J Toxicol Pathol 2022; 35: 149–157

Original Article

CD44 expression in the bile duct epithelium is related to hepatic fibrosis in nonalcoholic steatohepatitis rats induced by a choline-deficient, methionine-lowered, L-amino acid diet

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Abstract: Nonalcoholic steatohepatitis is a lifestyle-related disease and an increasing threat worldwide. Hepatic fibrosis, which results from chronic hepatic diseases including nonalcoholic steatohepatitis, is closely correlated with mortality among hepatic lesions, such as steatosis and inflammation. Thus, it is important to identify factors that can serve as diagnostic and therapeutic targets for hepatic fibrosis. In this study, we examined the function of CD44 in the development of hepatic fibrosis in choline-deficient, methionine-lowered, L-amino-acid diet-fed rats, especially with respect to the proliferation of bile duct epithelium. Male Fischer 344 rats were fed a choline-deficient, methionine-lowered, L-amino-acid diet for 2, 4, 13, or 26 weeks. This diet decreased the body weight; increased the levels of serum parameters indicating liver injury, such as aspartate and alanine aminotransferase; upregulated inflammation- and fibrosis-related gene expression in the liver; and resulted in the development of hepatic lesions, including fatty changes in hepatocytes, inflammatory cell infiltration, and fibrosis. Hepatic hyaluronan was synthesized and deposited in the liver tissue. The expression of both CD44 mRNA and protein was significantly increased throughout the experimental period. CD44 protein was observed in some of the bile duct epithelium, around which hyaluronic acid was deposited, and these bile duct lesions were concordant with the area of hepatic fibrosis. Thus, CD44 expressed in the bile duct epithelium may be a target for controlling nonalcoholic steatohepatitis-related hepatic fibrosis. (DOI: 10.1293/tox.2021-0069; J Toxicol Pathol 2022; 35: 149–157)

Key words: nonalcoholic steatohepatitis, fibrosis, hyaluronic acid, bile duct, choline

Introduction

Nonalcoholic fatty liver disease (NAFLD) is a liver phenotype associated with a metabolic syndrome that is divided into two categories: nonalcoholic fatty liver (NAFL), which is a simple fatty liver, and nonalcoholic steatohepatitis (NASH), which is characterized by hepatic inflammation and fibrosis. NAFLD is a major cause of liver disease with an increasing incidence worldwide[1]. In Japan, its prevalence was 29.7% between 2009 and 2010[2]. NAFLD/NASH is a threat to humans and should therefore be controlled on a mechanistic basis. The multiple parallel hit hypothesis has been proposed[3]. According to this hypothesis, various factors are implicated in pathogenesis and progression in parallel and include, but are not limited to, insulin resistance, oxidative stress, adipocytokines, nutritional factors, and the gut microbiome, which may be targets for disease control. Among NAFLD/NASH, the prevalence of the progressive form of the disease demonstrating severe hepatic fibrosis has increased[4], and the risk of mortality is closely related to the progression of hepatic fibrosis[5]. Hepatic fibrosis results from chronic hepatic diseases, including NASH, and its final stage is cirrhosis. A decisive therapy has not been established, and the only curative therapy currently available for cirrhosis is liver transplantation. Therefore, it is important to discover and develop preventive and therapeutic measures for hepatic fibrosis.

A rat model featuring chronic feeding with a choline-deficient, methionine-lowered, L-amino-acid-defined (CDAA) diet is one of the most widely used animal models

Received: 13 October 2021, Accepted: 22 November 2021
Published online in J-STAGE: 6 December 2021
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of NAFLD/NASH that can reproduce the phenotypic characteristics of the disease, especially prominent fibrosis. In the liver of non-genetically modified, intact male rats, the CDAA diet leads to hepatocellular fatty changes, inflammatory cell infiltration, and significant fibrosis, with extensive oxidative stress and a variety of signaling alterations, similar to the human counterpart of the disease[6, 7].

CD44 is involved in the infiltration of inflammatory cells and is known to play a role in liver regeneration[8, 9]. In addition, it serves as a marker of cancer stem cells and small hepatocytes and participates in tumor growth and spread[8, 10]. Recently, CD44 has also been reported to be a key factor in the development of NASH[11, 12]. Its expression in steatohepatitis induces the infiltration of macrophages and neutrophils via the interaction between CD44 and its ligand, hyaluronic acid[13]. Hyaluronic acid is a member of the extracellular matrix and is deposited in the fibrotic liver[14]. Serum hyaluronic acid is one of the markers of severe hepatic fibrosis and cirrhosis[15], and in the liver, CD44 is expressed in hepatic stellate cells which are closely related to hepatic fibrosis[16]. In severe lung fibrosis, CD44 regulates the invasive phenotype of fibroblasts through hyaluronic acid[17]. Taken together, the findings indicate that hyaluronic acid and CD44 are involved in liver fibrosis in NASH, but the underlying mechanisms remain unclear. While hepatic stellate cells are likely to be involved, the roles of other live constituents remain obscure.

Bile duct hyperplasia is observed in NASH patients with hepatic fibrosis[6]. Because severe hepatic fibrosis is frequently associated with the ductal reaction, such a reaction may be one of the factors contributing to hepatic fibrosis in NASH. In this context, the present study assessed the involvement of CD44 in hepatic fibrosis of CDAA diet-induced NASH in rats, with special interest in the bile duct epithelium.

Materials and Methods

Animals and treatments

A total of 36 male Fischer 344 (F344) rats 5 weeks of age were purchased from CLEA Japan Inc. (Tokyo, Japan) and housed at an average temperature of 23°C under air-controlled conditions in colony cages with a 12-h light/12-h dark cycle. The rats were allowed *ad libitum* access to food and water during both acclimation and treatment periods. At 6 weeks of age, the rats were randomly assigned to two groups, each consisting of 18 animals. One group received a standard diet (CE-2; CLEA Japan Inc.), and the other group was fed the CDAA diet (Research Diet Inc., New Brunswick, NJ, USA) for up to 26 weeks. Body weight, food consumption, and water intake were monitored weekly.

The rats in each group were sacrificed at the end of weeks 2, 4 (n=3), 13, and 26 (n=6) after overnight fasting. Before the scheduled sacrifice, blood samples were collected from the abdominal aorta of all animals to obtain serum samples for biochemical examinations. The rats were euthanized by exsanguination under isoflurane anesthesia. All organs were carefully studied during autopsy, and the liver was excised and weighed, if needed. Portions of the livers were immediately fixed in 10% neutrally buffered formalin for histopathological and immunohistochemical examinations, and the remaining samples were stored at −80°C for molecular biological assessments.

Serum biochemical examinations

Serum activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) and levels of triglyceride (TG) and total cholesterol (TC) were measured using an automatic analyzer (DRI-CHEM NX500V; Fujifilm, Tokyo, Japan).

Molecular biological examinations

Hepatic total RNA was extracted using Sepasol-RNA I Super G (Nacalai Tesque, Inc., Kyoto, Japan) and was reverse-transcribed to cDNA using ReverTra Ace qPCR Master Mix (Toyobo Co., Ltd., Osaka, Japan) and the Thermal Cycler Dice (Takara Bio Inc., Shiga, Japan). Quantitative real-time PCR (qPCR) was performed on the Thermal Cycler Dice Real Time System II (Takara Bio) using Thunderbird SYBR qPCR Mix (Toyobo Co., Ltd.). All procedures were performed according to the manufacturer's protocols. The primer sequences used for qPCR in this study are listed in Table 1.

Table 1. Real-time PCR Primers

	Forward Primer	Reverse Primer
MCP-1	CTTCCTCCACCACTATGCAGG	GATGCTACAGGCAGCAACTG
Collagen type 4	CTTCGTTGGCCTCTGTTTGC	TGCACTGGATTGCAAAAGGC
TGF beta	CCATGACATGAACCGACCCT	CTGCCGTACACAGCAGTTCT
CD44	TACTGGAGACCGGGATGACG	TGTTTCTGAGCTGTTGCATGG
HAS2	ACTGGGCAGAAGCGTGGATTATGT	AACACCCCAACCATCGGGTCTTCTT
HYAL2	ATCCTCCAAACACAGCCGCAA	ATGTGGGACGCGTGAAGACATACA
CK-19	GCCAGTACTTCAAGACCATC	ACTATTTCCTCCTCGTGGT
GAPDH	GTGCCAGCCTCGTCTCATA	AAGAGAAGGCAGCCCTGGTA

MCP1: monocyte chemotactic protein-1; TGF beta: transforming growth factor beta; CD44: cluster of differentiation 44; HAS2: hyaluronan synthase 2; HYAL2: hyaluronidase 2.

Histopathological and immunohistochemical examinations

Fixed liver samples were embedded in paraffin according to standard techniques and cut into 4-μm sections for hematoxylin-eosin (H&E) and Sirius Red (SR) staining.

In addition, immunohistochemical examinations were conducted using antibodies against cytokeratin 19 (CK19; 1:12000; Proteintech Japan, Inc., Tokyo, Japan), alpha-smooth muscle actin (α-SMA; 1:150; Abcam plc., Tokyo, Japan), and CD68 (1:100; Abcam plc.). Histofine Simple Stain Rat MAX-PO (MULTI) (Nichirei Bioscience Inc., Tokyo, Japan) was used as a secondary antibody, and the signals were visualized using 3,3′-diaminobenzidine (Wako Pure Chemical Industries, Ltd., Osaka, Japan). CD44 (1:100; Cell Signaling Technology, Inc., Danvers, MA, USA) and biotinylated hyaluronic acid-binding protein (HABP; 1:100, Sigma-Aldrich Japan K.K., Tokyo, Japan) were double-stained and visualized by immunofluorescence using Alexa Fluor 555 donkey anti-rabbit IgG (1:1000; Invitrogen, Thermo Fisher Scientific Inc., Waltham, MA, USA) and rhodamine streptavidin (1:100; Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA). Nuclear staining was performed with 4′,6-diamidino-2-phenylindole (SouthernBiotech, AL, USA). Using SR-stained specimens, the areas of fibrosis were measured using cellSens Dimension software (Olympus, Tokyo, Japan) in each period.

Statistical analysis

Statistically significant differences between the control and CDAA diet groups at each time point were determined using Welch-Aspin test. Statistical significance was set at a $p<0.05$.

Ethical considerations

All animal husbandry and experiments were conducted in compliance with the guiding principle of the Tokyo University of Agriculture and approved by the Animal Experiment Committee of the university. Consequently, this study complied with all related domestic and international laws, regulations, and guidelines. The animal experiments conducted in this study complied with the Animal Research: Reporting of In Vivo Experiments guidelines and were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments, and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978).

Results

Body and liver weights

Changes in the body, absolute, and relative liver weights are shown in Fig. 1. While body weight values were sequentially increased in both groups, those of the CDAA diet group were lower than those of the control diet group at each time point and after the end of week 2. Compared to that in the control diet group, the liver weight in the CDAA diet group was increased at the end of week 2. Although the absolute liver weight gradually decreased, it was higher in the CDAA diet group at weeks 2 and 4 and remained higher in the CDAA diet group until the end of weeks 4 and 26 than in the control diet group.

Serum biochemistry

Changes in the serum biochemical parameters are shown in Fig. 2. AST and ALT activities in the CDAA diet group were increased compared with those in the control diet group at the end of week 2. Although these values gradually decreased, AST and ALT activities remained higher in the CDAA diet group than in the control diet group until the end of weeks 13 and 26, respectively. AST activity was similar to that in the control diet group at week 26. TG and TC levels in the CDAA diet group tended to be lower than those in the control diet group at the end of week 2. While TG and TC levels gradually increased, they remained lower in the CDAA diet group than in the control diet group throughout the experimental period, and significant differences were observed at the end of weeks 13 and weeks 4 and 13 for TG and TC, respectively.

Hepatic gene expression profile

The changes in hepatic gene expression are shown in Fig. 3. The mRNA expression of monocyte chemoattractant protein-1 (*Mcp-1)* was higher in the CDAA group than in the control group at the end of week 2 and remained high until the end of week 26. Similarly, the mRNA expression of type IV collagen (*Col4a1*) and transforming growth factor beta *(Tgfβ)* was also higher in the CDAA group than in the control group at the end of week 2 and remained high until the end of week 26, after which it decreased.

The mRNA expression of *CD44* was higher in the CDAA group than in the control group at the end of week 2, and then prominently increased until the end of week 26. Regarding hyaluronic acid metabolism, the mRNA expression of hyaluronan synthase 2 (*Has2*) was higher in the CDAA diet group than in the control group at the end of week 13, but that of hyaluronidase-2 (*Hyal2*) was not affected by the CDAA diet. Regarding the bile duct epithelium, the mRNA expression of *CK19* in the CDAA diet group was higher than that in the control group at the end of week 4 and then further increased until the end of week 26.

Histopathological and immunohistochemical outcomes in the liver

The morphological changes in the liver are shown in Fig. 4. The CDAA diet induced NASH lesions, including fatty changes in hepatocytes, infiltration of macrophages, and hepatic fibrosis (Fig. 4A and B). Fatty changes in hepatocytes were observed within the first 2 weeks and maintained until the end of week 26. Infiltration of CD68-positive macrophages was also observed within the first 2 weeks (Fig. 4C and D) and progressed in a time-dependent manner until the end of week 26.

Hepatic fibrosis was slightly observed at the end of

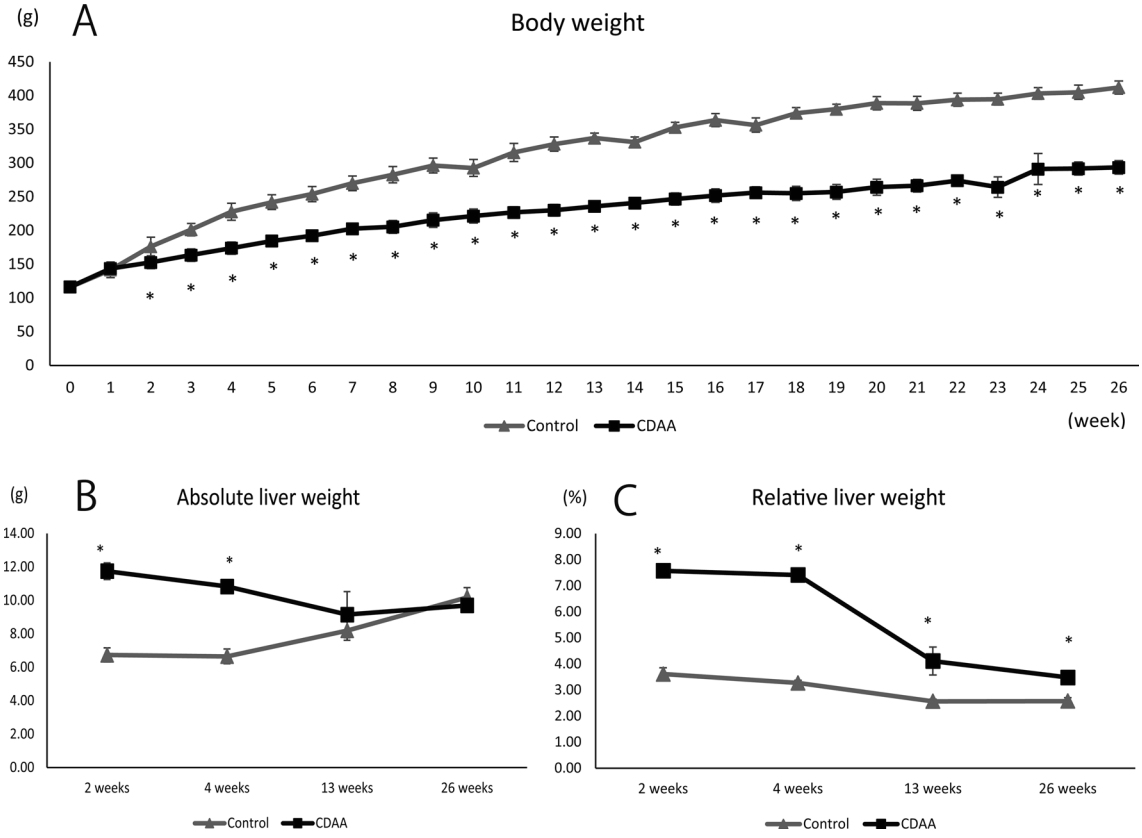

Fig. 1. Changes in body and liver weights. Changes in body weights (A), absolute liver weights (B), and relative liver weights (C) during the experiment. Data are presented as mean ± standard deviation. *Significantly different from the control group at the same time point.

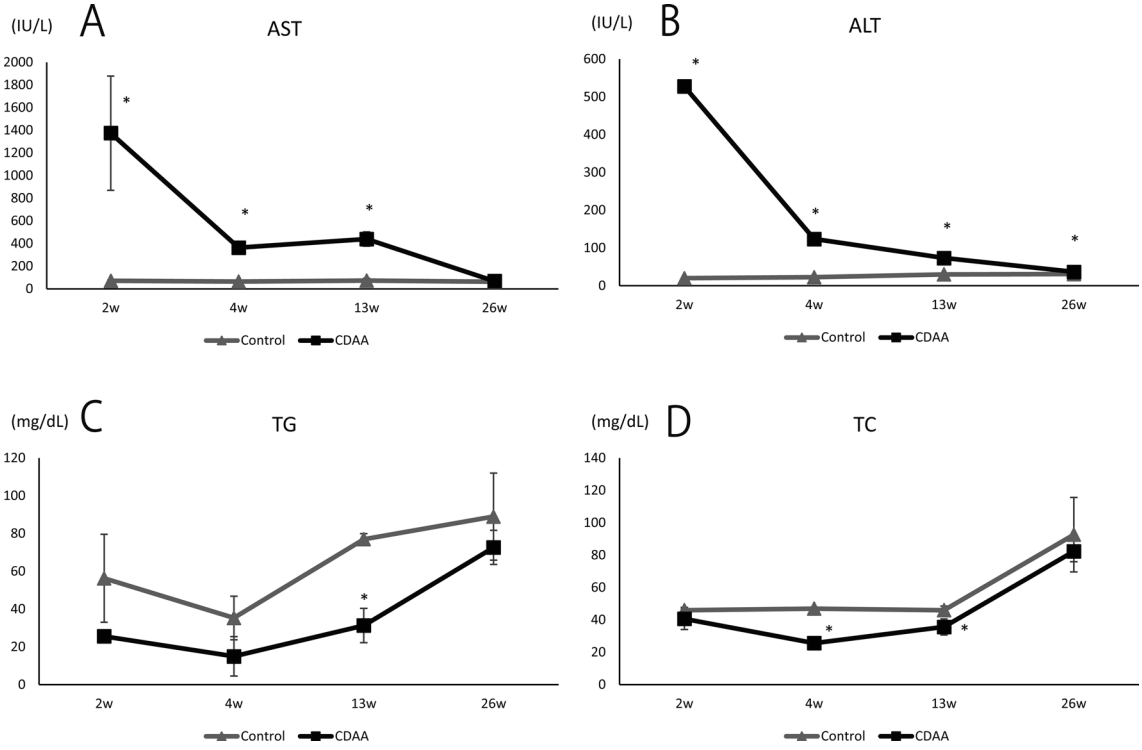

Fig. 2. Serum AST, ALT, TG, and TC changes. Changes in serum activities of AST (A) and ALT (B) and levels of TG (C) and TC (D). Data are presented as mean ± standard deviation. *Significantly different from the control group at the same time point.

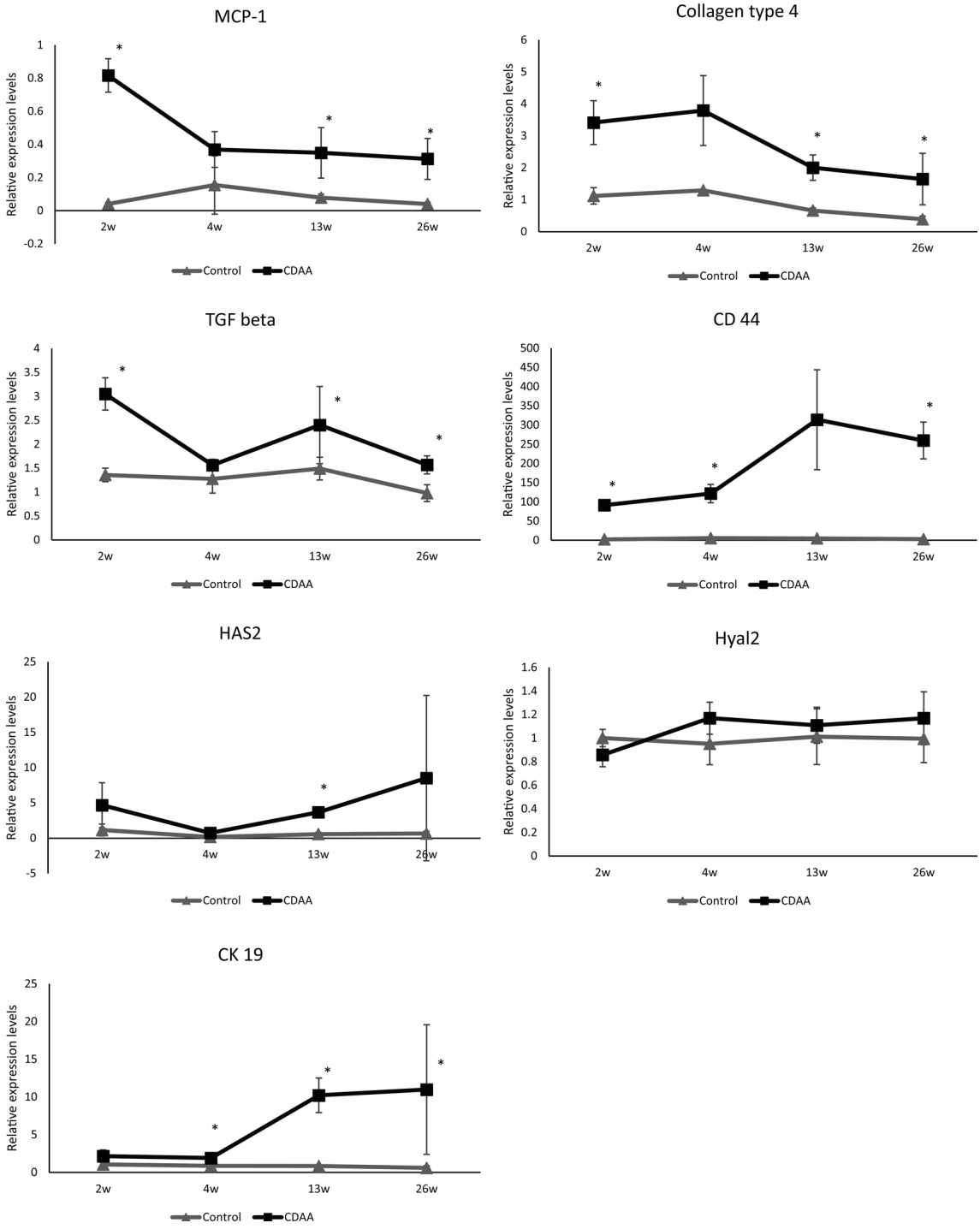

Fig. 3. Changes in hepatic gene expression. Changes in hepatic mRNA expression of *Mcp-1*, *Col4a1*, *Tgfβ*, *CD44*, *Has2*, *Hyal2* and, *CK19*. Data are presented as mean ± standard deviation. *Significantly different from the control group at the same time point.

week 4 and progressed to form the bird's nest-like appearance at the end of weeks 13 and 26. Correspondingly, the SR-positive fiber area in the liver slightly increased at the end of week 4 and significantly increased at the end of weeks 13 and 26 in the CDAA diet group compared with the control diet group (Fig. 4J). In addition, the activation of liver stellate cells was detected in the liver of the CDAA diet group at the end of week 2 based on the protein expression of α-SMA (Fig. 4F).

In the liver of the CDAA diet group, CD44 was immunohistochemically detected in lymphocytes and macrophages from early time points as part of the inflammatory sequence (Fig. 4E). At the later time points, CD44 was also present in bile duct epithelial cells, as confirmed by CK19

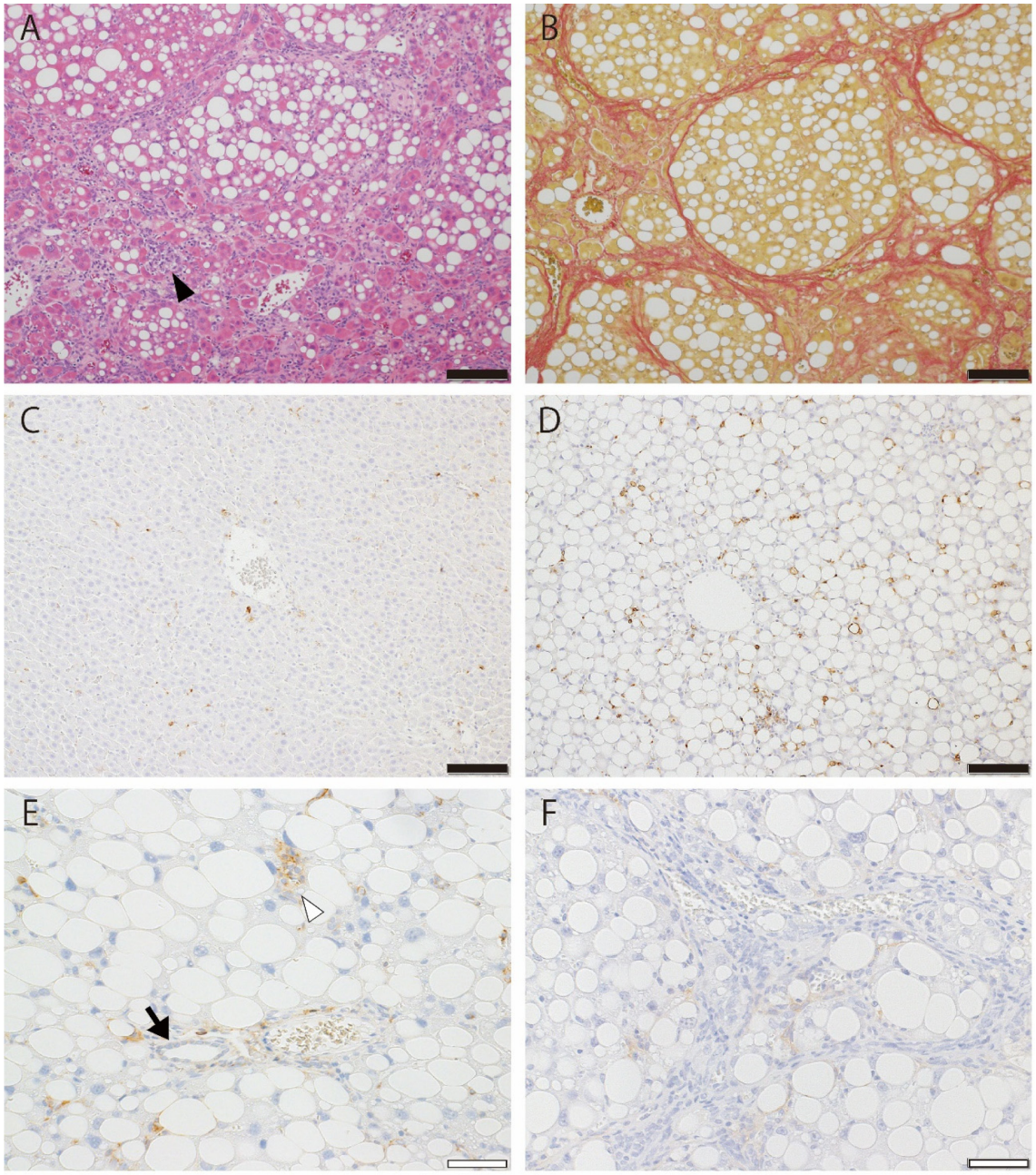

Fig. 4. Continued.

immunohistochemistry. Among the sequentially increased bile ducts as indicated by increased expression of the marker CK19 (Fig. 4G and H), CD44 and CK19 double-positive cells appeared concordantly with the fibrotic area (Fig. 4D). HABP was detected in the area surrounding the CD44-positive bile ducts (Fig. 4I), which supports the role of CD44 in the fibrotic sequence.

Discussion

NASH is a lifestyle-related disease that is considered life-threatening for humans. Therefore, controlling the disease by applying prevention and treatment strategies is important. However, it is difficult to facilitate the control of NASH as the precise underlying mechanisms have not yet been elucidated, making it difficult to develop a concrete treatment strategy. NASH exhibits diverse hepatic lesions, such as lipid accumulation in hepatocytes, infiltration of lymphocytes and macrophages, fibrosis resulting in cirrhosis with an increase in the extracellular matrix, and cancer. Among them, fibrosis/cirrhosis has received much attention[18]. Thus, it is important to assess the mechanisms underlying NASH-associated hepatic fibrosis and to identify relevant factors that can serve as preventive, diagnostic, and/or therapeutic targets.

In the CDAA diet group, the liver weights were in-

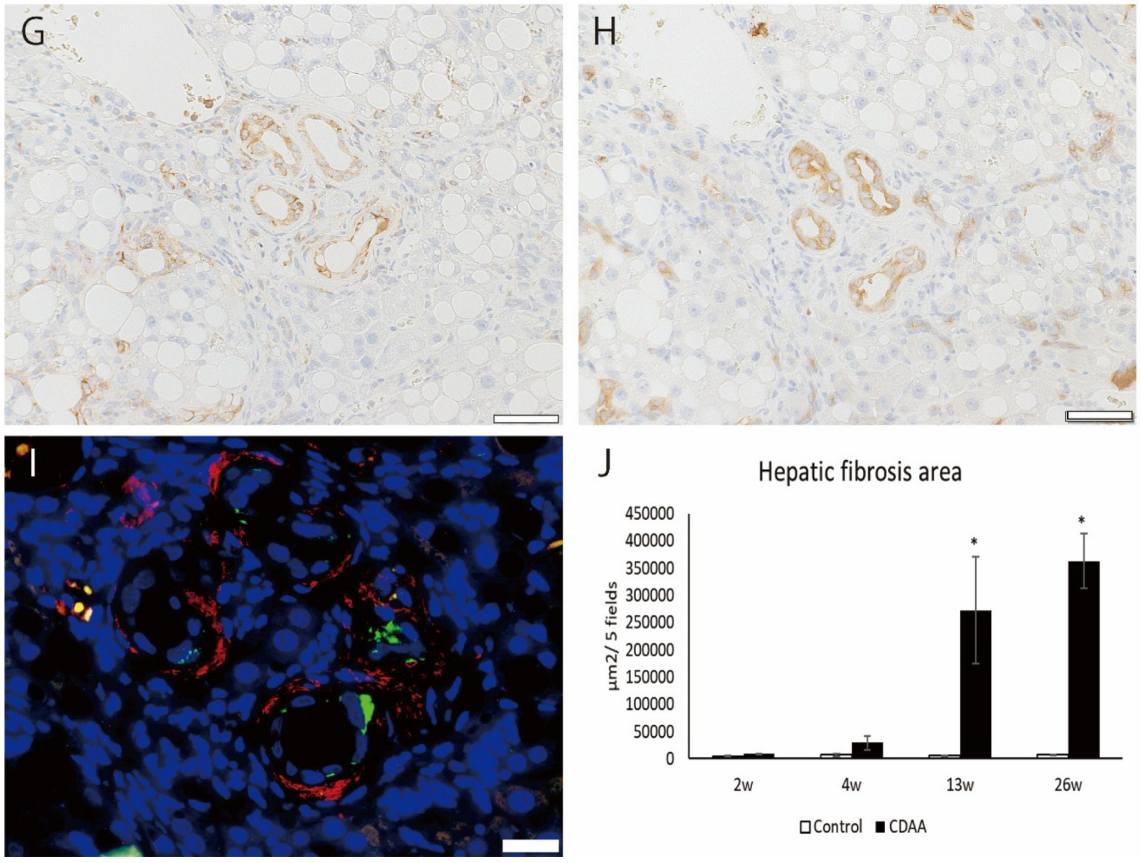

Fig. 4. Morphological changes in the liver. Representative outcomes in the liver of the CDAA diet group at week 13 for H&E (A) and SR (B) staining; black arrowhead, inflammatory cells infiltration, Representative CD68 immunohistochemistry of the control (C) and CDAA diet (D) groups at the end of week 2. Representative CD44 immunohistochemistry of CDAA diet group at the end of week 4 (E); black arrow: CD44-negative bile duct epithelial cell; white arrowhead: CD44-positive inflammatory cells. Representative outcomes in the liver of the CDAA diet group at the end of week 13 for α-SMA (F), CD44 (G), and CK19 immunohistochemistry (H), and immunohistochemical/fluorescent double staining for CD44 (green) and HABP (red; nucleic stain in blue) (I). The fibrotic area in the SR-stained liver in each period (J). Data are presented as the mean ± standard deviation. *Significantly different from the control group at the same time point. Black scale bar, 100 μm; white bar, 50 μm, and immunohistochemical/fluorescent double staining scale bar, 20 μm.

creased, serum TG and TC levels were decreased, and fatty liver developed at early time points. These changes reflect the altered lipid metabolism caused by the CDAA diet, such as the inhibition of synthesis and release of very low-density lipoprotein (VLDL)[19, 20]. A rat model of a choline-deficient, methionine-lowered diet is often criticized for being disadvantageous for human extrapolation because human NAFLD is thought to be mainly due to the enhancement of *de novo* lipogenesis. However, the inhibition of synthesis and release of VLDL from the liver can also serve as a key factor in the pathogenesis of human NASH[21]. The CDAA diet rat model in the present study is indeed useful for assessing the mechanisms underlying NASH and exploring evidence-based strategies to control the disease.

The CDAA diet induced the infiltration of CD68-positive macrophages and inflammation, as indicated by the upregulation of *MCP-1* mRNA in the liver of rats within 2 weeks, in association with an increase in serum AST and ALT activities. CD44 recruits macrophages and polarizes them to express a pro-inflammatory phenotype, which has been reported to be involved in the progression from NAFL to NASH[12]. In the present study, CD44 was expressed in the infiltrated inflammatory cells, with mRNA upregulation in the liver of rats fed the CDAA diet. Thus, CD44 is induced in the early phase as part of the hepatic inflammatory sequence.

Following early inflammation, hepatic fibrosis was observed in the CDAA diet group at the end of week 4 and progressed thereafter. Genes related to fibrosis, such as *COL4A1* and *TGFβ*, were upregulated in the liver before there was a histological manifestation of fibrosis and remained high throughout the experiment. CK19-positive bile ducts appeared and proliferated in association with hepatic fibrosis, and CD44 was expressed in the proliferated bile ducts, especially those in the fibrotic area. Hepatic injury induced by the CDAA diet was one of the reasons for the increased bile ducts. The CDAA diet is known to cause hepatocellular injury and causes liver regeneration[6]. Hepatic stem cells are

activated during liver regeneration, and oval cells, which are a hepatic stem cell population, have the potential to differentiate into both hepatocytes and bile duct epithelial cells[22]. It is therefore suggested that CD44 plays a role in the fibrotic sequence by virtue of bile duct proliferation during liver regeneration in CDAA-induced NASH rats. It has been reported that CD44-positive bile ducts are increased by hepatobiliary dysfunction and that the interaction between CD44 and its ligand hyaluronic acid is involved in biliary epithelial cell proliferation[23]. CD44 is a major receptor of hyaluronan, and its binding is enhanced during hepatic inflammation and fibrosis[17]. During fibrosis, hepatic stellate cells produce hyaluronan via activation of Has2[14]. In the present study, hepatic stellate cells were activated, and *Has2* mRNA expression was upregulated in the livers of rats that were fed the CDAA diet. These data, with unchanged *Hyal2* mRNA expression, indicate increased synthesis and deposition of HA. Furthermore, the decline in liver function due to advanced liver fibrosis may have induced a decline in overall hyaluronan resolution. Overproduced hyaluronan was deposited in the surrounding CD44-positive bile ducts in the fibrotic area, as demonstrated by HABP immunohistochemistry. Taken together, CD44 expression was increased throughout the period in the CDAA diet group. In all experimental periods, CD44 expression in inflammatory cells was observed, which indicated a relationship with upregulation of the expression until week 26. In addition, there was enhanced expression of CD44 in the bile ducts with advanced liver fibrosis. These results indicate that the expression of CD44 in the bile duct epithelium may be involved in the mechanisms underlying CDAA-induced hepatic fibrosis. Therefore, CD44 could be a target for controlling NASH-related hepatic fibrosis.

Disclosure of Potential Conflicts of Interest: The authors have no conflicts of interest directly relevant to the content of this article.

Acknowledgment: The present study was supported in part by the Tokyo University of Agriculture. The authors thank the members of our laboratories, with special thanks to Ms. Marika Matsumoto for her excellent assistance. We also want to thank our other co-authors in the present study for their discussion and helpful comments. We would like to thank Editage (www.editage.com) for editing the manuscript for language.

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