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Expression of Interleukin-10 by *in Vitro* and *in Vivo* Activated Hepatic Stellate Cells*

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Activated hepatic stellate cells (HSC) participate in matrix remodeling and deposition in liver fibrosis. The present study demonstrates that interleukin (IL)-10 is expressed by HSC upon activation *in vitro* or *in vivo* and that autocrine effects of this cytokine include inhibition of collagen production. Culture activation of HSC caused a distinct increase in IL-10 mRNA level compared with freshly isolated quiescent HSC. Treatment of cultured HSC with tumor necrosis factor-α, transforming growth factor-β, or lipopolysaccharide further increased IL-10 mRNA by 2-fold and resulted in the release of IL-10 protein into the medium. HSC isolated from rats after bile duct ligation (BDL) showed prominent increases in IL-10 mRNA (× 100) and protein (× 30) levels at 7 days after BDL, but such induction disappeared in advanced liver fibrosis (19 days after BDL). IL-10 expression correlated positively with mRNA expression of interstitial collagenase and inversely with that of α1(I) collagen. Addition of anti-IL-10 IgG to cultured HSC caused enhanced collagen production under a basal or stimulated condition with TGF-β, tumor necrosis factor-α, or lipopolysaccharide. These effects were associated with increased α1(I) collagen mRNA and reciprocally reduced collagenase mRNA levels. Co-transfection of HSC with an IL-10 expression vector and collagen reporter genes showed a 40% inhibition of α1(I) collagen promoter activity. These results demonstrate that activation of HSC causes enhanced autocrine expression of IL-10 which possesses a negative autoregulatory effect on HSC collagen production mediated at least in part by α1(I) collagen transcriptional inhibition and stimulation of collagenase expression. These findings, along with the demonstrated early induction of HSC IL-10 expression and its late disappearance during biliary liver fibrosis, suggest its *in vivo* role in matrix remodeling and a possibility that failure for HSC to sustain IL-10 expression underlies pathologic progression to liver cirrhosis.

Hepatic stellate cells (HSC)[1] are vitamin A-storing perisinusoidal cells in the liver. These cells participate in matrix remodeling and wound healing of the liver via their myofibroblastic activation (see Ref. 1 for review). Several plausible mechanisms have been proposed which underlie HSC activation (1). One such mechanism involves soluble factors such as cytokines and inflammatory mediators, which seem to induce different aspects of the cellular activation. Fox example, platelet-derived growth factor, IL-1, TNF-α, TGF-α are all mitogenic to HSC (2, 3), while TGF-β is a potent fibrogenic cytokine that not only induces expression of matrix genes (3–5) and tissue inhibitors of metalloprotease (TIMP) (6), but may also confer HSC a myofibroblastic phenotype by up-regulating α-smooth muscle actin expression (7, 8). These soluble factors are released by effector cells such as hepatic macrophages (9–11), endothelial cells (12), hepatocytes (13), or platelets (14) to establish a paracrine mode of action or produced by HSC to achieve autocrine effects (15). It also seems important to recognize that one of the primary activities of many of these cytokines resides in their modulation of inflammation and immune responses. As integral part of wound repair processes, monocytes, fibroblasts, and myofibroblasts are recruited to the injury site by platelet-derived growth factor (16) and TGF-β (17). HSC are shown to express MCP-1 (18), which chemoattracts monocytes; M-CSF (19), which induces proliferation and differentiation of monocytes; PAF (20) and CINC (21), which recruit neutrophils; and ICAM (22), which causes adhesion and transmigration of neutrophils. Thus, HSC may also actively participate in regulation of inflammation in the liver.

IL-10 is a cross-regulatory cytokine produced by Th2 cells, macrophages, mast cells, and B cells. It mediates several key functions of multiple cell types. IL-10 inhibits functions of Th1 cells and their expression of IL-2 and γ-interferon (23), suppresses macrophages, including antigen presentation to Th1 cells, cytokine production, and cytotoxic activities (24, 25). In contrast, IL-10 stimulates mast cells (26) and B cells (27). In addition, IL-10 has been shown recently to down-regulate type I collagen gene expression and to increase matrix metalloprotease-1 (interstitial collagenase) and matrix metalloprotease-3 (stromelysin-1) (MMP-1 and -3) expression in cultured skin fibroblasts, suggesting a role of IL-10 in the breakdown and remodeling of the extracellular matrix (28). In contrast, exogenous IL-10 inhibits synthesis of MMP-9 (92-kDa gelatinase) and blocks LPS-stimulated MMP-1 expression by human macrophages while it stimulates their TIMP-1 production (29). Thus, these findings suggest fibrogenic effects of IL-10 on macrophages, which seem to oppose the aforementioned effects on fibroblasts.

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[1] The abbreviations used are: HSC, hepatic stellate cells; IL, interleukin; TNF, tumor necrosis factor; TGF, transforming growth factor; TIMP, tissue inhibitors of metalloprotease; MMP, matrix metalloprotease; LPS, lipopolysaccharide; BDL, bile duct ligation; RT-PCR, reverse transcription-polymerase chain reaction; bp, base pair(s).

In this report, we demonstrate for the first time, induced expression of IL-10 by rat HSC upon activation *in vitro* by culturing on plastic dish and *in vivo* by cholestatic liver injury. IL-10 expression by HSC is up-regulated by TNF-α and TGF-β1 *in vitro* and induced conspicuously during the early phase of cholestatic injury followed by a disappearance of the induction at the late fibrogenic phase. *In vitro* neutralization experiments demonstrate autocrine stimulation of interstitial collagenase expression and inhibition of α1(I) collagen expression by IL-10 in HSC, suggesting its role in initiation of matrix remodeling.

MATERIALS AND METHODS

Cholestatic Liver Injury—Cholestatic liver injury was induced in male Wistar rats weighing 500–600 g by aseptic ligation and transection of the common bile duct (BDL) as described previously (30). Another group of the rats was sham-operated to serve as controls (Sham). The animal protocol described in this study was approved by the Institutional Care and Use Committee of the University of Southern California.

HSC Isolation and Culture—HSC were isolated from normal male Wistar rats, BDL, and Sham animals by *in situ* digestion of the liver and arabinogalactan gradient ultracentrifugation as reported previously (31, 32). The purity and the viability of the cells from all animals exceeded 98 and 97%, respectively. The cells from normal rats were cultured in RPMI with 10% fetal calf serum in 24-well plates for 5–6 days after isolation. For experiments testing effects of TNF-α (0.1–10 ng/ml), TGF-β (0.1–10 ng/ml), LPS (1–100 μg/ml), and anti-IL-10 IgG (20 μg/ml), the cells were washed with phosphate-buffered saline twice and incubated with serum-free RPMI and test substances for 42 h. TNF-α, TGF-β, and goat anti-mouse IL-10 IgG were purchased from R & D System (Minneapolis, MN), and LPS and non-immune goat IgG were from Sigma.

RNA Extraction, RT-PCR—Total RNA was extracted from freshly isolated and cultured cells by a method of Chomczynski and Sacchi (33). For RT-PCR, total RNA was reverse-transcribed using 600 units of Moloney murine leukemia virus reverse transcriptase and oligo(dT) at 37 °C for 60 min. The synthesized cDNA for IL-10, interstitial collagenase, α1(I) collagen, and β-actin were amplified using specific sets of primers designed from published cDNA sequences (34–36) as follow: IL-10, CTGGCTCAGCACTGCTAT and ATTCATGGCCTTGTAGACAC; rat interstitial collagenase, CGAACACTCAAATGGTCCCA and TCCACATGGTTGGGAAGTTC; collagen α1(I), ACAGCACGCTTGTGGAT and GTCTTCAAGCAAGAGGACCA; β-actin, GAGCTATGAGCTGCCTGACG and AGCACTTGCGGTCCACGATG. A competitive template containing the specific primer sequences for IL-10 was constructed using a PCR MIMIC™ (CLONTECH). For competitive PCR, sample cDNA was amplified in the presence of the increasing amount of the competitor (5×10^{-21} to 1.6×10^{-19} mol). The mRNA quantity was assessed by calculating the amount of the competitor added to achieve an equimolar amount of the PCR products of the competitor and IL-10.

IL-10 cDNA Fragment Subcloning and Sequencing—The 475-bp IL-10 cDNA fragment generated by PCR was isolated from 1.5% agarose gel with an elution buffer (0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, 0.1% SDS, pH 8.0). The eluted cDNA was ligated into a PCR™ vector from a TA cloning kit (Invitrogen Corp., San Diego, CA). After a large scale plasmid preparation, the sequence of the cDNA was determined by the chain termination method (U. S. Biochemical Corp.). A partial *Eco*RI fragment (326 bp) of the cDNA insert was purified from agarose gel as described above and used as a probe for Northern blot hybridization.

Northern Blot Analysis—For Northern blot analysis for IL-10, 10 μg of total RNA was electrophoresed in 1% agarose gel containing formaldehyde and transferred to nylon filter (Micron Separations, Westboro, MA) as described (31). The 326-bp IL-10 cDNA fragment was labeled with [α-^{32}P]dCTP using a random primer labeling kit (Life Technologies, Inc.). Prehybridization, hybridization, washing, and autoradiography were performed as described previously (31). Equivalent RNA loading was confirmed by rehybridization of the filter for 18 S rRNA.

Enzyme-linked Immunosorbent Assay and Western Blot Analysis—For determination of the IL-10 concentration in the HSC culture medium, the media from three wells were combined, dialyzed, lyophilized, and analyzed for IL-10 using a mouse IL-10 enzyme-linked immunosorbent assay kit (Quantikine™, R & D Systems). To detect IL-10 in HSC, Western blot analysis was performed. Freshly isolated HSC were subjected to protein extraction using a 2 × lysis buffer (100 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, and 2% β-mercaptoethanol). Samples (100 μg of protein per each sample) were separated by 8% polyacrylamide gel electrophoresis using reducing conditions and transferred to nitrocellulose filters (Bio-Rad). The filters were first treated with 10% non-fat milk in 20 mM Tris base, pH 7.6, 137 mM NaCl, and 0.1% Tween 20 (TBST) and incubated with goat polyclonal anti-mouse IL-10 antibodies (R & D Systems) at 1:300 dilution in TBST with 1% bovine serum albumin, followed by incubation with rabbit anti-goat IgG antibodies (1:2000) conjugated with horseradish peroxidase. The immobilized IL-10-antibody complex was detected by chemiluminescence using ECL kit (Amersham Corp.).

Collagen Synthesis Assay—To examine whether neutralization of IL-10 produced by HSC affects collagen production, the cultured cells were incubated for 48 h with serum-free RPMI with ascorbic acid (50 μg/ml) and β-aminopropionitrile fumarate (50 μg/ml) in the presence of anti-IL-10 IgG or nonimmune IgG (20 μg/ml) and incubated with [2,3,4,5-^{3}H]proline (10 μCi/ml) for the last 18 h to radiolabel newly synthesized collagen (3). The cell and media proteins were precipitated with 10% trichloroacetic acid. Collagen production was determined by a collagenase digestion method (37).

Transfection and Reporter Gene Assays—To examine whether IL-10 has any effects on collagen gene promoter activity, cultured HSC after the first passage were transiently co-transfected with a collagen reporter gene plasmid (pGLCO$_2$ or pGLCO3) (38) and a murine sense or antisense IL-10 expression vector (39), which was kindly provided by Dr. Lili Feng at the Scripps Research Institute. The collagen reporter gene, pGLCO$_2$, contains 2200 bp of the murine α1(I) collagen gene 5′-flanking region linked to the luciferase reporter gene, while pGLCOL3 contains 220 bp of α1(I) collagen gene 5′-flanking region. Liposomes were prepared using 3 μg of a reporter gene plasmid and 5 μg of the expression vector along with 32 μl of LipofectAMINE reagent (Life Technologies, Inc.). The cells were incubated with the liposomes for 8 h, the liposome mixture removed, and fresh medium containing 10% fetal calf serum added. The cells were incubated for additional 36 h, washed with phosphate-buffered saline, and lysed for luciferase assay as described previously (38).

Statistical Analysis—All data are expressed as means ± S.E. The significance for the difference between the groups was assessed using standard *t* test. For quantification of competitive RT-PCR data, a linear regression analysis was performed between the concentration of the competitive template used in the assay and the ratio of densitometric results for the competitor product over than of the target gene product.

RESULTS

IL-10 RT-PCR for Cultured HSC—RT-PCR analysis of RNA from freshly isolated HSC from normal rats showed no detectable product for IL-10 using 35 cycles of amplification (*first lane*, Fig. 1 (*upper panel*)). However, HSC cultured on plastic wells for 7 days showed an increase in IL-10 mRNA as indicated by a detectable PCR product (*second lane*, Fig. 1 (*upper panel*)). Furthermore, incubation of HSC with TNF-α or TGF-β, cytokines known to stimulate HSC (3), caused further increases in IL-10 mRNA (*lane 3–8*, Fig. 1 (*upper panel*)). Semi-quantitative analyses of the RT-PCR results were performed by scanning densitometry of the IL-10 PCR product and standardization with β-actin results (Fig. 1, *lower panel*). These analyses show 2-fold increases in IL-10 mRNA expression by TGF-β (10 ng/ml) or TNF-α (10 ng/ml) and a 50% increase by LPS (10 μg/ml).

IL-10 Release by Cultured HSC—To assess the levels of IL-10 released by cultured HSC following treatment with several agonists, and enzyme-linked immunosorbent assay was performed on the medium samples (Table I). No detectable IL-10 was measured in the medium from untreated HSC. However, TNF-α, TGF-β, and LPS all stimulated IL-10 release by HSC.

HSC IL-10 mRNA Expression in Cholestatic Liver Injury—To determine whether IL-10 is expressed by HSC during the course of cholestatic liver injury, HSC were isolated from rats at 2, 7, and 19 days after bile duct ligation or sham operation, and RT-PCR was performed on HSC RNA samples for detection of IL-10 mRNA (Fig. 2). Sham HSC show undetectable or minimal IL-10 mRNA levels at each time point. In

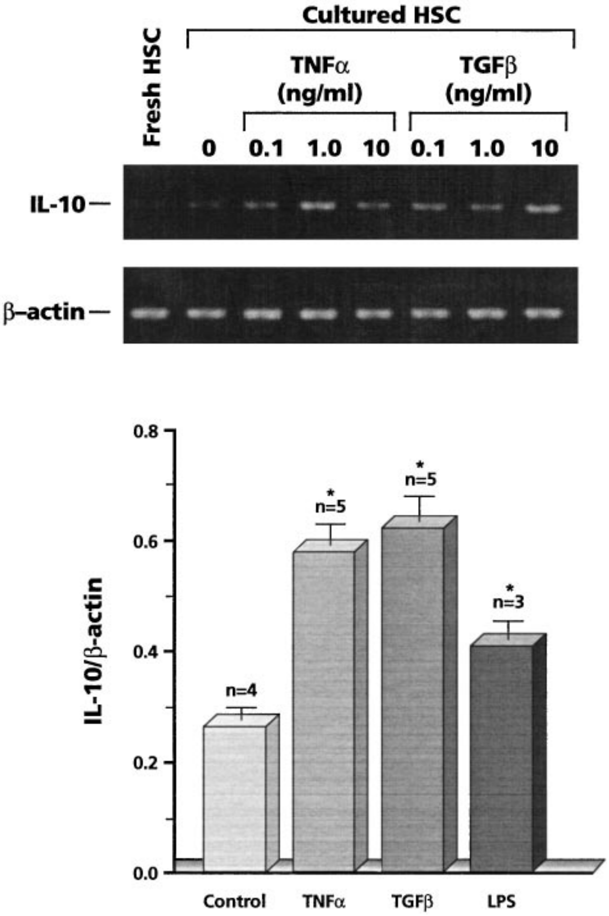

FIG. 1. *Upper panel*, IL-10 mRNA expression by culture activated HSC. IL-10 mRNA expression in HSC was assessed by RT-PCR. Freshly isolated HSC show very low levels of IL-10 (*first lane*), whereas culture-activated HSC grown in plastic dish for 6 days expressed appreciably more IL-10 mRNA (*second lane*). Treatment of cultured HSC with TNF-α and TGF-β further increased IL-10 mRNA levels (*third* to *eighth lanes*). *Lower panel*, densitometric analysis of IL-10 mRNA expression by cultured HSC. Densitometric RT-PCR data for IL-10 mRNA were standardized with β-actin signals and statistically compared between different treatment groups. Treatment of cultured HSC with TNF-α (10 ng/ml) and TGF-β (10 ng/ml) resulted in significant 2-fold increases in IL-10 mRNA expression, while LPS (10 μg/ml) caused a 40% increase. *, $p < 0.05$ as compared with control.

TABLE I

IL-10 released by cultured HSC in response to agonists

Condition	IL-10				
	Experiment				Mean ± S.E.
	1	2	3	4	
	pg/well				*pg/well*
TNF-α (10 ng/ml)	16.56	33.95	28.22	28.77	26.88 ± 3.67^a
TGF-β (10 ng/ml)	17.3	11.46	13.23	14.56	14.14 ± 1.23^a
LPS (10 μg/ml)	4.6	ND^b	13.89	18.73	12.41 ± 4.15^c
Control	UD^d	UD	UD	UD	UD

[a] $p < 0.05$.

[b] ND, not determined.

[c] $p = 0.09$.

[d] UD, undetected.

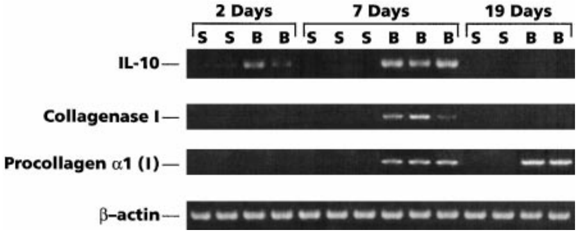

FIG. 2. **IL-10 mRNA expression in HSC isolated from rats with cholestatic liver injury.** IL-10 RT-PCR analysis was performed with HSC isolated from rats at 2 days, 7 days, and 19 days after ligation of bile duct (BDL) (*B*) or sham operation (*S*). IL-10 mRNA expression in HSC is slightly and prominently enhanced in BDL animals at 2 and 7 days, respectively, while such induction disappeared at 19 days. RT-PCR also demonstrates time-dependent induction of interstitial collagenase mRNA expression by HSC at 7 days after BDL, which coincides with IL-10 mRNA expression. α1(I) collagen mRNA levels were mildly increased in HSC from BDL animals at 7 days, followed by its more accentuated increase at 19 days when IL-10 expression ceased.

contrast, HSC from BDL show a distinct increase in IL-10 mRNA at 2 days, which was further accentuated at 7 days. Interestingly, this induction of IL-10 mRNA expression was completely abrogated at 19 days. To quantitatively assess the induction at 7 days, competitive PCR was performed using a specifically constructed competitive template. As shown in the *upper panel* of Fig. 3*A*, addition of an increasing amount of the competitor (from *right* to *left*) resulted in a progressive reduction in the level of IL-10 PCR product from the Sham sample while reciprocally raising the level of the competitor product. For the BDL samples, which are expected to have much lower levels, 40 cycles of amplification was used. Even though the level of IL-10 product was still lower, the similarly effective competition by the competitor was shown but with the amounts of the competitor that were approximately 2 orders of magnitude lower than those used for Sham. Linear regression analysis was performed for three pairs of competitive PCR data (Fig. 3*B*). As predicted, the level of IL-10 mRNA in 7-day BDL HSC was 100-fold higher than that in Sham HSC.

Subcloning and Sequencing of IL-10 PCR Product—To verify that the PCR product detected was truly a IL-10 cDNA fragment, we subcloned the product into a TA vector and sequenced a partial *Eco*RI fragment (326 bp) following a large scale plasmid preparation and cDNA purification. The sequence of the fragment showed a perfect match with the published nucleotide sequence of rat IL-10 cDNA (34), demonstrating that our PCR indeed detected IL-10 mRNA in HSC. Furthermore, we have utilized the purified 326-bp IL-10 cDNA fragment as a probe to perform Northern blot analysis on HSC RNA samples. Northern blot analysis clearly confirmed prominent induction of IL-10 mRNA expression in HSC from 7-day BDL as compared with corresponding Sham (Fig. 4).

Detection of IL-10 Protein in Activated HSC—Western blot analysis was performed to examine whether IL-10 protein level is coordinately increased in HSC from 7-day BDL (Fig. 5). The analysis detected an immunoreactive band with a distinct increased intensity in BDL (*last two lanes*), which corresponded to the molecular size (17 kDa) of authentic recombinant mouse IL-10 standard (*first lane*). As an internal control, desmin was immunoblotted using the same samples, which showed the relatively similar immunoreactivity between the two groups of the samples (*bottom panel*).

Relationship of IL-10 Expression to Collagen or Collagenase Expression in BDL—We were very intrigued by the time-dependent induction of IL-10 in HSC at 7 days in BDL animals. Since recent studies suggested regulation of collagen and collagenase genes by IL-10 in other cell types (28, 29), we examined α1(I) collagen and interstitial collagenase mRNA expression in the same HSC RNA samples used for IL-10 RT-PCR analysis (Fig. 2, *lower two panels*). Induction of interstitial collagenase mRNA expression was shown to coincide with that of IL-10 at 7 days as was the disappearance of induction of both genes at 19 days. On the contrary, marked induction of α1(I) collagen expression occurred when IL-10 expression ceased at 19 days.

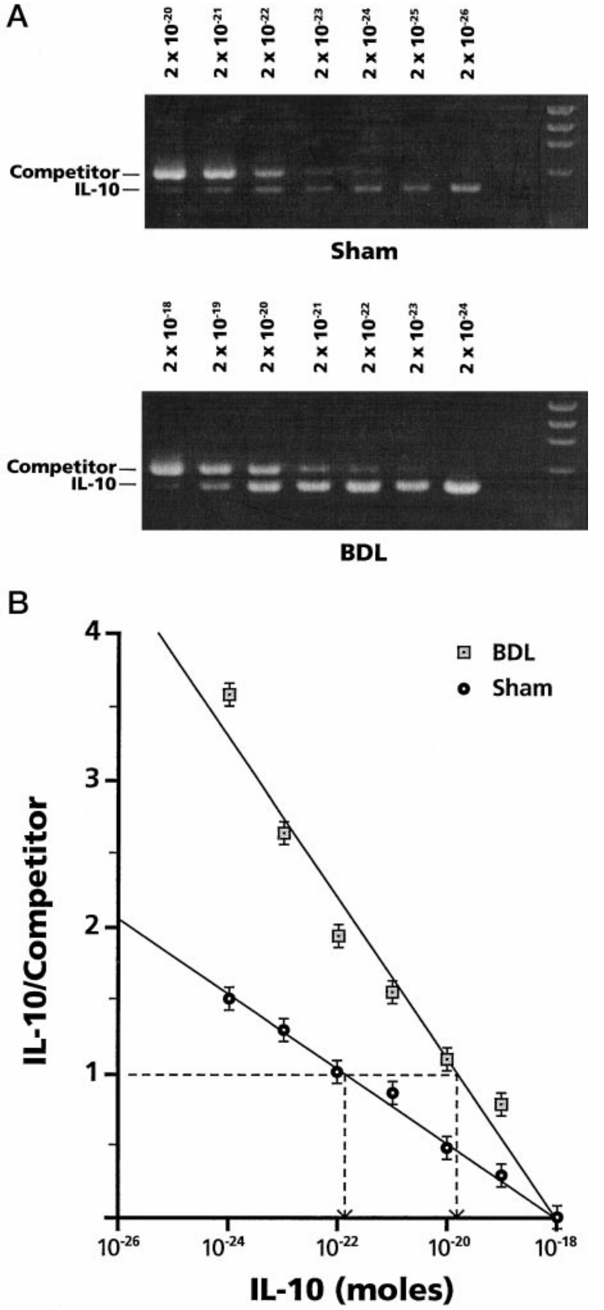

FIG. 3. *A*, representative data for IL-10 competitive PCR for HSC from rats at 7 days after bile duct ligation (*BDL*) or sham operation (*Sham*). As shown in this example, addition of an IL-10 competitor concentration-dependently suppressed the level of IL-10 PCR product while it increased the level of the competitor product in both samples from Sham and BDL animals. However, the BDL sample required higher concentrations of the competitor to suppress generation of IL-10 product than the Sham sample, indicating the higher IL-10 mRNA level in the BDL sample. *B*, data from three sets of competitive PCR were plotted and a linear regression analysis was performed. *Arrows* indicate the estimated levels of IL-10 mRNA as determined by the concentration of the competitor, which achieved generation of the equal amount of IL-10 and competitor products (the ratio of IL-10 over competitor product = 1). Note the estimated increase in IL-10 mRNA in BDL HSC is 100-fold.

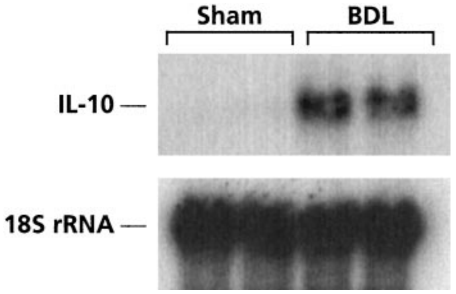

FIG. 4. **Northern blot analysis for IL-10 mRNA.** Using PCR-cloned IL-10 cDNA, we have performed Northern blot analysis on two sets of RNA samples. This analysis confirmed the prominent increase in IL-10 mRNA level in HSC from BDL animals.

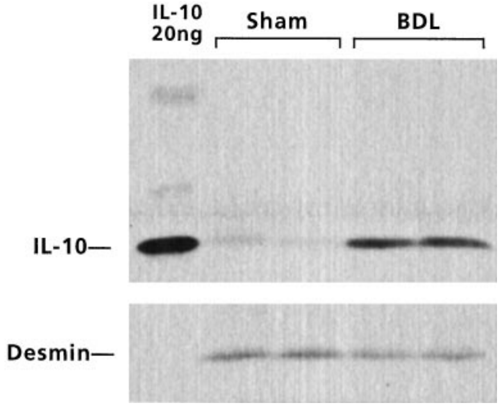

FIG. 5. **Western blot analysis of HSC protein extracts for IL-10.** HSC protein extracts (100 μg each lane) prepared from Sham and BDL animals were analyzed for IL-10 by Western blot analysis and a chemiluminescence detection method as described under "Materials and Methods." Note distinctly increased levels of IL-10 in BDL samples as compared with Sham samples. The *lower panel* shows desmin immunoblotting with relatively equal levels of this cytoskeletal protein in all four samples, indicating equal protein loading.

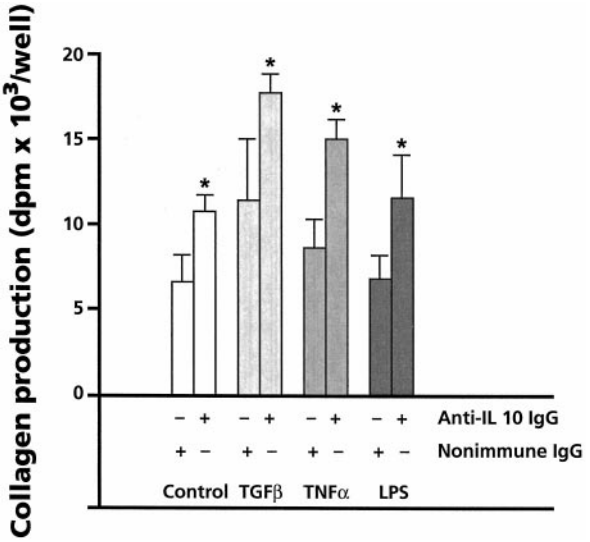

FIG. 6. **IL-10 neutralization stimulates HSC collagen production.** Cultured HSC were incubated with anti-IL-10 IgG (20 μg/ml) or nonimmune IgG (20 μg/ml) in the absence or presence of TGF-β (10 ng/ml), TNF-α (10 ng/ml), or LPS (10 μg/ml). Collagen production by these cells was determined by incorporation of [^{3}H]proline into collagenase sensitive peptides as described under "Materials and Methods." Addition of IL-10 antibodies alone resulted in a 50% increase in basal collagen production (*Control*) by HSC. TGF-β-mediated up-regulation of collagen production was enhanced by 2-fold by IL-10 neutralization. TNF-α or LPS, which did not stimulate HSC collagen production by itself, caused significant enhancement of collagen production if added together with anti-IL-10 IgG. *, $p < 0.05$.

IL-10 Neutralization Enhances HSC Collagen Production— The above results suggested a possible link between IL-10 expression by activated HSC and matrix homeostasis. To examine this possibility, collagen production was assessed by incorporation of [^{3}H]proline with cultured HSC exposed to

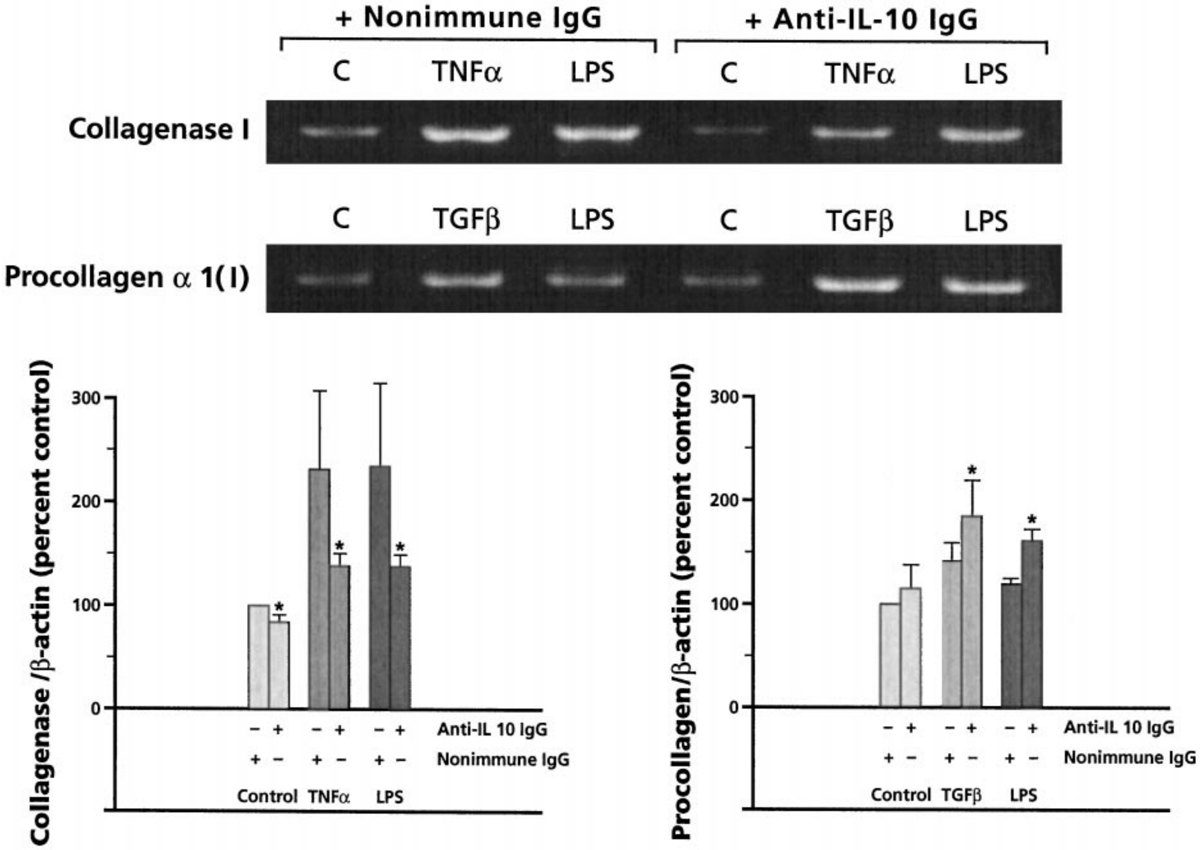

Fig. 7. *Upper panel*, RT-PCR for α1(I) collagen and interstitial collagenase mRNA in cultured HSC incubated with anti-IL-10 IgG. TNF-α (10 ng/ml)- or LPS (10 μg/ml)-stimulated collagenase mRNA expression by HSC (*upper left, first panel*), and this stimulation was suppressed by anti-IL-10 IgG (*upper right, first panel*). TGF-β (10 ng/ml) increased α1(I) collagen mRNA level (*upper left, second panel*), and this effect was further promoted by IL-10 neutralization (*upper right, second panel*). LPS (10 μg/ml), which marginally stimulated HSC α1(I) collagen mRNA expression alone, caused a distinct stimulatory effect if anti-IL-10 IgG is concomitantly added (*upper right, second panel*). *Lower panels*, densitometric analysis of RT-PCR data for interstitial collagenase and α1(I) collagen mRNA expression. Note anti-IL-10 IgG suppresses TNF-α- and LPS-stimulated interstitial collagenase mRNA expression by cultured HSC in addition to its inhibition of basal collagenase mRNA levels. *, $p < 0.05$ compared with HSC incubated with nonimmune IgG. Also note anti-IL-10 antibodies promote 2-fold the increased α1(I) collagen mRNA expression induced by TGF-β (10 ng/ml). Even though LPS (10 μg/ml) alone did not significantly increase α1(I) collagen mRNA levels, concomitant treatment of the cells with anti-IL-10 IgG caused a significant stimulation of α1(I) collagen mRNA expression.

TGF-β (10 ng/ml), TNF-α (10 ng/ml), and LPS (10 μg/ml) in the presence of anti-IL-10 IgG or nonimmune IgG (Fig. 6). The addition of anti-IL-10 IgG alone caused a 50% increase in basal collagen production. TGF-β-mediated stimulation of collagen production was doubled by the addition of the antibodies. Even though TNF-α or LPS alone did not increase collagen production, concomitant IL-10 neutralization resulted in significant enhancements in collagen synthesis. These results clearly demonstrate an inhibitory autocrine effect of IL-10 on collagen synthesis by culture-activated HSC.

IL-10 Neutralization Affects α1(I) Collagen and Collagenase mRNA Expression—To investigate mechanisms underlying the observed inhibitory role of IL-10 in HSC collagen production, we have examined effects of IL-10 neutralization on mRNA expression of α1(I) collagen and interstitial collagenase by cultured HSC. Exposure of HSC to TNF-α (10 ng/ml) or LPS (10 μg/ml) stimulated mRNA expression of collagenase (Fig. 7, *upper left panel*). However, addition of anti-IL-10 IgG clearly suppressed these stimulatory effects (Fig. 7, *upper right panel*). TGF-β (10 ng/ml) stimulated α1(I) collagen mRNA expression in cultured HSC and LPS marginally showed the effect (Fig. 7, *upper panel*). Addition of anti-IL-10 antibodies further promoted the increases in α1(I) collagen mRNA levels in TGF-β- or LPS-stimulated HSC (Fig. 7, *upper panel*). Densitometric data from at least three sets of experiments were standardized with β-actin results and statistically compared between the different treatments (Fig. 7, *lower panel*). Addition of anti-IL-10 antibodies slightly, but significantly, reduced basal interstitial collagenase mRNA expression. Furthermore, it suppressed an increase in interstitial collagenase mRNA levels induced by TNF-α and LPS (Fig. 7, *lower left panel*). On the contrary, IL-10 neutralization significantly enhanced by 2-fold the increase in α1(I) collagen mRNA expression by HSC exposed to TGF-β and LPS (Fig. 7, *lower right panel*). These results suggested that IL-10 secreted by culture-activated HSC not only induces basal expression of collagenase but also mediates up-regulation of collagenase expression caused by TNF-α and LPS. At the same time, IL-10 expression induced by TGF-β and LPS appears to exert an inhibitory effect on α1(I) collagen expression.

IL-10 Suppresses COLL Promoter Activity—To examine whether the observed inhibitory effect of IL-10 on collagen expression is mediated via its effect at transcriptional level, transient co-transfections were performed using an IL-10 expression vector (sense or antisense) with an α1(I) collagen promoter-luciferase construct (pGLCO2 or pGLCO3). As compared with transfection with the antisense IL-10 expression vector, collagen promoter activity was inhibited 40% with the sense IL-10 expression vector (Fig. 8). This suggests that IL-10's negative autoregulatory effects on HSC collagen expression is mediated at least in part via its transcriptonal inhibition of collagen gene.

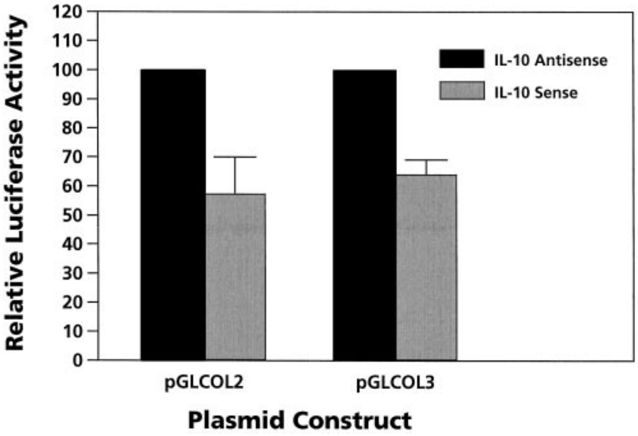

FIG. 8. **Effects of IL-10 expression on α1(I) collagen promoter activity.** Cultured HSC were co-transfected with a IL-10 expression vector (sense or antisense) and an α1(I) collagen promoter-luciferase construct (pGLCOL2 or pGLCOL3) to examine effects of IL-10 expression of α1(I) collagen promoter activity. The transfection with the antisense vector served as a control. Note that the promoter activity of both pGLCOL2 and pGLCO3 was suppressed by 40% by the IL-10 sense transfection, indicating expression of IL-10 suppresses α1(I) collagen promoter activity.

DISCUSSION

The present study is the first to demonstrate that HSC express IL-10 upon their activation *in vivo* and *in vitro*. HSC are considered as pericytes in the liver (40), which are also known to serve as principal cells to participate in liver fibrogenesis via their myofibroblastic activation (1). A recent report demonstrated expression of IL-10 by mesangial cells, the pericytes in the glomerulus, which are incriminated as the major source of fibroproliferative responses in glomerulonephritis (41). Since HSC and mesangial cells are considered analogous due to their similar functionality and pathophysiologic roles, we hypothesized that HSC may express IL-10. Our results demonstrate IL-10 is expressed by HSC upon activation in culture and during the early stage of biliary liver injury. Our RT-PCR specificity was verified by sequencing of the IL-10 PCR product, with which we further confirmed induced mRNA expression via Northern blot analysis. Western blot analysis of HSC protein extracts revealed a prominently expressed 17-kDa IL-10 protein at 7 days after BDL, and the cultured HSC were shown to express and release IL-10 in response to TNF-α, TGF-β, and LPS.

Our previous work showed enhanced expression of TNF-α by hepatic macrophages at 1 and 2 weeks after BDL but an almost complete disappearance of such induction at 3 weeks (42). Since our *in vitro* experiment demonstrates induction of IL-10 in HSC by TNF-α, it may be assumed that macrophage-derived TNF-α in the liver might have induced IL-10 expression by HSC in the time-dependent manner in the BDL model. However, the concomitant induction (7 days) and repression (19 days) of macrophage TNF-α and HSC IL-10 expression in this *in vivo* model also suggest IL-10 derived from HSC may not function as an anti-inflammatory cytokine toward hepatic macrophages. This assumption led us to think of other biological significance that HSC-derived IL-10 may possesses in the liver. To this end, we were intrigued by recent studies that showed IL-10-mediated regulation of the genes involved in matrix remodeling and homeostasis such as MMP-1, MMP-3, MMP-9, TIMP-1, and α1(I) collagen in skin fibroblasts (28) and macrophages (29). Indeed, our culture study clearly demonstrates IL-10 released by HSC suppresses their collagen production and this effect is mediated at least in part by transcriptional inhibition of collagen gene and enhanced expression of interstitial collagenase. These findings suggest a negative autoregulatory role of IL-10 in HSC collagen production in matrix remodeling. In support of this view, our *in vivo* data reveals concomitant induction of IL-10 and interstitial collagenase in HSC during the early stage of cholestatic liver fibrosis (7 days after BDL) and the lack of HSC IL-10 expression in association with marked α1(I) collagen induction in advanced liver fibrosis at 19 days. This raises an intriguing secondary hypothesis that the failure of HSC to continue their expression of IL-10 may underlie progressive fibrogenesis leading to liver cirrhosis.

Interplay between soluble factors of paracrine and autocrine sources is complex in regulation of HSC biology. Among several cytokines implicated in activation of HSC, TGF-β is considered a potent fibrogenic cytokine that seems capable of conferring HSC most aspects of cellular activation (3–8). This cytokine can be released by hepatic macrophages (9) or HSC by themselves (15), and the paracrine and autocrine interaction can be established via its ability to autoinduce its expression (15). In our *in vitro* study, TGF-β induced IL-10 in cultured HSC. However, IL-10 induction was abolished in HSC at 19 days after BDL despite HSC (43) and hepatic macrophages[2] continue to express TGF-β1 at this time point in this model. Thus, unlike the close *in vivo* association between IL-10 and TNF-α expression in the BDL model, this dissociation of IL-10 and TGF-β expression suggests complex regulation of IL-10 expression by TGF-β. Another discrepancy noted in the present study was the continued induction of IL-10 expression by culture-activated HSC as compared with the time-dependent induction seen *in vivo*. This obviously suggests cellular or molecular differences in *in vitro* and *in vivo* activated HSC or reflects the absence of other *in vivo* factors that may cause the time-dependent expression in the culture system. It is attractive to speculate that these factors may be derived from other cell types in the liver including hepatic macrophages. Additional studies are obviously needed to test this hypothesis.

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[2] S. C. Wang, M. Ohata, M. Lin, and H. Tsukamoto, unpublished observation.

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