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Monocyte chemoattractant protein-1 derived from biliary innate immunity contributes to the hepatic fibrogenesis

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Running title: MCP-1 in biliary epithelial cells

Key Words: hepatic stellate cell, hepatic fibrosis, biliary epithelial cells, Toll-like receptor, MCP-1, biliary innate immunity

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ABSTRACT

Aims: Monocyte chemoattractant protein-1 (MCP-1) is a major chemotactic factor for hepatic stellate cells (HSCs) associated with hepatic fibrosis. In this study, among several fibrogenetic factors derived from BECs, MCP-1 produced by the biliary innate immune system, was found to be most critical in the histogenesis of hepatic fibrogenesis. **Methods**: Using cultured human biliary epithelial cells (BECs), the expression of 5 fibrogenetic factors including MCP-1 on stimulation with Toll-like receptor (TLR) ligands, inflammatory cytokines, or bile acids was examined. Moreover, *in situ* detection of MCP-1 and α-smooth muscle actin (αSMA) proteins was performed using sections from normal and diseased livers by immunohistochemistry. **Results**: All fibrogenetic factors were detected in BECs, but only MCP-1 expression was upregulated, by all the TLR ligands, IL-1β, and TNF-α. Proliferating bile ductules in interface areas expressed MCP-1 in diseased livers accompanying αSMA-positive activated HSCs. **Conclusions**: Bile ductules proliferate in various hepatobiliary diseases and its significance is still unknown. This study demonstrated that BECs in bile ductules could produce MCP-1, particularly, via biliary innate immunity, suggesting that MCP-1 derived from BECs plays an important role in the recruitment of HSCs to interface areas and the activation of HSCs resulting in the progression of periportal fibrosis.

Introduction

Hepatic fibrosis is a major feature of advanced liver diseases and defines the prognosis. Hepatic fibrosis spreads within portal tracts and also periportal areas in patients with hepatitic and cholestatic liver diseases. Although periportal fibrosis is thought to be associated with the accumulation and activation of hepatic stellate cells (HSCs), its mechanisms are not fully understood. HSCs undergo differentiation toward an activated phenotype and this process is enhanced by soluble mediators such as platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF-β), which mediate the increase in cell proliferation and extracellular matrix production.[1, 2] Then, HSCs migrate into damaged areas in response to a chemokine, secreting monocyte chemotactic protein-1 (MCP-1/CCL2) that recruits monocytes and lymphocytes.[3] Moreover, MCP-1 triggers the migration of activated, but not quiescent, HSCs in a dose-dependent manner. Experiments *in vitro* using HSCs isolated from normal human livers revealed that MCP-1-dependent signals were not transduced by the chemokine receptor of MCP-1, CCR2, and may be mediated by alternative chemokine receptors.[3] Recently, Ramm *et al*.,[4] reported that hepatocyte-derived MCP-1 induced by a hydrophobic bile acid, taurocholate, could result in the recruitment of HSCs in cholestatic liver injury as the major fibrogenesis in a pediatric cholestatic liver disease such as biliary atresia. In contrast, biliary epithelial cells (BECs) may promote fibrogenesis by a number of mechanisms including the synthesis of matrix constituents and the release of mediators such as MCP-1, PDGF-BB, TGF-β, connective tissue growth factor (CTGF), and endothelin-1.[5] The role of BECs in the pathogenesis of hepatic fibrosis, particularly periportal fibrosis accompanying interface hepatitis in various hepatobiliary diseases including chronic viral hepatitis (CVH) and primary

biliary cirrhosis (PBC), is speculated to be important to the disease's progression, but its precise mechanism is still unknown.

Our previous study demonstrated that human BECs possess several inflammatory cytokine receptors and Toll-like receptors (TLRs) and produced cytokines and chemokines in response to inflammatory cytokines and pathogen-associated molecular patterns (PAMPs), respectively.[6-9] In particular, bile ductules located between interlobular bile ducts in portal tracts and bile canaliculi in hepatocytes are frequently increased in number under a variety of pathologic conditions of the liver and take part in various immunological responses and the pathogenesis of biliary diseases.[10-12] This ductular reaction is speculated to be closely associated with hepatic fibrosis, particularly periportal fibrosis accompanying interface hepatitis, but its precise mechanism is still unknown. In this study, we examined the possibility that BECs produce MCP-1 and play a role in fibrogenesis in periportal areas via MCP-1 production.

Materials and Methods

Cultured human BECs: Two human intrahepatic BEC lines were established from explanted livers with PBC as described previously.[7] These cell lines had been confirmed to be BECs by the expression of biliary-type cytokeratins.

Stimulation: Cultured BECs were stimulated with inflammatory cytokines, PAMPs, and bile acids (Table 1). As a NF-κB inhibitor, isohelenin (30μM, Calbiochem) was added to the culture medium before the stimulation We previously confirmed that BECs possess the receptors for all these cytokines and PAMPs.[6] Cell samples for the examination of mRNA as shown below were prepared at 2hrs after the stimulation.

RT-PCR and real-time PCR: For the evaluation of mRNAs of MCP-1, PDGF-B, CTGF, TGF-β1, endothelin-1, and GAPDH (internal control) in cultured BECs, total RNA was isolated from BECs and RT-PCR and real-time quantitative PCR were performed according to a standard protocol using specific primers (Table 1).

Enzyme-linked immunosorbent assay (ELISA): Cultured BECs were stimulated with LPS, Pam3CSK4, poly(I:C), and IL-1β for 24 hours and supernatants were tested for human MCP-1 by ELISA (Biosource International, Camarillo, CA, USA).

***Liver tissues*:** A total of 26 surgical or wedge liver biopsy specimens were obtained from patients with CVH (n=8, HCV-related, male/female=5/3, average age 58y.o., F1/F2=4/4), PBC (n=5, all female, average age 68y.o., Nakanuma's classification[13] Stage 2/3=4/1, Scheuer's classification Stage 1/2=2/3), and congenital hepatic fibrosis (CHF, n=5, used as a case of activated HSC-poor case[14], male/female 4/1, average age 23y.o.), and 6 cases with no significant histopathological change ("normal liver").

Immunohistochemistry: Deparaffinized sections were pretreated in Target Retrieval Solution (Dako, Tokyo, Japan) and incubated with a primary antibody against MCP-1 (10μg/ml; Abcam, Tokyo, Japan) or α-smooth muscle actin (αSMA, activated HSC marker) (1μg/ml, Dako) and then Envision-HRP (Dako) was used. No positive staining was obtained when the primary antibody was replaced with an isotype-matched, non-immunized immunoglobulin.

The simultaneous detection of combined CCR2 and αSMA, and CCR2 and CD68, was evaluated by double fluorescence immunohistochemical staining. After incubation with the antibodies for these combinations, rabbit antibody (CCR2, 1:400,

Epitomics, Burlingame, CA, USA) and mouse antibodies (αSMA and CD68, Dako) were visualized by Alexa Fluor 488 and 594 (Molecular Probe, Eugene, OR, USA), respectively.

Results

Detection of MCP-1 in cultured human BECs: RT-PCR revealed that all fibrogenic cytokines were detected in human BECs in the basal cultures (Fig.1). Quantitative PCR revealed that only MCP-1 was upregulated following stimulation with all PAMPs and two cytokines (IL-1β and TNF-α)(Fig2). Moreover, ELISA demonstrated that the concentration of MCP-1 protein in the supernatants was increased by these stimulants (Fig.3). None of the bile acids upregulated the expression of MCP-1 (Fig.2). The Pam3CSK4-induced upregulation of MCP-1 mRNA was significantly inhibited by pretreatment with the NF-κB inhibitor(Fig.2).

In situ *detection of MCP-1 and activated HSCs*: MCP-1 was mainly expressed in bile ducts and that the majority of proliferating bile ductules predominantly expressed MCP-1 in periportal and interface areas in cases of CVH and PBC (Fig.4). Moreover, αSMA-positive cells (activated HSCs) were scattered around these bile ductules (Fig.4). These characteristic findings were made in CVH and PBC accompanying interface hepatitis and ductular reaction, but rare or absent in normal livers. Periportal hepatocytes were also frequently positive for MCP-1, but the intensity was similar or less than that in bile ductules. In contrast, MCP-1-positive bile ductules were not found in CHF. Moreover, the expression of αSMA was limited in portal tracts and αSMA-positive HSCs were not found in CHF.

***Characterization of CCR2-expressing cells*:** Fluorescence double immunostaining revealed that cells double positive for CD68 and CCR2 were scattered mostly in interface areas of CVH and PBC, but cells double positive for αSMA and CCR2 were not found in any area (Fig.5).

Discussion

Chronic liver injury is generally characterized by inflammation and subsequent fibrosis and, regardless of the etiology, a cytokine-rich environment caused by inflammation and infection is closely associated with hepatic fibrosis. HSC is considered the most important effecter cell associated with fibrogenesis in hepatic parenchyma including the interface between portal tracts and periportal hepatocytes, and the fibrous enlargement of portal tracts and fibrous extension from portal areas are closely associated with activated HSCs and their transformed version, myofibroblasts. Within portal tracts, in contrast, fibroblasts and fibrocytes are also important to the histogenesis of portal fibrosis. Several cytokines such as PDGF-B, CTGF, TGF-β, endothelin-1, and MCP-1, are reported as fibrogenetic factors in liver.[5] These cytokines could chemoattract HSCs or directly activate the production of collagen in myofibroblasts.

We have previously reported that human BECs possess receptors for inflammatory cytokines (IL-1β, IL-6, IFN-γ, and TNF-α) and an innate immune system consisting of TLRs.[6, 8, 9] Therefore, in this study, we examined whether BECs contribute to hepatic fibrosis using cultured human BECs. Consequently, although mRNAs of all fibrogenic cytokines were detected in BECs, only the expression of MCP-1 was upregulated, by two proinflammatory cytokines (by IL-1β and to a lesser degree by

TNF-α) and all TLR ligands in an NF-κB-dependent manner. Because human BECs produced IL-1β when stimulated with TLR ligands,[15] the biliary innate immune response is suggested to be a critical trigger of MCP-1 production.

Bile ductules and their proliferation occur nonspecifically in various hepatobiliary diseases. In normal livers, a few bile ductules are recognizable in the portal tract, while in various hepatobiliary diseases, these ductular structures are often increased in number, to be termed "ductular proliferation" or "proliferating bile ductules". Because ductular proliferation was found accompanying interface hepatitis and periductal fibrosis in chronic liver diseases, these biliary elements are thought to take part in disease progression. However, the exact association between ductular proliferation and hepatic fibrogenesis is still unknown. Immunohistochemistry revealed that MCP-1 was expressed in bile ductules in areas of interface hepatitis, whereas normal livers lacked these findings. The ductular reaction predominantly corresponded to interface hepatitis in various chronic hepatobiliary diseases and these areas were rich in several cytokines caused by immune-mediated (necro)inflammatory reactions against virus-infected hepatocytes and bile-derived PAMPs. Therefore, these microenvironments are suitable for the production of MCP-1 in proliferating bile ductules at the interface.

In damaged liver, HSCs are activated, proliferate, and migrate into the injured area in response to the chemoattractive effects of chemokines. MCP-1 is a chemokine attracting monocyte/macrophages and plays a role in persistent inflammation in chronic liver diseases. Marra *et al*.,[3] reported that MCP-1 attracts HSCs, particularly activated HSCs, to the liver. Innate immunity is known to promote liver fibrosis and, as its mechanism, HSCs are reported to produce MCP-1 via TLR9 signaling.[16] In this study,

immunohistochemistry using liver sections from patients with CVH and PBC revealed that αSMA-positive activated HSCs (myofibroblasts) were scattered and accumulated around MCP-1-expressing bile ductules in areas showing interface hepatitis. This finding suggests that MCP-1 derived from BECs plays a role in the chemoattraction of HSCs.

A recent report demonstrated that hepatocytes are also a source of MCP-1 and that hepatocyte-derived MCP-1 induced by a hydrophobic bile acid, taurocholate, chemoattracts HSCs and is associated with the liver fibrosis under cholestatic conditions in cases of pediatric cholestatic liver disease such as biliary atresia. Our immunohistochemical study also confirmed the expression of MCP-1 in periportal hepatocytes, but, in the early stage of CVH and PBC, the MCP-1 derived from bile ductules is speculated to the major effecter, compared with that from hepatocytes, based on the intensity of MCP-1 immunostaining. We also examined the effect of bile acids on the production of MCP-1 in cultured BECs. No any bile acids affected the MCP-1 expression in BECs, suggesting that cholestasis could not directly induce the production of MCP-1 in BECs, differing from hepatocytes.

Portal fibroblasts located in portal tracts are fibrogenic cells distinct from HSCs and may be important mediators of biliary fibrosis and cirrhosis. Recently, Kruglov *et al*,[17] reported that portal fibroblasts express functional receptors for MCP-1 that are distinct from CCR2 and that the secretion of MCP-1 by BECs induces myofibroblastic transdifferentiation of portal fibroblasts. In fact, the expression of MCP-1 was found in some interlobular bile ducts as well as bile ductules within portal tracts in CVH and PBC (data not shown). This finding suggests that MCP-1-dervied from BECs of these interlobular bile ducts is associated with the migration and activation of portal

fibroblasts. However, we speculate that the fibrogenesis associated with portal fibroblasts is mainly associated with the histogenesis of portal sclerosis and expansive fibrous enlargement of portal tracts, not the fibrous extension accompanying interface hepatitis from portal tracts. The close correlation between MCP-1-positive bile ductules and αSMA-positive activated HSCs in the interface area shown in this study supports our contention. Moreover, we examined CHF as a control diseased liver in this study. CHF is different from cirrhosis in which no abnormal biliary channels are seen. In portal tracts of CHF, irregular and newly proliferating bile ducts and ductules rather than congenitally abnormal ductal plates, and cholestasis in these bile ductules are found. But, in parenchyma, the features of chronic cholestasis and interface changes are not prominent. Our previous study reported that the fibrogenesis of CHF is associated with intraportal heparan sulfate proteoglycan and CTGF, but not periportal HSCs.[14] It is true that although proliferating bile ductules were scattered within portal tracts, MCP-1 expression in bile ductules and αSMA-positive HSCs were not found in CHF, suggesting that the MCP-1-mediated migration of HSCs limited in proliferating bile ductules accompanying interface hepatitis of chronic inflammatory hepatobiliary diseases, but not associated with the fibrogenesis in sole cholestatic liver diseases lacking interface changes such as CHF.

Because the receptor of MCP-1, CCR2, is not expressed in human HSCs, HSC migration by MCP-1 occurs independent of CCR2 via an unknown receptor for MCP-1, instead of by CCR2.[3] In fact, double immunohistochemistry in this study revealed that the expression of CCR2 is found not in αSMA-positive HSCs, but in CD68-positive Kupffer cells, suggesting that MCP-1-dervied from BECs could chemoattract Kupffer cells. As mentioned, portal fibroblasts within portal tracts also lack CCR2, but could be

attracted by MCP-1 derived from BECs in a CCR2-independent manner.[17] In contrast, Seki et al.,[18] reported that both Kupffer cells and HSCs express CCR2 in mice, but that these differences might be explained by differences between humans and mice.

Various types of inflammation including infection-triggered inflammation are causative factors to induce hepatic fibrosis in chronic liver diseases. Particularly, HSCs are critical for hepatic fibrogenesis and MCP-1 is an important cytokine associated with HSC migration in fibrogenic areas. Therefore, the identification of MCP-1-producing cells and the clarification of the mechanism of MCP-1 production are mandatory to help regulate hepatic fibrosis and treat liver fibrosis. This study revealed that BECs are a source of MCP-1 in some hepatobiliary diseases and the production of MCP-1 is induced by inflammatory cytokines and biliary innate immune responses. Proliferating bile ductules are thought to be part of a non-specific reaction in various hepatobiliary diseases, but this study suggests that they are closely associated with the progression of periportal fibrosis via MCP-1 derived from biliary innate immunity. Because MCP-1 is thought to be a key mediator of hepatic fibrosis, it is a potential therapeutic target in inflammatory hepatobiliary diseases with hepatic fibrosis.

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Take home messages

- Biliary epithelial cells express several fibrogenic cytokines (MCP-1, PDGF-B, CTGF, TGF-β1, and endothelin-1), but only MCP-1 expression is upregulated by biliary innate immune reaction and proinflammatory cytokines (IL-1β and TNF-α).
- Proliferating bile ductules in interface areas expressed MCP-1 in diseased livers accompanying αSMA-positive activated HSCs.
- MCP-1 derived from biliary epithelial cells plays an important role in the recruitment of hepatic stellate cells (HSCs) to interface areas and the activation of HSCs resulting in the progression of periportal fibrosis.

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Table 1 Primers used for RT-PCR and quantitative PCR

Transcript	Primers	Product size (bp)
MCP-1	Forward 5'- CTGAATTTTGTTTGTTGATGTGAAA -3' Reverse 5'- GCAATTTCCCCAAGTCTCTG-3'	128
PDGF-B	Forward 5'- GAGGACCTCTCAGCATAGCC -3' Reverse 5'- GGGGTTTTCTCCAGTCTGTG -3'	135
CTGF	Forward 5'- GCAGGCTAGAGAAGCAGAGC-3' Reverse 5'- ATGTCTTCATGCTGGTGCAG -3'	153
TGF-β1	Forward 5'- CAGAAATACAGCAACAATTCCTGG-3' Reverse 5'- TTGCAGTGTGTTATCCCTGCTGTC-3'	186
endothelin-1	Forward 5'- CCATGAGAAACAGCGTCAAA -3' Reverse 5'- AGTCAGGAACCAGCAGAGGA-3'	213
GAPDH	Forward 5'- GCACCGTCAAGGCTGAGAAC-3' Reverse 5'- ATGGTGGTGAAGACGCCAGT-3'	142

Table 2 Stimulants

Stimulants	Concentration	Supplier
Cytokines		
IL-1β	1,000U/ml	PerpoTech
IL-6	1,000U/ml	PerpoTech
IFN-γ	1,000U/ml	PerpoTech
TNF-α	1,000U/ml	PerpoTech
PAMPs		
LPS (TLR4 ligand)	1μg/ml	Invitrogen
Pam3CSK4(TLR1/2 ligand)	100ng/ml	Invitrogen
poly(I:C) (TLR3 ligand)	25μg/ml	Invitrogen
Bile acid		
taurochenodeoxycholic acid (TCDC)	200μM	Calbiochem
taurocholic acid (TC)	200μM	Calbiochem
taurodeoxycholic acid (TDC)	200μM	Calbiochem
tauroursodeoxycholic acid (TUDC)	200μM	Calbiochem

PerpoTech PeproTech, London, UK. Invitrogen, San Diego, CA, USA. Calbiochem, Darmstadt, Germany.

Figures and legends

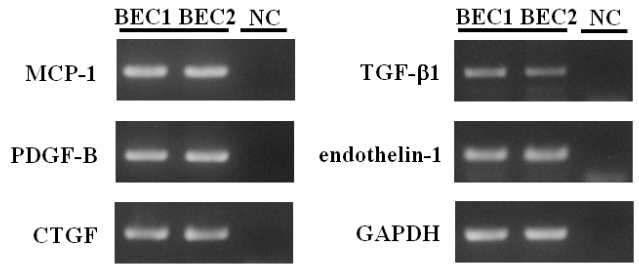
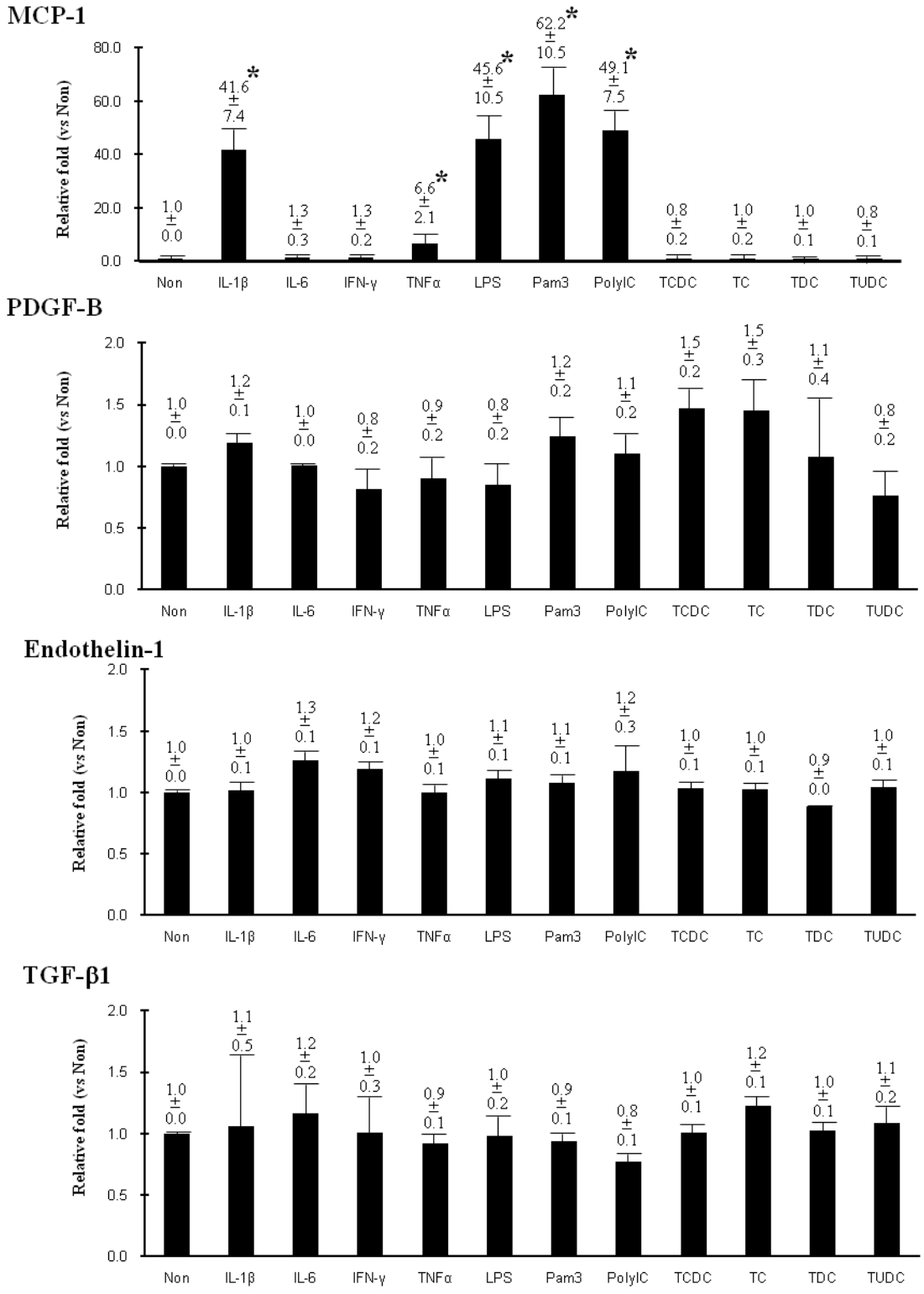


Fig.1

Fig.1 Detection of fibrogenic cytokines in cultured human BECs. The two human biliary epithelial cell lines (BEC1 and BEC2) express all mRNAs of fibrogenic cytokines (MCP-1, PDGF-B, CTGF, TGF-β1, and endothelin-1) and the internal control (GAPDH) under basal conditions. Each PCR product showed the predicted size as a single band. Negative controls (NC) were obtained by replacing reverse transcriptase with RNase- and DNase-free water.


MCP-1
Relative fold (vs Non)
80.0
60.0
40.0
20.0
0.0
1.0 ± 0.0
41.6 ± 7.4 *
1.3 ± 0.3
1.3 ± 0.2
6.6 ± 2.1 *
45.6 ± 10.5 *
62.2 ± 10.5 *
49.1 ± 7.5 *
0.8 ± 0.2
1.0 ± 0.2
1.0 ± 0.1
0.8 ± 0.1
Non
IL-1β
IL-6
IFN-γ
TNFα
LPS
Pam3
PolyIC
TCDC
TC
TDC
TUDC
PDGF-B
Relative fold (vs Non)
2.0
1.5
1.0
0.5
0.0
1.0 ± 0.0
1.2 ± 0.1
1.0 ± 0.0
0.8 ± 0.2
0.9 ± 0.2
0.8 ± 0.2
1.2 ± 0.2
1.1 ± 0.2
1.5 ± 0.2
1.5 ± 0.3
1.1 ± 0.4
0.8 ± 0.2
Non
IL-1β
IL-6
IFN-γ
TNFα
LPS
Pam3
PolyIC
TCDC
TC
TDC
TUDC
Endothelin-1
Relative fold (vs Non)
2.0
1.5
1.0
0.5
0.0
1.0 ± 0.0
1.0 ± 0.1
1.3 ± 0.1
1.2 ± 0.1
1.0 ± 0.1
1.1 ± 0.1
1.1 ± 0.1
1.2 ± 0.3
1.0 ± 0.1
1.0 ± 0.1
0.9 ± 0.0
1.0 ± 0.1
Non
IL-1β
IL-6
IFN-γ
TNFα
LPS
Pam3
PolyIC
TCDC
TC
TDC
TUDC
TGF-β1
Relative fold (vs Non)
2.0
1.5
1.0
0.5
0.0
1.0 ± 0.0
1.1 ± 0.5
1.2 ± 0.2
1.0 ± 0.3
0.9 ± 0.1
1.0 ± 0.2
0.9 ± 0.1
0.8 ± 0.1
1.0 ± 0.1
1.2 ± 0.1
1.0 ± 0.1
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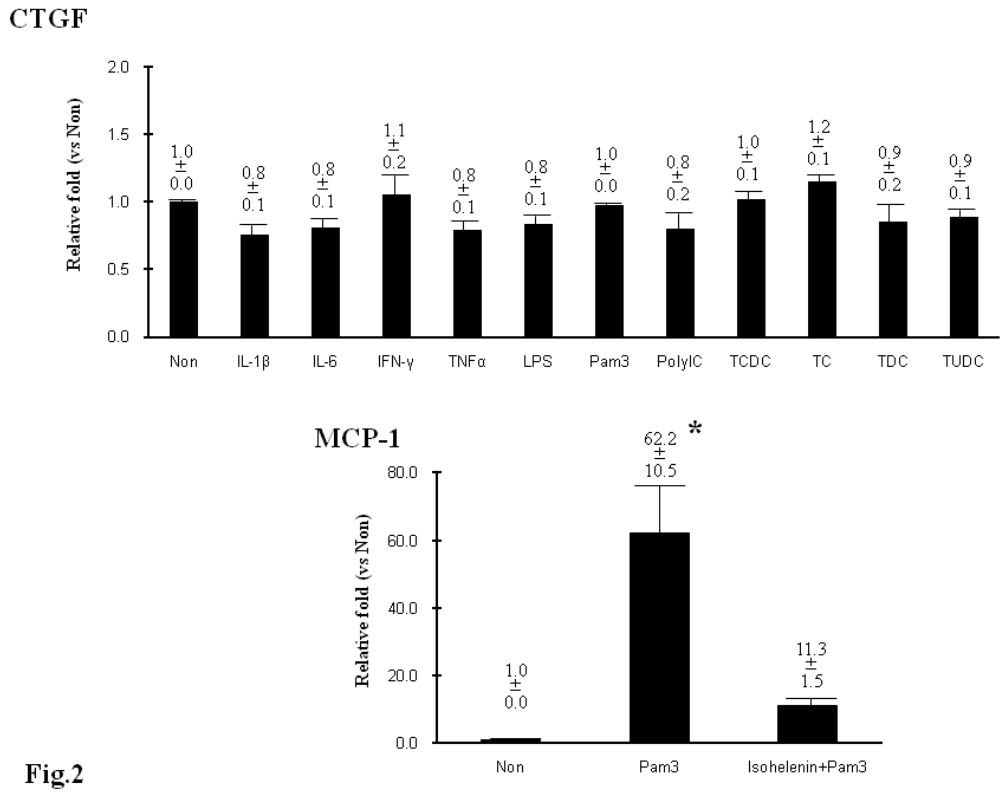

Fig.2

Fig.2 Quantitative PCR analysis for mRNA of fibrogenic cytokines (MCP-1, PDGF-B, CTGF, TGF-β1, and endothelin-1). MCP-1 expression alone was upregulated by stimulation with TLR ligands (LPS, Pam3CSK4, and poly(I:C)) and cytokines (IL-1β and TNF-α). No bile acids (TCDC, TC, TDC, or TUDC) significantly upregulated MCP-1 mRNA expression. Other fibrogenic cytokines were not affected by any stimulants. Lower figure denotes that Pam3CSK4-induced upregulation of MCP-1 mRNA expression was inhibited by pretreatment with an NF-κB inhibitor, isohelenin. Bars indicate the mean±S.E.M. *<0.05.

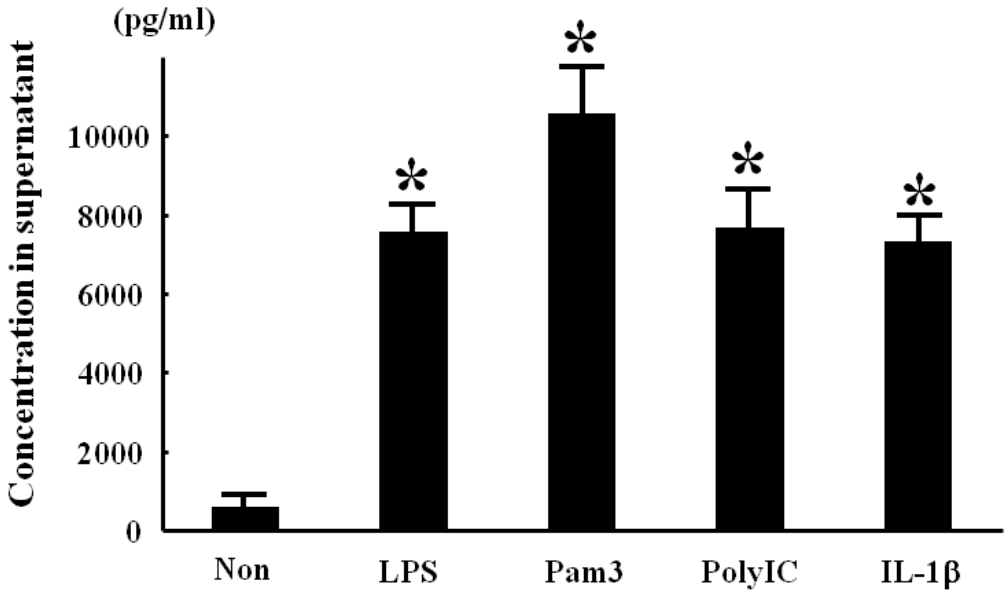

Fig.3

Fig.3 Measurement of MCP-1 protein by ELISA. After stimulation with LPS, Pam3CSK4 (Pam3), poly(I:C), and IL-1β for 24 hours, the concentration of MCP-1 protein in cultured supernatants of human biliary epithelial cells was significantly increased. Bars indicate the mean±S.E.M. *<0.05.

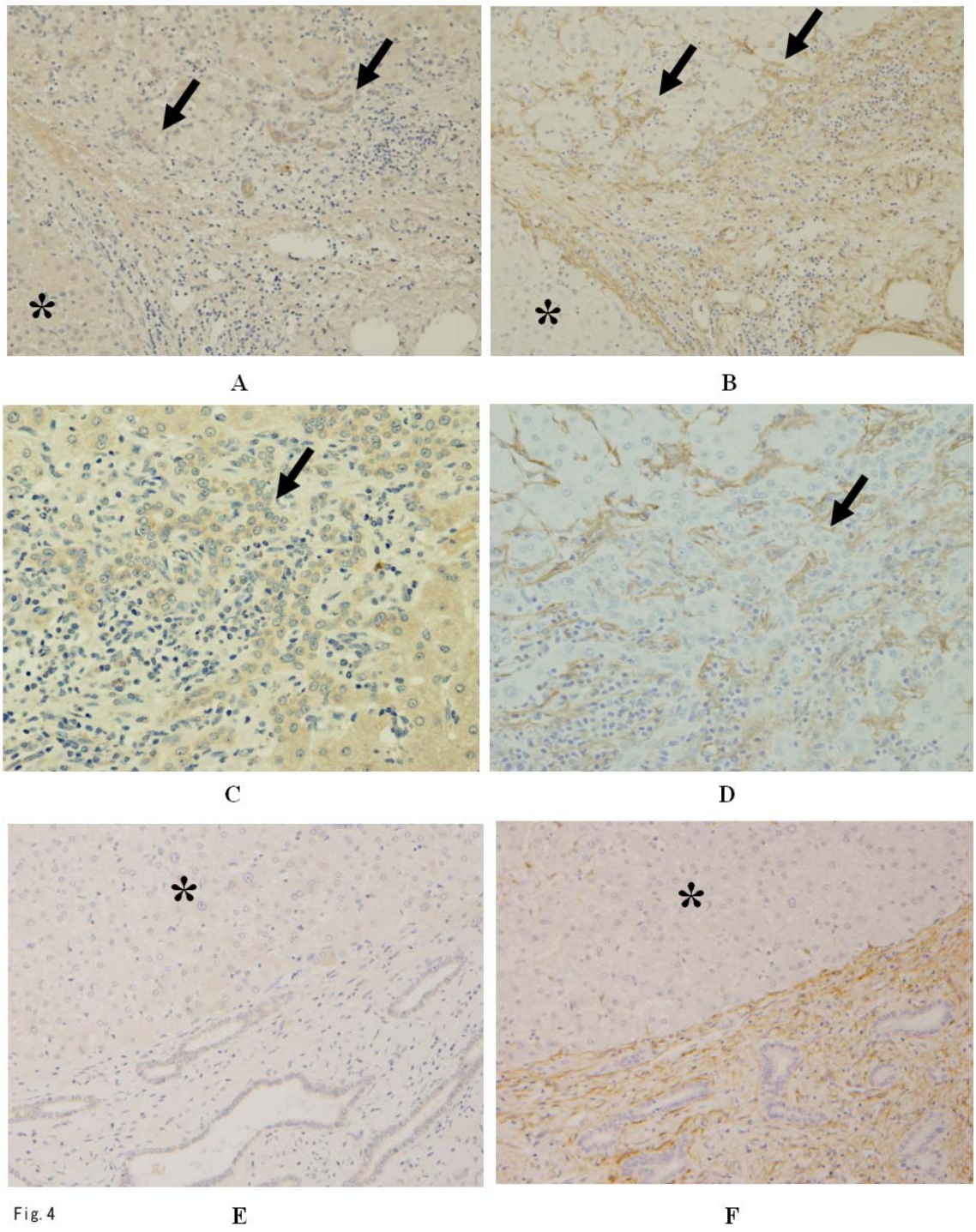

Fig.4 Immunohistochemistry for MCP-1 (A, C, and E) and αSMA (B, D, and F). A and B; HCV-related chronic viral hepatitis. Proliferating bile ductules at the interface of periportal areas express MCP-1(A, arrows) and αSMA-positive cells

showing hepatic stellate cell (HSC) morphology are found in the same area (B, arrows). In contrast, αSMA-positive HSCs are not found in periportal parenchyma lacking bile ductules (asterisks). C and D; primary biliary cirrhosis. MCP-1-positive proliferating bile ductules and αSMA-positive cells are intermingled (arrows). E and F; congenital hepatic fibrosis. No biliary bile ducts or ductules express MCP-1 and no αSMA-positive HSCs are found in periportal areas (asterisks). The expression of αSMA is limited in portal tracts (lower right).

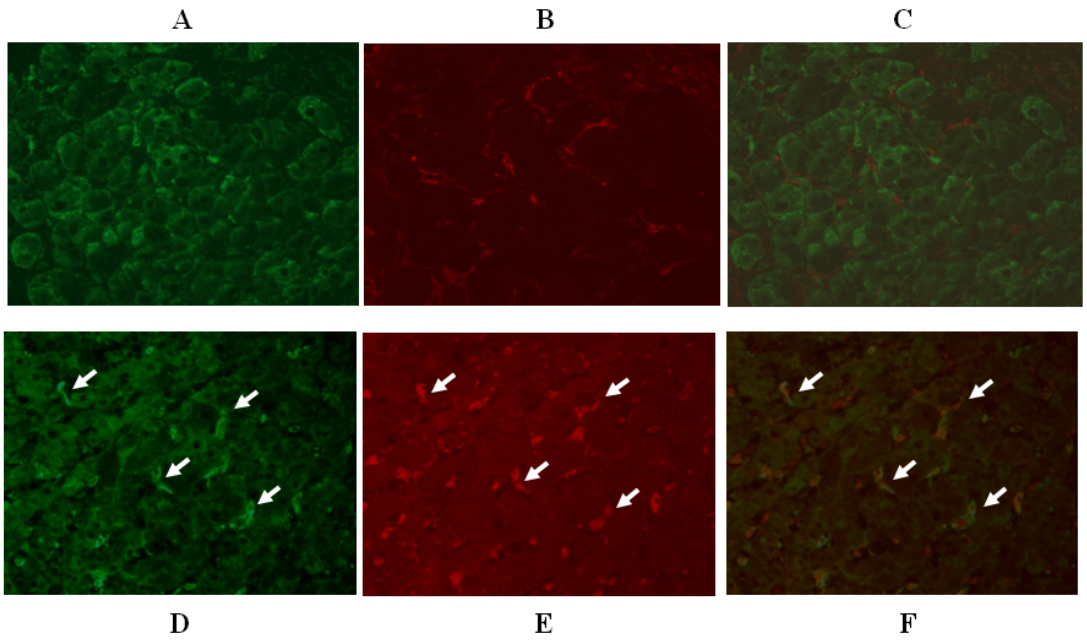

Fig.5

Fig.5 Double immunohistochemistry using CVH livers. A, B, C are MCP-1 receptor (CCR2, green), αSMA (red), and merged image, respectively, and double positive cells indicating CCR2-positive hepatic stellate cells (yellow) are not found. D, E, and F are CCR2 (green), CD68 (red), and merged image, respectively, and double positive cells indicating CCR2-positive Kupffer cells (yellow) are scattered (arrows).