Curcumin (Diferuloylmethane) Induces Apoptosis and Blocks Migration of Human Medulloblastoma Cells

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Abstract. *Medulloblastoma (MB) is the most common malignant brain tumor in children. Bcl-2 and MMP-9 promote the pathogenesis and progression of MB. The expression of both bcl-2 and MMP-9 is regulated by the transcription factor NF-κB. Curcumin, a natural food additive, has a potent anti-proliferative effect, presumably mediated through NF-κB suppression. The tumor-suppressing effects of curcumin are well documented, however, its effect on MB is unknown. Our objectives were to: a) examine the effect of curcumin on MB cell proliferation and apoptosis; b) characterize the mechanism that mediates the effect of curcumin; c) examine the effects of curcumin on MB cell migration. We report that curcumin inhibited cell proliferation and blocked clonogenicity of MB cells. Furthermore, curcumin down-regulated bcl-2 and bcl$_x$l, leading to caspase-mediated cell death. Finally, curcumin blocked migration of MB cells. Thus, we propose developing curcumin as a novel therapeutic agent for MB.*

Although progress has been made in the diagnosis and treatment of medulloblastoma (MB), current therapeutic combinations involving surgery chemotherapy and radiation are ineffective in a significant number of cases, and are accompanied by long-term toxic effects. Therefore, there is an urgent need to develop more effective and less toxic therapies. Disruption of several signaling pathways contribute to the development and progression of MB. These include the Hedgehog (Hh) signaling pathway, with mutations in *PTCH*, which encodes the receptor for Hh, detected in ~10% of MBs (1, 2). In addition to *PTCH*, mutations in a downstream signaling intermediary of the Hh signaling pathway, suppressor of fused (*SUFU*), have also been detected in MBs (3). Together these observations implicate activated Hh signaling pathway in MB progression. In addition to Hh, mutations in APC, and hence disruption of the Wnt signaling pathway has also been reported in MB (4, 5). Receptor tyrosine kinases that are believed to promote tumor growth and invasion of MB, include ErbB2 as well as IGF-1R (6, 7). Overexpression of the matrix metalloproteinase, MMP-9 is associated with increased invasiveness of MB (8). In an experimental MB model, shh (sonic hedgehog)-induced MB was enhanced by bcl-2 expression, suggesting that suppression of apoptosis along with increased cell proliferation leads to aggressive tumor formation (9). Expression of both MMP-9 and bcl-2 is regulated by the transcription factor NF-κB.

Curcumin, a commonly used food additive, has been shown to be an effective anti-proliferative and anti-inflammatory agent. Curcumin is believed to selectively target tumor cells by inhibiting signaling pathways that promote cell growth and survival. Curcumin targets several signaling pathways that are constitutively active in tumor cells. The suppression of NF-κB is believed to mediate the antiproliferative effect of curcumin (10). Several tumor cell types are susceptible to the growth inhibitory effects of curcumin, however, it is not known whether curcumin can inhibit proliferation of MB cells. Since the expression of both bcl-2 and MMP-9, which play an important role in MB tumor progression and metastasis, are regulated by NF-κB, we questioned whether curcumin would suppress the growth and migration of MB cells. We report that curcumin down-regulates the pro-survival proteins bcl-2 and bcl$_x$l, and induces caspase-mediated apoptosis in human MB cells. We also demonstrate that curcumin blocks migration of MB cells.

Materials and Methods

Chemicals and reagents. Curcumin was purchased from LTK laboratories (St. Paul, MN, USA). Anti -cleaved caspase 3 and -cleaved PARP, total PARP, bcl-2 and bclxl antibodies were purchased from Cell Signaling Technologies, (Danvers, MA, USA). Horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Upstate Biotechnology (Lake Placid, NY, USA).

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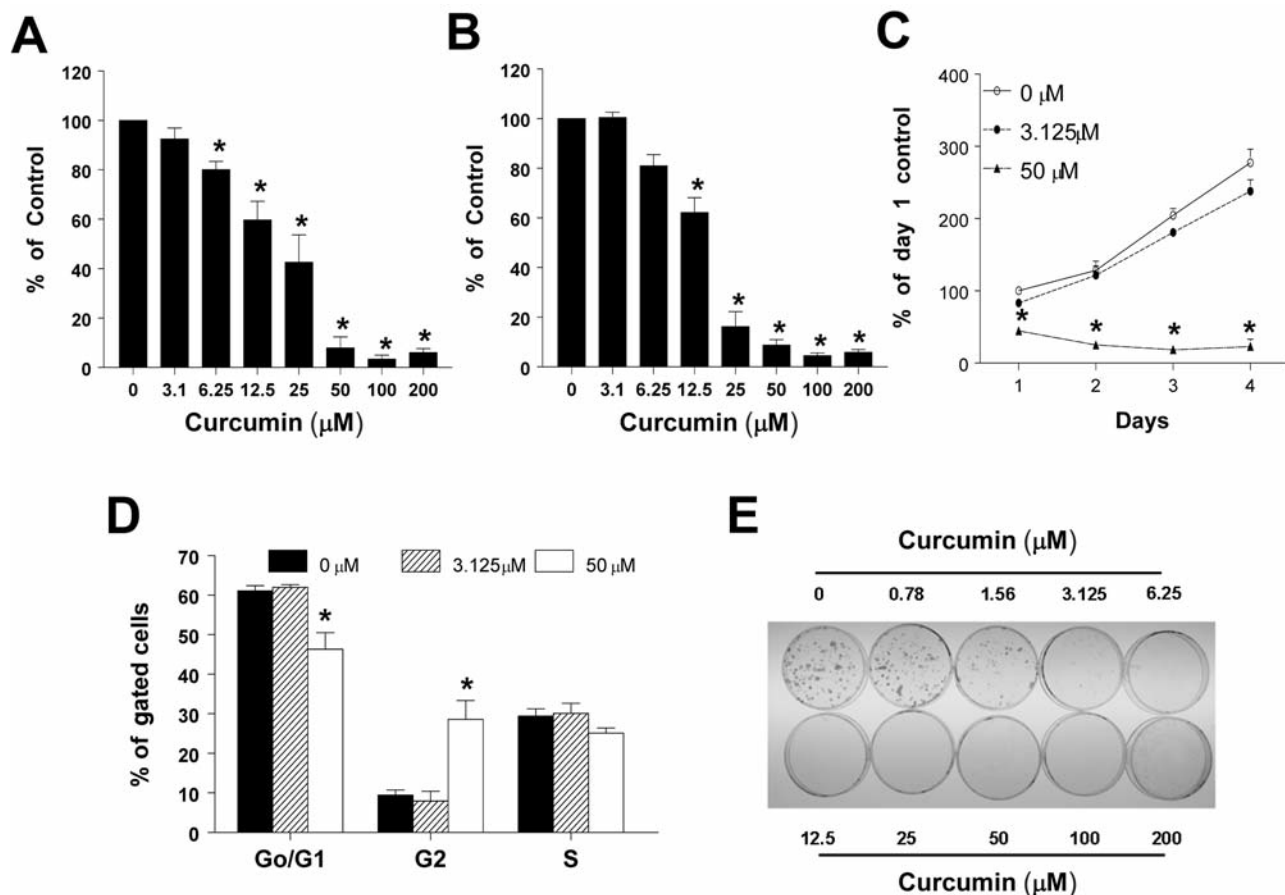
Figure 1. *Curcumin inhibits cell proliferation and blocks colony formation ability of MB cells. Daoy cells were treated with the indicated dose of curcumin, and cell proliferation was assessed after 4 days using either the MTT assay (A) or the BrdU incorporation assay (B) as described in Materials and Methods section. Data are calculated as % of vehicle control and expressed as a mean±SEM of at least 3 separate experiments. *designates significant differences (p<0.05) from control values. C: To assess the time-dependent effect of curcumin on MB cell proliferation, Daoy cells were treated with (0, 3.125, 50 μM) curcumin for the indicated times, and cell proliferation was assessed as above. Data are calculated as % of day 1 control and expressed as a mean±SEM of 3 separate experiments. D: Daoy cells were treated with (0, 3.125, 50 μM) curcumin for the indicated times. PI staining followed by flow cytometry was used to determine DNA content. Data are calculated as % of gated cells and expressed as a mean±SEM of at least 3 separate experiments. E: Daoy cells were seeded at a density of 1000 cells/treatment, in complete growth medium. After 24 h the medium was replaced with fresh complete growth medium containing the indicated concentrations of curcumin, and colony formation ability of Daoy cells was assessed after 10 days, as described in the Materials and Methods section. Data shown are from a single experiment and are representative of at least 3 experiments yielding similar results. *Designates significant differences (p<0.05) from control values.*

Cell culture. Daoy cells (a kind gift from Dr. S. Lakka, University of Illinois College of Medicine, Peoria, IL, USA) were maintained in complete medium [DMEM (Mediatech, Manassas, VA, USA) containing 10% FBS (Gibco/Invitrogen, Grand Island, NY, USA) and 5 U/ml Penicillin/ 5 μg/ml Streptomycin]. Complete medium was changed every 2-3 days, and cells were sub-cultured as required.

Assessment of cell proliferation. Daoy cells in log phase were seeded (5,000 cells/well) on 96-well plates in complete medium. The next day, cells were treated with the indicated concentrations of curcumin in medium containing 0.5% FBS. Proliferation was assessed using colorimetric MTT assay or BrdU incorporation assay as described previously (11).

Cell cycle analysis. Daoy cells were seeded and cultured in complete medium till ~70% confluence. After washing, cells were treated with the indicated concentrations of curcumin in medium containing 0.5% FBS. Cells were washed, fixed and stained with PI, and DNA content was determined by flow cytometry.

Colony formation assays. Colony formation assays were performed as described (12). Briefly, Daoy cells growing in log phase were seeded (1000 cells/well in a 6-well plate or in a 35 mm dish) in complete growth medium(10% FBS). Cells were allowed to adhere for 24 h, and medium was replaced with fresh complete growth medium containing the indicated concentrations of curcumin. Cells were cultured at 37°C for 10 days, with medium changes every third or fourth day. Colony formation was detected by crystal violet staining.
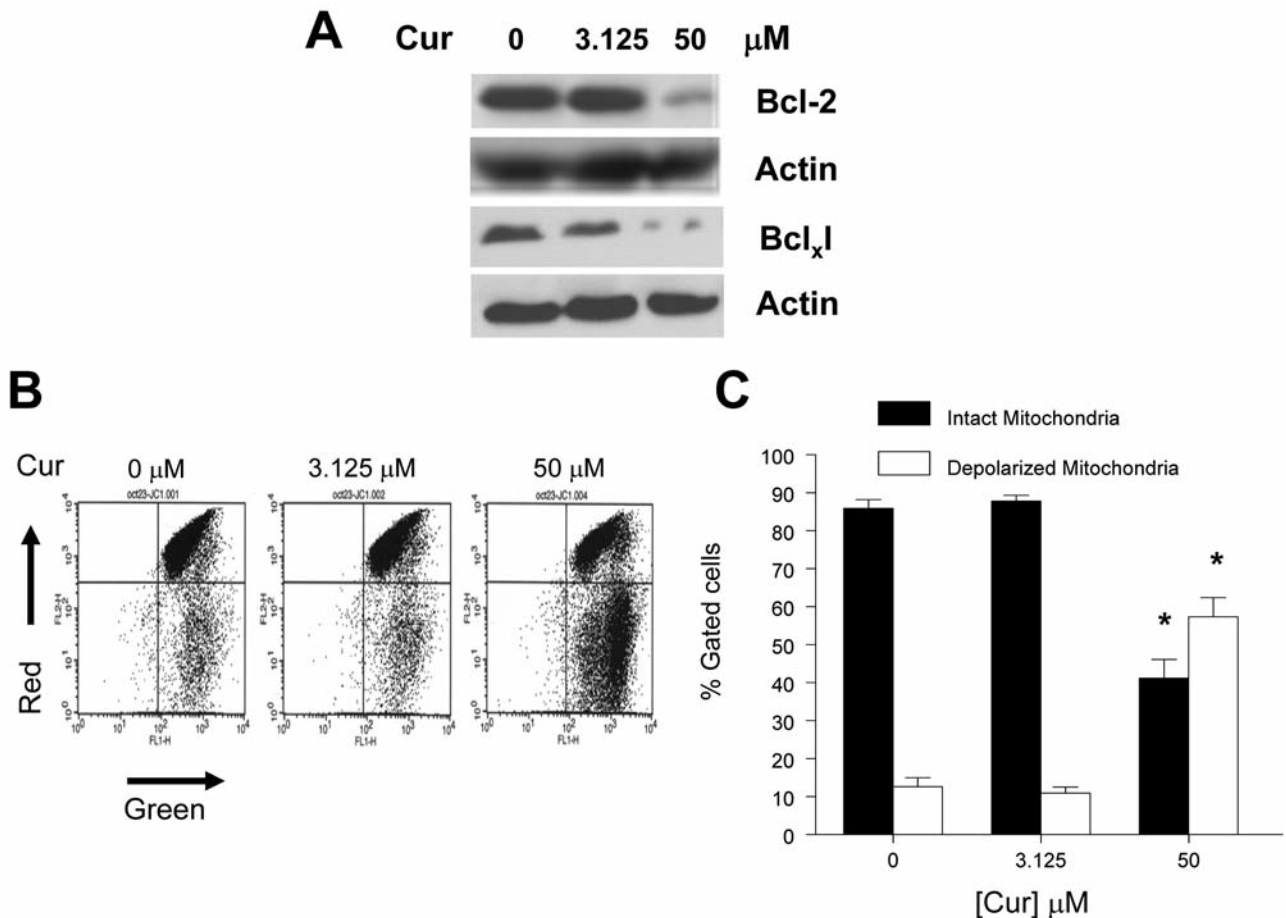
Figure 2. *Curcumin down-regulates pro-survival proteins bcl-2 and bcl$_x$l in MB cells. A: Daoy cells were treated with the indicated doses of curcumin for 48 h, and equal amount of cell lysates were subjected to Western blotting with: anti-bcl-2 Ab (top panel), or anti-actin Ab (second and fourth panels), or anti-bcl$_x$l Ab (third panel). Western blot data are from a single experiment that is representative of at least 3 independent experiments. Daoy were treated with indicated concentration of curcumin for 48 h, and cells were washed and labeled with JC-1 dye. Decreased red and increased green fluorescence intensities were measured by flow cytometry. B: Dot plot analysis from a single experiment which is representative of 3 independent experiments yielding similar results. C: Quantitation of dot plots. Data are presented as % of gated cells and are the mean±SEM of 3 independent experiments. *Designates significant differences (p<0.05) from control values.*

Annexin V staining assays. These were performed as described before (13). Briefly, Daoy cells were treated with the indicated amount of curcumin for 48 h, washed in PBS buffer, and stained with annexin V-FITC and propidium iodide, and analyzed by flow cytometry.

Mitochondrial membrane depolarization assays. Daoy cells were treated with the indicated amount of curcumin for 48 h, following which cells were washed and then stained with JC-1 dye, and analyzed by flow cytometry, according to the manufacturer's protocol (Cell Technologies Inc. Mountain View, CA, USA).

Western blotting. Preparation of cell lysates, protein content determination and western blotting was performed as described previously (13).

Scratch wound migration assay. Thiw was performed as described before (14). Briefly, Daoy cells were grown to monolayer confluence in complete medium in a 6-well plate. A scratch wound was inflicted by using a sterile p200 pipette tip. Cells were washed to remove debris, and phase contrast microscopy was used to capture the image. Cells were then incubated in medium containing 2.5% FBS with the indicated concentration of curcumin. After 24 h, wound closure was assessed.

Data analysis. Statistical significance was determined using Student's *t*-test.

Results and Discussion

Curcumin inhibits cell proliferation and blocks colony formation ability of MB cells. We first investigated the effect of curcumin on Daoy cell proliferation. Daoy cells were treated with curcumin (3.125-200 μM) and after 4 days cell proliferation was assessed by either the MTT assay (Figure 1A) or by BrdU
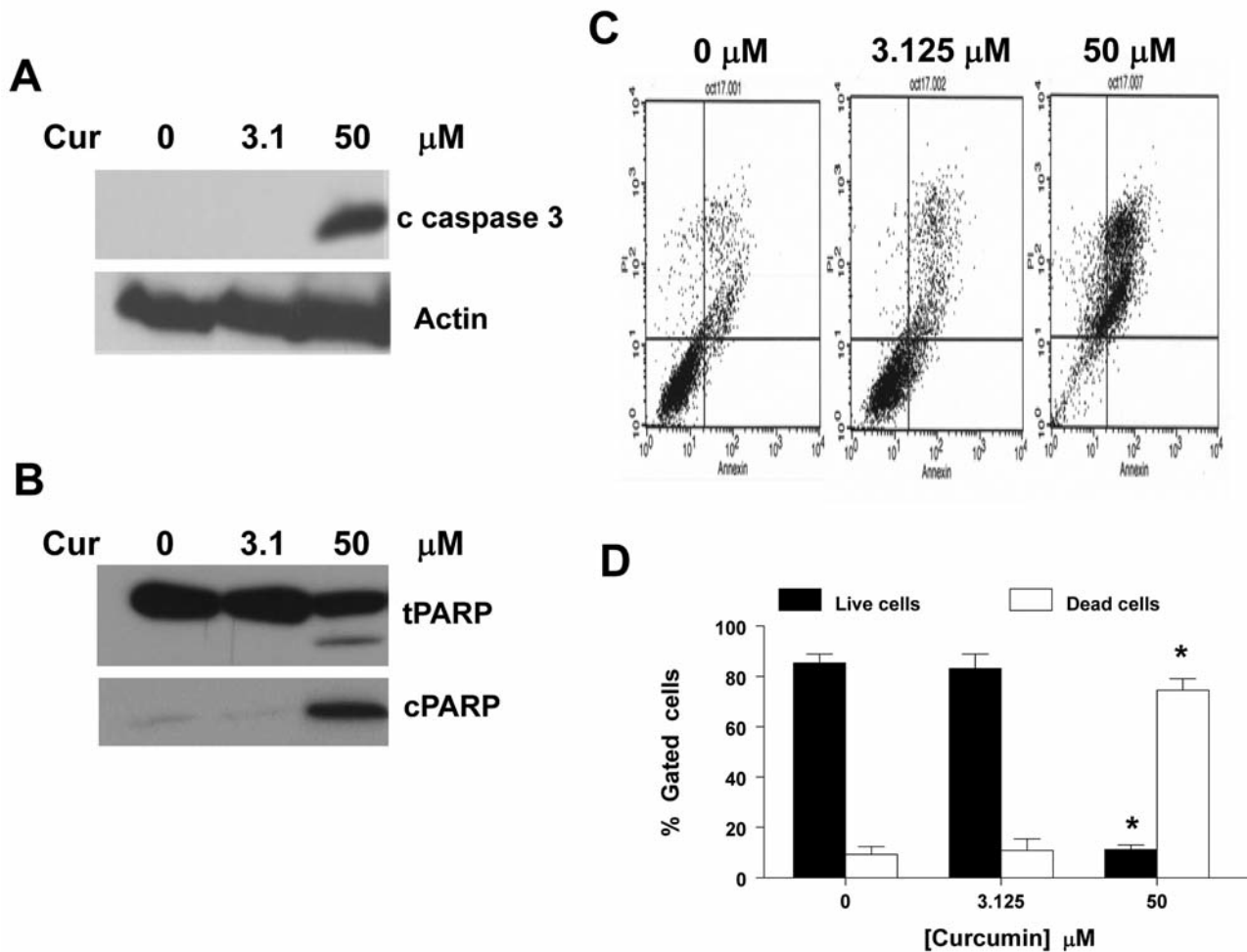
Figure 3. *Curcumin induces caspase-mediated apoptosis in MB cells. Daoy cells were treated with the indicated doses of curcumin for 48 h, and equal amount of cell lysates were subjected to Western blotting with (A) anti-cleaved caspase 3 Ab (top panel), or anti-actin Ab (bottom panel), or (B) anti-total PARP Ab (top panel) or anti-cleaved PARP Ab (bottom panel). Western blot data are from a single experiment that is representative of at least 3 independent experiments yielding similar results. Daoy cells were treated with the indicated amount of curcumin for 48 h. Annexin V-FITC and PI staining were analyzed by flow cytometry. C: A dot plot of curcumin treated Daoy cells stained with Annexin V-FITC and PI. Data shown are from a single experiment, and are a representative of 3 independent experiments with similar results. D: Quantitation of dot plots. Data are presented as % of control, and are the mean±SEM of 3 independent experiments. *Designates significant differences (p<0.05) from control values.*
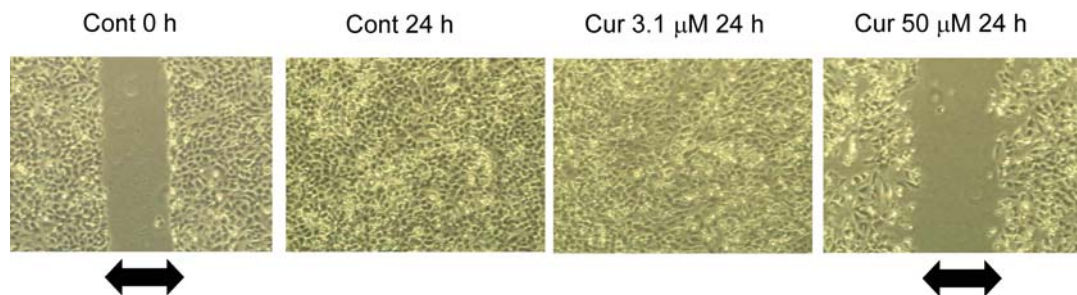

Figure 4. *Curcumin blocks migration of MB cells. Daoy cells in complete medium were grown to confluency. Scratch wound was inflicted as described in the Materials and Methods section. Phase-contrast microscopy was used to capture an image of the wound. After washing, cells were incubated in fresh medium containing 2.5% FBS and the indicated concentrations of curcumin. After 24 h, phase-contrast microscopy was used to capture an image of the wound. Data shown are from a single experiment and is representative of 3 independent experiments yielding similar results.*

incorporation assay (Figure 1B). Our results demonstrate that curcumin induced a dose-dependent suppression of cell proliferation in Daoy cells, with significant ($p<0.05$) inhibition detected in the range of 6.25-12.5 μM, while maximal inhibition (>90%) detected in the concentration range of 50-200 μM. We next determined the kinetics of growth suppression. Daoy cells were treated with the indicated amount of curcumin for 1-4 days. At 24-h intervals, cell proliferation was assessed and our results show (Figure 1C) that curcumin inhibited Daoy cell proliferation in a time-dependent manner, with an effective concentration of curcumin (50 μM) suppressing growth within 24 h, and persisting for up to 4 days.

We next questioned whether the curcumin-induced growth suppression was irreversible. To address this issue, Daoy cells were exposed to either vehicle or curcumin (50 μM) for 24 h. Cells were then washed to remove curcumin, and equal number of vehicle- or curcumin-treated cells were plated in complete growth medium containing 10% FBS. Cell proliferation was determined after 4 days. Our data demonstrate (data not shown) that Daoy cells exposed to vehicle proliferated robustly in response to FBS, however, cells that were exposed to curcumin failed to proliferate in 10% FBS-containing medium. These results suggest that curcumin induced growth suppression is irreversible.

We next examined the effects of curcumin on MB cell cycle progression. To address this issue, Daoy cells were treated with the indicated concentrations of curcumin, and after 48 h cells were stained with propidium iodide and DNA content was analyzed by flow cytometry. Our data show (Figure 1D) that consistent with the growth suppression that was observed in the MTT and BrdU incorporation assays, curcumin treatment lead to an increased accumulation of cells in the G_2/M phase, with a concomitant decrease in the G_1/S phase cells. We next questioned whether curcumin had any effect on the ability of Daoy cells to form colonies. The colony formation assays are an excellent indication of the long-term survival of tumor cells, and are an excellent *in vitro* assay to predict the antitumor effects of drugs. Daoy cells were seeded (1000 cells) in medium containing 10% FBS, and allowed to adhere for 24 h. The medium was then replaced with fresh medium containing 10% FBS together with the indicated concentration of curcumin, and colony formation was monitored over the next 10 days. We observed (Figure 1E) that Daoy cells have a robust ability to form colonies which is inhibited in a concentration-dependent manner by curcumin, with 6.25-200 μM curcumin completely abolishing colony formation of Daoy cells.

Together, these results demonstrate for the first time the suppressive effects of curcumin on MB cell proliferation.

Curcumin down-regulates pro-survival proteins bcl-2 and bcl$_x$l in MB cells. In addition to the growth suppressive and anti-

clonogenic effect of curcumin on Daoy cells, we observed that curcumin induced morphological changes (shrunken cells, loss of membrane integrity and dense intracellular granules) similar to those observed in apoptotic cells. Therefore, we next questioned whether curcumin had an effect on the pro-survival proteins, bcl-2 and bcl$_x$l. Daoy cells were treated as indicated with curcumin, and equal amount of cell lysates were subjected to Western blotting. Our data (Figure 2 A) show that curcumin in a concentration-dependent manner, decreases the expression of bcl-2 and bcl$_x$l. The concentration-dependent effect of curcumin on the down-regulation of the pro-survival proteins is in good agreement with the concentration-dependent inhibition of cell proliferation (Figure 1 A and B). These results lead us to hypothesize that curcumin inhibits cell proliferation and induces apoptosis in MB cells.

We next questioned whether the curcumin-induced decrease in bcl-2 and bcl$_x$l protein levels would shift the balance of pro-apoptotic and pro-survival proteins in favor of pro-apoptotic proteins, thereby depolarizing the mitochondrial membrane. Daoy cells were treated with the indicated concentrations of curcumin for 2 days, cells were washed and incubated with JC-1 dye (a mitochondrial-specific dual fluorescence probe). In intact cells, JC-1 dye coalesces in the mitochondria to emit a red fluorescence, however in apoptotic cells with a depolarized mitochondrial membrane, mitochondrial contents gain entry to the cytosol, and hence JC-1 dye cannot accumulate in the mitochondria and it enters the cytosol, emitting a green fluorescence. The dot plots (Figure 2B) clearly show that curcumin causes membrane depolarization in a concentration-dependent manner, as indicated by increased green fluorescence. In vehicle- and 3.125 μM curcumin-treated cells, 85.86% and 87.81%, respectively, of the cells emitted a red fluorescence while only 12.63% and 10.91%, respectively, emitted green fluorescence. However, in 50 μM curcumin-treated cells, only 41.18% cells emitted red fluorescence while 57.35% emitted green fluorescence (Figure 2C). These results demonstrate that upon curcumin treatment there is a significant reduction in the number of cells with intact mitochondrial membranes, supporting our hypothesis that curcumin induces mitochondrial apoptosis in MB cells.

Curcumin induces-caspase mediated apoptosis in MB cells. We next questioned whether the increase in mitochondrial membrane depolarization would lead to release of mitochondrial cytochrome *c*, which in turn would lead to activation of caspase 3 and ultimately cell death. To address this issue we treated Daoy cells with the indicated concentration of curcumin for 48 h, and activation of caspase 3 was determined by Western blotting using an antibody that specifically detects the activated (cleaved) form of caspase 3. Our results demonstrate that concentrations of curcumin that lead to down-regulation of bcl-2 and bcl$_x$l, and depolarization of the mitochondrial membrane potential also

resulted in increased caspase 3 activity (Figure 3A). Activation of caspase 3 resulted in cleavage and activation of its substrate PARP. Our results show (Figure 3B) that consistent with caspase 3 activation, we observed a dose-dependent decrease in total PARP together with a concomitant increase in cleaved PARP. Together these results suggest that curcumin induced mitochondrial apoptosis in MB cells. To confirm apoptosis, Daoy cells were treated with vehicle or indicated amount of curcumin for 48 h, and following trypsinization, annexin V-FITC binding (AV) and propidium iodide (PI) staining was analyzed by flow cytometry. The dot plots show (Figure 3C) that curcumin induced an increase in the cells that stained positive for annexin V-FITC and PI in a concentration-dependent manner. Quantitation of the data demonstrate (Figure 3D) that in vehicle- and 3.125 μM curcumin-treated cells, 9.25% and 10.79% cells, respectively, were undergoing apoptosis. However, treatment with 50 μM curcumin resulted in 74.53% of cells undergoing cell death.

Curcumin blocks migration of MB cells. At the time of diagnosis, most MBs have metastasized. Successful tumor metastasis is dependent upon the invasion of tumor cells into the surrounding microenvironment. Normally, the migration of cells is restricted by the basement membrane, however, metastatic tumors cells overcome this inhibition and infiltrate the lymphatic and vascular systems.

We next questioned whether curcumin would have any effect on the migration of Daoy cells. Daoy cells were cultured in complete growth medium and allowed to reach confluency. Cells were then serum starved for 24 h, and a scratch wound was inflicted. Cell migration was monitored over a 24 h period. Our data (Figure 4) clearly shows that in vehicle treated Daoy cells, migration of cells into the wound area results in a complete closure of the wound. However, curcumin blocked the migration of Daoy cells in a concentration-dependent manner.

Taken together, our data show for the first time that curcumin is effective at inducing MB cell apoptosis as well as blocking the migration of MB cells. The concentration of curcumin used in this study is comparable to that used in other *in vitro* studies examining the growth-suppressive effects of curcumin in tumor cells (15-17). Furthermore, in a Phase I clinical trial in humans, 8000 mg/ day of curcumin was found to be safe (18).

MB is the most common form of pediatric brain tumor and despite aggressive therapy, favorable treatment outcome still remains challenging. Since >80% of MBs occur in children when brain development is still in progress, both chemotherapy and radiation result in severe long-term toxic effects. In a vast majority of cases, recurring MBs are untreatable. Therefore, novel treatments that could selectively target MB without having toxic effects are highly desirable. Our results demonstrate that curcumin, a natural food additive with an excellent safety profile, was highly effective at suppressing growth and migration of MB cells. We propose developing curcumin as a novel therapeutic agent in the management of MB.

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