PANCREAS

A novel integrin $\alpha_5\beta_1$ binding domain in module 4 of connective tissue growth factor (CCN2/CTGF) promotes adhesion and migration of activated pancreatic stellate cells

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Background: Connective tissue growth factor (CCN2) is upregulated in pancreatic fibrosis and desmoplastic pancreatic tumours. CCN2 interacts with integrin $\alpha_5\beta_1$ on pancreatic stellate cells (PSC) in which it stimulates fibrogenesis, adhesion, migration, and proliferation.
Aim: To determine the structural domain(s) in CCN2 that interact with integrin $\alpha_5\beta_1$ to regulation PSC functions.
Methods: Primary activated rat PSC were tested for their adherence to isoforms of CCN2 comprising modules 1–4 ($CCN2_{1-4}$), modules 3–4 ($CCN2_{3-4}$), module 3 alone ($CCN2_3$), or module 4 alone ($CCN2_4$). Adhesion studies were performed in the presence of EDTA, divalent cations, anti-integrin $\alpha_5\beta_1$ antibodies, CCN2 synthetic peptides, or heparin, or after pretreatment of the cells with heparinase, chondroitinase, or sodium chlorate. CCN2 integrin $\alpha_5\beta_1$ binding was analysed in cell free systems. The ability of $CCN2_{1-4}$, $CCN2_{3-4}$, or $CCN2_4$ to stimulate PSC migration was evaluated in the presence of anti-integrin $\alpha_5\beta_1$ or heparin.
Results: PSC adhesion was stimulated by $CCN2_{1-4}$, $CCN2_{3-4}$, or $CCN2_4$ and supported by Mg^{2+} but not Ca^{2+}. $CCN2_4$ supported PSC adhesion or migration were blocked by anti-integrin $\alpha_5\beta_1$ antibodies or by treatment of cells with heparinase or sodium chlorate. A direct interaction between $CCN2_4$ and integrin $\alpha_5\beta_1$ was demonstrated in cell free assays. The sequence GVCTDGR in module 4 mediated the binding between $CCN2_4$ and integrin $\alpha_5\beta_1$ as well as $CCN2_4$ mediated PSC adhesion and migration.
Conclusions: A GVCTDGR sequence in module 4 of CCN2 is a novel integrin $\alpha_5\beta_1$ binding site that is essential for CCN2 stimulated functions in PSC and which represents a new therapeutic target in PSC mediated fibrogenesis.

Connective tissue growth factor (CCN2, also termed CTGF) is one of six structurally related molecules that comprise the CCN family.[1] CCN proteins regulate cell function (for example, cell cycle progression, division, chemotaxis, differentiation, apoptosis, adhesion, gene regulation, ion transport) by interacting contextually with cell surface receptors, cytokines, growth factors, and proteases.[1 2] CCN molecules participate in critical processes such as differentiation, development, angiogenesis, placentation, tumour growth, wound healing, and fibrosis,[1 2] the latter of which is the most common pathophysiological condition in which CCN2 has been implicated, often following its transcriptional activation or synergistic interaction with transforming growth factor β (TGF-β).[3]

In the pancreas, long term heavy alcohol consumption is associated with acute and chronic pancreatitis, the latter of which involves a significant fibrotic component.[4] Several recent reports have begun to link CCN2 overexpression with pancreatitis[5 6] and desmoplasia in pancreatic cancer.[7 8] Evidence from human clinical specimens and rat models has shown that CCN2 expression is associated with enhanced and concomitant expression of TGF-β and type collagen I in both acute and chronic pancreatitis.[5 6] CCN2 is produced by the remaining acinar, ductal, and fibroblastic cells in diseased tissue and is most abundant in severely damaged tissue adjacent to areas of necrosis. In pancreatic cancer, CCN2 mRNA expression was enhanced and positively correlated with the degree of tumour desmoplasia; CCN2 was implicated in the development of the desmoplastic stroma and was mainly produced by fibroblasts.[8] Although development of fibrosis during chronic pancreatitis clearly leads to additional tissue destruction and loss of function, pancreatic cancer patients with elevated pancreatic CCN2 mRNA expression have a better prognosis, possibly because a matrix rich desmoplastic stroma provides a growth disadvantage for pancreatic cancer cells.[9]

The principal fibrogenic cell type in the pancreas is the pancreatic stellate cell (PSC) which are localised around the acini and ducts in normal tissue.[10] When cultured, PSC undergo an activation process by which they become α smooth muscle actin expressing myofibroblast-like cells that are contractile, migratory, and proliferative, and produce high levels of collagen types I and III, laminin, and fibronectin (FN). These phenotypic changes are also proposed to occur during fibrosing pancreatic injury in vivo, resulting in deposition of a high density interstitial extracellular matrix (ECM) that severely compromises pancreatic function.[11 12] We recently performed a detailed analysis of the production of and response to CCN2 by PSC and discovered a central role for integrin $\alpha_5\beta_1$ as a novel CCN2 receptor that mediated adhesion, migration, mitogenesis, and fibrogenesis.[13] As integrins have emerged as receptors for several CCN

Abbreviations: BSA, bovine serum albumin; CCN2, connective tissue growth factor; ECM, extracellular matrix; FN, fibronectin; HSC, hepatic stellate cell; HSPG, heparan sulphate proteoglycan; MMPs, matrix metalloproteases; PSC, pancreatic stellate cell; TGF-β, transforming growth factor β

proteins,[2] CCN2-integrin interactions may offer a platform for developing new antifibrotic treatments. We now report that a unique region in the carboxy terminal domain of CCN2 plays a central role in functionally engaging integrin $\alpha_5\beta_1$ on activated PSC, highlighting this region of CCN2 as a potential therapeutic target.

MATERIALS AND METHODS

Reagents

Collagenase P and DNase I were purchased from Roche (Indianapolis, Indiana, USA). Heparinase I, chondroitinase ABC, bovine serum albumin (BSA; protease free and γ-globulin free), mouse IgG, protease XIV, and sodium chlorate ($NaClO_3$) were from Sigma (St Louis, Missouri, USA). Heparin was from USB (Cleveland, Ohio, USA). CyQUANT dye was from Molecular Probes (Eugene, Oregon, USA). FN, Dulbecco's modified Eagle's medium, and fetal bovine serum were purchased from Life Technologies (Grand Island, New York, USA). Purified human integrin $\alpha_5\beta_1$, mouse monoclonal anti-α_5, anti-β_1, rat monoclonal anti-$\alpha_5\beta_1$ blocking antibodies, and goat polyclonal anti-$\alpha_5\beta_1$ antibody were purchased from Chemicon Inc. (Temicula, California, USA).

The various forms of CCN2 used in these studies are shown in fig 1A. Intact recombinant 38 kDa human CCN2 containing all four modules ($CCN2_{1–4}$) and a C terminal fragment comprising essentially modules 3 and 4 ($CCN2_{3–4}$) were produced and purified as described previously.[14] CCN2 isoforms comprising either module 4 alone ($CCN2_4$) or module 3 alone ($CCN2_3$) were produced in *Escherichia coli* using the same approach as we have previously described.[15] Synthetic peptides spanning the entire 103 C terminal residues of CCN2 were produced as described previously.[16]

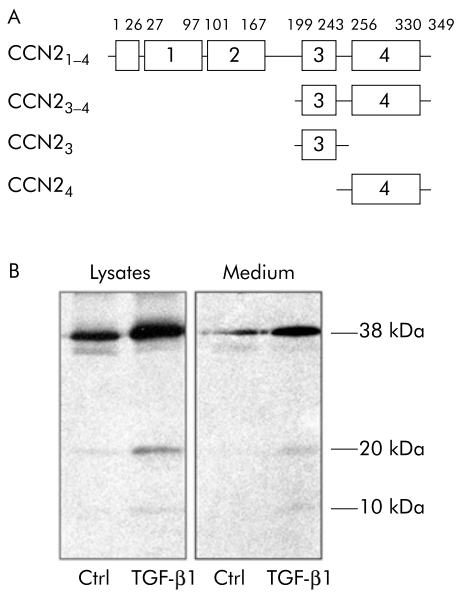

Figure 1 Structure of connective tissue growth factor (CCN2) isoforms and their production by pancreatic stellate cells (PSC). (A) Recombinant CCN2 isoforms used in these studies. (B) CCN2 isoforms detected in PSC lysates or medium by radioimmunoprecipitation, sodium dodecyl sulphate-polyacrylamide gel electrophoresis, and autoradiography of samples after labelling of cells with [^{35}S]cysteine/methionine for six hours in the presence or absence of transforming growth factor β (TGF-β_1) 20 ng/ml. The major immunoreactive proteins (10, 20, 38 kDa) are indicated.

Isolation and culture of PSC

Rat PSC were isolated from normal male Sprague-Dawley rats by in situ collaganese perfusion,[13] as approved by the Institutional Animal Care and Use Committee of the Children's Research Institute, Columbus, Ohio, USA. Activated PSC were split every three days at a ratio of 1:3 and used at passages 2–4. CCN2 levels in cell lysates or conditioned media were assessed by immunoprecipitation with anti-CCN2 antiserum after six hours of labelling of cells with [^{35}S]cysteine/methionine in the presence or absence of 20 ng/ml TGF-β.

Cell adhesion and migration assays

Activated PSC were used in adhesion and migration assays as described previously.[13] Briefly, for assessment of cell adhesion, 96 well plates (Costar, Corning, New York, USA) were precoated with CCN2 proteins or control cell adhesion molecules, prior to addition of 50 µl PSC (2.5×10^5 cells/ml), that were preincubated or coincubated with the various test substances, as detailed in the figure legends. Adherent cells were stained using CyQUANT GR dye. For PSC migration studies, PSC were suspended in cell culture inserts (2.5×10^4 cells/insert) that were contained within individual wells of a 12 well culture plates. The ability of PSC to migrate to the underside of the insert (8 µm pore size) in response to the presence of CCN2 proteins or FN was assessed after a six hour incubation. PSC on the upper and lower surfaces of the membrane were counted in 10 random 400× fields.[13]

Cell free CCN2-integrin $\alpha_5\beta_1$ binding assays

Complexes were allowed to form in solution between human integrin $\alpha_5\beta_1$ and each CCN2 isoform, prior to immunoprecipitation with anti-CCN2 antibody and protein A beads, essentially as described previously.[13] Immune complexes were separated on 8% sodium dodecyl sulphate-polyacrylamide gels, transferred to nitrocellulose, and then treated with goat antihuman $\alpha_5\beta$ antibody followed by horseradish peroxidase linked mouse antigoat IgG. Immunoreactive proteins were detected by chemiluminescence.

Binding of integrin $\alpha_5\beta_1$ to individual CCN2 isoform was further tested in a solid phase assay.[13] Briefly, microtitre wells (Dynex Technology, Chantilly, Virginia, USA) were precoated with CCN2 proteins or FN and, after extensive blocking and washing, were incubated with 1 µg/ml integrin $\alpha_5\beta_1$. The plate was developed using antihuman $\alpha_5\beta_1$ monoclonal antibody, as described previously.[13]

Statistical analysis

Values represent mean (SD) of measurements from at least four different PSC isolations. Statistical analysis of the data was performed using SPSS 11.5 for Windows. The Student's *t* test was used for paired data that were normally distributed. A p value of <0.05 was considered significant.

RESULTS

Multiple CCN2 isoforms were detected in PSC lysates and conditioned medium, levels of which were enhanced by treatment of cells with TGF-β (fig 1B). The size of the 38 kDa, 20 kDa, and 10 kDa proteins detected correspond to those of, respectively, $CCN2_{1–4}$, $CCN2_{3–4}$, and $CCN2_4$ (see fig 1A) that have previously been characterised in several in vitro and in vivo systems and that arise from limited CCN2 proteolysis.[14–17]

Adhesion of PSC was stimulated by $CCN2_{1–4}$, $CCN2_{3–4}$, and $CCN2_4$ and was divalent cation dependent, as shown by the ability of EDTA to block cell adhesion to all CCN2 isoforms (fig 2A). $CCN2_4$ mediated PSC adhesion was supported by Mg^{2+} but not by Ca^{2+} (fig 2B). There was however no effect on PSC adhesion in the presence of both EDTA and Ca^{2+}, likely reflecting their chelation with one another. Mg^{2+},

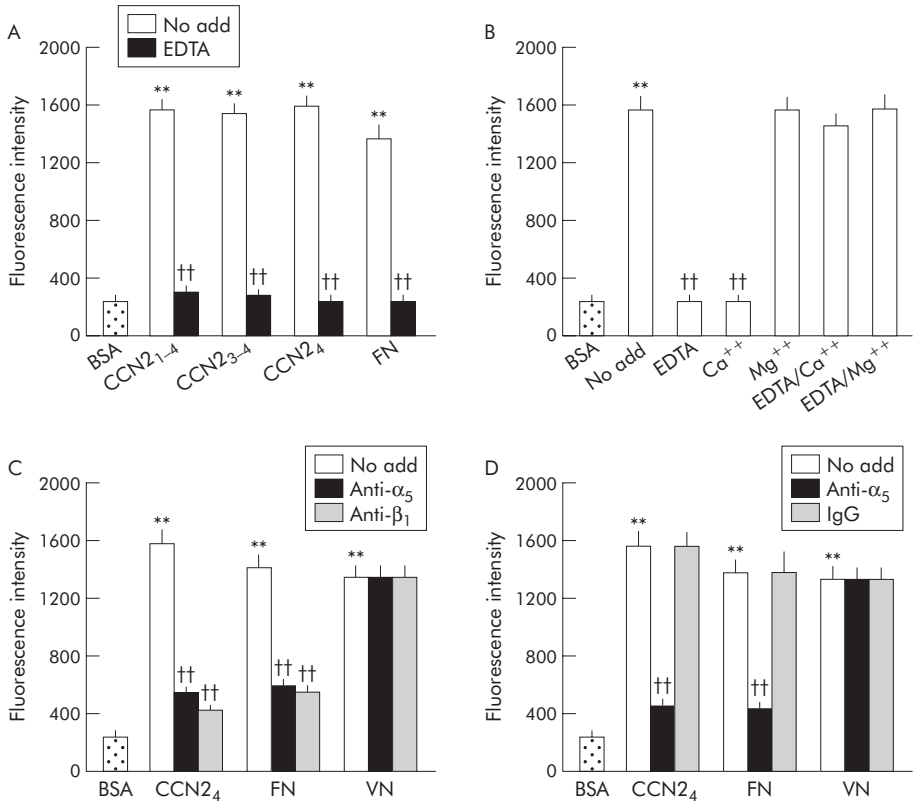

Figure 2 Connective tissue growth factor (CCN2) dependent pancreatic stellate cell (PSC) adhesion is mediated by interactions of module 4 with integrin $\alpha_5\beta_1$. (A) Microtitre wells were precoated at 4°C for 16 hours with phosphate buffered saline (PBS) or 2 μg/ml CCN2$_{1–4}$, CCN2$_{3–4}$, CCN2$_4$, or fibronectin (FN) and then blocked with PBS containing 1% bovine serum albumin (BSA) for one hour. Rat activated PSC (2.5×10^5 cells/ml) were preincubated in serum free medium for 30 minutes in vehicle buffer (no add) or EDTA (5 mM) prior to addition to individual wells at 50 μl/well. After incubation at 37°C for 20 minutes, adherent cells were washed, fixed, and stained by CyQUANT GR dye and quantified by measuring fluorescence intensity at an excitation of 485 nm and an emission of 530 nm. (B) PSC adhesion assays were performed using CCN2$_4$ following preincubation of the cells for 30 minutes with EDTA (5 mM) or with addition of Ca^{2+} (10 mM) or Mg^{2+} (10 mM) either alone or in combination. (C) PSC were preincubated with 25 μg/ml anti-integrin α_5 or anti-integrin β_1 monoclonal antibodies for 30 minutes prior to adding the cells to the wells that had been precoated with CCN2$_4$ (2 μg/ml), FN (2 μg/ml), or vitronectin (VN 4 μg/ml). (D) Microtitre wells were coated with CCN2$_4$, FN, or VN, as indicated, above prior to addition of PSC that had been preincubated at 37°C for 30 minutes with vehicle buffer (no add), 25 μg/ml monoclonal anti-$\alpha_5\beta_1$, or 25 μg/ml normal mouse IgG. Data are means (SD) of quadruplicate determinations and are representative of three experiments. **$p<0.01$ versus control; ††$p<0.01$ versus "no add" group.

which itself supported PSC binding, was able to block the inhibitory effect of EDTA (fig 2B).

As CCN2$_4$ appeared to be fully active in these assays, these data demonstrated that module 4 contributed fundamentally to PSC binding. Since CCN2$_{1–4}$ was previously shown to interact with integrin $\alpha_5\beta_1$,[13] we next tested whether this integrin was involved in adhesion of PSC to CCN2$_4$. Antibodies to the individual integrin α_5 or β_1 subunits or to integrin $\alpha_5\beta_1$ itself were effective in blocking CCN2$_4$ mediated PSC adhesion whereas normal IgG had no effect (fig 2C, D). The same pattern of binding was seen for FN, a well characterised ligand of integrin $\alpha_5\beta_1$, but not for vitronectin which does not bind to integrin $\alpha_5\beta_1$. Cell adhesion in response in CCN2$_4$ was dose dependent, reaching a plateau at coating concentrations of 2 μg/ml; this effect was heparin dependent, as shown by the inability of CCN2$_4$ to support PSC adhesion when the assays were performed in the presence of soluble heparin (fig 3A). Treatment of the cells with heparinase or sodium chlorate (an inhibitor of sulfation of heparin sulphate proteoglycans (HSPG)) reduced the ability of PSC to adhere to CCN2$_4$ by approximately 50% whereas adhesion to FN was not affected (fig 3B,C). Binding of PSC to CCN2$_4$ was not diminished by chondroitinase treatment, while the chlorate induced block was reversed by addition of sodium sulphate to the medium. Overall, these data suggest that heparin or heparin-like molecules such as cell surface HSPG contribute to the regulation of cell adhesion by CCN2$_4$.

To verify that an integrin $\alpha_5\beta_1$ binding site was located in module 4 of CCN2, a series of cell free binding assays were performed. We have previously shown that CCN2$_{1–4}$ is capable of engaging integrin $\alpha_5\beta_1$ in either solution based or solid phase cell free assays.[13] We found that this effect was mimicked by either CCN2$_{3–4}$ or CCN2$_4$ (the latter being divalent cation dependent) but not by CCN2$_3$ (fig 4A–C). These data thus pointed to the presence of an integrin $\alpha_5\beta_1$ binding site in module 4 of CCN2. When overlapping synthetic peptides from module 4 of CCN2 were analysed, we found that a peptide of the sequence GVCTDGR (corresponding to residues 285–291) was able to support PSC adhesion (fig 5A). Significantly, this peptide was also capable of blocking PSC adhesion to either CCN2$_4$ or FN (fig 5A) as well as blocking the interaction between integrin $\alpha_5\beta_1$ and either CCN2$_4$ or FN in a cell free system (fig 5B). Other peptides from module 4 were ineffective in these assays (data not shown). Overall, these data demonstrate the presence of an integrin $\alpha_5\beta_1$ binding motif within amino acids 285–291 of CCN2.

As we have recently shown that that CCN2$_{1–4}$ is chemotactic for activated PSC,[13] we next determined whether

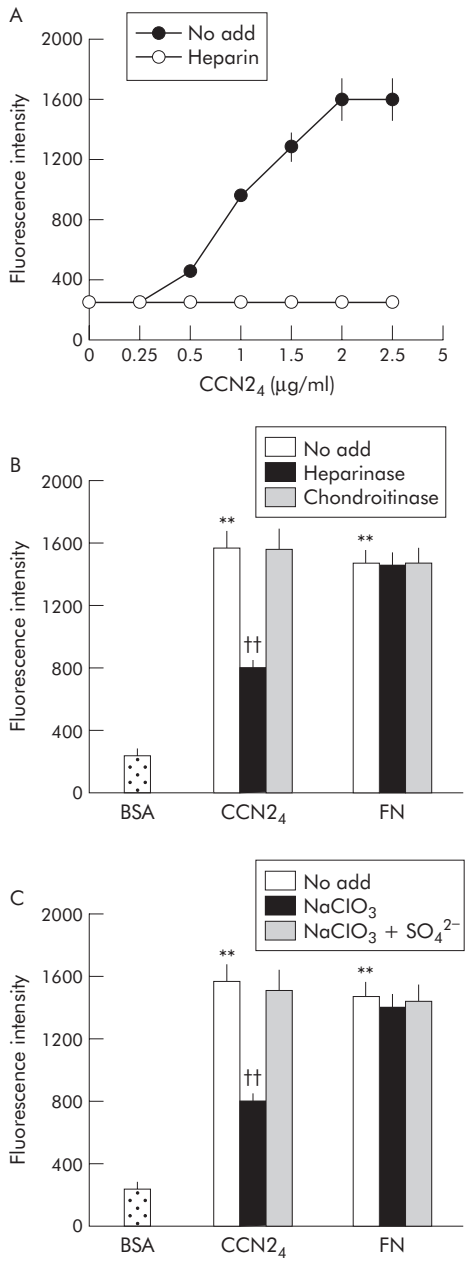

Figure 3 Role of cell surface heparan sulphate proteoglycan in connective tissue growth factor module 4 ($CCN2_4$) mediated pancreatic stellate cell (PSC) adhesion. (A) Cell adhesion assays were performed on $CCN2_4$ precoated microtitre wells using PSC that were treated with vehicle buffer (no add) or heparin (2 μg/ml) prior to plating. (B) Microtitre wells were coated with 2 μg/ml $CCN2_4$ or fibronectin (FN) prior to addition of PSC that had been pretreated at 37˚C for 30 minutes with vehicle buffer (no add), 2 units/ml heparinase I, or chondroitinase ABC. (C) PSC were cultured in complete medium containing 10 mM $NaClO_3$ for 48 hours in the presence or absence of 10 mM Na_2SO_4 prior to addition to $CCN2_4$ precoated wells. Data are means (SD) of quadruplicate determinations and are representative of three experiments. **p<0.01 versus control; ††p<0.01 versus ''no add'' group.

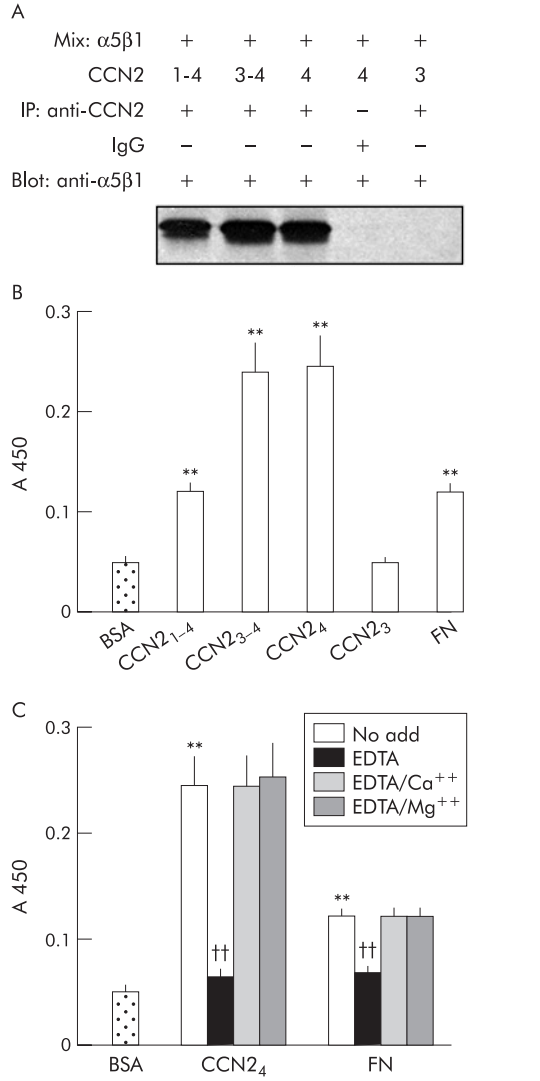

Figure 4 Integrin $\alpha_5\beta_1$ binds to connective tissue growth factor module 4 ($CCN2_4$) directly in cell free systems. (A) Integrin $\alpha_5\beta_1$ 2 μg were individually added to 4 μg $CCN2_{1-4}$, $CCN2_{3-4}$, $CCN2_4$, or $CCN2_3$ in 1 ml of NP40 buffer and mixed at 4˚C for two hours prior to treatment with immunoprecipitating polyclonal rabbit anti-CCN2 antibody or normal IgG. Samples were separated on 8% sodium dodecyl sulphate-polyacrylamide gels, and transferred onto a nitrocellulose membrane which was immunoblotted with anti-human $\alpha_5\beta_1$ before detection using enhanced chemiluminescence. (B) Microtitre wells were precoated with 2 μg/ml of $CCN2_{1-4}$, $CCN2_{3-4}$, $CCN2_4$, $CCN2_3$, or fibronectin (FN) at 4˚C for 16 hours. The wells were blocked and then incubated with 1 μg/ml integrin $\alpha_5\beta_1$ in blocking solution. The plate was developed by addition of antihuman $\alpha_5\beta_1$ monoclonal antibody followed by link antibody and horseradish peroxidase conjugated streptavidin. The colour reaction was developed using horseradish peroxidase substrate measured at A_{450}. BSA, bovine serum albumin. (C) Microtitre wells were precoated with $CCN2_4$ (2 μg/ml) or FN (2 μg/ml) at 4˚C for 16 hours, and the ability of integrin $\alpha_5\beta_1$ to subsequently bind to the plates was detected by ELISA following pretreatment of the integrin $\alpha_5\beta_1$ with either 5 mM EDTA alone or in combination with 10 mM Ca^{++} or 10 mM Mg^{++}. Data are means (SD) of quadruplicate determinations and are representative of three experiments. **p<0.01 versus control; ††p<0.01 versus ''no add'' group.

module 4 of $CCN2_4$ was involved in stimulating PSC migration. $CCN2_4$ was found to stimulate dose dependent migration of PSC in vitro, with a maximal response at 100 ng/ml (fig 6A). The migratory response of PSC to $CCN2_4$ was similar to that of $CCN2_{1-4}$, $CCN2_{3-4}$, or FN (fig 6B). PSC migration in response to either $CCN2_4$ or FN was blocked when the cells were treated with the P5 peptide or an anti-integrin $\alpha_5\beta_1$ monoclonal antibody, whereas treatment of the cells with soluble heparin caused PSC adhesion to be blocked only in the case of $CCN2_4$ (fig 6C).

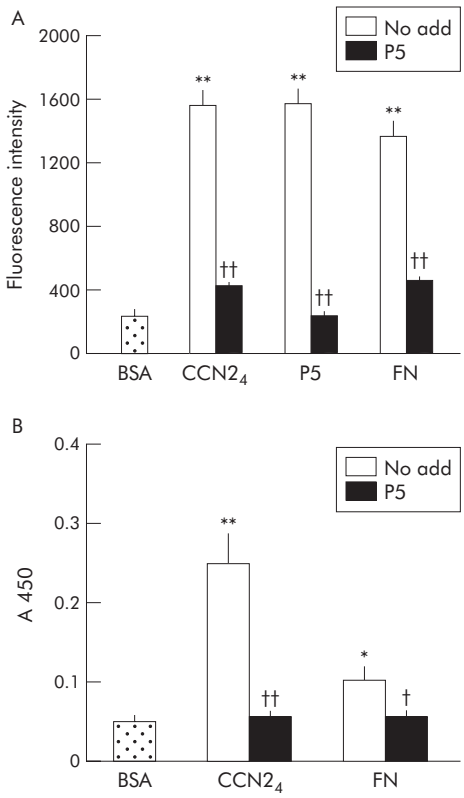

Figure 5 A synthetic connective tissue growth factor module 4 ($CCN2_4$) peptide, GVCTDGR, contains an integrin $\alpha_5\beta_1$ binding site. (A) Cell adhesion assays were performed on 96 well plates that had been coated at 2 μg/ml with $CCN2_4$, P5 (a synthetic peptide comprising the CVCTDGR, corresponding to residues 285–291 of CCN2) or fibronectin (FN). BSA, bovine serum albumin. (B) Microtitre wells were coated with $CCN2_4$ (2 μg/ml) or FN (2 μg/ml) at 4°C for 16 hours and then incubated with 1 μg/ml integrin $\alpha_5\beta_1$ alone or after its preincubation with 35 μM P5 for one hour. $CCN2_4$ binding by integrin $\alpha_5\beta_1$ was quantified by ELISA. Data are means (SD) of quadruplicate determinations and are representative of three experiments. *$p<0.05$, **$p<0.01$ versus control; †$p<0.05$, ††$p<0.01$ versus the ''no add'' group.

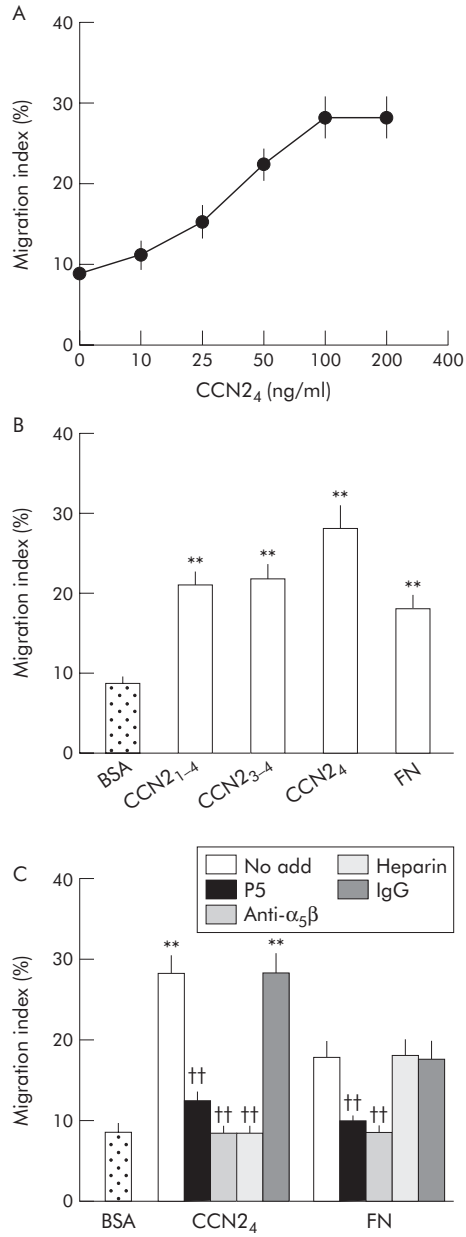

Figure 6 Connective tissue growth factor module 4 ($CCN2_4$) induces integrin $\alpha_5\beta_1$ dependent pancreatic stellate cell (PSC) migration. (A) PSC migration assays were performed by placing the cells in culture inserts (2.5×10^5 cells/insert) followed by incubation in a 12 well companion plate for six hours in the absence or presence of desired concentrations of $CCN2_4$ in the lower chamber. (B) PSC migration assays were performed in the presence of 100 ng/ml of $CCN2_{1-4}$, $CCN2_{3-4}$, $CCN2_4$, or 100 ng/ml fibronectin (FN) in the lower chamber. (C) Cell migration assays were performed following 30 minutes of preincubation of PSC with P5 (35 μM), anti-$\alpha_5\beta_1$ (25 μg/ml), mouse IgG (25 μg/ml), or heparin (2 μg/ml). Data are means (SD) of quadruplicate determinations and are representative of three experiments. **$p<0.01$ versus control; ††$p<0.01$ versus the ''no add'' group.

DISCUSSION

CCN2 is a recently described profibrotic factor that is produced in fibrosing tissues and organs and has fibrogenic properties in vivo and in vitro. Accumulating evidence suggests that CCN2 plays a role in acute and chronic pancreatitis as well as in pancreatic cancer. Of particular interest is the apparent involvement of CCN2 in driving excessive production of ECM proteins that are characteristic of pancreatic fibrosis and tumour desmoplasia. Based on our recent observations[13] as well as those in this report, we have shown that CCN2 is capable of stimulating responses in PSC (mitogenesis, chemotaxis, adhesion, proliferation, DNA synthesis, fibrogenesis) that are typically associated with the activated phenotype of this normally quiescent cell type, suggesting that CCN2 plays a key role in PSC mediated fibrogenesis. Moreover, CCN2 mRNA expression, along with that of FN, TGF-β1, and collagen I, is produced as a function of PSC activation in vitro, while CCN2 levels and promoter activity were enhanced following stimulation by TGF-β1, alcohol, or acetaldehyde.[13] CCN2 also mediates TGF-β stimulated collagen I production in PSC. Thus through its production by PSC themselves or by its production by other cell types such as ductular epithelium, acinar cells, fibroblasts, and endothelial cells,[5 6] CCN2 likely participates in autocrine and paracrine signalling pathways in response to pancreatic injury and, furthermore, is subject to limited proteolytic processing which generates bioactive C terminal isoforms. CCN2 proteolysis is promoted by matrix metalloproteases (MMPs), several of which are produced by PSC.[18–22] Although the significance of CCN2 processing has yet to be fully established, it likely has a major impact of the bioavailability and distribution of the protein. This is highlighted by the fact that CCN2 N terminal fragments in body fluids have been implicated as markers of fibrotic disease.[23–25]

On the other hand, C terminal fragments, which are bioactive and fibrogenic, may persist in tissues and act as matrix associated stimulants of fibrogenic pathways in target cells such as PSC.

Chronic pancreatitis and pancreatic cancer are associated with alterations in levels of various integrins and their ECM ligands.[26 27] PSC are believed to play a central role in the regulation of ECM levels as they not only regulate synthesis of ECM constituents such as FN or collagen but also produce MMPs, as well as tissue inhibitors of MMPs which collectively regulate the balance between ECM degradation and synthesis.[20–22] None the less, the manner in which PSC function is regulated by the interplay between ECM constituents, integrins, and other matrix molecules such as matricellular proteins (of which CCN2 is an example) has not been previously studied. While integrins have become recognised as receptors for various CCN proteins, the interaction is complex and involves a high degree of specificity with respect to the location of the CCN binding domain, the integrin partner, the cell type in question, and the functional readout. With respect to PSC, identification of integrin $\alpha_5\beta_1$ as the principal CCN2 receptor was unexpected yet this integrin was responsible for mediating several important biological properties of CCN2 in PSC, including adhesion and migration.[13] Interestingly, we have shown that hepatic stellate cells (HSC) also produce integrin $\alpha_5\beta$ but they appear not to exploit this integrin as an adhesion receptor for CCN2 (unpublished data). In addition, mutant $CCN2_4$ proteins in which the integrin $\alpha_v\beta_3$ site has been targeted show an unchanged ability to support PSC adhesion (unpublished data) while HSC binding is highly compromised.[28] Overall, the data suggest that CCN2 mediated cell adhesion involves principally integrin $\alpha_5\beta_1$ for PSC and integrin $\alpha_v\beta_3$ for HSC. Inhibition of CCN2 mediated PSC adhesion by Ca^{2+} is consistent with previously published data showing that ligand binding by integrin $\alpha_5\beta_1$ is not supported by millimolar concentrations of Ca^{2+} and is likely attributable to induction of an inactive integrin conformation by high concentrations of calcium.[29 30] In contrast, Mg^{2+} favours integrin activation and supports ligand-integrin $\alpha_5\beta_1$ interactions,[29] as was observed in our studies with CCN2.

The most significant findings now reported here are that a novel sequence in module 4 of CCN2 contains the principal integrin $\alpha_5\beta_1$ binding site and that an isoform of CCN2 that contains module 4 alone ($CCN2_4$) is able to support PSC adhesion and migration. Although it was previously shown that module 4 of CCN2 binds to integrin $\alpha_v\beta_3$[28] and that module 4 of CCN1 binds to integrin $\alpha_M\beta_2$,[31] the binding domains are clearly distinct from those used by CCN2 to engage integrin $\alpha_5\beta_1$. Whereas RGD motifs within integrin ligands are often used for binding their cognate integrin receptors, this motif is absent from CCN proteins. Even so, it is of interest that the GVCTDGR integrin $\alpha_5\beta_1$ binding sequence in module 4 contains a reverse RGD motif. While the precise role of this motif requires further study, a DGR sequence in fibroblast growth factor 2 was shown to be involved in its ability to bind to integrin $\alpha_v\beta_3$ on endothelial cells.[32] Whatever the precise role of the DGR sequence in residues 285–291, our data suggest that this domain is pivotal for integrin $\alpha_5\beta_1$ dependent functions of CCN2 in PSC.

Although integrin $\alpha_5\beta_1$ supported either FN or $CCN2_4$ mediated PSC adhesion or migration, responses of the cells were distinct in that the effects of $CCN2_4$ were HSPG dependent whereas those of FN were not. These data are consistent with the observation that $CCN2_4$ is heparin binding, a property that is attributed to the presence of one or more heparin binding domains in module 4.[14–16] Dependency on cell surface HSPG of several CCN2 bioactivities have been reported.[16 28 33] While the heparin binding properties of CCN2 may affect its bioavailability through interactions with HSPG in ECM, the ability of CCN2 to stimulate its fibrogenic target cells via integrins in vivo will likely be strongly influenced by their concomitant expression of HSPG. These data suggest that additional studies regarding HSPG production by PSC as a function of their activation may give further insight regarding the ability of these cells to interact with and respond to CCN2.

In the liver, sinusoidal accumulation of integrins and integrin ligands has been reported during fibrotic disease.[34 35] In addition, a variety of integrins are expressed by HSC during the activation process, and α and β_1 integrin subunits are more highly expressed by HSC in human fibrotic liver.[34–38] Furthermore, binding of some integrins (including integrin $\alpha_v\beta_3$, the principal CCN2 receptor) by their respective ligands is linked to the differentiated function and survival of HSC.[39 40] These data, coupled with the observation that liver fibrosis in mice can be blocked by RGD peptides,[41] highlight integrins as possible targets in fibrotic pathways, especially those that are driven by CCN2. There is growing optimism that anti-CCN2 strategies will offer new leads in the development of novel therapies for fibrosis.[42] However, mechanisms of CCN2 action in key fibrogenic cell types such as PSC in the pancreas, HSC in the liver, or mesangial cells in the kidney need to be thoroughly investigated so that rational CCN2 based therapeutic approaches can be developed. For example, mechanisms of injury and fibrosis are clearly different between the liver and pancreas, especially with respect to the role of pancreatic enzymes and ductal obstruction and these may influence CTGF receptor expression and signalling pathways in stellate cells. Interestingly, the GVCTDGR sequence was shown not to be involved in supporting adhesion of HSC cells, which instead preferentially use integrin $\alpha v\beta 3$ as a CCN2 receptor.[28] This difference highlights the fact that antifibrotic modalities may need to be developed for CCN2 on an organ by organ basis, taking into account the specific integrin subtypes that are used by CCN2 for binding to its respective target cells. Based on our observations, targeting of integrin $\alpha_5\beta_1$ on PSC and/or of its binding domain in module 4 of CCN2 provide a new platform for the development of novel antifibrotic strategies in pancreatic fibrosis.

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Conflict of interest: declared (the declaration can be viewed on the *Gut* website at http://www.gutjnl.com/supplemental).

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