Connective Tissue Growth Factor Confers Drug Resistance in Breast Cancer through Concomitant Up-regulation of Bcl-xL and cIAP1

Ming-Yang Wang,1,2,3 Pai-Sheng Chen,1,2 Ekambaranellore Prakash,1 Hsing-Chih Hsu,1,2,3 Hsin-Yi Huang,1 Ming-Tsan Lin,3,5 King-Jen Chang,2,3 and Min-Liang Kuo1

1Laboratory of Molecular and Cellular Toxicology, Institute of Toxicology, College of Medicine, and 2Angiogenesis Research Center, National Taiwan University; and Departments of ’Surgery, ’Pathology, and ’Primary Care Medicine, National Taiwan University Hospital, Taipei, Taiwan

Abstract

Connective tissue growth factor (CTGF) expression is elevated in advanced breast cancer and promotes metastasis. Chemotherapy response is only transient in most metastatic diseases. In the present study, we examined whether CTGF expression could confer drug resistance in human breast cancer. In breast cancer patients who received neoadjuvant chemotherapy, CTGF expression was inversely associated with chemotherapy response. Overexpression of CTGF in MCF7 cells (MCF7/CTGF) enhanced clonogenic ability, cell viability, and resistance to apoptosis on exposure to doxorubicin and paclitaxel. Reducing the CTGF level in MDA-MB-231 (MDA231) cells by antisense CTGF cDNA (MDA231/AS cells) mitigated this drug resistance capacity. CTGF overexpression resulted in resistance to doxorubicin- and paclitaxel-induced apoptosis by up-regulation of Bcl-xL and cellular inhibitor of apoptosis protein 1 (cIAP1). Knockdown of Bcl-xL or cIAP1 with specific small interfering RNAs abolished the CTGF-mediated resistance to apoptosis induced by the chemotherapeutic agents in MCF7/CTGF cells. Inhibition of extracellular signal-regulated kinase (ERK)-1/2 effectively reversed the resistance to apoptosis as well as the up-regulation of Bcl-xL and cIAP1 in MCF7/CTGF cells. A neutralizing antibody against integrin αβ3 significantly attenuated CTGF-mediated ERK1/2 activation and up-regulation of Bcl-xL and cIAP1, indicating that the integrin αβ3/ERK1/2 signaling pathway is essential for CTGF functions. The Bcl-xL level also correlated with the CTGF level in breast cancer patients. We also found that a COOH-terminal domain peptide from CTGF could exert activities similar to full-length CTGF, in activation of ERK1/2, up-regulation of Bcl-xL/cIAP1, and resistance to apoptosis. We conclude that CTGF expression could confer resistance to chemotherapy agents through augmenting a survival pathway through ERK1/2-dependent Bcl-xL/cIAP1 up-regulation.

Introduction

Connective tissue growth factor (CTGF), also known as CTGF/CCN2, belongs to the CCN family (1), consisting of six members: CTGF, NOVH, CYR61, WISP-1, WISP-2, and WISP-3 (2), all of which possessing an NH2-terminal signal peptide indicative of their secreted protein nature. The biological properties of CCN proteins include the stimulation of cellular proliferation, migration, adhesion, and extracellular matrix formation, as well as the regulation of angiogenesis and tumorigenesis. CTGF is a secreted growth factor that can bind to integrins on the cell surface (3) and also serves as an angiogenic factor in collaboration with matrix metalloproteinases (4). Elevated CTGF expression has been observed in breast cancers (5), pancreatic cancers (6), melanomas (7), and chondrosarcomas (8). Although CTGF shows multiple roles in various cancer types, in breast tumor cells CTGF overexpression has been linked to tumor size increase and lymph node metastasis (5, 9). Use of in vitro and in vivo selection models and large-scale microarray analysis has shown that CTGF is critically involved in the formation of osteolytic bone metastasis in breast cancer (10, 11). In our previous study, CTGF promoted metastasis of breast cancer cells via an integrin αβ3-extracellular signal–regulated kinase (ERK)-1/2-dependent, S100A4-upregulated pathway. Thus, CTGF may play an important role in breast cancer progression and metastasis.

Despite the proven survival benefit of systemic adjuvant chemotherapy in breast cancer patients, a significant number of them still develop metastatic diseases and respond only transiently to conventional treatments leading to eventual mortality. In Greenberg’s report, only 16.6% of metastatic breast cancer patients achieved complete response by cytotoxic chemotherapy (12). Even with targeted therapy using trastuzumab against HER2/neu overexpressing breast cancer, complete response was <10% in Slamon’s series (13). It has been proposed that metastatic cancer cells deregulate apoptotic modulators and that this plays a crucial role during the metastatic process (14–16). Because most of the conventional chemotherapeutic drugs induce apoptosis of cancer cells, we speculate that an increased resistance to drug-induced apoptosis in metastatic cancer cells might be the cause of poor response in metastatic breast cancer patients.

Taking into account that CTGF plays an important role in breast cancer metastasis and that metastatic cancer cells usually show resistance to apoptotic stress, we postulated that CTGF expression would increase drug resistance in breast cancer cells. It is still unclear whether CTGF expression would deregulate apoptotic modulators or would confer drug resistance in breast cancer cells. We herein examined the effects of CTGF on the survival and chemosensitivity of breast cancer cells and the signaling pathways involved therein.

Materials and Methods

Patients and specimens. Fifty-eight patients with locally advanced breast cancer who had received chemotherapy before surgery at National Taiwan University Hospital, Taipei, Taiwan, were included in this study. Their symptoms included breast mass, axillary lymphadenopathy, and skin invasion. In the present study, we examined whether CTGF expression could confer drug resistance in human breast cancer. In breast cancer patients who received neoadjuvant chemotherapy, CTGF expression was inversely associated with chemotherapy response. Overexpression of CTGF in MCF7 cells (MCF7/CTGF) enhanced clonogenic ability, cell viability, and resistance to apoptosis on exposure to doxorubicin and paclitaxel. Reducing the CTGF level in MDA-MB-231 (MDA231) cells by antisense CTGF cDNA (MDA231/AS cells) mitigated this drug resistance capacity. CTGF overexpression resulted in resistance to doxorubicin- and paclitaxel-induced apoptosis by up-regulation of Bcl-xL and cellular inhibitor of apoptosis protein 1 (cIAP1). Knockdown of Bcl-xL or cIAP1 with specific small interfering RNAs abolished the CTGF-mediated resistance to apoptosis induced by the chemotherapeutic agents in MCF7/CTGF cells. Inhibition of extracellular signal-regulated kinase (ERK)-1/2 effectively reversed the resistance to apoptosis as well as the up-regulation of Bcl-xL and cIAP1 in MCF7/CTGF cells. A neutralizing antibody against integrin αβ3 significantly attenuated CTGF-mediated ERK1/2 activation and up-regulation of Bcl-xL and cIAP1, indicating that the integrin αβ3/ERK1/2 signaling pathway is essential for CTGF functions. The Bcl-xL level also correlated with the CTGF level in breast cancer patients. We also found that a COOH-terminal domain peptide from CTGF could exert activities similar to full-length CTGF, in activation of ERK1/2, up-regulation of Bcl-xL/cIAP1, and resistance to apoptosis. We conclude that CTGF expression could confer resistance to chemotherapy agents through augmenting a survival pathway through ERK1/2-dependent Bcl-xL/cIAP1 up-regulation.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: Min-Liang Kuo or King Jen Chang, National Taiwan University, 1 Jen Ai Road, Section I, Room 544, Taipei 100, Taiwan. Phone: 886-2-23123456-5083; Fax: 886-2-23140127; E-mail: kuo@ntu.edu.tw or chang@ntu.edu.tw.

©2009 American Association for Cancer Research. doi:10.1158/0008-5472.CAN-08-2524

Cancer Res 2009; 69: (8). April 15, 2009 3482 www.aacrjournals.org

Research Article
Taiwan University Hospital were included. Paraffin-embedded, formalin-fixed surgical resection specimens were collected for immunohistochemical staining for CTGF. Tumor size, local invasion, and lymph node metastasis were acquired from pathology reports. Prechemotherapy tumor burden was estimated by tumor dimension acquired either by image or direct measurement of the breast tumor. Postchemotherapy tumor dimensions were acquired from the final pathology report after surgery. Chemotherapy response was determined as a change in tumor volume.

Cell culture, antibodies, and reagents. Human breast cancer cell lines MCF7 and MDA231 were obtained from the American Type Culture Collection. BT474, BT483, MDA-MB-453, and MDA-MB-435 cells were generously provided by the National Health Research Institute (Chunan, Taiwan). These cell lines were maintained in DMEM or MEM supplemented with 10% fetal bovine serum (Life Technologies, Inc.), 2 mmol/L L-glutamine (Life Technologies), 100 μg/mL streptomycin, and 100 units/mL penicillin in a humidified 5% CO2 atmosphere. Integrin αvβ3, α-tubulin, p-ERK1/2, ERK1/2, CTGF, Bcl-xL, phosphorylated focal adhesion kinase (p-FAK), FAK, p-Src, Src, p-AKT, AKT, poly(ADP-ribose) polymerase (PARP), and cellular inhibitor of apoptosis protein 1 (cIAP1) antibodies were purchased from Santa Cruz Biotechnology. CTGF antibody was from BD Biosciences. Goat anti-rabbit or anti-mouse IgG conjugated with either FITC or tetramethylrhodamine isothiocyanate were purchased from Jackson Immunoresearch. Antibody-bound protein bands were detected with enhanced chemiluminescence reagents (Amersham Pharmacia Biotech) and photographed with Kodak X-Omat Blue autoradiography film (Perkin-Elmer Life Sciences). The constitutively active mutant of mitogen-activated protein kinase/ERK kinase (MEK)-1 construct (a substitution of the regulatory phosphorylation sites, Ser218 and Ser222, with aspartic acid S218D/S222D mutant) was a gift from Dr. Ruey-Hwa Chen and has been described previously (17). The primer sequences for PCR are shown in Supplementary Table S3.

Immunohistochemical staining. For immunohistochemical staining, with the procedure previously described (18), CTGF, Bcl-xL, and cIAP1 expression were evaluated by our pathologist using a semiquantitative weighted histoscore method. We have randomly chosen five low-power views. The immunohistochemistry results were scored by taking the percentage of positivity and intensity of staining into account. The intensity score was given as follows: 0, no staining; 1, weak positivity; 2, moderate positivity; and 3, strong positivity. The percentage of positivity was multiplied by the intensity score to obtain a final value designated as the immunohistochemistry score. The sum of five immunohistochemistry scores represent the CTGF, Bcl-xL, or cIAP1 expression of breast cancer. The sum of an immunohistochemistry score ≥2 was defined as high expression.

Figure 1. Role of CTGF in chemotherapeutic response of human breast cancer. A, immunohistochemical staining for CTGF in breast cancer tissue from surgical resection specimens. CTGF protein was predominantly expressed in the cytoplasm and membrane of carcinoma cells. Staining intensity was scored as indicated. B, association between CTGF expression level and chemotherapeutic response of patients with breast cancer. C, clonogenic ability of MCF7/neo and MCF7/CTGF. Cells were treated with various concentrations of paclitaxel or doxorubicin for 24 h and then refed with fresh medium. After 2 wk of culture, the colonies were stained with crystal violet and counted. Each experiment was done in triplicate. D, cell viability of MCF7/neo and MCF7/CTGF on exposure to chemotherapy drugs. Cells were cultured in 96-well plates in the presence or absence of increasing concentrations of paclitaxel or doxorubicin until the untreated control cells reached confluence. Cell viability and IC50 values were then determined by using the MTT assay. P < 0.005, Student’s t test.
Detection of sub-G\textsubscript{1} cells by flow cytometry. Adherent and detached cells were collected after trypsin detachment. Cells were washed with ice-cold PBS and fixed in 70% ethanol at −20°C for at least 1 h. After fixation, cells were washed twice, incubated in 0.5 mL of 0.5% Triton X-100/PBS at 37°C for 30 min with 1 mg/mL of RNase A, and stained with 0.5 mL of 50 mg/mL propidium iodide for 10 min. Fluorescence emitted from the propidium iodide-DNA complex was quantified after laser excitation of the fluorescent dye by a FACSscan flow cytometer (BD Biosciences).

**Figure 2.** CTGF increased resistance to apoptosis induced by paclitaxel and doxorubicin in breast cancer cells. **A,** effect of CTGF on drug-induced apoptosis in MCF7 cells (left) and MDA231 cells (right). Cells were treated with 5 μmol/L paclitaxel or 10 μmol/L doxorubicin for 24 h and then assayed by TUNEL analysis. **Top,** immunofluorescence photomicrographs of cells undergoing apoptosis (green staining, arrowhead) and the corresponding propidium iodide–counterstained photomicrographs. **Bottom,** percentage of TUNEL-positive cells. *, \(P < 0.05\). **B,** detection of cleaved PARP induced by paclitaxel and doxorubicin. Cells were serum starved for 24 h and then treated with 5 μmol/L paclitaxel or 10 μmol/L doxorubicin for 24 h. Apoptosis was examined by PARP cleavage. **C,** effects of rCTGF on drug-induced apoptosis. Detection of apoptotic cells in MDA231 cells treated with paclitaxel and rCTGF. After serum starvation for 24 h, MDA231/AS cells were treated with 5 μmol/L paclitaxel and 200 ng/mL rCTGF. **D,** PARP cleavage in MDA231 cells treated with paclitaxel and rCTGF. Columns, mean of triplicate experiments; bars, SD. \(a, P < 0.05\), compared with MDA231/neu without treatment; \(b, P < 0.05\), compared with MDA231/AS without treatment; \(c, P < 0.05\), compared with MDA231/AS treated with paclitaxel (Student’s \(t\) test).
Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay. The terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) technology used was the DeadEnd Fluorometric TUNEL System (Promega). For TUNEL staining, all fixation and staining procedures were conducted at room temperature. Slides were prepared with 2 × 10^6 cells on each slide. Then these slides were treated according to the manufacturer’s instructions. The slides were counterstained with propidium iodide for 12 h at 4°C. The localized green fluorescence of apoptotic cells was detected by fluorescence microscopy.

Recombinant CTGF. Suspension FreeStyle 293-F cells (Invitrogen) at a density of 1.1 × 10^6/mL were transfected with CTGF expression vector with the FreeStyle 293 Expression System (Invitrogen) according to the manufacturer’s instructions. After culture at 37°C with 8% CO2 and 92% air in an orbital shaker for 48 h, conditioned medium was collected. To ultrafilter and concentrate the dilute protein sample, Amicon Ultra-15 devices (Millipore) were used for rapid separation of recombinant CTGF (rCTGF) protein without any loss of activity.

Small interfering RNA treatment for Bcl-xL. siRNA for Bcl-xL was designed and synthesized using the software and Stealth siRNA duplex oligoribonucleotides from Invitrogen. Of the small interfering RNA (siRNA) designed and synthesized using the software and Stealth siRNA duplex oligoribonucleotides from Invitrogen. We used si-GAPDH (Ambion) as the control siRNA. Inhibition of Bcl-xL expression was assessed by reverse transcription-PCR (RT-PCR) and Western blot analyses following (Ambion) as the control siRNA. Inhibition of Bcl-xL expression was assessed by reverse transcription-PCR (RT-PCR) and Western blot analyses following transfection of human breast cancer cells with Bcl-xL siRNA (siBcl-xL). Briefly; cells were grown in 6-cm dishes and transiently transfected with 50 pmol of siRNA using 8 μg of TransFast transfection reagent (Promega) in a total transfection volume of 2 mL of serum-free DMEM. After incubation at 37°C with 5% CO2 for 8 h, 2 mL of DMEM containing 20% normal growth medium were added. Samples were then prepared and analyzed by RT-PCR, Western blot, and invasion assays.

Statistical analysis. All observations were confirmed by at least three independent experiments. The data are presented as means ± SD. Student’s t test (paired and unpaired) was used to evaluate the statistical significance of mean values. Levels of statistical significance were P < 0.05 and P < 0.005. All P values were two-tailed.

Results

CTGF expression is inversely associated with chemotherapeutic response in breast cancer patients. CTGF has been shown to play an important role in breast cancer metastasis and also associated with increased tumor size and lymph node metastasis (5, 9). Because highly metastatic cancer cells exhibit a drug-resistant phenotype (14), we speculated that CTGF expression might increase drug resistance in breast cancer cells. To investigate the effect of CTGF expression and the chemotherapy response in breast cancer patients, 58 tumors from breast cancer patients receiving neoadjuvant chemotherapy were analyzed immunohistochemically for CTGF expression from their surgical resection specimen. Patients were divided into two groups: those with tumors expressing low levels of CTGF (scores 0 and 1, Fig. 1A) and those with tumors expressing high levels of CTGF (scores 2 and 3, Fig. 1A). Twenty-three (40%) tumors had low CTGF expression and 35 (60%) tumors had high CTGF expression. Chemotherapy response was defined as the quotient of postchemotherapy tumor burden divided by prechemotherapy tumor burden (Supplementary Fig. S1). Patients with low levels of CTGF expression had a better chemotherapy response than those with high levels of CTGF expression (response quotients, 0.16 ± 0.05 versus 0.91 ± 0.28; P < 0.005, t test), indicating that breast cancer patients with a higher CTGF expression had a poorer response to chemotherapy (Fig. 1B). We have similar results from diagnostic biopsy (data not shown). There was no difference in age, hormone status, HER2/neu, tumor grade, tumor size, and stage between these two groups (Supplementary Table S1).

CTGF enhanced clonogenic ability and viability in response to chemotherapeutic agents in human breast cancer cells. In our previous study, we found that MCF7 cells expressed low levels of CTGF, whereas MDA231 cells expressed abundant CTGF (9). To confirm that CTGF could confer drug resistance in breast cancer cells, we established MCF7/CTGF cells overexpressing CTGF and MDA231/AS cells in which CTGF expression was reduced by transfection with antisense-oriented CTGF. As previously reported, no appreciable difference in cell growth ability was evident among these cells at 24, 48, and 72 hours after plating (9), as measured by trypan blue exclusion assay (data not shown). Overexpressing CTGF in MCF7 cells significantly increased their clonogenic ability on exposure to paclitaxel and doxorubicin at various doses (Fig. 1C), whereas decreased CTGF expression in MDA231 cells drastically decreased their clonogenic ability on exposure to these drugs (Supplementary Fig. S2). Hence, we suggest that elevated CTGF levels can increase the clonogenic ability of breast cancer cells exposed to chemotherapeutic agents.

To determine the magnitude of CTGF expression affecting chemosensitivity toward paclitaxel and doxorubicin, the IC50 of proliferation was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in these breast cancer cells at 24 hours after exposure to paclitaxel or doxorubicin. MCF7/CTGF cells had greater cell viability than the MCF7/neo control cells in response to paclitaxel and doxorubicin (Fig. 1D). Similarly, reducing CTGF expression in MDA231 cells drastically reduced cell viability on exposure to these chemotherapeutic agents (Supplementary Fig. S3). The IC50 values of the MCF7/CTGF cells toward doxorubicin and paclitaxel were nearly 7 and 15 times higher than those of the MCF7/neo cells, respectively (10.43 ± 4.02 versus 1.48 ± 0.22 μmol/L and 6.30 ± 0.89 versus 0.41 ± 0.11 μmol/L; P < 0.01, t test). The IC50 values of the MDA231/neo cells toward doxorubicin and paclitaxel were nearly 17 and 9 times higher than those of the MDA231/AS cells, respectively (1.50 ± 0.39 versus 0.09 ± 0.01 μmol/L and 5.13 ± 0.87 versus 0.55 ± 0.08 μmol/L; P < 0.01, t test; Supplementary Table S2). These results indicate that expression of CTGF enhances resistance to chemotherapeutic agents in human breast cancer cells.

CTGF increased resistance to apoptosis induced by paclitaxel and doxorubicin in breast cancer cells. To confirm that CTGF-induced drug resistance is mediated through inhibition of apoptosis, we exposed cells to paclitaxel and doxorubicin and subjected them to apoptosis assays. MCF7/CTGF cells exhibited increased resistance to apoptosis induced by paclitaxel and doxorubicin as quantitatively determined by TUNEL assay (Fig. 2A, left). Thirty-seven percent of cells were induced into apoptosis in MCF7/neo cells after exposure to 5 μmol/L paclitaxel for 24 hours; in contrast, only 15% were induced in MCF7/CTGF cells (P < 0.05, t test). Similarly, the percentage of apoptotic cells was also higher in MCF7/neo cells than in MCF7/CTGF cells on exposure to 10 μmol/L doxorubicin for 24 hours (41 ± 5% versus 26 ± 5%; P < 0.05, t test). Reducing CTGF expression in MDA231 cells greatly increased the percentage of apoptotic cells induced by paclitaxel and doxorubicin. When exposed to paclitaxel and doxorubicin, apoptotic cells were 22% and 29% in MDA231/neo cells versus 47% and 44% in MDA231/AS cells, respectively (Fig. 2A, right; P < 0.05, t test). Apoptosis was further verified by PARP cleavage. Paclitaxel and doxorubicin treatment caused robust
PARP cleavage in MCF7/neo and MDA231/AS as compared with that of MCF7/CTGF and MDA231/neo cells (Fig. 2B). The above data confirmed that CTGF significantly increased resistance to drug-induced apoptosis.

Because CTGF is a secreted protein, we examined whether exogenous administration of CTGF could reverse the reduced CTGF expression effect. We treated MDA231/AS cells with 100 ng/mL rCTGF, then exposed them to paclitaxel and analyzed...
by apoptosis assays. Treatment of cells with rCTGF reduced paclitaxel-induced apoptotic cells from 75% to 49% in MDA231/AS and from 46% to 14% in MDA231/neo cells (Fig. 2C; P < 0.05, t test). We previously examined the expression levels of CTGF in several breast cancer cell lines and found that the BT483, T47D, MDA-MB-453, and MCF7 cell lines expressed extremely low

**Figure 4.** Role of integrin αvβ3 in CTGF-induced signal transduction. A, MCF7 cells were serum starved for 24 h and then treated with 10 μg/mL IgG or integrin αvβ3-blocking antibodies for 24 h. P-FAK, FAK, p-Src, Src, p-ERK1/2, ERK1, Bcl-xL, and cIAP1 expression were measured by Western blotting. B, cells (5 × 10^5) were seeded in 6-cm dishes, serum starved for 24 h, and then treated with 10 μg/mL of integrin αvβ3-blocking antibody or IgG and 2 μM/mL paclitaxel for 24 h. Cell viability was examined by MTT assay. C, involvement of ERK1/2 activation in the regulation of Bcl-xL/cIAP1 and drug-induced apoptosis. Top, cells were treated with 20 μmol/L PD98059 for 24 h. Bcl-xL and cIAP1 were examined by Western blot. Bottom, cells were pretreated with PD98059 for 24 h before adding paclitaxel. Apoptotic cells were stained with propidium iodide and analyzed by flow cytometry. D, top, MDA231 cells were transfected with constitutively active mutant MEK1. After 24 h, Bcl-xL and cIAP1 were examined by Western blot. Bottom, cells were transfected with constitutively active mutant MEK1. After 24 h, these cells were treated with paclitaxel. Apoptotic cells were detected by staining with propidium iodide and analyzed by flow cytometry. Columns, mean of experiments independently repeated thrice in triplicate; bars, SD. *, P < 0.05; #, nonsignificant (Student’s t test).
or undetectable CTGF mRNA, whereas MDA231 and MDA435 cells expressed it in abundant levels (9). Western blots revealed that protein expression was correlated with mRNA level in all these cells (Supplementary Fig. S4, top). To investigate the effects of endogenous CTGF in other breast cancer cells, we treated those cells with CTGF antiserum and examined the effects on drug resistance. Treatment with CTGF antiserum did not impair cell viability, suggesting that CTGF expression is not essential for cell growth. Administration of CTGF antiserum in combination with paclitaxel significantly decreased cell viability in MDA231 and MDA435 cells. Conversely, it did not increase the drug sensitivity in T47D, BT483, and MCF7 cells, all of which showed low or undetectable CTGF levels (Supplementary Fig. S4, bottom). These results suggest that a CTGF-induced outside-in signaling pathway contributed to the resistance to apoptosis induced by paclitaxel.

**Bcl-xL and cIAP1 are downstream effectors of CTGF.** To investigate the molecular mechanism of the antiapoptotic properties of CTGF, we examined the expression of Bcl-2 and inhibitor of apoptosis protein (IAP) family proteins by RT-PCR and Western blot. The mRNA and protein levels of Bcl-xL and cIAP1 were both up-regulated by CTGF (Fig. 3). To determine whether the up-regulation of Bcl-xL and cIAP1 were the crucial determinants of the resistance to drug-induced apoptosis in these cells, we assessed the effects of siRNA targeting Bcl-xL and cIAP1 on apoptosis sensitivity in MCF7/CTGF cells. The siRNA targeting Bcl-xL or cIAP1 effectively down-regulated expression of the target proteins, compared with the control siRNA (Fig. 3B, top). Then, the MCF7 cells...
cells were exposed to paclitaxel after siRNA treatment and were further analyzed by TUNEL and MTT assays. Paclitaxel induced apoptosis in 31% of MCF7/neu cells and 9% of MCF7/CTGF cells. Transfection with siRNA targeting Bcl-xL increased the apoptosis percentage from 9% to 20% in MCF7/CTGF cells but did not increase apoptosis in MCF7/neu cell cultures. The siRNA targeting cIAP1 had similar effects and increased apoptosis in MCF7/CTGF cells to 23%. Both siRNAs significantly increased apoptosis in MCF7/CTGF cells but did not manifest any effects in MCF7/neu cells (Fig. 3B, bottom). We also found that both siRNAs had similar effects on decreasing cell viability after exposure to paclitaxel in MCF7/CTGF cells (data not shown). According to these results, both Bcl-xL and cIAP1 are important downstream effectors in CTGF-enhanced drug resistance in MCF7 cells.

Furthermore, transfection with Bcl-xL or cIAP1 vectors successfully increased the corresponding protein levels in MDA231/AS cells (Fig. 3C, top), suggesting that down-regulation of Bcl-xL and cIAP1 contributed to increased paclitaxel-induced apoptosis, whereas restoring the levels of Bcl-xL or cIAP1 decreased apoptosis from 52% to 34% and 26%, respectively, after exposure to paclitaxel (Fig. 3C, bottom). These results suggest that CTGF expression conferred resistance to drug-induced apoptosis in breast cancer cells via up-regulation of Bcl-xL and cIAP1.

**Integrin αvβ3-dependent ERK1/2 activation is required for CTGF-conferred drug resistance.** CTGF exerts a range of diverse functions by binding with cell surface integrins, including isoforms αvβ3, αvβ5, αvβ6, αvβ3, and αvβ1 (19–22). Most integrins activate FAK and thereby also Src-family kinases, which causes the phosphorylation of and therefore signaling from p130CAS and paxillin (23). Here, we found that CTGF expression also dramatically induced FAK and Src phosphorylation (Fig. 4A). Similar results were also observed with rCTGF (Supplementary Fig. S5). Bcl-xL and cIAP1 protein expression were induced in ~120 minutes (data not shown). Earlier we have observed that integrin αvβ3 was essential for CTGF-induced ERK1/2 phosphorylation (9). In addition, integrin αvβ3–blocking antibody inhibited FAK and ERK1/2 phosphorylation as well as Bcl-xL and cIAP1 protein expression in MCF7/CTGF cells. Treatment with integrin αvβ3–blocking antibody also increased paclitaxel-induced cell death in MCF7/CTGF cells but not in IgG-treated cells (Fig. 4B).

The ERK1/2 pathway has previously been implicated in cellular protection from various apoptotic signals (19, 24–27). Inhibition of MEK1 activity has been reported to down-regulate Bcl-xL protein expression and promoter activity and to enhance susceptibility to cell death induction (28). The MEK/ERK pathway also mediates tumor necrosis factor-α–induced cIAP1 expression in human endothelial cells (29). To examine whether ERK1/2 activation contributed to the resistance to paclitaxel-induced apoptosis in these cells, the MEK1-specific inhibitor PD98059 and a constitutively active mutant MEK1 were used to modulate the ERK1/2 phosphorylation status. PD98059 effectively inhibited ERK1/2 phosphorylation and Bcl-xL and cIAP1 levels in MCF7/CTGF cells (Fig. 4C, top). The sub-G1 population of MCF7/CTGF cells induced by paclitaxel significantly increased from 15% to 22% after PD98059 treatment, whereas the control cells exhibited no change. There was no sub-G1 population difference in these cells after treatment with PD98059 (Fig. 4C, bottom). On the other hand, transfection with constitutively active MEK1 caused ERK1/2 rephosphorylation and restored Bcl-xL and cIAP1 expression in MDA231/AS cells (Fig. 4D, top). In addition, transfection with constitutively active MEK1 in MDA231/AS cells decreased sub-G1 cells from 44% to 26%, and there was no significant difference between MDA231/AS and MDA231/neo cells (Fig. 4D, bottom). These results indicated that the ERK1/2 pathway is essential for CTGF-mediated drug resistance and that the integrin αvβ3/FAK/ERK1/2 pathway is critical for CTGF-induced ERK1/2 activation and subsequent drug resistance.

**COOH-terminal domain is critical for CTGF function in drug resistance.** CTGF has four structural modules that resemble an insulin-like growth-factor-binding domain (module 1), a von Willebrand factor type C repeat (module 2), a thrombospondin type I repeat (module 3), and a COOH-terminal domain (CT) that contains a putative cysteine knot (module 4; Supplementary Fig. S6). A previous study suggests that the CT could bind to integrin αvβ3 (30). Hence, we hypothesized that the CT could confer drug resistance to breast cancer cells via binding to integrin αvβ3. We transfected MCF7 cells with plasmids expressing CT and found that both full-length CTGF and CT had similar effects on activating FAK and ERK1/2; moreover, expression of CT also up-regulated Bcl-xL and cIAP1 in MCF7 cells (Fig. S4). To examine whether CT expression could confer drug resistance in breast cancer cells, we performed an MTT assay in these stable cells after exposing them to paclitaxel. As we speculated, MCF7/CTGF and MCF7/CT had similar viability profiles after exposure to various concentrations of paclitaxel (Fig. 5B). We further synthesized a recombinant CT peptide (rCT) and found that rCT treatment activates FAK and ERK1/2 pathway and up-regulated Bcl-xL and cIAP1 expression in MCF7 cells (Fig. 5C). rCT could also reduce PARP cleavage dramatically in both MDA231/AS and MCF7 cells induced by paclitaxel (Fig. 5D). The above data indicated that the CT exhibits similar activity as full-length CTGF to induce drug resistance in breast cancer cells.

**CTGF expression is associated with Bcl-xL expression in breast cancer patients.** Having shown the inverse association of CTGF expression with chemotherapy response in breast cancer patients (Fig. 1), we further investigated whether the CTGF level correlated with Bcl-xL and cIAP1 in breast cancer patients. Hence, we examined Bcl-xL and cIAP1 expression by immunohistochemical staining in serial histologic sections of the same patient group studied in Fig. 1 (Fig. 6). Among patients who had a high CTGF expression level, 74% also highly expressed Bcl-xL, whereas in the low CTGF expression group, only 39% expressed high Bcl-xL (P = 0.05, Fisher’s exact test.). In addition, the CTGF level had a tendency to always be associated with the cIAP1 level, although the P value did not reach statistically significant levels (P = 0.29; Table 1). These data agreed with our in vitro results that CTGF expression up-regulated Bcl-xL and cIAP1 expression.

**Discussion**

In this study, we show that CTGF overexpression in human breast cancer cells enhances chemo-resistance through inhibition of apoptosis. The apoptosis inhibition was accompanied by decreased PARP cleavage and a reduction in the sub-G1 population as evinced by nuclear condensation and TUNEL assays. CTGF overexpression resulted in specific up-regulation of Bcl-xL and cIAP1, although the expression levels of the others remained unchanged. We also showed that activation of ERK1/2 via the integrin αvβ3/FAK pathway by CTGF is responsible for up-regulation of Bcl-xL and cIAP1, and that its inactivation effectively reverses both the resistance to drug-induced apoptosis and the up-regulation of Bcl-xL and cIAP1 in CTGF-overexpressing cells. Thus, CTGF...
confers drug resistance in breast cancer cells by increasing resistance to apoptosis through integrin αvβ3/FAK/ERK1/2-mediated up-regulation of the Bcl-xL and cIAP1 survival pathway.

Recently, CTGF has been reported to have an important role in wound healing and fibrotic disease, whereas CTGF is also reported to be critical in tumor development and progression (5, 31–33), as well as in tumor cell survival (34). In this study, we noticed that activation of ERK1/2 also up-regulated Bcl-xL and cIAP1, which are considered to be antiapoptotic proteins. The role of Bcl-xL in exerting resistance to chemotherapy treatment with conventional antineoplastic agents was first suggested when it was observed that the expression of Erb-B2 (Her-2/neu) in breast cancer cells increased the Bcl-xL level and rendered the cells resistant to tamoxifen-induced apoptosis (35). CTGF-induced Bcl-xL up-regulation is compatible with previous observations that CTGF is associated with a poor prognosis in breast cancer patients and the enhanced metastatic ability of breast cancer cells. IAPs are a family of antiapoptotic proteins characterized by the presence of baculoviral IAP repeat domains (36), which modulate apoptosis by binding and inactivating caspases (37). In the present study, we proposed a novel mechanism in which CTGF could up-regulate cIAP1 and thereby confer resistance to drug-induced apoptosis. Knockdown of Bcl-xL or cIAP1 in MCF7/CTGF cells increased drug-induced apoptosis. Likewise, restoring Bcl-xL and cIAP1 in MDA231/AS cells reduced drug-induced apoptosis. Thus, we concluded that both Bcl-xL and cIAP1 play important roles in CTGF-induced drug resistance.

Using immunohistochemical staining, we examined 58 breast cancer patients receiving neoadjuvant chemotherapy and observed a correlation between the CTGF level and a reduction in chemotherapy response. No pathologic complete response was found in our patient samples. Three patients in the low CTGF group had only residual tumor cells and one had no residual tumor cells after chemotherapy. In Mauri’s review article, rates of pathologic response are low among these studies (ranging from 4% to 29%; ref. 38). Absence of pathological complete response might be due to a limited number of patients involved in this study. Patients with high CTGF had a significantly lower chemotherapy response than those in the low CTGF group. Bcl-xL and cIAP1 expression were also examined in these patients by immunohistochemistry in serial sections. Both Bcl-xL and cIAP1 expression levels correlated with the CTGF level although cIAP1 expression did not reach statistically significant. This supports our in vitro experiment showing that CTGF up-regulated Bcl-xL and cIAP1 expression.

CTGF antiserum reduced cell viability when cotreated with chemotherapeutic agents such as doxorubicin or paclitaxel. Overexpression of CTGF in MCF7 cells significantly increased anchorage-independent growth in soft agar, and reducing CTGF expression in MDA231 cells decreased anchorage-independent growth. In Dornhöfer’s study, a specific monoclonal antibody against CTGF could suppress anchorage-independent growth in soft agar and inhibit tumor growth and lymph node metastasis in breast cancer patients.

| Table 1. Correlation of Bcl-xL or cIAP1 expression with CTGF expression in breast cancer patients |
|-----------------|-----------------|----------|
| Bcl-xL          | CTGF expression | P        |
| Low CTGF        | High CTGF       |          |
| Low             | 14              | 9        | <0.05   |
| High            | 9               | 26       |         |
| cIAP1           | CTGF expression |          |
| Low             | 15              | 17       | 0.29    |
| High            | 8               | 18       |         |

*Fisher’s exact test.
mouse xenograft model pancreatic cancer cells (39). In conformity to our previous data that a neutralizing antibody could reverse the effects of CTGF overexpression, CTGF neutralizing antibody may not only increase chemotherapy response in breast cancer but may also be to decrease micrometastasis and tumor recurrence.

We have shown that CTGF overexpression in breast cancer cells elicits an increase in resistance toward doxorubicin and paclitaxel, as well as the specific up-regulation of Bcl-xL and cIAP1. In view of its prominent expression in advanced breast cancer tissues as well as in breast cancer cell lines, coupled with its effect on the regulation of apoptosis, CTGF is evidently one of the important determinants of chemotherapeutic sensitivity in breast cancer. Hence, CTGF may be a novel predictive marker for chemosensitivity and a potential therapeutic target for breast cancer.

References


Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 7/2/08; revised 1/11/09; accepted 2/6/09; published OnlineFirst 4/7/09.

Grant support: National Science Council, Taiwan (NSC95-2314-B-002-198-), the National Health Research Institutes, Taiwan (NHRI-EX97-9728C), China Medical University, Taichung, Taiwan and National Taiwan University (97F008-111), and the Ministry of Economic Affairs, Taipei, Taiwan (99-EK-17A-19-01-D). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Dr. Tung-Tien Sun (Department of Urology, New York University School of Medicine) for revising the paper; Dr. Ruey-Hwa Chen (Institute of Biological Chemistry, Academia Sinica, Taipei, Taiwan) for providing the active MEK1 plasmid; and Dr. John Barrett [Department of Physiology and Biophysics (B130), University of Miami Miller School of Medicine] for generously providing us Bcl-xL plasmid.

Published OnlineFirst April 7, 2009; DOI: 10.1158/0008-5472.CAN-08-2524

www.aacrjournals.org


Cancer Research.
Connective Tissue Growth Factor Confers Drug Resistance in Breast Cancer through Concomitant Up-regulation of Bcl-xL and cIAP1
