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Transient Expression of Bone Morphogenic Protein-2 in Acute Liver Injury by Carbon Tetrachloride

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Acute liver injury induced by administration of carbon tetrachloride (CCl_4) was shown to be a model of wound-repair in rat liver. Albumin gene expression was significantly reduced at 24 h post injection with CCl_4, but recovered at 48 h. We also observed significant and transient expression of bone morphogenic protein-2 (BMP-2) at 6-24 h post treatment. This expression was also shown with depletion of Kupffer cell by $GdCl_3$, and immunostaining with anti-BMP-2 antibody showed BMP-2-producing cells interspersed in intralobular spaces of injured liver. These observations suggest that BMP-2 secreted from oval-like cells plays important roles in the wound healing response of injured liver.

Key words: BMP-2, carbon tetrachloride, gene expression, liver injury, wound-repair response.

Administration of carbon tetrachloride (CCl_4) hepatotoxicant to rodents is widely used to study the mechanisms of hepatic injury. CCl_4 is metabolized in mature hepatocytes by cytochrome P450 IIE1 (*1*) and converted to trichloromethyl radical, resulting in cell death with accumulation of lipid peroxidation and intracellular calcium ions. The wounded tissue induces a repair system through secretion of hepatocyte growth factor (HGF) from Kupffer or endothelial cells (*2*). HGF activates cell proliferation and differentiation of survived hepatocytes in wounded liver as well as in liver regeneration in partial hepatectomy (*3, 4*). In hypoplastic liver injury induced by administration of CCl_4 as well as the Solt-Farber regime by administration of 2-acetylaminofluorene (2-AAF) and partial hepatectomy, proliferation and differentiation of hepatocytes are induced in oval cells, hepatic stem-like cells (progenitor cells) derived from monocytes (*5–8*). Therefore, it is of interest to examine hepatogenesis in early embryos in connection with repair of wounded liver.

Bone morphogenetic protein-2 (BMP-2), which belongs to the transforming growth factor-β superfamily and is initially identified as a morphogenic factor in bone, is known to have a wide potential in development of not only bone but also numerous organs such as skin and the nervous system (*9*). In hepatogenesis in early embryos, BMP-2 signalling appears critical for morphogenetic growth of the hepatic endoderm into a liver bud (*10–12*). Hence, BMP-2 is thought to be involved in determination of stem (progenitor) cell fate, however, it is not known whether BMP-2 is related to regeneration of liver injury in adult rats. In the present study, we determined the expression of BMP-2 plus other factors involved in hepatogenesis in liver injury induced by CCl_4.

MATERIALS AND METHODS

Animals—Thirty male Wistar rats weighing 150 g were purchased from Nihon Crea (Japan). Acute liver injury was induced by intraperitoneal injection with 2 ml of CC14: olive oil (1:1 mixture) per kg of body weight then rats were sacrificed at 3, 6, 12, 24, 36 or 48 h, post injection for immunohistochemistry and total RNA preparation. Liver cirrhosis (chronic injury) was induced by intraperitoneal injection with 1 ml of $CC1_4$: olive oil (1:1 mixture) per kg of body weight twice a week for 4–8 weeks as described previously (*13, 14*). Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities in serum from the treated rats were determined by assay kits (Wako Pure Chemicals Co., Ltd., Tokyo, Japan). Depletion of macrophages from the rats was performed by gadolinium chloride ($GdCl_3$) administrated intraperitoneally (10 mg per kg of body weight) 24 h prior to CCl_4 treatment.

Parenchymal hepatocytes and non-parenchymal cells were isolated by *in situ* perfusion of the rat liver with 0.05% Collagenase (Sigma-Aldrich Co., St Louis, USA) as described elsewhere (*15*) and separated by differential centrifugation. All rats were fed at libitum and received human care in compliance with Tottori University's guidelines for the care and use of laboratory animals in research.

Cells—The rat oval cell line OC/CDE22 and mouse macrophage cell line RAW 264.7 were obtained from the American Type Culture Collection (ATCC). The rat myofibroblast cell line MFBY2 was isolated in our previous study (*16*), and the rat hepatoma cell lines Fao and C2 were obtained from Dr Mary C. Weiss,

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Table 1. **Nucleotide sequences of primers and PCR conditions in this study.**

Amplicon	Primers (forward/reverse)	Annealing temperature (°C)/cycle
BMP-2	5'-ACTGCGGTCTCCTAAAGGTC–3'/ 5'-TTTGCTGTACTAGCGACACC-3'	65/29
BMP-4	5'-CCTTTCCAGCAAGTTTGTTC–3'/ 5'-CCTCCGGACTGCCTGATTTC -3'	57.4/45
BMP-7	5'-CGCCGTCATTCCGGATTACAT–3'/ 5'-GGCCCAATCTCCACTCCCTT -3'	60/40
BMPR-1A	5'-AGCAGGACCAGTAATCAAGG-3'/ 5'-TGCAGCTATAAAACCAAGTA-3'	57/30
CD14	5'-TGTGGGACACGGAAGCAAATC-3'/ 5'-GGTGGCAAGGGCAGGGAAGA-3'	58/20
FGF-2	5'-CTCGGTCTCTCGGCTTCAGG-3'/ 5'-GCTCTACCACAGGGGACTGC-3'	60.3/30
GAPDH	5'-AAGGCTGTGGGCAAGGTCAT-3'/ 5'-CACCACCCTGTTGCTGTAGC-3'	60/24
GATA-4	5'-GTCCCAACTGCCAGACTACC-3'/ 5'-AGCCTTGTGGGGACAGCTTC-3'	58.3/25
HEX	5'-GCGGAGCAGCCATGCAGTTC-3'/ 5'-TCCCAGTTTGTCTGTAGCAG-3'	58/23
HGF	5'-TTATGGGGAATGAGAAATGC-3'/ 5'-CCACGACCAGGACACATGAC-3'	58/24
TNF-α	5'-ATGAGCACGGAAAGCATGATC-3'/ 5'-TCACAGAGCAATGACTCCAAA-3'	60/30

Institute Pasteur, France (*17, 18*). Cell lines except RAW 264.7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 150 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2. RAW 264.7 cells were maintained in α-modification of Eagle's medium (α-MEM).

RNA Preparation, Northern Blot Analysis and RT-PCR—Total RNAs were isolated from rat tissues and cell lines by acid phenol-guanidium thiocyanate-chloroform extraction as described previously (*19*). Northern blot analysis was performed as described by Goldberg. Briefly, 10μg of denatured total RNA was electrophoresed in 1% agarose gel and then transferred to Hybond-N^+ nylon membranes (Amersham Pharmacia Biotech, USA). The membranes were hybridized with cDNA probes labelled using the random primer method. The hybridized signals were detected using the BAS 1000 BioImage Analyzer (Fuji Film, Tokyo, Japan). Reverse transcription-polymerase chain reaction (RT-PCR) was carried out as described previously (*16*). One microgram of total RNA was converted to complementary DNA and amplified using the GeneAmp RNA PCR kit (Applied Biosytems Japan, Tokyo) in a PCR Thermal Cycler MP (TaKaRa Shuzo Kyoto, Japan). The primer sets used are shown in Table 1.

Western blot analysis—BMP-2, Smad1, and phosphorylated Smad1 (pSmad1) were determined by Western blot analysis as described previously (*20*). Fifty micrograms of lysates prepared from the livers of CCl_4-injured rats were separated on SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA). BMP-2 was detected with rabbit polyclonal antibody against human BMP-2/4 (Santa Cruz Biotechnology, Inc. CA, USA). Total Smad1 and activated Smad1 (phosphorylated) were detected with rabbit polyclonal antibody against human Smad1 (Cell Signaling Technology, Beverly, MA) and rabbit monoclonal antibody against human phospho-Smad1/5 (Cell Signaling Technology, Beverly, MA), respectively. Immunoactive protein bands were visualized using horseradish peroxidase-linked secondary anti-rabbit IgG antibody (Amersham Pharmasia Biotech, Buckinghamshire, UK) and ECM-peroxidase detection system (Amersham Pharmacia Biotech) according to the manufacture's instructions.

Immunohistochemistry—Livers were fixed in 10% paraformaldehyde and embedded in paraffin. Sections were then treated with 3% (v/v) H_2O_2 for 10 min at room temperature (RT) and blocked with 10% (v/v) goat serum or rabbit serum (Nichirei, Tokyo, Japan) for 30 min at RT. The sections were then incubated with rabbit polyclonal antibody against human BMP-2/4 (Santa Cruz Biotechnology, Inc. CA, USA) diluted 1:200 and mouse monoclonal antibody against rat Keratin19 (CHEMICON International, Inc. CA, USA) diluted 1:100, at 4°C overnight. The sections were then washed with PBS and incubated for 30 min with biotinylated goat anti-rabbit IgG (Nichirei) or biotinylated rabbit anti-mouse IgG (Nichirei) before being rewashed with PBS and incubated with a solution of avidin-conjugated horseradish peroxidase (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA, USA) for 15 min according to the manufacturer's recommendations, and then washed again with PBS for 5 min. Peroxidase activity was detected with H_2O_2/diaminobenzidine (DAB) substrate solution and the sections were counterstained with haematoxylin before dehydration and mounting.

RESULTS

Transient induction of BMP-2 in the early stage of acute liver injury by CCl_4—Mature hepatocytes in the rat liver were wounded by reactive oxygen species (ROS)

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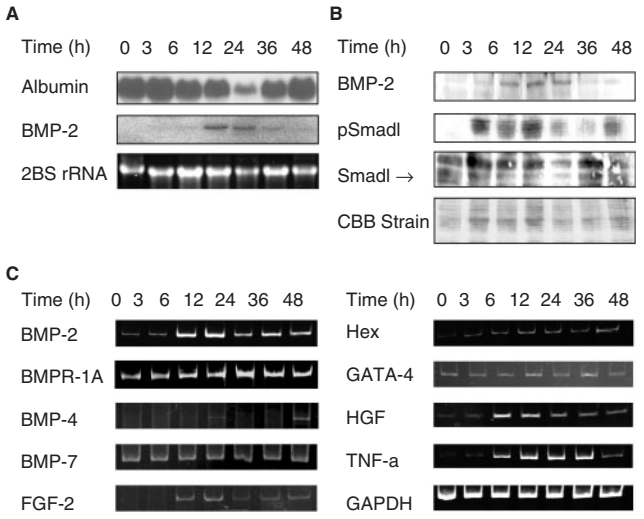

Fig. 1. **Expression of various genes in the response to CCl_4 liver injury. The time after CCl_4 injection is indicated at the top of the panels.** (A) Northern blot analysis was performed using albumin and BMP-2 cDNA as probes. (B) Western blot analysis of the injured liver using antibodies against BMP-2, Smad1 and phosphorylaed Smad1. CBB Stain was indicated controls for loading of proteins. (C) RT-PCR analysis of BMP-2, BMP receptor 1A (BMPR-1A), BMP-4, BMP-7, FGF-2, HEX, GATA-4, HGF and TNF-α was performed using primer set shown in Table 1.

generated during detoxication of the intraperitoneally injected CCl_4, resulting in a reduction in various hepatic functions. This acute liver injury was also observed using albumin gene expression as a liver function marker (Fig. 1A), as shown in a previous study (*13*). Albumin gene expression was significantly decreased at 24 h post treatment with CCl_4, followed by amelioration to a normal level at 48 h. Acute liver injury induced by CCl_4, therefore, provides a suitable animal model for examining the wound and repair response in liver injury.

Previously, HGF was reported to be involved in the wound healing response in injured liver *(21–23)*. The HGF produced by non-parenchymal cells such as Kupffer and hepatic stellate cells induces cell proliferation and differentiation in hepatocytes *(2, 24, 25)*. However, much less is known about regeneration of hepatocytes from immature tissue-specific stem-like cells in adult liver. We, therefore, determined BMP-2 expression in our model of liver injury in adult rats, because BMPs secreted from the cardiac mesoderm are involved in differentiation of hepatocytes in the liver bud during early embryogenesis. Interestingly, BMP-2 mRNA expression determined by Northern blot analysis was significantly and transiently induced at 12 to 24 h post injection of CCl4, but scarcely observed in normal-state liver (Fig. 1A). This observation was confirmed by Western blot analysis using anti-BMP-2 antibody (Fig. 1B). Activation of Smad1 in the injured liver was determined by Western blot analysis using antibodies against Smad1 and pSmad1 (Fig. 1B). As the result, induction of pSmad1 was observed before BMP-2 was increased. It is possible that rapid phosphorylation of Smad1 at 3 h post treatment was induced by protein(s) other than BMP-2, followed by activation of Smad1 by BMP-2. These results raise the possibility of BMP-2 involvement in the wound healing response in injured liver of adult rats.

Transient expression of genes involved in hepatogenesis and liver regeneration—The observed transient expression of BMP-2 during hepatogenesis in injured adult rat liver evoked our interest in expression of other genes involved in hepatogenesis and liver regeneration. Like BMP-2, FGF-2 generated and secreted by the cardiac mesoderm and septum transversum mesenchyme plays important roles in cell proliferation, patterning and morphogenesis of hepatic endoderm (ventral endoderm) of early embryos (*11, 12, 26*). Coincidentally with BMP-2, transient expression of FGF-2 was observed at 6–12 h post treatment with CCl_4 in injured adult rat liver (Fig.1C). In contrast, expression of BMPR-1A, which constitutes a receptor complex against BMP2 with BMPR-2, was constitutively activated in spite of treatment with CCl4. BMP-4 was quite a little induced and BMP-7 was constantly expressed in the liver injury (Fig. 1C). Furthermore, the divergent homeobox protein Hex and transcription factor GATA4 were expressed in foregut ventral endoderm and shown to be involved in hepatogenesis. Expression of Hex was slightly induced at an early stage of liver injury then maintained at this level. GATA-4, on the other hand, was expressed at a low level in both normal and injured livers (Fig. 1C). These results suggest that injury with the hepatotoxin might induce regeneration through a similar process as hepatogenesis in early embryos, although BMP-2 was involved in this process rather than BMP-4. Nevertheless, wound healing by HGF was also observed in the injured liver and induction of pre-inflammatory cytokine TNF-α production was indicated (Fig. 1C).

Characteristic induction of BMP2 in CCl_4 injury—We also attempted to reveal whether the transient expression of BMP-2 in liver injury is characteristic of acute liver injury induced by CCl_4. To address this question, we examined BMP-2 expression in chronic liver disease (cirrhotic liver) and in regenerating liver after partial hepatectomy. Cirrhotic liver was induced by intraperitoneal injection of CCl_4 twice a week for 5 or 7 weeks, and regenerating liver was generated by removal of two thirds of the whole liver. As shown in Fig. 2A, transient expression of BMP-2 was observed only in the acute liver injury at 12–24 h, not in the cirrhotic liver or regenerating liver, suggesting that BMP-2 is induced in the early stages of acute liver injury, but not in regenerating liver following partial hepatectomy.

To identify the BMP-2-producing cells in the injured liver, we fractionated liver cells obtained by collagenase perfusion into parenchymal and non-parenchymal cells by differential centrifugation. Prospectively, no BMP-2 mRNA was detected in parenchymal and non-parenchymal cells from normal rat liver, while the BMP-2 expression was observed in non-parenchymal cells from the injured liver, not in parechymal cells. This small cell fraction contains non-parenchymal cells such as Kupffer cells, hepatic stellate cells, hepatic

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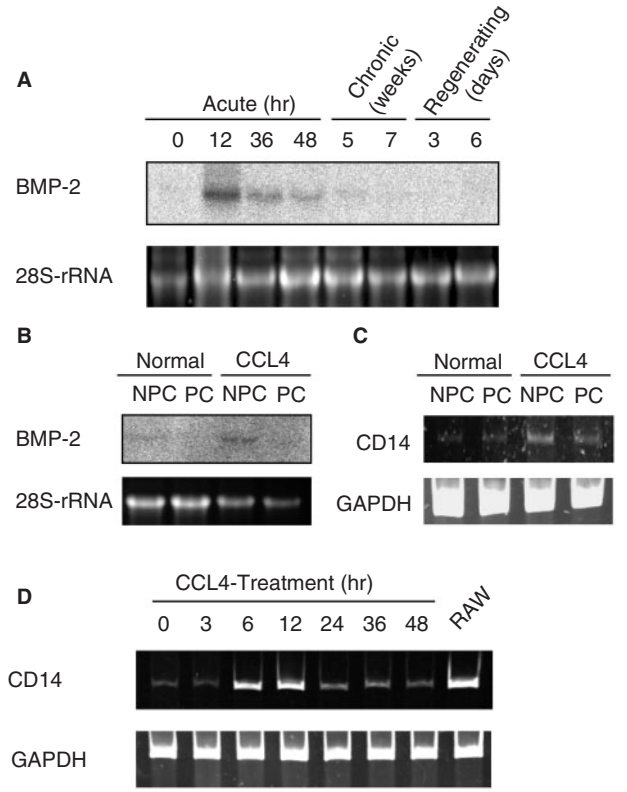

Fig. 2. **Expression of BMP-2 and CD14 mRNA in liver injury.** (A) Representative patterns of BMP-2 mRNA expression are shown by Northern blot analysis using the acute injured liver by CCl_4 exposure (Acute), the cirrhotic liver (Chronic) the regenerating liver generated by removal of two third of whole liver (Regenerating). The times after each treatment are indicated at the top of the panel. (B) (C) BMP-2 and CD14 mRNA expression of in isolated liver cells were determined by Northern blot analysis and RT-PCR analysis. Liver cells from normal rat (Normal) and CCl_4-treated rat (CCl_4) were separated to non-parenchymal cells (NPC), and parenchymal cells (PC) by differential centrifugation. (D) CD14 mRNA expression depend on the time CCl_4 treatment. RAW means mRNA from RAW 264.7 mouse macrophage cell line.

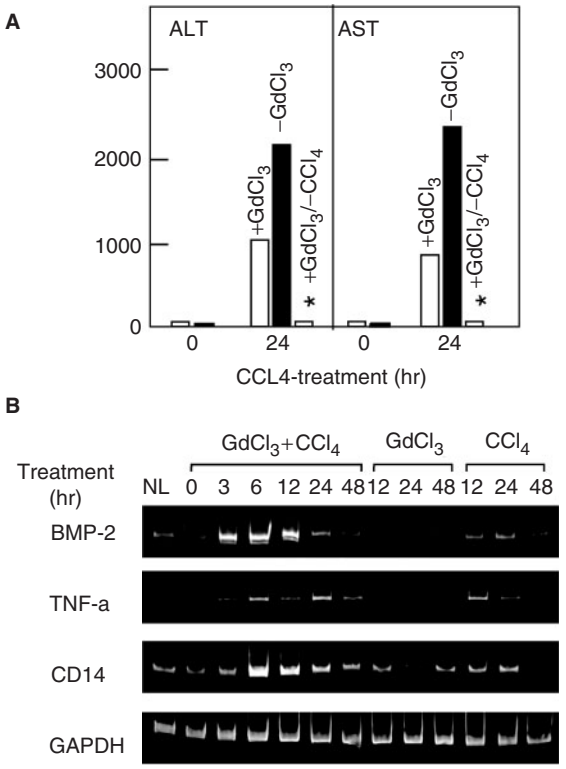

Fig. 3. **Effects of $GdCl_3$ treatment prior to CCl_4 exposure.** (A) Serum ALT and AST activities were determined with bloods from CCl_4-treated rats for 0 and 24 h with or without $GdCl_3$-pre-treatment (open column or dark column, respectively). As a control experiment, rats were administrated with $GdCl_3$ for 24 h followed 24 h pre-treatment (totally 48 h; * + $GdCl_3$ -CCl_4) (B) The effect of $GdCl_3$ treatment on expression of BMP-2, TNF-α, and CD14 mRNA. Rats ($GdCl_3 + CCl_4$) were administrated with CCl_4 for indicated time followed $GdCl_3$ pre-treatment for 24 h. Rats ($GdCl_3$) were continued with $GdCl_3$ treatment for indicated time followed $GdCl_3$ pre-treatment for 24 h. And rats (CCl_4) were treated CCl_4 for indicated time without pre-treatment. NL shows RNA from normal rat liver.

endothelial cells, small hepatocytes and other small cells derived from circulating blood cells. These results suggest that the non-parenchymal cells in injured liver produce and secrete BMP-2.

Participation of macrophage or Kupffer cell in production of BMP-2—Non-parenchymal cells fractionated with differential centrifugation included various small cells such as Kupffer cells, which are resident in normal liver, and macrophages, which infiltrated into the injured liver (*23, 27*). Interestingly, CD14 mRNA, which is a marker of macrophage cell lineage, was expressed in the non-parenchymal cell fraction (Fig. 2C). Moreover, expression of CD14 was observed at 6–12 h post-treatment with CCl_4 in line with BMP-2 expression (Fig. 2D). The association between expression of BMP-2 and CD14 suggests that activated Kupffer cells or macrophage cells infiltrated injured liver express BMP-2 and are involved in wound healing. This postulation was confirmed by depletion of macrophage cell lineages including Kupffer cells by pretreatment with $GdCl_3$ for 24 hr prior to CCl_4 administration (*28*). As shown in Fig. 3A, the liver-specific cytosolic enzyme activities of ALT and AST in the blood of the rats increased by CCl_4 injection; however, the ALT and AST activities were suppressed with depletion of macrophages by pre-treatment with $GdCl_3$. Injection of $GdCl_3$ did not influence liver function. In contrast to our expectation that depletion of macrophages by $GdCl_3$ may decrease BMP-2 expression, the BMP-2 mRNA level was significantly increased at 6–12 h post injection with CCl_4 in $GdCl_3$-pre-treated rat liver (Fig. 3B). Administration of $GaCl_3$ itself showed no effect on the expression of BMP-2. Interestingly, in the rat liver pre-treated with $GdCl_3$, CD14 and TNF-α were induced by CCl_4 injection, and likewise in the non-pre-treated liver (Fig. 3B). These results suggest that macrophages or Kupffer cells do not produce BMP-2, and that cells other than macrophages express BMP-2 and/or CD14.

Involvement of oval cells in BMP-2 production—To clarify the BMP-2 producing cells in injured liver,

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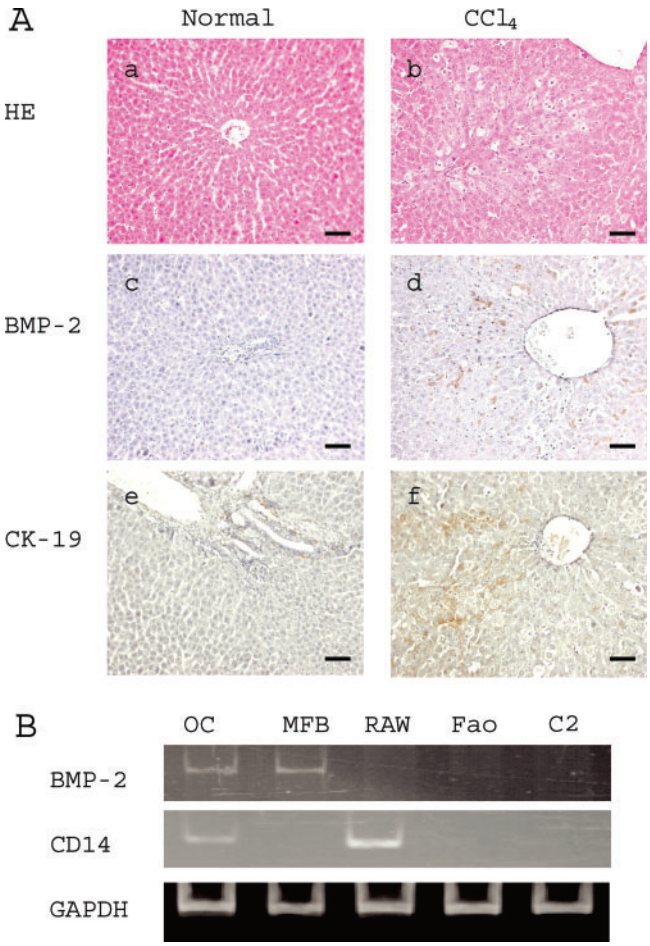

Fig. 4. **Localization of BMP-2 producing cell.** (A) Immunohistological analysis of normal (a, c, e) and CCl_4-injured liver (b, d, f). Injured liver was induced by CCl_4 exposure for 24 h. These sections were stained with haematoxylin and eosin (a, b), and immunostained with anti-BMP-2 antibody (b, d) and cytokeratin-19 (CK-19)(e, f). The scale bar is indicated 100 μm. (B) RT-PCR analysis of BMP-2 and CD14 expression were performed by use of oval cell line OC/CDE22 (OC), myofibroblast cell line MYBY2 (MFB), macrophage cell line RAW 264.7 (RAW) and rat hepatocyte cell lines (Fao and C2).

we performed immunostaining of tissue sections from the injured liver with anti-BMP-2 antibody. As shown in Fig. 4A, BMP-2 positive cells were observed in hepatocyte-like cells around the Glisson's capsule intralobular spaces of the injured liver (at 24 h post treatment with CCl_4), but not in normal liver. BMP-2 positive cells seem to be small cells such as small hepatocytes, progenitor cells for hepatocytes or wounded hepatocytes, but not Kupffer cells, stellate cells or sinusoidal endothelial cells.

To confirm this observation, we determined the expression of BMP-2 and CD14 in cultured cell lines. OC/CDE22, an oval cell line, is a progenitor of hepatocyte differentiation, and MFBY2 is a myofibroblast cell line derived from activated stellate cells isolated from CCl_4-treated rat liver. RAW264.7 is a mouse macrophage cell line, and Fao and C2 are well and poorly differentiated rat hepatocyte cell lines, respectively. As shown in Fig. 4E, OC/CDE22 oval cells showed BMP-2 and CD14 expression, while MFBY2 myofibroblast cells produced BMP-2 but not CD14. Moreover, RAW 264.7 macrophage cells expressed CD14 but not BMP-2, while the Fao and C2 hepatocyte cell lines generated neither BMP-2 nor CD14. These results suggest that activated oval cells or progenitor cells induce expression of BMP-2 mediated by injury signals from wounded hepatocytes. This observation was confirmed by immunohistochemical analysis that the distribution of BMP-2 positive cells was extremely resembled to that of cells expressing CK-19, an oval cell marker.

DISCUSSION

Chronic liver injury caused by hepatitis virus, an autoimmune response, intake of hepatotoxin, or cholestatic or metabolic disease, progresses to cirrhotic liver or fibrosis through stimulation of quiescent hepatic stellate cells to proliferate and transform into fibroblast cells (*29*). However, the earliest stage of these processes is thought to comprise of repeated cycles of injury and repair in liver cells (*30, 31*). Therefore, comprehension of the mechanism and regulation of the elementary process in the wound-healing response may lead to a novel therapeutic method for these liver diseases. In this study, we observed the transient expression of BMP-2, which is involved in hepatogenesis in early embryos, in adult rat liver. These findings suggest that the processes involved in development of the liver are tightly associated with repair of acute liver injury. BMP-4 was also involved in the hepatogenesis, and BMP-7 was reported to facilitate regeneration of injured kidney (*32*). However, these BMP-4 and -7 seemed to ignore the acute liver injury, BMP-2 was predominantly respond to repair process in liver injury.

BMP signalling derived from the cardiac mesoderm or septum transversum mesenchyme is required for morphogenetic movement of the liver bud, including hepatic competence and endodermal patterning in the foregut ventral endoderm expressing GATA-4 (*10, 11*). During hepatogenesis, autocrine BMP-2 signalling is also required for HEX and albumin expression in the endoderm (*33, 34*). Mesenchymal cells in the embryo play an important role in hepatogenesis, and likewise, hepatic stellate cells in the adult liver also have a role in hepatocyte function through production of extracellular matrix (*31*). However, stellate cells were shown to express BMP-2, but not CD14 *in vitro*. Activated Kupffer cells, which release cytokines and chemokines, perform a critical role in the toxicity of various hepatotoxins via secretion of preinflammatory cytokines, which attract blood neutrophils into the liver (*35*). However, transient expression of BMP-2 was also observed in liver depleted of Kupffer cells by $GdCl_3$. Furthermore, hepatocyte cell lines generated neither BMP-2 nor CD14. Taken together, these results suggest that these cells are not responsible for transient expression of BMP-2 in CCl_4-injured liver.

Oval cells, which are hepatic stem-like cells (progenitor cells) derived from bone marrow monocytes (*5–7, 12, 36*), appear to be the most likely candidate cells for expression of BMP-2 during liver injury. Monocytes are differentiated from haematopoietic cells and express

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CD14 (*37, 38*). In liver injury induced by CCl_4, activated oval cells or progenitor cells derived from monocytes seem to induce expression of BMP-2 mediated by injury signal from wounded hepatocytes. In conclusion, the repair of acute injury in adult liver seems to proceed through a similar process as hepatogenesis in early embryos, and BMP-2 signalling may, therefore, play important roles in development of hepatic progenitor cells into hepatocytes.

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