Effect of Wubeizi ointment aqueous solution on the expression of type I and III procollagen genes in keloid fibroblasts

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Abstract. We evaluated the effect of Wubeizi (WBZ) ointment on keloids. Keloid-derived fibroblast primary cultures were used to evaluate the effect of the different concentration of WBZ ointment on the expression of type I and III procollagen in keloid fibroblast primary cultures using dot blot assay. Type I and II precollagen cDNA probes labeled with non-radioactive digoxin were used for dot blot. Cell cultures were divided into 4 groups: The large dose group received 1 g/ml of WBZ, middle dose, and small dose groups received 0.5 and 0.25 g/ml of WBZ, respectively. The control group received serum-free medium without WBZ. Our results showed that type I and III procollagen mRNA expression was reduced significantly in the large dose and middle dose groups compared to the control group. Type I and III procollagen mRNA expression level in the small dose group had no statistically significant difference with the control group. However, the difference between the large dose group and the small dose group was statistically significant. We concluded that WBZ ointment aqueous solution restricted keloid fibroblast proliferation by downregulating the expression of type I and III procollagen and therefore reducing collagen deposition in keloid tissue.

Introduction

Keloid is formed by overreaction and hyperplasia of skin tissue trauma, The pathological changes mainly are fibroblast proliferation and excessive deposition of collagen based extracellular matrix, and it is a skin and connective tissue hyperplasia disease (1). Keloid often occurs after a burn, surgery or trauma, hyperplasia lesions are often beyond the original wound area, invade neighboring tissues with tumor like growth, not only affects the appearance, but can also have itching, pain and other symptoms, and even cause severe dysfunction (2), its prevention and cure in the field of skin surgery is an important issue.

The pathogenesis of the keloid is complicated, and has not been fully clarified yet. Keloid is due to fiber cell excessive proliferation and activity enhancement, resulting in large amount of type I and III collagen based extracellular matrix synthesis, and excessive deposition in the dermis layer of the skin (3). Under normal circumstances, the synthesis and degradation of collagen is in dynamic balance. This process is mainly involved in protein translation, modification and editing, and affected by cytokines, mechanical tension and many other types of factors, and many types of biological activity of enzymes are involved (4). Some cytokines can cause fibroblast proliferation, collagen synthesis is increased or decreased, resulting in a large amount of collagen deposition, resulting in the formation of keloid (5).

At present, the treatment of the keloid is difficult, as, although there are many methods, the results are not very satisfactory. Therefore, it is of great practical significance to study the mechanism of the occurrence of the keloid, and find a safe and effective treatment method. There is a long history of traditional Chinese medicine in prevention and treatment of keloid, people are treated with extraction components of the herbs which can promote blood circulation to remove blood stasis (such as *Salvia miltiorrhiza*, asiaticoside and *Radix notoginseng*) to prevent keloid that have made some progress and have demonstrated broad prospects for application (6). Our research team has been engaged in study of traditional Chinese medicine in the prevention and treatment of keloid, we have developed a compound traditional Chinese medicine preparation of Chinese gall ointment applied in the clinical treatment of keloid that has shown good effects. Based on the previous study, we demonstrated that Chinese gall ointment could inhibit the proliferation of keloid fibroblasts (7).However, whether there is an effect of Chinese gall ointment on fibroblast collagen synthesis remains to be determined. Based on this, the application of nucleic acid spot hybridization test in the detection of type I and III collagen mRNA expression in fiber cells was performed to elucidate the mechanism of Chinese gall ointment in the treatment of keloid, and provide a theoretical basis to its application in clinic.

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Materials and methods

Specimen. Keloid samples were taken directly from patients with keloids. Patients had the following characteristics: i) Keloids were in active hyperplasia period; ii) patients had no connective tissue disease or diseases which may affect the metabolism of connective tissue; iii) no lung, liver, kidney and other chronic diseases; iv) no use of steroid hormone drugs, penicillin and antitumor drugs; and v) patients with no history of radiotherapy.

Reagents. We used the following reagents during the course of this study: RPMI-1640 medium (batch no. SH30011.01; Hyclone, Logan, UT, USA), dimethyl sulfoxide (batch no. 1392B11; Beijing Solai Bao Technology Co., Ltd., Beijing, China), trypsin (subpackage, Gibco, Grand Island, NY, USA; Jinan Aibo Biology Co., Ltd., Jinan, China), fetal bovine serum (batch no. 021009; Hangzhou Evergreen Biological Engineering Materials Co., Ltd., Hangzhou, China), Triton and RNA enzyme (subpackage, both from Sigma-Aldrich, St. Louis, MO, USA; Jinan Aibo Biology Co.), PI reagent (Sigma-Aldrich), digoxin labeling and detection kit (Boehringer, Mannheim, Germany), type I and III precollagen cDNA probes (both from Labor Hygiene and Occupational Diseases Research Institution of Chinese Academy of Preventive Medicine), and TRIzol RNA extraction agents (Gibco).

Equipment. We used the following equipment during the course of this study: Carbon dioxide incubator (Heraeus, USA), XDS-LB inverted microscope (Chongqing Optical and Electrical Instrument Co., Ltd., Chongqing, China), double-row six-hole electric heated thermostatic water bath (Shandog Medical Instrument Institute, Shandong, China), XSZ-H ordinary optical microscope (Chongqing Optical and Electrical Instrument Co., Ltd.), YXQ-SG46-280SA stainless steel portable pressure steam sterilizer (Medical Equipment Factory of Shanghai Boxun Industrial Co., Ltd., Shanghai, China) BCM-1000A clean bench (Suzhou Antai Air Tech Co., Ltd., Suzhou, China), EL340i type ELISA detector (BioTek Instruments, Inc., Winooski, VT, USA), DU640 UV spectrophotometer (Beckman Coulter, Miami, FL, USA), and CS-930 lamina scanner (Shimadzu Co., Ltd., Kyoto, Japan).

Preparation of drugs. Pharmaceutical composition and extraction were the same as the method for preparing Wubeizi (WBZ) ointment for keloid (1). We mixed I and II at 1,000 g/l and stored it at -20˚C. Mixture was diluted in phosphate-buffered saline (PBS) and pH was adjusted to 7.4. Concentration used for the large dose group was 1,000 g/l, while for the middle dose and small dose groups 500 and 250 g/l was used, respectively.

Experimental groups. There were four experimental groups: the large dose, middle dose, small dose and control groups.

Experimental method

Fibroblast cultures. Keloid tissues were cut into blocks and washed in balanced salt solution to remove blood. Adipose and connective tissues were removed using integument. Tissues were transferred to culture dish filled with culture media, and were further cut into small pieces (1 mm^3) under the dissecting microscope. Tissues were rinsed again with balanced salt solution, and explants were drawn carefully with wet straws. Explants were then gently blown into a flask containing fetal calf serum. Subsequently, a small amount of culture media was added and flask was capped, turned over and placed in a CO_2 incubator for 6-8 h. After incubation, the flasks were taken out and proper amounts of culture media were added. They were incubated again, under the same condition, and media were changed every 2-3 days.

When culture reached 100% confluence, old media was removed and 2-3 ml of trypsin solution (0.25%) was added (just to cover the cell layer). After 3-5 min of digestion, protease was removed and 10 ml of complete media was added. Single cell suspension was prepared and cell counts were conducted under the microscope. Cells were sub-cultured in new flasks, and were incubated at 37˚C with 5% CO_2 in an incubator designed for axenic culture. Cell cultures were passaged once every 2-3 days. Cells in the logarithmic growth phase from 7th to 8th generation were used for different experiments.

Expression of type I and III procollagen mRNA in different medicine concentration. Recombinant plasmids used in this study were JM1b and S413. The inserted fragment sizes were 4.6 and 4.5 kb, the carrier was pUC8 and Bluescript, and the recombination sites were *Eco*RI. Type I and II precollagen cDNAs probes were labeled with non-radioactive digoxin. The labeling efficiency was determined after coloration and comparison with the standard.

The 7th and 8th generation keloid fibroblasts in logarithmic growth phase were digested using 0.25% trypsin, and cells were counted using blood cell counting plate. Cells were inoculated in culture flasks (inoculum density of 25 cm^2) and media were added to all flasks. Cultures were divided into 4 groups with 3 flasks in each group. Cultures were then incubated in a CO_2 incubator. Media were changed when cells adhered to the flask wall, and different concentrations of WBZ ointment aqueous solution were added. The large dose group received 1 g/ml of WBZ, while middle dose and small dose groups received 0.5 and 0.25 g/ml of WBZ, respectively. Serum-free medium without WBZ was added into cell cultures in the control group. The media were incubated for 24 h and then discarded and the cells were rinsed with PBS. TRIzol reagent was used to extract total RNA. RNA content and its purity were examined with UV spectrophotometry at 260 and 280 nm, and the integrity of RNA was observed using formaldehyde gel electrophoresis.

RNA samples (10 μg) were placed on a cellulose nitrate membrane. Prehybridization solution was added and the membrane were placed at 42˚C for 4 h. Specific cDNA probe labeled by digoxigenin (60 ng/ml) was then added on the membrane, and then anti-digoxin antibody (150 mU/ml) was added. It was developed in the dark after adding coloring liquid, and then studied with lamina scanner.

Statistical analyses. We used SPSS 11.0 software (SPSS, Inc., Chicago, IL, USA) for statistical analysis. Measurement data were presented as mean ± standard deviation. For comparison among the groups we used the Bonferroni method if data were normal. For homogeneity of variances, Tamhane's T2 method was employed to carry out variance test, and pair-wise comparison when we had heterogeneity of variances. $P<0.05$ was considered to indicate a statistically significant difference.

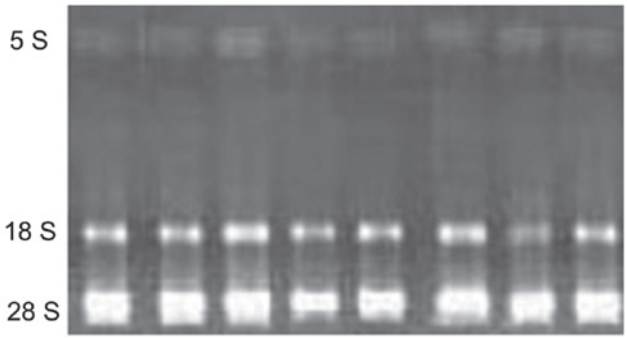

Figure 1. RNA denaturing agarose gel electrophoresis. No degradation was detected in 28S and 18S bands.

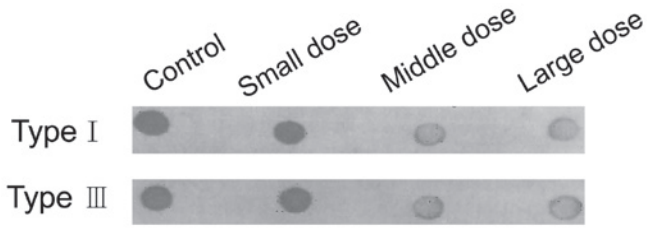

Figure 2. Dot-blot results with computer gray scale scanning.

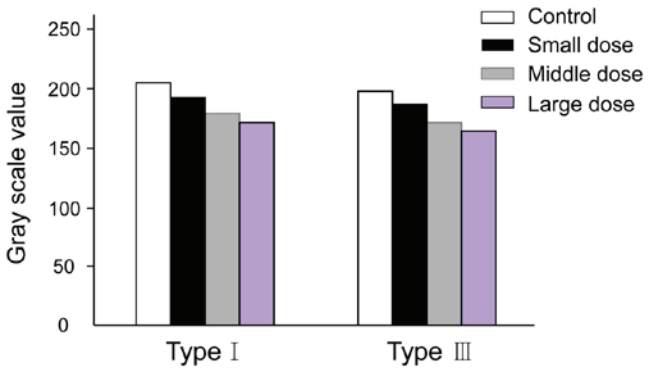

Figure 3. Histogram of gray value scanned of dot blot hybridization.

Table I. Gray value of dot blot hybridization (mean ± SD).

Groups	n	Type I gray value	Type III gray value
Control	6	204.33±4.50	197.67±7.55
Small dose	6	190.33±10.17	187.33±11.43
Middle dose	6	178.17±16.47[a]	169.17±7.08[a,c]
Large dose	6	171.67±7.61[a,b]	163.83±7.47[a,c]

Type I gray value F=32.15, P=0.000<0.01, type III gray value F=58.52, P=0.000<0.0001; [a]P<0.01 comparison with the control group; [b]P<0.05, [c]P<0.01 comparison with the small dose group.

Results

The type I and III procollagen probe concentrations used were 25 and 12 ng/l, respectively. Concentration and purity of total RNA extracted was verified using a UV spectrophotometer, and OD260/OD280 readings were between 1.8 and 2.0, indicating good purity. Results of RNA denaturing agarose gel electrophoresis is presented in Fig. 1. RNA integrity was good as each electrophoretic band in Fig. 1 shows 28S and 18S clearly with no degradation.

Type I and III procollagen mRNA expression levels in the large dose and the middle dose groups were considerably higher than the control group and the difference was statistically significant (P<0.05) (Fig. 2 and Table I). Type I and III procollagen mRNA expression levels in the small dose group had no statistically significant difference with the control group (P>0.05) (Fig. 3). However, the difference between the large dose group and the small dose group was statistically significant (P<0.05).

Discussion

Collagen is a joint name used for collagen fibers and collagen molecules, collagen generally refers to collagen fibers in clinical circles. Collagen fiber which is the product of collagen molecular polymerizing and crosslinking, is the main component of connective tissue, maintaining the integrity of tissues and organs. Collagen is mainly compounded of fibroblasts or cells from similar origin such as osteoblasts, and chondrocytes. The major amino acids found in collagen are glycine, proline and hydroxyproline, additionally there are a small number of hydroxylysine and tyrosine residues (8). Hydroxyproline is the characteristic amino acid in collagen, which is the product of hydroxylation of proline by prolylhydroxylase (9). The availability of proline hydroxylase is related to the rate of collagen synthesis. Collagen can be divided into four types according to the amino acids found in the fibres. Type I collagen is found abundantly in normal skin, and we can have up to 25% of type III collagen in normal skin. In a keloid, the balance between collagen synthesis and degradation is disturbed (the rate of collagen synthesis is high, while collagen decomposition rate stays at normal levels). This situation leads to keloid hypertrophy and hyperplasia (10).

In the keloid, expression of type I procollagen mRNA was significantly high, while the expression of type III procollagen was slightly up.

Results obtained from a previous study also revealed a significant increase in type I procollagen in keloids (7). Wei *et al* showed that the levels of type I collagen mRNA increased considerably in keloid fibroblasts (11). Another related study reported that compared with ordinary scar, the permutation, distribution and collagen content in keloid tissues containing mainly type III collagen were significantly different (12).

In the present study, we showed that WBZ ointment aqueous solution had a significant inhibitory effect on the expression of type I and III procollagen mRNAs. These results suggested that WBZ ointment influenced the synthesis of fibroblast collagen in the keloid by reducing the rate of transcription from type I and III procollagen genes, thus reducing the amount of collagen deposition in keloid tissue. However, this effect was dose-dependent and did not manifest itself when small doses of WBZ were used.

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