Expression of Connective Tissue Growth Factor in Experimental Rat and Human Liver Fibrosis

Valerie Paradis,[1,2] Delphine Dargere,[2] Michel Vidaud,[3] Anne-Charlotte de Gouville,[4] Stephane Huet,[4] Vincent Martinez,[4] Jean-Michel Gauthier,[4] Nathalie Ba,[1] Rodolphe Sobesky,[2] Vlad Ratziu,[2] and Pierre Bedossa[1,2]

Connective tissue growth factor (CTGF) stimulates *in vitro* fibroblast proliferation and extracellular matrix synthesis. The aim of this study was to assess the role of CTGF in liver fibrogenesis. CTGF expression was investigated both at the protein and mRNA level in biopsies of chronic liver diseases, in experimental models of liver fibrosis, and in hepatic stellate cells in culture. CTGF immunostaining was observed in most human liver biopsies with significant fibrosis. An increase of CTGF immunostaining was associated with a higher score of fibrosis both in the group of chronic hepatitis C ($\chi^2 = 9.3$; $P < .01$) and in the non–hepatitis C group ($\chi^2 = 7.2$; $P < .02$). *In situ* hybridization showed CTGF mRNA expression in spindle cells in both the fibrous septa and sinusoidal lining. In experimental models of liver fibrosis, CTGF accumulated in parallel with the development of septal fibrosis and cirrhosis. Quantification of CTGF mRNA by a real-time reverse-transcription polymerase chain reaction (RT-PCR) assay showed a significant increase of CTGF mRNA in both CCl_4-induced and bile duct–ligated rat models of liver fibrosis. Expression of CTGF protein and mRNA was definitively assigned to hepatic stellate cells, because CTGF was detected by Western blot both in lysate and supernatant of a hepatic stellate cell line derived from rats. These cells also displayed CTGF protein and mRNA as shown by immunohistochemistry and *in situ* hybridization. In conclusion, this study shows that CTGF is strongly expressed during liver fibrogenesis, and hepatic stellate cells seem to be the major cellular sources of CTGF in the liver. (Hepatology 1999;30:968-976.)

Connective tissue growth factor (CTGF) is a cysteine-rich peptide originally identified as a growth factor secreted by vascular endothelial cells in culture.[1] It belongs to a family of immediate early growth-responsive genes including *cyr61* and *fisp-12* from mouse, and *cef10* and *nov* from chicken.[2-5] These genes are thought to regulate cell proliferation, differentiation, and embryogenesis. The physiological function of CTGF both *in vitro* and *in vivo* has not yet been elucidated. However, CTGF is considered to be the major mitoattractant produced by human umbilical vascular endothelial cells.[1] Furthermore, results from experimental studies showed that CTGF may be one of the downstream effectors of transforming growth factor β (TGF-β). Indeed, CTGF mRNA is induced specifically by TGF-β, but not by platelet-derived growth factor (PDGF), epidermal growth factor, or basic fibroblast growth factor.[6,7] Furthermore, a novel TGF-β–responsive element was described in the CTGF promoter sequence.[8]

The role of CTGF in the fibrogenesis process has recently been proposed. First, Northern blot analysis showed that CTGF was expressed at higher levels in atherosclerotic vessels than in normal arteries.[9] Furthermore, the authors showed that the vascular smooth muscle cells that express CTGF were localized predominantly in areas with extracellular matrix accumulation.[9] Igarashi et al. showed that, during wound repair, CTGF participates in the process of tissue regeneration and skin fibrosis as a potential autocrine stimulator released in response to TGF-β.[7] Finally, it has recently been suggested that CTGF may be a common growth factor involved in human renal fibrosis.[10]

Liver fibrosis represents the main complication of most chronic liver diseases whatever their origin, and cirrhosis constitutes the ultimate stage of this process. The molecular mechanisms of liver fibrosis are not fully understood. However, fibrogenesis, characterized by cell proliferation and accumulation of extracellular matrix components, requires numerous mediators such as TGF-β and PDGF.[11-13] Aside from these key mediators, other molecules such as CTGF may play a role in the fibrogenesis process. The aim of this study was to investigate the possible involvement of CTGF in liver diseases and its relationship with liver fibrosis. To assess the role of CTGF, we evaluated the expression of CTGF in human biopsy specimens from various chronic liver diseases by immunohistochemistry and *in situ* hybridization. To gain further insight into the dynamics of CTGF production, we also studied the expression of CTGF at mRNA and protein levels in experimental models of liver fibrosis in rats. Because hepatic stellate cells (HSC) play a crucial role in the development of liver fibrosis through the production of several extracellular matrix components,[14] we studied CTGF expression in cultured HSC.

Abbreviations: CTGF, connective tissue growth factor; HSC, hepatic stellate cell; CCl_4, carbon tetrachloride; BDL, bile duct ligation; RT, room temperature; C_t, threshold cycle.

From the [1]Service d'Anatomie Pathologique, Hôpital de Bicêtre, Le Kremlin-Bicêtre; [2]CNRS UPRES-A8067, Faculté de Pharmacie, Paris V; [3]Laboratoire de Génétique Moléculaire JE 2195, Faculté de Pharmacie, Paris V; and [4]Centre de Recherche Glaxo Wellcome, Les Ulis, France.

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Address reprint requests to: Valérie Paradis, M.D., Service d'Anatomie Pathologique, Hôpital de Bicêtre, 78, rue du Général Leclerc 94275 Le Kremlin-Bicêtre, France. E-mail: vparadis@teaser.fr; fax: (33) 1-45-21-32-81.

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MATERIALS AND METHODS

Human Liver Biopsies

A total of 59 fixed, paraffin-embedded liver samples were retrospectively selected for the study. Hematein-eosin-safran and Masson's trichrome stainings were performed for histological diagnosis. There were 39 cases with chronic hepatitis C (mean age: 36 years; range: 17-63 years; 30 males and 9 females) and 20 cases with other chronic liver diseases (mean age: 49 years; range: 34-76 years; 15 males and 5 females). Three cases of histologically normal livers were also studied. Chronic hepatitis cases were graded according to METAVIR.[15] In the group with chronic hepatitis C, activity was graded A0 (absence) in 6 cases, A1 (mild) in 17 cases, A2 (moderate) in 7 cases, and A3 (severe) in 9 cases. The fibrosis score was F0 (no fibrosis) in 1 case, F1 (enlargement of portal tracts without bridging fibrosis) in 21 cases, F2 (portal fibrosis with rare fibrous septa) in 5 cases, F3 (septa without cirrhosis) in 7 cases, and F4 (cirrhosis) in 5 cases. For the purpose of the study, activity and fibrosis scores were dichotomized. In the second group, there were 18 cases with alcoholic liver disease and 2 cases with chronic hepatitis B. Among these, fibrosis was staged F1 in 2 cases, F2 in 5 cases, F3 in 6 cases, and F4 in 7 cases.

Experimental Models

CCl_4-Induced Liver Fibrosis in Rats. To assess the chronological expression of CTGF in early stages of fibrogenesis, liver fibrosis was induced in 24 Wistar Dawley rats (180 g) (Charles River Laboratories) by repeated (twice a week) intraperitoneal injections of CCl_4 in olive oil (vol/vol) at a dose of 2 mL/kg body weight. Seven groups of 4 rats were sequentially killed the day after the last injection (days 1, 5, 12, 15, 19, 23). Rats that were normal controls (1 additional rat per group) were given an equivalent volume of vehicle according to the same schedule. To assess CTGF expression at the late stage of cirrhosis, a group of 6 rats received 15 doses of CCl_4 (intragastric administration of 1 mL/kg of a vol/vol mixture in olive oil) every 5 days, and were killed 1 week after the last CCl_4 administration. Each liver was removed and cut into small pieces. Some pieces were fixed in formalin and embedded in paraffin for hematoxylin-eosin staining or immunohistochemical staining. Others were snap-frozen for RNA extraction. Fibrosis was graded according to the METAVIR score, and necrosis was semiquantitatively assessed.

Bile Duct Ligation Protocol. CTGF expression was also studied in a mechanistically different model of liver fibrosis. Male Sprague-Dawley rats were either bile duct–ligated (BDL) (n = 7) or sham-operated (n = 6). In brief, under light ether anesthesia, the common bile duct was double-ligated and sectioned between the ligatures. Sham-operated rats were treated in the same manner except that the bile duct was gently manipulated, but not ligated or sectioned. All animals were killed 1 and 3 weeks after the surgical procedure. Liver fragments were fixed in formalin and embedded in paraffin for hematoxylin-eosin staining and immunohistochemistry, while other pieces were snap-frozen for RNA extraction.

HSCs

The possible role of HSC in CTGF production was investigated *in vitro*. Rat HSC immortalized by SV40 (kindly provided by S. Friedman, Mount Sinai Hospital, New York, NY) were grown in P35 Petri dishes or incubation chambers (Labtek, Nalge Nunc International, Naperville, IL) at 37°C in an atmosphere of 5% CO_2. Cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum, L-glutamine 2 mmol/L, and penicillin (5,000 UI/mL)/streptomycin (5,000 μg/mL). Under these conditions, HSC had a pseudo-myofibroblastic phenotype as shown by the absence of vitamin A, characteristic cell shape, and α-smooth muscle actin expression. Some plates were fixed for immunohistochemical and *in situ* hybridization, while others were used to collect lysates and conditioned medium.

Immunohistochemistry

Production of Natural and Recombinant CTGF. For production of natural CTGF, endothelial cells were isolated from human umbilical cords (HUVEC) within 48 hours of delivery as described in Busso et al.[16] Cells were cultured using Endothelial Cell Medium (ECM) (Gibco BRL, Cergy Pontoise, France). For preparation of CTGF containing supernatant, 3 to 6 passaged HUVEC were plated in 25-cm^2 tissue-culture flasks precoated with purified human fibronectin. When cellular monolayers were confluent, the ECM was removed and 2 mL of fresh ECM was added. After a 24-hour incubation period, supernatants were collected and centrifuged for 15 minutes at 15,000*g* at 4°C to eliminate cellular debris.

For production of recombinant CTGF, the human CTGF cDNA was polymerase chain reaction (PCR)–amplified from a normal human liver cDNA library and cloned in a PFastBac1 plasmid (GIBCO BRL, Cergy Pontoise, France). After subcloning in a Baculovirus expression vector, Sf9 insect cells were infected and culture supernatants were collected after 24 hours. Recombinant CTGF was purified after passage on a Mono-S column and elution in 0.6 mol/L NaCl, followed by a second step of purification using a TSK gel G2000SW gel filtration column.

Anti-CTGF Antibody Preparation. For generation of anti-CTGF antibodies, rabbits were immunized by intradermal administration of a 27–amino acid CTGF peptide corresponding to positions 173 to 199 of the described CTGF protein sequence.[1] Serum immunoglobulins were purified on a protein A sepharose column before use in Western blotting or immunohistological experiments.

To check the specificity of CTGF antibody, Western blotting experiments were performed. Thirty microliters of HUVEC supernatants or 10 ng of purified recombinant CTGF was used for migration on 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane (Biorad). After transfer, membranes saturated in 2% skimmed milk were incubated overnight at 4°C with a 1/15,000 dilution of purified anti-CTGF antibodies. Fixation of the primary antibodies was detected using peroxidase-coupled goat anti-rabbit antibodies (Southern Technologies) and an ECL revelation kit (Amersham). The purified antibody preparation detected a 38-kd protein band for both recombinant CTGF and CTGF produced in 24-hour culture supernatants of HUVEC (Fig. 1). Furthermore, CTGF antibody did not cross-react with recombinant PDGF (data not shown)

Immunohistochemistry Procedure. The immunohistochemical procedure was performed either on paraffin-embedded sections for liver biopsies or on fixed HSC. For liver biopsies, immunohistochemistry

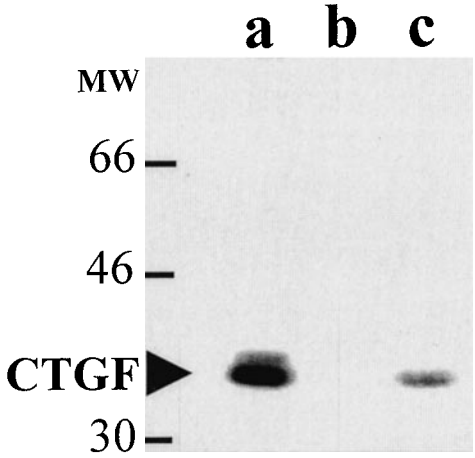

FIG. 1. Specificity of anti-CTGF antibody. Detection by Western blot using a 1/15000 dilution of purified anti-CTGF rabbit serum of recombinant human CTGF (a) and CTGF produced in 24-h culture supernatants of human vascular endothelial cells (HUVEC) (c). (b): No detection of CTGF in the medium alone used for endothelial cell culture.

was performed using an automated immunostainer (Techmate 500, Dako, Glostrup, Denmark) with the avidin-biotin-peroxidase method. To assess the cell type involved in the production of CTGF, serial sections were immunostained with anti-CTGF antibodies (1:100) and anti–α-smooth muscle actin antibodies (α-SMA) (Dako) to detect activated HSC. For HSC in culture, the same immunohistochemical procedure was run manually on cells fixed in acetone/methanol (vol/vol) for 10 minutes at 4°C.

As negative controls, we used either normal sheep or rabbit serum, protein A–purified immunoglobulins from non-immune rabbit, phosphate-buffered saline (PBS), or antibody preincubated with the synthetic peptide instead of primary antibodies.

Localization and intensity of staining were independently assessed by 2 pathologists who were not aware of the diagnosis. In cases of discordance, a consensual evaluation was performed by simultaneous reviewing of slides. For CTGF, the intensity of staining was graded as absent (no or mild staining) or present (moderate or strong staining). This evaluation involved the mean intensity of the signal on the whole slide. For α-SMA, staining was semiquantitatively assessed as absent or localized versus diffuse.

Western Blot

For Western blot, HSC lysate and conditioned media were collected. HSC were resuspended in lysis buffer containing Tris-HCl 50 mmol/L (pH 7.4), 1% Triton X-100, ethylenediaminetetraacetic acid (EDTA) 10 mmol/L, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,-tetraacetic acid (EGTA) 2 mmol/L, NaF 10 mmol/L, 2-Aminoethyl/benzene-sulfonyl fluoride (AEBSF) 0.5 mg/mL, Na_2-EDTA 5 mg/mL, leupeptine 1 μg/mL, pepstatine 1 μg/mL, $Na_4P_2O_7$ 1 mmol/L, and Na_2VO_4 2 mmol/L. After incubation for 30 minutes at 4°C, HSC were centrifugated at 14,000 rpm for 10 minutes, and the supernatant was collected. Conditioned media of HSC grown for 12 hours in a serum-free medium was also recovered to assess CTGF release in the supernatant. The protein concentration was measured by the Lowry procedure.

HSC lysate (80 μg) and conditioned medium (20 μg) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions on 10% acrylamide gels and transferred to nitrocellulose filters by electroblotting. The blot was incubated for 1 hour in Tween-PBS with 5% nonfat dry milk and incubated overnight at 4°C with the anti-CTGF antibody (1:1,000) diluted in Tween-PBS with 1% nonfat dry milk. The blot was then washed 6 times (15 minutes each) with Tween-PBS, and incubated with rabbit IgG for 1 hour at room temperature (Dako; 1:2,000). After washes with Tween-PBS, the immunoreactive bands were detected using the ECL method.

In Situ *Hybridization*

Probe Construction. A CTGF fragment encompassing nt 841-1050 (C-terminal part) was PCR-amplified from a vector containing the human CTGF cDNA. To generate sense and antisense riboprobes, this fragment was cloned between the *Sac*I and *Xba*I sites in the pGEM-3Zf(+) plasmid (Promega). Vectors were sequence-checked. Plasmids were linearized by *Hin*dIII digestion and were *in vitro*–transcripted using T7 RNA polymerase to obtain antisense riboprobes. As a control, sense RNAs were produced using SP6 RNA polymerase and EcoRI linearized vectors. The *in vitro* transcription DIG RNA Labelling kit (Boehringer Mannheim, Mannheim, Germany) was used to synthesize digoxigenin-labeled RNA according to the recommendations of the manufacturer.

In Situ Hybridization Procedure. For the human liver biopsy, paraffin-embedded tissue sections (3 μm) were cut onto Superfrost/Plus slides (Fisher Scientific, Pittsburgh, PA). The sections were deparaffinized, rehydrated in gradually decreasing concentrations of ethanol, and rinsed with 1× phosphate-buffered saline (PBS). Sections were treated with proteinase K (20 μg/mL) in 100 mmol/L Tris-HCl, 50 mmol/L EDTA for 20 minutes at 37°C. Sections were post-fixed in 4% paraformaldehyde in 1× PBS for 20 minutes at RT, rinsed in 1× PBS for 5 minutes, and acetylated in 0.25% acetic anhydride, 0.1 mmol/L triethanolamine for 10 minutes. The slides were dehydrated in gradually increasing concentrations of ethanol before hybridization. Sections were prehybridized 1 hour at 42°C in hybridization buffer (4× SSC, 10% dextran sulfate, 1× Denhart's solution, 2 mmol/L EDTA, 50% formamide, 500 μg/mL salmon sperm DNA). Hybridization was performed at 42°C overnight in the hybridization buffer containing 800 ng/mL probe. The tissue was washed at 42°C in 2× SSC for 5 minutes, at 42°C in 50% formamide, 2× SSC for 5 minutes, at 42°C in 0.2× SSC for 5 minutes. Following washes in 0.1× SSC for 5 minutes at RT, the slides were rinsed in buffer 1 (100 mmol/L Tris-HCl, 150 mmol/L NaCl, pH 7.5) for 5 minutes at RT and blocked for 30 minutes. Slides were then incubated with anti-digoxigenin antibody (1/200) for 2 hours at RT. After 2 rinses in buffer 1, slides were rinsed in buffer 3 (100 mmol/L Tris-HCl, 100 mmol/L NaCl, 50 mmol/L $MgCl_2$, pH 9.5) for 10 minutes. Alkaline phosphatase was detected using 5-bromo 4-chloro 3-indolyl phosphate and nitroblue tetrazolium chloride. Slides were then rinsed in buffer 4 (10 mmol/L Tris-HCl, 1 mnol/L EDTA, pH 8) and counterstained with nuclear red.

For HSC in culture, cells were rinsed in PBS 1× and fixed in paraformaldehyde 4% at room temperature for 20 minutes. After rinses in 1× PBS, cells were incubated with Triton X-100 1% for 30 minutes at room temperature. Before the prehybridization step, cells were rinsed with buffer 1. Sections incubated with the digoxigenin labeled sense probe or without the probe in the same conditions were used as negative controls.

Real-Time Reverse Transcription-PCR

Real-time RT-PCR is a sensitive, quantitative and highly reliable method for RNA quantitation. The theoretical base of the method have been previously described.[17,18] Briefly, reactions are characterized by the point during cycling when amplification of the PCR product is first detected, rather than the amount of PCR product accumulated after a fixed number of cycles. The parameter C_t (threshold cycle) is defined as the fractional cycle number at which the fluorescence generated by cleavage of the probe passes a fixed threshold above baseline. The CTGF target message in unknown samples is quantified by measuring C_t and by using a standard curve to determine the starting target message quantity. In addition, the level of mRNA for TBP a housekeeping gene encoding transcriptional factor was measured in each sample to control sample-to-sample variations in RNA concentrations.[19] Primers and probes for TBP, CTGF and Type I collagen were chosen with the assistance of computer programs Oligo 4.0 (National Biosciences, Plymouth, MN) and Primer Express (Perkin-Elmer Applied Biosystems, Foster City, CA). Nucleotide sequences for the oligonucleotide hybridization probes and primers are the following: TBP probe, TGT GCA CAG GAG CCA AGA GTG AAG A; TBP primers, GAA TAA GAG AGC CAC GAA CAA CTG and CCC AGC TTC TGC ACA ACT CT; Collagen (α1I probe, TTC TTG GCC ATG CGT CAG GA; Collagen (α1I primers, ATG TTC AGC TTT GTG GAC CT and CAG CTG ACT TCA GGG ATG T; CTGF probe, CTC CCC CGC CAA CCG CAA GAT; CTGF primers ATC CCT GCG ACC CAC ACA AG and CAA CTG CTT TGG AAG GAC TCG C.

Total RNA was extracted from frozen rat liver tissue by using the acid-phenol guanidium method. Reverse transcription of total RNA was performed in a final volume of 20 μL containing 1× RT buffer (500 μmol/L each dNTP, 3 mmol/L $MgCl_2$, 75 mmol/L KCl, 50 mmol/L Tris-HCl pH 8.3), 10 units of RNasin inhibitor (Promega, Madison, WI), 10 mmol/L dithiothreitol, 50 units of Superscript II Rnase H^- reverse transcriptase (Gibco BRL, Gaithersburg, MD), 1.5 μmol/L random hexamers (Pharmacia, Uppsala, Sweden) and 1 μg of total RNA. Samples were incubated at 20°C for 10 minutes, 42°C for 30 minutes, and reverse transcriptase was inactivated by heating at 99°C for 5 minutes and cooled at 5°C for 5 minutes. Amplification reactions were set up in a reaction volume of 50 μL by use of components (except primers and probes) supplied in a TaqMan PCR Core Reagent Kit (PE Biosystems). Ten microliters of diluted RT

samples were used for quantitative two step PCR (7700 Prism; PE Biosystems) with a 10 min step at 95°C, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 65°C in the presence of 200 nmol/L specific forward and reverse primers, 100 nmol/L specific fluorogenic probe, 5 mmol/L $MgCl_2$, 50 mmol/L KCl, 10 mmol/L Tris buffer (pH 8.3), 200 μmol/L dATP, dCTP, dGTP and 400 μmol/L dUTP and 1.25 units of AmpliTaq Gold DNA polymerase (Perkin Elmer Applied Biosystems). Each sample was analyzed in duplicate and a calibration curve constructed with a 10-fold serial dilution of a total RNA extracted from immortalised HSC was run in parallel with each analysis. The coefficient of correlation was $r = .99$ and slope was constant at each experiment. For each sample, the amounts of CTGF and Type I collagen were divided by the TBP amount to obtain normalized CTGF or Type I collagen values.

Statistical Analysis

Results were expressed as means ± SD. Differences in distributions between groups were assessed using the χ^2 test with Yates correction, supplemented by Fisher's exact test if needed. A level of $P < .05$ was accepted as statistically significant.

RESULTS

Connective Tissue Growth Factor Expression in Human Chronic Liver Diseases

Immunohistochemistry. In normal livers, only mild immunostaining was detected in portal tracts. There was no staining in the lobule and central vein. In chronic hepatitis, staining was observed in portal tracts and fibrous septa predominantly in the fibrogenesis areas, at the interface between the fibrous septa and liver parenchyma (Fig. 2A). CTGF was also detected in sinusoidal cells close to the fibrous septa (Fig. 2B, 2C). No or mild staining was observed in the lobular necroinflammatory foci. No staining was detected when pre-immune immunoglobulins or antibodies preincubated with synthetic peptide used for immunization were used instead of anti-CTGF antibodies (Fig. 2D). In the hepatitis C group, immunostaining of CTGF was absent in 8 cases, mild in 12 cases, moderate in 10 cases and strong in 9 cases. In all positive cases, furthermore, preincubation of tissue section with recombinant CTGF abolished any CTGF immunostaining. There was a significant association between CTGF immunostaining intensity and stage of fibrosis ($\chi^2 = 9.3$, $P = .01$). Moderate or strong CTGF staining was detected in 6 out of the 22 cases (27%) with F0 or F1, and in 13 out of the 17 cases (76%) with F2, F3 or F4. No relation was observed between the presence of CTGF and the grade of activity ($\chi^2 = 2$, not significant). Moderate or strong CTGF immunostaining was detected in 9 out of the 23 cases (39%) with no or mild activity and 10 out of the 16 cases (62%) with moderate or severe activity.

Staining of activated HSC was also performed on serial sections of 38 cases of chronic hepatitis using immunohistochemistry and α-SMA antibody. In 16 cases, α-SMA staining was absent or localized in perisinusoidal cells around portal tracts or centrilobular veins, and in 22 cases, the staining was diffuse all along the sinusoidal lining. A significant association was observed between the semiquantitative assessment of α-SMA positive cells and the staining intensity of CTGF ($\chi^2 = 5.5$, $P = .05$). CTGF staining was moderate or strong in 4 out of the 16 cases (25%) displaying no or localized α-SMA immunostaining and in 14 out of the 22 cases (63%) with extensive sinusoidal α-SMA immunostaining. It clearly appeared that regional colocalization of α-SMA and CTGF occurred in perisinusoidal spaces around portal tracts or central veins (Fig. 2E, 2F), but in cases displaying diffuse α-SMA staining, CTGF was more restricted than α-SMA immunostaining.

In the group of other chronic liver diseases, CTGF immunostaining was absent in 4 cases, mild in 8 cases, and considered as present (moderate or strong) in 8 cases (40%) (Fig. 2B and 2C). The prevalence of CTGF immunostaining was not significantly different between HCV and non-HCV groups. Localization of CTGF immunostaining was similar to that observed in the chronic hepatitis C group. Again, a significant correlation was observed between the presence of CTGF and the stage of fibrosis ($\chi^2 = 7.2$, $P = .02$). None of the 7 cases with fibrosis scored F0, F1 or F2 exhibited CTGF immunostaining, whereas 8 out of the 13 cases with fibrosis scored F3 or F4 expressed CTGF.

In Situ Hybridization. To assess the cell type involved in CTGF production, 4 cases of chronic hepatitis C showing overt cirrhosis were studied by *in situ* hybridization using an antisense CTGF riboprobe. In all cases, we observed labeling of a few sinusoidal cells predominantly located in the periportal area (Fig. 3). No staining was observed in hepatocytes. When a sense probe was hybridized, no staining was detected. Determination of a specific sinusoidal cell type involved in CTGF mRNA production was impossible on the basis of these morphological data.

Connective Tissue Growth Factor Expression in Experimental Models of Liver Fibrosis

Immunohistochemical Detection of CTGF in CCl_4- and Bile Duct Ligation Induced Liver Fibrosis. To assess the location and dynamics of CTGF expression in liver fibrosis, we studied, by immunohistochemistry, the expression of CTGF in rats treated with CCl_4 at different time points.

In rats sacrificed at an early stage, centrilobular necrosis but not fibrosis was observed, and CTGF immunostaining was absent or mild. When CTGF was present, it was only detected around the centrolobular vein (Fig. 4A). In the latter stages, when fibrosis progressed, CTGF was more abundant, localized both in the centrilobular areas and in the fibrous septa. In the group of CCl_4-induced cirrhosis, 5 out of the 6 livers showed strong CTGF expression. CTGF was localized along the fibrous septa. In the overall group, a significant correlation was observed between the fibrosis stage and CTGF expression ($\chi^2 = 4.2$, $P = .04$). Moderate or strong expression of CTGF was detected in 4 out of the 18 rats (22%) without septal fibrosis and in 3 out of the 4 rats with extensive fibrosis (75%). There was no correlation between CTGF immunostaining and the necrosis grade ($\chi^2 = 0.2$, not significant). In bile duct ligated rats, CTGF was strongly expressed along septa, with a significant increase around areas of ductular proliferation (Fig. 4B).

Quantitative CTGF mRNA Expression in CCl_4- and BDL-Induced Liver Fibrosis. We used the real time RT-PCR method to evaluate RNA amount. The standard curve performed with serial dilution of a sample showed a constant slope from experiment to experiment showing a constant efficiency of the PCR. We studied the quantitative levels of CTGF mRNA in liver tissue from CCl_4-treated or BDL-ligated animals compared with appropriate controls (olive oil and sham-operated respectively). To evaluate the fibrogenic process, type I collagen mRNAs were also quantified in all liver

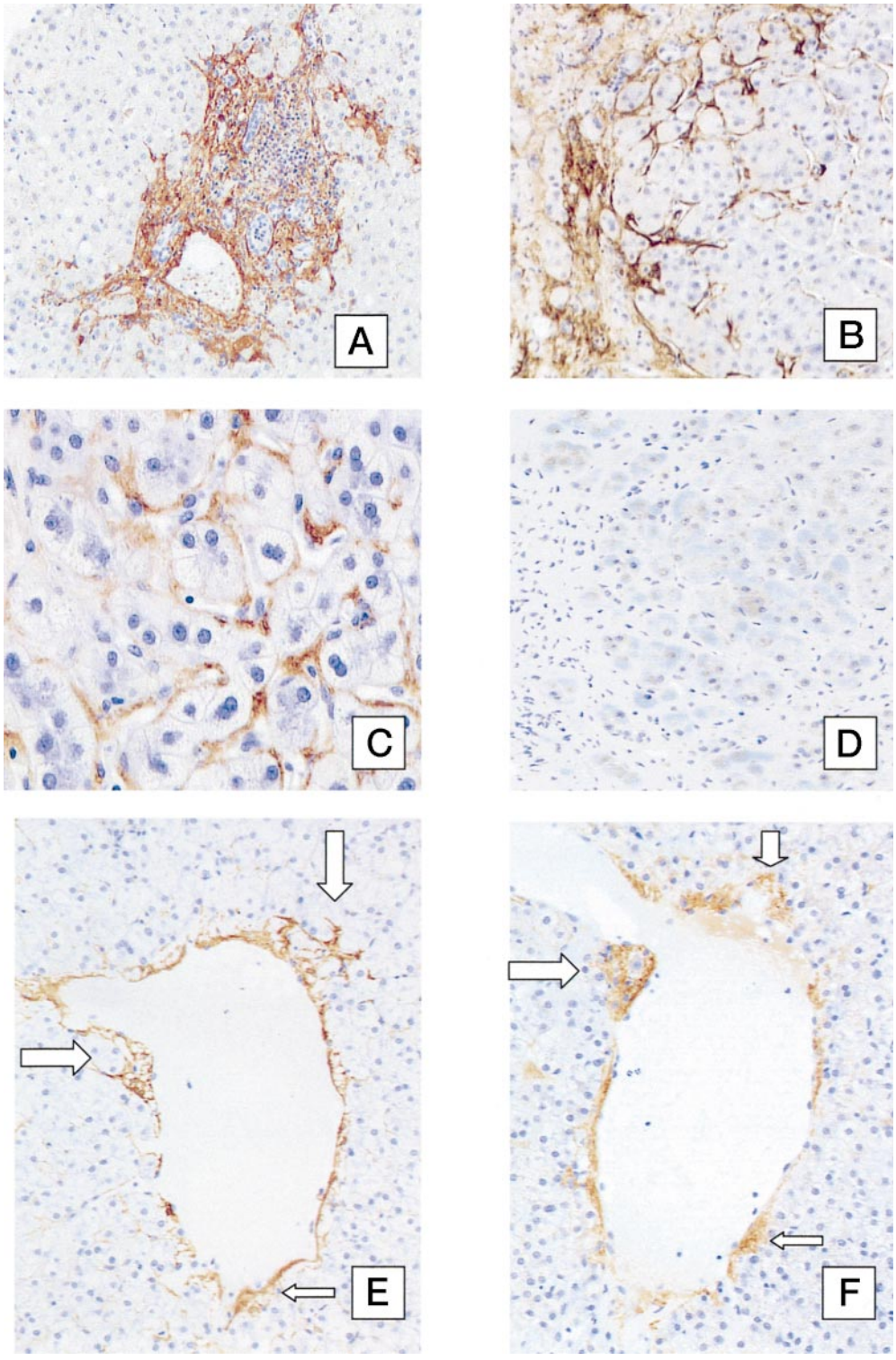

FIG. 2. Immunostaining for CTGF in human liver biopsies. (A) Chronic hepatitis C. Strong CTGF expression is detected in the extracellular matrix of the portal tract. (B) Hepatitis B cirrhosis. Immunostaining for CTGF is observed in the extracellular matrix of fibrous septa and in the sinusoidal lining at the interface between fibrous tissue and liver parenchyma (magnification × 10) (C) Hepatitis B cirrhosis. At higher magnification, CTGF expression is detected in the cytoplasm of sinusoidal cells of the Disse space. (D) Hepatitis B cirrhosis. Negative control. Serial section was incubated with purified immunoglobulin from non-immune serum instead of anti-CTGF antibody. No staining is observed. (E) Hepatitis C. Immunostaining for α-SMA showing a labeling of central vein with reinforcement in some perisinusoidal areas (*arrow*). (F) Hepatitis C, serial section with CTGF immunostaining showing a regional colocalization of the labeling. (Original magnification [A, D] ×6.3; [B, E, F] ×10; [C] ×20.)

samples. The type I collagen mRNA mean level was significantly higher in CCl_4 treated rats than in control rats (15.2 ± 4.2 versus 1.6 ± 0.9, $P < .01$). The same results were observed in BDL rats (14 ± 2.5 versus 1 ± 0.4, $P < .01$).

A dramatic increase in CTGF mRNA was also observed both in the group with CCl_4-induced fibrogenesis (42.2 ± 7 versus 15.7 ± 4, $P < .01$) and in the group with BDL-induced fibrogenesis (154.8 ± 29 versus 10.5 ± 3, $P < .001$). Results are shown in Fig. 5.

Connective Tissue Growth Factor Expression in Hepatic Stellate Cells

Since HSC is the major cell type involved in liver fibrogenesis, we studied CTGF expression in immortalized HSCs in their myofibroblast phenotype using Western blot, immunohistochemistry and *in situ* hybridization. By Western blot performed from the HSC lysate, a strong 38 kd band was observed. This band had a doublet pattern as already men-

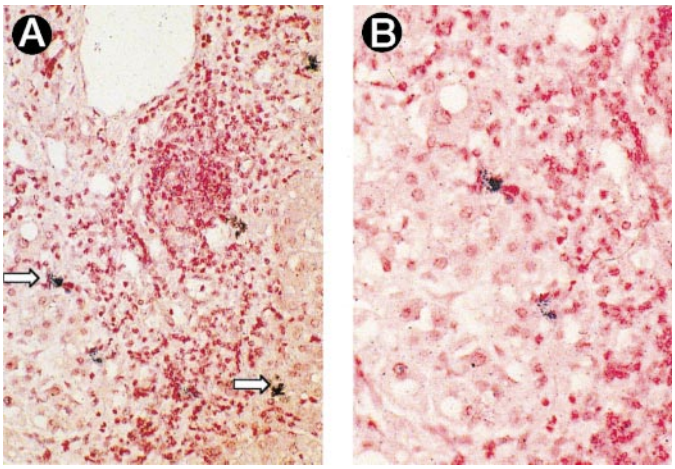

FIG. 3. *In situ* hybridization for CTGF mRNA in chronic hepatitis C. (A) CTGF mRNAs are detected in few cells in and around the portal tract. (B) At higher magnification, positive cells are detected at the interface between the portal tract and the parenchyma. No staining in hepatocytes. (Original magnification [A] ×6.3; [B] ×10.)

tioned in previous studies.[20-22] The same band was also observed in the conditioned medium, suggesting that CTGF is released by HSC in the supernatant (Fig. 6). Furthermore a 21 kd band was observed in lysate and supernatant in addition to the 38 kd CTGF doublet.

By immunohistochemistry, CTGF was detected in the cytoplasm of HSC. Staining was strong, granular and diffuse in the cytoplasm, with perinuclear reinforcement (Fig. 7A). With *in situ* hybridization, CTGF mRNA was also detected in cytoplasm of most HSC also staining was not as intense as with immunohistochemistry (Fig. 7B). No labeling was observed when the sense probe was applied to the slides (Fig. 7C).

DISCUSSION

Connective tissue growth factor is a recently isolated growth factor with platelet-derived growth factor-like biologic activities, including mitoattractant and mitogenic effects.[1] Recent studies showed that this factor *in vitro* induces an increase in extracellular matrix component gene expression by fibroblasts.[20] The role of CTGF has also been demonstrated *in vivo* in fibrotic changes in response to various injuries. Indeed, CTGF expression is up-regulated in advanced atherosclerotic lesions, scleroderma, wound healing, renal and pulmonary fibrosis.[7,9,10,23,24] Since liver fibrosis is the hallmark of all chronic liver diseases, we were

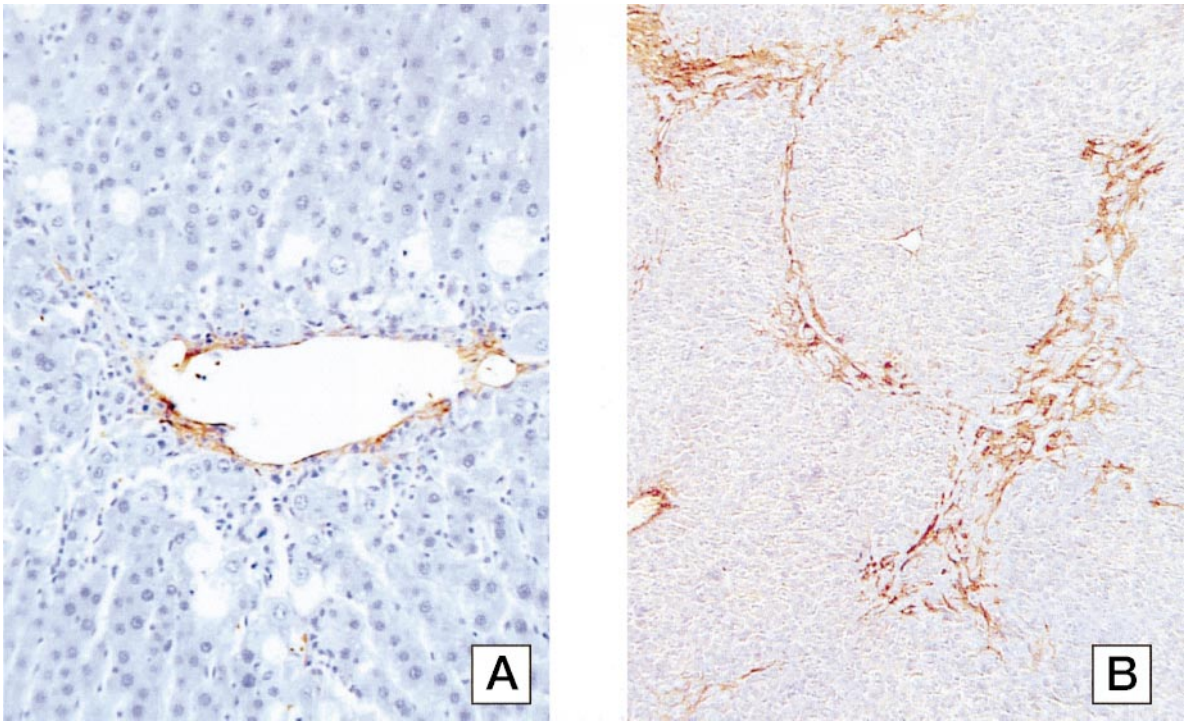

FIG. 4. Immunostaining for CTGF in CCl_4- and BDL-induced liver fibrosis. (A) In the early stage of CCl_4-induced liver fibrosis, mild staining was observed around the central vein. (B) In rat livers, 3 weeks after BDL, CTGF was detected in fibrosis septa with pronounced staining around ductular proliferation. (Original magnification [A] ×10; [B] ×4.)

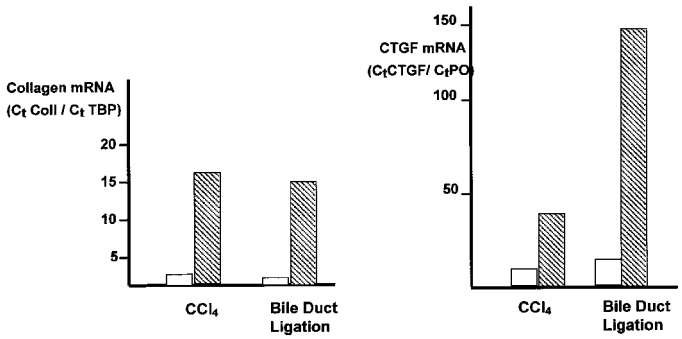

FIG. 5. Collagen and CTGF mRNA quantification in an experimental model of liver fibrosis. mRNAs were quantified using real-time RT-PCR. Results are expressed as threshold cycle (C_t) standardized by C_t for housekeeping gene. Mean values of BDL rats (n = 5) was compared with sham-operated rats (n = 4). Mean value of CCl_4-induced fibrosis in rats after 10 injections (n = 5) was compared with same dose of vehicles (n = 4).

interested in investigating the role of CTGF in liver pathology.

Our study provides evidence of the occurrence of an upregulation of CTGF during liver fibrosis and supports preliminary data.[25] Indeed, CTGF was detected at a significant level, by immunohistochemistry, in 28 out of the 59 (47%) human liver biopsies studied, and interestingly, the presence of CTGF was significantly correlated with stage of fibrosis. Although CTGF expression was more frequent and strong in chronic hepatitis C, CTGF was also detected at a significant level in biopsies of other chronic liver diseases. This strongly suggests that CTGF, like other growth factors involved in liver fibrogenesis regulation, is a ubiquitous mediator unrelated to any specific etiology. The overall involvement of CTGF is also supported by its detection and its correlation with development of fibrosis in experimental models of liver fibrogenesis. Indeed, CTGF was detected by immunohistochemistry in CCl_4-induced liver fibrosis, and a significant increase in CTGF mRNA is observed during the fibrogenic process related to CCl_4-induced liver fibrosis or the BDL procedure.

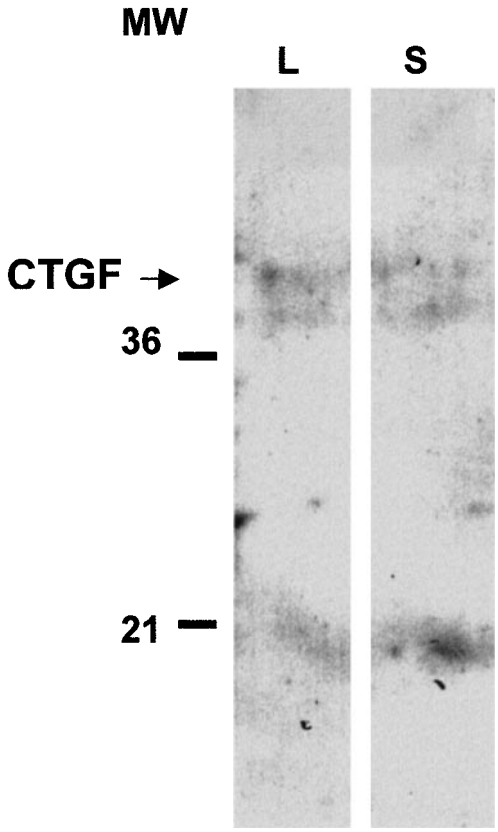

FIG. 6. CTGF expression in HSC lysate and supernatant by Western blot. Eighty micrograms of cell lysate (L) and 20 μg supernatant (S) of cultured HSC in their myofibroblastic phenotype were electrophoresed under reducing conditions on 10% acrylamide gels and transferred to nitrocellulose filters by electroblotting. The blot was incubated overnight at 4°C with the anti-CTGF antibody (1:1,000). A doublet 38-kd band was detected both in lysate and supernatant, as well as a lower-molecular-weight form around 21 kd.

Immunohistochemical detection of CTGF showed that it was predominantly located in the extracellular matrix of portal tracts and fibrous septa. The predominant extracellular localization of this molecule was expected, since CTGF was initially described as a peptide secreted by vascular endothelial cells in culture.[1] Detection of a 38 kd band in the supernatant of both HUVEC and HSC in their myofibroblastic phenotype suggests that CTGF is a secreted growth factor. This is also in accordance with molecular analysis of CTGF, since CTGF, like other members of its family, possess a secretory signal peptide at the N terminus.[1] It is noteworthy that CTGF appeared on Western-blot of HSC as a doublet that co-migrate at 38 kd. This pattern has already been observed in previous study and appears to be due to differences in glycosylation.[1,20,21] Why such a doublet pattern was not observed in HUVEC supernatant is unknown.

We also observed a lower molecular weight band (21 kd) in addition to the 38 kd CTGF bands. Previous reports have shown that these lower molecular weight forms correspond to biologically active cleaved form of 38 kd CTGF.[21,22] Once secreted in the milieu, CTGF might bind to liver extracellular matrix (ECM), as suggested by its immunohistochemical detection on ECM. Binding of CTGF fits well with the CTGF protein structure. Indeed, CTGF possesses a thrombospondin type 1 domain which is involved in binding to both soluble and matrix molecules, and in particular to sulfated glycoconjugates.[26] Thus, it is not surprising that CTGF was detected around areas of duct proliferation and on the outer part of portal tracts or fibrous septa, which represent areas of active and early fibrogenesis rich in proteoglycans. These results also fit with previous observation showing that the other members of the CTGF family such as *Fisp 12* and *nov* are secreted proteins associated with the extracellular matrix.[26,27] Extracellular CTGF bound to ECM could therefore constitute a local reservoir that can trigger competent cells. A similar scheme has been suggested for TGF-β and was recently shown for hepatocyte growth factor.[28]

Associated with labeling of the extracellular matrix, significant staining was also observed in spindle cells located either in the fibrous septa and in the lobule. Since myofibroblasts and activated HSC are the major sources of extracellular matrix components in the liver, the cellular origin of HSC was suspected. To further investigate this point, serial sections of biopsies immunolabeled with anti-CTGF were also immunostained with antibodies against α-SMA, a relevant marker of these cells in their activated phenotype. Results showed that α-SMA-positive cells were more numerous in cases displaying CTGF expression. Whether CTGF can activate HSC deserves

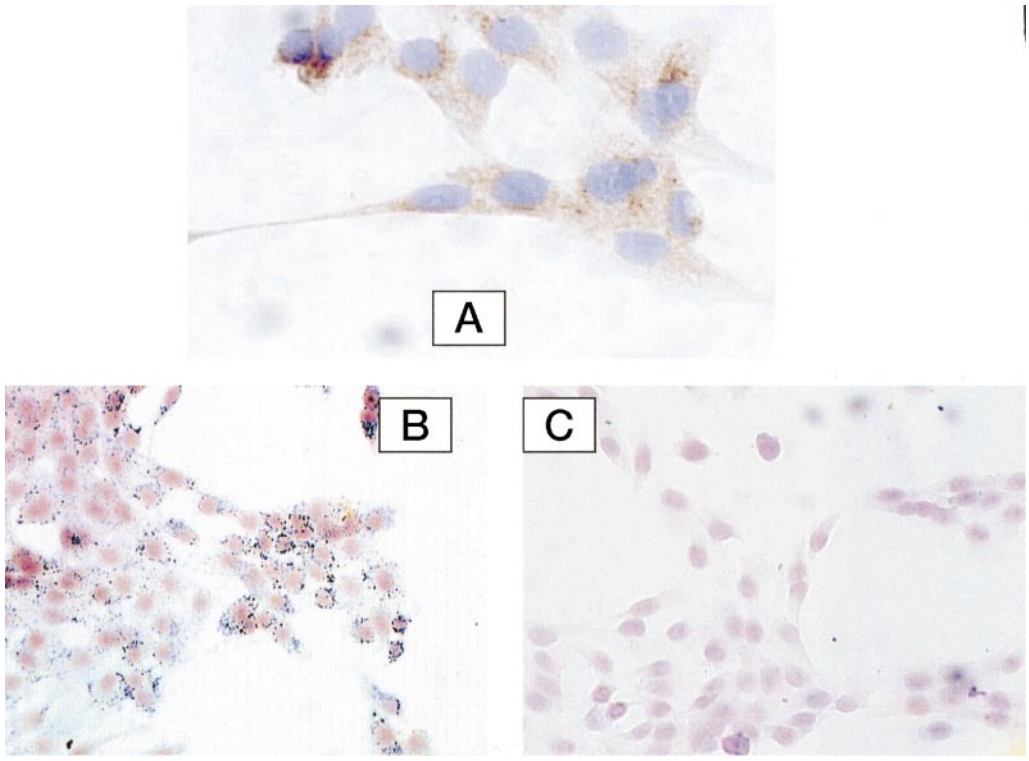

FIG. 7. Detection of CTGF in HSC in culture. (A) CTGF immunostaining in cultured HSC. CTGF is detected in the cytoplasm of the cells, with a perinuclear reinforcement. (B, C) *In situ* hybridization for CTGF mRNA in HSC in culture. (B) Cells express CTGF mRNA as shown by granular labeling of the cytoplasm of HSC. (C) No labeling was observed when the sense probe was used. (Original magnification [A] ×40; [B, C] ×20.) proliferation. (Original magnification [A] ×10; [B] ×4.)

further study. However, the close relationship between CTGF and α-SMA expression observed on serial sections also suggests that some activated HSC might produce CTGF. This is also supported by the results of *in situ* hybridization procedure on chronic hepatitis C liver biopsies showing the presence of CTGF mRNA in some fusiform cells in the fibrous septa and in sinusoidal spaces in these cases. To confirm the role of HSC, we studied CTGF production by cultured HSC in their myofibroblastic phenotype. Detection of a 38 kd doublet in lysate and supernatant of HSC by Western blot as well as results of CTGF immunostaining and *in situ* hybridization of cultured HSC are concordant, and show that HSC produce and release CTGF. CTGF production by HSC cells the phenotype of which is close to that of fibroblasts and myofibroblasts is in line with previous data. Indeed, Ito et al, by double immunostaining performed in human kidney with fibrosis, recently showed that the majority of CTGF positive cells in the tubulointerstitial area were myofibroblasts.[10] Furthermore, similar studies performed on scleroderma showed that CTGF was mainly expressed by fibroblasts.[23]

The overall regulation process of fibrogenesis is complex and requires a large number of factors, including cytokines and various growth factors. Although our results provide evidence for the involvement of CTGF in liver fibrogenesis, its role in the multifactorial model of ECM gene regulation remains to be clarified. Strong arguments suggest that TGF-β stimulate CTGF production.[6,7] Furthermore, a recent study described a TGF-β-responsive element in the CTGF promoter sequence.[8] Since TGF-β with its receptor is overexpressed by activated HSC, we hypothesize that CTGF expression might be upregulated in HSC through endogeneous TGF-β. These cells are then able to produce and secrete CTGF, which will accumulate in the surrounding ECM. Via a putative receptor present on the HSC cell membrane, CTGF could act as an autocrine stimulator to increase ECM component expression.

In conclusion, CTGF expression is induced in chronic liver diseases, suggesting a pathogenic role for this molecule in liver fibrogenesis. If further studies confirm the relative importance of CTGF in this situation, the development of selective antagonists of CTGF could specifically affect the fibrogenesis process.

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