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Effect of anti-fibrosis compound on collagen expression of hepatic cells in experimental liver fibrosis of rats

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INTRODUCTION

Liver fibrosis is mainly characterized by the excessive synthesis and decreased degradation of extracellular matrix (ECM), especially the synthesis and deposition of collagen. Almost all kinds of cells in the liver have participated in the production of collagen. The most important ones are hepatic stellate cells (HSC) and hepatocytes[1-3]. We have observed the collagen expression of normal and fibrotic rat liver, compared its expression in passaged HSC of the normal liver and in the primary HSC of the fibrotic liver. Serum pharmaco logical method was used to study the influence of anti-fibrosis compound prescription (ACP) on the production of collagen in hepatocytes and HSC of rats.

MATERIALS AND METHODS

Experimental animal

Wistar male rats of 250g-300g were used to separate the hepatocytes , of 300g-350g were used to separate HSC. The rats were provided by Shanghai Centre of Experimental Animals. Animal model of liver fibrosis was formed by intraperitoneal injection of DMN (10mg/kg) 3 times a week for three weeks.

Drugs and pharmacological serum preparation

ACP (*composed of Radix Astragali seu Hedysari, Herba Leonuri Radix Curcum ae*, etc.), prepared by the Pharmaceutical Department of Shuguang Hospital, contained 2g/mL crude drugs. Colchicine (Supplied by Serva Company) was prepared at the concentration of 0.3g/L. ACP and colchicine were instilled into the stomach of the rats once a day for 1 week. One hour after the last time of instillation, blood was drawn in aseptic condition and serum was separated. After 30 minutes of inactivation at 56℃, it was packed and refrigerated separately.

Separation and culture of rat hepatocytes

After anesthesia of the rats, a tube was inserted through the portal vein to infuse collagenase perfusion (0.05% of collagenase IV, 0.007% trypsin inhibitor magnesium-free Hank's medium, pH 7.4) to digest the liver and disperse the liver cells. Density gradient centrifugation was done with 49.2% (v/v) lymphocytic separation fluid to purify the liver cells. Then culture medium 199 with 10% calf serum (added with 10^{-8}mol/L of insulin and 10^{-8}mol/L of dexamethasone) was used to suspend the cells to 5×10^5/ mL and was inoculated to the 60mm culture plate and stored in the incubat or with 5% CO_2 and 37℃. After 4 hours, it was replaced by 5% calf serum in 199 medium for primary culture.

Separation and culture of the HSC

After anesthesia of the rats, a tube was inserted through the portal vein to infuse D-Hank's liquid to cleanse the blood in liver. Then the perfusion medium with enzyme (Hank's medium containing 0.05% of collagenase IV and 0.1% Pronase E) for cyclic infusion. The liver was cut into pieces, and re-digested . After centrifugation with 18% (w/v) Nycondenz density gradient centrifugation, the purified HSC was suspended in DMEM containing 20% calf serum attending 5×10^5/ mL for inoculation and culture. After full growth, the normal HSC was passaged while the HSC from the rat with fibrotic liver were processed in primary culture.

Assay of immunohistochemistry

The hepatocytes or HSC were inoculated in the plate covered with a piece of glass with a density of 5×10^5/mL. The hepatocytes after cultured for 2 days, 5% medicated serum or controlled serum was used to incubate for 48 hours. After 5 days, the HSC adhered to the plate were incubated in 10% medicated serum or controlled serum for 72 hours. The cells were then divided into ACP group with medicated serum of ACP, colchicine group with

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medicated serum of colchicine and control group with serum from normal rats. After culture, the glass slides were taken out to detect type I, III and IV collagen by ABC method of immunohistochemistry. After staining, the light density of the cells in each group was tested with the computer image analysis system. The higher the density, the deeper the positive staining.

RESULTS

Comparison of the production of collagen between normal and fibrotic hepatocytes

The immunocytochemical staining indicated that the collagen of the normal hepat ocytes was negative, the light density of the collagen type I, III and IV was 3.80, 4.30 and 3.90 respectively, the staining of those three kinds of colla gen in cytoplasma of the fibrotic hepatocytes was significantly increased. The staining of collagen type I was the deepest one, the next was type III. The light density of these three kinds of collagen was 42.40, 32.10 and 27.40 respectively. There was significant difference in the cells between the two groups(P<0.01).

Influence of the ACP on the collagen expression of type I, III and IV in hepatocytes from the fibrotic rat

The staining of the type I, III and IV collagen was positive in cytoplasma of hepatocytes from the fibrotic liver. After treatment with serum processed by the ACP, the staining obviously turned lighter as compared with the control group, indicating that the collagen expression of the type I, III and IV of the cells was reduced, The inhibition of colchicine serum group on collagen type I was close to the group of ACP, while the effect on expression of the collagen type III and IV was weaker (Table 1) than those of the ACP group.

Comparison of the collagen expression between passage HSC and fibrotic HSC

The immunochemical staining indicated that positive staining of the type I, III and IV collagen appeared in both of the normal passage HSC and the fibrotic HSC. The collagen expression of the cells in both groups was nearly similar. But the correlation analysis on type I and III showed that the collagen expression of the type III in passage HSC was significantly increased, type I/type III was 0.91. However, the collagen expression of type I in fibrotic HSC increased more significantly as compared with that of type III. TypeI/ type III was 1.22 (Figure 1).

Influence of the ACP on the collagen expression of type I, III and IV fibrotic HSC

ACP medicated serum could significantly reduce the staining of collagen type I, III and IV in fibrotic HSC and could remarkably inhibit the collagen type IV and I. The effect was better than that of the colchicine group. Compared with the control group, the positive staining of the HSC in the colchicine group became lighter. However, its inhibition on the expression of collagen type III was close to that of ACP (Table 2).

Table 1 Influence of ACP on the collagen expression of the fibroti c hepatocytes (density value, n=20, $\bar{x}$-±s)

	Inhibiting rate (%)					
	Col (I)		Col(III)		Col(IV)	
Control	45.6±2.29		38.0±2.76		27.9±2.39	
Colchicine	19.2±2.52[a]	25.29	15.2±1.75[a,b]	20.36	13.4±1.56[a,b]	11.87
ACP	18.3±2.61[a]	26.15	10.7±1.68[a]	24.37	7.9±2.55[a]	16.38

[a]P<0.01 *vs* control group; [b]P<0.01 *vs* ACP.

Table 2 Influence of the ACP on the collagen expression of fibrotic HSC (density value, n=20, $\bar{x}$±s)

	Inhibiting rate (%)					
	Col (I)		Col(III)		Col(IV)	
Control	26.8±1.99		21.9±1.97		33.3±2.19	
Colchicine	12.2±2.18[a,b]	11.85	9.0±2.00[a]	10.07	9.5±2.06[a,b]	20.39
ACP	8.4±1.50[a]	14.93	7.9±1.87[a]	10.93	5.8±1.40[a]	23.56

[a]P<0.01 *vs* control serum; [b]P<0.01 *vs* ACP.

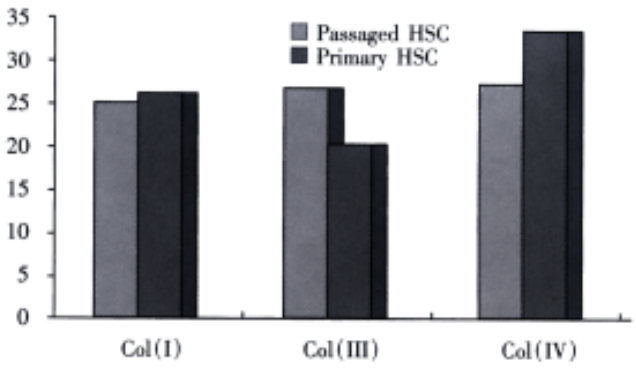

Figure 1 Comparison of the collagen expression between the passaged HSC and the fibrotic HSC.

DISCUSSION

The cells responsible for the production of collagen in the liver have aroused many scholars' attention. Recent studies have confirmed that HSCs are the key cells to produce collagen in the liver. The activated HSC can excessively synthesize collagen[4-6]. Through automatic secretion and multiplication, they maintain a constant progression of fibrosis and play very important roles in the course of liver fibrosis[7-10]. There are different ideas as to whether the hepatocytes can synthesize collagen. There are contradictory reports about whether there exits collagen mRNA or not in the hepatocytes[11-14]. The latest studies have proved that the hepatocytes can synthesize collagen[15-18]. Immunohistochemical studies have discovered that there is no collagen of the type III in the hepatocytes of normal liver. However, small quantity of it was found in the hepatocytes of fibrotic liver[19]. It can

be inferred that the excessive synthesis of collagen which may lead to liver fibrosis, is related to the constant abnormal regeneration of hepatocytes. Through *in situ* hybridization, we have discovered that the fibrotic rat liver induced by CCl_4 can express procollagen mRNA α1 (I) and α1 (III) in the hepatocytes of early, middle and advanced stages. This shows that the hepatocytes can synthesize the collagen type I and III, and also participate in the course of liver fibrosis[19,20].

The hepatocytes can synthesize at least the collagen of type I, III, IV and V while in the normal liver, collagen synthesis in hepatocytes is quite weak or even inhibited. In the process of liver fibrosis, the synthetic activity of collagen was remarkably increased[21]. This study indicates that in normal liver, hepatocytes do not express or seldom express collagen and that in the fibrotic liver the expression of collagen type I, III and IV in hepatocytoplasma is more significant. When the hepatocytes are impaired or under the stimulation of certain factors, the capacity of the hepatocytes to synthesize collagen will be activated[22,23]. As to a single liver cell, its capac ity to synthesize collagen is much lower than that of the HSC. However an absolute majority of the cells in the liver are hepatocytes, and their capacity to synthesize collagen should not be over looked. And in chronic liver disease, the degeneration and necrosis of the hepa tocytes can directly or indirectly stimulate non-parenchymal cells like HSC to increase collagen production, thus exerting great effort on liver fibrosis[24-27].

At the early stage of liver fibrosis, collagen type IV increases first and then gradually decreases, while the collagen type III increases and expresses predominantly. In the developing and advanced stages, the collagen type I gradually increased, and collagen of both type I and III significantly increased, especially the type I[11,28,29]. After passage culture of the HSC from normal liver, a series of changes have been observed in their features and the production of collagen and the expression scale is evidently increased. So HSC is regarded as *in vitro* cell model of fibrosis and is extensively used in the *in vitro* experiment of liver fibrosis[30-33]. In order to be close to the collagen expression of the *in vitro* HSC during the fibrosis of liver, the experiment was also made using the HSC of the primary culture from the fibrotic liver. The collagen expression of the HSC from passage culture and HSC from primary culture of the fibrotic liver was tested by immunocytochemical staining. Positive staining was observed in the collagen type I, III and IV.

When fibrosis occurs in the liver, the total amount and varieties of the collagen produced by HSC in the process of liver fibrosis are changed[34-36]. Such change is related to the course of liver fibrosis. Maker[37] has discovered that in the culture of HSC from normal SD rats, the gene expression of type I collagen is the weakest, while the gene expression of the coll agen type III or IV is comparatively stronger. However, The mRNA of type I collagen is 30 times that of normal and type III is 5 times that of normal due to difference in culture time and in the expression of procollagen mRNA in the HSC from both the normal liver and fibrotic liver. At the early stage of fibrosis,the expression of the collagen type III is more significant, while at the advanced stage the expression of the collagen I type is more remarkable. The passage culture for the HSC separated from the normal liver is about 2 weeks, while the time for *in vivo* modeling of the HSC in the fibrotic liver is 4 weeks. The experimental results show that the collagen expression of type III in passage HSC is significantly increased, while the increase of collagen expression of type I in passage HSC is more significant. It seems to show that these two kinds of cells bear the features of the HSC at the early and advanced stages of fibrosis.

Seropharmacological studies indicate that ACP can significantly inhibit the excessive synthesis of collagen by the hepatocytes and HSC under the condition of liver fibrosis[38]. *In vitro* experiment, medicated serum can elim inate the effect of impurity and acidity in the Chinese pharmaceutical preparation on the growth of cells, making it more closely mimic the absorption and metabolism of drugs *in vivo*[39,40], and bringing the pharmace utical effect into full play.

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