Suppression of Cell Proliferation and Signaling Transduction by Connective Tissue Growth Factor in Non–Small Cell Lung Cancer Cells

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Abstract

Connective tissue growth factor (CTGF) is a secreted protein that belongs to the CCN family. The proteins in this family are implicated in various biological processes, such as angiogenesis, adhesion, migration, and apoptosis. In this study, we explored the roles of CTGF in lung tumorigenesis. The expression levels of CTGF in 58 lung cancer samples were reduced by >2-fold in 57% of the samples compared with matched normal samples using real-time reverse transcription-PCR. These results were confirmed by immunohistochemical staining for CTGF in normal lung epithelia and lung cancer. Cellular proliferation was inhibited in non–small cell lung cancer (NSCLC) cell lines NCI-H460, NCI-H520, NCI-H1299, and SK-MES-1 by CTGF overexpression. Partially purified CTGF suppressed lung cancer cell growth. The growth inhibition caused by CTGF overexpression was associated with growth arrest at G0-G1 and prominent induction of p53 and ADP ribosylation factor. Most interestingly, overexpression of CTGF suppressed insulin-like growth factor–dependent Akt phosphorylation and epidermal growth factor–dependent extracellular signal-regulated kinase 1/2 phosphorylation. In summary, NSCLC cells expressed decreased levels of CTGF compared with normal lung cells; this lower expression has an effect on lung cancer cell proliferation and its cellular response to growth factors. Our data suggest that CTGF may behave as a secreted tumor suppressor protein in the normal lung, and its expression is suppressed in many NSCLCs.

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Introduction

Connective tissue growth factor (CTGF; CCN2) is a member of the CCN protein family, which is composed of cysteine-rich protein 61 (Cyr61), CTGF, nephroblastoma overexpressed, as well as WISP-1, WISP-2, and WISP-3 (Wnt-1 induced secreted proteins; refs. 1-3). The CCN proteins are multifunctional and are involved in cell adhesion, angiogenesis, cell differentiation, and tumorigenesis (4-8). CTGF was first identified as a mitogen from human umbilical vein endothelial cells, and expression of CTGF is inducible by tumor growth factor–β in fibroblasts (9). CTGF mediates wound healing, implantation, and fibrosis (10); hammerhead ribozymes targeting CTGF mRNA inhibit tumor growth factor–β–mediated cell proliferation (11). Northern blot analysis shows increases in CTGF mRNA in pancreatic cancer tissue compared with normal control, but in situ hybridization indicates that CTGF mRNA localizes to fibroblasts and not in the pancreatic cancer cells (12). Furthermore, survival rates for pancreatic cancer patients were directly correlated to their CTGF expression levels (12). Low levels of CTGF were associated with increased metastasis and poor prognosis in breast cancer patients (13, 14). Similarly, the metastatic breast cancer cell line, MDA-MB-231, expresses lower levels of CTGF than the less invasive MCF-7 cells (13). Forced expression of CTGF in MCF-7 cells induces apoptosis (15). In the progression of colorectal cancer, patients with high CTGF expression in their tumors have higher survival rates compared with patients with low CTGF expression in their tumors (16). Transfection of colorectal cancer cell lines with antisense CTGF increases their invasiveness both in vitro and in vivo (16).

Non–small cell lung cancer (NSCLC) represents ~80% of the total lung cancer cases, and the subtypes include adenocarcinoma, adenosquamous, squamous, and large cell carcinoma (17). A recent study showed that lower CTGF expression is associated with advanced stage and mortality in lung adenocarcinoma (18). Overexpression of CTGF in lung adenocarcinoma cell lines decreases invasive and metastatic
activities, perhaps mediated by a protein called collapsing response mediator protein 1 (18). In the current study, we found that expression levels of CTGF in lung cancer samples were reduced compared with matched normal samples. Force expression of CTGF in NSCLC cell lines NCI-H460, NCI-H520, NCI-H1299, and SK-MES-1 suppressed their proliferation. A similar effect was noted when partially purified CTGF was added to the cells. Overexpression of CTGF induced G1 cell cycle arrest concurrently with induction of p53 and p14 [ADP ribosylation factor (ARF)]. Kinase activation by the growth factors, insulin-like growth factor (IGF)-1 and epidermal growth factor (EGF), was suppressed by CTGF overexpression. In summary, blunted expression of CTGF may play a role in lung tumorigenesis, and the mechanism may be associated with lack of suppression of downstream signals of IGF-1 and EGF.

**Results**

**Differential Gene Expression of CTGF in Lung Cancers**

To determine the CTGF expression profile in lung tumors, total RNA was extracted from lung tumors and matched normal lung tissues from the same patients. Complementary DNA was prepared, and quantitative real-time PCR was used to measure the CTGF mRNA expressions. The relative CTGF expression levels were calculated in a panel of 58 tumor/normal pairs (Fig. 1A). Of the 58 pairs, 16 pairs (28%) showed <2-fold change in CTGF expressions, 9 pairs (16%) had increased CTGF expression in the tumors, and 33 pairs (57%) had >2-fold decreased CTGF expression in the tumors. To determine whether the CTGF mRNA expression levels corresponded to CTGF protein expressions, immunohistochemical staining for CTGF was evaluated in normal epithelia and lung tumors. Strong positive staining was found in bronchiolar normal epithelia (Fig. 1B). In contrast, only very weak CTGF staining occurred in the tumor cells (Fig. 1B). These results suggested that low expression of CTGF conferred a growth advantage to lung cancer cells.

**Growth Inhibition of NSCLC Cells by CTGF Overexpression**

To assess the effect of CTGF expression on lung cancer cell proliferation, four lung cancer cell lines (NCI-H1299 and NCI-H460: large cell lung cancer; NCI-H520 and SK-MES-1: squamous cell carcinoma) were transfected with either CTGF-pcDNA3.1 expression vector or empty vector pcDNA3.1. The effect of CTGF on clonogenic growth of these cells was evaluated. The empty vector–transfected cells (V) formed numerous colonies, whereas the number of colonies decreased after plating the cells transfected with CTGF-pcDNA3.1 (Fig. 2). G418-resistant clones were then screened and selected for CTGF expression by both real-time PCR and Western blot analysis. Two stable clones overexpressing CTGF were selected for each of two cell lines (NCI-H460, 4F1 and 4F2; NCI-H520, 5F1 and 5F2; Fig. 3A). These sublines were used for further study. Compared with 4V and 5V control cells, the two stable clones (4F1 and 4F2 and 5F1 and 5F2, respectively) expressing prominent levels of CTGF showed reduction in their growth rate as measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT; Fig. 3B).
Biologically active CTGF is both secreted and expressed on the cell membrane (19, 20). Conditioned medium prepared from 4F1 cells had prominent expression of CTGF compared with control 4V cells (Fig. 4A, left). After 48 hours of incubation, the CTGF-enriched conditioned medium from 4F1 cells (CTGF) inhibited cell growth compared with control conditioned medium from 4V cells (V) in both lung cancer NCI-H460 and breast cancer MCF-7 cells (Fig. 4A, right). MCF-7 cells were used as control because recombinant CTGF protein has been shown to cause growth inhibition in MCF-7 cells (15). Heparin affinity chromatography has been used in CTGF purification (21). Therefore, we used heparin agarose beads to deplete CTGF from the CTGF-conditioned medium and investigated the effect on cell growth using the CTGF-depleted conditioned medium. After heparin binding, CTGF expression was negligible in the conditioned medium (nonbinding; Fig. 4B, left), and the growth-inhibitory effect of the conditioned medium was lost (heparin nonbinding; Fig. 4B, right). No difference in growth (MTT assay) of NCI-H460 and MCF-7 cells occurred when they were grown in control conditioned medium either with or without heparin (Fig. 4C).

**CTGF Induced Growth Arrest and Expression of Genes Related to Growth Suppression**

To explore the mechanisms by which CTGF caused growth inhibition, we examined cell cycle profiles. The G0-G1 phase increased in CTGF-overexpressing cells compared with the control cells (4F1 versus 4V; 5F1 versus 5V). In contrast, the proportion of cells in S phase decreased in CTGF-overexpressing cells (Table 1). Expression of cyclin D1 is important in the promotion of cell cycle progression from G0 to S phase (22). We found that moderately high cyclin D1 was expressed in control cells (4V and 5V; Fig. 5); whereas in CTGF-overexpressing cells, cyclin D1 levels diminished (4F and 5F; Fig. 5).

**FIGURE 2.** Effect on clonogenic growth by the forced expression of CTGF in NSCLC cell lines. Cells were transfected with either a CTGF expression vector or empty vector (V) and grown under G418 selection. Colony formation was stained with crystal violet after 2 weeks and photographed. Results from one experiment. Similar findings were observed in an identical duplicate experiment. The staining was dissolved, and absorbance at 590 nm was measured and displayed in bar graph form. The number of cells showed a linear relationship to absorbance. NCI-H460 and NCI-H1299: large cell lung cancer cell lines; NCI-H520 and SK-MES-1: squamous cell carcinoma cell lines.

**FIGURE 3.** Suppression of lung cancer cell proliferation by overexpression of CTGF. A. Immunoblot analysis of CTGF expression. Two CTGF stably transfected clones from NCI-H460 (4F1 and 4F2) and NCI-H520 (5F1 and 5F2) cells were probed for CTGF expression. Glyceraldehyde-3-phosphate dehydrogenase was used as loading control. V, empty vector–transfected clones (4V and 5V). B. MTT assay. Growth rates were compared between the CTGF and control transfected cell lines. Viable cells were measured by MTT activity every 24 hours from day 0 (D0) to day 4 (D4). MTT activity measured at day 0 was set at 100%. Points, mean of two independent experiments done in triplicates; bars, SE.
Both NCI-H460 and NCI-H520 cells express the wild-type p53 tumor suppressor protein. We examined the effect of CTGF on p53 levels. Significantly increased p53 expression occurred in CTGF-overexpressing cells compared with control cells (Fig. 5). One important upstream regulator of p53 levels is ARF (23), which was also markedly induced in CTGF-overexpressing cells (Fig. 5). ARF can be activated by E2F1 (24), which was also increased in CTGF-overexpressing cells (Fig. 5).

**CTGF Suppression of IGF-1-Dependent Akt Phosphorylation and EGF-Dependent Extracellular Signal-Regulated Kinase 1/2 Phosphorylation**

Various signaling pathways inducible by growth factors are keys to regulation of tumorigenesis (25). We investigated the effect of CTGF on IGF-1- and EGF-dependent kinase activation. Phosphorylation of Akt was inducible by addition of IGF-1 to 5V cells (Fig. 6A, compare lane 2 with lane 1); in contrast, the induction was suppressed in CTGF-overexpressing 5F1 cells (Fig. 6A, compare lane 5 with lane 2). Similar results were found in 4F1 and 4F2 cells compared with 4V cells (data not shown). Because Akt is a downstream effector of phosphatidylinositol 3-kinase, we also used a phosphatidylinositol 3-kinase inhibitor, Wortmannin, to test whether it blocks Akt activation in these cells. Specific inhibition of Akt phosphorylation was mediated by Wortmannin (Fig. 6A, lanes 3 and 6). When exposed to EGF, phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) was found in 5V cells (Fig. 6, compare lane 2 with lane 1), and the level of phosphorylation was decreased in the CTGF-overexpressing cells 5F1 (Fig. 6B, compare lane 4 with lane 5). When the ERK1/2 inhibitor, PD98059, was added to the cells, it specifically blocked the ERK1/2 activation (Fig. 6B, lanes 3 and 6).
Table 1. Percentage of Cells in Different Phases of the Cell Cycle Analyzed by Flow Cytometry

<table>
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<th>DNA content (%)</th>
<th>NCI-H460</th>
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<td>40</td>
<td>32</td>
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<tr>
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NOTE: Cells were methanol fixed and stained with propidium iodide for flow cytometry. Results are mean ± SE of triplicate experiments.

Discussion

Several studies have shown the antigrowth activity of CTGF in cancer cells; however, the molecular mechanism by which CTGF exerts its effect is not well understood. The survival rates of colorectal and pancreatic cancer patients directly correspond to their CTGF expression levels (12, 16). In addition, breast cancer patients with a poor prognosis and mortality had significantly lower CTGF levels compared with their normal breast tissues (13). Forced expression of CTGF in MCF-7 breast cancer cells increased cell apoptosis (15). In addition, patients with lung adenocarcinoma with low CTGF expression more often had an advanced stage and a poorer prognosis (18).

In the current study, we initially examined the expression levels of CTGF in 58 pairs of matched lung tumor and normal samples. By quantitative real-time PCR, 57% of the samples expressed lower levels of CTGF in tumors compared with their matched normal tissues. Immunohistochemistry identified prominent CTGF protein localization in the normal bronchiolar epithelia but not in tumor cells. A similar decreased CTGF expression has been shown between normal breast tissue and tumor. CTGF was expressed highly in normal breast epithelia, endothelia, and stromal cells but weakly in breast cancer cells (14). This is similar to pancreatic cancer tissue, in which weak CTGF staining was found in tumor cells surrounded by fibroblasts staining strongly for CTGF (12). The growth disadvantage conferred by CTGF expression probably occurs via similar mechanisms in lung and breast and maybe in pancreas.

We evaluated the effect of CTGF on NSCLC cell growth by forced expression of CTGF. Both MTT and colony formation assays showed reduction in growth rates after CTGF overexpression in four NSCLC cell lines, including NSCLC line (NCI-H1299), large cell lung cancer line (NCI-H460), and two squamous cell carcinoma lines (NCI-H520 and SK-MES-1). This showed that CTGF is antiproliferative in these lung cancer cells. Inhibition of cell proliferation in vitro, however, may not translate into decreased tumorigenesis in vivo. CTGF may modulate expression of extracellular matrix components and promote angiogenesis and tumorigenesis. Expression of CTGF in prostate stromal fibroblasts promoted microvessel density (26); in MDA-MB-231 cells, hypoxia-stimulated CTGF induced neovascularization by modulating expression levels of matrix metalloproteinases and their inhibitors (27). Future studies will examine how CTGF-overexpressing lung cancer cells behave in vivo and what effect CTGF will have on the synthesis and degradation of extracellular matrix.

Although CTGF is antiproliferative in our lung cancer cells, a previous study showed that CTGF-transfected lung adenocarcinoma A549 cells lost their invasive ability but continued to have a comparable growth rate as control cells (18). Reason for these differences may relate to variability in the cell lines. This is shown from experiments with another CCN family protein, Cyr61 (8). Forced expression of Cyr61 had no effect on the growth rates of NCI-H125 (adenosquamous), NCI-H157 (squamous), and NCI-H1299 but inhibited proliferation of NCI-H460 (large cell) and NCI-H520 (squamous) carcinoma lung cancer cells (8). This growth inhibition by Cyr61 in NSCLC was p53 dependent. The cell growth of the NSCLC cell lines (NCI-H157, NCI-H125, and NCI-H1299) expressing mutant p53 was not suppressed by Cyr61 (28). Likewise, all four lung cancer cell lines in our study (NCI-H1299, NCI-H460, NCI-H520, and SK-MES-1) expressed wild-type p53. Future studies will explore if growth inhibition by CTGF is mediated in part by p53. Nevertheless, we found significant increase of p53 expression in NCI-H460 and NCI-H520 cells overexpressing CTGF compared with control cells. Up-regulated p53 can activate p21, inhibit cyclin-dependent kinase 2 kinase activity, and activate Rb tumor suppressor gene to promote cell cycle arrest (29). In NSCLC cells overexpressing Cyr61, p53 is induced by β-catenin/TCF4 complex activation and c-myc expression (8, 28).

The levels of p53 can be regulated by ARF, which is an antagonist of MDM2 by binding to this protein and preventing p53 degradation (23, 30, 31). In fibroblasts, ARF working through p53 induces cell cycle arrest at both G1 and G2 phases.

FIGURE 5. Growth arrest and induction of genes related to growth suppression by CTGF. Western blot analysis. Cell lysates were prepared and probed for the expression of cyclin D1, p53, ARF, and E2F1. Glyceraldehyde-3-phosphate dehydrogenase was used as loading control. Blots are representative of two separate experiments. 4F1 and 5F1 are CTGF-overexpressing NSCLC sublines of NCI-H460 and NCI-H520, and 4V and 5V are their control cell lines.
inhibited cell proliferation of the NSCLC NCI-H460 cells as well as the breast cancer MCF-7 cells; these latter cells have been shown to have enhanced apoptosis in the presence of recombinant CTGF (15). After the conditioned medium was incubated with heparin-coated beads, the inhibitory effect was reversed, whereas control conditioned medium showed no effect on cell proliferation either with or without heparin. Nevertheless, the heparin affinity beads might be expected to deplete not only CTGF but also other secretory heparin-binding protein, such as Cyr61, basic fibroblast growth factor, and vascular endothelial growth factor (44-46). One of these may also slow the growth of NSCLC cells. In addition, CTGF, like all other CCN proteins, has multiple modules: module I contains the IGF-binding protein domain; module II, the von Willebrand factor type C repeat; module III, the thrombospondin type I repeat; and module IV, the cystein knot. The multimodules provide the opportunity for the CCN proteins to interact with multiple proteins, which of modules is essential for the inhibitory function of CTGF in lung cancer cells requires further studies.

As noted above, CTGF protein contains an IGF-binding protein domain. In our study, IGF-I-dependent Akt phosphorylation and EGF-dependent ERK1/2 phosphorylation were suppressed by CTGF overexpression in NSCLC. Previous studies in inflammatory breast cancer showed that WISP3 (CCN6) was able to modulate IGF-I receptor signaling by interaction with IGF-1 (47). A similar mechanism may be feasible for CTGF; although studies have suggested that CTGF has a relatively low affinity toward IGF-1 (48). The Akt and the p53 pathways form a feedback loop. Akt activation leads to MDM2 phosphorylation resulting in inactivation of p53, whereas p53 activation suppresses Akt activity (49). CTGF expression may sway this cross-talk toward the p53 pathway, which concurrently will suppress the Akt pathway.

In summary, we showed that CTGF is able to suppress NSCLC cell growth. CTGF blunts cell growth probably by activation of E2F1, ARF, and p53 and by suppression of cyclin D1. Decreased expression of CTGF may play a role in lung tumorigenesis by allowing IGF-1 and EGF to have greater progrowth activity.

Materials and Methods
RNA Extraction, cDNA Preparation, and Real-time PCR
Lung tissues of matched tumor and normal samples were obtained from lung cancer patients after informed consent. RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) and cleanup with RNaseasy column (Qiagen, Valencia, CA). The first strand cDNA was synthesized using the SuperScript reverse transcription-PCR kit (Invitrogen). Quantitative real-time PCR was done using Taqman PCR Core Reagent kit (PE Biosystems, Foster City, CA) as described previously. Amplification of β-actin was used as control for cDNA normalization. The oligonucleotide primers (first two sequences) and probe (third sequence) were 5'−AGGATAGCCAGAGGTGCAAG, 5'−ATGTTCTTCATGCTGGTGCAG, and 5'−TGGGAAGCTGACCTGGAAGAGAAACA (CTGF) and 5'−GATCATTTGCTCCTCTGAGC, 5'−ACTCTCTGCTGCTGATCC, and 5'−CTGCTGCTCCACCTCCAGCAGAT (β-actin)
**Immunohistochemistry**

Immunohistochemical staining for CTGF was done with polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Heat-induced epitope retrieval was done with a pressure cooker and Tris buffer (pH 9.0) for 2 minutes. Localization was done with DAKO Envision (DAKO, Carpinteria, CA) conjugated to horseradish peroxidase followed by the diaminobenzidine reaction. Negative controls consisted of substitution of the primary antiseraum with normal rabbit serum at the same dilution.

**Cell Culture and Transfections**

Large cell lung cancer cell lines (NCI-H1299 and NCI-H460) and squamous cell lung carcinoma cell lines (NCI-H520 and SK-MES-1) were maintained in DMEM supplemented with 10% FCS. Cells were transfected with either 5 μg CTGF expression vector or empty vector pcDNA3.1 using LipofectAMINE 2000 (Invitrogen). Forty-eight hours after transfection, cells were replated in complete medium containing 600 μg/ml G418, and stable clones were selected after 2 weeks. Several G418-resistant stable clones after CTGF transfection were screened for CTGF expression with real-time PCR and Western blot analysis. Two clones with the most prominent CTGF expression were used for this study. A G418-resistant clone was also selected from an empty vector pcDNA3.1 transfection for each cell line and they were designated 4V for the one selected from NCI-H460 and 5V for the clone from NCI-H520 cells.

**Colony Formation Assay**

Cells were seeded at 1 × 10⁵ per well in 12-well plates. Triplicates of each cell line were plated. Cells were passaged under G418 selection for 2 to 3 weeks, fixed and stained with 0.1% crystal violet in 50% methanol, and photographed with an inverted phase-contrast microscope. The dye was then dissolved in 0.2% (v/v) Triton X-100, and absorbance was measured at 590 nm. The number of cells showed a linear relationship to absorbance.

**MTT Assay**

Cells were seeded at 1 × 10⁴ per well in 96-well plates. Triplicates of each cell line were plated. MTT reagents were added to each well, and absorbance was measured at 590 nm. The number of cells showed a linear relationship to absorbance.

**Western Blot Analysis**

Total cell lysates were prepared with lysis buffer [50 mmol/L Tris (pH 7.6), 5 mmol/L EDTA, 300 mmol/L NaCl, 0.1% NP40, 0.2 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L DTT, protease inhibitor mixture (Roche Molecular Biochemicals, Pleasanton, CA)] and resolved by SDS-PAGE followed by blotting. Membranes were probed with anti–glyceraldehyde-3-phosphate dehydrogenase (PharMingen, San Diego, CA) as loading control. Antibodies against CTGF, p53, ARF, and E2F1 were purchased from Santa Cruz Biotechnology. Signals were detected by West Pico reagents (Pierce, Rockford, CA).

**Conditioned Medium Preparation**

CTGF-pcDNA3.1 (4F1) and pcDNA3.1 (4V) transfected cells were grown to 80% confluency and replaced with serum-free medium for another 48 hours. The conditioned media were centrifuged at 3,000 g for 10 minutes, and the supernatants were aliquoted and stored at −80°C. Heparin agarose beads (Sigma) were prewashed, equilibrated with serum-free medium, and incubated with conditioned medium at 4°C overnight to remove CTGF from the conditioned media.

**Growth Factor Treatment**

Cells were plated in serum-free medium for 24 hours followed by treatment with either IGF-I (100 ng/ml; Sigma) or EGF (25 ng/ml; Sigma) for 15 minutes and then harvested for Western blot analysis. For inhibitor studies, cells were pretreated with either PD98059 (50 μmol/L) or Wortmannin (100 nmol/L) before addition of growth factors. Antibodies used for these studies included phosphorylated Akt, ERK1/2, as well as Akt and ERK1/2 (Santa Cruz Biotechnology).

**References**


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