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Original Article

Beneficial actions of the anti-inflammatory dimethyl fumarate in glioblastomas

Ali J. Ghods[1], Roberta Glick[1,2], David Braun[3], Douglas Feinstein[3]

[1]Departments of Neurosurgery, Rush University Medical Center, [2]Anatomy and Cell Biology, University of Illinois at Chicago, [3]Anesthesiology, University of Illinois Chicago, Chicago, IL, USA

E-mail: *Ali J. Ghods - alijghods@hotmail.com; Roberta Glick - rpglick@hotmail.com; David Braun - dbraun@hotmail.com; Douglas Feinstein -dlfeins@uic.edu
*Corresponding author:

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Abstract

Background: Dimethylfumarate (DMF), a drug used in the treatment of psoriasis and multiple sclerosis, has been shown to limit the growth of melanoma cells. The ability of DMF to inhibit the Rel protein has been used to explain the antioncogenic properties of this drug. Studies analyzing the effect of DMF in gliomas are limited. Therefore, we investigated the potential antitumor effects of DMF by assessing its effects on proliferation, cell death, and differentiation in gliomas in several glioma models.

Methods: Mouse glioma Gl261, human glioblastoma A172 and human glioblastoma cells from patients were exposed to DMF at therapeutic concentrations (100 μM) and supratherapeutic concentrations (300 μM) and studies to assess proliferation, cellular lysis, and differentiation undertaken. The 5-bromo-2'-deoxyuridine (BRDU) proliferation assay and lactate dehydrogenase LDH cell lysis assay were used. Immunocytochemistry was used to assess differentiation: CD133 (stem cell marker), Nestin (progenitor marker), Sox2 (progenitor marker), β-tubulin III (neuronal marker), glial fibrillary acidic protein (astrocytic marker), and myelin basic protein (oligodendrocytic marker). We also assessed cellular expression of nuclear factor kappa B (NF-κB) via immunocytochemistry.

Results: Proliferation significantly decreased and tumor cell lysis significantly increased in all tumor cell lines after exposure to DMF. The human glioblastoma cells expressed the Neuronal Stem Cell marker CD133, Progenitor Cell markers, Neuronal and Astrocytic Cell Markers *in vitro*. When exposed to DMF, a drastic decline in CD133 expression was observed in addition to a decrease in the expression of NF-κB.

Conclusion: DMF appears to have a promising role in the treatment of malignant brain neoplasms. DMF reduced proliferation rate, generated cell lysis, decreased the expression of NF-κB, and restricted the growth of CD133 cells in gliomas. This suggests that DMF may be considered for further antitumor studies, and provide a new treatment modality for brain tumors.

Key Words: Astrocytes, dimethylfumarate, glioma, glioblastoma, NF-κB

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INTRODUCTION

Dimethylfumarate (DMF), a fumaric acid ester and antiinflammatory medication, has been safely used since 1959 in the treatment of psoriasis, a T-cell mediated dermatological disease resulting from dysfunction of the immune system. Fumaderm, a European formulation containing predominantly DMF, has been used for more than 17 years and studies have shown the benefits of DMF stem from its reduction in T-cell mediated inflammatory gene expression, which includes inflammatory cytokines and chemokines. Additionally, DMF has proven to be a safe drug (therapeutic dose 50–100 μM) with limited short- and long-term side effects, more commonly consisting of flushing, gastrointestinal complaints, and leukocytopenia.[5,6,13,16,19] In view of its good safety profile, a DMF formulation BG-12 was developed for treatment of multiple sclerosis (MS). Clinical Phase II studies have shown that B-12 decreases the rate of new enhancing MS plaques, decreases relapse rate, downregulates T1 helper response, and inhibits macrophage activation.[7,13,23,24,27,29]

In addition to its actions on the adaptive immune response, DMF has been shown to suppress the innate inflammatory responses of glial cells via suppression of the nuclear factor kappa B (NF-κB) signaling pathways.[7,13,14,20] DMF works by inhibiting signals transmitted by the Rel proteins and inhibits the IκB kinase complex and NF-κB nuclear translocation, halting gene transcription. Normally, NF-κB complexes are located within the cell cytoplasm by the inhibitory IκB complex. However, with stimulation and via inflammatory mediators, phosphorylation of the complex occurs resulting in ubiquitin degradation, allowing for the translocation of NF-κB into the nucleus and subsequent downstream transcription of proteins.[10,25] As a result, DMF decreases inflammatory mediators (i.e. nitric oxide, tumor necrosis factor alpha (TNF-α), interleukin (IL)-1β, and IL-6). Via suppression of NF-κB, DMF can also induce apoptosis in several cell types including T-cells.[24] Recently, DMF has been demonstrated to limit the growth of melanoma *in vitro* and *in vivo*. Previous studies have shown a correlation of NF-κB activity and oncogenesis[18] and one proposed antitumor mechanisms of DMF is its ability to inhibit the Rel protein pathway, reduce nuclear translocation of NF-κB, and inhibit the transcription of inflammatory cytokines.[2-4,10,11,14,15,17,22,26] Also, research has shown that DMF may also limit tumor progression by inhibiting angiogenesis via inhibition of NF-κB in endothelial cells [1,5,6,10]

Studies analyzing the effects of DMF in gliomas are limited and have shown that pretreatment with DMF increased cytoxicity of other antitumor agents.[9] Therefore, we investigated the potential antitumor effects of DMF by assessing its effects on proliferation, apoptosis, and differentiation in gliomas.

MATERIALS AND METHODS

Cell culture

Mouse Gl261 and human A172 cell lines were obtained from ATCC (Manassas, VA). The cells were resuspended in Dulbecco's modified Eagle's medium/Ham's F-12 medium containing 5% fetal bovine serum (FBS) and B-27 supplement (Gibco, Grand Island, NY, www.invitrogen.com) and plated on 75-cm^2 flasks. The cells attached and grew as monolayers and were passaged upon confluence using mechanical separation with a cell spreader. Human glioblastoma cells were obtained from Rush University Hospital from patients harboring live tumor, and were cultured in 75-cm^2 flasks. GBM cells were grown in the same media as the Gl261 and A172 cells. The cells were passaged when they reached confluency.

Proliferation assay

Mouse glioma Gl261 cells, human glioblastoma A172 cells, and human GBM cells were grown on 96 well plates until confluent. The media was then changed, and cells were exposed to fresh media containing either 0 or 100 μM DMF. After 24 h, cellular proliferation was analyzed using the BrdU proliferation assay (Roche Applied Sciences).

Cell death assay

Mouse glioma Gl261cells, human glioblastoma A172 cells, and human GBM cells were grown on 96 well plates and grown until confluent. The media was then changed, and cells were exposed to fresh media containing either 0, 100, or 300 μM DMF. After 24 h, cellular death was determined measuring the release of LDH into the media (Promega).

Immunocytochemistry staining

To examine the expression of neuronal stem cell markers, progenitor markers, and lineage markers, and assess any changes that may occur by exposure to DMF, immuncytochemical staining was performed. Mouse glioma Gl261 cells, human glioblastoma A172 cells, and human GBM cells were grown at similar density in media or 50 μM DMF for 3 days on 16-well chamber slides. Cells were then fixed with 4% paraformaldehyde for 15 min at 4°C, treated with 5% normal horse serum/0.1% Triton X-100, and then incubated with the following antibodies: Rabbit anti-CD133 (1:200; Novus Biologicals, Littleton, CO), rabbit antiNestin (1:1000; Millipore, Temecula, CA), rabbit anti-Sox 2 (1:500; Novus Biologicals, Littleton, CO), rabbit anti-β-tubulin III (β3T) (1:500; Novus Biologicals, Littleton, CO), rabbit antiglial fibrillary acidic protein (anti-GFAP) (1:1000; Novus Biologicals, Littleton, CO), rabbit anti-NF-κB (1:200; Sigma-Aldrich, St. Louis, MO), and rabbit antimyelin basic protein (anti-MBP) (1:200; Sigma-Aldrich, St. Louis, MO). The primary antibodies were detected with rhodamine or fluorescein isothiocyanate (FITC)-conjugated

antirabbit IgG antibodies (1:200; Jackson Immunoresearch Labo-ratories, West Grove, PA). The cells were counterstained with 4,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA) to identify nuclei. The stained sections were examined and photographed using a Zeiss Axiophot II fluorescent microscope equipped with Axiovisoin 4.5 software.

Statistical analysis

All analyses were performed with Microsoft Excel 2007. A P value of 0.05 was considered statistically significant. Group comparisons were performed with Student's t-test. Error bars in figures are expressed as standard error (SE).

RESULTS

DMF reduces cell proliferation

Mouse glioma Gl261, human glioblastoma A172 cells, and human GBM cells exposed to DMF at a therapeutic concentration (100 μM) for 24 h showed a significant ($P < 0.05$) decrease in proliferation [Figure 1]. DMF reduced BrdU incorporation by 22% in the human GBM cells and A172 cells; and to a greater extent (38%) in the Gl261 cells.

DMF induces cell death

Mouse glioma Gl261 cells, human glioblastoma A172 cells, and human GBM cells exposed to DMF at a therapeutic concentration (100 μM), or a supra-therapeutic concentration (300 μM). After 24 h, [Figure 1] significant ($P < 0.05$) increased cell death

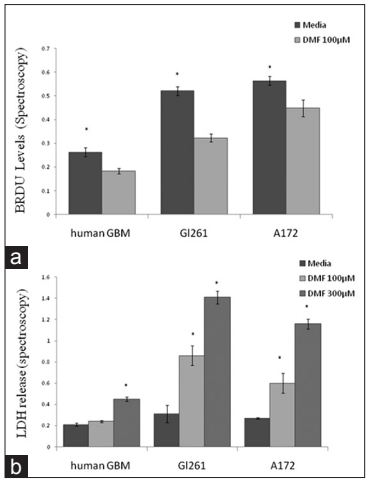

Figure 1: Effects of DMF on cell proliferation Mouse GL261 cells, human A172 cells, and human GBMs were incubated in the presence of the indicated doses of DMF. After 24 h, cell proliferation was determined using BrdU incorporation (a); and cell death determined by measurement of LDH release into the media (b). The data is mean ± SE of n=3–6 samples per group. DMF decreased proliferation of human GBMs and causes a dose dependent increase in LDH release. *, $P < 0.05$ versus media alone

was observed in all groups, except for the human GBM cells exposed to 100 μM of DMF, although there was a trend toward increased cell death ($P = 0.11$). Compared with media alone, DMF increased LDH release by 14% and 144% in the human GBMs; 122% and 329% in the A172 cells; and 177% and 343% in the Gl261 cells (at 100 and 300 μM DMF, respectively). The largest effect on cell death was observed in Gl261 cells. Interestingly, these cells showed a faster rapidly dividing time than the other two cell types.

Effects of DMF on cell maturation markers in GBM cells

To determine the maturation state of the human GBM population, we stained cells for various markers of neuronal and glial cell maturation. Untreated human GBM cells stained positive for the stem cell marker CD133, with greater staining in the neurospheres that were present [Figure 2a]. These cells also expressed the neural progenitor markers Sox 2 and Nestin. As expected, diffuse staining for the astrocytic marker GFAP was observed, as well as light staining for the neuronal marker β3T. We were not able to detect staining for the oligodendrocytic marker MBP.

After 24 h exposure to DMF, we observed a strong decrease in CD133 expression, which was associated with fewer numbers of neurospheres. However, staining for Nestin, Sox 2, β3T, GFAP, and MBP was unchanged by DMF [Figure 2a].

Effects of DMF on cell maturation markers in tumor cell lines

We carried out immunostaining of the mouse GL261 and human A172 cells using the same antibodies as described earlier. Neither of these two cell lines expressed the stem cell marker CD133 [Figure 2b and c], but did express the progenitor markers Sox 2 and Nestin. As expected, we could observe staining for the astrocytic marker GFAP, minimal staining for β3T, and absence of staining for MBP. In contrast to GBMs, following exposure to DMF no changes in CD133 or any other markers were noted.

DMF decreases NF-kb expression

Since DMF can influence the activity and expression of NF-κB, we stained the three cell lines for the presence of the NF-κB p65 subunit. After 3 days of exposure to 50 μM DMF, there was a modest decline in expression of the marker in all three cells lines. However, there was still mild expression even after exposure to DMF [Figure 3].

DISCUSSION

Our study showed that DMF decreased proliferation, increased cell lysis, decreased the expression of NF-κB in gliomas and restricted the expression of the stem

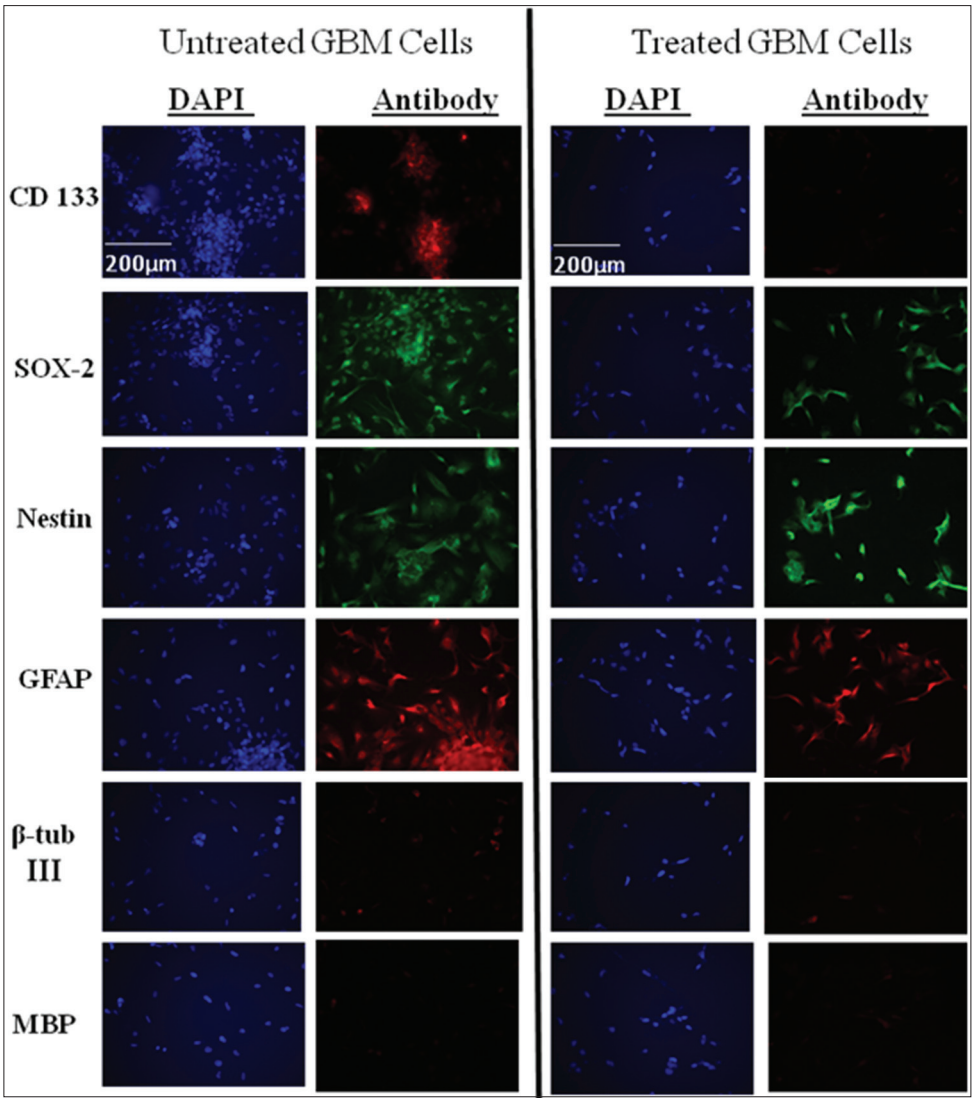

Figure 2: Effects of DMF on markers of neural differentiation (a) Human GBM cells, (b) human A172 cells, and (c) mouse GL251 cells were incubated in 0 or 50 µM DMF. After 24 h the cells were fixed and stained for the indicated markers

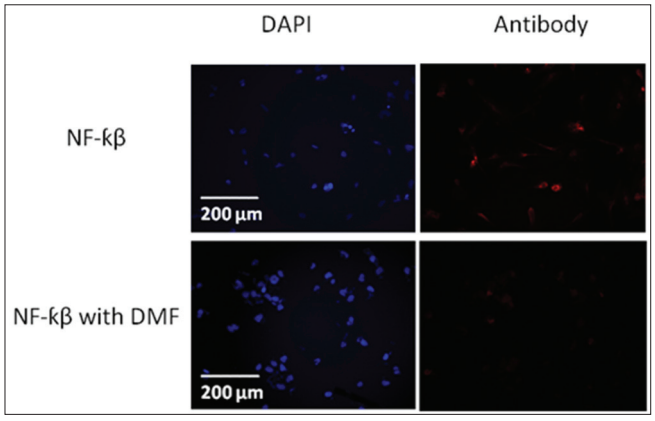

Figure 3: Effects of DMF on NF-κB After 3 days of exposure to 50 µM DMF, there was a modest decline in expression of this NF-κB

cell marker CD133 in this model. Unlike prior studies evaluating the effects of DMF on malignancy, we looked at a brain tumor model. These findings suggest a promising role for future studies investigating DMF as a treatment for gliomas.

Very few groups have looked at the role of DMF in tumors. Previous studies in noncentral nervous system (non-CNS) tumors demonstrated that one beneficial antitumor effect of DMF was via decreasing proliferation. Of particular interest, these findings were correlated with inhibition of the nuclear translocation of NF-κB.[4,15] DMF was first studied in melanoma models. Loewe *et al*. showed that DMF at three-times the dose used in psoriasis patients could inhibit the proliferation rate of melanoma cells both *in vitro* and *in vivo* by arresting the cell cycle in the G2-M phase and also generating apoptosis. This was associated with a decline in metastasis in SCID mice treated with DMF. These findings were correlated with a decline in the Rel protein cascade and the reduction in TNF and IL-8 expression.[15] In recent years, groups have looked at the role of NF-κB activity in oncogenesis and have shown

that by inhibiting the nuclear translocation of NF-κB, you can decrease proliferation, increase susceptibility to chemotherapy, decrease expression of adhesion molecules, prevent metastasis, and induce apoptosis.[2-4,10,11,14,15,17,22,26] Huang *et al*. looked at high grade melanomas *in vivo* and *in vivo* and demonstrated constitutive activity of NF-κB/relA. By transfecting cells with vectors containing the inhibitor of NF-κB, IκBα, the group showed a decrease in NF-κB expression *in vitro*. As a result, tumor growth was inhibited and there was a decline in lung metastasis *in vivo* in nude mice.[10] Similarly, Huber *et al*. demonstrated NF-κB is needed both *in vitro* and *in vivo* in the induction and metastasis of a mouse breast cancer model, and by inhibiting NF-κB, the group showed a decline in metastasis.[11]

More recently, a number of studies have demonstrated that DMF may limit tumor progression by inhibiting angiogenesis. DMF has been shown to decrease vascular endothelial growth factor receptor 2 (VEGFR2) expression via inhibition of NF-κB in endothelial cells, inhibit endothelial cell migration, and induce endothelial cell apoptosis, which may explain why DMF may be beneficial in proliferative diseases such as psoriasis, rheumatoid arthritis, and tumor growth and metastasis.[1,5,6,10] One group looked at high grade melanomas and showed a decrease in microvessel density *in vivo* in transfected tumors containing the inhibitor of NF-κB, IκBα, in nude mice.[10] This was also correlated with a decrease in the expression of the inflammatory cytokine IL-8.

In the present study, we demonstrate that DMF decreases proliferation rate and induce cell death. DMF appears to decrease the expression of NF-κB in our glioma model, which has also been described in other tumor studies. Additionally, we elucidate that DMF may play a role in decreasing the stem-like cell population residing in gliomas, which has not been described elsewhere. The cancer stem cell hypothesis proposes that a subpopulation of tumor cells proliferate, self-renew, and eventually differentiate into a phenotypically diverse and heterogeneous tumor cell population.[12,21] Furthermore, these cancer stem cells express the neural stem cell surface marker CD133 and can histologically recapitulate the original tumor.[8,28] In our study, we demonstrate a significant impact of DMF on decreasing the formation of both neurospheres and the expression of CD133 cells in human glioblastoma cells. These findings imply that DMF may inhibit tumor proliferation by disturbing the population of these CD133 stem-like tumor cells.

The present study possesses several limitations. Of importance, our study is entirely *in vitro*, and the effects of DMF *in vivo* and its translation into a clinical model remains to be performed. Additionally, we used several tumor variants in our experimental model—mouse glioma Gl261, human glioblastoma A172 cells, and human GBM cells—which showed phenotypic differences. The mouse glioma Gl261 and human glioblastoma A172 cells were similar in differentiation, yet failed to form neurospheres like the human GBM cells harvested from patients with diagnosed GBM or express the stem cell marker CD 133. Nonetheless, all three cell lines were still impacted by DMF with regard to proliferation and cell death.

In summary, DMF significantly reduced proliferation, generated cell death, decreased the expression of NF-κB, and restricted the growth of CD133 cells in gliomas. This suggests that this antiinflammatory drug, with minimal clinical side effects, may be considered for further antitumor studies, and provides a new treatment modality in gliomas. Future challenges for the clinical application of DMF in gliomas include *in vivo* analysis of the potential benefits of DMF in retarding the growth of GBM. These studies should also look at the effects of DMF on angiogenesis in animal models with GBMs treated with DMF.

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