The inhibition of proliferation and migration of glioma spheroids exposed to temozolomide is less than additive if combined with irradiation

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Abstract. The aim of this study was to investigate the effect of temozolomide (TZM) in combination with X-rays on proliferation and migration in human glioma spheroids. Multicellular spheroids were derived from GaMg and U87 cell lines. Spheroids were treated with various concentrations of TZM (5 μmol, 0.025 mmol, 0.05 mmol) and irradiation (RT). Proliferation and migration assays were performed. For GaMg spheroids, the proliferation inhibition was 30% (RT), 71%, 79%, 85% (for various TZM concentrations) and 78%, 83%, 90% following RT+TZM. For U87 spheroids, the inhibition of proliferation was 52% (RT), 62%, 78%, 88% (TZM), and 73%, 87%, 92% (RT+TZM). Inhibition of migration for GaMg was 30% (RT), 37%, 63%, 78% (TZM), and 56%, 75%, 84% (RT+TZM). For U87, migration inhibition was 29% (RT), 48%, 52%, 67% (TZM), and 62%, 67%, 73% (RT+TZM). Radiotherapy enhancement ratio (RER) of GaMg/U87 spheroid proliferation was 1.4/1.7 (5 μmol TZM), 1.3/1.8 (0.025 mmol TZM), and 1.4/1.4 (0.05 mmol TZM). RER for migration of GaMg/U87 was 2.2/1.9 (5 μmol TZM), 1.7/1.8 (0.025 mmol TZM), and 1.5/1.4 (0.05 mmol TZM). In terms of inhibition of proliferation and migration, irradiation can lead to an enhancement of the TZM effect in human glioma spheroids, which is less than additive.

Introduction

Gliomas are the most common primary human brain tumours and prognosis is poor, in particular with high-grade tumours such as glioblastoma multiforme (GBM). Intensive surgery followed by radiotherapy with or without adjuvant chemotherapy is considered as standard therapy for primary brain tumours (1). Treatment of brain tumours with adjuvant chemotherapy, mainly 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU), has only resulted in minor improvements (2-4). Phase I-II clinical studies with temozolomide (TMZ) have demonstrated that this drug crosses the blood-brain barrier and has activity against malignant glioma (5-8).

TMZ in the treatment of primary and recurrent malignant brain tumours is still a topic of clinical trials due to the promising pre-clinical and clinical reports. Recently, Stupp *et al* found a clinically meaningful increase, by a factor of 2.5, in the survival rate at two years, from 10% with radiotherapy alone to 27% with radiotherapy plus TZM in a randomized phase III trial. The addition of temozolomide to irradiation was associated with improvements in median progression-free survival (6.9 vs. 5.0 month) and overall survival (14.6 vs. 21.1 month) (9).

Since radiotherapy in combination with TMZ might be assumed as gold standard in the treatment of brain malignancies in future (10), it is of interest to investigate further what the pre-clinical effects are on various human glioma cell lines. In a study with the human glioma cell line U373 treated as monolayer cultures, an additive effect was reported for TMZ and X-rays (11). In glioma cells lines U251 and D384, combinations of TMZ and X-rays showed additional as well as supra-additional cytotoxic effects (12).

We have chosen spheroids of U87MG and GaMg glioma cell lines to study the proliverative and migrative behaviour of TZM and irradiation. However, the U87MG cell line contains a functional p53 protein and was described to be sensitive to cytostatic drugs, while the GaMg cell line was described to be relatively resistant to these compounds (13,14).

Moreover, most of the studies on TZM and its activity against glioma cells were carried out in monolayer cultures (15,16). The spheroid model is a three-dimensional cell culture system that more closely resembles the *in vivo* situation inside a tumour (17,18). Along the axis of spheroids, steep gradients can exist for cellular oxygen levels, glucose concentration, nutrients, serum-derived growth factors and pH (19,20). Individual tumour cells growing under these conditions face a different environmental situation depending on their position inside the three-dimensional framework of the spheroid.

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In a three-dimensional cell culture model of the glioma cell lines U87MG and GaMg, a TZM dose-dependent inhibition of tumour growth was found recently, but the role of the clinically relevant combination with irradiation was not investigated. There, the drug was able to induce apoptosis in the core region of glioma U87Mg spheroids, which harbours a functional p53 gene. Thus, TZM might act as an initiator of apoptosis in a glioma spheroid cell culture system (21).

Following these experiments, it was our aim to study the effects of irradiation on TZM-treated glioma cells in terms of tumour cell migration and proliferation in a well-defined three-dimensional spheroid culture system.

Materials and methods

Cell line spheroids. The human U87 cell line was obtained from Dr J. Ponten, University of Uppsala, Sweden. The GaMg cell line was kindly supplied by Dr R. Bjerkvig, University of Bergen, Norway (22). Both cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated newborn calf serum, 2% L-glutamine, 3.2% nonessential amino acids (alanine, asparagine, aspartic acid, glutamic acid, glycine, proline, serine), penicillin (100 IU ml^{-1}) and streptomycin (100 μg ml^{-1}) and kept in standard tissue culture conditions.

Spheroid cultures of both cell lines were formed by liquid-overlay technique (23). In short, exponentially growing monolayers were trypsinized and $5x10^6$ cells were seeded in 20 ml of growth medium into 0.8% agar-coated tissue culture flasks. After 10 days in culture, spheroids with diameter of ~250 μm were selected for the experiments. These spheroids do not present central necrosis and have very few hypoxic cells (24,25).

Treatment. Spheroids were irradiated in petri dishes (60 mm), containing 3 ml of culture medium at a temperature of 37˚C. Due to the known differences in response to irradiation, iso-effective single dose RT was performed using an orthovolt X-ray generator operating at 250 kV and 15 mA, with a 0.5-mm thick Cu filter and a tube opening of 8x8 cm: 10 Gy for GaMg spheroids and 5 Gy for U87 spheroids. Former experiments of our laboratory explored this RT dosages to be equally effective without sterilising the spheroids (26).

Temozolomide (Temodal®, TZM) was obtained from Essex Pharma (Munich, Germany). The drug was added to the culture medium and diluted serially prior to application. TZM was applied in a single dose in concentrations ranging from 5 μmol to 0.05 mmol to eight individual spheroids per treatment group. The drug was not removed after application as the half-life of TZM and the first derivative MTIC is ~2 h (27). Thereafter, equally sized spheroids were removed and exposed to irradiation. Three independent experiments were carried out.

Proliferation assay. Multicellular spheroids were aggregated from single-cell suspension and grown for 7-10 days in liquid overlay culture. Equally sized spheroids with diameters of ~250 mm were placed in 24-well culture dishes (NUNC, Roskilde, Denmark) base-coated with 0.8% agar. Each treatment group (sham, irradiation alone, TZM alone, and combinational treatment) consists of 24 glioma spheroids in three independent experiments. To study the spheroidal response to treatment, spheroids were incubated for 18 days. Spheroidal volume growth was monitored daily and spheroid volume was calculated using the equation: $4=3\pi(D1+D2)/4)^3$, where D1 and D2 are the maximal diameters of the spheroids measured in rectangular directions.

In order to measure growth delay, the average time required to reach five times the initial spheroid volume was determined. Specific growth delay (SGD) values were calculated by dividing growth delay of treated samples by the corresponding doubling times of control samples. Treatment-related changes in terms of reduction of the proliferation capacity of spheroids at day 18 were expressed in % of the control spheroids (100%). A relative reduction factor was calculated for all treatment groups of both assays (proliferation assay day 18, migration assay day 4) using the equation (control-treatment 1)/treatment 1 and (control-treatment 2)/treatment 2, thereafter (treatment 1/treatment 2). Statistical comparison of the original data was performed using a unpaired sample t-test after analysis of variance. P-values <0.05 were considered as significant.

Cellular migration. Each treatment group (sham, irradiation alone, TZM alone, and combinational treatment) consists of 24 glioma spheroids in three independent experiments. After 7 days of stationary culture in agar base-coated dishes, individual multicellular spheroids were placed in 24-well culture dishes. Upon adherence to the solid support, spheroids disassembled and released cells migrated away radially from their initial position. The area (mm^2) covered by cells was measured every 24 h over a period of 4 days. The area covered by the cells was taken as an indicator of cellular migration ability. Treatment-related changes in terms of reduction of the migration area compared to control spheroids were expressed in %.

Results

Proliferation assay. Proliferation capacity of different TZM concentrations on spheroids of both cell lines was determined. For GaMg spheroids, a significant reduction in spheroid growth was observed following 5 μmol TZM of 71% (specific growth delay, SGD 2.75), 0.025 mmol TZM of 79% (SGD 4.0), 0.05 mmol TZM of 85% (SGD 4.5) on day 18. For U87 spheroids, a significant reduction of the spheroid volume was observed following incubation of 5 μmol TZM of 62% (SGD 2.0), 0.025 mmol TZM of 78% (SGD 3.25), 0.05 mmol TZM of 88% (SGD 4.25) on day 18.

Following RT alone, GaMg and U87 spheroids showed a significant growth reduction of 30% (SGD 1.25) and 52% (SGD 1.75), respectively. Following combined modality treatment, GaMg and U87 spheroids showed an additively significant growth reduction of 78% (SGD 3.75) and 73% (SGD 2.7) incubated with 5 μmol TZM+RT, 83% (SGD 5.0) and 87% (SGD 5.25) incubated with 0.025 mmol TZM+RT, and 90% (SGD 5.5) and 92% (SGD 5.5) incubated with 0.05 mmol TZM+RT, respectively (Fig. 1).

Radiotherapy enhancement ratio (RER) of GaMg/U87 spheroid proliferation was 1.4/1.7 (5 μmol TZM), 1.3/1.8 (0.025 mmol TZM), and 1.4/1.4 (0.05 mmol TZM). Table I illustrates the overall outcome of experiments.

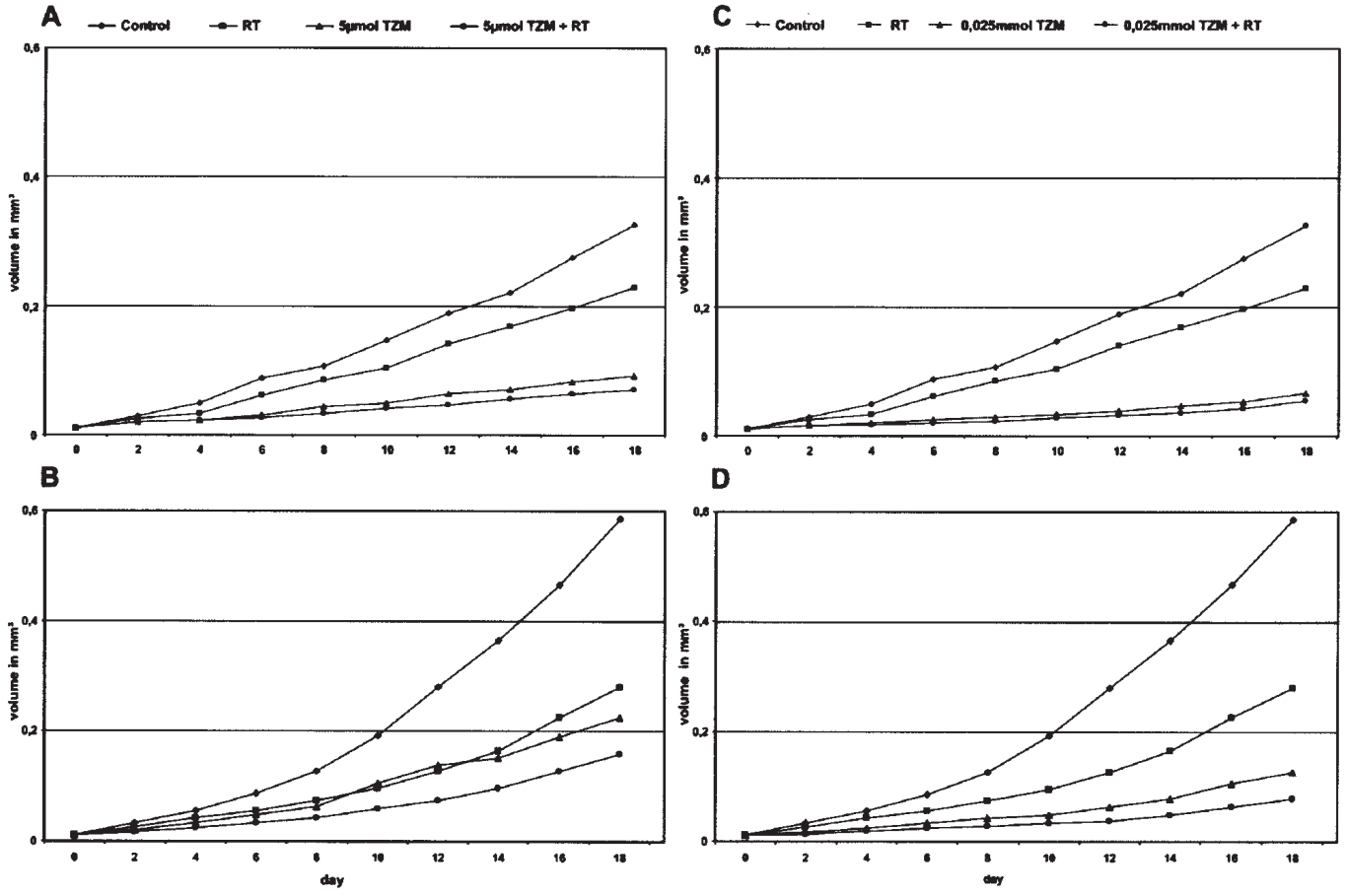

Figure 1. Spheroid proliferation of GaMg and U87 spheroids following single mode and combinational treatment. Each treatment group consists of 24 glioma spheroids in three independent experiments. Illustration of volume growth of GaMg spheroids (A and C) and U87 spheroids (B and D) following irradiation (RT) and temozolomide (TZM): 5 μmol TZM (left column), 0.025 mmol TZM (right column). Changes at day 18 were significant ($p<0.05$).

Table I. Overall treatment outcome (relative reduction factor at day 18).[a]

	Proliferation		Migration	
	GaMg	U87	GaMg	U87
RT	0.4	1.1	0.4	0.4
5 μmol TZM	2.5	1.6	0.6	0.9
0.025 mmol TZM	3.9	3.6	1.7	1.1
0.05 mmol TZM	6.2	7.5	3.5	2.0
5 μmol TZM+RT	3.6 (**1.4**)	2.7 (**1.7**)	1.3 (**2.2**)	1.7 (**1.9**)
0.025 mmol TZM+RT	4.9 (**1.3**)	6.5 (**1.8**)	2.9 (**1.7**)	2.0 (**1.8**)
0.05 mmol TZM+RT	8.9 (**1.4**)	10.9 (**1.4**)	5.3 (**1.5**)	2.7 (**1.4**)

[a]RT, irradiation; TZM, temozolomide. Bold numbers: radiotherapy enhancement ratio (RER).

Migration assay. The directorial cell migration from the spheroids was determined for treated and untreated spheroids. Following TZM treatment alone for GaMg spheroids, the tumour outgrowth area was reduced by 37% (5 μmol TZM), 63% (0.025 mmol TZM), and 78% (0.05 mmol), respectively. For U87 spheroids, the migration capacity was reduced by 48% (5 μmol TZM), 52% (0.025 mmol TZM), and 67% (0.05 mmol), respectively.

Following RT alone, GaMg and U87 spheroids showed a significant growth reduction of 30% and 29% after 4 days. Following combined modality treatment, GaMg and U87 spheroids showed an additively significant growth reduction of 56% and 62% incubated with 5 μmol TZM+RT, 75% and 67% (SGD 5.25) incubated with 0.025 mmol TZM+RT, and 84% and 73% incubated with 0.05 mmol TZM+RT, respectively (Fig. 2). RER for migration of GaMg/U87 was 2.2/1.9 (5 μmol TZM), 1.7/1.8 (0.025 mmol TZM), and 1.5/1.4 (0.05 mmol TZM).

Discussion

Local migration and proliferation activity of the remaining tumour cells following operation were inherent features of glioblastoma multiforme. Spheroid growth reflects the proliferation of tumour cells, while the migration assay measures the ability of the cells organised in three-dimensional structure to migrate and proliferate. Since modern radiotherapy is unable to inactivate glioblastoma growth for a prolonged interval, the temporary irradiation benefit has to be intensified by active drugs, such as temozolomide (TZM).

It is assumed that spheroid cultures of glioma cell lines can better predict the *in vivo* response than monolayer cultures,

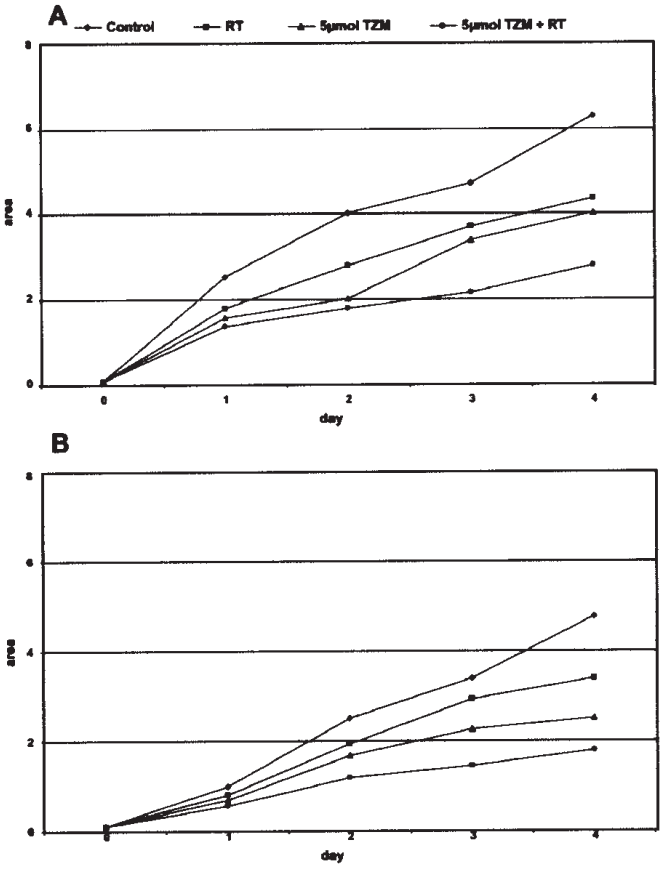

Figure 2. Example for tumor cell migration from GaMg (A) and U87 (B) spheroids following irradiation (RT), temozolomide (TZM), and combinational treatment (TZM+RT). Area of tumor cell migration (mm^2). Each treatment group consists of 24 glioma spheroids in three independent experiments. Changes at day 4 were significant ($p<0.05$).

since cell-cell contact, variation in cell cycle, altered metabolism, and diffusion of nutrients, oxygen or drugs may influence the outcome (20,28-31). The advantage of cell line spheroids is that they are relatively easy to obtain and to maintain in culture. Treatment-related changes of the growth kinetic of spheroids and the outgrowth of tumour cells present established and reproducible endpoints (25,32,33).

Using spheroids of two biologically different human glioblastoma cell lines, we have shown in this study that irradiation can enhance the cytotoxic effect of TZM, but the exerted effect is less than additive in terms of spheroid growth and migrational behaviour.

Tolerance to TMZ is primarily associated with the activity of the repair protein O6-methylguanine-DNA methyltransferase (MGMT) which removes alkylgroups from DNA (34). Spheroids of GaMg and U87 cells did not show a significant increase of TZM toxicity by co-application of the MGMT inhibitor O6-benzylguanine. Following TZM exposure, apoptosis was induced in spheroids of U87 cells, which express endogenous wild-type p53, but not in spheroids of GaMg cells (21). This study confirmed former experiments (26), that U87 glioma spheroids were more radiosensitive than GaMg spheroids, but TZM treatment (as well in combination irradiation) was more or less iso-effective in spheroids of both cell lines. Thus, the p53 status of glioma spheroids might influence the response to irradiation more than TZM. Cells with a known functional p53 gene, such as U87Mg spheroids, or an deficient MGMT detoxification system undergo either apoptosis or remain in a senescent state. Both pathways might be able to reduce glioblastoma spheroid growth in terms of cellular proliferation and migration.

In monolayer cultures of U373MG glioma cells, TZM and irradiation provided an additive effect of 2.5- to 3.0-fold increased cell kill (11). Our experiments using spheroid cell cultures led to the finding that irradiation induced an enhancement of various TZM concentrations of 1.3- to 1.8-fold growth inhibition for both cell lines. Following combination treatment, a 1.4- to 1.9-fold increased reduction of the tumour cell migration area was noted. However, the effects on spheroid growth and migration of combined treatment of TZM and irradiation are less than additive (<2.0-fold). This less than additive effect on the radiotherapy enhancement ratio might be called increased or enhanced, which reflects the current clinical situation at best (9).

Despite the progress in GBM treatment, the clinical responses are of short duration and barely influence the overall survival. Therefore, pre-studies of irradiation and TZM in combination with other classes of anti-neoplastic drugs are warranted.

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