Original

Macrophage Populations, Myofibroblastic Cells, and Extracellular Matrix Accumulation in Chronically-Developing Rat Liver Cirrhosis Induced by Repeated Injection of Thioacetamide

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Abstract: In order to clarify the cellular and molecular aspects in cirrhotic lesions, we investigated the response of macrophage populations, myofibroblastic cell development, and deposition of extracellular matrices (ECMs), as well as expression patterns of fibrogenic factors, in chronically-developing hepatic cirrhosis induced in rats by thioacetamide (TAA). As an animal model, male F344 rats were rendered cirrhotic by repeated intraperitoneal injection of TAA (100 mg/kg BW; twice a week), and were examined at post-first injection (PFI) weeks 1, 3, 5, 7, 10, 15, and 20. Histologically, hepatocyte degeneration became evident in the perivenular and periportal areas with time, and from PFI weeks 10 micronodular lesions separated by fibrous septa were developed. ECMs (collagen types I, III, and IV, fibronectin and laminin) deposited with advancing lesions. ED1-immunopositive exudate macrophages showed an increased number for 20 weeks. ED2-immunopositive Kupffer cells and antigen-presenting macrophages reacting to OX6 (MHC class II-recognized antibody) revealed a transiently increased number at PFI weeks 1 and 3; interestingly, Kupffer cells became hypertrophic with time. The number of myofibroblastic cells reacting to α-smooth muscle actin was increased from PFI week 1, with a peak at PFI week 10. The semiquantitative analysis by the reverse transcription polymerase chain reaction revealed that expressions of transforming growth factor-β1 (TGF-β1) and tumor necrosis factor-α (TNF-α) mRNAs could be increased for 20 weeks. These results indicated that different macrophage populations participated in chronically-developing rat hepatic cirrhosis with different kinetics patterns, and that these macrophages might be related to myofibroblastic cell development through the production of fibrogenic factors such as TGF-β1 and TNF-α.

Key words: macrophage populations, myofibroblastic cells, rat cirrhosis, transforming growth factor-β1, thioacetamide, tumor necrosis factor-α

Introduction

Liver cirrhosis is evoked by complex processes that require the participation of various inflammatory cells and abnormal accumulation of extracellular matrices (ECMs)1–4. Macrophages and hepatic stellate cells (HSCs) play the main roles in the fibrogenesis after liver injury, because macrophage-produced soluble factors such as transforming growth factor-β1 (TGF-β1) and tumor necrosis factor-α (TNF-α) induce the modulation of HSCs towards myofibroblastic cells capable of producing ECMs5–8.

Molecular and cellular events have been investigated in hepatic fibrosis induced in rats or mice by single or repeated injection of carbon tetrachloride (CCl4)9–11 or alcohol12,13. The bile duct legation rat model has also contributed to the development of such a lesion14. Moreover, the administration of thioacetamide (TAA) with hepatotoxicity to rats has been utilized to induce hepatic fibrosis. There is also sufficient evidence for the carcinogenicity of TAA in experimental animals15–17. The histology of liver cirrhosis produced by the long-term treatment with TAA bears a close resemblance to that of human micronodular cirrhosis18. The lesions are more prominent in TAA-induced cirrhotic rat model than in CCl4-treated rats15,18. However, molecular and cellular aspects in the TAA rat model have not yet been fully clarified.

In the present study, in order to shed some light on the pathogenesis we investigated the response of macrophage populations, myofibroblastic cell development, and deposition of ECM components, as well as expression...
patterns of fibrogenic factors (TGF-β1 and TNF-α) in chronically-developing hepatic cirrhosis induced in rats by repeated injection of TAA.

Materials and Methods

Animal model

Thirty 6-week-old male F344 rats (110–120 g; Charles River Japan, Hino, Japan) were housed in an animal room at 20 ± 2°C and with a 12 hour light-dark cycle, and fed a standard diet and tap water ad libitum. Rats in TAA group were injected intraperitoneally twice a week with TAA (Wako Pure Chemical, Osaka, Japan) dissolved in saline at a dose of 100 mg/kg body weight. Three rats were examined each at 1, 3, 5, 7, 10, 15, and 20 weeks after the first injection of TAA. Control rats received an equivalent volume of saline in the same manner, and three rats were examined each at 1, 10, and 20 weeks after the first injection. Body weights were measured at each examination week. This experiment was in compliance with our institutional guideline for animal care18.

In previous studies using TAA, Sprague-Dawley or Wistar rats (both, closed colony strains) have been often utilized and the drinking water was an administration route16,18. In such conditions, we have experienced difficulty in obtaining the homogeneous liver lesions19. In the present study, we therefore selected F344 rats (inbred strain) with genetically uniform nature and intraperitoneal route as an injection method. As a result, we could obtain homogeneous liver lesions as described below at each examination point.

Histological evaluation

All rats were euthanatized under ether anesthesia. At autopsy, blood samples were obtained from the abdominal aorta. The removed livers were fixed in 10% neutral buffered formalin (pH 7.4), Zamboni’s solution (0.21% picric acid, 2% paraformaldehyde, and 130 mM phosphate buffer, pH 7.4), and Methacarn solution (methanol: chloroform: acetic acid = 6: 3: 1)20. These tissues were embedded in paraffin, sectioned, and stained with hematoxylin-eosin (HE) and Azan-Mallory stain for collagens. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in serum were measured by a clinical analyzar 7170 (Hitachi, Tokyo, Japan) in rats under the condition without fasting.

Immunohistochemical analysis

Deparaffinized tissue sections were analyzed immunohistochemically using Labelled Histofine Simple Stain Kit (Nichirei, Tokyo, Japan). Primary antibodies and fixative solution available for each antibody are listed in Table 1. The detailed procedures were described elsewhere21. The following pretreatments were made; sections applied for ED1, ED2, OX6, α-smooth muscle actin (α-SMA), laminin, and fibronectin were treated with 0.1% trypsin for 10 min at 37°C and sections for collagen types I, III, and IV were incubated with 0.0004% pepsin for 15 min at 37°C. After treatments with 3% H2O2 in phosphate buffered saline (PBS) for 10 min to quench endogenous peroxidase and then with 5% skim milk in PBS for 30 min to inhibit non-specific reactions, these sections were incubated with primary antibody for 14 h at 4°C, followed by the application to the reagent (labeled polymer) for 30 min. Positive reactions were visualized with 3,3’-diaminobenzidine. Sections were counterstained lightly with hematoxylin. As negative controls, tissue sections were treated with mouse or rabbit non-immune serum instead of the primary antibody at the same concentrations.

ED1, ED2, and OX6 antibodies were used to differentiate macrophage subpopulations. ED1 labels blood monocytes and exudate macrophages in rats22,23. ED2 reacts with cell membrane antigens of rat resident macrophages (Kupffer cells)23. OX6 is an antibody against rat MHC class II antigen (Ia) that express on activated macrophages and dendritic cells24. α-SMA is known to be induced in developing myofibroblastic cells, and the antibody is used to detect the myofibroblasts in the fibrotic lesions8,14,25,26. As ECMs, collagen types I, III, and IV, fibronectin, and laminin were analyzed.

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from fresh liver tissues by being homogenized with Trizol Reagent™ (Invitrogen.}

<table>
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<th>Antibody</th>
<th>Poly/Mono</th>
<th>Fixative</th>
<th>Dilution</th>
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<tr>
<td>OX6</td>
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* Specification of primary antibodies is described in “Materials and Methods”.

Table 1. Primary Antibodies Used in This Study
The RNA was reverse-transcribed to cDNA using Super Script Preamplification System™ (Invitrogen Corp., Carlsbed, CA). cDNA was amplified by PCR with Taq DNA polymerase (TaKaRa Shuzo, Otsu, Japan) and each of the specific primers (sense and antisense) for rat TGF-β1, TNF-α or β-actin (control). The following conditions were used for the amplification: for TGF-β1, 25 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 61°C, and 1 min of synthesis at 72°C; for TNF-α, 28 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 57°C, and 1 min of synthesis at 72°C. The oligonucleotides used were as follows: for TGF-β1, sense 5’-CTTCAGCTCCACAGAGAAGAACTGC-3’ and antisense 5’-CACGATCATGTTGGACAACTGCTCC-3’; for TNF-α sense 5’-TGCTACTGAACACTCGGGGTG-3’ and antisense 5’-GAGGCTGACTTTCTCCTGGTA-3’; for β-actin, sense 5’-TAAAGACCTCTATGCCAACAC-3’ and antisense 5’-TGTCTACTGAACTTCGGGGTG-3’. The amplification method for β-actin was done along with the same method for TGF-β1 or TNF-α. The PCR products were subjected to electrophoresis in a 1% agarose gel. DNA was stained with ethidium bromide on the gel. The bands were semiquantitatively evaluated with an image analysis software (NIH Image 1.61, Bethesda, MD). The RT-PCR was done in triplicate using samples from one rat at each examination point.

Table 2. Accumulation of Extracellular Matrices (ECMs) in Thioacetamide (TAA)-induced Hepatic Cirrhosis

<table>
<thead>
<tr>
<th>ECMs</th>
<th>Control</th>
<th>TAA treatment (weeks after first injection)</th>
<th>1</th>
<th>3</th>
<th>5</th>
<th>7</th>
<th>10</th>
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<td>+++</td>
<td>+++</td>
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</tr>
<tr>
<td>Laminin</td>
<td>–</td>
<td>±</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
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<tr>
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<td>±</td>
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<td>+</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
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</tbody>
</table>

The immuno-reactivities for ECMs were assessed semiquantitatively as follows: –, negative staining; ±, less than faintly positive staining; +, faintly positive staining; ++, clearly evident staining; ++++, marked staining.

Statistical evaluation

Cells showing a distinct immunopositive-reaction for ED1, ED2, andOX6 were counted in five randomly selected areas (0.0625 mm²/area) in the following different regions at a magnification of ×40029,30; the perivenular, mid-zonal and portal areas of hepatic lobules. The mean ± standard deviation (SD) per area in each region or in total number was calculated at each examination point by post-first injection (PFI) week 7. From PFI week 10, as described in “Results”, the complete pseudolobules began to be formed by bridges of collagen bundles; thus, ED1-, ED2- andOX6-reacting cells were counted in the pseudolobules (0.0625 mm²/area) without distinguishing these regions. α-SMA-immunopositive cells with evident nuclei were counted in five randomly selected areas (0.0625 mm²/area) within hepatic lobules or pseudolobules. Cells expressing of an α-SMA antibody in the collagen bundles separating the pseudolobules were excluded from the counting in the immunohistochemistry.

Since no differences in AST and ALT values nor the immunohistochemical data were observed among three control groups (PFI weeks 1, 10, and 20), they were treated as one control group for statistical analysis31. Data were compared between controls and TAA group at each examination by Student’s t-test19–21. Values of P<0.05 were considered significant. The reactivities for ECMs were assessed semiquantitatively, as shown in Table 2.

Results

Hepatic failure

The body weight gain in the TAA group at PFI weeks 3 to 20 was significantly decreased than that in the control group (Fig. 1). AST values, except these at PFI weeks 3 and 5, were significantly increased in the TAA group at each PFI week; ALT values began to be significantly increased from PFI week 5 (Fig. 2). These findings indicated hepatic failure due to repeated injections of TAA.

At autopsy, the liver in the TAA group showed a roughened surface at PFI week 7. At PFI week 10 and subsequent weeks, a number of micronodules of variable sizes were seen both on the surface and cut surface.

Histopathology

In the TAA group, degenerating hepatocytes with eosinophilic, swollen cytoplasm were seen mainly in perivenular areas and occasionally in perportal areas; these hepatocytes often had hypertrophic nuclei. Such histological changes began to be observed at PFI week 1 to a less extent, and the severity became more evident with PFI weeks (Fig. 3A). At PFI weeks 5 and 7, collagen depositions, stained blue by Azan staining, started to occur among degenerating hepatocytes in the periportal and perivenular areas, forming thin fibrous septa; however, there had not yet been formation of bridges (collagen bundles) linking between central veins and Glisson’s sheath. At PFI week 10, the bridges were completely formed, thus separating the hepatic lobules into

Table 2. Accumulation of Extracellular Matrices (ECMs) in Thioacetamide (TAA)-induced Hepatic Cirrhosis
psuedolobules. At PFI weeks 15 and 20, the collagen bundles became more thickened, and micronodules of various sizes were frequently seen (Fig. 3B). No noticeable changes were observed in the control liver.

**Macrophage subpopulations**

In the control group, there were only a few ED1-positive cells in each lesion of hepatic lobules. The total number of ED1-positive cells in the TAA group had already reached a maximum level at PFI week 1, and it was gradually decreased by PFI week 20; however, the significantly
increased number persisted for 20-week observation period (Fig. 4A). At PFI weeks 1 to 7, ED1-positive cells showed a significantly increased number both in perivenular and periportal areas, and the number in the mid-zonal area at PFI weeks 1, 5 and 7 was also increased. ED1-positive cells appeared in and around the injured areas with degenerated hepatocytes (Fig. 5A). ED1-positive cells were also observed occasionally in the bundles at PFI weeks 10 to 20. ED1-reactivity appeared granular in the cytoplasm (Fig. 5A).

In the control group, ED2-positive cells were observed along the sinusoids. The total number of ED2-positive cells was significantly increased in the TAA group at PFI weeks 1 and 3 (Fig. 4B), but the increased level was smaller than that in the ED1-positive cells. At PFI week 20, the number was significantly decreased (Fig. 4B). In the injured perivenular area, ED2-positive cells showed a significantly increased number at PFI weeks 1 to 7, and at PFI week 7 these in the mid-zonal area were also increased significantly (Fig. 4B). Besides the injured perivenular areas, ED2-positive cells were often present along the sinusoids, indicative of activated Kupffer cells (Fig. 5B). Interestingly, ED2-positive cells in the control group and at PFI weeks 1 and 3 revealed small round or spindle morphology (Fig. 5C), whereas they at PFI weeks 5 to 20 became hypertrophic with PFI weeks (Fig. 5D). ED2-positive cells were rarely seen in collagen bundles.

In the control group, OX6-positive cells were present along the sinusoids. The total number of OX6-positive cells in the TAA group had already been maximum at PFI week 1, with a significant increase (Fig. 4C). The significant increase was also seen at PFI week 3; thereafter, the number was gradually decreased by PFI week 20, and at PFI weeks 15 and 20, it was significantly decreased (Fig. 4C). At PFI week 1, OX6-positive cells in all areas of hepatic lobules showed a significantly increased number, and the number in the perivenular area at PFI weeks 3 and 5 and in the periportal area at PFI week 3 were also significantly increased. These findings indicated that OX6-positive cells also appeared in response to tissue injury. They revealed various configurations such as large round, spindle and dendrite, and some cells were seen in Glisson’s sheath (Fig. 5E).

### Myofibroblastic cell development and ECM deposition

HSCs reacting with the anti-α-SMA antibody were rarely seen in the control group. The number of α-SMA-positive cells in hepatic lobules and pseudolobules began to be significantly increased at PFI week 1, and reached a peak at PFI week 10 (Fig. 6). On the other hand, the number in the pseudolobules at PFI weeks 15 and 20 was markedly decreased. The positive cells were mainly along the sinusoids, and similar cells were seen in fibrous septa at PFI weeks 10 to 20 (Fig. 5F). The HSCs showed spindle-shaped morphology at the early stages, and along the development of cirrhotic lesion they became to have slightly swollen cytoplasm.

As shown in Table 2, collagen type IV began to be deposited from PFI week 1, and other ECM elements from PFI week 3. All ECMs reached the greatest level (3+) at PFI weeks 7 to 15. The deposition of interstitial ECM elements (collagen types I and III, and fibronectin) were seen exclusively in the fibrous septa (Figs. 7A and 7B), whereas basement membrane-related ECM components such as
laminin and collagen type IV were deposited both along the sinusoids and in the fibrous septa (Figs. 7C and 7D).

**TNF-α and TGF-β1 mRNA expressions**

TNF-α mRNA expression was gradually increased from PFI week 1, and peaked at PFI week 10 (about two-fold greater than controls); then the level was slightly decreased, but did not return to that of the controls (Fig. 8A). TGF-β1 mRNA expression was about two-fold greater than that of the control as early as PFI week 1, and the higher expression...
Discussion

TAA-induced initial lesions in the early stages at PFI weeks 1 and 3 were degeneration of hepatocytes seen in the perivenular and periportal areas. In response to such an insult, many macrophages, especially ED1-positive exudate macrophages, were seen in these areas.

At PFI weeks 5 and 7, collagen deposition started to develop around degenerating hepatocytes in the perivenular and periportal areas. The hepatic lesions induced by CCl₄ were characterized mainly by degeneration/necrosis of perivenular hepatocytes and subsequent centrilobular fibrosis⁹⁻¹¹; the predilection sites appeared to be different from those caused by TAA¹⁵⁻¹⁸. At PFI week 10, pseudolobules began to be formed, and the cirrhotic lesions became more prominent at PFI weeks 15 and 20. The progressive hepatic lesions were confirmed by a decrease in body weight gain and increases in AST and ALT values. Therefore, we were able to analyze the cellular and

Fig. 6. The kinetics of α-SMA-immunopositive cells in hepatic lobules at PFI weeks 1 to 7 and in pseudolobules at PFI weeks 10 to 20. Values are means ± SD. *, significantly different from the controls at \( P<0.05 \).

Fig. 7. Immunohistochemistry with antibodies to collagen type III (A), fibronectin (B), collagen type IV (C), and laminin (D) in micronodular cirrhosis. At PFI week 10, deposition of collagen type III and fibronectin is seen exclusively in collagen bundles (arrows) (A and B), whereas basement membrane-related extracellular matrices such as collagen type IV and laminin are accumulated mainly along the sinusoids (C and D). Original magnification: A, B, C and D, × 50.
molecular aspects in chronically developing cirrhotic lesions following liver injury.

Myofibroblastic cells, that have features intermediate between those of fibroblasts and smooth muscles, act as fibrogenic cells in pulmonary, pancreatic and renal fibrosis. Similar cells have been identified in human liver lesions and CCl₄-induced rat hepatic fibrosis. In the hepatic fibrosis, the cells have been considered to be derived from HSCs. In the TAA group, thus, myofibroblastic cells expressing α-SMA appeared mainly along the sinusoids; the number of α-SMA positive cells began to be increased at PFI week 1, and reached a peak at PFI week 10 when complete pseudolobules began to be formed. In addition, in the fibrous septa, there were many cells reacting to α-SMA. Since myofibroblastic cells are a major cell group producing ECM components, the present findings suggest that these cells are responsible for the production of fibrotic lesions in this model. At PFI weeks 15 and 20, however, the number of myofibroblastic

Fibronectin was the first ECM component to be accumulated in hepatic fibrosis induced by CCl₄, followed by deposition of other ECM components. In the present study, abnormal deposition of collagen type IV first occurred, and then other components such as fibronectin, laminin, and collagens (types I and III) began to be accumulated. Although the reasons for the difference between TAA and CCl₄ models should be investigated further, the accumulation of different ECM components was confirmed in chronically-developing hepatic cirrhosis induced by TAA, as reported in CCl₄-induced rat fibrotic lesions.

Macrophages act as an early trigger in the genesis of hepatic fibrosis. In this study, we focused on the kinetics and subpopulations of macrophages. ED1 and ED2 are useful monoclonal antibodies to detect rat macrophage subpopulations. In the present TAA model, many ED1-positive exudate macrophages appeared as early as PFI week 1 with a plateau in number. The number of ED2-positive Kupffer cells was also significantly increased at PFI weeks 1 and 3. Studies on cholestatic and CCl₄-induced liver injury using rats have demonstrated that ED1- and ED2-positive cells were markedly increased in the early stages. These findings indicated that ED1-positive exudate macrophages and ED2-positive resident macrophages were quickly recruited in response to hepatic injury. Because these macrophage subpopulations emerged in perivenular and perportal areas where hepatocyte degeneration and subsequent collagen deposition occurred, they might play roles in the early production of TAA-induced hepatic fibrosis.

Additionally, although ED1-positive cell number began to be reduced from PFI week 3, higher values were retained by PFI week 20. It is likely that ED1-positive macrophages persistently contributed to the development of progressive cirrhotic lesions, resulting from repeated injection of TAA. On the other hand, ED2-positive cells no longer showed a significantly increased number at PFI weeks 5 to 15, and rather the number was significantly decreased at PFI week 20. However, it should be noted that ED2-positive cells in the pseudolobules became more hypertrophic. In acute phases after hepatic injury, Kupffer cells showed proliferation activity, resulting in quick expansion of macrophage populations in hepatic lesions. In chronic liver lesions as shown by the present study, it appears that Kupffer cells can alter their configuration. Recently, it was
reported that depressed Kupffer cell activity might play a role in augmented progression of hepatocellular carcinoma in cirrhotic livers. It is interestingly to pursue the biological significance of Kupffer cells appearing in cirrhotic lesions.

In agreement with the increment in ED1- and ED2-positive cells, OX6-positive cells showed a significantly increased number in the early stages at PFI weeks 1 and 3. OX6 is a monoclonal antibody against rat MHC class II antigen, and recognizes activated macrophages and dendritic cells as an antigen-presenting cell. Because OX6-positive cells appeared along the sinusoids or in perivenular and perportal areas with degenerated hepatocytes, it is likely considered that the OX6-positive cells were originated from exudate macrophages and Kupffer cells. The participation of OX6-positive cells has not been reported in experimentally-induced rat liver fibrosis. MHC class II-expressing cells present antigens to unprimed T cells, and in turn Th1/Th2 lymphocyte polarization will be determined; cytokines produced mainly by Th2 lymphocytes stimulate B cell proliferation, and Th1-derived cytokines activate macrophage populations. The increased number of OX6-positive cells in the early stages implies the presence of liver-derived antigens. The antigens and subtypes of lymphocytes in response to OX6-positive cells remain to be investigated.

TGF-β1 has been regarded as the most important factor in induction of myofibroblastic cells in fibrotic lesions. In the present study, greater expression of TGF-β1 mRNA (about two-fold greater than controls) was consistently seen during the 20-week observation period by the semiquantitative analysis of RT-PCR methods, indicating a significant number of ED2-positive cells. During the 20-week observation period, the cytokine expression levels of TNF-α mRNA could be increased during PFI weeks 1 to 20, with a peak at PFI week 10. TNF-α is also thought to act as a trigger for the fibrotic cascade. Additionally, TNF-α contributes to tissue damage via its cytotoxic effect. The increased expression of TNF-α might be related not only to the development of cirrhotic lesions but also hepatocyte degeneration. The major source of TGF-β1 and TNF-α is considered to be macrophages in response to tissue injury. The expansion of ED1-, ED2- and OX6-positive macrophages was seen in the early stages, and ED1-positive cells persisted significantly increased number for the 20-week observation period. These fibrogenic factors might be produced by infiltrating macrophages, in particular ED1-positive exudate macrophages. Besides, TGF-β1 and TNF-α may be released by other cell components including the activated HSCs in injured liver. Therefore, α-SMA-immunopositive HSCs might be also induced by these cytokines produced through autocrine or paracrine fashion in HSCs.

In conclusion, the present study showed for the first time that different macrophage populations participated in the development of chronically-developing rat hepatic cirrhosis induced by repeated injection of TAA. Macrophages exhibit heterogeneous functions such as phagocytosis, antigen presentation, and inhibition or killing of pathogens, as well as production of fibrogenic factors, depending on microenvironmental conditions induced by cell-cell or cell-matrix interaction. Such a cell type has not been known in other organs. Inhibition of Kupffer cell functions, that were caused by gadolinium chloride or dichloromethylene diphosphonate, ameliorated alcohol-induced liver injury or glucan-induced granulomas in rats. Such a cell type has not been seen in the present chronically-developing cirrhotic liver. Thus, there may be differences in macrophage populations participating in fibrotic lesions between organs, or between post-acute liver injury-fibrosis and chronically-developing cirrhotic lesions. The roles of macrophage populations in relation to myofibroblastic cell development should be investigated further, to understand the complicated pathogenesis of cirrhosis.

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