A Farnesoid X Receptor-Small Heterodimer Partner Regulatory Cascade Modulates Tissue Metalloproteinase Inhibitor-1 and Matrix Metalloprotease Expression in Hepatic Stellate Cells and Promotes Resolution of Liver Fibrosis

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ABSTRACT

The farnesoid X receptor (FXR) is expressed by and regulates hepatic stellate cells (HSCs). In the present study, we investigated whether 6-ethyl chenodeoxycholic acid (6-ECDCA or INT-747), a semisynthetic derivative of chenodeoxycholic acid (CDCA), modulates tissue metalloproteinase inhibitor (TIMP)-1 and matrix metalloprotease (MMP)-2 expression/activity in HSCs and in the liver of rats rendered cirrhotic by 4-week administration of CCl₄. Exposure of HSCs to FXR ligands increased small heterodimer partner (SHP) mRNA by 3-fold and increases basal and thrombin-stimulated expression of α(I)collagen, α-smooth muscle actin (α-SMA), TIMP-1, and TIMP-2 by 60 to 70%, whereas it increased matrix metalloproteinase (MMP)-2 activity by 2-fold. In coimmunoprecipitation, FXR activation/overexpression caused a SHP-dependent inhibition of JunD binding to its consensus element in the TIMP-1 promoter.

Hepatic fibrosis is a scarring process of the liver that includes components of both increased and altered deposition of extracellular matrix (ECM) and reduced breakdown of ECM components (Mann and Smart, 2002; Friedman, 2003).

In chronic liver disease, hepatic stellate cells (HSCs), the major source of ECM in the liver, undergo a process of transdifferentiation from a resting, fat-storing phenotype to a myofibroblast-like phenotype characterized by expression of fibroblastic cell markers such as α-smooth muscle actin (α-SMA) (Mann and Smart, 2002; Friedman, 2003). In addition, there is now considerable evidence that HSCs are a source of both matrix-degrading metalloproteinases (MMPs), including those that degrade type I collagen and the tissue inhibi-
tors of metalloproteinases (TIMPs) (Arthur, 2000). The prototypic member of the TIMP family, TIMP-1, is a broad inhibitor of MMPs, possesses growth factor-like and anti-apoptotic properties (Murphy et al., 2002), and, by promoting survival of HSCs, is mechanistically involved in the development of liver fibrosis (Iredale et al., 1998; Yoshiji et al., 2000, 2002; Smart et al., 2001).

Previous studies have shown that JunD, the predominant Jun family protein expressed in culture-activated rat HSCs, regulates TIMP-1 expression in HSCs (Bahr et al., 1999; Trim et al., 2000; Manning and Davis, 2003). The TIMP-1 promoter contains an activating protein (AP)-1 binding site, and mutation of this site results in greater than 90% reduction of expression of TIMP-1 promoter-reporter constructs in the transient transfection assays (Bahr et al., 1999; Trim et al., 2000; Manning and Davis, 2003).

The farnesoid X receptor (FXR) is a member of the nuclear receptor superfamily of ligand-activated transcription factors that functions as an endogenous sensor for bile acids (Forman et al., 1995; Makishima et al., 1999; Parks et al., 1999). FXR is bound to and activated by bile acid and chenodeoxycholic acid (CDCA) is the natural most active ligand (Forman et al., 1995; Makishima et al., 1999; Parks et al., 1999). Upon binding of a bile acid, FXR alters gene transcription by interacting with the 9-cis-retinoic acid receptor (RXR) (Wang et al., 1999). In liver cells, FXR activation leads to induction of an orphan nuclear receptor termed the short (or small) heterodimer partner (SHP) that mediates inhibition of FXR ligands on a cohort of genes that function to decrease the concentration of bile acids within hepatocytes (Wang et al., 1999; Goodwin et al., 2000; Lu et al., 2000; del Castillo-Olivares and Gil, 2001). FXR is expressed by HSCs, and FXR ligands function as negative regulators of α1(Ⅰ)collagen synthesis both in vitro and in vivo in rodent models of liver fibrosis (Pellicciari et al., 2002; Mi et al., 2003; Fiorucci et al., 2004, 2005); the transient transfection assays (Bahr et al., 1999; Trim et al., 2000; Manning and Davis, 2003). Here, we report that FXR functions as a negative regulator of TIMP-1 gene expression in HSCs. Activation of FXR causes an SHP-dependent inhibition of JunD binding to the AP-1 binding site in the TIMP-1 promoter, reducing TIMP-1 expression/function and increasing the susceptibility of HSCs to apoptotic stimuli. In addition, we provide evidence that in vivo activation of FXR decreases TIMP-1 expression/activity and promotes resolution of liver fibrosis.

**Materials and Methods**

**Isolation and Culture of HSCs.** In vitro studies were performed on primary cultures of rat HSCs; HSC-T6, a rat immortalized cell line; and LX2 cell lines, a human HSC line (Fiorucci et al., 2004a). Primary rat HSCs were isolated from control and cirrhotic rats according to techniques described previously (Fiorucci et al., 2004b) and cultured at 37°C in an atmosphere of 5% CO2 in Dulbecco’s modified minimal essential medium (Invitrogen, Carlsbad, CA) containing 10% fetal calf serum, 2 mM l-glutamine, and 5000 IU/ml penicillin/5000 g/ml streptomycin for 1 to 2 days before starting experiments.

To investigate the expression of FXR and FXR-regulated genes in HSCs, HSCs (days 0 and 7) and 24 h serum-starved HSC-T6 and LX2 cell lines were incubated for 18 h with the medium alone or with 1 to 50 μM CDCA; 0.1 to 10 μM 6-ECODCA, also indicated as INT-747 (Pellicciari et al., 2002; Mi et al., 2003; Fiorucci et al., 2004a, 2005); or 0.01 to 1 μM GW4064 (Maloney et al., 2000), a nonsteroidal FXR ligand; and FXR, α1(Ⅰ)collagen, α-SMA, SHP, TIMP-1, TIMP-2, MMP-2, and TGFB1 mRNA expression investigated by quantitative (q)RT-PCR (Fiorucci et al., 2004a, 2005).

**MMP-2 Activity and TIMP-1 ELISA.** MMP-2 levels were measured using an ELISA detection kit (Biotrak; Amersham Biosciences, Inc., Piscataway, NJ). This kit measures both the active and total (pro and active) forms of MMP-2. Only data on the activated form are shown. Secretion of TIMP-1 protein by HSCs was measured using a specific ELISA kit (Quantikine rat TIMP-1 immunoassay; R&D Systems, Minneapolis, MN).

**Generation of SHP-Overexpressing HSC-T6 (HA-SHP).** The SHP coding sequence was cloned from primary rat hepatocytes (Seol et al., 1996; Fiorucci et al., 2004a) induced with 6-ECODCA. For cell transfection, SHP was first cloned into the pcR2.1 vector (TOPO-TA cloning; Invitrogen) and then subcloned in the EcoRI site of the PINCO retroviral vector. 293T modified packaging cell line (293T) were transiently transfected with either the PINCO-SHP chimera or PINCO alone as a negative control. At 48 h post-transfection, the viral supernatant was recovered and used to infect HSC-T6. The PINCO vector contains the emerald green fluorescent protein gene that allows the separation of infected cells (green) from uninfected cells (Fiorucci et al., 2004a). HSC-T6 cells expressing either SHP or empty vector alone were obtained by cell sorting (Beckman Coulter, Milan, Italy).

**Silencing of SHP.** Selection of siRNAs was based on the characterization of siRNA by Elbashir et al. (2001). A 21-nucleotide RNA with 3′-dA overhangs was synthesized with Dharmacon 2′-ACE technology (Dharmacon Research, Lafayette, CO) in the “ready-to-use” option. The primers sequences were 5′-AAGGAGUACGCA-UACCUGAAA-3′ and 5′-AACAUCUCUGUACCCGUUG-3′ (corresponding to base pairs 498–518 and base pairs 684–704 of the coding sequence of the SHP gene). SHP-siRNAs (15 nM) were introduced into HSC-T6 cell line by transient transfection with TransIT TKO transfection reagent (Mirus, Madison, WI). The effect of siRNA was evaluated by measuring the relative expression of SHP mRNA by qRT-PCR (Fiorucci et al., 2004a).

**Transactivation Assay and Plasmid Construction.** The fragment corresponding to the promoter region of rat TIMP-1 (region between nucleotides –182 and –13) was amplified from genomic DNA obtained from the HSC-T6 cell line (DNAzol; Invitrogen) and then subcloned in the EcoRI site of the pGEM vector (Promega) was added to normalize the amounts of DNA transfected in each assay (2.5 pg). Twenty-four hours before transfection, HSC-T6 were seeded at ASPET Journals on March 31, 2017 jpet.aspetjournals.org Downloaded from
1 μM 6-ECDCA. Total lysates were prepared by solubilization of cells in sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, and 0.015% bromphenol blue) and separated by polyacrylamide gel electrophoresis. The proteins were then transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA) and probed with primary antibodies to c-Jun, JunD, SHP, FXR, α1(I)collagen, TIMP-1, and MMP-2 (Santa Cruz Biotechnology Inc., Santa Cruz, CA), HA epitope tag (Covance, Berkeley, CA), or α-SMA (Abeam, Cambridge, UK). The anti-immunoglobulin G horseradish peroxidase conjugate (Bio-Rad) was used as the secondary antibody, and specific protein bands were visualized using enhanced chemiluminescence (Amersham Biosciences, Inc.).

**Electrophoretic Mobility Shift Assays.** The synthesis of [γ-32P]ATP (Amersham Biosciences, Inc.) radiolabeled probes used for shift analysis (EMSAs) has been described previously (Fiorucci et al., 2004a). For EMSAs, 10 μg of nuclear extract from primary HSCs, HSC-T6, and HA-SHP were incubated with 50,000 cpm of the indicated γ-32P end-labeled probe in a total volume of 20 μl of binding buffer [50 mM NaCl, 10 mM Tris, pH 7.9, 0.5 mM EDTA, 1 μg of poly(dI-dC), and 10% glycerol] for 20 min at room temperature. For competition assays, excess unlabeled oligonucleotides were preincubated for 15 min before the addition of the radiolabeled probe (50,000 cpm). For antibody-mediated supershift assays, extracts were preincubated with 5 μl of either anti-SHP or anti-JunD antibodies at room temperature for 20 min before the addition of the radiolabeled probe. The reactions were loaded on a 6% polyacrylamide nondenaturing polyacrylamide gel electrophoresis gel in low ionic buffer electrophoresis gel in low ionic buffer electrophoresis (Fiorucci et al., 2004a). Apoptotic bodies were stained by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL). HSCs activation was detected by simultaneous staining of α-SMA, as a marker of activated HSCs, and of PCNA as a marker of S-phase nuclei as described previously (Fiorucci et al., 2004a). HSCs activation was detected by simultaneous staining of α-SMA, as a marker of activated HSCs, and of PCNA as a marker of S-phase nuclei (Fiorucci et al., 2004a,b).

Apoptosis on nonparenchymal cells in liver section was achieved by TUNEL staining of rat liver sections. The TUNEL staining was performed according to previously published methods (Iredale et al., 1998; Fiorucci et al., 1999). Each liver section was analyzed by a blinded observer who counted the number of nonparenchymal apoptotic figures/bodies present in 10 random high power (×40) fields from each specimen. Apoptotic bodies in a distribution compatible with parenchymal cells (hepatocytes) were not counted. Hepatic and urinary content of hydroxyproline (Fiorucci et al., 2004a) were determined by high-performance liquid chromatography (LC Varian Prostar high-performance liquid chromatograph; Varian, Rome, Italy).

**Statistical Analysis.** Analysis of variance or the Student’s t test was used for statistical comparisons. Statistical tests were performed using INSTAT statistical software (GraphPad Software, Inc., San Diego, CA).

**Results**

FXR is Expressed in HSCs and Functions as Negative Regulator of TIMP-1. FXR expression was found in both rodent and human HSCs (Fig. 1a). Thus, FXR, mRNA and protein were detected in primary cultures of HSCs, in HSC-T6, and in LX2 cells. FXR expression did not change significantly during the process of trans-differentiation of rats HSCs in primary cultures (data not shown) or after the addition of FXR ligands to the culture medium (Fig. 1a). As shown in Fig. 1, b through g, HSCs in 7-day culture demonstrated a significant increase in α1(I)collagen, α-SMA (not shown), TIMP-1, and MMP-2 compared with day 0 HSCs (n = 4–6; P < 0.01 versus day 0). HSC activation is associ-
ated with a significant increase of TIMP-1 release and MMP-2 activity (n = 4–6; P < 0.01 versus day 0). Whereas SHP expression did not change during the process of transdifferentiation, exposure of HSCs to 1 μM 6-ECDCA increased its expression 3-fold (Fig. 1b; n = 4–6; P < 0.01 versus medium) and reduced α1(I)collagen and TIMP-1 protein expression and secretion by 70 to 80% (Fig. 1, c, d, and f; n = 4–6; P < 0.01 versus medium). Finally, whereas 6-ECDCA had no effect on MMP-2 mRNA (Fig. 1e; n = 4–6; P > 0.05 versus medium), it significantly increased MMP-2 activity (Fig. 1g; n = 4–6; P < 0.01 versus medium). To investigate whether the effect of FXR activation on TIMP-1 expression was maintained in HSCs activated by growth factors, day-7 cultured HSCs were stimulated with 10 U thrombin for 18 h in the presence of natural and synthetic FXR ligands. Exposure to thrombin had no effect on FXR and SHP mRNA, but up-regulated α1(I)collagen, α-SMA, TIMP-1, TIMP-2, and MMP-2 gene expression and TIMP-1 release (not shown; n = 6; P < 0.01 versus control cells). Exposure to 1 μM 6-ECDCA caused a 2- to 3-fold induction of SHP (not shown; P < 0.01 versus thrombin) and reduced induction of α1(I)collagen, α-SMA, TIMP-1, TIMP-2 mRNA, and TIMP-1 release induced by thrombin by 50 to 80% (not shown; n = 6; P < 0.01 versus thrombin).

SHP Mediates Inhibition of FXR Ligands on TIMP-1. The qualitative and quantitative RT-PCR and Western blot analysis shown in Fig. 2, a and b, demonstrated that transfection of 15 nM SHP siRNA caused an ~90% reduction of SHP expression (n = 5; P < 0.001 versus untransfected cells). As shown in Fig. 2c, SHP-defective HSC-T6 demonstrated significantly higher levels of TIMP-1 mRNA and released higher amounts of TIMP-1 (not shown) compared with wild-

Fig. 1. a, FXR expressed in primary cultures of rat HSCs (lanes 1 and 2), HSC-T6 (lanes 3 and 4), and human LX2 cells (lanes 5 and 6). The RT-PCR shown represents three experiments. In lanes 1, 3, and 5, cells were left untreated. In lanes 2, 4, and 6, cells were treated for 18 h with 1 μM 6-ECDCA. b–e, Western blot analysis of SHP, α1(I)collagen, TIMP-1, and MMP-2 expression in freshly isolated HSC (day 0) and plastic-activated HSCs grown in 10% fetal calf serum for 7 days. FXR activation was achieved by incubating HSCs for 18 h with 1 μM 6-ECDCA. Data are mean ± S.E. of four experiments. *, P < 0.05 versus day 0; **, P < 0.05, 6-ECDCA versus day 7. f and g, exposure to 6-ECDCA reduces TIMP-1 release and increases MMP-2 activity in HSCs. Data are the mean ± S.E. of four experiments. *, P < 0.05 versus day 0; **, P < 0.05, 6-ECDCA versus day 7.
type HSC-T6. Furthermore, although abrogating the expression of SHP had no effect on MMP-2 mRNA expression (not shown), SHP-defective cells have significantly higher MMP-2 activity compared with wild-type HSC-T6 (Fig. 2d; \( n = 6; P < 0.01 \) versus wild type).

In contrast to these findings, SHP overexpression inhibits TIMP-1 expression. Thus, TIMP-1 mRNA and TIMP-1 release (not shown) was reduced by 70 to 90% in SHP-overexpressing cells (Fig. 2, e–h; \( n = 6; P < 0.001 \) versus wild type).

Although SHP overexpression had no effect on MMP-2 mRNA levels (not shown), it significantly increased MMP-2 activity (Fig. 2h; \( n = 6; P > 0.01 \) versus HSC-T6).

**FXR Activation Inhibits AP-1 Binding to the TIMP-1 Promoter.** Because SHP overexpression inhibits TIMP-1 mRNA expression and TIMP-1 release is induced by thrombin, a known AP-1 inducer, we then investigated whether SHP interacts with AP-1 and modulates its binding to the TIMP-1 promoter. For this purpose, lysates from wild-type and SHP-overexpressing HSC-T6 were immunoprecipitated with a monoclonal anti-HA or polyclonal anti-JunD antibodies and analyzed by Western blotting using the reverse antibody used for immunoprecipitation. As shown in Fig. 3A, exposure of HA-SHP to thrombin induces JunD expression. In coimmunoprecipitation experiments (Fig. 3A) HA-SHP immunoreactivities were detected in anti-JunD immunoprecipitates. Similarly, JunD immunoreactivities were found in HA-SHP immunoprecipitates (Fig. 3B), indicating that SHP directly interacts with AP-1. We next analyzed whether JunD-SHP interaction prevents binding of JunD to the AP-1 binding site in the TIMP-1 promoter. For this purpose, an EMSA was performed using nuclear extracts.
from wild-type and SHP-overexpressing HSC-T6 cells and a probe with the AP-1 response element obtained from the TIMP-1 promoter. As shown in Fig. 3C, AP-1 binding was found in wild-type HSC infected with the empty vector and in HA-SHP cells left untreated. SHP overexpression, however, was sufficient to reduce the binding of AP-1 to the TIMP-1 promoter. Addition of thrombin enhances AP-1 binding in wild-type cells, but not in HA-SHP cells. Exposure to 6-ECDCA, 1 μM, reduces AP-1 binding in HSC-T6, but not in HA-SHP cells likely due to the inability of these cells to further augment the level of SHP protein in response to FXR ligands. This was confirmed by real-time polymerase chain reaction of SHP mRNA (data not shown).

We have thus investigated whether FXR ligands directly modulate TIMP-1 gene transcription by transactivation assay using a sequence containing the AP-1 consensus from the TIMP-1 promoter cloned upstream of the luciferase reporter gene in the pGL3 vector. As shown in Fig. 3D, exposure to thrombin causes the transactivation of the TIMP-1 promoter in HSC-T6 cells transfected with pGL3-TIMP-1 chimeric. Specificity was evaluated using a mutant AP-1 and by adding an anti-JunD antibody. The AP-1 binding site mutant TIMP-1 promoter was only weakly activated by thrombin. Data are the mean ± S.E. of four experiments.
Fig. 4. FXR activation increases HSCs sensitivity to proapoptotic stimuli. a, HSC-T6 cells incubated with 100 μM cycloheximide alone or in combination with 1 μM 6-ECDCA, and apoptosis assessed by flow cytometry. The number of Annexin V+/PI− cells is shown. Data are the mean ± S.E. of six experiments. * P < 0.01 versus cells incubated with 6-ECDCA alone. ** P < 0.05 versus HSC-T6 incubated with cycloheximide alone. b, natural and synthetic FXR ligands increase the sensitivity of HSC-T6 to cycloheximide. Data are the mean ± S.E. of four experiments. * P < 0.01 versus incubated cells with 6-ECDCA alone. ** P < 0.05 versus HSC-T6 incubated with cycloheximide alone. c and d, recombinant 100 ng/ml TIMP-1 rescues HSC-T6 from apoptosis induced by FXR ligand and reduces caspase-3 activation. Data are the mean ± S.E. of six experiments. * P < 0.01 versus cells incubated with 6-ECDCA alone; ** P < 0.05 versus HSC-T6 incubated with cycloheximide alone or in combination with 6-ECDCA. e, Western blot analysis of cytochrome c translocation from the mitochondria to the cytosolic fraction. Lane 1, control cells; lane 2, HSC-T6 incubated with 50 μM cycloheximide; lane 3, HSC-T6 incubated with 50 μM cycloheximide in combination with 1 μM 6-ECDCA; and lane 4, HSC-T6 cells exposed to 100 ng/ml TIMP-1 and then incubated with cycloheximide plus 6-ECDCA. The blots shown represent three experiments. f and g, SHP overexpression (HA-SHP) increases, whereas SHP deficiency reduces, apoptosis induced by cycloheximide. SHP-deficient cells were obtained by transfection of HSCs with anti-SHP siRNA as described under Materials and Methods. Data are the mean ± S.E. of four experiments. * P < 0.05 versus medium alone; ** P < 0.05 versus HSC-T6 wild type exposed to cycloheximide.

coincubating 6-ECDCA with 50 μM cycloheximide increased the rate of apoptosis induced by the protein synthesis inhibitor by ~90% (from 23.5 ± 3.6 to 43.2 ± 4.2%; n = 6; P < 0.01). Similar to 6-ECDCA, 20 μM CDCA and 100 nM GW4064 increased the rate of apoptosis induced by cycloheximide (Fig. 4b; n = 6; P < 0.01 versus cycloheximide alone). Exposure of HSC-T6 to 100 ng/ml rTIMP-1 effectively reduced the rate of apoptosis induced by cycloheximide alone or in combination with 6-ECDCA (Fig. 4c; n = 6; P < 0.01). Furthermore, whereas cycloheximide increased caspase 3 activity (Fig. 4d), and this effect was significantly enhanced by coincubating the cells with 6-ECDCA (n = 6; P < 0.05 versus cycloheximide alone), exposure of HSC-T6 to 100 ng/ml rTIMP-1 significantly reduced caspase 3 activity (Fig. 4d; n = 6; P < 0.05). Translocation of cytochrome c from the mitochondria to the cytosol correlates with loss of potential of the mitochondrial membrane (Fiorucci et al., 2002) and is considered a marker of mitochondrial injury. Although exposure to cycloheximide, alone or in combination with 6-ECDCA, correlated with relocation of cytochrome c in the cytosol, this event was prevented by incubating cells with rTIMP-1 (Fig. 4e; n = 4).

SHP-overexpressing HSC-T6 were significantly more sensitive to apoptosis induced by cycloheximide than wild-type cells (Fig. 4, f and g; P < 0.05). In contrast, SHP-deficient HSC-T6 cells, generated by siRNA, were protected against apoptogenic stimuli as assessed by the measurement of Annexin V+/PI− cells and caspase 3 activity (P < 0.05 versus wild-type cells).

In Vivo FXR Activation Prevents Fibrosis Development and TIMP-1 Induction. CCl4 administration up-regulated liver expression of a number of genes involved in liver fibrosis development (Fig. 5, a–e) and caused a rapid induction of TIMP-1 and TIMP-2 mRNA (Fig. 5, f and g; n = 4; P < 0.01 versus control). The expression of TIMP-1 mRNA increased by ~30 fold in response to a single administration of CCl4 and declined at later time points (n = 4; P < 0.01 versus control rats). Consistent with these findings TIMP-1 protein, as measured by ELISA in liver homogenates, was found maximally elevated in liver samples collected 3 to 5 days after the first CCl4 administration and declined in a later phases. In contrast, α1(I)collagen and α-SMA mRNA expression increased over time reaching the peak at 4 weeks after the first dose of CCl4 administration. Administration of rats rendered cirrhotic by CCl4 with 3 mg/kg 6-ECDCA caused a 60 to 80% reduction of TIMP-1 and TIMP-2 mRNA expression at days 3 and 5 (Fig. 5; P < 0.05 versus CCl4 alone),...
whereas it had no effect on MMP-2 gene expression. However, MMP-2 activity at days 3, 5, and 28 increased significantly in response to the FXR ligand (Fig. 5; n/H11005 /n/H11021 4; /P/H11021 0.01 versus CCL4). Consistent with these findings, 4-week administration of 6-ECDCA reduced /H9251 1(I)collagen and /H9251 -SMA by /H11015 70%. In contrast to 6-ECDCA, 15 mg/kg UDCA had no effect on /H9251 1(I)collagen, /H9251 -SMA, TIMP-1, and TIMP-2 mRNA expression, nor did it modulate the activity of either TIMP-1 or MMP-2 (Fig. 5).

The morphometric analysis (Fig. 6, a–f) of liver samples obtained from rats administered CCL4 after 28 days revealed a significant increase in the fibrotic surface compared with control rats (Fig. 6b), resulting in a continuous meshwork of connective tissue infiltrating the hepatic parenchyma with central-central, central-portal, and portal-portal bridging. In addition, CCL4 increased liver hydroxyproline content 5-fold (/P/H11021 0.01 versus control rats) and urinary excretion of hydroxyproline by 3-fold (Fig. 6, g and h; /P/H11021 0.01 versus control rats). Administering rats with 6-ECDCA protected against liver fibrosis development. Upon morphometric analysis the area of liver parenchyma occupied by fibrotic tissues was reduced by /H11015 70% after 6-ECDCA treatment (/P/H11021 0.01 versus CCL4) and as shown in Fig. 6c, bridging was significantly attenuated by 6-ECDCA. 6-ECDCA administration also reduced liver and urinary hydroxyproline content (Fig. 6, a–h) and significantly attenuated the increase in body/liver weight ratio caused by CCL4 administration (Table 1). In contrast to 6-ECDCA, UDCA failed to reduce liver fibrosis (Fig. 6). Attenuation of liver fibrosis in animals treated with the FXR ligand was not due to a reduced liver toxicity of CCL4. Consistent with this view, analysis of aspartate aminotransferase and alanine aminotransferase plasma levels measured on days 3, 5 (not shown), and 28 demonstrates a similar liver injury in rats treated with CCL4 alone or in combination with 6-ECDCA or UDCA.

These findings were further confirmed by analysis of TIMP-1 and MMP-2 expression in lysates obtained from HSCs isolated from CCL4-treated rats. Administering rats with 3 mg/kg 6-ECDCA significantly reduced /H9251 -SMA and TIMP-1 expression caused by CCL4 (Fig. 6i), whereas it had no effect on MMP-2 expression. In vivo administration of 6-ECDCA increased SHP and FXR expression in HSCs (Fig. 6i). In contrast, no effect on /H9251 -SMA, TIMP-1, MMP-2, SHP, and FXR was detected in HSCs obtained from animals treated with UDCA.
FXR Ligand Promotes Liver Fibrosis Resolution. In rats administered CCl₄ for 4 weeks, resolution of liver fibrosis became evident as early as 5 to 7 days after the last dose of CCl₄. The qRT-PCR analysis shown in Fig. 7 demonstrates that a significant reduction in α1(I)collagen and α-SMA expression was detectable 5 to 7 days after the last dose of CCl₄ (n = 4; P < 0.05 versus peak). In contrast to these changes, neither TIMP-1 nor MMP-2 mRNA or protein changed in the first postadministration week (Fig. 7, f and g). Similarly, we found no increase in the number of nonparenchyma apoptotic cells during this time (Fig. 7i). Administration of 6-ECDCA caused a notable increase in

**Table 1**

Effect of 6-ECDCA and UDCA on liver injury caused by CCL₄ administration

<table>
<thead>
<tr>
<th></th>
<th>Liver/Body Weight Ratio</th>
<th>ALT</th>
<th>AST</th>
<th>g-GT</th>
<th>Bilirubin</th>
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<td>UI/l</td>
<td>mg/dl</td>
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<td>Day 3</td>
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<td>Control</td>
<td>2.2 ± 0.6</td>
<td>55.0 ± 5.0</td>
<td>108.0 ± 12.4</td>
<td>5.2 ± 0.2</td>
<td>0.01 ± 0.01</td>
<td>22.3 ± 3.7</td>
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<tr>
<td>CCL₄</td>
<td>2.4 ± 0.4</td>
<td>1490.0 ± 215.7*</td>
<td>2021.0 ± 147.3*</td>
<td>8.3 ± 1.4</td>
<td>0.4 ± 0.1*</td>
<td>26.4 ± 4.2</td>
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<tr>
<td>CCL₄ + 6-ECDCA, 1 mg</td>
<td>2.4 ± 0.3</td>
<td>1248.3 ± 169.4*</td>
<td>1892.0 ± 238.4*</td>
<td>7.2 ± 0.3</td>
<td>0.3 ± 0.03*</td>
<td>24.2 ± 3.8</td>
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<tr>
<td>CCL₄ + UDCA, 15 mg</td>
<td>2.5 ± 0.2</td>
<td>1392.5 ± 149.8*</td>
<td>2316.2 ± 173.8*</td>
<td>8.5 ± 1.1</td>
<td>0.3 ± 0.1*</td>
<td>25.5 ± 2.9</td>
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<td>Day 28</td>
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<tr>
<td>CCL₄</td>
<td>4.6 ± 0.3*</td>
<td>54.2 ± 5.2</td>
<td>121.0 ± 23.0</td>
<td>7.3 ± 0.4</td>
<td>0.2 ± 0.05*</td>
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<td>CCL₄ + 6-ECDCA, 1 mg</td>
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<td>49.5 ± 6.3</td>
<td>98.0 ± 17.2</td>
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<td>0.1 ± 0.02</td>
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<td>CCL₄ + UDCA, 15 mg</td>
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ALT, alanine aminotransferase; AST, aspartate aminotransferase.
* P < 0.05 versus control rats; ** P < 0.05 versus CCL₄.

**Fig. 6.** Sirius red staining of a liver section obtained from a control rat (a), rat administered CCl₄ alone for 4 weeks (b), rat administered 3 mg/kg/day 6-ECDCA (c), and rat administered CCl₄ combined with 15 mg/kg UDCA (d). Original magnification, 40×. e and f, effect of 6-ECDCA on morphometry of Sirius red-stained liver and HSCs proliferation (α-SMA/PCNA-positive cells). Data are the mean ± S.E. of four to six rats per group. *, P < 0.01 compared with control rats. **, P < 0.01 compared with CCl₄. g and h, effect of 6-ECDCA on liver and urinary hydroxyproline. Data are mean ± S.E. of four to six rats.

**Table 2**

FXR Ligand Promotes Liver Fibrosis Resolution. In rats administered CCL₄ for 4 weeks, resolution of liver fibrosis became evident as early as 5 to 7 days after the last dose of CCL₄. The qRT-PCR analysis shown in Fig. 7 demonstrates that a significant reduction in α1(I)collagen and α-SMA expression was detectable 5 to 7 days after the last dose of CCL₄ (n = 4; P < 0.05 versus peak). In contrast to these changes, neither TIMP-1 nor MMP-2 mRNA or protein changed in the first postadministration week (Fig. 7, f and g). Similarly, we found no increase in the number of nonparenchyma apoptotic cells during this time (Fig. 7i). Administration of 6-ECDCA caused a notable increase in

**Fig. 7.** α-SMA, TIMP-1, MMP-2 FXR, and SHP in HSCs isolated from a control rat (lane 1), a rat treated with CCL₄ for 4 weeks (lane 2), a rat treated with 3 mg/kg/day 6-ECDCA (lane 3), and a rat treated with 15 mg/kg/day UDCA (lane 4).
the velocity of fibrosis resolution. At 3 to 5 days of administration of 3 mg/kg 6-ECDCA, there was a 50 to 60% reduction of liver hydroxyproline content, \( \alpha \)-SMA, TIMP-1, and TIMP-2 mRNA expression compared with fibrosis peak (Figure 7, a–e). Furthermore, 6-ECDCA decreased TIMP-1 protein content and although it had no effect on MMP-2 mRNA expression, it significantly increased the MMP-2 activity (Fig. 7h). Finally, administration of rats with 6-ECDCA led to an increase in the number of apoptotic figures in nonparenchyma cells (Fig. 7). TUNEL-positive cells had similar distribution to that of \( \alpha \)-SMA-positive cells, although we failed to achieve good double immunostaining with \( \alpha \)-SMA and TUNEL.

Discussion

The FXR is a ligand-regulated transcription factor that functions as an endogenous sensor for bile acids (Makishima et al., 1999; Parks et al., 1999; Wang et al., 1999; Goodwin et al., 2000; del Castillo-Olivares and Gil, 2001; Lu et al., 2003). We have reported previously that modification of CDCA, the most potent FXR ligand, by addition of an ethyl group in position 6 results in a semisynthetic bile acid, namely, 6-ECDCA, which has potent FXR agonist activity (Mi et al., 2003). The resolution of the crystal structure of the FXR ligand binding domain complexed with 6-ECDCA and the coactivator peptide Src-1 (Pellicciari et al., 2002; Mi et al., 2003; Fiorucci et al., 2004) demonstrates that in the presence of 6-ECDCA, helix 12, the activation function 2 of the receptor, adopts the agonist conformation and stabilizes the binding of coactivator peptides. This would provide a mechanism for enhanced binding of coactivators through intermolecular contacts between their LXXLL sequences, explaining the enhanced potency of 6-ECDCA compared with the parent compound (Pellicciari et al., 2002; Mi et al., 2003; Fiorucci et al., 2004). 6-ECDCA has an \( EC_{50} \) value of 90 nM in the coactivator recruitment assay (Pellicciari et al., 2002; Mi et al., 2003; Fiorucci et al., 2004) and is 100-fold more potent than CDCA in activating FXR in transactivation assays (Pellicciari et al., 2002; Mi et al., 2003; Fiorucci et al., 2004). Similarly to GW4064 (Maloney et al., 2000), 6-ECDCA protects the liver in a rodent model of cholestasis (Lu et al., 2003).
In the present study, we have shown that FXR activation in HSCs leads to SHP-dependent inhibition of TIMP-1 expression/activity and increases the susceptibility of HSCs to apoptotic stimuli. SHP is an atypical member of the nuclear receptor superfamily since it lacks a DNA-binding domain (Lee et al., 2000). It is mostly expressed in the liver where it binds to and inhibits the function of other nuclear receptors. SHP activation by FXR ligands in hepatocytes leads to the repression of cholesterol 7-α-hydroxylase expression, the rate-limiting enzyme in bile acid production from cholesterol (Goodwin et al., 2000). SHP is also known to repress the activity of many nuclear hormone receptors in vitro, including estrogen receptors, androgen receptor, hepatocyte nuclear factor 4, constitutive androstane receptor, RXR, liver X receptor α, and liver receptor homologous protein-1 (Lee et al., 2000; Lai et al., 2003). Here, we demonstrated that 6-ECDCA induces SHP in HSCs and that SHP induction is essential for FXR ligands to suppress TIMP-1 expression. Support for this concept comes from the observation that exposure of HSCs to FXR ligands increases SHP mRNA and that FXR ligands are unable to suppress TIMP-1 expression in SHP-deficient HSCs. Furthermore, we found that SHP overexpression is sufficient to cause a 70 to 80% reduction of TIMP-1 mRNA in resting HSCs and abrogates the up-regulation of TIMP-1 induced by thrombin. Together, these data indicate that SHP is an essential part of the inhibitory pathway activated by FXR ligands in HSCs.

In this study, we have also provided evidence that SHP interacts with AP-1 by preventing its binding to the TIMP-1 promoter (Trim et al., 2000). The mechanism through which SHP inhibits TIMP-1 expression involves its direct interaction with JunD, the main AP-1 constituent in HSCs, and the formation of a SHP-JunD protein complex. Support for this concept comes from results of coimmunoprecipitation experiments carried out in SHP-overexpressing HSCs. In these experiments, we demonstrated that JunD is induced by thrombin and coimmunoprecipitates with SHP. Sequestration of JunD in a protein complex with SHP is likely responsible for prevention of JunD binding to the TIMP-1 promoter as demonstrated by the results of our EMSA experiments. Thus, we found that exposure of wild-type HSC-T6 to 6-ECDCA or SHP overexpression abrogates AP-1 binding to the TIMP-1 promoter. The results of the transactivation assay were also consistent with this concept, since SHP overexpression in HA-SHP was sufficient to abrogate the transactivation of the TIMP-1 promoter. Together, these results indicate that an FXR-SHP regulatory cascade mediates TIMP-1 inhibition caused by FXR ligands. We have now provided evidence that exposure of HSCs to FXR ligands prevents the physical interaction between JunD with SHP. The possibility of direct interaction of a nuclear receptor with JunD in HSCs has already been shown (Hazra et al., 2004) since peroxisome proliferator-activated receptor-γ overexpression leads to formation of a JunD-peroxisome proliferator-activated receptor-γ complex, which prevents JunD binding to the AP-1 binding site (Hazra et al., 2004).

Exposure to FXR ligands increases HSCs susceptibility to apoptotic stimuli through a mechanism that involves SHP-mediated inhibition of TIMP-1 expression (Canbay et al., 2004). Thus, not only do FXR ligands decrease the expression of TIMP-1 but also addition of rTIMP-1 to HSCs attenuates apoptosis induced by 6-ECDCA and cycloheximide. The role of SHP in modulating HSCs sensitivity to apoptotic stimuli is further shown by the fact that exposure of SHP-deficient cells, which express increased levels of TIMP-1, to cycloheximide greatly attenuated the apoptotic potential of 6-ECDCA. In contrast, treating SHP-overexpressing cells, in which TIMP-1 expression is reduced by ~80%, increased the apoptotic potential of cycloheximide and 6-ECDCA.

Our in vivo data demonstrate that treatment of rats with 6-ECDCA protects against liver fibrosis development and accelerates liver fibrosis resolution induced by CCl4. The results presented in Fig. 7 show that administration of 6-ECDCA results in a rapid (3–5-day) induction of SHP expression that associates with a dramatic down-regulation of TIMP-1 induction caused by CCl4. Thus, whereas a single dose of CCl4 causes an ~30-fold induction of TIMP-1 mRNA, simultaneous administration of 3 mg/kg 6-ECDCA, a dose of FXR ligand that protects against fibrosis development in bile duct ligated, and porcine serum administered rat reduced TIMP-1 induction by ~60%, whereas 15 mg/kg UDCA had no protective effects (Paumgartner and Beuers, 2002). Furthermore, 6-ECDCA administration reduced the early, 3- and 5-day, up-regulation of α-SMA and α1(1)collagen mRNA induced by CCl4, suggesting that the FXR ligand prevents the phenotype switch that associated with activation of HSCs in this model.

Administration of CCl4 for 4 weeks is an established model of liver fibrosis. Using this model, we have shown that 3 mg/kg 6-ECDCA caused a 70 to 90% reduction of collagen deposition as assessed by liver morphometry and measurement of hepatic hydroxyproline content and α-SMA and α1(1)collagen mRNA expression. The reduction in collagen deposition caused by 6-ECDCA treatment was associated with the reduction of the parenchymal area occupied by α-SMA-positive cells, suggesting a causal relationship between the decreased number of activated HSCs and the reduced accumulation of ECM components in CCl4-intoxicated rats.

Here, we demonstrated that FXR activation in rats with established cirrhosis leads to accelerated resolution of liver fibrosis. The results shown in Fig. 7 demonstrate that administration of 3 mg/kg 6-ECDCA to rats leads to a rapid decrease (within 3–7 days) of α-SMA, α1(1)collagen, and TGFβ1 mRNA. This effect correlates with reduction of TIMP-1 mRNA and a 100% increase in the activity of MMP2. Previous studies have shown that spontaneous resolution of liver fibrosis in the CCl4 model is accompanied by an increased rate of apoptosis in HSCs, leading to a reduction of the number of activated HSCs. In accordance with our in vitro data, we found that administration of 6-ECDCA to rats with established cirrhosis increases the number of apoptotic figures in nonparenchymal cells, suggesting that an increased rate of HSCs apoptosis mediates the proresolusion effect of 6-ECDCA in this model.

In conclusion, we have shown that FXR regulates the balance between profibrotic and antifibrotic mediators in HSCs. By demonstrating that a regulatory cascade involving FXR-SHP promotes the development of a quiescent phenotype and increases apoptosis of HSCs, this study establishes that FXR ligands may be beneficial in treatment of liver fibrosis.