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Original Article/Liver

Monocyte-derived fibrocytes elimination had little contribution on liver fibrosis

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ABSTRACT

Background: Monocyte-derived fibrocytes play an important role in the progression of fibrosis in the skin, lungs, heart and kidney. However, the contribution of fibrocytes to liver fibrosis is unclear. The aim of this study was to investigate whether fibrocytes contributed to fibrosis progression in the livers of carbon tetrachloride (CCl_4)-treated mice.

Methods: C57BL/6J mice were divided into 4 groups: normal control group, CCl_4-treated group, CCl_4 + control liposome-treated group, and CCl_4 + clodronate liposome-treated group. For the elimination of systemic monocyte and monocyte-derived fibrocyte, one group was treated with clodronate liposome, and another group with control liposome as a control. After 4 weeks of treatment, hepatic mononuclear cells were subjected to immunofluorescent (IF) staining and fluorescence-activated cell sorter (FACS) analysis to detect fibrocytes. Measurement of collagen-positive Sirius red stained area and collagen-I mRNA expression in the liver were performed to evaluate the degree of liver fibrosis quantitatively.

Results: In the liver of the CCl_4-treated and CCl_4 + control liposome-treated groups, the number of fibrocytes, the area positive for Sirius red staining and collagen-I mRNA expression significantly increased compared with those in the normal control group. In the liver of the CCl_4 + clodronate liposome-treated group, few fibrocytes was observed as in the normal control group, but Sirius red staining positive area and collagen-I mRNA expression were increased and equivalent to the CCl_4-treated and CCl_4 + control liposome-treated groups.

Conclusion: Monocyte-derived fibrocytes play a minimal role in CCl_4-induced liver fibrosis. Cells other than fibrocytes such as hepatic stellate cells play a central role in liver fibrosis.

Introduction

Liver fibrosis occurs as a result of chronic liver diseases such as alcoholic liver disease, non-alcoholic steatohepatitis, and hepatitis caused by the hepatitis B and C viruses [1]. Liver fibrosis results from the accumulation of extracellular matrix (ECM), mostly collagen type I that is generated from collagen-producing myofibroblasts. In liver fibrosis, myofibroblasts were reported to arise from hepatic stellate cells (HSCs), portal fibroblasts, epithelial cells, mesenchymal stromal cells, fibrocytes and mesothelial cells [2]. Fibrocytes are monocyte-derived cells with features of both monocytes and fibroblasts. They were first identified in 1994 as a result of studies using a model of wound repair [3]. This cell type expresses the hematopoietic stem cell marker CD34, the pan-hematopoietic marker CD45 and monocyte markers such as CD14 and CD11, and produces components of the connective tissue matrix, including collagen-I, collagen-III and vimentin [4]. Although fibrocytes constitute approximately 0.5% of circulating leukocytes in the normal human host [3], this proportion increases in response to certain cytokines and chemokines and to the presence of underlying fibrotic or inflammatory conditions [5,6]. The contribution of fibrocytes to tissue fibrogenesis has recently been shown in many fibrotic disease models, including those involving the skin [7], lungs [8–11], heart [12–14] and kidney [15,16]. However, the

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contribution of fibrocytes to liver fibrosis remains unclear. Therefore, we carried out the present study to investigate whether fibrocytes contribute to fibrosis progression in the livers of carbon tetrachloride (CCl_4)-treated mice.

Methods

Animals

Ten-week-old female C57BL/6J mice (SLC Japan, Shizuoka, Japan) were used in all experiments. Mice were housed under controlled humidity and temperature conditions, and under a 12-h light/dark cycle. All experiments were approved by the Animal Experiment Committee of the University of Miyazaki (2017-519).

CCl_4 treatment and clodronate administration

CCl_4 (Wako Pure Chemical Industries, Osaka, Japan) was administered at 2000 μL/kg (10% in olive oil) twice per week for 4 weeks by intraperitoneal injection. We treated mice with clodronate because fibrocytes are derived from monocytes and clodronate has been reported to efficiently induce monocyte apoptosis [17]. For the elimination of systemic monocytes, mice were administered 200 μL of clodronate liposome (Anionic) (FormuMax Scientific, Sunnyvale, CA, USA) or 200 μL of control liposomes (FormuMax Scientific) by intravenous injection starting 7 days prior to initiation of CCl_4 administration and then every 4 days for 4 weeks. Mice were divided into 4 groups: normal control group ($n=5$), CCl_4-treated group ($n=5$), CCl_4+control liposome-treated group ($n=8$), and CCl_4+clodronate liposome-treated group ($n=8$).

Fluorescence-activated cell sorter (FACS) analysis of hepatic mononuclear cells

Hepatic mononuclear cells were prepared as previously described [18]. Briefly, livers were removed, cut into small pieces with scissors, pressed through a 100-μm cell strainer and suspended in phosphate buffered saline (PBS). The cell suspension was subjected to density gradient centrifugation using Percoll (GE Healthcare, Chicago, IL, USA) at 2000 rpm for 20 min at room temperature. Supernatant containing hepatocytes was removed and the pellet was resuspended in erythrocyte-lysing solution. Cell pellets were washed once more with FACS buffer (PBS with 2% fetal bovine serum) and cells were counted. The cells were stained with APC/Cy7-labelled anti-mouse CD45 antibody (103116; BioLegend, San Diego, CA, USA), then permeabilized with a BD Cytofix/Cytoperm Kit (554714; BD Biosciences, San Diego, CA, USA) and stained with biotin-conjugated anti-collagen I antibody (600-406-103; Rockland, Gilbertsville, Pennsylvania, USA) followed by PerCP Cy5.5-conjugated streptavidin (405214; BioLegend). FACS analysis was performed using a BD FACSCanto II (BD Biosciences).

Histological examination

Liver samples were fixed in 4% paraformaldehyde (Wako Pure Chemical Industries) for histological examination. Paraffin sections 5-μm in thickness were deparaffinized, rehydrated, and stained with hematoxylin and eosin (HE) to examine morphological changes, or Sirius red to detect hepatic fibrosis. The collagen-positive red stained area was measured quantitatively using ImageJ software (NIH, Bethesda, MD, USA).

Immunofluorescence

Deparaffinized liver sections were rehydrated, and then boiled in 10 mmol/L citrate buffer, pH 6.0, for 10 min to retrieve the antigenic sites. After several rinsing in PBS, the sections were incubated in 5% normal goat serum/1% bovine serum albumin (BSA) in PBS for 20 min, and then incubated with rat monoclonal antibody against the mouse CD45 (ab25386, Abcam, Cambridge, MA, USA; 1:100 diluted with 1% BSA in PBS) and biotin-conjugated anti-rat IgG (BA-9400; Vector Laboratories, Burlingame, CA, USA; 1:200 diluted with 1% BSA in PBS) as the secondary antibody, followed by Alexa Fluor 488-conjugated streptavidin (S11223; Invitrogen, Carlsbad, CA, USA; 1:200 diluted with 1% BSA in PBS). Subsequently, these sections were incubated with rabbit polyclonal antibody against the mouse collagen-I (PA1-85319, Invitrogen, USA; 1:100 diluted with 1% BSA in PBS), followed by Alexa Fluor 594-conjugated goat anti-rabbit IgG (ab150080; Abcam; 1:200 diluted with 1% BSA in PBS) as the secondary antibody. These sections were then rinsed in PBS and mounted with coverslips with the use of Vectashield (H-1000; Vector Laboratories). Microscopy images were acquired using a Zeiss LSM 700 confocal microscope (Athens, GA, USA).

Quantitative real-time reverse transcriptase-polymerase chain reaction

RNA was extracted using the RNeasy mini kit (74106; QIAGEN, Valencia, CA, USA). Complementary DNA was generated from 1 μg RNA using the QuantiTect reverse transcription kit (205311; QIAGEN). Universal PCR Master Mix and appropriate probes were purchased from Roche (Basel, Switzerland). The primer sequences and probes used for real-time PCR were as follows: colagen-I, 5′-CATGTTCAGCTTTGTGGACCT-3′, 5′-GCAGCTGACTTCAGGGATGT-3′, and universal probe #15.

Statistical analysis

All data are expressed as the mean ± standard error. Statistical significance was determined using the one-way analysis of variance. P values < 0.05 were considered statistically significant. Data were analyzed using SPSS version 20.0 software (SPSS Inc., Chicago, IL, USA).

Results

Clodronate liposomes eliminate fibrocytes in livers from CCl_4-treated mice

CCl_4 is one of the oldest and most widely used toxins to induce liver fibrosis in murine models [19]. Repeat CCl_4 administration results in liver fibrosis progression. Since a mouse model involving CCl_4 administration showed that monocyte-derived fibrocytes migrated from bone marrow to the portal area of the injured liver [20], we adopted this model in this study.

The experimental design is shown in Fig. 1. Mice were divided into 4 groups: normal control group, CCl_4-treated group, CCl_4 + control liposome-treated group, and CCl_4 + clodronate liposome-treated group. After 4 weeks of treatment, we analyzed the number of fibrocytes in the liver. The representative pictures of IF staining are presented in Fig. 2A. Around portal areas, CD45 positive (green) cells are observed in all four groups, and collagen-I positive (red) cells were increased in three groups of mice received CCl_4 treatment (Fig. 2A upper panel). Monocyte-derived fibrocytes (CD45 and collagen-I double-positive cells) were observed in the liver of the CCl_4-treated and CCl_4 + control liposome-treated groups. On the other hand, in the liver of the normal control group and CCl_4 + clodronate-treated group, few such double positive cells were observed (Fig. 2A lower panel). As shown in Fig. 2C, the number of fibrocytes per 5 random

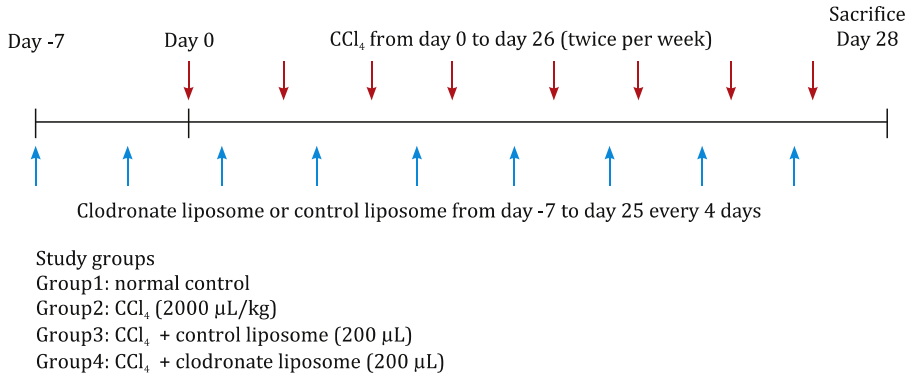

Fig. 1. Outline of experimental design. CCl_4 was administered at 2000 μL/kg twice per week for 4 weeks. Control liposomes and clodronate liposomes were administered in a 200-μL volume starting 7 days prior to initiation of CCl_4 administration and then every 4 days for 4 weeks. Each experiment was performed with at least 5 mice per group. CCl_4: carbon tetrachloride.

high-power fields (HPF) was significantly increased in the CCl_4-treated group (41.0±1.9) and CCl_4 + control liposome-treated group (39.3±2.1) compared with the normal control group (0.7±0.3). In the liver of the CCl_4 + clodronate liposome-treated group, this increase was dramatically attenuated (6.7±0.9). The results of FACS analysis were similar to those of IF staining analysis. The proportion of fibrocytes in the liver was increased in the CCl_4-treated group and CCl_4 + control liposome-treated group compared with that in the normal control group. On the other hand, in the CCl_4 + clodronate liposome-treated group, the proportion of fibrocytes hardly increased and was about the same as that of the normal control group (Fig. 2B). The absolute number of fibrocytes in the liver was increased from $0.5\pm0.1\times10^4$ cells (normal control) to $2.7\pm0.7\times10^5$ cells (CCl_4 alone) and $2.6\pm0.4\times10^5$ cells (CCl_4 + control liposome). Clodronate liposome treatment attenuated this increase to $0.6\pm0.1\times10^5$ cells (Fig. 2D).

CCl_4 treatment induced liver fibrosis that was minimally affected by clodronate

We next evaluated the effect of clodronate on CCl_4-induced liver fibrosis. Necrotizing regions (HE) and collagen-positive Sirius red stained regions were observed in all three groups of mice received CCl_4 (Fig. 3A). To evaluate the degree of liver fibrosis objectively, semi-quantification of Sirius red-positive areas was performed by Image-J software. Sirius red-positive area in the liver of CCl_4 + clodronate liposome-treated group was almost comparable with that of the CCl_4-treated and CCl_4 + control liposome-treated groups (Fig. 3B). In addition, we evaluated collagen-I mRNA expression in the liver. Collagen-I mRNA expression in the liver of the CCl_4 + clodronate liposome-treated group was also comparable to that of the CCl_4-treated and CCl_4 + control liposome-treated groups (Fig. 3C). Therefore, fibrocyte elimination by clodronate did not ameliorate CCl_4 induced liver fibrosis.

Discussion

We demonstrated here that CCl_4 treatment increased the number of liver fibrocytes and induced liver fibrosis. Clodronate treatment attenuated the increase of liver fibrocytes in CCl_4-treated livers, but it had little effect on liver fibrosis. This is the first report showed that removal of fibrocytes in the liver does not affect drug-induced liver fibrosis *in vivo*.

Fibrosis is one of the leading causes of many chronic diseases in the lungs, kidneys, heart, and liver. Chronic infections, toxic and metabolic injuries, and idiopathic inflammatory disease can promote the development of fibrosis [21]. In many cases, patients with progressive fibrosis have poor prognosis and often require organ transplantation. Although fibrosis is a part of the normal pathophysiologic response to injury in many tissues, extended exposure to chronic injury results in tissue fibrosis, massive deposition of ECM, scar formation, and organ failure [21]. Myofibroblasts are considered to be the dominant collagen-producing cells in many organ fibrosis, and they synthesize and accumulate interstitial ECM components such as type I and III collagens and fibronectin during wound healing and at sites of scarring and fibrosis [22].

In lung and kidney fibrosis, the main collagen-producing cells are resident fibroblasts [23,24]; however recent findings demonstrated that fibrocytes played an important role in the development of fibrosis. Fibrocytes were first described by Bucala et al. [3] and are defined by the simultaneous expression of CD45 and collagen-I. Fibrocytes possess dual characteristics of fibroblasts (expression of collagen-I, collagen-III, fibronectin, and vimentin) and hematopoietic cells (CD45, CD34, CD11b, Gr1, Ly6C, CD54, CD80, CD86, CCR1, CCR2, CCR5, and CCR7) [4]. Under physiological conditions, only a few fibrocytes can be detected in the peripheral blood or tissues [19]. However, in response to injury, fibrocytes downregulate expression of hematopoietic markers and rapidly differentiate into myofibroblasts [19].

Fibrocytes were reported to account for 25%–50% of collagen-producing cells in lung fibrosis and 14%–15% in kidney fibrosis [21]. Inomata et al. [25] reported that pirfenidone, which is approved for patients with idiopathic pulmonary fibrosis, attenuated the fibrocyte pool size in bleomycin-treated mouse lungs and ameliorated pulmonary fibrosis. Moreover, Sakai et al. [26] reported that valsartan, which is administered to patients with hypertension, reduced the degree of renal fibrosis and the number of fibrocytes in a mouse model of unilateral ureteral obstruction.

Unlike the lung and kidney, HSCs were reported to account for the majority (82%–96%) of collagen-producing cells in liver fibrosis [27]. On the other hand, fibrocytes were reported to account for 4%–6% of collagen-producing cells in liver fibrosis mice models [21]. This proportions of fibrocytes account for collagen-producing cells in liver fibrosis is smaller than those of fibrocytes in lung and kidney fibrosis. However, the data on the fibrocytes and liver fibrosis are scarce, thus, we conducted the present study to investigate whether fibrocytes contribute to liver fibrosis to the same degree as in lung and kidney fibrosis.

In this study, CCl_4 administration increased the number of liver fibrocytes of CCl_4-treated mice in both IF and FACS analyses. And this increase of liver fibrocytes was significantly attenuated by clodronate administration. This indicates that clodronate induced apoptosis of fibrocytes in liver in CCl_4-treated mice.

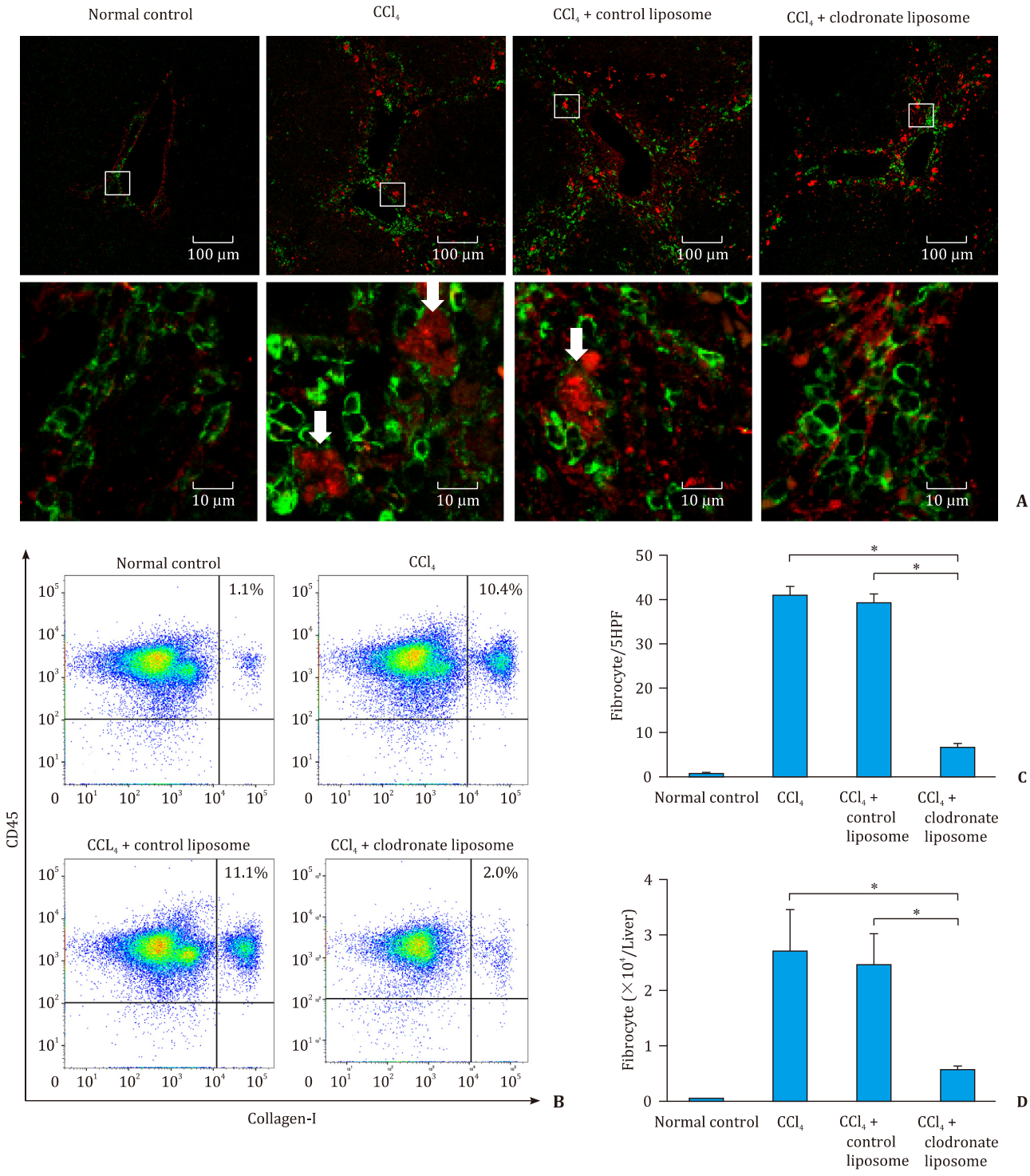

Fig. 2. Clodronate liposomes inhibited accumulation of fibrocytes in the livers of CCl_4-treated mice. **A**: CD45 (green) and collagen-I (red) staining of liver sections on day 28. Upper row: merged low-magnification images of periportal area (original magnification × 100); Lower row: merged high-magnification images of periportal area (original magnification × 800). The white arrows show co-staining with CD45 and collagen-I, indicating fibrocytes; **B**: Liver mononuclear cells were isolated from mice (3–5 mice in each category) after each treatment. Fibrocytes were examined via fluorescence-activated cell sorter (FACS) analysis. A representative FACS image from each treatment is shown. **C**: The number of fibrocytes per 5 random high-power fields (HPFs) was determined in liver sections obtained on day 28; **D**: Absolute numbers of liver fibrocytes were calculated by FACS analysis. Data are expressed as mean ± standard error. *: $P < 0.05$. HPF: high-power fields.

We did not investigate the effects of clodronate regarding the number of liver fibrocytes on mice without CCl_4 administration. This is because the number of fibrocytes in the normal liver was very few. Likewise, in the previous reports about the relationship between organ fibrosis and fibrocytes, only a few fibrocytes can be detected in the normal lung and kidney, and inhibition of fibrocytes by therapeutic agents had no effects on the number of fibrocytes [28,29].

Moreover, CCl_4 treatment resulted in development of bridging fibrosis in the mice liver by Sirius red staining. Likewise, CCl_4-treated mice had a higher collagen-I mRNA expression. However, clodronate administration did not attenuate the progression of liver

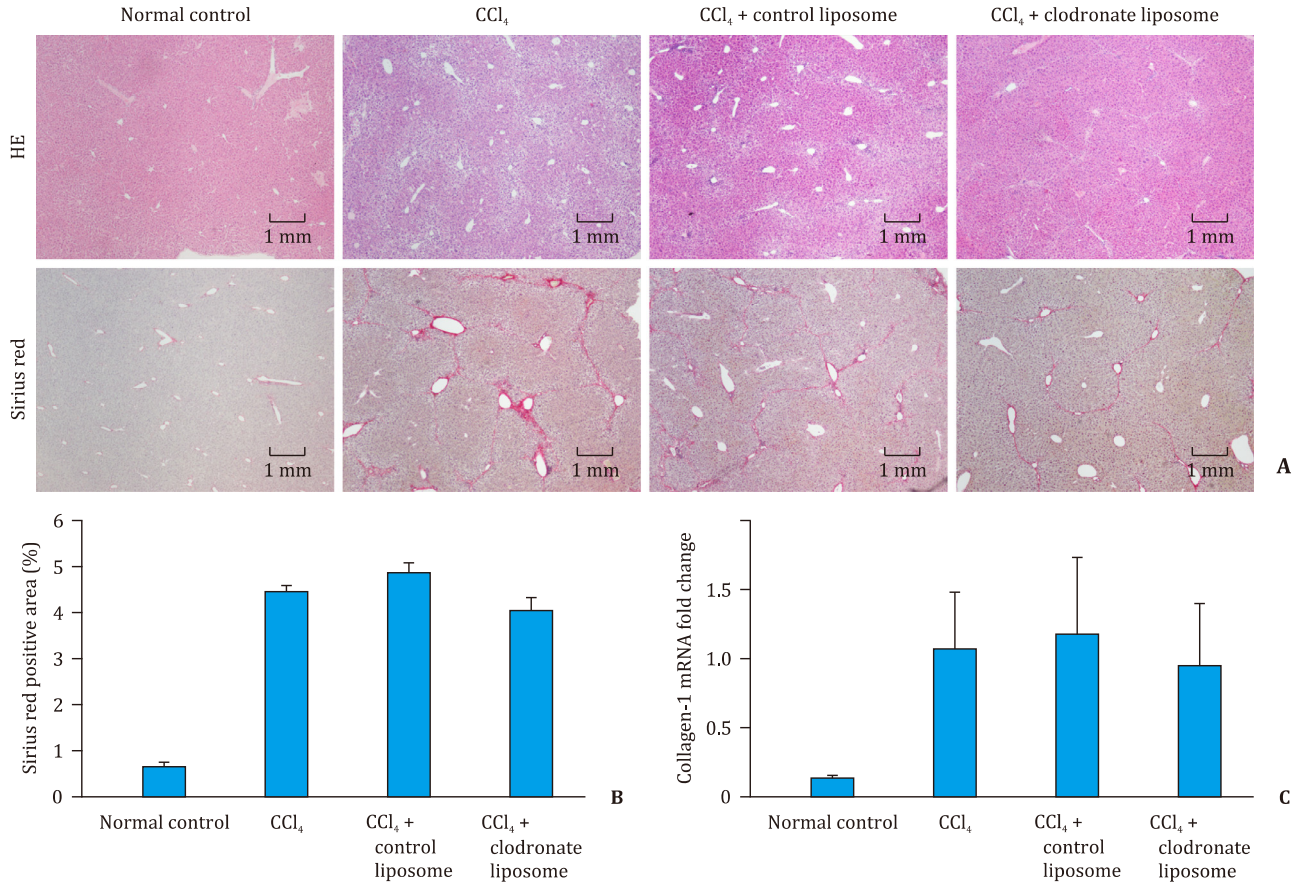

Fig. 3. Liver histological data obtained on day 28. **A**: Upper row: hematoxylin and eosin (HE) staining (original magnification × 40). Lower row: Sirius red staining (original magnification × 40); **B**: Semi-quantification of Sirius red-positive areas was performed by Image-J software; **C**: Quantitative real-time polymerase chain reaction was performed to evaluate collagen-I messenger RNA (mRNA) expression. Data are expressed as mean ± standard error.

fibrosis and collagen-I mRNA expression in the CCl_4-treated liver. These results suggest that fibrocytes had little contribution on liver fibrosis, and liver fibrosis is mainly induced by HSCs.

It was reported that depletion of HSCs using gliotoxin, which induce selective apoptosis of HSCs by subverting NF-κB-mediated survival, resulted in attenuation of liver fibrosis in CCl_4-treated mice [30]. Puche et al. [31] developed a novel mouse model for depleting HSCs using transgenic mice expressing the herpes simplex virus-thymidine kinase gene driven by the mouse GFAP promoter, which were used to render proliferating HSCs susceptible to killing in response to ganciclovir. This approach also resulted in marked attenuation of CCl_4-induced liver fibrosis. However, depletion of liver fibrocytes did not attenuate CCl_4-treated liver fibrosis in our study. This is because fibrocytes accounted for a small population of collagen-producing cells in the liver compared to HSCs.

In conclusion, monocyte-derived fibrocytes played a minimal role in CCl_4-induced liver fibrosis. Cells other than fibrocytes, such as HSCs, play a central role in liver fibrosis.

Contributors

OY and Shide K contributed to the study concept and design; TY, TM, KA, KT, Nakamura K, MT, KK, IH, HS, and Nagata K contributed to data analysis and interpretation; OY and Shide K contributed to drafting of the manuscript; Shide K and Shimoda K contributed to critical revision of the manuscript; TF and SA contributed to technical support; all authors have given final approval of the manuscript. Shide K is the guarantor.

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Ethical approval

This study was approved by the Animal Experiment Committee of the University of Miyazaki (2017-519).

Competing interest

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

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