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

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Figure 3 Figure 4

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 the temozolomide (TMZ) or both could inhibit cell invasion, angiogenesis, and growth of human glioblastomas U251MG and LN18 cells in vivo. The cells were stably transduced with human CTGF siRNA DNA and then treated with 10 mM TMZ for 48 h. Semi-quantitative 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, both subcutaneously and orthotopically. 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 inhibitor p21 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-91404 and NS-57811) from the National Institutes of Health (Bethesda, MD).

Introduction

Glioblastomas are the most common and very heterogenous form of malignant brain tumors in adults that cause significant mortality and morbidity. The prognosis of patients with glioblastomas 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 specific genetic alterations, which result in the activation of oncogenes and/or in the inactivation of tumor suppressor genes. A major challenge in patients with glioblastomas is the propensity of the tumor 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. Higher invasive glioblastoma cells escape surgical removal and, because of their increased resistance to apoptosis, they are relatively resistant to radiation and chemotherapy. The current therapeutic regimens warrant development of explicit treatment strategies targeting the specific molecular alterations that underlie tumorigenesis. The current standard of care includes maximal safe surgical resection, followed by a combination of radiation and chemotherapy. Higher invasive glioblastoma cells escape surgical removal and, because of their increased resistance to apoptosis, they are relatively resistant to radiation and chemotherapy. The current therapeutic regimens warrant development of explicit treatment strategies targeting the specific molecular alterations that underlie tumorigenesis.

Results

Figure 2

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. In this investigation, we examined whether knockdown of CTGF at the mRNA level and treatment with the temozolomide (TMZ) or both could inhibit cell invasion, angiogenesis, and growth of human glioblastomas U251MG and LN18 cells in vivo. The cells were stably transduced with human CTGF siRNA DNA and then treated with 10 mM TMZ for 48 h. Semi-quantitative 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, both subcutaneously and orthotopically. 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 inhibitor p21 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-91404 and NS-57811) from the National Institutes of Health (Bethesda, MD).

Figure 3

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 (OGT) or O-6-alkylguanine DNA alkyltransferase (MGMT) or O-6-alkylguanine-DNA alkyltransferase (ALTR) or ALGAT. 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 alkylation of glioblastoma cells is associated with the upregulation of several molecules including CTGF. Overexpression of CTGF caused the U87 GBM cells to survive for longer. The data indicate that TMZ induced alkylating DNA lesions, which can be repaired by OGG1, leading to cell death. Therefore, free mediators and resistant tumor cells may have increased resistance to TMZ. The present study aimed to knockdown the upregulated CTGF and simultaneous treatment with TMZ in two highly invasive glioblastoma cell lines, U251MG and LN18, and to examine whether such a combination could inhibit cell invasion, angiogenesis, and tumor growth in in vitro and orthotopic xenograft models.

Small interfering RNAs (siRNAs) can silence the expression of a particular gene by its complementary binding and cleavage of mRNA, 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 (OGT) or O-6-alkylguanine DNA alkyltransferase (MGMT) or O-6-alkylguanine-DNA alkyltransferase (ALTR) or ALGAT. 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 alkylation of glioblastoma cells is associated with the upregulation of several molecules including CTGF. Overexpression of CTGF caused the U87 GBM cells to survive for longer. The data indicate that TMZ induced alkylating DNA lesions, which can be repaired by OGG1, leading to cell death. Therefore, free mediators and resistant tumor cells may have increased resistance to TMZ. The present study aimed to knockdown the upregulated CTGF and simultaneous treatment with TMZ in two highly invasive glioblastoma cell lines, U251MG and LN18, and to examine whether such a combination could inhibit cell invasion, angiogenesis, and tumor growth in in vitro and orthotopic xenograft models.

Figure 4

Figure 5

Figure 6

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.
- Gene silencing treatment with CTGF siRNA and TMZ resulted in inhibition of cell migration from spheroids and decreased cell proliferation.
- Combination treatment with CTGF siRNA and TMZ resulted in marked decrease of tumor cell migration on matrigel.
- Combination treatment with CTGF siRNA and TMZ resulted in inhibition of cell invasion in vitro and in vivo angiogenesis.
- Temozolomide treatment with CTGF siRNA attenuated tumor angiogenesis and subcutaneous xenograft tumorigenesis in nude mice.
- Combination treatment with CTGF siRNA and TMZ resulted in indicated decrease in solid tumor growth in the subcutaneous xenograft of nude mice.