The Anti-angiogenesis Properties of Two Tumstatin Synthetic Peptides in Infant Hemangioma

Yi-lun Liu[1*], Tuan-jie Hou[2*], Wei-hua Liu[1], Jing Sun[3], Yue-ming Liu[1], Hai-rong Liu[1], Peng Ruan[1]

[1] Department of Burns Surgery and Experiment Research Center, the First Affiliated Hospital of Chengdu Medical College, Chengdu, Sichuan 610500,China
[2] Plastic and Cosmetic Surgery Center, Subei People's Hospital, Clinical Medical College, Yangzhou University, Yangzhou, Jiangsu 225001, China
[3] Department of Pathology, Chengdu Medical College, Chengdu, Sichuan 610083, China.
[*] These authors contributed equally to this work. E-mail: 13375280848@163.com

Abstract: The aim of this paper was to describe the anti-angiogenesis properties of the two tumstatin synthetic peptides, T3 and T7, which corresponded to 69-88, 74-98 respectively of amino acid sequence of tumstatin, on infantile hemangioma endothelial cells in vitro. The MTT assay results demonstrated that the tumstatin peptides, T3 and T7, could inhibit the proliferation of hemECs in the culture media with 0.025 volume fraction of FBS in a dose dependent manner. When the volume fraction of FBS in the culture media rose, it was deduced that more gowth factors exerted their effect promoting proliferation to the extent that the difference between different dose groups were covered up. Additionally, it was observed in the tumstatin peptides groups that the cell morphology had changed, the cells became contracted and sparse compared with the control group.Further, the cell cycle assay demonstrated that the two tumstatin peptides could arrest the cell cycle at the G0/G1 phase in the hemECs.This results were consistent with the prior MTT assay results, and showed the reasonable cause why the two peptides could inhibit the hemECs proliferation. Moreover, it was demonstrated that the two tumstatin peptides could inhibit the migration of hemECs by the in vitro wound healing assay.
[Liu YL, Hou TJ, Liu WH, Sun J, Liu YM, Liu HR, Ruan P. **The anti-angiogenesis properties of two tumstatin synthetic peptides in infant hemangioma**. *Life Sci J.* 2012;9(4):325-329] (ISSN:1097-8135). http://www.lifesciencesite.com. 49

Keywords: tumstatin; anti-angiogenesis; infant hemangioma; endothelial cells

1. Introduction

Infantile hemangioma (IH), a benign vascular tumor, is the most common tumor of infancy, with an incidence of 5%~10% at the end of the first year. There is increased risk in premature neonates under the weight of 1500 g. The abnormal growth of blood vessels, was considered to be the main pathological process of infantile hemangioma. Thus, it should be reasonable that the disease--infantile hemangioma could be treated by the same therapeutic strategy used to treat tumor by anti-angiogenesis theory.

Vascular basement membrane (VBM) is an important constituent of blood vessels providing structural support. VBM was also speculated to modulate capillary endothelial cell behavior especially during sprouting of new capillaries. During matrix re-organization, several short protein fragments are generated from VBM by proteases. Some of these fragments were identified as inhibitors of angiogenesis. At present there are about 25 endogenous angioinhibitors in clinical trials and many more in preclinical studies for the treatment of cancer. These angioinhibitors fall into two general categories: (a) antibodies or small molecules that target pro-angiogenic factors of tumor cells such as VEGF, bFGF or PDGF, and (b) endogenous angioinhibitors such as thrombopondin-1, angiostatin, interferons, endostatin and some of the non collagenous (NC1) domains of Type IV collagen that target vascular endothelial cells. Tumstatin (the NC1 domain of α3 chain of type IV collagen) and its deletion mutant tum-5 possess anti-angiogenic activity. It was demonstrated that the anti-angiogenic activity of tumstatin and tum-5 is independent of disulfide bond requirement. This property of tum-5 allowed researchers to use overlapping synthetic peptide strategy to identify peptide sequence(s) which possess anti-angiogenic activity. Among these peptides, only the T3 peptide (69–88 amino acids) and T7 peptide (74–98 amino acids) inhibited proliferation and induced apoptosis specifically in endothelial cells.

In this work， we tried to identify the properties of T3 and T7 peptide on the cultured infant hemangioma endothelial cells. It was observed that the changes of the cultured infant hemangioma endothelial cells on its proliferation and apoptosis on the condition that the synthetic tumstatin peptide T3 and T7 was appplied. It was desired that this work should shed light on the prevention and control of the infantile hemangioma by providing the experimental base for the new therapeutic strategy.

2. Materials and methods

2.1 Synthetic peptides

T3 peptides covering 69–88 amino acids of tumstatin, LQTFTTMPFLFCNVNDVCNF, T7 peptides covering 74–98 amino acids of tumstatin，TMPFLFCNVNDVCNFASRNDYSYWL, and the corresponding scrambled peptide, SRNLSFYASCYPEWNNSRSCNLNYV were purchased from ChinaPeptides Co.Ltd. (Shanghai, China).

2.2 HE staining

The hemangioma tissues derivеded from surgical resection specimen of Chinese infant cavernous hemangioma patients was resected, fixed in 10% neutral formaldehyde for 12 hours, followed by decalcification, dehydration, rendering transparency, paraffin embedding, slicing, spreading, drying. It was then stained by hematoxylin solution for 5 min, differentiated in 0.5％acid alcohol for 5 s, rinsed with diluted ammonia for 30 s, counterstained with 0.5％aqueous eosin for 2 min, cleared and mounted in neutral resin.

2.3 Isolation of endothelial cells and cell culture

Primary cultures of hemangioma endothelial cells (hemEC) were established from Chinese infant hemangioma patients. These hemEC were selected for the studies because they are derived from typical proliferating hemangioma, exhibit clonality and cryopreserved cells at low population doublings are available. All properties we have examined have remained constant over several generations in culture. Briefly, ECs were isolated from hemangiomas by the method described previously by Eileen Boye, et al [1].Tissue samples of infant hemangioma were digested with trypsin, the cells were resuspended in EBM-A (EBM131 (Clonetics, USA), 20% heat-inactivated FBS (MinhaiBio, China), 2 mM glutamine, 100U/ml penicillin, 100 mg/ml streptomycin, 0.5 mM N-6,2'-O-dibutyryladenosine 3':5'-cyclic monophosphate, 1.0 mg/ml hydrocortisone (Sigma,USA) and grown to preconfluence. The ECs were purified from such primary cultures using Ulex europaeus I lectin-coated magnetic beads (Vector Laboratories Inc., USA), then, were grown in EGM-2 (Clonetics) on AF-coated dishes (Cascade Biologics), with 10% heat-inactivated FBS, in 5% CO_2 at 37 °C. Prior to specific experiments, cells were cultured in the absence of serum and supplemental growth factors for 12−24 hours. Collection and handling of all human material was according to guidelines of Ethics Committee of the First Affiliated Hospital of Chengdu Medical College on Human Studies and Clinical Investigation for protection of research subjects，and informed consent was obtained from all subjects.

2.4 MTT assay

Cells were trypsinized, seeded at 1×10^3 cells/well in 96-well plates, and treated with T3、T7 peptide at various concentrations (0, 15, 30, 45, and 60 μg/mL) in the culture media with various volume fraction of FBS (0, 0.025, 0.05, 0.10). Twenty-four hours later, the effects on cell growth were examined by MTT assay: 20 μL of MTT (Sigma,USA) 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 490 nm was recorded using an ELISA reader (BioTec,USA).

2.5 Cell cycle assay

Cells were treated with T3, T7 and the corresponding scrambled peptide (34 μg/mL). 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.

2.6 *In vitro* wound healing assay

Subconfluent cells were detached and 5×10^5cells were seeded in six-well tissue culture plates.After 24 h, the cells， which grown to confluence to form a monolayer，were cultured in serum-free medium for 24 h. Scratch wounds were created by scraping confluent cell monolays with a steril pipette tip to make an approximately 0.8mm gap.The cells scraped down were washed by serum-free medium,and the cells were then treated with T3 、T7 and the control peptide (34 μg/mL in each group) or with out peptide in the medium with 10% FBS. After 48 h, migration was quantified by counting cell number that had advanced into the cell-free space from 10 randomly chosen segments(0.8mm/segment) along with the initial wound border.

2.7 Statistical analysis

SPSS11.0 for Windows (SPSS Inc) was used to analyze the data. Pearson's chi square test and t-test were used to compare the statistical significance of the differences in data. A level of $P<0.05$ was considered statistically significant.

3 Results

3.1 Histologic appraisal of hemangioma

The histologic appraisal of infant cavernous hemangioma tissues includes the following characters ： grossly large dilated blood vessels and large vascular channels, less well circumscribed, with a single layer of endothelium. These thinly walled

vessels resemble sinusoidal cavities filled with blood cells such as figure 1.

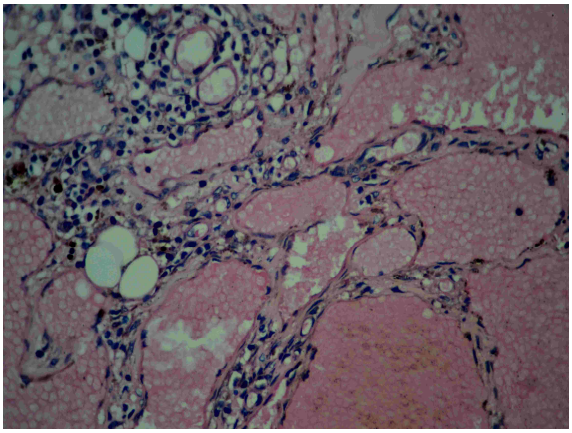

Figure 1. The histologic appraisal of infant cavernous hemangioma tissues. The hemangioma characterized by grossly large dilated blood vessels and large vascular channels, less well circumscribed, with a single layer of endothelium filled with blood cells.

3.2 Effects of tumstatin peptides on proliferation of hemECs

The MTT assay was used to measure the viabilities of hemECs after a 24-h treatment of tumstatin peptide (T3, T7), and the corresponding scrambled peptide (control peptide). The results show that peptide T3, T7 significantly inhibited the proliferation of hemECs in the condition that the hemECs were cultured in culture media with 0.025 volume fraction of FBS,compared with the control peptide group(P<0.05), in a dose dependent manner. While in the culture media with 0, 0.05, 0.10 volume fraction of FBS, there were no significant difference among the different dose groups (Figure 2).

3.3 Effects of tumstatin peptides on cell cycle of hemECs

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 T3, T7 and control peptide for 24 h. Conventional DNA FCM showed that 43.6% of the hemECs treated with control peptides were found in G0/G1 phase, 26.3% in S phase, and the remaining cells in G2/M phase. However, treatment with T3, T7 peptide resulted in an accumulation of cells in the G0/G1 phase. After treatment with T3, T7 peptide, the fraction of G0/G1 DNA content increased to 60.7%, 57.7% respectively compared with the control peptide group and the contro group(P < 0.05), indicating that tumstatin peptides could arrest the cell cycle at the G0/G1 phase in the hemECs (Table 1).

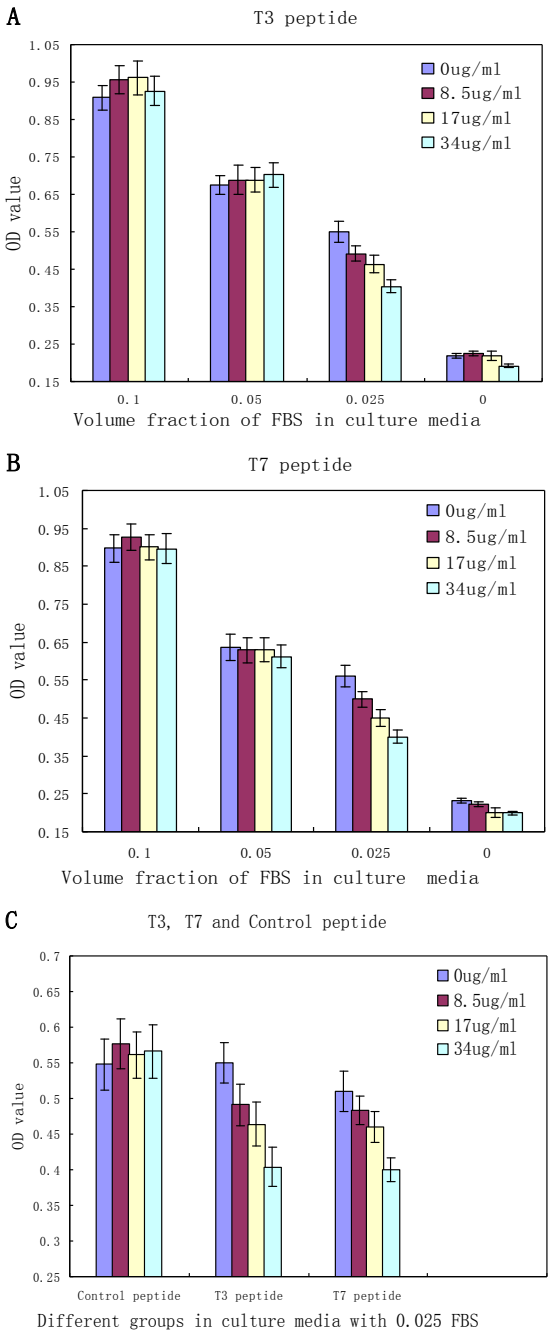

Figure 2. Effects of tumstatin peptides on proliferation of hemECs. Peptide T3, T7 significantly inhibited the proliferation of hemECs in the condition that the hemECs were cultured in the culture media with 0.025 volume fraction of FBS, compared with the control group(P<0.05). While in the culture media with 0, 0.05, 0.10 volume fraction of FBS, there were no significant differences among those peptides.

Table 1. Effects of tumstatin peptides on the hemECs cycle distribution of by flow cytometry assay.

Groups	G0/G1(%)	S(%)	G2/M(%)
T3 peptide	60.4±2.1 *†	17.4±2.3	21.6±3.4
T7 peptide	57.7±1.8 *†	20.9±1.9	21.5±3.0
Control peptide	43.6±3.1	26.3±2.7	30.4±2.9
Control	40.5±2.5	29.4±3.2	30.3±4.5

*P < 0.05 vs Control peptide, †P <0.05 vs Control

3.4 Effects of tumstatin peptides on migration of hemECs

Peptide T3, T7 could significantly inhibited the migration of hemECs compared with the control group and control peptide group(P<0.05) at a dose of 34 μg/mL added into the medium with 10% FBS in the corresponding group for 48 h, while there were no significant differences between the T3 peptide group and T7 peptide group, the control peptide group and control group (Figure 3). This results indicated that the tumstatin peptides—T3 and T7, have the potential capability to inhibit angiogenesis of infantile hemangioma through inhibiting the migration of hemECs. Additionally, it was observed in the tumstatin peptides groups that the cell morphology had changed—the cells became contracted and sparse compared with control group, which is according with the results of the MTT assay.

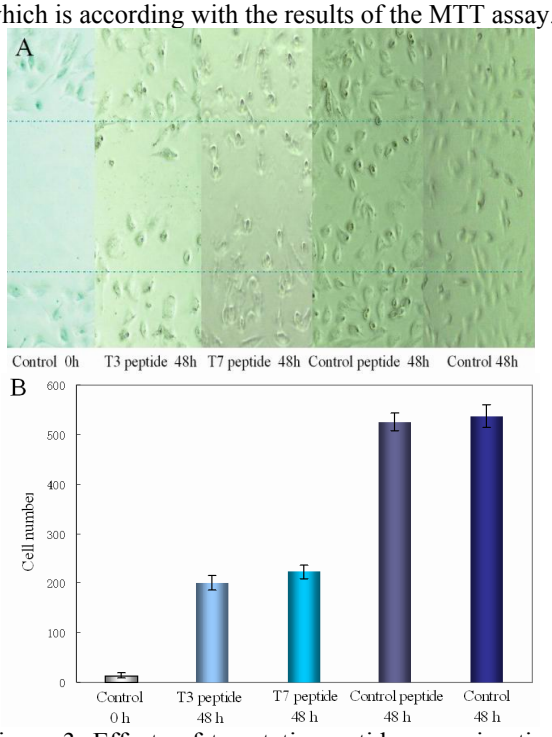

Figure 3. Effects of tumstatin peptides on migration of hemECs. Peptide T3,T7 significantly inhibited the migration of hemECs compared with the control group and control peptide group(P<0.05) after added into the medium with 10% FBS at a dose of 34 μg/mL in the corresponding group,while there were no significant differences between the T3 peptide group and T7 peptide group, the control peptide group and control group.

4 Discussion

Angiogenesis is involved in various pathological disorders including infantile hemangioma as well as other tumor. To inhibit angiogenesis, a number of inhibitors have been identified, and certain factors such as angiostatin [2], endostatin [3], canstatin [4], arresten [5], and tumstatin [6] are tumor-associated angiogenesis inhibitors which are potentially generated in vivo. Tumstatin and its deletionmutant tum-5 possess powerful anti-angiogenic activity. As one of the endogenous angiogenesis inhibitors (EAIs), tumstain's roles in tumor are remain to be fully elaborated. It was demonstrated that EAIs are part of a balance mechanism regulating tumor angiogenesis, serving as intrinsic microenvironmental barriers to tumorigenesis [7]. T3 and T7 peptide are synthetic peptides according to the different segment of tum-5 peptide sequence, and both been reported that could inhibited proliferation specifically in endothelial cells[8]. However, the role of tumstatin on the infantile hemangioma have not been demonstrated to date. And understanding the mechanisms in cellular fate of the hemangioma will help to establish more effective eradicating therapies.As the most common tumor of infancy, there still exists many obstacles in the treatment process of hemangioma. At present, many infant and young children suffered from this disease still could not been rescued because of the limited effect of the treatment methods of infantile hemangioma including surgical operation and pharmacotherapy such as steroidal hormone, etc[9,10]. The abnormal growth of blood vessels, was considered to be the main pathological process of infantile hemangioma. Thus, it should be reasonable that the disease—infantile hemangioma could be treated by the same therapeutic strategy used to treat tumor by anti-angiogenesis theory.As were reported, tumstatin could inhibit the proliferation of endothelial cells, specially on the dividing cells. Due to the superiority of specificity and targeting effects compared with traditional therapy, tumstatin have the potential applying value to become a novel drug in the treatment of infantile hemangioma .

In the present study, we demonstrate the anti-angiogenesis property of two tumstatin synthetic peptides, T3 and T7, which corresponds to 69–88、74–98 respectively of amino acid sequence of tumstatin, on infantile hemangioma endothelial cells in vitro. The MTT assay results demonstrated that tumstatin peptide (T3, T7) could inhibit the proliferation of hemECs in the culture media with

0.025 volume fraction of FBS in a dose dependent manner. When the volume fraction of FBS in the culture media rose, it was deduced that more gowth factors exerted their effect promoting proliferation to the extent that the difference between different dose groups were covered up. Additionally, it was observed in the tumstatin peptides groups that the cell morphology had changed, the cells became contracted and sparse compared with the control group.Further, the cell cycle assay demonstrated that the two tumstatin peptides could arrest the cell cycle at the G0/G1 phase in the hemECs.This results were consistent with the prior MTT assay results, and showed the reasonable cause why the two peptides could inhibit the hemECs proliferation. Moreover，it was demonstrated that the two tumstatin peptides could inhibit the migration of hemECs by the *In vitro* wound healing assay.

It has been reported that the T3 peptide and T7 peptide could induced apoptosis specifically in endothelial cells. We also tried to identified the property of those peptides on the hemECs apoptosis. But there was no difference being found between those peptides and control groups in the apoptosis detection assay by a annexin-v-fluos staining kit(Roche). So, there is much more work to be done to explain the results. Interestingly, it has been reported that a synthetic peptide fragments of tumstatin, peptide 21 (MPFLFCNVNDVCNFASRN DYS), couldn't demonstrated its effect on apoptosis of HUVEC-12 cells in the TUNEL assay[11].

Acknowledgements

This work was supported by grants from Scientific Research Project of Sichuan Provincial Health Department (100103) and Youth Fund Project of Sichuan Provincial Education Department (07ZB124).

Corresponding Author:
Tuanjie Hou
Plastic and Cosmetic Surgery Center, Subei People's Hospital, Clinical Medical College, Yangzhou University, Yangzhou, Jiangsu 225001, China
E-mail：13375280848@163.com

References

1. Boye E, Yu Y, Paranya G, Mulliken JB, Olsen BR, Bischoff J. Clonality and altered behavior of endothelial cells from hemangiomas. J. Clin. Invest. 2001; 107(6):745–52.
2. O'Reilly MS, Holmgren L, Shing Y, Chen C, Rosenthal RA, Moses M, Lane WS, Cao Y, Sage EH, Folkman J. Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. Cell. 1994; 79(2):315-28.
3. O'Reilly MS, Boehm T, Shing Y, Fukai N, Vasios G, Lane WS, Flynn E, Birkhead JR, Olsen BR, Folkman J. Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. Cell. 1997; 88(2):277-85.
4. Kamphaus GD, Colorado PC, Panka DJ, Hopfer H, Ramchandran R, Torre A, Maeshima Y, Mier JW, Sukhatme VP, Kalluri R. Canstatin, a novel matrix-derived inhibitor of angiogenesis and tumor growth. J Biol Chem. 2000; 275(2):1209-15.
5. Colorado PC, Torre A, Kamphaus G, Maeshima Y, Hopfer H, Takahashi K, Volk R, Zamborsky ED, Herman S, Sarkar PK, Ericksen MB, Dhanabal M, Simons M, Post M, Kufe DW, Weichselbaum RR, Sukhatme VP, Kalluri R. Anti-angiogenic cues from vascular basement membrane collagen. Cancer Res. 2000; 60(9):2520-6.
6. Maeshima Y, Colorado PC, Torre A, Holthaus KA, Grunkemeyer JA, Ericksen MB, Hopfer H, Xiao Y, Stillman IE, Kalluri R. Distinct antitumor properties of a type IV collagen domain derived from basement membrane. J Biol Chem. 2000; 275(28):21340-8.
7. Xie L, Duncan MB, Pahler J, Sugimoto H, Martino M, Lively J, Mundel T, Soubasakos M, Rubin K, Takeda T, Inoue M, Lawler J, Hynes RO, Hanahan D, Kalluri R. Counterbalancing angiogenic regulatory factors control the rate of cancer progression and survival in a stage-specific manner. Proc Natl Acad Sci USA. 2011; 108(24):9939-44.
8. Maeshima Y, Yerramalla UL, Dhanabal M, Holthaus KA, Barbashov S, Kharbanda S, Reimer C, Manfredi M, Dickerson WM, Kalluri R. Extracellular matrix-derived peptide binds to alpha(v)beta(3) integrin and inhibits angiogenesis. J Biol Chem. 2001; 276(34):31959-68.
9. Maguiness SM, Frieden IJ. Management of difficult infantile hemangiomas. Arch Dis Child. 2012; 97(3):266-71.
10. Neri I, Balestri R, Patrizi A.Hemangiomas: new insight and medical treatment. Dermatol Ther. 2012; 25(4):322-34.
11. Li YJ, Sun LC, He Y, Liu XH, Liu M, Wang QM, Jin XM. The anti-tumor properties of two tumstatin peptide fragments in human gastric carcinoma. Acta Pharmacol Sin. 2009; 30(9):1307-15.

9/19/2012