Effect of Tumstatin on Hypertrophic Scar in the Rabbit Model

Yi-lun Liu [1,2*], Jing Sun [3*], Tuan-jie Hou [4], Shu-rong Li [3], Feng-mei Deng [3], Hai-rong Liu [1], Yue-ming Liu [2], Xin-lian Liu [3], Juan Li [3]

[1] Experiment Research Center, First Affiliated Hospital, Chengdu Medical College, Chengdu, Sichuan 610500, China
[2] Departments of Burns Surgery, First Affiliated Hospital, Chengdu Medical College, Chengdu, Sichuan 610500, China
[3] Department of Pathology, Chengdu Medical College, Chengdu, Sichuan 610083, China.
[4] Plastic and Cosmetic Surgery Center, Subei People's Hospital of Jiangsu Province, Yangzhou 225001, China
* These authors contributed equally to this work, e-mail:lucysunmoon@126.com

Abstract: This paper aimed to investigate the effect of tumstatin on hypertrophic scar in the rabbit model to explore a new treatment method for hypertrophic scar. An animal model of hypertrophic scar on the rabbit ears was established and was intervened with tumstatin via local injection. The rabbits were randomly assigned into tumstatin group, endostatin group, triamcinolone acetonide group and the saline control group. At 5 days after epithelization, 4 ml of tumstatin, endostatin, triamcinolone acetonide and the saline with concentration of 200 ug/ ml were injected into tissue of scar along the perimeter of the scar in the four experimental groups, respectively. After 30 days of injection, the gross appearance of scar was observed and the blood flow of hyperplastic scar was assessed by laser Doppler flowmetry. The scar tissues on the bilateral ears of each rabbits were resected afterwards. The histological features of scar tissue were examined with HE staining, and the scar thickness was also measured under microscope. The blood flow, vessel density and the scar thickness in the tumstatin group were significantly lower than that in the other three groups, and the difference is statistically significant ($P<0.05$). Tumstatin may inhibit angiogenesis in hypertrophic scar by means of blocking endothelium proliferation and promoting its apoptosis, have a promising application in the prevention of human hypertrophic scar, which may serve as a promising role in the prevention of human hypertrophic scar.

Keywords: Hypertrophic scar; tumstatin; microvascular endothelial cells; anti-angiogenesis

1. Introduction

Hypertrophic scaring is one of the most common types of pathological scars, and excessive scars form as a result of aberrations of physiologic wound healing and may develop following any insult to the deep dermis, including burn injury, lacerations, abrasions, surgery and piercings. By causing pruritus, pain and contractures, excessive scarring can dramatically affect a patient's quality of life, both physically and psychologically (Bock et al., 2006). Over the past decades, great progress had been made in hypertrophic scars development, but no breakthrough has yet occurred in the specific pathogenic mechanism (Ferrara et al., 2003). At present, it is recognized that scar formation contains an important mechanism, that is, scar angiogenesis, and aberrant vascularization may be a critical factor to the abnormal scar formation. In recent years, the role of angiogenesis in scar formation has been a hot spot in studying mechanism of scar formation (Lakos et al., 2004; Galiano et al., 2004; Takeda et al., 2004). The studies demonstrated that the active angiogenesis was certainly associated with excessive scar formation, and it might affect wound healing by regulating wound healing process. Therefore, the study of the scar tissue angiogenesis, and exploration on therapeutic and preventive methods based on interrelationship between vascularization and scar formation, may become an important research direction in hypertrophic scars area.

Tumstatin peptide is an endothelial cell-specific inhibitor of protein synthesis and probably one of the most effective angiogenesis inhibitor derived from type IV collagen (Maeshima et al., 2002). Its antiangiogenesis activity is significantly stronger than that of endostatin. The researches found that it exhibited good efficacy in antiangiogenic treatment in tumor, without drug resistance and any side effects. And the studies also revealed that it played an important role in tumor angiogenesis. Currently, many researches on antiangiogenic effect of tumstatin in tumor have been reported. However, the report about the effect of tumstatin on scar angiogenesis has not been found. In the present attempt, the hypertrophic scar formed in the rabbit ear was interfered using tumstatin peptide, whose

efficacy was compared with that of endostatin peptide and triamcinolone acetonide (a conventional medicine), respectively. We observed its anti-angiogenic effect and impact on scar formation, and sought to explore a novel modality for the treatment and prevention of hypertrophic scars.

2. Material and Methods
Main reagents and instruments

New Zealand white rabbits of either sex weighting 2.0-2.5 kg were purchased from experimental animal center of Sichuan University (Chengdu, Sichuan province, China) and were maintained in separate cages. Tumstatin and endostatin peptides were all synthesized by Shanghai Kehua Bio-engineering Co. Ltd (Shanghai, China). Tumstatin peptide (74-98 amino acids) contained 25 amino acids and was referred to as T7 peptide (TMPFLFCNVNDVCNFASRNDYSYWL). The nucleotide sequence encoding amino acids 1-30 of endostatin termed as peptide 30 was used with amino acid sequence: PGARIFSFDGKDVLRHPTWPQKSVWHGSDPN. Triamcinolone acetonide injection was purchased from Shanghai General Pharmaceutical Co., Ltd (Shanghai, China). Moor FLPI laser Doppler flowmetry (Moor, UK) and Motic stereomicroscope (Motic Xiamen) was provided by Experiment Research Center of first affiliated hospital of Chengdu medical college.

Establishment of hypertrophic scar model in rabbit ears

All experimental rabbits were anesthetized with ketamine (15mg/kg) with administered intravenously via the ear vein, and then fixed on an operating table in the supine position. The routine preoperative preparation was performed and the procedure was conducted under an operating microscope. The full-thickness skin of the inside aspect of each ear was resected, and perichondrium was scraped away. Four 8 mm-diameter round wound model were generated in each rabbit ear. The wound area was exposed to the air after the bleeding was completely stopped. Each rabbit was singly housed under standard caging conditions. The wound exudates and scab was removed every two days. Twenty-five days after surgery, the wound epithelialization occurred and the scab fall off naturally, and an early phases of model of hypertrophic scar was obtained (Kloeters et al., 2007).

Experiment grouping design and drug delivery methods

The rabbits were randomly divided into four groups: tumstatin group（A）, endostatin group（B）, triamcinolone acetonide group （C）and the saline control group （D）. The ears whose epithelialization time was not consistent with the others was excluded the experiment.

Drug injection was administered posterior to 5 days of epithelialization. Tumstatin (200 ug/ml), endostatin (200 ug/ml), triamcinolone acetonide (20 mg/ml) of total volume of 0.4 ml was injected at multiple points of the scar tissue. After the intralesional injection, the scar turned pale white. The saline control group received saline injection, with the same volume and method of above.

The gross morphology observation and perfusion detection

The gross morphology, that is, the thickness, color and texture of the scar tissue was observed every other day. The blood flow of the scar tissue was detected 30 days after drug injection. The rabbits were anesthetized 30 min posterior to measurement in the same manner described above. Avoid unnecessary stimulation of the animals. The blood perfusion of the whole ear was assessed using Moor FLPI laser Doppler flowmetry and the quantitative local perfusion was recorded.

HE staining and scar thickness measurement

The subsequent operation was performed immediately after the perfusion detection, in which the scar tissue 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. The maximum thickness of the scar in slices was taken as the scar thickness under the Nikon TIS microscope (×100 times), which was detected using NIS-Elements D microscope imaging software for real-time measurements.

Effect of tumstatin on MECs proliferation detected by MTT assay

The subcutaneous tissue was removed from specimens, and then the specimens were cut into narrow strips of 3 mm wide and placed in 20 ml sterile antibiotic bottle, followed by adding of appropriate amount of dispase. After being cultured for 24 h at 4°C, the dermis and epidermis were carefully separated, and the former was put in a new antibiotic bottle. Then the samples were digested

with 1.25 g / L trypsin for 6-20 h at 4°C and washed with PBS. Add 10-20 ml DMEM or PBS to Petri dish, and the MECs were separated by mechanical extrusion method (Wang et al., 2008). The supernatant was removed after centrifugation, and the cells were adjusted to 2 x 10^4 cells/ml. The cells were purified and subcultured, and their proliferative activity was measured by MTT assay.

Statistical analysis
        The paired t-test was performed using SPSS 12.0 software, and analysis of variance was used to compare the mean of several groups with each other. The results were expressed as $\bar{x} \pm s$, and a P value of less than 0.05 was considered as statistically significant.

3. Results
Gross morphological changes
        Thirty days after drug injection, significant degeneration of the scar tissue was observed in the tumstatin group, whose color was close to the normal color of rabbit ears. It was slightly higher than the surrounding skin with relatively flat surface and soft touch. For the control groups, the scar tissue was light red, and obviously higher than the surrounding skin with hard texture and varied thickness (Figure 1). The results showed significant difference in gross morphology between the tumstatin group and the other three groups ($P<0.05$).

Perfusion detection
        Microcirculatory perfusion of the tumstatin group was significantly lower compared with the controls ($P<0.05$), indicating tumstatin can significantly inhibit blood flow of the hypertrophic scar (Figure 2, 3).

HE staining of the scar tissue
        Microscope morphology observation demonstrated that the microvessels and fibroblasts in the tumstatin group were significantly reduced, and the inflammation cells were less infiltrated in the tumstatin group compared with the other groups (Figure 4).

Scar thickness measurement
        The average scar thickness was 0.39 mm in the tumstatin group, and was 0.43 mm, 0.50 mm and 0.62 mm in the endostatin group, triamcinolone acetonide group and the saline control group, respectively (Figure 5).

Effect of tumstatin on MECs proliferation detected by MTT assay

        The optical density and OD values are linearly proportional to the number of living cells. As shown in Table 1, the OD value was significantly decreased in the tumstatin group compared with the other three groups, and the difference among them was statistically significant (Table 1, $P<0.05$). The results suggested that tumstatin can inhibit endothelium cells proliferation in vitro.
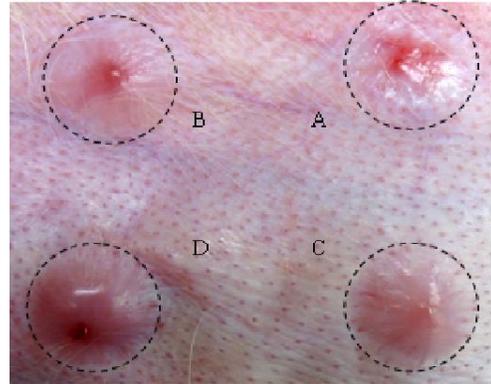

Figure 1. Gross morphological changes of hypertrophic scar. A: tumstatin group, B: endostatin group, C: triamcinolone acetonide group, D: saline control group.
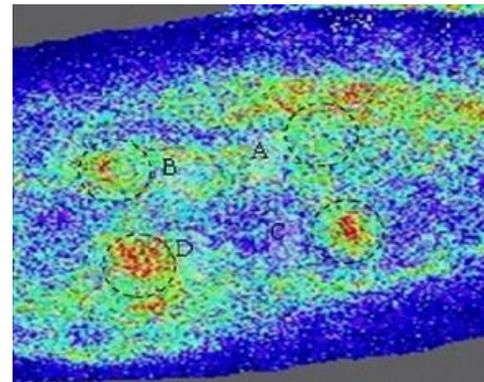

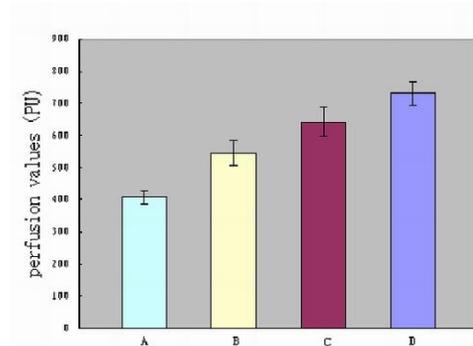**Figure 2. Laser Doppler Perfusion Images of the scar tissue.**
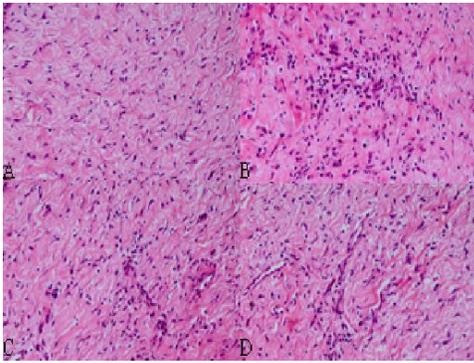

Figure 3. Perfusion values of the scar tissue.

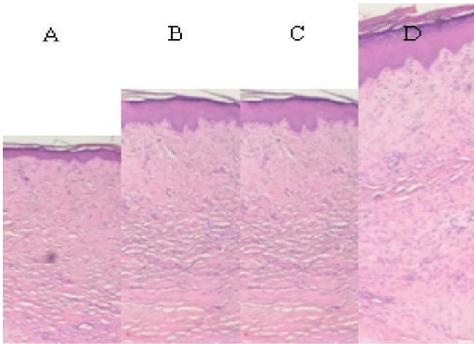**Figure 4. HE staining of the scar tissue.**

Figure 5. Thickness of the scar tissue.

Table 1. MECs proliferative activity in hypertrophic tissue in each group ($\bar{x} \pm s$).

Grouping	n	OD values
Saline control group	8	0.340±0.048
Triamcinolone acetonide group	8	0.320±0.036
Endostatin group	8	0.265±0.050 [*] [#]
Tumstatin group	8	0.235±0.056 [*] [#]

[*] $P<0.05$, vs saline control group;
[#] $P<0.05$, vs triamcinolone acetonide group.

4. Discussions

Hypertrophic scarring can occur after thermal injury, surgical incision, or other traumatic injury. These lesions are raised, pruritic erythematous scars that may widen, but which remain within the confines of the original wound. Histologically, hypertrophic scars appear hyperplastic and manifest a thickened epidermis, a dermis lacking dermal papillae, and the presence of collagen nodules in an abnormally increased vascular wound matrix (Harunari et al., 2006 and van der Veer et al., 2009). Hypertrophic scarring is a difficult clinical problem, and its management poses a great challenge. Treatments have progressed from rudimentary methods such as gross excision and radiotherapy to less invasive techniques such as intralesional injections and topical preparations. However, through this evolution of therapies, success rates have not significantly improved (Kelly，2004 and Chung et al., 2006). Additional research needs to be done to explore more effective treatment method, since there is no universally accepted treatment regimen.

Modern research has lead to an increased understanding of the pathophysiologic process of wound healing and scar formation. Nevertheless, the definite etiology of this abnormal scarring process is, for the most part, unknown. Many researches conducted both at home and abroad have showed that the hypertrophic process of scar is closely associated with development of angiogenesis (Kilarski，2009 and Jacobi et al., 2006). It is revealed that the average number of blood vessels and blood flow in hypertrophic scar was significantly higher than that in normal skin tissue.

The study on anti-angiogenesis therapy of hypertrophic scarring demonstrated that, the blood vessels were significantly less in hypertrophic scarring (Vranckx et al., 2005). Furthermore, decreased scar volume, fibroblast necrosis and apoptosis were also observed. A large number of experimental results indicated that impeding angiogenesis would inhibit scar formation

Tumstatin is a 28-kilodalton fragment of type IV collagen that displays both anti-angiogenic and proapoptotic activity (Floquet et al., 2004). Because tumstatin interacts with the $\alpha v \beta 3$ integrin in two separate locations, it has two distinct functions. The first is to inhibit angiogenesis and the second is to increase the amount of cellular apoptosis, or programmed cell death. By affecting the apoptotic pathway, tumstatin inhibits the proliferation of endothelial cells （Hamano et al.，2003）. At present, the role of tumstatin in various cancers has been fully described. It has been suggested that tumstatin can inhibit MECs proliferation and promote their apoptosis, thereby effectively impairing angiogenesis in tumor tissues, which is considered to be the most powerful anti-angiogenic factor. Nevertheless, studies on scar formation have not yet been seen, and thus in our attempt we sought to explore the role of tumstatin played in hypertrophic scarring.

The results obtained revealed that the scar tissue was significantly shrunk with relatively more flat surface after been treated with tumstatin in contrast to the other control groups ($P<0.05$), suggesting tumstatin can greatly reduce the scar volume. In addition, the microcirculatory perfusion of the tumstatin group was significantly lower compared with the controls ($P<0.05$), indicating tumstatin can significantly inhibit blood flow of the

hypertrophic scar. Furthermore, decreased microvessels and fibroblasts as well as less infiltrated inflammation cells were observed in the tumstatin group under microscopy. After been interfered with tumstatin, the average scar thickness (0.39 mm) was significantly smaller than that of the controls (0.43 mm, 0.50 mm and 0.62 mm) with statistical significance. Additionally, the OD value was significantly decreased after tumstatin injection compared with the other three groups, and the difference among them was statistically significant ($P<0.05$).The results suggested that tumstatin can markedly inhibit endothelium cells proliferation in vitro.

In conclusion, tumstatin can more effectively, than endostatin and triamcinolone acetonide, inhibit angiogenesis and significantly induce endothelium cells apoptosis, thereby inhibiting the formation of hypertrophic scars.

Corresponding Author:
Jing Sun,
Department of Pathology,
Chengdu Medical College,
Chengdu, Sichuan 610083, China,
Email: lucysunmoon@126.com

References
1. Bock O, Schmid-Ott G, Malewski P, Mrowietz U. Quality of life of patients with keloid and hypertrophic scarring. Arch Dermatol Res 2006; 297(10):433-8.
2. Ferrara N, Gerber HP, Le Couter J. The biology of VEGF and its receptors. Nat Med 2003; 9(6):669–76.
3. Lakos G, Takagawa S, Chen SJ, et al. Targeted disruption of TGF-beta/Smad3 signaling modulates skin fibrosis in a mouse model of scleroderma. Am J Pathol 2004; 165(1):203-17.
4. Galiano RD, Tepper OM, Pelo CR, et al. Topical vascular endothelial growth factor accelerates diabetic wound healing through increased angiogenesis and by mobilizing and recruiting bone marrow-derived cells. Am J Pathol 2004; 164(6):1935-47.
5. Takeda H, Katagata Y, Hozumi Y, et al. Effects of angiotensin II receptor signaling during skin wound healing. Am J Pathol 2004; 165(5):1653–62.
6. Maeshima Y, Sudhakar A, Lively JC, et al. Tumstatin, an endothelial cell-specific inhibitor of protein synthesis. Science 2002; 295(5552):140-3.
7. Kloeters O, Tandara A, Mustoe TA. Hypertrophic scar model in the rabbit ear: a reproducible model for studying scar tissue behavior with new observations on silicone gel sheeting for scar reduction. Wound Repair Regen 2007; 15 (Suppl 1):S40-5.
8. Xi-Qiao Wang,Ying-Kai Liu, Zhi-Gang Mao, et al. Isolation, Culture and Characterization of Endothelial Cells from Human Hypertrophic Scar. Endothelium 2008; 15(3):113-9.
9. Harunari N, Zhu KQ, Armendariz RT et al. Histology of the thick scar on the female, red Duroc pig: final similarities to human hypertrophic scar. Burns 2006; 32(6):669-77.
10. van der Veer W.M., Bloemen MC, Ulrich MM,et al. Potential cellular and molecular causes of hypertrophic scar formation. Burns 2009; 35(1):15-29.
11. Kelly AP. Medical and surgical therapies for keloids. Dermatol Ther 2004; 17(2): 212-218.
12. Chung V, Kelley L, Marra D., Jiang S.B.: Onion extract gel versus petrolatum emollient on new surgical scars: prospective double-blinded study. Dermatol Surg 2006; 32(1):193-198.
13. Kilarski WW, Samolov B, Petersson L, et al. Biomechanical regulation of blood vessel growth during tissue visualization. Nat Med. 2009 ;15(6):657-64.
14. Jacobi J, Tam BY, Sundram U, et al. Discordant effects of a soluble VEGF receptor on wound healing and angiogenesis. Gene Ther 2004; 11(3):302–9.
15. Vranckx JJ, Yao F, Petrie N, et al. In vivo gene delivery of Ad-VEGF121 to full-thickness wounds in aged pigs results in high levels of VEGF expression but not in accelerated healing. Wound Repair Regen 2005; 13(1):51–60.
16. Floquet N, Pasco S, Ramont L, et al.The antitumor properties of the alpha 3(IV)-(185-203) peptide from the NC1 domain of type IV collagen (tumstatin) are conformation-–dependent. J Biol Chem 2004; 279 (3):2091-100.
17. Hamano Y, Zeisberg M, Sugimoto H, et al. Physiological levels of tumstatin, a fragment of collagen IV alpha3 chain, are generated by MMP-9 proteolysis and suppress angiogenesis via alphaV beta3 integrin. Cancer Cell 2003; 3(6): 589-601.

2/20/2012