Expression of connective tissue growth factor (CTGF/CCN2) in breast cancer cells is associated with increased migration and angiogenesis

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Abstract. Connective tissue growth factor (CTGF/CCN2) belongs to the CCN family of matricellular proteins, comprising Cyr61, CTGF, NovH and WISP1-3. The CCN proteins contain an N-terminal signal peptide followed by four conserved domains sharing sequence similarities with the insulin-like growth factor binding proteins, von Willebrand factor type C repeat, thrombospondin type 1 repeat, and a C-terminal growth factor cysteine knot domain. To investigate the role of CCN2 in breast cancer, we transfected MCF-7 cells with full-length CCN2, and with four mutant constructs in which one of the domains had been deleted. MCF-7 cells stably expressing full-length CCN2 demonstrated reduced cell proliferation, increased migration in Boyden chamber assays and promoted angiogenesis in chorioallantoic membrane assays compared to control cells. Deletion of the C-terminal cysteine knot domain, but not of any other domain-deleted mutants, abolished activities mediated by full-length CCN2. We have dissected the role of CCN2 in breast tumorigenesis on a structural basis.

Introduction

Connective tissue growth factor (CTGF/CCN2) belongs to the CCN family of proteins (1-4) which has five additional members: cysteine rich 61 (Cyr61/CCN1), nephroblastoma overexpressed (NOV/CCN3) and the Wnt-inducible secreted proteins (WISP1-3/CCN4-6). With the exception of WISP2, CCN proteins contain an N-terminal signal peptide followed by four conserved modular domains, each encoded by a separate exon. Module I shares sequence similarities with the insulin-like growth factor binding proteins (IGFBPs). Module II has sequence homology with the von Willebrand factor type C repeat (VWC). Module III shares sequence similarity with the thrombospondin type 1 repeat (TSP). Module IV resembles the C-terminal growth factor cysteine knot domain (CT). Different isoforms of CCN proteins lacking one or more domains have been detected under both normal and disease conditions, arising from either alternative mRNA splicing or post-translational processing (5-7). Several lines of evidence demonstrate that these variants possess significant biological activity, suggesting that the production of truncated forms of CCN proteins influences the eventual physiological or pathophysiological outcome. Indeed, transformation of chick embryonic fibroblasts was induced by forced expression of amino-truncated, but not intact, CCN3 (8). In addition, plasmin cleavage of CCN1 released an N-terminal form into the culture media of MDA-MB-231 breast cancer cells, which stimulated endothelial cell migration at equivalent levels to that produced by the full-length protein (9). A plasmin digestion site is also present in the central hinge region of CCN2, and cleavage of the protein generates fragments that have distinct biological activities. The N-terminal portion containing modules I and II promoted myofibroblast differentiation and collagen production, whereas the C-terminal fragment containing modules III and IV stimulated fibroblast DNA synthesis (10). Additionally, a 10 kDa C-terminal fragment including the CT domain of CCN2 is present in uterine secretions, and was found to be mitogenic for fibroblasts and smooth muscle cells (6). In contrast to its mitogenic effects on fibroblasts, CCN2 has tumor suppressive activities in lung cancer (11,12).

The wide-range and often conflicting effects of CCN family members have been attributed in part to their multimodular structure, which enables different regions of the protein to fulfill distinct functions (3). Our laboratory has identified that module 4 of CCN1 is involved in cell spreading, migration and proliferation in breast cancer cells (13). In the present study, we generated a series of deletion mutants, containing CCN2 isoforms in which each of the modular domains had been deleted. These CCN2 deletion mutants were stably expressed
in MCF-7 cells, and the cellular activities of these deletion mutants were compared to that of the full-length CCN2 in structure-function analysis. Additional experiments examined the influence of CCN2 on growth in vitro and in vivo of human breast cancer. Notably, CCN2 was important in breast cancer migration and angiogenesis.

Materials and methods

Cell culture. The breast cancer cell lines MCF-7, T47D, BT-474 were obtained from the American Type Tissue Culture Collection (Rockville, MD). Cells were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies) supplemented with 10% fetal calf serum (Gemini Bio-Products, Calabasas, CA) and 10 units/ml penicillin-streptomycin (Life Technologies) and incubated at 37°C in 5% CO₂. Deletion mutants of CCN2 were generated by PCR amplification using a full-length CCN2 expression vector as a template. Breast cancer cells were stably transfected with the constructs using Lipofectin (Invitrogen) according to manufacturer’s protocols. Stable cells were grown in complete culture medium with 500 µg/ml of G418.

Cell proliferation and apoptosis assays. For measurement of apoptosis, cells were labeled with FITC-conjugated Annexin V antibody and propidium iodide using Annexin V-FITC Apoptosis Detection kit I (BD Biosciences, San Jose, CA) according to the instructions from the manufacturer. Positive cells were detected by fluorescence-activated cell sorting. For cell cycle analysis, cells were washed and suspended in ice-cold phosphate-buffered saline (PBS), fixed in 75% chilled methanol at 4°C, and stained with propidium iodine. Cell cycle status was analyzed on a Beckton-Dickinson flow cytometer (BD Biosciences). To measure growth, cells were placed into 96-well plates at 1x10³ cells per well. After incubation for the indicated time, cell number was assessed by the MTT assay (Sigma-Aldrich). Recombinant protein containing only the CT domain (CT-CCN2) was obtained from PeproTech (Rocky Hills, NJ). To measure DNA synthesis, [³H]-thymidine (0.25 µCi) was added to each well for the last 6 h of culture; cells were harvested onto filters, which were assayed for [³H]-thymidine content using the beta plate liquid scintillation counter (Amersham Pharmacia Biotech).

Western blot analysis. Protein from cells harvested for Western blotting was extracted with RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH 7.5) containing a protease inhibitor cocktail (Roche Molecular Biochemicals). Protein lysates (40 µg) were boiled in Laemmli sample
buffer (Bio-Rad), resolved by electrophoresis on 4-15% SDS-polyacrylamide gels, and transferred to PVDF membranes. Membranes were probed with antibodies (Santa Cruz) and developed using the SuperSignal West Pico Chemiluminescent Substrate (Pierce). For heparin-pull down experiments, cells were lysed in IP buffer (50 mM Tris pH 7.4, 150 mM NaCl, 0.5% NP-40), and 500 μg of protein was incubated with heparin-agarose beads (Sigma) at 4°C overnight with rotation. Beads were washed 3 times in PBS, boiled in Laemmli sample buffer and subjected to Western blotting as described above.

**In vivo tumorigenesis assay.** MCF-7 cells (1x10⁶) were suspended in 100 μl Matrigel (BD Biosciences) for each tumor. Cells were injected into the mammary fat pad of immunodeficient BNX mice. Animals were supplemented with 17-estradiol pellets (Innovative Research of America, FL) that were implanted subcutaneously one week in advance. The size of the tumors was measured at regular intervals, and their volume was calculated by the following formula: A (length) x B (width) x C (height) x 0.5236. All mice were euthanized at the end of the study, and the final mass of the tumors was determined by weight after their dissection. Data were analyzed by Student’s t-test.

**Cell adhesion, wound healing, and Boyden chamber assays.** For cell adhesion assay, 96-well plates were coated with 50 μg/ml of fibronectin, vitronectin, or collagen overnight at 4°C. Cells were resuspended in serum-free medium and allowed to adhere to ECM-coated wells for 30 min. After the incubation period, wells were washed with PBS 3 times, and adherent cells were quantified by crystal violet staining and OD measurement at OD 540 nm. For wound healing assays, cells were grown to confluence and serum-starved overnight. Monolayer of cells was scratched with a sterile razor and images were captured at a fixed point along the cut surface. Width of the wound was calculated from five measurements of the distance between the migrating fronts in the captured images using the Datinf Measure software (Tubingen, Germany). The relative rates of total wound closure were calculated from 100% minus the percentage of (width of wound at 24 h/width of wound at 0 h). Boyden Chambers (Nunc, Rochester NY) with 8 mm pore size were coated on the underside of the membrane with 50 μg/ml fibronectin, vitronectin, or collagen for 1 h at room temp. Cells (2x10⁶) in 300 μl serum-free media were added to the top of each chamber with the lower chamber containing 300 μl of serum-free medium plus 0.5% BSA. Chambers were placed in 24-well plates, and cells were allowed to migrate to the under-side of the top chamber for 12 h. The migratory cells that attached to the bottom surface of the membrane were stained with 0.25% crystal violet in 50% methanol for 15 min. The stained cells were extracted with 10% acetic acid. The number of migratory cells per membrane was determined by absorbance at 540 nm.

**Chorioallantoic membrane angiogenesis assay.** MCF-7 cells were seeded on the chorioallantoic membrane (CAM) of 7-day old quail embryos. After incubation for three additional days, CAMs were dissected and mounted on slides. The pattern of vessel branching was assessed by measurement of the fractal dimension (Df) in order to quantify the degree of angiogenesis.

We first determined the impact of CCN2 on proliferation of human breast cancer cells *in vitro* and *in vivo*. Three breast cancer cell lines (MCF-7, T47D and BT-474) were transfected with either vector containing full-length CCN2 (pcDNA3.1-CCN2) or control empty vector (pcDNA3.1-Neo). Transfected cells were grown in selection medium, and cell number was assessed by measuring the absorbance of cells stained with crystal violet. CCN2 expression decreased the proliferation of all three cell lines (Fig. 1A). We then made stable CCN2-expressing breast cancer cells. MCF-7 cells in which CCN2 was stably expressed (MCF-7/CCN2) were used for further analysis in comparison to control cells (MCF-7/Neo). Two stable clones for each construct (Neo and CCN2) were selected, and their increased expression of CCN2 was demonstrated in these cells (Fig. 1B, left panel, #1 and #2). These cells also had decreased cell numbers similar to Fig. 1A (data not shown). We examined if this was caused by cell apoptosis. Annexin V

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**Results**

Figure 2. Stable expression of CCN2 deletion mutants in MCF-7 cells and their effect on human breast cancer cell proliferation. (A) Schematic diagram of CCN2 deletional mutants in which one of the modules [IGFBP (dI), von Willebrand factor type C repeat (dV), thrombospondin type 1 repeat (dT), and cysteine knot domain (dC)] has been deleted. (B) Forced expression of each of the mutant CCN2 genes in MCF-7 cells was detected by Western blot analysis using a HA antibody that recognizes the HA-tag on each protein. Cell lysates were incubated with heparin-agarose beads to pull down heparin-binding proteins and detected by HA antibody. (C) MTT assays showed CCN2 deletion mutants expressed in MCF-7 breast cancer cells. Results represent the mean ± SD of three experiments done in triplicates.

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**Cell proliferation**

Day 1  Day 2  Day 3  Day 4  Day 5  Day 6  Day 7

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staining showed no difference in the degree of apoptosis in control (Neo, 5%) vs. CCN2 expressing cells (CCN2, 6%) (Fig. 1B, right panel). On the other hand, cell cycle analysis showed a significant increase in the proportion of CCN2-transfected cells in the G₀-G₁ phase (52%, CCN2 transfected vs. 36%, control) and decrease in the S phase (34%, CCN2 transfected vs. 51%, control) (Fig. 1C). These results demonstrated that CCN2-mediated growth inhibition of MCF-7 cells is primarily due to cell cycle arrest. The tumorigenic potency of the stably CCN2 transfected MCF-7 breast cancer cells was investigated in an orthotopic xenograft model in nude mice. In contrast to in vitro study, no significant difference was observed in the growth rate of CCN2-transfected MCF-7 cells orthotopically implanted into the breast tissue of mice compared to controls (Fig. 1D).

To investigate the contribution of each of the structural domains of CCN2 to its biological properties, we generated four mutant constructs in which one of the modules (I, V, T, C) was deleted (Fig. 2A). A HA-tag was added to the C-terminus of the protein, enabling detection of each of the isoforms using an anti-HA antibody (Fig. 2B). Characteristic doublets of protein bands were detected, as previously observed by other investigators (14). CCN2 is a secreted heparin-binding protein. We used heparin-agarose beads to investigate the heparin-binding ability of each deletion mutants. Western blot analysis showed heparin-agarose beads could pull-down the CCN2 protein that was either full-length or missing module I, V, or T (Fig. 2B, FL, dI, dV, and dT), but not the MCF-7 CT-domain deletion mutant (Fig. 2B, dC). Overexpression of deletion mutants missing I, V, or T modules in MCF-7 cells showed comparable growth suppression to that of the full-length protein (Fig. 2C, dI, dV, dT, and dC vs. FL). In contrast, deletion of the CT domain prevented the CCN2-mediated growth inhibition of MCF-7 cells (Fig. 2C, dC vs. FL).

Cell adhesion of MCF-7 to extracellular matrix proteins was assessed on plates coated with either fibronectin, vitronectin, or collagen. Overexpression of CCN2 in these cells decreased their cell adhesion to both collagen and vitronectin, but not to fibronectin (Fig. 3A, Neo vs. FL). Western blot analysis showed heparin-agarose beads could pull-down the CCN2 protein that was either full-length or missing module I, V, or T (Fig. 2B, FL, dI, dV, and dT), but not the MCF-7 CT-domain deletion mutant (Fig. 2B, dC). Overexpression of deletion mutants missing I, V, or T modules in MCF-7 cells showed comparable growth suppression to that of the full-length protein (Fig. 2C, dI, dV, dT, and dC vs. FL). In contrast, deletion of the CT domain prevented the CCN2-mediated growth inhibition of MCF-7 cells (Fig. 2C, dC vs. FL).

To examine whether overexpression of CCN2 and its deletion mutants have an effect on the migratory ability of the MCF-7 breast cancer cells, ECM-coated Boyden chambers assays were performed. Overexpression of full-length CCN2 increased MCF-7 cell migration in both fibronectin- and vitronectin-coated Boyden chambers, with more significant increase on vitronectin-coated chambers (Fig. 3B, Neo vs. FL).
Deletion of the TSP domain of CCN2 enhanced even more the migration of the MCF-7 cells (Fig. 3B, FL and dT). The presence of the IGFBP, VWC, and CT domains were important for CCN2-mediated migration, as mutants in which these domains had been deleted migrated more slowly than those cells expressing full-length CCN2 (FL) (Fig. 3B, Neo, dI, dV, and dC). Next, wound healing assays were also used to measure motility of MCF-7 breast cancer cells. Overexpression of full-length CCN2 increased the migration of MCF-7 cells (Fig. 3C, upper panel). This increased migratory phenotype occurred with each of the deletion mutants dI, dV, and dT, except the mutant missing the CT domain (Fig. 3C, bottom panel).

To verify the significance of the CT domain in cell growth and migration, we treated MCF-7 cells with recombinant protein containing only the CT domain. This recombinant CT protein inhibited DNA synthesis of MCF-7 cells, as determined by $[^3H]$-thymidine incorporation (Fig. 4A). Furthermore, recombinant CT protein promoted migration of MCF-7 cells in a collagen-coated Boyden chamber assay (Fig. 4B).

To assess the effect of CCN2 on angiogenesis, the chorioallantoic membrane angiogenesis assay was used. Compared with blood vessel formation in fertilized quail eggs of control MCF-7 breast cancer cells (Fig. 5, panel Neo), blood vessels were prominently stimulated to grow when the MCF-7 cells overexpressed full-length CCN2 (Fig. 5, panel FL). MCF-7 breast cancer cells that overexpressed CCN2 with a deletion of the CT domain no longer had enhanced angiogenesis (Fig. 5, panel dC).

**Discussion**

Breast cancer is a multistep process in which the cells acquire a series of malignant characteristics, including increased proliferation and greater cell motility (15). In this study we demonstrated that CCN2 expression in breast cancer produced divergent effects within the same cell population. MCF-7 cells transfected with CCN2 proliferated less rapidly than control cells, yet their ability to migrate was enhanced. CCN2 is a TGFβ responsive gene (14), and as such, our results are consistent with the ambiguous role of TGFβ in breast cancer progression, as TGFβ possesses both tumor-suppressing and tumor-enhancing effects (16-18). The actions of TGFβ on breast cancer are both cell type- and context-dependent. TGFβ signaling inhibits the proliferation of epithelial cells, but it promotes invasion and metastasis as the cancer progresses. CCN2 expression is up-regulated by TGFβ in MCF-7 cells,
where it contributes to the anti-proliferative actions of TGFβ (19). In addition, high levels of both CCN2 and TGFβ were associated with breast cancer metastasis to bone in an animal model (20,21). Similarly to CCN2, stable transfection of a CCN3 expression vector, both reduced proliferation and increased migration of an Ewing's sarcoma cell line (22). However, divergent from our observations, a prior study found that overexpression of CCN2 in MCF-7 cells increased motility without affecting cell growth (23). Although we observed an anti-proliferative effect of CCN2 in MCF-7 cells (23), growth of orthotopic human xenografts of MCF-7/CCN2 breast cancer cells was only slightly slower than for control tumors, but results were not statistically significant, suggesting that other factors present in vivo can overcome CCN2-mediated growth suppression. Since CCN2 expression is elevated in breast cancer patient samples and correlates with more advanced features of disease (24,25), the overall effect of CCN2 up-regulation is likely advantageous for the cancer. Moreover, a recent study showed that drug resistance was conferred in MCF7 cells over-expressing CTGF (26).

Biological activities of CCN proteins are domain-specific. Examples include: a novel variant of CCN4 lacking a VWC module was associated with progression of scirrhous gastric carcinoma (27). In myofibroblast, the N-terminal domain of CCN2 mediates migration, while the C-terminal region mediates proliferation (10). In contrast, the C-terminal fragment of CCN2 inhibits the proliferation of chondrocytes (28). CT module of CCN3 is involved in inhibition of cell growth in gliomas (29). The CT domain is important for the anti-metastatic properties of CCN2 in lung cancer, where a CT-domain deleted mutant had a reduced ability to inhibit lung cancer cell invasion compared to the full-length protein (11).

Our data highlight the importance of the C-terminal CT domain in mediating CCN2 activities in breast cancer cells. MCF-7 cells expressing CCN2 in which the CT domain was deleted, lost both the suppression of growth, and the enhancement of migration and angiogenesis. Furthermore, a recombinant CT domain peptide effectively reproduced the effects of the full-length protein as measure by DNA synthesis and migration. The C-terminal portion of CCN2 contains heparin-binding sites; and we show that deletion of this region, in particular the CT domain, prevents binding to heparin-agarose. Interaction of CCN2 with the cell surface is important for its regulation of growth factor signaling, as CCN2 chaperones other growth factors regulate their receptor-binding ability (12,30). Furthermore, heparin sulfate proteoglycans act as co-receptors for other integrin ligands, including CCN1 (31).

The CT domain of CCN2 contains a binding site for integrin αβ3 (32). Integrin activation plays an important role in the development and progression of breast cancer, achieved through intracellular signaling via activation of downstream kinases such as ILK and MAPK (33-35). CCN1 has been shown to promote an aggressive breast cancer phenotype in an integrin αβ3-dependent manner (36). A recent study in chondrosarcoma cells showed that CCN2 stimulates up-regulation of MMP-13 and migration via integrin αβ3 (37). Anti-Integrin αβ3 antibody has been shown to block CTGF-induced migration in MCF-7 cells (23).

Taken together, we have previously shown that high expression of CCN2 is associated with advanced breast cancer including metastasis to lymph nodes and a worse prognosis (25). Consistent with these findings, we now report that forced expression of CCN2 in breast cancer cells is associated with their increased migration and stimulation of angiogenesis. The migratory activity is dependent on the CT domain of CCN2. Furthermore, recombinant CT-CCN2 recapitulates the activity of the full-length protein. Therefore, the CT domain behaves as a critical regulator of CCN2 function and is a valid therapeutic target for breast cancer.

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