Matrix Protein CCN1 Is Critical for Prostate Carcinoma Cell Proliferation and TRAIL-Induced Apoptosis

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Abstract

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) plays an important role in immune surveillance and preferentially induces apoptosis in cancer cells over normal cells, suggesting its potential in cancer therapy. However, the molecular basis for its selective killing of cancer cells is not well understood. Recent studies have identified the CCN family of integrin-binding matricellular proteins as important regulators of cell behavior, including cell adhesion, proliferation, migration, differentiation, and survival. We show here that CCN1 (CYR61) supports the adhesion of prostatic carcinoma cells as an adhesion substrate through integrins and heparan sulfate proteoglycans. Knockdown of *CCN1* expression in PC-3 and DU-145 androgen-independent prostate cancer cells strongly inhibited their proliferation without causing apoptosis, indicating that *CCN1* promotes their growth. However, CCN1 also significantly enhances TRAIL-induced apoptosis through interaction with integrins $\alpha_v\beta_3$ and $\alpha_6\beta_4$ and the cell-surface heparan sulfate proteoglycan syndecan-4, acting through a protein kinase Cα-dependent mechanism without requiring *de novo* protein synthesis. Knockdown of *CCN1* expression in PC-3, DU-145, and LNCaP cells severely blunted their sensitivity to TRAIL, an effect that was reversed by exogenously added CCN1 protein. These findings reveal a functional dichotomy for CCN1 in prostate carcinoma cells, because it contributes to both cell proliferation and TRAIL-induced cell death and suggest that *CCN1* expression status may be an important parameter in assessing the efficacy of TRAIL-dependent cancer therapy. (Mol Cancer Res 2009;7(7):1045–55)

Introduction

Prostate cancer is the most commonly diagnosed malignancy in men and the second leading cause of cancer-related deaths in the United States (1, 2). In early stages of the disease, prostate cancer cells are dependent on androgens that increase proliferation and inhibit apoptosis. Although androgen ablation therapy often results in cancer regression, this process also selects for androgen-independent cancer cells, which can develop into later stage cancers that are metastatic, resistant to apoptosis, and recalcitrant to therapy (1). Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a type II transmembrane protein of the TNF superfamily that binds five known receptors. Whereas DcR1, DcR2, and OPG are thought to be decoy receptors, ligation of TRAIL to DR4 or DR5 triggers receptor trimerization, recruitment of FADD to the receptor via the receptor death domain, and activation of the apoptotic initiators, caspase-8 and -10 (3). TRAIL is expressed mainly in immune cells and plays a critical role in immune surveillance by natural killer cells (4), and TRAIL-deficient mice are more susceptible to autoimmune diseases and more prone to tumor metastasis (5, 6). TRAIL preferentially induces apoptosis in a variety of cancer cells but exhibits limited cytotoxicity in normal cells. Thus, using TRAIL to induce apoptosis has emerged as a promising cancer therapeutic approach, especially as an adjuvant therapy for advanced prostate cancer in combination with irradiation or chemotherapy (7-9). However, the molecular basis for the susceptibility of prostate cancer cells to TRAIL-induced apoptosis is not well understood and may involve multiple contributing factors that function in a cell type-dependent manner (10, 11).

CCN1 (CYR61) is a secreted, integrin-binding protein that regulates multiple cellular activities, including cell adhesion, migration, proliferation, survival, and apoptosis (12), and is considered a matricellular protein given its predominantly regulatory rather than structural roles (13). Whereas CCN1 is a potent angiogenic inducer and is essential for cardiovascular development during embryogenesis, its expression in the adult is associated with pathologic conditions in which angiogenesis and inflammation play important roles, such as wound healing, restenosis, atherosclerosis, and tumorigenesis (12, 14-16). Consistent with its angiogenic activity, overexpression of *CCN1* in cancer cells promotes tumor growth and vascular density in immunodeficient mice (14), and overexpression of *CCN1* has been associated with human breast cancers, gliomas, gastric adenocarcinomas, and melanomas (17, 18). In the prostate, *CCN1* is overexpressed in benign prostatic hyperplasia and promotes prostate epithelial and stromal cell proliferation (19). Down-regulation of *CCN1* by staurosporine in prostate cancer cells is associated with neuronal differentiation and suppression of malignancy (20), implicating *CCN1* in prostate cancer. Furthermore,

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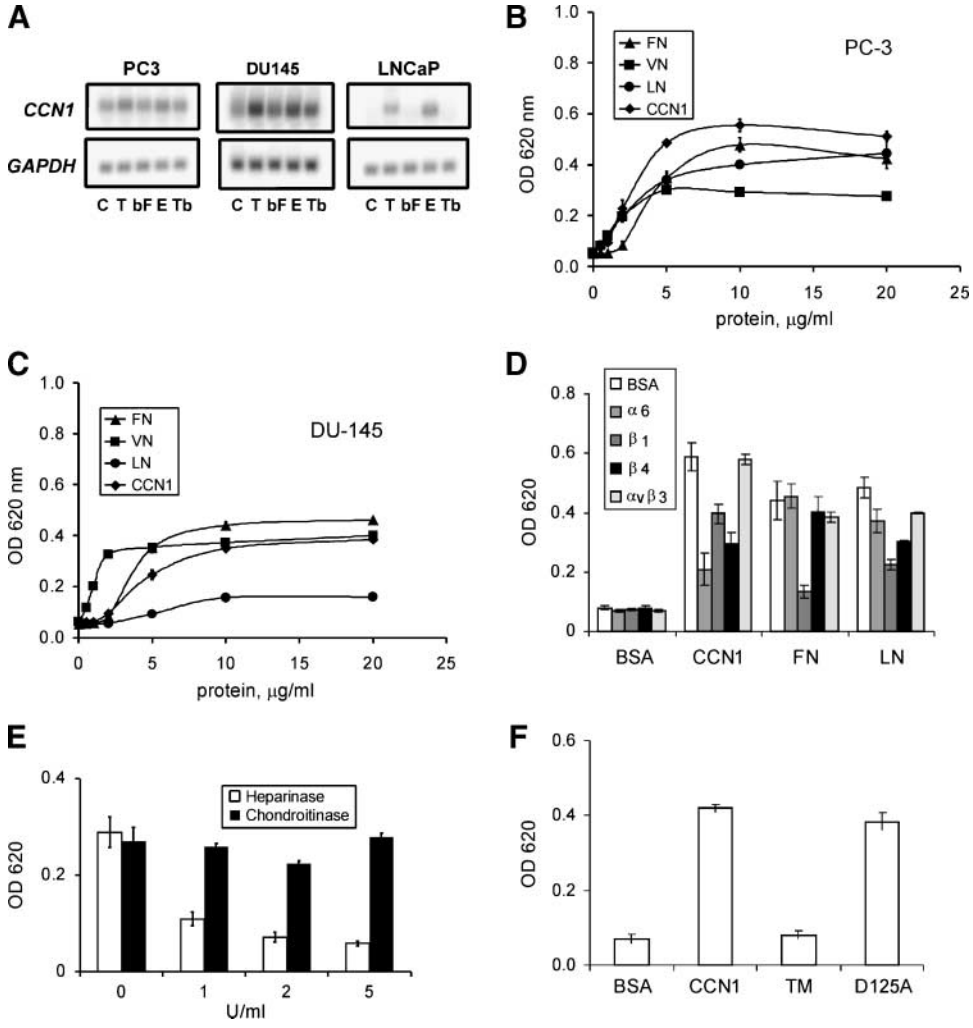

FIGURE 1. CCN1 is expressed in prostate cells and supports prostatic cell adhesion through integrins. **A.** RNA blot of total RNA from PC-3, DU-145, and LNCaP cells treated with TPA (*T*; 10 nmol/L), basic fibroblast growth factor (*bF*; 10 ng/mL), EGF (*E*; 3 ng/mL), or transforming growth factor-β (*Tb*; 10 ng/mL) for 1 h and hybridized to cDNA probes for *CCN1* and *GAPDH*. **B.** PC-3 cells adhered on plates coated with various concentrations of CCN1, fibronectin, vitronectin, and laminin for 30 min. Adherent cells were stained with methylene blue and extracted dye was quantified by absorbance at 620 nm. **C.** Adhesion of DU-145 cells on various substrates as described above. **D.** PC-3 cells were pretreated with antibodies against α_6, β_1, β_4, or $\alpha_v\beta_3$, integrins before adhering to plates coated with indicated substrates and cell adhesion was measured. **E.** PC-3 cells were treated with 1 to 5 units/mL heparinase or chondroitinase and their adhesion to CCN1-coated plates was measured. **F.** PC-3 cell adhesion to plates coated with bovine serum albumin, CCN1, or the CCN1 mutants TM ($\alpha_6\beta_1$-HSPG binding-defective) and D125A (α_v binding-defective) measured by methylene blue staining.

stromal expression of *CCN2*, a close homologue of *CCN1*, has been shown to promote prostate cancer angiogenesis and tumorigenesis (21).

Here, we show that *CCN1* expression in prostate carcinoma cells is a double-edged sword: it enhances both cell proliferation and TRAIL-induced cytotoxicity. Knockdown of constitutive *CCN1* expression in the androgen-independent prostate carcinoma cell lines PC-3 and DU-145 severely inhibits cell proliferation and curtails TRAIL-dependent apoptosis. Mechanistically, CCN1 cooperates with TRAIL in a protein kinase C (PKC) α-dependent manner, triggered by its interaction with integrins $\alpha_v\beta_3$ and $\alpha_6\beta_4$ and syndecan-4. These findings indicate that prostate carcinoma cell growth and apoptosis are regulated by the extracellular matrix (ECM) microenvironment through integrin-mediated signaling and point to *CCN1* expression as a critical factor in both prostatic cell proliferation and sensitivity to TRAIL.

Results

CCN1 Supports Adhesion and Promotes Cell Proliferation of Prostate Cancer Cells

To investigate the potential role of *CCN1* in prostate carcinoma cells, we examined its expression. *CCN1* is expressed in the malignant, androgen-independent PC-3 and DU-145 prostate carcinoma cells, and its expression is induced to a higher level by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and epidermal growth factor (EGF) in DU-145 cells (Fig. 1A). By contrast, *CCN1* mRNA is undetectable in the androgen-dependent LNCaP cells but is induced on by stimulation of cells with TPA

or EGF (Fig. 1A). Because CCN1 is a cell-adhesive ECM protein that induces adhesive signaling in various cell types in endothelial cells through integrin $\alpha_v\beta_3$ and fibroblasts through $\alpha_6\beta_1$ and heparan sulfate proteoglycans (HSPG; ref. 12), we tested the ability of prostate carcinoma cells to adhere to CCN1-coated surfaces. Indeed, CCN1 supported the adhesion of PC-3 and DU-145 cells with similar efficiency compared with other ECM proteins, including fibronectin, vitronectin, or laminin (Fig. 1B and C). Furthermore, inhibitory monoclonal antibodies (mAb) against integrins α_6 (GoH3), β_1 (JB1A), or β_4 (ASC-3), but not mAb against integrin $\alpha_v\beta_3$, blocked the adhesion of PC-3 cells to CCN1 (Fig. 1D). Similar results were observed for DU-145 cells (data not shown). Because CCN1 supports cell adhesion through integrin $\alpha_6\beta_1$ and HSPGs as coreceptors in several cell types including fibroblasts and smooth muscle cells (12), we tested the requirement of HSPGs. Treatment of cells with heparinase, but not chondroitinase, abolished cell adhesion to CCN1 (Fig. 1E). To corroborate these results, we tested the functions of the CCN1 mutants TM and D125A, which contain missense mutations targeting specific receptor binding sites and are unable to bind $\alpha_6\beta_1$-HSPG or $\alpha_v\beta_3$, respectively (22, 23). Whereas TM was completely unable to support cell adhesion, D125A was as active as wild-type CCN1 (Fig. 1F). These results show that CCN1 supports PC-3 prostate carcinoma cell adhesion through integrins $\alpha_6\beta_1$ and $\alpha_6\beta_4$ and HSPGs.

CCN1 is known to enhance growth factor-induced DNA synthesis in some cell types and to stimulate the proliferation of prostate epithelial and stromal cells (19, 24). The basal expression of *CCN1* in PC-3 and DU-145 cells suggests that *CCN1* expression may promote cell proliferation in these aggressive prostate carcinoma cells. To test this possibility, we knocked down *CCN1* expression in PC-3 and DU-145 cells using antisense oligonucleotide and counted the total number of cells at 24, 48, and 72 h post-transfection (Fig. 2). Transfection of antisense oligonucleotide, but not the sense oligonucleotide, significantly reduced *CCN1* expression in PC-3 and DU-145 cells (Fig. 2A and D). Knockdown of *CCN1* in both PC-3 and DU-145 cells resulted in substantially slower rates of growth compared with control cells transfected with the sense oligonucleotide (Fig. 2B and E). Cell division was essentially negligible within the first 48 h in *CCN1*-depleted cells, whereas control cells proliferated substantially. No apoptosis was observed in these cells; thus, the inhibition of cell proliferation by *CCN1* knockdown was not due to apoptotic cell death (Fig. 2C and F). Together, these results show that, as expected, endogenous *CCN1* expression promotes cell proliferation and confers a substantial growth advantage to prostate cancer cells in culture.

CCN1 Cooperates with TRAIL to Induce Apoptosis in Prostate Cells

As a matricellular protein, CCN1 regulates cell survival and cell death through integrin-mediated signaling (24, 25). Recently, we found that CCN1 allows TNF-α to induce apoptosis in human skin fibroblasts by overriding the antioxidant effect

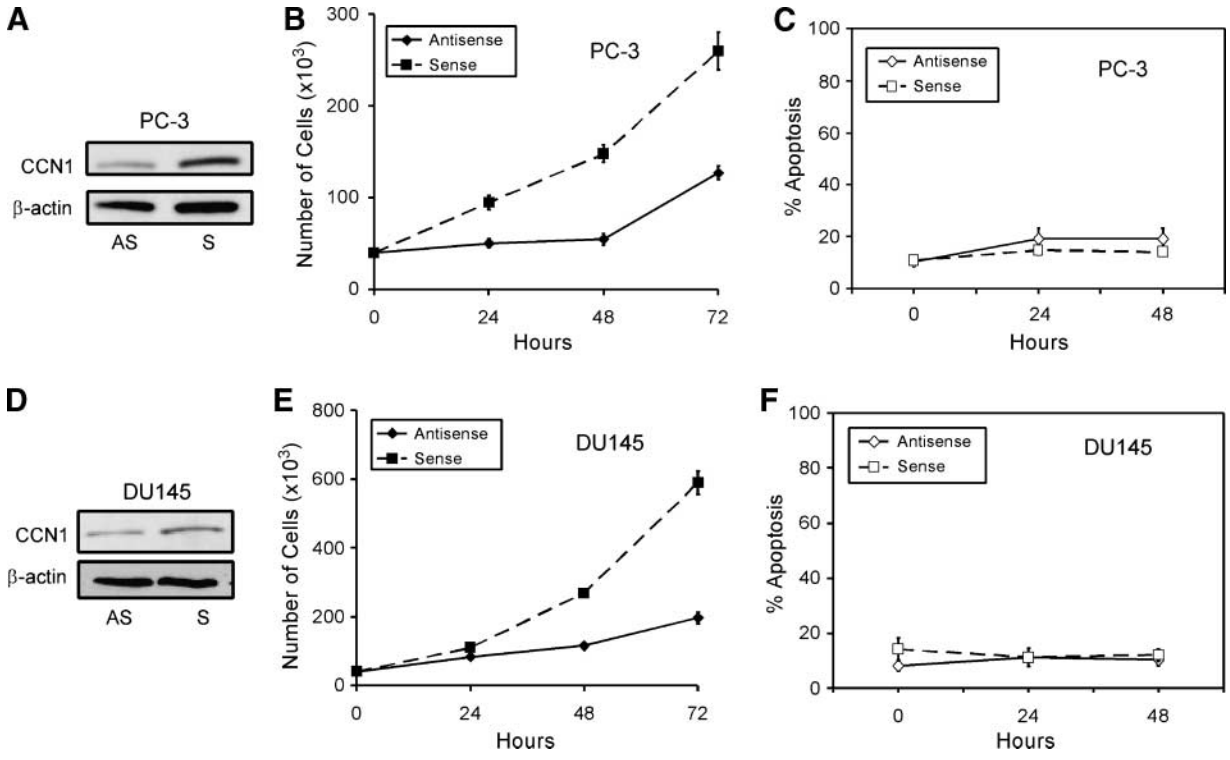

FIGURE 2. CCN1 is critical for proliferation of prostate carcinoma cells. PC-3 (**A**) and DU-145 (**D**) cells were treated with antisense (*AS*) or sense (*S*) *CCN1* oligonucleotides. To show *CCN1* knockdown, cell lysates were collected 24 h after transfection and electrophoresed on 10% SDS-PAGE followed by immunoblotting with antibodies against CCN1 and β-actin. Transfected PC-3 (**B**) and DU-145 (**E**) cells were cultured in growth medium, and total cell numbers were counted 24, 48, and 72 h later. Parallel plates of PC-3 (**C**) and DU-145 (**F**) cells were scored for apoptosis at each time point.

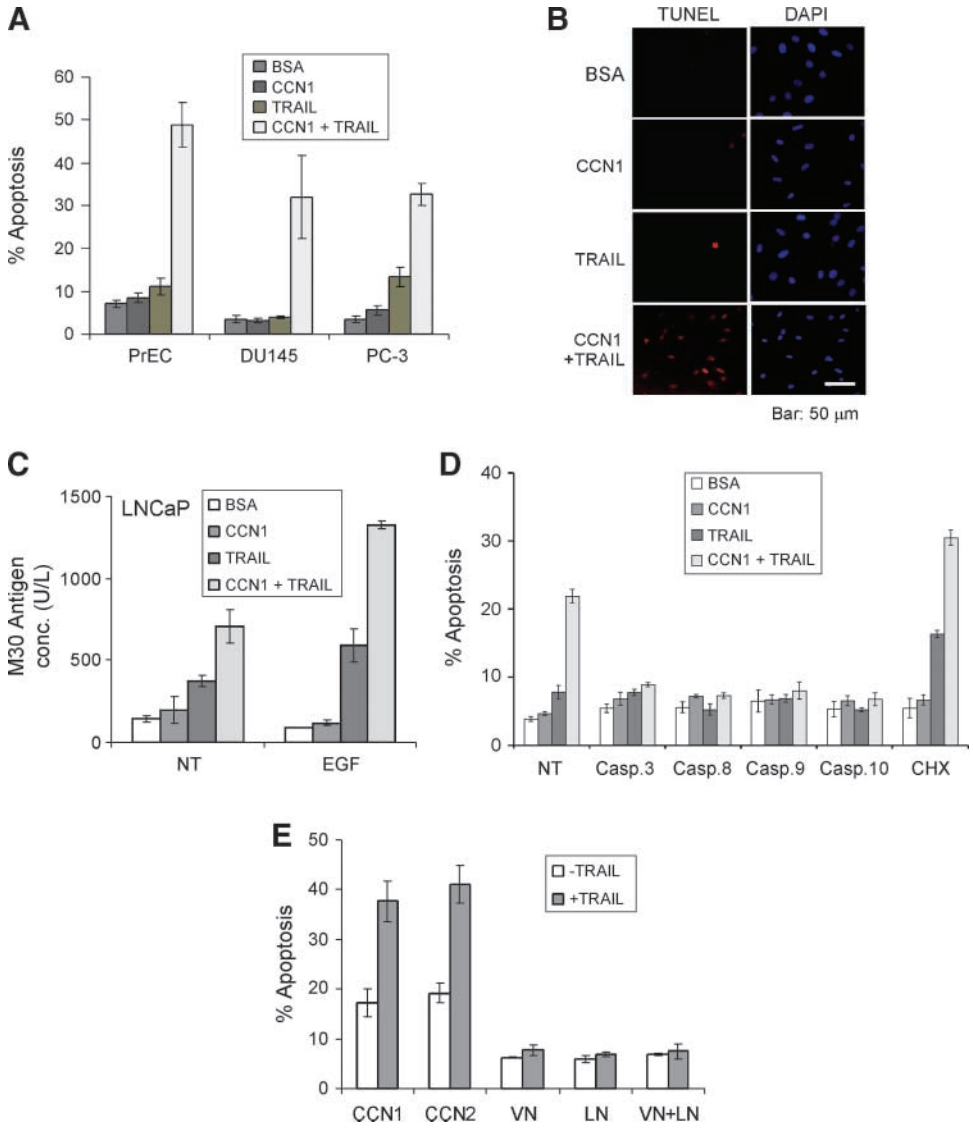

FIGURE 3. CCN1 cooperates with TRAIL to induce apoptosis in prostate cells. **A.** Prostate epithelial cells, DU-145 cells, and PC-3 cells were treated with CCN1 (10 μg/mL) and/or TRAIL (5 ng/mL) for 6 h and scored for apoptosis. **B.** PC-3 cells were treated with CCN1 and/or TRAIL as above followed by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay and counterstaining with DAPI. **C.** LNCaP cells were either untreated (*NT*) or treated with EGF for 60 min before stimulation with CCN1 and/or TRAIL for 6 h and subjected to M30 apoptosis assay. **D.** Cells were pretreated with cycloheximide (5 μmol/L) or caspase inhibitors for 30 min, including 50 μmol/L Z-DEVD (caspase-3), Z-IETD (caspase-8), Z-LEHD (caspase-9), or Z-AEVD (caspase-10), then treated with CCN1 and/or TRAIL for 6 h as above and scored for apoptosis. **E.** PC-3 cells were adhered to plates coated with CCN1, CCN2, vitronectin, laminin (10 μg/mL each), or vitronectin and laminin and cultured in serum-free medium for 6 h with or without TRAIL (5 ng/mL). Cells were then fixed and scored for apoptosis.

of nuclear factor-κB (NF-κB) without requiring the inhibition of protein synthesis or NF-κB signaling (26). Given the potential application of the TNF family member TRAIL as a cancer therapeutic, we tested the possibility that CCN1 might regulate the susceptibility of prostatic cancer cells to TRAIL-induced apoptosis. Whereas CCN1 (even as high as 10 μg/mL) or a low dose of TRAIL (5 ng/mL) alone did not induce appreciable cell death in normal prostate epithelial cells or in DU-145 and PC-3 prostate carcinoma cells, the combination of both proteins triggered ~30% to 50% cell death within 6 h (Fig. 3A and B). Thus, although TRAIL alone can also induce apoptosis in DU-145 and PC-3 cells when used at high concentrations (>10 ng/mL; data not shown; ref. 27), CCN1 strongly enhances TRAIL-induced prostatic cell death and does so in a dose-dependent manner (Fig. 3A; data not shown). Likewise, exogenously added CCN1 cooperated with TRAIL to induce apoptosis in the androgen-dependent LNCaP cells as judged by the M30 assay for caspase-3 activity (Fig. 3C) and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay for cell viability (Supplementary Fig. S1). Because *CCN1* expression in LNCaP cells is inducible by TPA or EGF (Fig. 1A), pretreatment with EGF to induce *CCN1* expression enhanced TRAIL-induced apoptosis (Fig. 3C), consistent with CCN1 cooperation with TRAIL.

Inhibitors against caspase-3, -8, -9, and -10 each abrogated CCN1/TRAIL-mediated cell death (Fig. 3D). These results, together with terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling-positive staining (Fig. 3B), indicate that CCN1/TRAIL-induced cell death is apoptotic in nature. Furthermore, the caspase requirements suggest that apoptotic signals initiated through the TRAIL receptors (caspase-8 and -10) are amplified through the mitochondria, resulting in the activation of caspase-9 (28). CCN1 also cooperates with agonistic antibodies against DR4 and DR5 to induce cell death, indicating that CCN1 can cooperate with apoptotic signaling through either death receptor of TRAIL (Supplementary Fig. S2). Treatment of cells with cycloheximide enhanced TRAIL-induced apoptosis as expected (29) and did not prevent CCN1/TRAIL cooperation (Fig. 3D). Thus, *de novo* protein synthesis is not required for CCN1/TRAIL-dependent cell death.

Because CCN1 is an ECM-associated cell-adhesive molecule, we tested its ability to promote TRAIL-induced apoptosis as a cell adhesion substrate. PC-3 cells were adhered to plates coated with CCN1, the closely related family member CCN2 (connective tissue growth factor; ref. 30), or ECM proteins such as vitronectin and laminin. Cells adhered to CCN1- or CCN2-coated surfaces were highly apoptotic in the presence of TRAIL, whereas cells adhered to vitronectin or laminin did not undergo apoptosis (Fig. 3E). Furthermore, a combination of vitronectin and laminin, which can bind HSPGs and several integrins including $\alpha_6\beta_4$ and $\alpha_v\beta_3$, the CCN1 receptors required for cooperation with TRAIL (see below), was unable to promote TRAIL-mediated apoptosis (Fig. 3E). These results indicate that CCN proteins appear unique among ECM cell-adhesive molecules in being able to cooperate with TRAIL.

Endogenous CCN1 Expression Drives TRAIL Sensitivity

Because exogenously added CCN1 protein can enhance TRAIL-induced apoptosis, we postulated that endogenously expressed *CCN1* in PC-3 and DU-145 cells may sensitize these cells to TRAIL-induced apoptosis. To test this hypothesis, we knocked down the expression of *CCN1* in these cells as described above and then treated them with various concentrations of TRAIL in the presence or absence of exogenously added CCN1 24 h post-transfection. Remarkably, knockdown of *CCN1* by antisense oligonucleotide greatly diminished the sensitivity of cells to TRAIL-induced apoptosis. When *CCN1* was depleted, there was essentially no response to TRAIL at lower doses, and it was not until TRAIL concentrations reached 50 ng/mL that appreciable levels of apoptosis were observed (Fig. 4A and B). By contrast, control cells transfected with sense oligonucleotide were still responsive to TRAIL. Exogenously added CCN1 reversed the effect of antisense oligonucleotides, restoring the apoptotic response to a level similar to that in control cells transfected with sense oligonucleotides. CCN1 protein

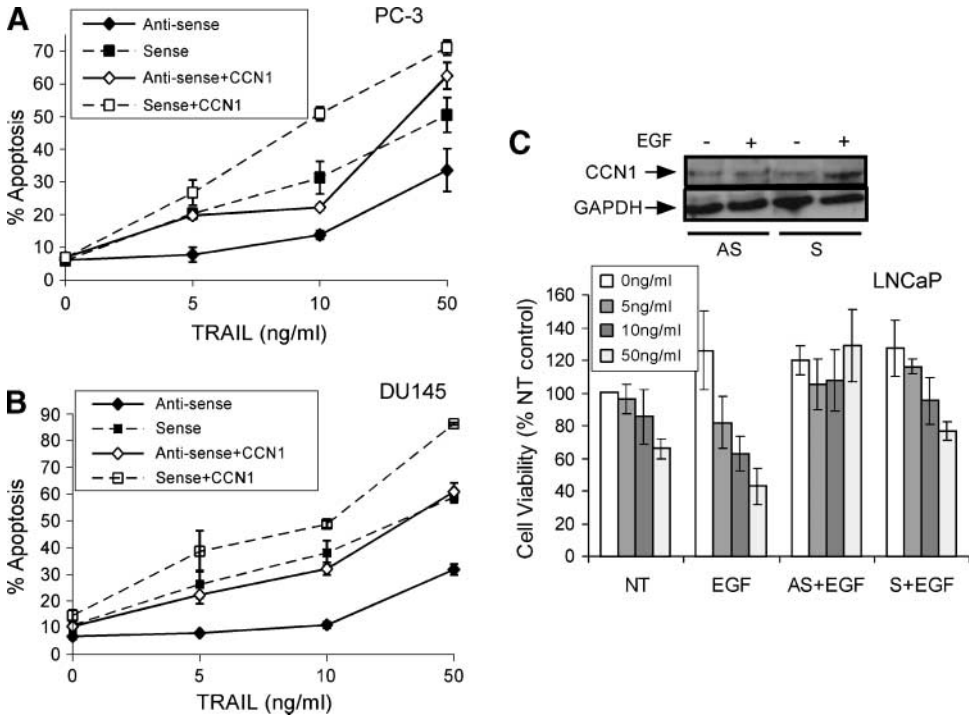

FIGURE 4. Down-regulation of *CCN1* in prostate carcinoma cells inhibits TRAIL-induced apoptosis. PC-3 (**A**) and DU-145 (**B**) cells were transfected with sense or antisense *CCN1* oligonucleotides as described in Fig. 2 and treated with various concentrations of TRAIL (0-50 ng/mL), as indicated, in either the presence or the absence of soluble CCN1 for 6 h before scoring for apoptosis by DAPI staining. **C.** LNCaP cells were transfected with sense or antisense *CCN1* oligonucleotides and treated with EGF (3 ng/mL), where indicated, for 1 h before exposure to various concentrations of TRAIL and CCN1 (10 μg/mL) for 6 h. Cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. To show EGF induction of CCN1 and antisense knockdown, cell lysates were electrophoresed on 10% SDS-PAGE followed by immunoblotting with antibodies against CCN1 and GAPDH (*top*).

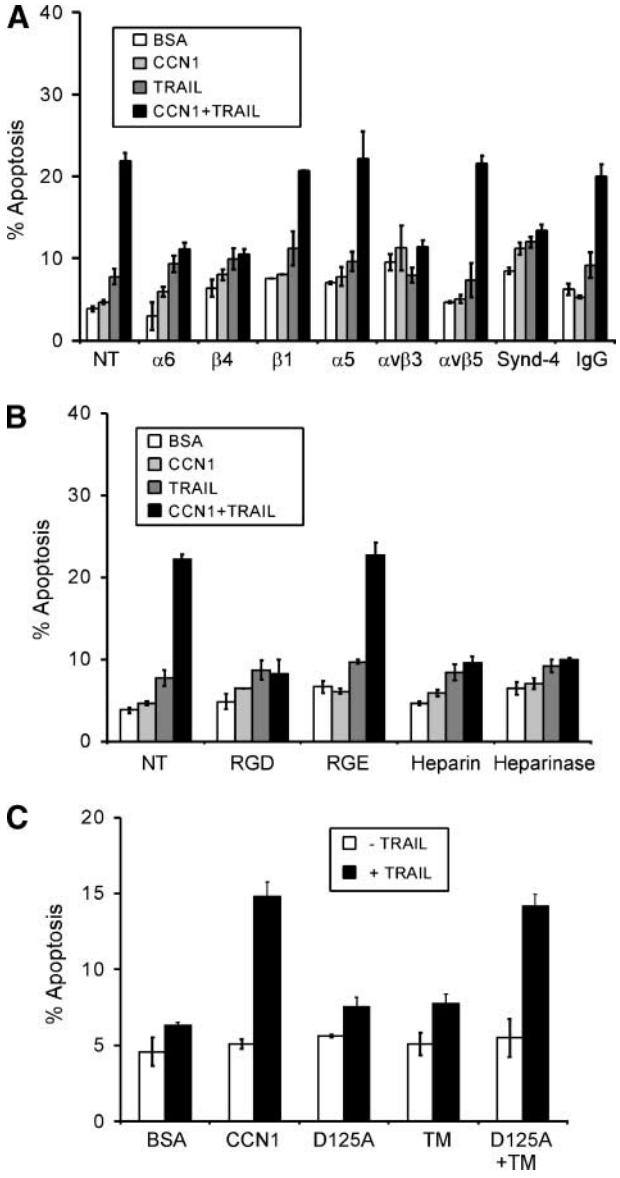

FIGURE 5. CCN1/TRAIL cooperation is mediated through $\alpha_v\beta_3$, $\alpha_6\beta_4$, and syndecan-4. PC-3 cells were scored for apoptosis following indicated treatments. **A.** Cells were pretreated with mAbs (50 μg/mL each) against α_6 (GoH3), β_4 (ASC-3), β_1 (JB1A), $\alpha_5\beta_1$ (JB-55), $\alpha_v\beta_5$ (P1F6), $\alpha_v\beta_3$ (anti-VNR-1), or syndecan-4 or mouse IgG for 1 h before stimulation with CCN1 (10 μg/mL) and/or TRAIL (5 ng/mL). **B.** Cells were pretreated for 30 min with GRGDSP or GRGESP peptides (0.2 mmol/L each), soluble heparin (1 mg/mL), or heparinase (20 units/mL) for 24 h before addition of CCN1 and/or TRAIL. **C.** Cells were treated with wild-type CCN1, D125A (α_v binding-defective mutant), TM ($\alpha_6\beta_1$-HSPG binding-defective mutants), or D125A and TM with or without TRAIL and scored for apoptosis.

added to control cells further enhanced apoptosis in the presence of TRAIL. These results show that endogenously expressed *CCN1* is critical for the susceptibility of PC-3 and DU-145 cells to TRAIL-induced apoptosis. Similarly, antisense oligonucleotide eliminated EGF-induced *CCN1* expression in LNCaP cells, and concomitantly, the EGF enhancement of TRAIL-dependent apoptosis was abolished (Fig. 4C). Thus, endogenous expression of *CCN1* drives TRAIL sensitivity, and *CCN1* knockdown in PC-3, DU-145, and LNCaP prostate carcinoma cells significantly reduced TRAIL-induced apoptosis.

CCN1 Cooperates with TRAIL through Interaction with Integrins and HSPGs

To analyze the mechanism of CCN1/TRAIL cooperation, we have focused on PC-3 cells in subsequent studies. Because PC-3 cells adhered to CCN1 through $\alpha_6\beta_1$, $\alpha_6\beta_4$, and HSPGs, we investigated whether these adhesive receptors for CCN1 also play a role in CCN1/TRAIL cooperation. Pretreatment of cells with mAbs against integrins α_6 (GoH3) or β_4 (ASC-3) subunits blocked CCN1/TRAIL-induced apoptosis, whereas mAbs against β_1 (P1D6) and α_5 (JB1A) had no effect (Fig. 5A). Because α_6 heterodimerizes with β_1 or β_4 chains, these results indicate that although both $\alpha_6\beta_1$ and $\alpha_6\beta_4$ are involved in mediating adhesion to CCN1, only $\alpha_6\beta_4$, but not $\alpha_6\beta_1$, is critical for mediating CCN1 enhancement of TRAIL activity in PC-3 cells. Furthermore, pretreatment of PC-3 cells with function-blocking anti-$\alpha_v\beta_3$ mAb abrogated apoptosis, although $\alpha_v\beta_3$ is not required for adhesion to CCN1. Function-blocking anti-$\alpha_5\beta_1$ or anti-$\alpha_v\beta_5$ mAbs had no effect on CCN1 enhancement of TRAIL activity (Fig. 5B). Consistently, the peptide GRGDSP, which inhibits ligand binding to α_v integrins, $\alpha_5\beta_1$, and $\alpha_8\beta_1$, obliterated CCN1/TRAIL-induced apoptosis in PC-3 cells, whereas the control peptide GRGESP had no effect (Fig. 5B). These results indicate that $\alpha_v\beta_3$ and $\alpha_6\beta_4$ play a critical role in CCN1/TRAIL cooperation, whereas $\alpha_v\beta_5$, $\alpha_5\beta_1$, and $\alpha_6\beta_1$ are not important for this process.

To evaluate the potential role of cell-surface HSPGs, we added soluble heparin in the culture medium or treated the cells with heparinase before apoptosis assay. Either treatment blocked CCN1 enhancement of TRAIL-induced cell death, indicating that cell-surface HSPGs are involved (Fig. 5B). Antibodies against syndecan-4 abrogated CCN1/TRAIL cooperation, whereas control IgG had no effect (Fig. 5A), in agreement with the role of syndecan-4 as the HSPG receptor for CCN1 (25, 26). Together, these results indicate that CCN1 sensitizes PC-3 cells to TRAIL-induced apoptosis through interaction with integrins $\alpha_v\beta_3$ and $\alpha_6\beta_4$ and syndecan-4. Consistent with this interpretation, CCN1 mutants that are either defective for binding α_6 integrin and HSPGs (TM) or $\alpha_v\beta_3$ (D125A) are incapable of apoptotic cooperation with TRAIL (Fig. 5C). Interestingly, the combination of D125A and TM reconstituted wild-type activity, indicating that the mutant proteins are biologically active and that interaction of CCN1 with $\alpha_v\beta_3$ and α_6-HSGPs need not occur through the same CCN1 molecule. However, a combination of vitronectin and laminin, which can bind HSPGs and several integrins including $\alpha_6\beta_4$ and $\alpha_v\beta_3$, the receptors for CCN1 cooperation with TRAIL, was unable to enhance TRAIL activity (Fig. 3E). These results indicate that CCN proteins are unique among ECM molecules in being able to cooperate with TRAIL.

CCN1 Cooperates with TRAIL in a PKCα-Dependent Manner

Published studies have implicated the roles of reactive oxygen species (ROS) and the stress-induced kinases, including c-Jun NH_2-terminal kinase (JNK) and p38 mitogen-activated

protein kinase, in TRAIL-mediated apoptosis (31-34). Recently, we have found that CCN1 is a potent inducer of ROS in fibroblasts (26) and confirmed that CCN1 induces ROS accumulation in PC-3 cells (Supplementary Fig. S3). However, the presence of the ROS scavengers *N*-acetylcysteine or butylated hydroxyanisole had no effect on CCN1/TRAIL-induced apoptosis in PC-3 cells (Supplementary Fig. S3). Further, neither a JNK inhibitory peptide nor a chemical inhibitor of JNK (SP600125) nor the p38 mitogen-activated protein kinase inhibitor SB202190 had any effect on CCN1/TRAIL-mediated apoptosis in PC-3 cells (Supplementary Fig. S4). Therefore, ROS, JNK, and p38 do not appear to be involved in mediating CCN1/TRAIL apoptotic cooperation.

Because TRAIL-induced apoptosis is dependent on PKC activity in LNCaP cells (33), we tested whether PKC plays a role in CCN1/TRAIL cooperation. We found that each of three different inhibitors of classic PKC, Gö6976, bisindolylmaleimide I, and chelerythrine chloride, blocked CCN1-enhanced apoptosis in PC-3 cells (Fig. 6A). Treatment of cells with CCN1 and TRAIL resulted in enhanced phosphorylation of PKC substrates compared with stimulation with either CCN1 or TRAIL alone, indicating that CCN1 and TRAIL cooperate to induce PKC activity (Fig. 6B). This phosphorylation event was blocked by bisindolylmaleimide I, indicating its dependence on PKC. Among the various PKC isoforms, PKCα is uniquely activated by syndecan-4 (35, 36), a coreceptor required for CCN1/TRAIL cooperation (Fig. 5A). Thus, we investigated the role of PKCα by using a well-characterized antisense oligonucleotide to down-regulate its expression (37). Transfection of PKCα antisense oligonucleotide, but not the sense oligonucleotide, reduced the level of PKCα in PC-3 cells (Fig. 6C). Remarkably, knockdown of PKCα completely abrogated CCN1 enhancement of TRAIL-induced apoptosis (Fig. 6C). These results indicate that CCN1-TRAIL cooperation proceeds through a PKCα-dependent mechanism.

Discussion

The present study reveals a surprising functional dichotomy for the matricellular protein CCN1 in prostatic cells. Whereas expression of *CCN1* promotes prostatic cell proliferation, it also enhances the cytotoxicity of TRAIL. Thus, expression of *CCN1* in prostate carcinoma cells appears to be a double-edged sword, conferring a growth advantage on the one hand, while sensitizing them as targets of TRAIL-induced apoptosis on the other (Fig. 7). These findings indicate that the ECM microenvironment may play critical roles in prostate carcinoma cell growth and survival and implicate *CCN1* expression as a

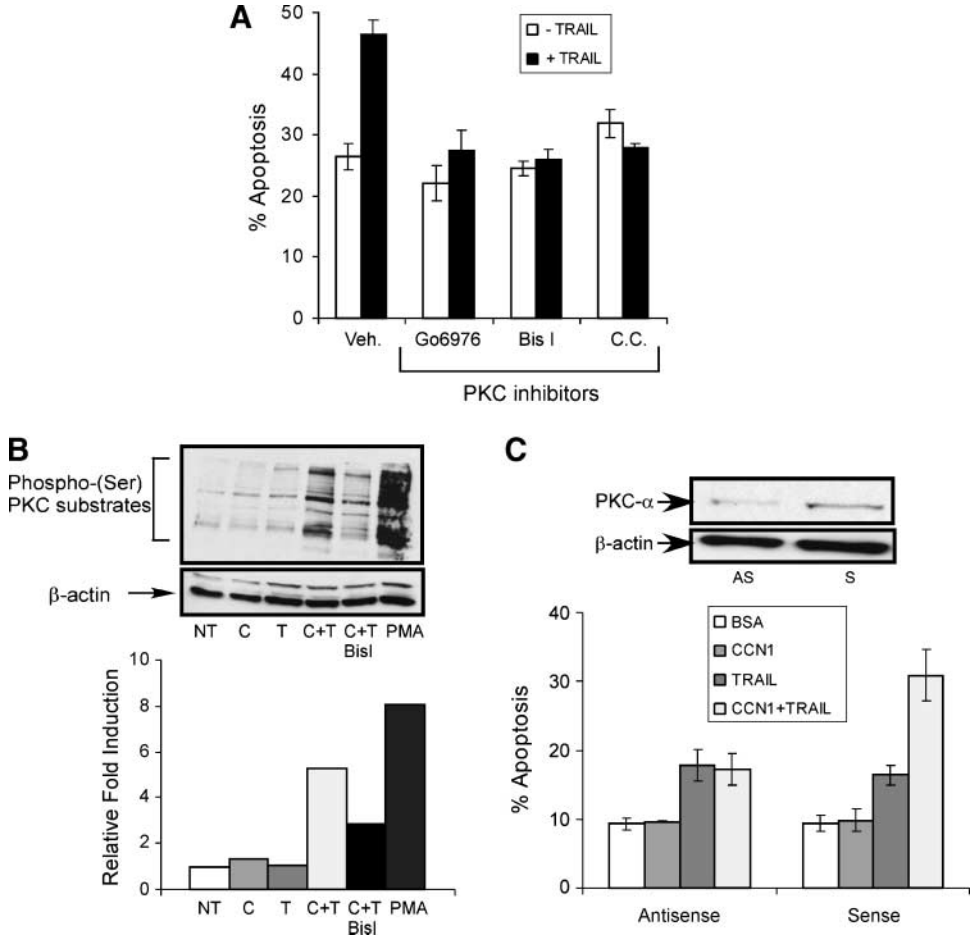

FIGURE 6. PKCα is required for CCN1/TRAIL cooperation. **A.** PC-3 cells were pretreated with Gö6976 (1 μmol/L), bisindolylmaleimide I (1 μmol/L), or chelerythrine chloride (1 μmol/L) or a vehicle control (*Veh*) and adhered to surfaces coated with CCN1 (10 μg/mL). TRAIL was then added and incubated for 6 h and scored for apoptosis. **B.** Adherent PC-3 cells were treated with CCN1 (*C*), TRAIL (*T*), CCN1 and TRAIL (*C+T*), or phorbol 12-myristate 13-acetate (*PMA*) for 30 min, with preincubation with bisindolylmaleimide I for 30 min where indicated. Cell lysates were resolved on 10% SDS-PAGE and immunoblotted with antibodies against phospho-serine PKC substrates and β-actin. Quantification of signals using ImageJ software from NIH. **C.** PC-3 cells were transfected with antisense or sense oligonucleotides against PKCα, treated with CCN1 and/or TRAIL for 6 h, and scored for apoptosis. To analyze the oligonucleotide knockdown, cell lysates were collected 48 h after transfection and resolved on 10% SDS-PAGE followed by immunoblotting with antibodies against PKCα or β-actin (*top*).

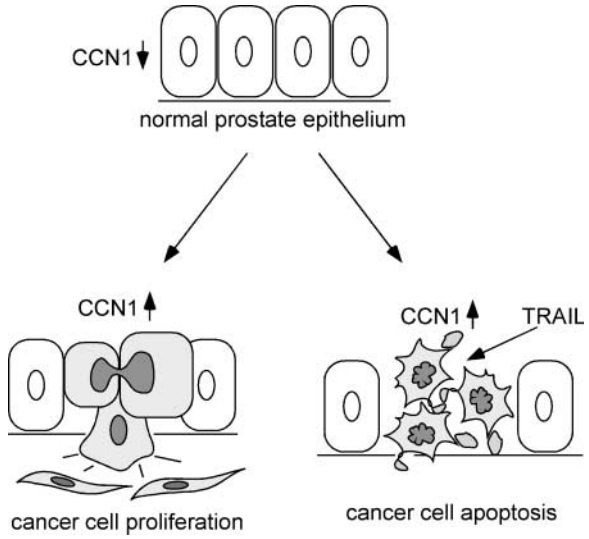

FIGURE 7. A model of CCN1 functions in prostate carcinoma cells. In the normal prostate epithelium, *CCN1* expression is low. Elevated *CCN1* expression (cells in gray) promotes prostate cancer cell proliferation, thus enhancing tumor growth. CCN1 is also a known angiogenic inducer and can up-regulate the expression of matrix metalloproteinases (14, 47), attributes that can promote tumor growth invasion. However, the expression of *CCN1* makes the prostate cancer cells hypersensitive to TRAIL-induced apoptosis.

potentially important parameter in assessing the efficacy of TRAIL-dependent cancer therapy.

CCN1 has been shown to promote DNA synthesis and proliferation of various cell types, including prostate epithelial and stromal cells (19). Thus, it is not surprising that endogenous expression of *CCN1* elevates cell proliferation in PC-3 and DU-145 cells (Fig. 2). Whereas CCN1 supports endothelial cell survival through interaction with integrin $\alpha_v\beta_3$, it can also induce apoptosis in some cell types, including fibroblasts, through an $\alpha_6\beta_1$-HSPG-dependent pathway (24, 25). Although CCN1 does not induce cell death in prostatic cells on its own, it cooperates with TRAIL to trigger apoptosis through interaction with integrins $\alpha_v\beta_3$ and $\alpha_6\beta_4$ and syndecan-4 (Fig. 5). The ability of CCN1 to promote TRAIL-induced cell death in both p53-null (PC-3) and p53 mutant (DU-145) cells indicates that it functions through a p53-independent pathway and suggests that it may be involved in apoptosis of other cancer cells, many of which have lost p53 expression or function. The requirement of multiple CCN1 receptors for cooperation with TRAIL may serve to specify the target cells, ensuring that only those expressing the correct receptors are eliminated. It is interesting to note that a combination of vitronectin and laminin, which together can bind $\alpha_6\beta_4$, $\alpha_v\beta_3$, and HSPGs, is unable to enhance TRAIL cytotoxicity (Fig. 3E), suggesting that unique functions of CCN1 are required. It is possible that CCN1 might engage an additional receptor or that ligation to CCN1 may trigger a unique assembly of receptors that is necessary for crosstalk with the TRAIL-induced signaling pathway. Aside from CCN1, the related family member CCN2 is also able to enhance the cytotoxicity of TRAIL (Fig. 3E), suggesting that this activity may be common among multiple CCN family members. Because CCN2 is also expressed in PC-3 and DU-145 cells (data not shown), it is likely that knockdown of both CCN1 and CCN2 may produce a greater cytoprotective effect against TRAIL-induced apoptosis than depletion of CCN1 alone.

TRAIL is structurally related to TNF-α, a proinflammatory cytokine and a potent activator of NF-κB. TNF-α also induces apoptotic signals that are normally suppressed by NF-κB activity, leading to cell death only when NF-κB signaling is blocked (38). Recently, we have found that CCN1 is able to unmask the cytotoxicity of TNF-α, converting it from a cytokine that normally promotes cell proliferation in fibroblasts into one that induces rapid apoptosis without perturbation of NF-κB signaling (26). Although TNF-α and TRAIL are related cytokines, they act through distinct receptors and signaling mechanisms. CCN1 enables the cytotoxicity of TNF-α in fibroblasts by inducing a high level of ROS, thereby overriding the antiapoptotic effects of NF-κB to achieve a prolonged activation of JNK that is necessary for TNF-α-induced apoptosis (26). By contrast, we show that CCN1/TRAIL cooperation does not require ROS or JNK activation but is instead dependent on the activation of PKCα (Fig. 6; Supplementary Figs. S3 and S4).

The requirement of PKCα for CCN1 to enhance TRAIL activity is somewhat unexpected, because PKCα signaling is generally associated with cell survival (39). Syndecan-4 can directly activate PKCα (35), suggesting that CCN1 may activate PKCα through its interaction with syndecan-4. PKCα is promitogenic in many cancer cells, and antisense approaches targeting PKCα are undergoing clinical trial as cancer therapy (40). Nevertheless, PKCα has also been shown to mediate apoptosis in some cell types. For example, in LNCaP prostate carcinoma cells, phorbol ester-induced apoptosis proceeds through a PKCα-dependent pathway through a mechanism requiring p38 mitogen-activated protein kinase (33). By contrast, the PKCα-dependent apoptotic cooperation of CCN1 and TRAIL does not require activation of p38 mitogen-activated protein kinase or JNK. Future studies will be required to elucidate precisely how PKCα signaling mediates apoptosis in this context.

The expression of *CCN1* has been detected in stromal cells of prostate carcinomas (ref. 41; data not shown). Interestingly, stromal expression of *CCN2*, a close homologue of *CCN1*, has been shown to promote prostate cancer angiogenesis and tumorigenesis (21). Both CCN1 and CCN2 are potent angiogenic inducers (14, 42) and may promote tumorigenesis through angiogenesis. Although *CCN1* expression may confer a growth advantage in prostatic carcinoma cell lines, it may also enhance tumor suppression through TRAIL-induced apoptosis. It has been observed that *CCN1* is down-regulated in some prostate carcinomas (43), suggesting the possibility that low *CCN1* expression may help evade immune surveillance in some instances.

Because TRAIL preferentially targets a variety of cancer cells over normal cells for elimination, its deployment has emerged as a promising approach of cancer therapy (7-9). TRAIL shows no systemic toxicity in nonhuman primates, and phase I clinical trials using agonistic mAbs against DR4 and DR5 have also found no significant toxicity, supporting the notion that inducing TRAIL signaling may be a useful therapeutic strategy (44). Thus, understanding the molecular basis of TRAIL sensitivity may be of potential therapeutic significance. Our findings indicate that CCN proteins may function as novel matrix regulators of TRAIL-induced

apoptosis, providing contextual cues for the induction of cell death. Thus, the expression status of *CCN1* may be an important consideration in assessing the efficacy of TRAIL-dependent cancer therapies.

Materials and Methods

Cell Culture

Normal human prostate epithelial cells were obtained from Cambrex along with the basal medium and growth medium supplements. PC-3, DU-145, and LNCaP cells were obtained from the American Type Culture Collection and grown in RPMI 1640 (Life Technologies) with 10% fetal bovine serum (Intergen). All cells were grown at 37°C with 5% CO_2.

Proteins, Reagents, and Antibodies

Wild-type CCN1, CCN2, and mutant CCN1 proteins were purified from a baculovirus expression system as described (14, 22). Mouse mAb against PKCα (clone 3), fibronectin, and laminin were from BD Biosciences. Heparin (sodium salt), 4′,6-diamidino-2-phenylindole (DAPI), human vitronectin, TPA/phorbol 12-myristate 13-acetate, *N*-acetylcysteine, butylated hydroxyanisole, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, bovine serum albumin, heparinase, chondroitinase, and anti-β-actin mAb (AC-15) were from Sigma. EGF and basic fibroblast growth factor were supplied by Collaborative Biomedical Products. Transforming growth factor-β was from R&D Systems. Recombinant soluble human TRAIL was from Axxora. Synthetic peptides GRGDSPK and GRGESPK were purchased from American Peptide Company. Function-blocking mAbs against integrins, including GoH3 (anti-α_6), anti-β_4 (ASC-3), JB1A and P5D2 (anti-β_1), P1D6 (anti-α5), and PiF6 (anti-$\alpha_v\beta_5$), were purchased from Chemicon. Function-blocking anti-$\alpha_v\beta_3$ antibody (anti-VNR-1) was a generous gift from Dr. Stephen Lam (University of Illinois). Polyclonal phospho-serine PKC substrate antibodies were from Cell Signaling. Rabbit polyclonal syndecan-4 antibodies were from Santa Cruz Biotechnology. H_2DCF-DA was obtained from Molecular Probes. Anti-mouse and anti-rabbit secondary antibodies were purchased from Amersham. Rabbit polyclonal anti-CCN1 antibodies were raised in rabbits as described previously (14). Caspase-3 inhibitor Z-DEVD-fmk, caspase-8 inhibitor Z-IETD-fmk, caspase-9 inhibitor Z-LEHD-fmk, caspase-10 inhibitor Z-AEVD-fmk, cycloheximide, JNK inhibitor I (cell-permeable inhibitory peptide from the JNK-binding domain of JIP), JNK inhibitor II (SP600125), p38 inhibitor (SB202190), and PKC inhibitors (Gö6976 and bisindolylmaleimide I, and chelerythrine chloride) were from Calbiochem. Rabbit polyclonal antibodies against GAPDH and agonistic mAbs against DR4 (DR-4-02) and DR5 (79103) were from Abcam and R&D Systems, respectively.

Northern Hybridization

Cell were grown to subconfluency in RPMI 1640 without phenol red with 5% delipidated fetal bovine serum and starved in serum-free RPMI 1640 24 h before treatment with TPA, basic fibroblast growth factor, EGF, or transforming growth factor-β for 1 h at 37°C, 5% CO_2. Total RNA was isolated and electrophoresed on 1% agarose gel followed by RNA blotting and hybridization with radioactively labeled human CCN1 and GAPDH cDNA probes using standard protocols.

Adhesion Assays

Adhesion assays were done as described previously (30). Briefly, CCN1, fibronectin, vitronectin, laminin, or bovine serum albumin were coated on 96-well plate (50 μL protein/well in 1× PBS) and kept at 4°C overnight. The wells were then blocked for 1 h with 1% bovine serum albumin at room temperature. Cells were brought to suspension in 1× PBS, 2.5 mmol/L EDTA, 0.1% glucose. Following two washes in PBS, cells were suspended in serum-free medium and allowed to adhere to the protein-coated wells at 37°C for 10 to 15 min. Where indicated, antibodies, heparin, or heparinase were added to the cell suspension followed by a 30 min gentle agitation at room temperature before plating. Adherent cells were fixed overnight with formalin, washed, and stained with 1% methylene blue in sodium borate buffer (10 mmol/L, pH 6.0) for 30 min at room temperature. The cells were then washed with boric acid buffer, and the dye was extracted with 50% ethanol/0.1 N HCl and quantified by absorbance at 620 nm.

Apoptosis Assays

For DAPI staining, cells were plated in 24- or 48-well plates (50,000 or 25,000 per well, respectively) in culture medium and allowed to attach overnight. Cells were rinsed with PBS and incubated at 37°C with serum-free medium with test molecules for 6 h. Cells were fixed with 4% formaldehyde overnight and then stained with DAPI at 1 μg/mL in PBS for 5 min. Using a fluorescent microscope, three randomly selected fields (~150 cells per field) per well were counted. For terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay, PC-3 cells were plated on cover slips (in a 24-well plate, 50,000 per well). Apoptosis was carried out as described above and fixed with 1% paraformaldehyde for 10 min at room temperature. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay was done according to the manufacturer's instructions (ApopTag, Red; Chemicon). Samples were counterstained with DAPI before mounting.

Apoptosis in LNCaP cells were scored by the M30 assay, which detects the caspase-3 cleaved form of keratin XVIII (45, 46). The M30 Apoptosense ELISA was done according to the manufacturer's instruction (DiaPharma). Absorbance was measured at 450 nm. Cell viability was determined with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay for metabolic activity, carried out as described (31).

Oligonucleotide Transfection

PC-3 and DU-145 cells were plated in 24-well plates (20,000 per well) overnight and transfected with phosphorothiolated oligonucleotides (Sigma Genosys) using Lipofectamine reagent (Invitrogen) according to the manufacturer's instructions. Briefly, 0.4 μg oligonucleotide was complexed with 0.5 μL Lipofectamine in the presence of 4 μL Plus reagent (Invitrogen) in a total volume of 250 μL serum- and antibiotic-free RPMI 1640. Transfection was carried out for 3 h, at which time medium was added to make a final volume of 500 μL RPMI 1640 with 10% fetal bovine serum. Cells were washed with PBS 24 h post-transfection, and normal growth medium was added. After 24 or 48 h, cell lysates were collected

for immunoblotting. The *CCN1* antisense sequence was 5′-GCAGGCACGGGGCAGGTGG-3′ and the sense sequence was 5′-CCACCTGCCCGCTGCCTGC-3′ (19). The antisense sequence for PKCα was 5′-CAGCCATGGTTCCCCCCAAC-3′ and the sense sequence was 5′-CCAGTCACTCGCAC-CATCGC-3′ (37).

Growth Curves

PC-3 and DU-145 cells were plated in 6-well plates and transfected with oligonucleotides as described above, and growth was assessed at 24, 48, and 72 h. At each time point, cells were collected by trypsin and the number of cells was counted using a hemocytometer. Cell death was measured in parallel at each time point with DAPI staining.

Immunoblotting

Immunoblotting was done according to standard protocols. Samples represent an equivalent amount of protein from 250,000 cells and were resolved on 10% SDS-PAGE before transfer to nitrocellulose membranes.

Measurement of ROS

ROS generation was measured as described previously (26). Briefly, intracellular peroxide was measured using the cell-permeable dye, H_2DCF-DA, which becomes fluorescent on oxidation by intracellular peroxide/hydroperoxides. PC-3 cells (50,000 per well) plated on glass coverslips were serum starved in phenol red-free medium for 30 min before treatment. Medium was then replaced after 1 h of treatment with PBS containing 5 μmol/L H_2DCF-DA and incubated for 10 min at 37°C. Coverslips were then mounted on slides and imaged by fluoresce microscopy. The integrated densities were measured using NIH ImageJ software.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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