The effect of curcumin on low-passage glioblastoma cells in vitro

ABSTRACT

Background: Plant extract therapy has been the cornerstone of cancer treatment for many years. The natural component curcumin demonstrated antineoplastic effects on different type of tumor cells. In this study, we explored the effectiveness of curcumin against low-passage human primary glioblastoma (GB) cell cultures.

Materials and Methods: Early passage GB cell cultures (GB3B, GB4B, and GB5B) were established from fresh samples tissue obtained from GB patients. Growth rate (GR) and doubling time (DT) was determined for each cell line. The cytotoxic effect of curcumin was quantified by hemocytometer cell counting, using trypan blue. To study the changes in cell shape, GB cells exposed to a concentration corresponding to inhibitory concentration 50 (IC50) of curcumin were studied by phase-contrast microscopy by capturing images during the treatment.

Results: Our results showed that GB cells proliferate with a GR of 0.2872 and a DT of 2.41 days for GB3B, a GR of 0.2787 and a DT of 2.49 days for GB4B, and a GR of 0.2787 and a DT of 2.49 days for GB5B. Curcumin induced cell death in GB cells in a time- and dose-dependent manner. The IC50 for GB3B was 46.4 µM, for GB4B was 78.3 µM, and for GB5B was 47.7 µM. Phase contrast microscopy showed that cultures treated with curcumin in a concentration corresponding to IC50 contained rounded cells and cell fragments, 72 h after the treatment.

Conclusions: The results of the present investigation proved that curcumin is a natural compound potentially useful in the fight against GB.

KEY WORDS: Curcumin, glioblastoma, therapy

INTRODUCTION

Glioblastoma (GB) is the most common type of brain tumor in adults. GB may occur de novo or by malignant progression from astrocytomas.[1,2] The current therapeutic methods for this severe tumor are represented by multimodal, targeted, and aggressive regimen which includes the surgical resection, radiation therapy, and chemotherapy. However, their success is very limited, and no present therapeutical approaches are curative. Consequently, the overall survival is extremely poor with a median of 9–15 months.[3] In addition, the effect of existing brain tumor chemotherapeutic drugs is limited by the blood-tumor barrier. Therefore, there is a great effort from the medical scientific community to develop new therapeutic approaches to treat this devastating disease.

Natural compounds extracted from plants are more used in cancer therapy or as chemopreventive drugs. In fact, many of the antineoplastic drugs used during the last decades are either directly obtained from plants or synthetic products derived from some natural structures. Drugs like vinca alkaloids (e.g., vincristine, vinblastine, vinorelbine, and vindesine) extracted from Catharanthus roseus, taxanes (e.g., paclitaxel) extracted from the bark of the Pacific yew tree, inhibitors of the topoisomerase I and II (e.g., irinotecan, etoposide, doxorubicin, and so on) proved to be some of the most effective anticancer medications.[4,5] Others, like catechins (e.g., epigallocatechin-3-gal extracted from green tea, isothiocyanates found in cruciferous vegetables, isoflavones (e.g., pomiferin) and so on, have been shown to possess anti-tumor effects, both in vitro and in vivo.[6,4]

longa, popularly known as turmeric. Turmeric has been used since ancient times (over 2000 years ago) in Indian Ayurvedic medicine for treatment of different disorders like infections, burns, allergies, rheumatism, liver disorders along with others.[9,10] Nowadays, researchers discovered new properties of curcumin such as Antiproliferative, antimetastatic, antiangiogenic, and antimutagenic capacity.[11-13] The efficacy of turmeric treatment was studied in various types of solid tumors, including GB. Since 2003, several research groups investigated curcumin effect in various GB cell lines and the compound was shown to be effective in killing cancer cells.[11,14-18] After that, many other studies have shown the efficacy of turmeric on GB, in vivo and in vitro.[19-21]

Several mechanisms have been reported for curcumin induced cancer cell death: Cyclin D1, tumor necrosis factor, cyclooxygenase 2 and matrix metalloproteinase (MMP)-9 downregulation, nuclear factor kappa B, and activator protein 1 inactivation.[12,22] Curcumin was suggested to induce apoptosis in several types of cancers by downregulating Bcl-2 and Bcl-XL and by activation of caspase-3, caspase-9, and caspase-8.[23]

In GB, curcumin was reported to induce apoptosis by activation of pro-apoptotic and suppression of anti-apoptotic signals, by activating a nonapoptotic autophagy and also by inducing differentiation cascade signaling.[24-26] Turmeric inhibits MMPs and glucose-6-phosphate transporter gene expression and also activates proteolytic pathways.[27,28]

Despite a limited number of in vivo studies that used curcumin as a treatment in GB tumors, the results are encouraging.[29-31] One clinical trial that used turmeric in association with other treatments for a personalized and targeted therapy in pediatric brain tumors has also shown promising preliminary results.[32]

Although curcumin induced high cytotoxicity in various types of brain tumor cells, in human normal cells its cytotoxicity was much lower.[33]

In this study, we examined the in vitro antineoplastic effects of curcumin on human primary GB cells. Compared to established cell lines, low passage cell lines are reported to better preserve features of cancer like phenotype or genotype. Low-passage cell lines should better mimic the tumor heterogeneity in vivo because of their mixture of several cell populations. In fact, they are supposed to have better value as tumor models. In our experiments, we analyzed the potential of curcumin to induce death in three low-passage GB cell lines.

MATERIALS AND METHODS

Cell lines and cell culture
The human primary GB cell lines GB3B, GB4B and GB5B are low passage primary brain tumor cell lines derived from GB tumors. We established these cell lines in our laboratory from fresh samples of tumor biopsy collected from GB diagnosed patients, surgically operated, following standard procedures. The informed consent of the patients and the ethical approval for the project were obtained prior to surgery and any experiment. The cell lines were grown in minimum essential medium (MEM) containing 10% fetal bovine serum, 2 mM glutamine and antibiotic (100 UI/ml penicillin and 100 UI/ml streptomycine). The cells were grown in tissue culture flasks maintained in a 95% air/5% carbon dioxide (CO₂) atmosphere at 37°C in a humified incubator. Cells at the same passages were used for the experimental purpose.

Curcumin preparation
Curcumin was purchased from Sigma Aldrich. The curcumin powder was dissolved in dimethylsulfoxide in order to obtain a 100 mM stock solution. The solution was stored at −20°C, protected from light. We used different concentrations (0.1 µM, 1 µM, 2 µM, 5 µM, 10 µM, 20 µM, 50 µM, 100 µM, and 150 µM) of curcumin by diluting the stock solution with standard medium.

Growth rate and doubling times
Human GB cells were seeded into 6-well plates at a concentration of 2 × 10^5 cells/well and were grown in standard MEM medium for 72 h. At each time point, cells were trypsinized, centrifugated, and resuspended in standard medium, and a uniform cell suspension was counted in a Bürker hemocytometer. Dye exclusion with trypan blue has been used to quantify living cells. Each experiment was performed in triplicate and repeated 3 times. Growth rate (GR) and doubling times (DTs) were determined for each cell line, using a publicly available algorithm.[34,35]

The assay was done in triplicate or quadruplicate for each time point.

Cell treatment
The cells were grown in tissue culture flasks maintained in a 95% air/5% CO₂ atmosphere at 37°C in a humified incubator. For cell viability assay, cells were seeded in 96-well culture plates (3 × 10^3 cells/well) and treated with various concentrations of curcumin 0.1, 1, 2, 5, 10, 20, 50, 100, and 150 µM) for 3 days. For Western blotting assay, the cells were transferred to cell culture bottles, treated with the concentration corresponding to inhibitory concentration 50 (IC50) and incubated for 3 days.

Cell viability and inhibitory concentration 50
The cells were treated as mentioned above (“cell treatment” section) and the inhibitory effect was quantified by performing a cell count with a hemocytometer, using trypan blue. The cell viability was reported as a percentage of living cells compared to untreated control cells, by using the formula: Percentage of Living cells = Nb of treated cells/Nb of control cells × 100. An untreated control was considered as 100% living cells.
Appropriate control groups with diluents only and blank control were included. The assay was done in triplicate or quadruplicate for each data point.

To estimate the IC that kills 50% of cells ([IC50]), the formula used was: $IC50 = \frac{[50 - X]}{[Y - X]} \times (W - Z) + Z$, where $X$ is the first point on the curve, expressed as percent inhibition, that is, $\leq 50$%; $Y$ is the first point on the curve, expressed as percent inhibition, that is, $\geq 50$%; $Z$ is the concentration of inhibitor that gives $X\%$ inhibition; and $W$ refers to the concentration of inhibitor that gives $Y\%$ inhibition.

Morphological features were studied under an inverted microscope at $\times 10$ magnification.$^{[36,37]}$

**Statistical analysis**

Statistical analysis was performed using Microsoft Excel (Microsoft Corp., Redmond, WA, USA), the XLSTAT and IBM SPSS statistics version 20.0 (IBM Corporation, Armonk, NY, USA). To test the normality of the data we used the Anderson–Darling test. None of the numerical variables investigated had a normal distribution of data ($P > 0.05$), so nonparametric statistical tests had to be used. We used the nonparametric Spearman’s rank correlation test to measure the strength of association between two ranked variables, considering a statistically significant correlation. Analysis of variance and the $t$-test were used to analyze the significance of differences between the study groups. $P < 0.05$ values were considered statistically significant. All data are represented as a mean $\pm$ standard deviation.

**RESULTS**

**Glioblastoma cell growth patterns, growth rate and doubling time**

Several research studies showed that low-passage cell cultures isolated from tumor are different from high-passage established cell lines of similar origin. Many disadvantages of the use of immortal cell lines in cancer studies have been discussed in the literature. For example, cells maintained in culture over a long period of time (immortal cell lines) were shown to accumulate mutations that may produce changes in the cell genotype and phenotype that have initially been detected at earlier passages. Low-passage primary cultures may better reflect the properties of original tumor and are considered much more relevant models for studying the malignant diseases *in vitro*.

In this study, we used three low-passage GB cell lines: GB3B, GB4B, and GB5B. The cell lines were established from fresh samples of brain tumor tissues, were cultured in standard conditions and frozen after passage three. After thawing in standard conditions, adherent monolayer cells showed continuous growth and could recover. The cell growth patterns of GB3B, GB4B, and GB5B cell lines are depicted in Figure 1. GR and DT were assessed for each cell line used in the study. GB3B cells proliferate with a GR of 0.2872 and a DT of 2.3 days [Figure 1a], GB4B cells proliferate with a GR of 0.2787 and a DT of 2.5 days [Figure 1b], and GB5B cells proliferate with a GR of 0.2787 and a DT of 2.7 days [Figure 1c]. The DT of GB3B cells was determined to be 5 h less than that of GB4B and

![Figure 1: Glioblastoma cell growth. Growth rate doubling time. Cells seeded at 1.5 x 10^4 cells/well were grown in standard minimum essential medium for 3 days. Cell growth was determined for glioblastoma 3B (a), glioblastoma 4B (b) and glioblastoma 5B (c) cell lines by hemocytometric counting, using trypan blue. Doubling time was determined using a publically available (34, 35) (d). Each experiment was repeated three times. Data are reported as the mean ± standard deviation.](#)
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10 h less that of GB5B, but the difference was not statistically significant ($P > 0.005$) [Figure 1d].

**The effect of curcumin on human glioblastoma cell viability**

Many studies to date have demonstrated beneficial effect of curcumin on cancer cells such as breast, colon, lung, and prostate cancer treatment in vivo and in vitro.[38-40] Curcumin was also reported to suppress malignant glioma growth in vitro and in vivo as a single agent or to sensitize malignant glioma cells to conventional therapy, making it a good candidate for individual or combined therapy.[38-40,41-43]

This natural yellow, orange dye has been found to be safe, exhibiting minimal toxicity in human; no drug-related toxicities has been reported when administered at doses up to 10 g/day.

In this study, we analyzed the effect of curcumin on three low-passage glioblastoma cell lines: GB3B, GB4B, and GB5B. To examine the effect of the drug on cell viability, exponential growing cells were exposed to increasing doses of curcumin (0.1, 1, 2, 5, 10, 20, 50, 100, and 150 µM) for three days and the inhibitory effect was quantified by performing a cell count with hemocytometer [Figures 2-4]. All cell lines studied answered in a dose-dependent manner to the treatment [Figures 2-4]. The lowest concentration of curcumin used in this study (0.1 µM) did not induce cell death in GB cells.

Minimum inhibitory concentration was 1 µM for all cell lines and provoked 3–4% cell death in GB cell lines, whereas the high concentrations of curcumin (150 µM) drastically reduced cell viability to 24.5% cell viability in GB3B, 11.8% viability in GB4B cells and 11% cell viability in GB5B cells [Figures 2-4].

The half inhibitory concentration to induce 50% cell death (IC50) was determined in each cell line for curcumin compound. The IC50 value is very important in the evaluation of the drug cytotoxic potency. The IC50 value is also important when comparing drug effect on different cell lines. We found that IC50 value was 46.4 µM for GB3B, 78.3 µM for GB4B cells, and 47.7 µM for GB5B cells.

Next, we set out to examine whether curcumin may produce changes in cell shape. The shape of the cells and the anchoring condition of the cells were observed under a phase contrast microscope. Phase contrast microscopy showed that cultures treated with curcumin in a concentration corresponding to IC50 contained rounded attached cells, cells detached from their substrate and cell fragments, 72 h after the treatment [Figures 2-4].

**Correlations between doubling time and inhibitory concentration 50 values in glioblastoma cell lines**

The DT was shown to be correlated with the sensitivity to the conventional chemotherapeutic agents that usually target fast dividing cells. The IC50 values are also important when comparing drug effect on different cell lines. We next correlated the DT of each cell lines with IC50. Using the

Figure 2: Curcumin effect on glioblastoma 3B cells. Cells seeded at $1.5 \times 10^4$ cells/well were grown in standard medium and treated with increasing doses of curcumin (0.1, 1, 2, 5, 10, 20, 50, 100, and 150 µM) for 3 days. Cytotoxic effects of curcumin were evaluated after 3 days by hemocytometric counting, using trypan blue (a). Results are expressed as a percentage of control. Data are mean and standard error of three separate experiments. Phase contrast microscopy pictures (b) were taken at initial culture day, 48 and 72 h after the treatment with 46.4 µM curcumin (×10)
nonparametric Spearman's rank correlation test we did not observe a statistically significant correlation between DT and curcumin IC50 values ($P = 0.2$, $P = 0.552$) [Figure 5]. For example, the cell line GB4B that demonstrated greater IC50 value than GB3B and GB5B cell lines did not display prolonged DT compared to these cell lines, while the cell line GB5B that displayed the longest DT, were not the least sensitive to curcumin.

Figure 3: Curcumin effect on glioblastoma 4B cells. Cells seeded at $1.5 \times 10^4$ cells/well were grown in standard medium and treated with increasing doses of curcumin (0.1, 1, 2, 5, 10, 20, 50, 100, and 150 µM) for 3 days. Cytotoxic effects of curcumin were evaluated after 3 days by hemocytometric counting, using trypan blue (a). Results are expressed as a percentage of control. Data are mean and standard error of three separate experiments. Phase contrast microscopy pictures (b) were taken at initial culture day, 48 and 72 h after the treatment with 78.3 µM curcumin ($\times 10$).

Figure 4: Curcumin effect on glioblastoma 5B cells. Cells seeded at $1.5 \times 10^4$ cells/well were grown in standard medium and treated with increasing doses of curcumin (0.1, 1, 2, 5, 10, 20, 50, 100, and 150 µM) for 3 days. Cytotoxic effects of curcumin were evaluated after 3 days by hemocytometric counting, using trypan blue (a). Results are expressed as a percentage of control. Data are mean and standard error of three separate experiments. Phase contrast microscopy pictures (b) were taken at initial culture day, 48 and 72 h after the treatment with 47.7 µM curcumin ($\times 10$).
Overall, these data indicate that the sensitivity to curcumin is not dependent on the GB cell cultures DT.

**DISCUSSION**

In recent years, curcumin as other dye compounds, either natural or synthetic, have been shown promise both as potential antitumor agents either alone or in combination with conventional treatment for several types of malignancies.

Since 2003, several research groups investigated curcumin effect in various GB cell lines and the compound was effective in killing cancer cells.

Here, we found that curcumin killed GB cells in vitro, in a dose-dependent manner. Our results are consistent with other studies that demonstrated the beneficial effect of turmeric treatment, not only on brain tumor but also on other types of solid tumor cell lines like: Breast, colon, pancreas, and lung.

In phase II clinical trials, curcumin treatment showed promising clinical results in patients with pancreatic and colorectal cancer. Although the results are encouraging, there are only a few in vivo studies that used curcumin as a treatment for malignant diseases and the substance is not yet used as a standard treatment for humans. Often, in vitro studies may lead to results that do not correspond to the in vivo findings. One of the frequent deficiencies of experiments that use established tumor cell lines is that they fail to reproduce the in vivo tumor heterogeneity. The association between tumor heterogeneity, cell survival and response to treatment has been confirmed by several studies. Another barrier in using high-passage established cell lines is that all long-time culture cancer cells accumulate a series of mutations that may produce alterations in GRs, cells morphology, protein expression, signaling, or their response to mitogenic stimuli. It has been proposed that low passage tumor cell cultures may provide a better model for testing the response to drug treatment because they preserve original tumor phenotype and genotype.

To address these questions, we used in our study three low-passage primary cell lines isolated from GB tumors (GB3B, GB4B, and GB5B). The proliferation rate was approximately the same for all three cell lines. Actually, the DT of GB3B cells was found to be 5 less than that of GB4B and 10 h less that of GB5B but the difference between them was not statistically significant. All cell lines studied answered in a dose-dependent manner to the treatment. The lowest concentration of curcumin that induced cell death in GB cells was 1 µM for all cell lines, but the treatment did not kill more than 4% cells. Used in everyday diet of many people the turmeric has been found to be safe, exhibiting minimal toxicity in human. In fact, no drug-related toxicities have been reported when administered at doses up to 10 g/day. The highest concentrations of curcumin used in our study were 150 µM that drastically reduced cell viability to 24.5% in GB3B, 11.8%, in GB4B cells and 11% in GB5B cells. Other preclinical studies have indicated that it is possible to anticipate the cell response to the drug treatment based on correlating IC50 with measurements of cell DT. Unfortunately, we did not observe a statistically significant correlation between DT and curcumin IC50 values for GB cells used in this study. For example, the cell line GB4B that demonstrated greatest IC50 value did not display prolonged DT than GB3B and GB5B cell lines. Moreover, the cell line GB5B that displayed the longest DT was not the least sensitive to curcumin. The curcumin mechanism of action on GB cells can perhaps explain this result. It is well demonstrated that several antineoplastic targeted agents act by inactivation such survival-linked molecules (e.g., growth factor receptors, apoptosis activators, etc.) that may not be directly related to cell GR or to cell DT. In this context, curcumin induced cell death in glioma cells was reported to interfere with individual molecules necessary for tumor growth and progression. In 2007, Liu et al. demonstrated the inhibitory capacity of curcumin on glioma cell growth and proliferation through cell cycle arrest and the possible involvement of ING4 in the signaling pathways. In 2015, Qian et al. proved that curcumin is able to kill U87 GB cells in vitro, in a dose and time-dependent manner. The drug also enhanced the radiosensitivity of the human GB cells and apoptosis.

The results of this investigation proved once more that curcumin is a natural compound potentially useful in the fight against GB.

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Conflicts of interest
There are no conflicts of interest.

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