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Establishment of the Enzyme-Linked Immunosorbent Assay for Connective Tissue Growth Factor (CTGF) and Its Detection in the Sera of Biliary Atresia

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Connective tissue growth factor (CTGF) is a mitogenic, chemotactic, and cell matrix-inducing factor for fibroblasts. We generated murine monoclonal antibodies against CTGF and established a sandwich enzyme-linked immunosorbent assay (ELISA) for detection of CTGF. By using the ELISA, we confirmed that CTGF was specifically induced in human fibroblasts by TGF-β but not by PDGF, FGF, IGF-I, or EGF. We also found that the serum levels of CTGF were significantly correlated with the progression of hepatic fibrosis in biliary atresia. These results indicated that CTGF is potentially a useful parameter for monitoring certain types of fibrotic disorders.

Fibrosis can occur in any organ and is a major medical problem ranging from dysfunction to progressive disability and death. Transforming growth factor (TGF)-β is likely to be one of the most significant cytokines for fibrotic disorders (1). TGF-β induces the synthesis of most matrix molecules, blocks the degradation of matrix by inhibiting the secretion of proteases and by inducing the production of protease inhibitors, and modulates the expression of integrin matrix receptors.

Connective tissue growth factor (CTGF) (2,3) is a member of the CCN (CTGF, Cyr61/Cef10 and Nov) family which is characterized by a modular architecture, with domains homologous to (1) insulin-like growth factor-binding proteins (IGFBPs), (2) von Willebrand Factor type C domain, (3) thrombospondin type I repeat, and (4) the C-terminal domains of some types of collagens and mucins. CTGF has been shown to be involved in cell adhesion, migration, and proliferation (2,3,4) and specifically induced by TGF-β-stimulated mesenchymal cells. It has been recently reported that both TGF-β and CTGF can induce connective tissue cell proliferation in vitro and in vivo and stimulate extracellular matrix synthesis (5). In addition, there is a TGF-β-responsive element in the CTGF promoter sequence (6). Moreover, CTGF mRNA is overexpressed in a large number of fibrotic conditions including, atherosclerosis (7), renal fibrosis (8), scleroderma (9) and keloid (10). These findings indicate that CTGF may play an important role in fibrosis as one of the downstream effectors of TGF-β.

Biliary atresia (BA) is defined as the end result of a progressive fibrotic obstruction of preexisting bile ducts. Surgical treatment consists of excision of the entire extrahepatic biliary structure up to the level of the porta hepatis to find patent biliary channels and hence restore bile drainage from the liver (Kasai portoenterostomy) (11). Progressive hepatic fibrosis, in spite of a successful Kasai portoenterostomy, is a major problem in patients with BA. Thus, determination of the prognosis for patients with progressive fibrosis after a Kasai procedure has important clinical implications. The ability to reliably prognosticate would be invaluable when planning management of BA.

In this study, using a sensitive assay for CTGF, we confirmed that CTGF was specifically induced by TGF-β from human fibroblasts. Screening the sera from patients with BA indicated that the serum levels of CTGF were significantly correlated with the progression of hepatic fibrosis.

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Abbreviations used: CTGF, connective tissue growth factor; ELISA, enzyme-linked immunosorbent assay; TGF, trnsforming growth factor; PDGF, platelet-derived growth factor; FGF, fibroblast growth factor; IGF, insulin-like growth factor; EGF, epidermal growth factor; BA, biliary atresia; mAb, monoclonal antibody; HCS, human chondrosarcoma-derived chondrocyte.

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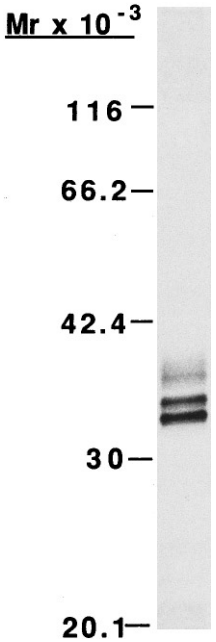

FIG. 1. SDS-PAGE analysis of immunoaffinity-purified CTGF. Recombinant human CTGF was purified by MHCT2-conjugated affinity chromatography, electrophoresed on 10-20% gradient polyacrylamide gels, and detected by silver staining.

MATERIALS AND METHODS

Preparation of recombinant CTGF. For construction of a CTGF-expression vector, a DNA fragment of CTGF that contained the whole coding region of CTGF cDNA (12) was cloned into pcDNA3.1(-) vector and CTGF was expressed under the control of the CMV promoter. This expression vector was introduced into HeLa cells by electroporation using a Gene Pulser (Bio-Rad Lab, Hercules, CA). The stable transfectants were maintained in ASF104 serum-free medium (Ajinomoto, Tokyo, Japan). The conditioned medium was loaded onto a heparin affinity chromatography (HiTrap Heparin column; Pharmacia Biotech, Uppsala, Sweden). After washing with PBS containing 0.2 M NaCl, bound proteins were eluted with PBS containing 0.5 M NaCl. The fractions were dialyzed with PBS. The partially purified rCTGF was used as an immunogen. rCTGF was subsequently purified from the heparin column products by anti-CTGF MHCT2 mAb affinity chromatography. Its purity was analyzed by SDS-PAGE and silver staining (Fig. 1). The banding pattern was fully compatible with observation obtained with native human CTGF (5).

Hybridoma production. Female BALB/c mice (Shizuoka Laboratory Animal Center, Shizuoka, Japan) were immunized every 7 days with foot pad (f.p.) injection of partially purified rCTGF. Complete Freund's adjuvant was included in the first inoculum. The booster injection was made f.p. 2 days before the fusion. Popliteal LN cells were fused with mouse myeloma cells using PEG 1500 (Boehringer Mannheim, Mannheim, Germany) and hybridomas were screened by their ability to react with human CTGF in ELISA.

Sandwich ELISA. To detect human CTGF, a sandwich ELISA with two different anti-human CTGF mAbs (MHCT1 and MHCT2; mouse IgG1) was developed. ELISA plates (96-well, Corning) were coated with 50 μl of MHCT1 mAb or isotype-matched control mAb (WT.3; anti-rat CD18) (13) at 10 μg/ml in PBS for 1 h at room temperature (RT). After washing, unbound sites were blocked by incubation with 200 μl of blocking reagent (Block Ace; Dainippon Pharm. Co., Osaka, Japan) for 2 h at RT. After washing three times with 0.1% Tween 20 in PBS (PBS-T), 50 μl of samples were added to the wells and incubated for 1 h. After washing three times, 50 μl of biotinylated MHCT2 mAb at 2 μg/ml in 1% BSA-PBS-T was added. The plates were

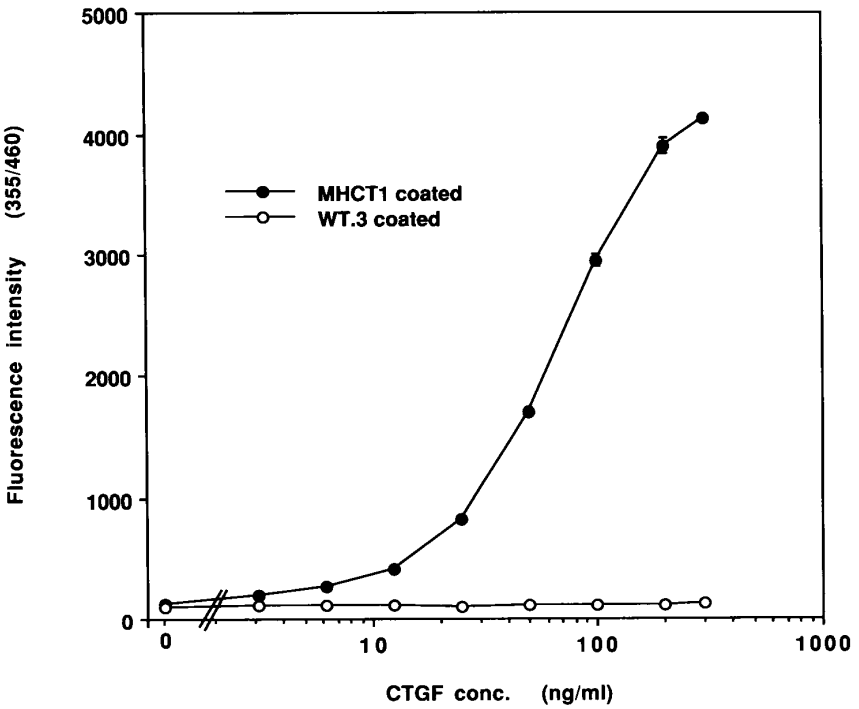

FIG. 2. Detection of human CTGF by a sandwich ELISA system. Serial dilutions of human CTGF were added to WT.3 or MHCT1-coated wells. Captured Ag were stained with biotinylated MHCT2, streptavidin-β-D-galactosidase, and 4-methyl-umbelliferyl-β-D-galactoside, and the fluorescence intensity of the wells was determined.

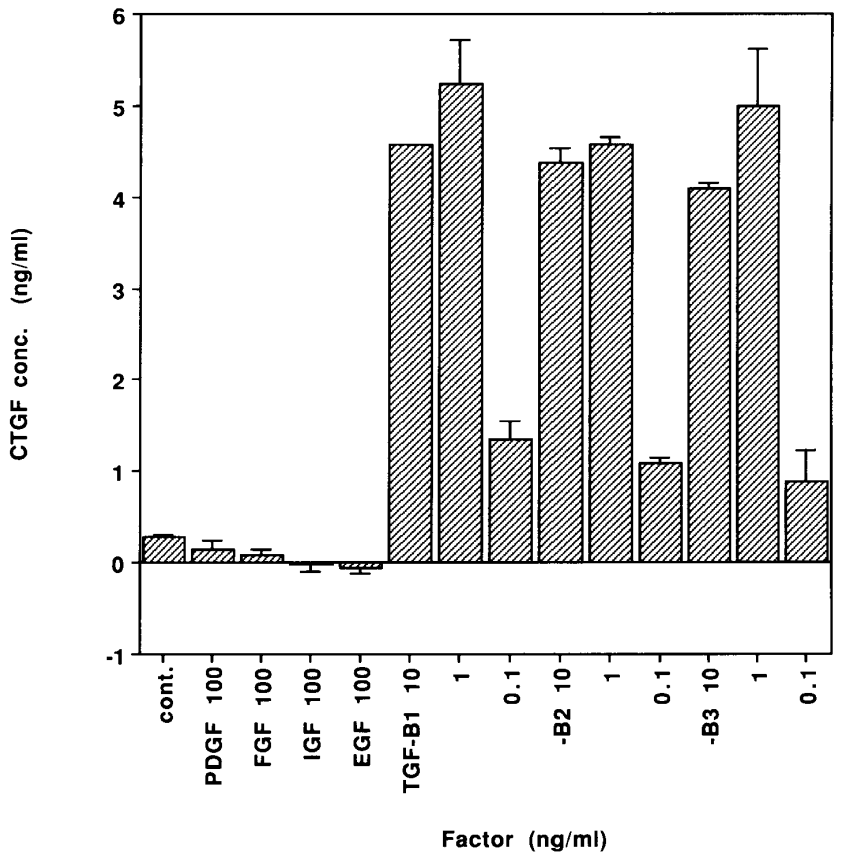

FIG. 3. TGF-β-induced CTGF production in human fibroblasts. Human dermal fibroblasts were incubated for 24 h in the absence or presence of PDGF, EGF, IGF-I, FGF, TGF-β1, TGF-β2 or TGF-β3. Each cell culture supernatant was tested for CTGF by the sandwich ELISA system. Values represent the mean concentration of CTGF ± SD obtained in triplicate determinations for each sample.

incubated for 1-h at RT and subsequently washed three times, and 50 μl of a 1/1000 dilution of streptavidin-β-D-galactosidase (Gibco BRL, Gaithersburg, MD) were added. After a 1 h incubation, the plates were washed three times, 50 μl of 1% 4-methyl-umbelliferyl-β-D galactoside (Sigma) was added, and the fluorescence intensity of the wells was determined after 10 min at 460 nm (excitation; 355 nm) by a Fluoroskan II microplate fluorometer (Lab Systems, Hampshire, UK).

Measurement of fibroblast CTGF production. Neonatal human dermal fibroblasts were cultured in triplicate in flat-bottom 96-well plates (Becton Dickinson, Oxnard, CA) in DMEM supplemented with penicillin, streptomycin, and 10% FCS in 5% CO2 at 37 °C with or without recombinant human PDGF-BB, EGF, IGF-I, FGF, TGF-β1, TGF-β2, or TGF-β3 (R & D Systems, Inc., Minneapolis, MN). After 24 h, the supernatants were harvested. The concentration of CTGF in the supernatants was measured by a sandwich ELISA.

Patients. Thirty-six patients with biliary atresia after a successful Kasai procedure and 10 healthy volunteers were investigated after obtaining their parents' informed consent to participate in this study. Patients were divided into three groups for this study according to their outcome based on four liver function tests (14): total bilirubin (T-Bil), glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), and γ-glutamyl transpeptidase (γGTP). Serum T-Bil, GOT, GPT, and γGTP activity were measured by routine methods. Group I (n = 17), were jaundice free, with normal liver function (T-Bil, = 12) had moderate liver dysfunction (T-Bil, 40 IU/L; GPT, 35 IU/L; γGTP, 55 IU/L). Group III (n = 7), the "unfavorable prognosis group," had severe liver dysfunction (T-Bil, 1.5 mg/dL: GOT, 40 IU/L; GPT, 35 IU/L; γGTP, 55 IU/L).

RESULTS

Generation of anti-CTGF mAb. We have previously cloned human CTGF from a human chondrocyte cell line (HCS-2/8) (15) by a differential cloning strategy (12). Recombinant CTGF was expressed in stable transfectants and partially purified from the conditioned medium by heparin affinity chromatography. BALB/c mice were immunized with the recombinant CTGF, and the hybridomas were generated. Hybridoma supernatants were then screened for their ability to specifically react with human CTGF in ELISA. Two of the hybridomas were subsequently cloned and designated as MHCT1 and MHCT2 (mouse IgG1), respectively. Human CTGF could be purified by MHCT2 mAb affinity chromatography (Fig. 1).

Detection of CTGF by sandwich ELISA. The anti-CTGF mAbs were used to establish an enzyme-linked immunosorbent assay (ELISA) for CTGF. The sandwich ELISA successfully detected human CTGF in a dose-dependent manner (Fig. 2) but not PDGF, FGF, EGF, IGF-I, TGF-β1, -β2, or -β3 (100 ng/ml; data not shown). When an isotype-matched control mAb was used as a primary antibody in the ELISA system, no

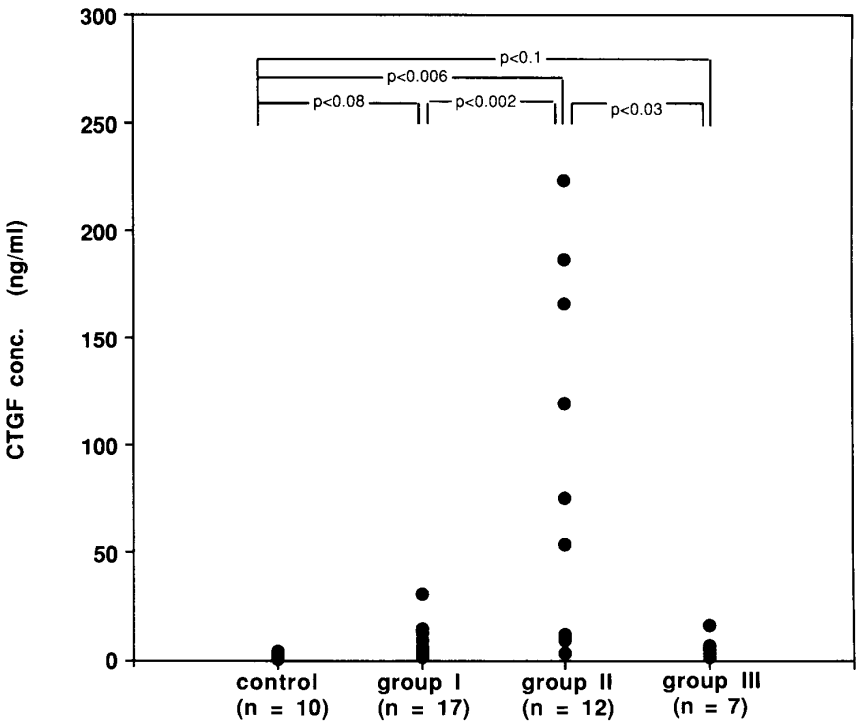

FIG. 4. The serum levels of CTGF in patients with BA. Serum from healthy volunteers or patients with various prognoses of biliary atresia (BA) were assayed by ELISA for CTGF. Concentrations of CTGF were determined by comparison with the standard curve in Fig. 2.

signal was obtained, demonstrating the specificity of this assay. The standard curve using the affinity-purified human CTGF indicated that the sensitivity of this assay was about 25 pg (0.5 ng/ml).

First we used this ELISA system to examine whether CTGF was induced by stimulation with TGF-βs. We found that detectable levels of CTGF were present in culture supernatants from TGF-β1, -β2, or -β3-stimulated human dermal fibroblasts, but not in supernatants from untreated and PDGF-, EGF-, IGF-I-, or FGF-stimulated fibroblasts (Fig. 3). These results indicate that CTGF was specifically induced by TGF-β from human fibroblasts.

CTGF in human sera. The serum CTGF levels of healthy volunteers and patients with various prognoses of biliary atresia (BA) were quantified in a blind manner (Fig. 4). The BA patients were divided into three groups according to liver function (14). There was evidence of severe cirrhosis in group III and progressive cirrhosis in group II. None of the sera from the healthy volunteers contained detectable levels of CTGF (less than 0.5 ng/ml). In patients with normal liver function (group I), CTGF serum levels (4.31 ng/ml; range: $<$0.5-27.95 ng/ml) were slightly but significantly increased as compared with normal controls. On the other hand, the serum levels of CTGF in group II (moderate liver dysfunction) were strongly increased (82.94 ng/ml; range: 0.66-220.90 ng/ml). Interestingly, the serum levels in group III (severe liver dysfunction) (2.68 ng/ml; range: $<$0.5-13.59 ng/ml) were not different from those in group I. These results indicated that the serum CTGF levels of CTGF were significantly correlated with the progression of hepatic fibrosis.

DISCUSSION

It is a widely accepted paradigm that TGF-β up-regulation is an important factor responsible for various organ fibroses (1). In experimental glomerulonephritis, antibodies to TGF-β (16), antisense oligonucleotides against TGF-β (17) and TGF-β binding protein (decorin) (18), have effectively reduced the fibrosis. Glomerulosclerosis (19) and interstitial pneumonia (20) were induced by in vivo transfection of TGF-β. In TGF-β transgenic mice (21), hepatic fibrosis and glomerulosclerosis have been induced. However, TGF-β is a multi-functional factor with many other effects that are not directly related to fibrosis, including immunosuppressive and anti-inflammatory activities. Blocking of TGF-β activity may therefore have conflicting effects. A downstream effector of TGF-β that is directly involved in the proliferation of connective tissue cells and the accumulation of matrix may provide a better target for fibrotic diseases.

In this study, we showed that CTGF peptides were specifically induced by TGF-β but not by PDGF, FGF, IGF-I or EGF. In addition, we have also reported that only TGF-β and its related protein, bone morphological protein-2, increased the mRNA level of CTGF in hu-

man chondrocytic cells (12). In an in vivo model for wound healing in the rat, TGF-β and CTGF mRNAs have been shown to be coordinately overexpressed during wound repair (22). It has been reported that both TGF-β and CTGF increased the amount of transcripts encoding α1 type I collagen, α5 integrin, and fibronectin in vitro, and also induced connective tissue cell proliferation in vivo (5). Inhibition of CTGF synthesis or action could block TGF-β-stimulated anchorage-independent growth of NRK fibroblasts (23). In addition, there is a TGF-β-responsive element in the CTGF promoter sequence (6). All these results indicate that CTGF may play an important role in fibrosis as one of the downstream effectors of TGF-β.

In patients with biliary atresia (BA), progressive hepatic fibrosis, in spite of a successful Kasai portoenterostomy (11), is a major problem. Determination of the prognosis for patients with BA has important clinical implications. Therefore, simple, sensitive, and non-invasive procedures are needed to assess the prognosis of BA patients. In the present study, we showed that serum levels of CTGF in the patients were significantly correlated with the progressiveness of hepatic fibrosis in BA. This is the first evidence that serum levels of CTGF are upregulated in fibrosis. These results indicate that serum levels of CTGF may be useful in the long term follow-up of BA patients after Kasai's portoenterostomy. It has been reported that CTGF mRNA is overexpressed in a large number of fibrotic conditions (7,8,9,10), demonstrating that CTGF may be potentially a useful parameter for monitoring certain types of fibrotic disorders.

We conclude that CTGF must be an important parameter for fibrotic events and also an attractive clinical target for anti-fibrosis treatment.

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