Connective tissue growth factor inhibits gastric cancer peritoneal metastasis by blocking integrin $\alpha 3\beta 1$ -dependent adhesion

Chiung-Nien Chen · Cheng-Chi Chang · Hong-Shiee Lai ·
Yung-Ming Jeng · Chia-I Chen · King-Jeng Chang ·
Po-Huang Lee · Hsinyu Lee

Received: 19 March 2014 / Accepted: 11 June 2014 / Published online: 2 July 2014
© The International Gastric Cancer Association and The Japanese Gastric Cancer Association 2014

Abstract

Background Connective tissue growth factor (CTGF) plays important roles in normal and pathological conditions. The aim of this study was to investigate the role of CTGF in peritoneal metastasis as well as the underlying mechanism in gastric cancer progression.

Methods CTGF expression levels for wild-type and stable overexpression clones were determined by Western blotting and quantitative polymerase chain reaction (Q-PCR). Univariate and multivariate analyses, immunohistochemistry, and survival probability analyses were performed on gastric cancer patients. The extracellular matrix components

involved in CTGF-regulated adhesion were determined. Recombinant CTGF was added to cells or coinoculated with gastric cancer cells into mice to evaluate its therapeutic potential.

Results CTGF overexpression and treatment with the recombinant protein significantly inhibited cell adhesion. In vivo peritoneal metastasis demonstrated that CTGF-stable transfectants markedly decreased the number and size of tumor nodules in the mesentery. Statistical analysis of gastric cancer patient data showed that patients expressing higher CTGF levels had earlier TNM staging and a higher survival probability after the surgery. Integrin $\alpha 3\beta 1$ was the cell adhesion molecule mediating gastric cancer cell adhesion to laminin, and blocking of integrin $\alpha 3\beta 1$ prevented gastric cancer cell adhesion to recombinant CTGF. Coimmunoprecipitation results indicated that CTGF binds to integrin $\alpha 3$. Coinoculation of recombinant CTGF and gastric cancer cell lines in mice showed effective inhibition of peritoneal dissemination.

Conclusions Our results suggested that gastric cancer peritoneal metastasis is mediated through integrin $\alpha 3\beta 1$ binding to laminin, and CTGF effectively blocks the interaction by binding to integrin $\alpha 3\beta 1$, thus demonstrating the therapeutic potential of recombinant CTGF in gastric cancer patients.

Keywords CTGF · Peritoneal dissemination · Gastric cancer · Integrin · Adhesion

C.-N. Chen (✉) · H.-S. Lai · C.-I. Chen · P.-H. Lee
Department of Surgery, National Taiwan University Hospital,
Taipei, Taiwan
e-mail: cnchen@ntu.edu.tw

C.-N. Chen · C.-C. Chang · C.-I. Chen · K.-J. Chang ·
H. Lee (✉)
Angiogenesis Research Center, National Taiwan University,
Taipei, Taiwan
e-mail: hsinyu@ntu.edu.tw

C.-C. Chang
Graduate Institute of Oral Biology, School of Dentistry, College
of Medicine, National Taiwan University, Taipei, Taiwan

C.-C. Chang
Department of Dentistry, National Taiwan University, Taipei,
Taiwan

Y.-M. Jeng
Department of Pathology, National Taiwan University Hospital,
Taipei, Taiwan

H. Lee
Department of Life Science, National Taiwan University, Taipei,
Taiwan

one of the noncurative factors in gastric cancer, and many efforts have been made to prevent this condition with limited success [2]. Extensive metastasis of the lymph nodes and liver is an important factor in the poor prognosis of gastric cancer. In addition, peritoneal dissemination has been considered a major contributing factor through the direct spillage of cancer cells during surgery [3, 4]. Development of peritoneal metastasis is a multistep process that begins with the detachment of cancer cells from the primary tumor, progressing to the attachment of free tumor cells to peritoneal mesothelial cells, the retraction of mesothelial cells to expose the basement membrane, attachment to the basement membrane, degradation in the extracellular matrix, proliferation of cancer cells, and, finally, angiogenesis [5]. Since peritoneal metastasis is one of the most common causes of noncurative surgery in gastric cancer therapy, prevention of cancer cell adhesion to the peritoneum is an important treatment strategy. Therefore, developing a new therapeutic method for this mode of metastasis is desirable.

We have previously demonstrated that calreticulin (CRT), a multifunctional protein that plays an important role in calcium regulation in the endoplasmic reticulum [6], is a prognostic marker in gastric cancer that contributes to angiogenesis, lymph node metastasis, and survival in human gastric cancer [7]. Our microarray analysis data demonstrated that the CRT expression level is inversely correlated with the expression level of an important growth factor [i.e., connective tissue growth factor (CTGF)]. CTGF has been found to play a diverse role in a variety of cancers and is known to be an important regulator for cell adhesion. Therefore, we hypothesize that CTGF may play an important role in the progression and prognosis of gastric cancer.

CTGF is a secretory protein first identified in 1991 [8] and belongs to the CCN family [cysteine-rich 61, CTGF, and nephroblastoma-overexpressed gene (NOV)]. It is a multifunctional growth factor involved in wound healing, inflammation, cell adhesion, chemotaxis, apoptosis, tumor growth, and fibrosis [9]. Elevated CTGF expression has been detected in various tumors [10–13]. Additionally, CTGF can promote angiogenesis by regulating endothelial cell growth, migration, adhesion, and survival [14, 15]. However, recent studies have shown that overexpression of CTGF in human oral squamous cell carcinoma (OSCC) reduces cell growth and invasion [16, 17]. CTGF has also been reported to be a key regulator of colorectal cancer invasion and metastasis. Moreover, it appears to be a good prognostic factor [18], and recombinant CTGF was suggested to be a potential therapeutic agent in colorectal cancer therapy [19]. However, the underlying mechanisms and functions of CTGF in gastric cancer have not yet been clarified.

In this study, we assessed the clinical significance of CTGF expression in gastric cancer patients by using immunohistochemistry, determined the role of CTGF in the adhesive ability of gastric cancer cells (in vitro and in vivo), and delineated the molecular mechanism of peritoneal dissemination and how CTGF affects the capacity of peritoneal metastasis in severe combined immunodeficient (SCID) mice. Our results suggest a potential therapeutic use for recombinant CTGF in gastric cancer therapy.

Materials and methods

Patients

Written informed consent was obtained from all patients, and this study was monitored and approved by the institutional review board of the research ethics committee at National Taiwan University Hospital (approval no. 200906054R). For CTGF expression profiling and survival probability analysis, a total of 107 patients with gastric cancer who had undergone radical gastrectomy at the National Taiwan University Hospital from January 1995 to September 2004 were included in this study. They included 70 males and 37 females with an average age of 63.8 years and staged according to the TNM system based on postoperative pathological reports. The criteria for consideration as a curative resection included the complete removal of a primary gastric tumor, D2 dissection of regional lymph nodes, and absence of any residual macroscopic tumors. No other previous or concomitant primary cancers were present. The patients had not received chemotherapy or radiotherapy before the surgery. Clinicopathologic factors, including age, sex, gross tumor type (Borrmann classification), histologic tumor type (Lauren classification), depth of tumor invasion, status of lymph node metastasis, vascular invasion, and *Helicobacter pylori* infection documented with histological findings were reviewed and stored in the patient database. Vascular invasion was considered to be definite only when tumor cells and red blood cells were noted together in an endothelium-lined vascular space or when tumor cells were found in an endothelium-lined vascular space with a definite smooth muscle layer. Tissues were considered positive for *H. pylori* if faintly blue-staining curved bacilli were seen in the mucus of crypts adjacent to the tumor by hematoxylin and eosin staining. Peritoneal metastasis was defined as metachronous peritoneal recurrence, which can be detected by radiological and/or reoperative findings. The patients were subjected to a follow-up 6–143 months after surgery. The follow-up intervals were calculated as survival intervals after surgery.

Immunohistochemical staining of CTGF

The CTGF protein was detected on formalin-fixed, paraffin-embedded sections by using the labeled streptavidin-biotin (LSAB) method after antigen retrieval according to the manufacturer's instructions (Dako Corp., Carpinteria, CA). The results of the immunohistological staining were classified using the extent of the cell stained [i.e., level 0 (negative staining), level 1 (< 5 % of tumor cells stained), level 2 (< 50 % of tumor cells stained), and level 3 (> 50 % of tumor cells stained)]. Levels 0 and 1 were grouped as low CTGF expression levels, and levels 2 and 3 were grouped as high CTGF expression levels.

Cell culture and stable clone selection

For CTGF expression in wild-type gastric cancer cells, the human gastric cancer cell lines MKN45, N87, and AGS were grown in RPMI 1640 medium (Biological Industries, Israel) supplemented with 10 % fetal bovine serum and a penicillin/streptomycin/amphotericin B antibiotic cocktail (Biological Industries, Israel) in a humidified atmosphere of 5 % CO₂ at 37 °C. For lentivirus packaging and viral titer determination, 293T and A514 cells were maintained under the same conditions as the MKN45 and AGS cell lines.

The pCDNA3/CTGF plasmid was provided by Prof. M.-L. Kuo's laboratory at the Institute of Toxicology, College of Medicine, National Taiwan University. The stable transfectant clones were selected according to a previous study [20] CTGF stable knockdown clones were generated by the cotransfection of the packaging plasmids pCMVΔR8.91, pMD.G, and pLKO.1-shCTGF (RNAi Core, Academia Sinica, Taipei, Taiwan) into the 293T cell line using Lipofectamine 2000 according to the manufacturer's instructions. The virus-containing medium was harvested at 24 and 48 h post transfection of packaging plasmids. AGS cells were grown to confluence and infected using a lentivirus-containing medium and polybrene. Stable transfectants were selected in puromycin at a concentration of 8 μg/ml. Thereafter, the medium was replaced every 3 days. Two weeks after selection, puromycin-resistant clones were maintained in 4 μg/ml puromycin. Knockdown efficiency was assessed with Western blot analysis.

Western blot analysis

For CTGF protein expression levels in gastric cancer cell lines, 30 μg of the total cell lysate was separated on a 10 % SDS-PAGE gel and electroblotted onto a PVDF membrane (Millipore, USA). The levels of the CTGF protein were analyzed via Western blot analysis using an anti-CTGF

(GTX124232, Genetex, Taiwan) at a concentration of 1:1000, a horseradish peroxidase [labeled as a secondary antibody (Chemicon, USA)] at a concentration of 1:2500, and ECL as the chemiluminescent detection reagent (Millipore, USA). The gel was exposed to an X-ray film (Kodak), and the film was developed using an automated developer (Kodak X-OMAT 1000 processor).

In vitro adhesion assay

To determine the in vitro adhesive ability of wild-type gastric cancer cell lines as well as CTGF overexpressed and knockdown cells, Matrigel (BD bioscience, USA) was coated onto 96-well cell culture plates, and an in vitro adhesion assay was carried out as described by Lin et al. [19].

To determine the therapeutic potential of the recombinant CTGF protein (rCTGF), 50, 100, and 200 ng of recombinant CTGF (Peprotech) were added to 1×10^6 cells, and the cells were harvested at 6 h post-rCTGF treatment. Harvested cells were subjected to an in vitro adhesion assay using Matrigel, as described above. Dose-dependent rCTGF treated cells were counted under a phase contrast microscope to calculate the number of surviving cells 6 h post-rCTGF treatment. Each treatment was done in triplicate, and the *p* value was determined using Student's *t* test. All experiments were performed at least three times.

To confirm the interaction between CTGF and cell surface integrin, 1×10^4 MKN45 cells were treated with 0.5 μg/μl of the anti-integrin α3 subunit antibody and incubated at 37 °C for 1 h. Cells were then subjected to an adhesion assay using rCTGF-coated 96-well culture plates coated with 200 ng rCTGF (Genetex, Taiwan) and analyzed as described above.

In vitro adhesion assay of differing ECMs and antibody blocking against integrin subunits

To determine the extracellular matrix target involved in the adhesion of gastric cancer cells, collagen type I-, fibronectin-, and laminin beta 1-coated plates were used (Millipore, USA). In total, 5×10^3 cells were seeded in each ECM substratum, incubated at 37 °C for 1 h followed by fixing in 4 % paraformaldehyde, and stained with 0.05 % crystal violet. Cells were washed and lysed for determination of optical density at 540 nm. All experiments were performed in triplicate at least three times. To determine the integrin subunits that are involved in the adhesion of gastric cancer cells to laminin beta 1, integrin blocking antibodies were obtained from Millipore, USA. In total, 5×10^3 cells were treated by blocking antibodies at 0.5 μg/μl and incubated at 37 °C for 1 h followed by the

above-described adhesion assay. IgG was used as the negative control in all antibody-blocking experiments.

In vivo peritoneum metastasis assay of CTGF overexpressed and knockdown gastric cancer cell lines

To confirm the in vitro adhesion assay results, in vivo peritoneal metastasis was carried out using a mouse model. CB17/Icr-Prkdc^{scid}/CrI SCID (severe combined immunodeficient) mice were purchased from BioLASCO Taiwan Co., Ltd., housed in microisolator cages, and fed autoclaved water and chow ad libitum. All animal experiments were carried out in the animal facilities of the National Taiwan University Hospital, and they adhered to the guidelines approved by the Institutional Animal Care and Use Committee (IACUC) of the National Taiwan University Medical College, Center of Experimental Animals. In total, 2×10^6 MKN45/Neo or MKN45/CTGF cells were injected intraperitoneally. Mice were killed and dissected at 45 days after cancer cell inoculation or when moribund. The number of nodules was counted and analyzed.

Coimmunoprecipitation of integrin $\alpha 3$ and CTGF

To further determine the direct interaction of CTGF and integrin $\alpha 3$, coimmunoprecipitation was carried out using the Catch and Release Immunoprecipitation System (Millipore, USA) according to the manufacturer's instructions. Briefly, 500 μ g of the total cell lysate of MKN45/CTGF cells was added to the resin column in addition to 5 μ g of the anti-integrin $\alpha 3$ subunit monoclonal antibody (Millipore, USA). The reaction was incubated at 4 °C overnight on a rotating wheel. The resin was washed and elution carried out to obtain the immunoprecipitated products. The immunoprecipitated products were subjected to Western blot analysis using the anti-CTGF polyclonal antibody and IgG as control (catalog no. GTX124232, Genetex, Taiwan) following the above-mentioned procedures.

Statistical analysis

Experimental data were statistically analyzed using Student's *t* test. Each result was obtained from at least three independent experiments, and a value of $p < 0.05$ was considered statistically significant. Fisher's exact probability test and Chi square test were used for the statistical analysis of the expression of CTGF with traditional clinical outcome. Survival was calculated using the Kaplan–Meier method, and differences were analyzed by the log-rank test. A multivariate survival analysis was performed using the Cox proportional hazards model to investigate the independent prognostic factors. Statistical significance was defined as $p < 0.05$.

Results

CTGF expression profiling and clinical correlation by immunohistochemical staining of gastric cancer patient tissues

To investigate the localization of CTGF expression in gastric cancer tissues, CTGF protein expression in normal and tumor specimens was stained immunohistochemically. CTGF protein expression was high in normal gastric epithelium and moderate in diffuse- or intestinal-type gastric adenocarcinoma tissues (Fig. 1a–c). In tumor tissues, the CTGF protein was predominantly localized in the cytoplasm. Analyzing tissue samples from 107 patients who underwent gastrectomy for gastric cancer, the CTGF proteins were positively stained in 64 % (69/107) of the gastric cancer specimens.

The correlation between the expression of CTGF and clinicopathological factors is shown in Table 1. There was no significant statistical relationship between CTGF expression and age, sex, serosal invasion, lymph node metastasis, vascular invasion, Borrmann type, or Lauren classification. However, a significant correlation was found between CTGF expression and peritoneal metastasis ($p < 0.001$) and stage ($p = 0.037$) by univariate analysis. Multivariate analyses showed that only CTGF was significantly correlated with peritoneal metastasis ($p = 0.002$) (Table 2).

The survival probability was calculated using the Kaplan–Meier survival and Cox-regression probability test. Patients who expressed serosal invasion ($p = 0.038$), lymph node metastasis ($p = 0.006$), advanced Borrmann type ($p = 0.023$) and Lauren classification ($p = 0.025$) had lower survival rates in the postoperative follow-up (Table 3). In contrast, only higher levels of CTGF were found to have a higher survival probability postsurgery ($p = 0.0014$) (Fig. 1d).

This strongly suggests that CTGF expression is correlated with the survival of gastric cancer patients and that it may act as a prognostic marker for patients who have undergone gastrectomy. The data also reveal a correlation between two factors (i.e., peritoneal dissemination and survival probability) and CTGF expression levels. This is an indication that CTGF levels had an effect on peritoneal dissemination. Patients with high CTGF expression had a better prognosis than those with lower expression levels.

CTGF expression and adhesive ability

In our previous study, CTGF expression was inversely correlated with calreticulin levels [7]. Therefore, CTGF expression and the in vitro adhesive ability of three different gastric cancer cell lines were determined by Western blot analysis and RT-PCR. AGS, N87, and MKN45 were found to

Fig. 1 Immunohistochemical staining of CTGF protein expression in human normal gastric tissues and gastric cancer tissues and Kaplan-Meier survival probability of CTGF-positive and -negative patients. Higher expression of CTGF was observed in normal gastric tissues, and higher survival probability was observed in CTGF-positive patients. **a** Normal tissue taken from gastric cancer at a distance from

tumor site ($\times 200$ magnification). **b** Paired diffuse-type gastric cancer tissue ($\times 400$ magnification). **c** Paired intestinal type gastric cancer tissue ($\times 400$ magnification). **d** Survival probability of CTGF-positive ($n = 69$) and CTGF-negative ($n = 38$) gastric cancer patients at 144 months postsurgery ($p = 0.0014$)

have different levels of endogenous CTGF expression (Fig. 2a, left panel). N87 had the highest level of CTGF expression, which was followed by AGS and lastly MKN45. The adhesive ability of the three cell lines was inversely correlated with their CTGF expression levels. N87 cells had the lowest adhesive ability, and this was significantly lower than that of the AGS cell lines ($p = 0.0145$) and MKN45 cells ($p = 0.015$). AGS cells also had a significantly lower

adhesive ability than the MKN45 cells ($p = 0.043$) (Fig. 2a, right panel). This result confirms our hypothesis and clinical observations that CTGF expression is a factor that determines the adhesive ability of gastric cancer cells. MKN45 was chosen for CTGF overexpression as it is one of the most commonly used gastric cancer cell lines and has a comparatively low endogenous CTGF level. Full-length CTGF cDNA was transfected into MKN45 cells, and a stable cell

Table 1 Relationship between the expression of CTGF and clinicopathological factors in 107 patients with gastric cancer

	CTGF(−)	CTGF(+)	<i>p</i> Value
Age	63.71 ± 11.91	63.86 ± 12.36	0.059
Sex			1
Males	25	45	
Females	13	24	
Depth of tumor invasion (T)			0.62
T1	5	15	
T2	6	12	
T3	25	37	
T4	2	5	
Nodal status (N)			0.352
Node negative	10	28	
Node positive	28	41	
Gross tumor morphology			0.349
Early cancer	4	14	
Borrmann type I	2	1	
Borrmann type II	5	11	
Borrmann type III	26	38	
Borrmann type IV	1	5	
Lauren classification			0.843
Intestinal type	16	30	
Diffuse type	18	34	
Mixed type	4	5	
Vascular invasion			0.306
Negative	13	32	
Positive	25	37	
Peritoneal metastasis			<0.001
Negative	13	50	
Positive	25	19	
Stages			0.037
I	8	19	
II	3	12	
III	15	31	
IV	12	7	

line was selected using G418. The adhesiveness of the transfected cells was monitored by an in vitro Matrigel adhesion assay. Full length CTGF and control vectors were transfected in MKN45 cells (Fig. 2b, left panel), and the adhesiveness to Matrigel in MKN45/CTGF cells was decreased by 60 % compared to the Neo control clone ($p = 0.022$) (Fig. 2b, right panel). Since MKN45 has lower endogenous CTGF expression, AGS cells were chosen for knockdown experiments because of their comparatively higher endogenous CTGF expression. CTGF was knocked down using the lentivirus system, and adhesion of virus-infected cells was tested (Fig. 2c, left panel). A 30 %

Table 2 Expression of connective tissue growth factor and clinicopathological factors: multivariate analysis

Variable	B	SE	Exp(β)	Sig	Odds ratio
Age	−0.015	0.02	0.54	0.462	0.985
Sex	0.094	0.486	0.038	0.846	1.099
Borrmann type	−0.081	0.305	0.071	0.789	0.922
Lauren classification	0.469	0.412	1.296	0.255	1.598
Serosal invasion	0.487	0.472	1.065	0.302	1.627
Lymph node metastasis	−0.170	0.377	0.202	0.653	0.844
Distant metastasis	−0.720	0.673	1.145	0.285	0.487
Vascular invasion	0.206	0.612	0.114	0.736	1.229
Peritoneal metastasis	−2.01	0.640	9.865	0.002	0.134

B β regression coefficient, *SE* standard error, *Exp(β)* exponent β , *Sig* significance (*p* value)

Table 3 Clinicopathological factors affecting survival rate: multivariate analysis

Variable	B	SE	Exp(β)	Sig	Odds ratio
Age	−0.002	0.013	0.032	0.859	0.998
Sex	0.056	0.303	0.034	0.854	1.058
Borrmann types	0.659	0.290	5.143	0.023	1.932
Lauren classification	0.561	0.251	5.009	0.025	1.752
Serosal invasion	1.021	0.493	4.290	0.038	2.777
Lymph node metastasis	1.467	0.535	7.519	0.006	4.336
Vascular invasion	0.818	0.431	3.603	0.058	2.265
CTGF	−0.720	0.288	6.244	0.012	0.487

B β regression coefficient, *SE* standard error, *Exp(β)* exponent β , *Sig* significance (*p* value)

increase in adhesion was observed in the CTGF knockdown AGS cell line (Fig. 2c, right panel). To determine whether the adhesive ability was affected in vivo, we inoculated SCID mice with CTGF-overexpressed MKN45 and its control cell lines intraperitoneally. There was a significant decrease in peritoneal nodules in MKN45/CTGF inoculated mice (Fig. 2d, e) ($p = 0.000077$) when compared to MKN45/Neo inoculated mice. For the peritoneal metastasis animal model, the data of overexpressed CTGF in MKN45 cells was well correlated with the in vitro data, which suggested that CTGF modulates gastric cancer cell adhesion to further support the potential therapeutic role of CTGF in advanced gastric cancer.

CTGF inhibits gastric cancer cell peritoneal dissemination by blocking the interaction of integrin $\alpha 3 \beta 1$ to laminin

To investigate the molecular mechanism of CTGF-induced inhibition of gastric cancer cells to the

Fig. 2 Adhesion of gastric cancer cells in vitro and in vivo is affected by the CTGF expression level and recombinant CTGF blocks cell adhesion via blocking integrin $\alpha 3 \beta 1$ in vitro. **a** Wild-type AGS, N87, and MKN45 gastric cancer cells. Higher CTGF mRNA and protein levels of AGS and N87 cells significantly reduced adhesion in vitro, while lower CTGF mRNA and protein of MKN45 have significantly higher adhesion in vitro ($p = 0.043$ for AGS vs. MKN45; $p = 0.015$ for N87 vs. MKN45). **b** CTGF overexpression in MKN45 gastric cancer cells significantly inhibited adhesion in vitro compared to pCDNA3 control cells ($p < 0.05$). **c** Lentivirus CTGF knockdown in AGS gastric cancer cells elevated adhesion in vitro ($p < 0.05$). **d** SCID mice IP inoculated with MKN45/neo or MKN45/CTGF cells. In vivo tumorigenicity has shown that the number of mesenteric tumor nodules is significantly decreased in the MKN45/CTGF

inoculated group ($p < 0.05$). **e** SCID mice inoculated with MKN45/Neo or MKN45/CTGF. The number of peritoneal tumor nodules was significantly reduced in the MKN45/CTGF group. **f** CTGF overexpression significantly inhibited MKN45 cells to fibronectin and laminin substratum in vitro compared to wild-type MKN45 gastric cancer cells. **g** Integrin $\alpha 3$ and $\beta 1$ blocking antibodies significantly inhibited MKN45 gastric cancer cell adhesion to laminin substratum. **h** Integrin $\alpha 3$ subunit blocking antibody inhibits CTGF knockdown AGS gastric cancer cell adhesion to laminin. **i** MKN45 cells adhere significantly to the recombinant CTGF-coated plate, and adhesion is inhibited by integrin $\alpha 3$ blocking antibody. **j** Coimmunoprecipitation using integrin $\alpha 3$ antibody as IP antibody followed by detection using CTGF antibody showed that CTGF and integrin $\alpha 3$ bind to each other in vitro

peritoneum, we first screened the major extracellular matrix component present in Matrigel to determine the possible adhesion substratum. Adhesion of CTGF overexpressed MKN45 cells to collagen type I, fibronectin, and laminin was examined, and CTGF significantly inhibited adhesion in fibronectin- and laminin-coated culture plates (Fig. 2f). This is consistent with previous reports that the adhesion of gastric cancer cells is mediated through the binding of integrin $\alpha 3 \beta 1$ to laminin [21, 22]. We therefore chose laminin beta 1-coated plates to

investigate the role of CTGF in substrate adhesion. Next, we determined the integrin subunits involved in CTGF-mediated adhesion inhibition in MKN45 gastric cancer cells by using integrin subunit blocking antibodies. We observed a significant inhibition of MKN45 cell adhesion to laminin-coated cell culture plates by antibodies against the integrin $\alpha 3$ and $\beta 1$ subunits (Fig. 2g). To further confirm adhesion inhibition, CTGF knocked-down AGS gastric cancer cell lines were treated with antibodies against the integrin $\alpha 3$ subunit, and the adhesive ability

Fig. 2 continued

Fig. 2 continued

was measured. The results showed a significant inhibition of adhesion to laminin-coated plates when CTGF-knocked down cells were treated with the integrin $\alpha 3$ blocking antibody (Fig. 2h). In order to confirm the interaction between CTGF and the integrin $\alpha 3$ subunit, a cell culture plate was coated with a fragment of recombinant CTGF. MKN45 cells that had higher adhesive ability were blocked by the antibody against the integrin $\alpha 3$ subunit, and significant inhibition of adhesion to the rCTGF-coated plate was observed (Fig. 2i). Previous studies have shown that the integrin $\alpha 3$ subunit is involved in the recognition of laminin-5 [23]. Therefore, we speculate that it is also involved in the recognition of CTGF. To confirm the interaction between integrin $\alpha 3$ and CTGF, a coimmunoprecipitation experiment was carried out using an anti-integrin $\alpha 3$ antibody to immunoprecipitate the cell lysate; subsequently, CTGF was detected by Western blot analysis. The results showed that CTGF was detected in anti-integrin $\alpha 3$ pulled-down lysates (Fig. 2j). These results suggested that adhesion inhibition by CTGF expression was through direct binding to integrin $\alpha 3\beta 1$.

CTGF is a potential therapeutic target for the inhibition of peritoneal dissemination

Previous studies have shown that the CT domain of the CTGF protein binds to a variety of members from the integrin family in different cell types [24–27]. In order to assess the potential therapeutic effect of recombinant CTGF, in vitro adhesion and in vivo peritoneal metastasis of MKN45 cells were tested in the presence of the CT domain of recombinant CTGF. In the in vitro adhesion assay, dose-dependent adhesion inhibition was observed. At 6 h post treatment, 50 % adhesion inhibition was observed at 50 ng of recombinant CTGF treatment; 70 and 75 % inhibition for 100 and 200 ng of recombinant CTGF was also observed, respectively (Fig. 3a). To determine whether recombinant CTGF caused cytotoxicity, cell density was determined, and no significant change in the number of cells was observed when compared to the control at 6 h post treatment (Fig. 3b). To further determine whether recombinant CTGF inhibits adhesion in an animal model, we co-inoculated MKN45 cells and recombinant CTGF intraperitoneally and monitored peritoneal metastasis in SCID

Fig. 3 Recombinant CTGF protein effectively blocks gastric cancer cell adhesion in vitro and in vivo as well as increases survival probability in vivo. **a** The 0-, 50-, 100-, and 200-ng/ml recombinant CTGF-treated cells showed decreased adhesive ability at 6 h post treatment in vitro. ($p < 0.01$) **b** Recombinant CTGF treatment does

not cause cell death at 0, 50, 100 and 200 ng/ml at 6 h post treatment. **c** Recombinant CTGF treatment in mice showed a decreased number of tumor nodules on the mesentery compared to nontreated mice. **d** Recombinant CTGF-treated mice have significantly higher survival probability than nontreated mice. ($p = 0.0056$). NS = no significance

mice. The survival probability at 45 days post inoculation was determined. We observed that the buffer control group had more tumor nodules when compared to the recombinant CTGF-treated group (Fig. 3c) and that the buffer control group had a significantly lower survival probability at 45 days post inoculation ($p = 0.0056$) (Fig. 3d). In vitro and in vivo data suggest that recombinant CTGF may be potential therapeutic targets for preventing postsurgical peritoneal dissemination in gastric cancer therapy.

Discussion

Peritoneal dissemination has long been an obstacle for the treatment of gastrointestinal cancers. Our study has successfully addressed the hypothesis that CTGF prevents

peritoneal dissemination both in vitro and in vivo in gastric cancer. In addition, we also identified the molecule involved in gastric cancer cell adhesion to the peritoneum. We have demonstrated that overexpression of CTGF effectively inhibited gastric cancer cell adhesion to the peritoneum that resulted in peritoneal metastasis blockage. Moreover, CTGF expression levels were associated with patient survival. This is the first study indicating that CTGF is an antiadhesion protein in peritoneal metastasis for gastric cancer, and we suggest a therapeutic use for recombinant CTGF in the late stage of this disease.

Previous studies in abdominal cancers, such as colorectal cancer, have demonstrated a negative correlation between CTGF expression and cancer progression (survival as well as metastasis) [18, 19]. These results are consistent with our findings that CTGF expression is

negatively correlated with gastric cancer, which is also an abdominal-origin cancer. Our in vivo results indicate that recombinant CTGF treatment is an effective prevention strategy for peritoneal dissemination as well as an independent prognostic marker for gastric cancer patients. Although previous studies have shown that blocking CTGF using antibodies has shown some promising results in inhibiting pancreatic tumor growth and metastasis in nude mice [28], it was found that the CTGF recombinant protein had a significantly therapeutic effect in advanced colorectal cancer [19]. However, previous studies have reported that CTGF may contribute positively to gastric cancer progression and attenuate adhesion of gastric cancer cells to cultured human peritoneal mesothelial cells [29–31]. The cells used were unavailable for further testing, and the differences in patient survival may due to genetic background variations between patient groups. Taken together, these results indicate that CTGF may have different roles in different cancers.

Integrins are cell-surface glycoproteins composed of heterodimers of α and β subunits linked by noncovalent bonds. They have been implicated in the development of different types of cancer [32, 33]. In our study, we first demonstrated that CTGF is a ligand that binds to integrin to prevent cell adhesion in gastric cancer peritoneal metastasis; therefore, it may act as an autocrine factor capable of regulating cell adhesion to different substrates.

Many integrin inhibitors have been identified and are currently being actively investigated in clinical trials for cancer diagnosis and therapy [34, 35]. However, integrin blocking strategies have only been used with limited success in animal models. A radiolabeled high-affinity peptidomimetic antagonist that selectively targets $\alpha_v\beta_3$ has been developed and tested in a mouse model for the molecular imaging of tumor-induced angiogenic vessels [36]. An anti-integrin gastric cancer therapy has not been reported to date. Therefore, our results may contribute to the first anti-integrin gastric cancer therapy that may effectively block cancer cell adhesion to the peritoneum and thus prevent peritoneal metastasis.

Despite the identification of the therapeutic potential of recombinant CTGF, many questions remain to be answered. For example, our study has demonstrated that the intraperitoneal coinjection of cancer cells and recombinant CTGF successfully prevented peritoneal dissemination. However, determination of the proper timing, route of administration for recombinant CTGF, and safety of its therapeutic use in peritoneal metastasis of gastric cancer requires further investigation. Therefore, a gastric cancer animal model to further identify the dosage, route of administration, and course of therapy will be useful for future clinical investigations on the role of CTGF for peritoneal metastasis in gastric cancer therapies.

Acknowledgments We would like to thank the Second Core Laboratory at the National Taiwan University Hospital and Angiogenesis Research Center of National Taiwan University for providing technical assistance and laboratory equipment. This research was supported by grants from the National Taiwan University Hospital (95A06), National Science Council (NSC95-2314-B002-151-MY3), and Department of Industrial Technology, Ministry of Economic Affairs (95-EC-17-A-19-S1-016), Taipei, Taiwan.

References

1. Wagner AD, Grothe W, Haerting J, Kleber G, Grothey A, Fleig WE. Chemotherapy in advanced gastric cancer: a systematic review and meta-analysis based on aggregate data. *J Clin Oncol*. 2006;24:2903–9.
2. Gonzalez-Moreno S. Peritoneal dissemination: a pending issue in gastric cancer worth exploring. *Ann Surg Oncol*. 2009;16:3217–8.
3. Maehara Y, Kakeji Y, Takahashi I, Okuyama T, Baba H, Anai H, et al. Noncurative resection for advanced gastric cancer. *J Surg Oncol*. 1992;51:221–5.
4. Baba H, Okuyama T, Hiroyuki O, Anai H, Korenaga D, Maehara Y, et al. Prognostic factors for noncurative gastric cancer: univariate and multivariate analyses. *J Surg Oncol*. 1992;51:104–8.
5. Yonemura Y, Endou Y [Molecular mechanisms of peritoneal dissemination]. *Nippon Shokakibyo Gakkai Zasshi*. 2000;97:680–90.
6. Michalak M, Groenendyk J, Szabo E, Gold LI, Opas M. Calreticulin, a multi-process calcium-buffering chaperone of the endoplasmic reticulum. *Biochem J*. 2009;417:651–66.
7. Chen CN, Chang CC, Su TE, Hsu WM, Jeng YM, Ho MC, et al. Identification of calreticulin as a prognosis marker and angiogenic regulator in human gastric cancer. *Ann Surg Oncol*. 2009;16:524–33.
8. Bradham DM, Igarashi A, Potter RL, Grotendorst GR. Connective tissue growth factor: a cysteine-rich mitogen secreted by human vascular endothelial cells is related to the SRC-induced immediate early gene product CEF-10. *J Cell Biol*. 1991;114:1285–94.
9. Brigstock DR. Regulation of angiogenesis and endothelial cell function by connective tissue growth factor (CTGF) and cysteine-rich 61 (CYR61). *Angiogenesis*. 2002;5:153–65.
10. Wenger C, Ellenrieder V, Alber B, Lacher U, Menke A, Hammeier H, et al. Expression and differential regulation of connective tissue growth factor in pancreatic cancer cells. *Oncogene*. 1999;18:1073–80.
11. Shakunaga T, Ozaki T, Ohara N, Asaumi K, Doi T, Nishida K, et al. Expression of connective tissue growth factor in cartilaginous tumors. *Cancer*. 2000;89:1466–73.
12. Pan LH, Beppu T, Kurose A, Yamauchi K, Sugawara A, Suzuki M, et al. Neoplastic cells and proliferating endothelial cells express connective tissue growth factor (CTGF) in glioblastoma. *Neurol Res*. 2002;24:677–83.
13. Chen PS, Wang MY, Wu SN, Su JL, Hong CC, Chuang SE, et al. CTGF enhances the motility of breast cancer cells via an integrin- $\alpha_v\beta_3$ -ERK1/2-dependent S100A4-upregulated pathway. *J Cell Sci*. 2007;120:2053–65.
14. Brigstock DR. The connective tissue growth factor/cysteine-rich 61/nephroblastoma overexpressed (CCN) family. *Endocr Rev*. 1999;20:189–206.
15. Planque N, Perbal B. A structural approach to the role of CCN (CYR61/CTGF/NOV) proteins in tumorigenesis. *Cancer Cell Int*. 2003;3:15.
16. Moritani NH, Kubota S, Nishida T, Kawaki H, Kondo S, Sugahara T, et al. Suppressive effect of overexpressed connective

- tissue growth factor on tumor cell growth in a human oral squamous cell carcinoma-derived cell line. *Cancer Lett.* 2003;192:205–14.
17. Yang MH, Lin BR, Chang CH, Chen ST, Lin SK, Kuo MY, et al. Connective tissue growth factor modulates oral squamous cell carcinoma invasion by activating a miR-504/FOXP1 signalling. *Oncogene.* 2011;31(19):2401–11.
 18. Lin BR, Chang CC, Che TF, Chen ST, Chen RJ, Yang CY, et al. Connective tissue growth factor inhibits metastasis and acts as an independent prognostic marker in colorectal cancer. *Gastroenterology.* 2005;128:9–23.
 19. Lin BR, Chang CC, Chen RJ, Jeng YM, Liang JT, Lee PH, et al. Connective tissue growth factor acts as a therapeutic agent and predictor for peritoneal carcinomatosis of colorectal cancer. *Clin Cancer Res.* 2011;17:3077–88.
 20. Chang CC, Shih JY, Jeng YM, Su JL, Lin BZ, Chen ST, et al. Connective tissue growth factor and its role in lung adenocarcinoma invasion and metastasis. *J Natl Cancer Inst.* 2004;96:364–75.
 21. Takatsuki H, Komatsu S, Sano R, Takada Y, Tsuji T. Adhesion of gastric carcinoma cells to peritoneum mediated by alpha3beta1 integrin (VLA-3). *Cancer Res.* 2004;64:6065–70.
 22. Saito Y, Sekine W, Sano R, Komatsu S, Mizuno H, Katabami K, et al. Potentiation of cell invasion and matrix metalloproteinase production by alpha3beta1 integrin-mediated adhesion of gastric carcinoma cells to laminin-5. *Clin Exp Metastasis.* 2010;27:197–205.
 23. Zhang XP, Puzon-McLaughlin W, Irie A, Kovach N, Prokopi-shyn NL, Laferte S, et al. Alpha 3 beta 1 adhesion to laminin-5 and invasin: critical and differential role of integrin residues clustered at the boundary between alpha 3 N-terminal repeats 2 and 3. *Biochemistry.* 1999;38:14424–31.
 24. Shi Y, Wang W, Tu Z, Zhang L, Qiu J, Li Q, et al. The C-terminal peptide of connective tissue growth factor blocks the full molecule binding to tubular epithelial cell. *Transplant Proc.* 2006;38:2187–9.
 25. Hoshijima M, Hattori T, Inoue M, Araki D, Hanagata H, Miyauchi A, et al. CT domain of CCN2/CTGF directly interacts with fibronectin and enhances cell adhesion of chondrocytes through integrin alpha5beta1. *FEBS Lett.* 2006;580:1376–82.
 26. Gao R, Brigstock DR. Connective tissue growth factor (CCN2) induces adhesion of rat activated hepatic stellate cells by binding of its C-terminal domain to integrin alpha(v)beta(3) and heparan sulfate proteoglycan. *J Biol Chem.* 2004;279:8848–55.
 27. Gao R, Brigstock DR. A novel integrin alpha5beta1 binding domain in module 4 of connective tissue growth factor (CCN2/CTGF) promotes adhesion and migration of activated pancreatic stellate cells. *Gut.* 2006;55:856–62.
 28. Dornhofer N, Spong S, Bennewith K, Salim A, Klaus S, Kambham N, et al. Connective tissue growth factor-specific monoclonal antibody therapy inhibits pancreatic tumor growth and metastasis. *Cancer Res.* 2006;66:5816–27.
 29. Jiang CG, Lv L, Liu FR, Wang ZN, Liu FN, Li YS, et al. Downregulation of connective tissue growth factor inhibits the growth and invasion of gastric cancer cells and attenuates peritoneal dissemination. *Mol Cancer.* 2011;10:122.
 30. Jiang CG, Lv L, Liu FR, Wang ZN, Na D, Li F, et al. Connective tissue growth factor is a positive regulator of epithelial-mesenchymal transition and promotes the adhesion with gastric cancer cells in human peritoneal mesothelial cells. *Cytokine.* 2013;61:173–80.
 31. Cheng TY, Wu MS, Hua KT, Kuo ML, Lin MT. Cyr61/CTGF/Nov family proteins in gastric carcinogenesis. *World J Gastroenterol.* 2014;20:1694–700.
 32. Zigler M, Dobroff AS, Bar-Eli M. Cell adhesion: implication in tumor progression. *Minerva Med.* 2010;101:149–62.
 33. Makrilia N, Kollias A, Manolopoulos L, Syrigos K. Cell adhesion molecules: role and clinical significance in cancer. *Cancer Invest.* 2009;27:1023–37.
 34. Tucker GC. Inhibitors of integrins. *Curr Opin Pharmacol.* 2002;2:394–402.
 35. Cox D, Brennan M, Moran N. Integrins as therapeutic targets: lessons and opportunities. *Nat Rev Drug Discov.* 2010;9:804–20.
 36. Jang BS, Lim E, Hee Park S, Shin IS, Danthi SN, Hwang IS, et al. Radiolabeled high affinity peptidomimetic antagonist selectively targets alpha(v)beta(3) receptor-positive tumor in mice. *Nucl Med Biol.* 2007;34:363–70.