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Glucose Influences the Response of *Glioblastoma* Cells to Temozolomide and Dexamethasone

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Abstract

Objective: Current research indicates that weakness of glucose metabolism plays an important role in silencing of invasiveness and growth of hypoxic tumors such as GBM. Moreover, there are indications that DXM, frequently used in treatment, may support GBM energy metabolism and provoke its recurrence.

Methods: We carried out in vitro experiments on the commercial T98G cell line and two primary GBM lines (HROG02, HROG17) treated with TMZ and/or DXM in physiological oxygen conditions for GBM (2.5% oxygen) and for comparison, in standard laboratory conditions (20% oxygen). The influence of different glucose levels on selected malignancy features of GBM cells-cellular viability and division, dynamic of cell culture changes, colony formation and concentration of InsR have been elevated.

Results: Under 2.5% oxygen and high glucose concentration, an attenuated cytotoxic effect of TMZ and intensification of malignancy features in all glioblastoma cell lines exposed to DXM was seen. Furthermore, preliminary retrospective analysis to assess the correlation between serum glucose levels and Ki-67 expression in surgical specimens derived from patients with GBM (IV) treated with radio-chemotherapy and prophylactic DXM therapy was performed.

Conclusion: The data suggest a link between the in vitro study results and clinical data. High glucose can influence on GBM progression through the promotion of the following parameters: cell viability, dispersal, InsR expression and cell proliferation (Ki-67). However, this problem needs more studies and explain the mechanism of action studied drugs.

Keywords

glioblastoma, brain tumor, glucose in cancer, chemotherapy in brain cancer, adjuvant treatment in brain cancer

Introduction

GBM is the most common and aggressive primary brain tumor in adults. Although treatment options have been improved during the last few years, prognosis for patients with diagnosed GBM still remains dismal and median survival following standard treatment invariably ranks in the range of about 14 months. For this reason, new approaches to design optimized therapies are urgently needed.[1]

GBM exhibits an aberrant glucose metabolism based on a high glycolytic rate what opens the door for potential metabolic interventions.[2] Despite the results of some in vitro studies,

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preclinical *in vivo* experiments and clinical studies demonstrated a correlation between high glucose levels and tumor malignancy; however, this issue remains neglected in clinical practice and is controversially discussed. It was shown that limiting glucose availability along with increased production of ketone bodies prolongs survival in *in vivo* models and clinical studies.[3,4] This beneficial effect is the result of some processes induced under these conditions, including inhibition/reduction of proliferation, tumor cell vitality, angiogenesis and blockade of the cell cycle, diminished production of ROS, suppression of brain edema, reduced cell motility, attenuation of gene expression to patterns seen in non-tumor cells, induction of apoptosis and autophagy, enhancement of radiation and chemotherapy efficacy and even neuroprotection.[5,6] The anticancer effect induced in GBM cells by glucose deprivation as explained in the "starving cancer cells of sugar" hypothesis which is related to triggering voltage differences across the cancer cell membrane and increasing calcium levels in cells, thus inducing apoptosis.[7] On the other hand, Schwartbaum et al[8] demonstrated an inverse association between blood glucose levels and GBM occurrence, suggesting that hyperglycemia may paradoxically confer protection against GBM through the inhibition of cerebral circulation.

The exact mechanisms through which the compromised glucose metabolism affects tumors and makes glucose the "sweet kiss of death" are unclear. One plausible explanation is the increased insulin levels and resulting in augmented signaling network via InsR and IGF-1R which play a role in formation, maintenance, and progression of many types of tumors.[9] Almost all GBM patients are routinely treated with GC, which modulate InsR activity.[10] Chronic use of these drugs is known to induce insulin resistance, which upregulates gluconeogenesis and leads to hyperglycemia.[11] Acute hyperglycemia is managed with insulin and insulin analogs, raising concerns about insulin oncogenic activity.[12]

One of the main GC used in GBM patients is DXM, a strong anti-inflammatory drug, being the "gold standard" in therapy of brain edema, pain and neurologic deficits occurring during intracranial mass effect creation.[13] However, DXM´s effect on glioblastoma cells is controversial, especially during simultaneous use of radiotherapy and chemotherapy with TMZ. There are conflicting results between *in vitro* and *in vivo* studies ranging from antagonism, to no interaction, to even synergy between DXM and TMZ.[14-16] Moreover, there is also data indicating that DXM may significantly decrease survival of GBM patients by impairment of immune functions or induction of hyperglycemia.[17]

Since our previous *in vitro* studies[18] demonstrated that higher glucose concentrations create an aggressive GBM cell phenotype including resistance to TMZ, we decided to study here the association between glucose levels, DXM effects and TMZ treatment on cellular viability, division, dispersal, cohesion, morphology, adhesion to the substrate, and concentration of InsR. The study was conducted on three GBM cell lines: commercial T98G and two primary, molecularly characterized lines: HROG02—with EGFR amplification, mutation in TP53 gene, methylated of MGMT promotor, no hot spot mutations in the genes IDH 1 and 2—or B-RafHROG17—relapse, EGFR amplification, methylated of MGMT promotor, no hot spot mutations in the genes IDH 1 and 2 or B-Raf. Experiments were carried out under tumor physiological oxygen conditions (2.5% oxygen) in direct comparison with standard laboratory conditions (20% oxygen). The latter oxygen concentration is not achieved in GBM or in the central nervous system, but is used in most studies. We also performed a retrospective analysis of medical records of 40 patients with GBM at the Oncology Institute in Gliwice. Patients with normo- and hyperglycemia, treated with TMZ/RT and DXM were analyzed to investigate whether there is an association between the effects observed in the *in vitro* study and in clinic.

Material and Methods

In vitro Studies: Cell Cultures, Drugs, Solutions and Study Design

In vitro experiments were carried out on the: T98G cell line (Sigma-Aldrich, St Louis, MO USA) and primary cell lines: HROG02 (CLS order no. 300931) HROG017 (CLS no. 300938) derived from patients with GBM WHO grade IV operated at the University Medicine Rostock. GBM samples were molecularly characterized and then cryopreserved as described by Mullins et al[18,19]. Medium for cell cultures, gentamicin and fetal bovine serum were purchased from Gibco-BRL (Waltham, Massachusetts, USA). Plasticware was from Falcon (Lexington, TN, USA) and Eppendorf (Hamburg, Germany). TMZ and DXM were obtained from Sigma-Aldrich.

Cell density, cytotoxic concentration of TMZ and viability of GBM cells exposed to different concentrations of glucose in our experimental model were described previously.[20] Since the therapeutic oral dose of DXM is between 4 and 16 mg/day and the predicted concentration of DXM in the brain after oral administration in a dose of 0.5 mg is 5×10^{-3} µg/mL we used a concentration of 10 µM. T98G, HROG02, and HROG17 cell cultures were trypsinized and passaged after achieving 90% confluency. On the second day following trypsinization, the medium was replenished with a fresh portion containing glucose at different doses (0.6 g/L; 1 g/L; 4.5 g/L), TMZ (1 mM) with or without DXM (10 µM). Cultures were placed in the incubator for 48 hours. The glucose concentrations used in the experiments were chosen to reflect 3 clinical states: 0.6 g/L, low physiologic concentration; 1 g/L, normoglycemia; 4.5 g/L, high concentration that corresponds with hyperglycemia in diabetic patients. Experiments were conducted in two types of CO_2 incubators: (1) CO_2 incubator, NuAir (Fernbrook Lane, Centerville, Massachusetts) for experiments conducted in standard laboratory conditions (5% CO_2, 20% oxygen, and 97% humidity) and (2) New Brunswick Galaxy 48R (Eppendorf-MG Scientific, Hamburg, Germany) for experiments conducted in 2.5% oxygen (5% CO_2 and 94% N_2) to reflect the hypoxia that exists in glioblastoma.

The results for each analyze came from 3 independent experiments.

Cell Viability. Three methods were used for evaluation of viability of GBM cells exposed to DXM (10 μM), with or without TMZ (1 mM), in different concentrations of glucose in culture medium (0.6 g/L; 1 g/L; 4.5 g/L): a MTT assay as previously described,[21] the Burker chamber method and an assay using the Eve Automatic Cell Counter (NanoEntek, South Korea) which was used according to the manufacturer's instructions.

Cell Division Assay. The degree of cell division was evaluated using an immunoenzymatic assay kit based on determination of bromodeoxyuridine (BrdU) level (Abcam, Cambridge, UK), which is incorporated into DNA of dividing cells. The determination was conducted according to the manufacturer's instructions and was described previously.[20] Absorbance was measured in a Multiscan RC microplate reader (Labsystems, Helsinki, Finland).

InsR Expression. InsR expression was determined using a Human InsR1 Elisa Kit (Abnova) according to the manufacturer's instructions. Absorbance was measured with an excitation wavelength of 450 nm and emission wavelength of 540–570 nm.

Phenotype Analyses of Glioblastoma Cell Lines - Microphotography. Dynamics of changes in the cell culture (cohesion and dispersal) and morphology of GBM cells were observed using a JuLI cell analyzer and fluorescent microscope (Nikon Eclipse TS 100, Minato, Tokyo, Japan). No convolution was carried out on the pictures.

The results for each experiment came from three independent repetitions.

Retrospective Clinical Analysis

300 GBM patients treated at the Oncology Institute in Gliwice in years 2006–2016 were included. Patients were treated with definitive RT to a total dose of 54 to 60 Gy in 30 daily weekday fractions from 1.8 to 2 Gy per day in combination with TMZ (75 mg/m^2). This therapy was followed by adjuvant treatment with TMZ 150–200 mg/m^2. Based on the inclusion criteria (glioblastoma IV) confirmed histologically, determination of Ki-67 activity (%) and glycemia, full documentation of radiochemotherapy and DXM usage), 40 patients were included in the present study: 17 women and 23 men. The exclusion criteria included mixed diagnosis of GBM (grade III/IV), other malignancy, other radiation doses or fraction schedule, no concurrent TMZ administration, the presence of an concurrent illness that could interfere with protocol treatment, age ≤18 and overall survival >2 years from the time of GBM diagnosis.

The retrospective study based on a standard treatment according to our Institutional rules does not need a specific Bioethical Committee approval. All patients gave an informed, written consent to undergo a treatment. The personal data of the patients for the experiment were blinded.

Ki-67, Glycemia and Steroid Assessment. Expression of the Ki-67 marker was evaluated in the surgical specimens by immunohistochemical staining. Serum glucose levels were measured throughout therapy and the mean glucose level was calculated for each patient using all available results. Steroids (DXM, Dexaven) were prescribed as needed clinically, mainly for postoperative anti-edematous prophylaxis.

Statistical Analyses

Statistical analyses were performed using GraphPad Prism 7.01 software system (GraphPad Software Inc, San Diego, California). The values are expressed as the means and standard deviation (SD) for normally distributed data. A one-way ANOVA test, post hoc a Tukey's or Newman Keuls Multiple Comparison Test was applied to evaluate differences in the examined groups. The level of significance was set at $P < .05$ for all of the statistical tests.

In a retrospective study statistical analysis was performed using Spearman's correlation coefficient and Mann-Whitney test.

Results

In vitro Studies—Primary Cell Lines HROG02 and GROG17 Versus T98

(Figures showing viability, division and InsR expression data for primary lines: HROG02 and HROG17 are presented in attached Supplemental files).

Cell Viability. Physiological oxygen conditions for glioblastoma (2.5% oxygen) - despite the different dynamics in achieving confluency, the trend of changes observed in the primary lines was a similar to those observed in the commercial line T98G.

The only difference observed between the cell lines regarded their sensitivity towards TMZ. The strongest cytotoxic effect under physiological glucose conditions for hypoxic tumor cells (2.5%), was observed for the T98G line (about 80% dead cells, 0.6 g/L glucose). Primary lines were less sensitive to the cytotoxic effects of TMZ (approximately 60% dead cells for HROG02 and HROG17 in 2.5% oxygen condition). This effect, however, was observed only under low glucose conditions. e 0.6 g/L. For higher glucose concentrations primary GBM cell lines have shown resistance to TMZ mechanisms. Especially for HROG17 we observed viability levels of 85% live cells (and 70% live cells HROG02, respectively).

In the *physiologic oxygen condition for glioblastoma (2.5% oxygen)* we observed reduced viability of cells exposed to TMZ and DXM in comparison to the control cultures but this effect was weaker in cells of T98G line cultured in medium with increasing glucose concentrations,

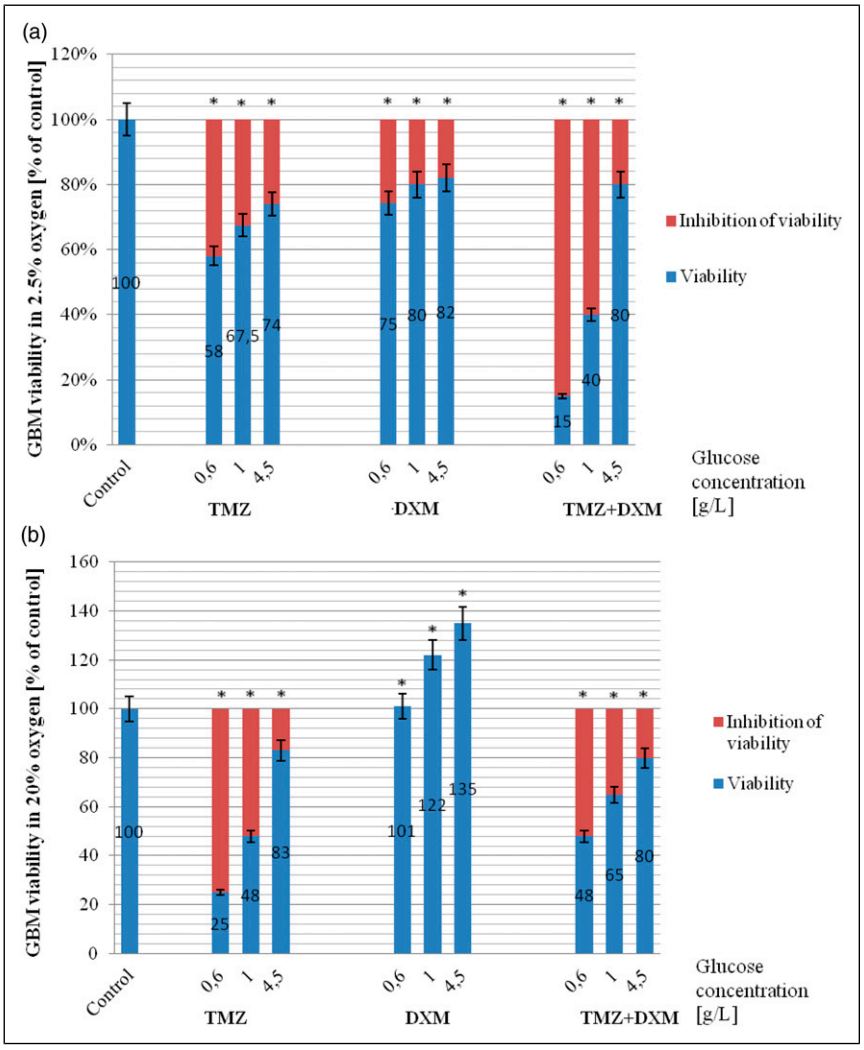

Figure 1. (A and B) Effect of TMZ, DXM and combination of TMZ+DXM on viability of T98G cells cultured in medium containing different glucose concentrations (0.6 g/L; 1 g/L; 4.5 g/L) in 2.5% physiological hypoxia (A) and 20% oxygen (standard laboratory conditions) (B). Each bar represents the mean ± SEM of at least three independent experiments. Values were analyzed by one-way ANOVA, followed by Tukey post hoc test, $^*P < .05$ vs control (untreated cells). Correlation between groups was tested by calculating the correlation coefficient (Pearson's test).

as in our previous study.[18] The cytotoxic effect of TMZ was stronger than DXM. Simultaneous administration of TMZ and DXM in culture medium enhanced the cytotoxic action of TMZ in cells cultured in the lower glucose medium (0.6 and 1 g/L glucose) but not in the high glucose medium (4.5 g/L) (Figure 1A).

Standard laboratory conditions (20% oxygen)—the cytotoxic effect of TMZ on T98G cells was much stronger in low glucose medium (viability was decreased by about 75%) than in the media with higher glucose concentration (1 or 4.5 g/L glucose). Furthermore, this effect was also stronger than the effect induced by TMZ in low glucose medium under 2.5% oxygen conditions, where the viability of cells was decreased by only 42%. Unexpectedly, DXM increased the viability of glioblastoma cells about 35% of the control cells in high glucose medium. TMZ and DXM administrated simultaneously decreased the viability of cells in a glucose concentration dependent manner but these effects were weaker than those observed of TMZ alone. Only in cells cultured with the high glucose medium TMZ and DXM effect was similar to TMZ effect (Figure 1B).

Division Intensity. Physiological oxygen conditions for glioblastoma (2.5% oxygen)—in comparison to control cultures, the amount of glioblastoma cell division was decreased in cultures exposed to TMZ, DXM or the combination of DXM and TMZ conducted in media with the increasing glucose concentration. The strongest effect was seen in the cultures treated with TMZ in the lower glucose media (53% for T98G, 46% respectively for HROG02, and 48%, respectively, for HROG17).

Standard laboratory conditions (20% oxygen)—when compared to the control cultures, TMZ decreased glioblastoma division by about 49% for T98G; 40% for HROG02 and 34% for HROG17) in the low glucose medium. In contrast, DXM increased proliferation at the highest rate (by about

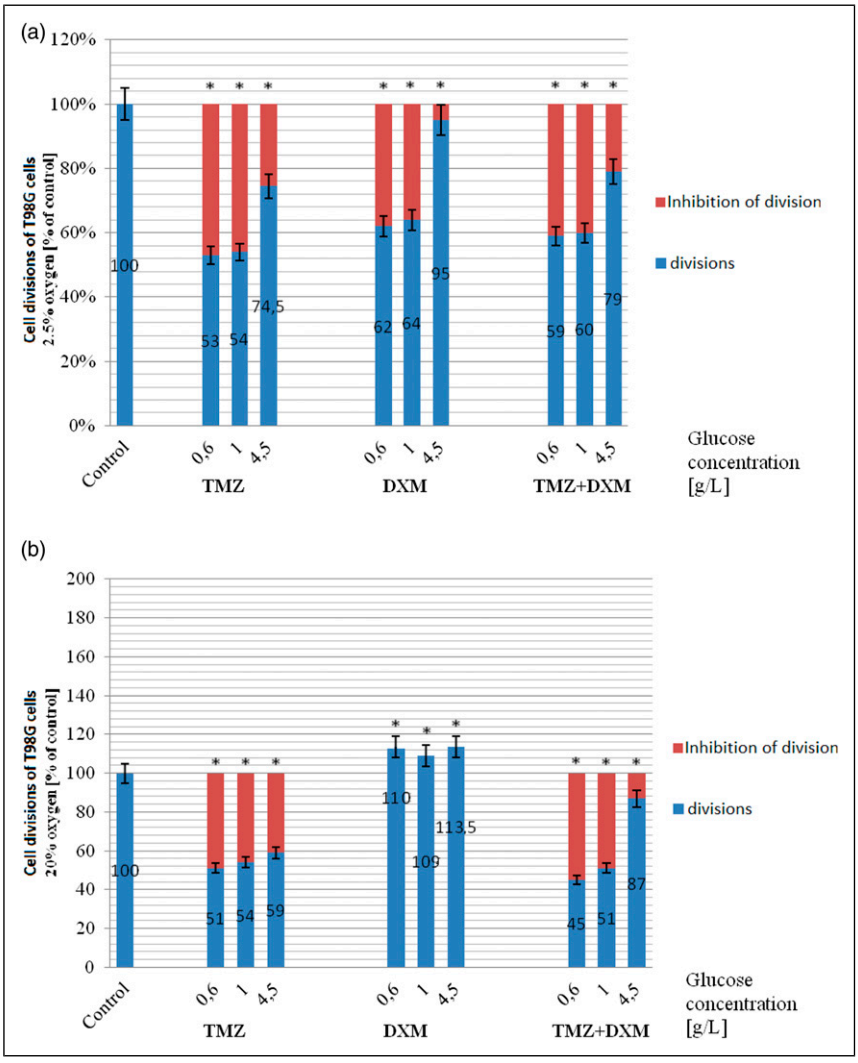

Figure 2. (A and B) Cell divisions of T98G cells exposed to TMZ, DXM and combination of TMZ+DXM cultured in different glucose concentrations (0.6 g/L; 1 g/L; 4.5 g/L) in 2.5% (A) and 20% oxygen (B). *Each bar* represents the mean ± SEM of at least three independent experiments. Values were analyzed by one-way ANOVA, followed by Tukey post hoc test, *$P < .05$ vs control-TMZ, DXM, combination of TMZ+DXM. Correlation between groups was tested by calculating the correlation coefficient (Pearson's test).

13.5% for T98G line and by 15%/17%, respectively, for HROG02 and HROG17 cell lines as compared to control cells) in cultures grown in low glucose medium. DXM administrated together with TMZ merely diminished the TMZ effects in the high glucose medium.

The influence of DXM and TMZ on the intensity of GBM cell divisions is presented in Figure 2A and 2B.

InsR *Expression*. *Physiological oxygen conditions for glioblastoma (2.5% oxygen)*—in control (=untreated) T98G cells an increase of InsR levels was observed in a glucose dose dependent manner. Compared to control conditions, administering TMZ decreased InsR expression, especially for 1 and 4.5 g/L glucose concentrations. However, the trend of the glucose concentration dependent increased expression of this receptor was maintained. It is interesting that DXM, in comparison to TMZ settings, increased InsR expression in T98G cell culture by about 54%, 50%, and 36%, respectively, in HROG17 by about 61%, 60%, and 40%, in HROG02 by about 55%, 58%, and 63% in all media (1 and 4.5 g/L). Simultaneous administration of TMZ and DXM decreased concentration of InsR, compared to control, but this effect was weaker than the effect of TMZ alone.

Standard laboratory conditions (20% oxygen)—in each group, an increased concentration of InsR was associated with the increased glucose concentration in the culture medium. In comparison to the control cultures and in contrast to the 2.5% oxygen conditions, DXM did not alter InsR concentration whereas the TMZ induced decrease of this parameter (by about 29% in medium with 1 g/L glucose, and 32% in medium with 4.5 g/L glucose). TMZ and DXM administrated together decreased the concentration of the receptor in medium

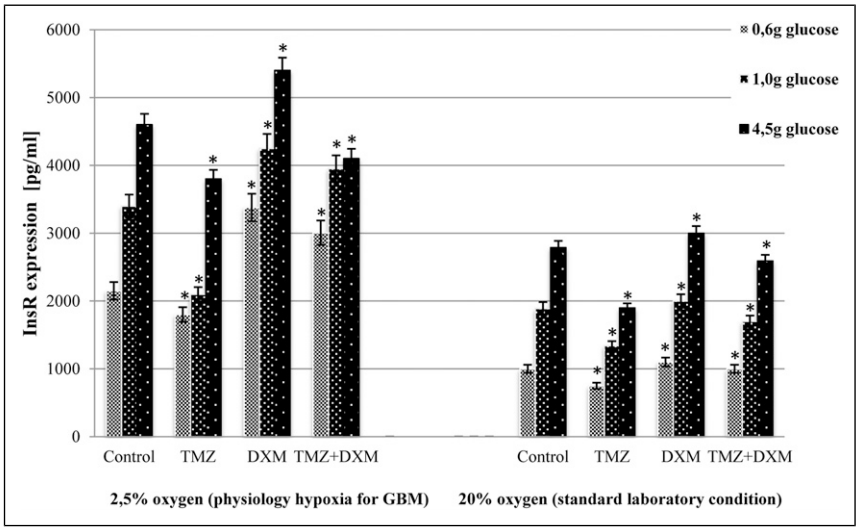

Figure 3. InsR expression in T98G cells exposed to: TMZ, DXM and combination of TMZ+DXM. Glioblastoma cells were cultured in medium containing different glucose concentration (0.6 g/L; I g/L; 4.5 g/L) in 2.5% oxygen and 20% oxygen. Data are represented as means ± SEM of triplicate samples. *P < .05 vs. control (untreated cells). Each bar represents the mean ± SEM of at least three independent experiments. Values were analyzed by one-way ANOVA, followed by Tukey post hoc test, *P < .0 5 vs control (untreated cells). Correlation between groups was tested by calculating the correlation coefficient (Pearson's test).

containing 1 and 4.5 g/L glucose, about 10% and 7%, respectively, as compared to DXM effect (Figure 3).

The influence of TMZ, DXM, and TMZ+DXM on the InsR levels in T98G line is presented in Figure 3

Microscopic Analyses. Microscopic observations of cultures conducted under different conditions demonstrated various morphological alterations of glioblastoma cells exposed to DXM and/or TMZ. Similar morphological changes were observed for the T98G (Figure 4A and 4B) and primary cell lines HROG02 (Figure 4C and 4D) and HROG17 (Figure 4E and 4F).

Physiological oxygen conditions for glioblastoma (2.5% oxygen)—control (untreated) GBM cells cultured in medium supplemented with 0.6 g/L glucose exhibited typical morphology with preserved division figures, dispersal, and adhesion. The addition of DXM into the medium induced inhibition of cell proliferation and dispersal, created cell aggregation, cell shrinking and detachment of cells from the substrate. TMZ elicited cytotoxic damage of GBM cells manifested by swelling of individual cells and inhibition of cell division, but it did not influence their dispersal in the culture. Simultaneous administration of TMZ and DXM initiated mass swelling of the cells, cohesion and degeneration of the nucleus. The latter was weaker than the effect induced by DXM alone.

An increase in glucose supplementation in culture medium (1 g/L, 4.5 g/L) caused a change in morphology of the cells. In the control groups, enhanced viability, adhesion, and tight connections between cells were visible. In medium containing 1 g/L glucose, DXM still initiated formation of aggregates from viable cells and detaching of the cells from the substrate. Moreover, it stimulated variability of morphologic forms in active and resting cells. In medium containing 4.5 g/L glucose, glioblastoma cells exposed to DXM were dispersed like control cells cultured with 0.6 g/L glucose, but some detached cells, beyond the culture monolayer, formed a second, top layer of aggregates. TMZ in medium with 4.5 g/L glucose induced cytotoxic damage of cells, but this effect was weaker than in cells cultured in medium with lower glucose concentration (1 or 0.6 g/L glucose). The effects of TMZ given together with DXM were weaker than those of TMZ treatment alone. Cells in medium with 1 g/L glucose demonstrated aggregation. An increase in cellular cohesion and a phenotype of detaching from the substrate were still observed. In culture medium containing 4.5 g/L glucose, cells showed typical dispersal, and adhesion patterns, similar to the untreated cells (cultured in 0.6 g/L glucose) (Figure 4A, A-L).

Standard laboratory conditions (20% oxygen)—remarkable changes in arrangement of GBM cells exposed to DXM were observed. In cells cultured in medium containing 0.6 or 1 g/L glucose, DXM significantly inhibited dispersal of cells and induced their cohesion leading to the formation of large aggregates. However, in contrast to the DXM effect observed in the 2.5% oxygen condition, these clusters were stably attached to the substrate. This effect was not observed in cells cultured in medium containing 4.5 g/L glucose, where cells maintained the typical dispersal, as observed for the control (untreated) cells. TMZ in the low glucose medium (0.6 g/L) induced cell shrinking and detachment from the substrate. The observed cytotoxic TMZ effects were stronger than in media with higher glucose (1 or 4.5 g/L). Namely, in media with

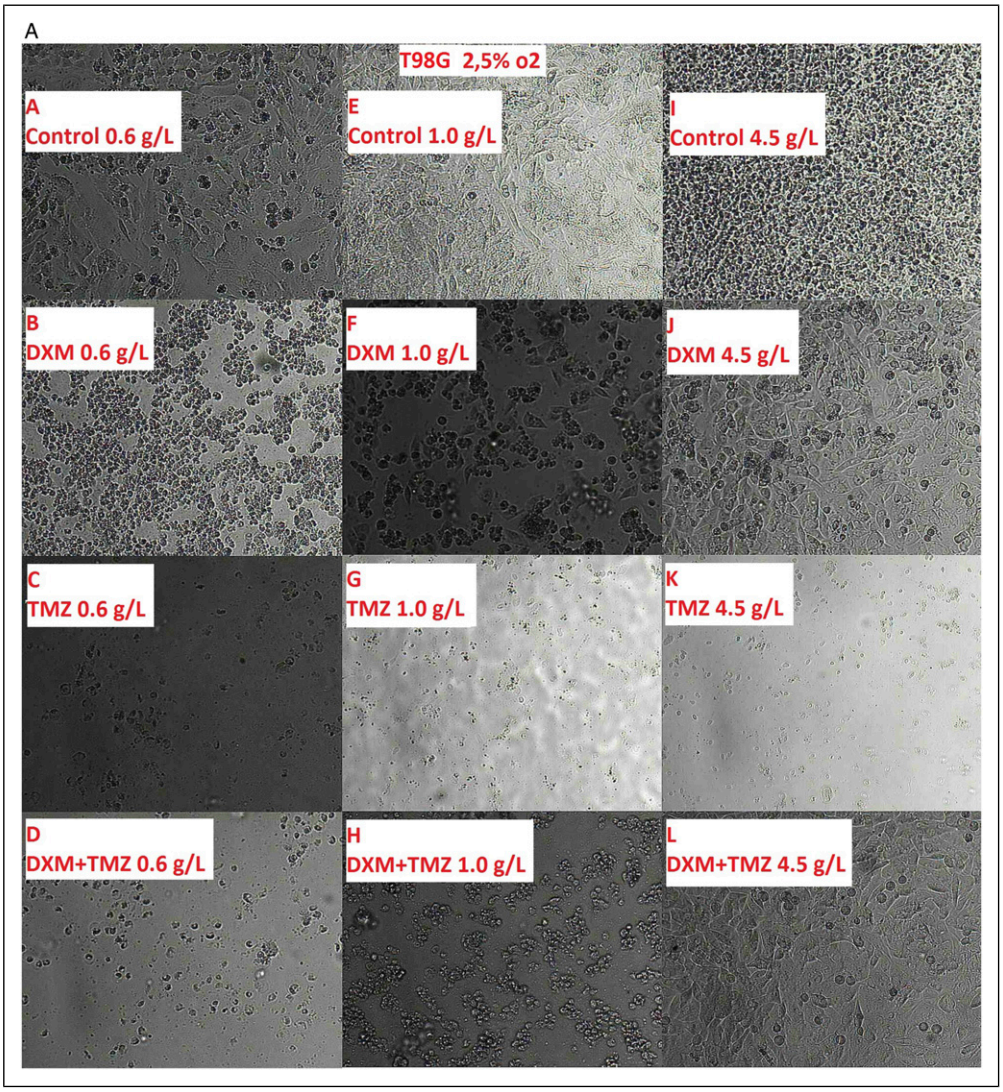

Figure 4. Microphotographs presenting three GBM cell lines cultured in medium containing different glucose concentration: 0.6 g/L (A–D); 1 g/L (E–H); 4.5 g/L (I–L) and exposed to: DXM (B, F, J), TMZ (C, G, K) or combination of DXM+TMZ (D, H, L); A, E, I—T98G control group of cells not exposed to drugs. A—T98G cells cultured in 2.5% (physiologic conditions for GBM) and 20% oxygen (B) (standard laboratory conditions) C—Cells of primary line HROG02 cultured in 2.5% and 20% oxygen (D) E—Cells of primary line HROG17 cultured in 2.5% oxygen and 20% (F) Analyses were conducted using the Juli cell analyzer (magnification ×20). No convolution was carried out on the pictures.

higher glucose, cell cultures were characterized by increased dispersal and tight density of living cells. In media containing 1 or 4.5 g/L glucose, TMZ + DXM did not significantly influence dispersal of GBM cells in comparison to the effect of DXM alone, but detracted cell-cell contact. The strongest effect was observed in cells cultured in low glucose medium where GBM cells tended to attach to the substrate and formed colonies (Figure 4B, A-L).

Retrospective Clinical Analysis

The study included 40 patients, 17 women and 23 men, with the mean age 54.3 years (SD = 7.65), with confirmed glioblastoma (IV) diagnosis, subjected to treatment with radio-chemotherapy (TMZ) and prophylactic steroid therapy. To facilitate the analysis of data, patients were divided into groups in respect to (1) mean glucose level during treatment and (2) correlation between glycemia level and Ki-67 marker level evaluated in the surgical specimens of patients with normoglycemia or diabetes in their medical history.

Results show that in patients with glioblastoma, increased glucose level is positively correlated with an increased expression of Ki-67 proliferation index (Figure 5). Furthermore, the highest expression of Ki-67 was noted in patients in whom high glucose level during therapy was induced by steroid administration (posteroid diabetes).

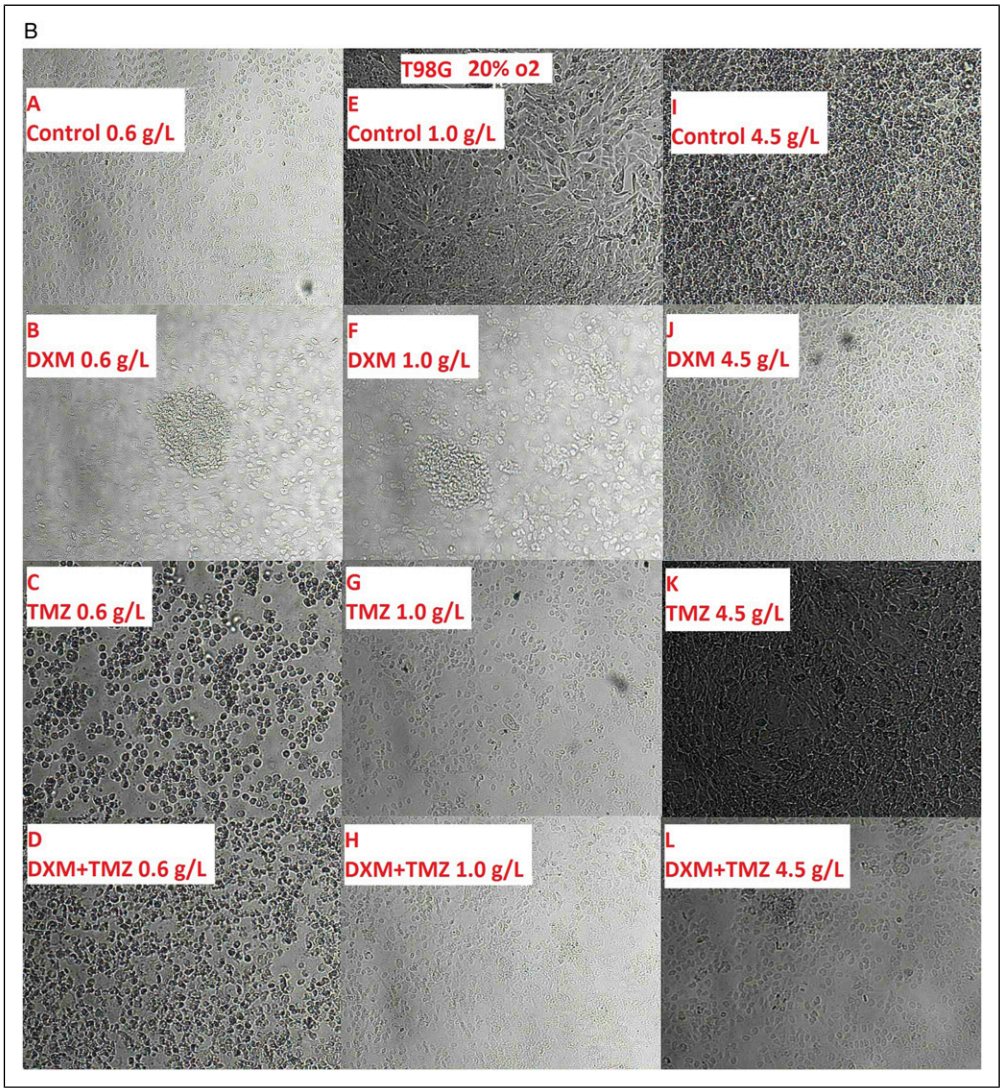

Figure 4. Continued.

Discussion

Since glucose is the main energetic fuel for cancer cells, fluctuations in its concentration are relevant for tumorigenic abilities.[20] Using three concentrations of glucose in *in vitro* model (0.6, 1.0, and 4.5 g/L) we observed that only high glucose medium (corresponding to hyperglycemia in patients) boosted the metastatic glioblastoma phenotype to varying degrees both in T98G as well as primary glioblastoma lines (HROG02 and HROG17) as demonstrated by increased cell viability, proliferation, density and dispersal, chemoresistance or enhanced expression of InsR.

It is interesting that despite different genetic profiles and different intensification of alterations of studied GBM lines: commercial and primary (including line derived from the patient with recurrence) we observed a common direction of changes in all studied parameters. It shows that modulations in glucose concentration in tumor microenvironment it is a common point for all GBM lines, independently from their genetic profile or recurrence.

Therefore, bearing in mind heterogeneity and strong genetic variation observed in other studies it seems, that influence to metabolism of GBM cells can meet with a greater therapeutic success than molecular implications.

Temozolomide, Dexamethasone Chemoresistance and Drug Interaction in In Vitro Study

Our study confirms the results of others concerning the association of glucose concentrations with TMZ effectiveness. As in our previous study,[20] we observed that an increased glucose availability in glioblastoma cultures exposed to TMZ promoted division of cells and chemoresistance mechanisms, especially in the hypoxia conditions (2.5% oxygen). Compared to control cells and DXM effects, TMZ inhibited InsR expression.

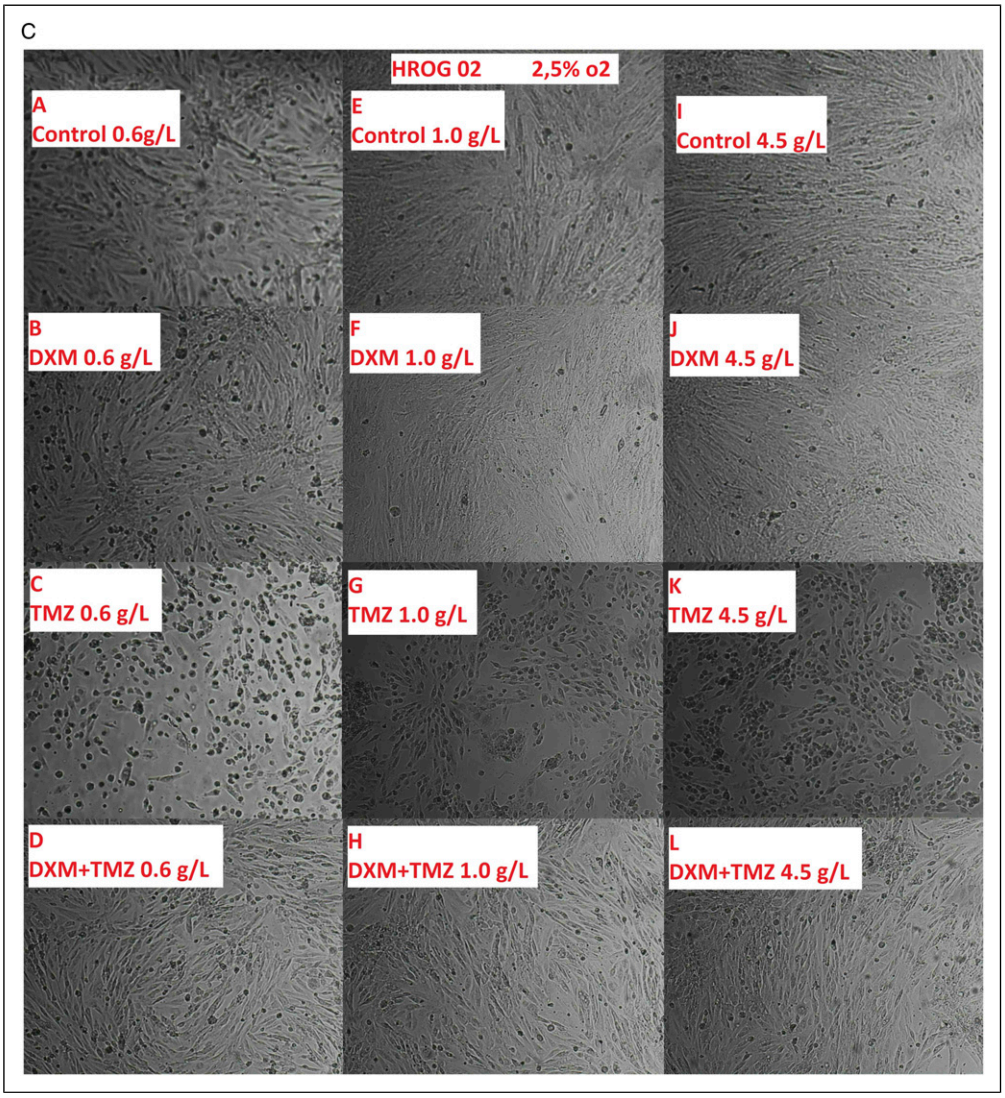

Figure 4. Continued.

In both oxygen conditions, TMZ preserved dispersal cells of all glioblastoma lines in the cultures and induced visible damage as well as cell shrinkage. Only under the conditions of 20% oxygen and high glucose medium, tight cohesion of glioblastoma cells subjected to TMZ was visible. Simultaneous administration of TMZ and DXM weakened the effect of TMZ but only in cells cultured in media with high glucose concentrations. Such negative interaction between TMZ and DXM was also observed in other studies.[21,22]

The conflicting effects of DXM on cancer cells reported in studies conducted in recent years raised an ethical question about legitimacy of its use as an adjuvant therapy in patients with GBM. Using DXM at one concentration in an *in vitro* model, we observed that its effects were dependent on extracellular circumstances, namely this drug was able to inhibit in 2.5% oxygen, or increase in 20% oxygen, glioblastoma cell viability and cell division. It should be stressed that only in low glucose medium (0.6 g/L) and physiological for glioblastoma cells, oxygen conditions (2.5% oxygen) DXM induced the strongest suppression. DXM also exerted differential influences on InsR expression. Under the 2.5% oxygen condition with an increasing glucose level in the culture medium, expression of InsR significantly increased in cells exposed to DXM. However, in the 20% oxygen model, DXM did not influence this parameter. Furthermore, we noticed two distinct pictures of T98G and primary glioblastoma cell lines exposed to DXM in an oxygen and glucose dependent manner. In both oxygen conditions, but only in cells cultured in low glucose medium, DXM inhibited dispersal of cells in all lines, weakened their adhesion to the substrate, inhibited motility and increased formation of cell aggregates.

Bearing in mind that in clinical practice aggressiveness and progression of glioblastoma is associated with a lack of radical, effective treatments, the influence of glucose on

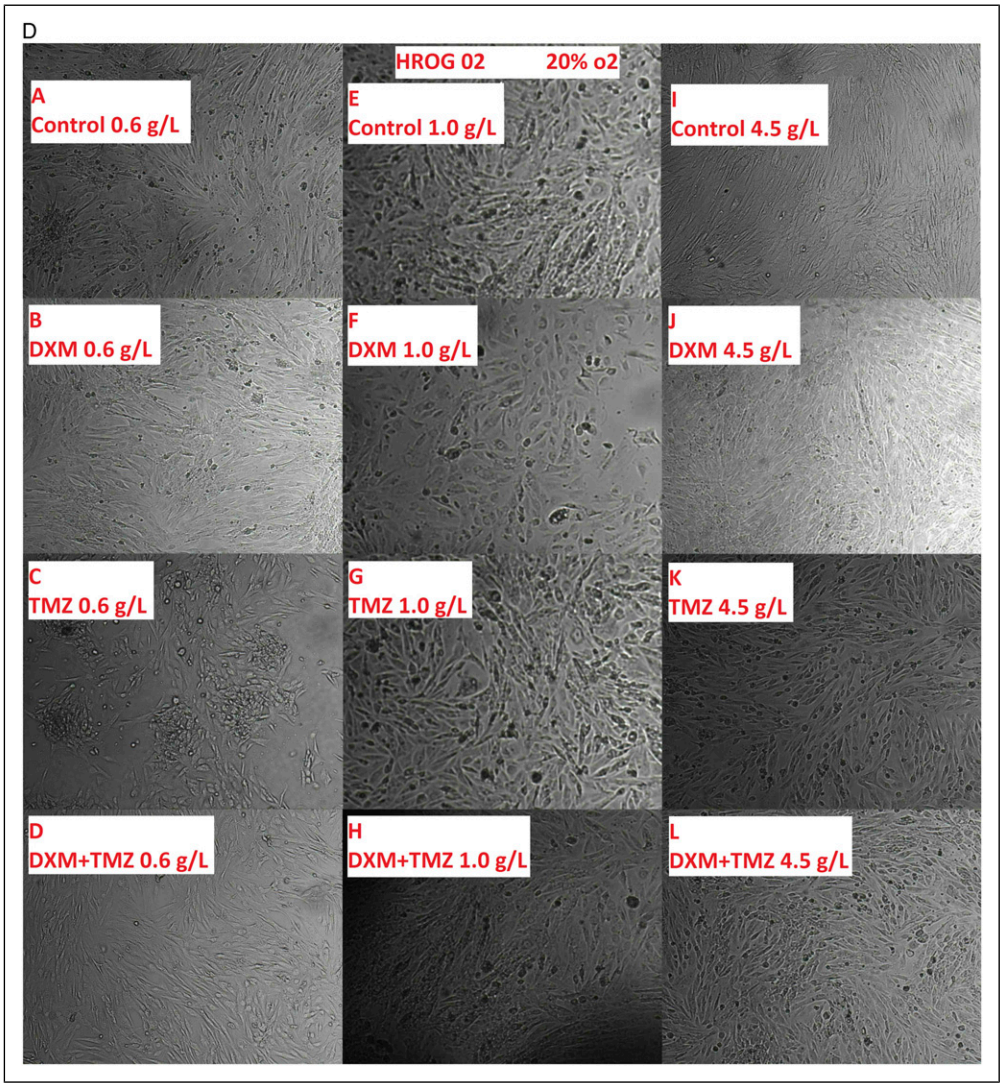

Figure 4. Continued.

(especially) DXM action against tumor cells and their behavior observed in this study seems to be important for suppression of an infiltrative glioblastoma phenotype and recurrence. Studies on glioblastoma behavior dynamics in cell-cell contacts present also new insights into potential targets for future therapies**.** Undoubtedly, DXM action against cancer cells is a complex phenomenon guided by microenvironment conditions. However, there are several hypotheses explaining its mechanism of action for causing cell dispersal inhibition and stimulation of cell aggregate formation. One hypothesis is related to (1) an increased expression of adhesive molecules, including cadherins, integrins and laminins, which are engaged in key cellular processes such as survival, migration, cell-substrate and cell-cell contact strength and (2) modulation of actin that participates in cell movement and changes of cell shape.[23,24] A second hypothesis concerns AQP1, a membrane bound protein with an important role in transmembrane transport of water molecules. Up-regulation of AQP1 in gliomas is induced inter alia by DXM, glucose, hypoxia, and is closely associated with brain edema and tumor metastasis. Moreover, AQP1 binding to Lin7/β catenin causes translocation of actin to plasma membrane and mediates rapid transport of water molecules in pseudopodia, promotes rapid renewal of plasma membranes, which, in effect, supports glioblastoma cell migration. These data regarding AQP1 regulation in gliomas are in line with our presented findings which indicate that a trend for glioblastoma cell dispersal was visible only in cells cultured in media supplemented with a high concentration of glucose.[25] According to the third hypothesis, DXM decreases activity of Cxn43, a protein of gap junction channels, acting as a tumor suppressor in gliomas (it regulates expression of n-cadherins) and allows communication between the tumor and its surrounding microglia-immune cells. Moreover, DXM, acting as an anti-inflammatory drug, inhibits expression of Cxn43 on the surface of microglial cells and decreases their activation/

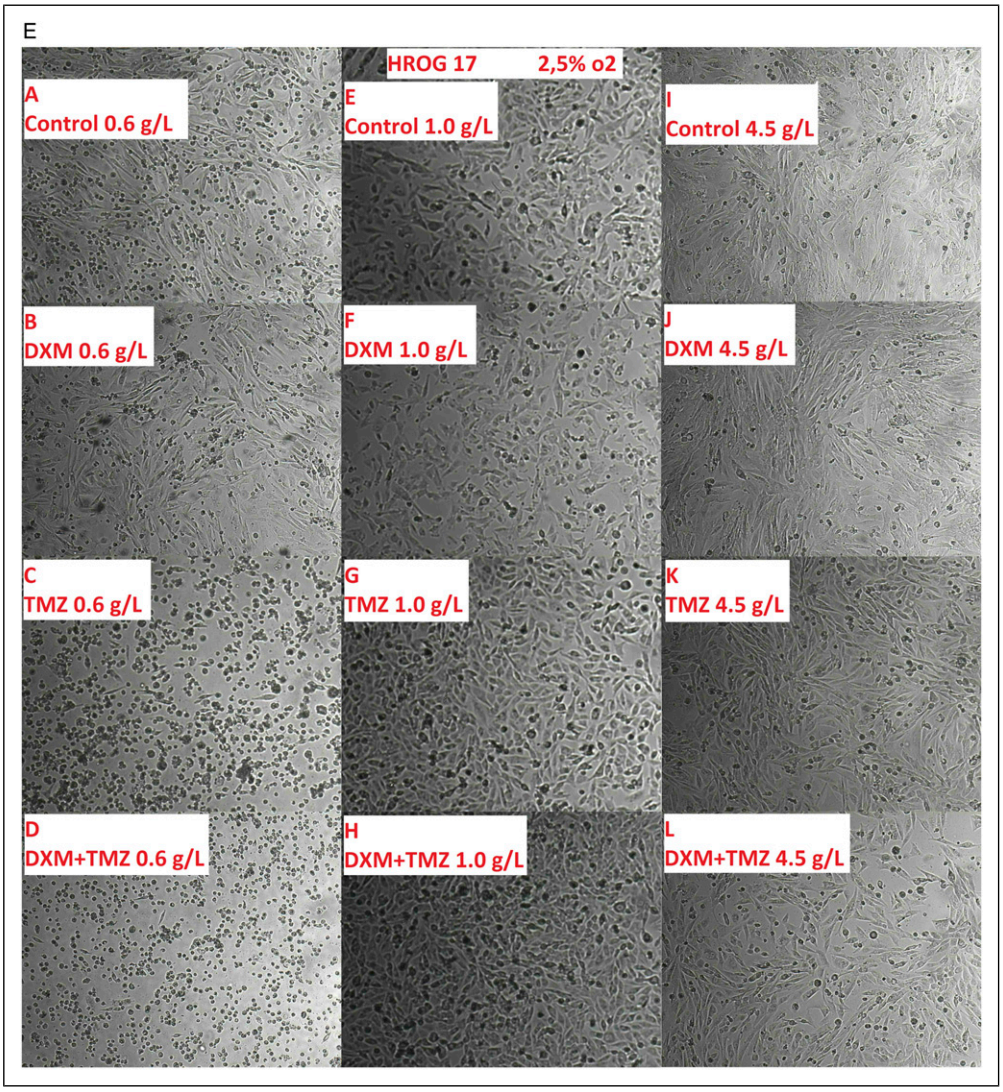

Figure 4. Continued.

migration/recruitment which occurs in inflammatory conditions induced inter alia by hyperglycemia.[26,27] Furthermore, DXM´s influence on cytoskeleton organization, migration and proliferation of glioblastoma cells may be dependent on Akt/mTOR/RhoA pathway acting as a down/upstream regulator of PI3K that plays a crucial role in glucose metabolism.[28,29] Another possibility underlying the mechanism of action of DXM is associated with its inhibition of macrophage migration inhibitory factor (MIF), a proinflammatory cytokine acting as an endogenous antagonist of GCr that is able to promote proliferation/migration/invasion of tumor cells and interact with AP-1, NF-kappa B and ERK1/2 MAPK pathways.[30-32] However, one should remember that activity of some signaling pathways such as ERK1/2 MAPK depend on various factors (cell stress and glucose),[34,35] which might provide an explanation for the opposing effects of DXM on key cellular functions observed in our study in media with different glucose concentration. Finally, it is also known that DXM affects the IGF receptors displaying oncogenic functions. Due to up-regulation of their expression in glioblastoma cells, they are closely associated with cancer progression and were proposed to be a future therapeutic target.[36]

Increase in of glucose levels in the tumor microenvironment seems to be a method for shaping glioblastoma cell behavior and InsR expression modulation, thus providing the connecting link between *in vitro* data and the retrospective study.

Clinical Data

The clinical data analyzed also suggests that glucose is involved in modulation of the malignant glioblastoma "face" because a positive correlation between Ki-67 expression in tumor specimens and hyperglycemia was found. The highest

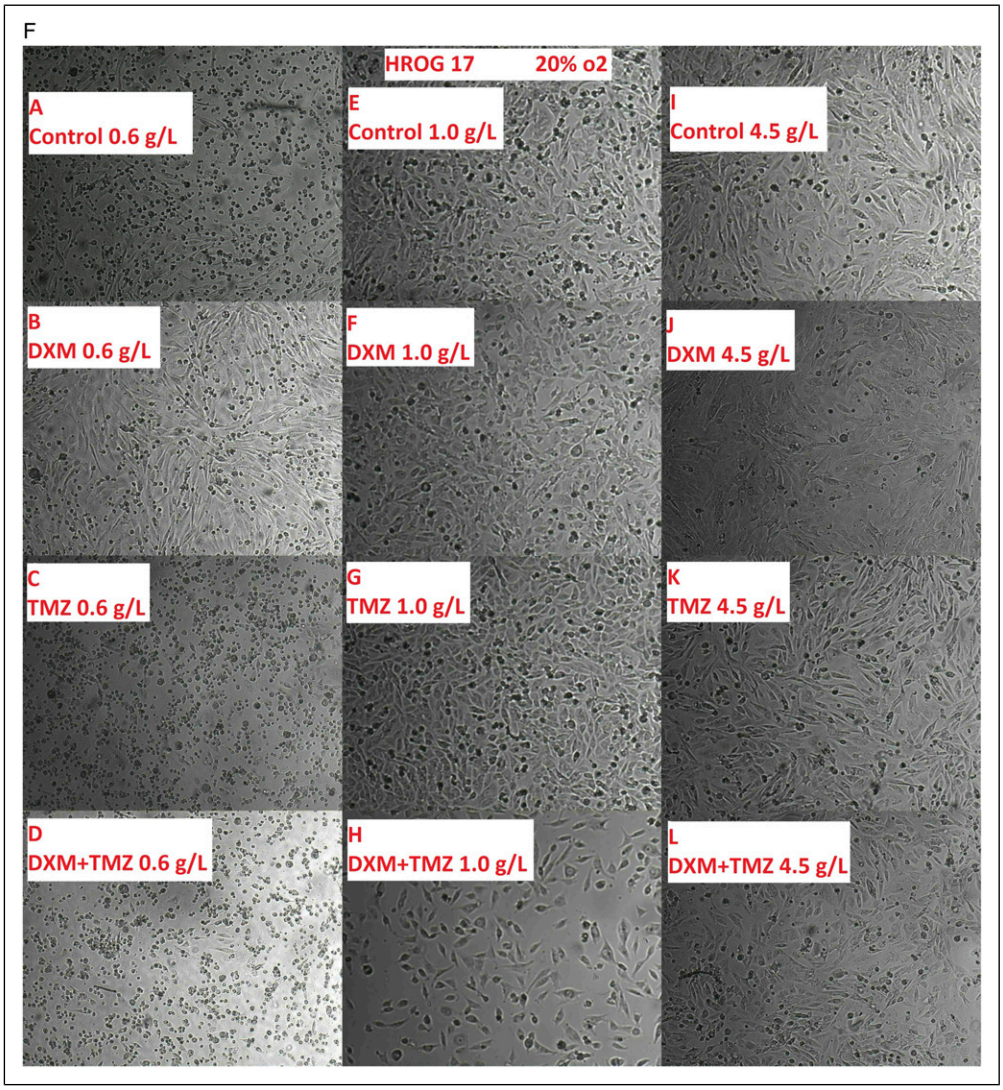

Figure 4. Continued.

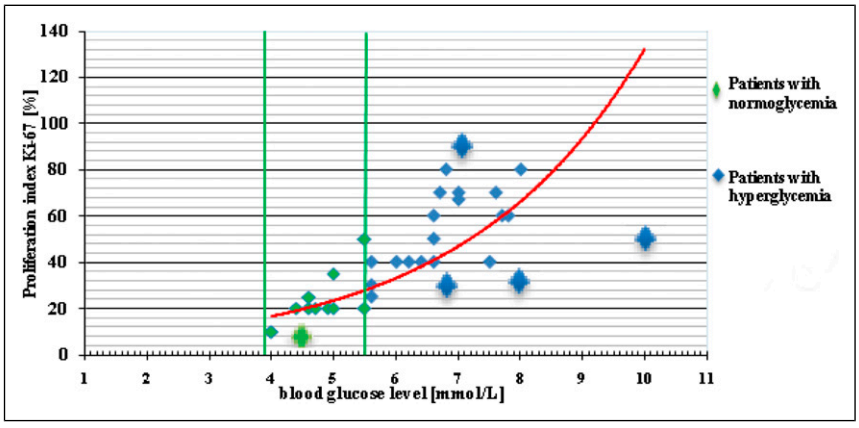

Figure 5. Correlation between blood glucose levels and the Ki-67 index in patients. Patients were classified into 3 categories according to glucose concentration and Ki-67 expression. The subgroups were compared using the long-rank test. Analysis were performed using Spearman's correlation coefficient and Mann-Whitney test. All differences were considered statistically significant at the level of $P < .05$.

percentage of proliferation index was observed in patients with posteroid diabetes and with type II diabetes, which are both characterized by glycemia peaks and unstable glycemia levels. The main mechanism responsible for the Ki-67 up-regulation is probably related to hyperinsulinemia induced in response to hyperglycemia that acts as cell stressor. Metabolic stress elicited by hyperglycemia (also stimulated by DXM) can upregulate expression of about 41 genes involved in the ROS system and mechanism of cell proliferation.[37] This stress triggers a cascade of several reactions such as (1) elevation of circulating insulin levels and free IGF-1, (2) stimulation of TNF-α and IL-6 production that, in effect, induces an inflammatory response, insulin resistance and gluconeogenesis; (3) Akt pathway activation; (4) alteration of immune responses through impairment of ascorbic acid transport into immune cells, which in turn is necessary for effective phagocytosis; and (5) chemotherapy resistance.[38-40] Therefore maintenance of stabilized normoglycemia in glioblastoma patients throughout therapy and/or replacement of DXM by other drugs with anti-edema potential like, for instance, VEGF inhibitors, seems to be a reasonable approach to improve efficacy of anticancer treatments.[41]

Future Perspectives

Since high-grade tumors (compared to healthy cells) exhibit high sensitivity to a decrease of glucose concentration in the extracellular microenvironment, implementation of adjuvant metabolic therapy into the standard scheme of glioblastoma treatment may be suggested.[42] The main aim of such a therapy is the restriction of glucose supply and induction of physiological ketosis, leading to production of ketone bodies as a primary energy source for cells. In recent years, a few case reports presenting promising results of this form of therapy applied in glioblastoma patients have been published. The authors reported clinical improvement characterized by elimination of brain edema, relief of pain, nausea and vomiting, prolongation of overall survival and improvement of life comfort described by patients. These effects were achieved without use of glucocorticoids. Moreover, they observed synergistic effects of diet and cytostatics, alkylating drugs and radiotherapy and finally, a better tolerance of cytostatic treatments by glioblastoma patients.[43-45]

Role of Oxygen in In Vitro Studies

In our *in vitro* study we tried to mimic *in vivo* conditions, therefore, the *in vitro* experiments were conducted in 2.5% oxygen, which is the average oxygen concentration in glioblastoma tumors, and compared to standard laboratory conditions (20% oxygen). On the basis of the results generated in the present study, we can confirm both the pro- and antitumorigenic actions of DXM reported by other authors due to differences in DXM effects induced in cells cultured in 2.5% or 20% oxygen concentration in the atmosphere. However, in our opinion only results obtained in the physiological oxygen condition for glioblastoma may have translational value, since standard laboratory conditions do not exist in the tumor *in vivo*.

Limitations

Although an *in vitro* model is an important platform for understanding glioblastoma biology and we tried to mimic certain microenvironmental variables (glucose and oxygen), we are aware of this studies' limitations. Cells were cultured on flat bottom plates (not in a 3-D model) without an environment of normal human astrocytes, oligodendrocytes or neurons, and in a single hypoxic condition (2.5%). Since cells were merely exposed to TMZ/DXM for 48 h, it is difficult to draw conclusions about long-term effects on glioblastoma cells. In the retrospective analysis of medical records, we did not find information about BMI, extend of tumor resection (total, subtotal) and glycemia measurements were not a standard procedure. Thus at this point we aimed at verifying if glucose concentrations are somehow related to the aggressiveness of glioblastoma (represented by the marker Ki-67) without detailed assessment of other clinical prognostic factors and their intercorrelations.

Conclusion

Our results (despite the limitations) are in line with those of other studies concerning hyperglycemia in patients and high glucose concentrations in *in vitro* models. It is a significant, but neglected factor which contributes to glioblastoma progression.[46] Our investigations shed new light on the lability of glioblastoma cells in response to TMZ and DXM mediated by microenvironmental conditions. To the best of our knowledge, this is the first study presenting a link between *in vitro* models and clinical results indicating a significant role for glucose in the response of glioblastoma cells to TMZ and DXM. For many years, studies of glioblastoma were focused mainly on the molecular characterization and the essential role of the microenvironment in shaping the malignant phenotype of glioblastoma has not been sufficiently stressed. Now we suppose based on results of these studies that influence on GBM metabolism or microenvironment can be universal therapeutic strategy for all GBM tumors, independently from molecular profile. However, this problem needs more studies and explain the mechanism of action studied drugs. The present studies also suggest that a regular control of glycemia and the maintenance of low physiological glycemia levels in patients with glioblastoma would lead to the weakening of the cancer´s energy metabolism and thus help modulate the efficacy of applied drugs (TMZ and DXM). This is why the analysis of glycemia levels and its appropriate modulation should be an integrated part of conventional anticancer therapies.

Appendix

Abbreviations

GBM	glioblastoma multiforme
DXM	dexamethasone
TMZ	temozolomide
INSR	insulin receptor
ROS	reactive oxygen species
IGF-1R	insulin-like growth factor 1 receptor
GC	glucocorticoids
EGFR; MGMT	(O[6]-methylguanine-DNA methyltransferase) DNA repair enzyme
RT	radiotherapy
AQP1	aquaporin
Cxn43	conexin 43
GCr	glucocorticoid receptor
VEGF	vascular endothelial growth factor

Author Contributions

Anna Bielecka-Wajdman—corresponding author, designed the experiment, wrote an article
Tomasz Ludyga-responsible for figures, photos and statistics, assistance in conducting cell cultures
Wojciech Smyk—4 Students of the Pharmacology Circle, assistance in conducting cell cultures, experiments and determinations, collecting data about patients
Christina Suzanne Mullins—Native speaker, assistance in translation, with Prof. Linnenbacher assistance in cell cultures conducting, they lent primary lines from patients with glioblastoma
Ewa Obuchowicz—substantive help, assistance with writing the article.

Declaration of Conflicting Interests

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Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors

Informed consent

No human participants were used in this study.

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Supplemental Material

Supplemental material for this article is available online.

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