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Research paper

Modulation of extracellular matrix proteins and hepatate stellate cell activation following gadolinium chloride induced Kuffer cell blockade in an experimental model of liver fibrosis/cirrhosis

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ABSTRACT

Background: Hepatic fibrosis results from an imbalance between fibrogenesis and fibrolysis in the liver. The process of Ito cell activation, which is thought to be the central pathogenic mechanism in liver fibrogenesis/cirrhosis, may involve distinct interactions with Kupffer cells (KCs) mediated by various cytokines and growth factors. The aim of this study was to determine whether targeting KC function using $GdCl_3$, which specifically acts on Kupffer cells, interferes with the manifastation of carbon tetrachloride (CCl_4) and dimethylnitrosamine (DMN) induced hepatic fibrosis/cirrhosis.

Methods: We assesed the change in distribution of ECM proteins, laminin and fibronectin and the marker of HSC activation, alpha-smooth muscle actin (α-SMA) after liver injury and after KC inactivator, $GdCl_3$-treatment with light microscope immunohistochemistry. Using light microscopy, characteristic changes of fibrosis/cirrhosis were seen in the hepatotoxin-administrated groups.

Results: The immunohistochemical profile of anti-laminin was significantly altered in hepototoxin-treated groups ($p < 0.05$) and $GdCl_3$ blocked this effect. The immunoproducts of anti-fibronectin and anti-SMA antibody were not significantly altered in the CCl_4-treated group. In contrast, after DMN-induced flbrosis/cirrhosis, laminin, fibronectin and α-SMA staining were significantly increased ($p < 0.05$) and were present along the sinusoids in cirrhotic liver tissue. However, those ECM proteins and α-SMA staining in the parenchyma and fibrotic nodules decreased but not significantly after $GdCl_3$ treatment.

Conclusions: The results suggest that $GdCl_3$ suppressed the activation of lipocytes and their transition from hepatic lipocytes to myofibroblast-like cells in cirrhotic livers in CCl_4-treated mice. These results support that treatment with the selective Kupffer cell toxicant $GdCl_3$ prevents stellate cell activation and prevents liver fibrosis/cirrhosis

Keywords: immunohistochemistry, laminin, fibronectin, alpha-smooth muscle actin, cirrhosis

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BACKGROUND

Hepatic fibrosis is now regarded as a common response to chronic liver injury; regardless of its nature (viral infections, alcohol abuse, and metal overload). It is also characterized by excessive deposition of extracellular matrix (ECM) components.[1] The ECM is a dynamic complex of macromolecules that includes collagens, glycoproteins, and proteoglycans such as laminin and fibronectin; recently it has been shown that it does not only support the tissue structure but also plays a major role in cell adhesion, proliferation, and differentiation.[2]

Remodelling of the ECM may be the signal that facilitates lobular reorganization during liver regeneration after a liver injury. Much work has been done concerning the ECM synthesis and protein contents.

The quiescent hepatic stellate cells (HSCs) synthesize low levels of matrix proteins, but as a result of injury, HSCs proliferate and transform to myofibroblast-like phenotype, a process termed activation.[1] This process, which is ought to be the central pathogenic mechanism in liver fibrogenesis/cirrhosis, may involve distinct interactions with Kupffer cells (KCs) mediated various cytokines and growth factors. These cells are involved in the deposition of basement membrane proteins,[1] thus playing a pivotal role in sinusoidal capillarization.[3] Activated HSCs expressing smooth muscle actin (α-SMA) is known to be the major source of collagens and other matrix proteins that are deposited in fibrosis.[4] Accumulation of matrix, therefore, occurs as a consequence of both an increase in the numbers of HSCs, in addition to their increased synthesis and secretion of matrix proteins when in the activated phenotype.[5] Resolution of liver fibrosis could be associated with a reversal of activated HSCs to a quiescent phenotype, or a decrease in ECM protein contents.

In chronic liver disease tissue, excessive deposition of ECM is observed.[6] It has been suggested that hepatocytes,[6–11] HSCs,[1,10,11] endothelial cells[12] or proliferating myofibroblast-like cells[13] are responsible for the production of ECM. Particularly, the synthesis of ECM proteins takes place mainly in HSC and their differentiated offspring, the myofibroblasts[13] content contribute to the hepatic liver diseases.[14] As fibronectin has multiple binding sides, it has a wide variety of biological functions involving the interaction of cells with extracellular substances. In tissues, fibronectin is found in ECM where it served as a cell adhesion protein modulating cell binding to the extracellular tissue matrix.[15] Laminin is also known as a major basement membrane protein which possesses several biological activities.[16] In normal liver, although small amounts of focally-deposited fibronectin has been reported, continuous basement membranes and laminin are not observed along the sinusoids.[17]

It is reported that activated KCs could contribute to the development of the hepatic injury as they could release cytotoxic mediators outside the cell that cause hepatocyte damage.[18] Adachi et al.[19] demonstrated that destruction of Kupffer cells with gadolinium chloride ($GdCl_3$) during chronic exposure to alcohol blocked early alcoholic liver injury. The aim of this study was to determine whether targeting KC function using $GdCl_3$, which specifically acts on Kupffer cells[19] interferes with the manifestation of carbon tefrachloride (CCl_4)- and dimethylnitrosamine (DMN)-induced hepatic fibrosis/cirrhosis. $GdCl_3$ exhibits an inhibitory effect, including phagocytosis, on the Kupffer cells.[20] Therefore, we assessed the change in distribution of ECM proteins, laminin and fibronectin and the marker of HSC activation, α-SMA after liver injury and after KC inactivator, $GdCl_3$-treatment.

METHODS

Materials

All materials used in the immunohistochemical staining of paraffin sections were as follows: mouse monoclonal antibody to 1aminin, fibronectin and α-SMA from Neomarkers, CA; DAB chromogen, ultra Ab diluent, ultra V block, biotinylated goat anti-rabbit, and streptavidin peroxidase from Lab Vision, USA; protease, citrate buffer, and EDTA; formaldehyde 37% and 3-aminopropyl-triethoxysilane from Sigma Chemical Co., St. Louis, MO. CCl_4 and DMN were purchased from Sigma Chemical Co., St. Louis, MO.

Animals and treatments

65 adult male BALB/c mice weighed from 23 to 32 g were studied. The mice were housed, three to five per cage and maintained under constant room temperature (25°C) and provided with free access to standard chow and tap water under a 12 h light (08.00–20.00) and 12 h darkness (20.00–08.00).

All experiments were started between 11 AM and 12 AM and were conducted according to institutionally approved protocols.

Five groups of mice were studied with CCl_4, DMN, $GdCl_3$-treatment after CCl_4, $GdCl_3$-treatment after DMN, and vehicles. All animals were killed 24 h after treatment with hepatotoxins, $GdCl_3$ and vehicles.

CCl_4-treatment; mice were treated by intraperitoneal injection of 0.20 ml of a mixture of CCl_4 with mineral oil (v/v) 3 times per week for 7 weeks, increasing the concentration of CCl_4 progressively until the fourth week of treatment.[20] The percentages of CCl_4 in mineral oil (v/v) were as follows: Week 1, 13%; Week 2, 16%; Week 3, 20%; and Week 4 to 7, 25%.

DMN treatment; DMN was administrated (1% in saline, 1 microliter/100 g body weight, intraperitoneally) on three consecutive days a week for 4 weeks.[21]

$GdCl_3$ treatment; As fibrosis/cirrhosis was maintained, it is stopped to give those hepatotoxins and they received $GdCl_3$ (7 mg/kg bw intraperitoneally) dissolved in saline for two weeks, or equal volume of sterile saline (1 ml/kg bw) as a control for two times weekly to examine the involvement of Kupffer cells.[22]

At intervals of 1, 3, and 7 weeks after CCl_4 administration, 1 and 4 weeks after DMN administration, and two weeks after $GdCl_3$, five of mice were killed following a light anesthesia with either by decapitation and exsanguinated from carotid arteries 24 h after the last administrations. Control animals were also prepared in batches of five mice each, killed at the same time points, and evaluated postmortem in an identical manner.

Histological Examination

Light microscopic examination was performed in formalin fixed and paraffin embedded specimens of the liver tissue, cut into 5-μm sections, and stained with H&E for morphological evaluation and Mallory's trichrome, reticulin silver and Gomori's stains for evaluation of fibrosis. To avoid potential differences in the response of the various lobes, the same lobe was always harvested for each type of specimen. Histological evaluation was performed by an independent pathologist (in a blinded fashion) using liver preparations from all the animals in our experimental groups.

Immunuhistochemical Analysis

The livers were rapidly removed and then about 0.5 g of liver was cut from each mouse for immunohistochemical examination of laminin, fibronectin and α-SMA. The liver specimens were fixed in neutral formalin, embedded in paraffin and sectioned. To avoid potential differences in the response of the various lobes, the same lobe was always harvested for each type of specimen, including those from the control animals. The indirect immunoperoxidase method was used.[23] Tissues were deparaffinized with a graded xyhelen series at 37°C for 50 min. After the removal of endogenous peroxidase, the sections were washed with phosphate buffer saline (PBS) and incubated at room temperature, for 60 min with primary antibodies, i.e. rabbit anti-human laminin antibody, rabbit anti-human fibronectin antibody, anti-SMA antibody, and non-immunized rabbit serum for the control (all antibodies were diluted 100 times with PBS containing 1% bovine albumin. After being washed with PBS, the specimen was incubated with the second antibody, a 100-fold dilution of peroxidase-conjugated goat anti-rabbit IgG, at room temperature for 50 min. After further washes with 0.005% H_2O_2-3,3′-diaminobenzidine at room temperature for 90s, then washed with PBS. After being stained with hematoxylin, the specimens were embedded in glycerinated gelatin and observed under a light microscope. All slides were evaluated at random by one of the authors. The trend of immunostaining was classified semi-quantitatively by means of a four-degree score[24];

- (−), no staining − 0
- weakly, trace staining covering $<$5% of hepatic lobules − 1
- (+), trace staining covering %5−20 of hepatic lobules − 2
- (++), moderate staining covering %20−50 of hepatic lobules − 3
- (+++), strong staining covering $>$%50 of hepatic lobules − 4.

Control experiments were performed using non-immune rabbit IgG or phosphate buffered saline as a substitute for each primary antibody. Control tissues, which considered of the omission of the primary antibody or the substitution of the primary antibody by normal mouse IgG, were invariably negative.

Ethical Considerations

This experiment was reviewed by the committee of the Ethics on Animal Experiment in Marmara Univercity School of Medicine and carried out under the control of the Guideline for Animal Experiment in Marmara Univercity School of Medicine and The Law (No: 12.2000.mar) of the Government.

Statistical Methods

Group means of the test groups were evaluated for statistically significant differences with (Statistical Package for Social Sciences) for Windows 7.0 the Mann-Whitney U test (significant levels of 5%, 10% and 0.1%). ECM protein concentrations were given as median, because of the limited number of animals. Wilcoxon signed rank test was used with two related variables to test the hypothesis that the two variables have the same distribution.

RESULTS

Histology of progressive fibrotic liver injury. Cirrhosis/fibrosis for each model was confirmed by macroscopic and histological analysis before using liver tissue samples for experiments. In spite of the heterogeneity in the hepatotoxin-induced cirrhotic models examined, the results described here were unchanged as long as the degree of fibrosis was nearly the same. Liver morphology showed fibrosis in all groups, but to a different extent. Livers from animals that were not treated with hepatotoxins showed occasional single cell necrosis and slight increase in connective tissue around the portal tracts (data not shown).

CCl_4-induced liver cirrhosis. Histological analyses of the livers harvested at first week after CCl_4 demonstrated perivenular ballooning degeneration of hepatocytes; there was an increase in the inflammatory infiltrate in the necrotic areas. By 3 weeks there was evidence of early fibrosis with increased numbers of cells with a fibroblast-like morphology (data not shown) in each section. After 3 weeks, the connective tissue was increased and newly produced thin fibers were found by Masson's trichrome along some sinusoids or small blood vessels (including capillarized sinusoids). Trichrome staining confirmed the increased collagen content of the strome (Figure 1). Postmortem macroscopical examination after 7 weeks of CCl_4 treatment showed coarse parenchymal nodules on the surface of the livers of mice (Figure 2). By week 7, accumulated connective tissues formed bridging fibrosis between central and portal area dividing the hepatic tissue into pseudolobules of different sizes. Morphological

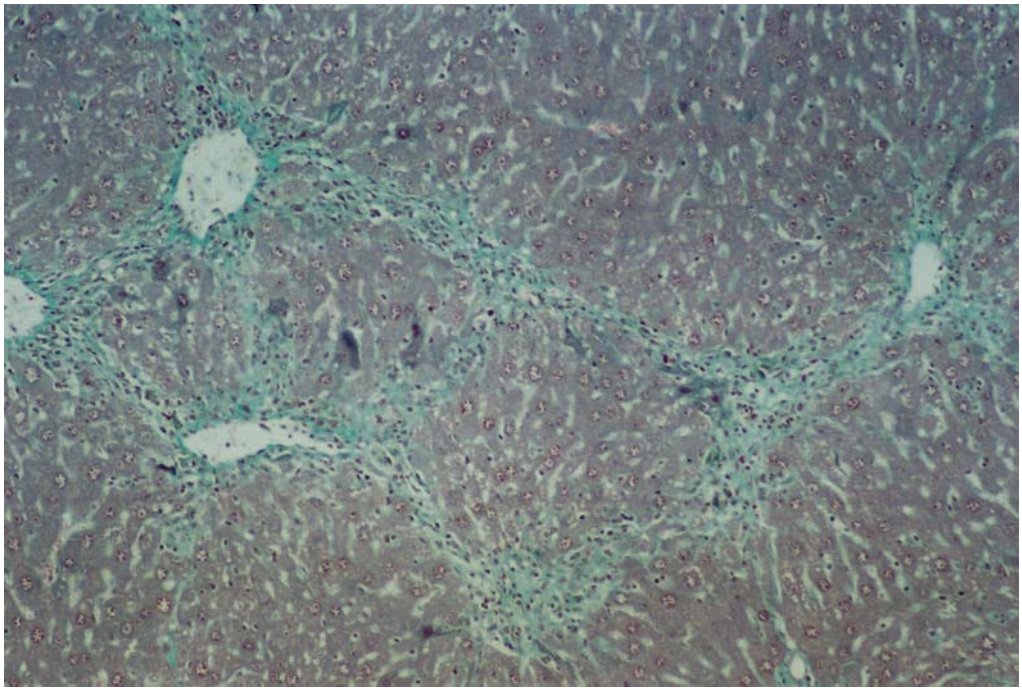

Figure 1. Porto-portal bridging necrosis and diffuse mixed inflammation in the portal area with a fibroblast-like morphology in each section, by week 3, after CCl_4 administration (H&E × 100).

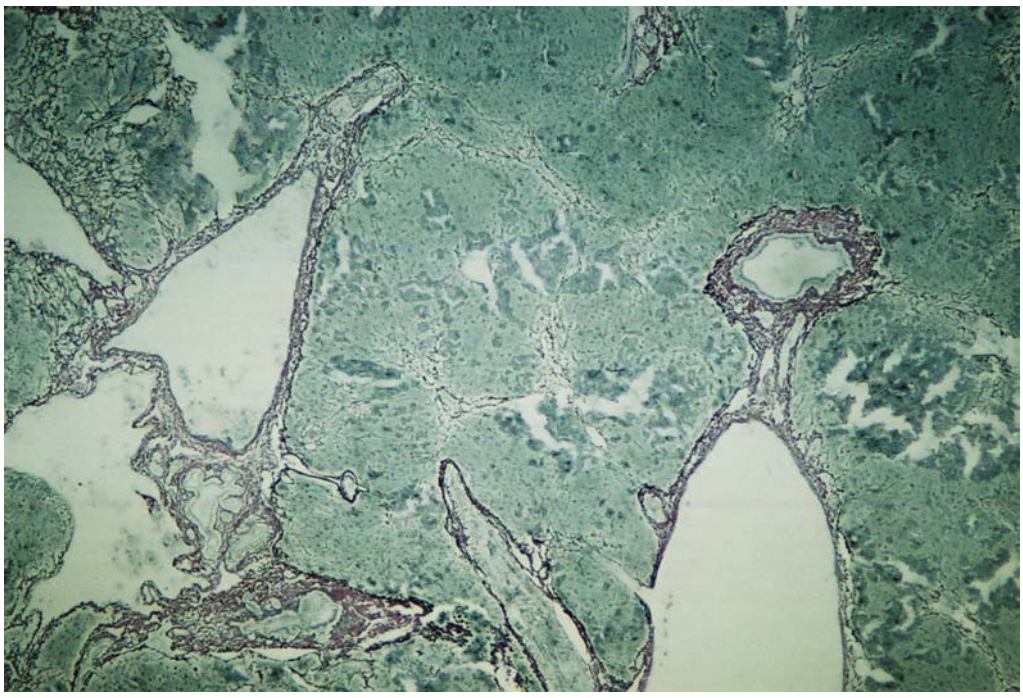

Figure 2. By week 7, after CCl_4 administration, accumulated connective tissues formed bridging fibrosis between central and portal areas, dividing the hepatic tissue into pseudolobules of different sizes (Gommori reticulin × 100).

changes produced by chronic treatment of mice with CCl_4 are shown in Figure 2 with extensive distribution of the parenchymal organization. Microscopic examination of control livers showed a preserved lobular architecture with liver cell plates of normal thickness. By contrast, all treated animals exhibited micronodular cirrhosis with complete septal fibrosis surrounding regenerative hepatocyte nodules.

DMN-induced liver cirrhosis. The livers of mice treated with DMN showed a progressive derangement of the lobular architecture with the formation of slender and later broad centro-central fibrous septa in the livers of all experimental animals. After 1 week, connective tissues accumulated in the Glisson's sheath, reticulin fibers spread radially throughout the liver, and pseudo-lobules formed after the last administrations. By the fourth week of treatment, nodular transformation with both narrow and broad bands of fibrous stroma was present in virtually all animals. Fibrosis have been usually extensive and remained present until the completion of experiments, at which times the animals frequently show ascites. A prominent liver cirrhosis was present at week 4.

Histology of $GdCl_3$ treated groups after hepatotoxin administrations. We sought to determine the role of gadolinium chloride ($GdCl_3$), which specific acts on Kupffer cells in hepatic fibrosis/cirhosis. Histological analyses confirmed that the mature collagen fibers bridging vascular structures present after hepatotoxin treatments became remodeled during $GdCl_3$ treatment period.

Immunohistochemical examinations. In spite of the different hepatotoxin treatments, the results described here were unchanged as long as the degree of fibrosis was nearly the same. In normal liver; reaction staining for fibronectin was seen along hepatic sinusoids (+++). Hepatocytes, central veins, Kupffer cells and portal tractus showed weak staining (++). In contrast, laminin showed only a fine filamentous deposition along hepatic sinusoids (−). Laminin thinly lined the sinusoidal walls but did not appear to intercalate among clusters of proliferating hepatocytes. In portal triads, basement membranes around portal vessels and bile-duct systems revealed a strong reaction for laminin (++), but very weak for fibronectin. The fibrous stroma in this area showed a positive reaction for fibronectin in the first weak and diffusely spread pattern.

Staining of control liver tissue with those monoclonal antibodies yielded faint, but specific, reaction with portal triads. The immunohistochemical profile of anti-laminin was significantly altered in hepototoxin-treated groups ($p < 0.05$) (Table 1). The immunoproducts of anti-fibronectin and anti-SMA antibody were not significantly altered in CCl_4-treated group. In contrast, after DMN-induced

Table 1. The immunostaining of laminin, fibronectin and α-SMA after CCl_4 administration, and the effect of $GdCl_3$ treatment on the stains.

Week	Protein	Median (n = 5)
1. week	Laminin	2.1667*
	Fibronectin	3.4032
	α-SMA	3.0000
3. week	Laminin	1.8333$^{\alpha}$
	Fibronectin	3.1667
	α-SMA	2.8333
7. week	Laminin	3.5000
	Fibronectin	3.4815
	α-SMA	3.0000
After $GdCl_3$	Laminin	3.1667$^{\beta x}$
	Fibronectin	3.1667
	α-SMA	3.1667

*$p < 0.05$ 1. wk vs 7. wk
$^{\alpha}$ $p < 0.05$ 3. wk vs 7. wk
$^{\beta}$ $p < 0.05$ 1.wk vs after $GdCl_3$ treatment
^{x} $p < 0.05$ 3. wk vs after $GdCl_3$ treatment

fibrosis/cirrhosis, those proteins were significantly increased ($p < 0.05$) and were present along the sinusoids in cirrhotic liver tissue (Table 2). Of the ECM components studied, laminin was the one demonstrating the most dramatic changes.

Table 2. The immunostaining of laminin, fibronectin and α-SMA after DMN administration, and the effect of $GdCl_3$ treatment on the stains.

Week	Protein	Median (n = 5)
Basal	Laminin	1.6667
	Fibronectin	3.0000
	α-SMA	2.0000
1. week	La Laminin	1.8333*
	Fibronectin	3.3333$^{\delta}$
	α-SMA	2.3333$^{\bullet}$
4. week	Laminin	3.8333
	Fibronectin	4.0000
	α-SMA	3.6667
After $GdCl_3$	Laminin	3.3333$^{\beta}$
	Fibronectin	3.6667
	α-SMA	2.8333

*$p < 0.05$ 1. wk vs 4. wk
$^{\delta}$ $p < 0.05$ 1. wk vs 4. wk
$^{\bullet}$ $p < 0.05$ 1. wk vs 4. wk
$^{\beta}$ $p < 0.05$ 1. wk vs after $GdCl_3$

After CCl_4,-induced cirrhosis, staining for laminin was especially pronounced in the fibrotic areas around the central veins. By week 1 in DMN-treated group, laminin appeared in the hepatic sinusoids reaching a maximum staining intensity at 4 weeks. Intracellular laminin was prominent in numerous nonparenchymal cells, with many having the morphology, location, and desmin content characteristic of HSCs. Laminin was also abundant on basement membranes of proliferating bile ducts or ductules, but faint in the blood vessels in fibrosing portal triads. Treatment with $GdCl_3$ attenuated the increased staining of laminin at week 7 after CCl_4, and at week 4 after DMN-induced cirrhosis, but it was not significant.

Fibronectin was demonstrated in portal interstitium and in a sinusoidal pattern in the early stages of hepatotoxins-induced liver disease. In late stages of fibrosis, this extracellular matrix protein was found in developing fibrous septa. In fibrotic liver; fibronectin was detectable and abundantly deposited in necrotic areas and newly fibrosing areas. Although it was not significant, fibronectin staining was decreased in CCl_4-induced cirrhosis at week 7 below the healthy group. Fibronectin staining in the parenchyma and fibrotic nodules decreased, but not significantly after $GdCl_3$ treatment.

Sections of liver stained with H&E at week 7 after CCl_4 treatment demonstrated large numbers of nonparenchymal cells with a morphology consistent with activated myofibroblast-like HSCs clustered

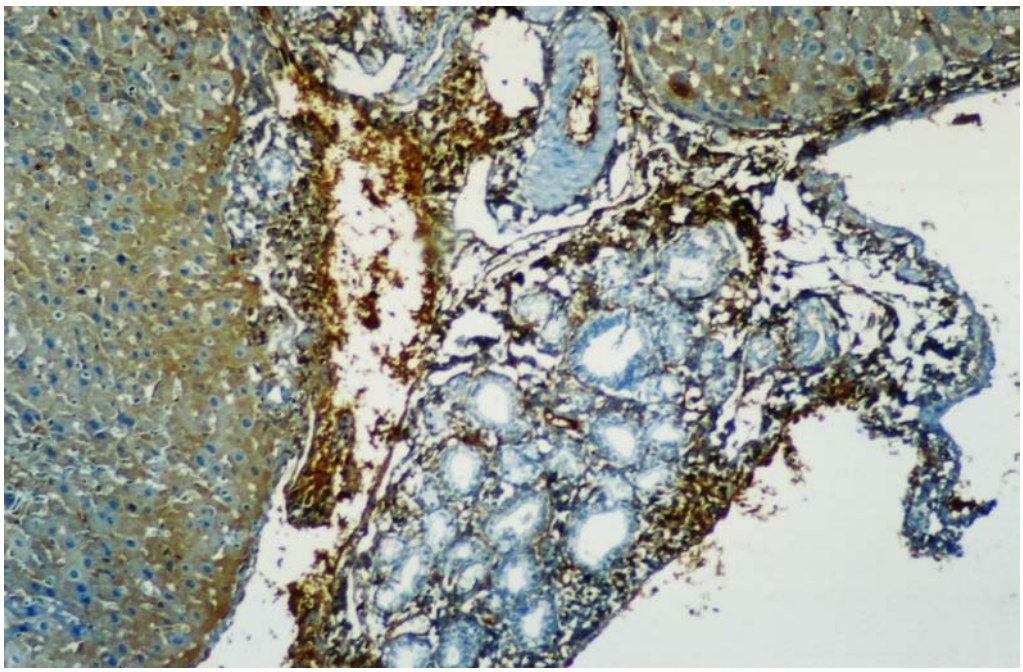

Figure 3. After hepatotoxin-induced cirrhosis was formed, staining for laminin was especially pronounced in the fibrotic areas around the central veins, in the hepatic sinusoids and on the basement membranes of proliferating bile ducts or ductules. Staining for laminin was faint in the blood vessels in fibrosing portal triads (anti-laminin × 200).

around the fibrotic septa. Kupffer cells were clustered around the fibrous septum (Figure 3), consistent with the view that Kupffer cells are concerned with fibrogenesis. Because activation of HSC is associated with expression of α-SMA (in the majority of cells),[25] immunostaining for this protein was used to detect and quantify the activated HSC in each specimen of liver during liver fibrosis/cirrhosis and after $GdCl_3$ treatments. In normal (untreated) liver, only very few α-SMA-reactive cells can be seen within the parenchyma. The staining of α-SMA was more prominent in the livers of mice treated with hepatotoxins. In cirrhotic group, numerous α-SMA-reactive cells were present along the sinusoidal profiles and in the space of Disse. There were significantly higher numbers α-SMA-positive cells around central veins in the livers of mice treated with DMN at 4 weeks ($p < 0.05$). The increased staining of α-SMA after DMN-induced cirrhosis was decreased after $GdCl_3$, but it was not significant.

DISCUSSION

The structure and function of hepatic ECM have been gradually clarified by recent investigations. Changes in composition, distribution, and arrangement of hepatic ECM components and their regulatory systems are of interest in research into hepatic fibrogenesis. Numerous studies have dealt with the alterations of the ECM following chronic liver injury. A close relationship between liver injury and the hepatic ECM was suggested by reports of studies of chronic liver injury in rats induced with CCl_4.[26] Regarding the hepatic ECM in mouse experimental fibrotic/cirrhotic model, few studies of its changes after Kupffer cell inactivation have been described.[26]

We used the mouse model of DMN and CCl_4-induced hepatic injury. 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 hepatocyte injury.[27] CCl_4–induced liver damage is a well characterized experimental model of fibrosis and cirrhosis.[28] Fibrin deposition and endothelial cell damage are found in massive hepatic necrosis in rats given CCl_4.[29] In the present study, CCl_4 caused a severe hepatic damage ended with cirrhosis, as confirmed by the histological examination. The changes of hepatic ECM in rat liver after CCl_4 administration have been studied biochemically and immunohistochemically.[30] Actin mRNA was increased after acute and chronic treatments of CCl_4, which is associated with the increase of fibronectin.[31] After chronic exposure to the DMN, an extensive fibrosis was observed and a prominent liver cirrhosis was present at week 4. The fibrin deposition was mostly seen in the hepatic sinusoids as reported.[32]

Kupffer cell is assumed to play an important role in fibrogenesis. In recent years, $GdCl_3$, a rare earth metal, has frequently been used to study the role and function of Kupffer cells under physiological and pathological conditions.[33] Although the precise mechanism of $GdCl_3$ action on Kupffer cells is not known, this compound is frequently used in studies of Kupffer cell's role in the regulation of Kupffer cell repopulation of the liver. This study was performed to elucidate the consequences of $GdCl_3$-induced Kupffer cell blockade for extracellular matrix proteins and HSC integrity. The Kupffer cell inhibitor, gadolinium chloride protects the liver from a number of toxicants that require biotransformation to elicit toxicity such as CCl_4 through reducing total microsomal P450 activity.[34] The pretreatment of rats with substances previously shown to affect hepatic reticuloendothelial system function (gadolinium, zymosan, and sheep erythrocytes), found that it selectively modulated the clearence and/or killing of radioactive E. Coli from the liver.[35] Kupffer cells participated in the mechanism of toxicity of CCl_4 in vivo, possibly by the release of chemoattractants for neutrophils.[36] The inhibition of Kupffer cells with $GdCl_3$ prevented both lipid peroxidation and CCl_4-induced liver injury.[37] $GdCl_3$ is shown to destroy exclusively activated Kupffer cells, by damaging the plasma membrane.[38] It was suggested that such macrophages are responsible for endothelial cell destruction in the hepatic sinusoids. Its action has a biphasic pattern; during an initial phase, it inhibits phagocytosis by Kupffer cells, whereas during a later phase it induces destruction of large Kupffer cells.[38] The reason the frequency of administration of $GdCl_3$ was determined to be twice weekly is that this selectively eliminates Kupffer cells from the liver for 2-3 days[31] 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.[31]

Hepatic stellate cells are a major source of ECM in normal and pathological conditions.[1,39] During fibrogenesis, HSCs undergo a process of activation, developing a myofibroblast-like phenotype associated with increased proliferation[40] and collagen synthesis.[41] As pathways leading to intralobular hepatic fibrosis and sinusoidal capillarization are mediated by ECM-producing cells physically situated in the space of Disse, we evaluated α-SMA reactive-cells located in the perisinusoidal space.

In CCl_4-induced cirrhosis, an increase in lipocytes is prominent and they are α-SMA positive.[42] In DMN – induced liver fibrosis/cirrhosis, most of the cells around the fibrotic area were α-SMA-positive, indicating DMN-induced fibrosis is associated with an acceleration of the activation of lipocytes.[42] In the present study, we examined the expression of α-SMA in mice by immunohistochemistry and confirmed previous reports.[43] Data consistently showed that there were numerous α-SMA-positive cells around central veins in the livers of mice treated with hepatotoxins and the increase of α-SMA-positive is hepatotoxin-associated with hepatotoxin-induced liver fibrosis. $GdCl_3$ attenuated the α-SMA-positive cells in CCl_4-treated mice. The results suggested that $GdCl_3$ suppressed the activation of lipocytes and their transition from hepatic lipocytes to myofibroblast-like cells in cirrhotic livers in CCl_4-treated mice. $GdCl_3$ induced hepatocte proliferation and this action of $GdCl_3$ suggested to modify the development of CCl_4-induced liver injury.[44] Modulation of Kupffer cells with $GdCl_3$ prevented pig-serum induced liver fibrosis with increased expression of interstitial collagenase.[26]

The loss of activated HSCs is not itself sufficient to allow a remodelling of the existing excess collagens. For this to occur, matrix degradation must be upregulated. It has been reported that Kupffer cells, which are considered deeply involved in HSCs activation via various cytokines, growth factors, and other soluble mediators could produce interstial collagenase resulting in the reduction of ECM.[26]

While excessive collagen deposition in cirrhotic livers has been known for several decades, only recently, it has been appreciated that other ECM proteins, such as laminin and fibronectin, are also deposited in large amounts in cirrhotic livers and suspected to actually contribute to the disease process.[39]

In normal hepatic lobules, fibronectin and small amounts of laminin was present in the space of Disse.[45] The small amounts of laminin in the space of Disse may account for the lack of basal membrane, since the increased amount of laminin in the space may cause the basal lamina visible in fibrotic human liver as described previously by Schaffner & Popper.[50] This change is called sinusoidal capillerization. Laminin might act as an organizer, and is increased in the space of Disse when intrahepatic fibrogenesis proceeds. The HSCs and endothelial cells are the major sites of production of laminin in normal liver, while hepatocyte may express laminin during the fibrotic process.[46] Our results agree with the demonstration of the presence of laminin in HSCs of fibrotic liver following CCl_4 injury.[23,47] Laminin was observed in the basement membranes of portal veins, hepatic arteries and bile ducts in the portal tracts. However, it was faint in the portal tracts that shıowed marked inflammatory cell infiltration or the areas of focal necrosis. From these findings, laminin is considered to be involved to a lesser degree than the other components of the ECM in fibrogenesis associated with necrotic and inflammatory reactions in the early phase of hepatic injury. CCl_4 treatment increased laminin staining

and $GdCl_3$ blocked this effect. DMN-induced fibrosis/cirrhosis showed a marked increased staining of laminin between the first and the fourth weeks. However, $GdCl_3$ treatment could not effect the staining of this protein in DMN-treated group. Although the high median values of week 4 were decreased after $GdCl_3$-treatment, it was not significant statistically. The administration period of $GdCl_3$ might not be enough to get the exact effect in DMN-induced liver injury group.

Fibronectin is one of the most abundant ECM proteins in the interstitial space of Disse in normal liver.[17] It is distributed in all extracellular hepatic compartments including the sinusoidal walls and basement membranes.[16] It plays an important role as a mediator of subsequent deposition of collagen. Among the earliest detectable changes in the ECM of the injured liver is an increase in fibronectin.[17] Also, it is one of the first major matrix proteins to accumulate during hepatic fibrosis and cirrhosis, during which is speculated to act as a template-organizer for other matrix molecules.[39,48] Martines-Hernandez et al.[49] reported that hepatic cirrhosis is associated with enhanced deposition of several matrix proteins, most notably fibronectin. Failure of the liver to remove ECM-bound fibronectin may allow its excessive accumulation in the hepatic ECM.

Fibronectin also induces taxis of fibroblasts to the injury site and increases adhesion of each component of the ECM. Fibronectin quickly accumulates at the injury site and appears to play an important role in wound healing. In our study fibronectin was found around inflammatory cells over the entire areas of focal necrosis that showed marked inflammatory cell infiltration. Taken together, these findings suggest that fibronectin appears early in the process of fibrogenesis associated with tissue repair and the production of fibronectin by endothelial cells in fibrosing injury is independent to the type of hepatotoxins and likely to be broadly relevant to the repair of the response in wound healing. CCl_4- and DMN-induced fibrosis/cirrhosis showed a marked increased staining of fibronectin whereas treatment with $GdCl_3$ after hepatotoxins could not blocked this staining. Although the high median values of week 4 were decreased after $GdCl_3$-treatment, it was not significant statistically. The administration period of $GdCl_3$ might not be enough to get the exact effect.

Our data have highlightened the key areas in the process of Kupffer cell inhibition with $GdCl_3$ resulting in HSCs inactivation and diminishing the ECM components in fibrotic liver. The development of treatment against hepatic fibrosis as a part of therapy for chronic liver disease has has important implications for future development of therapeutic antifibrotic strategies in the liver.

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