Connective Tissue Growth Factor–Specific Monoclonal Antibody Therapy Inhibits Pancreatic Tumor Growth and Metastasis

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Abstract

Pancreatic cancer is highly aggressive and refractory to most existing therapies. Past studies have shown that connective tissue growth factor (CTGF) expression is elevated in human pancreatic adenocarcinomas and some pancreatic cancer cell lines. To address whether and how CTGF influences tumor growth, we generated pancreatic tumor cell lines that overexpress different levels of human CTGF. The effect of CTGF overexpression on cell proliferation was measured in vitro in monolayer culture, suspension culture, or soft agar, and in vivo in tumor xenografts. Although there was no effect of CTGF expression on proliferation in two-dimensional cultures, anchorage-independent growth (AIG) was enhanced. The capacity of CTGF to enhance AIG in vitro was linked to enhanced pancreatic tumor growth in vivo when these cells were implanted s.c. in nude mice. Administration of a neutralizing CTGF-specific monoclonal antibody, FG-3019, had no effect on monolayer cell proliferation, but blocked AIG in soft agar. Consistent with this observation, anti-CTGF treatment of mice bearing established CTGF-expressing tumors abrogated CTGF-dependent tumor growth and inhibited lymph node metastases without any toxicity observed in normal tissue. Together, these studies implicate CTGF as a new target in pancreatic cancer and suggest that inhibition of CTGF with a human monoclonal antibody may control primary and metastatic tumor growth. (Cancer Res 2006; 66(11): 5816-27)

Introduction

Pancreatic cancer continues to be one of the most lethal cancers. More than 30,000 new cases of pancreatic cancer are diagnosed annually in the U.S. Mortality rates for this disease have not changed significantly in 30 years and approach 100% within 5 years after diagnosis. Current treatment options are limited due to the lack of diagnostic biomarkers and a lack of obvious symptoms until the tumor stage is already advanced. Approximately 10% to 20% of patients have surgically resectable disease at presentation, but even in these cases, the 5-year survival rate is only 20% (1). In patients with advanced disease, gemcitabine is considered to be a first-line option. However, gemcitabine only modestly improves survival with a 1-year survival rate of <20% (1). Clearly, there exists a need for more effective targeted therapies to treat pancreatic cancer by targeting gene products that will alter the malignant progression of pancreatic cancer or its response to therapy.

Connective tissue growth factor (CTGF) is a member of the CCN (Cysteine-rich 61, CTGF, NOV [nephroblastoma overexpressed]) family of secreted proteins, which are characterized as cysteine-rich matricellular proteins that each contain four modular domains displaying homology to insulin-like growth factor–binding proteins (domain 1), a von Willebrand factor type C repeat (domain 2), a thrombospondin type 1 repeat (domain 3), and a cysteine knot domain (domain 4), respectively (2). The cysteine knot domain contains heparin-binding sites that mediate binding to extracellular matrix and cell surface heparan sulfate proteoglycans (3). CTGF is an immediate early gene that is potently induced by a variety of stimuli that regulate extracellular matrix deposition, tissue remodeling, and neovascularization, including platelet-derived growth factor, transforming growth factor (TGF)-β, basic fibroblast growth factor, vascular endothelial growth factor (VEGF), and hypoxia in fibroblasts or endothelial cells (4–7). CTGF exhibits a diverse range of cellular functions including cell adhesion, stimulation of cell migration, and potentiation of growth factor–induced DNA synthesis (8).

CTGF interacts with integrin receptors including α5β1, αvβ3, αvβ5, and αvβ6 (3, 9–11) and has been reported to be a ligand for low-density lipoprotein-related protein 1 (LRP-1); interacts with LRP-5 to inhibit Wnt signaling (12–14) and can interact directly with several growth factors including TGF-β (15). Taken together, past studies indicate that the mechanism of action of CTGF relates to its capacity to modulate and amplify a variety of biological processes by binding directly to mitogenic, fibrogenic, and angiogenic factors that are important in inflammation, fibrosis, tumor growth, and tumor metastasis.

Elevated CTGF levels have been detected in a number of cancers including pancreatic (15), breast (16), glioblastoma (17, 18), esophageal (19), melanoma (20), chondrosarcoma (21), oral squamous cell cancer (22), acute lymphoblastic leukemia (23), rhabdomyosarcoma (24), and hepatocellular carcinoma (25), but its direct role in tumor suppression or progression has not been investigated in pancreatic cancer nor with therapeutic agents with the capacity to inhibit CTGF function in vivo. An increase in CTGF was reported to be associated with decreased survival of patients with breast cancer (16), glioblastoma (18), or adenosquamous of the esophagus (19), and increased breast cancer bone metastasis in a mouse model (26). In contrast, high levels of CTGF were associated with better survival in patients with esophageal
squamous cell carcinoma (19) and chondrosarcoma (21), and decreased metastasis in a colon cancer mouse model (27). Likewise, in lung adenocarcinoma, reduced CTGF expression was correlated with advanced disease stage and decreased survival, and expression of CTGF in lung cancer cell lines suppressed metastasis in a mouse tail vein injection model (28).

Whereas elevated levels of CTGF have been detected in the above tumor types, pancreatic carcinomas are especially noteworthy because a hallmark of their histopathology is desmoplasia. In a previous study by Wenger et al. (15, 19), 15 of 19 samples from pancreatic tumors exhibited an average 59-fold enhancement of CTGF mRNA expression, compared with a 4.5-fold increase in chronic pancreatitis (15). CTGF transcript levels were grossly correlated with the degree of fibrosis and collagen expression, consistent with known CTGF bioactivity. In a study of 25 patients with pancreatic cancer, Hartel et al. reported a 46-fold increase in CTGF mRNA levels in pancreatic cancer tissue compared with normal tissue (29). Furthermore, they found a positive correlation between desmoplastic reaction and CTGF mRNA level and concluded that the desmoplastic reaction might account for better survival of patients with elevated CTGF expression observed in this study. In contrast, Ryu and coworkers identified CTGF as a member of a pancreatic cancer invasion–specific gene cluster (30). In this study, the CTGF message was localized to pancreatic tumor cells rather than to stromal or endothelial cells (31). However, CTGF expression has been seen in both tumor cells and associated fibroblasts, endothelial cells, pancreatic stellate cells, and vascular smooth muscle cells (15, 29), not permitting clear insight into the significance of the cellular origin of CTGF expression. However, because CTGF has been found to be expressed in both the stromal and tumoral compartments and is a potential driver of desmoplasia, CTGF represents a unique target in pancreatic tumorigenesis. Although we and others (15) have found that most patients with pancreatic cancer exhibit elevated levels of CTGF compared with controls, no study has directly investigated the role of CTGF in pancreatic cancer growth or the potential of targeting CTGF for therapy.

In this study, we investigated the influence of overexpressing CTGF on pancreatic cancer cell growth in vitro and in vivo, and the effect of inhibiting CTGF to control pancreatic tumor growth. Our data suggest that tumor cell–derived CTGF enhances pancreatic tumor growth, whereas inhibition of CTGF with a human monoclonal antibody (mAb) reduces pancreatic tumor growth and metastasis. Therefore, CTGF may be a new target for the treatment of pancreatic cancer.

Materials and Methods

**Patient samples.** Human tissue from patients with pancreatic cancer was obtained during Whipple procedures at Stanford University. All patients signed an informed consent approved by the Stanford Institutional Review Board (in accord with an assurance filed with and approved by the U.S. Department of Health and Human Services). Representative paraffin blocks with carcinoma and normal pancreas were selected from each of eight cases. The immunohistochemical stains were done using rabbit polyclonal antibody directed against mouse CCN2/Flap 12 which cross-reacts with human CTGF (provided by L. Lau; ref. 32). Serial sections of 4 μm/L were obtained from the selected paraffin blocks, deparaffinized in xylene, and hydrated in a graded series of alcohols. Heat-induced antigen retrieval was carried out by microwave pretreatment in citric acid buffer (10 mmol/L, pH 6.0) for 10 minutes. The CCN2 antibody was used at a dilution of 1:400. The endogenous peroxidase was blocked and the DAKO Envision System (DAKO Corporation, Carpinteria, CA) was used for detection; diaminobenzidine was used as a chromogen.

The intensity of the staining was scored on a scale of 0 to 3+: where 0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining. If the staining in a particular case was variable, the percentage of tumor and normal tissue with a specific intensity score was also recorded. Scoring was done separately for normal pancreas, areas of chronic pancreatitis, and carcinoma; both epithelial elements and stroma were scored.

**CTGF ELISA.** Cell supernatants were collected in DMEM supplemented with 1% penicillin/streptomycin, 100 μg/mL low molecular weight heparin, and 0.25% bovine serum albumin. CTGF levels in culture supernatants, blood plasma, and urine were measured using a sandwich ELISA that detects whole CTGF and the NH2-terminal fragment of CTGF that persists in body fluids and cell culture supernatants after proteolytic cleavage of the hinge domain (33).

**Cell lines and tissue culture.** The human pancreatic cancer cell lines, MIA PaCa-2 and Panc-1, were grown in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin, whereas Sub86.86 pancreatic cancer cells were grown in RPMI 1640 containing 10% FBS. To generate pancreatic cancer cell lines overexpressing CTGF, we stably transfected MIA PaCa-2 cells with pShuttle-CMV-Puro-CTGF vector (an adenoval construct encoding full-length CTGF and the puromycin antibiotic-resistance gene) using LipofectAMINE 2000 reagent (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer’s instructions. Control transfections were done with the same vector lacking the CTGF cDNA sequence. Individual clones were selected based on their resistance to puromycin, and CTGF mRNA and protein levels were determined by quantitative real-time PCR (qRT-PCR) and ELISA or Western blot assay, respectively. A series of CTGF-expressing or vector control clones were isolated and characterized, and representative clones that exhibited different levels of CTGF expression and protein production were identified. Stable transfectants were cultured in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin and 1.5 μg/mL puromycin. All cell lines were cultured in a 5% CO2 humidified atmosphere at 37°C.

**CTGF mAb.** FG-3019 is a fully human IgG1, mAb recognizing domain 2 of human and rodent CTGF, and was obtained from FibroGen, Inc. (South San Francisco, CA). FG-3019 was purified under cyclic guanosine 3′,5′-monophosphate conditions and formulated in a 25 mmol/L histidine buffer (pH 6.0). In some experiments, polyclonal human IgG from Cohin’s fraction of human serum (Sigma, St. Louis, MO) purified by protein A sepharose was used as a control.

**In vitro growth curve.** Cells were plated at a density of 5 × 103 in 6 cm dishes. Every 3 or 4 days, cells were trypsinized, counted and 5 × 104 cells were replated. To investigate the effect of a CTGF-specific antibody (FG-3019) on tumor cell growth, growth curves were also determined in the presence of 20 and 40 μg/mL of FG-3019 or control human IgG. For growth in suspension, 2.5 × 103 cells were plated on ultra-low cluster plates (Costar, Cambridge, MA) which have a covalently bound hydrogel layer that effectively inhibits cellular attachment. Photographs were taken 4 days after cells were plated using a Leica MZ6 microscope with 10× and 40× objectives. Growth curves were obtained by plating 2.5 × 103 tumor cells into each of 12 wells, allowing the aggregates to form over a 48-hour period, and trypsinizing the tumor cell aggregates in triplicate wells daily with subsequent cell counting.

**Soft agar assay.** In duplicate experiments, ~3000 cells from each clone were resuspended in 2 mL of 0.33% Noble agar (Difco Laboratories, Detroit, MI) containing 10% FBS (Life Technologies) and 10% newborn calf serum (Life Technologies). Each embedded cell mixture was overlaid on 1.5 mL of 0.7% Noble Agar in six-well plates, and a 1.5 mL top layer of 0.7% Noble Agar was added to each well to prevent evaporation. In some experiments, human control IgG or FG-3019 was added at a concentration of 100 μg/mL to the cell layer containing 1,500 cells in 2 mL. Plates were incubated for 11 days in a humidified incubator at 37°C, 5% CO2. The number of colonies was enumerated by counting a 1.5 × 1.5 cm grid under a microscope. Total colony counts were extrapolated to the entire plate based on the ratio of the surface area of each well to the surface area of the grid. Colony morphologies were captured with a Nikon camera mounted onto an inverted microscope that was set at ×20 magnification.
qRT-PCR. For confirmation of CTGF mRNA expression, we did qRT-PCR. We obtained cDNA by reverse transcription of 1 μg of DNase-treated total RNA from each sample using random hexamer priming in 50 μL reactions according to the manufacturer's recommendations (TaqMan reverse transcription reagent kit; Applied Biosystems, Foster City, CA). We proceeded with qRT-PCR using the Applied Biosystems Prism 7900HT sequence detection system. A nonmultiplexed SYBR Green assay in which each cDNA sample was evaluated at least in triplicate and 20 μL reactions was used for all target transcripts. Expression values were normalized to human glyceraldehyde-3-phosphate dehydrogenase. qRT-PCR primers were designed using Primer Express version 2.0.0 (Applied Biosystems) and tested to confirm appropriate product size and optimal concentrations. All primer sequences are available on request.

Animal experiments/s.c. pancreatic tumor growth. To establish s.c. xenografts, 6- to 8-week-old male nude mice (nu/nu, 25 g) or severe combined immunodeficient mice (28 g; Charles River Breeding Laboratories, Wilmington, MA) were used. Cells were grown to subconfluence and 2 × 10⁷ or 1 × 10⁷ cells in 0.1 mL DMEM + 10% FBS were s.c. injected into the flank of the animals. Mice were maintained in a pathogen-free environment; food and water were given ad libitum. Housing and all procedures were done with approval of the Institutional Animal Care and Use Committee at Stanford University. Tumor size was measured in three dimensions with a caliper ruler and tumor volume was calculated by multiplication of the three dimensions divided by 2 (volume = a × b × c / 2). At the end of the experiments, mice were sacrificed using a CO₂ chamber consistent with the 2000 report of the American Veterinary Medical Association Panel on Euthanasia. Xenografts were then excised and fixed in 10% neutral buffered formaldehyde or embedded in optimal cutting temperature compound (Sakura Finetek USA, Inc., Torrance, CA) and stored at −80°C until processing.

Immunohistochemistry (Ki-67 and CD31). Sequential 4 μmol/L paraffin sections were stained with rabbit anti-Ki-67 antibody (1:50; Zymed, San Francisco, CA). The detection was done by using biotinylated secondary antibodies in combination with horseradish peroxidase–coupled streptavidin (Jackson ImmunoResearch, West Grove, PA) and the substrate 3,3'-diaminobenzidine (Research Genetics/Invitrogen). CD31 staining was done on frozen optimal cutting temperature–embedded tumor sections using rat anti-mouse (platelet/endothelial cell adhesion molecule 1; 1:50; BD PharMingen, Bedford, MA). All sections were counterstained with hematoxylin, dehydrated, and mounted using synthetic nonaqueous mounting medium.

Terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling staining for apoptotic cells in tumor sections. Apoptosis in formalin fixed, paraffin-embedded tumor slides was assessed by the principle of terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) to detect fragmented DNA in apoptotic cells. The DeadEnd Fluorometric TUNEL System (Promega, Madison, WI) was used. Sections were treated according to the manufacturer's recommendations. Briefly, sections were deparaffinized and rehydrated, permeabilized in protease K, and treated with terminal deoxynucleotidyl transferase incubation buffer at 37°C for 60 minutes in the dark. Sections were counterstained with 4'-6-diamidino-2-phenylindole (DAPI; Sigma).

Evaluation of immunohistochemical slides. Using the antimouse Ki-67 antibody and the TUNEL reagent, we determined the percentage of Ki-67- and TUNEL-positive tumor cells in relation to all tumor cells. For this purpose, four representative areas (high-power fields) of tumor sections were randomly selected. Evaluation was done with a Nikon Eclipse E800 microscope with ×400 magnification. The percentage of Ki-67- and TUNEL-positive cells was determined by manually counting at least 400 cells per high power field. Indices of cell proliferation and apoptosis were expressed.
as the ratio of Ki-67- and TUNEL-positive cells, respectively, and normalized to the vector-control-transfected cell line, VA2. Using CD31 staining, the number of vessels was counted in equivalent areas of a low power field \((\times 100)\) in at least four randomly selected low-power fields per tumor section. Mean values for all five areas and SDs were calculated.

Statistical analysis. Statistical evaluation was done using unpaired Student's t test for comparison between two values where appropriate. All statistical tests were two-sided. \(P < 0.05\) was considered statistically significant.

Results

CTGF is overexpressed in pancreatic cancer tissue. Several studies have shown CTGF to be elevated in pancreatic cancer tissue samples. We did immunohistochemical staining of CTGF expression in noninvolved normal pancreas tissue (Fig. 1A) and carcinoma tumor tissue (Fig. 1B) to evaluate whether CTGF expression was present in all pancreatic cancer samples. In five of eight patients, CTGF staining was scored as level 3 (levels 0-3) in pancreatic cancer cells, whereas staining was scored as levels 1 to 2 in healthy pancreatic tissue or pancreatitis. Interestingly, the highest CTGF staining was found in the cytoplasm of pancreatic cancer cells, rather than in the surrounding stromal cellular and connective tissue elements.

Generation of MIA PaCa-2-derived cell lines overexpressing CTGF. To understand the effect of CTGF on pancreatic tumor growth, we used a genetic approach in which we compared isogenic pancreatic tumor cells that lack constitutive CTGF expression in vitro with those in which the CTGF gene had been introduced under the control of a CMV promoter. In this manner, the effects of CTGF on the same cells could be determined and differences due to other genetic alterations were minimized. We used the human MIA PaCa-2 pancreatic cancer cell line to examine CTGF expression because it does not exhibit TGF-\(\beta\)-responsiveness (34) and does not constitutively produce endogenous CTGF (15). Stable clones of MIA PaCa-2 cells overexpressing different levels of CTGF (CE8, CA9, C9, and CB4) or vector control cells (VA2, VA3, VB4, VA6, and VB1) that do not express CTGF in vitro as confirmed by CTGF-specific ELISA were generated (see Fig. 2A and B). We also determined VEGF levels produced by the selected clones because VEGF is known to be overexpressed in many tumor cell types (35), to enhance tumorigenicity in pancreatic tumor models (36) and to interact with CTGF (ref. 37; Fig. 2A and B). To rule out effects of differing VEGF levels on CTGF-dependent tumor cell growth, we picked three clones with comparably low VEGF secretion (VA2, CE8, and CA9, Fig. 2A) for further studies and confirmed expression of CTGF mRNA in these clones by qRT-PCR (Fig. 2C). As expected, no CTGF mRNA expression was found in the vector control cell line VA2, whereas there were 40-fold and 180-fold inductions of mRNA, respectively, in the clones transfected with CTGF. MIA PaCa-2 clones with similar VEGF levels but differing CTGF expression levels were then tested for growth in vitro and in vivo.

CTGF expression does not influence in vitro (monolayer) proliferation rate of pancreatic cancer cells. To study the influence of CTGF expression on cell proliferation in vitro, we determined the monolayer growth rate of the VEGF-matched, non-CTGF-expressing cell line (VA2), and the two CTGF-expressing cell lines (CE8 and CA9). Over 13 days, there were no significant differences in in vitro cell growth rates (Fig. 3A). Therefore, CTGF does not have any apparent paracrine or autocrine effect on MIA PaCa-2 tumor cell proliferation in two-dimensional growth assays.

CTGF promotes anchorage-independent tumor cell growth of MIA PaCa-2 cells. Anchorage-independent growth (AIG) is a common characteristic of cancer cells. Although unable to induce AIG independently, CTGF is required for the induction of AIG by TGF-\(\beta\) (38). MIA PaCa-2 cells reportedly have a colony-forming efficiency in soft agar of \(~19\%\) (39). To determine the effect of CTGF expression on AIG in MIA PaCa-2 cells, VEGF-matched pairs of vector-transfected control cells and CTGF-expressing cells (VA2, CA9, and VA6, CD2) were analyzed for their ability to grow in soft agar (Fig. 3B); \(*, P < 0.001\) and in ultra-low attachment plates (Fig. 3C). The latter have a covalently bound hydrogel layer that inhibits cell attachment, allowing cells to grow in suspension (40). Although CTGF had little effect on monolayer growth, both the number and size of colonies were increased when the same cells were grown in soft agar (Fig. 3B). Multicellular masses also formed.
in the ultra-low attachment plates after 48 hours of culture, with the CTGF-expressing tumor cell aggregates displaying increased proliferation over time (Fig. 3C; *, \( P < 0.001 \)). These results indicate that CTGF could increase the AIG of MIA PaCa-2 cells.

**CTGF overexpression supports tumor xenograft growth.** Because the transfected clones showed different levels of VEGF expression, we next investigated the influence of VEGF expression on tumor growth in our system. We implanted three vector control clones (VA2, VA3, and VB4) expressing different VEGF levels in nude mice. As shown in Fig. 4A, the growth of MIA PaCa-2 vector control clones was VEGF-dependent in the absence of CTGF. These data indicate that CTGF-deficient tumor cells such as MIA PaCa-2 grow in a VEGF-dependent manner. To determine the effects of elevated CTGF expression on tumor cell growth in vivo, three low VEGF-expressing MIA PaCa-2 clones with differing CTGF expression levels were injected s.c. into the flanks of nude mice. As shown in Fig. 4B, there was little growth of the vector control clone that does not express CTGF. Clones expressing CTGF exhibited significantly enhanced tumor growth that was directly related to CTGF protein levels (*, \( P \leq 0.01 \)). Representative photographs of tumors are shown in Fig. 4C. Similar results were obtained with severe combined immunodeficient mice (data not shown) indicating no difference in CTGF-enhanced tumor growth with the host strain. To determine whether the expression levels of CTGF observed in vitro translated into higher circulating levels of CTGF in vivo, CTGF levels were measured in the plasma (41) and urine (33) of hosts bearing tumors derived from MIA PaCa-2 clones expressing different levels of CTGF. The level of CTGF present in both urine and plasma of tumor-bearing hosts paralleled the in vitro CTGF expression levels observed in culture supernatants of the respective MIA PaCa-2 clones (Fig. 4D). Animals implanted with high CTGF-expressing clones exhibited high levels of circulating and urinary CTGF. CTGF levels were undetectable in hosts implanted with the non–CTGF-expressing vector control cells. Taken together, these data suggest that CTGF promotes tumor growth in immunodeficient mice, and that CTGF produced by pancreatic cancer cells could be detected in the plasma and urine of pancreatic tumor-bearing mice.

**CTGF increases proliferation and decreases apoptosis in pancreatic tumors.** We examined tumor sections for effects on proliferation using Ki-67 staining, apoptosis by TUNEL staining, and neovascularization by CD31 staining. We found that the levels of Ki-67-positive cells correlated with tumor growth and CTGF levels (Fig. 4E). The percentage of Ki-67-positive cells in tumors

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**Figure 3.** Effect of CTGF expression on in vitro monolayer and AIG of MIA PaCa-2–derived clones. **A,** influence of CTGF on monolayer growth rate of MIA PaCa-2–derived clones. Vector-transfected (VA2) and CTGF-expressing (CE8 and CA9) clones were tested for their proliferation rates in vitro. Cells were plated in triplicate at the same starting cell density (5 \( \times \) 10⁴) and counted in regular intervals over a period of 13 days. **B,** effect of CTGF expression on tumor cell growth in soft agar. Vector control (VA2 and VA6) and CTGF-expressing (CA9 and CD2) MIA PaCa-2 cells were plated in soft agar and allowed to form colonies over an 11-day period before counting the colonies. The VA2-CA9 and VA6-CD2 pairs are matched for similar VEGF expression (see Fig. 2A and B). Bars, SE; *, \( P < 0.001 \) (Student’s t test). **C,** effect of CTGF expression on AIG of cells plated in ultra-low attachment plates. Photographs were taken after 72 hours with \( \times 10 \) and \( \times 40 \) magnification. Tumor cell aggregates were trypsinized and counted daily for the growth curves; points, mean from triplicate wells; bars, ±SE; *, \( P < 0.001 \) (Student’s t test).
Figure 4. Influence of CTGF expression on tumor xenograft growth. A, s.c. tumor xenograft growth of MIA PaCa-2 clones transfected with empty vector–expressing different levels of VEGF. B, s.c. tumor xenograft growth of MIA PaCa-2 transfectants expressing either empty control vector (VA2) or human CTGF (CE8 and CA9) in nude mice. Tumor volume was calculated at weekly intervals with caliper measurements. Points, mean; bars, ±SE; *, \( P < 0.01 \) (Student’s t test). C, macroscopic photographs of vector-transfected (VA2) and CTGF-expressing (CE8 and CA9) s.c. xenografts excised from nude mice. D, CTGF levels detected in urine and plasma samples from mice implanted with non–CTGF-expressing (VA2) or CTGF-expressing (CE8 and CA9) xenografts. CTGF levels were determined by sandwich ELISA. E, Ki-67 staining of paraffin-embedded tumor sections from vector control (VA2) and CTGF-expressing (CE8) xenografts were used to assess tumor cell proliferation (brown, proliferating cells; ×200 magnification). Columns, mean percentage of proliferating cells; bars, ±SE; *, \( P < 0.01 \) (Student’s t test). F, TUNEL and DAPI staining of paraffin-embedded tumor sections of vector control (VA2) and CTGF-expressing (CE8) xenografts were used to assess apoptosis (green, ×200 magnification). Columns, mean percentage of apoptotic cells; bars, ±SE; *, \( P < 0.01 \) (Student’s t test). G, CD31 staining of paraffin-embedded tumor sections of vector control (VA2) and CTGF-expressing (CE8 and CA9) xenografts were used to assess blood vessel density (brown, endothelial cells; ×100 magnification). Columns, mean number of blood vessels per field of view; bars, ±SE.
generated with the non–CTGF-expressing cell line (VA2) was 11% compared with 18% (P < 0.01) in tumors of the CTGF-expressing cell line CE8. The percentage of apoptotic cells in tumors generated with the VA2 cell line was 2.2%, whereas for tumors generated with CE8, the percentage of apoptotic cells was significantly lower at 0.7% (Fig. 4F; P < 0.01). Thus, the ratio of proliferation to apoptosis for VA2 is 5 (11/2.2) and for CE8 is 26 (18/0.7). No difference in neovascularization was found between the three cell lines (Fig. 4G).

Taken together, CTGF expression induces a significant increase in proliferation and a significant inhibition of apoptosis in pancreatic tumor xenografts, leading to a nearly 4-fold increase in the proliferation/apoptosis ratio. These data suggest that the tumor growth advantage of CTGF-overexpressing cells is due to enhanced cell growth and diminished cell death.

The neutralizing CTGF-specific mAb FG-3019 does not influence monolayer tumor cell proliferation in vitro. To confirm that increased tumor growth and progression in vivo are CTGF-dependent, we investigated the effects of CTGF inhibition in vivo and in vitro using a CTGF-specific human mAb FG-3019 (FibroGen). We first determined the monolayer proliferation rate of the three cell lines we had previously assessed in xenograft transplant studies (VA2, CE8, and CA9) in the presence of FG-3019 or control human IgG. As shown in Fig. 5A, FG-3019 did not exhibit a significant effect on monolayer tumor cell growth over a time period of 10 days in all three cell lines, consistent with the finding that CTGF overexpression had little effect on cell growth on tissue culture plates.

FG-3019 inhibits AIG. Because CTGF expression supports AIG, we further examined the influence of CTGF inhibition by FG-3019 on cells growing in soft agar. Soft agar assays were therefore done in the presence of FG-3019 or control human IgG over 10 days. As shown in Fig. 5B (right columns), colony formation of a CTGF-overexpressing cell line can be inhibited down to the level of non–CTGF-expressing clones by treatment with FG-3019 compared with control human IgG (*, P < 0.001). No effect of FG-3019 was observed on the growth of vector controls that do not express CTGF (left columns). This further supports the hypothesis that CTGF promotes AIG and shows that this effect could be inhibited by blocking CTGF using a neutralizing human mAb.

FG-3019 has a cytostatic effect on MIA PaCa-2-derived tumor xenografts. As the expression of CTGF promotes tumor xenograft growth, and the in vitro soft agar experiments showed an inhibition of AIG with FG-3019, we further sought to investigate the effect of CTGF inhibition on tumor growth in vivo. Based on previous pharmacokinetic data generated with FG-3019 in normal mice (data not shown), we gave FG-3019 twice a week to animals that had been implanted with CTGF-expressing MIA PaCa-2 cells (CE8). When mean tumor size reached 150 to 200 mm³, animals were stratified on the basis of tumor volume (such that the mean tumor volume in each group was uniform) into a treatment or control group. Three of six mice in each group were injected i.p. with FG-3019 (40 mg/kg) twice per week. As shown in Fig. 6C, PANC-1 tumor growth was significantly decreased when treated with neutralizing CTGF-specific antibody. After 6 weeks of treatment, the mean tumor size of the treatment group was reduced by >50% relative to the control group treated with control human IgG (*, P < 0.01). A tumor growth delay of 2.5 weeks was also observed when comparing the growth curves at the half-maximal control tumor size. Su86.86 tumor growth was even more affected by FG-3019 treatment (Fig. 6D), with treated tumors 75% smaller than control tumors after 6 weeks of treatment, and a tumor growth delay of 5 weeks at the half-maximal control tumor size. The delay of Su86.86 tumor growth induced by inhibition of CTGF was of comparable magnitude to that induced by three injections of 100 mg/kg of gemcitabine in a separate experiment (Fig. 6E).

FG-3019 treatment inhibits tumor growth and increases apoptosis, but does not alter proliferation. To analyze the potential mechanisms for the inhibitory effect of FG-3019, we examined whether antibody treatment alters the proliferative, apoptotic, or angiogenic status of treated tumors. We excised tumors from animals implanted with CTGF-expressing MIA PaCa-2 cells that were treated or not treated with FG-3019. Tumor sections were analyzed for effects on apoptosis or proliferation as before, and for neangiogenesis by staining for the endothelial cell marker CD31. No significant differences were seen in proliferation markers or CD31 staining in tumor xenografts from mice treated with or without FG-3019 (Fig. 5D and F). However, the percentage of apoptotic cells increased 3-fold in the FG-3019 treatment group (Fig. 5E; P < 0.01). The ratio of proliferation to apoptosis was 3-fold higher in the control versus the FG-3019 group [30 (20.7/0.7) compared to 10 (23.5/2.4)]. Taken together, these data indicate that antibody treatment inhibits tumor growth by decreasing the ratio of proliferation to apoptosis by 67%, mainly by inducing apoptosis.

FG-3019 inhibits the pancreatic cancer tumor growth of two endogenous CTGF-expressing pancreatic tumor cell lines. Thus far, all experiments described have been done with genetically engineered tumor cell clones originating from the pancreatic cancer cell line Mia PaCa-2. We next investigated the effect of inhibiting CTGF on the wild-type pancreatic tumor cell lines Su86.86 and PANC-1, both of which endogenously express CTGF (Fig. 6I) in a TGF-β-inducible manner (ref. 15; data not shown). As seen previously with MIA PaCa-2 clones that overexpress CTGF, the in vitro monolayer growth rate of PANC-1 cells was not affected using CTGF antibody concentrations up to 100 μg/mL (Fig. 6B). For in vivo studies, PANC-1 or Su86.86 cells were s.c. injected into the flanks of nude mice. After a mean tumor size of 150 to 200 mm³ was reached, animals were stratified on the basis of tumor volume (such that the mean tumor volume in each group was uniform) into a treatment or a control group such that the starting tumor volume in each group was uniform. The treatment group was injected i.p. with FG-3019 (40 mg/kg) twice per week. As shown in Fig. 6C, PANC-1 tumor growth was significantly decreased when treated with neutralizing CTGF-specific antibody. After 6 weeks of treatment, the mean tumor size of the treatment group was reduced by >50% relative to the control group treated with control human IgG (*, P < 0.01). A tumor growth delay of 2.5 weeks was also observed when comparing the growth curves at the half-maximal control tumor size. Su86.86 tumor growth was even more affected by FG-3019 treatment (Fig. 6D), with treated tumors 75% smaller than control tumors after 6 weeks of treatment, and a tumor growth delay of 5 weeks at the half-maximal control tumor size. The delay of Su86.86 tumor growth induced by inhibition of CTGF was of comparable magnitude to that induced by three injections of 100 mg/kg of gemcitabine in a separate experiment (Fig. 6E).
Figure 5. Influence of neutralizing CTGF-specific mAb on CTGF-expressing cells in vitro and in vivo. A, vector-transfected (VA2) and CTGF-expressing (CE8 and CA9) clones were grown in the presence of the CTGF-specific mAb FG-3019 (20 or 40 μg/mL) or human IgG (Control IgG) and the in vitro proliferation rates were determined over 10 days. Antibody concentrations up to 100 μg/mL were used, showing comparable results. B, vector-transfected (VA6) and CTGF-expressing MIA PaCa-2 cells (CD2) were plated in soft agar in the presence of the CTGF-specific mAb FG-3019 (100 μg/mL) or control human IgG and allowed to form colonies over a 10-day period before counting the colonies. Columns, mean number of tumor cell colonies; bars, ±SE; *, \( P < 0.001 \) (Student’s \( t \) test). C, CTGF-expressing MIA PaCa-2 clone CE8 were s.c. inoculated into nude mice. At a mean tumor size of 150 to 200 mm\(^3\), animals were stratified on the basis of tumor volume (such that the mean starting tumor volume in each group was uniform) into treatment and control groups, and i.p. injections of the neutralizing CTGF-specific mAb FG-3019 or vehicle control were done twice a week (40 mg/kg). Tumor volume was calculated at weekly intervals with caliper measurements. Columns, mean; bars, ±SE; *, \( P < 0.05 \) (Student’s \( t \) test). D, Ki-67 staining of paraffin-embedded tumor sections of CTGF-expressing (CE8) xenografts treated with either CTGF-specific mAb FG-3019 or vehicle were used to assess the influence of CTGF antibody treatment on tumor cell proliferation (brown, proliferating cells; ×200 magnification). Columns, mean percentage of proliferating cells; bars, ±SE. E, TUNEL of paraffin-embedded tumor sections of CTGF-expressing (CE8) xenografts treated with either CTGF-specific mAb FG-3019 or vehicle were used to assess the influence of CTGF antibody on apoptosis (green, TUNEL-positive apoptotic cells; ×200 magnification). Columns, mean percentage of apoptotic cells; bars, ±SE; *, \( P < 0.01 \) (Student’s \( t \) test). F, CD31 staining of paraffin-embedded tumor sections of CTGF-expressing (CE8) xenografts treated with either CTGF-specific mAb FG-3019 or vehicle, were used to assess blood vessel density (brown-green, endothelial cells, ×100 magnification). Columns, mean number of blood vessels per field of view; bars, ±SE.
The most extensive literature to date regarding CTGF defines its role in wound-healing and fibrotic disease. However, several recent studies implicate CTGF in tumor development and progression (16–19, 25, 26) and tumor cell survival (24). Our results show that CTGF promotes anchorage-independent pancreatic cancer cell growth, which translates in vivo to enhanced tumor growth. Furthermore, anti-CTGF treatment with FG-3019 inhibited AIG in vitro, primary tumor growth in vivo, and the development of macroscopic lymph node metastases. Thus, CTGF may represent a novel target in pancreatic cancer and blocking its activity may also inhibit the growth of distant metastasis. This is especially relevant because patients are frequently diagnosed with advanced metastatic disease and there are few effective therapies for pancreatic cancer metastasis.

Discussion

The most extensive literature to date regarding CTGF defines its role in wound-healing and fibrotic disease. However, several recent studies implicate CTGF in tumor development and progression (16–19, 25, 26) and tumor cell survival (24). Our results show that CTGF promotes anchorage-independent pancreatic cancer cell growth, which translates in vivo to enhanced tumor growth. Furthermore, anti-CTGF treatment with FG-3019 inhibited AIG in vitro, primary tumor growth in vivo, and the development of macroscopic lymph node metastases. Thus, CTGF may represent a novel target in pancreatic cancer and blocking its activity may also inhibit the growth of distant metastasis. This is especially relevant because patients are frequently diagnosed with advanced metastatic disease and there are few effective therapies for pancreatic cancer metastasis.

These results with CTGF in the context of pancreatic adenocarcinomas are particularly noteworthy because a hallmark of their histopathology is desmoplasia, and overexpression of CTGF is associated with increased tissue fibrosis. To date, only one small clinical study has examined the relationship between CTGF, desmoplasia, and prognosis of pancreatic cancer (29). In this study, CTGF mRNA was overexpressed mostly in connective tissue cells and high CTGF levels in tumor samples were associated with increased tumor differentiation and patient survival. The authors hypothesized that elevated CTGF resulted in a better prognosis by inducing fibrosis and inhibiting metastasis. However, the fact remains that >90% of pancreatic tumors have prominent desmoplasia (30), and the aggressive nature of these tumors suggests that this reaction may facilitate invasion rather than prevent it (31, 42). In situ hybridization studies by Iacobuzio-Donahue et al. have shown CTGF mRNA expression within the tumor cells in one pancreatic cancer tumor specimen (31). Although our immunohistochemical and transplanted tumor data suggest that tumor-cell–derived CTGF is important for tumor growth and metastasis, other reports suggest that CTGF produced by stromal fibroblasts mediate tumor growth (15, 29). Our xenograft studies indicate that CTGF overexpression could promote tumor growth and metastasis, but does not exclude the possibility that stromal CTGF may have a significant role in tumor progression. Although the production of CTGF by the stroma could explain why pancreatic tumors which possess low levels of CTGF still grow and metastasize, our data suggest that elevated levels of

Figure 6. Influence of CTGF-specific antibody FG-3019 on the growth of xenografts derived from the pancreatic cancer cell lines, PANC-1 and Su86.86. A, the pancreatic cancer cell line PANC-1 expresses CTGF endogenously. B, the CTGF-specific mAb FG-3019 does not influence in vitro monolayer growth of PANC-1 cells (concentrations up to 100 μg/mL). C, nude mice were s.c. inoculated with 10^6 PANC-1 cells, a pancreatic cancer cell line with endogenous CTGF expression. At a mean tumor size of 150 to 200 mm^3, animals were stratified on the basis of tumor volume (such that the mean starting tumor volume in each group was uniform) and control and treatment groups and i.p. injections of the neutralizing, CTGF-specific mAb FG-3019, or control IgG were done twice a week (40 mg/kg). Tumor volume was calculated at weekly intervals with caliper measurements. D, nude mice were s.c. implanted with 10^7 Su86.86 cells, a second pancreatic cancer cell line with endogenous CTGF expression. At a mean tumor size of 150 to 200 mm^3, animals were randomized into treatment and control groups and i.p. injections of FG-3019 (40 mg/kg) or vehicle control were done twice per week. Tumor volume was calculated at weekly intervals with caliper measurements. E, nude mice bearing Su86.86 tumors were given 100 mg/kg of gemcitabine on days 1, 5, and 9 of the experiment. The growth delay induced by gemcitabine was of comparable magnitude to that obtained with 40 mg/kg of FG-3019. Tumor volume was calculated twice weekly with caliper measurements.
VEGF could compensate for a lack of CTGF production by the tumor. Consistent with this idea, preclinical studies have shown that VEGF inhibition with a modified soluble VEGF receptor (VEGF-Trap) inhibited both s.c. and orthotopic pancreatic tumor growth and metastasis (43). CTGF may also act as a biostore for angiogenic factors, e.g., CTGF can bind to VEGF and inhibit its signaling, but VEGF function is restored upon cleavage of CTGF by matrix metalloproteases. Thus, a combination of anti-VEGF therapy (43) with FG-3019 could be a highly effective treatment strategy.

Our data suggest that CTGF mediates both cell survival and proliferation; in vitro in AIG assays and in vivo in pancreatic tumors. This is consistent with earlier reports which showed that CTGF mediates the effects of TGF-β on AIG of NRK fibroblasts (38). However, the role of CTGF in regulating cell survival or proliferation is likely to be highly cell type–specific and dependent on what other signaling pathways are also activated in the target cell. For example, CTGF is co-mitogenic for fibroblast proliferation in combination with epidermal growth factor or basic fibroblast growth factor (32), but will induce differentiation into myofibroblasts in the absence of epidermal growth factor (44). In rhabdomyosarcoma-derived cell lines that secrete CTGF, inhibition of CTGF results in increased apoptosis, suggesting that CTGF establishes an essential autocrine loop in these cells that is necessary for cell survival (24). This is consistent with the increased levels of pancreatic tumor cells undergoing apoptosis in vivo after FG-3019 administration (Fig. 5E). Furthermore, the increase of soft agar colony number, colony size, and apparent cell density indicates that CTGF expression may lead to enhanced deregulation of cell-cell contact inhibition of growth.

CTGF may also modulate the expression of metastasis by mechanisms dependent on the microenvironment and growth factor availability. CTGF was one of four genes significantly up-regulated in bone metastatic populations of mammary gland tumors (26, 45), but was differentially necessary according to the expression levels of other metastatic genes such as osteopontin,

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**Figure 7.** Influence of CTGF-specific antibody FG-3019 on the development of macroscopic lymph node metastases derived from s.c. PANC-1 xenografts. A, macroscopic lymph node metastases were observed in mice bearing s.c. PANC-1 tumor xenografts. Axillary and/or inguinal lymph node metastases were macroscopically visible in five of six mice treated with control antibody and one of five mice in the FG-3019-treated group. B, lymph nodes were removed and paraffin-embedded sections were stained with H&E (×100 and ×400 magnification). Tumor cell infiltration into lymph node tissue is evident.
interleukin-11, and CXCR4 that increase bone metastases. CTGF has also been shown to be elevated in breast cancer patients with positive lymph nodes compared to patients with negative nodes (16, 46).

Several studies have shown a role for the TGF-β pathway in pancreatic cancer growth and metastasis (47). Domain 2 of CTGF has also been shown to bind and potentiate TGF-β effects, suggesting that one possible mechanism for CTGF is through amplifying the protumorigenic actions of TGF-β (48). However, this may not entirely explain how CTGF promotes tumor growth and metastasis in pancreatic tumor cells like MIA PaCa-2 that are defective in TGF-β signaling. Therefore, it is likely that other domains of CTGF may affect pancreatic tumor growth. The domain structure of CTGF has been associated with various interactions with other growth factors and cell receptors. Domain 1 contains homology to insulin-like growth factor (IGF)–binding proteins and could bind to IGF-I and IGF-II; domain 3 possesses thrombospondin type 1–like repeats and could bind VEGF, whereas domain 4 could bind to heparan sulfate proteoglycans and integrins (9, 23, 49, 50). Thus, there are a number of possible mechanisms whereby CTGF interactions with other growth factors and cell surface receptors might affect pancreatic tumorigenesis.

Overall, this study supports the clinical investigation of anti-CTGF therapy. However, it will first be important to test the effects of combining FG-3019 with standard treatments like gemcitabine and radiotherapy to determine the potential additive or synergistic effects on primary tumor growth inhibition. We have already shown that FG-3019 treatment alone produces an inhibition of Su86.86 tumor growth that is similar to gemcitabine (Fig. 6E), and recent clinical studies have indicated that combining targeted agents with chemotherapy or radiotherapy are resulting in improved outcomes for patients. Thus, targeting CTGF with FG-3019 in combination with a cytotoxic agent such as gemcitabine or radiation could be more efficacious than either treatment alone. A small animal study addressing this question is currently ongoing. In addition, one could consider anti-CTGF treatment either as an adjuvant treatment after initial surgical or radiation therapy, or as a second-line treatment for gemcitabine-refractory patients, to prevent or inhibit metastasis of disseminated tumor cells by inhibiting AIG. In addition to therapy, another implication of this study would be the use of CTGF as a biomarker. Because circulating CTGF levels in mouse plasma and urine paralleled CTGF expression levels in the tumor cell lines, CTGF might be useful as a marker of disease. Consistent with this idea, CTGF expression has been found to be prognostic for tumor progression and survival in patients with glioma (18).

In summary, CTGF produced by pancreatic cancer cells seems to play an important role in pancreatic tumor growth and metastasis. Our data clearly shows that CTGF promotes AIG in vitro as well as tumor xenograft growth in vivo. Furthermore, treatment with the CTGF-specific antibody, FG-3019, inhibited the growth of tumor xenografts and metastases, without exhibiting noticeable side effects. These data provide a sound scientific rationale for further investigation into targeting CTGF in pancreatic cancer.

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References


CTGF in Pancreatic Cancer

In the article on CTGF in pancreatic cancer in the June 1, 2006 issue of Cancer Research (1), the correct spelling of the tenth author's name is Randall Nacamuli.

Connective Tissue Growth Factor–Specific Monoclonal Antibody Therapy Inhibits Pancreatic Tumor Growth and Metastasis

Nadja Dornhöfer, Suzanne Spong, Kevin Bennewith, et al.


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