Combination hyperbaric oxygen and temozolomide therapy in c6 rat glioma model[1]

Terapia combinada de oxigênio hiperbárico e temozomida no modelo C6 de glioma em ratos

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ABSTRACT

PURPOSE: Temozolomide (TMZ) has anti-tumor activity in patients with malignant glioma. Hyperbaric oxygen (HBO) may enhance the efficacy of certain therapies that are limited because of the hypoxic tumor microenvironment. We examined the combined effects of TMZ–HBO in a rat glioma model.

METHODS: After stereotactic injection of C6/LacZ rat glioma cells into the Wistar rats brain, the rats were randomly assigned to three treatment groups [group 1, control treatment; group 2, TMZ alone; group 3, a combination of TMZ and HBO]. Rats were sacrificed 18 days after treatment, and number of intra-/peri-tumoral vessels, microendothelial proliferations, immunohistochemistry and necrotic area were evaluated.

RESULTS: Tumoral tissue was stained only sparsely with GFAP. Temozolomide treatment was significantly decreased in tumor tissue intratumoral vessel number / total tumor area level. The level of Ki67 was significantly decreased in the tumor tissue of the group 3. Additionally, the total necrotic area / total tumor volume (%) was decreased significantly in tumor tissue of the group 3 rats compared to group1 and 2.

CONLUSION: The combination of hyperbaric oxygen with temozolomide produced an important reduction in glioma growth and effective approach to the treatment of glioblastoma.

Key words: Glioma. Glioblastoma. Temozolomide. Hyperbaric Oxygenation. Rats.

RESUMO

OBJETIVO: A temozolomida (TMZ) tem atividade anti-tumoral em pacientes com glioma maligno. Oxigênio hiperbárico (HBO) pode aumentar a eficácia de terapias que são limitadas devido a um microambiente do tumor hipóxico. Foram examinados os efeitos combinados de TMZ-HBO em um modelo de glioma em rato.

MÉTODOS: Após a injeção estereotáxica de células de glioma de rato C6/LacZ no cérebro de ratos Wistar, os ratos foram distribuídos aleatoriamente em três grupos de tratamento: Grupo 1: tratamento de controle. Grupo 2: TMZ sozinho. Grupo 3: uma combinação de TMZ e HBO. Os ratos foram sacrificados 18 dias após o tratamento. Foram avaliados o número de vasos intra-/peri-tumoral, proliferação microendotelial, imunohistoquímica e área necrótica .

RESULTADOS: O tecido tumoral foi marcado apenas esparsamente com GFAP. O tratamento com temozolomida diminuiu significativamente o tecido intratumoral e a área total do tumor. O nível de Ki67 foi significativamente diminuído no tecido do tumor do grupo 3. Além disso, a superfície necrótica total / volume total do tumor (%) diminuiu significativamente no tecido do tumor do grupo 3 em comparação com grupo 1 e 2.

CONCLUSÃO: A combinação de oxigênio hiperbárico com temozolomida produziu uma redução importante no crescimento do glioma podendo ser abordagem eficaz para o tratamento do glioblastoma.

Descritores: Glioma. Glioblastoma. Temozolomida.Oxigenação Hiperbárica. Ratos.

Introduction

Glioblastomas are the most frequent types of intracranial tumors, and locally infiltrating, aggressive and hypervascularized tumors with a median survival rate of less than one year. The current therapy is cytoreductive surgery followed by radiotherapy, with a more limited role for adjuvant chemotherapy. Glioblastomas are dependent on angiogenesis, as proliferation of microvascular endothelial cells[1–4].

Temozolomide (TMZ), in many cancers including gliomas, has broad-spectrum anti-tumor activity[5]. Temozolomide penetrated excellent into the brain tissue is agent an oral imidazotetrazinone methylating. Also, inhibition of protein kinase C has been implicated as a possible mechanism of action[6].

In high-grade gliomas have been found to be areas hypoxic. In particular, the C6 line has demonstrated tumor hypoxia when used in experimental models. Hypoxia may decrease the effectiveness of adjuvant therapy[7,8]. Hyperbaric oxygen treatment (HBO), which in turn will improve the radiation response in solid tumors, including gliomas, will enhance the oxygen tension in tumors[9-11].

In the present study, we evaluated the effects of the combination treatment with temozolomide and hyperbaric oxygen in a rat brain tumor model using C6 glioma cell line.

Methods

Glioma cell lines and culture condition

The rat glioblastoma cell line C6 was obtained from ATCC (Rockville, USA) and serial passages were made at the Histology and Embryology Department of Istanbul Medical School in modified Eagle medium of Dulbecco, which contained 15% heat inactivated fetal calf serum, 0.2 mM glutamine, 50 mg/ml neomycin, and 100 mg/ml streptomycin. Culture flasks were kept in electronic incubator (Sanyo) under humidified atmosphere with 5% CO_2 at 37°C.

Animals and implantation procedure

Cells were harvested via 2 ml trypsin- EDTA solution C (Biological Industries, Israel) and centrifuged after the addition of 1.5 cc of F12 medium and fetal calf serum mixture at 1x103 rpm for three minutes. After removing the supernatant, pellet was resuspended with 2 cc of medium. Cell suspension was concentrated; so that 5 microliters of any injection volume would contain $5x10^5$ cells, and then placed in a microcentrifuge tube, and kept in a water-ice mixture environment during the whole implantation procedure, which always lasted less than 2 hours.

Male Wistar rats, weighing between 260 and 300 grams, were anesthetized by intramuscular injection of a solution containing 42.8 mg/ml of 10% ketamine and 8.6 mg/ml of 2% xylazine (this dosage did not exceed 0.7 mg/kg of body weight in total). The rats were secured into a stereotaxis apparatus (Trent Welles Inc., South Gate, Ca, USA). After antiseptic preparation of the scalp with betadine, the skin was incised on the mid-sagittal line for 1 cm. A burrhole (1.5 mm wide) was made on the right side 3 mm lateral to midline, and 2 mm proximal to the bregma using a dental drill. Injection of the C6 glioma cell suspension was made using a Hamilton syringe with a 27 gauge needle, which was fixed to the manipulation arm of the stereotaxis apparatus and advanced to the center of the right caudate nucleus which was 5 mm deep to the surface of the brain. A total of 5 ml solution was injected over a 5 minute of time in 0.5 µl aliquo. The needle was kept in same place for an additional 3 minutes and then withdrawn very gently over two minutes. The operation area was irrigated with saline and the burrhole was covered with bone-wax. The scalp was sutured using 5-0 vicryl.

Therapy for established C6 rat glioma growing in the brain of rats

All animals underwent implantation of guide screws and after one week they were divided into three groups. For all animals (21 rats), $5x10^5$ C6/LacZ glioma cells were injected and the animals were sacrificed 18 days later following specific treatment. Animals in group 1 (Control, seven rats) were treated by saline (23 ml/kg/day) p.o. daily from 11th to 15th day. Animals in group 2 (TMZ treated, n=7) were treated by temozolomide, which was administered at a dose of 150 mg/m^2/day by gastric catheter (6°F) doses of temozolomide (Temodar, Scherling-Plough) on days 11–15 after tumor implantation. Animals in group 3 (TMZ and HBO combination treated n=7) were treated by the combination of hyperbaric oxygen (2.0 atm, 7–12 exposures, seven days) and temozolomide.

Histopathological evaluation

Brains fixed in 10% buffered formalin were dissected in 10 mm coronal slices via taking inoculation hole as origin. All samples were photographed. Glass slides of 4 microns thickness were prepared. Histology sections from these were stained with conventional haematoxylin-eosin technique for routine analysis. Number of intra-/ peri-tumoral vessels and glomeruloid microendothelial proliferations were counted in five high power field (HPF) area (0.238 mm^2); according to these data, two

indexes were calculated, i1and i2 respectively. i1 = Intratumoral vessel number / total tumor area, i2 = Peritumoral vessel number / intratumoral vessel number.

For immunohistochemistry

Primary antibodies to glial fibrillary acidic protein (GFAP), and Ki-67 (clone SP6, Lab Vision, Fremont, CA, USA), were used. Immunoreactivity was visualized by a biotin-streptavidin-peroxidase kit (Nichirei, Tokyo, Japan) and 3,3′-diaminobenzidine solution. To assess Ki-67 labeling index, more than 1000 neoplastic cells from each histologic type of brain tumor were counted.

Necrotic area quantification

Hematoxylin and eosin (H&E) sections (4 micron thick, paraffin embedded) of each tumor were analyzed by a pathologist. For tumor *necrotic area* quantification (μm²), images were captured using a digital camera (Olympus Camedia 7070) and analyzed using SPOT version 3.2.4 software. (Diagnostic Instruments, Inc.). Total necrotic area / total tumor volume was given as a percentage (%).

Statistics

Data are expressed as mean ± S.D. and analyzed for statistical significance by one-way analysis of variance (ANOVA) followed by post-hoc for multiple comparisons (Tukey's test) using an Instat software package (GraphPad Software, San Diego, CA, USA). Differences between mean values were considered significant when $p<0.05$.

Results

The i1, i2, Ki67 and necrotic area values for the different groups are shown in Table 1.

TABLE 1 - Number of intra-/ peri-tumoral vessels, glomeruloid microendothelial proliferations and total necrotic area.

Groups	i1	i2	Ki67	Total necrotic area (%) /total tumor volume
Group 1	5.27±0.9	3.54±0.6	75.4±9.8	0.248±0.10
Group 2	4.14±0.7*	3.40±0.7	46.6±7.2*	0.188±0.18
Group 3	4.68±0.6	3.46±0.3	32.1±4.3*†	0.066±0.04▲

Values are as mean±SD. Abbreviations are: i1 = Intratumoral vessel number / total tumor area, i2 = Peritumoral vessel number / intratumoral vessel number. Group 1: Control, Group 2: Temozolomide treated, Group 3: Temozolomide and hyperbaric oxygen treated. *P < 0.05 vs group 1. ▲p<0.05 vs. group 1, 2 and group 3.
† p<0.05 vs. group 2 and group 3.

Histopathologically, all tumors contained pleomorphic cellular elements, mostly with vesicular nuclei and moderate amounts of eosinophilic cytoplasm; mitotic figures and atypical nuclei were medium, as well as necrosis with pseudopalisading. Tumoral tissue was stained with GFAP (Figure 1a).

The i1 levels in the group 1 and 3 were increased (Figure 1b); however, Temozolomide treatment was significantly decreased in tumor tissue i1 level ($p<0.05$) (Figure 1c). For i2 level in group 2 and 3 was found to be not different than that of the control group.

The level of Ki67 was significantly increased in the tumor tissue of the group 1 ($p<0.001$) compared to the group 2 and 3 (respectively $p<0.05$, $p<0.05$) (Figure 1d). Additionally, the level of Ki67 was significantly decreased in the tumor tissue of the group 3 ($p<0.001$) compared to the group 2 ($p<0.05$) (Figure 1e).

The total necrotic area / total tumor volume (%) was decreased significantly in tumor tissue of the group 3 rats (Figure 1f) compared to group1 and 2 (Figure 1g) ($p<0.05$, $p<0.05$, respectively).

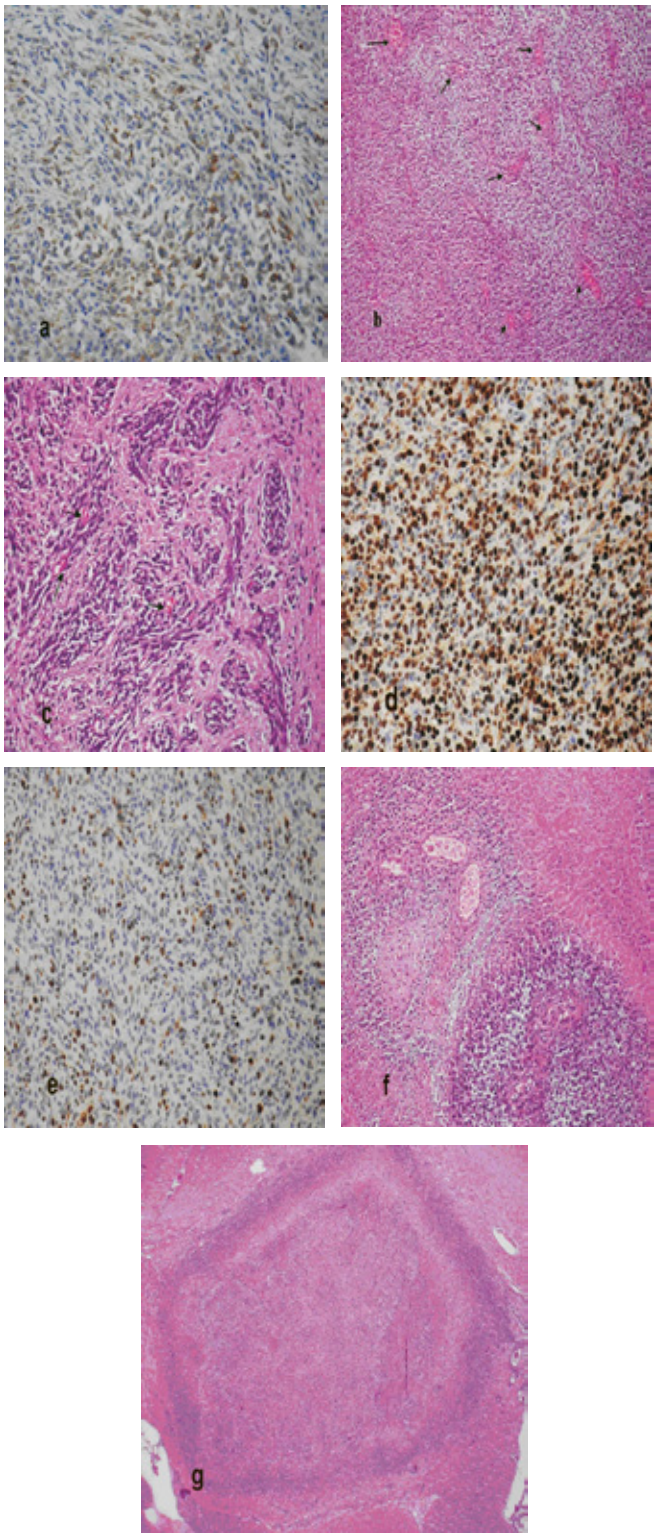

FIGURE 1 - (a) Immunohistochemically, the neoplastic cells react positively for GFAP (100x). **(b)** Intense vascular endothelial proliferation in Temozolomide (TMZ) treated group (arrows) (H&E, 200x). **(c)** Mild vascular endothelial proliferation in TMZ treated group (arrows) (H&E, 200x). **(d)** Glioma cell proliferation in control group was assessed by immunostaining for Ki67 intense positive glioma cell nuclei (200x). **(e)** In combination TMZ and HBO was stained Ki67 mild positive (200x). **(f)** Mild Necrosis with pseudopalisading in group 3 (H&E, 200x). **(g)** Necrosis with pseudopalisading in control group (H&E, 200x).

Discussion

Data obtained from the rat glioma model of this study indicate that the combination of TMZ and HBO significantly inhibits cell proliferation, angiogenesis and induce the stimulation of apoptosis compared with the effect of TMZ alone.

TMZ suggests capability as an effective chemotherapeutic agent for the treatment of patients with malignant glioma[12]. TMZ is quickly absorbed after oral administration and is cleaved in vivo to monoethyl triazenoimidazole carboxamide, a reactive DNA methylating species[13]. TMZ has excellent central nervous system entry, and reaches the brain in therapeutical concentrations[14]. Son *et al*.[15] found that treatment with TMZ alone significantly decreased the number of proliferating cell nuclear antigen-positive proliferating cells and increased the number of TUNEL-positive apoptotic cells. Kim *et al*.[16] demonstrated that temozolomide inhibited the growth of tumors and was accompanied by a reduction in tumor cell proliferation and induction of apoptosis. In the present study, TMZ treatment was significantly decreased in tumor tissue intratumoral vessel number. This result is similar to the reports by other authors that temozolomide inhibits angiogenesis.

Hypoxia in tumor tissue restricts the efficacy of treatment. Malignant cells in hypoxic fields are exposed to lower drug concentrations because of the limited access of parenterally administered drugs[17]. Additionally, these cells in hypoxic regions are less susceptible to radiotherapy, which target rapidly proliferating cells. Furthermore, hypoxia directly influences the expression of numerous gene products that are involved in angiogenesis, apoptosis, and glycolysis[17]. Tumor hypoxia in human malignant glioma has been studied by direct and indirect measurements. In direct pO^2 measurement, Kayama *et al*.[18] found that the intratumoural pO^2 value was significantly lower than that of the surrounding brain. Hypoxia is suggested to contribute to the resistance of gliomas to radiosurgery, radiation therapy, and chemotherapeutic agents such as temozolomide[19]. Kohshi *et al*.[20] in study multivariate analysis a small series revealed that combination with HBO was a good predictive prognostic factor for survival. Radiotherapy after HBO exposure is one of the initial treatment options for patients with malignant gliomas. Stuhr *et al*.[21] shown that repeated HBO induces an anti-angiogenic effect in the dimethylbenzanthracene induced tumors. The Ki-67 protein is a cellular marker for proliferation[22]. Ki-67 immunostaining of a brain tumor associated with a high proliferative rate. In our study, the level of Ki67 was significantly decreased in the tumor tissue of the group 3 compared to the group 2. Additionally, the total necrotic area / total tumor volume (%) was decreased significantly

in tumor tissue of the group 3 rats compared to group1 and 2.

Conclusions

The combination of hyperbaric oxygen with temozolomide produced an important reduction in glioma growth and effective approach to the treatment of glioblastoma. The treatment demonstrated an inhibition to rat glioma tumors, a significant decrease in cell proliferation and an increase in apoptosis.

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