

Original Research

TSG-6 treatment promoted apoptosis in human fibroblasts of pathological scar

H. Wang^{1#}, Z. Chen^{1#}, X. Li^{1*}, Y. Tang², X. Li¹, S. Zhang¹, L. Ma³, X. Huang⁴

¹ Department of Plastic Surgery, First Affiliated Hospital of Anhui Medical University, No. 218 Jixi Road, Hefei, 230022, China

² Intensive Care Unit, The Second Affiliated Hospital of Xi'an Medical College, No. 167 Fangdong Road, Xi'an, 710000, China

³ Aesthetic Medicine Research Office, College of Nursing, Anhui University of Chinese Medicine, Hefei, 230038, China

⁴ Department of Anatomy, Anhui Medical University, 81 Meishan Road, Hefei 230032, China

Abstract: Pathological scars are characterized by excessive fibrosis and extracellular matrix (ECM) deposition and can be functionally and cosmetically problematic; however, there are few satisfactory treatments for controlling pathological scars. The proliferation of fibroblast during wound healing play an important role in pathologic scar formation. Therefore, the promotion of fibroblast apoptosis may be a new point to inhibit scar formation. In this study, pathological scar fibroblasts were treated with TSG-6 in monolayer culture and subjected to examination for cell apoptosis and associated proteins involved in cell apoptosis. We found that TSG-6 significantly promoted cell apoptosis of pathological scar fibroblasts. Meanwhile, expression level of cell apoptosis related cytokine protein Bax increased and cell proliferation related signal transduction pathways are blocked since the expression level of Bcl-2 was decreased.

Key words: TSG-6, pathological scar fibroblast, apoptosis, Bax, Bcl2.

Introduction

Scar formation is an inevitable result of wound healing. Pathological scar is a fibrotic skin disease that is characterized by the abundant production and deposition of extracellular matrix which is usually secreted by fibroblast. Clinical data showed that pathological scar hyperemia, bright red color, active hyperplasia, local thickening and hardening, significantly higher than the surrounding normal skin, not only affect beautiful, but also appeared with symptoms such as itching, pain, and even contracture, especially happened on joints, and often cause severe joint dysfunction (1, 2). Pathological scars significantly affect physical and psychological aspects of quality of life, as clinical management remains problematic, a more efficient therapy is needed.

As histological studies showed, fibroblasts were the most important cells involved in tissue repair and scar formation, collagen and other extracellular matrix were mainly synthesized and secreted by fibroblasts (3, 4). Therefore, inhibiting fibroblasts proliferation or inducing fibroblast apoptosis is the key way to improve pathological scar (1, 5). Many genes such as Bcl-2, Bax, p53, caspase-3 are the important genes involved in regulation of cell apoptosis. As research showed, the expression of anti-apoptotic proteins Bcl-2 in the scar tissue increased (6, 7), the increased expression of caspase-3 could promote the apoptosis of fibroblasts in hyperplastic scar (8). So the study of the relation between the pathological scar formation and fibroblast apoptosis is of great significance.

TSG-6 (tumor necrosis factor, alpha-stimulated gene-6 protein) is encoded by the TNFAIP6 (tumor necrosis factor, alpha-induced protein 6) gene, which is a new gene isolated by differential screening of a cDNA library prepared from tumor necrosis factor-treated human diploid FS-4 fibroblasts (9). TNFAIP6 expression can be induced by numerous signaling molecules, principally TNF- α and IL-1 (10). TSG-6 contains a hyalu-

ronan-binding link domain and can form a stable covalent complex with inter-alpha-inhibitor (I α I), which is important in the protease network associated with inflammation. This anti-inflammatory effect of TSG-6 has been confirmed in many studies (11-13). In 2010, Tan showed that the anti-inflammatory factor TSG-6 was differentially distributed in keloid scars, normal scars and unscarred skin and said the loss of TSG-6 may lead to the formation of pathological scar and the significantly increase of TSG-6 may cause no scar repair (14). Seidita showed that there was not TSG-6 expression in the cells without p53 gene, and TSG-6 expression level raised in the p53 gene relied apoptosis pathway, and in p53 mediated cell cycle G1 phase stationary, TSG-6 is regulated by the downstream effect factor of p53 directly (15). Thus, Milner thought p53 mediated apoptosis pathway may be another biology effect of TSG-6 (16). These studies indicated the promoting apoptosis effect of TSG-6.

However, the effect of TSG-6 on apoptosis of fibroblasts in pathological scar issue has scarcely been previously reported. This study aimed to evaluate the effects of TSG-6 on fibroblasts cell cultures isolated from samples of pathological scars on cell proliferation.

Materials and Methods

Patients and Samples

All pathological scars (6 men and 4 women, ave-

Received November 24, 2015; Accepted May 5, 2016; Published May 30, 2016

* **Corresponding author:** Xiaojing Li, Department of Plastic Surgery, First Affiliated Hospital of Anhui Medical University, No. 218 Jixi Road, Hefei, Anhui Province, 230022, China. Email: xiaojingli6@yahoo.com

These authors contributed equally to this work.

Copyright: © 2016 by the C.M.B. Association. All rights reserved.

rage age 20 years, average disease duration 2.5 years) got from the first affiliated hospital of Anhui Medical University plastic surgery organizations obtain patients consent and signed an informed consent form. Selected specimens did not undergo any treatment. After surgical resection in saline immediately after cleansing and trimming skin, wash 3 times with 75% alcohol after cleaning with saline, mount containing culture medium (containing 20% fetal bovine serum) in sterile culture flasks, transfer to the laboratory for standby.

Cell culture and drug treatment

Using the explant method (17, 18), primary fibroblast cultures were initiated from pathological scars. Growth medium was changed every second day, fibroblasts could be found surrounded tissue within day 10~14. The cells were grown to confluence and then to passage maximum of three times prior to further experiments. Fibroblasts which were seeded on 6-well plates were subjected to different treatment: control groups, fibroblasts were cultured with DMEM only; experiment group, 0.01ng/mL TSG-6 was added in the culture medium.

Flow cytometry

Fibroblasts were cultured in 25cm² cell culture flasks. After culture for 48 h, cells were harvested and washed in ice-cold PBS. The cells were stained with propidium iodide (2 mg/ml, Pharmacia) in 4 mM sodium citrate buffer containing 3% Triton X-100 and RNase A (100 mg/ml). Fibroblasts were counted for each histogram, and cell cycle distributions were analyzed with the ModFitLT V2.0 (BD FACSCalibur, USA).

Transmission electron microscopy

The hypertrophic scars were fixed in 2% glutaraldehyde and 2% paraformaldehyde for 2 hours, rinsed with PBS three times, conventionally dehydrated, embedded in paraffin and cut into 0.1 mm sections. The sections were stained with uranyl acetate and lead citrate, and examined with a transmission electron microscopy (TEM; JEM-1230 electron microscope, JEOL Ltd., Tokyo, Japan).

Immunohistochemistry

Immunohistochemistry was performed to detect Bcl-2, Bax and PCNA (proliferating cell nuclear antigen). Fibroblasts were seeded on 6-well plates in which the primed cover slips were placed. The cover slips were removed out and washed three times with PBS, and then fixed with cold acetone for 6 min. After inactivating endogenous peroxidase (POD) with deionization by 3% H₂O₂ (Zhongshan Goldenbridge Biotechnology Co. Ltd., China) for 10 min and blocking with normal goat serum for 15 min at room temperature, the slides were incubated with the primary antibody at 4°C overnight. The following primary antibodies were used in this assay: rabbit anti Bcl-2, rabbit anti Bax, and rabbit anti PCNA. All the antibodies were purchased from Cell Signaling Technology. Then, the slides were incubated with goat anti-rabbit secondary antibody working solution for 20 min and Horseradish enzyme labeled streptavidin avidin working solution for 30 min at 37°C. The slides were then washed three times with PBS and the

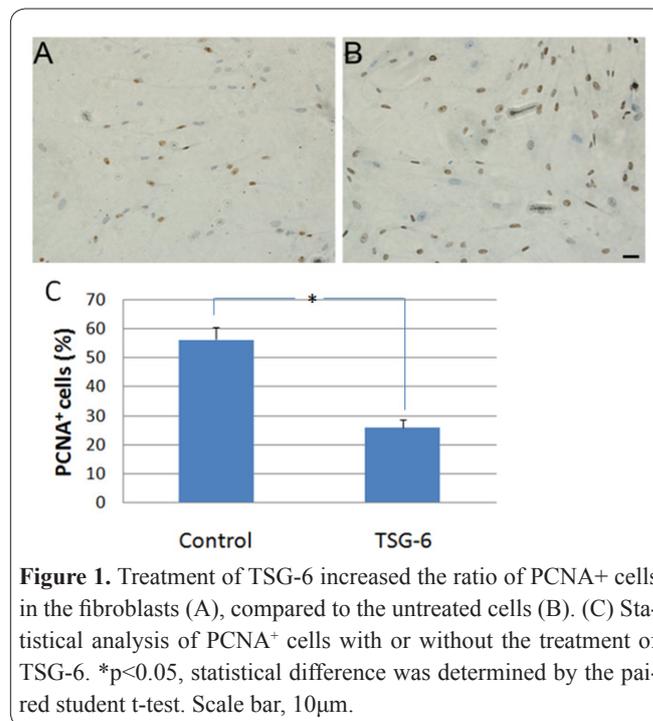


Figure 1. Treatment of TSG-6 increased the ratio of PCNA⁺ cells in the fibroblasts (A), compared to the untreated cells (B). (C) Statistical analysis of PCNA⁺ cells with or without the treatment of TSG-6. **p*<0.05, statistical difference was determined by the paired student t-test. Scale bar, 10μm.

color was developed with DAB for 10 min.

TdT-mediated dUTP-biotin nick end-labeling (TUNEL)

Transferase-mediated dUTP nick end-labeling (TUNEL) assays were performed using an assay kit (In Situ Apoptosis Detection Kit, Trevigen Inc., Gaithersburg, Maryland, USA) according to the manufacturer's instructions. The number of apoptotic cells was counted under an optical microscope. Apoptosis was evaluated by counting the TUNEL-positive cells (brown) compared with the total number of cells in a randomly selected area.

Real time PCR

Total RNA was isolated using TRIZOL reagent (Life Technologies). Synthesis of cDNA was performed by using one-step RT-PCR kit from Takara. SYBR Green (Toyobo) RT-PCR amplification and real time fluorescence detection were performed using the PRISM 7300 sequence detection system (Applied Biosystems). Relative gene expression was calculated by the $\Delta\Delta C_t$ method.

Statistical analysis

Statistical analysis was performed using SPSS software, version 18 (SPSS Inc., Chicago, USA). Comparisons between the groups were made with unpaired Student's t-test. The data are presented as the mean \pm SD. Differences were considered significant at *p*<0.05.

Results

Apoptosis was promoted by TSG-6

The apoptotic cells could be distinguish obviously by PCNA staining, a marker for proliferative cells. As shown in Fig.1A, the normal fibroblast cultures (treated with PBS) were more abundant with PCNA positive cells than the TSG-6 treated cultures (Fig.1B). Statistic analysis showed the significantly less expression of

PCNA in TSG-6 treated cells (Fig. 1B). The apoptosis rate in the TSG-6 treated and control groups were measured using TUNEL assays (Fig. 2). The apoptosis rates were significantly higher in fibroblasts treated with TSG-6 (Fig.2B, 21±2%) than the control group (Fig.2A, 11±3%). The statistical analysis was shown in Fig.2C ($p<0.05$, paired student t test). To evaluate cell cycle distributions of fibroblasts subjected to TSG-6, cells were stained with propidium iodide. Fibroblasts treated with TSG-6 were blocked mainly at G1 phase of the cell cycle and S phase markedly decreased in contrast to that of the control (Fig. 3).

The morphological changes of apoptotic cell induced by TSG-6

The number of microvillus of TSG-6 treated cells was significant decreased compared to the untreated group (Fig. 4A, 4C, $p<0.01$, marked by arrows). Furthermore, the number of apoptotic bodies (marked by cycles) was drastically increased in the cytoplasm (Fig. 4B, 4D, $p<0.01$). The fibroblasts in the control group appeared with normal microvillus (arrows in Fig.4A) and chromatin was much even. This was another piece of evidence to show that TSG-6 induced apoptosis in the fibroblasts.

Effects of TSG-6 on apoptosis-related cytokines

The expression of Bcl-2 and Bax were detected by immunohistochemistry assay. As shown in Fig. 5B, Bcl-2 (an anti-apoptotic protein) expression was suppressed in the TSG-6 group when compared to that of the control group (Fig.5B), while the Bax expression (an apoptotic activator) in the TSG-6 group was up-regulated (Fig.5C 5D). The mRNA level alteration of BCL2 and BAX showed the similar tendency (Fig.5E). This

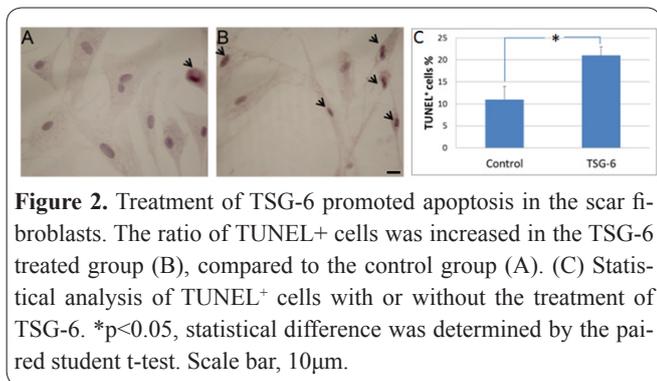


Figure 2. Treatment of TSG-6 promoted apoptosis in the scar fibroblasts. The ratio of TUNEL+ cells was increased in the TSG-6 treated group (B), compared to the control group (A). (C) Statistical analysis of TUNEL+ cells with or without the treatment of TSG-6. * $p<0.05$, statistical difference was determined by the paired student t-test. Scale bar, 10µm.

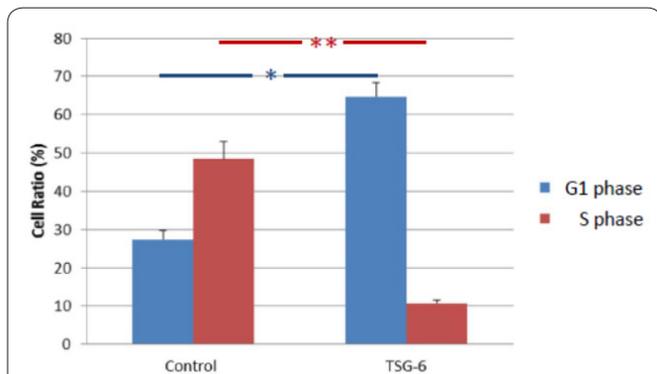


Figure 3. TSG-6 affected the cell cycle. More cells were in the G1 phase in the treatment with TSG-6, compared to the control ($p<0.05$), while less cells were in the S phase ($p<0.01$). Statistical difference was determined by the paired student t-test.

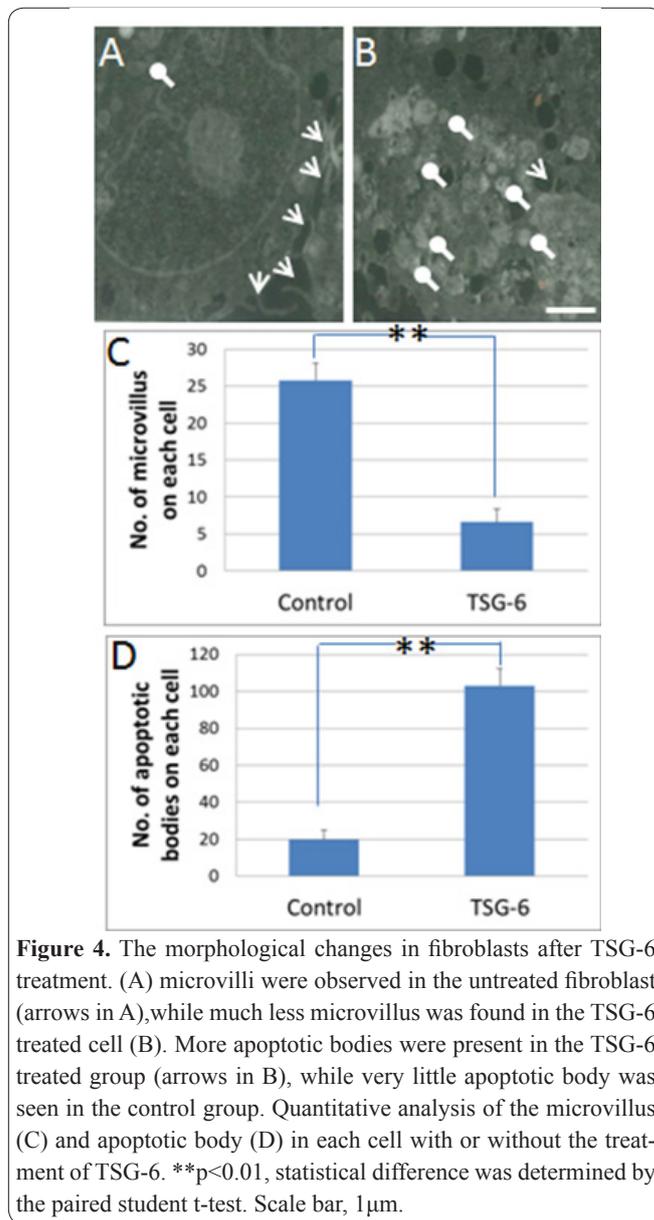


Figure 4. The morphological changes in fibroblasts after TSG-6 treatment. (A) microvilli were observed in the untreated fibroblast (arrows in A),while much less microvillus was found in the TSG-6 treated cell (B). More apoptotic bodies were present in the TSG-6 treated group (arrows in B), while very little apoptotic body was seen in the control group. Quantitative analysis of the microvillus (C) and apoptotic body (D) in each cell with or without the treatment of TSG-6. ** $p<0.01$, statistical difference was determined by the paired student t-test. Scale bar, 1µm.

was consistent with the conclusion that TSG-6 induced apoptosis in the fibroblasts.

Discussion

Pathological scar, a common proliferative disorder of dermal fibroblasts, results from an overproduction of fibroblasts and excessive collagen deposition. Patients with pathological scars often experience major physical and psychological problems. Numerous treatments, including pressure, silicone gel sheets, intralesional steroids, 5-fluorouracil, cryotherapy, surgical excision and lasers, have been applied to prevent and treat pathological scar, but the treatments remain unsatisfactory. Hence, it is necessary to explore new effective therapeutic methods for pathological scars. As fibroblasts play an important role in tissue repair and scar formation, the drug which has a promoting of apoptosis effect on fibroblast may be useful in treating pathological scar. In this study, we first aimed to evaluate the inhibitory effect on cell proliferation, expression of Bcl-2, Bax, p53 and caspase-3 in fibroblasts that derived from pathological scars exposed to TSG-6. We observed that in the presence of TSG-6, cell proliferation was significantly inhibited in TSG-6 treated group.

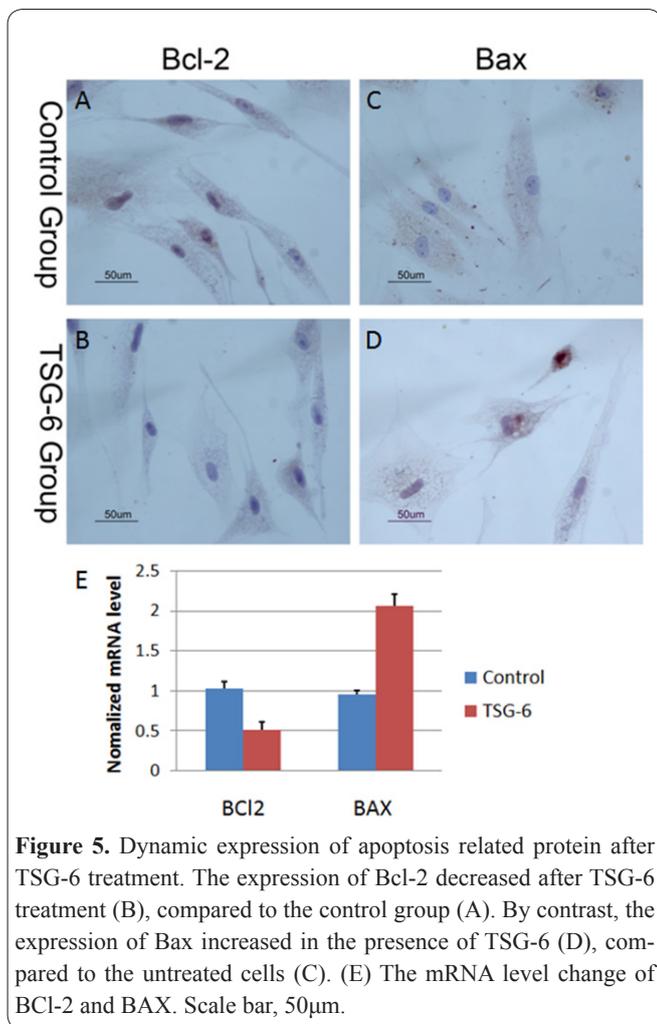


Figure 5. Dynamic expression of apoptosis related protein after TSG-6 treatment. The expression of Bcl-2 decreased after TSG-6 treatment (B), compared to the control group (A). By contrast, the expression of Bax increased in the presence of TSG-6 (D), compared to the untreated cells (C). (E) The mRNA level change of BCL-2 and BAX. Scale bar, 50µm.

TSG-6 is a new gene that was identified by Lee (19) from a cDNA derived from TNF-treated fibroblasts. The 277 amino acid protein encoded by TSG-6 belongs to the hyaluronic acid-binding protein family, contains multiple adjacent functional domains and a CUB domain and binds to HA, chondroitin sulfate, polysaccharide and the G1 chain of proteoglycan (20). The anti-inflammatory effect of TSG-6 has previously been confirmed in many studies (21-25). The expression of TSG-6 is also related with pathological scarring (26).

Considering the long-hypothesized possibility that the hypercellular nature (and potentially the whole pathology) of pathological scarring is caused by a failure of apoptosis, we therefore investigated whether a reduced susceptibility of fibroblasts to this particular form of apoptosis might underlie the pathology of this condition. As study showed, reversible and irreversible p53-mediated G1 cell cycle arrest can be induced in human fibroblasts by the antimetabolite Nphosphoacetyl-L-aspartate (PALA) and g-irradiation, respectively. cDNA representational difference analysis has revealed that TSG-6 mRNA is substantially upregulated in embryonic skin fibroblasts by PALA, but not g-irradiation, indicating that TSG-6 might be a novel component of the reversible arrest pathway (27). Furthermore, the absence of TSG-6 expression in p53-defective cells suggests that TSG-6 is directly controlled by p53. p21WAF1/CIP1 is a downstream effector in p53-mediated G1 arrest, but can also be upregulated and drive apoptosis in a p53-independent manner. Wu *et al.* have used adenovirus-vector-mediated transduc-

tion of p53 (rAd-p53) or p21WAF1/CIP1 (rAd-p21) to mimic p53-dependent and -independent upregulation, respectively, of p21WAF1/CIP1 in human ovarian cancer cell lines (28). TSG-6 is not induced by rAd-p53 but is significantly upregulated within 4-8 hours of rAd-p21 infection. And p53-independent apoptosis might thus be another process in which TSG-6 functions. We designed this study to detect the pro-apoptosis effect of the product of TSG-6 *in vitro*.

In the present study, we investigated the anti-proliferation effect of TSG-6 on fibroblasts of pathological scars, which has scarcely been previously reported. To investigate the promoting apoptotic effect of TSG-6, the level of PCNA was determined by immunohistochemistry. We demonstrated that PCNA level significantly decreased in TSG-6-treated scars compared with control group. More apoptotic cells were observed in the TSG-6-treated scars, as evidenced by TUNEL. Morphological changes of fibroblasts were observed by TEM as karyopyknosis, mitochondrial swelling and apoptotic bodies were founded in the TSG-6 treated group clearly. The phase of cell cycle was detected by flow cytometry, fibroblasts treated with TSG-6 were blocked mainly at G1 phase of the cell cycle and S phase markedly decreased in contrast to that of the control. The anti-proliferation and promote apoptosis effects of TSG-6 were confirmed on these date. We also examined cell apoptosis and proliferation related cytokine by immunohistochemistry. Protein Bax increased and cell proliferation related signal transduction pathways are blocked as the level of Bcl-2 lowered. So we deduced that TSG-6 predominantly suppressed fibroblasts proliferation and induced fibroblasts apoptosis by increasing the p53 protein level, activate the expression of Bax which is p53 signaling pathways downstream, to block the cell cycle in G2 / M phase, and inducing apoptosis by activating Bcl-2 and caspase-3.

Although the application of TSG-6 to induce fibroblast apoptosis was confirmed, many questions remain unanswered. As the cells *in vitro* is different to that *in vivo*, future studies investigating the application of TSG-6 on scar tissue are needed. The anti-proliferation effect of TSG-6 was confirmed in our study; however, the mechanisms of all of the different types of cytokine intracellular signal transduction and gene regulation are unclear. Clearly, further research to test this hypothesis is needed.

In a summary, these results suggest that this protein may be used as a preventive and therapeutic agent for pathological scar. Further investigations are required to elucidate the other potential mechanisms involved and to validate the mechanism of TSG-6 *in vivo* model. The current study raises the possibility of testing TSG-6 in both future laboratory and clinical trials.

Acknowledgements

This work was supported by a National Science Foundation of China grant (No. 81272107) and the National Natural Science Foundation of China (No. 81171819).

References

- Gauglitz GG, Korting HC, Pavicic T, Ruzicka T, Jeschke MG. Hypertrophic scarring and keloids: pathomechanisms and current and emerging treatment strategies(J). *Mol Med*, 2011, 17(1-2):113-

- 25.
2. Bloemen MC, van der Veer WM, Ulrich MM, van Zuijlen PP, Niessen FB, Middelkoop E. Prevention and curative management of hypertrophic scar formation(J). *Burns*, 2009, 35(4): 463-75.
3. Wang J, Dodd C, Shankowsky HA, Scott PG, Tredget EE. Wound Healing Research Group. Deep dermal fibroblasts contribute to hypertrophic scarring(J). *Lab Invest*, 2008, 88(12): 1278-90.
4. Hinz B. Formation and function of the myofibroblast during tissue repair(J). *J Invest Dermatol*, 2007, 127(5): 526-37.
5. Willem M. van der Veer, Monica C.T. Bloemen, Magda M.W. Ulrich, Grietje Molema, Paul P. van Zuijlen, Esther Middelkoop, Frank B. Niessen. Potential cellular and molecular causes of hypertrophic scar formation(J). *burns*, 2009(35): 15-29.
6. Lu F, Gao J, Ogawa R, Hyakusoku H, Ou C. Fas-mediated apoptotic signal transduction in keloid and hypertrophic scar(J). *Plast Reconstr Surg*, 2007, 119(6): 1714-21.
7. Zhu G, Cai J, Zhang J, Zhao Y, Xu B. Abnormal nuclear factor (NF)-kappaB signal pathway and aspirin inhibits tumor necrosis factor alpha-induced NF-kappaB activation in keloid fibroblasts(J). *Dermatol Surg*, 2007, 33(6): 697-708.
8. Akasaka Y, Ishikawa Y, Ono I, Fujita K, Masuda T, Asuwa N, Inuzuka K, Kiguchi H, Ishii T. Enhanced expression of caspase-3 in hypertrophic scars and keloid: induction of caspase-3 and apoptosis in keloid fibroblasts in vitro(J). *Lab Invest*, 2000, 80(3): 345-57.
9. Lee TH, Lee GW, Ziff EB, Vilcek J. Isolation and characterization of eight tumor necrosis factor-induced gene sequences from human fibroblasts. *Mol Cell Biol*. 1990;10:1982-1988.
10. Klampfer L, Lee TH, Hsu W, Vilcek J, Chen-Kiang S. NF-IL6 and AP-1 cooperatively modulate the activation of the TSG-6 gene by tumor necrosis factor alpha and interleukin-1. *Mol Cell Biol*. 1994;14:6561-6569.
11. Getting SJ, Mahoney DJ, Cao T, et al. The link module from human TSG-6 inhibits neutrophil migration in a hyaluronan- and inter-alpha -inhibitor-independent manner. *J Biol Chem*. 2002;277:51068-51076.
12. Lin QM, Zhao S, Zhou LL, Fang XS, Fu Y, Huang ZT. Mesenchymal stem cells transplantation suppresses inflammatory responses in global cerebral ischemia: Contribution of TNF-alpha-induced protein 6. *Acta Pharmacol Sin*. 2013;34:784-792.
13. Nagyri G, Radacs M, Ghassemi-Nejad S, et al. TSG-6 protein, a negative regulator of inflammatory arthritis, forms a ternary complex with murine mast cell tryptases and heparin. *J Biol Chem*. 2011;286:23559-23569.
14. Tan KT, McGrouther DA, Day AJ, Milner CM, Bayat A. Characterization of hyaluronan and TSG-6 in skin scarring: Differential distribution in keloid scars, normal scars and unscarred skin. *J Eur Acad Dermatol Venereol*. 2011;25:317-327.
15. Seidita G, Polizzi D, Costanzo G, Costa S, Di Leonardo A. Differential gene expression in p53-mediated G(1) arrest of human fibroblasts after gamma-irradiation or N-phosphoacetyl-L-aspartate treatment(J). *Carcinogenesis*, 2000, 21(12): 2203-10.
16. Caroline M. Milner and Anthony J. Day. TSG-6: a multifunctional protein associated with inflammation (J). *Journal of Cell Science*, 2003, 116 (10): 1863-1873.
17. Bi D, Chen FG, Zhang WJ, et al. Differentiation of human multipotent dermal fibroblasts into islet-like cell clusters. *BMC Cell Biol*. 2010;11:46.
18. Bauer M, Su G, Beebe DJ, et al. 3D microchannel co-culture: method and biological validation. *Integr Biol (Camb)*. 2010; 2(7-8): 371-378.
19. Lee TH, Lee GW, Ziff EB, Vilcek J. Isolation and characterization of eight tumor necrosis factor-induced gene sequences from human fibroblasts. *Mol Cell Biol*. 1990;10:1982-1988.
20. Milner CM, Higman VA, Day AJ. TSG-6: A pluripotent inflammatory mediator? *Biochem Soc Trans*. 2006;34:446-450.
21. Milner CM, Day AJ. TSG-6: A multifunctional protein associated with inflammation. *J Cell Sci*. 2003;116:1863-1873.
22. Danchuk S, Ylostalo JH, Hossain F, et al. Human multipotent stromal cells attenuate lipopolysaccharide-induced acute lung injury in mice via secretion of tumor necrosis factor-alpha-induced protein 6. *Stem Cell Res Ther*. 2011;2:27.
23. Choi H, Lee RH, Bazhanov N, Oh JY, Prockop DJ. Anti-inflammatory protein TSG-6 secreted by activated MSCs attenuates zymosan-induced mouse peritonitis by decreasing TLR2/NF-kappaB signaling in resident macrophages. *Blood*. 2011;118:330-338.
24. Prockop DJ, Oh JY. Mesenchymal stem/stromal cells (MSCs): Role as guardians of inflammation. *Mol Ther*. 2012;20:14-20.
25. Wang N, Li Q, Zhang L, et al. Mesenchymal stem cells attenuate peritoneal injury through secretion of TSG-6. *PLoS One*. 2012;7:e43768.
26. Lin QM, Zhao S, Zhou LL, Fang XS, Fu Y, Huang ZT. Mesenchymal stem cells transplantation suppresses inflammatory responses in global cerebral ischemia: Contribution of TNF-alpha-induced protein 6. *Acta Pharmacol Sin*. 2013;34:784-792.
27. Seidita, G., Polizzi, D., Costanzo, G., Costa, S. and di Leonardo, A. (2000). Differential gene expression in p53-mediated G1 arrest of human fibroblasts after g-irradiation or N-phosphacetyl-L-aspartate treatment. *Carcinogenesis* 21, 2203-2210.
28. Wu, Q., Kirschmeier, P., Hockenberry, T., Yang, T. Y., Brassard, D. L., Wang, L., McClanahan, T., Black, S., Rizzi, G., Musco, M. L. et al.(2002). Transcriptional regulation during p21WAF1/CIP1-induced apoptosis in human ovarian cancer cells. *J. Biol. Chem*. 277, 36329-36337.