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• BASIC RESEARCH •

Expression of exogenous rat collagenase *in vitro* and in a rat model of liver fibrosis

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Abstract

AIM: The present study was conducted to test the hypothesis that the introduction of the collagenase gene into tissue culture cells and into a rat model of liver fibrosis would result in the expression of enzymatically active product.

METHODS: FLAG-tagged full-length rat collagenase cDNA was PCR amplified and cloned into a mammalian expression vector. NIH3T3 cells were then transiently transfected with this construct. Expression of exogenous collagenase mRNA was assessed by RT-PCR, and the exogenous collagenase detected by Western blotting using anti-FLAG monoclonal antibody. Enzymatic activity was detected by gelatin zymography. To determine the effects of exogenous collagenase production *in vivo*, the construct was bound to glycosyl-poly-L-lysine and then transduced into rats that had developed liver fibrosis as a result of CCl_4 plus ethanol treatment. The hepatic expression of the construct and its effect on the formation of liver fibrosis were demonstrated using RT-PCR and immunohistochemistry.

RESULTS: It was found that exogenously expressed rat collagenase mRNA could be detected in NIH3T3 cells following transfection. Enzymatically active collagenase could also be detected in the culture medium. The recombinant plasmid was also expressed in rat liver after *in vivo* gene transfer. Expression of exogenous rat collagenase correlated with decreased deposition of collagen types I and III in the livers of rats with experimentally induced liver fibrosis.

CONCLUSION: The expression of active exogenous rat collagenase could be achieved *in vitro* and *in vivo*. It was suggested that *in vivo* expression of active exogenous collagenase may have therapeutic effects on the formation of liver fibrosis.

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INTRODUCTION

Matrix metalloproteinases (MMPs) are a group of proteolytic enzymes that require zinc ions for their activities, they take part in a variety of normal and disease-associated matrix lysis and remodeling events[1-3]. For example, interstitial collagenase, one member of MMPs, also called collagenase, mainly initiates the degradation of collagen types I, II, III, V, VII, and X, which are fibrillar collagens[4-6]. Interstitial collagenase probably contributes importantly to tissue repair, embryogenesis, bone renewal, and to some pathogenic processes such as rheumatoid arthritis, tumor metastasis, and also organ fibrosis[7-10]. Changing the expression or the activities of interstitial collagenase by regulatory factors or by gene transfer may have therapeutic effects on those disease processes.

Liver cirrhosis often shortens life expectancy and decreases the quality of life. In liver cirrhosis, a major pathological feature is the accumulation of extracellular matrix (ECM). Within the major components of ECM, collagen types I and III constitute more than 95 % of the total content of increased collagen in fibrotic liver. The abnormal accumulation of ECM may reflect alterations in the synthesis of matrix proteins, their degradation, or both[11,12]. The reversibility of liver fibrosis/ cirrhosis has been found in patients with various chronic hepatopathy[13, 14] and in experimental cirrhotic animals[15], indicating a therapeutic approach of this disease condition through improving ECM degradation. It has been reported that the mRNA transcription of interstitial collagenase is not up-regulated in experimental hepatic fibrosis[16,17] and the interstitial collagenase proteins in cirrhotic human livers and in serum of chronic hepatitis patients decrease[18,19]. Previous study also shows that the activity of collagenase decreases in advanced liver fibrosis[20-22] and in serum from patients with chronic liver disease[23,24]. Therefore, we proposed that exogenous gene expression of active collagenase might have therapeutic effects on the chronic liver diseases processes. The present experiments were performed to test the hypothesis that the introduction of a collagenase expression vector into tissue culture cells resulted in the enzyme expression, and that similar introduction of exogenous collagenase into a rat model of chemically induced liver fibrosis, altered the development of fibrosis. The *in vitro* results suggest that rat collagenase could be transiently expressed in NIH3T3 cells, and that rat liver fibrosis could be delayed by transducing the collagenase gene *in vivo*.

MATERIALS AND METHODS

Vector construction

A 6 amino acids deleted rat collagenase cDNA containing plasmid (UMR5.4) was kindly provided by Dr John J. Jeffrey[25] and used as starting material. Full-length rat collagenase cDNA was PCR amplified using the primers RIC-UP1 and RIC-DP1 with UMR5.4 as the DNA template (Table 1). The sense primer RIC-UP1 contained the sequences of the deleted 6 amino acids. The PCR fragments were then inserted into the PCR2.1 vector (Invitrogen, Carlsbad, CA, USA) to form PCR2.1-RIC. Using PCR2.1-RIC as template, the cDNA was further PCR amplified with RIC-UP2 and RIC-DP2 as primers (Table 1). The FLAG epitope was added to the C-terminus of the rat collagenase

gene in this construct by using RIC-DP2 that contained the FLAG sequence at the 3' end adjacent to the translation stop codon TAA (Table 1). The PCR reaction was performed as follows: the total reaction volume was 100 μl which contained 1ng DNA template (PCR2.1-RIC), 10 pmol· L^{-1} of each primer of RIC-UP2 and RIC-DP2, 2.5 μmol· L^{-1} dNTP, 10×PCR buffer and 5U Taq polymerase; 35 PCR cycles at 95 ℃ for 1.5 min, 55 ℃ for 1.5 min, and 72 ℃ for 2 min. The 1400 kb PCR product was purified and inserted into mammalian expression vector pTarge™ by the method of TA cloning following the protocol suggested by manufacture (Promega, Madison, Wis, USA) and named as pCMV-RC-F (Figure 1, Map of the plasmid pCMV-RC-F). The pCMV-RC-F was DNA sequenced using T7 primer. The large-scale of plasmid DNA was prepared using Qiagen tip100 (Qiagen, Hilden, Germany). The vector pTarge™ that was self-ligated without any inserted DNA was also isolated and used as control in the transfection study.

Table 1 Primer pairs for PCR and RT-RCR reactions

RIC-UP1: 5'-CATGCATTCAGCTATCCTGGCCACCTTCTTCTTGTTG-3'
RIC-DP1: 5'-CTCCATAGATGAACTCCCC-3'
RIC-UP2: 5'-CCACCATGCATTCAGCTATCCTGG-3'
RIC-DP2: 5'-GTTACTTATCATCGTCGTCCTTGTAGTCACACCAC AATAAGGAATTC-3'
RIC-UP3: 5'-GAATCCAGTCTCTCTATGGTCCAG-3' (785-808)
RIC-DP3: 5'-TCATCGTCGTCCTTGTAGTCACAC-3' (1413-1436)
IC-UP4: 5'-TGGAGCCCTGATGTTTCCCATCTA--3' (693-717)
IC-DP4: 5'-ACCCAAATACTCTTTGGGAGATAA--3' (1423-1446)
β-UP5: 5'-AACCGCGAGAAGATGAACCAGATCATGTTT-3' (2135-2164)
β-DP5: 5'-AGCAGCCGTGGCCATCTCTTGCTCGAAGTC-3' (2456-2485)

( ): binding location of primer in relative cDNA sequence

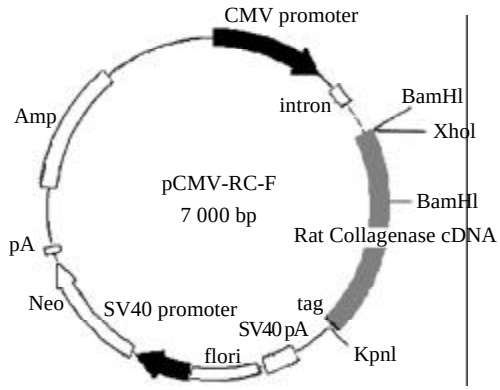

Figure 1 Map of plasmid pCMV-RC-F**.** The FLAG tagged full length rat collagenase cDNA was inserted into a mammalian expression pasmid vector Ptarge™ which carries the human cytomegalovirus (CMV) immediate-early enhancer / promoter region.

In vitro transfection

Exponentially growing NIH 3T3 cells were seeded into 6-well tissue culture dishes at 5×10^5 cells/well and grown overnight to 60 % confluency in culture medium supplemented with 10 % FCS. Each well was then transfected with 5 μg DNA constructs using lipofectamine according to the instruction provided by the manufacturer (Gibico BRL, Eggenstein, Germany). Encapsulated DNA was incubated for 5 h on cells in serum free medium, then in medium containing 10 % bovine serum. After further incubation of the cells for 24 h, the culture medium was discarded and replaced by normal culture medium. Fifty-six h after transfection, the NIH 3T3 cells and their media were harvested for further analysis.

RT-PCR analysis for mRNA expression

The collected cells were washed twice with ice-cold phosphate-buffered saline (PBS), and the mRNA extracted with mRNA Capture Kit (Boehringer Mannheim). The samples were further incubated with biotin-labeled oligo $(dT)_{20}$ working solution for 5 min to hybridize the mRNA with oligo $(dA)_n$. The mixture was added to a streptavidin-coated PCR tube, and incubated for 3 min at 37 ℃ to immobilize the poly $(A)_n$ RNA. Tubes were then washed three times and amplified by RT-PCR. The RT-PCR reaction system contained 0.3 μmol· L^{-1} each of the primer pairs RIC-UP3 and RIC-DP3 (corresponded to nucleotides 785-808 of the collagenase encoding region and the FLAG domain coding region, amplificated a PCR product of 652 bp), 5 mmol· L^{-1} DTT, 6 U RNAsin, 1.5 mmol· L^{-1} $MgCl_2$, 5×RT-PCR buffer and 1 μl Titan™ Enzyme mix provided with the Titan™ RT-PCR System (Boehringer Mannheim). Samples were placed in a thermocycler equilibrated at 50 ℃ for 30 min, at 94 ℃ for 5 min, then for 34 cycles at 94 ℃ for 1 min, 55 ℃ for 2 min, and 72 ℃ for 3 min, with a final extension at 72 ℃ for 10 min. The amplification products were then analyzed on 10 g· L^{-1} agarose gels containing 0.5 mg· L^{-1} ethidium bromide.

Western blot analysis of FLAG-tagged rat collagenase expression

The amount of protein in the collected culture medium of transfected cells was determined with Dye-binding method (Bio-Rad, Hercules, CA, USA). For each samples 30 μg total protein was electrophoresed on sodium dodecyl sulfate-polyacrylamide gel and then transferred to polyvinylidene difluoride (PVDF) membrane (Gelman Inc, Ann Arbor, MI, USA) by electroblotting. Pre-stained rainbrow recombinant protein molecular weight markers (Amersham International plc, Little Chalfont, Buckinghamshire, England) were used for molecular weight determinations. Membranes were blocked with a blocking buffer containing 50 g· L^{-1} non-fat milk powder, 10 mmol· L^{-1} Tris/HCl (pH 7.5), 100 mmol· L^{-1} NaCl and 1 g· L^{-1} Tween 20 and incubated with 1:100 diluted solution of anti-FLAG M_2 McAb[26] (Kodak, New Haven, CT, USA) at 37 ℃ for 1 h. After washing for 30 min the membranes were treated with HRP-conjugated secondary antibody (1:5000) (Bio-Rad) for 1 h at room temperature followed by another 30 min of washings. The ECL Western blotting system (Amersham Life Sciences) was used in accordance to the manufacturer' s instructions for chemiluminescence of proteins, and the blots were then exposed to photographic films (Fuji Photo Film Co., Tokyo, Japan).

Gelatin zymography assay for the enzyme activity

The activity of collagenase was detected by gelatin zymography as previously described[27, 28] with minor modifications. Culture supernatants that contained 60 μg /lane total protein were prepared and mixed with 5× zymogram sample buffer consisting of 0.4 mol· L^{-1} Tris (pH 6.8), 50 g· L^{-1} SDS, 200 g· L^{-1} glycerol, and 0.3 g· L^{-1} bromphenol blue, then electrophoresed with protein molecular weight markers as described before on 100 g· L^{-1} sodium dodecyl sulfate-polyacrylamide gels containing 10 g· L^{-1} gelatin under an ice water cycle. After electrophoresis, the position of molecular weight markers were marked and the gels were rinsed in washing buffer consisting of 50 mmol· L^{-1} Tris, pH 7.6, 1 μmol· L^{-1} $ZnCl_2$, 5 mmol· L^{-1} $CaCl_2$, 250 g· L^{-1} TritonX-100 at 4 ℃ on a rotary shaker. The Triton X-100 solution was decanted and replaced with rinsing

buffer consisting of 50 mmol· L^{-1} Tris (pH 7.6), 1 μmol· L^{-1} $ZnCl_2$, 5 mmol· L^{-1} $CaCl_2$ for further washing. The gel was then incubated with enzyme buffer containing 50 mmol· L^{-1} Tris (pH 7.6), 1 μmol· L^{-1} $ZnCl_2$, 5 mmol· L^{-1} $CaCl_2$, 0.2 g· L^{-1} Brij-35 at 37 ℃ for 42 h. Each gel was stained with 0.5 g· L^{-1} Coomassie blue G-250 in an aqueous solution of 300 g· L^{-1} methanol and 100 g· L^{-1} acetic acid and destained with three changes of 300 g· L^{-1} methanol, 100 g· L^{-1} acetic acid. Areas of digestion were visualized as non-staining regions of the gel and photographed.

Animal model of liver fibrosis

Thirty-six male Wistar rats (Shanghai Experimental Animal Center, Chinese Academy of Sciences) weighing 130±10 g were randomly distributed into four groups, 6 in a normal control group (A), 10 in an experimental liver fibrosis model without plasmid treatment as disease control group (B), and 10 in each of two pCMV-RC-F plasmid transfection as treatment groups (C1 and C2). Animals in the normal control group were treated with olive oil and received food and water *ad libitum*. Rats in the other three groups received a subcutaneous injection of CCl_4 solution (500 g· L^{-1} in olive oil) twice a week for eight weeks at a dose of 0.3 ml per 100 g of body weight after an initial dose of 0.6 ml per 100 g. The only source of fluid for the rats was 100 g· L^{-1} ethanol in water during the entire period of experiment. In addition, animals in the C1 group were intravenous injected with of 100 μg of galactosyl poly-L-lysine (G-PLL, obtained from Dr. Wen Shouming, The Air Force General Hospital of Chinese Peoples Liberation Army, Beijing, China) encapsulated recombinant plasmid through tails vein every two weeks, beginning two weeks after the start of treatment with CCl_4. G-PLL was included to accelerate the targeting effect of the transduced plasmid to the livers[29-31]. Animals in the C2 group received the same treatment as those in group C1 but the plasmid was administrated four weeks after starting the treatment with CCl_4. Animals in the B group received G-PLL encapsulated pTarge™ empty plasmid vector at the same dosage and the same time as those in the C1 group. The molecular ratio of galactose and poly-L-lysine is 15:28 and the average molecular weight of the G-PLL is M_r 8 500.

After weeks 8 treatment, the surviving 6 or 7 rats in each of the C1, C2, B groups and all the 6 rats in the A group were narcotized with 20 g· L^{-1} pentobarbital sodium. The middle lobes of the livers were removed and specimens were fixed in Carnoy' s fixative (glacial acetic acid, chloroform, and ethanol in volume ratio of 1:3:6) and then embedded in paraffin for histological analysis. The remaining tissue was quickly partitioned and immediately frozen and stored under liquid nitrogen for RNA extraction and RT-PCR analysis. Then the animals were humanly killed.

Collagenase mRNA expression in rat liver

Total RNA was isolated from the liver tissues using Trizol (Gibco BRL) according to the manufacturer' s directions. Integrity of RNA was confirmed by visual examination on an ethidium bromide-stained 10 g· L^{-1} Tris-acetate and EDTA agarose gel. Total RNA of 1 mg from each sample was used for reverse transcription and amplification using a RT-PCR kit (Sino-American Biotechnology Co. Shanghai. China). The RT-PCR reaction contained 10 pmol each of the primer pairs RIC-UP3 and RIC-DP3 for detecting the recombinant collagenase mRNA (a product of 652 bp), or each primer pairs of IC-UP4 and IC-DP4 (designed within nucleotides 711-734 of the coding region and nucleotides 1440-1463 of 3' untranslated region of collagenase, Table 1) for amplifying the endogenous collagenase mRNA (a product of 753 bp)[25]. The later primer pairs could not amplify the expression of recombinant collagenase that was lack of the nature 3' untranslated region of collagenase gene. Additionally primer pairs β-UP5 and β-DP5 were included for amplifying the expression of b-actin 335 bp DNA fragment) as internal control[32]. Samples were placed in a thermocycler with the incubation programme of 37 ℃ for 40 min, 94 ℃ for 7 min, then 36 cycles of 94 ℃ for 1 min, 56 ℃ for 1 min, and 72 ℃ for 1 min, with a final extension of 72 ℃ for 10 min. Products of RT-PCR were electrophoresed on a 20 g· L^{-1} agarose gel to show the amplified bands.

Histology and Immunohistochemistry

For immunohistochemical staining, 5 mm sections of each group were treated with anti-FLAG M_2 McAb to assay the expression of recombinant collagenase. After dewaxed with xylene and rehydrated through a graded alcohol series, sections were digested with 4 g· L^{-1} trypsin. Nonspecific binding was blocked with calf serum 1:5 in PBS. The sections were incubated with mouse anti-FLAG M_2 McAb (1:100) overnight at 4 ℃ and then with biotinylated horse anti-mouse IgG (Vector) for 30 min, avidin-peroxidase complex for 30 min, and the substrate solution (3,3-diaminobenzidine tetrahydrochloride in H_2O_2 in Tris buffer, pH 7.4) for 10 min, followed by counterstaining with hematoxylin. The sections were washed with 0.01 mol· L^{-1} PBS (pH 7.4) three times after each step. Finally, ten high power fields of each section were observed under microscope and the positive signals were counted.

Some sections of each group were treated with the monoclonal antibodies against collagen types I and III (1:100), then with secondary antibody and the procedure of the immunostaining were same to the above-mentioned except without counterstaining with hematoxylin. The slides were then analyzed with an Image Analyzing system (TJTY 300 System) to obtain the integral light density (ILD) of stained collagen types I and III.

Morphological examination was performed with Hematoxylin and Eosin staining (H&E) for histological changes of liver fibrosis.

Statistical analysis

The ILD values of collagen types I and III were analyzed by one-way ANOVA. The statistics were calculated by software SPSS 7.0 for Windows. The data are expressed as mean ±S. E. M. Values of $P<0.05$ were considered statistically significant.

RESULTS

In vitro expression and secretion of active FLAG-tagged rat collagenase

After *in vitro* transfection of plasmid in NIH 3T3 cells, the transcription of recombinant collagenase gene was confirmed by RT-PCR amplification of 652 bp cDNA fragment from cells transfected with pCMV-RC-F, but not from cells transfected with control plasmid pTarge™ (Figure 2). This product was of the size expected from mRNA of the tagged collagenase. Further demonstration of transfected gene expression was performed by Western blot analysis using anti-FLAG McAb. The immunoreactive band at about M_r 55 000 was observed in the culture supernatant of cells transfected with pCMV-RC-F but not in that with pTarge™ transfection or in culture medium alone (Figure 3). Collagenase activities were also found around the M_r 55 000 in gelatin zymogram analysis of the culture supernatant of pCMV-RC-F transfected cells and still could be observed after 1:64 dilution. A weak activity of collagenase was also observed in the culture supernatant of pTarge™

transfected cells but was almost undetectable after 1:2 dilution. No enzyme activity of M_r 55 000 collagenase could be detected in culture medium alone (Figure 4).

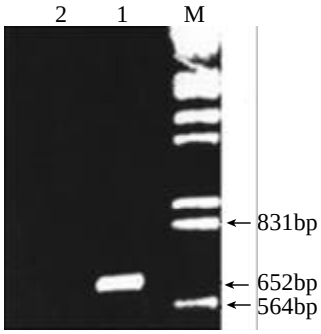

Figure 2 RT-PCR assay of the mRNA expression of recombinant collagenase in NIH 3T3 cells. The PCR-amplified 652 bp fragment of recombinant collagenase cDNA derived from their respective mRNA was found in NIH 3T3 cells which were transfected with pCMV-RC-F (lane 1), while no signal was detected in cells which transfected with control plasmid pTarge™ (lane 2). Lane M is the molecular weight marker (DNA / *Hind* III, *BamH* 1).

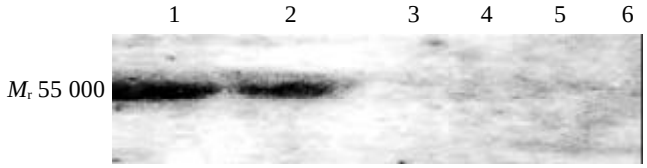

Figure 3 Westernblot analysis of the FLAG-tagged rat collagenase expression using mouse anti-FLAG M_2 McAb. The immunoreactive band with anti-FLAG M_2 McAb at about M_r 55 000 was observed in the culture supernatant of cells transfected with PCMV-RC-F (lane 1) and the supernatant of 1:2 dilution (lane 2). No immunoreactive was found in the supernatant of cells transfected with pTarge™ (lane 3, 4) or in culture medium alone (lane 5, 6).

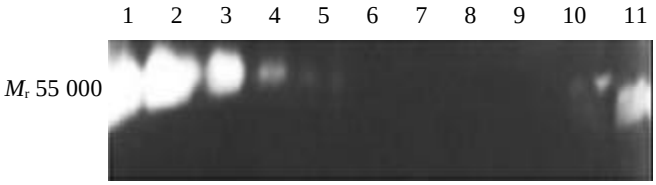

Figure 4 Gelatin zymography analysis for the activity of collagenase. The enhanced gelatin degradation activity was found in the culture supernatant of pCMV-RC-F transfected cells (lane 1-7 represent the zymograph of 1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64 diluted solutions of the supernatant) when compared with pTarge™ transfected cells (lane 10,11 represent the zymograph of 1:2 and 1:1 diluted solutions of the surpernatant). No activity was found at about M_r 55 000 in the culture medium (lane 8, 9).

In vivo expresson of FLAG-tagged rat collagenase in rat livers and its effects on liver fibrosis

The 650bp amplified product was detected in the RT-PCR samples from pCMV-RIC-F plasmid transduced rat groups (C1 and C2 group) but not in the control animal groups (A and B groups) (Figure 5). The 753 bp amplified product by primer IC-UP4 and IC-DP4 which represent the endogenous collagenase gene expression was detected in all samples (Figure 6). In addition to RT-PCR, the expression of exogenous collagenase was observed in C1 and C2 groups by immunostaining with anti-FLAG antibody (Figure 7). Nearly about 30 percent of total cells presented positive signals in C1 and C2 groups, and the distributions of positive signals were found in both the hepatocytes and the perisinusoidal cells.

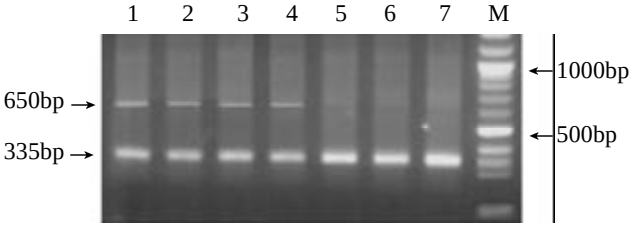

Figure 5 RT-PCR assay for the expression of recombinant interstitial collagenase in rat livers. The 652bp PCR amplified fragment of recombinant collagenase cDNA which was derived from their respective mRNA could be detected in liver samples from pCMV-RIC-F plasmid transduced rat groups C1 (lane 1, 2) and C2 (lane 3, 4) but not in the normal control group A (lane 5, 6) and disease control group B (lane 7). 335 bp fragment was PCR amplified b-actin cDNA. Lane M was 100 bp-1200 bp DNA marker ladders.

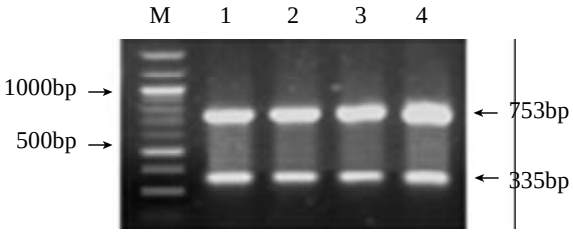

Figure 6 RT-PCR assay for the endogenous expression of collagenase in rat livers. The 753bp PCR-amplified fragment of endogenous collagenase cDNA which was derived from their respective mRNA could be detected in all liver samples from the normal control groups A (lane 1) and disease control group B (lane 4), as well as pCMV-RIC-F plasmid transduced rat groups C1 (lane 3) and C2 (lane 4). 335 bp fragment was PCR amplified b-actin cDNA. Lane M was 100bp-1200bp DNA marker ladders.

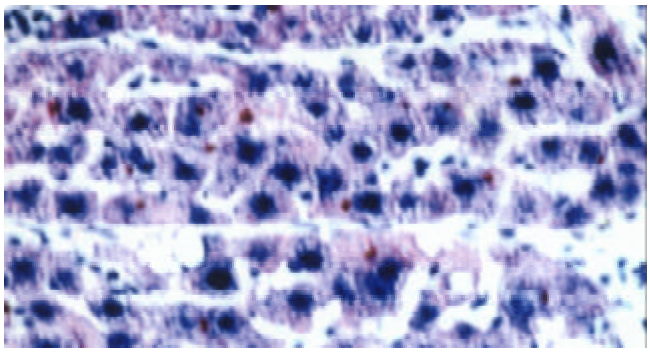

Figure 7 Immunohistochemical analysis of the expression of recombinant plasmid with anti-FLAG antibody in pCMV-RIC-F plasmid transduced group C1 (×400). Nearly about 30 percent of total cells presented positive signals. The distributions of positive signals were found in both the hepatocytes and the perisinusoidal cells.

The accumulation of collagen types I and III in the livers of animals in group B was more severe then that in group C1 and C2 according to the quantitative analysis of the immunohistochemical findings (P<0.05) (Table 2,Figure 8A-D).

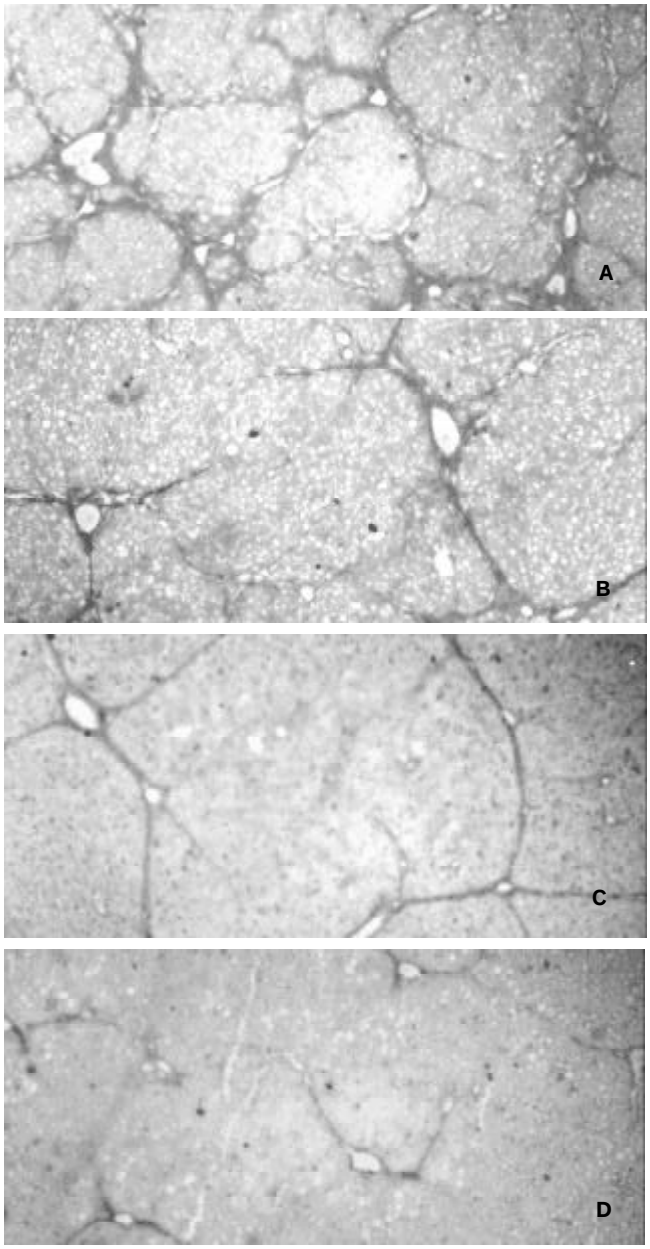

Figure 8 Immunohistochemical analysis of collagen types I (A, B) and III (C, D) in livers (×100). The accumulations of collagen in the disease control group (A, C) were more severe than that in the pCMV-RIC-F plasmid transduced C1 group (B, D).

Table 2 Effects of in vivo transfection of FLAG-tagged rat collagenase on the accumulation of collagen type I and III in experimental liver fibrosis

Group	*n*	ILD of collagen I	ILD of collagen III
A	6	17.35±2.62	18.94±3.78
B	6	86.83±11.93[a]	75.21±10.07[a]
C1	7	69.25±12.31[ab]	62.10±9.72[ab]
C2	7	72.18±14.18[ab]	64.80±11.69[ab]

[a]P<0.05 when compared with normal control group (group A); [b]P<0.05 when compared with disease control group (group B). ILD: Integral Light Density; N: number of rats in each group

DISCUSSION

In the present study the expression of FLAG-fusion rat collagenase was detected in pCMV-RC-F transfected NIH 3T3 cells and the secreted FLAG-fusion collagenase was found in the culture supernatant. This recombinant collagenase also exhibited enhanced gelatin degradation activity. These results suggest that the construction of recombinant collagenase plasmid is successfully function *in vitro* and could be used in further study *in vivo*. NIH 3T3 cell is a cell line of mouse embryonic fibroblasts. The mouse and the rat interstitial collagenase share highly homology when comparison was made between the amino acid sequences of their active forms[33]. Constitutive expression of collagenase gene had been found in NIH 3T3 cells before[34]. In our study, the weak activity of collagenase found in the culture supernatant of pTarge™ transfected cells in our gelatin zymography study might reflect this constitutive expression of mouse collagenase. Similar situation may also exist in liver tissues *in vivo*. Using anti-FLAG McAb we can easily distinct the recombinant rat collagenase that is tagged with FLAG domain from the natural mouse collagenase or the natural rat collagenase.

Collagen types I and III constituted the main components found in the increase of ECM[35, 36]. It has been proposed that the degradation of collagen types I and III is very important in the reversion of liver fibrosis. Theoretically the degradation of collagen could be enhanced if the expression of active collagenase increases. In this study, it was found that the recombinant plasmid could be delivered to liver by G-PLL and could be expressed in the tissue of liver. It was observed immunohistochemically that there were significant decrease of collagen types I and III deposition after transducing the recombinant collagenase plasmid into fibrostic livers. This suggests that the exogenous collagenase gene was able to degrade collagen.

Experimental gene therapies of liver cirrhosis have been tried by other research groups with different gene transfer methods and targeted genes[37-42]. In our study glycosyl-poly-L-lysine was used in the recombinant collagenase gene transfer into the livers since hepatocytes possess receptors that recognize galactose-terminal (asialo-) glycoproteins and thus they are particularly well suited for receptor-mediated methods of gene transfer. In our previous study it was proved that the recombinant plasmid could be delivered to liver and hepatocytes more specifically by G-PLL than lipofectamine[33]. In the present study it was also found that the positive signals of anti-FLAG McAb binding protein were in the hepatocytes of the rats of C1 and C2 groups. The less amounts of perisinusoidal cells which had positive signals as well might be the activated kupffer cells that had the function of phagocytosis. In addition, the previous study also observed the obvious expression of recombinant FLAG-tagged rat collagenase in liver at 24 h after plasmid transfection and persisting for longer than three weeks[31]. No detrimental effects of the transfection and expression of plasmid on important organs of normal rats such as liver, lung, heart and kidney were found by monitoring the serum levels of alanine transaminase, aspartic transaminase and creatinine and by observing the histological manifestation of these organs before and after transfection. These results indicated that the *in vivo* transfection of recombinant collagenase in liver that was mediated by glycosyl-poly-L-lysine was functionally expressed in liver and safe for other tissues.

The regulation of collagenase activity was affected by multiple factors[43, 44]. These include regulation of gene-transcription and protein-biosynthesis by cytokines or other factorsm, transformation of proenzymes into active forms, the

influence of specific or non-specific inhibitors on the activity of activated enzyme. Some studies have shown that the level of tissue inhibitor of metalloproteinase-1 (TIMP-1), which is a specific inhibitor of metalloproteinase, became very high during progressive liver fibrosis[45-49]. However the changes of regulation mechanisms such as the expression of TIMP-1 after collagenase gene transduction are still unknown. Whether the treatment effect on liver fibrosis by transferring an antisense gene of TIMP-1 is better than directly transferring collagenase gene as it was performed in this study worth further investigation.

In summary, the exogenous FLAG-tagged rat collagenase can be transferred by a recombinant plasmid and is expressed functionally in NIH3T3 cells and in a rat liver fibrosis model. As the result demonstrated that *in vivo* transduction of exogenous FLAG-tagged rat collagenase reduced liver fibrosis, this may stimulate the effort for which, the new therapeutic strategies in the management of liver fibrosis through regulation of collagenase activity and/or its inhibitors.

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REFERENCES

1 **Friedman SL**. The cellular basis of hepatic fibrosis: mechanisms and treatment strategies. *N Engl J Med* 1993; **323**: 1828-1835
2 **Matrisian LM**. The matrix-degrading metalloproteinases. *Bioessays* 1992; **14**: 455-463
3 **Ries C**, Petrides PE. Cytokine regulation of matrix metalloproteinase activity and its regulatory dysfunction in disease. *Biol Chem Hoppe Seyler* 1995; **376**: 345-355
4 **Varghese S**, Rydziel S, Jeffrey JJ, Canalis E. Regulation of interstitial collagenase expression and collagen degradation by retinoic acid in bone cells. *Endocrinology* 1994; **134**: 2438-2444
5 **Sires UI**, Schmid TM, Fliszar CJ, Wang ZQ, Gluck SL, Welgus HG. Complete degradation of type X collagen requires the combined action of interstitial collagenase and osteoclast-derived cathepsin-B. *J Clin Invest* 1995; **95**: 2089-2095
6 **Nikkari ST**, OBrien KD, Ferguson M, Hatsukami T, Welgus HG, Alpers CE, Clowes AW. Interstitial collagenase (MMP-1) expression in human carotid atherosclerosis. *Circulation* 1995; **92**: 1393-1398
7 **Arthur MJP**. Collagenases and liver fibrosis. *J Hepatol* 1995; **22** (Suppl. 2): 43-48
8 **Woessner JF**. Matrix metalloproteinases and their inhibitors in connective tissue remodeling. *FASEB J* 1991; **5**: 2145-2154
9 **Sodek J**, Overall CM. Matrix metalloproteinases in periodontal tissue remodelling. *Matrix Suppl* 1992; **1**: 352-362
10 **Okazaki I**, Watanabe T, Hozawa S, Arai M, Maruyama K. Molecular mechanism of the reversibility of hepatic fibrosis: with special reference to the role of matrix metalloproteinases. *J Gastroenterol Hepatol* 2000; **15**(Suppl.): D26-D32
11 **Scott JE**, Bosworth TR, Cribb AM, Gressner AM. The chemical morphology of extracellular matrix in experimental rat liver fibrosis resembles that of normal developing connective tissue. *Virchows Arch* 1994; **424**: 89-98
12 **Kovalszky I**. Fibrogenesis in the liver fibrosis, cirrhosis. *Orv Hetil* 1993; **134**: 59-64
13 **Defour JF**, Delellis R, Kaplan MM. Regression of hepatic fibrosis in hepatic fibrosis in hepatitis C with long-term interferon treatment. *Dig Dis Sci* 1998; **43**: 2573-2576
14 **Dufour JF**, Delellis R, Kaplan MM. Reversibility of hepatic fibrosis in autoimmune hepatitis. *Ann intern Med* 1997; **127**: 981-985
15 **Fort J**, Pilette C, Veal N, Oberti F, Gallois Y, Douay O, Rosenbaum J, Cales P. Effects of long-term administration of interferon alpha in two models of liver fibrosis in rats. *J Hepatol* 1998; **29**: 263-270
16 **Kossakowska AE**, Edwards DR, Lee SS, Urbanski LS, Stabbler AL, Zhang CL, Phillips BW, Zhang YK, Urbanski SL. Altered balance between matrix metalloproteinases and their inhibitors in experimental biliary fibrosis. *Am J Pathol* 1998; **153**: 1895-1902
17 **Watanabe T**, Niioka M, Hozawa S, Kameyama K, Hayashi T, Aria M, Ishikawa A, Maruyama K, Okazaki I. Gene expression of interstitial collagenase in both progressive and recovery phase of rat liver fibrosis induced by carbon tetrachloride. *J Hepatol* 2000; **33**: 224-235
18 **Lichtinghagen R**, Breitenstein K, Arndt B, Kühbacher T, Böker KHW. Comparison of matrix metalloproteinase expression in normal and cirrhotic human liver. *Virchows Arch* 1998; **432**: 153-158
19 **Murawaki Y**, Ikuta Y, Idobe Y, Kawasaki H. Serum matrix metalloproteinase-1 in patients with chronic viral hepatitis. *J Gastrol Hepatol* 1999; **14**: 138-145
20 **Maruyama K**, Feinman L, Fainsilber Z, Nakano M, Okazaki I, Lieber CS. Mammalian collagenase increases in early alcoholic liver disease and decreases with cirrhosis. *Life Sci* 1982; **30**: 1379-1384
21 **Perez-Tamayo R**, Montfort I, Gonzalez E. Collagenolytic activity in experimental cirrhosis of the liver. *Exp Mol Pathol* 1987; **47**: 300-308
22 **Giménez A**, Parés A, Alié S, Camps J, Deulofeu R, Caballería J, Rodés J. Fibrogenic and collagenolytic activity in carbon-tetrachloride-injured rats: beneficial effects of zinc administration. *J Hepatol* 1994; **21**: 292-298
23 **Murawaki Y**, Koda M, Yamada S, Kawasaki H, Shima H, Burkhardt H. Serum collagenase activity in patients with chronic liver disease. *J Hepatol* 1993; **18**: 328-334
24 **Murawaki Y**, Kawasaki H, Burkhardt H. Serum collagenase activity in chronic liver diseases. *Pathol Res Pract* 1994; **190**: 929-933
25 **Quinn CO**, Scott DK, Brinckerhoff CE, Matrisian LM, Jeffrey JJ, Partridge NC. Rat collagenase cloning, amino acid sequence comparison, and parathyroid hormone regulation in osteoblastic cells. *J Biol Chem* 1990; **265**: 22342-22348
26 **Brizzard BL**, Chubet RG, Vizard DL. Immunoaffinity purification of FLAG epitope-tagged bacterial alkaline phosphatase using a novel monoclonal antibody and peptide elution. *Biotechniques* 1994; **16**: 730-735
27 **Kleiner DE**, Stetler-Stevenson WG. Quantitative zymography: Detection of picogram quantities of gelatinases. *Anal Biochem* 1994; **218**: 325-329
28 **Tyagi SC**, Matsubara L, Weber KT. Direct extraction and estimation of collagenase (s) activity by zymography in microquantities of rat myocardium and uterus. *Clin Biochem* 1993; **26**: 191-198
29 **Perales JC**, Ferkol T, Beegen H, Ratnoff OD, Hanson RW. Gene transfer in vivo: sustained expression and regulation of genes introduced into the liver by receptor-targeted uptake. *Proc Natl Acad Sci USA* 1994; **91**: 4086-4090
30 **Nishikawa M**, Takemura S, Takakura Y, Hashida M. Targeted delivery of plasmid DNA to hepatocytes *in vivo*: optimization of the pharmacokinetics of plasmid DNA/galactosylated poly (L-lysine) complexes by controlling

their physicochemical properties. *J Pharmacol Exp Ther* 1998; **287**: 408-415
31 **Yang C**, Wang J, He B, Liu J, Guo J. Glyco-poly-L-lysine is better than liposomal delivery of exogenous genes to rat liver. *World J Gastroenterol* 2000; **6**: 526-531
32 **Nudel U**, Zakut R, Shani M, Neuman S, Levy Z, Yaffe D. The nucleotide sequence of the rat cytoplasmic beta-actin gene. *Nucleic Acids Res* 1983; **11**: 1759-1771
33 **Henriet P**, Rousseau GG, Eeckhout Y. Cloning and sequencing of mouse collagenase cDNA divergence of mouse and rat collagenases from the other mammalian collagenases. *FEBS L* 1992; **310**: 175-178
34 **Aho S**, Rouda S, Kennedy SH, Qin H, Tan EM. Regulation of human interstitial collagenase (matrix metalloproteinase-1) promoter activity by fibroblast growth factor. *Eur J Biochem* 1997; **247**: 503-510
35 **Rojkind M**. Role of metalloproteinases in liver fibrosis. *Alcohol Clin Exp Res* 1999; **23**: 934-939
36 **Arthur MJ**. Degradation of matrix proteins in liver fibrosis. *Pathol Res Pract* 1994; **190**: 825-833
37 **Garcia-Banuelos J**, Siller-Lopez F, Miranda A, Aguilar LK, Aguilar-Cordova E, Armendariz-Borunda J. Cirrhotic rat livers with extensive fibrosis can be safely transduced with clinical-grade adenoviral vectors. Evidence of cirrhosis reversion. *Gene Ther* 2002; **9**: 127-134
38 **Salgado S**, Garcia J, Vera J, Siller F, Bueno M, Miranda A, Segura A, Grijalva G, Segura J, Orozco H, Hernandez-Pando R, Fafutis M, Aguilar LK, Aguilar-Cordova E, Armendariz-Borunda J. Liver cirrhosis is reverted by urokinase-type plasminogen activator gene therapy. *Mol Ther* 2000; **2**: 545-551
39 **Rudolph KL**, Chang S, Millard M, Schreiber-Agus N, DePinho RA. Inhibition of experimental liver cirrhosis in mice by telomerase gene delivery. *Science* 2000; **287**: 1253-1258
40 **Ukei T**, Kaneda Y, Tsutui H, Nakanishi K, Sawa Y, Morishita R, Matsumoto K, Nakamura T, Takahashi H, Okamoto E, Fujimoto J. Hepatocyte growth factor gene therapy of liver cirrhosis in rats. *Nat Med* 1999; **5**: 226-230
41 **Qi Z**, Atsuchi N, Ooshima A, Takeshita A, Ueno H. Blockage of type beta transforming growth factor signaling prevents liver fibrosis and dysfunction in the rat. *Proc Natl Acad Sci USA* 1999; **96**: 2345-2349
42 **Ueno H**, Sakamoto T, Nakamura T, Qi Z, Astuchi N, Takeshita A, Shimizu K, Ohashi H. A soluble transforming growth factor beta receptor expressed in muscle prevents liver fibrogenesis and dysfunction in rats. *Hum Gene Ther* 2000; **11**: 33-42
43 **DeCarlo AA Jr**, Windsor LJ, Bodden MK, Harber GJ, Birkedal Hansen B, Birkedal Hansen H. Activation and novel processing of matrix metalloproteinases by a thiol-proteinase from the oral anaerobe Porphyromonas gingivalis. *J Dent Res* 1997; **76**: 1260-1270
44 **Schwartz JD**, Monea S, Marcus SG, Patel S, Eng K, Galloway AC, Mignatti P, Shamamian P. Soluble factor(s) released from neutrophils activates endothelial cell matrix metalloproteinase-2. *J Surg Res* 1998; **76**: 79-85
45 **Benyon RC**, Iredale JP, Goddard S, Winwood PJ, Arthur MJ. Expression of tissue inhibitor of metalloproteinase 1 and 2 is increased in fibrotic human liver. *Gastroenterology* 1996; **110**: 821-831
46 **Murawaki Y**, Ikuta Y, Idobe Y, Kitamura Y, Kawasaki H. Tissue inhibitor of metalloproteinase-1 in the liver of patients with chronic liver disease. *J Hepatol* 1997; **26**: 1213-1219
47 **Dudas J**, Kovalszky I, Gallai M, Nagy JO, Schaff Z, Knittel T, Mehde M, Neubauer K, Szalay F, Ramadori G. Expression of decorin, transforming factor –beta 1, tissue inhibitor metalloproteinase 1 and 2, and type IV collagenases in chronic hepatitis. *Am J Clin Pathol* 2001; **115**: 725-735
48 **Lichtinghagen R**, Michels D, Haberkorn CI, Arndt B, Bahr M, Flemming P, Manns MP, Boeker KH. Matrix metalloproteinase (MMP)-2, MMP-7, and tissue inhibitor of metalloproteinase-1 are closely related to the fibroproliferative process in the liver during chronic hepatitis C. *J Hepatol* 2001; **34**: 239-247
49 **Boeker KH**, Haberkorn CI, Michels D, Flemming P, Manns MP, Lichtinghagen R. Diagnostic potential of circulating TIMP-1 and MMP-2 as markers of liver fibrosis in patients with chronic hepatitis C. *Clin Chim Acta* 2002; **316**: 71-81

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