IL-10 receptor and coreceptor expression in quiescent and activated hepatic stellate cells

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Received 10 July 2001; accepted in final form 23 December 2001

Mathurin, Philippe, Shigang Xiong, Kusum K. Harbanda, Nary Veal, Takeo Miyahara, Kenta Motomura, Richard A. Rippe, Max G. Bachem, and Hidekazu Tsukamoto. IL-10 receptor and coreceptor expression in quiescent and activated hepatic stellate cells. Am J Physiol Gastrointest Liver Physiol 282: G981–G990, 2002. First published January 9, 2002; 10.1152/ajpgi.00293.2001.—Interleukin (IL)-10 expression is induced in activated hepatic stellate cells (HSC) in vitro and in vivo. We analyzed expression of IL-10 receptor (IL-10R) and coreceptor cytokine receptor family (CRF2–4) in HSC. We aimed to clone and sequence partial cDNA for rat IL-10R and CRF2–4, determine their expression in activated rat HSC in vivo and in vitro, and examine the biological responsiveness of HSC to exogenous IL-10. PCR cloning and sequencing of partial rat IL-10R and CRF2–4 cDNAs revealed 86% homology with corresponding mouse sequences. In hepatic macrophages, Northern blot with cloned IL-10R cDNA detected an expected 3.5-kb transcript, and IL-10R and CRF2–4 mRNAs showed steady constitutive expression after in vitro lipopolysaccharide treatment or cholestatic liver injury. IL-10R mRNA expression, as confirmed by immunohistochemistry, was induced 20.1- and 8.6-fold in HSC from cholestatic livers and 7-day culture-activated HSC, respectively but CRF2–4 mRNA levels were unchanged. Under serum-free conditions, IL-10 had minimal effects on collagen production but reduced DNA synthesis, matrix metalloprotease-2 mRNA levels, and activity in HSC. With serum, IL-10 inhibited both collagen production and DNA synthesis but had no effect on procollagen-α1(I) mRNA levels. This shows concomitant induction of IL-10R but not CRF2–4 to that of IL-10 by activated HSC in vitro and in vivo and associated acquisition of the responsiveness to IL-10, entailing complex effects on HSC.

interleukin-10; cytokine receptor family 2–4; procollagen-α1(I); matrix metalloprotease-2; matrix metalloprotease-13; monocyte-chemoattracting protein-1; liver fibrosis

INTERLEUKIN (IL)-10 was originally identified as a molecule from Th2 cells that downregulates Th1 cell functions. IL-10 is mainly produced by Th2 CD4+ cells, CD5+ B cells, and macrophages and inhibits inflammatory and cell-mediated immune responses while enhancing humoral immunity (26).

Protective effects of IL-10 on the liver have previously been demonstrated in different animal models (20–22, 33) in which IL-10 inhibited the release of tumor necrosis factor (TNF)-α, a critical factor implicated in a direct cytotoxic or indirect neutrophil-dependent mechanism of liver injury (10, 25, 27). In galactosamine/lipopolysaccharide (LPS) liver injury, administration of recombinant IL-10 decreased the serum levels of TNF-α and alanine aminotransferase, hepatocyte necrosis, expression of adhesion molecules, neutrophil infiltration, and lethality (20, 33). Conversely, antibodies against IL-10 accentuated the increases in serum TNF-α and alanine aminotransferase and the severity of hepatic necrosis in this model (20). IL-10 was also shown to protect mice from immune-mediated hepatitis induced by administration of concanavalin A (21).

In addition to anti-inflammatory effects, IL-10 may possess regulatory activities toward matrix homeostasis. In vitro, it downregulates type I collagen expression and increases expression of matrix metalloprotease-1 (MMP)-1 and -3 by cultivated skin fibroblasts (31). In vivo, IL-10 knockout mice developed excessive skin scar formation after exposure to irritant oil (32). A recent pilot trial demonstrated reduction of fibrosis in patients with chronic hepatitis C infection who received IL-10 (28), but it still remains to be determined whether this response is due to a direct antifibrotic effect or is secondary to IL-10’s anti-inflammatory effect.

Hepatic stellate cells (HSC), the vitamin A-storing perisinusoidal cells, participate in matrix remodeling and wound healing via their myofibroblastic activation (4). HSC also express IL-10 on activation (43). In vitro,
addition of anti-IL-10 antibodies to culture-activated HSC was shown to increase collagen production via induction of procollagen-α1(I) and inhibition of MMP-13 expression (43). Moreover, IL-10 knockout mice developed more severe carbon tetrachloride-induced fibrosis than wild-type animals (22, 38). Together, these results supported the hypothesis that IL-10 may act as a regulator of liver fibrogenesis (40, 41).

Like other cytokines, IL-10 exerts its effects through cell surface receptors. Human and mouse IL-10 receptors (IL-10R) have been cloned, and their functional domains have been analyzed (11, 13, 35–37). Interestingly, the lower sensitivity to human IL-10 of mouse Ba/F3 cells expressing the recombinant human IL-10R compared with those expressing the recombinant mouse IL-10R led to the identification of another IL-10R subunit required for IL-10 signaling (12, 19). This coreceptor, named cytokine receptor family (CRF2–4), was shown to be an accessory chain essential for the ligand-activated IL-10R to initiate its signal transduction (17). Indeed, CRF2–4-deficient mice show the phenotype of IL-10-deficient mice and the lack of responsiveness to IL-10 (34).

Even though an accumulating body of evidence exists in support of the regulatory role of IL-10 in liver fibrogenesis, the mechanisms underlying this regulation are still elusive. To our knowledge, virtually nothing is known about the expression of IL-10R and coreceptor in HSC. In the present study, we pursued the following specific aims: 1) to partially clone and sequence rat IL-10R and rat CRF2–4; 2) to examine regulation of these genes in activated HSC in vivo and in vitro; and 3) to determine the biological responsiveness of cultured HSC to IL-10 in relation to changes in their IL-10 expression.

MATERIALS AND METHODS

RNA extraction and RT-PCR. Total RNA from freshly isolated and cultured cells was extracted by the guanidium-phenol-chloroform method of Chomczynski and Sacchi (1). Total RNA was reverse transcribed by adding 30 μl of a master mix of RT buffer (Perkin-Elmer, Norwalk, CT), 0.5 mmol/l dNTP mixture, 1 U/ml RNase inhibitor, 600 units of Moloney murine leukemia virus RT, and 2 μl oligo(dT) at 37°C for 60 min. The RT was heat inactivated at 99°C for 5 min and cooled at 5°C for 5 min. The synthesized cDNA were amplified using a specific set of primers designed from published sequences (6, 8, 29, 43) as follows: IL-10, CTG-GCTCAGGACTGCTAT and ATTCATGGCCCTGGAGACAC; procollagen-α1(I), ACAGCACTGTTGGAAGTAGTGTCT and GTCTTC-AAGCAAGAGGACCA; MMP-13, GGACACCTCAATGGTCCA-CCA and TCCACATGGTTGGAGAAGTTC; MMP-2, GAGATC-TTTGTTTACACCTGAC and GTATCCGCGCCAGCAG; and β-actin, GAGCTTAGAGCTGCAAGG and GAGCCTGCGCCAGACAGAT.

PCR. Mouse IL-10R and CRF2–4 sequences were downloaded from the GenBank database using MacDNASIS software (Hitachi). We designed three independent sets of primers in different locations of the mouse IL-10R and CRF2–4 sequences using MacDNASIS. The primer sequences, the nucleotide position in mouse sequences, and the expected sizes of PCR products are given in Table 1. Each set of primers was used to perform PCR amplification on RNA extracted from culture-activated HSC according to the protocol described above. The size of each PCR product was tested on agarose gel with ethidium bromide staining. The fragments generated by PCR were isolated from agarose gel with an elution buffer and ligated into a TA cloning vector followed by cloning procedures according to the TA cloning kit (One-Step PCR cloning; Invitrogen, San Diego, CA). After a large-scale plasmid preparation, the sequence of each cDNA was determined by using the chain determination method (US Biochemical). A partial EcoRI fragment of the clone obtained with the second set of IL-10R primers was purified as above and used as a probe for Northern blot hybridization.

Competitive RT-PCR. Competitive RT-PCR is one of the approaches to overcome the variability in quantification by PCR. In the present study, we performed the competitive

Table 1. Mouse primers for IL-10R and CRF2–4

<table>
<thead>
<tr>
<th>Nucleotide Sequences</th>
<th>Nucleotide Position in Mouse Sequence</th>
<th>Size of PCR Product, nt</th>
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<td>5’-CCTCTCATGTTGTTGATGAAGGCCTCC-3’</td>
<td>164–187</td>
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<tr>
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<td>5’-CATCAGCTGTTGGAAGGACTCC-3’</td>
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<td>302–324</td>
</tr>
<tr>
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<td>5’-AAGCAGAGAGCAGAACGAGTCACCC-3’</td>
<td>850–828</td>
</tr>
<tr>
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<td>119–211</td>
</tr>
<tr>
<td>Primer IL-10R backward 3</td>
<td>5’-CCATCTGGTCTTGTCTTCTGGTTAGCC-3’</td>
<td>1426–1403</td>
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<tr>
<td>Primer CRF2–4 forward 1</td>
<td>5’-CCTGCTTTTTCACAAAGGAC-3’</td>
<td>129–149</td>
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<tr>
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<td>5’-TCACTCCATCTTACTCCTCCAGGTTCC-3’</td>
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<tr>
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<td>123–147</td>
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<tr>
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<td>5’-CGTTTTTCCATTGGCATGCACTC-3’</td>
<td>341–318</td>
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<tr>
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<td>5’-TCTCTGTTCCCTGGAAAGGACACCC-3’</td>
<td>167–188</td>
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<td>5’-GTGAGAAAGAAGCCCTTGAACCTTG-3’</td>
<td>447–424</td>
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IL, interleukin; IL-10R, IL-10 receptor; CRF, cytokine receptor family.
PCR using an RNA competitive template containing the specific primer sites for rat IL-10R (primer sequences are given in RESULTS). This RNA competitor was generated by in vitro transcription using a competitive RNA transcription kit (Takara’s, Panvera, Madison, WI). For competitive PCR, sample RNA was amplified in the presence of the increasing amount of the RNA competitor.

**Northern blot analysis.** For Northern blot analysis for IL-10R and procollagen-α₁(I), ~5–20 μg of total RNA was electrophoresed in 1% agarose gel containing formaldehyde and transferred to a nylon filter (Micron Separations, Westboro, MA) as described (43). PCR-cloned rat IL-10R cDNA obtained with the second set of primers and rat procollagen-α₁(I) cDNA were labeled with [α-32P]dCTP using a random primer labeling kit (Life Technologies). The filter was prehybridized and hybridized with the 32P-labeled cDNAs at 50°C and then washed at a high stringency at 50°C as described previously (42).

**Bile duct ligation.** Bile duct ligation was performed on male Wistar rats weighing 550–650 g by aseptic ligation and scissors of the common bile duct (BDL) as previously described (29). Another group of rats was sham-operated by the adherence method as previously described (29). HSC were isolated 1 wk after the surgery. The animal protocol in this study was approved by the Institutional Care and Use Committee of the University of Southern California.

**HSC isolation and biochemical assays.** HSC were isolated from normal, BDL, and Sham Wistar rats by in situ digestion of the liver and arabinogalactan gradient ultracentrifugation as reported previously (29, 42). The purity of the cells was examined by Trypan blue exclusion. The viability was examined by phase-contrast microscopy, and the purity and the viability of the cells from the animals was examined by Trypan blue exclusion. The cells were cultured in RPMI or DMEM with 10% fetal calf serum for 3 or 7 days according to the design of the experiment. For measurement of collagen production by 3- or 7-day cultured HSC, the conditioned medium was collected and centrifuged at 12,000 rpm for 1 min. The supernatant was stored at ~−70°C until assay. MMP activity in HSC-conditioned medium was determined using zymographic analysis under denaturing but nonreducing conditions. In brief, each sample (10–20 μl) was applied onto a denaturing 8% SDS-PAGE gel (1 g/100 ml) containing 0.1% gelatin (Sigma, St. Louis, MO) for MMP-2 and 9 (23) or 0.1% collagen I (Sigma) for MMP-13 (7). Electrophoresis was performed at 25 mA constant current for 2 h at room temperature, followed by equilibration in distilled water containing 2.5% Triton X-100 for 1 h to remove SDS. The gel was then incubated in enzyme buffer containing 50 mM Tris·HCl (pH 8.0), 200 mM NaCl, 5 mM CaCl₂, and 0.02% Brij 35 for 18 h at 37°C. Bands of enzymatic activity were visualized by negative staining with standard Coomassie brilliant blue dye solution (Bio-Rad, Hercules, CA). Molecular sizes of bands displaying enzymatic activity were identified by comparison with prestained standard proteins (Bio-Rad).

**IL-10R immunofluorescence.** The 3 and 7 day cultured HSC on chamber slides were fixed by ice-cold acetone for 10 min and air dried. The culture slides were then rehydrated with PBS. Nonspecific binding was blocked with a TNB buffer (50 mM Tris, 150 mM NaCl, 0.5% bovine serum albumin, and 0.5% sodium azide, pH 7.7). Goat anti-murine IL-10R antibodies (R&D Systems) were diluted 1:1,000 in TBS (50 mM Tris and 150 mM NaCl, pH 7.4) and applied onto the slides overnight. After three washes with a TNT buffer (50 mM Tris, 150 mM NaCl, and 0.05% Tween-40, pH 7.4), biotinylated rabbit anti-goat IgG (DAKO, Glostrup, Denmark), diluted 1:500 in TNB, was applied and incubated for 1 h. After three washes with TNT buffer, horseradish peroxidase-conjugated streptavidin (DAKO), diluted 1:100 in TNB, was added and incubated for another hour. Thereafter, the slides were washed again three times using TNT buffer, and tyramid signal amplification (TSA) reagent (NEN-Life Science Products, Boston, MA), diluted 1:50, was applied for 20 min. After three washes, Streptavidin-Red613 (GIBCO-BRL Life Technologies, Eggernstein, Germany), diluted 1:100 in TBS, was applied for 20 min. After the final washes, nuclei were stained by bisbenzimide (Hoeschst-33258; Sigma, Munich, Germany). Fluorescence was observed and photographed with a fluorescence microscope (C. Zeiss, Oberkothen, Germany) equipped with epilumination at the magnification of ×200 or ×400.

**Hepatic macrophage isolation and culture.** Hepatic macrophages were isolated from normal, BDL, and Sham Wistar rats as described before (14). The purity and viability always exceeded 85% and 90%, respectively. Freshly isolated hepatic macrophages were immediately processed for RNA extraction. For the culture experiments, hepatic macrophages were seeded at 30 × 10⁵ cells/100-mm dish and further purified by the adherence method as previously described (5, 14). After the adherence purification, the purity of hepatic macrophages exceeded 95% as assessed by latex bead (1 μm) phagocytosis. The cells were incubated with RPMI 5% fetal calf serum for 2 days following the adherence method for in vitro experiments. For stimulation with LPS, the cells were washed twice with PBS, incubated in serum-free RPMI, and exposed to LPS (500 ng/ml; Escherichia coli 026:B6, cell culture grade, prepared by TCA precipitation and gel filtration chromatography; Sigma) for 1, 2, 6, 8, 18, and 24 h.

**RESULTS**

**Partial sequences of rat IL-10R and CRF2–4 genes.** We first tested whether the primers designed from the mouse sequences led to PCR products with the expected sizes. Following ~30–35 cycles of PCR on RNA from culture-activated HSC, the use of three sets of primers (Table 1) yields products with sizes matching
those predicted from the sequence (data not shown). RT-PCR for CRF2–4 was also performed using the three sets of primers, and it also produced three expected sizes of the product. RT-PCR of RNA from hepatic macrophages produced detectable products for both IL-10R and CRF2–4 but required only 20–25 cycles of amplification, suggesting higher expression of these genes in hepatic macrophages. The PCR fragments from HSC RNA were subsequently cloned and sequenced. Analysis of the rat sequence from each PCR product for IL-10R revealed 85% homology with the mouse sequence. A partial sequence of 1,215 nt for rat IL-10R was obtained after a combination of three independent sequencings. For CRF2–4, a partial rat sequence of 449 nt disclosed a similar pattern of homology (86%) to that of the mouse sequence. Using the obtained sequence of rat IL-10R, we designed a new specific set of primers to yield a 319-nt PCR product: 5’-CCAACTGGACCATCAGTAAA-3’ and 5’-GCCTTGTTAATTCGGGATTCCAC-3’. Northern blot analysis was performed using a PCR-cloned IL-10R cDNA. This detected a 3.5-kb transcript in hepatic macrophage RNA, a size identical to the mouse IL-10R mRNA (Fig. 1). The levels of IL-10R mRNA in cultured HSC were too low to be detected by this method (Fig. 1).

**IL-10R and CRF2–4 expression by hepatic macrophages.** Regulation of hepatic macrophage expression of IL-10R and its coreceptor CRF2–4 was examined next by RT-PCR. In vitro, the treatment of cultured hepatic macrophages with LPS resulted in expected, time-dependent induction of TNF-α and IL-10 (Fig. 2). IL-10R mRNA showed a tendency to be increased, but this change was not significant and reproducible (Fig. 2). This finding corroborates steady constitutive expression of IL-10R previously demonstrated in hepatic macrophages (16). CRF2–4 mRNA levels in hepatic macrophages were also unaffected by LPS challenge (data not shown). We also examined the expression of IL-10R in hepatic macrophages isolated from rats with cholestatic liver injury. Northern blot analysis of hepatic macrophage RNA from BDL rats showed similar levels of IL-10R mRNA to those from Sham animals (Fig. 3).

**IL-10R and CRF2–4 expression by culture-activated HSC.** We next examined regulation of IL-10R and CRF2–4 expression in activation of HSC in culture. Six independent experiments revealed a reproducible increase in IL-10R mRNA in 7-day culture-activated HSC compared with freshly isolated HSC (day 0) or 3-day cultures of quiescent HSC. A representative set of data is shown in Fig. 4. Culture activation of HSC on day 7 was clearly observed under phase contrast microscopy and was confirmed by conspicuous upregulation of the procollagen-α(I) gene (Fig. 4). On the other hand, the level of CRF2–4 mRNA was not changed in culture-activated HSC (Fig. 4). To quantitatively determine the induction of IL-10R mRNA in culture-activated HSC, a competitive RT-PCR was performed...
using a constructed RNA competitive template. As shown in Fig. 5, an increasing amount of the competitor resulted in a progressive reduction in the level of IL-10R product while reciprocally raising the level of the competitor product. However, the range of the competitor concentration required for this competition was much higher with the samples from 7-day HSC than those from 3-day HSC. In fact, the computation of our results revealed that the level of IL-10R mRNA was 20.1-fold higher in 7-day culture-activated HSC than in quiescent, 3-day HSC (1.438 × 10^6 vs. 0.716 × 10^5 copies/μl, P = 0.02).

To verify the observed induction of IL-10R in culture-activated HSC at the protein level, immunostaining was performed on 3-day HSC (Fig. 6, A–C) and 7-day HSC (Fig. 6, D–F). Immunofluorescence staining for IL-10R is minimal in 3-day HSC (Fig. 6, B and C), but it is clearly intensified in 7-day HSC (Fig. 6, E and F). Note that a contaminating lymphocyte in the 3-day HSC culture is positively stained for IL-10R, serving as an inadvertent positive control. Figure 6, A and D, are negative controls for staining without the primary antibodies.

**IL-10R and CRF2–4 expression by HSC from cholestatic liver injury.** We next examined regulation of IL-10R and CRF2–4 expression in rat HSC on activation in vivo induced by cholestatic liver injury. As expected, HSC isolated from BDL rats had induced expression of activation marker genes such as procollagen-α(1) and IL-10 (Fig. 7) (43). In these cells, mRNA expression of IL-10R was induced, whereas that for CRF2–4 was unchanged (Fig. 7). These results were reproducible in HSC isolated from six pairs of BDL and Sham rats. Competitive RT-PCR for IL-10R was performed (Fig. 8) and demonstrated an 8.6-fold induction in BDL compared with Sham rats (5.876 × 10^5 ± 0.477 × 10^5 vs. 6.834 × 10^4 ± 0.989 × 10^4 copies/μl, P < 0.0001).
Again, no effects were observed in 3-day HSC (Fig. 10A), the effects of IL-10 on MMP-13 and -2 expression, regardless of whether the media contained serum or not, IL-10 had no effects on procollagen-α(1)(I) mRNA expression, and MCP-1 production were assessed. As shown in Fig. 9A, IL-10 modestly but consistently decreased collagen production in 7-day HSC under serum-stimulated conditions (Fig. 9A). DNA synthesis was not affected by IL-10 in 3-day HSC but was decreased in 7-day HSC cultured in serum-free medium with 100 ng/ml of IL-10 (Fig. 9B). Under serum-supplemented conditions, this inhibition was more consistently observed even with lower concentrations of IL-10, suggesting that serum-stimulated HSC are more sensitive to inhibitory effects of IL-10 (Fig. 9B). Effects of IL-10 on procollagen-α(1)(I) mRNA levels were next examined by Northern blot analysis. As shown in Fig. 9C, regardless of whether the media contained serum or not, IL-10 had no effects on procollagen-α(1)(I) mRNA in either 3- or 7-day cultured HSC. Next, we examined the effects of IL-10 on MMP-13 and -2 expression. Again, no effects were observed in 3-day HSC (Fig. 10, A and B). The 7-day HSC showed higher basal expression of MMP-13 mRNA compared with 3-day HSC, and this was further increased by the treatment with IL-10 (Fig. 10A). However, we could not detect MMP-13 activity in the medium of 7-day HSC with or without IL-10 treatment by a zymography assay. MMP-2 mRNA level was increased in 7-day HSC compared with 3-day HSC and was reduced twofold by IL-10 in 7-day HSC (Fig. 10B). This inhibition was also confirmed at the activity level because the zymography analysis demonstrated a twofold reduction in MMP-2 activity (Fig. 10C). IL-10 failed to affect basal or TNF-α induced MCP-1 production by either 3- or 7-day HSC (Table 2).

Thus these results demonstrate a correlation of induced IL-10R expression in 7-day HSC with their acquisition of the biological responsiveness to IL-10. They also show the complexity of IL-10-mediated effects on HSC functions, which are significantly influenced depending on whether HSC are tested under serum-free or supplemented conditions. Under serum-stimulated conditions, IL-10's inhibitory effects on DNA synthesis and collagen production become apparent in addition to its suppression of MMP-2 expression.

**DISCUSSION**

Enhanced autocrine expression of IL-10 by activated HSC was demonstrated by others and us (39, 43) and was suggested to regulate matrix expression. Because IL-10 induces its biological response in cells by interacting with its specific receptors expressed on the target cell surface, our primary objective was to evaluate regulation of the expression of IL-10R and coreceptor in activation of HSC in vitro and in vivo. To achieve this objective, we first cloned and sequenced rat IL-10R and CRF2−4 cDNAs. In support of the authenticity of the cDNAs, we generated the following results: 1) all of the PCR products matched the sizes expected from the known mouse sequences; 2) rat IL-10R and CRF2−4 clones revealed 86% homology with the corresponding mouse sequences; and 3) Northern blot analysis using a cloned IL-10R cDNA detected a transcript with a very similar if not identical size to the mouse IL-10R mRNA. Macrophages are one of the known target cell types for IL-10 that induces profound inhibition of expression of proinflammatory cytokines such as TNF-α, IL-1α, IL-1β, IL-6, and IL-8 (2, 9, 15). IL-10 also reduces monocyte expression of major histocompatibility complex class II (3). To further test our sequence-specific primers and Fig. 8. Representative data for IL-10R competitive PCR for RNA extracted from cholestatic liver injury (BDL HSC) vs. sham-operated animals (Sham HSC). Note that higher concentrations of the competitor were used for BDL HSC.
IL-10R AND CRF2–4 IN HSC

A

Day 3 HSC

Day 7 HSC

FCS-

FCS-

FCS+

Collagen Production (% of control)

IL-10 (ng/ml)

0 1 10 100

0 1 10 100

0 1 10 100

B

Day 3 HSC

Day 7 HSC

FCS-

FCS-

FCS+

DNA Synthesis (% of control)

IL-10 (ng/ml)

0 1 10 100

0 1 10 100

0 1 10 100

C

Day

FCS

IL-10

procollagen alpha1(I)

28S

18S

Fig. 9. Effects of IL-10 on HSC collagen production, DNA synthesis, and procollagen-alpha1(I) mRNA expression. The 3- or 7-day cultured HSC were treated with IL-10 under serum-free (FCS−) or serum-supplemented (FCS+) conditions, and the effects on collagen production (A), DNA synthesis (B), and procollagen-alpha1(I) mRNA levels (C) were analyzed. Note that only in 7-day HSC was a modest (+22%) but significant decrease in DNA synthesis by IL-10 (100 ng/ml) observed under serum-free conditions. In contrast, in serum-stimulated cells on day 7, IL-10 consistently inhibits collagen production and DNA synthesis but had no effects on procollagen-alpha1(I) mRNA levels.

**P < 0.05, ***P < 0.01 vs. control (no IL-10).

cDNAs, we first examined the expression of IL-10R and CRF2–4 in hepatic macrophages. Indeed, we demonstrated that the levels of IL-10R and CRF2–4 mRNA were substantially higher in hepatic macrophages than in HSC. We further showed that neither LPS stimulation in vitro nor activation in vivo by cholestatic liver injury resulted in alterations in the expression of IL-10R mRNA, corroborating the previous reports on LPS-stimulated hepatic macrophages (16) and RAW 264.7, a murine macrophage cell line (44). In addition, we also demonstrated that the expression of CRF2–4, the IL-10 coreceptor, was constitutive and unaffected by the activation state of hepatic macrophages.

In contrast to hepatic macrophages, IL-10R expression was conspicuously upregulated in culture or in vivo activated HSC, and this was concomitant with induction of IL-10 (Fig. 7) (39, 43), supporting an induced autocrine loop involving this cytokine in activated HSC. With regard to CRF2–4, the IL-10 coreceptor, we did not observe any significant changes in its expression.
mRNA expression in HSC in response to activation either in vivo or in vitro. These results suggest that CRF2-4 and IL-10R do not share the same mechanisms of regulation in HSC. Since IL-10-induced signal transduction occurs only when both IL-10R and CRF2-4 are present (17), we can speculate that the weak expression of IL-10R in quiescent HSC is a major limiting factor for their lack of the responsiveness to IL-10 and that induction of IL-10R in activated HSC confers the responsiveness.

Indeed, our biological data support this notion. In 7-day culture-activated HSC with induced IL-10R expression only, the treatment with rat recombinant IL-10 caused inhibition of DNA synthesis, upregulation of MMP-13, and downregulation of MMP-2 expression. Our previous study (43) using antibodies against murine IL-10 showed that neutralization of IL-10 produced by culture-activated HSC stimulated collagen and inhibited MMP-13 expression, suggesting antifibrotic effects of HSC-derived IL-10. The present study demonstrated no effects on collagen production under serum-free conditions but showed inhibition of this parameter in serum-stimulated HSC. Even though the magnitude of inhibition is modest, these results highlight that the culture condition is a critical determinant for IL-10’s effects. IL-10 also had an inhibitory effect on HSC DNA synthesis, the effect which was also accentuated in serum-stimulated HSC. MMP-13 mRNA expression was clearly induced by IL-10, and this effect corroborates our previous observation using the IL-10-neutralizing antibodies. However, the biological implication of this finding is not certain since we failed to detect MMP-13 activity in the culture media by a zymography assay. In contrast, MMP-2 expression was inhibited by IL-10 at both mRNA and activity levels. This effect may also be considered antifibrotic because MMP-2 is implicated in matrix remodeling and HSC migration through degradation of collagen IV, laminin, and proteoglycans in the initiation of liver fibrogenesis.

Even though IL-10 consistently inhibited collagen production by serum-stimulated HSC, IL-10 did not suppress the steady-state mRNA levels for procollagen-α(I). This is contrary to upregulated procollagen-α(I) mRNA expression by HSC treated with anti-IL-10 antibodies (43). Reasons for this discrepancy are not known presently but may include problematic nonspecific effects of the antibodies used in the previous study, differential responses to low endogenous concentrations of IL-10 and higher concentrations of exogenous IL-10 used in the present study, or different culture durations used in the two studies (~3–5 days vs. 7 days in the previous vs. present studies). Furthermore, the different activation state of cultured HSC may result in the differential sensitivity to IL-10’s effects. As highlighted in the present study, serum stimulation alone can profoundly increase HSC’s sensitivity to IL-10. Since we did not observe any effects on procollagen-α(I) mRNA, IL-10’s inhibition of collagen production is likely mediated by translational or posttranslational effects.

Table 2. MCP-1 production by HSC

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<th>IL-10, ng/ml</th>
<th>TNF-α, ng/ml</th>
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<tr>
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<tr>
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</tbody>
</table>

Amount of monocyte-chemoattractant protein (MCP)-1 released from basal (0 ng/ml) or tumor necrosis factor (TNF-α)-stimulated (10 ng/ml) hepatic stellate cells (HSC) was not affected by IL-10.
Another important mediator expressed by activated HSC is MCP-1. In our study, IL-10 treatment did not affect either basal or TNF-α-induced MCP-1 production by cultured HSC. This is in contrast to inhibition of MCP-1 production by IL-10 in activated intestinal epithelial cells (18) and inhibition of the endotoxin-induced release of macrophage inflammatory protein-1 and MCP-1 by IL-10 in healthy subjects, which was shown to be independent from IL-10’s inhibitory effect on TNF-α production (30).

Collectively, these results show rather complex and multiple effects of IL-10 on the fibrogenic potential of cultured HSC. In summary, the present study demonstrated coordinated induction of IL-10R and IL-10 in activated HSC in vitro and in vivo, suggesting that establishment of this autocrine loop may be of physiological relevance. The responsiveness to exogenous IL-10 was conferred by IL-10R induction in culture-activated HSC, but IL-10-mediated regulation of HSC biology was multifarious with induction of both MMP-13 mRNA and suppressed DNA synthesis and MMP-2 activity. Furthermore, under serum-stimulated conditions, IL-10’s inhibitory effects on DNA synthesis and collagen production become apparent. Further investigation of IL-10’s translational and posttranslational regulation of collagen expression and of the molecular mechanisms underlying IL-10’s inhibitory effects on DNA synthesis and MMP-2 are required to fully understand the mechanisms and significance of induced IL-10 autocrine loop in HSC activation and liver fibrogenesis.

This work was supported by National Institutes of Health Grants R01-AA-06603 (to H. Tsukamoto), R01-DR-34987 (to R. A. Rippe), R01-AA-10459 (to K. K. Kharbanda and H. Tsukamoto), R37-AA-06603 (to R. A. Rippe), R50-AA-11999 (to USC-UCLA Research Center for Liver Diseases) and by the Medical Research Service, Department of Veterans Affairs (to K. K. Kharbanda and H. Tsukamoto). Dr. Mathurin’s research fellowship was supported by French grants (Bourse Tournat, Laboratoire Glaxo Wellcome, and Institut Lilly).


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