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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計畫主持人：黃冠棠

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行政院國家科學委員會專題研究計畫成果報告

Expression of Matrix Metalloproteinases in Spontaneous Regression of Liver Fibrosis

計畫編號：NSC 89-2315-B-002-026
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一、Abstract

BACKGROUND/AIMS: Spontaneous regression of liver fibrosis would depend on the degradation of the excessive matrix in the liver. In this study, we tried to determine the kinetics of the expression of genes for matrix metalloproteinase-2 and -13.

METHODOLOGY: Liver fibrosis induced by carbon tetrachloride was resolved after withdrawal of this toxin. Histological staining for fibrous septa and determination of liver collagen content were used to evaluate the extent of liver fibrosis. Expression in liver of matrix metalloproteinase-2 and -13 was determined by reverse transcription-polymerase chain reaction.

RESULTS: The fibrous septa became thinner and interrupted and liver fibrosis resolved rapidly within 10 days. Expression of matrix metalloproteinase-2 and -13 was elevated to 2.5- and 8.7-fold, respectively, at peak fibrosis. The former was maintained at 88% ~ 76% and the later dropped rapidly to 30% ~ 20% in the recovery periods.

CONCLUSIONS: Resolution of liver fibrosis began within 10 days but only to 70%. Gene expression kinetics suggested metalloproteinase-13 might play a more important role in the resolution because it surged more markedly at peak fibrosis and returned to nearly basal levels in the recovery periods in parallel with liver collagen content.

Key words: liver fibrosis, regression, MMP

二、Introduction

Liver fibrosis is characterized by excessive synthesis and deposition of extracellular matrix (ECM) in the liver. Liver fibrosis has often been regarded as irreversible. However, several lines of evidence suggested it was reversible (1). In animal models, regression of fibrosis was observed after effective treatment of Schistosomiasis mansoni, reconstruction of biliary obstruction, or cessation of CCl_4 injection (2-4). Clinically, regression of liver fibrosis or even cirrhosis had also frequently been reported when the underlining diseases were well controlled (5-10). Nevertheless, most of those studies were only based on morphometric analyses.

It is now known fibrogenesis is a complex process involving ECM turnover. In the liver, a family of matrix metalloproteinase (MMP) is responsible for ECM degradation. According to their major substrates, MMPs can be divided into collagenases, gelatinases and stromelysins. Collagenases can specifically cleave native fibrillar type I and type III collagens. Gelatinases have degrading activities on collagen IV and V, and also on fibrillar collagen fragments already cleaved by collagenases. Stromelysins have a broad substrate profile including noncollagen glycoproteins, glycosaminoglycans, proteoglycans and elastin (11).

The aforementioned MMPs are very likely responsible for the spontaneous regression of liver fibrosis. In the present report, we studied in mice both the kinetics of resolution of liver collagen content and the kinetics of gene expression of MMP-2, a gelatinase, and MMP-13, a collagenase, during the course of spontaneous regression of carbon tetrachloride (CCl_4)-induced liver fibrosis.

三、Materials and Methods

Reagents

Taq polymerase, deoxynucleotides, random primers were purchased from Promega Corp. (Madison, WI, USA). CCl_4 was obtained from Merck & Co., Inc. (Whitehouse, NJ, USA), Sirius red was from Fluka Chemicals (Milwaukee, WI, USA), guanidium thiocyanate, phenol, chloroform, Fast green and saturated picric acid were from Sigma Chemical Co. (St. Louis, MO, USA) and Moloney murine leukaemia virus-reverse transcriptase was from Roche Molecular Biochemicals (Indianapolis, IND, USA).

Animals

Fifteen female BALB/c mice weighing 15-20 g were divided into 5 groups. They were provided with food and water *ad libitum*. All animals received humane care in compliance with the institution's guidelines, which is following the guidelines of National Institutes of Health. Three control mice (received corn oil injection only) were sacrificed 4 wk later. The other 12 mice received twice weekly i.p. injection with 1 μl/g body weight of CCl_4 in a 1:5 ratio with corn oil. The treatment was lasted for 4 wk. Three mice were then killed 24 hr after the last CCl_4 treatment. The rest of mice were kept free from the liver toxin and were sacrificed 10, 20, and 30 days after the last CCl_4 injection (Fig. 1). When animals were killed, their livers were removed. Part of the liver was fixed in 10 % neutralized formalin and then embedded in paraffin for further sectioning. The remaining parts were frozen immediately and kept in liquid nitrogen until used for extraction of total RNA.

Histological examination and determination of collagen content

The formalin-fixed paraffin-embedded blocks were sectioned into 5-μm tissue slices. The slices on glass slides were heated in oven to 50℃ for 30 min and then deparaffinized. They were then stained with 0.1% Sirius red and 0.1% Fast green in saturated picric acid.

The method to determine total collagen content in the liver tissue was as described previously (12). Briefly, 5-μm slices of tissue were placed in 1.5ml tubes, deparaffinized by xyline, and covered with saturated picric acid containing 0.1% Fast green and 0.1% Sirius red for 30 min. The tissue slices in the tubes were rinsed several times with distilled water until the fluid became colorless. One ml of 0.1N NaOH in methanol (1:1, v/v) was then added to elute the dye from the tissue slices. The absorbance at 540 and 605nm of individual elutes were determined by a spectrophotometer. Concentrations of collagen and total protein were calculated from the values of absorbance accordingly (12).

Total RNA isolation and RT-PCR analysis

Total RNA in the liver tissues were isolated with acid guanidinium thiocyanate as described elsewhere (13). One μg of total RNA was reverse transcribed for 60 min using 200U of MMLV-RT at 42°C. Polymerase chain reaction (PCR) was then performed with specific primers as below: MMP-2, forward, 5'-CTATTCTGTCAGCA CTTTGG-3', reverse, 5'-GAGACTTTGGTTCTCCAACT T-3'; MMP-13, forward, 5'-GCCCTGAATGGGTATGACAT-3', reverse, 5'-GCAT GACTCTCACAATGCG A-3'; β-actin, forward, 5'-TCCTTCCTGGGTAAGTTGTA-3', reverse, 5'-ACTCATC GTACTCCTGCTTG-3'. The PCR conditions were 35 cycles of 30 sec at 95°C, 30 sec at 55°C, and 1.5 min at 72°C with a final elongation at 72°C for 10 min. The PCR products were then electrophoresed in 2% agarose gels, stained with ethidium bromide. The intensities of respective bands were measured by IS-1000 digital imaging system (Alpha Innotech Corp., San Francisco, CA, USA) and normalized to the intensities of β-actin from respective RNA samples.

To confirm the specificity of the PCR reactions, individual bands were cut and the eluted DNA fragments were directly sequenced by ABI autosequence analyzer (Applied Biosystems, Foster City, CA, USA).

四、Results

Morphometric assessment of liver fibrosis-histologic staining

After 4 wk of CCl_4 administration,

bridging fibrous septa appeared in the liver parenchyma as stained by Sirius red (Fig. 2A). These septa were continuous and interweaving between vascular structures. They became thinner and more or less interrupted after recovery (Fig. 2B). Yet, none of the livers showed complete resolution of the bridging fibrous septa in the whole recovery periods.

Quantitative assessment of liver fibrosis-determination of liver collagen content

Assessment of liver fibrosis was also quantitatively determined. The mean value of total collagen content in the livers of untreated control mice was 8.2±0.3μg collagen/mg total protein. At peak fibrosis, the mean value was increased to 14.5±0.8μg collagen/mg total protein. After 10, 20, and 30 days of recovery, the values were decreased respectively to 10.1±0.4, 9.1±0.5, and 10.3±0.3μg collagen/mg total protein. At peak fibrosis, liver collagen content was significantly elevated compared with that of untreated controls (1.8-fold increase, $P < 0.005$). After 10 days of recovery, it decreased dramatically to a level representing 70% resolution of increased collagen fibers in peak fibrosis. The drop of collagen content after 10 days of recovery was statistically significant as compared with that at peak fibrosis ($P< 0.005$). But this level was still significantly higher than that of controls ($P < 0.05$, Fig. 3). There was no difference between the values of the 3 recovery groups. These results were in consistency with the histological studies. They all suggested that the first 10 days were the most critical period for spontaneous regression of liver fibrosis. From Fig. 3, the limitation of spontaneous regression from increased fibrosis in this model was about 70%.

Expression of MMP-2 and MMP-13

Expression of the mRNA of MMP-2 and MMP-13 was determined by RT-PCR. The expression of both genes was elevated to peaks in peak fibrosis, but their sustenance in the recovery stages was not the same (Fig. 4). Compared to untreated controls, MMP-2 expression level was increased to a peak of 2.5-fold in peak fibrosis, and maintained at 2.2-, 1.9-, and 2.1-fold (i.e., 88%, 76%, and 84% of that in peak fibrosis) after 10, 20, and 30 days, respectively, of recovery. For MMP-13, the expression level compared to controls was elevated to a peak of 8.7-fold in peak fibrosis, but quickly declined to 2.4-, 2.6-, and 1.7-fold (i.e., 28%, 30%, and 20% of that in peak fibrosis) in the 3 recovery stages.

五、Discussion

ECM in the body is not an inert component. Instead, it is subjected to "turnover", a dynamic process of balance between deposition and degradation (11). ECM degradation should be an indispensable mechanism for the spontaneous resolution of liver fibrosis. Without the degradation capability, shutdown of the over synthesis and over deposition of ECM may only stop the progression but not induce the resolution of fibrosis. As mentioned above, a number of MMPs are responsible for ECM degradation. Among them, collagenases are the key enzymes capable of cleaving native fibrillar collagen type I and type III. In human, there are 3 members of collagenase, i.e. interstitial collagenase (MMP-1), neutrophil collagenase (MMP-8) and collagenase-3 (MMP-13). In rodent, only one collagenase (MMP-13) has been identified. It has a high degree of functional and sequence homology with human MMP-13 and is distinct from MMP-1 (14).

In the present study, we assessed tissue mRNA expression of MMP-2 and MMP-13 in both the induction and recovery phases of CCl_4-induced liver fibrosis. As shown in Fig. 4, expression of both genes was all significantly up-regulated at peak fibrosis. MMP-2 was persistently elevated during the whole follow-up period of 30 days, while MMP-13 declined rapidly and remarkably after 10 days of recovery and was maintained thereafter at a level slightly higher than untreated controls. These results suggested that collagenases (herein MMP-13) might play an important role in the early stage of resolution of liver fibrosis. While in the later stage, gelatinases (herein MMP-2) might have some role.

A similar expression pattern of MMP-2 during the recovery phase of CCl_4-induced

liver fibrosis has been reported by Takahara et al. (15). They found that elevation of MMP-2 expression was persisted for as long as 42 days after CCl_4 withdrawal. In another report by Iredale et al. (16), they also demonstrated a rapid decline of liver collagen content to a comparable level with untreated controls after 7 days of recovery. But they found that during the recovery phase of 28 days, the expression of MMP-13 was kept as high as in peak fibrosis. We have carried out the experiment twice. But both experiments revealed that MMP-13 expression was rapidly and significantly decreased after 10 days of recovery. Although we used mice while Iredale et al. used rats for experiments, the cause of the discrepancy was unclear.

In the present study, we found the changing pattern of liver collagen content was temporally best correlated with that of MMP-13 expression (Fig. 3 vs. Fig. 4B). It was quite distinct from expression of MMP-2. Previous studies had demonstrated that collagenase activities in the liver was increased in the early stages of liver injury, but decreased when fibrosis became more advanced (17-19). This may explain the clinical observations that early stage cirrhosis may be reversible while advanced cirrhosis is probably irreversible (8) and emphasize on the important role of collagenase activity in the reversibility of liver fibrosis as suggested in the present study.

It is interesting that differential expression patterns between MMP-2 and MMP-13 may represent different mechanisms of their regulation in the induction and resolution of liver fibrosis. What is the scenario they would act in the resolution of liver fibrosis remains unknown. More insights into the mechanisms and limitations for the spontaneous resolution of liver fibrosis may ultimately lead to identification of drugs capable of enhancing or extending the resolution of established liver cirrhosis.

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Fig. 1

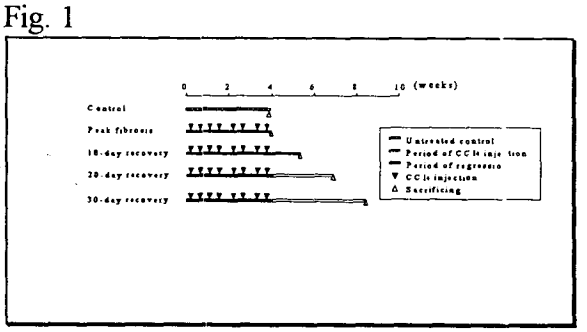

Fig. 2

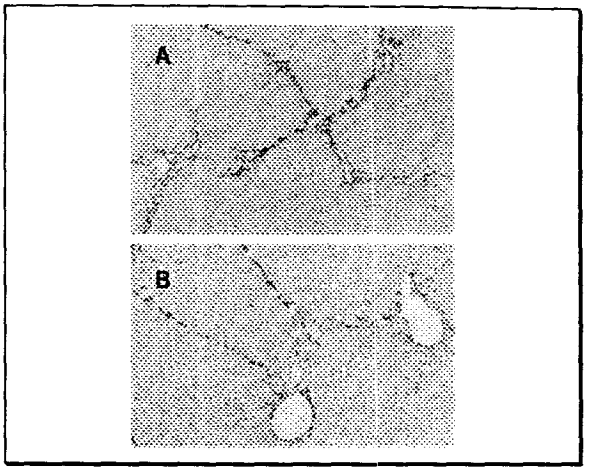

Fig. 3

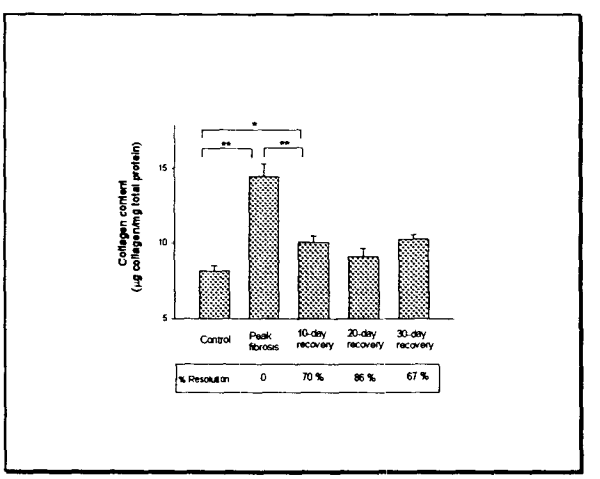

Fig. 4