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Enhanced Interstitial Collagenase (Matrix Metalloproteinase-13) Production of Kupffer Cell by Gadolinium Chloride Prevents Pig Serum-Induced Rat Liver Fibrosis

Koji Hironaka,*,† Isao Sakaida,*,[1] Yasuhiro Matsumura,* Seiji Kaino,* Koji Miyamoto,† and Kiwamu Okita*

**First Department of Internal Medicine and †Department of Bioregulatory Function, School of Medicine, Yamaguchi University, Ube, Yamaguchi Prefecture 755-8505, Japan*

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Hepatic fibrosis results from an imbalance between fibrogenesis and fibrolysis in the liver. It remains uninvestigated whether Kupffer cells produce matrix metalloproteinase-13 (MMP-13), which mainly hydrolyzes extracellular matrix (ECM). We sought to determine the role of Kupffer cells in fibrogenesis/fibrolysis. *In vivo,* we used the rat model of pig serum-induced liver fibrosis. A subset was treated with gadolinium chloride ($GdCl_3$), which specifically acts on Kupffer cells. Administration of $GdCl_3$ remarkably decreased the hydroxyproline content of the liver and increased the expression of MMP-13 mRNA in the liver without a difference in procollagen type I and tissue inhibitors of metalloproteinase-1 (TIMP-1) mRNA expression on Northern blot analysis with the elimination of ED2-positive cells. *In vitro,* addition of $GdCl_3$ to isolated Kupffer cells showed increased type I collagen-degrading activity in a dose-dependent manner as well as MMP-13 mRNA expression on Northern blot analysis. It is concluded that Kupffer cells are a major source of MMP-13 and modulation of Kupffer cells by $GdCl_3$ prevents liver fibrosis with increased expression of MMP-13 mRNA and protein, whereas procollagen type I and TIMP-1 mRNA, which encode two major effectors of fibrogenesis, were unchanged. This is the first report showing that Kupffer cells produce interstitial collagenase (MMP-13) resulting in the reduction of ECM. This discovery may provide new insights into therapy for hepatic fibrosis. © 2000 Academic Press

Key Words: **matrix metalloproteinase-13; Kupffer cell; liver fibrosis; matrix degradation; gadolinium chloride.**

The development of treatment against hepatic fibrosis as part of therapy for chronic liver diseases has been awaited with keen interest. Hepatic fibrosis results from an imbalance between fibrogenesis and fibrolysis in the liver, and much work has been done concerning the extracellular matrix (ECM) synthesis and the main ECM-producing cells in the liver, i.e., the hepatic stellate cells (HSCs) (1).

Recently there has been much interest in ECM-splitting enzymes, so importance is attached to matrix metalloproteinases (MMPs) that play a major role in the hydrolysis of ECM (2).

The activity of MMPs is increased in the early stage of fibrosis, but is decreased in the stage of liver cirrhosis (3, 4). Interstitial collagenase is thought to act as a key enzyme in fibrolysis. However, it has been unknown whether Kupffer cells, which are considered to be deeply involved in HSCs activation via various cytokines, growth factors, and other soluble mediators, can produce interstitial collagenase (5).

In this study the effect on hepatic fibrosis of gadolinium chloride ($GdCl_3$) (6, 7), which specifically acts on Kupffer cells, was determined in the rat model of pig serum-induced liver fibrosis without necrosis or inflammation (8, 9). This study clearly demonstrated that Kupffer cells are a major source of the interstitial collagenase (MMP-13 or collagenase-3) resulting in the prevention of liver fibrosis and that they play an important role in fibrolysis. This evidence suggests a new concept of therapy for hepatic fibrosis.

MATERIALS AND METHODS

In Vivo

Animals. Male Wistar rats, 6 weeks of age and weighting 140 to 150 g (Nippon SLC Co., Ltd., Shizuoka, Japan), were obtained, quarantined for 1 week, and housed in a room under controlled temperature (25°C), humidity, and lighting (12 h light, 12 h dark). Access to food and tap water was *ad libitum* throughout the study

Abbreviations used: ECM, extracellular matrix; MMPs, matrix metalloproteinases; TIMPs, tissue inhibitors of metalloproteinases; HSC, hepatic stellate cell; KC, Kupffer cell; HBSS, Hanks' balanced salt solution; APMA, 4-aminophenyl mercuric acetate; G3PDH, glyceraldehyde-3-phosphate dehydrogenase.

[1] To whom correspondence should be addressed. Fax: 81-836-22-2240. E-mail: sakaida@po.cc.yamaguchi-u.ac.jp.

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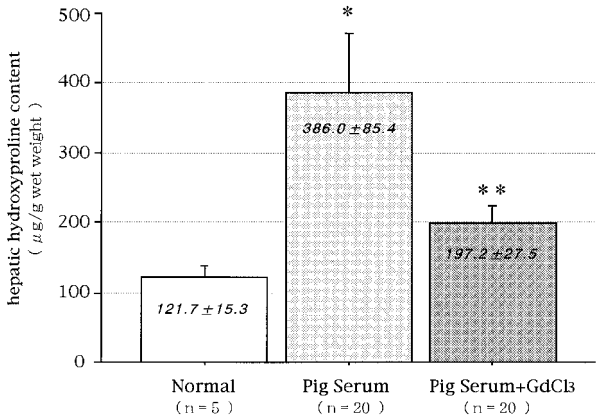

FIG. 1. Effect of $GdCl_3$ on hepatic hydroxyproline content. Liver collagen content (measured as hydroxyproline content in liver) in rats received pig serum alone for 8 weeks and in rats received pig serum with $GdCl_3$ (mean ± SD). *$P < .0001$ versus normal rats. **$P < .0001$ versus rats received pig serum alone.

period. After a 1-week acclimation period on a basal diet (Oriental MF Diet; Oriental Yeast Company, Japan), the rats were divided into experimental groups.

Experimental protocols. Animals ($n = 45$) were divided into the following 3 groups. The experiment was started on Monday (Day 1) and the total study period was 8 weeks. The 3 experimental groups for assessing the effect of $GdCl_3$ on hydroxyproline content comprised 20 or 5 rats each. Two groups of 20 rats received 0.5 ml of pig serum (Cosmo Bio Co., Ltd., Tokyo) twice weekly [every Monday (Days 1, 8, 15, . . .) and Thursday (Days 4, 11, 18, . . .)] by intraperitoneal (i.p.) injection for 8 weeks. Gadolinium chloride hexahydrate ($GdCl_36H_20$; Wako Pure Chemical Industries, Osaka, Japan) in sterile isotonic saline was administered intravenously (i.v.) through the tail vein at a dose of 10 mg/kg body weight (bw), corresponding to 7 mg $GdCl_3$/kg bw, twice weekly without anesthesia. This method was a modification of that of Husztik *et al.* (6). The dose of $GdCl_3$ was set at 7 mg/kg bw because $GdCl_3$-induced effects on liver enzyme activity were not observed using only 7 mg/kg bw and this dose was reported to reduce Kupffer cells phagocytosis by 75% of normal (10). The remaining rats were injected with identical volumes of normal saline i.v. twice weekly.

GdCl3(−) group ($n = 20$): [pig serum 0.5 ml i.p. + saline i.v.] twice weekly

GdCl3(+) group ($n = 20$): [pig serum 0.5 ml i.p. + GdCl3 (10 mg/kg) i.v.] twice weekly

Control group ($n = 5$): [no treatment]

At the end of the study, all rats were killed under ether anesthesia. Blood was obtained from the bifurcation of the abdominal aorta, and the liver was excised. The livers were immediately frozen for hydroxyproline measurements or fixed in 10% formalin for 24 h and embedded in paraffin for picro-sirius red and ED1/ED2 staining.

Histology and immunohistochemical examination. Sections of 5-mm thickness from the right lobe of all rat livers were routinely fixed in 10% formalin and embedded in paraffin. Then 4-μm-thick sections were stained with hematoxylin-eosin-saffran and 0.1% picro-sirius red solution (11). ED1/ED2 for the detection of Kupffer cells was immunohistochemically assessed by the avidin-biotin-peroxidase complex method as previously described (12, 13). ED1/ED2 monoclonal antibody (Serotec, Ltd., Oxford, UK) was used. After counterstainig with hematoxylin, sections were microscopically examined and the number of ED1/ED2 positively stained cells was counted. Data were determined on a random evaluation (24 fields per specimen) and expressed as the number of ED1/ED2 positive cells per 0.125 square millimeter.

Imaging analysis. The percentage area of sirius red positive fiber was determined as previously reported (14). Briefly, we assessed the mean values of sirius red positive area in 6 ocular fields per specimen, which were randomly selected at 40× magnification using an image analysis system (Personal Image Analysis System LA-555, Pias, Ltd., Osaka, Japan) and sirius red positive fiber was expressed as a percentage of the total area of the specimen.

Hydroxyproline content. Hydroxyproline content was determined by the modified as previously reported (13). The hydroxyproline content of the liver was expressed as micrograms per gram of wet weight.

Hybridization probes. The following probes were used in this study. G3PDH (glyceraldehyde-3-phosphate dehydrogenase) (14) was purchased from American Type Culture Collection (Rockville, MD). Type I procollagen alpha 2 (15) and TIMP-1 cDNAs were used as described (16). MMP-13 cDNA was the generous gift of Dr. Cheryl O. Quinn (Pediatric Research Institute, Department of Pediatrics, Health Sciences Center, Saint Louis University, MO) (17).

Northern blot analysis. Northern blot analysis was performed after the isolation of 6 μg poly(A)$^+$ RNA for each sample from whole liver (15) or 7 μg of total RNA from isolated Kupffer cell described previously (14). Each signal strength of mRNA was determined after normalization by relevant G3PDH mRNA levels.

In Vitro

Kupffer cell isolation. Kupffer cells were isolated from normal rat liver (male Wistar rats 150 to 200 g) using a modified collagenase perfusion technique, as described previously using elutriation rotor (18). Cell viability was always over 95%, as determined by the trypan blue exclusion test. Cell purity was more than 90%, as determined by nonspecific esterase staining and the ability to ingest latex beads of 4.58 μm in diameter (Polysciences Inc., Warrington, PA).

Preparation of conditioned media. Freshly isolated Kupffer cells were suspended in serum-free RPMI 1640 (Nissui Pharm. Co. Ltd., Tokyo, Japan) on uncoated 60-mm plastic dishes (Iwaki Glass Co., Ltd., Tokyo, Japan). All cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. After 30 min of incubation Kupffer cells were cultured for 12 h in serum-free RPMI 1640 containing varying concentrations of $GdCl_3$ (0, 1, 10, 100, 500, 1000 μg/ml). The supernatants and Kupffer cells were collected, and 0.5% Triton X was added to induce cell lysis. The Kupffer cells were centrifuged for 15 min at 10,000*g* at 4°C to remove cellular debris.

Quantitative analysis of type I collagen-degrading activity. Conditioned media were preincubated in 1 nmol/l of 4-aminophenyl mercuric acetate (APMA) (Sigma Chemical Co., Poole, UK) at 37°C for 4 h to measure type I collagenase activity using a type I collagenase activity assay kit (YU-16001, Yagai Co., Yamagata, Japan) (19). The principle of type I collagenase activity assay kit is based on fluorescent measurement of collagen fragmants upon cleavage by interstitial collagenase. It is known that collagen decomposition fragments differ from whole collagen in their temperature of denaturation and ethanol solubility. Upon interstitial collagenase cleavage of fluorescently labeled collagen type I, decomposition fragments are produced. These fragments are selectively denatured and extracted with ethanol. Fluorescence intesity of the extracted product is measured and correlates with type I collagen-degrading activity.

Statistical analysis. Results are presented as the mean ± SD. Differences between groups were analyzed by one-way analysis of variance (ANOVA).

Ethical considerations. This experiment was reviewed by the Committee of Animal Experiment Ethics in Yamaguchi University School of Medicine and was carried out under the Guidelines for Animal Experiment in Yamaguchi University School of Medicine (No. 105) and Notification (No. 6) of the Japanese government.

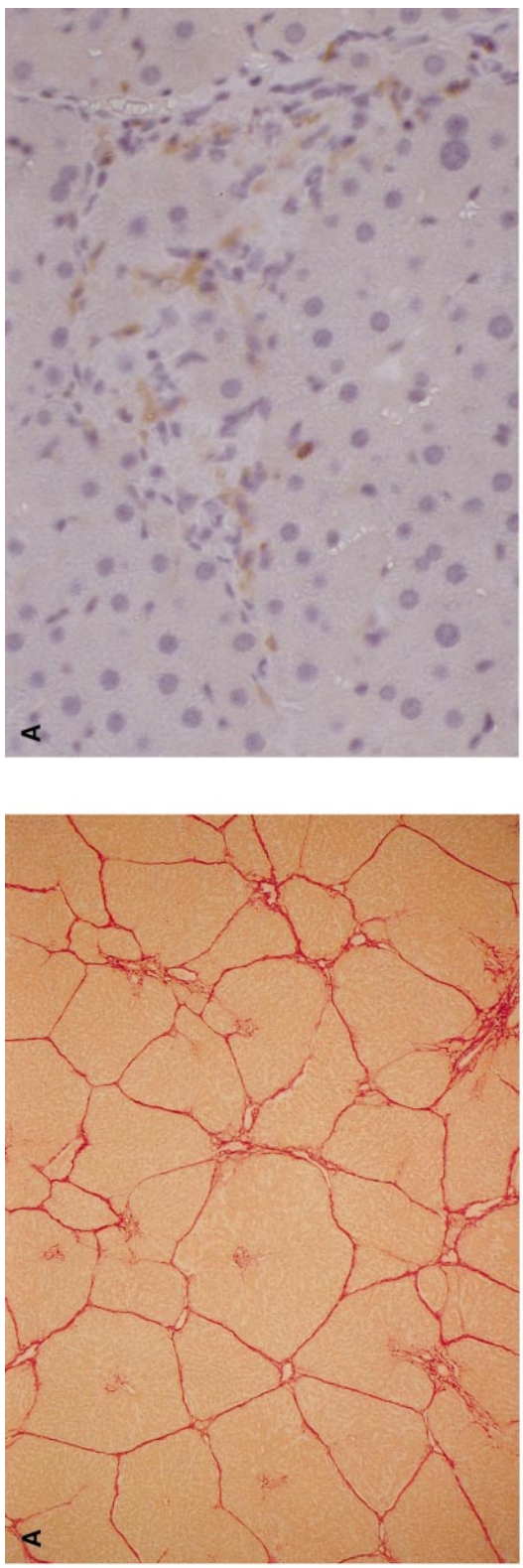

FIG. 2. Photomicrograph of a liver section stained with picro-sirius red staining from a rat that received pig serum alone for 8 weeks (A) and from a rat that received pig serum with $GdCl_3$ (B). Magnification 40×.

FIG. 3. Photomicrograph of a liver section stained with anti-ED2 antibody from a rat that received pig serum alone for 8 weeks (A) and from a rat that received pig serum with $GdCl_3$ (B). Magnification 400×.

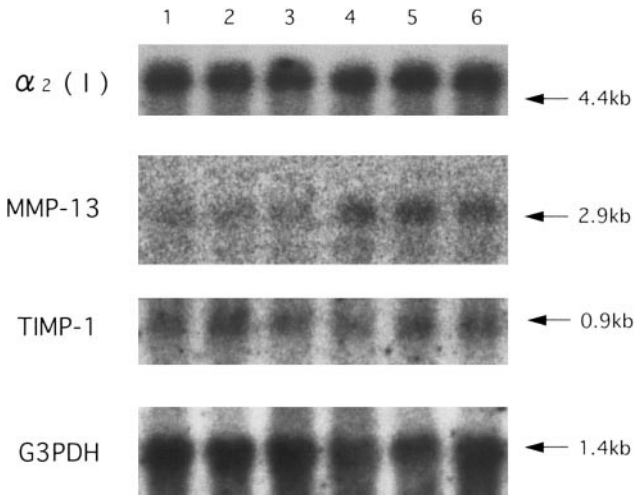

FIG. 4. mRNA expression of α2 (I) procollagen, MMP-13, TIMP-1, and G3PDH in the liver of rats that received pig serum alone for 8 weeks (lanes 1–3) or pig serum with $GdCl_3$ (lanes 4–6). The figure shows a representative example of 3 independent Northern blots.

RESULTS

In Vivo

The content of hydroxyproline in the liver tissue was increased in the pig serum group, compared to the control group ($P < 0.01$) (Fig. 1). The sirius red positive area was also significantly increased to 4.46 ± 2.50% ($P < 0.01$) in the pig serum group (Fig. 2A), compared to the control group (0.25 ± 0.11%). Kupffer cells were found clustering around the fibrous septum (Fig. 3A), consistent with the view that Kupffer cells are concerned with fibrogenesis. When the Kupffer cells were counted after ED2 staining, the cell count was significantly higher for the pig serum group than for the $GdCl_3$ group (14.4 ± 6.8/0.125 mm^2 vs 3.9 ± 1.6/0.125 mm^2, $P < 0.01$) (Fig. 3). However, there was no difference in ED1-positive cell count between the pig serum and the $GdCl_3$ groups (46.3 ± 4.1/0.125 mm^2 vs 43.1 ± 5.4/0.125 mm^2, $P = 0.302$). The liver tissue content of hydroxyproline was significantly decreased after $GdCl_3$ administration ($P < 0.01$) (Fig. 1), and the sirius red positive area was narrowed by 0.77 ± 0.59% ($P < 0.01$) (Fig. 2B). These changes confirmed the inhibitory effect of $GdCl_3$ on hepatic fibrosis.

The levels of MMP-13 mRNA identified as interstitial collagenases (23, 27), TIMP-1 as inhibitor of MMP-13, and procollagen type I were compared in the whole liver between the pig serum and the $GdCl_3$ groups. Whereas there was no difference in the expression of procollagen type I mRNA or TIMP-1 mRNA, MMP-13 mRNA expression was significantly increased in the $GdCl_3$ group (Fig. 4).

These results suggests that $GdCl_3$ inhibits hepatic fibrosis not through collagen synthesis inhibition but through a relative increase in collagen decomposition.

In Vitro

$GdCl_3$ was administered to isolated rat Kupffer cells to evaluate its effect on interstitial collagenase production. Type I collagen-degrading activity by isolated Kupffer cells with $GdCl_3$ clearly increased dose-dependently and their activity reached a peak at the concentration of 500 μg/ml of $GdCl_3$ (Fig. 5).

Also MMP-13 mRNA expression was increased after the administration of 500 μg/ml $GdCl_3$ (Fig. 6) in accordance with increased collagenase activity.

DISCUSSION

Pig serum-induced rat hepatic fibrosis is a model that shows an intense immune response to the administration of heterologous serum, and the Kupffer cell is assumed to play an important role in fibrogenesis. Histologically, the changes are characterized by mononuclear cell infiltration and fibrotic response in the periportal area, followed by the septum formation connecting portal tract with central veins without hepatocytes injury (9, 21).

ED1 and ED2 stains are widely used in the identification of macrophages including hepatic resident macrophages (Kupffer cells) (29). There was no difference in ED1-positive cell count between the $GdCl_3$ and the pig serum groups, but the number of ED2-positive cells was significantly decreased in the $GdCl_3$ group (Fig. 3). This may indicate that repopulation of hepatic macrophages other than Kupffer cells is caused by an influx of blood monocytes, known to be positive for ED1 and negative for ED2 (23), and regeneration of Kupffer cells, known to be negative for ED1 and positive for ED2 (22), was eliminated by repeated administration of $GdCl_3$. The reason the frequency of administration was determined to be twice weekly is that this selectively eliminates Kupffer cells from the liver for 2–3

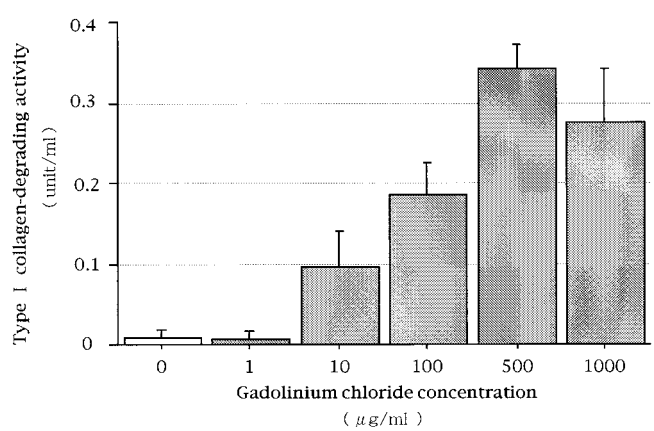

FIG. 5. Effect of $GdCl_3$ on interstitial collagenase production by isolated Kupffer cells. Shown are means ± SD of 5 experiments each performed in duplicates. 1 unit = 1 μg type I collagen degradation per 1 min.

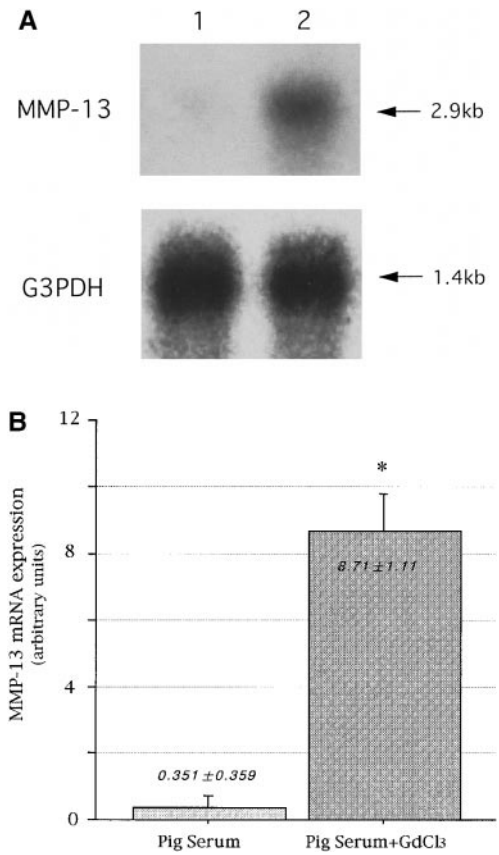

FIG. 6. (A) mRNA expression of MMP-13 and G3PDH in isolated Kupffer cells with $GdCl_3$ (500 μg /ml) treatment (lane 2) and control (without $GdCl_3$) (lane 1). The figure shows a representative example of 5 independent Northern blots. (B) Graphic representation of MMP-13 mRNA expression in isolated Kupffer cells with $GdCl_3$ (500 μg /ml) treatment (lane 2) and control (without $GdCl_3$) (lane 1) shown in (A). The results of densitometric analysis after normalization against hybridization signals for G3PDH (A) are shown (mean ± SD). $*P < 0.0001$ compared with the group of rats treated only with pig serum.

days (24) and that the liver begins to be repopulated by immature macrophages (influx of blood monocytes or regeneration of residual Kupffer cells) 3–4 days after $GdCl_3$ injection (25).

Several mechanisms may account for the $GdCl_3$-induced Kupffer cells elimination. $GdCl_3$ is soluble at low pH, but possibly turns turbid near physiologic pH, and it is likely that colloidal aggregates are formed in the bloodstream after an intravenous injection. These aggregates, probably complexed with serum proteins, are taken up from the circulation by Kupffer cells. Once internalized by Kupffer cells, because the colloidal gadolinium aggregates dissolve again below pH 6.0, gadolinium ions originating from the complex at the acid pH of the endosomal–lysosomal compartment of the cell will attach to components of this compartment (26). Recycling of endosomes to the plasma membrane may gradually change this membrane, finally resulting in cellular disintegration (25).

The present study was conducted with the hypothesis that $GdCl_3$ may prevent pig serum-induced liver fibrosis by the elimination of Kupffer cells involved in the activation of stellate cells leading to fibrogenesis. However, the mechanism of prevention of fibrosis was different from what expected, i.e., perhaps the activation of fibrolysis through MMP-13 of Kupffer cells shown *in vivo* and *in vitro* studies.

$GdCl_3$ exhibits an inhibitory effect, including phagocytosis, on the Kupffer cell. The increase in interstitial collagenase production observed after the administration of $GdCl_3$ may be explained by the following reports. The serum levels of *N*-acetyl-β-glucosaminidase and *N*-acetyl-β-galactosaminidase (lysosomal enzymes), markers for the Kupffer cell, increase after the administration of $GdCl_3$ (27). The Kupffer cell causes the labilization of lysosomal membranes so that the liver tissue levels of cathepsin B (main cysteine proteinase of macrophages) increases (28). In the present study the Kupffer cells were ultimately eliminated by phagocytosis of $GdCl_3$ aggregates, but $GdCl_3$ possibly induced activation of Kupffer cells with cytokine release (29) and an increase in carbohydrate metabolism (30).

The presence of TIMPs is important in the determination of MMPs activity (2), because TIMPs regulate MMPs activity (32). Our study indicates no difference of TIMP-1 mRNA expression in pig serum-treated livers with or without $GdCl_3$ administration. Thus at least TIMP-1 does not seem to be involved in the prevention of fibrosis by $GdCl_3$.

The cell that produces interstitial collagenase in the liver has not been determined yet. There are reports that transient expression of interstitial collagenase (MMP-1 in humans and MMP-13 in rats) is observed in HSCs activated by the primary culture on plastic (33) and that MMP-13mRNA is increased after the administration of TNF-α (34). These reports suggest the possibility that HCS also produces interstitial collagenase as do MMP-2 (34) and stromelysin (35).

On the other hand, it is also conceivable that the Kupffer cell, the same system as the macrophage and monocyte, produces interstitial collagenase (36). As a matter of fact, it has been suggested that the Kupffer cell does so (37), but this must be interpreted cautiously as these earlier studies used cell preparation methods that would have resulted in mixed sinusoidal liver cell cultures (38).

The present study is the first to document that the Kupffer cell certainly exhibits the ability to produce MMP-13 when treated with $GdCl_3$. It has also been shown that the Kupffer cell inhibits hepatic fibrosis by releasing the interstitial collagenase or MMP-13 without the effect on type I collagen and TIMP-1 mRNA expression in the liver.

In summary, the present study suggests that the regulation of interstitial collagenase produced by the

Kupffer cell may provide a clue to new therapy for hepatic fibrosis.

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