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CTGF Associated with Oncogenic Activities and Drug Resistance in Glioblastoma Multiforme (GBM)

Dong Yin^{1,2,*}, Weikai Chen², James O’Kelly², Daning Lu², Michelle Ham², Ngan B Doan³, Dong Xie⁴, Charles Wang^{5,8}, Jay Vadgama⁶, Jonathan W. Said³, Keith L Black⁷, and H. Phillip Koeffler²

¹ Shantou University Medical College, Guangdong, China

² Division of Hematology/Oncology, Cedars-Sinai Medical Center, UCLA School of Medicine, Los Angeles, CA 90048

³ Department of Pathology, UCLA School of Medicine, Los Angeles, CA 90024

⁴ Institute for Nutritional Science, SIBS Chinese Academy of Science, Shanghai, China

⁵ Functional Genomics Core, Beckman Research Institute, City of Hope Comprehensive Cancer Center, Duarte, CA 91010

⁶ Department of Medicine, Charles R. Drew University of Medicine and Science, UCLA School of Medicine, Los Angeles, CA 90024

⁷ Maxine Dunitz Neurosurgical Institute, Cedars-Sinai Medical Center, UCLA School of Medicine, Los Angeles, CA 90048

⁸ Department of Medicine, David Geffen School of Medicine at UCLA, Los Angeles, CA 90024

Abstract

Connective tissue growth factor (CTGF or CCN2) is a secreted protein that belongs to the CCN [cysteine-rich CYR61/connective tissue growth factor/nephroblastoma overexpressed gene] family. These proteins have been implicated in various biological processes, including stimulation of cell proliferation, migration, angiogenesis and tumorigenesis. In a previous study, we found that CTGF mRNA was elevated in primary gliomas and a significant correlation existed between CTGF mRNA levels *versus* tumor grade, histology, and patient survival. In the present study, the role of CTGF in glioma tumorigenesis was explored. Forced expression of CTGF in glioblastoma multiforme (GBM) cells accelerated their growth in liquid culture and soft agar, stimulated cells migration in Boyden chamber assays and significantly increased their ability to form large, vascularized tumors in nude mice. CTGF induced the expression of the anti-apoptotic proteins, Bcl-xl, Survivin, and Flip. Over-expression of CTGF caused the U343 GBM cells to survive for longer than 40 days in serum-free medium and resist anti-tumor drugs including tumor necrosis factor (TNF), TNF-related apoptosis-inducing ligand (TRAIL), VELCADE (Bortezomib, Proteasome Inhibitor), and Temozolomide. Our data suggest that CTGF plays an important role in glioma progression, by supporting tumor cells survival and drug resistance.

Keywords

CTGF; CCN; Drug resistance; Glioblastoma Multiforme (GBM)

* Address correspondence to: Dong Yin, Ph.D, 8700 Beverly Boulevard, Davis Building 5022 Room, Cedars-Sinai Medical Center, UCLA School of Medicine, Los Angeles, California, 90048, USA, Phone: (310) 423-7740, Fax: (310) 423-0225; Dong.Yin@cshs.org.

Introduction

Glioblastoma multiforme (GBM) is the most common primary brain tumor and is among the deadliest of human cancers¹. Despite advances in diagnosis and treatment, mean survival times are less than two years. These tumors typically have a very high proliferative rate with wide-spread microvascular proliferation and areas of focal necrosis. They develop as a result of multiple genetic alterations, which result in the activation of oncogenes and/or the inactivation of tumor suppressor genes. Major pathways that are frequently dysregulated include PI3 kinase/AKT/mTOR/PTEN²⁻⁴, p16(INK4A)-CDK4/6-pRb^{5, 6} and p14(ARF)-MDM2/4-p53⁷. Nevertheless, microarray studies reveal that expression of hundreds of gene transcripts are altered in gliomas compared with normal brain tissue⁸.

CTGF is a member of a family of growth factors termed the CCN (Cysteine-rich 61 (Cyr61), Connective tissue growth factor (CTGF), Nephroblastoma overexpressed (Nov)) gene family that is characterized by a high degree of amino acid sequence homology ranging from 50–90%. This family consists of six distinct members: CYR61(CCN1), CTGF(CCN2), NOV(CCN3), WISP-1(wnt-1 inducible gene, CCN4), WISP-2 (CCN5) and WISP-3(CCN6). All of the members of the CCN gene family are secreted. Each contains four distinct structural modules: insulin-like growth factor-binding protein (IGFBP), Von Willebrand type C (VWC), thrombospondin type 1 (TSP1), and COOH-terminal cysteine-rich domain (CT). These distinct modules exhibit homology to conserved regions in a variety of extracellular matrix proteins⁹. Each module is involved in protein binding and contains conserved cysteines, as well as, hydrophobic and polar residues. CTGF modulates Wnt signaling and interacts with the Wnt receptor complex¹⁰. The biological properties of CCN proteins include stimulation of cell proliferation, migration, adhesion, and extracellular matrix (ECM) formation. They also regulate more complex biological processes such as angiogenesis and tumorigenesis¹¹⁻¹³.

Recent studies have shown that CCN genes are involved in tumorigenesis. Overexpression of CTGF occurs in acute lymphoblastic leukemias¹⁴, esophageal squamous cell carcinomas¹⁵, pancreatic cancers¹⁶, prostate cancers¹⁷, and gliomas^{18, 19}. In our previously study¹⁹, CTGF mRNA levels in primary gliomas were elevated, and high levels of CTGF mRNA were directly correlated with advanced tumor stage, less differentiation and poor survival. This study explores the unique ability of CTGF to enhance growth of GBM cells both in vitro and in vivo.

Material and Methods

Cell culture

GBM cell lines U87, U118, U343, T98G and their various clones were maintained in Dulbecco's modified Eagle's medium (Gibco, BRL) with 10 % fetal calf serum (Gemini Bio-Products, Calabasas, CA), 10 U/ml penicillin-G, and 10 mg/ml streptomycin (Gemini Bio-Products, Calabasas, CA). All cells were incubated at 37° C in 5% CO₂.

Cell proliferation and colony formation in vitro

The expression vector pcDNA-CTGF-HA was constructed by placing full-length human CTGF cDNA (with HA tag) into the pcDNA3.1 eukaryotic expression vector containing the neomycin gene (Invitrogen). The constructs were transfected into U87, U118, U343, T98G human glioblastoma multiforme cells by using lipofectamine, and transfectants were selected for G418 resistance (500 ug/ml). The selected clones were confirmed by western blot analysis. Cells which had been stably transfected with either pcDNA3.1-CTGF-HA or pcDNA3.1 empty vector, were placed into 96 well plates at 1000 cells/well. After culturing for various durations, cell numbers were measured by MTT assay according to the protocol

provided by Roche Molecular Biochemicals (Basel, Switzerland). For clonogenic assays, cells were plated into 24-well flat-bottomed plates using a two-layer soft agar system with a total of 1000 cells/well in a volume of 400 μ l/well. After 14 days of incubation, the colonies were counted. All experiments were done three times using quadruplicate plates per experimental point.

Cell migration assays

Cell migration assays were performed according to the protocol from CHEMICON (Temecula, CA). 5×10^4 cells were added to the top of each Boyden chamber (10 μ m thickness and 8 μ m pores) containing polycarbonate membranes (6.5 mm diameter). Cells were allowed to migrate to the underside of the top chamber for 8 hrs. The migratory cells attached to the bottom surface of the membrane were stained with 0.1% crystal violet in 0.1 M borate, pH 9.0 and 2% ethanol for 20 min at room temperature. The stained cells were extracted using extraction buffer (CHEMICON, Temecula, CA). The number of migratory cells per membrane was determined by absorbance at 540 nm.

Actin filaments and universal focal adhesion staining

U343-V and U343-CTGF-1 cells were plated on chamber slides for 12 hours and fixed in 4% Paraformaldehyde/PBS, permeabilized with 0.1% Triton X-100 in PBS, and stained with Phalloidin-TRITC for actin filaments and Vinculin for universal focal adhesion (UFA). Confocal images were collected on a Leica microscope (Wetzlar, Germany).

Western blot analysis

Cells were harvested for total cell lysates with RIPA buffer (1 % Nonidet P-40, 0.5 % sodium deoxycholate, 0.1 % SDS, 50 mM Tris-HCl, pH 7.5) containing a mixture of protease inhibitors (Roche Diagnostics GmbH, Mannheim, Germany), as well as, 1 mM NaF and 1 mM NaVO₄. Cell lysates were centrifuged at 13,000 rpm for 10 min at 4° C. Supernatants were collected, and protein concentrations were measured. Lysates (30 μ g) were denatured in sample buffer (10 % glycerol, 5 % β -mercaptoethanol, 2.3 % SDS, 62.5 mM Tris-HCl, pH 6.8) by boiling and then subjected to 4–15 % SDS-PAGE followed by electro-transfer to polyvinylidene difluoride membrane. Immune complexes were visualized with Supersignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL), and normalized by internal control (GAPDH).

Chemicals

Proteasome inhibitor VELCADE was obtained from Millennium Pharmaceuticals (Cambridge, MA), and dissolved in DMSO at a stock concentration of 10^{-2} M and stored at –20 °C. Fresh dilutions in medium were made for each experiment. Temozolomide was obtained from Merck & Co.. TNF-alpha and Trail were bought from PeproTech (Rocky Hill, NJ).

Assessment of apoptosis

To study induction of apoptosis, annexin V assays (Annexin V-FITC Apoptosis Detection Kit; Pharmingen, San Diego, CA) were performed according to the manufacturer's instructions. Briefly, cells were harvested after exposure to reagents, washed twice with PBS, incubated with FITC-conjugated annexin V and Propidium Iodide (PI) for 15 minutes, and measured by FACSscan (Becton Dickinson).

RNA isolation and reverse transcription

Extraction of total RNA was carried out using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, DNA was removed by DNase. 2 μ g of RNA

was reverse-transcribed with random primers and Superscript[™] III reverse transcriptase (Invitrogen). The cDNA was used for real-time PCR.

Real-time RT-PCR

Real-time PCR was performed on the iCycler iQ[™] system (Bio-Rad, Hercules, CA). The PCR product was measured via a fluorescent signal generated by the binding of the fluorophore SybrGreen during the PCR. The amplification was followed by 45 cycles with 94°C for 20 s, 60°C for 10 s, 72°C for 25 s, and a fluorescence read step at 87°C (for DR5), and 84°C (for GAPDH) for 25 s. We used the GAPDH cDNA to normalize the expression data of DR5. The gene expression in control samples was given the value of 1. We examined RNA levels of four different housekeep genes (beta-actin, GAPDH, 18S and beta-2 microglobulin) to use as internal control for real-time RT PCR (Supplementary Figure 1A). Comparable results were obtained; therefore, GAPDH was chosen as an internal control. GAPDH primers: agccacatcgctcagacac (forward), gaatttgccatgggtgga (reverse); DR5 primer: aagaccctgtgctcgtgtg (forward on exon 1), aggtggacacaatccctctg (reverse on exon 3 and 4). All experiments were done in triplicate.

Microarray procedures

Vector control GBM cells (U343-V) and CTGF stably transfected cells (U343-CTGF: A combination of 50% U343-CTGF-1 and 50% U343-CTGF-2) were harvested and total RNA was extracted using RNeasy purification kit (Qiagen Inc., Valencia, CA) following the manufacturer's advice. The detailed protocol for the sample preparation and microarray processing is available from Affymetrix Inc. (Santa Clara, CA). Biotinylated cRNA was hybridized to a HG-U133A microarray (Affymetrix Inc.). The fluorescence intensity was measured for each microarray and normalized to the average fluorescence intensity for the entire microarray. GeneChip image analysis was performed using the Microarray Analysis Suite 5.0 (Affymetrix, Inc.). The experiments were done in duplicates for each sample. Data were analyzed by the GeneSpring software version 4.2 (Silicon Genetics, San Carlos, CA).

In vivo tumor studies

U343-V and U343-CTGF cells (50% of U343-CTGF-1 and 50% of U343-CTGF-2) (1×10^6 /ml) were injected s.c. into 8-week-old nude mice Beige Nude XID (BNX, NIH III from Harlan Laboratories), and tumor growth was measured after 7 weeks. Tumors derived from the U343-CTGF and U343-V cells were analyzed immunohistochemically for angiogenesis using a monoclonal antibody against CD31.

Immunohistochemistry for CD31

The sections were deparaffinized with xylene and rehydrated through ethanol. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 10 min. Heat-induced antigen retrieval (HIER) was carried out for all sections in 0.01M sodium citrate buffer, pH 6 using a vegetable steamer at 95°C for 25 min. Murine monoclonal antibody to CD31 (Dako Corporation, Carpinteria, CA) was applied at a dilution of 1:400 for 45 min at room temperature. The signal was detected using the murine DAKO horseradish peroxidase EnVision kit (DAKO) and visualized with the diaminobenzidine reaction. The sections were counterstained with hematoxylin.

Statistical Analysis

Differences between the results of experimental treatments were evaluated by the *t*-test. Differences were considered significant at values of $P < 0.05$.

Results

CTGF stimulated GBM cell migration

In our previous clinical analysis, over-expression of CTGF occurred in 78% (31 of 40) of GBM samples, and elevated levels were associated with a more advance disease and worse survival¹⁹. The results suggested that CTGF may play a role in the progression of the disease. To determine whether increased expression of CTGF enhanced cell movement, clones stably overexpressing CTGF were established using U343, U87, U118, T98G GBM cell lines. As expected, CTGF was highly expressed in CTGF-vector-transfected clones (U343-CTGF-1, -2, -3), but not in the pcDNA3.1 empty vector transfected cells (U343-V) as examined by Western blot analysis (Figure 1A). Immunofluorescence showed that the CTGF localized to the cytoplasm of the U343-CTGF cells (Supplementary Figure 1B). Migration assays of clones from 3 GBM cell lines (U343, U118, T98G) either with or without overexpression of CTGF were performed in Boyden chambers. The CTGF stably transfected clones of each cell line had a significantly increased migration compared with the empty vector-transfected cells ($P < 0.05$) (Figure 1B). Also, we examined the ability of GBM cells to adhere to collagen. When the U343 wild type and vector control U343-V cells were seeded on collagen coated plates, these cells quickly attached within 15 to 60 mins. In contrast, under the same conditions, the U343-CTGF clones did not adhere (Figures 1C). In further studies, the actin filaments and focal adhesion were examined by staining the cells with Phalloid-TRITC (actin filaments) and Vinculin (universal focal adhesion, UFA). U343 control cells had UFA evenly localized over the entire cells, and actin microfilaments were clearly formed. In contrast, UFA accumulated on the outer perimeter of U343-CTGF and their microfilaments were less structured (Figure 1D). The overall shape of the U343-CTGF cells suggested that they were structurally capable of greater mobility than the control cells.

CTGF stimulated GBM cell growth

To study if CTGF can promote proliferation, the ability of CTGF to enhance growth of these cells was also investigated by MTT assay. The clones stably transfected CTGF clones grew faster than the control cells at 5 days of liquid culture in four GBM cell lines, U87, U118, U343, and T98G (Figure 2A). CTGF also enhanced anchorage-independent growth. The U343-CTGF clones developed 2.5- to 3-fold more colonies in soft agar than the U343-V control cells ($p < 0.05$) (Figure 2B). These colonies were also substantially larger than those formed by U343-V (data not shown).

CTGF supported long-term survival of GBM cells in serum-free medium

We noted that the CTGF-expressing cells required very infrequent changing of medium compared to the U343 control cells. To examine this observation further, the U343-V and U343-CTGF cells were grown to confluence in regular DMEM medium with 10% serum. Cells were wash three times and then cultured only in DMEM medium without serum. After 10 days, 39% of U343-V cells and 15–19% of U343-CTGF cells (U343-CTGF-1/U343-CTGF-2) had either undergone apoptosis and/or death as measured by Annexin V and PI using FACS (Figure 3A). By day 30 of serum starvation, most of the U343-V cells were no longer viable and had detached from the plate, while the U343-CTGF cells were still attached and appeared healthy (Figure 3B). After 40 days of serum-free conditions, plates were stained with crystal violet; many more U343-CTGF cells remained on the plate compared with U343-V (Figure 3C). These experiments were repeated numerous times, and each time the U343-CTGF cells showed similar long-term survival. Western blot studies showed that the anti-apoptosis protein Bcl-xl was strongly expressed in U343-CTGF clones and were nearly undetectable in the U343 vector control cells that were cultured in DMEM medium with 10% serum (Figure 3D).

CTGF caused resistance to multiple drugs in GBM cells

To explore further how CTGF expression alters GBM cells, gene expression profiles of U343-V *versus* U343-CTGF (50% mixture of U343-CTGT-1 and U343-CTGF-2 cells) were measured using microarray expression analysis (data not shown, and see Material and Methods for details). Expression of anti-apoptotic proteins, Bcl-2, Bcl-xl, Birc5/Survivin, and CFLAR/Flip was increased more than 3-fold, and the expression of pro-apoptotic proteins p21, PTEN, DR5, P53AP1 was decreased more than 3-fold in the U343-CTGF cells (data not shown). DR5 is the receptor of TRAIL (TNF-Related Apoptosis-Inducing Ligand). Real-time PCR confirmed that the expression of DR5 was down-regulated 2.5- to 5- fold in the stably transfected U343-CTGF clones (Figure 4A). After exposure to TRAIL (20 ng/ml) (12 hours), only 5% of U343-CTGF cells and 11% of U87-CTGF cells underwent either apoptosis or death, compared to 32% and 28% of the U343-V and U87-V control cells, respectively (Figure 4B). These data showed that over-expression of CTGF caused GBM cells to become resistant to TRAIL. The U343-CTGF and U87-CTGF cells were also markedly resistant to the combination of VELCADE and TRAIL, with 8% and 66% of U343-CTGF and U343-V, 12% and 64% of U87-CTGF and U87-V, respectively being either apoptotic or dead (Figure 4B). The cleaved PARP protein was detected by western blot after culture with VELCADE (10^{-7} M) and TRAIL (20 ng/ml) for 16 hours in the U343-V cells, but not the U343-CTGF-2 cells (Figure 4C). Also, the expression of the anti-apoptotic proteins Survivin and Flip was markedly increased after exposure of U343-CTGF to the combined therapy, but after exposure to the same agents, these proteins did not increase in expression in the U343-V cells (Figure 4C). Blocking the degradation of I κ B is a major mechanism by which VELCADE induces apoptosis of cancer cells. Paradoxically, the treatment with VELCADE caused a decreased level of I κ B in the U343-CTGF but not in the U-343-V control cells (Figure 4C). In addition, the U343-CTGF clones were profoundly resistant to the anti-proliferative effects of TNF- α (50–200 ng/ml) (Figures 5A) and Temozolomide (25–600 μ M) (Figures 5B) compared to the control U343-V cells. In further experiments, we determined that U118 had relatively higher endogenous expression of CTGF compared to the other glioma cell lines as detected by real-time RT-PCR (Figure 5C). Using the U118 cell line, levels of CTGF were decreased by CTGF siRNA (middle panel of Figure 5C). These cells were cultured with Temozolomide. The siRNA CTGF treated U118 cells were more sensitive to growth inhibition by the drug than the cells containing scrambled control siRNA (bottom panel of Figure 5C).

CTGF promoted tumorigenesis and vascularization of human GBM cells grown in vivo

Our *in vitro* studies suggested that overexpression of CTGF promoted both anchorage-independent clonogenic proliferation in soft agar and liquid culture. Therefore, we examined whether overexpression of CTGF in U343 cells (U343-CTGF) could enhance their ability to form tumors and stimulate neovascularization in nude mice compared with U343-V controls. Cells were subcutaneously injected into the flanks of 8-week-old nude mice, and tumor growth was measured after 7 weeks. The U343-CTGF cells developed much large tumors compared to the control U343-V cells ($p < 0.05$) (Figure 6A). To examine angiogenic activity, tumors were analyzed immunohistochemically using an antibody against CD31. Robust blood vessel formation occurred in the U343-CTGF tumors compared to the U343-V derived tumors (Figure 6B).

Discussion

In our previous study, we found that CTGF was frequently highly expressed in GBMs (31 of 40, 78%), and the clinical samples with highest levels of expression were associated with more advanced disease¹⁹. Statistical analysis showed that the level of expression of CTGF in the GBM at the time of diagnosis provided compelling prognostic information. Patients

with high expression of CTGF in their GBM had a significantly shorter survival than those whose GBM had low expression¹⁹. To begin to gain an understanding of how CTGF effects formation and progression of GBM, we choose to develop and study clones of GBM cell lines having forced expression of CTGF. For example, forced expression of CTGF in U343 clones promoted their cell growth in liquid culture, their clonogenic proliferation in soft agar culture and their robust tumor formation in nude mice associated with prominent formation of blood vessels. These cells were easily able to survive over 40 days in the absence of serum and growth factors. CTGF also enhanced migration of the GBM associated with changes in their cellular structure. In additional, CTGF caused glioma cells to become resistant to the growth inhibitory affects of several anti-proliferative drugs, associated with increased expression of anti-apoptosis proteins and decreased levels of apoptosis-related proteins.

The involvement of CTGF in tumorigenesis has been examined by several other investigators. Individuals whose gastric carcinoma expressed a high level of CTGF had significantly lower survival rates than those with a low CTGF expression²⁰. However, we and others noted that patients with lung²¹, breast²², and gallbladder²³ cancers, as well as cartilaginous malignancies²⁴, whose tumors had high CTGF expression, had significantly better survivals. These data suggest that the role of CTGF in tumor progression is dependent on the tumor type.

In this study, we found that the cells with over-expression of CTGF migrated faster than the control cells. Confocal microscopic analysis clearly showed that the U343-CTGF cells accumulated universal focal adhesion (UFA) towards their periphery, and they had a less structured microfilaments. Taken together, these cells were structurally capable of greater mobility than the control GBM cells. Extracellular matrix proteins play a key role in migration of tumor cells. Forced expression of CTGF markedly reduced the ability of GBM cells to bind to collagen-coated plates. Another investigative group also suggested that CTGF is one of the important genes associated with glioma cell migration. Reducing the expression of CTGF by siRNA blocked *in vitro* migration and *ex vivo* invasion of these cells²⁵. The chief difficulty in surgically curing GBM is that the malignant cells migrate widely making it nearly impossible to remove all of these cells. The prominent expression of CTGF in these cells may in part explain their migratory behavior.

Cancer cells have a growth advantage over their normal counterparts because they often have decrease apoptosis allowing them to remain in the proliferation pool. The apoptotic pathway appears to be very aberrant in CTGF expressing GBM cells. We found, for the first time, that these cells can remain viable for at least 40 days in the absence of fetal bovine serum or any added cytokines. The CTGF is a secrete protein that binds to the surrounding stroma. We have previously shown that the CCN1 (Cyr61) protein in a paracrine and autocrine fashion, can activate integrins which are able to stimulate the PI3K/AKT/mTOR and beta-catenin/Wnt/LEF pathways to enhance expression of anti-apoptotic proteins²⁶. Recently, Wang *et al* reported that CTGF expression was associated with Bcl-xl expression and weak chemotherapy response in breast cancer patients and over-expression CTGF caused breast cancer cells to become drug resistance through up-regulation of Bcl-xl/cIAP1²⁷. In the present studies, we found that CTGF over-expression also dramatically induced anti-apoptotic protein BCL-xl expression in GBM cells. GBMs are often resistant to chemotherapeutic drugs²⁸, which contribute to the poor prognosis of these patients. Their resistance in part may be explained by stimulation of the anti-apoptotic pathway by CTGF. We found that these cells were resistant to TRAIL, TNF α and Temozolomide, as well as, the combination of VELCADE and TRAIL. Investigators have suggested that NF- κ B contributes to the enhanced survival, radioresistance, and chemoresistance of gliomas²⁹. Proteasome inhibitors (e.g. VELCADE) block the nuclear activity of NF- κ B by inhibiting

the degradation of I κ B. Studies have shown an enhanced apoptosis of selected glioma cells by the combination of VELCADE and TRAIL^{30, 31}. In contrast, we show that GBM cells with prominent expression of CTGF, down-regulate their levels of I κ B, probably associated with NF- κ B stimulating the anti-apoptotic pathway producing resistance to various therapies, including VELCADE.

In summary, our present studies taken together with our prior clinical data suggest that prominent expression of CTGF in GBM promotes an aggressive phenotype. These cells proliferate more rapidly in liquid culture, have anchorage-independent growth in soft agar, and have increased migration. They form bigger, more vascularized tumors in vivo. They have increased expression of the anti-apoptotic proteins, BCL-xL, Survivin and Flip. These CTGF-expressing GBM cells have a remarkable ability to survive prolonged durations in adverse conditions such as the lack of fetal bovine serum. Furthermore, GBM highly expressing CTGF are resistant to various therapeutic agents. Taken together, prominent level of CTGF provides GBM cells oncogenic activities and drug resistance and our study helps explain why high expression of CTGF is associated with a poor clinical course and identifies CTGF as a therapeutic target.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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The abbreviations used are

CTGF	Connective tissue growth factor
CCN	Cysteine-rich 61/Connective tissue growth factor/Nephroblastoma overexpressed
TRAIL	TNF-Related Apoptosis-Inducing Ligand
GBM	glioblastoma multiforme

References

- DeAngelis LM. Brain tumors. *N Engl J Med*. 2001; 344:114–23. [PubMed: 11150363]
- Duerr EM, Rollbrocker B, Hayashi Y, Peters N, Meyer-Puttlitz B, Louis DN, Schramm J, Wiestler OD, Parsons R, Eng C, von Deimling A. PTEN mutations in gliomas and glioneuronal tumors. *Oncogene*. 1998; 16:2259–64. [PubMed: 9619835]
- Li J, Yen C, Liaw D, Podsypanina K, Bose S, Wang SI, Puc J, Miliareis C, Rodgers L, McCombie R, Bigner SH, Giovanella BC, et al. PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science*. 1997; 275:1943–7. [PubMed: 9072974]
- Steck PA, Pershouse MA, Jasser SA, Yung WK, Lin H, Ligon AH, Langford LA, Baumgard ML, Hattier T, Davis T, Frye C, Hu R, et al. Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. *Nat Genet*. 1997; 15:356–62. [PubMed: 9090379]

5. Schmidt EE, Ichimura K, Reifenberger G, Collins VP. CDKN2 (p16/MTS1) gene deletion or CDK4 amplification occurs in the majority of glioblastomas. *Cancer Res.* 1994; 54:6321–4. [PubMed: 7987821]
6. Ueki K, Ono Y, Henson JW, Efird JT, von Deimling A, Louis DN. CDKN2/p16 or RB alterations occur in the majority of glioblastomas and are inversely correlated. *Cancer Res.* 1996; 56:150–3. [PubMed: 8548755]
7. Ghimenti C, Fiano V, Chiado-Piat L, Chio A, Cavalla P, Schiffer D. Deregulation of the p14ARF/Mdm2/p53 pathway and G1/S transition in two glioblastoma sets. *J Neurooncol.* 2003; 61:95–102. [PubMed: 12622447]
8. Ljubimova JY, Khazenon NM, Chen Z, Neyman YI, Turner L, Riedinger MS, Black KL. Gene expression abnormalities in human glial tumors identified by gene array. *Int J Oncol.* 2001; 18:287–95. [PubMed: 11172594]
9. Bork P. The modular architecture of a new family of growth regulators related to connective tissue growth factor. *FEBS Lett.* 1993; 327:125–30. [PubMed: 7687569]
10. Mercurio S, Latinkic B, Itasaki N, Krumlauf R, Smith JC. Connective-tissue growth factor modulates WNT signalling and interacts with the WNT receptor complex. *Development.* 2004; 131:2137–47. [PubMed: 15105373]
11. Brigstock DR. The CCN family: a new stimulus package. *J Endocrinol.* 2003; 178:169–75. [PubMed: 12904165]
12. Perbal B. CCN proteins: multifunctional signalling regulators. *Lancet.* 2004; 363:62–4. [PubMed: 14723997]
13. Planque N, Perbal B. A structural approach to the role of CCN (CYR61/CTGF/NOV) proteins in tumorigenesis. *Cancer Cell Int.* 2003; 3:15. [PubMed: 12969515]
14. Sala-Torra O, Gundacker HM, Stirewalt DL, Ladne PA, Pogossova-Agadjanyan EL, Slovak ML, Willman CL, Heimfeld S, Boldt DH, Radich JP. Connective tissue growth factor (CTGF) expression and outcome in adult patients with acute lymphoblastic leukemia. *Blood.* 2007; 109:3080–3. [PubMed: 17170128]
15. Deng YZ, Chen PP, Wang Y, Yin D, Koeffler HP, Li B, Tong XJ, Xie D. Connective tissue growth factor is overexpressed in esophageal squamous cell carcinoma and promotes tumorigenicity through beta-catenin-T-cell factor/Lef signaling. *J Biol Chem.* 2007; 282:36571–81. [PubMed: 17951630]
16. Kwon S, Munroe X, Crawley SC, Lee HY, Spong S, Bradham D, Gum JR Jr, Sleisenger MH, Kim YS. Expression of connective tissue growth factor in pancreatic cancer cell lines. *Int J Oncol.* 2007; 31:693–703. [PubMed: 17786299]
17. Yang F, Tuxhorn JA, Ressler SJ, McAlhany SJ, Dang TD, Rowley DR. Stromal expression of connective tissue growth factor promotes angiogenesis and prostate cancer tumorigenesis. *Cancer Res.* 2005; 65:8887–95. [PubMed: 16204060]
18. Pan LH, Beppu T, Kurose A, Yamauchi K, Sugawara A, Suzuki M, Ogawa A, Sawai T. Neoplastic cells and proliferating endothelial cells express connective tissue growth factor (CTGF) in glioblastoma. *Neurol Res.* 2002; 24:677–83. [PubMed: 12392205]
19. Xie D, Yin D, Wang HJ, Liu GT, Elashoff R, Black K, Koeffler HP. Levels of expression of CYR61 and CTGF are prognostic for tumor progression and survival of individuals with gliomas. *Clin Cancer Res.* 2004; 10:2072–81. [PubMed: 15041728]
20. Liu LY, Han YC, Wu SH, Lv ZH. Expression of connective tissue growth factor in tumor tissues is an independent predictor of poor prognosis in patients with gastric cancer. *World J Gastroenterol.* 2008; 14:2110–4. [PubMed: 18395916]
21. Chen PP, Li WJ, Wang Y, Zhao S, Li DY, Feng LY, Shi XL, Koeffler HP, Tong XJ, Xie D. Expression of Cyr61, CTGF, and WISP-1 correlates with clinical features of lung cancer. *PLoS ONE.* 2007; 2:e534. [PubMed: 17579708]
22. Jiang WG, Watkins G, Fodstad O, Douglas-Jones A, Mokbel K, Mansel RE. Differential expression of the CCN family members Cyr61, CTGF and Nov in human breast cancer. *Endocr Relat Cancer.* 2004; 11:781–91. [PubMed: 15613452]
23. Alvarez H, Corvalan A, Roa JC, Argani P, Murillo F, Edwards J, Beaty R, Feldmann G, Hong SM, Mullendore M, Roa I, Ibanez L, et al. Serial analysis of gene expression identifies connective

- tissue growth factor expression as a prognostic biomarker in gallbladder cancer. *Clin Cancer Res.* 2008; 14:2631–8. [PubMed: 18451226]
24. Shakunaga T, Ozaki T, Ohara N, Asaumi K, Doi T, Nishida K, Kawai A, Nakanishi T, Takigawa M, Inoue H. Expression of connective tissue growth factor in cartilaginous tumors. *Cancer.* 2000; 89:1466–73. [PubMed: 11013359]
25. Demuth T, Rennert JL, Hoelzinger DB, Reavie LB, Nakada M, Beaudry C, Nakada S, Anderson EM, Henrichs AN, McDonough WS, Holz D, Joy A, et al. Glioma cells on the run - the migratory transcriptome of 10 human glioma cell lines. *BMC Genomics.* 2008; 9:54. [PubMed: 18230158]
26. Xie D, Yin D, Tong X, O'Kelly J, Mori A, Miller C, Black K, Gui D, Said JW, Koeffler HP. Cyr61 is overexpressed in gliomas and involved in integrin-linked kinase-mediated Akt and beta-catenin-TCF/Lef signaling pathways. *Cancer Res.* 2004; 64:1987–96. [PubMed: 15026334]
27. Wang MY, Chen PS, Prakash E, Hsu HC, Huang HY, Lin MT, Chang KJ, Kuo ML. Connective tissue growth factor confers drug resistance in breast cancer through concomitant up-regulation of Bcl-xL and cIAP1. *Cancer Res.* 2009; 69:3482–91. [PubMed: 19351859]
28. Lefranc F, Kiss R. The sodium pump alpha1 subunit as a potential target to combat apoptosis-resistant glioblastomas. *Neoplasia.* 2008; 10:198–206. [PubMed: 18323016]
29. Dhandapani KM, Mahesh VB, Brann DW. Curcumin suppresses growth and chemoresistance of human glioblastoma cells via AP-1 and NFkappaB transcription factors. *J Neurochem.* 2007; 102:522–38. [PubMed: 17596214]
30. Yin D, Zhou H, Kumagai T, Liu G, Ong JM, Black KL, Koeffler HP. Proteasome inhibitor PS-341 causes cell growth arrest and apoptosis in human glioblastoma multiforme (GBM). *Oncogene.* 2005; 24:344–54. [PubMed: 15531918]
31. Kasuga C, Ebata T, Kayagaki N, Yagita H, Hishii M, Arai H, Sato K, Okumura K. Sensitization of human glioblastomas to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) by NF-kappaB inhibitors. *Cancer Sci.* 2004; 95:840–4. [PubMed: 15504253]

D

Fig. 1.

CTGF stimulates cell migration in U343 cells. **(A)** Human CTGF cDNA with HA tag in pcDNA3.1 eukaryotic expression vector was transfected into GBM cell line U343, and stable transfectants were selected for G418 resistance (500 ug/ml). Single colonies were isolated, and CTGF expression was confirmed by western blot. **(B)** Clones of 3 GBM cell lines (U343, U118, and T98G) either without or with stable overexpressed CTGF (5×10^4 /well) were seeded in Boyden chambers, allowed to migrate for 8 h, and quantified as described in the QCM-VN protocol (Chemicon). Number of cells that migrated through the membrane was determined by absorbance at 540 nm. Each bar represents the mean $\pm$ SD of triplicate chambers of three independent experiments. **(C)** U343, U343-V, U343-CTGF-1 and U343-CTGF-3 cells were seeded on plates that had been coated with collagen. After either 15 or 60 mins, cells were washed twice with PBS and stained with crystal violet (Panel C, left figure). Stained cells were solubilized with 10 % acetic acid, and the optical density of the extracts was measured by a spectrophotometer at an OD540 nm (Panel C, right figure). Results represent the mean $\pm$ SD of triplicate wells and experiments were repeated three times. **(D)** U343-V and U343-CTGF-1 cells were plated on chamber slide for 12 hours, fixed and stained with Phalloidin-TRITC for actin filaments and Vinculin for universal focal adhesion (UFA). The stained cells were observed using a confocal microscope. Photograph displays representative cells of the entire population.

Fig. 2. CTGF stimulates growth of GBM cells. **(A)** Cell growth rates of stably over-expressing CTGF clones (U343-CTGF, U87-CTGF, U118-CTGF, and T98G-CTGF) and their controls (U343-V, U87-V, U118-V, T98G-V) were measured by MTT assay. Results represent the mean $\pm$ SD of 3 experiments performed in triplicate. **(B)** Soft agar assays of U343-CTGF (clones -1, -2 and -3). A total of 1000 cells/well were seeded in soft agar for 2 weeks, and colonies were enumerated. Results represent the mean $\pm$ SD of quadruplicate plates of three independent experiments. U343-V cells are control cells with neo vector.

Fig. 3. CTGF supports the long-term survival of GBM cells in serum-free medium. **(A)** After U343-V and U343-CTGF became confluent in DMEM medium with 10% serum, cells were wash three times with PBS followed by culturing in serum-free DMEM medium for 10 days, apoptosis was then measured using Annexin V. Figures show a representative experiment. The % apoptotic (Annexin V, LR) and/or dead (PI, Propidium Iodine, UR/UL) cells are shown. **(B)** On day 30 of serum-free culture, U343-V and U343 CTGF (clone-1 and -2) cells were microscopically examined. **(C)** Cells were stained with crystal violet after 40 days

culture in serum-free medium. The experiments were repeated three times with similar results. **(D)**. Expression of Bcl-x1 proteins were examined by Western blot in U343-CTGF cells, neo-control U343-V and wild type U343 cells cultured in DMEM medium with 10% serum.

A

B

Fig. 4. CTGF produces drug resistance in GBM cells. (A) Expression of DR5 in U343-CTGF clone-1 and -2 and vector control cells (U343-V) was measured using Real-time PCR (normalized using GAPDH). (B) U343-V and U343-CTGF, U87-V and U87-CTGF cells were exposed to TRAIL (20 ng/ml) and/or VELCADE (3×10^{-8} M) for 12 hours, harvested and examined for apoptosis and cell death using Annexin V (LR) and propidium iodine (PI, UR and UL). (C) Western blot was used to detect cleaved PARP protein (lower arrow) after U343-V and U343-CTGF cells were treated either without or with VELCADE (3×10^{-8} M) and TRAIL (20 ng/ml) for 16 hours (left panel). Expression of Survivin, Flip and IκB was detected by Western blot in U343-V and U343-CTGF cells after combined treatment either without or with VELCADE (3×10^{-8} M) and TRAIL (20 ng/ml) for 16 hours. GAPDH was used as an internal loading control (right panel).

Fig. 5.

(A) U343-V and U343-CTGF-1 and -2 cells were treated with TNF-alpha (50–200 ng/ml) for 3 days, stained with crystal violet (up panel), solubilized and absorption was measured by a spectrophotometer at OD540 nm (low panel). Results represent the mean $\pm$ SD of triplicate wells, and three experiments were performed with similar results. (B) U343, U343-V, U343-CTGF-1, U343-CTGF-2 cells were exposed to Temozolomide (25–600 uM) for 3 days, cell growth was measured by MTT assay. Experiments were performed in triplicate; and results represent the mean $\pm$ SD of three experiments. (C) Upper panel: Endogenous

expression of CTGF in GBM cell lines was examined using real-time RT-PCR; human brain microvascular endothelial cells (HBMEC) were used as control (=1) (normalized using GADPH). Middle panel: siRNA against CTGF was transfected into U118 glioma cells, and the expression of CTGF was measured using Western blot. Lower panel: U118 glioma cells with either control siRNA or CTGF siRNA were exposed to Temozolomide (300 or 1000 uM) for 2 days, cell growth was measured by MTT assay. Results expressed as a mean $\pm$ SD of triplicate plates. Experiments were repeated twice with similar results (*, $p < 0.05$).

Fig. 6. CTGF promotes GBM tumorigenicity in nude mice. **(A)** U343-V and U343-CTGF cells ($1 \times 10^6/\text{ml}$) were injected subcutaneously into 8-week-old nude mice; and tumor growth was measured after 7 weeks. Solid line indicates the medium tumor volume ($P < 0.05$). **(B)** Tumors derived from the U343-CTGF and U343-V cells were analyzed immunohistochemically for angiogenesis using monoclonal antibody against CD31. Tumor microvessels (endothelial cells stained brown) are more numerous in the U343-CTGF derived tumors (lower) compared with control U343-V (upper). (Hematoxylin counterstain X150).