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Original Article

Time-course of changes in fibrous components in a thioacetamide-induced liver fibrosis model in cynomolgus monkeys

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Abstract: In liver fibrosis, the possible causes of irreversibility include the accumulation of collagen I during extracellular matrix remodeling, together with the deposition of elastic fibers in later stages. Drug development targeting liver fibrosis should preferably employ models that closely mimic human diseases. To better understand the progress of fibrosis in a cynomolgus monkey liver fibrosis model, we evaluated the time-course of changes in the fibrosis score, collagens, and elastic fibers. The animals were subcutaneously administered thioacetamide twice a week (experiment 1) or once every 2 weeks (experiment 2). Liver tissues were collected at 8 and 16 (experiment 1) or 10 and 20 (experiment 2) weeks of administration, and 12 weeks after withdrawal (experiments 1 and 2). The fibrosis score was evaluated by Masson's trichrome staining. Immunohistochemistry for collagen Ia1, III, and IV, and Elastica van Gieson staining were also performed. Fibrosis was observed from week 8 (experiment 1) or 10 (experiment 2), and in most animals, it progressed during the administration period. After withdrawal, the fibrosis scores tended to decrease. Collagen IV was predominant in the early stage but was replaced by collagen I after 20 weeks in both experiments. Collagen III distributed mostly along with collagen I throughout the study period. The elastic fibers deposition was markedly limited throughout the experiment. Fibrous component examination showed that the main collagen type contributing to fibrosis shifted from collagen IV to I after 20 weeks or later and revealed that the fibrosis status is not fully reflected in the fibrosis score. (DOI: 10.1293/tox.2024-0084; J Toxicol Pathol 2025; 38: 155–160)

Key words: liver fibrosis, reversibility, collagen type I, collagen type IV, elastin, cynomolgus monkey

Introduction

Liver fibrosis and cirrhosis are serious health issues worldwide. They can be induced by chronic liver injury, regardless of the causality, such as hepatitis viral infection, alcohol consumption, and metabolic-associated fatty liver disease. Although liver fibrosis has recently been recognized as reversible after the removal of causative agents, recovery remains difficult in advanced cases[1]. This condition is characterized by excessive synthesis and deposition of extracellular matrix (ECM), leading to liver stiffness. In the normal liver, collagen (col) IV is a normal matrix component of the space of Disse, but disruption of this matrix after injury and replacement by fibrillar collagens, such as col I and III, can occur[1–4]. In addition to a dramatic increase in collagen, elastic fibers are known to accumulate in the late stages of fibrosis. Notably, elastin is a very stable component that is highly resistant to degradation, suggesting that it may be responsible for the irreversibility of liver fibrosis[5, 6].

Efforts to understand the pathogenic mechanism of liver fibrosis and development of targeted drugs have been primarily based on animal models, mainly rodents[7]. However, rodent models are often unsuitable for the use of antibody drugs, and their limited lifespan makes them unsuitable for long-term observation, assuming disease progression over time. Therefore, recent efforts have focused on the development of liver fibrosis models using nonhuman primates[8]. Masson's trichrome (MT) and Sirius red staining are commonly used to score or quantify the amount of fibrous components. However, these staining methods cannot distinguish between individual ECM components or collagen types. To adequately evaluate novel drugs and differentiate between drug efficacy and spontaneous recovery, it is important to use animal models that are equivalent to the targeted patients.

To better understand the progression of fibrosis, we evaluated the time-course of changes in the fibrosis score, collagens, and elastic fibers in a liver fibrosis model in cynomolgus monkeys.

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Materials and Methods

Animals

Eleven adult male cynomolgus monkeys were used in the present study. The animals were individually housed in metal cages in animal rooms under controlled conditions and were fed a solid diet with free access to water. All animal experiments were approved by the Animal Experiment Committee of Astellas Pharma Inc. (Tsukuba, Japan).

Liver fibrosis and tissue sampling

In experiment 1, five animals were subcutaneously administered thioacetamide (TAA, Sigma-Aldrich, MO, USA) twice a week for 8 weeks and once or twice a week for another 8 weeks. The TAA dose was started at 30 mg/kg and increased to 120 mg/kg based on the condition of each animal. The dosing regimen and dose levels were designed according to Yasuda *et al*[8]. A liver biopsy was conducted at 8 and 16 weeks of administration, and 12 weeks after withdrawal (28 weeks of the experiment). A biopsy was performed 4 days after the last drug administration at 8 weeks and 4 or 7 days after the last drug administration at 16 weeks. Under anesthesia, the edge of the liver was cut with a scalpel, and the tissues obtained were fixed in 10% neutral buffered formalin[8]. As controls, two untreated animals were biopsied in a similar manner.

In experiment 2, four animals were administered 30 mg/kg TAA subcutaneously once every 2 weeks for 10 weeks, and a liver biopsy was conducted in the same manner as in experiment 1. Two animals were continuously administered TAA for another 10 weeks and two animals were kept without administration. After subjected to liver biopsy again at week 20, all animals were kept without administration for 12 weeks, animals were autopsied and liver tissue was collected from the same region that was subjected to the biopsy at week 32 of the experiment.

Special stains and immunohistochemistry

Fixed tissues were embedded in paraffin, sectioned, and stained with MT and Elastica van Gieson (EVG) using Sirius red instead of acid fuchsin.

For immunohistochemistry, antigen retrieval was conducted by heating sections in citrate buffer (pH 6, LSI Medience Corporation, Tokyo, Japan) for col Ia1 and col III or Target Retrieval Solution (Dako Denmark A/S, Glostrup, Denmark) for col IV using an autoclave at 121°C for 3 min. Sections were then incubated with antibodies against col Ia1 (1:800, Novus Biologicals, CO, USA), col III (1:1000, Abcam, Cambridge, UK), and col IV (1:500, Abcam) for 1 hour at room temperature. Secondary antibody reactions were performed using Envision+ System-HRP-labeled Polymer Anti-Rabbit (Dako Denmark A/S). A Liquid DAB+Substrate Chromogen System (Dako Denmark A/S) was used for visualization.

Histopathological evaluation

The progress of fibrosis was evaluated using MT staining, with reference to the Ishak fibrosis score[8], modified to account for TAA-induced centrilobular necrosis (Table 1). It has been reported that the binding capacity of aniline blue of MT for col IV is lower than that of col I and III, whereas Sirius red binds to col I, III, and IV equally[9]. To avoid the underestimation of col IV, EVG staining sections were also observed to confirm the difference from MT staining.

As for col I, III, and IV, the contribution ratio to fibrosis area was assessed by comparison between immunohistochemistry and MT staining as follows: —, within normally localized area; ± , less; +, partly; and ++, mostly consistent with fibrosis area. Newly formed elastic fibers within the fibrosis area were detected by EVG staining as follows: —, not found; ± , very slight.

Results

Fibrosis score

No obvious differences were found in fibrosis score between MT and EVG stains.

In experiment 1 (Table 2), fibrosis was observed from week 8 of TAA administration. Fibrosis was mainly located in the centrilobular area and partly expanded to the portal area. The fibrosis scores were 1 or 2 at week 8, and some animals showed bridging fibrosis (scores of 3 or 4) at week 16. Fibrosis scores decreased in most animals after withdrawal.

In experiment 2 (Table 3), the animals showed a fibrosis score of 3 or 4 at week 10 of TAA administration. At week 20, fibrosis remained constant in the animals administered with TAA for 20 weeks (Animals F and G), but scores decreased in the animals administered with TAA for 10 weeks followed by 10 weeks withdrawal (Animals H and I). After 12 weeks of withdrawal, the fibrosis scores tended to decrease at the autopsy conducted at week 32 in animals administered with TAA for 20 weeks. The scores further decreased or returned to the normal range after 10 weeks of drug administration, followed by 22 weeks of withdrawal.

Table 1. Fibrosis Score

Score	Description
0	No fibrosis (normal)
1	Fibrosis expansion of some central and/or portal areas
2	Fibrosis expansion of most central and/or portal areas
3	Fibrosis expansion of most central and/or portal areas with occasional bridging
4	Fibrosis expansion of most central and/or portal areas with marked bridging
5	Marked bridging with occasional nodules
6	Cirrhosis

Table 2. Time-course Changes in Fibrosis Score, Types of Collagen Contributing to Fibrosis, and Amount of Elastic Fibers in Experiment 1

Treatment	Thioacetamide, subcutaneous injection, 2 times/week				
Liver/Findings Animal	A	B	C	D	E
8 weeks					
Fibrosis score	**1**	**2**	**2**	**2**	**1**
Col Ia1[a]	—	+	±	+	+
Col III [a]	—	±	±	—	±
Col IV [a]	++	++	++	+	+
Collagen type [b]	IV	IV	IV	I / IV	I / IV
Elastic fibers [c]	—	—	—	—	—
16 weeks					
Fibrosis score	**1**	**3**	**2**	**4**	**3**
Col Ia1[a]	+	±	+	+	+
Col III [a]	±	±	±	—	±
Col IV [a]	+	++	++	+	++
Collagen type [b]	I / IV	IV	IV	I / IV	IV
Elastic fibers [c]	—	±	±	±	±
28 weeks (16 weeks +12 weeks withdrawal)					
Fibrosis score	**1**	**2**	**1**	**3**	**2**
Col Ia1[a]	+	++	++	++	+
Col III [a]	+	±	±	+	+
Col IV [a]	±	+	+	±	±
Collagen type [b]	I / III	I	I	I	I / III
Elastic fibers [c]	—	±	±	±	±

a) Ratio of immunostained area to fibrosis area: ±: less, +: partly, ++: mostly consistent with the area stained with MT, —: within normally localized area. b) Collagen type mainly contributing to fibrosis score. c) Amount of newly formed elastic fibers, —: not found, ±: very slight.

Table 3. Time-course Changes in Fibrosis Score, Types of Collagen Contributing to Fibrosis, and Amount of Elastic Fibers in Experiment 2

Treatment	Thioacetamide, subcutaneous injection, once every 2 weeks			
Liver/Findings Animal	F	G	H	I
10 weeks				
Fibrosis score	**3**	**3**	**4**	**3**
Col Ia1[a]	+	+	+	±
Col III [a]	±	±	±	±
Col IV [a]	++	++	+	++
Collagen type [b]	IV	IV	I / IV	IV
Elastic fibers [c]	—	—	±	—
20 weeks			(10 weeks + 10 weeks withdrawal)	
Fibrosis score	**2**	**3**	**2**	**1**
Col Ia1[a]	++	+	++	+
Col III [a]	+	±	+	+
Col IV [a]	±	+	±	±
Collagen type [b]	I	I / IV	I	I / III
Elastic fibers [c]	—	±	±	—
32 weeks	(20 weeks + 12 weeks withdrawal)		(10 weeks + 22 weeks withdrawal)	
Fibrosis score	**2**	**2**	**1**	**0**
Col Ia1[a]	+	+	+	—
Col III [a]	+	±	±	—
Col IV [a]	±	±	±	—
Collagen type [b]	I / III	I	I	NE
Elastic fibers [c]	±	±	±	NE

a) Ratio of immunostained area to fibrosis area: ±: less, +: partly, ++: mostly consistent with the area stained with MT, —: within normally localized area. b) Collagen type mainly contributing to fibrosis score. c) Amount of newly formed elastic fibers, —: not found, ±: very slight, NE: not examined as fibrosis area was not observed (fibrosis score 0).

Time-course changes in fibrous components

In the livers of untreated normal animals, col I and col III were detected at the edges of the central veins and portal area (Fig. 1A). Col IV was located together with the sinusoid and was also observed in the basement membrane of the vessels and bile ducts. In experiment 1 (Fig. 1A), at weeks 8 and 16, the area positive for col IV was mostly consistent with the fibrotic area, whereas those of col I and III were partly negative in the fibrotic area. At these time points, the collagen type making the major contribution to fibrosis was considered to be col IV in most animals, while in some animals, col I also contributed to fibrosis together with col

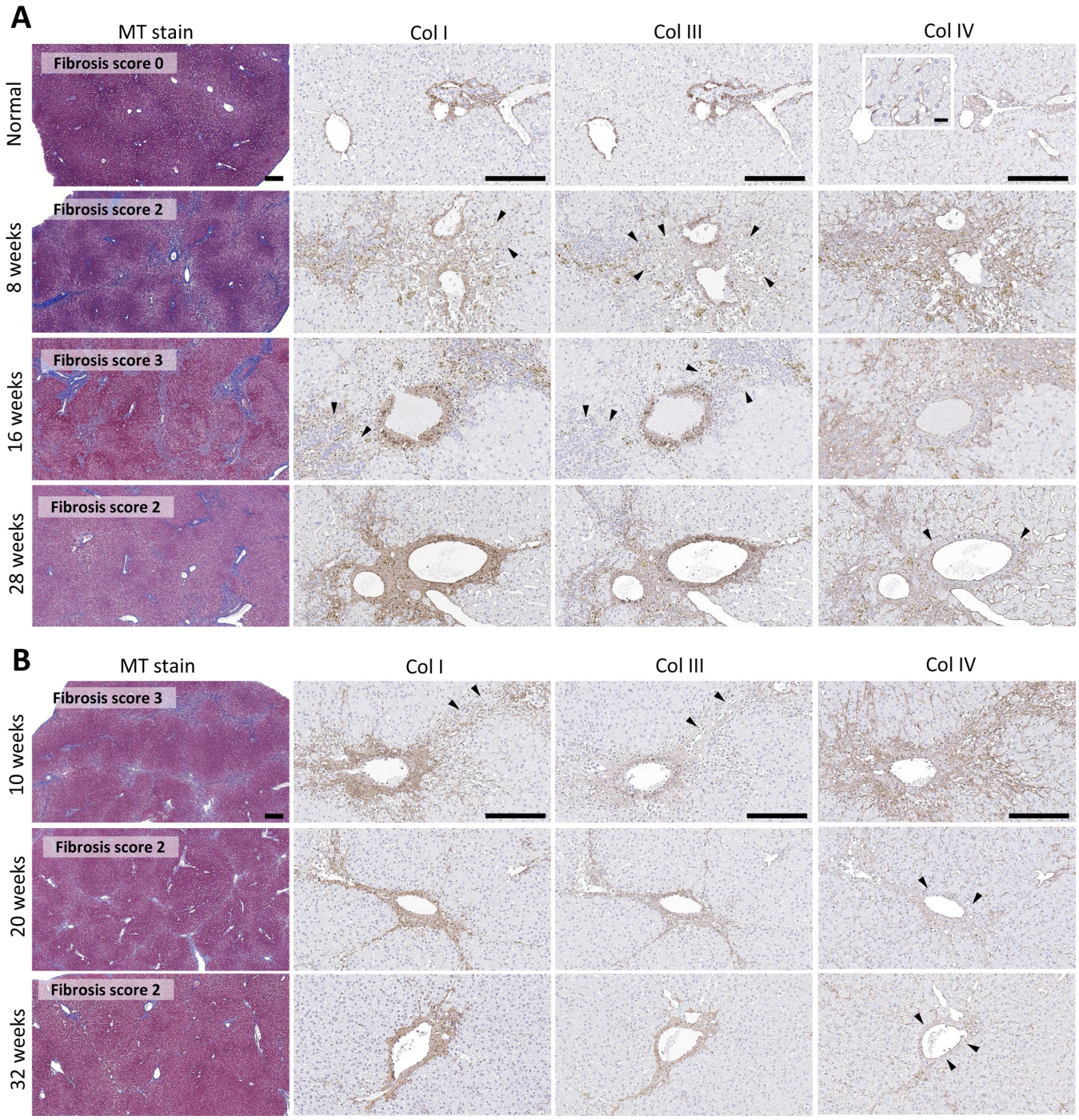

Fig. 1. Sequential liver histopathology of MT staining and immunohistochemistry for col Ia1, III, and IV in animal B and the control from experiment 1 (A) and animal F from experiment 2 (B). The insert shows a high-magnification image of col IV located with a sinusoid. Arrowheads indicate partial negativity in the fibrotic areas on immunohistochemistry. MT: Masson's trichrome. Scale bars: 200 μm in low magnification images and 20 μm in the insert, respectively.

IV. In contrast, the areas positive for col I or col I and III coincided with the fibrotic area at week 28, whereas col IV became partially negative in the fibrotic area, particularly in mature fibrous bundles. In experiment 2 (Fig. 1B), the collagen type that made the major contribution to fibrosis was col IV at week 10, which shifted to col I at week 20. At week 32, the areas positive for col I or col I and III matched the fibrotic area. Col III showed a similar distribution as col I in both experiments but was not predominant at any time point.

In the normal liver, elastic fibers stained black were detected in the portal area, elastic lamina in the artery, the relatively large central veins, and capsules (Fig. 2). Newly formed elastic fibers were found in the fibrotic area, but the amount was limited and the distribution was restricted to small areas.

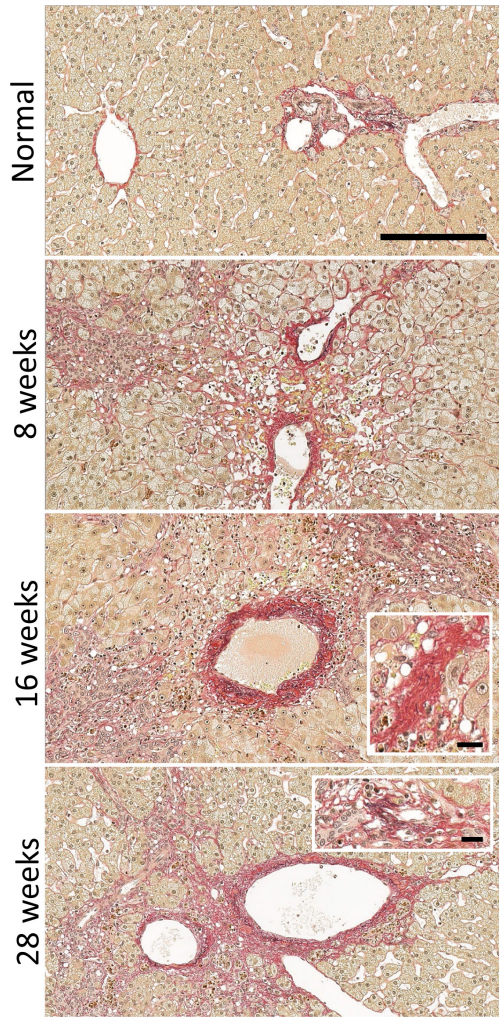

Fig. 2. Representative photographs of EVG staining in animal B and control from experiment 1. The inserts show high magnification images of newly formed elastic fibers in the fibrous area. EVG: Elastica van Gieson. Scale bars: 200 μm in low magnification images and 20 μm in the inserts, respectively.

Discussion

In this study of the time-course of changes in fibrosis score, collagens, and elastic fibers in the liver of cynomolgus monkeys subcutaneously administered with TAA, we found TAA-induced liver fibrosis in both experiments 1 and 2, and some animals showed bridging fibrosis. TAA require metabolic activation to induce toxicity. Hepatotoxicity may not occur in a dose-dependent manner because TAA inhibits the formation of its reactive metabolite[10]. This supports the fact that severe hepatotoxicity was not induced despite the high dose in experiment 1 and that the fibrosis scores in experiment 2 were higher than those in experiment 1. In both experiment, the major contributor to fibrosis was col IV, a matrix component of the space of Disse at weeks 8 to 16, suggesting an early response to tissue injury. Subsequently, col IV in the fibrotic areas was replaced with col I at 20 weeks or later. Although the fibrosis score decreased after withdrawal, immunohistochemistry revealed not only a decrease in the fibrous amount but also a switching of collagen types. This change was observed in both experiments, and replacement with col I was prominent at 20 weeks and later. At week 20 in experiment 2, col I was prominent in both animals administered with TAA for 20 weeks and 10 weeks followed by 10 weeks of withdrawal. These findings suggest that withdrawal was not required to produce col I, but that the accumulation of col I takes a certain amount of time, regardless of the TAA administration method. Stiffness was positively correlated with the content of collagen type I[11]. Accordingly, we assumed that the liver tissues became stiffer after 20 weeks, even though the fibrosis score was reduced. Col III is known as a main component of the ECM was not predominant at any time point in the present study. Generally, col III is upregulated together with col I during the wound healing process, and the ratio of col I, with stiffer fibrils than col III, increases in a healed wound[12], which is consistent with our results.

The findings of cirrhosis in humans include abundant col I and dense elastic bundles in the fibrous septa. Elastin is stable and highly resistant to degradation and is therefore considered a possible cause of irreversible liver fibrosis[5]. Although elastic fiber formation in the liver has also been reported in animal models, it appears later than col I in the progression of liver fibrosis[13]. In the present study, although some animals showed bridging fibrosis, the deposition of elastic fibers was markedly limited, and we considered that the fibrosis examined did not reach an irreversible status. Bridging fibrosis resembles human fibrosis, but the fibrous components and maturity status are not consistent with those in human patients. In addition to collagen disposition, many factors, including organization and cross-linking of the ECM and an imbalance in matrix metalloproteinases and their inhibitors, are thought to contribute to the progression of fibrosis[11]. The fibrous score was judged by the area positive for MT or Sirius red staining only and might accordingly have been overestimated, particularly in the early phase of fibrosis, in which matrix collagen actively responds

to tissue injury. Therefore, assessment by fibrosis score alone might be insufficient, and detailed examination of the fibrous component will be helpful in understanding the fibrosis status in animal models compared to human patients.

In summary, immunohistochemistry of collagen fibers revealed that the main contributor to fibrosis was col IV in the early phase, which was subsequently replaced by col I. It took more than 20 weeks to accumulate collagen type I to account for stiffness. In the present study, fibrosis was considered reversible because the amount of newly formed elastic fibers was markedly low. A single assessment by fibrosis score may not allow the comparison of fibrosis stages between humans and animal models, and additional col I staining might be useful to confirm the fibrosis status. Understanding these differences among fibrosis stages will help establish animal models that mimic human patients and allow the appropriate evaluation of pharmacological effects.

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