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Knockdown of connective tissue growth factor and treatment with temozolomide inhibited invasion, angiogenesis, and tumorigenesis of human glioblastomas

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Abstract

Connective tissue growth factor (CTGF) is a putative proto-oncogene and plays a crucial role in endothelial cell migration and tumor angiogenesis. CTGF is highly upregulated in proliferating endothelial cells and glioblastoma cells. In this investigation, we examined whether knockdown of CTGF at the mRNA level and treatment with temozolomide (TMZ) could inhibit cell invasion, angiogenesis, and growth of human glioblastoma U251MG and LN18 cells in vivo. The cells were stably transfected with a plasmid vector carrying the human CTGF siRNA cDNA and then treated with 10 μM TMZ for 48 h. Semiquantitative PCR, Western blotting, and immunohistochemical staining demonstrated 80% downregulation of CTGF both at mRNA and protein levels after stable transfection. Matrigel invasion, cell migration from spheroids, and cell proliferation studies demonstrated significant inhibition of cell invasion, migration, and proliferation, respectively, of both cell lines after downregulation of CTGF and treatment with TMZ. In vitro and in vivo angiogenesis assays demonstrated inhibition of network formation of endothelial cells and neovascularization under the dorsal skin of nude mice, respectively, after knockdown of CTGF and treatment with TMZ. Both subcutaneous and intracerebral tumorigenesis in nude mice was markedly reduced in CTGF downregulated cells after TMZ treatment. Mechanistic studies demonstrated significant reduction of PCNA, VEGF, c-Myc, CDK2, CDK4, and cyclin D1 and upregulation of the cell cycle inhibitors p21Waf1 and p27Kip1. Flow cytometric analysis showed cell cycle arrest at G2/M phase in both cell lines after CTGF knockdown and TMZ treatment. Taken together, our study indicates that the CTGF knockdown and TMZ treatment effectively prevents cell invasion, migration, angiogenesis, and growth of glioblastomas in vivo. Therefore, CTGF knockdown during TMZ treatment offers a novel and potential therapeutic strategy for controlling the growth of glioblastomas. This investigation was supported in part by the R01 grants (CA-91460 and NS-57811) from the National Institutes of Health (Bethesda, MD).

Introduction

Glioblastomas are the most common and very heterogenous form of malignant brain tumor in adults that causes significant mortality annually. The prognosis of patients with glioblastoma is extremely poor despite multimodal treatments including surgery, chemotherapy, and radiotherapy. The exact etiology of glioblastoma is unknown, but mostly related to genetic factors. Glioblastomas occur due to multiple genetic alterations, which result in the activation of oncogenes and/or the inactivation of tumor suppressor genes. A major challenge in patients with glioblastomas is the propensity of the tumor cells to invade rapidly deep into the surrounding tissues. The current standard of care includes maximal safe surgical resection, followed by a combination of radiation and chemotherapy. Highly invasive glioblastoma cells escape surgical removal and, because of their increased resistance to apoptosis, they are relatively resistant to radiation and chemotherapy. Limitations of current therapeutic regimens warrant development of explicit treatment strategies targeting the specific molecular aberrations that underlie the pathogenesis of glioblastoma. Development of appropriate combination therapeutic strategies involving gene therapy and chemotherapy would help to alleviate the aggressive behavior of this fatal tumor and thereby an effective treatment.

Connective tissue growth factor (CTGF or CCN2) is a cysteine-rich, matrix-associated, heparin-binding, secreted protein that belongs to the CCN family. CTGF is a putative proto-oncogene and plays a crucial role in endothelial cell migration and tumor angiogenesis. CTGF is remarkably upregulated in proliferating endothelial cells and glioblastoma cells. There is growing body of evidence that CTGF regulates cancer cell migration, invasion, angiogenesis, and tumor progression. CTGF mRNA levels were elevated in primary glioblastomas, and high levels of CTGF mRNA were directly correlated with advanced tumor stage and less differentiation and patient survival. Forced expression of CTGF in glioblastoma multiforme (GBM) cells accelerated their growth in liquid culture and soft agar, stimulated cells migration in Boyden chamber and significantly increased their ability to form large, vascularized tumors in nude mice. Appropriate strategies to downregulate CTGF would be beneficial to improve the efficacy of current therapeutic agents and/or for the effective treatment of glioblastomas.

Temozolomide (TMZ) is an alkylating agent , which is currently in use for the treatment of GBM. TMZ alkylates/methylates DNA, which often occurs at the N-7 or O-6 positions of guanine residues and damages DNA and triggers tumor cell death. However, certain tumor cells are able to repair TMZ mediated DNA damage by expressing O-6-alkylguanine-DNA alkyltransferase (MGMT) or O-6-alkylguanine-DNA alkyltransferase (AGT or AGAT). Epigenetic silencing of the MGMT gene by promoter methylation compromises DNA repair and has been associated with longer survival in glioblastoma patients who receive TMZ. The TMZ induced autophagy of glioblastoma cells is associated with the upregulation of several molecules including CTGF. Overexpression 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), and TMZ. Knockdown of Galectin-1 expression in Hs683 orthotopic xenografts by siRNA administration caused endoplasmic reticulum stress and enhanced the therapeutic effects of TMZ.

Small interfering RNAs (siRNAs) can silence the expression of a particular gene by its complementary binding and cleavage of mRNA, which results in significant disruption of translation of that particular gene. We have demonstrated effective knockdown of several upregulated genes in glioblastoma cell lines as well as in glioblastoma orthotopic xenograft models. The present study was aimed to knockdown the upregulated CTGF and simultaneous treatment with TMZ in two highly invasive human glioblastoma cell lines, U251MG and LN18, and to examine whether such a combination could inhibit cell invasion, angiogenesis, and tumor growth in subcutaneous and orthotopic xenograft models.

Figure 1

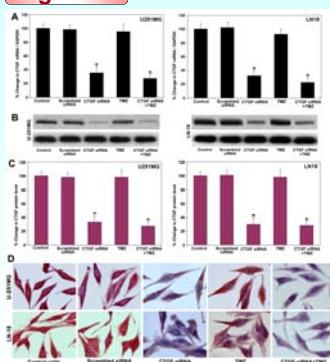

Figure 1. Percentage changes in CTGF mRNA and protein levels in U251MG and LN18 cells after transfection with a plasmid encoding CTGF siRNA cDNA or treatment with 10 μM TMZ or both agents together for 48 h. **(A)** Quantitative RT-PCR analysis for CTGF mRNA. Values are mean ± S.D. of 6 assays in each group (*p<0.001 compared to the control mean values). **(B)** Western blotting for CTGF. The blots were reprobed for GAPDH content to demonstrate that all samples were loaded with equal amount of proteins. The data are representative of 6 independent experiments. **(C)** Quantitative evaluation of Western blotting. Values are mean ± SD of 6 assays in each group (*p<0.001 compared to the control mean values). **(D)** Immunohistochemical staining for CTGF after transfection with a plasmid encoding CTGF siRNA cDNA or treatment with TMZ or both agents together for 48 h. Staining for CTGF was completely absent in cells transfected with CTGF siRNA.

Figure 2

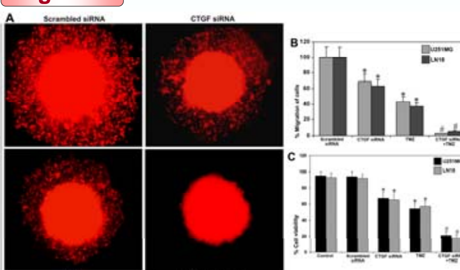

Figure 2. **(A)** Inhibition of cell migration from spheroids after transfection with CTGF siRNA or treatment with 10 μM TMZ or both agents combined. Spheroids were prepared after treatment from U251MG and LN18 cells stably expressing RFP, transferred to a 24-well chamber, and allowed to migrate for 24 h. The migration of cells from the spheroids were observed under a fluorescent microscope and photographed. **(B)** Percentage migration of cells from the center of the spheroids to the monolayers. The data represented as mean ± SD of 6 independent experiments from each group (*p<0.001 compared to the scrambled siRNA mean values and #p<0.001 compared to CTGF siRNA or TMZ mean values). **(C)** MTT assay for cell viability. MTT assay was performed in U251MG and LN18 cells after transfection with CTGF siRNA or treatment with temozolomide or both together for 48 h. Data are mean ± SD of 6 independent experiments in duplicate (*p<0.001 compared to the control mean values and #p<0.001 compared to CTGF siRNA or TMZ mean values).

Figure 5

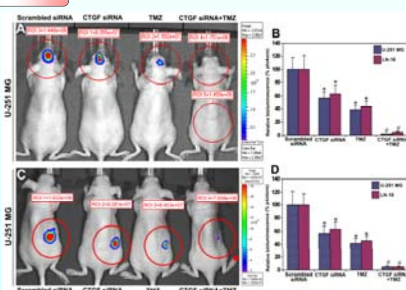

Figure 5. Inhibition of intracerebral **(A)** and subcutaneous **(C)** tumorigenesis in immunosuppressed mice after treatment with CTGF siRNA and/or TMZ. U251MG and LN18 cells were stably transfected with luciferase gene and injected into the intracerebrum (1x10⁶ cells) or in the subcutaneous area (2x10⁶ cells) of mice. Beginning from day 7, the mice were injected either with CTGF siRNA plasmid vector (5 μg DNA/injection/mouse) or TMZ (10 μg/injection/mouse) or both agents together until day 20 on alternate days at the site of tumor cell implantation. On day 21, the mice were injected intraperitoneally with 100 μl (50 mg/ml) of luciferin and the resultant bioluminescence was visualized for the effect of treatments using Xenogen IVIS–200 imaging system. The data are representative of 6 sets of mice in each group. The background bioluminescence signal from an untreated mouse is about 1.5e+05 photons on Xenogen IVIS-200 imaging machine. **(B & D)** Quantitative representation of relative bioluminescence as percentage photons after treatment with CTGF siRNA and/or TMZ in intracerebral and subcutaneous tumorigenesis, respectively. Data are mean ± S.D. of 6 animals in each group (*P < 0.001 compared to the scrambled siRNA mean values and #P < 0.001 compared with CTGF siRNA or TMZ mean values).

Figure 3

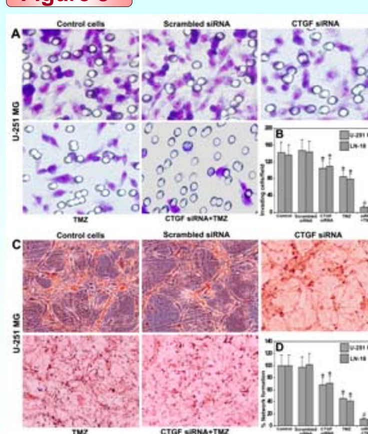

Figure 3. **(A)** Tumor cell invasion assay for U251MG and LN18 cells transfected with a plasmid encoding CTGF siRNA or treated with 10 μM TMZ or both agents together. Invasion assays were carried out in 12-well transwell inserts of polycarbonate filters with 12.0 μm pores coated with 200 μl of 0.1% matrigel. After a 48 h incubation period, the transwell membranes were collected and stained. A significant reduction in the number of invaded cells indicates the decreased invasive potency of the treated cells. **(B)** Quantitative evaluation of matrigel invasion assay. The data are mean ± SD of 10 randomly selected microscopic fields from three independent wells in each group (*p<0.001 compared to the control mean values and #p<0.001 compared to CTGF siRNA or TMZ mean values). **(C)** Effect of CTGF siRNA and/or temozolomide on in-vitro network formation of HME cells. U251MG and LN18 cells on chamber slides were transfected with CTGF siRNA or treated with TMZ or both agents together. After 24 h, HME cells were co-cultured. The cultures were terminated at 72 h of co-culture, and stained for Von Willebrand factor immunohistochemically and photographed **(D)** Quantitative representation of in-vitro network formation by HME cells. The relative networking was measured using Image-pro Discovery software. Values are mean ± S.D. of 6 samples in each group of both U251MG and LN18 cells (*p<0.001 compared to the control mean values and #p<0.001 compared to CTGF siRNA or TMZ mean values).

Figure 4

Figure 4. **(A)** In vivo angiogenesis assay. U251MG and LN18 cells transfected with a plasmid encoding CTGF siRNA cDNA or treated with TMZ or both agents together were suspended in 200 μl of serum-free medium, injected into a diffusion chamber, implanted under the dorsal skin of nude mice and left for 10 days. Strong development of new microvessels (arrows, TN) arising from pre-existing vessels as curved thin structures in zigzag pattern was observed in U251MG and LN18 control and scrambled siRNA transfected cells. The formation of such microvasculature was considerably reduced in both CTGF siRNA and TMZ treated cells and almost completely inhibited after combination treatment with both agents. **(B)** Quantitative representation of in vivo angiogenesis. Tumor-induced neovasculature was measured with the help of an ocular micrometer. Values are mean ± SD of 6 samples from each group (*p<0.001 compared to the scrambled siRNA mean values and #p<0.001 compared to CTGF siRNA or TMZ mean values). *TN: tumor-induced neovasculature; PV: pre-existing vasculature.* **(C)** An immunocompromised mice bearing a diffusion chamber implanted under the dorsal skin for the assay of in vivo angiogenesis.

Figure 6

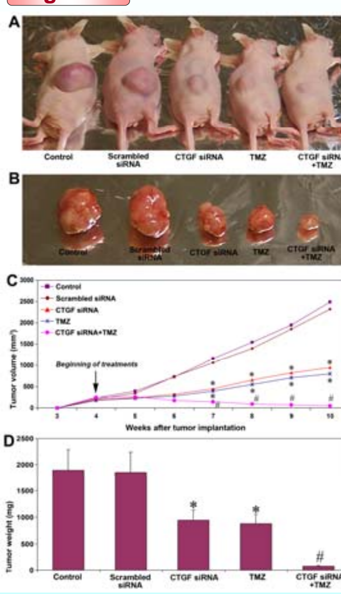

Figure 6. **(A)** Inhibition of subcutaneous solid tumor development in nude mice after the treatment with CTGF siRNA and/or TMZ. LN18 cells were harvested, counted, suspended in an equal volume of high concentrated Matrigel and 100 μl of the suspension (5x10⁶ cells) was injected under the dorsal skin of nude mice. The animals were left for 4 weeks without any treatment for uniform development of visible tumors. Afterwards, the mice were injected at the tumor site with either CTGF siRNA plasmid vector (50 μg DNA/injection/mouse) or TMZ (100 μg/injection/mouse) or both agents together on alternate days for 6 weeks. At the end of the 10th week, the animals were anesthetized with ketamine and xylazine and then photographed. **(B)** The tumors were surgically removed, weighed, and photographed. The data are representative of 6 sets of mice in each group. **(C)** Longitudinal measurement of tumor volume in nude mice using a digital vernier caliper after the combination treatment with CTGF siRNA and/or TMZ. Data are mean ± SD of 6 animals in each group. (*P<0.001 compared to the control mean values and #P<0.001 compared to CTGF siRNA or TMZ mean values). **(D)** Quantitative representation of tumor weight. Data are mean ± SD of 6 animals in each group (*P<0.001 compared to the control mean values and #P<0.001 compared to CTGF siRNA or TMZ mean values).

Conclusions

- *Knockdown of CTGF using cognate siRNA resulted in about 70% decrease in CTGF mRNA and protein levels in both U-251MG and LN-18 cells.*
- *Combination treatment with CTGF siRNA and TMZ resulted in inhibition of cell migration from spheroids and decreased cell viability.*
- *Combination treatment with CTGF siRNA and TMZ resulted in marked decrease of tumor cell invasion on matrigel.*
- *Combination treatment with CTGF siRNA and TMZ resulted in inhibition of both in vitro and in vivo angiogenesis.*
- *Simultaneous administration of CTGF siRNA and TMZ significantly decreased orthotopic and subcutaneous tumorigenesis in nude mice.*
- *Combination treatment with CTGF siRNA and TMZ resulted in marked decrease in solid tumor growth in the subcutaneous of nude mice.*

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