Connective Tissue Growth Factor and Its Role in Lung Adenocarcinoma Invasion and Metastasis

Cheng-Chi Chang, Jin-Yuan Shih, Yung-Ming Jeng, Jen-Liang Su, Been-Zen Lin, Szu-Ta Chen, Yat-Pang Chau, Pan-Chyr Yang, Min-Liang Kuo

Background: Tumor invasion and metastasis cause most deaths in cancer patients. Connective tissue growth factor (CTGF), a secreted protein that binds to integrins, modulates the invasive behavior of certain human cancer cells, but few mechanistic details are known. We investigated the roles of CTGF and collapsin response mediator protein 1 (CRMP-1) in metastasis and invasion of human lung adenocarcinoma. Methods: We compared vector control–transfected cells with corresponding CTGF gene-transfected cells. Invasive activity was measured with a modified Boyden chamber assay, and metastatic activity was measured in an animal model. We used CTGF deletion mutants, CTGF and CRMP-1 antisense oligonucleotides, and anti-integrin and anti-CRMP-1 antibodies to investigate the functional relationship between CTGF and CRMP-1. Expression of CTGF protein in 78 lung adenocarcinoma specimens was investigated immunohistochemically. All statistical tests were two-sided. Results: Invasive (both \( P < .001 \)) and metastatic (\( P < .001 \) and \( P = .003 \), respectively) activities were lower in cells that overexpress CTGF than in vector control cells. Expression of CRMP-1 was higher in CTGF-transfected clones than in vector control cells, and its level decreased after cells were treated with anti-integrin \( \alpha_\beta_3 \) and \( \alpha_\beta_5 \) antibodies. Reduced levels of CRMP-1 protein after the transfection of CRMP-1–specific antisense oligonucleotides, but not sense oligonucleotides, increased the invasiveness of CTGF-transfected cells (mean numbers of invasive CTGF-transfected cells treated with 20 \( \mu M \) CRMP-1–specific sense and antisense oligonucleotides were 327 and 516 cells, respectively; [difference = 189 cells, 95% confidence interval (CI) = 156 to 221 cells; \( P < .001 \)]. The CT module of CTGF was the region primarily responsible for the increased expression of CRMP-1 and the inhibition of invasion (mean numbers of invasive cells expressing full-length CTGF and CT module–deleted mutant were 148 and 385 cells, respectively; [difference = 237 cells, 95% CI = 208 to 266 cells; \( P < .001 \)]. Reduced expression of CTGF in lung cancer specimens was statistically significantly associated with the risk of more advanced-stage disease (stages III and IV versus stages I and II; \( P = .001 \)), lymph node metastasis (\( P = .014 \)), and shorter survival (median survival with high levels of CTGF = 66.7 months and median survival for low levels = 18.2 months; difference = 48.5 months, 95% CI = 33.5 to 63.5 months; \( P = .02 \)). Conclusion: CTGF inhibits metastasis and invasion of human lung adenocarcinoma by a CRMP-1–dependent mechanism. [J Natl Cancer Inst 2004;96:364–75]

Lung cancer is by far the most common cause of cancer death in the world (1). This high mortality is probably attributable to early metastasis, especially for non–small-cell lung carcinoma (NSCLC). NSCLCs are subdivided into adenocarcinomas (the most common type), squamous-cell carcinomas, and large-cell carcinomas (2). Tumor metastasis involves detachment of tumor cells from the primary tumor mass, microinvasion of tumor cells into stromal tissue, intravasation of tumor cells into blood vessels, and extravasation and growth of tumor cells in secondary sites (3,4). To become metastatic, tumor cells must coordinate the increased expression of metastasis-promoting genes and/or the decreased expression of metastasis-suppressing genes. A number of metastasis-suppressing genes, such as NM23, KAI1, MKK4, KiSS, and RhoGDI2, have been identified in different types of cancer (5–15). These metastasis suppressors appear to operate at various points in the metastatic process, but their mechanisms are not clear. Many approaches have been developed to dissect and identify genes that regulate metastatic processes. One straightforward approach is to establish a panel of cells with progressive invasiveness or metastasis and use it to define which genes are functionally involved in the process of invasion and metastasis.

Connective tissue growth factor (CTGF, also known as CCN2) is a member of the CCN family, which includes CTGF, cysteine-rich 61 (Cyr61, also known as CCN1), and nephroblastoma overexpressed (Nov, also known as CCN3), as well as Wisp-1/elm1 (CCN4), Wisp-2/cop1 (CCN5), and Wisp-3 (CCN6) (16,17). The CTGF gene encodes a 38-kd cysteine-rich heparin-binding protein, first identified as a mitogen found in conditioned medium from human umbilical vein endothelial cells (18). CTGF is a secreted growth factor that can bind to integrins on the cell surface (16). CTGF mRNA is expressed in adult human heart, brain, placenta, liver, muscle, kidney, and lung (19) and may have physiologic functions in these human tissues. CTGF is involved in chondrocyte growth, proliferation (20,21), and differentiation (20–22); endothelial cell proliferation and migration (23,24); and regulation of apoptosis in human breast cancer cells and aortic smooth muscle cells (25,26). The level of CTGF protein is increased in patients with various

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human diseases, including systemic scleroderma (27,28), atherosclerosis (29), renal diseases (30,31), and hepatic fibrosis in biliary atresia (32). Although elevated CTGF expression has been observed in breast cancers (33), pancreatic cancers (34), melanomas (35), and chondrosarcomas (36), a definitive role of CTGF in the development or progression of these human cancers has not yet been established.

Recent findings indicated that several members of the CCN family can affect tumor development and progression. For example, Cyr61 (CCN1) overexpression appears to inhibit the tumorigenicity of NSCLC cells in SCID mice (37). Wisp-1 (CCN4) expression appears to reduce the in vitro invasive ability of NSCLC cells (38) and the in vivo metastasis of mouse melanoma cells (39). Thus, CCN proteins appear to display different biologic properties in distinct types of cancer. Our goal in this study was to elucidate the role of CTGF and its possible downstream effectors in the metastasis and invasion of human lung adenocarcinoma.

MATERIALS AND METHODS

Cell Culture

Lung adenocarcinoma cells were grown in RPMI-1640 medium with 10% fetal bovine serum and 2 mM l-glutamine (all from Life Technologies, Rockville, MD) at 37 °C in a humidified atmosphere of 5% CO₂/95% air. Lung adenocarcinoma cell lines CL1-3 and CL1-5 are sublines that were selected from parental CL1-0 cultures with a Matrigel-coated polycarbonate membrane (Collaborative Biomedical, BD Biosciences, Bedford, MA). After the blot was blocked in a solution of 5% skim milk, 0.1% Tween 20, and PBS, membrane-bound proteins were probed with primary antibodies against CTGF, Cyr61, β-actin, or α-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA) or against CRMP-1 (41). The membrane was washed and then incubated with horseradish peroxidase–conjugated secondary antibodies for 30 minutes. Antibody-bound protein bands were detected with enhanced chemiluminescence reagents (Amersham Pharmacia Biotech, Piscataway, NJ) and photographed with Kodak X-OMat Blue autoradiography film (Perkin Elmer Life Sciences, Boston, MA).

Reverse Transcription–Polymerase Chain Reaction

Reverse transcription of RNA isolated from cells was performed in a final reaction volume of 20 μL containing 5 μg of total RNA in Moloney murine leukemia virus (MMLV) reverse transcriptase buffer (Promega, Madison, WI), which consists of 10 mM dithiothreitol, all four deoxynucleoside 5'-triphosphates (dNTPs; each at 2.5 mM), 1 μg of (dT)₁₂–₁₈ primer, and 200 U of MMLV reverse transcriptase (Promega). The reaction mixture was incubated at 37 °C for 2 hours, and the reaction was terminated by heating at 70 °C for 10 minutes. One microliter of the reaction mixture was then amplified by polymerase chain reaction (PCR) with the following pairs of primers: CTGF primers, 5'-GCTTACCGACTGGAAGACGCTT-3' (sense) and 5'-TATGCCCATTGTCTCGATACAT-3' (antisense), to produce a 500-base-pair (bp) fragment of the CTGF gene; Cyr61 primers, 5'-CAGGAAAGCTGTTGACGAGAAAC-3' (sense) and 5'-AGGACTGGATCATCATGACCATGT-3' (antisense), to produce a 450-bp fragment of the Cyr61 gene; CRMP-1 primers, 5'-CACGATGGATCATGACGCAATGTC-3' (sense) and 5'-GGGATTAATCAGCAGAGATTG-3' (antisense) to produce a 320-bp fragment of the CRMP-1 gene; and β-actin primers, 5'-GATGATATCGGACGCTGTT-3' (sense) and 5'-GGGATTAATCAGCAGAGATTG-3' (antisense) to produce a 200-bp fragment of the β-actin gene, which was used as the internal control. The PCR amplification was carried out in a reaction buffer containing 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl₂, all four dNTPs (each at 167 μM), 2.5 U of Tag DNA polymerase, and 0.1 μM primers. The reactions were performed in a Biometra Thermoblock (Biometra, Hamburg, Germany) with the following program: denaturing for 1 minute at 95 °C, annealing for 1 minute at 58 °C, and elongating for 1 minute at 72 °C for a total of 23 cycles; the final extension took place at 72 °C for 10 minutes. Equal volumes of each PCR sample were subjected to electrophoresis in a 1% agarose gel, which was then stained with ethidium bromide and photographed under UV illumination.

Western Blot Analysis

Proteins in the total cell lysate (40 μg of protein) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis in 10% gels and electrotransferred to a polyvinylidene difluoride membrane (Immobilon-P membrane; Millipore, Bedford, MA). After the blot was blocked in a solution of 5% skim milk, 0.1% Tween 20, and PBS, membrane-bound proteins were probed with primary antibodies against CTGF, Cyr61, β-actin, or α-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA) or against CRMP-1 (41). The membrane was washed and then incubated with horseradish peroxidase–conjugated secondary antibodies for 30 minutes. Antibody-bound protein bands were detected with enhanced chemiluminescence reagents (Amersham Pharmacia Biotech, Piscataway, NJ) and photographed with Kodak X-OMat Blue autoradiography film (Perkin Elmer Life Sciences, Boston, MA).
Construction of CTGF/wt Expression Plasmid and CTGF Deletion Mutants

Total RNA was extracted from CL1-0 cells. CTGF cDNA was cloned and amplified by reverse transcription–polymerase chain reaction with primers 5'-ATGACGGCCTGCAGTATGG-3' and 5'-TCAAGCTTACTCCGATCATCTT-3' (PubMed serial number XM-037056), and the product was subcloned into a pcDNA3/V5-His TOPO TA vector (Invitrogen, San Diego, CA). The CTGF-expression vector (CTGF/wt) was used in transient and stable transfections of human lung adenocarcinoma cells in vitro and in vivo. Three serial deletion mutants of CTGF were generated by deleting the CT domain; the CT and TSP-1 domains; or the CT, TSP-1, and VWC domains. These constructs were designated CTGF/d3, CTGF/d2, or CTGF/d1, respectively. Deletion constructs were generated with the reverse primer 5'-CGAATTCTAGACGGCCTGCAGTATGG-3' and the forward primers 5'-GCTCTAGATCTAGCAAGTGCACCTTTTCTCC-3', 5'-GCTCTAGATCTAGCAGTCGGGGCCACCGTGTCT-3', and 5'-GCTCTAGATCAGCAAGGAGCACCATCTTTG-3', respectively.

Plasmids and Transient Transfection

The CTGF expression vectors were transiently transfected into CL1-5 and A549 cells with TransFast transfection reagent (Promega). Briefly, 3 μg of plasmid DNA (CTGF/wt, CTGF/d3, CTGF/d2, CTGF/d1, or pcDNA3) and 8 μg of transfection reagent were mixed, and the transfection protocol was carried out according to the manufacturer's instructions (Promega). One hour after transfection, the cells were cultured in normal complete medium for another 8 hours. The transfected cells were harvested and subjected to an invasion assay and western blot analysis.

Stable Transfected Clone Selection

Purified plasmid DNA (3 μg) was transfected into CL1-5 and A549 cells with TransFast transfection reagent (Promega). Twenty-four hours after transfection, stable transfecants were selected in Gentamicin (G418; Life Technologies) at a concentration of 600 μg/mL. Thereafter, the selection medium was replaced every 3 days. After 2 weeks of selection in G418, clones of resistant cells were isolated and allowed to proliferate in medium containing G418 at 100 μg/mL. Integration of transfected plasmid DNA was confirmed by reverse transcription–polymerase chain reaction and western blot analysis.

In Vitro Cell Growth Assay

Control vector (pcDNA3), the respective CTGF/wt expressing vector, and several truncated deletion plasmids (3 μg/μL) were added to 6-cm dishes initially containing 10⁶ cells per well. At regular intervals, cells were trypsinized and resuspended, and the cell numbers were counted with a hemacytometer.

Boyden Chamber Assays

For invasion assays, we used modified Boyden chambers with filter inserts (pore size, 8 μm) coated with Matrigel (40 μg; Collaborative Biomedical, Becton Dickinson Labware) in 24-well dishes (Nucleopore, Pleasanton, CA). Approximately 2.5 × 10⁶ cells in 100 μL of complete RPMI-1640 medium were placed in the upper chamber, and 1 mL of the same medium was placed in the lower chamber. After 48 hours in culture, cells were fixed in methanol for 15 minutes and then stained with 0.05% crystal violet in PBS for 15 minutes. Cells on the upper side of the filters were removed with cotton-tipped swabs, and the filters were washed with PBS. Cells on the underside of the filters were viewed and counted under a microscope (type 090-135.001; Leica Microsystems, Wetzlar, Germany). Each clone was plated in triplicate in each experiment, and each experiment was repeated at least three times.

Antisense Oligonucleotide Experiments

CRMP-1 antisense or sense oligonucleotides were introduced into stable transfected clones CL1-5/neo and CL1-5/CTGF by incubation with Lipofectamine (TransFast transfection reagent; Promega) (oligonucleotide/Lipofectamine ratio = 10:1 [wt/wt]) in starvation medium for 8 hours. After the indicated time, cells were transferred to complete RPMI-1640 medium for another 48 hours. Total cell extracts were then collected and subjected to western blot analysis for CRMP-1 and α-tubulin proteins. In the invasion experiments, CL1-5/neo and CL1-5/CTGF cells were transfected with CRMP-1 antisense (or sense) oligonucleotides by use of Lipofectamine in starvation medium. After 8 hours of incubation, cells were trypsinized; 2.5 × 10⁶ cells were seeded in the upper chamber, and 1 mL of the RPMI-1640 medium was placed in the lower chamber. After another 48 hours, cells were fixed and counted as described above.

Experimental Metastasis

Cells were washed and resuspended in PBS. Subsequently, a single-cell suspension containing 10⁶ cells in 0.1 mL of PBS was injected into the lateral tail vein of 6-week-old SCID mice (supplied by the animal center in the College of Medicine, National Taiwan University, Taipei, Taiwan). Mice were killed after 8 weeks. (Our preliminary study in this animal model indicated that CL1-5 cells developed numerous lung metastasis nodules by 8 weeks.) All organs were examined for metastasis formation. The lungs were removed, weighed, and fixed in 10% formalin. The number of lung tumor colonies was counted under a dissecting microscope. Representative lung tumors were resected and embedded in paraffin. Embedded tissue was sectioned into 4-μm sections, and the sections were stained with hematoxylin–eosin for histologic analysis. All animal work was performed under protocols approved by the Institutional Animal Care and Use Committee of the College of Medicine, National Taiwan University.

Patients and Specimens

Lung adenocarcinoma specimens were obtained from a total of 78 consecutive patients who underwent surgical resection at the National Taiwan University Hospital from September 1, 1993, to August 31, 1997. Patients who had previous history of cancers or had been treated with neoadjuvant chemotherapy and/or radiation therapy were not included in this study. Only patients who provided lung adenocarcinoma specimens were included in this study. Paraffin-embedded, formalin-fixed surgical specimens were collected for immunohistochemical staining for CTGF. The group consisted of 39 men and 39 women with an age of 62 ± 11 years (mean ± standard deviation). Written informed consent was obtained from all patients. The histologic identification of lung cancer was determined as recommended.
by the World Health Organization (42). Tumor size, local invasion, and lymph node metastasis were determined at pathologic examination. The final disease stage was determined by a combination of surgical and pathologic findings, according to the current tumor–node–metastasis staging system for lung cancer (43). Follow-up data were obtained from the patients’ medical charts and from our tumor registry service. The survival time of patients was calculated from the date of surgery to the date of death. The relapse time was calculated from the date of surgery to the date of local recurrence or distant metastasis. Median follow-up was 37.4 months (range 1–117 months).

Immunohistochemistry

After rehydration, 4-μm sections of paraffin-embedded tissue on glass slides were incubated in 3% hydrogen peroxide to block the endogenous peroxidase activity. After trypsinization, sections were blocked by incubation in 3% bovine serum albumin in PBS. The primary antibody, a polyclonal goat anti-human CTGF antibody (Santa Cruz Biotechnology) was applied to the slides at a dilution of 1:100 (diluted in 3% bovine serum albumin) and incubated at 4 °C overnight. After three washes in PBS, the samples were treated with donkey anti-goat IgG biotin-labeled secondary antibodies (Vector Laboratories, Burlingame, CA) at a dilution of 1:500 (diluted in 0.05% PBS–Tween 20) for 1 hour at room temperature. Bound antibodies were detected with an ABC kit (Vector Laboratories). The slides were stained with diaminobenzidine, washed, counterstained with Delafield’s hematoxylin, dehydrated, treated with xylene, and mounted. A scoring system was devised to assign a staining intensity score for CTGF expression from 0 (no expression) to 3 (highest intensity staining). Immunostaining was classified into one of two groups according to both intensity and extent: low expression was defined as no staining present (staining intensity score = 0) or positive staining detected in less than or equal to 10% of the cells (staining intensity score = 1) and high expression was defined as positive immunostaining present in 10%–50% of the cells (staining intensity score = 2) or more than 50% of the cells (staining intensity score = 3).

Statistical Analysis

Statistical evaluation of the data was performed with a two-tailed Student’s t test for simple comparison between two values when appropriate. All statistical analyses were performed with SPSS, version 10.0 (SPSS, Chicago, IL). Pearson chi-square tests and Student’s t tests were used to compare the clinicopathologic characteristics of tumors (and patients) with high expression of CTGF and those with low expression of CTGF. Survival was analyzed by the Kaplan–Meier method, and the log-rank test was used to test the difference in relapse time and survival between patients with tumors that had high expression of CTGF and those with low expression of CTGF. The median survival times with 95% confidence intervals (CIs) were calculated as described (44). Multivariable analyses with the Cox proportional hazards model were used to estimate the simultaneous effects of prognostic factors on survival (45). After confirmation that the data met the assumptions for a proportional hazards analysis, stepwise selection was used. Variables were retained in the model if the associated two-sided P values were .10 or less. All statistical tests were two-sided. P values of less than .05 were considered statistically significant.

RESULTS

Expression of CTGF and Invasiveness of Human Lung Adenocarcinoma Cancer Cell Lines

To explore the possible role of CTGF in invasiveness of lung adenocarcinoma cells, we first examined the expression of CTGF in a panel of cell lines, CL1-0, CL1-3, and CL1-5, with either low or high invasive ability, as previously described (41). CTGF mRNA was highly expressed in CL1-0 cells that have low invasive and low metastatic ability but was almost undetectable in highly invasive CL1-3 and CL1-5 cells (Fig. 1, A, upper right panel). The invasive ability of CL1-3 and CL1-5 was fourfold to sixfold higher than that of CL1-0 cells (Fig. 1, A, lower panel). The level of Cyr61 mRNA, another member of the CCN family, was the same in cell lines with low or high invasive ability (Fig. 1, A, upper right panel). CL1-0 cells expressed a high level of CTGF protein, and CL1-3 and CL1-5 cells expressed extremely low levels of CTGF protein (Fig. 1, A, upper left panel); the level of Cyr61 protein appeared constant in CL1-0, CL1-3, and CL1-5 cells (Fig. 1, A, upper left panel). We tested other lung adenocarcinoma cell lines (A549, NICH520, and H928) to determine whether this relationship between the level of CTGF and invasive ability was also present. A549 cells were more invasive than NICH520 and H928 cells (Fig. 1, B, lower panel). The highly invasive A549 cells expressed a very low or undetectable level of CTGF protein, whereas the less invasive NICH520 and H928 cells expressed high levels of CTGF protein (Fig. 1, B, upper panel). Thus, expression of CTGF was inversely associated with an invasive and/or metastatic phenotype of lung adenocarcinoma cell lines. Although Cyr61 has been shown to act as a tumor suppressor in NSCLC cells (37), the same level of Cyr61 expression was detected in A549, NICH520, and H928 lung adenocarcinoma cells. Thus, Cyr61 does not appear to be associated with invasion and metastasis in lung adenocarcinoma cells.

Overexpression of CTGF Inhibits Invasion and Metastasis

To clarify the direct role of CTGF expression in the invasiveness of lung adenocarcinoma cells, human CTGF cDNA expression vectors or control vectors were transfected into the highly invasive CL1-5 cells. After G418 selection, we isolated two single clones (CL1-5/CTGF-3 and CL1-5/CTGF-10), a clonal mixture (CL1-5/CTGF-M), and vector control clone (CL1-5/neo), and then we assessed the levels of CTGF expression in each. We detected 3.6-fold to 5.5-fold more CTGF protein in CTGF-overexpressing cell lines than in vector control cells (Fig. 1, C). Invasive capacity was much lower in CTGF-transfected CL1-5 cells than in CL1-5/neo control cells (Fig. 1, D) (percentage of invasive CL1-5/neo control cells: CL1-5/CTGF-M cells = 52.7% [95% CI = 23.9% to 70.8%], CL1-5/CTGF-3 cells = 44.0% [95% CI = 47.0% to 65.0%], and CL1-5/CTGF-10 cells = 54.0% [95% CI = 36.9% to 55.2%]; all P<.001). We also transfected the CTGF expression vector or the control vector into highly invasive A549 cells and examined the invasive ability of the transfected cells. After transfection and selection in G418, a pool of cells (A549/CTGF-M), two single clones (A549/CTGF-4 and A549/CTGF-5), and a vector control clone (A549/neo) were isolated, and their expression of CTGF protein was assessed by western blotting. CTGF-transfected A549 cells (A549/CTGF-M, A549/CTGF-4, and A549/
Fig. 1. Connective tissue growth factor (CTGF) expression and in vitro invasion ability in human lung cancer cell lines. A) Upper left panel: western blot analysis of the expression of CTGF and cysteine-rich 61 (Cyr61) proteins. Total proteins were extracted from CL1-0, CL1-3, and CL1-5 cells and probed with polyclonal antibody specific for CTGF or Cyr61. Each lane contains 40 μg of total protein. β-actin was used as an internal loading control. Upper right panel: reverse transcription-polymerase chain reaction analysis for CTGF and Cyr61 mRNAs. The 500-base-pair coding regions of CTGF and Cyr61 cDNA were used as probes. A β-actin probe was used as an internal control for RNA quantity. Lower panel: The invasion activity of each clone was measured in vitro with the Boyden chamber after 48 hours. Each cell subline was assayed in three experiments carried out in triplicate. Error bars correspond to 95% confidence intervals. B) Upper panel: western blot analysis of CTGF and Cyr61 proteins in human lung adenocarcinoma cancer cell lines H928, NICH520, and A549. Lower panel: in vitro invasion activity of human lung adenocarcinoma cancer cell lines measured with the Boyden chamber after 48 hours. These experiments were repeated four times. Data are the mean; error bars show the corresponding upper 95% confidence interval. C) CTGF protein expression in mock-transfected and CTGF-transfected CL1-5 clones assessed by immunoblot analysis to facilitate comparison of the relative CTGF protein expression between the mock-transfected clone CL1-5 neo and the CTGF-transfected clones CL1-5/CTGF-M, CL1-5/CTGF-3, and CL1-5/CTGF-10. D) In vitro invasion activity of CL1-5 cells stably transfected with CTGF or control vectors. In vitro invasion was measured with the Boyden chamber after 48 hours by determining the percentage of cells that migrated through Matrigel-coated filters (8-μm pore size) in Transwell chambers. The invasive activity was statistically significantly lower in CTGF-transfected clones than in the CL1-5 neo vector control clone, as indicated by the asterisks (P = 0.003, two-tailed Student’s t test). Each clone was assayed in three experiments carried out in triplicate. E) CTGF overexpression and the invasion ability of A549 cells. Upper panel: immunoblot analysis of CTGF and α-tubulin expression in mock-transfected A549 (A549/neo) cells, CTGF-overexpressed mixture clone A549/CTGF-M, and clonal A549/CTGF-4 and A549/CTGF-5 cells. Lower panel: expression of CTGF and the in vitro invasion activity of A549 cells stably transfected with CTGF or control vectors. To compare the relative invasiveness among A549/neo, A549/CTGF-M, A549/CTGF-4, and A549/CTGF-5 cells, values were normalized to that of A549/neo cells. Error bars are the corresponding upper 95% confidence intervals. CTGF-transfected clones had statistically significantly lower invasive activity than A549/neo clones, as shown by asterisks (P < 0.001, two-tailed Student’s t test). F) CTGF overexpression and the growth properties of the vector (neo) and CTGF-transfected CL1-5 cells in monolayer culture cell lines. All experiments were carried out in triplicate on separate occasions with similar results. Error bars correspond to 95% confidence intervals.
CTGF-5) clearly expressed CTGF protein (Fig. 1, E, upper panel), and the invasive ability of CTGF-overexpressing A549 cells was only about 40% that of the control A549/neo cells (percentage of invasive A549/neo cells: A549/CTGF-M cells = 37.7% [95% CI = 37.0% to 87.7%], A549/CTGF-4 cells = 32.9% [95% CI = 30.9% to 88.4%], A549/CTGF-5 cells = 41.0% [95% CI = 32.7% to 85.3%]; all \( P < .001 \) ) (Fig. 1, E, lower panel).

To rule out the possibility that the effect of CTGF on in vitro cell invasiveness was caused by different proliferation rates among the cell lines, we compared the growth rates of CTGF-overexpressing cells with those of the corresponding vector control cells. The growth curves for the vector control cells (10^6 control vector- or CTGF-transfected tumor cells (CL1-5/CTGF-M or A549/CTGF-M) in 0.1 mL of phosphate-buffered saline and injected them into the lateral tail vein of SCID mice (10 mice per group). Animals were killed 2 months after intravenous injection, and lungs were excised and photographed after fixation. White arrows = metastatic tumor nodules. B) Histologic analysis of lung metastasis of CL1-5/neo control cells and CL1-5/CTGF-M cells. Lungs were embedded in parafin, parafin-embedded tissue was sectioned into 4-μm thick sections, and the sections were stained with hematoxylin–eosin. A metastatic tumor (T) is shown within the lung parenchyma of a CL1-5/neo tumor (left). The CL1-5/CTGF tumor (right) had fewer foci. Scale bar = 100 μm.

The effect of CTGF overexpression on metastatic colonization was further assessed by intravenous injection of 1 × 10^6 cells into the lateral tail vein of male SCID mice. Each clonal cell line was injected into 10 mice. Eight weeks later, the mice were killed, and the number of metastatic tumors formed in the lungs was counted. Mice injected with CL1-5/neo or A549/neo control clones had numerous large lung metastases, whereas mice injected with CL1-5/CTGF-M or A549/CTGF-M cells had fewer and smaller metastatic nodules in the lung (Fig. 2, A). Metastatic tumors formed in the lungs by CL1-5/neo cells had the morphology of a typical adenocarcinoma (Fig. 2, B). Mice injected with CL1-5/neo cells had 77.5 metastatic lung nodules, and mice injected with CL1-5/CTGF had 12.3 metastatic lung nodules (difference = 65.2 nodules, 95% CI = 48.9 to 81.6 nodules; \( P < .001 \)). Mice injected with A549/neo had 61.3 metastatic lung nodules, and mice injected with A549/CTGF had 21.7 metastatic lung nodules (difference = 39.6 nodules, 95% CI = 23.4 to 56.0 nodules; \( P = .003 \)). Thus, overexpression of CTGF in CL1-5 and A549 cells suppressed the ability of these cells to form metastatic nodules in the lungs.

The weight of lungs from mice injected with CL1-5/CTGF cells was 390 mg and that from mice injected with CL1-5/neo vector control cells was 448 mg (difference = 58 mg, 95% CI of the difference = 24 to 91 mg; \( P = .005 \)). The weight of lungs from mice injected with A549/CTGF cells was 473 mg and that from mice injected with A549/neo vector control cells was 617 mg (difference = 144 mg, 95% CI of the difference = 39 to 247; \( P = .008 \)). Thus, lung weight was decreased 12.9% in mice injected with CL1-5/CTGF cells and decreased 23.3% in mice injected with A549/CTGF cells, compared with lung weight in mice injected with vector control cells. Data from these two experiments were summarized in Table 1.

**Action of CRMP-1 as a Downstream Effector of CTGF**

CTGF is a secreted growth factor that binds to integrins on the cell surface (16). The binding of CTGF to integrins could activate intracellular pathways that regulate invasion and metastasis. It has been reported that CRMP-1, an intracellular molecule, inhibits the invasion of lung cancer cells (40). To characterize the relationship between CTGF and CRMP-1, we first used western blot analysis to determine the level of CRMP-1 protein in CL1-5/CTGF stable clones and found that CRMP-1 protein expression was threefold to sixfold higher in CTGF-overexpressing CL1-5 cells than in vector control cells (Fig. 3, A, upper panel). A similar increase of CRMP-1 protein was also

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*Each group contained 20 mice. Statistical evaluation of the data was performed with a Student’s \( t \) test. Test for simple comparison between two values was also used when appropriate. All statistical tests were two-sided.

†P values of less than .05 were considered statistically significant (CL1-5/neo versus CL1-5/CTGF; A549/neo versus A549/CTGF).
expression vector or a control vector, the level of CRMP-1 protein was substantially lower in cells transfected with the antisense vector than with the control vector (Fig. 3, A, upper panel). In addition, the expression of CRMP-1 was greatly reduced in CL1-5/CTGF-M cells incubated with antibodies that specifically block the functions of integrin αβ3 or αβ5 compared with a control IgG antibody (Fig. 3, A, lower panel). Thus, it appears that CTGF induces CRMP-1 expression through integrins αβ3 and αβ5.

To exclude the possibility that artificial drug selection enhanced the expression of CRMP-1 protein in these CTGF stable clones, we transiently transfected CL1-5 cells with various concentrations of the CTGF expression vector and then determined the level of CRMP-1 protein. An apparent dose-dependent increase in the expression of CRMP-1 protein was detected in CL1-5 cells transfected with various concentrations of CTGF expression vector but not in CL1-5 cells transfected with the control vector (Fig. 3, B). Accordingly, CTGF appears to regulate the expression of CRMP-1 directly.

To further assess the role of CRMP-1 in the CTGF-mediated inhibition of cell invasion, CL1-5/CTGF-M cells were treated with 10 or 20 μM antisense or sense oligonucleotides specific for CRMP-1. The level of CRMP-1 protein was effectively reduced in CL1-5/CTGF-M cells by treatment with 20 μM CRMP-1-specific antisense oligonucleotides but not by treatment with its sense oligonucleotides (Fig. 3, C, upper panel). Treatment with antisense oligonucleotides specific for CRMP-1 did not alter the invasiveness of CL1-5/neo cells (mean cell number = 578 cells; Fig. 3, C, lower panel). In addition, treatment of CL1-5/CTGF cells with 20 μM CRMP-1-specific sense oligonucleotide also did not alter their invasion activity (mean cell number = 327 cells). However, the invasive ability of CL1-5/CTGF-M cells was statistically significantly increased by treatment with 20 μM CRMP-1 antisense oligonucleotides (mean numbers of CL1-5/CTGF cells treated with 10 μM or 20 μM CRMP-1-specific antisense oligonucleotides = 354 cells [difference from control cells = 27 cells, 95% CI of the difference = 1 to 52 cells; P<.001] and 516 cells [difference from control cells = 189 cells, 95% CI of the difference = 156 to 221 cells; P<.001]). Therefore, CTGF-mediated inhibition of invasiveness in lung

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**Fig. 3.** Expression of collapsin response mediator protein 1 (CRMP-1) protein in connective tissue growth factor (CTGF)-transfected cells. A) Western blot analysis of CTGF. α-tubulin was the loading control. Upper panel: Protein level of CRMP-1 in CL1-5/CTGF-M, CL1-5/CTGF-3, CL1-5/CTGF-10, A549/CTGF-M, A549/CTGF-4, and A549/CTGF-5 cells was compared with that in vector control cells, and the protein level of CRMP-1 in CL1-0/CTGF (antisense) cells was compared with that in CL1-0/neo cells. Lower panel: Integrins αβ3 and αβ5 and CTGF-induced CRMP-1 protein induction. CL1-5 cells transfected with either control or CTGF vectors were treated with anti-αβ3 antibody (LM609), anti-αβ5 antibody (B1F6), or immunoglobulin G (IgG) control antibody (each at 10 μg/mL) for 24 hours. B) Western blot analysis of CRMP-1 protein expression in CL1-5 cells transiently transfected with CTGF expression vector. Cells were transiently transfected with 3 μg of control vector or various concentrations of CTGF expression vectors as indicated. Forty-eight hours after transfection, whole-cell extracts were subjected to western blot analysis with antibodies against CRMP-1, CTGF, or α-tubulin. C) Antisense oligonucleotides to CRMP-1 and the invasion ability of CL1-5/CTGF cells.
adenocarcinoma cells appears to be mediated, at least in part, by enhanced CRMP-1 expression.

CTGF and other CCN proteins have a structure with several domains or modules. To determine which module participates in the induction of CRMP-1 and inhibition of invasion, we produced and characterized a series of CTGF deletion constructs. Because CTGF is a secretory protein, the signal sequence at the amino-terminal end was retained and sequences at the carboxyl-terminal end were sequentially deleted. The products were subcloned into a pcDNA3 expression vector, and isolated clones were designated CTGF/wt, CTGF/d3, CTGF/d2, and CTGF/d1 (Fig. 4, A). The invasive capacities of transiently transfected CL1-5 cells expressing the full-length CTGF (CTGF/wt) or its deletion mutants CTGF/d3, CTGF/d2, or CTGF/d1 were determined. CL1-5 cells expressing CTGF/d3, which lacks the CT module, had a high invasive capacity that was equivalent to that of CL1-5 cells expressing the control vector. The mean numbers of invasive CL1-5 cells transfected with CTGF/wt and CTGF/d3, a deletion of the CT module, were 148 cells and 385 cells of invasive CL1-5 cells transfected with CTGF/wt and CTGF/d3 (Fig. 4, A). The invasive capacities of transiently transfected CL1-5 cells expressing the full-length CTGF (CTGF/wt) or its deletion mutants CTGF/d3, CTGF/d2, or CTGF/d1 were determined. CL1-5 cells expressing CTGF/d3, which lacks the CT module, had a high invasive capacity that was equivalent to that of CL1-5 cells expressing the control vector. The mean numbers of invasive CL1-5 cells transfected with CTGF/wt and CTGF/d3, a deletion of the CT module, were 148 cells and 385 cells (difference = 237 cells, 95% CI = 208 to 266 cells; P < 0.001), respectively (Fig. 4, B). CL1-5 cells transfected with CTGF/d2 or CTGF/d1 also had a relatively high invasive capacity compared with cells transfected with CTGF/wt. Western blot analysis showed statistically significantly higher expression of CRMP-1 protein in CTGF/wt-transfected CL1-5 cells than in CTGF/d3-, CTGF/d2-, or CTGF/d1-transfected CL1-5 cells, which was either very low or undetectable (Fig. 4, C). Thus, the CT module appears to be essential for CTGF-induced inhibition of tumor invasion and metastasis.

Association of CTGF Protein Expression With Lung Adenocarcinoma Tissue Stage, Tumor Status, Lymph Node Status, and Survival of Patients With Lung Cancer

To investigate the involvement of CTGF in the progression of human lung adenocarcinoma, normal and tumor specimens from 78 patients with lung adenocarcinoma were analyzed immunohistochemically for the expression of CTGF. Expression of CTGF protein was high (intensity level 3) in the normal lung epithelium (Fig. 5, A) and moderate to high (intensity levels 2 and 3) in stage I lung adenocarcinoma cells (Fig. 5, B). In these tumors, the protein was predominately localized to the cytoplasm. Expression of CTGF was reduced (intensity level 1) in low-grade metastatic epithelial tumor cells (Fig. 5, C). CTGF was not detected in adenocarcinoma cells but was detected in the normal fibroblast and epithelial components of the same field, which were used as the corresponding positive staining control (Fig. 5, D).

We next determined whether the level of CTGF protein was related to any prognostic factors for lung adenocarcinoma (Table 2). When the patients with lung adenocarcinoma were divided into two groups, those with tumors expressing low levels of CTGF (levels 0 and 1) and those with tumors expressing high levels of CTGF (levels 2 and 3), the low expression of CTGF was found to be statistically significantly associated with higher grade lymph node metastasis (N1-3 versus N0, P = 0.014), larger tumor size (T2-4 versus T1, P = 0.031), and advanced-stage disease (stage III and IV versus stage I and II, P = 0.001). The median time to postoperative disease recurrence was longer for patients whose tumors expressed a high level of CTGF (28.8 months, 95% CI = 8.0 to 49.6 months) than for patients whose tumors expressed a low level of CTGF (10.0 months, 95% CI = 3.3 to 16.7 months) (two-sided log-rank test, P = .07; Fig. 6, A). The 5-year disease-free survival rate was 35% (95% CI = 15% to 54%) in the high expression group and 22% (95% CI = 10% to 33%) in the low expression group. In addition, the median survival time was longer for patients whose tumors expressed a high level of CTGF (66.7 months, 95% CI = 47.9 to 85.6 months) than for patients whose tumors expressed a lower level.
of CTGF (18.2 months, 95% CI = 5.2 to 31.1 months) (difference = 48.5 months, 95% CI = 33.5 to 63.5 months; two-sided log-rank test, \( P = .02 \), Fig. 6, B). The 5-year overall survival rate was 54% (95% CI = 34% to 74%) in the high expression group and 26% (95% CI = 14% to 38%) in the low expression group. Thus, the expression of CTGF was statistically significantly associated with lymph node metastasis and overall survival.

**DISCUSSION**

Only a limited number of metastasis suppressor genes have been identified to date, and mechanistic details for most of these genes are largely unknown. In this article, we have demonstrated, to our knowledge for the first time, that CTGF has novel anti-invasive and anti-metastatic activities on human lung adenocarcinoma. We showed that CTGF expression was associated with the suppression of human lung cancer cell metastasis in a mouse model and that reduced CTGF expression was also associated with clinical metastasis and patient survival. Furthermore, we showed that a metastasis suppressor gene, CRMP-1, was functionally involved in the CTGF-mediated invasion and metastasis inhibition of human lung adenocarcinoma. Our results not only delineate a functional linkage between two metastasis suppressors, CTGF and CRMP-1, in lung adenocarcinoma but also provide insights into the mechanisms of CTGF action.
noma but also provide additional information about the mechanistic process of tumor metastasis of this cancer. We have demonstrated that the level of CTGF protein was statistically significantly higher in normal lung type I and II epithelial cells than in the majority of metastatic adenocarcinoma specimens, suggesting that the level of CTGF protein decreases during clinical disease when cells acquire the ability to grow at a metastatic site. This type of an effect has also been observed with other members of the CCN family in different types of cancer. For instance, expression of Cyr61 mRNA is higher in breast cancers but is lower in hepatoma (46), prostate cancer (47), and NSCLC (48) than their corresponding normal tissues. Although overexpression of Cyr61 in breast cancer cells increased tumor growth (49), overexpression of Cyr61 in lung cancer cells decreased tumor growth in mice (37). Expression of human Wisp-1 (CCN4) increased tumorigenesis in normal rat kidney fibroblasts (50) but decreased tumorigenesis and metastasis in melanoma cells (39) and lung cancer cells (38). Thus, the relationship between these proteins and cancer progression cannot be generalized across different types of cancers.

CTGF-mediated increase in CRMP-1 expression was abolished by treatment with antibodies that specifically block the function of integrins α5β1 and α5β3. In addition, antisense CRMP-1 oligonucleotides essentially abolished the CTGF-mediated inhibition of cell invasion. These data indicate that CRMP-1 acts downstream of CTGF and is regulated by an integrin-related signaling pathway. CRMP-1 is a member of the CRMP family, whose members are involved in axonal guidance and neuronal differentiation (51). CRMPs can function as inhibitors of axon extension during development and can decrease cell motility by the depolymerization of filamentous actin in filopodia. The level of CRMP-1 protein was much lower in more invasive CL1-5 cells than in less invasive CL1-0 cells (41). Overexpression of CRMP-1 in CL1-5 cells decreased the invasiveness of CL1-5 cells. These CRMP-1-overexpressing cells displayed a rounded and less invasive epithelial morphology with fewer filopodia containing filamentous actin (41). CTGF-transfected CL1-5 cells had all the typical rounded epithelial cell morphology with less filamentous actin, but CL1-5 cells had a flat, spindle- and fibroblast-like morphology (data not shown). Hall et al. (52) and Leung et al. (53) showed that CRMP-2 can be regulated by Rho kinase and Rho GTPase and can, in turn, promote neurite outgrowth and dynamic cell shape changes. In addition, small G proteins are involved in the integrin-mediated modulation of cell migration and invasion (54). These findings lead us to suspect that CTGF affects CRMP-1 expression by modulating the activity of integrin-coupled small G proteins. Indeed, our preliminary data indicate that RhoA activity is lower in CTGF-transfected CL1-5 cells than in vector control CL1-5/neo cells (data not shown). Our study has identified a critical role of CRMP-1 in CTGF-mediated tumor invasion and metastasis and also identified the regulatory mechanism of the metastasis suppressor gene CRMP-1. Details of the CTGF-induced signaling pathway leading to the increased expression of the CRMP-1 gene are currently under investigation.

Integrins are important receptors for CCN proteins, and receptor activation may produce a variety of effects. CTGF protein can bind directly to integrins α5β1 and α5β3 (24,55). Interaction of CTGF with integrin α5β3 promotes endothelial cell adhesion, migration, and survival and also induces angiogenesis in vivo (24). CTGF also stimulates human skin fibroblast migration and proliferation through integrin α5β1 (56). In contrast, we show that both α5β1 and α5β3 integrins were required for the inhibition of CTGF-mediated invasion. Our findings are supported by those of Soon et al. (38) that Wisp-1 (CCN4) inhibits the invasion of lung cancer cells through a pathway dependent on integrins α5β1 and α1-dependent pathway. It is puzzling that interaction of CCN proteins with the same cell surface integrins exerts contrasting biologic outcomes in different cell types. CCN proteins interact with a wide variety of cell surface glycoproteins, such as heparan sulfate proteoglycan (57), decorin (58,59), and biglycan (59), which might serve as co-receptors to modulate the signaling and function of integrins and in turn engage diverse biologic effects. In addition, CTGF protein forms complexes with various growth factors, such as vascular endothelial growth factor (60), bone morphogenetic proteins, and transforming growth factor β (61). CTGF could alter biologic functions by the selective interactions with possible co-receptors of integrins α5β1 or α5β3 or other soluble factors. Investigations are underway to identify additional factors that could interact with CTGF in human lung adenocarcinoma cells.

Members of the CCN family have high structural homology (62), sharing four conserved cysteine-rich modular domains with sequence similarity to insulin-like growth factor–binding protein, von Willebrand factor, thrombospondin, and growth factor cysteine knots (CT module). To determine which domain of CTGF is responsible for invasion inhibition, we established a series of constructs by sequential deletion of sequences at the CT module of CTGF. Cells transfected with and expressing CTGF constructs lacking the CT module could not induce increased CRMP-1 expression or inhibit cell invasion (data not shown). The CT module may contribute a dimerization motif and, in turn, bind with cell surface integrins, heparan sulfate proteoglycan, or Notch receptor (63,64), suggesting that the CT module may play a role in CTGF-mediated outside-in signaling. Interestingly, the CT module is also essential in mitosis and increases adhesive properties of fibroblasts and endothelial cells (65,66).

In summary, CTGF appears to be a suppressor of lung tumor invasion and metastasis. Our studies directly demonstrated that overexpression of CTGF not only suppressed the ability of lung adenocarcinoma cells to invade Matrigel in vitro but also strongly inhibited tumor metastasis in an animal model. At the mechanistic level, we found that CRMP-1 acts downstream of CTGF and that its regulation is mediated by integrins α5β1 and α5β3. Decreased CTGF expression in tumor tissues was associated with advanced tumor stage, lymph node metastasis, early postoperative relapse, and shorter patient survival. We document a functional linkage between two metastasis suppressors, CTGF and CRMP-1, in the same tumor.

REFERENCES


Note

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