Expression of Basic Fibroblast Growth Factor in Rat Liver Fibrosis and Hepatic Stellate Cells

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Summary: The expression of basic fibroblast growth factor (bFGF) in rat liver fibrosis and hepatic stellate cells (HSCs) and the relationship between the expression of bFGF and rat liver fibrogenesis were studied. Sixty male SD rats (230—260 g) were divided into 4 groups randomly (the 0 week group, 1 week group, 4 week group and 8 week group). Liver fibrosis was induced by subcutaneous injection of carbon tetrachloride. The sections of rats' liver in each group were tested by Van-Gieson (V-G) staining and immunohistochemistry. The expression of bFGF mRNA was detected by reverse transcription polymerase chain reaction (RT-PCR). HSCs were isolated by the combined methods of collagenase IV perfusion and density gradient centrifugation. The expression of bFGF protein in cultured HSCs was detected by Western blot. Images of immunohistochemistry detection, agarose gel electrophoresis of RT-PCR and SDS-polyacrylamide gel electrophoresis of Western blot were analyzed semiquantitatively by image-analyzing system. The results were analyzed by statistics. The results showed that the fibers were gradually increased in the sections of rat liver with the prolongation of the model induction. At the end of the 8th weeks, liver fibrosis was formed. The expression of bFGF detected by immunohistochemistry showed a similar tendency of gradual increase. At the end of the 8th weeks, the bFGF expression could be observed in many regions in sections and the strongest expression was in interstitial cells including HSCs and some hepatocytes in regions around the portal area and central veins. Also there was moderate expression widely in extracellular matrix (ECM). In RT-PCR detection and Western blot detection of HSCs cultured in vitro, the similar tendency of gradual increase was evident either. It is suggested that bFGF is related with liver fibrosis of rats closely and may be a fibrogenesis factor of liver. bFGF possibly regulates liver fibrogenesis through regulating metabolism of extracellular matrix (ECM) by autocrine and paracrine stimulation.

Key words: basic fibroblast growth factor; rat; hepatic stellate cells; liver fibrosis

Fibrosis represents the consequences of a sustained injury healing response to chronic liver injury from a variety of causes including virus, autoimmune, drug, cholestatic and metabolic diseases, etc. Regardless of the causes, the fibrosis is characterized by an accumulation in extracellular matrix (ECM) constituents that collectively form hepatic scars[1]. Activated hepatic stellate cells (HSCs) are identified to be the most important source of ECM and fiber. Thus, activation of HSCs are proved to be the dominant event in fibrogenesis[2]. During activated HSCs are developed to myofibroblasts (MFBs) in inflammation and necrosis tissues, a large mass of ECM is produced and accumulated in liver sinus. Thus, fibrosis was developed gradually[3]. There are a series of cytokines participating in regulating the whole course[4].

Basic fibroblast growth factor (bFGF), a kind of peptide molecule identified in several tissues of human and animal, has been found to play many roles in organism, such as mitrogen of several type of cell, stimulator of blood vessel, etc. It has also been found to be elevated in liver of cirrhotic patients[5]. But action and change of bFGF in liver fibrosis is still unrevealed. We tried to explore it by immunohistochemistry, RT-PCR and western blot in this article and contribute to advanced research of molecular mechanism of liver fibrogenesis.

1 MATERIALS AND METHODS

1.1 Induction of Fibrosis

Sixty male SD rats (230—260 g) were obtained from the Animal Center of Tongji Medical College of Huazhong University of Science and Technology and maintained on routine laboratory rat chow. Then the rats were divided into 4 groups (0 week group, 1 week group, 4 week group and 8 week group) randomly, 15/group, and fibrosis was induced by subcutaneous injection of carbon tetrachloride (CCL4), 150 μL/100 g body weight in an equal volume of vegetable oil twice a week.

1.2 Histology and Immunohistochemistry

Liver samples were fixed in 4 % formaldehydum polymerisatum, paraffin-embedded and sectioned at 5 μm. Parts of tissue sections were stained with Von-Gieson (V-G) staining. bFGF detection was performed using immunohistochemistry SP kit (Zhongshan Bioengineering Company, Beijing). Microwave antigen retrieval (750 W, 15 min in citrate buffer 0.01 mol/L, pH=6.0) was performed after tissues sections were dewaxed. Before incubated with polyclonal antibodies against human bFGF (1 : 100) over night at 4 ℃, endoge-

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nous peroxidase activity was inhibited with 3 % hydrogen peroxide for 10 min and endogenous biotin was blocked by 10 % goat serum. Then the sections were incubated with biotinylated rabbit anti-goat IgG for 15 min at 37 ℃ and complement Horseradish peroxidase conjugated Streptavidin solution for 15min at 37 ℃. Color development was achieved with DAB. 0 week groups were obtained by omitting the first antibody. Results were scanned by multimedia pathology imaging analysis system (MPIAS-1000). Five views of each section were selected randomly and their average optical density scanned. Means were made to represent intensity of the bFGF expression.

1.3 Reverse Transcription Polymerase Chain Reaction Analysis of bFGF mRNA

Total RNAs were extracted by Trizol (Gibco, USA). 5 μg of RNAs were reverse transcribed using a commercial kit (Promega, USA). Samples of cDNA were subjected to bFGF amplification combined with GAPDH co-amplification by polymerase chain reaction (PCR). The bFGF and GAPDH oligonucleotide primers were designed based on published rat cDNA sequences in GENBANK database. The bFGF sense primer was 5′-GGAGAAGAGCGACCCACA-3′ (position on cDNA: 54 — 71) and the antisense primer was 5′-CCAGTTCGTTTCAGTGCC-3′ (position on cDNA: 260—287). The GAPDH sense primer was 5′-TCACCACCATGGAGAAGG-3′ (position on cDNA: bp298 — bp315), antisense primer was: 5′-TGGGAGTTGCTGTTGAAG-3′ (position on cDNA: bp848—bp865). PCR was performed in a 50-μL reaction mixture containing: 3.0 μL cDNA; 4.0 μL MgCl2; 2.0 μL dNTPs; 2.0 μL of each bFGF primer; and 1.0 μL of each GAPDH primer. Reaction mixture was cooled on ice for 1 min after predegenerated at 94 ℃ for 5 min. Then 1 μL Taq DNA polymerase and 5 μL 10×buffer of Taq DNA polymerase enzyme was added. The PCR conditions were as follows: 31 cycles of 1 min denaturation at 94 ℃, 45 s annealing at 52 ℃ and 1 min extension at 72 ℃ and a 8-min terminal extension at 72 ℃, cooled at 4 ℃ and preserved at −20 ℃. Agarose gel electrophoresis was used to analyze the product of PCR.

Taking GAPDH for standard, average absorbency of images of agarose gel electrophoresis was scanned and analyzed semiquantitatively. Take ratio of average absorbency of target DNA/GAPDH to represent relative level of the expression of target DNA.

1.4 Cell Isolation and Culture

Under chloral hydrate anesthesia, a cross incision was made in the abdomen of the rat. The portal vein was cannulated with a polyethylene tube, and the liver was injected in situ with D- Hanks' solution 20 mL containing 12 500 U heparin rapidly and was subsequently perfused at 20 mL/min with D-Hanks' solution warmed up to 37 ℃ beforehand until the liver became light yellow appearance. The anesthetized rat was killed by exsanguination during perfusion. Then the liver was isolated from the abdomen and was perfused circularly with 80 mL of D-Hanks' solution at 37 ℃ containing 0.05 % collagenase IV (Sigma, USA) for 40—60 min. Immediately after the liver was softened completely, envelope of liver was removed using scissors. Digested liver tissue was suspended in the same collagenase solution and filtered through a mesh in combination with grinding by the core of injection syringe. Then the cell suspension was centrifuged at 500×g for 7 min at 20 ℃. The deposition was resuspended in D-Hanks' solution and added two volume of 18 % Nycodenz (Sigma, USA). Then the cell suspension was separated into two glass centrifuge tubes and 1 mL non-serum DMEM (Gibco, USA) was added carefully into each glass centrifuge tube later. Stellate cells were isolated by density gradient centrifugation at 1500×g for 17 min at 20 ℃. The white layer of cells were imbibed carefully and resuspended in DMEM. After centrifugation at 500×g for 7 min at 20 ℃, the deposition of cells was resuspended in DMEM supplemented with 10 % fetal bovine serum (Sigma, USA) and was cultured in culture bottle in incubator at 37 ℃ and 5 % CO_2. Stellate cells were identified by the autofluorescence under fluorescence microscopy. Immediately after isolation, the viability of cells was assessed by trypan blue exclusion test.

1.5 Western Blot Analysis of bFGF Protein

According to the method described in reference[6], 0.5 mL ice-cooled lysis buffer was added to cells washed with ice-cooled PBS. After 20 min, the cellular debris suspension was treated by 2-min centrifugations (12 000 g) at 4 ℃ twice. Then the protein sample was electrophoresed on SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Western transfer to nitrocellulose filter was performed at 350 mA for 1 h. After the nonspecific sites were blocked overnight with 4 % (wt/vol) nonfat milk in PBS, the membrane was incubated with polyclonal antibodies against human bFGF (1:1000 dilution in PBS) (Santa Cruz Biotechnology, USA) over night at 4 ℃. After washed with TBS-T solution, reaction with secondary antibody conjugated to peroxidase was performed for 40 min at 37 ℃. Finally, the membrane developed by ECL until the color developed.

Absorbency of sections stained by immunohistochemistry detection and gray scale of figures of agarose gel electriphoresis was analyzed semiquantitatively by image analysis system.

2 RESULTS

Different levels and extents of fibrosis were found in sections of rat liver in different phases injected with carbon tetrachloride and vegetable oil.

Only very few fibrosis silks stained as light red were found in portal area and central veins in normal livers. At the end of the 1st week of injection, the fibers were found to be increased in portal area and central veins and some were found in surrounding region. At the end of the 4th week, the fibers become obvious and fibrous bands extended to liver tissue. In some regions, tissue was inserted and separated by these fibrous bands. At the same tissue, steatosis of hepatocyte was evident (fig. 1). At the end of 8th week, the fibers become thicker and disarranged. The normal structure of liver tissue was destroyed and became orderless. Fibrous bands extended into hepatic lobules. Damaged hepatic lobules and some reproduced hepatocyte mass were surrounded and separated by thick fibrous bands. But pseudo lobule didn't formed yet. Steatosis of hepatocyte was severe (fig. 2).

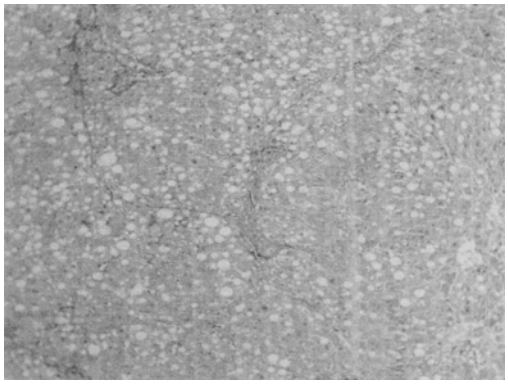

Fig. 1 Section of rat liver of 4 week group stained by V-G staining (×100)

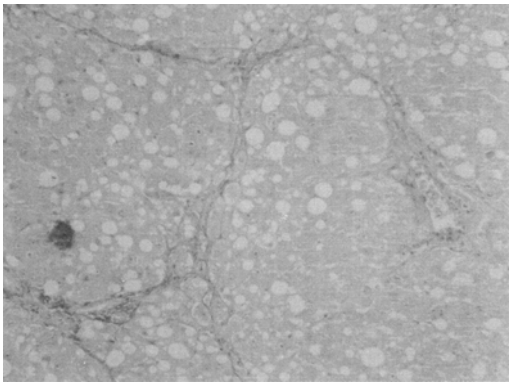

Fig. 2 Section of rat liver of 8 week group stained by V-G staining (×100)

Almost no apparent bFGF expression was found in normal liver sections by immunohistochemistry. At the end of the 1st week, no or minimal bFGF expression could be found yet. Occasionally it occured in cytoplasm of a few Mesenchymal cells. At the end of the 4th week, moderate bFGF expression could be found in mesenchymal and stronger in regions near the fibrotic partition, space of Disse, cytoplasm of HSCs and a few hepatocytes around portal area and central veins (fig. 3). At the end of the 8th week, bFGF expression was increased dramatically. It was observed in most regions in sections and the strongest expression was still in regions around the portal area and central veins. Those were regions near the fibrotic partition, space of Disse, cytoplasm of HSCs, Kupffer cells, sinusoidal endothelial cells and many hepatocytes. Also there was moderate expression widely in ECM (fig. 4).

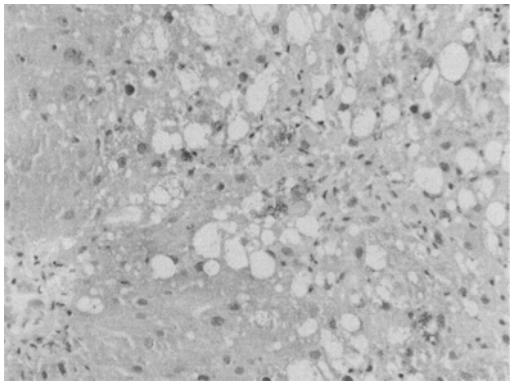

Fig. 3 Section of rat liver of 4 week group stained by immunohistochemistry (×200)

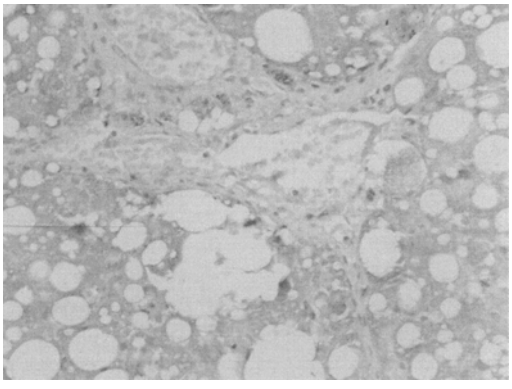

Fig. 4 Section of rat liver of 8 week group stained by immunohistochemistry (×400)

The expression of bFGF in liver tissue detected by RT-PCR (fig. 5) and in HSCs cultured *in vitro* detected by Western blot (fig. 6) showed similar tendency of gradual increase either. The expression of bFGF in 4 week group and 8 week group was increased apparently, especially in 8 week group.

The detecting results of the bFGF expression both in tissues and HSCs showed there were significant differences between 4 week group, 8 week group and 0 week group both in tissues and HSCs (table 1, *t*- test).

Table 1 Expression of bFGF in liver tissue detected by immunohistochemistry and RT-PCR $\bar{x}\pm s$

Groups	Immunohistochemistry	RT-PCR (Ratio of gray scale: bFGF/GAPDH
0 week	0.1054±0.0335	0.07±0.01
1 week	0.1077±0.0213	0.10±0.02
4 week	0.5366±0.1134*	0.34±0.07*
8 week	1.462±0.2275*	0.66±0.04*

* $P<0.01$ as compared with 0 week group

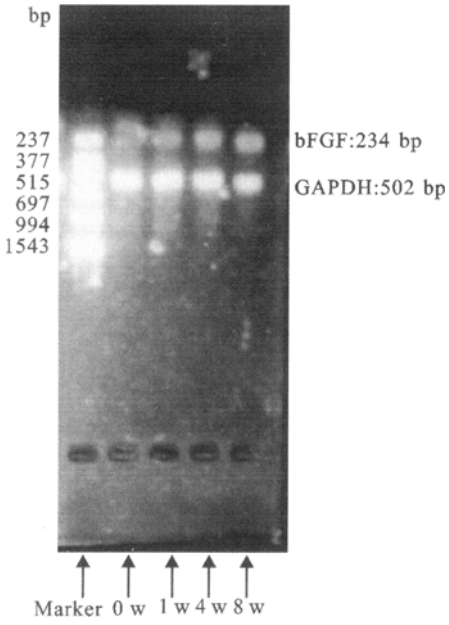

Fig. 5 bFGF expression detected by RT-PCR

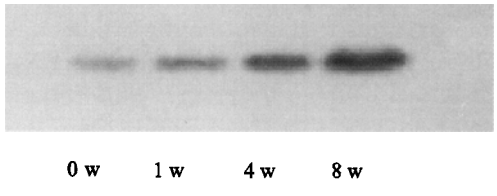

Fig. 6 bFGF expression detected by Western blot

3 DISCUSSION

bFGF, a kind of peptide molecule regulating growth of cells by binding specific receptor of cell membrane, has been found to have many functions, including mitogen activity to various cells originated from mesoderm and neural ectoderm (such as fibroblast, endothelial cell, smooth muscle cell, neuron. etc), chemotaxis to various mesoderm cells (such as endothelial cell, fibroblast, macrophage), angiogenesis stimulator[7], restore of injury[8], embryogenesis inducer and an important role in growth of tumor[9]. Reports indicated promotion roles of bFGF in fibrosis of several organs, such as lung[10] and kidney[11]. In these tissues, bFGF was identified to interact with some cytokines (TGF-β, PDGF[10], VEGF[12], etc). Some of these cytokines were found to contribute to fibrogenesis of liver fibrosis powerfully. So it is a reasonable suspect that bFGF may be associated closely with fibrogenesis of liver fibrosis. Presently, there are still few reports about it.

In this study, the bFGF expression in liver tissues of fibrosis was detected by immunohistochemistry and RT-PCR. Tendency of the expression is accordance with fibrosis tested by V-G staining. In fact, this provides evidence that bFGF is associated closely with rat liver fibrosis induced by carbon tetrachloride and bFGF will possibly be identified to be a stimulator for fibrogenesis of liver. In immunohistochemical test, stronger expression of bFGF occurs in regions near the fibrotic partition, space of Disse, cytoplasm of HSCs, Kupffer cells, sinusoidal endothelial cells, some hepatocytes and ECM, indicating that HSCs, Kupffer cells, sinusoidal endothelial cells are possible locus synthesizing and excreting bFGF and bFGF may participate in metabolism of ECM and play an important role. Previous researches show Kupffer cells, hepatocytes[13], sinusoidal endothelial cells[14] and mastocytes[15] are important sources of bFGF. Our research finds so were HSCs.

According to Western blot, bFGF expression was also increased gradually in HSCs isolated and cultured *in vitro*. The expression of bFGF in HSCs was enhanced with the increase of liver fibrosis and was accordance with the increased tendency of fibrosis. It confirms our previous hypothesis that HSCs are regions synthesizing and excreting bFGF. Numerous researches show HSCs are the key fibrogenesis cells in fibrogenesis of liver[16]. It is believed that fibrogenesis activity of HSCs is associated with bFGF and bFGF is an important fibrogenesis cytokine synthesized and excreted in HSCs. On the other hand, bFGF may also play an important role in regulating activation, proliferation or migration of HSCs and regulating synthesis and metabolism of ECM further, therefore promoting the fibrogenesis of rat liver.

Previous research indicated that the 22-kD (1 kD=0.992 ku) bFGF was the main isoform expressed in experimental liver fibrosis model[13], which was consistent with the result in this study. Some scholars believed that the low molecular weight form (18 kD) might modulate cell motility and proliferation through interaction with its cell surface receptor, while the high molecular weight isoforms (22 to 24 kD) might act as a mitogen through an intracellular mechanism[17]. Thus there

(Continued on page 222)

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are possibly complex interaction and mechanisms between bFGF and liver fibrosis.

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