Published in final edited form as:

Hepatology. 2015 February ; 61(2): 678–691. doi:10.1002/hep.27425.

Connective Tissue Growth Factor and Integrin $\alpha\text{v}\beta 6$: a New Pair of Regulators Critical for Ductular Reaction and Biliary Fibrosis

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Abstract

Connective tissue growth factor (CTGF) is a matricellular protein that mediates cell-matrix interaction through various subtypes of integrin receptors. This study investigated the role of CTGF and integrin $\alpha\text{v}\beta 6$ in hepatic progenitor/oval cell activation, which often occurs in the form of ductular reactions (DRs) when hepatocyte proliferation is inhibited during severe liver injury. CTGF and integrin $\alpha\text{v}\beta 6$ proteins were highly expressed in DRs of human cirrhotic livers and cholangiocarcinoma. Confocal microscopy analysis of livers from *Ctgf* promoter driven GFP reporter mice suggested that oval cells and cholangiocytes were the main sources of CTGF and integrin $\alpha\text{v}\beta 6$ during liver injury induced by 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC). Deletion of exon 4 of the *Ctgf* gene using tamoxifen inducible Cre-loxP system down-regulated integrin $\alpha\text{v}\beta 6$ in DDC damaged livers of knockout mice. *Ctgf* deficiency or inhibition of integrin $\alpha\text{v}\beta 6$ by administering the neutralizing antibody 6.3G9 (10 mg/kg body weight) caused low levels of *EpCAM* and *CK19* mRNAs. Also, there were smaller oval cell areas, fewer proliferating ductular epithelial cells, and lower cholestasis serum markers within two weeks after DDC treatment. Associated fibrosis was attenuated as indicated by reduced expression of fibrosis-related genes, smaller areas of α smooth muscle actin staining and low collagen production based on hydroxyproline content and the Sirius red staining. Finally, integrin $\alpha\text{v}\beta 6$ could bind to CTGF mediating oval cell adhesion to CTGF and fibronectin substrata and promoting transforming growth factor (TGF)- $\beta 1$ activation *in vitro*.

Conclusions: CTGF and integrin $\alpha\text{v}\beta 6$ regulate oval cell activation and fibrosis, probably through interacting with their common matrix and signal partners, fibronectin and TGF- $\beta 1$. CTGF

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Disclosures: PHW and SMV are employees and shareholders of Biogen Idec, Inc. The other authors have nothing to disclose.

and integrin $\alpha v \beta 6$ are potential therapeutic targets to control DRs and fibrosis in related liver disease.

Keywords

CTGF; integrin $\alpha v \beta 6$; ductular reaction; liver fibrosis; hepatic progenitor/oval cells

INTRODUCTION

The liver contains a small subset of hepatic progenitor cells (HPCs, also termed oval cells in rodents) that reside within canals of Hering at terminal branches of the biliary tree and become activated in periportal niches to mediate the regenerative response when hepatocyte proliferation is inhibited during severe liver injury.¹ Expansion of the progenitor compartment, which is known as ductular reactions (DRs), involves a heterogeneous population of transient amplifying cells capable of differentiation into biliary and hepatocytic lineages.^{2,3} This reactive process occurs not only in biliary disorders such as cholangiocarcinoma (CC) but also in many acute and chronic liver diseases resulting from insults such as alcohol toxicity and submassive parenchymal necrosis. Despite involvement of central lobular injury in many of these diseases, DRs are often accompanied with stellate cell and myofibroblast activation, as well as excessive deposition of extracellular matrix (ECM) around the portal areas, suggesting a direct correlation between DRs and periportal fibrosis.⁴ Liver cirrhosis arises from severe liver fibrosis, eventually leading to portal hypertension and end-stage liver disease.

Several signaling pathways have been shown to regulate DRs and associated fibrosis. Ligands such as Jagged 1, Wnt, and tumor necrosis factor-like weak inducer of apoptosis (TWEAK) can act through their receptors promoting HPC activation, differentiation, and fibrogenesis.^{1,5,6} Transforming growth factor (TGF)- β is a potent mitogen for fibroblasts that promotes ECM formation and expression of other pro-fibrogenic mediators. TGF- β inhibits hepatocytes proliferation whereas HPCs remain proliferative in presence of TGF- β protein due to glycosylation of the TGF β RII.⁵ Connective tissue growth factor (CTGF) of the CCN (Cyr61/CTGF/Nov) protein family, also termed CCN2, is transcriptionally activated by TGF- β in various types of fibrotic disorders.⁷ Hepatocyte-specific overexpression of CTGF in transgenic mice renders livers susceptible to actions of other fibrotic stimuli.⁸ CTGF contains four evolutionally conserved domains capable of modulating external signal transduction into cells through interaction with ECM proteins like fibronectin, cell surface integrins, and growth factors including TGF- β and other members of the cysteine knot superfamily.⁹⁻¹² Our previous studies demonstrated that CTGF is induced during oval cell mediated liver regeneration. Administration of iloprost, a prostacyclin analogue that reduces CTGF production and impairs rat oval cell response.¹³ Furthermore, CTGF binds to integrin $\alpha 5 \beta 1$ and fibronectin promoting migration and adhesion of both primary rat oval cells and mouse stellate cells.^{9,10}

In this study, we focused on integrin $\alpha v \beta 6$ that can bind to fibronectin and the latency associated peptide (LAP) leading to activation of TGF- β .^{14,15} The integrin $\beta 6$ subunit only combines with the ubiquitously expressed αv chain and forms an epithelial specific integrin

$\alpha\text{v}\beta 6$ that is induced during wound healing and cancer progression. Neutralization of integrin $\alpha\text{v}\beta 6$ activity has been shown to decrease tumor growth and fibrosis in multiple tissues.¹⁶⁻¹⁹ Here, we demonstrated a close functional association between integrin $\alpha\text{v}\beta 6$ and CTGF during HPC activation after liver injury. CTGF and integrin $\alpha\text{v}\beta 6$ were not only highly expressed in DRs of human cirrhotic livers and CCs but also co-induced during mouse oval cell activation and biliary fibrosis induced by 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC). Deletion of *Ctgf* gene in conditional knockout mice or administration of neutralizing antibody 6.3G9, which has been reported to effectively block integrin $\alpha\text{v}\beta 6$ action,²⁰ affected oval cell response and associated fibrosis.

MATERIALS AND METHODS

Human Tissue and Animal Experimentation

Human liver tissues were obtained at Shands Hospital according to approved protocol by the institutional review board at the University of Florida. Written informed consents were obtained from all subjects. Tumor or adjacent non-tumor parts of CC containing livers were separated after dissection and snap-frozen before RNA and protein analyses. For animal experimentation, all protocols and procedures were approved by University of Florida IACUC and were in accordance with National Institutes of Health guidelines. Transgenic mice carrying *Ctgf* promoter driven enhanced GFP (*Ctgfp-GFP*), mice carried two loxP sites that flanked exon 4 of *Ctgf* gene (*Ctgf^{fl/f}*), and *Ctgf* conditional knockouts (*Ctgf^{fl/k}*), were described previously.^{11,21,22} Detailed animal experimental designs including integrin $\alpha\text{v}\beta 6$ neutralization using mouse monoclonal integrin $\alpha\text{v}\beta 6$ -neutralizing antibody 6.3G9 or isotype control immunoglobulin (Ig) G (clone 1E6, Biogen Idec) are in Supplemental Materials and Methods.

Histology, Immunostaining, Morphometry, Hepatic Hydroxyproline and Serum Marker Measurement

Human cirrhotic liver sections were from BioChain (Newark, CA) and human CC tissue arrays were from US Biomax, INC (Rockville, MD). Antibodies used for staining are listed in Supporting Table 1. Procedures for histology, morphometry, hepatic hydroxyproline and serum marker measurement are in Supplemental Materials and Methods.

RNA Isolation and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis

Total RNAs were extracted using RNeasy Mini kit (Qiagen, Valencia, CA). RT-PCR analysis is described in Supplemental Materials and Methods.

Hepatic Cell Isolation and the Immunofluorescent Staining for Co-localization of CTGF and Integrin $\alpha\text{v}\beta 6$

Isolation of primary hepatic cells, generation of WB-F344 stable cell lines, and dual staining for CTGF and integrin $\alpha\text{v}\beta 6$ are described in Supplemental Materials and Methods.

Western Analysis and Immunoprecipitation Assays

Total protein lysates were extracted from human samples, mouse livers or cultured cells using cold RIPA buffer containing proteinase inhibitors. Western analysis and Immunoprecipitation assays are described in Supplemental Materials and Methods.

Cell Adhesion Assay and TGF- β 1 Bioassay

Cell adhesion was assessed as described previously.⁹ Procedures for cell adhesion assay, TGF- β 1 bioassay, and TMLC cells that were stably transfected with a plasmid containing a luciferase cDNA downstream of a sensitive TGF- β portion of plasminogen activator and inhibitor 1 promoter are in Supplemental Materials and Methods.

Statistical Analysis

Microsoft Excel software (Microsoft Corp, Redmond, WA) was used for statistical analysis. Statistical significance ($P < 0.05$) was evaluated using the Student *t*-test and one-way analysis of variance (ANOVA).

RESULTS

CTGF and Integrin α v β 6 are Highly Expressed in DRs of Cirrhotic Human Livers and CC

To test whether CTGF and integrin α v β 6 are involved in DRs, we first histologically analyzed human cirrhotic livers that expressed abundant integrin α v β 6 protein and had extensive collagen deposition (Fig. 1A and insert, Supporting Figure 1A,B). CTGF was found in reactive ducts at the interface of portal and parenchymal compartments expressing biliary marker cytokeratin (CK) 19 in human cirrhotic livers by immunofluorescent analysis (Fig. 1B and Supporting Figure 1C). We also analyzed human tissue microarrays that contained 24 specimens of CCs by histology because integrin α v β 6 has been shown to be a highly specific marker for this type of malignant liver tumor.²³ As shown in Fig. 1C and Supporting Figure 2, co-expression of both integrin α v β 6 and CTGF was found in CC samples that contained reactive ducts ranging from fully differentiated to poorly differentiated ductular epithelial cells in fibrous septa. RT-PCR and Western analyses detected specific expression of integrin β 6 mRNA and protein in CC tumors, whereas CTGF mRNA and protein were induced ubiquitously in both non-tumor and tumor areas (Fig. 1D,E). Immunoreactive bands corresponding to CTGF protein from CC homogenates (~42 kDa) migrated faster than those from non-tumor parts of the CC patients (~50 kDa) in the Western analysis, indicating differential post-translational modification of CTGF protein among CC tumors and adjacent non-tumor tissues. Immunofluorescent staining detected distribution of CTGF protein in subsets of myofibroblast cells that expressed α smooth muscle actin (α SMA) and were closely associated with reactive ducts (Supporting Figure 3). In contrast, abundant CTGF protein was predominantly localized in CK19⁺ reactive ducts of CC tumors (Fig. 2), supporting the notion that CTGF is co-expressed together with integrin α v β 6 in HPCs and cholangiocytes during DRs in cirrhotic livers and CCs.

Expression Pattern of CTGF and Integrin α v β 6 in Normal and Injured Mouse Livers

To determine the cellular sources of *Ctgf* gene, we utilized *Ctgfp-GFP* mice and introduced two kinds of liver injury. One injury activated oval cells around periportal regions through feeding mice a porphyrinogenic agent, DDC, which has been known to trigger oval cell response as early as day 3 and oval cell peak around three to four weeks after treatment.²⁴ The second liver injury was through CCl₄ intoxication that mainly caused hepatocyte damage and liver fibrosis characterized by myofibroblast activation. Sustained co-induction of *Ctgf* and *integrin β 6* mRNAs were observed from days 7 to 33 after DDC treatment in comparison to untreated livers (Fig. 3A). In contrast, there was a five-fold increase of *Ctgf* mRNA and little *integrin β 6* up-regulation in damaged livers caused by CCl₄ intoxication for 4-6 weeks. Thus, co-induction of *Ctgf* and *integrin β 6* mRNAs mainly occurred in the DDC mouse model involving oval cell activation.

Confocal microscopy was performed to determine *Ctgf* promoter activity indicated by GFP fluorescence in normal and injured *Ctgfp-GFP* livers. Constitutive *Ctgf* promoter activity was found in vascular cells lining α SMA⁺ walls of blood vessels around central veins and portal areas in normal and injured livers even after DDC treatment or chronic administration of CCl₄ (Supporting Figure 4). Some subsets of small ducts expressing oval cell marker A6 antigen had clear GFP signal in normal livers (Fig. 3B), although integrin α v β 6 staining was very faint in normal condition (Data not shown). DDC treatment gave rise to strong GFP signal, together with positive integrin α v β 6 staining, in Ki67⁺ atypical ductal (oval) cells and proliferating cholangiocytes that expressed A6 or another oval cell marker, Epithelial cell adhesion molecule (EpCAM) antigen, around periportal regions (Fig. 3C-E, Supporting Figure 5,6). This contrasted the basal level of *Ctgf* promoter activity in EpCAM⁺ cholangiocytes after 6-week CCl₄ administration (Supporting Figure 5). Unexpectedly, α SMA⁺ or desmin⁺ myofibroblast cells had weak GFP signal in reporter livers following DDC or CCl₄ treatment (Supporting Figure 7 and 8). F4/80⁺ macrophages/Kupffer cells were co-localized with GFP signal nearby fibrous cords around central veins after CCl₄ intoxication (Fig. 4A). However, *Ctgf* induction in F4/80⁺ macrophages/Kupffer cells was not apparent in DDC damaged livers at day 14 after treatment (Supporting Figure 7), indicating different roles of *Ctgf* and macrophages/Kupffer cells during DRs/biliary fibrosis and central lobular fibrosis. High levels of *Ctgf* gene in hepatic oval cells during DDC-induced liver injury and F4/80⁺ macrophages/Kupffer cells in response to CCl₄ intoxication were confirmed by semi-quantitative RT-PCR analysis from purified cells (Fig. 4B,C).

Integrin α v β 6 is Down-regulated in *Ctgf* Conditional Knockout Mice During DDC Induced Liver Injury

To determine the function of CTGF *in vivo*, we developed a new mouse line carrying *Ctgf^{fl/fl}* and *ubc-Cre/ERT2* transgenes to systemically conditionally knockout the *Ctgf* gene in adult tissues.²¹ In this system, the ERT2 fusion protein contained ligand-binding domain of a mutated estrogen receptor and brought fused Cre recombinase into the nucleus where floxed exon 4 in *Ctgf^{fl}* locus was excised in response to tamoxifen as confirmed in PCR based genotyping (Fig. 5A). We designated the resulting conditional knockout mice as *Ctgf^{k/k}*. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) showed that the full-length *Ctgf* mRNA containing exon 4 was barely detectable in *Ctgf^{k/k}* livers even after

DDC feeding (Fig. 5B). Western analysis also confirmed the loss of the full-length CTGF protein (~40 kDa) in hepatic extracts of *Ctgf^{fl/fl}* mice despite its sustained induction in DDC damaged *Ctgf^{fl/fl}* livers (Fig. 5C). This is consistent with a previous report that Cre mediated excision in exon 4 of *Ctgf* gene caused early frameshift, translation termination, and quantitative loss of CTGF protein in conditional knockout mouse line carrying tamoxifen inducible Cre under control of fibroblast/smooth muscle cell-specific regulatory sequence from the *pro α 2(I) collagen* gene.²²

Integrin α v β 6 expression was examined in the *Ctgf* conditional knockout mice during DDC induced liver injury. There was an approximately two-fold reduction of *integrin β 6* mRNA in *Ctgf* deficient livers in comparison to control livers at days 7 and 14 after DDC treatment (Fig. 5B,C). Immunohistochemical and quantitative analyses of stained areas indicated 69% and 26% reduction of integrin α v β 6 immunoreactive areas around periportal regions in *Ctgf^{fl/fl}* compared to *Ctgf^{fl/fl}* mice. In contrast, there were comparable low levels of integrin α v β 6 protein in untreated livers from both strains of mice (Fig. 5D). Therefore, deletion of exon 4 in *Ctgf* gene was associated with down-regulation of integrin α v β 6 expression in conditional knockout mice during DDC induced injury.

Ctgf Deficiency or Neutralization of Integrin α v β 6 Activity Decreases Fibrotic Response During DDC Induced Liver Injury

Since integrin α v β 6 is an activator of TGF- β 1 and is involved in biliary fibrosis,¹⁴⁻¹⁹ we suspected that down-regulation of integrin β 6 caused a decrease in fibrosis of *Ctgf^{fl/fl}* mice during DDC induced liver injury. We measured transcriptional levels of other fibrosis-related genes in total liver extracts using qRT-PCR analysis. Down-regulation of *TGF- β 1*, *TGF- β 2*, *α SMA*, and *Procollagen α 1(I)* mRNAs were found in *Ctgf^{fl/fl}* livers compared to *Ctgf^{fl/fl}* livers at days 7 and 14 after DDC treatment (Fig. 6A). Measurement of active and total TGF- β 1 by the enzyme-linked immunosorbent assay (ELISA) showed that relative amounts of active TGF- β 1 protein in *Ctgf^{fl/fl}* liver homogenates were only 67% and 78% of *Ctgf^{fl/fl}* controls at days 7 and 14 after DDC treatment. These data indicate that a lack of *Ctgf* gene is associated with decreased activation of TGF- β 1 in DDC damaged *Ctgf^{fl/fl}* livers.

Smaller α SMA⁺ areas and fewer Sirius red-stained collagen fibrils were found in *Ctgf^{fl/fl}* livers than in *Ctgf^{fl/fl}* livers using histological analysis (Fig. 6D). Quantification of these areas showed 36% decrease of α SMA⁺ areas and 47% reduction of Sirius red-stained collagen fibrils in *Ctgf^{fl/fl}* livers compared to *Ctgf^{fl/fl}* livers (Fig. 6E). Biochemical collagen assessment by way of hydroxyproline assay detected ~30% reduction of hepatic collagen in *Ctgf^{fl/fl}* livers compared to *Ctgf^{fl/fl}* livers at day 14 after DDC treatment, even though the two types of livers from untreated animals had comparable levels of collagen (Fig. 6E, right panel). These results indicate that deletion of the *Ctgf* gene reduces fibrotic response during DDC induced liver injury.

To further investigate the role of integrin α v β 6 in biliary fibrosis induced by DDC, we took advantage of the 6.3G9 antibody shown to effectively inhibit integrin α v β 6 activity in experimental models.¹⁶⁻¹⁹ Administering this function-blocking antibody at high dose (10 mg/kg body weight) was associated with down-regulation of *TGF- β 1*, *TGF- β 2*, *α SMA* and *Procollagen α 1(I)* mRNAs as compared to the treatment with the same amount of control

IgG (clone 1E6) at 14 days after DDC treatment (Fig. 6C). Areas that were stained positive for α SMA and Sirius red were decreased to 59% and 62% (Fig. 6D). A 25% reduction of collagen content was also found according to hydroxyproline measurements (Fig. 6E). These observations show that inhibition of integrin $\alpha\beta 6$ activity affects fibrotic response during DDC induced liver injury.

Ctgf Deficiency or Neutralization of Integrin $\alpha\beta 6$ Activity Impairs Oval Cell Activation During DDC Induced Liver Injury in Mice

We analyzed the effect of *Ctgf* deficiency or integrin $\alpha\beta 6$ neutralization on oval cell activation. Transcriptional levels of *EpCAM* and *CK19* were assessed in total liver extracts using qRT-PCR analysis. As shown in Fig. 7A, there were 80% and 85% reduction of *EpCAM* mRNA as well as 78% and 82% decrease of *CK19* mRNA in *Ctgf^{Δ/Δ}* livers compared to *Ctgf^{+/+}* livers at days 7 and 14 after DDC treatment, respectively. Further immunofluorescent staining showed that *Ctgf^{Δ/Δ}* mice had ~50 to 60% decrease in *EpCAM*⁺ or *A6*⁺ areas compared to *Ctgf^{+/+}* mice after 7 to 14 days of DDC treatment (Fig. 7C,D). Dual staining for Ki67 and CK19 detected 63-65% decrease of proliferating CK19⁺ epithelial cells around periportal regions in *Ctgf^{Δ/Δ}* livers (Fig. 7E,F). The number of Ki67⁺ hepatocytes in *Ctgf^{Δ/Δ}* livers was also slightly decreased at days 7 and 14 after DDC treatment. These results indicated that conditional deletion of *Ctgf* gene affected oval cell response and hepatic proliferation during DDC induced liver injury.

We characterized oval cell response in animals after administration of 6.3G9 neutralizing antibody. High dose of 6.3G9 antibody caused 52% and 46% decrease of *EpCAM* and *CK19* mRNAs (Fig. 7B), 33% to 44% decrease of *A6*⁺ or *EpCAM*⁺ in oval cell areas (Fig. 7C,D), and 13% reduction in the number of proliferating biliary ductular epithelial cells (Fig. 7E,F). About 1.78-1.88% hepatocytes were Ki67⁺ in IgG treated mice at day 14 after DDC treatment. Administration with 6.3G9 antibody did not significantly affect the number of proliferating hepatocytes (Fig. 7E,F). These results indicate that blocking integrin $\alpha\beta 6$ activity affects oval cell activation induced by DDC in mice.

Ctgf Deficiency or Neutralization of Integrin $\alpha\beta 6$ Activity Improves Serum Markers of Cholestasis During DDC Induced Liver Injury

Serum levels of alkaline phosphatase (ALP), total bilirubin level, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were measured to determine liver function. *Ctgf^{+/+}* and *Ctgf^{Δ/Δ}* mice without DDC treatment had normal liver function tests (Supporting Table 2). In contrast, *Ctgf^{+/+}* mice had higher levels of these cholestasis markers in sera than *Ctgf^{Δ/Δ}* animals at days 7 and 14 after DDC treatment. Administration of integrin $\alpha\beta 6$ antibody was associated with lower levels of ALP, total bilirubin, ALT, and AST than IgG controls at day 14 after DDC treatment.

CTGF is Associated With Integrin $\alpha\beta 6$ in Vitro

Induction of integrin $\alpha\beta 6$ and CTGF in reporter mice during DDC induced liver injury led us to test whether the two proteins were able to interact with each other in oval cells. We overexpressed the two proteins in WB-F344 line, an oval cell line derived from normal rat livers capable of differentiation into both hepatocytes and bile ductular epithelial cells *in*

vitro, by co-transfecting plasmids carrying CTGF:3xFLAG and integrin $\beta 6$ cDNAs. We isolated primary mouse oval cells with more than 85% purity based on CK19 and A6 staining (Supporting Figure 9). Immunofluorescent analysis detected co-localization of integrin $\alpha \nu \beta 6$ and CTGF on surface of primary mouse oval cells and transfected WB-F344 line (Fig. 8A). Immunoprecipitation assays showed that integrin $\alpha \nu \beta 6$ was specifically co-precipitated with CTGF protein from total protein lysates of oval cells by rabbit CTGF antibody but not by IgG control (Fig. 8A). These results demonstrate that integrin $\alpha \nu \beta 6$ is associated with CTGF *in vitro*.

Integrin $\alpha \nu \beta 6$ Mediates Oval Cell Adhesion to CTGF and Fibronectin in Vitro

Immunofluorescent staining for matrix proteins revealed that *Ctgf* expressing epithelial cells in DRs were closely associated with provisional matrix that were rich in laminin and fibronectin (Supporting Figure 10). Since both CTGF and integrin $\alpha \nu \beta 6$ are known to interact with fibronectin, we hypothesized that these two molecules cooperated to regulate oval cell attachment. This hypothesis was tested *in vitro* adhesion assay using WB-F344 cells and mouse oval cells. Recombinant CTGF protein at various concentrations was used to coat the plastic surface. Attached cells were stained fluorescently and quantified by ELISA. CTGF was able to induce adhesion of mouse oval cells and WB-F344 cells (Fig. 8B). When mixed with same amount of fibronectin, CTGF-coated wells had more attached mouse oval cells and WB-F344 cells than bovine serum albumin (BSA)-coated wells. Furthermore, adhesion activities of CTGF and fibronectin were largely blocked by pre-incubation with the 6.3G9 antibody ranging from 100-300 $\mu\text{g/ml}$ (Fig. 8C), indicating the importance of integrin $\alpha \nu \beta 6$ in oval cell adhesion to CTGF and fibronectin.

Integrin $\alpha \nu \beta 6$ Promotes TGF- $\beta 1$ Activation in Cultured Oval Cells

We investigated whether levels of integrin $\alpha \nu \beta 6$ and CTGF expression influenced TGF- β activation in WB-F344 cells. As shown in Fig. 8D, stable WB-F344 cell lines expressing empty vector, rat CTGF-3xFLAG or together with human integrin $\beta 6$ subunit were generated. TGF- β activation was measured in co-cultured TMLC cells based on transcriptional activation of the plasminogen activator inhibitor 1 promoter in luciferase reporter assays. Relative endogenous TGF- β activation indicated by relative luciferase activity appeared to be highest in cells overexpressing both integrin $\alpha \nu \beta 6$ and CTGF. Endogenous TGF- β activation was significantly higher in cells overexpressing CTGF alone than those expressing empty vector control despite of comparable integrin $\alpha \nu \beta 6$ in the two cell types (Fig. 8E). Measurement of spontaneously activated endogenous TGF- β or total TGF- β proteins in conditioned media by ELISA showed similar results (Fig. 8F). Addition of 6.3G9 antibody (300 $\mu\text{g/ml}$) significantly decreased endogenous TGF- β activation in all the tested cells (Fig. 8F). These results indicate that integrin $\alpha \nu \beta 6$ promotes TGF- β activation on oval cells and overexpression of CTGF enhances integrin $\alpha \nu \beta 6$ mediated TGF- β activation.

DISCUSSION

The availability of conditional knockout animals allowed us to comprehensively investigate the *in vivo* function of CTGF through tissue- or developmental-specific deletion of this gene.

Exon 4 encodes module III in CTGF that has intrinsic biological activities including stimulation of dose-dependent stellate cell adhesion and activity of p42/44 mitogen activated protein kinase as well as production of fibronectin and pro-collagen type IV.²⁵ Deletion of exon 4 in the *Ctgf* gene throughout the whole body using a Cre/loxP system to constitutively express Cre recombinase under the control of the cytomegalovirus promoter/enhancer causes lethality without any alteration in skin during embryonic development in mice.²⁶ In contrast, deletion of *Ctgf* exon 4 in fibroblast cells affects myofibroblast recruitment and decreases skin fibrosis.²² We have recently showed that conditional deletion of *Ctgf* exon 4 affects corneal epithelial wound closure.²¹ Considering that multiple hepatic cells express CTGF in adult mice, we sought to systemically delete exon 4 of the *Ctgf* gene using a tamoxifen-inducible Cre system prior to liver injury. Consistent with a dispensable role of CTGF in liver homeostasis and a critical role of full CTGF protein in liver repair, we observed little change of hepatic structure and function in untreated conditional knockout animals, whereas, *Ctgf* deficiency was associated with significant reduction of oval cell activation and fibrogenic response after DDC treatment. This is in agreement with our previous observations that CTGF is differentially expressed in Thy1⁺ rat oval cells and inhibition of CTGF synthesis by iloprost impairs liver regeneration following 2-acetylaminofluorescence and partial hepatectomy.¹³ Moreover, *Ctgf* was predominantly expressed in activated oval cells and proliferating cholangiocytes during DDC induced liver injury and in macrophages/Kupffer cells after chronic CCl₄ intoxication. Macrophages/Kupffer cells are key players in orchestrating the resolution of inflammation and restoring integrity/function of the tissue. Future studies using cell specific knockout are needed to elucidate the importance of each cell type expressing *Ctgf* during liver injury.

CTGF and other CCN members have been shown to mediate cell and matrix interaction through various types of integrins. The nature of binding between CCNs and specific integrins is complex and involves engagement of unique non-RGD motifs. This study showed that CTGF was associated with epithelial specific integrin $\alpha\beta6$. Although the exact nature of interaction between the two proteins remains to be determined, a recent study reported that CTGF secretion in kidney proximal tubule cells is induced through integrin $\alpha\beta6$ -dependent activation of TGF- β .²⁷ CTGF interacts with TGF- β and potentiates TGF- β signaling.¹² Integrin $\alpha\beta6$ binds to the RGD amino acid sequence present in LAP and promotes TGF- β activation through cytoskeleton organization.¹⁵ This study showed that integrin $\alpha\beta6$ was expressed during DRs in human cirrhotic livers and CCs. It was also specifically activated in mouse oval cells and cholangiocytes. In agreement with the established role of integrin $\alpha\beta6$ in biliary fibrosis,¹⁷⁻¹⁹ we observed that neutralizing integrin $\alpha\beta6$ activity reduced the fibrotic response during DDC induced liver injury in mice. Thus, integrin $\alpha\beta6$, like CTGF, is critical for oval cell activation and DRs. Down-regulation of integrin $\alpha\beta6$ during oval cell activation in *Ctgf* deficient livers after DDC treatment could be a result of an overall slower regenerative response caused by the loss of CTGF function.

Together, CTGF and integrin $\alpha\beta6$, may have a profound effect on cell-cell or cell-matrix crosstalk during HPC activation and DRs. HPC expansion is often accompanied with myofibroblast activation and fibrosis. CTGF promotes proliferation, adhesion, migration and

ECM production in fibroblast cells. Thus, HPC derived CTGF may act in a paracrine manner and regulate myofibroblast cells that are closely associated with the progenitor cell compartment. In addition, CTGF can bind to fibronectin in provisional matrix during liver repair.⁹ Coincidentally, integrin $\alpha\beta6$ is a receptor for fibronectin and mediates TGF- β 1 activation through mechanical tension.¹⁴ Fibronectin is critical for cholangiocyte adhesion during biliary fibrosis.¹⁷ Hence, it is easy to postulate that engagement of integrin $\alpha\beta6$ on CTGF/fibronectin enriched provisional matrix can effectively produce mechanical tension leading to efficient TGF- β 1 activation. Through binding to CTGF and fibronectin, integrin $\alpha\beta6$ may activate intracellular signal molecules and regulate the proliferative state of HPCs and cholangiocytes through transduction of the adhesive signaling. We speculate that the integrin $\alpha\beta6$ -CTGF mediated adhesion to fibronectin is responsible for enhanced proliferation of HPCs *in vivo*. Blocking integrin $\alpha\beta6$ activity using 6.3G9 neutralizing antibody may inhibit integrin $\alpha\beta6$ binding to the CTGF/fibronectin complex and affect the generation of mechanical tension leading to a decrease in TGF- β 1 activation, reducing intracellular signal transduction that ultimately lead to alleviation of DRs. Future studies are needed to investigate intracellular downstream signaling molecules that are activated by integrin $\alpha\beta6$ /CTGF/fibronectin complex during HPC activation, DRs and biliary fibrosis.

In summary, this study presents a functional connection between CTGF and integrin $\alpha\beta6$ during hepatic progenitor/oval cell activation and associated fibrosis. CTGF appears to be ubiquitously expressed in intrahepatic vasculature and biliary trees while integrin $\alpha\beta6$ receptor is specifically induced in hepatic progenitor/oval cells and cholangiocytes. Deletion of *Ctgf* gene or neutralization of integrin $\alpha\beta6$ activity can significantly inhibit mouse oval cell activation and associated liver fibrogenesis during DDC induced liver injury. Therapeutic interventions that inhibit biological activities of CTGF and integrin $\alpha\beta6$ individually have been tested in clinical trials for fibrosis in other organs.²⁸ This study provides a basis for therapeutics designed to target both CTGF and integrin $\alpha\beta6$ for the treatment of DRs and fibrosis in liver disease in the future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENT

We would like to thank Drs. Valentina Factor, William B. Coleman and Daniel B. Rifkin for sending A6 antibody, WB-F344 oval cells and TMLC cells.

Financial Support This study is supported by National Institutes of Health grants DK058614 and DK065096 awarded to BEP and EY05587 awarded to GSS. This study is also partly supported by Children's Miracle Network research awards to LP.

Abbreviations

CTGF	connective tissue growth factor
DRs	ductular reactions
DDC	3,5-diethoxycarbonyl-1,4-dihydrocollidine

TGF-β	transforming growth factor- β
HPCs	hepatic progenitor cells
CC	cholangiocarcinoma
ECM	extracellular matrix
TWEAK	tumor necrosis factor-like weak inducer of apoptosis
CCN	Cyr61/CTGF/Nov
LAP	latency associated peptide
<i>Ctgfp-eGFP</i>	<i>Ctgf</i> promoter driven enhanced GFP
ubc	human ubiquitin C promoter
IgG	immunoglobulin
CK19	cytokeratin 19
αSMA	α smooth muscle actin
<i>EpCAM</i>	<i>Epithelial cell adhesion molecule</i>
qRT-PCR	quantitative reverse transcription polymerase chain reaction
ELISA	enzyme-linked immunosorbent assay
BSA	bovine serum albumin
ALP	alkaline phosphatase
ALT	alanine aminotransferase
AST	aspartate aminotransferase

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Fig. 1.

CTGF and integrin $\alpha v \beta 6$ are highly expressed in DRs of cirrhotic human livers and CCs. (A) Trichrome staining detected collagen fibrils (blue) in fibrous septa of human cirrhotic livers, in which plenty of integrin $\alpha v \beta 6$ (brown) was detected by the 3,3'-Diaminobenzidine (DAB) staining (insert). (B) Immunofluorescent staining detected CTGF and CK19 in the cirrhotic livers. Areas in (B) correspond to boxed regions in (A) from consecutive sections. (C) Representative images from CC tissue arrays showed fibrosis indicated by heavy collagen deposits (blue) in Trichrome staining. CTGF protein (red) was detected by alkaline phosphatase substrate and integrin $\alpha v \beta 6$ protein (brown) was stained by DAB. Nuclei were labeled with hematoxylin (purple) in (A and C) and with DAPI (blue) in (B). Scale bar: 0.5 mm. Arrows point to some reactive ducts. (D and E) Integrin $\beta 6$ was specifically expressed in CC tumors whereas CTGF was expressed in both nontumor and tumor parts of the CC samples by RT-PCR analysis (D) and Western analysis (E). "N" stands for non-tumor portion of the CC samples while "T" represents CC tumor tissues. Notably, CTGF protein detected from total protein lysates of CC tumor tissues migrated faster than those of non-tumor tissues, probably due to a difference in post-translational modifications. NSP: nonspecific product.

Fig. 2.

CTGF protein is located in CK19+ HPCs and cholangiocytes during DRs in human CC tumors. Dual staining of CTGF and CK19 was carried out on hepatocholangiocarcinoma tissue microarray. Representative images are from three different CC samples. White arrows point to co-localization of CTGF and CK19. Scale bar: 50 μ m.

Fig. 3.

(A) The qRT-PCR analysis detected Ctgf and $\beta 6$ mRNAs in injured livers after DDC feeding or CCl₄ intoxication. Relative transcripts in each time point were calculated relative to untreated Ctgfp-GFP livers (UL). Data were expressed as means $\pm$ SEM (n=3-6/group). **P<0.01 and *P<0.05 versus UL group. (B and C) The dual staining for A6 and GFP indicate CTGF promoter activity in Ctgfp-GFP mice under normal condition (B) or at d14 after DDC treatment (C). Boxed areas in (B) are shown in inserts with increased magnification on the right. (D and E) Livers from the DDC treated Ctgfp-GFP mice were further stained for integrin $\alpha v \beta 6$ using ch2A1 antibody in combination with GFP (D) or A6 antibodies (E). OC: oval cells; BD: bile ducts; PV: portal vein. Scale bar: 50 μ m.

Fig. 4.

Ctgf gene is highly up-regulated in F4/80+ macrophages/Kupffer cells after chronic CCl₄ intoxication and in mouse oval cells that are purified from DDC damaged livers. (A) Dual staining for GFP and F4/80 was carried out to analyze liver sections after administering CCl₄ to mice. Scale bar: 100 μm. Arrows indicate the same areas around central veins (CV). (B) Semi-quantitative RT-PCR analysis detected *Ctgf* and cell-specific genes in isolated stellate cells (S), hepatocytes (H), macrophages (M), oval cells (O) from mice that were injected with CCl₄ for 6 weeks (A) or treated with DDC diet for 14 days. Actin was amplified as controls. All the PCR products were resolved on 1.2% agarose gels. (C) Quantification analysis was carried out based on relative band intensity of PCR products of *Ctgf* and Actin in (A) using Image J software. Relative fold increase of *Ctgf* transcript in each group was compared to total cell populations from normal livers (N). **p*<0.05; ***p*<0.01 in comparison to N group.

Fig. 5.

Integrin $\alpha v \beta 6$ is down-regulated in *Ctgf* conditional knockout mice during DDC induced liver injury. (A) PCR-based genotyping detected a 578-bp *Ctgf* product when exon 4 was deleted from conditional knockout (*Ctgfk/k*) mice and a 1003-bp product with intact exon 4 from floxed homozygotes (*Ctgff/f*) mice. (B) qRT-PCR analysis showed loss of exon 4 of *Ctgf* gene and down-regulation of integrin $\beta 6$ gene in DDC treated livers of *Ctgfk/k* mice. Relative transcripts in each time point were calculated relative to untreated livers (UL) from *Ctgff/f* mice controls. Data were expressed as means $\pm$ SEM (n=6/group). **P<0.01. (C) Western analysis detected CTGF and integrin $\alpha v \beta 6$ proteins from hepatic protein lysates of treated mice. (D) The IHC using ch2A1 antibody detected less integrin $\alpha v \beta 6$ immunoreactive areas in liver sections of *Ctgfk/k* mice than *Ctgff/f* animals after DDC treatment. Scale bar: 150 μ m.

Fig. 6.

Ctgf deletion or integrin $\alpha v \beta 6$ neutralization attenuates fibrotic response after DDC treatment. (A and C) qRT-PCR analysis determined the mRNA levels of fibrotic-related genes in livers of transgenic mice at day 7 and 14 after DDC treatment. The relative amount of each transcript was expressed as fold change versus untreated livers (UL) in each set of groups. Data were expressed as means $\pm$ SEM (n=6/group). *P<0.01. (B) The ratio between active and total TGF- $\beta 1$ proteins was decreased in liver lysates of Ctgfk/k mice in comparison to Ctgff/f animals at days 7 and 14 after DDC treatment. Active TGF- $\beta 1$ protein was measure without activation with 1N HCl, whereas total TGF- $\beta 1$ was measured with activation using 1N HCl by ELISA. (D) Representative images show the IHC of α SMA (blue) and the Sirius red staining for collagen (red) on liver sections from the transgenic mice DDC treated mice. Dark brown spots are porphyrin pigments due to DDC feeding. Scale bar: 200 μ m. (E) Graphs are quantification of α SMA+ (left panel) or Sirius red stained areas (middle panel) based on image analysis of at least five random fields (200x magnification) per slide. In addition, hepatic hydroxyproline content in treated animals was measured (right panel). Six animals per group were scored. Data are means $\pm$ SEM. *P<0.05.

Fig. 7.

Ctgf deletion or integrin $\alpha v\beta 6$ neutralization inhibits oval cell response during DDC induced liver injury. (A and B) qRT-PCR analysis determined relative induction of EpCAM and CK19 after DDC treatment. Results were means $\pm$ SEM (n=6/group). **P<0.01. (C and D) Representative images show the immunofluorescent staining for A6 (C) or EpCAM (D) on indicated liver sections. Corresponding immunoreactive areas were quantified based on intensity of green fluorescence on images (100x magnification) using ImageJ software. Total positive areas were calculated as a percentage of the total pixels in each image. Five random-fields were taken per slide, and six animals per group were scored. Values are means $\pm$ SEM. *P<0.05. (E) Representative images show dual staining for Ki67 and CK19 on indicated liver sections. PD: proliferating ductular epithelial cell; HC: hepatocyte. Scale bar: 100 μ m for (CE). (F) Quantification of PDs and Ki67+ hepatocytes. Biliary proliferation was indicated by a ratio between the number of Ki67+ CK19+ cells and total number of CK19+ bile epithelial cells per field. Hepatocyte proliferation was determined by counting the number of Ki67+ hepatocytes per field. Five fields were assessed per liver section (200x magnification) from six animals per groups. Values are means $\pm$ SEM.

Fig. 8.

Integrin $\alpha v \beta 6$ is associated with CTGF, mediates oval cell adhesion to CTGF and fibronectin substrates, and promotes TGF- $\beta 1$ activation in vitro. (A) The first three columns show co-localization of CTGF and integrin $\alpha v \beta 6$ proteins on the surfaces of primary mouse oval cells or WB-F344 oval cells that were transfected with plasmids overexpressing CTGF: 3xFLAG and integrin $\beta 6$ by immunofluorescent staining. IgG staining was shown as controls in the fourth column. Scale bar: 20 μm . In the last column, the immunoprecipitation assays detected that integrin $\beta 6$ subunit was co-immunoprecipitated in protein A/G beads with CTGF protein from total protein lysates of transfected WB-F344 cells and mouse oval cells by a rabbit CTGF antibody (Ab) but not by IgG control. Equal amounts of CTGF and integrin $\beta 6$ subunit were used in the assays and shown as total input in the lower panels. (B) Cell adhesion assays were carried out to determine attachment of mouse oval cells and WB-F344 oval cells to plastic surfaces that were coated with either recombinant CTGF protein ranging from 0.1-2.5 μg (the first two graphs) or mixtures that contained fibronectin (0.5 μg) and variable concentration of CTGF ranging from 0.05-2.0 μg (the last two graphs). BSA was used as control. (C) shows the effect of 6.3G9 antibody (Ab) on the adhesion of WB-F344 cells and mouse oval cells to surfaces that were coated with a mixture containing 0.5 μg fibronectin and 1.5 μg CTGF. Clone 1E6 was used as IgG control. Results in (B and C) were expressed as means $\pm$ SD (n=3/group). *P<0.05. (D) Western analysis determined the levels of CTGF and integrin $\alpha v \beta 6$ in WB-F344 cells that stably expressed empty vector and CTGF with or without human $\beta 6$. (E) Integrin $\alpha v \beta 6$ and CTGF promoted TGF- $\beta 1$ activation. WB-F344 stable cell lines were co-cultured with TMLC cells for 24 hours. Luciferase activity for each cell line was measured and compared with the cells expressing empty vector to calculate relative luciferase activity (RLU). Data are means $\pm$ SD (n=3/group). (F) Total and active TGF- $\beta 1$ in conditioned media of WB-F344 stable cell lines were measured

in ELISA assays with or without chemical activation by 1N HCl, respectively. The ratio of active to total TGF- β 1 was expressed as the percentage of endogenous TGF- β 1 activation. The α v β 6 Ab or IgG (300 μ g/ml) was added in the conditioned media to inhibit integrin α v β 6 activity. Results were expressed as means $\pm$ SD (n=3/group). *P<0.05.