CCN1 promotes vascular endothelial growth factor secretion through $\alpha_v\beta_3$ integrin receptors in breast cancer

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Introduction

Accumulating evidence supports an association between angiogenesis and breast cancer invasion and metastasis (Locopo et al. 1998; Rayson et al. 1999; Saaristo et al. 2000; Leek 2001). Several angiogenic factors and their receptors have been identified as important mediators of angiogenesis (Boudreau and Myers 2003; Sledge 2002; Gasparini 2001; Kern and Lippman 1996). CCN1, a cysteine-rich, heparin-binding protein that is secreted and associated with the cell surface and the extracellular matrix (Yang and Lau 1991), belongs to the Cysteine rich 61/Connective tissue growth factor/Nephroblastoma overexpressed (CCN) gene-family of angiogenic and growth regulators (Grote et al. 2007; Chen et al. 2001, 2004; Ivkovic et al. 2003; Hashimoto et al. 2002; Dean et al. 2007; Brigstock 2002). CCN1 is differentially expressed in invasive/metastatic human breast cancer cells (Tsai et al. 2000).

CCN1 has been shown to mediate functions as diverse as cell proliferation, migration, adhesion, cell survival, differentiation, and extracellular matrix formation. CCN1 also regulates more complex processes such as angiogenesis and tumorigenesis (Planque and Perbal 2003; Babic et al. 1998,

1999; Xie et al. 2001a; Tsai et al. 2002). Expression of CCN1 is sufficient for acquisition of hormone independence and anti-estrogen resistance in human breast cancer cells (Tsai et al. 2002). Indeed, CCN1 enhances a metastatic phenotype by promoting cell proliferation in soft agar, cell migration and invasion, and Matrigel outgrowth of breast cancer cells (Tsai et al. 2000). CCN1 overexpression is correlated, in patient samples, with advanced stages of malignancy (Tsai et al. 2000; Xie et al. 2001b). This evidence indicates that CCN1 plays an important role in breast cancer development and could serve as a valuable biomarker for monitoring tumor status. The molecular mechanism by which CCN1 promotes aggressive breast cancer phenotypes is still unknown.

We showed previously that CCN1 overexpression enhances tumorigenicity by increasing tumor size and vascularization of human breast cancer xenografts (Tsai et al. 2002). CCN1-induced tumors in ovariectomized athymic nude mice resembled human invasive carcinomas with increased vascularization and overexpression of VEGF (Tsai et al. 2002). These findings prompted us to hypothesize that CCN1 is an important regulator of the vascular compartment in breast cancer, with stimulating effects on tumor neovascularization that, in turn, promote the progression and dissemination of breast carcinoma. Through direct binding to integrin $\alpha_v\beta_3$, CCN1 can recapitulate angiogenic events in vitro by promoting endothelial cell adhesion, migration, proliferation, and tubule formation (Babic et al. 1999; Lin et al. 2003; Shimo et al. 1999; Leu et al. 2002). CCN1 stimulates integrin-dependent recruitment of CD34+ progenitor cells to endothelial cells, thereby enhancing endothelial proliferation and neovascularization (Grote et al. 2007). Additionally, CCN1 regulates the expression and activities VEGF-A and VEGF-C (Chen et al. 2001; Ivkovic et al. 2003; Hashimoto et al. 2002; Dean et al. 2007).

Considering that CCN1 is an angiogenic ligand for the $\alpha_v\beta_3$ integrin receptor in endothelial cells (Chen et al. 2001, 2004; Brigstock 2002; Leu et al. 2002), it is reasonable to

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suggest that CCN1 mediates breast cancer angiogenesis in a paracrine manner through its binding to the $\alpha_v\beta_3$ integrin receptor. However, little is known about the regulatory role of CCN1 in the secretion of VEGF in the epithelial compartment of breast carcinoma. We speculate here that CCN1 may promote VEGF-dependent breast cancer angiogenesis in an autocrine fashion. Thus, we examined whether CCN1-induced overproduction of VEGF in breast cancer cells was associated with increased CCN1- $\alpha_v\beta_3$ integrin signaling in the epithelial compartment of breast carcinomas.

Materials and methods

Reagents Small peptidomimetic antagonists of $\alpha_v\beta_3$ containing the RGD (Arg-Gly-Asp) motif (SC56631, S-247) were developed using structure-activity relationship-based rational medicinal chemistry as previously described (Shannon et al. 2004; Carron et al. 1998). Briefly, human placenta-derived $\alpha_v\beta_3$ or human platelet-derived $\alpha_{bII}\beta_3$ were bound to 96-well microtiter plates and incubated with human plasma-derived vitronectin conjugated to biotin for detection purposes. Densitometric determination after coincubation of the labeled ligand with a competitor provided data that, when analyzed using four-parameter fitting techniques, yielded the IC₅₀. In all cases, 2 mM stock solutions in sterile water were stored at 4 °C until use. LY29400, a specific inhibitor of the p110 catalytic subunit of PI-3K, and the MEK1/MEK2 inhibitor U0126 were purchased from Calbiochem (San Diego, CA, USA), dissolved in DMSO, and stored as 10 mM stock solutions in the dark at -20 °C until use. For experimental use, SC56631, S-247, LY294002, and U0126 were freshly prepared from stock solutions and diluted with growth medium. Control cells were cultured with the same concentrations of DMSO (v/v) or sterile water (v/v) as the experimental cultures with LY294002 and U0126 or S-247, respectively. Addition of DMSO or sterile water to the culture had no effects.

Cell culture MCF-7, Hs578T, and MDA-MB-231 human breast cancer cells were obtained from the American Type Culture Collection (ATCC). Cells were routinely grown in phenol red-containing improved MEM (IMEM, Biosource International, Camarillo, CA, USA) supplemented with 5 % (v/v) fetal bovine serum (FBS) and 2 mM L-glutamine at 37 °C in a humidified atmosphere of 95 % air and 5 % CO₂.

CCN1 expression in MCF-7 cells MCF-7 human breast cancer cells were engineered to overexpress CCN1 as previously described and CCN1-positive clones were selected (Tsai et al. 2002). Briefly, MCF-7 cells were stably transfected by electroporation with a eucaryotic expression vector pcDNA3.1/zeocine (-) containing the full-length cDNA of the human

CCN1 gene, or with an empty vector as a negative control. Transfected MCF-7 cells were selected in the presence of the antibiotic zeocine (200 µg/ml) for 2 weeks. CCN1 expression (mRNA and protein) and cellular behavior was similar among the different clones, a representative vector (MCF-7/pcDNA3.1) and three clones (C2-2, C2-6, and C2-9) were selected for further studies. Cells were culture in the presence of 200 µg/ml of zeocine. A pool MCF-7/CCN1 cell line was generated by transducing MCF-7 cells with a retroviral vector (pBABE) containing the full-length cDNA for CCN1. Cell lines were selected with puromycin (3 µg/ml) (MCF-7/CCN1), and a control cell line was generated in parallel, under similar conditions using the empty retroviral vector (MCF-7/pBABE) alone.

Silencing of CCN1 in MDA-MB-231 cells Knockdown of CCN1 expression in MDA-MB-231 breast cancer cells was performed using a lentiviral construct (pLKO.1 vector) carrying shRNA against CCN1 (MDA-MB-231/shRNA #6). Control cells were infected with the pLKO.1 vector (V) (MDA-MB-231/shRNA-V). Stable cells lines were generated after 3 weeks of selection in puromycin.

Enzyme-linked Immunosorbent assay (ELISA) analysis of secreted VEGF protein concentrations Breast cancer cell lines were seeded on 100-mm plates and cultured in complete growth medium. Upon reaching ~75 % confluence, the cells were washed twice with pre-warmed phosphate-buffered saline (PBS) and cultured in serum-free medium overnight. Cells were either untreated or exposed to $\alpha_v\beta_3$ antagonists U0126 or LY294002 in 0.1 % FBS-IMEM, and incubation was carried out at 37 °C for up to 48 h. After incubation, the conditioned medium was aspirated, centrifuged at 1100×g for 10 min at 4 °C to remove debris, and stored at -80 °C until analysis. The VEGF protein level in the conditioned media was determined by a VEGF enzyme-linked immunosorbent assay (R & D Systems, Minneapolis, MN), as per the manufacturer's instructions. Data presented are the mean (columns) ± S.D. (bars) from three independent experiments performed in duplicate. A paired Student's *t*-test was used to evaluate statistically significant differences in VEGF protein levels (pg/mg protein⁻¹) between different cell lines or between treatment groups and the vehicle control group. *P* < .05 (*) and *P* < .005 (**) were selected as the statistically significant values. All statistical tests and corresponding *P* values were two-sided.

Results

CCN1 expression correlates with VEGF secretion in human breast cancer cells Figure 1a shows the basal level of the

VEGF secretory isoform, VEGF₁₆₅, in human breast cancer cell lines naturally overexpressing CCN1 (MDA-MB-231, Hs578T) when compared to VEGF₁₆₅ secretion levels in MCF-7 breast cancer cells, which express undetectable levels of CCN1. CCN1-overexpressing MDA-MB-231 and Hs578T breast cancer cell lines showed VEGF₁₆₅ secretion levels (20.2 ± 3 and 28.8 ± 0.5 pg VEGF/ μ g protein, respectively) that were significantly higher than low CCN1-expressing MCF-7 cells (4.2 ± 0.2 pg VEGF/ μ g protein). These results show a clear correlation between CCN1 expression and VEGF secretion in breast cancer cells. We next evaluated whether forced expression of CCN1 could modify basal VEGF levels in MCF-7 cells (Fig. 1b). Indeed, MCF-7 cells engineered to overexpress CCN1 demonstrated VEGF₁₆₅ secretion levels (6 – 10.2 ± 0.1 pg VEGF/ μ g protein) significantly higher than those found in MCF-7 control cells (4.2 ± 0.8 pg VEGF/ μ g protein). Moreover, the level of VEGF secretion in the MCF-7/CCN1 cell line was significantly increased ($11.986.7 \pm 1.1$ pg VEGF/ μ g protein) compared to the MCF-7/pBabe control cell line. These results strongly suggest that CCN1 expression positively correlates with VEGF secretion levels in human breast cancer cells. Given that, as we recently demonstrated, CCN1 overexpression dramatically up-regulates the expression of its own receptor $\alpha_v\beta_3$ in MCF-7 cells (Menendez et al. 2005), the findings of this study suggest that CCN1 overexpression is *sufficient* to up-regulate VEGF₁₆₅ secretion in a $\alpha_v\beta_3$ -related manner.

The CCN1/ $\alpha_v\beta_3$ interaction up-regulates VEGF secretion in breast cancer cells through aMEK1/MEK2 $\rightarrow$ ERK1/ERK2 signaling To demonstrate that the expression of CCN1 correlates with VEGF secretion in breast cancer cells, we stably silenced CCN1 in MDA-MB-231 cells (MDA-MB-231/shRNA-CCN1-C6). The basal levels of VEGF secreted from the CCN1-knockdown cells (MDA-MB-231/C6) were significantly lower (2.9 ± 0.5 pg VEGF/ μ g protein) than the parental MDA-MB-231-WT cells and the vector MDA-MB-231/V cells (6.7 ± 0.72 and 7.5 ± 0.49 pg VEGF/ μ g protein, respectively) (Fig. 2a). To further demonstrate the active involvement of CCN1 and $\alpha_v\beta_3$ in the maintenance of high VEGF levels in breast cancer cells, we assessed VEGF secretion levels in MCF-7/CCN1 cells following exposure to a novel group of small peptidomimetic antagonists of $\alpha_v\beta_3$ (SC56631, SC68448, S-247, S-197, and S-205; Oncology Pharmacology, Discovery Research, Pharmacia Corporation, St. Louis, MO). We demonstrated that forced expression of CCN1 in MCF-7 cells notably upregulates the expression of $\alpha_v\beta_3$ (Menendez et al. 2005). Interestingly, MCF-7/C2-6 cells (CCN1-overexpressing MCF-7 cells) incubated in the presence of S-247, a specific $\alpha_v\beta_3$ RGD peptidomimetic antagonist with high affinity and specificity for $\alpha_v\beta_3$ (Menendez et al. 2005), significantly decreased the levels of VEGF₁₆₅ secretion (Fig. 2b). These results demonstrate that the

Fig. 1 VEGF₁₆₅ expression correlates with CCN1 expression in breast cancer cells: Cells were serum starved overnight and then cultured in 0.1 % FBS-IMEM for 48 h and supernatants were collected to determine the level of VEGF₁₆₅. **a)** Supernatants were collected from human breast cancer cell lines with constitutive overexpression of CCN1 (MDA-MB-231 and Hs578T) and from CCN1-negative MCF-7 cells. **b)** Supernatants were collected from human breast cancer cell lines engineered to overexpress CCN1 (clones C2-2, -6, and -9), empty vector (MCF-7/pCDNA3.1), and from a cell line expressing CCN1 generated using a retroviral expression vector (MCF-7/CCN1). The control vector MCF-7/pBabe and MCF-7 wild type cells were used as controls. The level of VEGF₁₆₅ in the supernatant was determined by the ELISA assay, normalized to the amount of protein in the cell extracts

interaction between CCN1 and $\alpha_v\beta_3$ is *necessary* for the optimal stimulation of VEGF₁₆₅ secretion in CCN1-overexpressing breast cancer cells. Therefore, it is reasonable to suggest that a CCN1/ $\alpha_v\beta_3$ autocrine loop maintains high levels of VEGF secretion in breast cancer cells.

The CCN1- $\alpha_v\beta_3$ signaling network activates several signaling pathways that promote enhanced endothelial cell survival and proliferation (Menendez et al. 2005; Vellon et al. 2005). Next, we examined whether the PI-3'K $\rightarrow$ AKT or MEK1/MEK2 $\rightarrow$ ERK1/ERK2 pathways were actively involved, downstream of $\alpha_v\beta_3$, in the CCN1-promoted release of VEGF from breast cancer cells. To link CCN1-induced activation of PI-3'K or MAPK cascades to VEGF secretion, we treated CCN1-overexpressing MCF-7 cells with nontoxic concentrations of LY294002 and U0126, specific inhibitors of PI-3'K and of MEK1/MEK2 enzymatic activities, respectively (Fig. 2c). Interestingly, the use of equimolar concentrations of LY294002 and U0126 revealed a more prominent involvement of CCN1-activated MEK1/MEK2 $\rightarrow$ ERK1/ERK2 signaling on the maintenance of high levels of VEGF secretion in CCN1-overexpressing breast cancer cells. Of note, we previously described how optimal concentrations of $\alpha_v\beta_3$ antagonists completely abolished hyperactivation of ERK1/ERK2 MAPK in MCF-7 cells engineered to overexpress CCN1

Fig. 2 CCN1/ $\alpha_v\beta_3$ interaction promotes increase in VEGF secretion in breast cancer cells: **a**) CCN1 was silenced in MDA-MB-231 cells using a lentivirus containing shRNA against CCN1 (MDA-MB-231/C6). Levels of VEGF_A secreted by MDA-MB-231/C6 cells, cells infected with the empty vector (MDA-MB-231/V), or wild-type cells (MDA-MB-231) were determined by ELISA and normalized to the amount of protein in the cell extracts. Prior to media collection, infected cells were grown for 24 h and then serum starved for an additional 48 h. **b**) MCF-7/CCN1 cells were treated with the $\alpha_v\beta_3$ antagonist S-247 (1 μ M in 0.1 % FBS-

IMEM). After 48 h of treatment, levels of VEGF₁₆₅ in the supernatants were determined by ELISA assay, normalized to the amount of protein in the cell extracts, and compared to VEGF secretion in untreated cells. **c**) MCF-7/CCN1 cells and MCF-7/C2-6 and MCF-7/C2-9 clones were treated with increasing concentrations of U0126 (MEK1/MEK2 inhibitor) or LY294002 (PI-3'K inhibitor) in 0.1 % FBS-IMEM. After 48 h of treatment, levels of VEGF₁₆₅ in the supernatants were determined by the ELISA assay, normalized to the amount of protein in the cell extracts, and compared to VEGF secretion in untreated cells

and in naturally CCN1-overexpressing MDA-MB-231 cells, whereas the activation status of AKT did not decrease (Menendez et al. 2005; Vellon et al. 2005). These earlier findings indicated that the $\alpha_v\beta_3$ integrin specifically regulates cell survival and proliferation of CCN1-overexpressing breast cancer cells through activation of ERK1/ERK2 MAPK signaling, with minimal involvement of AKT activity. Similarly, our current results strongly suggest that the MEK1/MEK2 $\rightarrow$ ERK1/ERK2 transduction cascade, not PI-3'K $\rightarrow$ AKT, is the main signaling pathway involved in CCN1-regulated VEGF secretion in breast cancer cells.

Discussion

This study provides new insights on the role of CCN1 in breast cancer progression. Several oncogenes, growth factors, hormones, and hypoxia have been shown to upregulated VEGF expression, an angiogenic factor of reference. CCN1 is differentially expressed in breast cancer cells (Tsai et al. 2000). CCN1 stimulates tumor vascularization by acting as an angiogenic inducer of endothelial cells, as VEGF does (Planque and Perbal 2003; Babic et al. 1998, 1999; Xie et al. 2001a; Tsai et al. 2002). Considering that CCN1 is an angiogenic ligand for the $\alpha_v\beta_3$ integrin receptor in endothelial cells (Chen et al. 2001, 2004; Brigstock 2002; Leu et al. 2002),

CCN1 might mediate breast cancer angiogenesis in a paracrine manner through its binding to the $\alpha_v\beta_3$ integrin receptor. Furthermore, our current results support the notion that the up-regulatory actions of CCN1 on the secretion of VEGF are not restricted to the endothelial compartment of breast carcinomas but occur also, in an autocrine-dependent manner, in the epithelial compartment. Interestingly, it seems that CCN1 can drive VEGF secretion in both cellular compartments via the integrin receptor $\alpha_v\beta_3$. These findings strongly suggest that CCN1- $\alpha_v\beta_3$ -regulated pro-metastatic signaling occurs in breast carcinomas.

Since CCN1 overexpression activates the expression of its own $\alpha_v\beta_3$ integrin receptor (Menendez et al. 2005), upregulation of CCN1 expression in the epithelial compartment of breast carcinoma may manage breast tumorigenesis and malignant progression in several concerted ways: 1) by directing breast tumor epithelial cell migration as a chemokinetic factor; 2) by promoting breast cancer epithelial cell proliferation in an autocrine/paracrine fashion, thereby augmenting the bioactivity of other growth factors; 3) by enhancing breast cancer epithelial cell survival and chemoresistance through activation of pro-survival signaling pathways (e.g., ERK1 $\rightarrow$ ERK2 MAPK) downstream of $\alpha_v\beta_3$; 4) by regulating endothelial cell survival and recruitment during tumor neovascularization in a paracrine fashion through an $\alpha_v\beta_3$ -dependent mechanism; and 5) by synergistically enhancing CCN1-stimulated secretion of VEGF via the $\alpha_v\beta_3$ integrin receptor in breast

cancer epithelial cells. From a clinical perspective, our description of a novel CCN1-triggered “CCN1- $\alpha_v\beta_3$ autocrine loop” in breast cancer epithelial cells that regulates VEGF secretion strongly suggests that antagonists of specific integrins, such those used in this study directed against $\alpha_v\beta_3$, or other anti-CCN1 strategies have the potential to suppress tumorigenicity and metastasis of CCN1-overexpressing breast carcinomas.

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