

Connective tissue growth factor–specific antibody attenuates tumor growth, metastasis, and angiogenesis in an orthotopic mouse model of pancreatic cancer

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Abstract

Connective tissue growth factor (CTGF) plays an important role in fibrosis by modulating cell migration and cell growth but may also modify tumor growth and metastasis. Because CTGF is overexpressed in pancreatic ductal adenocarcinoma, we investigated the *in vitro* effects of CTGF on the proliferation and invasiveness of PANC-1 pancreatic cancer cells and examined the consequences of its *in vivo* inhibition on the growth and metastasis of these cells using a fully human CTGF-specific monoclonal antibody (FG-3019) in an orthotopic nude mouse model. Although PANC-1 cells expressed relatively high levels of endogenous CTGF mRNA, the addition of CTGF to conditioned medium increased the proliferation and invasiveness of PANC-1 cells. Moreover, transforming growth factor- β 1 caused a further increase in CTGF expression in these cells. *In vivo*, the twice weekly i.p. administration of FG-3019 decreased tumor growth and metastasis and attenuated tumor angiogenesis and cancer cell proliferation. FG-3019 did not enhance apoptosis and did not attenuate the inhibitory effects of gemcitabine on tumor growth and metastasis. These findings suggest that CTGF may contribute to aberrant autocrine and paracrine pathways that promote pancreatic cancer cell growth, invasion, metastasis, and angiogenesis. Therefore, blocking CTGF actions with FG-3019 may represent a novel therapeutic approach in pancreatic ductal adenocarcinoma. [Mol Cancer Ther 2006;5(5):1108–16]

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer death in the United States (1). Despite recent advances, the prognosis of patients with PDAC is extremely poor, with a median survival of ~6 months (1). Although survival is often limited to patients who had surgery at an early stage of the disease, the diagnosis of PDAC is established at an advanced stage in ~85% of cases, precluding these patients from surgery. This biological aggressiveness is due, in part, to the propensity of the tumor to metastasize (2). The current standard of care for metastatic pancreatic cancer patients is gemcitabine (Gemzar), with a median survival time of 5.7 months and a 1-year survival rate of 18%. Erlotinib (Tarceva), which targets the epidermal growth factor receptor and was recently approved for use in combination with gemcitabine in pancreatic cancer patients, showed a median survival of 6.4 versus 5.9 months with gemcitabine alone. There is an urgent need, therefore, for an improved understanding of the molecular mechanisms that contribute to pancreatic tumor growth and metastasis to allow for the design of better therapeutic modalities for this disorder.

At the molecular level, PDAC is characterized by multiple alterations, including mutations in the *K-ras*, *p53*, *p16*, and *Smad4* genes (3). In addition, there is overexpression of multiple mitogenic and angiogenic growth factors and their tyrosine kinase receptors (4, 5), high levels of transforming growth factor- β (TGF- β) isoforms (6), which are known to up-regulate the expression of connective tissue growth factor (CTGF) in multiple cell types (7), and an abundance of CTGF (8, 9).

CTGF, also known as CCN2, is a 38-kDa multifunctional growth factor that is a member of the CCN cysteine-rich family of proteins (10, 11) and that has been implicated in chondrogenesis, cell migration, proliferation, and differentiation and in the modulation of extracellular matrix deposition and angiogenesis (12–17). It was originally identified as a mitogen in conditioned medium from human umbilical vein endothelial cells, and its expression, in addition to being up-regulated by TGF- β , is enhanced by vascular endothelial growth factor-A (VEGF-A) and hypoxia (7, 12–14). It is important in embryonic development as underscored by the finding that CTGF-null mice exhibit an angiogenic defect in bone that leads to perinatal death due to respiratory failure associated with marked skeletal defects (18).

Additional members of the CCN family include Cyr61/CCN1, NOV/CCN3, Elm1/WISP1/CCN4, rCop1/WISP2/CCN5, and WISP3/CCN6 (10, 11). With the exception of CCN5, which has three modules, CCN family members are

characterized by four modular domains that participate in protein-protein interactions (10, 11). Module I, encoded by exon 2, shares partial identity with the NH₂-terminal part of insulin-like growth factor-binding proteins. Module II, encoded by exon 3, shares partial identity with the von Willebrand factor type C repeat. Module III, encoded by exon 4, shares partial identity with the thrombospondin type 1 repeat and can bind to VEGF-A (19). Module IV, encoded by exon 5, is the least conserved of the four domains but contains six cysteines that form a “cysteine knot” motif (20), which is characteristic of diverse growth factors, such as TGF- β , platelet-derived growth factor, and nerve growth factor. This module binds to integrins and contains the heparin-binding site that mediates binding to heparin sulfate proteoglycans in the extracellular matrix or on the cell surface (21).

CTGF is expressed at high levels in several types of human malignancies, and in some instances, its expression has been shown to correlate with tumor stage and/or patient prognosis (7, 22–30). In brain tumors, high levels of CTGF are associated with highly invasive glioblastomas and low levels of CTGF are seen with low-grade astrocytomas (23). In the glioblastomas, CTGF is especially abundant in proliferating endothelial cells (23), the presence of CTGF in the cancer cells correlates with an enhanced capacity to proliferate (23), and increased CTGF mRNA levels in the tumors are associated with decreased patient survival (24). In breast cancers, increased CTGF levels are associated with a more aggressive disease (22), and CTGF has been shown to mediate the metastasis of osteolytic breast cancer (31, 32). However, in colorectal cancer (33) and non-small cell lung cancer (34), high CTGF expression levels have been associated with improved survival. Furthermore, engineered CTGF overexpression inhibits metastasis and invasion of human lung cancer cells (34) and attenuates the growth and tumorigenicity of oral squamous cell carcinoma cells (35).

The important contributions of TGF- β with respect to tumor growth and metastasis in PDAC have been confirmed by using a soluble TGF- β receptor strategy in nude mouse models of PDAC (36, 37). By contrast, the potential role of CTGF in PDAC has not been clearly delineated, although TGF- β s are known to up-regulate CTGF expression (7). Therefore, in the present study, we sought to characterize the effects of CTGF inhibition on pancreatic tumor growth and metastasis in an orthotopic nude mouse model, in which s.c. derived pancreatic tumor fragments are implanted in the pancreas of athymic nude mice (37, 38). We chose to use the orthotopic model because metastatic lesions do not develop in the s.c. model (36–38). We also examined the effects of CTGF on pancreatic cancer cell proliferation and invasiveness *in vitro*. We now report that FG-3019, a fully human monoclonal CTGF-specific antibody, markedly attenuates pancreatic tumor growth, angiogenesis, and metastasis in an orthotopic mouse model, raising the possibility that CTGF may be a novel therapeutic target in PDAC and that FG-3019 may have a therapeutic potential in this disorder.

Materials and Methods

Materials

The following materials were purchased as follows: DMEM and trypsin-EDTA (Mediatech, Inc., Herndon, VA); fetal bovine serum (Omega Scientific, Inc., Tarzana, CA); anti-CD31 rat anti-mouse monoclonal antibody (PharMingen, San Diego, CA); anti-proliferating cell nuclear antigen (PCNA) monoclonal antibody (Novocastra, Newcastle upon Tyne, United Kingdom); ApopTag *in situ* apoptosis detection kit (Chemicon, Temecula, CA); Matrigel-coated invasion chambers (BD Biosciences, Bedford, MA); oligonucleotide primers, Taqman Universal PCR Master Mix, and Taqman Assays-on-Demand (Applied Biosystems, Foster City, CA); gemcitabine (Eli Lilly, Indianapolis, IN); and PANC-1 pancreatic cancer cells (American Type Culture Collection, Manassas, VA). All other reagents were from Sigma Chemical Co. (St. Louis, MO). Recombinant human TGF- β 1 was from Genentech, Inc. (South San Francisco, CA). Recombinant human CTGF (rhCTGF), which was expressed and purified from CTGF baculovirus-infected insect cells (39), and FG-3019, a fully human IgG1 κ monoclonal antibody recognizing domain 2 of rodent and human CTGF and directed against an epitope (amino acids 142–157) that is not conserved between CCN family members (40), were gifts from FibroGen, Inc. (South San Francisco, CA). An article detailing the high degree of specificity of FG-3019 is currently in preparation.

Cell Growth and Invasion Assays

Human pancreatic cancer cell lines were grown in DMEM (COLO-357, PANC-1, and MiaPaCa-2) or RPMI (BxPC3, CAPAN-1, and T3M4). All media were supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μ g/mL streptomycin (complete medium). For cell growth assays, PANC-1 cells were seeded at a density of 20,000 per well, and following specified additions and times, cells were counted with a hemocytometer.

To assess the effects of CTGF on cell invasion capacity, PANC-1 cells were incubated for 24 hours in serum-free DMEM, and 2.5×10^4 cells were then added to the top chamber of Matrigel-coated invasion chambers (8 μ m filters). Incubation was continued for 18 hours at 37°C in the absence or presence of CTGF, which was added to the lower chamber. The membranes were fixed in methanol and stained with H&E (37). Noninvasive cells on the upper surface of the filters were scraped off with a cotton swab, and the cells that had migrated through the membrane to the lower surface of the filter were counted in nine different fields using a light microscope (magnification, $\times 200$). Invasion assays were done in triplicate, and each experiment was repeated thrice.

In vivo Tumorigenicity and Metastasis

PANC-1 cells were injected s.c. into the flank region on one side of 4- to 6-week-old, female, athymic (nude) mice (Harlan, Indianapolis, IN). The animals were killed 8 to 15 weeks after injection when the tumor diameter approached 15 mm. Tumors were rapidly excised and two small fragments were implanted orthotopically into a new group

of 6-week-old, female, nude mice as described previously (37). For most experiments, 2 weeks following implantation, mice were given twice weekly i.p. injections of either carrier buffer or 20 mg/kg FG-3019, which were given for 6 weeks. The mice were then killed and the size of the intrapancreatic tumors was calculated as $\pi / 4 \times \text{width} \times \text{height} \times \text{length}$ of the tumor as reported previously (37). In addition, the number of metastatic lymph nodes was counted. The intrapancreatic tumors were rapidly excised and divided into three fragments. One fragment was snap frozen in liquid nitrogen and stored at -80°C , another was embedded in OCT, frozen in liquid nitrogen, and stored at -80°C for subsequent staining with anti-CD31 antibody (38), and a third tumor fragment was fixed in formalin and embedded in paraffin for subsequent immunohistochemical staining.

To assess potential interactions with the nucleoside analogue gemcitabine, a separate series of experiments was done in which, 3 weeks following tumor fragment implantation, mice were given twice weekly i.p. injections of either carrier buffer or the indicated doses of FG-3019, which were continued for 3.5 weeks. Some mice were given once weekly injections of 300 mg/kg gemcitabine for a total of three injections that were given alone or in combination with twice weekly injections of FG-3019. Tumors were then excised and tumor weights were measured using an electronic balance.

All studies with mice were approved by Dartmouth Medical School Institutional Animal Care and Use Committee.

Immunohistochemistry

Paraffin-embedded tumor tissues were sectioned (5 μm thick), mounted on poly-L-lysine-coated glass slides, and allowed to dry overnight at 23°C . These sections were used for detection of PCNA and apoptosis by the ApopTag terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay (38, 41). PCNA immunostaining was done using the streptavidin-peroxidase technique (Vectastain ABC kit). For antigen retrieval, sections were heated in a microwave oven (95°C) for 5 minutes in 10 mmol/L citrate buffer (pH 6.0). Sections were then incubated overnight at 4°C with a monoclonal anti-PCNA antibody (2 $\mu\text{g}/\text{mL}$). Bound antibody was detected with biotinylated anti-mouse IgG and Vectastain ABC reagent complex using 3,3'-diaminobenzidine as the substrate (38). Counterstaining was with Mayer's hematoxylin. Some sections were incubated without primary antibodies and did not yield positive immunoreactivity. The TUNEL assay was done by detecting digoxigenin residues catalytically bound to the DNA 3'-OH ends by terminal deoxynucleotidyl transferase using antidigoxigenin antibodies conjugated with peroxidase and 3,3'-diaminobenzidine as the substrate followed by counterstaining with methyl green (41). Omission of terminal deoxynucleotidyl transferase enzyme did not yield a signal.

To assess microvessel density, tissues frozen in OCT (5 μm) were mounted on poly-L-lysine-coated glass slides, fixed in acetone, and incubated overnight (4°C) with

1 $\mu\text{g}/\text{mL}$ of a monoclonal anti-CD31 antibody (38). Bound antibody was detected with biotinylated rabbit anti-rat IgG secondary antibody and streptavidin-peroxidase complex. Mayer's hematoxylin was used for counterstaining.

Quantitative morphometry was done by analyzing slides with Image-Pro Plus (Media Cybernetics, Silver Spring, MD) as reported previously (38). Three fields at $\times 100$ magnification were chosen randomly from each tumor for CD31 staining, and five fields at $\times 400$ magnification were analyzed following TUNEL and PCNA staining.

Quantitative Real-time PCR

To perform quantitative real-time PCR (Q-PCR), RNA extraction from cultured pancreatic cancer cell lines, reverse transcription-PCR, and first-strand cDNA synthesis for Q-PCR were carried out as described previously (42) using an ABI PRISM 7300 sequence detection system (Applied Biosystems) and target gene sequences were from the National Cancer for Biotechnology Information Genbank databases. RNA expression was calculated based on a relative standard curve representing 4-fold dilutions of human cDNA. Q-PCR data were expressed as a relative quantity based on the ratio of the fluorescent change observed with the target gene to the fluorescent change observed with rRNA.

CTGF ELISA

To assess the amount of CTGF released by the cells, 1×10^5 BxPC3 and PANC-1 cells were seeded into 24-well plates and cultured for 48 hours in complete medium (DMEM + 10% fetal bovine serum). The medium was then changed to serum-free medium containing 0.25% bovine serum albumin and 100 $\mu\text{g}/\text{mL}$ heparin, and incubation was continued for 48 hours. Cell-free culture supernatants were then analyzed for CTGF expression levels by ELISA (43).

Statistical Analysis

Except when indicated, the two-sided Student's *t* test was used for statistical analysis, with $P < 0.05$ taken as significant.

Results

Expression of the CCN Family of Genes in Human Pancreatic Cancer Cells

To characterize the expression of all the members of the CCN family in human pancreatic cancer cell lines, Q-PCR was carried out using RNA prepared from BxPC3, CAPAN-1, COLO-357, MiaPaCa-2, PANC-1, and T3M4 cells (Fig. 1). This analysis revealed that PANC-1 cells, in addition to expressing relatively high levels of CTGF mRNA, expressed relatively high levels of Cyr61, NOV, WISP1, WISP2, and WISP3 mRNA levels. MiaPaCa-2 cells expressed relatively low levels of CTGF mRNA, but the other cell lines, with the exception of PANC-1 cells, expressed even lower levels of CTGF. By contrast, all the cell lines, with the exception of MiaPaCa-2 cells, expressed relatively high levels of NOV (Fig. 1).

To assess whether there was a correlation between CTGF mRNA levels and CTGF release by the cells, a sensitive and specific ELISA assay (43) was used to measure CTGF levels

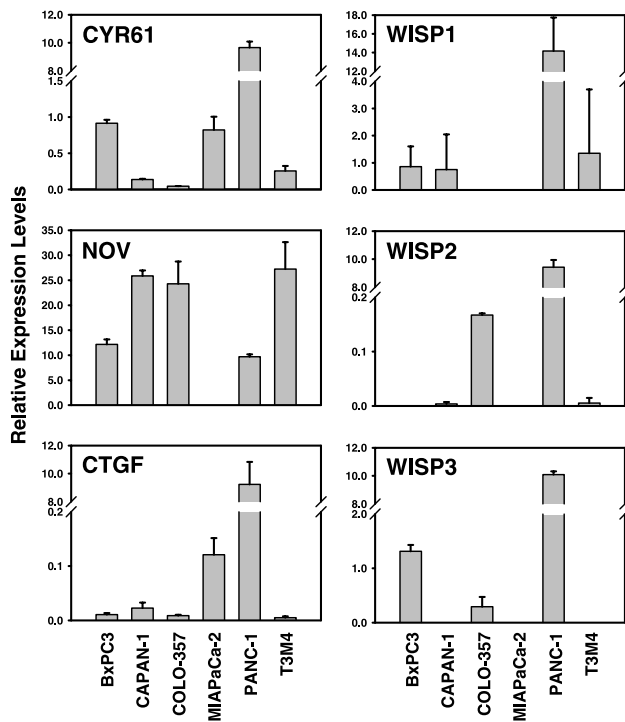


Figure 1. Expression of CCN family members in pancreatic cancer cell lines. RNA from the indicated cell lines was subjected to Q-PCR as described in Materials and Methods. Relative expression levels for the indicated CCN family member as described in Materials and Methods. *Columns*, mean of triplicate determinations from a representative experiment; *bars*, SE.

in PANC-1 cells, which expressed the most CTGF at the mRNA level, and BxPC3 cells, which expressed very low CTGF mRNA (Fig. 1). This analysis revealed that PANC-1 cells secreted 53 ng/mL CTGF during a 48-hour incubation period, whereas BxPC3 cells did not release any detectable CTGF.

PANC-1 cells are poorly differentiated and are known to harbor *K-ras* and *p53* mutations and to express high levels of the epidermal growth factor receptor. These alterations are frequent in PDAC (3, 4), indicating that PANC-1 cells represent an excellent model for studying potential therapeutic targets in PDAC. Inasmuch as PANC-1 cells also expressed high levels of CTGF, all subsequent studies were carried out with this cell line.

Effect of CTGF on Cancer Cell Proliferation and Invasion *In vitro*

To determine whether CTGF could exert direct effects on pancreatic cancer cells, its effects on cell proliferation and invasion were tested in PANC-1 cells. rhCTGF increased the proliferation of these cells in a dose-dependent manner (Fig. 2A). Thus, following a 48-hour incubation period, rhCTGF enhanced cell number at a concentration of 1 and 5 nmol/L ($P < 0.02$). The same concentrations of rhCTGF also significantly enhanced the invasiveness of PANC-1 cells (Fig. 2B), which was determined following an 18-hour incubation, to avoid any confounding influence by the mitogenic actions of CTGF.

Effect of TGF- β 1 on CTGF Expression in PANC-1 Cells

TGF- β 1 is known to up-regulate CTGF expression in fibroblasts and endothelial cells (7). To determine whether TGF- β 1 modulated CTGF expression in PANC-1 cells, time-course and dose-response studies were carried out using Q-PCR to measure CTGF mRNA levels. At a concentration of 0.5 nmol/L, TGF- β 1 rapidly increased the relative expression of CTGF (Fig. 3A). This effect was maximal at 2 hours (>8-fold increase) and persisted for at least 24 hours (Fig. 3A). At the 2-hour time point, TGF- β 1 enhanced CTGF expression in a dose-dependent manner, with a maximal effect (12-fold increase) occurring at the 5 nmol/L concentration (Fig. 3B).

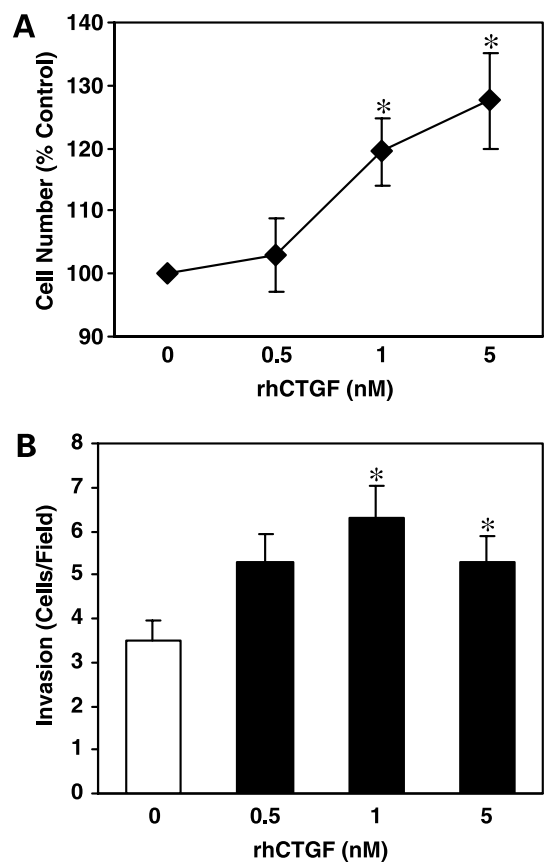


Figure 2. Effects of rhCTGF on the proliferation and invasiveness of PANC-1 cells. **A**, effects on cell proliferation. PANC-1 cells were seeded in six-well plates (20,000 per well) and incubated for two sequential 24-h periods in complete medium, and then in serum-free medium, before the addition of the indicated concentrations of CTGF. Cell number was counted by a hemocytometer 2 d later. Percentage change from the respective controls. *Points*, mean of three determinations per experiment from four separate experiments; *bars*, SE. *, $P < 0.02$, compared with the respective controls. **B**, effects on invasion. PANC-1 cells were incubated for 18 h in the absence (*open column*) or presence (*filled columns*) of the indicated concentrations of rhCTGF as described in Materials and Methods. The number of invading cells was then determined. *Columns*, mean number of invading cells per field of three determinations per experiment from four separate experiments; *bars*, SE. *, $P < 0.05$, compared with the respective controls.

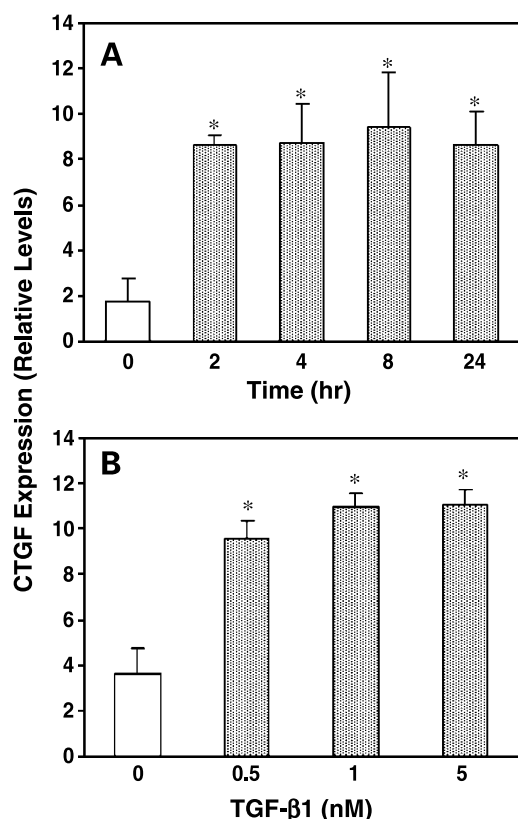


Figure 3. Effects of TGF- β 1 on CTGF RNA levels. **A**, time course. PANC-1 cells were incubated for the indicated times in the absence or presence of 0.5 nmol/L TGF- β 1. **B**, dose response. Cells were incubated for 2 h in the absence (*open columns*) or presence (*stippled columns*) of the indicated concentrations of TGF- β 1. RNA was subjected to Q-PCR. Relative expression levels were determined in triplicate. *Columns*, mean of three separate experiments; *bars*, SE. *, $P < 0.01$, compared with the respective controls.

Effect of FG-3019 on Tumor Growth

An orthotopic model of PANC-1 pancreatic cancer cells was used to assess the consequences of CTGF inhibition on pancreatic tumor growth and metastasis. Accordingly, 2 weeks following intrapancreatic implantation of small fragments of PANC-1-derived s.c. tumors, mice received twice weekly i.p. injections of FG-3019 (20 mg/kg) or vehicle control for 6 weeks. By comparison with mice injected with vehicle control, mice receiving FG-3019 developed significantly smaller tumors (Fig. 4), and 7 of 13 control mice developed a total of 18 visible metastatic lesions, whereas only 2 of 15 mice treated with FG 3019 developed a total of 3 metastatic lesions. Furthermore, 2 mice in the control group were found dead a few days before termination of the experiments. Both mice had extensive ascites and a large tumor burden but could not be studied in detail due to postmortem changes. None of the mice receiving FG-3019 exhibited any drug-related side effects. Moreover, four mice that were given nonspecific IgG under the same conditions as mice receiving either FG-3019 or buffer alone exhibited tumor growth and

metastasis that was comparable with that observed with buffer alone.

To assess whether lower doses of FG-3019 were effective at suppressing tumor growth and whether the antitumor effects of FG-3019 were additive or synergistic with the effects of the nucleoside analogue gemcitabine, an additional experiment was done in which tumor weight was measured following monotherapy or combination therapy (Fig. 5). In this experiment, treatment began 3 weeks after tumor implantation to assess whether FG-3019 could attenuate growth and metastasis after the implanted tumors have had a longer time to become established. In addition, treatment was continued for only 3.5 weeks to avoid the possibility of losing mice in the vehicle control group. The intrapancreatic tumors excised from vehicle-treated mice weighed 2.8 ± 0.5 g, whereas tumors from FG-3019-treated mice exhibited reduced tumor burden at all tested doses (Fig. 5A). Gemcitabine alone, at a dose of 300 mg/kg once weekly for 3 weeks, also exerted a marked inhibitory effect on tumor growth with an apparent additional decrease in tumor weight when mice received the combination of both FG-3019 and gemcitabine (Fig. 5A). An apparent additive antitumor effect was also observed with FG-3019 and gemcitabine combination therapy when the weight of the individual tumors was examined (Fig. 5B).

As with our previous experiments, FG-3019 significantly reduced tumor metastasis to mesenteric lymph nodes compared with the vehicle-treated group, with 2 of 10 mice (5 or 10 mg/kg dose), 1 of 10 mice (20 mg/kg), and 7 of 10 mice (vehicle control) exhibiting metastasis (Fig. 5B). None of the mice receiving gemcitabine alone developed metastasis; therefore, additive effects of FG-3019 and gemcitabine on tumor metastasis were not evaluable.

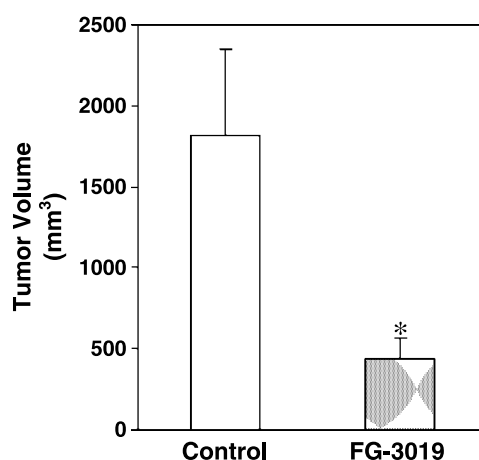


Figure 4. Inhibitory effects of FG-3019 on tumor growth in an orthotopic model of pancreatic cancer. FG-3019 (20 mg/kg) or vehicle (control) were administered i.p. twice weekly for 6 wks beginning at day 14 after tumor inoculation. *Columns*, mean of four different experiments with a total of 13 mice in the control group and 15 mice in the antibody-treated group; *bars*, SE. *, $P < 0.02$, compared with vehicle control.

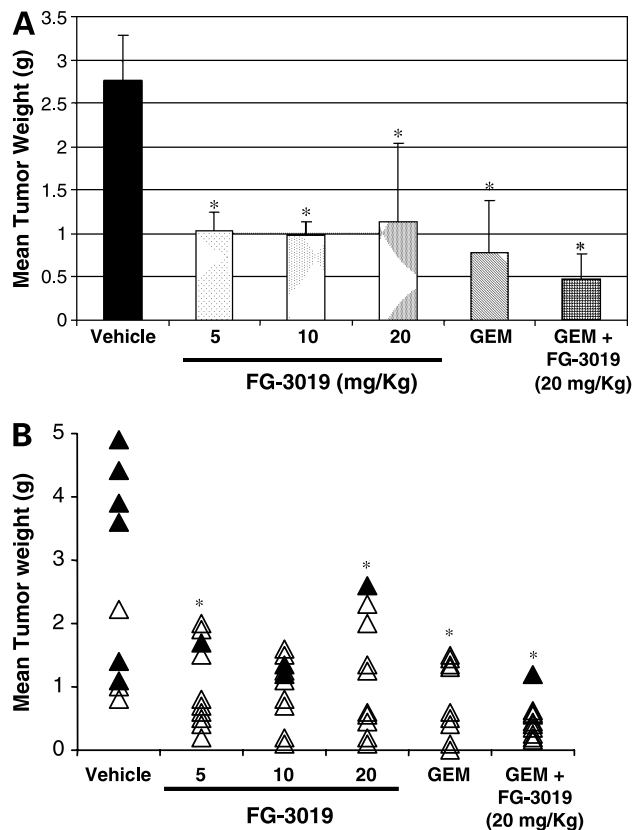


Figure 5. Inhibitory effects of FG-3019 and gemcitabine in an orthotopic model of pancreatic cancer. FG-3019 (5, 10, or 20 mg/kg) or vehicle (control) were administered i.p. twice weekly for 3.5 wks beginning at day 23 after tumor inoculation. Gemcitabine (GEM; 300 mg/kg) was given i.p. once weekly for 3 wks alone or in combination with 20 mg/kg FG-3019. **A**, tumor weight. Columns, mean of 10 mice per group; bars, SE. *, $P < 0.005$, compared with vehicle control, which due to the multiple comparisons was based on ANOVA with Tukey's test. **B**, lymph node metastasis. Δ , individual tumors; \blacktriangle , primary tumors that gave rise to mesenteric lymph node metastasis that was confirmed by histology. *, $P < 0.05$, metastatic incidence compared with vehicle control based on Fisher's exact test for dichotomous variables.

Effect of FG-3019 on Tumor Angiogenesis

To assess the effects of FG-3019 on tumor angiogenesis, tumors from sections of the experiments in which mice were treated for 6 weeks were immunostained with anti-CD31 antibodies. There was a clear reduction in the number of blood vessels within the tumor mass of FG-3019-treated mice by comparison with mice treated with vehicle control (Fig. 6). Quantitative morphometry indicated that the mean microvessel density (CD31⁺ structures) was significantly less in tumors treated with FG-3019 (Fig. 7A).

Effect of FG-3019 on Cancer Cell Proliferation and Apoptosis *In vivo*

To evaluate the effects of FG 3019 on cancer cell proliferation and apoptosis *in vivo*, tumor samples were sectioned and either immunostained with anti-PCNA antibodies or subjected to a TUNEL assay (41). PCNA immunoreactivity was more abundant in the tumors of

vehicle-treated mice by comparison with the cancer cells of FG-3019-treated mice. By quantitative morphometry, this decrease was statistically significant (Fig. 7B). By contrast, there was no significant difference in the number of apoptotic cells between the two groups as determined using the TUNEL assay (data not shown).

Discussion

In the present study, inhibition of CTGF by FG-3019 caused a significant decrease in intrapancreatic tumor volumes and attenuated the metastatic potential of the tumors. FG-3019 is a fully human IgG1 κ monoclonal antibody that binds in a highly specific manner to domain 2 of CTGF and that has shown efficacy previously in preclinical models of fibrosis (44, 45). Thus, FG-3019 has shown efficacy in models of early-stage renal disease that incorporates aspects of tissue remodeling that involve not only TGF β but also VEGF and advanced glycation end product-mediated pathologies, such as hyperfiltration and proteinuria (44, 46). In the present study, the inhibitory effect of FG-3019 was observed, although PANC-1 cells also expressed relatively high levels of all the known members of the CCN family, suggesting that there was no redundancy between CCN family members to drive tumor progression and overcome the antitumorigenic activity of FG-3019 in this orthotopic model. Moreover, quantitative morphometry revealed that there was a marked concomitant decrease in microvessel density in the tumors from FG-3019-treated mice by comparison with control mice, indicating that CTGF is an important proangiogenic factor in PDAC. This conclusion is in agreement with previous studies, which revealed that CTGF exerts proangiogenic

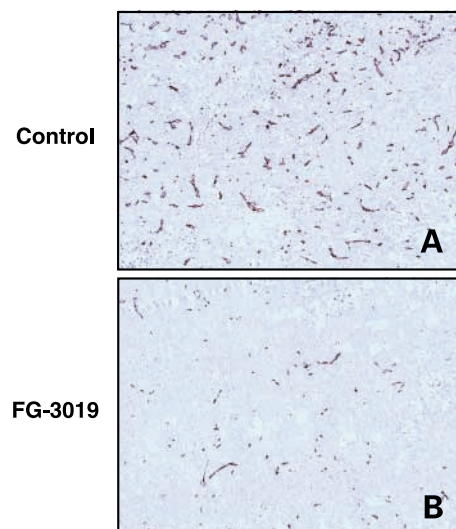


Figure 6. Inhibitory effects of FG-3019 on CD31 immunoreactivity. Immunohistochemical staining for CD31 was done in the primary tumors from control (**A**) and FG-3019-treated (**B**) mice. Decreased immunoreactivity was readily evident in FG-3019-treated tumors relative to those in control tumors. Magnification, $\times 100$.

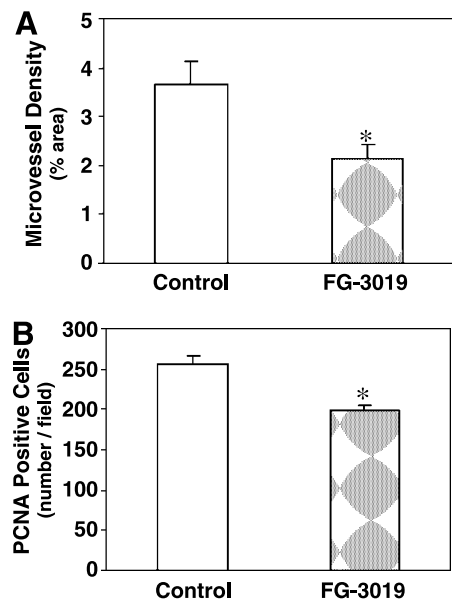


Figure 7. Quantitative morphometry. **A**, CD31 immunoreactivity. Immunohistochemical staining for CD31 from 13 tumors in the control group and 14 tumors in the FG-3019 group was subjected to quantitative morphometry as described in Materials and Methods. Columns, mean; bars, SE. *, $P < 0.01$, compared with control. **B**, PCNA immunoreactivity. Immunohistochemical staining for PCNA from 12 tumors in the control group and 12 tumors in the FG-3019 group was subjected to quantitative morphometry as described in Materials and Methods. Columns, mean; bars, SE. *, $P < 0.001$, compared with the control.

effects (15–17) and that FG-3019 can ameliorate VEGF-dependent pathologies associated with kidney disease (44, 45). In this regard, it is interesting to note that most PDAC express high levels of matrix metalloproteinases, especially MMP7 and MMP9 (46), and both matrix metalloproteinases are known to release VEGF-A from a complex with CTGF (47).

Several mechanisms may underlie the ability of FG-3019 to suppress intrapancreatic tumor growth. First, inhibition of angiogenesis would be expected to place constraints on the growth efficiency of these tumors. Second, FG-3019 caused a significant decrease in PCNA immunoreactivity, indicating that it also acted to decrease cell proliferation, which is consistent with our finding that rhCTGF directly enhances the growth of cultured PANC-1 cells (Fig. 2A) and with other reports that CTGF is mitogenic toward hepatic oval cells and cultured fibroblasts (48, 49). Third, the TUNEL assay failed to reveal any evidence for enhanced apoptosis in the tumors of the FG-3019-treated mice, indicating that the smaller tumor volumes were likely not due to enhanced apoptosis. Taken together, these findings suggest that CTGF promotes cancer growth by at least two mechanisms, the stimulation of cancer cell proliferation and the enhancement of angiogenesis.

In vitro, CTGF stimulated the invasiveness of PANC-1 cells in Boyden-type chambers, and TGF- β 1 induced CTGF expression. These observations suggest that in addition to

inhibiting metastases by suppressing angiogenesis FG-3019 may act directly on pancreatic cancer cells to suppress their invasiveness. CTGF may signal through the TrkA receptor, which is activated by CTGF (50) and which is expressed at relatively high levels in PANC-1 cells and at variable levels in PDAC (42). CTGF may also signal through the α_2 -macroglobulin receptor, which also functions as a receptor for CTGF (39). Our findings thus suggest that CTGF may exert deleterious effects in pancreatic cancer independently of TGF- β and that some of the deleterious effects of TGF- β may be mediated via its ability to up-regulate CTGF expression. Inasmuch as CTGF and TGF- β may cooperate in promoting tumor metastasis (31), it is also possible that FG-3019 may attenuate the metastatic potential of pancreatic cancer cells *in vivo* by interfering with this cooperative interaction that promotes cancer spread and metastasis.

PDAC is often characterized by the presence of an intense desmoplastic reaction, which has been attributed to the presence of high levels of profibrotic and tumorigenic factors, such as platelet-derived growth factor, insulin-like growth factor-I, and TGF- β , in these tumors and the proliferation of fibroblasts and pancreatic stellate cells within the tumor stroma (51, 52). It is noteworthy, therefore, that CTGF is not only present in the cancer cells within the pancreatic tumor mass (8, 9) but also in the tumor stroma and is most abundant in pancreatic stellate cells within the stroma (9). Pancreatic stellate cells have been shown to promote angiogenesis and to produce large amounts of collagen type I (52, 53), which is specifically the type of collagen that has been implicated in facilitating cancer growth (54). It is possible, therefore, that FG-3019 also attenuates tumor growth and metastasis by interfering with stroma formation and function, including pancreatic stellate cell activation, thereby attenuating collagen I deposition, suppressing further stromal interactions with the tumor, and decreasing stroma-derived tumorigenic factors, such as VEGF-A. By contrast, FG-3019 did not interfere with the efficacy of the cytotoxic agent gemcitabine.

CTGF cooperates with, or is a downstream mediator of, multiple growth factors that are frequently overexpressed in pancreatic cancer. Thus, in addition to its up-regulation by TGF- β , CTGF can be induced by epidermal growth factor (8), VEGF (12), platelet-derived growth factor (55), and fibroblast growth factor-2 (56). CTGF also binds and cooperates with insulin-like growth factor-I in the up-regulation of collagen expression (57). Taken together with our current findings, these observations indicate that CTGF may participate in aberrant autocrine and paracrine pathways in PDAC that contribute to altered stromal-epithelial interactions and that promote both directly and indirectly pancreatic cancer cell growth, invasion, and metastasis while enhancing the proangiogenic imbalance that is known to occur in this deadly disease (5). Thus, CTGF may be an attractive therapeutic target in a disease where monotherapy is unlikely to be successful, especially in those subsets of patients whose pancreatic cancers express high levels of CTGF. FG-3019, by blocking CTGF actions,

may therefore represent a novel therapeutic option in PDAC and may ultimately have a clinical role in this disease either alone or in combination with other chemotherapeutic agents, such as gemcitabine.

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