse xenograft model[23].

The antiangiogenic activity of tumstatin is localized to two distinct integrin binding regions that are separate from the region responsible for its anti-tumor activity[9, 24]. αVβ3 integrin binds to the NH_2-terminal end, amino acids 54–132, which is presumably associated with cap-dependent translation inhibition and antiangiogenic activity[12]. α3β1 integrin binds to the C-terminal region, residues 185–203, which is associated with antitumor activity[25, 26]. When tumstatin binds to αVβ3 integrin in endothelial cells it inhibits phosphorylation of FAK. Inhibition of FAK activation leads to inhibition of the FAK/PI-3K/Akt/mTOR/eIF4E/4E-BP1 signaling axis that mediates cap dependent translation, resulting in activation of apoptosis. The binding of α3(IV)NC1 to α3β1 integrin transdominantly inhibits αVβ3 expression in cells. Under hypoxic conditions, this inhibits NFκB mediated signaling and leads to inhibition of COX-2/VEGF/bFGF expression, resulting in inhibition of hypoxic tumor angiogenesis. We found that the expression of VEGF and bFGF was low in gastric cancer tissues of mice treated with peptide 21. Peptide 21 has no effect on apoptosis of tumor cells and may prevent angiogenesis by suppressing the activity of VEGF and bFGF. However, in gastric cancer tissues of mice treated with peptide 19 alone or peptide 19 and peptide 21 together, the expressions of Fas, FasL, and caspase3 were high. The expressions of Bcl-2 and Bax were similar between the treated and control groups. These results show that peptide 19 may induce apoptosis of tumor cells through the Fas pathway but not through the Bc1-2 family.

There are distinct mechanisms that mediate the anti-angiogenic and anti-proliferative activities of these tumstatin peptides. The data reported here suggest that two tumstatin synthetic peptide fragments together facilitate two unique antitumor activities, which could make them valuable therapeutic agents for inhibition of tumor growth.

Acknowledgments

This work was supported by a grant from the National Natural Science Foundation of China (No 30472035), the Key Program of Heilongjiang Science and Technology Foundation (ZJY04-0102), Heilongjiang Innovation Program in Graduate Education (YJSCX2007-0201HLJ), a grant from the Department of Education of Heilongjiang Province (11531089), and the Youth Program of Heilongjiang Science and Technology Foundation (QC08C07).

Author contribution

Xiao-ming JIN and Xing-han LIU designed research; Ying-jie LI and Yan HE performed research; Li-chun SUN contributed analytical tools, analyzed data, and wrote the paper; Miao LIU and Qi-min WANG contributed some reagents and assisted to establish orthotopic tumor model.

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