

Combination treatment with temozolomide and thalidomide inhibits tumor growth and angiogenesis in an orthotopic glioma model

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Abstract. The chemotherapeutic agent temozolomide (TMZ) and the anti-angiogenic agent thalidomide (THD) have both demonstrated anti-tumor activity in patients with recurrent malignant glioma. Combination treatment with TMZ and THD in patients with glioblastoma multiforme (GBM) appears to be more effective than treatment with either drug alone. To investigate the mechanism of this anti-tumor effect, we examined the combined effects of TMZ and THD in a rat glioma xenograft model. We found that combination treatment markedly inhibited the growth of tumors that were orthotopically implanted into rat brains. Using proliferating cell nuclear antigen (PCNA) staining, we observed a significant decrease in cell proliferation in these tumors. CD31 staining of the microvasculature revealed a significant decrease in angiogenesis. We also found increased apoptosis in treated tumors by terminal deoxynucleotidyl-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay. We further demonstrated that the expression of angiogenic factors, such as vascular endothelial cell growth factor (VEGF) and basic fibroblastic growth factor (bFGF), were inhibited by THD. THD also decreased the number of ED1-positive, activated macrophages or microglial cells, which produce pro-angiogenic molecules around the glioma. Taken together, these results

suggest that combination treatment with TMZ and THD inhibits tumor growth via the induction of apoptosis and the inhibition of angiogenesis in a rat model and may be a promising therapy for malignant gliomas.

Introduction

Malignant gliomas are the most common primary brain tumors associated with a high degree of morbidity and mortality (1). Gliomas are non-metastasizing but locally infiltrating, highly invasive, hypervascularized tumors with a poor prognosis (2). The current management of gliomas is based on a combination of surgery, radiation therapy, and chemotherapy (3). Despite this multidisciplinary approach, gliomas remain difficult to treat. Recently, molecular mechanisms of tumor progression have been clarified, and such studies have led to the re-evaluation of chemotherapies and the development of new drugs. Many agents are now being tested in phase I and II clinical trials and have shown some promising results (3-5).

Thalidomide (THD), which fell out of favor because of severe teratogenic side effects, has been shown to have anti-angiogenic properties (6). It has been tested in a number of preclinical animal models of different tumors, with variable effects on tumor growth, metastasis, and angiogenesis (7). High-grade gliomas are a potentially responsive target for anti-angiogenic therapies. These tumors are highly vascularized, apparently through the acquisition of a number of genetic alterations that enable them to overexpress inducers of angiogenesis or to down-regulate natural angiogenesis inhibitors (8).

Even tumors initially responsive to chemotherapy generally develop chemoresistance upon recurrence of the disease (9). Recent clinical trials have indicated that combination therapy has promise for overcoming resistance in the treatment of patients with gliomas (3). THD has minimal effects on tumor growth in patients with recurrent high-grade gliomas [5-6% partial response (10)]; however, THD may provide additive or synergistic anti-tumor effects when given concurrently with

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temozolomide (TMZ). Such synergy between anti-angiogenic and cytotoxic agents has been reported (11,12). TMZ, an imidazotetrazine-derived DNA alkylating agent, exhibits broad-spectrum anti-tumor activity and has shown significant penetration into the CSF and brain tissue (3). The combination of TMZ and THD was evaluated in a phase II study, where it demonstrated anti-tumor activity that was significantly more potent than either agent alone. It also had a favorable safety profile with minimal non-hematologic toxicities (3). However, the mechanisms of action of THD and TMZ on tumor growth and angiogenesis have not been established.

In the present study, we evaluated the effects and mechanisms of action of THD and TMZ on tumor growth and angiogenesis, examining each drug alone and in combination treatment, in a rat orthotopic brain tumor model using the C6 glioma cell line.

Materials and methods

Cell culture. C6/LacZ rat glioma cells (ATCC) were grown in DMEM (Cambrex) supplemented with 10% fetal bovine serum and antibiotics (Life Technologies).

Animals and orthotopic implantation of tumor cells. Specific pathogen-free Sprague Dawley (SD) rats were used. All animal work was approved by the Animal Care and Use Committee of Samsung Medical Center. Orthotopic implantation of glioma cells was performed as described previously (13). Briefly, male SD rats (200-250 g) were anesthetized and shaved. They were secured in a rodent stereotactic frame, and a hollow guide screw was implanted into a small drill hole made 3 mm left lateral and 1 mm anterior to the bregma (14). Tumor cells (1×10^5 cells/ $10 \mu\text{l}$) were injected through this guide screw into the white matter at a depth of 5 mm.

Drug administration. Temozolomide (Temodal) and thalidomide were obtained from Schering-Plough and Sigma, respectively, and both were dissolved in 10% DMSO. One day after intracranial implantation, the rats were randomized into four groups ($n=14$ per group). THD (16 mg/kg) was then administered per oral every day for 17 days, and TMZ (7.5 mg/kg) was administered intraperitoneally for 5 days, between the 7th and 11th day after tumor cell inoculation.

Harvesting of specimens. Seventeen days after inoculation of tumor cells, the rats were sacrificed, and the brains were removed and sectioned axially. One section was fixed in 10% buffered formalin and embedded in paraffin, and the other was embedded in OCT compound (Miles, Inc.), frozen rapidly in liquid nitrogen, and stored at -70°C . The tumor volume was calculated by measuring the section with the largest tumor portion and applying the formula: $\text{width}^2 \times \text{length} \times 0.5$.

Immunohistochemistry. Immunohistochemistry was performed as described previously (13). For PCNA, the sections were stained with mouse anti-PCNA, clone PC10 (Dako). The DeadEnd fluorometric TUNEL system (Promega) was used to assay apoptosis. Mouse anti-rat CD31/PECAM-1 (BD Pharmingen), rabbit anti-VEGF (Santa Cruz Biotechnology), rabbit anti-bFGF (Sigma Chemical Co.), and mouse anti-rat

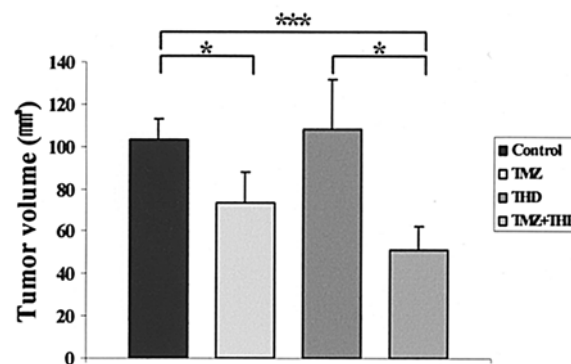


Figure 1. Combination treatment with temozolomide and thalidomide inhibits tumor growth in an orthotopic rat glioma model. C6 rat glioma cells were orthotopically inoculated into the brains of rats. Oral THD (16 mg/kg) was administered daily for 17 days, starting on the day after intracranial implantation. TMZ (7.5 mg/kg) was intraperitoneally administered daily between the 7th and 11th day after tumor cell inoculation. Tumor volumes are shown as the mean $\pm$ SE from 13 to 14 rats. * $P<0.05$ and *** $P<0.001$.

macrophage (ED1, Serotec) antibodies were used to stain their respective antigens.

Quantification of immunostaining. For the quantification of immunostaining for PCNA and TUNEL assay, the number of stained cells was counted in ten random fields at $\times 400$ magnification. For the CD31 and ED1, the number of stained cells was counted in ten random fields at $\times 200$ magnification. To quantify the immunohistochemical reaction intensities for bFGF and VEGF, the absorbance of stained cells was measured using the Optima image analysis software.

Data analysis and statistics. Values are presented as means $\pm$ SE or $\pm$ SD. Statistical comparisons between groups were performed using Student's t-test, ANOVA, or multiple comparison tests. Values of $P<0.05$ were considered statistically significant.

Results

Combination treatment with TMZ and THD inhibits tumor growth in an orthotopic rat glioma model. To determine the effects of combination treatment with TMZ and THD, we monitored tumor size after treatment in an orthotopic rat glioma model (Fig. 1). The C6 rat glioma cells were injected into the white matter of SD rat brains. The rats received oral THD daily for 17 days following the intracranial implantation of tumor cells and received TMZ intraperitoneally for 5 days, from day 7 through day 11 post-implantation. There were no signs of drug toxicity, such as weight loss (data not shown). The average tumor volume in TMZ-treated rats was 29% smaller than that in control rats ($P=0.017$) (Fig. 1). The tumor volume in THD-treated rats was not altered, but the average volume in TMZ and THD combination-treated animals was 50% smaller than that of controls ($P=0.0003$). The volume was also significantly reduced relative to THD alone ($P=0.017$). These results demonstrate that the combination treatment of TMZ and THD was more effective in tumor growth inhibition than either TMZ or THD alone in this model, suggesting synergistic effects ($P=0.038$).

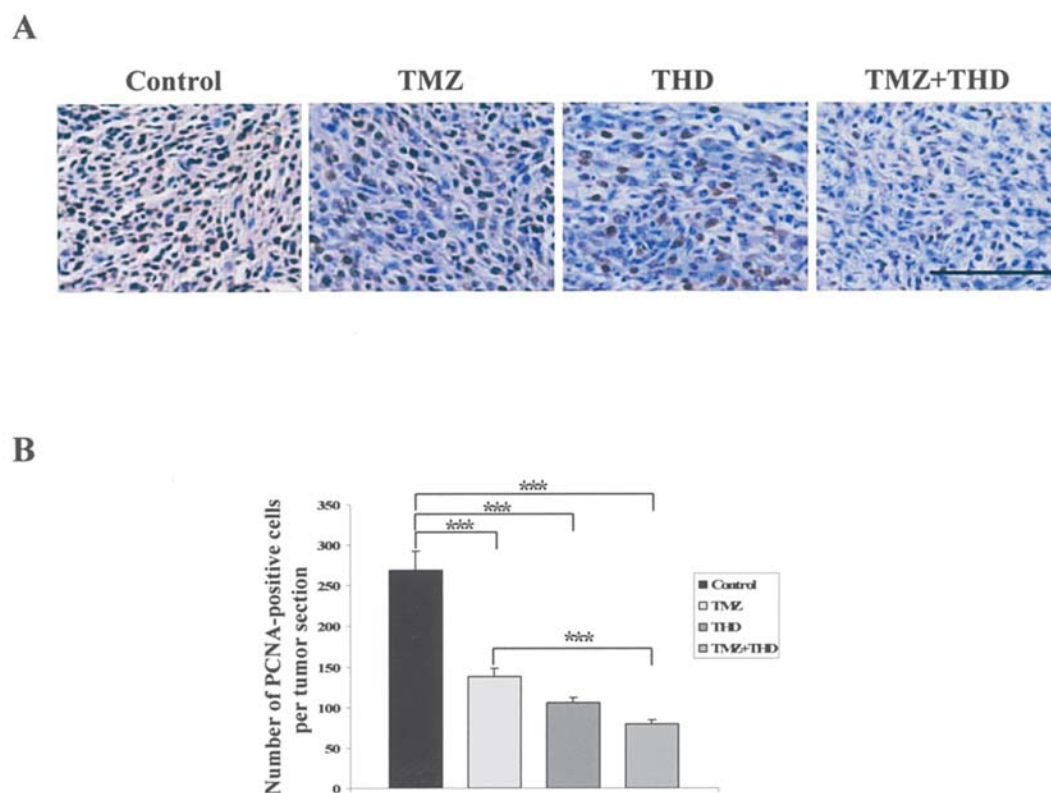


Figure 2. Combination treatment with temozolomide and thalidomide inhibits cell proliferation in glioma xenografts. A, Paraffin sections of tumors stained for proliferating cells using an anti-PCNA antibody. Scale bar: 100 μ m. B, Mean numbers of PCNA-positive cells are shown (n=10). ***P<0.0001.

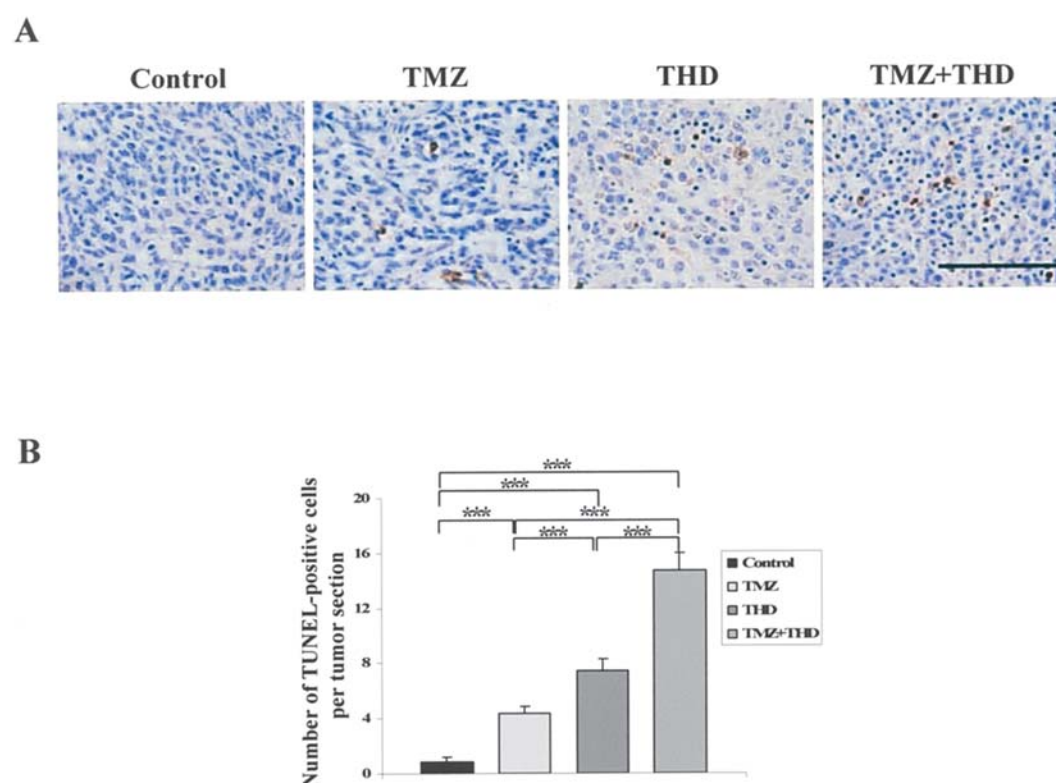


Figure 3. Combination treatment with temozolomide and thalidomide increases apoptosis in glioma xenografts. A, Paraffin sections of the tumors were stained for apoptotic cells using TUNEL assay. Scale bar: 100 μ m. B, Mean numbers of TUNEL-positive cells are shown (n=10). ***P<0.0001.

Combination treatment of TMZ and THD inhibits cell proliferation and induces apoptosis. As the combination treatment inhibited tumor mass, we examined cell proliferation

and apoptosis in this model by immunostaining for PCNA (Fig. 2) and by TUNEL assay (Fig. 3), respectively. The number of strongly stained PCNA-positive cells (Fig. 2A)

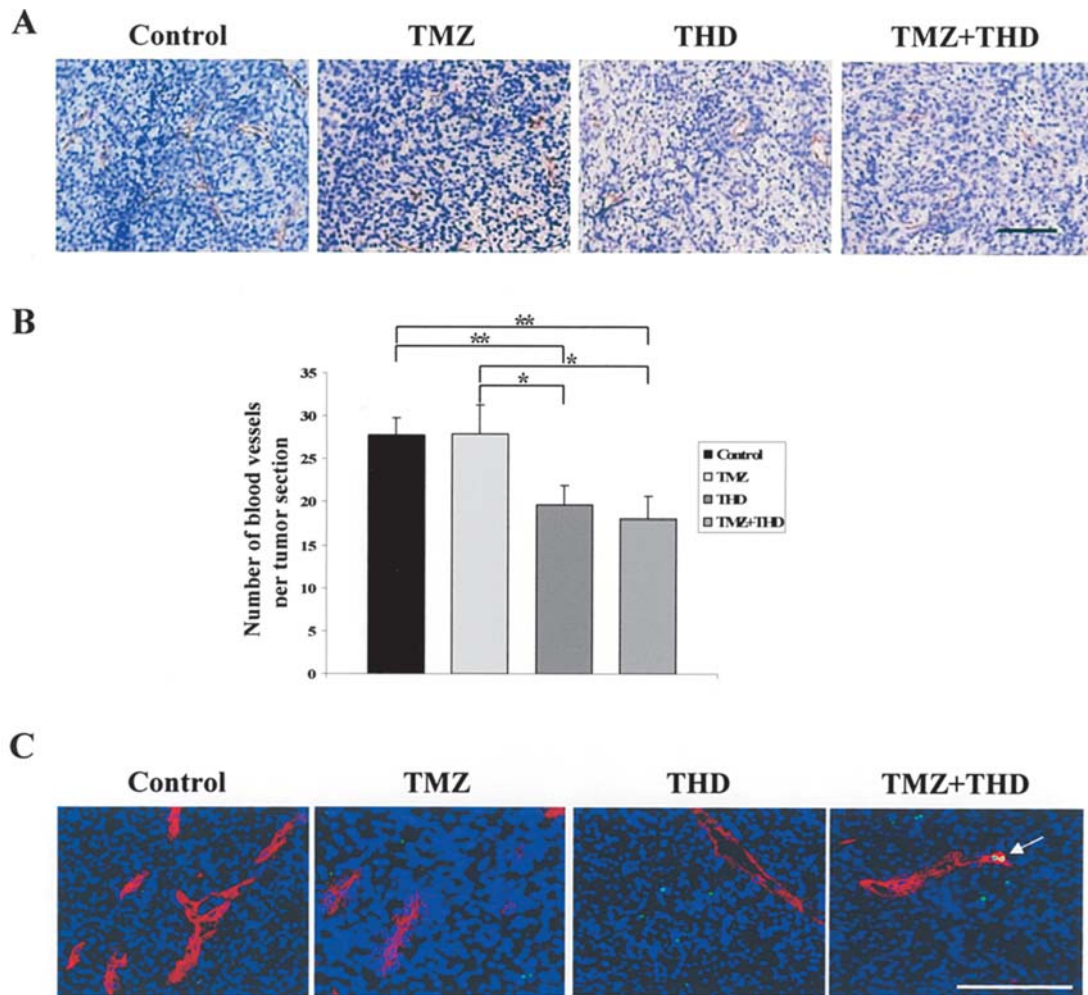


Figure 4. Combination treatment with temozolomide and thalidomide inhibits microvessel density and increases endothelial apoptosis in glioma xenografts. A, Frozen sections of tumors were stained for endothelial cells using anti-CD31 antibody. Scale bar: 100 μ m. B, Mean numbers of blood vessels are shown (n=10). * $P<0.05$ and ** $P<0.01$. C, Immunofluorescence staining for blood vessels (red), apoptotic cells (green), and nuclei (blue). Apoptotic endothelial cells are seen as yellow in merged images (white arrow).

seen in control tumors was reduced in both TMZ- and THD-treated rats, by 48% ($P<0.0001$) and by 61% ($P<0.0001$) relative to controls, respectively. The combination of TMZ and THD dramatically decreased the number of PCNA-positive cells by 70% ($P<0.0001$) (Fig. 2B).

The number of apoptotic cells stained in the TUNEL assay (Fig. 3A) was increased by treatment with either TMZ (5.2-fold increase, $P=0.0002$) or THD (8.9-fold, $P<0.0001$) compared with controls. The combination of TMZ and THD increased the number of apoptotic cells 17.6-fold compared with controls ($P<0.0001$) (Fig. 3B). Synergistic effects between TMZ and THD were observed in proliferation ($P=0.0003$), but not in apoptosis.

Combination treatment with TMZ and THD inhibits angiogenesis. Given that gliomas are hypervascularized brain tumors and are dependent upon angiogenesis for their growth, we examined the anti-angiogenic potential of the combination treatment, using immunohistological staining of CD31 for vessel detection (Fig. 4). With THD treatment, the number of CD31-stained vessels (Fig. 4A) was decreased by 29% compared with controls ($P=0.003$), while tumors treated with TMZ showed a number of blood vessels similar to that of

controls. TMZ and THD in combination decreased by 35% the number of blood vessels stained with CD31 ($P=0.004$) (Fig. 4B). Moreover, immunofluorescence staining for CD31 and TUNEL showed that the combination treatment induced apoptosis of endothelial cells as well as tumor cells (Fig. 4C).

THD inhibits the expression of VEGF and bFGF and reduces the number of activated microglia/monocytes. We examined the expression of angiogenic factors and the presence of activated macrophages or microglial cells by immunostaining. Such activated cells in the vicinity of necrotic tumors induce inflammation and angiogenesis. The major angiogenic activators, VEGF and bFGF, were both strongly expressed in control tumors (Fig. 5). The expression of each was dramatically decreased by THD treatment ($P<0.0001$).

Activated microglia were detected by immunostaining with ED1 (Fig. 6). ED1-positive cells were detected in control tumors and were somewhat increased by TMZ treatment. Treatment with THD or with the combination of TMZ and THD led to an 82% ($P<0.0001$) or 50% ($P<0.0001$) decrease in the number of ED1-positive cells relative to controls, respectively. The combination treatment produced a 43%

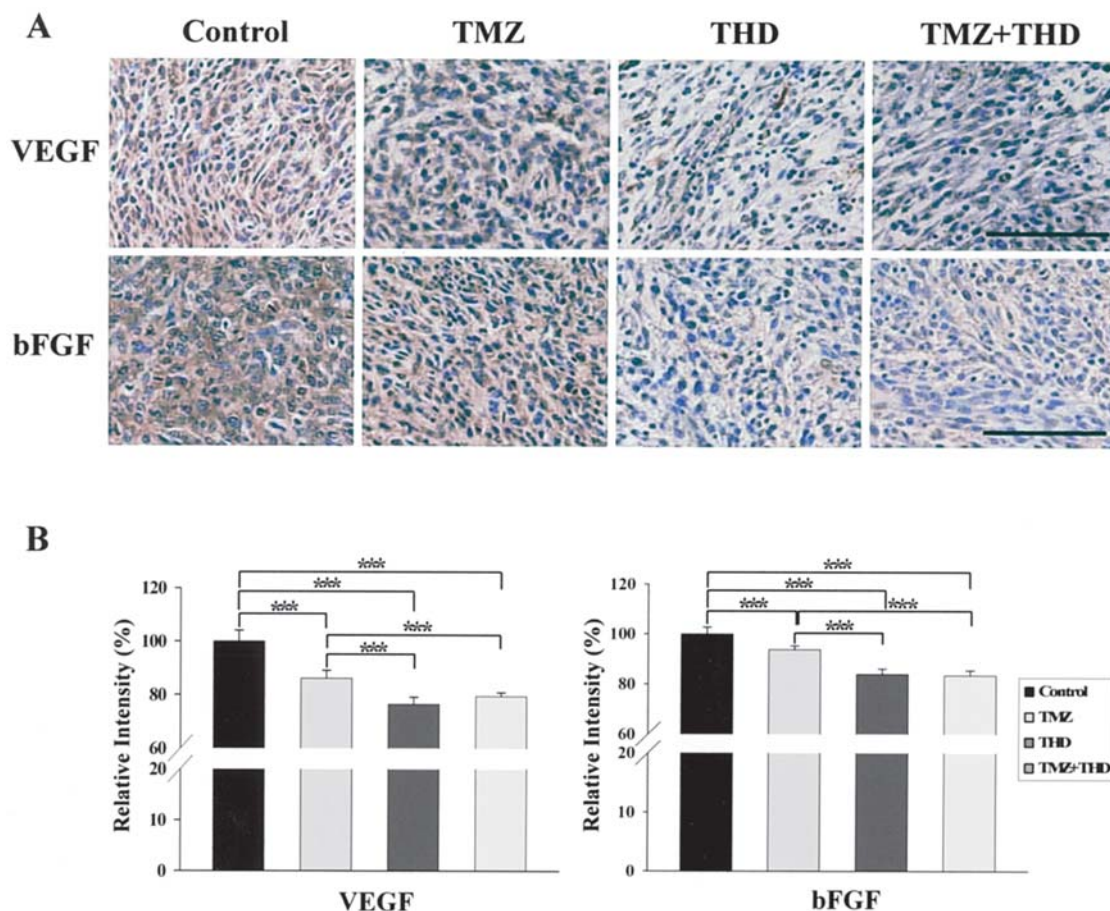


Figure 5. THD inhibits the expression of VEGF and bFGF in glioma xenografts. A, Frozen sections of tumors were stained with anti-VEGF or anti-bFGF antibodies. Scale bar: 100 μ m. B, Absorbance of stained cells was measured, and the immunohistochemical reaction intensity relative to controls is represented (%). ***P<0.0001.

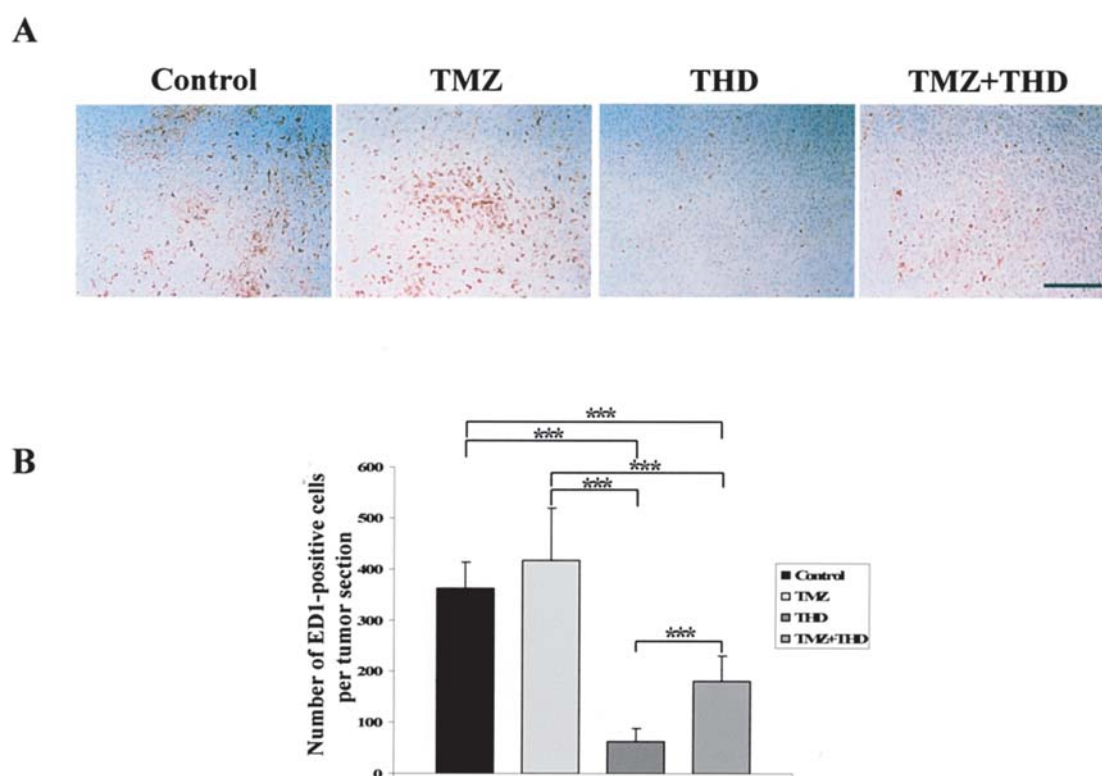


Figure 6. THD reduces the number of activated microglia in glioma xenografts. A, Paraffin sections of tumors were stained for activated microglia using anti-ED1 antibody. Scale bar: 100 μ m. B, Mean numbers of ED1-positive cells are shown (n=10). ***P<0.0001.

decrease in the number of ED1-positive cells compared with the number produced by TMZ treatment alone ($P=0.0002$).

Taken together, these results indicate that THD decreased the expression of both VEGF and bFGF. The inhibition of angiogenesis seen with THD might be, at least in part, associated with an observed decrease in ED1-positive cells, which produce pro-inflammatory molecules, including TNF- α .

Discussion

Data obtained in this study of an orthotopic rat glioma model indicate that the combination of TMZ and THD significantly inhibits tumor growth compared with the effect of either agent alone. The mechanism by which such potentiation is achieved is multifactorial, involving the inhibition of cell proliferation, the stimulation of apoptosis, and the inhibition of angiogenesis.

Treatment with THD alone significantly decreased the number of PCNA-positive proliferating cells (Fig. 2), increased the number of TUNEL-positive apoptotic cells (Fig. 3), and decreased microvascular density (Fig. 4). However, THD alone was not sufficient to suppress tumor growth (Fig. 1). These results are consistent with those of others; THD treatment did not significantly alter tumor growth in neuroblastoma xenografts (7) and, while it could stabilize recurrent GBM, THD did not have a dramatic effect on tumor growth in phase I trials (3-5). We found that, instead, THD inhibited tumor angiogenesis (Fig. 4), as other studies have found (6,7), and also inhibited the expression of angiogenic activators (Fig. 5) and reduced the number of activated microglia (Fig. 6). This indicates that THD is cytostatic and might thereby improve the cytotoxic effects of TMZ in combination treatment.

TMZ shows promise as an effective chemotherapeutic agent for the treatment of patients with malignant glioma (3). We found that treatment with TMZ alone significantly decreased the number of PCNA-positive proliferating cells (Fig. 2) and increased the number of TUNEL-positive apoptotic cells (Fig. 3). TMZ alone differed from THD in that it caused the inhibition of tumor growth (Fig. 1) without the inhibition of angiogenesis (Fig. 4).

ED1-positive cells were detected in necrotic areas of control tumors (Fig. 6). ED1 is a marker for activated macrophages and microglia (15), and the implanted tumor itself induces humoral and cell-mediated host immune responses (16). The cytotoxicity of TMZ may induce immune responses and inflammation, as the number of activated microglia was somewhat increased by treatment with TMZ.

Aggressive tumor growth is associated with increased expression of pro-inflammatory and pro-angiogenic factors (17). Inflammatory cells fully participate in the angiogenic process by secreting cytokines/chemokines such as IFN- γ , IL-2, and tumor necrosis factor- α (TNF- α) that may affect endothelial cell function (18-20). TNF- α up-regulates the expression of potent angiogenic factors such as VEGF, bFGF, IL-8, metalloproteinases, and plasminogen activators in endothelial cells and thereby induces angiogenesis (21). Recently, it was reported that drugs having anti-inflammatory effects, such as curcumin and cyclooxygenase-2 (COX-2) inhibitors, inhibit tumor growth and angiogenesis (22,23). Curcumin, a pharmacologically safe chemopreventive agent, has been shown to suppress NF- κ B activation induced by various

inflammatory stimuli and to suppress COX-2 expression (22). COX-2 is a key enzyme in inflammatory cytokine-induced angiogenesis (24). It was also reported that selective COX-2 inhibitors decreased the expression of VEGF (25) and suppressed bFGF-induced angiogenesis (26). We have also reported that the COX-2 inhibitor celecoxib inhibits glioma cell growth via the inhibition of the EGF signaling pathway and the induction of apoptosis in an orthotopic glioma model.

THD inhibits the inflammation induced by aggressive tumor growth and the cytotoxicity of TMZ and thereby suppresses angiogenesis by the inhibition of pro-inflammatory and pro-angiogenic factors. THD has shown potent anti-inflammatory activity through the inhibition of TNF- α synthesis by activated monocytes (27). THD might be a good candidate in therapy for hypervascular tumors that are accompanied by necrosis, such as glioblastomas.

Treatment with TMZ alone did not decrease the expression of VEGF or bFGF, and TMZ in combination with THD reduced the inhibitory effect of THD on this expression (Fig. 5). The combination of TMZ and THD did, however, induce endothelial apoptosis (Fig. 4). TMZ may increase endothelial apoptosis in combination with THD, which functioned to inhibit the expression of angiogenic activators acting as endothelial survival factors. In this way, TMZ and THD may act through different anti-tumor mechanisms: TMZ as a cytotoxic agent inhibiting tumor growth and THD as a maintenance agent to prevent tumor progression supported by new vessel formation. That THD is likely to enhance a tumor's sensitivity to a cytotoxic drug is consistent with other studies (27). Combination treatment may allow these drugs to complement each other and may enhance their anti-tumor activities.

In conclusion, greater tumor inhibition efficacy was achieved by combination treatment with TMZ and THD. This resulted from a) synergistic growth inhibition by TMZ and THD, b) induction of apoptosis of tumor cells as well as endothelial cells by TMZ and THD, c) inhibition of angiogenesis by THD, and d) reduction in the number of activated microglia by THD. These results provide an experimental basis for clinical studies combining TMZ and THD, which may be a promising strategy for the management of malignant gliomas.

Acknowledgements

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