CCN2 (Connective Tissue Growth Factor) Promotes Fibroblast Adhesion to Fibronectin

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In vivo, CCN2 (connective tissue growth factor) promotes angiogenesis, osteogenesis, tissue repair, and fibrosis, through largely unknown mechanisms. In vitro, CCN2 promotes cell adhesion in a variety of systems via integrins and heparin sulfate proteoglycans (HSPGs). However, the physiological relevance of CCN2-mediated cell adhesion is unknown. Here, we find that HSPGs and the mitogen-activated protein kinase kinase/extracellular signal-regulated kinase (ERK) mitogen-activated protein kinase cascade are required for adult human dermal fibroblasts to adhere to CCN2. Endogenous CCN2 directly binds fibronectin and the fibronectin receptors integrins α4 β1 and α5 and syndecan 4. Using Ccn2−/− mouse embryonic fibroblasts, we show that loss of endogenous CCN2 results in impaired spreading on fibronectin, delayed α-smooth muscle actin stress fiber formation, and reduced ERK and focal adhesion kinase phosphorylation. These results suggest that a physiological role of CCN2 is to potentiate the ability of fibroblasts to spread on fibronectin, which may be important in modulating fibroblast adhesion to the provisional matrix during tissue development and wound healing. These results are consistent with the notion that a principal function of CCN2 is to modulate receptor/ligand interactions in vivo.

INTRODUCTION

Human skin, the largest organ in the body, acts as a protective barrier between the internal organs and the external environment (Chuong et al., 2002). The barrier function of skin is provided by both the epidermis, which consists of extracellular lipid-like substances and intracellular insoluble keratin (Fuchs and Byrne, 1994), and the dermis, which consists of fibroblasts that produce extracellular matrix (ECM; Kiety and Shuttleworth, 1997).

As a response to environmental insults or trauma, or as a consequence of local inflammatory processes, structural damage to skin can occur. The resultant wound-healing response consists of an integrated series of biochemical, immunological, and structural changes culminating in the de novo synthesis of new epithelia, blood vessels, and connective tissue (Werner and Grose, 2003). During this process, fibroblasts synthesize new ECM components, such as collagen and fibronectin (Badylak, 2002). In addition, fibroblasts proliferate and repopulate the wound. These fibroblasts attach to, remodel, and contract the newly synthesized ECM, providing the tensile strength required for the support function of the dermis (Clark, 1985). Thus, understanding the mechanism underlying the ability of fibroblasts to adhere to ECM components such as fibronectin is a necessary prerequisite to understanding how the new dermis and other connective tissues are being synthesized.

Connective tissue growth factor (CCN2), a member of the CCN family of matricellular proteins (Bork, 1993; Lau and Lam, 1999; Moussad and Brigstock, 2000; Perbal, 2003; Leask and Abraham, 2003), is a cysteine-rich protein that is secreted via a 37-amino acid signal sequence (Chen et al., 2001b). Secondary sequence structure predictions have suggested that CCN2 comprises four modules sharing identity with insulin-like growth factor binding proteins, Von Willebrand factor, thrombospondin domain, and a cysteine knot-containing family of growth regulators (Bork, 1993; Lau and Lam, 1999; Moussad and Brigstock, 2000; Perbal, 2001). CCN2 is expressed by mesenchymal cells during development (Ivkovic et al., 2003). In adult mammals, CCN2 is not normally expressed in skin fibroblasts unless induced, such as in response to wounding or injury (Igarashi et al., 1993; Leask and Abraham, 2003; Leask et al., 2004). One of the most potent inducers of CCN2 is transforming growth factor β (TGFβ), which promotes CCN2 expression in dermal fibroblasts, but not in epidermal keratinocytes, through a complex network of transcriptional interactions, requiring Smads, protein kinase C, and ras/mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK), and a consensus transcription enhancer factor binding element in the CCN2 promoter (Holmes et al., 2001; Chen et al., 2002; Stratton et al., 2002; Leask et al., 2003). In contrast to the situation in normal dermal fibroblasts, CCN2 is constitutively expressed by fibroblasts present in fibrotic lesions.
Igarashi et al., 1995; Abraham et al., 2000; Holmes et al., 2003; Leask and Abraham, 2003; Leask et al., 2004). Because CCN2 has profibrotic activity in vivo (Mori et al., 1999), CCN2 has been proposed to be a key mediator of the profibrotic effects of TGFβ (Grotendorst, 1997; Mori et al., 1999; Leask et al., 2004; Leask and Abraham, 2004).

Although CCN2 was initially identified over a decade ago, the precise physiological role of CCN2 is not clear. The recent generation of Ccn2−/− mice has been a key initial step in the identification of the function of CCN2 in vivo (Ivkovic et al., 2003). Ccn2−/− mice die soon after birth primarily due to an inability of the rib cage to properly ossify (Ivkovic et al., 2003). Ccn2−/− mice also display decreased expression of extracellular matrix components and matrix metalloproteinases, suggesting that matrix remodeling is also defective in these animals (Ivkovic et al., 2003). However, the precise effect(s) of removing CCN2 on connective tissue biology and in isolated fibroblasts has not yet been investigated.

As might be expected of a secreted protein, exogenous CCN2 possesses activity. One activity of CCN2 revolves around the ability to promote cell adhesion through the integrins (Babic et al., 1999) and cell surface heparin sulfate proteoglycans (HSPGs) (Babic et al., 1999; Chen et al., 2001a; Schober et al., 2002; Gao and Brigstock, 2004). Recently, it was shown that the 10-kDa heparin-binding carboxyl-termin-also known as domain of human CCN2, previously shown to promote cell proliferation (Brigstock et al., 1997), was sufficient to promote fibroblast cell adhesion (Ball et al., 2003). However, two key issues remain. First, the signal transduction cascades required for CCN2-promoted adhesion are unknown. Second, and perhaps more important, the precise biological relevance of CCN2-mediated cell attachment is unclear.

In this report, we explore the biological function of CCN2-promoted fibroblast adhesion. We address two crucial outstanding questions concerning CCN-mediated fibroblast adhesion. First, we investigate the downstream signaling pathways required for the ability of CCN2 to promote fibroblast adhesion. Second, we use Ccn2−/− embryonic fibroblasts to evaluate the biological significance of CCN2-promoted fibroblast adhesion to ECM. Our results provide new insights into the mechanism of action and function of CCN2 action in fibroblast biology and tissue repair.

MATERIALS AND METHODS

Isolation of Recombinant CCN2

A 1073-base pair CCN2 cDNA fragment was amplified (from a plasmid provided by Dr. Gary Grotendorst, University of Miami, Miami, FL) by polymerase chain reaction (PCR) with Pfu polymerase (New England Biolabs, Beverly, MA). Primer sets were designed such that a 24-base pair sequence (Igarashi et al., 1995; Abraham et al., 2000, 10 µg/ml CCN2 or 10 µg/ml fibronectin or 10 µg/ml bovine serum albumin (Sigma-Aldrich) in PBS. When appropriate, cells were preincubated in the presence or absence of 50 µg/ml FLAG-Cy3 in PBS for 1 h, at 4°C. Before adding cells, nonspecific binding sites on proteins were blocked with a 1-h incubation with 0.5% BSA, PBS, at 37°C. To detect phospho-ERK antibodies, cells were fixed for 10 min in 3% paraformaldehyde/PBS, blocked in 10% normal goat serum (Jackson Immunoresearch Laboratories, West Grove, PA). To detect phosphorylated-local adhesion (FAK) or α-smooth muscle actin (SMA), cells were fixed for 10 min in 3% paraformaldehyde/PBS, blocked in 10% normal goat serum (Jackson Immunoresearch Laboratories, and subjected to staining with either anti-α-SMA antibody (1:100; Sigma-Aldrich) or anti-phospho-FAK (1:100; Cell Signaling Technology, Beverly, MA) and Texas Red-conjugated anti-rabbit (Jackson ImmunoResearch Laboratories, respectively). To detect F-actin, cells were similarly treated and stained with rhodamine-phalloidin (Molecular Probes, Eugene, OR). Cells were photographed using an Axioskop microscope and Axiovision software (Carl Zeiss, Welwyn Garden City, United Kingdom). and images were subsequently exported into Adobe Photoshop version 7.0 (Adobe Systems, San Jose, CA).

Western Blot Analysis

Fibroblasts were grown in monolayer until 80% confluent, serum starved cells for 24 h, and then removed by treatment with versene. Cells were then allowed to adhere to fibronectin as described above. After various lengths of time, cell layers were washed in PBS and harvested in 2% SDS. The resultant cell pellets were then extracted and subjected to SDS-PAGE. Gels were electrophoretically transferred to nitrocellulose (Immobilon). Membrane was blocked with 5% nonfat dry milk (Bio-Rad) in TBS (5 mM Tris buffered saline, 0.1% Tween 20; Sigma-Aldrich), and immunoblotting was performed using anti-phospho-ERK, anti-ERK anti-FAK, or anti-phospho-FAK antibodies (Cell Signaling Technology) or anti CCN2 (Abcam, Cambridge, United Kingdom) antibody as described previously. Blots were then probed overnight with biotinylated anti-rabbit or mouse antibodies (1:1000; Vector Laboratories, Burlingame, CA) as secondary antibodies, followed by incubation with ABC regent (Vector Laboratories). Signal was detected using a luminescence kit (ECL kit; Amersham Biosciences UK, Little Chalfont, Buckinghamshire, United Kingdom) and x-ray film. Densitometry was performed using Gel Base/Gel-Blot Pro (Synoptics, Cambridge, United Kingdom). Normal adult human dermal fibroblasts were isolated and cultured as described previously (Ivkovic et al., 2003). Mouse embryonic fibroblasts were isolated and cultured as described previously (Ivkovic et al., 2003). Adhesion assays were performed by coating wells of 96-well plates overnight, at 4°C, with 10 µg/ml CCN2 (Babic et al., 1999) or 10 µg/ml fibronectin (Sigma-Aldrich), 1% PBS or 10 µg/ml bovine serum albumin in 1× PBS. Non-specific binding sites on substrates were blocked for 1 h in 1% BSA (Sigma-Aldrich) in PBS at room temperature before addition of cells. Fibroblasts were cultured in DMEM 0.5% fetal calf serum for 24 h, and then were harvested with 2 mM EDTA in PBS (20 min, room temperature), washed twice with PBS, and then resuspended at a density of 1.5×105 cells/ml. 105 cells/ml were subjected to SDS-PAGE. Nonspecific binding sites on proteins were blocked with a 1-h incubation with 0.5% BSA, PBS, at 37°C. To detect phospho-ERK antibodies, cells were fixed for 10 min in 3% paraformaldehyde/PBS, blocked in 10% normal goat serum (Jackson Immunoresearch Laboratories, and subjected to staining with either anti-α-SMA antibody (1:100; Sigma-Aldrich) or anti-phospho-FAK (1:100; Cell Signaling Technology, Beverly, MA) and Texas Red-conjugated anti-rabbit antibody (Jackson Immunoresearch Laboratories, respectively). To detect F-actin, cells were similarly treated and stained with rhodamine-phalloidin (Molecular Probes, Eugene, OR). Cells were photographed using an Axioskop microscope and Axiovision software (Carl Zeiss, Welwyn Garden City, United Kingdom). and images were subsequently exported into Adobe Photoshop version 7.0 (Adobe Systems, San Jose, CA).
**Immunoprecipitation Assay**

Cells were cultured for 24 h in DMEM, 0.5% fetal bovine serum (FBS), and membrane preparations were isolated (Chen et al., 2001b). Equal amounts of protein were subjected to immunoprecipitation analysis with rabbit anti-CCN2 antibody (Abcam) or mouse IgG (Sigma-Aldrich) by using protein G-Sepharose (Pharmacia, Amersham, United Kingdom) as described previously (Holmes et al., 2001). Protein was detected using anti-CCN2 (Abcam), anti-a5 integrin, anti-b1 integrin, anti-a4 integrin (Zymed Laboratories, South San Francisco, CA) anti-a6 integrin anti-endoglin (all from BD Biosciences), anti-syndecan 4, and anti-syndecan 2 antibodies (Zymed Laboratories). Blots were then developed by incubation with biotinylated anti-rabbit or mouse antibodies (1:1000; Vector Laboratories) as secondary antibodies, followed by incubation with ABC regent (Vector Laboratories). Signal was detected using a luminescence kit (ECL kit; Amersham Biosciences UK) and x-ray film.

**125I-CCN2 Binding Assay**

Recombinant CCN2 was iodinated with 125I (Amersham Biosciences UK) as described previously (Segarini et al., 2001). Wells of a 96-well plate were coated overnight at 4°C with PBS, 50 μg/ml BSA suspended in PBS, or 50 μg/ml fibronectin suspended in PBS. Wells were blocked in 1% BSA/PBS for 1 h at room temperature and then 125I-CCN2 (500,000 cpm/ml) was added for 1 h at room temperature. Unbound 125I-CCN2 was then removed by washing three times with 1% BSA/PBS. Remaining bound 125I-CCN2 was measured using a gamma counter (N ~ 6).

**RESULTS**

The Ability of CCN2 to Bind to the Cell Surface of Adult Human Dermal Fibroblasts Is HSPG Dependent

Normal dermal fibroblasts cultured in 0.5% serum express little appreciable CCN2 expression (Abraham et al., 2000; Leask et al., 2003). Thus, these cells provide a useful model system to assess the contribution of endogenous CCN2 to fibroblast biology without the complication of having to consider the effects of endogenous CCN2. When adsorbed to tissue culture plates, CCN2 promotes adhesion of a variety of cell types (Babic et al., 1999; Chen et al., 2001a). However, the signal transduction mechanisms required for CCN2-mediated cell adhesion are not known. To begin to explore the signaling requirements involved with the ability of CCN2 to promote adhesion of primary adult dermal fibroblasts, we bound varying amounts of CCN2 to tissue culture plates. After blocking nonspecific binding sites on CCN2 with BSA, we added fibroblasts that had been cultured in 0.5% serum that had been detached from tissue culture plates with EDTA. After cells had been allowed to adhere to CCN2 for 40 min, the tissue culture plates were washed to remove unbound cells. Western blot analysis of cell lysates prepared from fibroblasts adhered to CCN2 showed that normal dermal fibroblasts cultured in 0.5% serum and allowed to adhere to CCN2 for 40 min did not show appreciable CCN2 expression (Figure 1A). In addition, we found that CCN2, when coated on 96-well plates, dose dependently promoted adhesion of normal adult dermal fibroblasts (Figure 1B). The ability of CCN2 to promote fibroblast adhesion was dependent on cell surface HSPGs as a 45-min preincubation of cells with heparin or heparin sulfate, but not keratin sulfate, chondroitinase, or chondroitin A, B, or C, blocked the ability of cells to adhere to CCN2 (Figure 1C). Thus, adhesion of adult human dermal fibroblasts to CCN2 was HSPG dependent.

**Attachment of Adult Human Dermal Fibroblasts to CCN2**

To begin to probe the physiological relevance of CCN2-promoted adhesion, we compared the adhesion of adult dermal fibroblasts to CCN2 and fibronectin. To confirm that adult human dermal fibroblasts were capable of binding to CCN2 in a dose-dependent manner, wells of a 96-well plate were incubated overnight with BSA (10 μg/ml) or different concentrations of CCN2, and cell adhesion was assessed as described in Materials and Methods (6 wells/data point; average ± SEM [error bar] is shown). The values obtained from the BSA control experiment, which were not significantly above background, were subtracted from the experimental values. (C) Adhesion of fibroblasts to CCN2 is dependent on cell surface heparin-containing proteoglycans. Adhesion assays were repeated, but cells were incubated with or without heparinase (HEPase) or chondroitinase ABC (ChaseABC), or soluble chondroitin A, B, or C, keratin sulfate, or heparin (50 μg/ml; 6 wells/data point; average ± SEM [error bar] are shown).
fibronectin, we performed adhesion assays, identical to those described above, except that cells were allowed to adhere for 40 min to increasing amounts of fibronectin. We showed that fibronectin promoted fibroblast attachment in a dose-dependent manner (Figure 2). To compare the abilities of CCN2 and fibronectin to promote fibroblast attachment, we coated tissue culture plates with 10 µg/ml CCN2 or 10 µg/ml fibronectin, and allowed suspended normal adult dermal fibroblasts to adhere to substrata for either 40 or 120 min. After removing nonadherent cells, attached cells were fixed in 4% paraformaldehyde and examined cells under a light microscope. Cells also were stained with phallolidin to detect F-actin (Figure 3, actin), anti-phospho-focal adhesion kinase (Figure 3, p-FAK), and anti-phospho-p42/p44 (Figure 3, p-ERK) antibodies. Fibroblasts adhered to CCN2 for 40 min were attached but filopodia did not form (Figure 3A, CCN2). By 120 min after attachment, fibroblasts adhered to CCN2 displayed lamellopodia (Figure 3B, CCN2, actin, arrow with l). Conversely, normal adult human dermal fibroblasts adhered to fibronectin for either 40 or 120 min displayed filopodial extensions (Figure 3A, FN, actin, arrow with f). In addition, cells adhered to fibronectin, but not to CCN2, showed formation of actin stress fibers (denoted by an arrow in Figure 3A, FN, actin), and induction of focal adhesions as visualized by punctuate cell surface anti-phospho-FAK staining (denoted by an arrow in Figure 3A, FN, p-FAK). These results are consistent with the known involvement of integrin-mediated FAK phosphorylation in

**Figure 2.** Fibronectin promotes adhesion of normal adult dermal fibroblasts in a dose-dependent manner. Wells of a 96-well plate were incubated overnight with different concentrations of fibronectin, as indicated. After a blocking step, primary adult dermal fibroblasts were allowed to adhere to fibronectin for 40 min, and cell adhesion was assessed as described in *Materials and Methods* (3 wells/data point; average [open diamond] ± SEM [error bar] are shown). The values obtained from a control adhesion experiment with BSA, which were not statistically significantly above background, were subtracted from the experimental values.

**Figure 3.** Comparison of adult dermal fibroblast adhesion to CCN2 and fibronectin. Staining of cells adhered to CCN2 and fibronectin with phalloidin (ACTIN), anti-phospho-FAK (p-FAK), and anti-phospho-ERK (p-ERK) antibodies are shown. Fibroblasts were allowed to adhere to substrates, as described in *Materials and Methods*, for (A) 40 or (B) 120 min before fixation of cells in paraformaldehyde. (A) Fibroblasts adhered to fibronectin for 40 min showed extension of filopodia (arrow with f, actin), actin stress fibers, and punctuate phospho-FAK staining (arrow, p-FAK, FN), consistent with the induction of focal adhesions. Conversely, fibroblasts adhered to CCN2 did not show extended filopodia, displayed disorganized actin staining, and did not show clustered, localized phospho-FAK staining (400×). Fibroblasts adhered to CCN2 and fibronectin showed punctuate foci of phospho-ERK staining (arrow, CCN2, FN, p-ERK), consistent with an induction of cellular signaling in fibroblasts adhered to both substrates. Conversely, cells adhered to BSA showed no phospho-ERK induction. Nuclei were detected by DAPI staining (blue).
Figure 4. Adhesion of adult dermal fibroblasts to fibronectin results in ERK phosphorylation. Normal adult dermal fibroblasts were cultured and detached as described in Materials and Methods and in the legend for Figure 1. Fibroblasts were allowed to adhere to six-well plates that had been coated with CCN2 (10 μg/ml) as described above. Protein extracts were prepared after adhesion at the times indicated. The resultant protein extracts (20 μg/lane) were subjected to SDS-PAGE and Western blot analysis with anti-ERK and anti-phospho-ERK antibodies. Densitometry was performed on the resultant autoradiograms, and ratio of phosphorylated/non-phosphorylated ERK is shown.

fibronectin-mediated fibroblast adhesion (Zamir and Geiger, 2001). Fibroblasts adhered to both fibronectin and CCN2 for 40 min showed induction of cellular signaling as visualized by punctuate phospho-ERK staining (denoted by arrows, Figure 4). Western blot analysis of lysates prepared from fibroblasts adhered to CCN2 verified that adhesion of fibroblasts to CCN2 resulted in ERK phosphorylation (Figure 4). The very small number of cells (>100-fold fewer than those adhering to CCN2 or FN) nonspecifically binding BSA showed no induction of phospho-ERK staining (Figure 3A, BSA, p-ERK). Overall, our data suggest that normal adult human dermal fibroblast adhesion to CCN2 and fibronectin seemed to be fundamentally distinct, in that adhesion of normal adult human dermal fibroblasts onto CCN2 does not result in the extension of filopodia, the induction of focal adhesions, or the formation of actin stress fibers.

Adult Human Dermal Fibroblast Attachment to CCN2 Requires MEK/ERK Activity

Because fibroblast attachment to both CCN2 and fibronectin resulted in the induction of the MEK/ERK pathway, we decided to compare the requirements for the MEK/ERK pathway in CCN2- and fibronectin-promoted fibroblast adhesion. Thus, we treated fibroblasts for 45 min with or without the MEK1/2 inhibitor U0126 (10 μM; Favata et al., 1998) before performing adhesion assays on fibronectin or CCN2. We found that preincubation with U0126 completely blocked the ability of CCN2 to promote attachment of adult fibroblasts (Figure 5). Conversely, U0126 did not appreciably affect the ability of adult human dermal fibroblasts to adhere to fibronectin (Figure 5). Because we had previously shown that the ability of CCN2 to promote attachment of adult dermal fibroblasts was HSPG dependent, we determined whether soluble heparin affected fibroblast attachment to fibronectin. We found that whereas, as shown above, soluble heparin blocked the ability of adult fibroblasts to adhere to CCN2, soluble heparin did not appreciably impact the number of fibroblasts that adhered to fibronectin (Figure 5). These data support the notion that attachment of adult fibroblasts to CCN2 and fibronectin possess different requirements, in that attachment of adult fibroblasts to CCN2, but not fibronectin, required both MEK/ERK activity and HSPGs.

CCN2 Binds Fibronectin, α4 α5, and β1 Integrins and Syndecan-4

Although it has been previously shown that CCN2 promotes fibroblast adhesion, the functional relevance of this activity has not yet been clarified. CCN2 has been proposed to modulate the ability of receptors to bind ligands (Abreu et al., 2002; Leask and Abraham, 2003). Thus, we hypothesized that the biological significance of CCN2-promoted fibroblast adhesion might be to enhance the ability of fibroblasts to interact with extracellular matrix components through cell surface receptors. To begin to test this hypothesis, we reasoned that CCN2 might bind the extracellular matrix component fibronectin. We coated wells of a 96-well tissue culture plate with BSA or fibronectin overnight, and, after a blocking step, added ¹²⁵I-labeled CCN2 for 1 h. After unbound ¹²⁵I-CCN2 was removed by washing, ¹²⁵I-CCN2 bound to fibronectin was detected using a gamma counter. We found that ¹²⁵I-CCN2 bound fibronectin, but not BSA (Figure 6A). These results were consistent with the notion that a physiological role of CCN2 might be to enhance the ability of fibroblasts to interact with fibronectin.

To continue our investigation into the role of CCN2-mediated adhesion to fibroblast biology, we cultured mouse embryonic fibroblasts, which constitutively express CCN2 (Holmes et al., 2001; Figure 6B) and thus can be used as a basis to probe the function of endogenous CCN2. We reasoned that if a function of CCN2 is to potentiate the ability of fibroblasts to adhere to fibronectin, endogenous cell-surface associated CCN2 might be in a complex with fibronectin and cell-surface fibronectin receptors. We cultured mouse embryonic fibroblasts to confluence and isolated membrane preparations from these cells. The resultant protein preparations were subjected to immunoprecipitation analysis with an anti-CCN2 antibody. The immunoprecipitated protein was then subjected to Western blot analysis with antibodies to fibronectin and the fibronectin receptors integrin α4, integrin β1, integrin α5, and syndecan 4. The latter protein is an HSPG that acts with integrin α5β1 to promote cell spreading on fibronectin (Saoncella et al., 1999). We found that endogenous membrane-bound CCN2 was present in a complex with fibronectin, and the fibronectin receptors syn-

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decan 4 and integrin α4, integrin α5, integrin β1, and syndecan-4 (Figure 6C). CCN2 did not interact with the protein endoglin (Figure 6C), an accessory TGFβ receptor (Leask et al., 2002). This latter suggests that CCN2 is not linked to TGFβ signaling via endoglin. CCN2 also was present in a complex containing syndecan 2 and integrin α6 (our unpublished data), consistent with the notion that CCN2 can interact with a variety of integrins and HSPGs (Gao and Brigstock, 2003; Leu et al., 2003; Leask and Abraham, 2003; Gao et al., 2004).

Ccn2−/− Fibroblasts Show Impaired Spreading on Fibronectin
Our immunoprecipitation results were consistent with the hypothesis that a physiological role of CCN2 might be to potentiate the ability of cell surface membrane proteins to adhere to fibronectin. Consequently, we wished to test the effect of the loss of CCN2 on fibroblast attachment and spreading on fibronectin, by comparing the ability of Ccn2+/+ and Ccn2−/− mouse embryonic fibroblasts to adhere to fibronectin. That Ccn2−/− mouse embryonic fibroblasts lacked CCN2 expression was confirmed by Western blot analysis (Figure 6, B and C). Due to culture conditions, fibroblasts can often become spontaneously differentiated into myofibroblasts, as visualized by α-SMA expression (Masur et al., 1996). Myofibroblasts mediate matrix contraction during wound healing and fibrosis (Gabbiani, 2003; Shi-wen et al., 2004). Indeed, immunofluorescence analysis showed that ~90% of Ccn2+/+ and Ccn2−/− fibroblasts had differentiated into myofibroblasts, as visualized by expression of α-SMA (our unpublished data). Because we were interested in exploring the contribution CCN2 may play in wound healing and fibrosis, we wanted to evaluate the impact of loss of CCN2 on the induction of α-SMA stress fibers upon cell attachment and spreading on fibronectin.

First, to detect whether both Ccn2+/+ and Ccn2−/− mouse embryonic fibroblasts showed similar levels of CCN2 receptors, we compared the ability of Ccn2+/+ and Ccn2−/− mouse embryonic fibroblasts to adhere to fibronectin.
We found that Ccn2+/+ and Ccn2−/− mouse myofibroblasts showed similar abilities to bind to CCN2 (Figure 7). To compare the abilities of Ccn2+/+ and Ccn2−/− mouse embryonic fibroblasts to induce α-SMA stress fibers, Ccn2+/+ and Ccn2−/− mouse embryonic fibroblasts were allowed to adhere to fibronectin for 40 min. Cells were then fixed with paraformaldehyde and subjected to indirect immunofluorescence with an anti-α-SMA antibody. After 40 min of attachment, Ccn2+/+ fibroblasts showed extensive spreading, extension of filopodia, and α-SMA localization at the cell periphery (denoted by an arrow with f, Figure 8, Ccn2+/+). A few stress fibers were observed (denoted by an arrow with a, Figure 8, Ccn2+/+). Ccn2+/+ fibroblasts showed a few α-SMA stress fibers after 40 min of attachment on fibronectin. Conversely, 40 min after attachment on fibronectin, Ccn2−/− fibroblasts showed neither extensive filopodia nor organization of α-SMA along the cell periphery or into stress fibers (Figure 8, Ccn2−/−). To assess whether the spreading defect displayed by Ccn2−/− fibroblasts could be rescued by recombinant CCN2, we subjected Ccn2−/− fibroblasts to a 1-h preincubation of Ccn2+/− fibroblasts at 4°C prior with recombinant CCN2. We then placed cells on fibronectin and examined the appearance of Ccn2−/− fibroblasts 40 min after attachment. We found that preincubating Ccn2−/− fibroblasts with recombinant CCN2 at least partially reversed the spreading defect of Ccn2−/− fibroblasts, because Ccn2+/− fibroblasts pretreated with CCN2 showed both α-SMA localization along the cell periphery and filopodial extensions (denoted by an arrow with f, Figure 8, Ccn2+/− + CCN2), and a few stress fibers (denoted by an arrow with a, Figure 8, Ccn2−/− + CCN2).

Collectively, our results are consistent with the hypothesis that CCN2 potentiates, but is not required for, the ability of embryonic fibroblasts to spread on fibronectin.

Figure 7. Comparison of the adhesive abilities of Ccn2+/+ and Ccn2−/− mouse embryonic fibroblasts on CCN2. Ccn2+/+ and Ccn2−/− mouse embryonic fibroblasts were cultured in DMEM, 0.5% FBS for 24 h; detached in EDTA; and allowed to adhere to CCN2 that had been adsorbed to 96-well plates as described in Materials and Methods and the legend to Figure 1. Cell adhesion after 40 min was determined (N = 6; average ± SEM is shown) as described in Materials and Methods and in the legend to Figure 1B.
immunofluorescence analysis, Ccn2-/- fibroblasts showed substantially reduced levels of FAK and ERK phosphorylation (Figure 10, A and B). To confirm these results, we incubated Ccn2+/+ and Ccn2-/- embryonic fibroblasts for up to 2 h on fibronectin and subjected cell lysates to Western blot analysis with anti-phospho-FAK and anti-phospho-ERK antibodies. Giving support to our immunofluorescence data, we found that although antibodies. Giving support to our immunofluorescence data, up to 2 h on fibronectin and subjected cell lysates to Western incubation (Figure 10, A and B). To confirm these results, we substantially reduced levels of FAK and ERK phosphorylation (Figure 10C). Thus, CCN2 was required for maximal induction of FAK and ERK phosphorylation upon the adhesion of Ccn2+/+ mouse embryonic fibroblasts to fibronectin.

**Soluble Heparin and U0126 Impede Fibroblast Spreading on Fibronectin**

Because we had shown that fibroblast adhesion to CCN2 was blocked by soluble heparin and U0126, we tested the impact of adding soluble heparin or U1026 to Ccn2+/+ mouse embryonic fibroblasts before placing cells on fibronectin. We found that heparin or U1026 potently impeded the ability of Ccn2+/+ fibroblasts to spread on fibronectin. Even 120 min after adding Ccn2+/+ fibroblasts to fibronectin, cells pretreated with U1026 or soluble heparin showed markedly reduced numbers of both filopodia and α-SMA stress fibers (Figure 11). Intriguingly, heparin or U0126 did not affect the ability of cells to attach to fibronectin (our unpublished data). Our results showed that the effect on cell spreading of adding soluble heparin or U1026 to Ccn2+/+ fibroblasts was more severe than the spreading phenotype displayed by Ccn2-/- fibroblasts. Collectively, these results are consistent with the notion that the ability of CCN2 to interact with HSPGs and induce the MEK/ERK cascade is not absolutely required for fibroblast spreading or α-SMA stress fiber induction. Rather, these results strongly suggest that signaling through CCN2 results in the enhancement of spreading of Ccn2+/+ mouse embryonic fibroblasts on fibronectin. Our results support the hypothesis that the biological significance of CCN2-promoted fibroblast adhesion is to enhance the ability of fibroblasts to interact with the matrix component fibronectin through cell surface receptors.

**DISCUSSION**

Although CCN2 was discovered over a decade ago, the precise biological function of CCN2 remains elusive. When coated on glass or plastic, CCN2 is to promote cell attachment. However, the physiological significance of this activity has not been previously elucidated. In this report, we uncover several novel findings. First, we show that inhibition of the MEK/ERK cascade prevents CCN2-promoted fibroblast adhesion. Second, we show that CCN2 binds fibronectin and fibronectin receptors. Third, we show that endogenous CCN2 potentiates the cell adhesive properties of fibronectin. A novel implication of our results is that a biological role of CCN2 might be to promote cell attachment to fibronectin. In development, CCN2 is expressed during tissue remodeling (Ivkovic et al., 2003). Similarly, in adults, although CCN2 is not normally expressed by adult dermal fibroblasts, CCN2 is potently induced in this cell type during normal tissue repair and fibrotic disease (Igarashi et al., 1993, 1995; Shi-wen et al., 2000; Leask and Abraham, 2003; Leask et al., 2004). Accordingly, we hypothesize that CCN2 may promote tissue remodeling and repair in vivo by facilitating the adhesion of cells to the provisional matrix. This activity may be crucial for the enhanced ability of fibrotic fibroblasts to contract extracellular matrix (Shi-wen et al., 2004). Direct adhesion of adult dermal fibroblasts to CCN2 did not result in appreciable filopodia, FAK phosphorylation, or induction of stress fibers. However, adhesion of human foreskin fibroblasts to CCN2 was recently shown to result in extension of filopodia, FAK phosphorylation, and stress fiber formation (Chen et al., 2001a). The differences in our results may have arisen due to the different cell types used for our respective studies, because fibroblasts taken from different parts of the body show different expression profiles (Chang et al., 2002). Supporting this latter notion, Chen et al. (2001a) also showed that the CCN2-related protein CCN1 (Cyr61) promoted adhesion of human foreskin fibroblasts, resulting in the extension of filopodia and FAK phosphorylation. However, CCN1 (Cyr61) prepared by the same laboratory has been shown to promote the adhesion of cells isolated from *Xenopus* blastulae, without resulting in the extension of filopodia (Latinkic et al., 2003). Instead, in these series of experiments CCN1 (Cyr61)-promoted adhesion of *Xenopus* blastulae cells resulted in the formation of lamellipodia (Latinkic et al., 2003), similar to our results obtained.
when we examined CCN2 (CTGF)-promoted attachment of normal adult human dermal fibroblasts. These cell type-dependent variations in responses may ultimately arise due to differences in the receptors engaged by CCN2 (and CCN1) on these particular cell types.

That CCN family members may engage different receptors depending on the cell type is supported by a series of observations suggesting that although recombinant CCN2 adsorbed to plastic promotes cell adhesion through integrins and HSPGs (Babic et al., 1999; Chen et al., 2001a; Ball et al., 2003), the actual integrins through which CCN2 mediates adhesion vary depending on the cell type (Babic et al., 1999; Chen et al., 2001a; Schober et al., 2002; Leu et al., 2003). Similarly, the HSPGs that CCN2 interacts with may vary depending on the system (this report; Gao and Brigstock, 2003; Nishida et al., 2003). The notion that CCN2 can interact with a variety of integrins and HSPGs is supported by our observations that CCN2 is present in

Figure 10. CCN2−/− mouse embryonic fibroblasts show impaired phosphorylation of FAK and ERK. Ccn2+/+ and Ccn2−/− mouse embryonic fibroblasts were allowed to adhere to fibronectin (FN), as described in Materials and Methods, for up to 120 min (min on FN). (A and B) Indirect immunofluorescence analysis. Cells were fixed as described in Materials and Methods and in the legend to Figure 7 and were stained with anti-phospho-ERK (A) and anti-phospho-FAK (B) antibodies. Whereas Ccn2+/+ MEFs showed abundant phospho-ERK (arrow, A, Ccn2+/+ 40) and phospho-FAK (arrow, Ccn2+/+ 120) staining, Ccn2−/− MEFs showed markedly reduced phospho-ERK and phospho-FAK staining (A and B, Ccn2−/−). Nuclei were detected by DAPI staining (blue). (C) Western blot analysis. Experiments were performed as described above, except cell layers were harvested at different time points postadhesion (min on FN), and equal amounts of protein were subjected to Western blot analysis with anti-phospho-FAK and anti-FAK antibodies or anti-phospho-ERK and ERK antibodies, as indicated. Densitometry values showing ratio between phosphorylated epitopes and total protein, are indicated.
a complex in the cell surface of fibroblasts with several different integrins and HSPGs (Figure 6). Although CCN1 has not been as extensively studied as CCN2, CCN1 may also bind a variety of integrins and HSPGs (Grzeszkiewicz et al., 2002; Schober et al., 2002). Adding further complexity to this issue, CCN2 promotes adhesion of hepatic stellate cells through integrin αvβ3 HSPGs and low-density lipoprotein-receptor related protein (Gao and Brigstock, 2003, 2004), which had previously been shown to be involved with internalization and degradation of CCN2 (Segarini et al., 2001). We hypothesize that CCN2 (or CCN1) might not promote adhesion through a specific receptor but exert its function through a variety of proadhesive cell surface receptors, albeit through specific domains in the CCN2 molecule (Brigstock et al., 1997; Ball et al., 2003; Leu et al., 2003; Gao and Brigstock, 2004).

Fibronectin promotes adhesion, spreading, and attachment via integrins α5β1 and α4β1 and the HSPG coreceptor syndecan 4 (Saoncella et al., 1999; Zamir and Geiger, 2001; Wilcox-Adelman et al., 2002). Whereas the ability of fibroblasts to attach on fibronectin depends on integrins, the ability of fibroblasts to spread on fibronectin requires the heparin binding domain of fibronectin and syndecan 4 (Saoncella et al., 1999; Zamir and Geiger, 2001; Wilcox-Adelman et al., 2002). However, in this report, for the first time we show, that ERK activation is required for spreading of fibroblasts on fibronectin, and for the induction of α-SMA stress fibers. That spreading on fibronectin requires HSPGs and ERK is consistent with our hypothesis that, although CCN2 is not absolutely required for the spreading of fibroblasts and induction of α-SMA stress fibers, CCN2 potentiates the ability of fibroblasts to spread on fibronectin through ERK activation and by modulating the interaction between integrins α4, α5, β1, syndecan 4, and fibronectin (Figure 12). We would anticipate that CCN2 might generally act by potentiating the ability of cells to adhere to other components of the extracellular matrix. Intriguingly, CCN2 has been recently shown to promote the ability of hepatic stellate cells to proliferate through ERK, suggesting that CCN2 may generally function through MEK/ERK activation (Gao et al., 2004). We have found that ERK activation may be a key component of fibrogenesis (Stratton et al., 2002). Thus, the CCN2-mediated activation of ERK may play a role in this process.

It is possible that the defects in cell spreading observed with our Ccn2−/− fibroblasts could be explained due to the fact that Ccn2−/− fibroblasts display reduced levels of receptors for CCN2 or fibronectin. However, we...
Figure 12. Schematic diagram summarizing CCN2-promoted fibroblast adhesion. CCN2 supports cell adhesion by binding to fibronectin, cell surface proteoglycans, and integrins; potentiates the phosphorylation of FAK and ERK; and thus enhances focal adhesion formation, cell spreading, and myofibroblast formation.

believe that this interpretation is unlikely because 1) Ccn2+/+ and Ccn2−/− fibroblasts show similar abilities to adhere to CCN2 and 2) a 1-h preincubation at 4°C of Ccn2−/− fibroblasts with recombinant CCN2 before allowing spreading on fibronectin at least partially rescued the spreading defect of the Ccn2−/− fibroblasts (Figure 8). Although beyond the scope of this current study, we are currently in the process of comparing the gene expression profiles of Ccn2+/+ and Ccn2−/− fibroblasts. Preliminary data suggest that Ccn2−/− fibroblasts do not show reduced levels of fibronectin receptor transcription (A. Holmes, D.J.A, X. Shi-wen, C.M.B., K.L., A.L., unpublished data). However, “rescued” Ccn2−/− cells did not quite show the same degree of spreading as Ccn2+/+ fibroblasts. The full repertoire of CCN2 binding proteins has not yet been identified; Ccn2−/− cells may possess some qualitative aberrations in integrin/HSPG organization or may show altered expression of signal transduction components required for CCN2 action. Addressing this issue requires further investigations.

Collectively, our results are consistent with the recently proposed hypothesis that CCN2 may generally function as an adaptor molecule that promotes binding of extracellular proteins to their cell surface receptors (Leask and Abraham, 2003). For example, a recent report showed that CCN2 bound TGFβ through a binding site in the amino-terminal Von-Willebrand factor domain of CCN2 and assisted in presenting TGFβ to the high-affinity TGFβ type II receptor (Abreu et al., 2002). We hypothesize CCN2 may generally act by modulating the activity of specific receptor/ligand interactions and thus may influence a range of signaling events by binding cell surface HSPGs via the heparin-binding carboxyl-terminal domain and by interacting with other receptors or ligands, whose identity may be specified depending on the context, through other domains in the CCN2 protein (Leask and Abraham, 2003). In this manner, CCN2 may play a broad role in matrix organization and play diverse roles in signal transduction through promoting cell adhesion and/or potentiating the signaling mediated by other receptors and by acting as a molecular bridge aiding in the integration between extracellular and intracellular signaling networks.

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REFERENCES


