CCN3 (NOV) Is a Novel Angiogenic Regulator of the CCN Protein Family*

Received for publication, February 26, 2003, and in revised form, April 9, 2003
Published, JBC Papers in Press, April 13, 2003, DOI 10.1074/jbc.M302028200

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CCN3 (NOV) is a matricellular protein of the CCN family, which also includes CCN1 (CYR61), CCN2 (CTGF), CCN4 (WISP-1), CCN5 (WISP-2), and CCN6 (WISP-3). During development, CCN3 is expressed widely in derivatives of all three germ layers, and high levels of expression are observed in smooth muscle cells of the arterial vessel wall. Altered expression of CCN3 has been observed in a variety of tumors, including hepatocellular carcinomas, Wilms's tumors, Ewing's sarcomas, gliomas, rhabdomyosarcomas, and adrenocortical carcinomas. To understand its biological functions, we have investigated the activities of purified recombinant CCN3. We show that in endothelial cells, CCN3 supports cell adhesion, induces directed cell migration (chemotaxis), and promotes cell survival. Mechanistically, CCN3 supports human umbilical vein endothelial cell adhesion through multiple cell surface receptors, including integrins αβ2, αβ3, αβ5, and heparan sulfate proteoglycans. In contrast, CCN3-induced cell migration is dependent on integrins αβ2 and αβ5, whereas αβ1 does not play a role in this process. Although CCN3 does not contain a RGD sequence, it binds directly to immobilized integrins αβ2 and αβ5, with half-maximal binding occurring at 10 nM and 50 nM CCN3, respectively. Furthermore, CCN3 induces neovascularization when implanted in rat cornea, demonstrating that it is a novel angiogenic inducer. Together, these findings show that CCN3 is a ligand of integrins αβ2 and αβ5, acts directly upon endothelial cells to stimulate pro-angiogenic activities, and induces angiogenesis in vivo.

CCN3 (NOV, nephroblastoma overexpressed) was originally identified as an aberrantly expressed gene in avian nephroblastomas induced by myeloblastosis-associated virus (1, 2). It is a member of the CCN family, which also includes CCN1 (CYR61), CCN2 (CTGF), CCN4 (WISP-1), CCN5 (WISP-2), and CCN6 (WISP-3). During development, CCN3 is expressed widely in derivatives of all three germ layers, and high levels of expression are observed in smooth muscle cells of the arterial vessel wall. Altered expression of CCN3 has been observed in a variety of tumors, including hepatocellular carcinomas, Wilms's tumors, Ewing's sarcomas, gliomas, rhabdomyosarcomas, and adrenocortical carcinomas. To understand its biological functions, we have investigated the activities of purified recombinant CCN3. We show that in endothelial cells, CCN3 supports cell adhesion, induces directed cell migration (chemotaxis), and promotes cell survival. Mechanistically, CCN3 supports human umbilical vein endothelial cell adhesion through multiple cell surface receptors, including integrins αβ2, αβ3, αβ5, and heparan sulfate proteoglycans. In contrast, CCN3-induced cell migration is dependent on integrins αβ2 and αβ5, whereas αβ1 does not play a role in this process. Although CCN3 does not contain a RGD sequence, it binds directly to immobilized integrins αβ2 and αβ5, with half-maximal binding occurring at 10 nM and 50 nM CCN3, respectively. Furthermore, CCN3 induces neovascularization when implanted in rat cornea, demonstrating that it is a novel angiogenic inducer. Together, these findings show that CCN3 is a ligand of integrins αβ2 and αβ5, acts directly upon endothelial cells to stimulate pro-angiogenic activities, and induces angiogenesis in vivo.

This paper is available on line at http://www.jbc.org
nist to CCN1 and CCN2, and play antithetical roles in similar biological processes. Since CCN1 and CCN2 have been shown to be angiogenic inducers (12, 16, 17), we speculated that CCN3 might work as an angiogenic inhibitor. In this study, we show that purified recombinant CCN3 interacts with multiple integrin receptors and binds directly to integrins $\alpha_5\beta_1$ and $\alpha_6\beta_1$. Contrary to expectation, CCN3 is pro-angiogenic in endothelial cells, supports cell adhesion and survival. Furthermore, CCN3 induces neovascularization in a corneal micropocket assay. These results identify CCN3 as a novel angiogenic factor and suggest its possible functions in development and tumor growth.

MATERIALS AND METHODS

Cell Culture—HUVECs were grown as described by the supplier (Cascade Biologics, Inc., Portland, OR) and used before passage 16. BACEs (a generous gift from Dr. Judah Folkman, Harvard Medical School) were grown in plates coated with 1.5% gelatin (Difco Laboratories, Detroit, MI) and maintained in Dulbecco's modified Eagle's medium (DMEM, JRH Bioscience, Lenexa, KS) supplemented with 10% fetal bovine serum (Intergene, Purchase, NY) and 3 ng/ml bFGF (In vitrogen, Carlsbad, CA); they were used before passage 16.

Antibodies, Peptides, and Reagents—Function-blocking mAbs against $\alpha_5\beta_1$ and $\alpha_6\beta_1$ were purchased from Chemicon (Temecula, CA) IV1 (anti-$\alpha_5$), LM609 (anti-$\alpha_6\beta_1$), and JB55 (anti-$\alpha_6\beta_1$). Beckman-Coulter, Inc. (Fullerton, CA) [NK1-GoH3 (anti-$\alpha_3$), and SAM-1 (anti-$\alpha_3$)], and Invitrogen [PC10 (anti-$\alpha_3$)]. Normal mouse IgG was from Zymed Laboratories, Inc. (South San Francisco, CA) and normal rabbit serum was from Sigma-Aldrich. GRGDSP and GRGESP peptides were purchased from Invitrogen. RGDS and RGE5 peptides were from American Peptide Company, Inc. (Sunnyvale, CA). Heparin (sodium salt, from porcine intestinal mucosa) was from Sigma-Aldrich. bFGF, FN, VN, and LN were obtained from Invitrogen. Purified integrins $\alpha_5\beta_1$ and $\alpha_6\beta_1$ were from Chemicon.

Purification of Recombinant CCN3—Human CCN3 cDNA was constructed by ligation of a 5' (nt 72–654, GenBank37 X696584) and 3' (nt 654–1653) fragments, and the resulting full-length cDNA was cloned into pK5+ and verified by sequencing. The 5' fragment (nt 72–654) was obtained by reverse transcriptase-polymerase chain reaction using total RNA isolated from serum-starved human skin fibroblasts using the primer set 5'-AGCACTGCACATCTACAGC-3' and 5'-CAGCATCTCA-CATTGAGCGG-3'. The RT-PCR product was digested with SphI and StyI to yield a fragment containing nt 72–654. The 3' fragment (nt 654–1653) was generated by restriction digestion of IMAGE clone 49415 (human neuronal brain, nt 590–1653) with SphI and XhoI. To produce recombinant CCN3 protein, the full-length CCN3 cDNA was cloned into the baculovirus expression vector pBlueBac 4.5 (Invitrogen). The vector was modified to encode an enterokinase histidine tag linked to the C terminus of CCN3 in a manner similar to that previously described for expression of CCN1 (21). CCN3 was produced in serum-free medium containing 0.5% BSA. Coverslips were coated overnight at 4 °C with 2.5 mg/ml mouse LN (ultrapure grade; Becton-Dickinson Biosciences, Bedford, MA) and blocked with 1% heat-inactivated BSA. Cells were loaded into wells of the lower chamber; the wells were then covered with a gelatinized polycarbonate filter (5 μm pore diameter, Nuclepore) followed by the membrane modified Eagle's medium containing 0.1% BSA. Cells were loaded into wells of the lower chamber; the wells were then covered with a gelatinized polycarbonate filter (5 μm pore diameter, Nuclepore) followed by the upper chamber. Where indicated, cells were either mixed with EDTA, Mg2+, or peptides prior to plating, or incubated with function-blocking monoclonal antibodies for 30 min at 4 °C prior to plating. After washing, bound integrins were detected with polyclonal anti-integrin $\alpha_5$ (AB1930) or $\alpha_6$ antibodies (AB1926) followed by staining with anti-CCN3 antibodies, mAbs against integrin $\alpha_3$ (P3G8) or $\beta_1$ (HUTS-4) followed by goat anti-mouse antibody and AP-labeled secondary antibody (1:5000), and color reaction was developed using a non-immunoperoxidase DAB kit (Zymed Laboratories, Inc.) with absorbance measured at 420 nm.

Cell Migration Assay—A 48-well modified Boyden chamber (Neuro Probe, Inc., Gaithersburg, MD) was used to assay cell migration as described with modifications (16). BACEs were harvested with trypsin, washed, and resuspended at 5 × 105 cells/ml in Dulbecco's modified Eagle's medium containing 0.1% BSA. Cells were plated into wells of the lower chamber; the wells were then covered with a gelatinized polycarbonate filter (5 μm pore diameter, Nuclepore) followed by the upper chamber. Where indicated, cells were either mixed with peptides or incubated with antibodies (1 h at room temperature) prior to loading. The chamber was inverted to allow cells to attach (2 h at 37 °C) and fixed in 10% formalin in Dulbecco's modified Eagle's medium containing 0.1% BSA, and incubated at 37 °C for 1 h. The membrane was removed and stained using a Diff-Quik Kit (Dade-Behring, Deerfield, IL). Cell migration was monitored by counting the total number of (BACE) cells that migrated in 10 randomly selected microscopic fields at ×400 magnification.

Measurement of Apoptosis and DNA Synthesis—Apoptosis and mitogenesis was assessed largely as described (14). To measure apoptosis, HUVECs were starved for 16 h, harvested, and resuspended in serum-free medium containing 0.5% BSA. Coverslips were coated overnight at room temperature with 10 μg/ml mouse TNF-α (Amersham Biosciences). Coverslips were coated overnight at room temperature with 10 μg/ml mouse TNF-α (Amersham Biosciences) and incubated with 1% heat-inactivated BSA. Cells were plated for 1 h at 37 °C. Soluble CCN3, serum (20%) or GRGESP peptide (50 μM) was added, and incubation continued for 24 h.
another 16 h at 37°C. Where indicated, CCN3 was incubated with anti-CCN3 antibodies for 1 h at room temperature prior to addition to cells. After incubation, cells were fixed with 4% paraformaldehyde (pH 7.4) and apoptosis was detected by TUNEL assay using the in situ cell death detection kit POD (Roche Applied Science, Indianapolis, IN). Cells were lightly stained with hematoxylin and apoptotic nuclei counted. A total of 500 cells were counted from random fields in each coverslip, and the number of apoptotic cells was represented as a percentage of the total cells counted. To assess proliferation, cells were grown as described above except that 10 nM bromodeoxyuridine (BrdUrd) was included in the medium for 16 h in the presence or absence of CCN3. BrdU incorporation was detected using the BrdUrd staining kit (Calbiochem Novabiochem Corp., San Diego, CA).

**Rat Corneal Pocket Angiogenesis Assay**—CCN3-induced neovascularization was examined *in vivo* by implanting Hydron pellets, formulated with test substances, into rat corneas essentially as described (16). Briefly, male Sprague-Dawley rats were anesthetized and Hydron pellets (Interferon Sciences, Inc., New Brunswick, NJ) containing test substances were implanted into micropockets made in the corneal stroma 1 to 1.5 mm from the corneal limbus. Where indicated, CCN3 and bFGF were incubated with anti-CCN3 antibodies for 1 h at room temperature prior to being incorporated into the Hydron pellet. 7 days postimplantation, rats were perfused with India ink with heparin (100-U bolus), and neovascularization was examined and scored.

**RESULTS**

**CCN3 Mediates Endothelial Cell Adhesion through Integrins**

CCN3 and CCN2 are known to mediate HUVEC adhesion through integrin αvβ3 (19) and fibroblast adhesion through integrin α6β1 and heparan sulfate proteoglycans (8, 20). HUVEC adhesion to CCN3, as well as to FN or VN, was inhibited by EDTA and restored by the addition of Mg2+ or Ca2+ (Fig. 2C). These results are consistent with the notion that divalent cation-dependent cell adhesion molecules, such as integrins, mediate cell adhesion to CCN3 (Fig. 2C). To define the specific integrins that might be involved, we investigated the inhibitory effect of RGD peptide. The GRGDSP peptide, but...
not the control GRGESP peptide, was able to partially block cell adhesion to CCN3 and completely block adhesion to VN (Fig. 2D), indicating the involvement of RGD-sensitive integrins. Since integrin αvβ5 is known to be inhibited by a relatively low concentration of RGD peptide (0.2 mM) as shown in Fig. 2D, we examined the role of this integrin. Cells were incubated with LM609, a mAb against integrin αvβ5, prior to plating. LM609 partially blocked HUVEC adhesion to CCN3 and VN but not to FN (Fig. 3B). We also tested the possible involvement of integrin αvβ3 given its RGD sensitivity, even though no other CCN protein is known to bind this integrin. To our surprise, mAb against integrin αvβ3 (JSB5) was also able to partially block HUVEC adhesion to CCN3 and FN (Fig. 3C). As expected, the same mAb had no effect on HUVEC adhesion to VN. These results indicate that both integrins αvβ1 and αvβ3 play a role in HUVEC adhesion to CCN3.

Since it was previously shown that CCN1 and CCN2 mediate adhesion of fibroblasts through integrin αvβ3 and heparan sulfate proteoglycans acting as co-receptors (8, 20), we investigated whether these two receptors also mediate HUVEC adhesion to CCN3. To investigate the potential role of integrin αvβ3, cells were incubated with mAbs against either integrin αv (GoH3) or β3 (P4C10) subunit prior to plating. These mAbs partially blocked adhesion to CCN3, indicating that integrin αvβ3 also plays a role in HUVEC adhesion to CCN3 (Fig. 3A). As expected, these mAbs partially blocked cell adhesion to LN, which binds integrins αvβ1 and αvβ3, but not to VN, which binds αv integrins. Soluble heparin is known to block fibroblast adhesion to CCN1 and CCN2 by saturating the heparin binding sites located in the CT domain, thereby preventing them from binding cell surface heparan sulfate proteoglycans (8, 20). Likewise, soluble heparin partially blocked HUVEC adhesion to CCN3, but not to FN or VN (Fig. 2D). Thus, it is likely that CCN3 may also engage heparan sulfate proteoglycans as a co-receptor when interacting with integrin αvβ3.

Given that antagonists of integrins αvβ3, αvβ1, and αvβ3 were able to partially inhibit HUVEC adhesion to CCN3, we tested whether the combination of antagonists of these integrins were sufficient to account for endothelial cell adhesion to CCN3. When cells were treated with GRGDS peptide and soluble heparin, CCN3 adhesion was obliterated (Fig. 2D). In contrast, adhesion of the same cells to FN was not affected. Also, when HUVECs were incubated with function-blocking mAbs against integrins αvβ1, αvβ3, and α5β1, cell adhesion to CCN3 was completely abolished (Fig. 3D). As expected, HUVEC adhesion to FN, VN, and laminin was only partially blocked by these antibodies. Together, these results indicate that endothelial cell adhesion to CCN3 is mediated through the combined actions of integrins αvβ3, αvβ1, and α5β1.

**CCN3 Binds Directly to Integrins αvβ3 and αvβ1 in Vitro—**

Since CCN3 mediates HUVEC adhesion through integrins αvβ3, αvβ1, and α5β1 (Fig. 3D), we investigated whether CCN3 can bind integrin receptors directly. Purified integrins αvβ3 or αvβ1 were immobilized on microtiter wells, then CCN3 was added in varying concentrations, and binding was detected using anti-CCN3 antibodies. As shown in Figs. 4A and 5A, CCN3 was able to bind immobilized integrins αvβ3 and α5β1 in a dose-dependent and saturable manner, with half-maximal binding occurring at 0.4 μg/ml (10 mM) and 1 μg/ml (50 mM) CCN3, respectively. Conversely, CCN3 was immobilized on microtiter wells and allowed to interact with integrins αvβ3 or αvβ1. Binding of integrins to immobilized CCN3 was observed using antibodies against integrin αv or α5 subunits (Figs. 4, B–D and 5, B–D).

We employed a variety of antagonists to address the specificity of the interaction between CCN3 and integrins αvβ3 and αvβ1. When CCN3 was incubated with affinity-purified anti-CCN3 polyclonal antibodies, binding to integrins αvβ3 and αvβ1...
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CCN3 binds directly to integrin \( \alpha_\beta_2 \). A, microtiter wells were coated with purified integrin \( \alpha_\beta_2 \) (1 \( \mu \)g/ml) and blocked with 1% BSA. Binding of varying concentrations of CCN3 was detected using anti-CCN3 antibodies. B–D, microtiter wells were coated with CCN3 (10 \( \mu \)g/ml) or VN (1 \( \mu \)g/ml) and blocked with BSA. Effects of preincubation of coated proteins with anti-CCN antibodies (B), 20 \( \mu \)g/ml LM609 (D), or 20 \( \mu \)g/ml normal mouse IgG (D) prior to addition and binding of integrin \( \alpha_\beta_2 \) was observed. C, integrin \( \alpha_\beta_2 \) was incubated with 5 mM EDTA, EDTA + 10 mM MgCl\(_2\), 0.2 mM RGDPS peptide, or 0.2 mM RGES peptide for 30 min at 4 °C prior to addition into microtiter wells. Binding was detected using anti-\( \alpha_\delta \) antibodies. Data shown are from three separate experiments and represented as mean ± S.D. of duplicate determinations in each experiment.

was abolished (Figs. 4B and 5B). By contrast, incubation of FN (Fig. 5B) or VN (Fig. 4B) with the same antibodies had no effect, as expected. Divalent cations are required for integrin function, and both integrins \( \alpha_\beta_2 \) and \( \alpha_\beta_1 \) can be inhibited by RGD-containing peptides (41). As shown in Figs. 4C and 5C, EDTA completely abrogated the binding of integrins \( \alpha_\beta_2 \) and \( \alpha_\beta_1 \) to CCN3, as well as to VN and FN. As expected, binding was restored upon the addition of MgCl\(_2\), RGDPS peptide, but not RGES peptide, was able to inhibit binding of integrins \( \alpha_\beta_2 \) and \( \alpha_\beta_1 \) to CCN3 and to positive controls, VN or FN. Furthermore, mAb against integrin \( \alpha_\beta_1 \) (LM609) blocked binding of integrin \( \alpha_\beta_2 \) to CCN3 and VN (Fig. 4D), and mAb against integrin \( \alpha_\beta_1 \) (JBS5) inhibited binding of integrin \( \alpha_\beta_2 \) to CCN3 and FN (Fig. 5D). Taken together, these results show that CCN3 is a novel ligand of integrins and binds directly and specifically to integrins \( \alpha_\beta_2 \) and \( \alpha_\beta_1 \).

BACECs Migrate to CCN3 Through Integrins \( \alpha_\beta_2 \) and \( \alpha_\beta_1 \)

Cell migration is an integral part of the angiogenic process, and induction of endothelial cell chemotaxis correlates with angiogenic activity (42). We thus investigated whether CCN3 can stimulate migration of endothelial cells. Using a modified Boyden chamber assay, we found that CCN3 was able to stimulate migration of BACECs (Fig. 6A). CCN3-induced migration was dose-dependent and reached maximal level at 0.25 \( \mu \)g/ml. A higher concentration of CCN3 was less effective in inducing cell migration, resulting in a bell-shaped dose response curve observed for many chemotactic factors. Incubation of CCN3 protein with affinity-purified anti-CCN3 antibodies abolished CCN3-induced migration, while incubation with the same antibodies did not affect FN-stimulated migration (Fig. 6B), indicating that cell migration can be attributed to CCN3.

Stimulation of cell migration can be due to a chemotactic (directed cell movement) or a chemokinetic (random cell movement) response. In order to determine whether CCN3 induces chemotaxis or chemokinesis, a checkerboard analysis was performed. CCN3 was placed in the upper chamber (no cells), in the lower chamber (with cells), and in both or neither chambers (Fig. 6C). Addition of CCN3 to the lower chamber did not enhance BACEC migration to the upper chamber, indicating that CCN3 did not induce a chemokinetic response. Addition of CCN3 to the upper chamber induced the maximal level of migration, consistent with a chemotaxis. Addition of CCN3 to both chambers reduced the level of BACEC migration, suggesting that BACECs are sensitive to a CCN3 gradient. Together, these results show that CCN3 induces directed endothelial cell migration.

Since we have shown that CCN3 is a ligand for integrins \( \alpha_\beta_2 \) and \( \alpha_\beta_1 \), both of which can mediate cell migration, we tested whether CCN3 promotes BACEC migration through these integrins. Incubation of cells with GRGDS peptide completely inhibited migration to CCN3 and VN (Fig. 6D). No effect on migration was observed with cells incubated with GRGES control peptide. Consistent with the involvement of integrin \( \alpha_\beta_2 \), anti-integrin \( \alpha_\delta \) mAbs (AV1), and anti-integrin \( \alpha_\beta_1 \) mAbs (LM609) partially block BACEC migration to CCN3 (Fig. 7A). As expected, these antibodies inhibited BACEC migration to VN but had no effect on cell migration to FN. In addition, anti-integrin \( \alpha_\delta \) mAb (SAM-1) partially inhibited CCN3-stimulated cell migration but not VN-stimulated cell migration (Fig. 7B), indicating that integrin \( \alpha_\beta_2 \) may also be involved. Moreover, a combination of mAbs against integrin \( \alpha_\beta_2 \) and \( \alpha_\delta \) mAbs (LM609 and SAM-1) completely abolished BACEC migration to CCN3. As expected, these mAbs partially inhibited cell migration to VN and FN and had no effect on cell migration to laminin (Fig. 7C). To address whether integrin \( \alpha_\beta_1 \) might also play a role, we examined the inhibitory activity of the anti-\( \alpha_\delta \) mAb, GoH3 (Fig. 7D). BACEC migration to CCN3 or FN was unaffected by the presence of GoH3, whereas cell migration to
laminin was partially inhibited. Taken together, these results show that of the three integrins known to interact with CCN3 (Figs. 2–5), integrins \( \alpha_5\beta_1 \) and \( \alpha_6\beta_1 \) mediate endothelial cell migration to CCN3, whereas integrin \( \alpha_5\beta_1 \) is not involved in this process.

**CCN3 Promotes Endothelial Cell Survival**—During angiogenesis, endothelial cells require survival signals in order to migrate, proliferate, and interact with the remodeling ECM. Ligation of integrin \( \alpha_5\beta_1 \) has been shown to induce survival signals in endothelial cells (43). To investigate the possibility that CCN3 may promote cell survival, HUVECs were plated on laminin and maintained in serum-free medium. Under these conditions, endothelial cells were susceptible to apoptosis (44). GRGDSP peptide, 20% serum, or varying concentrations of CCN3 were then added to cells, and apoptosis was determined using a TUNEL assay after 16 h (Fig. 8A). Under these conditions, CCN3 was able to promote endothelial cell survival in a dose-dependent manner. Serum also protected cells from apoptosis, while GRGDSP peptide promoted apoptosis. Preincubation of CCN3 with affinity-purified anti-CCN3 antibodies abol-
Fig. 7. Integrins α₁β₁ and α₅β₁ mediate migration of BACEC to CCN3. Migration assays were performed using a modified boyden chamber. As chemotactic agents, CCN3 (0.25 μg/ml), vitronectin (10 μg/ml), and FN (10 μg/ml) were placed in the top chamber. A, cells were treated with anti-integrin α₁ (AV1, 60 μg/ml) or anti-integrin α₅β₁ (LM609, 60 μg/ml) for 1 h prior to chamber loading. Cells were also preincubated with either anti-integrin α₁ (SAM-1, 40 μg/ml) or both SAM-1 (12.5 μg/ml) and LM609 (25 μg/ml) (C), or anti-integrin α₅ (GoH3, 25 μg/ml) (D) for 1 h prior to chamber loading. Data shown are mean ± S.D. of triplicate determinations and are representative of three experiments.

Fig. 8. CCN3 protects HUVECs from apoptosis. HUVECs were serum-starved prior to attachment to coverslips pre-coated with 20 μg/ml LN. A, cells were then incubated in serum-free medium for 4 h. This was followed by addition of serum, various concentrations of CCN3 protein or 50 μM GRGDS peptide and incubation at 37 °C for an additional 16 h. Cells were fixed and apoptosis was monitored by using a TUNEL assay; the number of apoptotic cells were then counted. In the last bar, 10 μg/ml CCN3 was preincubated with anti-CCN3 antibodies prior to addition into the medium. B, HUVECs treated as described in panel A were labeled with BrdUrd for 16 h, and cells incorporating label in the absence or presence of CCN3 are quantified by colorimetric determination. Data shown are mean ± S.D. of triplicate determinations and are representative of three experiments.

DISCUSSION

Although CCN3 was first identified more than 10 years ago, little is known about its biochemical activities and biological functions. In this study, we show that purified CCN3 is capable of pro-angiogenic activities in endothelial cells. CCN3 supports endothelial cell adhesion, stimulates directed cell migration, and promotes cell survival. Furthermore, CCN3 induces neovascularization in vivo in a corneal micropocket assay. Mechanistically, CCN3 acts as a ligand of integrin receptors and mediates endothelial cell adhesion through integrins α₁β₁, α₅β₁, and α₅β₃, whereas its chemotactic activity is mediated through integrins α₅β₁ and α₁β₃. Despite lacking a RGD sequence motif, CCN3 binds directly to integrins α₁β₃ and α₅β₃. These findings establish CCN3 as a novel integrin ligand and angiogenic inducer, provide insights into its mechanism of action, and suggest biological functions for CCN3 both in normal development and in pathological conditions where its aberrant expression has been observed.

Angiogenesis, or the formation of new blood vessels from pre-existing ones, is a complex process requiring the coordinated execution of multiple cellular events (45). The sprouting of vessels requires degradation of the basement membrane
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Members of the CCN family of matricellular proteins clearly surrounding the parental vessel, migration of vascular endothelial cells toward the angiogenic stimulus, proliferation of endothelial cells and their alignment into tubular structures, and coalescence of new vessels into circular loops to provide blood supply to the target tissue (45). Angiogenesis is essential for embryogenesis, and in the adult, it is important in the female reproductive cycle and in wound healing. Angiogenesis may underlie a number of pathological conditions including diabetic retinopathy, arthritis, atherosclerosis, psoriasis, and cancer (46). It is now clear that angiogenesis is regulated by a network of multiple inducers and inhibitors (47, 48), and CCN3 may be part of this network of regulators. Our conclusion that CCN3 is a novel angiogenic inducer is based on the observations that it acts directly upon endothelial cells to promote cell adhesion, migration, and cell survival in vitro (Figs. 2, 6, and 8), and induces angiogenesis in vivo (Fig. 9). These activities are inhibited by antibodies specific for CCN3, showing that they are intrinsic properties of the CCN3 polypeptide. In this study, we have employed HUVECs and BACECs to examine the angiogenic functions of CCN3. It is important to note that there is considerable heterogeneity among vascular endothelial cells, differing in morphology and function, matrix environment, and responses to growth factors (49–51). Thus, it is possible that other endothelial cells may exhibit different responses to NOV in culture.

The discovery that CCN3 is an angiogenic factor helps to shed light on its functions in development and disease. CCN3 is expressed in hypertrophic cartilage (26), where vessel growth is required for the formation of a scaffold onto which the osteoblasts settle and deposit bone matrix (52). Thus, CCN3-induced angiogenesis may be important in endochondral ossification. During nephrogenesis, CCN3 is localized to the metanephric mesenchyme into which endothelial cells are recruited (32). These endothelial cells then proliferate and form a capillary network as the metanephric mesenchyme develops to form the glomeruli, the basic units of filtration (53). The presence of CCN3 might help serve as a chemotactic and survival factor for endothelial cells. In addition, CCN3 expression is correlated with various tumors, including Wilms’ tumors, and benign adenocortical tumors (27, 32, 54). It is well established that tumor growth beyond –1 mm in size requires the growth of new vessels to provide the necessary blood supply (47, 55). Thus, the expression of CCN3 in tumors is consistent with its angiogenic activity. Furthermore, the Wilms’ tumor suppressor gene (WT1) was shown to negatively regulate CCN3 expression (55). It is possible to speculate that, as part of its function, WT1 down-regulates the angiogenic inducer CCN3 to help suppress tumor growth.

We have also identified the cell surface receptors that mediate CCN3 functions in endothelial cells. CCN3 supports endothelial cell adhesion through integrins αvβ3, αvβ1, and αvβ6, whereas chemotaxis is mediated through αvβ3 and αvβ1 (Figs. 3 and 7). Furthermore, CCN3 binds directly to integrins αvβ3 and αvβ1 (Figs. 4 and 5). Although it is well established that integrins are important in developmental and pathological angiogenesis (57), it is currently unclear which integrins play a greater role in CCN3-induced angiogenesis. Interestingly, CCN3 is the only member of the CCN protein family known to bind integrin αvβ1 to date. By contrast, integrin αvβ1 plays no role in CCN1-mediated endothelial cell adhesion (7), and we have found that CCN1 does not bind purified αvβ1 in a solid phase binding assay (data not shown). The role of integrin αvβ1 in developmental angiogenesis has been established genetically in mice, where targeted gene disruptions in integrins αv or β1 resulted in embryonic lethality with prominent angiogenic defects (58–60). Inasmuch as CCN3 acts directly on endothelial cells through integrin αvβ1 to promote cell adhesion and migration, the binding of CCN3 to αvβ1 may be critical to CCN3-mediated angiogenesis.

A wealth of data also supports the notion that integrin αvβ3 plays a critical role in angiogenesis. Importantly, antagonists of integrin αvβ3 effectively block angiogenesis both in vitro and in vivo, and inhibit tumor formation in animal models (61, 62). A humanized monoclonal antibody against integrin αvβ3, Vitaxin, is currently undergoing clinical trial as an anti-cancer drug (63). This therapeutic approach is predicated on the premise that integrin αvβ3 acts as a pivotal regulator of angiogenesis. However, recent studies have raised questions challenging this view (64). Human or mouse deficient in the integrin β3 subunit display normal developmental angiogenesis and are viable and fertile, although they have a bleeding disorder due to defects in the platelet integrin αIIbβ3 (65). This finding indicates that αvβ3 is not absolutely required for developmental angiogenesis. Furthermore, mice with targeted disruptions in integrin β3 or both β3 and β5 subunits grow larger tumors than wild-type, suggesting that integrins αvβ3 and αvβ5 may actually be negative regulators of angiogenesis (66). These observations indicate that the roles of αv integrins in angiogenesis may be more complex than previously thought. Nevertheless, the ability of CCN3 to bind integrin αvβ3 and to promote endothelial cell adhesion and migration through this integrin shows that angiogenic actions may be mediated through αvβ3. In this context, it is possible to contemplate the actions of CCN3 both as an inducer via direct binding to αvβ3, and as a modulator by competition with other ligands that bind integrin αvβ3.

Table I

| Test substance | Vascularized (+) and unvascularized (−) corneas |
|---------------|-----------------------------------------------|
| CCN3          | 13 and 1                                       |
| bFGF          | 7 and 1                                        |
| CCN3 buffer   | 0 and 7                                        |
| CCN3 + anti-CCN3 antibodies | 0 and 8                                 |

Fig. 9. CCN3 induces neovascularization in rat corneas. Hydron pellets containing test substances were made and implanted into rats corneas (Table I). Blood vessel formation was visualized by perfusion with colloidal carbon 7 days after implantation. Vessel formation due to Hydron pellets containing CCN3 storage buffer (A), bFGF (B), CCN3 protein (C), and CCN3 protein preincubated with anti-CCN3 antibodies (D), are shown.

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serve important developmental functions. Deficiency in CCN6 causes progressive pseudohematoiod dysplasia in humans, a juvenile-onset degenerative disease of the cartilage (67). Although both CCN1 and CCN2 are angiogenic inducers, mutations in their structural genes result in distinct phenotypes related to angiogenic defects. Targeted gene disruption of CCN1 in mice resulted in embryonic lethality with vascular defects in both the placenta and the embryo (23). CCN2-null mice, on the other hand, are perinatal lethal as a consequence of respiratory failure due to skeletal malformations (18). Interestingly, angiogenic defects in the growth plates underlie part of the skeletal defects in CCN2 mutants. These findings indicate that although proteins of the CCN family share extensive sequence homology and overlapping activities in vitro, they serve non-redundant developmental functions. Thus, although CCN3 induces angiogenesis, its biological roles may be distinct from those of CCN1 and CCN2. The observation that CCN3 is related to angiogenic defects. Targeted gene disruption of CCN6 from those of CCN1 and CCN2. The observation that CCN3 is non-redundant developmental functions. Thus, although angiogenic defects in both the placenta and the embryo (23). CCN2-null mice, the on the other hand, are perinatal lethal as a consequence of respiratory failure due to skeletal malformations (18).

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