Secretion of MCP-1/CCL2 by bile duct epithelia induces myofibroblastic transdifferentiation of portal fibroblasts

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Submitted 6 July 2005; accepted in final form 6 November 2005


Biliary fibrosis and resultant cirrhosis are among the most important causes of chronic liver disease. Biliary cirrhosis accounts for nearly 20% of adult liver transplants (Organ Procurement and Transplantation Network database; www.optn.org) and is the leading indication for pediatric liver transplantation (14). Despite recent advances in the understanding of liver fibrosis, relatively little is known about the molecular mechanisms regulating biliary fibrosis.

Portal fibroblasts (PF) are potentially fibrogenic cells restricted to the portal area of the liver (5, 10). Mounting evidence suggests that PF are important regulators of biliary fibrosis. Our group (7) and others (15, 33) have shown that PF transdifferentiate into myofibroblasts in experimental models of fibrosis in rodents and in human biliary cirrhosis. Myofibroblastic transdifferentiation of PF likely results in the bile ductular hyperplasia characteristic of cholangiopathies such as primary sclerosing cholangitis, primary biliary cirrhosis, and related conditions (13). PF express the ectonucleotide triphosphate diphosphohydrolase (NTPDase2) and are, in fact, the only cells in the normal liver to do so (8). Loss of NTPDase2 is a cardinal event in biliary cirrhosis (7) and seems to be among the earliest events in myofibroblastic transdifferentiation of PF (unpublished observation).

The inflammatory cytokine monocyte chemoattractant protein-1 (MCP-1)/CCL2 was first identified as a chemotactant for monocytes released by blood cells (34, 35). More recently, evidence has suggested that MCP-1 may be an important regulator of liver fibrosis. Secretion of MCP-1 by hepatic stellate cells (HSC) is upregulated in several forms of fibrotic liver injury (6, 23) and may directly induce HSC migration (21). MCP-1 may be of particular importance in biliary fibrosis, because bile duct epithelia (BDE) secrete MCP-1 (22) and upregulate MCP-1 secretion in biliary injury (11, 17). Thus MCP-1 may be an important mediator of liver fibrosis/cirrhosis. However, the actions of MCP-1 on PF have not been determined.

In the present study we have examined the effects of MCP-1 on PF transdifferentiation and proliferation. We have shown that MCP-1 induces both myofibroblastic transdifferentiation and proliferation of PF. Furthermore, we have demonstrated that release of MCP-1 by injured BDE is sufficient to induce these changes, suggesting that this pathway may be important in the pathogenesis of biliary fibrosis.

EXPERIMENTAL PROCEDURES

Animals and reagents. Male adult Sprague-Dawley rats (180–250 g) were used for all experiments. MCP-1 was purchased from Calbiochem (San Diego, CA). MCP-1 blocking antibody was purchased from R&D Systems (Minneapolis, MN). Fluo-4 AM was purchased from Molecular Probes (Eugene, OR). All other chemicals, including polyacrylamide, were of the highest quality available.

Bile duct ligation. Rats underwent bile duct ligation (BDL) for 1 wk (7). Briefly, rats were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg). The abdomen was washed with isopropyl alcohol, and a midline laparotomy was performed. The common bile duct was exposed using blunt dissection and was ligated with dual sutures. Treatment of animals was within the prescribed guidelines of the Yale University Institutional Animal Care and Use Committee.

Isolation of PF. PF were isolated as described previously (16). Briefly, rat nonparenchymal cell fractions were obtained by collage- nase and Pronase digestion of rat livers. Cell suspensions were separated using serial mesh filtration. The resulting suspension of nonparenchymal cells was plated in medium containing DMEM/F-12 containing 2% penicillin-streptomycin, 10% fetal calf serum, 0.3% gentamicin, and 0.1% fungizone. Cells were used 2–24 h after isolation, at which time cell purity approaches 100%. Cell isolation has been defined as day 0. Therefore, day 1 PF are PF that were observed 24 h after isolation.

Isolation of HSC. HSC were isolated from retired breeder rats (500–700 g) by in situ pronase/collagenase perfusion followed by density gradient centrifugation, as described previously (1). Primary cells were >95% pure. Cells were grown on standard tissue culture plastic dishes in M199 medium with 10% fetal calf serum and antibiotics. Primary cells were used at 4 days after isolation, at which time they are known to be myofibroblastic (18).

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Isolation of BDE. Single BDE were prepared and characterized as described previously (12). This preparation results in a BDE preparation that is $>98\%$ pure as assessed by positive staining for the biliary epithelial markers $\gamma$-glutamyl transpeptidase, cytokeratin-19, and cytokeratin-7 (3, 28). Experiments using isolated bile duct cells were performed 12 h after plating.

Reverse transcriptase-polymerase chain reaction. Reverse transcriptase-polymerase chain reaction (RT-PCR) was used to determine whether CCL2 and the MCP-1 receptor CCR2 were expressed in PF. PF RNA was isolated using chaotropic methods (RNAqueous; Ambion, Austin, TX). Day 1 and day 7 HSC cDNA was produced using MMLV reverse transcriptase (BD Biosciences Clontech, Palo Alto, CA). Specific oligonucleotide primers were designed on the basis of cloned rat CCL2 (5'-GGCAAGATGATCCCAATGAGTC-3' and 5'-GCCTGAGCCTGGTGTTGGAAAG-3') and CCR2 (5'-TGATCTCTGCCCCCTACTTGTCACT-3' and 5'-ATGGCCGTTGCTAAAGGGCCA-TGT-3') mRNA sequences by using the following thermal cycling parameters: 94°C for 1 min; 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, and then 72°C for 5 min. Control reactions were performed with DNase-treated RNA that was treated with RNase but not subjected to RT. Products were evaluated using agarose gel electrophoresis, and PCR products were directly sequenced to ensure identity to the cloned rat genes.

Immunoblot. Alterations in expression of $\alpha$-smooth muscle actin ($\alpha$-SMA) were determined by immunoblotting using a mouse monoclonal antibody directed against rat $\alpha$-SMA (Sigma, St. Louis, MO) (9). PF were treated with MCP-1 (0.1 or 10 ng/ml), and protein homogenates were isolated. Equal amounts of protein for each group were electrophoresed using SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane (Immobilon; Millipore, Bedford, MA). The membrane was blocked with nonfat milk (5% in PBS), hybridized to the anti-$\alpha$-SMA antibody and

Fig. 1. Monocyte chemoattractant protein-1 (MCP-1) induces cytosolic $\text{Ca}^{2+}$ increases in portal fibroblasts (PF). A: representative confocal micrographs. PF were plated on glass coverslips and loaded with the cell-permeant $\text{Ca}^{2+}$-sensitive fluorophore fluo-4 AM. Changes in fluo-4 fluorescence were determined using confocal video microscopy. As shown in the representative images, perfusion with MCP-1 (1–100 ng/ml) induced increases in cytosolic $\text{Ca}^{2+}$ in PF. B: representative tracing of cytosolic $\text{Ca}^{2+}$ changes in cells shown in A. The representative tracing demonstrates that there were more frequent and qualitatively larger increases in cytosolic $\text{Ca}^{2+}$ in PF after stimulation with MCP-1 at 100 ng/ml than at 1 ng/ml. These images and tracing are representative of $>20$ experiments.

Fig. 2. PF express MCP-1/CCL2 but not the typical MCP-1 receptor CCR2. A: RT-PCR. Freshly isolated PF were plated, and RNA was isolated from cells at day 1, when cells are $\alpha$-smooth muscle actin ($\alpha$-SMA) negative, and day 7, when cells are $\alpha$-SMA positive (7). RT-PCR was performed to determine whether these cell populations express transcripts of CCL2 and CCR2. As shown in the agarose gel, PF expressed CCL2 at days 1 and 7. However, PF did not express CCR2 transcripts, although the positive control, rat whole blood, did express a CCR2 transcript. MWM, molecular weight marker.

B: effect of pertussis toxin (PTX) on MCP-1-sensitive cytosolic $\text{Ca}^{2+}$ changes. PF were plated on glass coverslips, treated overnight with PTX (100 ng/ml), and then loaded with fluo-4 AM and perfused with MCP-1 (10 ng/ml) as described in Fig. 1. Pretreatment with PTX completely inhibited MCP-1-sensitive increases in cytosolic $\text{Ca}^{2+}$ ($n=8$ experiments per condition). $P=0.0002$. 
then anti-mouse secondary antibody (1:1,000), and developed using enhanced chemiluminescence (ECL plus, Amersham, Amersham, UK).

Alterations in expression of NTPDase2 were determined by immuno blotting using a rabbit polyclonal antibody directed against NTPDase2/CD39L1 (8, 30). Protein was isolated as described above and subjected to electrophoresis and blot transfer. The membrane was blocked with nonfat milk (5% in PBS), hybridized to the anti-NTPDase2 antibody (1:500) then anti-rabbit secondary antibody, and developed using enhanced chemiluminescence. Changes in α-SMA and NTPDase2 expression were normalized to β-actin or GAPDH expression.

Confocal immunofluorescence. Changes in distribution of α-SMA after MCP-1 treatment were determined using confocal immunofluorescence. Fluorescence immunohistochemistry was performed on PF grown on glass coverslips. The cells were fixed by perfusion with 2% (wt/vol) paraformaldehyde in 0.075 M sodium phosphate. After un-grown on glass coverslips, the cells were fixed by perfusion with 2% after MCP-1 treatment were determined using confocal immunofluorescence microscopy.

Confocal video microscopy. Functional expression of MCP-1 receptors in PF was determined using confocal video microscopy to examine nucleotide-induced changes in cytosolic Ca²⁺ (9). PF were grown on glass coverslips, loaded with the Ca²⁺-sensitive fluorophore fluo-4 AM (Molecular Probes), and mounted on a specially designed stage for use on a confocal microscope. Cells were perfused initially with HEPES buffer and then with buffer containing MCP-1 (0.1–100 μM). Changes in fluo-4 fluorescence were monitored using a Bio-Rad MRC 600 confocal imaging system. Fluo-4 fluorescence was excited using a krypton-argon laser at 488 nm; emitted fluorescence at >515 nm was collected. Changes in fluorescence over time were expressed as peak fluorescence (f) divided by initial fluorescence (f₀). In separate experiments, PF were plated as described above and exposed to pertussis toxin (PTX; 100 ng/ml) overnight before loading with fluo-4 AM and perfusion with MCP-1.

Real-time RT-PCR. Alterations in expression of α₁-procollagen mRNA in PF were determined using real-time RT-PCR (9). PF were treated with either vehicle alone (control) or MCP-1 (0.1 or 10 ng/ml) overnight. Total RNA was isolated as described and subjected to real-time PCR using ABI-PRISM 7700 (Applied Biosystems, Foster City, CA). Detection of α₁-procollagen was accomplished by labeling with 6-FAM normalized to a VIC-labeled GAPDH probe. PCR was performed using the following cycling parameters: reverse transcription at 48°C for 30 min, activation of AmpliTaq polymerase (Applied Biosystems) at 95°C for 10 min, PCR cycling comprising 40 cycles at 95°C for 15 s (denaturation) and 60°C for 1 min (annealing/extension).

Bromodeoxyuridine uptake. Cell proliferation was measured using bromodeoxyuridine (BrdU) ELISA according to the manufacturer’s instructions (Cell Proliferation ELISA, BrdU Colorimetric; Roche Diagnostics, Nonnenwald, Germany). Cells were plated in 96-well culture plates and treated for 48 h with BrdU labeling solution with either control buffer or MCP-1 (0.1–100 ng/ml). Cells were then fixed and denatured, and anti-BrdU antibody was added. Colorimetric substrate was added, and BrdU incorporation was quantitated using a multplate reader.

Statistical analysis. Data are expressed as means (SD) where appropriate. Comparisons between individual groups were made with two-tailed t-tests.

![Fig. 3. MCP-1 induces PF proliferation. Proliferation of PF was determined by bromodeoxyuridine (BrdU) uptake. Cells were treated overnight with MCP-1 (0.1–100 ng/ml), and BrdU uptake was determined using ELISA. Although MCP-1 at 0.1 ng/ml did not change PF proliferation, MCP-1 at 1–100 ng/ml increased PF proliferation by roughly 30–40% (n = 12 for each condition). *P < 0.01 (two-tailed t-test).](http://ajpgi.physiology.org/)

![Fig. 4. MCP-1 induces α-SMA expression in day 1 PF. α-SMA expression by PF was determined using immunoblotting. PF were treated overnight (day 1), for 3 nights (day 4), or for 6 nights (day 7) with either buffer alone (negative control) or MCP-1 (0.1 or 10 ng/ml). As shown in the blot, MCP-1 at 10 ng/ml induced α-SMA expression in day 1 PF, whereas MCP-1 at 0.1 ng/ml did not. By days 4 and 7, all PF expressed high levels of α-SMA. Results are compared with GAPDH expression. These experiments were repeated 3 times to confirm results.](http://ajpgi.physiology.org/)
Fig. 5. MCP-1 induces redistribution of α-SMA expression in day 1 PF. α-SMA expression in PF was determined using confocal immunofluorescence. PF were treated overnight (day 1), for 3 nights (day 4), or for 6 nights (day 7) with either buffer alone (negative control) or MCP-1 (0.1 or 10 ng/ml). Images are labeled with anti-α-SMA (green) and the nuclear stain TOPRO (blue). A: low-power micrographs. Images (×100) reveal that MCP-1 at 0.1 or 10 ng/ml upregulated the number of α-SMA-positive cells and the extent of α-SMA expression outside of the perinuclear cytoplasm in day 1 PF. By days 4 and 7, all cells expressed α-SMA in a typical stress fiber distribution. B: high-power micrographs. Images (×630) reveal that control day 1 PF expressed a small amount of α-SMA that was restricted to the perinuclear cytoplasm, whereas MCP-1-treated PF expressed α-SMA throughout the cell. Some stress fibers appear to be present but are fewer than the organized α-SMA stress fibers shown on untreated control day 4 PF. MCP-1-treated day 4 and day 7 PF and control day 7 PF appear identical to the representative day 4 PF shown. C: control micrographs. Day 4 hepatic stellate cells (HSC) were observed for positive and negative control α-SMA staining because the distribution of α-SMA in these cells is well established. As shown in the positive control images obtained using identical confocal settings to the images in A and B, α-SMA staining is in a typical stress fiber distribution; however, no α-SMA staining is observed in cells labeled with secondary antibody but without primary anti-SMA antibody. All images shown are representative of more than 40 images collected from at least 2 experiments per treatment.
RESULTS

**PF express a functional receptor for MCP-1.** MCP-1-mediated responses in cells are mediated by cytosolic Ca\(^{2+}\)/H\(^{+}\) signals (21, 29). Thus, to determine whether MCP-1 induced downstream responses in PF, we examined MCP-1-sensitive cytosolic Ca\(^{2+}\)/H\(^{+}\) signals in PF using confocal video microscopy (Fig. 1). MCP-1 at concentrations of 0.1–1 ng/ml induced occasional modest sustained increases in cytosolic Ca\(^{2+}\)/H\(^{+}\), whereas MCP-1 at concentrations of 10–100 ng/ml induced sustained increases in cytosolic Ca\(^{2+}\) in all cells examined. These data suggest that PF express a functional MCP-1 receptor that is linked to increases in cytosolic Ca\(^{2+}\).

**Molecular identity of PF MCP-1 receptor is not CCR2.** Expression of both CCL2/MCP-1 and the MCP-1 receptor CCR2 was determined in PF by using RT-PCR (Fig. 2A). Although PF expressed CCL2 mRNA, they did not express the typical MCP-1 receptor CCR2 that has been described in monocytes and other cells (27). Note that this finding is identical to results of analogous experiments in human hepatic stellate cells (21). These results demonstrate that the PF MCP-1 receptor is distinct from CCR2. To determine whether the PF MCP-1 receptor was coupled to G\(\alpha\) proteins, we assessed the effect of PTX on MCP-1-sensitive changes in cytosolic Ca\(^{2+}\). As shown in Fig. 2B, PTX completely inhibited MCP-1-sensitive increases in cytosolic Ca\(^{2+}\), suggesting that the PF MCP-1 receptor is G\(\alpha\) coupled (2).

**PF proliferation and myofibroblastic transdifferentiation are regulated by MCP-1.** MCP-1-sensitive proliferation of PF was determined using BrdU ELISA (Fig. 3). Treatment of PF with MCP-1 at 1–100 ng/ml induced a 29–42% increase in PF proliferation (\(P < 0.01\) for each condition), whereas MCP-1 at 0.1 ng/ml had no effect on PF proliferation. Thus, MCP-1 stimulates proliferation of PF.

MCP-1-sensitive myofibroblastic transdifferentiation of PF was determined using immunoblot detection of \(\alpha\)-SMA. As shown in Fig. 4, MCP-1 at 10 ng/ml induced \(\alpha\)-SMA expression in PF, whereas MCP-1 at 0.1 ng/ml had no effect after 1 night of treatment. By day 4, all PF in each treatment group were \(\alpha\)-SMA positive. To confirm that this increase in \(\alpha\)-SMA expression was consistent with myofibroblastic transdifferentiation, we determined the subcellular distribution of \(\alpha\)-SMA in PF using confocal immunofluorescence (Fig. 5). In day 1 control (untreated) PF, \(\alpha\)-SMA staining was weak and restricted primarily to the perinuclear cytoplasm. In day 1 MCP-1 (0.1–10 ng/ml)-treated PF, \(\alpha\)-SMA staining was prominent and found in a primitive stress fiber distribution. By day 4, all PF expressed \(\alpha\)-SMA in an organized stress fiber distribution typical of myofibroblasts (24).

MCP-1-sensitive PF fibrogenesis was determined using real-time RT-PCR for \(\alpha\)-procollagen. As shown in Fig. 6, MCP-1 at 0.1 or 10 ng/ml markedly upregulated \(\alpha\)-procollagen transcription, suggesting that MCP-1 upregulates the fibrogenic potential of PF.

Because activation of PF is associated with decreased expression of the ectonucleotidase NTPDase2 (7), MCP-1-sensitive changes in NTPDase2 expression in PF were determined using immunoblotting. As shown in Fig. 7, MCP-1 at 0.1–10 ng/ml markedly downregulated expression of NTPDase2.

**Fig. 6.** MCP-1 markedly upregulates \(\alpha\)-procollagen transcription in PF. The effect of MCP-1 on transcription of \(\alpha\)-procollagen in day 1 PF was determined using real-time RT-PCR and normalized to GAPDH. MCP-1 (0.1 or 10 ng/ml) increased \(\alpha\)-procollagen transcription by \(\sim 14\)-fold (\(n = 3\) for each condition). \(P = 0.05\) for MCP-1 (0.1 ng/ml). \(P < 0.01\) for MCP-1 (10 ng/ml).

**Fig. 7.** MCP-1 attenuates expression of the ectonucleotidase triphosphate diphosphohydrolase (NTPDase2) in PF. NTPDase2 expression by day 1 PF was determined using immunoblotting. PF were treated overnight with either buffer alone (negative control) or MCP-1 (0.1 or 10 ng/ml). As shown in the blot, MCP-1 (0.1 or 10 ng/ml) markedly reduced expression of NTPDase2 relative to control. Results are compared with \(\beta\)-actin expression to ensure equal protein loading.
We hypothesized that MCP-1/CCL2 may be an important mediator of PF to portal myofibroblast (PMF) transdifferentiation for several reasons. First, investigators in our laboratory (7, 13) have previously shown that extrahepatic BDL induces PF to PMF transdifferentiation. Second, and perhaps more importantly, BDE markedly upregulate MCP-1 production in biliary fibrosis (19). Finally, there is evidence that HSC express functional MCP-1 receptors (20). Together, these observations suggest that biliary injury may induce a fibrogenic response via a paracrine pathway: BDE upregulate MCP-1 production, leading to “activation” of PF.

We found first that PF express functional MCP-1 receptors. Interestingly, these receptors are distinct from the previously characterized MCP-1 receptor CCR2. This finding replicates similar findings in HSC, which express a functional MCP-1 receptor molecularly distinct from CCR2 (20). Next, we noted that MCP-1 induces proliferation and myofibroblastic transdifferentiation of PF. Together, these findings would be necessary to support the paracrine signaling pathway that we have proposed. We also observed that MCP-1 induces downregulation of NTPDase2 expression by PF. This finding provides further evidence that the myofibroblastic cells observed were indeed PMF, because activated HSC express high levels of NTPDase2, and activated PMF express low levels of NTPDase2 (7). Finally, we demonstrated that media containing secretions from BDE isolated from rats that had undergone BDL induced transdifferentiation of PF. Furthermore, blockade of MCP-1 with a blocking antibody was sufficient to abrogate this response completely. This finding suggests that release of MCP-1 is likely a primary mechanism for paracrine regulation of PF by BDE. Thus the hypothesis that we have proposed is supported by the data presented.

This finding complements the recent report by our group (13) that PF regulate proliferation of BDE in a paracrine fashion through expression of NTPDase2. Hence, it seems that PF and BDE are constituents of a paracrine cross-talk loop. In this loop, injury to BDE induces MCP-1 production and activation of PF. Loss of NTPDase2 by PMF then allows extracellular ATP to stimulate BDE proliferation via activation of P2Y receptors. We do not know, however, whether extracellular ATP induces further production of MCP-1 by PF. We also do not know which is the initiating event in biliary fibrosis: injury of BDE with subsequent MCP-1 or transdifferentiation of PF and loss of NTPDase2. Further experiments should clarify this potentially important question as to which event is primary (4, 31). In either case, we now have several novel potential targets for the prevention and/or treatment of biliary fibrosis.

GRANTS
This project was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-066287 and DK-02379 (to J. A. Dranoff) and by the Yale Liver Center (DK-45710).
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