Connective Tissue Growth Factor Acts as a Therapeutic Agent and Predictor for Peritoneal Carcinomatosis of Colorectal Cancer

Been-Ren Lin1,4, Cheng-Chi Chang2,3, Robert Jeen-Chen Chen6, Yung-Ming Jeng5, Jin-Tung Liang4, Po-Huang Lee5, King-Jen Chang3,4, and Min-Liang Kuo1,3

Abstract

Purpose: Here, we aimed to investigate the role of connective tissue growth factor (CTGF) in peritoneal carcinomatosis (PC) associated with colorectal cancer (CRC) and to characterize the underlying mechanism of CTGF mediating adhesion.

Experimental Design: A cohort of 136 CRC patient specimens was analyzed in this study. CRC cell lines were used for in vitro adhesion assay and in vivo peritoneal dissemination experiment. Recombinant CTGF protein treatment, transfection of CTGF expression plasmids, and knockdown of CTGF expression in CRC cells were utilized to evaluate the integrin α5, which served as a target of CTGF in inhibiting peritoneal seeding.

Results: The analysis of CRC tissues revealed an inverse correlation between CTGF expression and prevalence of PC. Lower CTGF level in CRC patients was associated with higher peritoneal recurrence rate after surgery. Inducing CTGF expression in cancer cells resulted in decreased incidence of PC and increased rate of mice survival. The mice received intraperitoneal injection of recombinant CTGF protein simultaneously with cancer cells or following tumor formation; in both cases, peritoneal tumor dissemination was found to be effectively inhibited in the mouse model. Functional assay revealed that CTGF significantly decreased the CRC cell adhesion ability, and integrin α5 was confirmed by reverse transcriptase PCR and functional blocking assay as a downstream effector in the CTGF-mediated inhibition of CRC cell adhesion.


Introduction

Peritoneal carcinomatosis (PC) in colorectal cancer (CRC) has long been considered to be a fatal clinical entity and treated palliatively. It is defined as the implantation of tumor cells throughout the peritoneal cavity, frequently resulting in locoregional morbidity without systemic metastasis. Previous clinical studies have reported that approximately 8% to 15% of the patients simultaneously have isolated PC at the time of initial surgery (1), and that 10% to 35% of the patients exhibit metachronous recurrence after curative resection of primary tumor (2–4). Over the past decade, novel therapeutic approaches such as aggressive cytoreductive surgery combined with hyperthermic intraperitoneal chemotherapy have been shown to improve the median survival rate up to 32.4 months (4). Nevertheless, in half of the studies that employ these novel approaches, the postoperative mortality was found to be greater than 5% with morbidity of approximately 25% to 35% (1, 5–8), suggesting that the above treatment is more effective only in patients with minimum carcinomatosis. To develop effective therapeutic strategies, an understanding of the unique characteristics of the individual tumor is urgently required.

Peritoneal dissemination of cancer cells involves multiple steps. First, the cancer cells invade the entire thickness of the colon or rectum, evading anoikis in adhesion-free status, following which they readhere onto the peritoneal surface, proliferate, and grow in distal size. Among these hallmarks of seeding, the cancer cells adhering toward the peritoneum were considered to play a key role in gastrointestinal cancers. Therefore, adhesion molecular markers, such as integrin α3β1 (9), α6β4 (10), or CD44 (11), have been reported to affect the peritoneal seeding ability of gastric cancer cells, and they could be significant predictors for peritoneal dissemination of gastric cancer. In CRC, little information is available on the molecular markers that can predict peritoneal recurrence in CRC.
Translational Relevance

Peritoneal carcinomatosis (PC) in patients with colorectal cancer (CRC) patients has a dismal prognosis. Comprehending the molecular mechanisms that mediate CRC peritoneal seeding may enable identification of novel therapeutic targets. Here, for the first time, we reported the usefulness of connective tissue growth factor (CTGF) as a biological marker for the prediction of PC in CRC patients with T3 and T4 stages as well as the role of integrin α5-dependent pathway in the adhesion ability of CTGF-modulating colon cancer cells. These findings suggest that therapies targeting tumor cell adhesion may diminish the propensity for PC in CRC patients. Thus, this newly defined function of CTGF has important implications in clinical therapies that utilize this promising biological protein with other agents and such tailored treatment strategies for treating PC will improve the long-term survival in CRC patients.

Connective tissue growth factor [(CTGF) CCN2] is an extracellular matrix (ECM)-associated molecule and a member of the CCN family, which includes cysteine-rich 61 [(Cyr61) CCN1], nephroblastoma overexpressed [(Nov) CCN3], Wisp-1/elm1 (CCN4). Wisp-2/rCop1 (CCN5), and Wisp-3 (CCN6) cells. It has been known to influence several important cellular functions such as cell-cycle regulation, apoptosis, ECM production, angiogenesis, and migration (12–14). Recently, growing evidences have suggested that CTGF expression is highly associated with tumor progression including breast cancer–induced bone metastasis (15), glioblastoma growth (16), or poor prognosis of esophageal cancer (17). However, we have previously shown that CTGF inhibits the ability of CRC (18) and non–small cell lung cancer (19) cells to metastasize and invade the neighboring tissue. Thus, CTGF probably plays a tissue-specific role in the inhibition of the adhesion ability of CRC cells.

To determine whether CTGF could influence the formation of peritoneal seeding, recombinant CTGF (rCTGF) proteins were utilized in animal models of peritoneal dissemination and the therapeutic potential of CTGF was determined. On the basis of our immunohistochemical data, we successfully documented the relationships between PC recurrence rate and CTGF expression in patients with T3 and T4 CRC. Finally, we investigated the adhesion abilities of several human colon cancer cells in vitro and the underlying mechanism of CTGF-mediated adhesion.

Materials and Methods

Study subjects and surgical specimens

We obtained cancer tissue samples from 136 consecutive patients who visited National Taiwan University (NTU) Hospital from December 2001 to July 2003. All the patients presented with CRC invading the subserosa (T3) or perforating the visceral peritoneum or the peripheral organ (T4). There were 68 male and female patients and their average age was 62.4 ± 12.9 years (median, 63 years; range, 31–89 years). These patients underwent complete surgical resection, and their clinical and pathologic data have been recorded. Patients with histologically proven peritoneal metastases of CRC diagnosed at the first visit were defined as synchronous peritoneal seeding. These patients were followed up with periodic examinations comprising serum blood chemistry panels, carcinoembryonic antigen (CEA) level, endoscopy and abdominal ultrasonography, and radiograms of the thorax. The median duration of follow-up was 34.9 months. Patients with metachronous carcinomatosis were deemed to be free of peritoneal disease at the initial curative colorectal resection but subsequently became symptomatic on follow-up and were diagnosed with peritoneal recurrence following computed tomography or laparotomy.

3,3'-Diaminobenzidine immunohistochemistry

Colorectal tissue sections were deparaffinized in xylene, rehydrated in ethanol, and washed in PBS. Antigen retrieval was carried out by autoclaving for 10 minutes in 10 mmol/L citric acid buffer (pH 6.0). Endogenous peroxidase activity was blocked using with H2O2. The sections were subsequently blocked by incubation with 3% bovine serum albumin (BSA) in PBS for 2 hours at room temperature. The primary antibodies, polyclonal goat anti-human CTGF antibodies (Santa Cruz Biotechnology), were applied to the slides at a dilution of 1:100 and incubated at 4°C overnight. After the samples were washed, they were treated with biotin-labeled secondary antibody (Santa Cruz Biotechnology) at a dilution of 1:250 for 1 hour at room temperature. The antigens were detected using an ABC kit (DakoCytomation). The slides were developed with 3,3'-diaminobenzidine (DAB) chromogen, and the nuclei were counterstained with hematoxylin (BioGenex Laboratories Inc.). The sections were dehydrated in ethanol and cleared in xylene before mounting in DPX (di-N-butyl phthalate in xylene) for observation. The results of immunohistochemical staining were classified into the following level: 0 (negative staining), 1 (<5% of all tumor cells stained), 2 (<50% of all tumor cells stained), and 3 (>50% of all tumor cells stained). The pathologist who assessed immunostaining intensity was blinded to all patients’ information.

Cell cultures

There were 4 human colon cancer cell lines (Caco-2, HT29, LoVo, and HCT116) and 1 murine colon cancer cell line (CT26) used in this study. HCT116 cells or their transfectants (HCT116/Neo and HCT116/CTGF-mixed pool) and LoVo cells were maintained in Dulbecco’s modified Eagle medium (DMEM; Life Technologies Inc.) with the addition of 4 mmol/L l-glutamine and 10 mmol/L sodium pyruvate (Sigma Chemicals). HT-29 and CT26 cells were cultured in RPMI 1640 medium (Life Technologies).
The media used for cell culture were supplemented with 10% FBS and 1% penicillin (10,000 units/mL) solution. Caco-2 cells were maintained in MEM (minimum essential medium) with 20% FBS and 1% penicillin solution. Cells were maintained at 37°C in the presence of 5% CO2 in air. All the cells were passaged into fresh medium every 2 to 3 days and before confluence.

Transient transfection and established stable clone cells
The CTGF (sense) expression vectors were transiently transfected into HCT116 cells and the small interfering CTGF (siCTGF) nucleotides into CT26 cells by using TransFast transfection reagents (Promega Corporation). Briefly, 3 µg of plasmid DNA [CTGF (sense, antisense) or pcDNA3] or 200 nmol/L of nucleotide (siCTGF or scramble RNA) was mixed with 8 µg of transfection reagents. The transfection protocol was carried out according to the manufacturer’s instructions (Promega Corporation) and checked by Western blot analysis. After 24 hours of transfection, the cells were plated in fresh media with 10% fetal calf serum and 1,000 µg/mL G418. G418-resistant clones were selected and cultured.

Severe combined immunodeficient mouse colon cancer xenograft studies
HCT116 cells or their transfectants were harvested in 0.25% trypsin/PBS/EDTA, washed once each in medium and PBS, and resuspended in PBS at the concentration of 1 million cells per 200 µL. In the first part, the 5-week-old female severe combined immunodeficient (SCID) mice were intraperitoneally injected with HCT116 cells and the mice were sorted into 3 groups. The control group was treated with an equal volume of dimethyl sulfoxide (DMSO; 200 µL). The cotreatment group and posttreatment groups received rCTGF protein (BioVender Lab Med; 1.5 mg/kg) intraperitoneally once every 2 days for 14 days. However, mice in the posttreatment group were injected with CTGF protein 3 days after they were injected with HCT116 cells. In the second part, CTGF-stable transfectants, HCT116/Neo or HCT116/CTGF cells, were injected intraperitoneally in mice. The mice were sacrificed when they appeared moribund. Postmortem examinations included measuring the body weight and abdominal circumference, examining the peritoneal cavity of mice, and counting peritoneal seeding nodules. These studies were approved by the Institutional Review Board and Institutional Animal Care and Use Committee of NTU and conducted in the NTU hospital.

Adhesion assay
For the adhesion assay, the cells were sorted into 3 parts. The first part included all wild-type colon cancer cells and their own transfectants (HCT116/Neo, HCT116/CTGF-mixed pool, and CT26/siCTGF-transient cells), the second group contained HCT116 cells with rCTGF, and the last group contained CT26/siCTGF-transient cells with integrin α5–blocking antibody (Millipore). The adhesion assay was executed in the same way for all 3 groups. Firstly, a 96-well plate was coated with 50 µl Matrigel per well and incubated in 37°C for an hour. The plate contained 100 µL complete medium and were seeded with approximately 5 × 103 cells. Simultaneously, cells of the second group were treated with rCTGF. In the group of CT26/siCTGF-transient cells, the cells were transfected for 48 hours and pretreated with integrin α5–blocking antibody for 6 hours before they were used for adhesion assay. The cells were incubated in the plates for 1 hour, following which they were fixed in 4% paraformaldehyde for 15 minutes, and gently washed with PBS. The cells were stained with 0.5% crystal violet in PBS for 1 hour and then washed. Then, the cells were viewed and counted using a microscope system (type 090-135.001; Leica Microsystems). Each clone was plated in duplicate in each experiment, and each experiment was repeated at least 3 times.

Ex vivo cell adhesion to the peritoneum
The excised peritoneum (approximately 1.6 cm2) was placed in a 6-well culture plate, which was filled with 1.0 mL of 1% BSA/RPMI 1640 medium. The cells were fluorescently labeled with 5-chloromethylfluorescein diacetate (CMFDA; 20 µmol/L) at 37°C for 30 minutes and washed twice with 1% BSA/RPMI 1640. A cell suspension (2 × 105 cells/mL in 1% BSA/RPMI 1640; 0.5 mL) was overlaid on the peritoneum in a 6-well plate and then incubated at 37°C for 30 minutes. After gentle washing with PBS, the cells that adhered to the peritoneum were observed and counted under a fluorescence microscope (Olympus IX70; Olympus) equipped with an NIBA filter unit (Ex = 470–490 nm, Em = 515–550 nm) for CMFDA.

Statistical analysis
The background data of the low and high CTGF expression groups were compared. Scale variables (expressed as mean ± SD) were compared using the Mann–Whitney test and nominal variables, using Fisher’s exact test. Survival and recurrence data were analyzed using the Kaplan–Meier method. Kaplan–Meier curves were compared by a log-rank test. P values were 2 sided and the significance level was 0.05.

Results
CTGF acts as a predictive molecular marker for PC in human CRC
To investigate the possible roles of CTGF in subserosal and serosa-invasive CRC (including T3 and T4 tumors), tumor specimens were immunohistochemically stained to determine the presence of CTGF. High expression levels of CTGF were observed in the basal membrane and cytoplasm of well-differentiated cancer epithelium [Fig. 1A (II, staining level 3)] and the normal epithelium [Fig. 1A (III, right side)], as indicated by the arrowhead. In contrast, weak CTGF immunoreactivity levels were detected in advanced poorly differentiated colorectal tumors [Fig. 1A (III, as indicated by the arrow; staining level 0)] and the section
Figure 1. CTGF acts as a predictor of peritoneal seeding in CRC patients. A, representative CTGF immunohistochemical staining of human colorectal carcinomas. I, negative control IgG staining of colon adenocarcinoma. High CTGF expression is observed in differentiated adenocarcinoma tissue, and CTGF is distinctly localized in the apical cytoplasm and membrane of the tumor cells (arrowhead; II). Low CTGF protein expression is noted in a primary poorly differentiated colon adenocarcinoma specimen, as indicated by the arrow (III); the adjacent normal colon tissue with high CTGF expression is located in the right side, as indicated by arrowhead. The peritoneal seeding nodule is shown in IV.

Kaplan-Meier plots of recurrence with metachronous PC (B) and of overall survival (C) in 99 patients without peritoneal dissemination at initial surgery (significance of differences between low and high CTGF group in B and C was \( P < 0.001 \) by log-rank test). “Number at risk” was the number of individuals who neither recurred nor were censored before the setting time.
containing peritoneal seeding nodules (Fig. 1A, IV). This result indicates that advanced colon cancer is caused by loss of CTGF expression. Clinical features and CTGF expression profiles are summarized in Table 1.

Of the 136 CRC specimens analyzed, 55% (75 of 136) and 45% (61 of 136) had low (levels 0 and 1) and high expression (levels 2 and 3) levels, respectively. CRC tumors were distributed as follows: cecum and ascending and transverse colon (right side of the colon), 33% (45 of 136); descending and sigmoid colon (left side), 32% (44 of 136); and rectum, 35% (47 of 136). There was no significant statistical relationship between CTGF expression and age, sex, tumor site, preoperative CEA level, or intratumor invasion status; however, CTGF expression was significantly associated with the degree of tumor differentiation, tumor stage, and lymph node metastasis. Next, 99 patients without synchronous peritoneal seeding, 17 of 50 patients with low CTGF and 3 of 49 patients with high CTGF expression were found to have metachronous peritoneal recurrence ($P = 0.001$; Table 1). In other words, low CTGF expression in tumors was strongly associated with an 8-fold increase in the peritoneal recurrence rate compared with tumors with high levels CTGF expression (5-year recurrence risks: 48% vs. 6%, $P < 0.001$; Fig. 1B). Pathologic predictors were further analyzed and CTGF expression was found to be the only significant predictor for intraperitoneal recurrence (Supplementary Table S1). Meanwhile, multivariate logistic regression analysis revealed that CTGF expression level is the only significant factor for the prediction of intraperitoneal recurrence (OR: 0.133, 95% CI: 0.035–0.504, $P = 0.003$).

Finally, the disease-free interval in the form of metachronous peritoneal dissemination (Fig. 1B) and the overall survival rates (Fig. 1C) between these 2 CTGF expression groups were found to be significantly different ($P < 0.001$). In addition, by utilizing real-time PCR to analyze 30 tumor samples, descriptive statistics showed that CTGF mRNA levels were significantly lower in the presence of recurrent PC than in samples without recurrent PC ($P < 0.001$; Supplementary Fig. S1). These results indicate that CTGF acts as a potent diagnostic predictor of metachronous peritoneal seeding in CRC patients.

Forced expression of CTGF reduces the formation of PC in animal models

To assess whether CTGF might interfere with the development of PC in CRC, CTGF-stable transfectants, which were injected intraperitoneally, were established and confirmed by Western blotting (Fig. 2A, left). After the animals were sacrificed, the hemoperitoneum and the number of nodules were noted in the group injected with control HCT116/Neo cells (Fig. 2A, I and II) and compared with those in the HCT116/CTGF-M group (Fig. 2A, III and IV). Of 10 mice injected intraperitoneally with HCT116/Neo cells, 6 of 10 mice formed nodules at the diaphragm and 7 of 10 exhibited local encasement of the bowel loop caused by seeding tumors or hepatic surface seeding (Fig. 2A, II). Interestingly, the number of peritoneal nodules were significantly fewer in the HCT116/CTGF-M group than the HCT116/Neo group (Table 2; $P = 0.001$). Moreover, overall survival was considerably shorter in the HCT116/Neo group than in the HCT116/CTGF-M group (mean survival period = 58 days vs. 104.8 days, $P = 0.001$;}

<table>
<thead>
<tr>
<th>Feature</th>
<th>CTGF expression</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number</td>
<td>Low</td>
<td>High</td>
<td></td>
</tr>
<tr>
<td>Patients</td>
<td>136</td>
<td>75</td>
<td>61</td>
<td></td>
</tr>
<tr>
<td>Mean age, y</td>
<td></td>
<td>62.6</td>
<td>62.1</td>
<td>0.820</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>68</td>
<td>33</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>68</td>
<td>35</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>Tumor site</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right</td>
<td>45</td>
<td>23</td>
<td>22</td>
<td>0.823</td>
</tr>
<tr>
<td>Left</td>
<td>44</td>
<td>25</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>Rectum</td>
<td>47</td>
<td>27</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Tumor differentiation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poor</td>
<td>26</td>
<td>21</td>
<td>5</td>
<td>0.007b</td>
</tr>
<tr>
<td>Moderate</td>
<td>106</td>
<td>52</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td>Well</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>47</td>
<td>20</td>
<td>27</td>
<td>0.040b</td>
</tr>
<tr>
<td>III</td>
<td>63</td>
<td>36</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>26</td>
<td>19</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Lymph node</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>56</td>
<td>24</td>
<td>32</td>
<td>0.017b</td>
</tr>
<tr>
<td>N1</td>
<td>43</td>
<td>24</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>N2</td>
<td>37</td>
<td>27</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Intratumor invasion</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>63</td>
<td>38</td>
<td>25</td>
<td>0.301</td>
</tr>
<tr>
<td>Absent</td>
<td>73</td>
<td>37</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>CEA level, ng/mL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤3</td>
<td>44</td>
<td>23</td>
<td>21</td>
<td>0.582</td>
</tr>
<tr>
<td>&gt;3</td>
<td>92</td>
<td>52</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>Synchronous peritoneal seeding</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>37</td>
<td>25</td>
<td>12</td>
<td>0.084</td>
</tr>
<tr>
<td>Absent</td>
<td>99</td>
<td>50</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td>Metachronous peritoneal seeding</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>21</td>
<td>17</td>
<td>3</td>
<td>0.001b</td>
</tr>
<tr>
<td>Absent</td>
<td>78</td>
<td>33</td>
<td>46</td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Clinical and pathologic characteristics for high and low CTGF expression in T3 and T4 CRC

aRight, cecum to splenic flexure; left, splenic flexure to sigmoid colon.
bStatistical significance ($P < 0.05$).
cPresent, if pathologic report revealed one of venous, lymphovessel, or perinurial invasions.

Of the 136 CRC specimens analyzed, 55% (75 of 136) and 45% (61 of 136) had low (levels 0 and 1) and high expression (levels 2 and 3) levels, respectively. CRC tumors were distributed as follows: cecum and ascending and transverse colon (right side of the colon), 33% (45 of 136); descending and sigmoid colon (left side), 32% (44 of 136); and rectum, 35% (47 of 136). There was no significant statistical relationship between CTGF expression and age, sex, tumor site, preoperative CEA level, or intratumor invasion status; however, CTGF expression was significantly associated with the degree of tumor differentiation, tumor stage, and lymph node metastasis. Next, 99 patients without synchronous peritoneal seeding, 17 of 50 patients with low CTGF and 3 of 49 patients with high CTGF expression were found to have metachronous peritoneal recurrence ($P = 0.001$; Table 1). In other words, low CTGF expression in tumors was strongly associated with an 8-fold increase in the peritoneal recurrence rate compared with tumors with high levels CTGF expression (5-year recurrence risks: 48% vs. 6%, $P < 0.001$; Fig. 1B). Pathologic predictors were further analyzed and CTGF expression was found to be the only significant predictor for intraperitoneal recurrence (Supplementary Table S1). Meanwhile, multivariate logistic regression analysis revealed that CTGF expression level is the only significant factor for the prediction of intraperitoneal recurrence (OR: 0.133, 95% CI: 0.035–0.504, $P = 0.003$). Finally, the disease-free interval in the form of metachronous peritoneal dissemination (Fig. 1B) and the overall survival rates (Fig. 1C) between these 2 CTGF expression groups were found to be significantly different ($P < 0.001$). In addition, by utilizing real-time PCR to analyze 30 tumor samples, descriptive statistics showed that CTGF mRNA levels were significantly lower in the presence of recurrent PC than in samples without recurrent PC ($P < 0.001$; Supplementary Fig. S1). These results indicate that CTGF acts as a potent diagnostic predictor of metachronous peritoneal seeding in CRC patients.

** Forced expression of CTGF reduces the formation of PC in animal models

To assess whether CTGF might interfere with the development of PC in CRC, CTGF-stable transfectants, which were injected intraperitoneally, were established and confirmed by Western blotting (Fig. 2A, left). After the animals were sacrificed, the hemoperitoneum and the number of nodules were noted in the group injected with control HCT116/Neo cells (Fig. 2A, I and II) and compared with those in the HCT116/CTGF-M group (Fig. 2A, III and IV). Of 10 mice injected intraperitoneally with HCT116/Neo cells, 6 of 10 mice formed nodules at the diaphragm and 7 of 10 exhibited local encasement of the bowel loop caused by seeding tumors or hepatic surface seeding (Fig. 2A, II). Interestingly, the number of peritoneal nodules were significantly fewer in the HCT116/CTGF-M group than the HCT116/Neo group (Table 2; $P = 0.001$). Moreover, overall survival was considerably shorter in the HCT116/Neo group than in the HCT116/CTGF-M group (mean survival period = 58 days vs. 104.8 days, $P = 0.001$;...
Fig. 2B). In contrast, endogenous knockout CTGF expression of murine colon cancer cells CT26 by transfection with antisense CTGF-expressing vectors could significantly increase the number of peritoneal seeding nodules in BALB/c mice (Supplementary Fig. S2 and Table S2). Taken together, these data suggest that overexpression of CTGF effectively abolishes the process of peritoneal dissemination in CRC cells in vivo.
Suppression of peritoneal implantation by exogenous rCTGF in SCID mice

To further clarify the role of CTGF in the PC process, HCT116 cells were injected and administered intraperitoneally with DMSO (CTL) or rCTGF (1.5 mg/kg, Co-Tx group) every 2 days for 14 days. In addition, to mimic the clinical situation of CRC patients with early established microscopic implants, mice in the posttreatment group (post-Tx group) received rCTGF treatments on the third day after injection with cancer cells. All the CTL mice were moribund within 70 days with copious bloody ascites and increased body weight, which were recorded during the dissection of the abdominal cavity (Fig. 2C, I and II). Numerous peritoneal nodules were also observed (Fig. 2C, III), indicating that advanced PC occurred in the CTL group. In contrast to the rCTGF treatment groups, mice (3 and 2 in the Co-Tx and post-Tx groups, respectively) showed absolutely no PC in the abdominal cavity (Fig. 2C, VI). Moreover, abdominal circumference and body weight were significantly decreased in the rCTGF treatment groups in comparison with CTL group (Fig. 2D and E). The number of peritoneal seeding nodules was also significantly decreased in rCTGF treatment groups (Fig. 2F). Taken together, these results suggest that CTGF may be a potent and attractive therapeutic strategy for high-risk patients with peritoneal dissemination.

CTGF inhibits the adhesion abilities of CRC cells

Because adhesion is the first and most crucial step of PC, it was speculated that CTGF may affect adhesion capacities. To test this hypothesis, 4 human colon cancer cell lines (Caco-2, HT29, LoVo, and HCT116) and 1 murine colon cancer cell line (CT26) were used. Figure 3A shows the strong correlation between adhesion ability and endogenous CTGF expression. It was further shown that the adhesion abilities of HCT116/CTGF cells were significantly decreased by 41.85% in Matrigel (Fig. 3B, left) and 52.61% in ex vivo rat peritoneum (Fig. 3B, right) compared with the Neo control. Furthermore, endogenous CTGF was transiently knocked down by siRNA (siCTGF) in CT26 cells, resulting in 1.25- to 1.7-fold increase in adhesion ability compared with control cells (Fig. 3C). These data all support the notion that CTGF could inhibit CRC cell adhesion.

To the best of current knowledge, CTGF has been proven to enhance the adhesion status of different normal cells (20-24), including fibroblasts. To clarify whether CTGF alters cellular adherence to the matrix is cell-type specific, fibroblast 293T cells were included in models and rCTGF treatment was found to significantly enhance adhesion capacities in fibroblast by 40% (Fig 3D). However, treating CRC cells with rCTGF resulted in a 20% to 40% decrease in cell adhesion (Fig. 3D) and significantly reduced the dose-dependent adhesion ability of HCT116 cells (Fig. 3E). These data indicate that CTGF has different physiologic functions depending on the cell type. In addition, to test whether poor cell–matrix adhesion was the cause of the slow growth observed in vivo, the growth rates of CTGF transfectants cultured in Petri dish or Matrigel were compared. As shown in Supplementary Figure S3, CTGF did not regulate cancer cell proliferation in Petri dish under the subconfluent conditions, but CTGF transfectants did exhibit slower proliferation rates due to “contact inhibition” in confluent conditions after more than 3 days of culture. However, CTGF clones produced fewer colonies in Matrigel during the initial stage (day 1) compared with Neo clones (Fig. 3F). Once these 2 clones adhered, the colony diameters would not have made a difference. The evidence provided here supports the notion that the status of cell adhesion plays a crucial role during the initial stage of CRC cells seeding in the abdominal cavity, and CTGF could negatively regulate this process.

Integrin α5 is a downstream effector of the CTGF-inhibited adhesion pathway

Integrins play a prominent role in the ECM interactions (25-28). Therefore, we analyzed the presence of integrin subunits in HCT116 and LoVo colon cancer cells by reverse transcriptase (RT)-PCR assay. Both cell lines expressed integrin β1, α2, and α5 subunits (Fig. 4A). In addition, integrin α3, α4, α6, and αl subunits were absent in both cell lines (data not shown). To examine integrins affected by CTGF, CRC cells were treated with rCTGF doses (Fig. 4A, top) or time courses (Fig. 4A, bottom). It is noteworthy that only the integrin α5 subunit was dramatically decreased in both dose- and time-dependent manners. A similar experiment was shown in CTGF-stable

Table 2. Results of peritoneal seeding of HCT116/Neo and CTGF transfectants in SCID mice

<table>
<thead>
<tr>
<th>Peritoneal seeding</th>
<th>Diaphragm seeding</th>
<th>Local bowel invasion</th>
<th>Nodule count</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Involved no.</td>
<td>P</td>
<td>Involved no.</td>
</tr>
<tr>
<td>HCT116/Neo</td>
<td>6/10</td>
<td>0.057</td>
<td>7/10</td>
</tr>
<tr>
<td>HCT116/CTGF-M</td>
<td>1/10</td>
<td></td>
<td>2/10</td>
</tr>
</tbody>
</table>

*P value derived from Fisher’s exact test.

*P value from Mann-Whitney test.
transfectants (Fig. 4A, top right). Quantitative real-time RT-PCR analysis was carried out next to test how long the effects of rCTGF treatment would continue. The integrin α5 expression was markedly decreased after 2 hours of rCTGF treatment and this effect continued until 48 hours later (Supplementary Fig. S4, top). In contrast, integrin α5

**Figure 3.** Adhesion abilities are reversely correlated with CTGF expression in colon cancer cells. **A,** top, immunoblot analysis of CTGF and β-actin in colon cancer cell lines. Protein extracts (40 μg per lane) from the indicated cell lines were electrophoresed, transferred, and used for immunodetection of CTGF and β-actin. **A,** bottom, in vitro adhesion was measured by determining the portion (percentage) of cells adherent to the Matrigel as compared with HCT116 wild-type cells. **B,** adhesion assay for HCT116 stable-transfected clones on Matrigel (left) and excised rat peritoneum (right). Briefly, these transfectants in serum-free DMEM were seeded in a different plate. After incubation at 37°C for 30 minutes, wells were washed by 1× PBS, and then adherent cells were fixed and counted. **C,** CTGF expression (top) and adhesion assay (bottom) in CT26 cells treated with siRNA-mediated knockdown of CTGF (siCTGF) as described under Materials and Methods. Each cell was assayed in 3 separate experiments. **D,** adhesion assay for 293T fibroblast and HCT116, LoVo colon cancer cells pretreated with 100 ng/mL exogenous CTGF proteins. **E,** the change of adhesion ability of HCT116 cell lines treated with different doses of exogenous rCTGF protein. **F,** growth rates of stable transfectants, HCT116/CTGF and its Neo control using the proliferation assay on Matrigel. The cells were incubated in the plates for different days, following which they were fixed in 4% paraformaldehyde for 15 minutes. The cells were stained with 0.5% crystal violet in PBS for 1 hour, and then counted using a microscope system. *, statistically significant difference (P < 0.05), as compared with each control group.
in vivo mice in turn, plays a critical role in prolonging the survival of which also inhibits the peritoneal dissemination, which, down CT26 cells, as shown in Figure 4B. To confirm the expression was significantly increased in CTGF knockdown CT26 cells, as shown in Figure 4B. To confirm the role of integrin α5 in CTGF-modulated CRC cell adhesion, CT26/siCTGF cells were established and pretreated with integrin α5–blocking antibodies for 6 hours. The adhesion ability of CT26/siCTGF cells was significantly decreased in a dose-dependent manner after treatment with the integrin α5–blocking antibodies (Fig. 4C). Altogether, these results indicate that CTGF-decreased CRC cell adhesion is mediated by its downstream effector, integrin α5, which also inhibits the peritoneal dissemination, which, in turn, plays a critical role in prolonging the survival of mice in vivo.

Discussion

Although recent studies have shown that the combination of intraperitoneal chemotherapy and peritonectomy procedures can improve the survival rate of CRC patients, the high incidence of postoperative mortality and morbidity make these therapeutic approaches suitable only for certain patient populations (1, 5–8). Therefore, identifying CRC patients at increased risk of peritoneal recurrence and early intervention with efficient molecular targeting drugs are crucial. To date, several reports have tried to identify intraperitoneal recurrence with various clinicopathologic parameters. For example, Shepherd and colleagues (29) identified local peritoneal involvement as an independent prognostic predictor of subsequent intraperitoneal recurrence in patients with colon cancer. Kanellos and colleagues (30) and Yamamoto and colleagues (31) found that tumor-positive cytology is significantly correlated with risk of intraperitoneal recurrence; furthermore, the relative risk of intraperitoneal recurrence in patients with tumor-positive cytology and in those with tumor-negative cytology was 16.5 and 2.9, respectively. However, Koppe and colleagues (1) mentioned that multivariate analyses, as a means to determine whether tumor-positive cytology acts as an independent factor or just as a confounding prognostic indicator, were not carried out in any of these studies. In addition, Katoh and colleagues (32) advocated that intraperitoneal tumor cell positivity is associated with distal recurrence rather than subsequent PC. Therefore, the prognostic value of tumor-positive cytology for PC remains controversial.

CTGF is an ECM-associated protein and believed to be a multifunctional signaling modulator involved in a wide variety of biological and pathologic processes (12–19). In this study, CTGF was shown to inhibit the peritoneal dissemination of colon cancer cells by reducing cancer cell adhesion to the peritoneum. First, in this clinical study, CTGF was shown to inhibit the peritoneal dissemination of CT26 transfectants. CT26/siCTGF transient cells were pretreated with integrin α5 neutralizing antibody (5 μg/mL) for 6 hours. After another 1 hour of incubation, the adhesive cells were fixed in 4% paraformaldehyde and stained with 0.5% crystal violet. Analysis of each clone was carried out in triplicate for each experiment. *, statistically significant difference (P < 0.05), as compared with each control group. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Figure 4. Integrin α5 acts as a downstream target gene in CTGF-mediated adhesion. A, RT-PCR analysis of HCT116, LoVo, and CTGF-stable transfectants was carried out with integrin β1, α2, α5, or CD44. Among these, HCT116 cell lines were treated with different doses and time courses of rCTGF. B, Western blot analysis of integrin α5 in CT26 cell lines, which is treated with siCTGF. C, adhesion assay of integrin α5 neutralizing antibody treatment of CT26 transfectants. CT26/siCTGF transient cells were pretreated with integrin α5 neutralizing antibody (5 μg/mL) for 6 hours. After another 1 hour of incubation, the adhesive cells were fixed in 4% paraformaldehyde and stained with 0.5% crystal violet. Analysis of each clone was carried out in triplicate for each experiment. *, statistically significant difference (P < 0.05), as compared with each control group. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
with advanced depth of tumor invasions is that such patients are more likely to develop peritoneal dissemination, as reported in previous studies (29, 30, 33). Indeed, these reports concur with the findings of this study, wherein the incidence of synchronous and metachronous peritoneal dissemination was 27.2% and 20.2%, respectively (Table 1). Our results indicate that patients with lower CTGF expression in the tumors are at a significantly higher risk of developing metachronous PC. Furthermore, CTGF acts as an independent biological marker for the prediction of subsequent PC recurrence, which has been confirmed by multivariate analyses. Second, it was found that both CTGF treatment and overexpression of CTGF could extend the survival rates of mice (Fig. 2B) and reduce the numbers of intraperitoneal seeding nodules (Table 2 and Fig. 2F), even in the posttreatment group where cancer cells had been implanted into the peritoneal cavity 3 days prior to CTGF administration. CTGF knockouts of CT26 cells were shown to consistently increase the number of intraperitoneal nodules (Supplementary Fig. S2 and Table S2). This indicates that CTGF has important implications in chemoprevention as well as therapies concomitant with other agents. In addition, previous clinical and animal studies were supported by a series of experimental approaches designed to confirm that CTGF plays a cell-type–specific role in adhesion inhibition, which is a crucial role for the initial process of CRC cells seeding in the abdominal cavity.

Integrins belong to a large family of cell surface glycoproteins that mediate cellular and ECM interactions including cell anchorage, migration, proliferation, adhesion, differentiation, and angiogenesis (25–28). They are formed by the noncovalent associations of an α subunit with a β subunit. Both subunits are transmembrane proteins with large extracellular domains for ligand binding and a relative short cytoplasmic domain that interacts with cytoskeletal structures or other intracellular proteins for activating signal cascades. Several previous studies have suggested that integrins expression and colon cancer invasion are correlated. In one study, Conti and colleagues reported that integrin αv inhibition in CRC specimens from hepatic metastases caused a significant reduction in tumor cell proliferation on collagen plates (34). A previous study utilizing animal models showed that the resection of CRC dramatically increased malignant cell adhesion within the liver, and blocking of α2 integrin prevented the increase of colorectal hepatic metastases (35). In addition, anti-β1 integrin antibodies have been reported to reduce surgery-induced adhesion of murine colon cancer cells (36). These reports coincide with the findings of this study, wherein the reduction of α5 integrin expression is, at least in part, mediated by CTGF, acts to inhibit the adhesion abilities of CRC cells, and prevents PC in the abdominal cavity.

Particularly interesting is the finding that CTGF is found to enhance the adhesion status of different cells, including endothelial cells (20), rat hepatic stellate cells (21), fibroblasts (22), platelets (23), and chondrocytes (24), by binding with respective integrins such as αvβ3 (20, 21), α6β1 (22), α1β1β3 (23), and α5β1 (24). On the other hand, previous reports have shown the upregulation of α5 transcripts in NRK (normal rat kidney) fibroblasts treated with CTGF (37), and CTGF levels were shown to correlate with α5 levels in the ileum from patients with inflammatory bowel diseases (38). In contrast, the results of this study suggest that CTGF in colon cancer cells could transcriptionally downregulate the α5 integrin subunit and, in turn, reduce adhesion capacity. In prior publications (24), CTGF was shown to increase chondrocyte adhesion to fibronectin through direct interaction of its CT domain with fibronectin, and only integrin α5β1 was involved in this mechanism. Chen and colleagues (22) proved that both Cyr61 and CTGF both induce adhesive signaling in primary skin fibroblasts via FAK (focal adhesion kinase), Rac, and MAPK (mitogen-activated protein kinase). In CRC cancer cells, CTGF was found to impair adhesion only by downregulating integrin α5 expression in screened integrin candidates. This finding is supported by the evidence that integrin α5–blocking antibodies could restore the adhesion abilities in CRC models. Although integrin α5 is found on most epithelial and mesenchymal cells, including normal and cancer cells, the involvement of integrins other than α5 still cannot be excluded. Therefore, it is still unknown whether direct protein–protein interactions in CTGF-mediated adhesion inhibit the CRC peritoneal seeding mechanism. It is also possible that indirect effects of CTGF may be partially responsible. Further clarification is necessary to determine whether CTGF, either through direct binding or a cross-talk mechanism, alters integrin α5 expression, as its mRNA is significantly diminished, as shown in Figure 4A and Supplementary Data section.

As far as can be determined, integrin α5β1 is a fibronectin-binding receptor that engages in various biological and pathologic processes such as facilitating the cellular adhesion of chondrocytes (24) and mediating TGF-β–induced fibronectin deposition in human mesangial cells (39). At the underlying molecular level, there is accumulating evidence that indicates that the promoter activity of integrin α5 can be transactivated by transcriptional factors such as activator protein 1 (AP-1; refs. 40, 41), SP-1 (40, 41), and CCAAT/enhancer binding proteins (C/EBP; refs. 40, 42). Preliminary data from high-throughput transcriptome analysis show that C/EBP mRNA was markedly downregulated in CTGF transfectants (data not shown). After confirmation of that observation using real-time RT-PCR analysis, possible microRNA mechanisms that could regulate CTGF-inhibited integrin α5 expression through downregulation of C/EBP mRNA levels are now being investigated in CRC cell models. However, this is a working hypothesis and requires further investigation.

In conclusion, our study is the first to indicate that CTGF not only influences cancer cell adhesion activities but also inhibits the formation of peritoneal seeding nodules. Moreover, CTGF can serve as a biomarker for predicting metachronous peritoneal dissemination in patients with
CRC. These results could aid in the development of therapeutic modalities by incorporating CTGF administration into treatment for CRC patients, which could potentially prevent peritoneal recurrence in certain subgroups of patients at increased risk of PC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Reference

34. Conti JA, Kendall TJ, Bateman A, Armstrong TA, Papa-Adams A, Xu Q, et al. The desmoplastic reaction surrounding hepatic colorectal...


Connective Tissue Growth Factor Acts as a Therapeutic Agent and Predictor for Peritoneal Carcinomatosis of Colorectal Cancer


Clin Cancer Res 2011;17:3077-3088. Published OnlineFirst May 10, 2011.

Updated version  Access the most recent version of this article at: doi:10.1158/1078-0432.CCR-09-3256
Supplementary Material  Access the most recent supplemental material at: http://clincancerres.aacrjournals.org/content/suppl/2011/05/18/1078-0432.CCR-09-3256.DC1

Cited articles  This article cites 42 articles, 16 of which you can access for free at: http://clincancerres.aacrjournals.org/content/17/10/3077.full.html#ref-list-1

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.