Effects of N-6 essential fatty acids on glioma invasion and growth: experimental studies with glioma spheroids in collagen gels


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Object. Intracranial infusions of gamma-linolenic acid (GLA), an essential fatty acid, have been used as an adjuvant therapy following malignant glioma resection; however, little is known about the dose response of glioma cells to this therapy. In this in vitro study the authors address this important pharmacological question.

Methods. Glioma spheroids derived from U87, U373, MOG-G-CCM, and C6 cell lines were grown in collagen gel and exposed to a range of GLA concentrations (0–1 mM) for 5 days. The diameter of glioma spheroids was measured, the apoptotic index was assessed using both the terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling technique and cell morphological testing, and the levels of proliferating cell nuclear antigen were also measured.

Conclusions. The dose–response patterns were similar for all four glioma spheroids. Low concentrations of GLA (< 100 μM) increased both apoptosis and proliferation with a net increase in tumor growth and invasion, whereas high-dose GLA (> 100 μM) significantly impaired spheroid cell growth. The proliferative effects of low-dose GLA could be a hazard in the clinical treatment of malignant glioma; however, because of the low toxicity of GLA against normal cells, local delivery of millimolar doses of GLA could significantly reduce tumor size.

KEY WORDS • glioma spheroid • gamma-linolenic acid • essential fatty acid • proliferation apoptosis • tumor cell invasion

Despite significant advances in the diagnosis and treatment of malignant gliomas, they remain incurable. The invasive properties of malignant gliomas render radical surgery ineffective, and these tumors have intrinsic resistance to both chemotherapy and radiotherapy. The latter two treatments are also of limited usefulness because of their toxicity to systemic tissues and peritumoral brain. A novel alternative approach would be to use neurotoxic therapies that selectively increase tumor cell death by stimulating apoptotic pathways. In in vitro studies in which monolayer cultures and cell suspensions from primary brain tumors and the C6 glioma cell line were used it was shown that essential fatty acids (EFAs) such as gamma-linolenic acid (GLA) and arachidonic acid (ArA) induced cell death and apoptosis. The mechanisms by which EFAs induce cell death and apoptosis are unclear, but activation of macrophages, free radical production, and lipid peroxidation is thought to play a role, because the membranes of transformed cells are more susceptible to lipid peroxidation than normal cells. Infusion of GLA into cavities after glioma resection has been described as being without complication; however, little is known about the dose–response characteristics of glioma cells and tissue to GLA.

To further evaluate the effects and mechanisms of action of GLA, we have used glioma spheroids grown in collagen gel. The advantages of the use of collagen gel matrix compared with a monolayer culture system to assess tumor growth, individual cell migration from the spheroid into the gel matrix, and cellular proliferation are well described. In addition, spheroids offer several advantages over monolayer culture, including similarity to three-dimensional malignant glial tumors, enhancement of cell–cell interactions in the biomass, and the induction of diffusion gradients within the tumor cell mass. The culture of glioma cell lines as spheroid suspensions in collagen gels results in a tissue mass that can be fixed and neuropathologically evaluated using conventional and immunohistochemical techniques.

Using this model we tested the hypothesis that GLA, the N-6 EFA, will inhibit both growth and invasion of glioma spheroids by signaling proapoptotic and antiproliferative pathways. We also wished to evaluate the dose–response effects of GLA on tumor spheroids in view of the enhanced proliferation of some malignant cells that have been reported at low levels of EFA (unpublished data). We also evaluated the uniformity of the GLA dose–response curve for a range of human and rodent glioma cells.
to determine if idiosyncratic responses were present in particular glioma cell lines. These experiments are particularly pertinent if the therapeutic potential of EFA infusion directly into the brain of patients with malignant tumors is to be optimized.  

Materials and Methods

Cell Culture

We used four cell lines in this study: the C6 rodent glioma cell line and the human cell lines MOG-G-CCM (anaplastic astrocytoma), U373 (glioblastoma), and U87 (glioblastoma). All cell lines were supplied by the European Collection of Cell Cultures and were tested with anti-glial fibrillary acidic protein antibodies to confirm their glial origin. The cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS), penicillin, and streptomycin.

Spheroid Formation

When they reached confluence in monolayer culture, the cell lines were trypsinized and seeded into spinner culture flasks at a density of $3 \times 10^6$ cells/100 ml of medium (DMEM with 10% FCS), and centrifuged at 180 rpm for 3 weeks or longer until spheroids of 500 to 600 µm were obtained. Individual spheroids were then injected into 600 µl of type I collagen gel in 24-well plates overlaid with 500 µl of medium (DMEM with 10% FCS). A concentration of 3 mg/ml of type I collagen gel was used with a 10-fold concentrated solution of phosphate-buffered saline in a ratio of 8:1.

Incubation of Spheroids in GLA

An assessment of the effects of continuously administered GLA on the invasive capacities of the cell lines was made by removing the medium daily and replacing it with medium containing varying concentrations of GLA (0, 10, 25, 50, 100, 250, and 500 µM, and 1 mM). Four spheroids from each cell line were tested with each concentration of GLA. The spheroids were examined daily to observe cellular and morphological features and to record differences in invasion distance from the spheroid margin. To ascertain the total invasion distance into a spheroid, the radius of the mass was measured and this was added to the distance from the periphery of the solid tumor mass to the edge of the surrounding halo of invading cells. These measurements were taken separately and the whole diameter was calculated (the whole spheroid cannot be seen in one field of view by using the lowest objective lens). The growth rates of glioma spheroids were plotted with time against the spheroid diameter, and growth kinetics were assessed using the residual means squared to calculate the best fit by using various linear curve fit models. Changes in the density and shape of the spheroids and any changes in the morphological features of the invading cell population were recorded. After 5 days the collagen matrices and incorporated spheroids were fixed in 4% buffered formaldehyde and embedded in paraffin.

Immunohistochemical Analysis of Proliferative Index by Using Proliferative Cell Nuclear Antigen

Sections from the fixed spheroids were cut at 5-µm thickness, mounted on slides, and dried overnight at 60°C. The slides were deparaffinized in xylene for 5 minutes and rehydrated in water following immersion in 70% alcohol. After immersion in 3% H2O2, and blocking in rabbit serum, the sections were incubated at 4°C overnight with a monoclonal antibody to proliferating cell nuclear antigen (PCNA) at a dilution of 1:200 in 1:5 rabbit serum/Tris-buffered saline (TBS). After removal of the primary antibody, the sections were washed in TBS and incubated in rabbit anti–mouse biotinylated secondary antibody for 30 minutes at room temperature at a dilution of 1:200 in 1:5 rabbit serum/TBS. They were then washed with TBS followed by a 30-minute incubation with peroxidase-labeled avidin–biotin complex, and then rinsed in distilled water and visualized using 0.1% diaminobenzidine and 0.003% H2O2. The sections were washed in tap water, counterstained with hematoxylin, dehydrated in alcohol, and mounted on a coverslip with Depex after immersion in xylene. Proliferative activity was measured by calculating the percentage of PCNA-positive against normal cells. Four 50-µm squares were counted across the spheroid radius and an average was taken for each cell line and concentration group. Areas of central necrosis were excluded.

Analysis of Apoptotic Index by Using Nick-End Labeling and Cell Morphological Studies

Additional sections from the fixed spheroids were cut, deparaffinized, and rehydrated as described earlier and then incubated with proteinase K for 20 minutes at a concentration of 20 µg/ml in 10 mM/HCl, pH 7.6. After washing in phosphate-buffered saline, the sections were incubated in a terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL) reaction mixture. This was followed by the addition of a secondary detection system that contained a biotinylated antibody to fluorescein to visualize the fluorescent markers for light microscopy studies according to the instructions for the in situ cell detection kit. Once the substrate (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium tablets) was added, the sections were washed, dehydrated, counterstained, and mounted as described earlier. The overall apoptotic index of each spheroid was measured by counting the number of TUNEL-stained and normal cells present within four 50-µm squares spanning the spheroid radius, not including areas of necrosis. Two spheroids were used for each concentration and cell line for these measurements. To assess the accuracy of the TUNEL technique, hematoxylin and eosin–stained slides were used to calculate an apoptotic index by morphological observation of the cellular features of apoptosis. One in two of the aforementioned...
TUNEL-stained 50-μm squares was chosen for each concentration, and squares were counted using adjacent serial sections.

Sources of Supplies and Equipment

The anti-glial fibrillary acidic protein antibodies, PCNA, and avidin–biotin complex kit were purchased from DAKO, High Wycombe, UK. The European Collection of Cell Cultures (CAMR), which supplied the cell lines, is located in Salisbury, UK. The DMEM, FCS, penicillin, and streptomycin were all obtained from Gibco-BRL Life Technologies, Paisley, Scotland. The Vitrogen 100 gel was acquired from the Collagen Corp., Fremont, CA. The GLA (lithium gamolenate) was a gift from Scotia Pharmaceuticals, Stirling, Scotland. The rabbit serum was obtained from the Scottish Antibody Production Unit, Paisely, Scotland. The DePex mounting material was purchased from Merck Ltd., Poole, Dorset, UK. The in situ cell detection kit was acquired from Boehringer Mannheim, Mannheim, Germany. The 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium tablets were purchased from Sigma Chemical Co., St. Louis, MO.

Results

Kinetics of Glioma Spheroid Growth

All cell lines (C6, MOG, U87, and U373) were confirmed to be of glial origin and were phenotypically similar in monolayer cultures. When spheroids made from these cell lines were incubated in the absence of GLA very similar rates of increase in diameters (0.5–2.5 mm) were observed over a 5-day period (Fig. 1). Although statistically significant differences between patterns of spheroid growth were observed at certain time points, this is probably not biologically significant, because in all cell lines the kinetics of growth fitted the same linear regression model. In all cell lines, the best regression function describing spheroid growth was a polynomial third-degree curve giving the best fit ($r^2 = 0.975–0.9992$).

Effects of GLA on Glioma Spheroid Growth

When the effects of GLA on glioma spheroid invasion into collagen gel were measured, concentrations in the range of 10 to 50 μM produced invasion distances significantly greater than those in the spheroids grown in control media (Fig. 2). The effect of 100 μM of GLA was similar to the control medium in three of the cell lines (MOG, C6, and U373). More rapid spheroid growth was observed in the U87 cell line at 100 μM GLA after Day 3.
At GLA concentrations between 250 and 1000 μM, the invasive capacities of the glioma spheroids in all four cell lines were significantly impaired, with only very small increases in diameter occurring with the size at implantation (Fig. 2). During incubation in the 1-mM solution of GLA the C6 glioma spheroids were induced to fragment into necrotic clusters and were measured at “zero” from Day 2 until the end of the experiment.

When examined using light microscopy before fixation, there were no noticeable differences in the morphological features of the invading cell population between the control and low-dose GLA gels. The control and lower-dose (10–50 μM) GLA experiments showed a characteristic thinning of the cell population at the periphery of the spheroid mass, where cells move from the main spheroid body to become part of the invading cell population (Fig. 3). In the three highest GLA concentrations (250–1000 μM) an absence of this thinning was shown, and detailed histological examination of the cells on the surface of the spheroid revealed that many were necrotic in appearance.

Spheroid Cross-Section Examination With Apoptotic and Proliferative Markers

**The PCNA Marker for Proliferating Cells.** The spheroids that were growing in the absence of GLA had a thin outer rim of PCNA-positive cells and a mean count of 5% PCNA positivity across the spheroid radius, excluding areas of central necrosis (Table 1). When exposed to concentrations of up to 100 μM GLA, the overall number of PCNA-positive cells increased. Table 1 shows the mean PCNA indices for each cell line when exposed to increasing concentrations of GLA. The greatest increase in PCNA staining was observed within the peripheral band of cells. As the band of cells became larger there was a corresponding decrease in the viability of the cells within the center of the tumor mass (Fig. 4), where few PCNA-positive cells were observed. As the concentrations of GLA were increased to more than 100 μM, the PCNA-positive peripheral band was seen to decrease in size. The overall trend in PCNA positivity is shown in Fig. 5.

**Use of TUNEL Staining to Detect Apoptotic Index.** The control TUNEL experiments showed a consistent apoptot-

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**TABLE 1**

<table>
<thead>
<tr>
<th>GLA Dose (μM)</th>
<th>TUNEL (8 spheroids)</th>
<th>H&amp;E (4 spheroids)</th>
<th>PCNA (4 spheroids)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell Line</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C6 0</td>
<td>4.2</td>
<td>2.8</td>
<td>2.6</td>
</tr>
<tr>
<td>50 31.4</td>
<td>22.6</td>
<td>10.2</td>
<td></td>
</tr>
<tr>
<td>100 47.6</td>
<td>38.4</td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td>500 83.2</td>
<td>72.0</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>U87 0</td>
<td>5.8</td>
<td>4.0</td>
<td>4.8</td>
</tr>
<tr>
<td>50 24.6</td>
<td>15.8</td>
<td>11.1</td>
<td></td>
</tr>
<tr>
<td>100 39.3</td>
<td>30.2</td>
<td>6.1</td>
<td></td>
</tr>
<tr>
<td>500 90.2</td>
<td>60.8</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>U373 0</td>
<td>3.9</td>
<td>2.8</td>
<td>3.2</td>
</tr>
<tr>
<td>50 28.6</td>
<td>20.0</td>
<td>11.1</td>
<td></td>
</tr>
<tr>
<td>100 50.0</td>
<td>40.0</td>
<td>4.2</td>
<td></td>
</tr>
<tr>
<td>500 79.0</td>
<td>68.3</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>MOG-G-CCM 0</td>
<td>5.1</td>
<td>3.8</td>
<td>6.2</td>
</tr>
<tr>
<td>50 30.1</td>
<td>22.2</td>
<td>11.1</td>
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<td>100 50.9</td>
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</tr>
<tr>
<td>500 88.3</td>
<td>67.8</td>
<td>1.2</td>
<td></td>
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</table>

* Morphological assessment with hematoxylin and eosin (H&E) staining.
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Fig. 4. Photomicrograph showing a cross section of an MOG-G-CCM spheroid treated with 50-μM of GLA. Immunohistochemical studies with PCNA were used to label all cells in the S phase (brown cells). An area of necrosis (N), a proliferative band (P), and an invading cell population (I) are seen. Bar = 50 μM.

Fig. 5. Graph showing the comparative trends for apoptosis and proliferation by combining the apoptotic indices from glioma spheroids grown from the four cell lines (C6, MOG-G-CCM, U87, and U373) over a range of GLA concentrations. Data points represent the mean ± SE for spheroids (one for each cell line) and eight spheroids (two for each cell line) for PCNA and TUNEL techniques, respectively.

periphery of each spheroid, where maximum PCNA labeling was observed. A summary of the effect of GLA concentration on apoptosis, combining results in all four cell lines, is presented in Fig. 5.

Discussion

There is considerable current interest in the proapoptotic properties of EFAs, and these have been evaluated using both primary tumor cultures and established tumor cell lines.1,13,15,23 In this report we have shown that when GLA was added to the culture medium containing spheroids derived from four established glioma cell lines, three distinct effects were observed. First, low concentrations of GLA (10–50 μM) significantly enhanced spheroid growth and increased the number of proliferating cells around its surface. Second, concentrations exceeding 100 μM inhibited cellular invasion and reduced the proliferative capacity of the spheroid, and third, the overall number of apoptotic cells within the spheroid progressively increased with GLA concentrations in the range 10 to 500 μM. The observed invasive properties of spheroids derived from the C6 cell line were similar to those found in other studies,8,21 and the four cell lines examined all showed similar growth characteristics and responses to GLA therapy.

Tumor cells have been found to contain lower levels of membrane-bound EFAs compared with levels found in normal cells.2,12,18,24 When c-unesterified fatty acids are added to the culture medium of tumor cells in monolayer cultures, lipid peroxidation and free-radical production are initiated, which eventually result in tumor cell death.3,19 In studies in which TUNEL and flow cytometry were used, it has been shown that C6 glioma cells and cells from primary human glioblastoma suspensions can be stimulated to undergo apoptosis within minutes of incubation with
concentrations of ArA as low as 20 to 40 μM. Recently it has been shown that EFAs significantly increased the production of reactive oxygen intermediates in glioma compared with normal tissue. Some EFAs, such as ArA and eicosapentaenoic acid, have been found to have in vitro effects similar to GLA, although different types of tumor cells vary in their sensitivity to various EFAs. However, preparations of primary human glioma cell were particularly sensitive to GLA in their peroxidative response, and lipid peroxidation activity was found to correlate with antiproliferative activity in various tumor cell lines.

Spheroid Cell Death and GLA

The exclusive use of TUNEL in the absence of other methods of identifying apoptotic cells has recently been criticized. However, in all spheroid types, the assessment of apoptosis by using both TUNEL and morphological criteria were consistent in showing apoptotic indices that were always higher with increasing GLA concentrations in the concentration range of 10 to 500 μM. The higher apoptotic index detected using TUNEL arises partly because with TUNEL techniques we have the ability to detect apoptotic cells earlier and partly because of the inclusion of some necrotic cells as TUNEL positive. Thus we found that certain cells labeled positive with the TUNEL technique did not have the classic morphological features of apoptosis.

Although we found a steady increase in overall cell death with increasing concentrations of GLA, the increases in apoptotic index and volume of necrosis in the low-dose (10–50 μM) experiments were confined to the center of the spheroid, not the periphery where the cells were directly exposed to the drug. It is therefore unlikely that these cytological observations were the result of increased reactive oxygen intermediate formation because the products of lipid peroxidation can readily pass between cells. Additionally, an increase in reactive lipid peroxide formation should have been generated peripherally, where GLA exposure is maximal, yet the cells on the outside of the spheroids remained unaffected. Because low concentrations of GLA increased peripheral glioma cell proliferation, the observed increase in central apoptosis and necrosis were most likely to have been caused by intrasphenoid hypoxia resulting from inadequate oxygen and nutrient delivery. Central hypoxic regions containing necrosis are common features of larger C6 spheroids and glioblastomas multiforme.

Although the apoptotic index of the invading cell population was not measured because of cell loss during tissue processing, it is likely that there was a dose-dependent increase in the apoptotic index in this population. At low GLA exposures, increases in cell death within the invading cell population were not sufficient to decrease either the invasion distance or peripheral sphenoid proliferation. Studies in which monolayer cultures of neuroblastoma and breast cancer cell lines were observed over a 5-day period have shown that there is significant cell loss with direct exposure to GLA at concentrations similar to those used in our low-dose experiments (10–50 μM). This indicates that the invading cell population, which has been
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shown to contain very few proliferating cells (R Del Maestro, personal communication, 1998), may have relative resistance to GLA therapy. Doses greater than 100 μM pf GLA were required before the distance traveled by the invading cell population was reduced and any overall spheroid growth inhibition was observed.

Spheroid Cell Proliferation and GLA

Paraffin-fixed blocks were stained with PCNA, which strongly labels cells in the S phase, and this provided a relative measure of proliferation between spheroid groups that was easy to quantify. There has been little investigation into the corresponding proliferation indices associated with the addition of EFAs to glioma cell cultures. We have found that the addition of low-dose GLA (10–50 μM) produced not only large increases in maximum invasion distance but significantly increased the proliferative index of the glioma cells on the periphery of the spheroid. The addition of GLA to culture media may increase proliferation simply by supplying required membrane-bound substrates that optimize tumor cell growth. Such substrates are present in larger amounts in normal cells.12,13 As with the TUNEL experiments, it was not possible to label the invading cell population with the PCNA marker because of cell loss during fixation. The proliferative indices for this region would be significantly lower than in other regions of the spheroid because invading cells are generally thought of as being nonproliferative.21

By creating a diverse cellular microenvironment containing the three-dimensional characteristics of a solid tumor, we have identified changes in cell death and proliferation that could be potentially relevant to local GLA therapy in vivo. Our in vitro experiments with low-dose GLA indicated that an intratumoral infusion of high-dose GLA could create a diffusion gradient encouraging peripheral growth where the concentration of this EFA has become lower. However, low toxicity against normal cells and tumor selectivity allows GLA to be introduced in very high doses. In recent in vivo studies in which the investigators used the rodent C6 implantation glioma model and intratumoral infusions of GLA through miniosmotic pumps, it has been shown that concentrations as high as 2 to 4 mM were required for significant antitumoral effects against established 7-day implanted glioma.14 In these in vivo experiments it was also observed that the GLA had little effect on the surrounding neuropil. Additionally, preliminary clinical experience with EFA infusions in patients with malignant gliomas indicates that proliferative effects are not significant and neurotoxicity is minimal.17

Conclusions

From this glioma spheroid model it is clear that GLA concentrations approximately three times higher than those found to significantly increase the apoptotic rate in monolayer culture and 10 times lower than those exhibiting antitumoral activity in vivo are required to kill invading glioma cells, reduce the size of the proliferative rim, and significantly increase the apoptotic index of the central core of tumor cells.

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References


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