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Research Paper

Effects of connective tissue growth factor and collagen type I scleroderma

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Abstract

Objective: To investigate the effects of connective tissue growth factor(CTGF) and collagen type I(COL-I) on the pathogenesis of scleroderma and explore the relationship between the level of COL-I and CTGF. **Methods**: 12 mice model of scleroderma was established by the injection of Bleomycin. The level of CTGF and COL-I were detected by immunohistochemical method. The relationship was analyzed between CTGF and COL-I level. As control group, 12 healthy mice were selected. **Results**: The levels of CTGF and COL-I in sclerotic models were higher than in normal controls ($P < 0.05$). It was found that there was a correlation between the level of CTGF and COL-I. **Conclusion**: CTGF and COL-I played an important role in the hardening process of the skin lesions of the mice model, which may be involved in the pathogenesis of scleroderma.

Keywords: connective tissue growth factor; collagen type I; scleroderma

INTRODUCTION

Systemic sclerosis(SSc) is a connective tissue disease characterized by fibrosis of the skin and various internal organs, with an unknown cause [1]. Subcutaneously injection of bleomycin solution to induce sclerotic modal mice was first reported by the Japanese scholar Yamamoto [2]. We have chosen this formula, as it is one of the most replicated methods in the study of scleroderma, and it has been extensively repeated by Lubing ZHU[3] and Wenzhen TU[4]. We employed the use of an immunohistochemical test to examine the level of CTGF and COL-I in the sclerotic mice model, and to investigate the effect on the occurrence of scleroderma allowing us to discuss the pathogenesis.

MATERIALS AND METHODS

Experimental animals

24 female SPF level BALB/c mice(six-week old, 20 ± 5 g) were purchased from medical the animal experimental center at Xi'an Jiao Tong University, fed with non specific food and water in the clean room at temperature of 20-24℃.

Main medicines

Bleomycin powder, 1 Vial × 15 mg, which was the product of Japanese Medicine Style Commercial Firm, batch number: 450260.

Scleroderma animal model

The BALB/c mice were divided into 2 groups randomly. There were 12 mice in each group. All of them had the central dorsal seta shaved. Bleomycin solution (0.1 ml) prepared in the concentration of 200 μg/ml was injected subcutaneously in the model group once a day for 3 weeks. In the control group, 0.1 ml PBS (0.01 mol/L pH 7.4) was injected for 3 weeks at the same site. After that, the mice were sacrificed with the manipulation of cervical vertebral dislocation. The skin samples were taken from the injection spot of each mouse, then embedded in the 10% formaldehyde solution and fixed by paraffin.

Histopathology in skin and lungs

The slices were obtained from model skin, and dyed by HE. Any histological changes which oc-

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curred in the skin, lungs and the blood vessels were observed.

Immunohistochemical test

The slices were put into the hot repaired box with standard saline-citrate(SSC) and treated in the medical microwave oven at 92~97℃ for 10 minutes after having been dyed and hydrated. Thereafter 50μl 3% hydrogen peroxide solution were added, the samples were fostered for 10 minutes at room temperature. Then washed with PBS for 3 minutes 3 times, the samples were added antibody CTGF and COL-I 50 μl respectively and left overnight at 4℃. Polymer strengthener was added to samples ands also fostered at the room temperature for 20 minutes. Once again, PBS washed for 3 minutes 3 times, the slices were added 50 μl enzyme against mouse or hare polymer and fostered for 30 minutes. After having been washed by PBS for 3 minutes 3 times, the slices were added a drop of DAB coloration fluid and flushed with running water, counterstained with hematoxylin, flushed with 0.1% hydrochloric acid differentiated tap water. Finally, the following procedures were overseen, returning to blue by PBS, dehydrating by gradient ethanol, drying and blocking the slices by neutral gum. In the negative comparison, PBS substituted the first antibody.

Skin tissue dyeing analysis

The desired result (a buffy or yellow brown dyeing with positive reaction) was analyzed by MIAS 2000 software (the automatic colour pathologic photo analyze system) to test the positive dyeing square and intensity on each display screen under a microscope magnifying 100 diameters. 5 fields of vision from each slice were chosen randomly, then they were analyzed with semi-quantity. Buffy was known as positive. On each field of vision after calculating the amount of COL-I or CTGF and immunohistochemically positive expressed strength, measuring its square, the average could be obtained. In addition, immunohistochemical index could be calculated. Immunohistochemical index = (positive square value × positive intensity).

Lung tissue quantitative analysis

According to Lingjuan DAI [5] and Szapiel [6] (the chosen method of pulmonary tissue quantitative analysis), the pulmonary tissue slice was divided into 3 areas: (1) the areas without dyeing express the squares of alveolar cavities. (2) the areas with dark dyeing represent the squares occupied by cellular nucleus. (3) the areas with light dyeing, representing the squares occupied by fibrous connective tissue and collagenic fibers. The slices were put under microscope of photographic analyzer, 6 fields of vision were chosen from each slice to handle with photograph under HE(× 100). The samples in each group was analyzed 36 times, measuring the squares automatically, and then an average taken (μm^2).

Statistical analysis

All analyses were performed at least in triplicate. Statistical tests were done using SPSS 12.0. All values are expressed as mean ± SD. Statistical analysis was performed using analysis of variance followed by t' test, which was also applied to analyze the relativity of CTGF and COL-I. The P value of less than 0.05 was considered statistically significant.

RESULT

Model group mice ordinary instance observation

After bleomycin injection, the mice in the model group lost weight and became decreased activity with the pelage all over the body becoming paler. The skin especially at the back region with injection was thickening and hardening obviously. Its elasticity became worse, and the superficial areas involved became hairless. The routine tissue slices were performed by HE dyeing. We could find that the total thickening of the dermis was remarkably increased, the collagen was increased in size and number, and the whole dermis was uniformly pink with loss of spaces between the collagen bundles. The hair follicles became atrophic, and the blood vessels were increasing in diameter and thickening in walls. Narrow inner caves were present and accompanied infiltration by inflammatory cell. The thickening membrane between alveoli and infiltration of monocytes were present in pulmonary tissue, and there was a small quantity of fibroblast with proliferation and thickening in capillary walls.

Comparison of CTGF and COL-I immunohistochemical index

As is shown in ***Tab 1***, we found that there were two bands in the comparison between model group and control group. COL-I immunohistochemical in-

Tab 1 **Comparison of CTGF and COL-I immunohistochemical index between the model group and control group** ($\bar{x} \pm s$)

group	n	CTGF	COL- I
control group	12	42256.74 ± 2447.86	28456.50 ± 3112.62
model group	12	164003.65 ± 7824.88	159948.20 ± 8965.22

dex in the model group (159948.20 ± 8965.22) was higher than that in the control group (28456.50 ± 3112.62) and the differences had highly significant ($P < 0.05$); CTGF immunohistochemical index was also increased in the model group (164003.65 ± 7824.88) compared with the control group (42256.74 ± 2447.86). The differences had highly significant($P < 0.05$). (***Fig 1-4***)

The result of form quantitative analysis is shown in ***Tab 2***, the squares of the dark dyeing areas in model group were the largest. The highly significant difference,($P < 0.01$)indicated that the inflammatory cells were increasing and obvious,especially in alveoli. When the squares in light dyeing areas were increased, the difference had highly significance($P < 0.05$). The squares in the areas without dyeing were decreased. The difference also had highly significant ($P < 0.05$). It was implied that the pulmonary interstitial fibrosis was increased, and the alveolar cavity volume was decreased. (***Fig 5-8***)

Tab 2 **Comparison in quantitative analysis of the pulmonary tissue between the model group and control group** ($\mu m^2, \bar{x} \pm s$)

Group	n	the areas without dyeing	dark dyeing	light dyeing
control group	12	4241. 10 ± 1128.71	2359. 72 ± 822. 40	9514.72 ± 1635. 74
model group	12	4965. 83 ± 1214.72*	3212.64 ± 894. 86**	8496.12 ± 1744. 92**

Compared with control group, *$P < 0.05$; compared with control group, **$P < 0.01$.

SPSS statistical software was applied to the analysis of the relationship between CTGF and COL-I. It was illustrated that there was a remarkably correlation between CTGF and COL-I expression in the skin of model mice ($R = 0.8818$, $P < 0.05$).

DISCUSSION

Scleroderma is a fibrotic condition characterized by immunologic abnormalities,vascular injury and increased accumulation of matrix proteins in the skin [7]. CTGF is a kind of new cell factor that may promote fibroblast fission and collagenic deposition. Connective tissue growth factor (CCN2/CTGF) is a cysteine-rich protein with a molecular weight of 36-38 kDa [8-10] and belongs to the CCN family, which stands for CTGF, CEF10/Cyr61, and Nov [11]. It is one sort of instant early gene that widely exists in various tissue and organs, such as heart, lungs, skin and connective tissue. CCN2/CTGF increased the expression of extra-cellular matrix molecules such as type I collagen, fibronectin, and integrin [12], and was overexpressed in fibroblasts in the dermis of patients with scleroderma [13]. CCN2/CTGF is suggested to have some physiological function, playing a role in the organization and maintenance of periodontal tissues[14]. However, in pathology CTGF excessive expression is closely related to the certain proliferation and occurrence of some fibrosis diseases, such as pulmonary fibrosis and scleroderma etc. Igarashi A[15] had been testing CTGF mRNA expression in the skin lesions of the patients with systemic scleroderma through in-situ hybridization. It was found that there was a strongly positive expression in the skin lesions but not in the normal skin of the same patient. Querfeld [16] discovered that TGF-β expression only occurred at the early stage of inflammation in skin lesions, while CTGF expression would last to the later period of fibrosis. What's more, there was a correlation between the extent of increased expression sclerosis. Sato[17] found that the level of CTGF was remarkably increased in the serum of the patients with systemic scleroderma, and the increased degree was positively related to the fibrosis degree in the skins and lungs. Frazier [18] and his partners had observed the feature of CTGF mRNA expression during each stage of the experiment that bleomycin induced the mice and rats to make fibrosis models. For the first time, they had proved that lung fibroblast was also able to express CTGF. Therefore the rules applied to TGF-β expression and pulmonary fibrosis.

This study utilized Japanese scholar Yamamoto's technique to establish sclerotic skin mimicking scleroderma model by local injecting bleomycin at the back of BALB/c mice. After 3 weeks later, there were typical histopathological changes to the skins and lungs of scleroderma. This indicated that injections of bleomycin induced mimicking scleroderma model was convenient procedure and produced fine repetition. This study was firstly used immunohistochemical method to test the content of CTGF and COL-I in the skin and lungs of the mice of scleroderma. Compared with the normal control group, we found that the expression value was remarkably higher in the model group. It was accorded with Zhaohui LIAO [19]. The study was using in-situ-hybridiza-

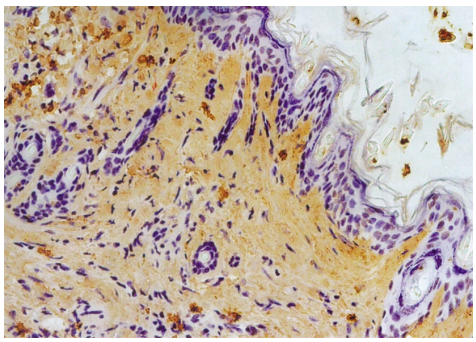

Fig 1 CTGF of mice skin tissue in model group (immunohistochemical dyeing,× 40)

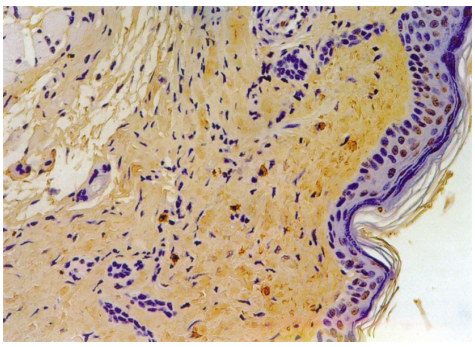

Fig 2 COL-I of mice skin tissue in model group (immunohistochemical dyeing,× 40)

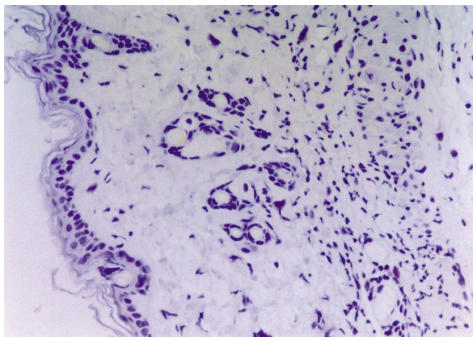

Fig 3 CTGF of mice skin tissue in control group (immunohistochemical dyeing,× 40)

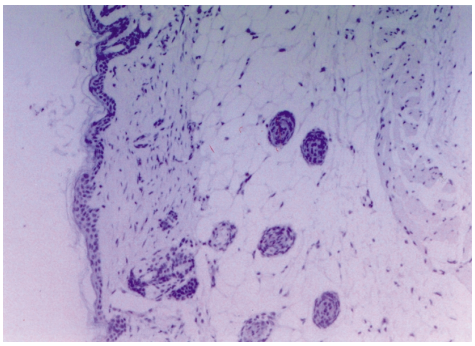

Fig 4 COL-I of mice skin tissue in control group (immunohistochemical dyeing,× 40)

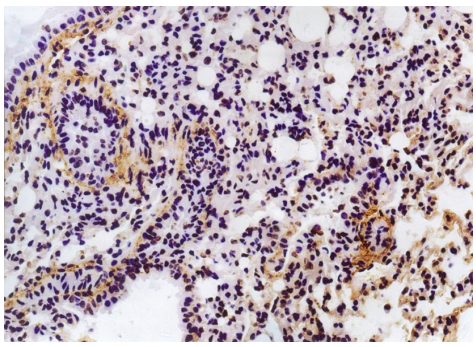

Fig 5 CTGF of mice lungs tissue in model group (immunohistochemical dyeing ,× 40)

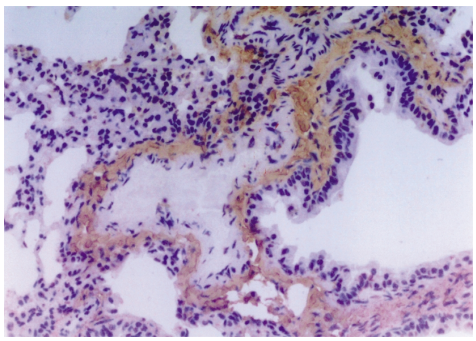

Fig 6 COL-I of mice lungs tissue in model group (immunohistochemical dyeing,× 40)

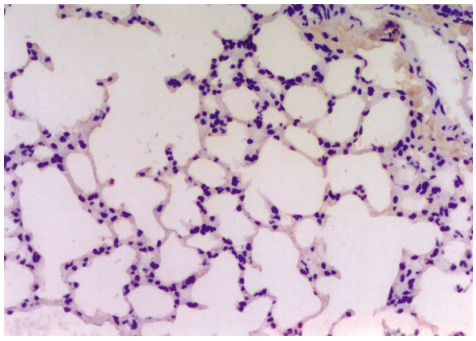

Fig 7 CTGF of mice lungs tissue in control group (immunohistochemical dyeing, × 40)

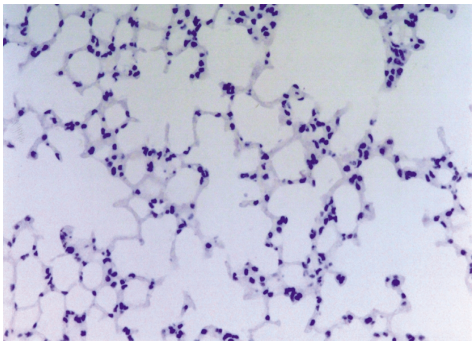

Fig 8 COL-I of mice lungs tissue in control group (immunohistochemical dyeing, × 40)

tion to test the expression of CTGF mRNA in sclerotic skin of mice with bleomycin-induced scleroderma. Moreover, we found there was relativity between CTGF and COL-I. It was shown that CTGF and COL-I had played an important role in the course of model skin fibrosis of the mice with scleroderma. CTGF expression, which was related to form and development of scleroderma in the skin and lungs, was an important medium in the course of fibrosis. CTGF, which induced transfer of COL-I mRNA with the track of protein kinase C, contributed to increase of collagen fibers synthesis, activation in fibroblast to multiplicate differentiate secrete extracellular matrix and participated in the sclerodermic progress. The SSc skin without treatment is characterized by marked accumulation of type I collagen fibres in the reticular dermis[20].

It was thought that CTGF is a downstream medium of TGF-β. And that blocking or inhibiting CTGF expression, may be a certain effective method to cure fibrosis disease. Consequently, a new method was provided in the treatment of fibrosis. That was to block or inhibit CTGF and COL-I expression in the skin and lungs of scleroderma.

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