Liver fibrosis is a common complication associated with transient myeloproliferative disorder (TMD) in Down syndrome (DS). The exact molecular pathogenesis that regulates disease progression is largely unknown. We recently found serum and/or urinary monocyte chemoattractant protein-1 (MCP-1) as a novel biomarker of liver fibrosis. This study was an in vitro analysis to investigate the fibrogenic activity of MCP-1 using the collagen-producing LX-2 human hepatic stellate cell line. We also examined the fibrogenic activity of serum from a male neonate with DS in whom late-onset liver fibrosis developed even after the resolution of TMD. MCP-1 stimulated both cell growth and collagen synthesis of LX-2 in a dose-dependent manner. Patient serum obtained during the active disease phase significantly up-regulated fibrogenic activity, which was suppressed in the presence of MCP-1-blocking antibody. Transient transforming growth factor beta 1 stimulation primed LX-2 to induce prolonged hypersecretion of MCP-1 in the culture supernatant and in collagen synthesis, which was suppressed with MCP-1 blocking antibody as well.

Conclusion: MCP-1 accounts for the prolonged activation of collagen-producing hepatic stellate cells in both a paracrine and autocrine manner, thereby promoting liver fibrosis. Anti-cytokine therapy targeting the fibrogenic cytokines of MCP-1, for example, herbal medicine, could provide a new therapeutic intervention for liver fibrosis associated with TMD in DS. (Hepatology Communications 2018;2:230-236)

Introduction

Transient myeloproliferative disorder (TMD) in neonates with Down syndrome (DS) is a self-limited disorder, but a small proportion of these infants suffer from life-threatening complications, such as liver fibrosis. TMD originates from fetal liver hematopoiesis, and it is believed that the liver complication is driven by direct interaction of megakaryoblast and/or blast-derived proinflammatory cytokines, i.e., platelet-derived growth factor, tumor growth factor β1 (TGF-β1), but the exact pathogenesis is unknown. Furthermore, the co-occurrence of the live-birth complication is not always associated with the severity of the hematologic condition, indicating that another mechanism other than direct megakaryoblast invasion takes place in the progression of liver fibrosis.
It is widely accepted that hepatic stellate cells (hSCs) play a critical role in the pathogenesis of liver fibrosis as the main source of the fibrotic extracellular matrix. Accordingly, hSC-derived CXC and CC profibrogenic chemokines have been identified as a target motif for anti-cytokine therapy for liver diseases. Among them, monocyte chemoattractant protein-1 (MCP-1) is one of the best studied chemokines as a biomarker of liver cirrhosis and/or posttraumatic liver failure. Thus, the understanding of molecular pathogenesis of liver fibrosis, especially the fibrogenic activation of hSCs by MCP-1, is a major focus of current research on liver fibrosis associated with TMD in patients with DS.

We encountered a male neonate with DS in whom liver fibrosis developed even after the resolution of TMD. The liver biopsy showed little infiltration of megakaryoblasts. Interestingly, we identified the characteristic expression of the profibrogenic cytokines of MCP-1 in the expanding hSCs and also found that circulating and urinary MCP-1 are novel biomarkers of liver fibrosis associated with TMD in DS.

Materials and Methods

Written informed consent was obtained from parents of the neonate who was treated in Hyogo Kenritsu Amagasaki Sogo Iryo Center for liver fibrosis associated with TMD in Down syndrome. This study was approved by the institutional review board of Hyogo Kenritsu Amagasaki Sogo Iryo Center.

HUMAN STELLATE CELL CULTURE

LX-2 human hSC line was routinely grown in Dulbecco’s modified Eagle’s medium (D5796; Sigma, St. Louis, MO) supplemented with 2% fetal calf serum (MP Biomedicals, Santa Ana, CA) not otherwise specified. Cells were stimulated with either recombinant human MCP-1 (Z028029, GenScript, Piscataway, NJ) or TGF-β1 (240-B-002, R&D Systems, Minneapolis, MN) and then incubated with either 1 μg/mL MCP-1 blocking antibody (M2420; Sigma, St. Louis, MO) or mouse IgG (278-810; Ancell, Bayport, MN). After culturing, the viable cell count was obtained by staining with trypan blue dye. For evaluation of mitogen-activated protein kinase (MAPK) activity, cells were transferred to serum-free medium for 24 hours and stimulated with an increasing dose of MCP-1 for 30 minutes at 37°C.

EVALUATION OF PATIENT SERUM ACTIVITY WITH AN EX VIVO EXPERIMENT USING THE LX-2 CELL LINE

Prior to evaluating patient serum activity, stably propagated LX-2 cells were transferred to serum-free medium for 2 days. Cells were then stimulated with 10% patient serum for 5 days with or without MCP-1 blocking antibody. At the end of the cell culture, cells were analyzed for both cell growth and de novo type IV collagen protein level.

WESTERN BLOT ANALYSIS

Equal amounts of cell lysates were resolved by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride.
membranes. After blocking with 1X tris(hydroxymethyl)aminomethane-buffered saline containing 5% weight/volume nonfat dry milk and 0.1% Tween-20, the membranes were incubated with the primary antibody (overnight at 4°C) followed by horseradish peroxidase (HRP)-conjugated secondary antibody (1 hour at room temperature). Protein bands were visualized using Amersham enhanced chemiluminescence (RPN2232; GE Healthcare, Piscataway, NJ). Primary antibodies for p44/42 MAPK (9102) and p38 MAPK (9212) were purchased from Cell Signaling Technology (Danvers, MA), and primary antibodies for beta actin (A5316) and type IV collagen (c7521) were purchased from Sigma. Secondary antibodies were anti-mouse IgG (heavy + light chains) HRP conjugate (81-6520; Zymed Laboratories, South San Francisco, CA) and anti-rabbit IgG, HRP-linked antibody (7047; Cell Signaling Technology).

GENE EXPRESSION ANALYSIS

Total RNA was isolated using RNeasy Plus mini kit (74104; Qiagen, Valencia, CA) and reverse transcribed using Superscript III First-Strand Synthesis Supermix (18080400; Invitrogen, Carlsbad, CA). Quantitative real-time polymerase chain reaction was performed using the SYBR green detection kit (4385612; Invitrogen). Expression values were normalized with glyceraldehyde 3-phosphate dehydrogenase before calculating expression ratios. Primer sequences used in expression analysis are available upon request.

EVALUATION OF MCP-1 CONCENTRATION

Culture supernatant was collected as indicated, i.e. 0, 1, 24, 72, 120 hours after the initiation of cell culture. Specimens were stored at –80°C until analysis. Concentrations of MCP-1 were determined by cyto-metric bead array (Becton Dickinson, San Jose, CA).

IMMUNOHISTOCHEMISTRY

Formalin-fixed paraffin-embedded tissue specimens were processed according to standard protocols. For the detection of MCP-1-producing cells, sections were incubated with primary antibodies of MCP-1(73680; Abcam), 1:200 dilution; MCP-1 (505905; BioLegend, San Diego, CA), 1:500 dilution; C-C chemokine receptor type 2(21667; Abcam), 1:1,000 dilution; and type IV collagen (c1926; Sigma), 1:200 dilution. The secondary antibodies were as follows Alexa Fluor 488-conjugated anti-mouse IgG (H+L) (A-11001; Molecular probes, Carlsbad, CA), Alexa Fluor 546-conjugated anti-rabbit IgG (A-11035; Molecular Probes), and Alexa Fluor 546-conjugated anti-hamster IgG (H+L) (A-21111; Molecular Probes). The nucleus was stained with 4’,6-diamidino-2-phenylindole.

STATISTICAL ANALYSIS

Each in vitro experiment was performed at least 3 times. The Student t test was used to compare cellular effects. Data are shown as means ± SD. All tests were two-tailed, and P < 0.05 compared to control was considered statistically significant.

Results

MCP-1 was initially identified as a chemoattractant for monocytes(12) and was later found in a wide array of pathogenesis, such as diabetic nephropathy, atherosclerosis, and liver disease.(6,8-10) We identified MCP-1 as a biomarker of liver fibrosis associated with TMD in DS(11) and then hypothesized that MCP-1 might play fundamental roles during the fibrogenic activation of hSCs, thereby promoting liver fibrosis (Fig. 1A). Having shown the characteristic expression of MCP-1 in the expanding hSCs within affected liver tissue (Fig. 1B), we explored the functional relevance of MCP-1 using an LX-2 human hSC line that was established from normal liver tissue without any genetic modification.(13) We verified the co-expression of MCP-1 and its receptor C-C chemokine receptor type 2 (Fig. 1B) and then stimulated the cells with an increasing dose of MCP-1. First, we found that MCP-1 dose dependently stimulated cellular growth along with the induction of both p42/p44 extracellular signal-regulated kinase and p38 MAPK phosphorylation (Fig. 1C). We also found that the mitogenic response paralleled the up-regulation of.
FIG. 1. Monocyte chemoattractant protein-1 induces fibrogenic activation of hepatic stellate cells. (A) Research hypothesis of the study. Following the identification of extracellular MCP-1 level as a novel biomarker of liver fibrosis associated with TMD in DS, we hypothesized that prolonged hSC activation by paracrine and/or autocrine MCP-1 mediation plays a fundamental role during the progression of liver fibrosis associated with TMD. (B) MCP-1 expression in hSCs. Left, representative image of an affected liver specimen showing characteristic MCP-1 expression in the hSCs. Original magnification ×200. Right, co-expression of CCR2 and MCP-1 in LX-2 cells. Scale bar, 100 μm. (C) LX-2 cells were treated with an increasing dose of MCP-1 (1 ng/mL to 100 ng/mL). Cell growth was assessed 3 days later. Upper panel shows their significant growth rate compared to unstimulated control cells (# P < 0.01). Bottom panel shows a dose-dependent increase of phosphorylated p38 and p42/44. ACTB expression was used as a protein loading control. Representative images of three independent experiments are shown. (D) qRT-PCR analysis of profibrogenic genes. Graphs show a 1.89-fold increase of type IV collagen and a 1.78-fold increase of α-SMA expression compared to unstimulated cells (# P < 0.01). (E) Ex vivo evaluation of the patient's serum activity. LX-2 cells were stimulated with the patient's serum obtained during the active stage (postnatal day 90) and the remission stage (postnatal day 144). Cell growth ratio and type IV collagen protein expression levels were examined after serum stimulation. Note the strong fibrogenic activity of the patient serum obtained at the active phase and prominent inhibition of serum activity in the presence of MCP-1 blocking antibody (#### P < 0.01). Data in C-E represent mean ± SD. Abbreviations: α-SMA, α-smooth muscle actin; ACTB, beta actin; CCR2, C-C chemokine receptor type 2; mRNA, messenger RNA; qRT-PCR, quantitative reverse-transcription polymerase chain reaction.
of profibrogenic gene expression levels, such as type IV collagen and alpha smooth muscle actin (Fig. 1D). Next, we examined whether the patient’s serum could by itself stimulate hSCs \textit{ex vivo}. As expected, we found that the serum obtained at day 99 during the active disease phase significantly activated hSCs to induce both cell growth and type IV collagen synthesis compared to serum obtained at day 144 during the remission phase (Fig. 1E). Serum activity was suppressed in the presence of MCP-1 blocking antibody, indicating the functional interaction of MCP-1 in the progression of liver fibrosis associated with TMD.

Because our patient developed liver fibrosis even after the resolution of TMD, we examined whether a
megakaryoblast-derived cytokine, such as TGF-β1, could prime hSCs to induce hypersecretion of MCP-1, thereby promoting collagenogenesis. We stimulated LX-2 cells with a single pulse of TGF-β1 and analyzed the cellular effects (Fig. 2A). Interestingly, we found that 1 hour of TGF-β1 stimulation was sufficient to induce prolonged hypersecretion of MCP-1 for at least 120 hours in vitro (Fig. 2B); this paralleled the induction of both cell growth and type IV collagen synthesis. More importantly, these cellular effects were completely suppressed in the presence of MCP-1 blocking antibody (Fig. 2C,D). On the whole, these findings show the possible involvement of MCP-1, at least in part, for prolonged activation of collagen-producing hSCs to promote liver fibrosis in both a paracrine and autocrine manner.

**Discussion**

Following our identification of MCP-1 as a novel biomarker of liver fibrosis associated with TMD, we found that MCP-1 mediates fibrogenic activation of hSCs both in vitro and ex vivo. More importantly, we showed that the fibrogenic cytokine is potentiated to be a future molecular target of liver fibrosis associated with TMD.

Several therapeutic interventions, such as steroids, exchange blood transfusion, and low-dose cytarabine, have been established for TMD, especially during the acute phase of hyperleukocytosis, but little is known about pathogenesis-oriented therapy for liver fibrosis associated with TMD. In this regard, we suggest that inchin-ko-to (ICKT), a Japanese herbal medicine, can be included as a therapeutic choice for liver fibrosis associated with TMD. ICKT has been prescribed for hepatobiliary diseases, such as hepatotocerized adult patients and infants with biliary atresia, with favorable clinical responses. It is noteworthy that the pharmacologic effect of ICKT is shown to suppress both cytokine-induced hepatocellular apoptosis and fibrogenic activity of hSCs. The beneficial roles of ICKT for liver fibrosis associated with TMD have been reported. Although limited data are available for the clinical heterogeneity of liver fibrosis associated with TMD, we believe that antifibrogenic cytokine therapy, i.e., an ICKT herbal medicine, might be included as one of the supportive measures for TMD in patients with DS. In fact, liver fibrosis associated with TMD in patients with DS is a unique pathologic condition. We put forward the possibility that hSC activation by MCP-1 mediation might play an important role not only in liver fibrosis associated with TMD in DS but also in other liver diseases of adults.

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