Cyclosporine Inhibits Profibrotic Effects of Interleukin-4 and Transforming Growth Factor β on Human Intrahepatic Fibroblasts Cultured In Vitro

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ABSTRACT

Background. Hepatic fibrosis, an outcome of chronic liver diseases, is characterized by an accumulation of collagen, which is produced by activated human intrahepatic fibroblasts (HIF). Transforming growth factor (TGF) β is an important inducer of fibrogenesis, in collaboration with other cytokines, such as interleukin (IL) 4. IL-4 is overexpressed in severe recurrent hepatitis C after liver transplantation, exerting profibrotic effects. In contrast, cyclosporine (CsA) had been shown to decrease fibroblast activation and collagen production. We therefore investigated the effects of CsA on TGF-β and IL-4 profibrotic activities on HIF in vitro.

Methods. Isolated HIF were cultured without or with human TGF-β, human IL-4, CsA, or combined TGF-β + CsA or IL-4 + CsA. We performed real-time polymerase chain reaction for collagen types I, III, and IV and alpha-SMA, a marker of fibroblast activation we also measured total collagen in supernates. TGF-β and IL-4 increased the expressions of alpha smooth muscle actin (SMA) collagen I, III, and IV mRNAs (P < .05 vs untreated cells) as well as the overall collagen level in the supernates (P < .01). CsA decreased the expression of mRNAs encoding alpha-SMA and collagens (P < .01). Expressions of alpha-SMA and collagens I, III, and IV mRNAs were significantly lower under combined treatments (TGF-β vs TGF-β + CsA [P < .01] and IL-4 vs IL-4 + CsA [P < .01]). Collagen level was decreased by combined treatments (TGF-β vs TGF-β + CsA [P < .05] and IL-4 vs IL-4 + CsA [P = .05]).

Conclusion. CsA inhibited the profibrotic effects of TGF-β and IL-4 by decreasing the activation and production of collagen by HIF. CsA may decrease fibroblast activation and collagen accumulation, exerting beneficial effects on fibrosis progression, particularly among patients with recurrent hepatitis C.
Kupffer cells, or infiltrating lymphocytes. Although transforming growth factor \( \beta \) (TGF-\( \beta \)) is an important inducer of liver fibrogenesis,\(^6\) cytokines other than TGF-\( \beta \) may also be involved in the fibrotic process.

Liver transplantation (LT) has become an accepted therapy for patients with end-stage liver diseases, including hepatitis C virus (HCV)–related cirrhosis.\(^7\) Uniformly, HCV persists or recurs after LT, resulting in accelerated progression toward fibrosis and impaired patient and allograft survival\(^8\) mediated by poorly understood mechanisms. After transplantation, immunosuppressive drugs (mainly calcineurin inhibitors) markedly reduce interleukin (IL) 2 but not IL-4 production. We have recently shown that IL-4 is overexpressed in patients with severe recurrent hepatitis C after LT.\(^9\) We have also recently shown that IL-4 exerts profibrotic effects by activating HIF, which induces collagen production. IL-4 may thus play a role in the accelerated course of fibrogenesis during recurrent hepatitis C.

Immunosuppression may also play a role in the accelerated course, but clinical studies comparing immunosuppression protocols are controversial.\(^10\) Berenguer et al observed that tacrolimus therapy was associated with higher probability of developing cirrhosis,\(^11\) and in some studies, cyclosporine (CsA) seems to be associated with slower progression of fibrosis.\(^12\) Furthermore, in vitro studies have shown that CsA inhibits activation and decreases collagen production in HIF.\(^13\) The aim of the present study was to investigate the effects of CsA on TGF-\( \beta \) and IL-4 on the profibrotic activity in HIF in vitro.

**MATERIAL AND METHODS**

HIF were isolated from normal liver tissue obtained from patients undergoing partial heptectomy for metastases or benign tumors. This procedure complied with the ethical guidelines stipulated by French legislation. The dissociation was based on a 2-step collagenase perfusion method followed by centrifugation over gradients.\(^14\) Freshly isolated HIF were seeded in 5% fetal calf serum (FCS)/Dulbecco Modified Eagle Medium (DMEM) (Gibco) supplemented with penicillin (100 U/mL) and streptomycin (100 mg/mL) for culture at 37°C in a 5% CO\(_2\) atmosphere. Cells between the second and sixth passages were used for the experimentation. Into each of 6-well plates HIF(3 × 10\(^5\)) cells were seeded in DMEM (5% FCS). Medium was replaced with FCS-DMEM (5% FCS) and the cells cultured for 48 hours without (for control) or with recombinant human TGF-\( \beta \) (10 ng/mL; R&D Systems, Lille, France), recombinant human IL-4 (50 ng/mL; R&D Systems), or CsA (1 \( \mu \)g/mL; Novartis Pharma, Basel, Switzerland).

Types I, III, and IV collagen and \( \alpha \)-SMA gene expressions in HIF were analyzed by real-time polymerase chain reaction (PCR).\(^14\) The reaction was initiated using the DNA Fast Start Sybr Green Kit (Roche Diagnostics, Grenoble, France) with LightCycler instruments and technology (Roche Diagnostics). PCR amplification was performed in a total volume of 20 mL in glass capillaries containing 20 ng of each primer (Sigma-Genosys), 3 mmol/L MgCl\(_2\), 2 mL LightCycler Fast Start DNA Master Sybr Green (containing 1.25 U Fast Start Taq polymerase, 10 × Taq buffer, 2 mmol/L each deoxyribonucleoside triphosphate, 10 × Sybr Green; Roche Diagnostics), and 2 mL cDNA (previously diluted to 1:10). The PCR amplification protocol consisted in 1 step of initial denaturation for 10 minutes at 94°C, followed by 40 cycles of denaturation (95°C for 10 seconds), annealing for 5 seconds (54°C for 28 seconds, 58°C for collagen I or 65°C for collagen III, IV, \( \alpha \)-SMA), and extension (72°C for 5 seconds).

The mRNA level was calculated by normalizing the threshold cycle (Ct) of STAT6, GAPDH, type I, III, and IV collagens, or \( \alpha \)-SMA to the Ct of the housekeeping gene 28S ribosomal RNA, according to the following formula: the average 28S Ct was subtracted from the average type I, III, and IV collagens or \( \alpha \)-SMA Ct, with the result representing \( \Delta \)Ct. This \( \Delta \)Ct was specific and could be compared with the \( \Delta \)Ct of a calibration sample (control HIF). The subtraction of control \( \Delta \)Ct from the \( \Delta \)Ct of the fibroblasts being processed is referred to as \( \Delta \Delta \)Ct. We determined relative quantification compared with the controls for the expression of STAT6, GAPDH, types I, III, and IV collagens, or \( \alpha \)-SMA by using the value of \( 2^{-\Delta \Delta \text{Ct}} \).

The supernate was then assayed for soluble collagen using the Sircol collagen assay, according to the manufacturer’s protocol (Sigma). This assay uses Sirius red, an anionic dye with sulfonic acid side chains, which reacts with the basic amino acids present in collagen. Briefly, after the Sircol reagent was added to the medium, it was gently agitated for 30 minutes to allow collagen-dye complexes to form. The samples were then centrifuged at 12,000 rpm for 10 minutes; the collagen-dye complex precipitates were collected for resolubilization in 0.5 mmol/L sodium hydroxide for dye concentrations to be estimated by spectrophotometry at 540 nm.

**RESULTS**

As expected the incubation of HIF with TGF-\( \beta \), increased mRNA expressions of \( \alpha \)-SMA and collagens I and IV (\( P < .05 \) vs untreated cells) as well as the collagen level in supernates (\( P < .01 \) vs untreated cells; Fig 1). IL-4 also increased the mRNA expression in HIF of \( \alpha \)-SMA as well as collagens I, III, and IV (\( P < .05 \) vs untreated cells); collagen levels were increased in the supernates (Fig 1). The results of TGF-\( \beta \) and IL-4 were similar, except for collagen III, which was significantly increased only with IL-4 (Fig 1C), and collagen IV, for which TGF-\( \beta \) was a more potent inducer than IL-4 (Fig 1D). CsA decreased the mRNA expression of \( \alpha \)-SMA and collagens (\( P < .01 \) vs untreated cells). mRNA expression of alpha-SMA was significantly lower under combined treatment with TGF-\( \beta \)+CsA or IL-4+CsA than under TGF-\( \beta \) or IL-4 alone (TGF-\( \beta \) vs TGF-\( \beta \)+CsA \( P < .01 \) and IL-4 vs IL-4+CsA \( P < .05 \)).

Expressions of collagen I, III, and IV mRNA were significantly decreased in cells treated with combinations compared with TGF-\( \beta \) or IL-4 alone: collagen I: for TGF-\( \beta \) versus TGF-\( \beta \)+CsA (\( P < .01 \)) and for IL-4 versus IL-4+CsA (\( P < .05 \)); collagen III: for TGF-\( \beta \) versus TGF-\( \beta \)+CsA and for IL-4 versus IL-4+CsA (\( P < .05 \)); and collagen IV: TGF-\( \beta \) versus TGF-\( \beta \)+CsA and IL-4 versus IL-4+CsA (\( P < .01 \)). Collagen levels in supernates were also significantly decreased by combined treatments: TGF-\( \beta \) versus TGF-\( \beta \)+CsA (\( P < .05 \)) and IL-4 versus IL-4+CsA (\( P = .05 \)).
DISCUSSION

HCV-related cirrhosis is a common indication for LT, although recurrence of HCV infection is universal, characterized by accelerated hepatic fibrosis. TGF-β is an important inducer of liver fibrogenesis, as are other cytokines. We have recently shown that IL-4 expression is increased in severe recurrent hepatitis C and that IL-4 induces activation and collagen production by HIF. Although clinical studies are controversial immunosuppressive therapy may also play a role, because CsA exerts a protective role in vitro.

In the present study we have shown that CsA inhibits the profibrotic effects of TGF-β and IL-4 in vitro by decreasing HIF activation and inhibiting collagen production. Recently, our group showed that IL-4 was as potent as TGF-β as an HIF activator of probably exerting an important role in the accelerated fibrogenesis process after LT, in patients receiving immunosuppressive therapy aimed to reduce IL-2 production.

CsA, as well as tacrolimus, inhibits IL-2 production. Some studies have reported that patients receiving CsA-based immunosuppression show less severe recurrences of hepatitis C than those receiving tacrolimus, whereas an other evaluation was inconclusive. CsA reduces HCV replication in vitro, but the viral load was similar among patients treated with CsA versus tacrolimus, even among studies that reported a beneficial effect of CsA on fibrosis.

In a model of cirrhosis in the rat, CsA inhibited TGF-β activation of HIF by a mechanism involving down-regulation of transglutaminase and collagen expressions. The present results showed that CsA also decreases the activation of HIF induced by IL-4. This effect could contribute to reduce the fibrosis during recurrent hepatitis C among patients treated with CsA. Actually, CsA inhibits the profibrotic effects of TGF-β and IL-4 by decreasing the activation of and inhibiting the collagen production by HIF. CsA may decrease fibroblast activation and collagen accumulation, thus exerting beneficial effects on fibrosis progression, particularly among patients with recurrent hepatitis C.

REFERENCES