行政院國家科學委員會補助專題研究計畫成果報告

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※　　藥物治療對肝臟纖維化恢復的影響　　　　　　※

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計畫編號：NSC　89－2315－B－002－038－

執行期間：　89 年 8 月 1 日至 90 年 7 月 31 日

計畫主持人：黃冠棠

共同主持人：

本成果報告包括以下應繳交之附件：

□赴國外出差或研習心得報告一份

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□國際合作研究計畫國外研究報告書一份

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一、摘要

Abstract

BACKGROUND/AIMS: It was generally believed, but not proved, that early cirrhosis may be reversible, while advanced cirrhosis may not. This present study is to compare in mice the spontaneous regression of liver fibrosis between early and more advanced stage.

METHODOLOGY: Liver fibrosis in mice was induced by intraperitoneal injection of carbon tetrachloride for 4, 10, and 16 weeks. After the last dose of each schedule, mice were sacrificed 1 day later (progression model) or left untreated for 10, 20, and 60 days (regression model). Tissue sections were stained by Sirius red. Liver hydroxyproline levels were determined to assess severity of fibrosis. Gelatinases in tissue extracts were assayed by zymography.

RESULTS: During regression, diminution of fibrotic bands was more prominent in the 4-week group than the others. Liver hydroxyproline levels in the progression model increased and resolution of liver fibrosis in regression model decreased as carbon tetrachloride injection prolonged. Liver matrix metalloproteinase-2 and -9 activities in the progression model also decreased as the injection prolonged.

CONCLUSIONS: These data demonstrated that reversibility of liver fibrosis would be gradually lost as liver injuries prolonged. Gradual loss of the expression of matrix metalloproteinases may be responsible for the loss of reversibility.

Keywords: liver fibrosis, regression, MMP

二、緣由與目的

Liver fibrosis/cirrhosis was usually regarded as an irreversible process. In a scoring system for liver histology, fibrosis was defined as an irreversible item (1). Clinically, most of the cirrhotic patients went downhill relentlessly without regression. On the contrary, a few case reports demonstrated by follow-up liver biopsies that fibrosis, or even cirrhosis, had resolved after successful medical treatment (2,3). In animal models, liver fibrosis induced by several toxins or bile duct ligation had been shown by morphological studies to regress after the toxic agents were removed or bile duct reconstructed (4-7).

There has been an unproved hypothesis that early cirrhosis may be reversible while advanced one not. Thus, there may be a "point of no return" during the process of fibrogenesis. It is not clear yet what is this point and what determines this point.

Recently, resolution of CCl_4-induced liver fibrosis in rats has been demonstrated objectively by determination of liver hydroxyproline (HOP) level or morphometric analysis of fibrotic area (8,9). These models were based on observations of regression after cessation of a single period of CCl_4 treatment (4 weeks and 8 weeks, respectively, in these two studies). In the present study, we compared the reversibility of liver fibrosis after different periods of CCl_4 treatment to test the above hypothesis of "point of no return".

三、內容

METHODOLOGY

Experimental design

Fifty-five female BALB/c mice

weighing 15-20 g were used in this experiment. They were provided with food and water *ad libitum*. They received humane care in compliance with institution's guidelines, which are following the guidelines of National Institutes of Health. The experimental schedules of treatment for these mice are shown in Fig. 1. Four control mice received corn oil injection only and were sacrificed 4 weeks later. The other mice were divided into 3 groups (17 in each group) and received intraperitoneal injection of CCl_4 (1 μl/g body weight mixed with 5x corn oil) twice weekly. These 3 groups of mice were treated with CCl_4 for 4, 10, and 16 weeks, respectively. Five mice in each group were sacrificed 1 day after their respective last dose of CCl_4 injection and designated as "peak fibrosis" subgroups. Thereafter, 5, 5, and 2 mice in each group were sacrificed 10, 20, and 60 days after their respective last CCl_4 dose. An anterior segment of removed liver was excised and stored at -70℃ until used for determination of HOP level. Another segment was fixed in 10% neutralized formalin, paraffin-embedded, and sectioned (7 μm). The other part of liver was stored at -70℃ until used for extraction of tissue protein.

Histological analysis

The sections were deparaffinized and then stained with 0.1% Sirius red and 0.025% Fast green in saturated picric acid.

Determination of HOP concentrations in liver samples

Samples were weighed and hydrolyzed in 6N HCl (20 μl/ mg wet tissue) at 120℃ for 16 hours. Equal moles of NaOH were added to neutralize the hydrolysates. After centrifugation for 10 min at 12000 rpm, the supernatants were collected for spectrophotometric determination of HOP according to Bergman and Loxley (10). Briefly, HOP in solution was oxidized by Chloramine T and condensed in Ehrlich's solution (p-dimethylamino-benzaldehyde dissolved in 60% perchloric acid) to give red color products, and then determined by a spectrophotometer at 560 nm. Resolution of fibrosis in percentage was defined as below: % resolution of fibrosis = (HOP in peak fibrosis - HOP in time points of regression/ HOP in peak fibrosis - HOP in control) × 100%.

Tissue protein extraction and gelatin zymography

The tissues were cut into small pieces and ground in phosphate-buffered saline (PBS) containing 1 mM of phenylmethylsulfonyl fluoride (PMSF). After centrifugation at 12000 rpm for 30 min at 4 ℃, the supernatants were collected and protein concentration in each sample was determined by a protein assay kit (Bio-Rad). The extracts (100 μg of protein) were electrophoresed in 10% polyacrylamide gels containing 1 mg/ml of gelatin. After electrophoresis, gels were washed twice (1 hour each time) in 2.5% Triton X-100 and incubated for 16 hours at 37℃ in an assay buffer containing 50mM Tris (pH 7.4) and 5mM $CaCl_2$. After incubation, gels were stained with 0.25% coomassie blue in a solution containing 45% methanol and 10% acetic acid for 4 hours and then destained in a solution containing 10% methanol and 10% acetic acid. The gelatinolytic bands were scanned for determination of intensities.

Statistical analysis

Comparison of means between groups was performed using *t* test. P values less than 0.05 were considered statistically significant.

RESULTS

Histology

As shown in Fig. 2, at peak fibrosis, 16-week injection of CCl_4 induced more prominent septa than 4-week or 10-week injection (Fig. 2E vs. 2 C & 2A). On the contrary, following 10-day regression after 4-week administration of CCl_4, the septal collagen fibers became thinner and tend to be interrupted. The difference between peak fibrosis and 10-day regression after 10-week and 16-week injection were not so striking (Fig. 2B vs. 2D & 2F).

Regression of liver fibrosis assessed by liver HOP level

Liver HOP level in control mice was 106.5 ± 8.4 μg/g wet weight. At peak fibrosis after 4-week, 10-week, and 16-week CCl_4 injection, the levels were significantly increased to 272.4 ± 15.8, 306.8 ± 10.3, and 320.4 ± 24.0 μg/g wet weight, respectively. The level increased as the CCl_4-injection period prolonged from 4 weeks to 16 weeks, but the differences between these 3 peak fibrosis subgroups were mild and statistically not significant (Fig. 3A). During regression in the 4-week group, HOP levels declined significantly to 191.6 ± 13.1 μg/g wet weight after 10-day regression. This represented a 48.7% resolution of fibrosis (Fig. 3B). The levels did not decline further even after 60-day regression. In the 10-week group, significant resolution of fibrosis did not occur until 20-day regression (28.2% resolution) and 60-day regression (41.3% resolution). In the 16-week group, resolution of 24.4%, 16.5%, and 29.8% occurred (but not statistically significant) after 10-, 20-, and 60-day regression, respectively.

Gelatinolytic activities in liver tissues

Zymograms demonstrating gelatinolytic activities in liver tissues (controls and peak fibrosis subgroups) are shown in Fig. 4A. Three major activities (92 kDa, 65 kDa, and 62 kDa) are clearly shown. For standardization, the 4 control samples were loaded in every gel. The activities corresponding to these bands have been shown to be latent matrix metalloproteinase (MMP)-9, latent MMP-2 and active MMP-2, respectively (11). Two of the major bands (92 kDa and 65 kDa) were scanned to represent MMP-9 and MMP-2 expressions in these tissues. The intensities were compared to the mean of 4 controls in respective gel and shown in bar charts in Fig. 4B. Expressions of both MMP-9 and MMP-2 increased dramatically to 5.74 ± 0.37-fold and 4.56 ± 0.49-fold of control, respectively, after 4 weeks of CCl_4 injection. But the levels decreased significantly ($P<0.005$) to 3.92 ± 0.25- and 2.03 ± 0.16-fold, respectively, at 10 weeks, and to 2.07 ± 0.36- and 1.32 ± 0.06-fold of control, respectively, at 16 weeks.

DISCUSSION

Clinical observations suggested more advanced cirrhosis, less possible of reversal. Although several reports have confirmed the reversibility of liver fibrosis clinically or in animal experiments (2-7), none has compared the reversibility between early and more advanced fibrosis. This present study is the first to do the comparison in an animal model.

Using the BALB/c mouse model, we confirmed our previous study that resolution of liver fibrosis occurred rapidly to a steady level within 10 days after cessation of a 4-week injection of CCl_4 (12). The limitation of resolution was ~70% in the last study and ~50% in the present study. The reasons for the inability of complete resolution were unknown yet.

This present study further demonstrated that more prolonged induction of CCl_4 was correlated with less resolution of liver fibrosis after removal of this toxin (Fig. 3). The severity of fibrosis might be an important determinant of reversibility. But because the severity of fibrosis in the 3 peak-fibrosis groups was not significantly changed (Fig. 3A), factors other than severity may also be important. Chronicity of liver damage may be an alternative. In this present

experimental model, we did not demonstrate a clear-cut "point of no return". Instead, loss of reversibility seemed to be progressive as fibrogenesis progressed. Even after 16-week injection of CCl_4, regression still occurred although mildly and statistically nonsignificant. We do not know whether there is such a point beyond 16 weeks of CCl_4 treatment.

MMPs are a family of extracellular matrix-degrading enzyme (13). They play important roles in the reversal of liver fibrosis (14). Previous studies had shown that hepatic collagenase activity increased in the early stage of liver fibrosis but decreased as fibrosis progress (15,16). Were this the case, gradual loss of reversibility in the present study might be resulted from reduced production of collagenase during progression of liver fibrosis. As shown by Watanabe et al, MMP-13 mRNA expression increased dramatically at 4 weeks of CCl_4 treatment, but decreased thereafter at 8 weeks and 12 weeks (9). These findings prompted us to investigate MMP activities in the livers during progression of liver fibrosis in our model. We determined MMP activities by gelatin zymography. As shown in Fig. 4, both MMP-9 and MMP-2 activities increased markedly, compared with controls, after 4-week CCl_4 injection. The activities were reduced significantly and progressively as the injection was prolonged to 10 weeks and 16 weeks. The progressive loss of MMP expression was highly correlated with progressive loss of reversibility of liver fibrosis. Taken together, our data demonstrated not only interstitial collagenase but also gelatinases decreased during progression of liver injury.

In contrast to our data, Takahara et al (11) have shown that MMP-2 in the liver increased during progression of fibrosis from 7 to 14 weeks. The reason for this discrepancy was not clear. Their samples loaded in gels were not normalized based on total protein amount but on the volume of the samples. This might be a source of error. The trends of the alteration of MMP-9 and -2 in our study were in consistence with that of MMP-13 in the study by Watanabe et al (9).

In clinical situations, patients with chronic liver disease (e.g. chronic viral hepatitis) developed cirrhosis after prolonged liver insults. Reduced expression of MMPs in the prolonged injuries is probably an important factor for the loss of reversibility of liver cirrhosis in these patients. Nowadays, as effective anti-viral therapies are becoming possible, further insights into mechanisms of and efforts in enhancing reversibility are mandatory.

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