Original Article

Effect of Temozolomide Treatment on the Adenine Nucleotide Hydrolysis in Blood Serum of Rats with Implanted Gliomas

Fernanda B Morrone;[4*] Fernando Spiller;[1*] Maria Isabel A Edelweiss;[3] Louise Meurer;[3] Paula Engroff;[1,4] Carlos H Barrios;[5] Alan A Azambuja;[5] Diogo L Oliveira;[1] Guido Lenz;[2] Ana Maria O Battastini.[1]

1 Department of Biochemistry, ICBS Federal University of Rio Grande do Sul, UFRGS

2 Department of Biophysics, Institute of Bioscience,Federal University of Rio Grande do Sul, UFRGS

3 Department of Pathology Federal University of Rio Grande do Sul, UFRGS

4 School of Pharmacy, Federal University of Rio Grande do Sul,UFRGS

5 School of Medicine, PUCRS - Pontifícia Universidade Católica of Rio Grande do Sul

* Both are first authors

Abstract

Objective: Adenine nucleotides and adenosine have many important functions in the physiological and pathological conditions. The measurement of these nucleotides in serum may be an auxiliary tool in the identification of cellular damage in many pathological conditions. The aim of this study is to examine the effect of chemotherapy treatment on nucleotide hydrolysis in the serum of rats following glioma implantation. **Methods:** C6 glioma cells were injected in the right striatum of 60 day-old Wistar rats, and 20 days after the induction of gliomas, blood serum samples were prepared for measurement of ATP and AMP hydrolysis. **Results:** The pathological analysis showed that the malignant gliomas induced by C6 injection and treated with temozolomide exhibited a reduction in malignant characteristics. The results demonstrated that the rats that underwent temozolomide treatment had a significant decrease ($p<0.05$) in blood serum hydrolysis of ATP and AMP when compared with the glioma group. None of the animals included in this study presented significant alterations in the activities of the serum enzymes alanine aminotransferase, aspartate aminotransferase, and alkaline phosphatase. **Conclusion:** The decrease in the enzymatic hydrolysis of the ATP and AMP is probably related to the diminished malignant characteristics caused by temozolomide treatment on the gliomas *in vivo*.

Keywords: Malignant gliomas; Temozolomide; Ectonucleotidases; Brain tumors

Introduction

Gliomas are the most common primary brain tumors. They are distinct from other solid tumors and invade the surrounding normal brain tissue, which makes gliomas a major challenge for clinical intervention. First-line therapy is surgery, although total surgical resection of gliomas is not possible and recurrence of tumor growth is common.[1-2] Historically, chemotherapy for gliomas has used cytotoxic drugs whose mode of action is cell cycle-independent.[3] Previous studies showed that the proliferation of malignant gliomas can be blocked by alkylating agents, such as Temozolomide.[4] This drug is the most commonly used chemotherapeutic agent in therapy for glioblastomas and is usually well-tolerated.[5]

ATP and other nucleotides and nucleosides are very important signaling molecules under physiological and pathological conditions in the central nervous system.[6] The events induced by extracellular adenine nucleotides are controlled by the action of ectonucleotidases, which hydrolyze ATP into adenosine in the extracellular space.[7]

Our group has shown the involvement of the purinergic system in glioma proliferation in different cell types.[8] Low concentrations of ATP induced proliferation

Correspondence
Fernanda B Morrone
Faculdade de Farmácia Pontifícia Universidade Católica do RS
Av. Ipiranga, 6681 P. 12 Bl. C Sala 148
90619900 Porto Alegre,Brasil
Phone: +55 51 33203512
E-mail: fbmorrone@gmail.com

of glioma cells and also induced the death of normal brain cells. Moreover, glioma cells degraded ATP very slowly when compared to astrocytes, potentially resulting in the accumulation of extracellular ATP around the gliomas.[9] Therefore, during glioma development, the extracellular ATP could induce the death of normal cells and induces the proliferation of glioma cells. In accordance with this hypothesis, recently we demonstrated that the administration of the enzyme apyrase decreases malignant characteristics and tumor size in a rat glioma model. These results indicate that the participation of extracellular ATP and the decrease of ectonucleotidases activities may be associated with the development of gliomas *in vivo*.[10,11] Two main mechanisms can liberate ATP into the extracellular space around the invasive tumor cells: first, the excitotoxic death of the normal host cells, and second, the injury caused by tumor resection, as occurs during surgery. The measurement of the ATP and AMP enzymatic hydrolysis rate in serum could reflect the ATP/AMP content in the local tissue and provide an auxiliary tool for cancer diagnosis. In fact, studies have demonstrated that the serum 5'-NT levels seem to be correlated with disease status in some ovarian carcinoma patients[12] and patients with neck cancer.[13] In this context, serum derived from breast cancer patients was found to be a rich source of 5-NT.[14] Moreover, the level of exogenous ATP may be increased in various inflammatory and shock conditions, mainly as a consequence of nucleotide release from platelets, endothelial and blood vessel cells.[15]

Since evidence suggests that purinergic signaling is involved in the growth and progression of gliomas, the aim of this study is to examine the effect of chemotherapy treatment with temozolomide on ATP and AMP hydrolysis in the serum of rats following glioma implantation. This measurement is a simple biochemical method that is easy to perform and may be used as a marker for the accompaniment of treatment and tumor recurrence. Moreover, this work may provide some insights about the mechanism of glioma development *in vivo*.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM) and fungizone were purchased from Gibco-BRL (Grand Island, NY, USA); fetal calf serum (FCS) was from Cultilab (Campinas, SP, Brazil). Temozolomide (Temodar in the United States, Temodal globally) was from the Schering-Plough Corporation (Kenilworth, NJ, USA). Nucleotides (ATP and AMP), HEPES, streptomycin, penicillin G, Trizma base, and EDTA were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Cell Culture

The rat glioma cell line C6 (derived from N-nitrosomethylurea-induced glioma in rat) was obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 0.5 U/ml of penicillin/streptomycin antibiotics, and supplemented with 5% (v/v) FCS. Cells were kept at a temperature of 37°C, a minimum relative humidity of 95%, and an atmosphere of 5% CO_2 in air. All the experiments of this study were conducted in serum supplemented DMEM.

Glioma induction and treatment with temozolomide

The study was performed in accordance with the University Ethics Committee guidelines for experiments with animals (approval nº 03-050). Male Wistar rats weighing 200-300 g were anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg) and placed in a stereotactic frame. Glioma cells were injected (10^6 cells in a volume of 2-3 μl) at a depth of 6 mm into the right striatum with a Hamilton syringe. Injection coordinates with regard to bregma were 0.5 mm posterior and 3.0 mm lateral.[16] After 10 days of induction of the gliomas, rats were treated with 5 mg/kg/day (i.p.) of temozolomide (TMZ) dissolved in dimethyl sulfoxide (DMSO) at a final concentration of 10% (200 μL)[17]. For control experiments, rats were treated only with 10% (200 μL) DMSO for 5 consecutive days. The groups were divided as follows: naive rats, C (n=5); glioma (induction of glioma), G (n=8); DMSO (temozolomide vehicle alone), negative control, n=4; TMZ, temozolomide treated rats, n=4.

Tumor Size and Survival Rate in the C6 Glioma Model

Twenty days after glioma induction, the rats were euthanized and the tumor size was quantified. Images were captured using a digital camera connected to the microscope and analyzed using Image Tool Software.

The total volume (mm^3) of the tumor was computed by summing the segmented areas and by the multiplication of the slice resolution. Survival rate was evaluated by percentage of days of survival (± standard deviation, SD) to a maximum of an 8-week observation period.

Pathological Analysis

At least three hematoxylin and eosin (H&E) sections (4 μm thick, paraffin embedded) of each tumor were analyzed by two independent pathologists blinded to the experimental data.

Measurement of ATP and AMP Hydrolysis in Rat Blood Serum

Twenty days after induction of the gliomas, blood samples were drawn after decapitation of the rats, and were immediately centrifuged at 3,000 *g* for 10 min at room temperature. The serum samples obtained were stored at –20°C for up to 10 days and used for the measurement of nucleotide hydrolysis. ATP hydrolysis was determined using the method previously described by Oses et al. (2004).[18] The reaction mixture containing ATP as substrate (3 mM), 112.5 mM Tris-HCl, pH 8.0, was incubated with approximately 1.0 mg of serum protein at 37°C for 40 minutes in a final volume of 0.2 mL. The reaction was stopped by the addition of 0.2 mL of 10% trichloroacetic acid. The samples were chilled on ice and the amount of liberated inorganic phosphate was measured by the malachite green method.[19] All samples were centrifuged at 5,000 *g* for 5 minutes and the supernatant was used for the colorimetric assay. AMP hydrolysis was determined under the same conditions for ATP, except that the substrate was AMP and at pH 7.5. For all enzyme assays, incubation times and protein concentration were chosen to ensure the linearity of the reactions. All samples were run in duplicate. The addition of the enzyme preparation after the addition of TCA was used as a control to correct for nonenzymatic hydrolysis of the substrates. Enzyme activities were expressed as μmol of Pi liberated/min/ per liter (U/L).

Protein was measured by the Coomassie Blue method[20] using bovine serum albumin as standard.

Determinations of ALT, AST, and ALP

The activity of alanine aminotransaminase (ALT), aspartate aminotransaminase (AST)[21] and alkaline phosphatase (ALP)[22] in the serum were determined using commercial diagnostic kits, manufactured by Labtest Diagnostic, Brazil. Enzyme activities were expressed as U/L.

Statistical Analysis

The results are expressed as mean ± SD. Multiple group comparisons were performed by ANOVA followed by a Tukey-Kramer *post hoc* test. P values < 0.05 were taken to indicate statistical significance.

Results

In the present study, we analyzed the changes in the hydrolysis of nucleotides in the blood serum of rats implanted with C6 glioma cells compared with rats with gliomas treated with the drug temozolomide. First, we implanted the glioma cells using stereotactic surgery. Twenty days after the surgery, malignant tumors with a high mitotic index, necrosis, intratumoral hemorrhage, parenchymal invasion, and lymphocytic infiltration were identified during the pathological analysis of the implanted tumors (Table 1). According to the literature, these are characteristics of high-grade glioblastoma multiform (grade IV).[23-25] Results of the pathological analysis showed that the malignant gliomas induced by C6 injection and treated with temozolomide presented a reduction in the mitotic index and in the other malignant characteristics (Table 1).

Furthermore, rats treated with temozolomide presented a significant decrease in tumor size and a relative benefit of 100% in survival, when compared with its vehicle (10% DMSO) and rats with implanted glioma (Table 2). To confirm the anti-tumor effect of temozolomide, we

Table 1 - Pathological characteristics of gliomas and temozolomide-treated gliomas

	Glioma	Glioma + TMZ
	(n=4)	(n=4)
Coagulative necrosis	1/4	0/4
Intratumoral Hemorrhage	3/4	0/4
Lymphocytic infiltration	4/4	0/4
Peritumoral edema	3/4	0/4
Peripheric pseudopalisading	3/4	1/4
Mitotic index: mitosis/HPF	19.6 ± 5.3	12.2 ± 3.8

HPF – high power field

Table 2 - Tumor size and survival rate in the C6 glioma model (n=4)

	Glioma	DMSO	TMZ
Tumor size#	86.7 ± 23	85.2 ± 17.9	27.6 ± 11.9⋆
Days of survival⋆⋆	38.7 ± 8.7	37.1 ± 0.6	NM

mm3
⋆ p< 0.05, ANOVA, followed by Tukey-Kramer test
⋆⋆ Days of survival (± standard deviation)
NM = no mortality during an 8-week observation period

tested this drug on glioma cell proliferation in vitro. The results demonstrated that treatment of C6 cells for [24, 48] and 72 h with temozolomide (1μM and 3μM), caused a significant inhibition of cell proliferation in all times evaluated (data not shown).

The level of exogenous ATP may be increased under various inflammatory and shock conditions, mainly as a consequence of nucleotide release from platelets, endothelial and blood vessel cells.[15] Thus, the measurement of the ATP enzymatic hydrolysis rate in the blood may serve as an auxiliary tool in the diagnosis of cellular damage in various pathophysiological conditions.[26] Therefore, we investigated the effect of chemotherapy treatment with temozolomide on the hydrolysis of nucleotides in rat serum. The hydrolysis of ATP and AMP were evaluated in the blood serum of control (naive rats), glioma implanted rats (G), DMSO rats (vehicle), and rats treated with TMZ. The blood serum of rats treated with TMZ demonstrated a significant decrease in ATP hydrolysis when compared with the glioma group (p <0.001) or DMSO group (p <0.05). Interestingly, the ATP hydrolysis in serum from DMSO group also showed a significantly decrease when compared with the glioma group (Figure 1A).

It is important to highlight that the DMSO group did not change the characteristics of high-grade glioblastoma multiform (grade IV) and also did not improve the survival rate.

Since previous studies have already found an increase in the 5'-nucleotidase activity in the serum of patients with head and neck cancers,[13] it seems to be important to analyze this enzyme activity in a glioma model. As also shown in Figure 1B, 5'-nucleotidase activity was increased in the serum of rats with implanted brain tumors (53.7 ± 11.4 units/L). The TMZ group presented a significant decrease (p<0.001) in the AMP

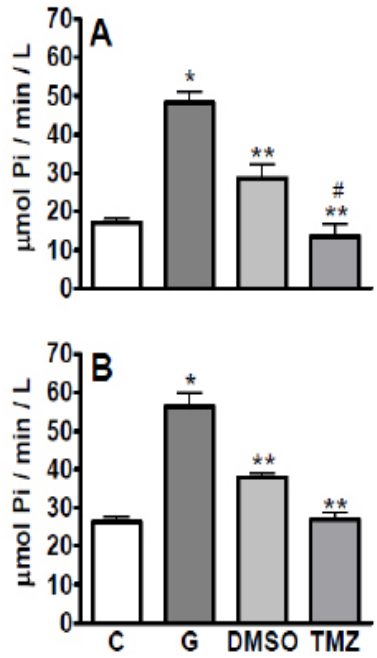

Figure 1 - Nucleotide hydrolysis activities in the serum of rats treated with temozolomide and DMSO Treatment with temozolomide and with 10% DMSO was performed as described in Material and Methods. Twenty days after induction of the gliomas, blood samples were collected and ATP (A) and AMP (B) hydrolysis was measured as described in Material and Methods. C = naive rats (n=5); G = glioma (induction of glioma, n=8); DMSO = negative control (temozolomide vehicle alone) (n=4); TMZ = temozolomide treated rats (n=4). The data are expressed as the mean SD. Statistics as in Figure 1. ⋆ p<0.005, T vs DMSO ⋆⋆ p<0.01, T and DMSO vs G.

hydrolysis in comparison with the glioma group and DMSO group (p<0.05), indicating that gliomagenesis could be an important cause of the elevation of this enzyme activity in the blood serum (Figure 1B).

It is well known that some of the enzymes studied here can be elevated in the presence of other pathological events, particularly hepatic disorders.[27-28] Thus, to evaluate the possible liver damage in the rats studied, we measured the levels of hepatic enzymes, alanine aminotransaminase (ALT), aspartate aminotransaminase (AST), and alkaline phosphatase (ALP) in the rat blood serum. As shown in Figure 2, none of the animals included in this study presented significant alterations in the AST, ALT, and ALP enzymes, eliminating the possibility of hepatic alterations in the animals studied.

Discussion

In this study, we confirmed that TMZ treatment decreased the tumor size, improved the survival rate and decreased glioma cells proliferation *in vitro* as previously shown by Raymond et al (1997).[29] Next, we have shown,

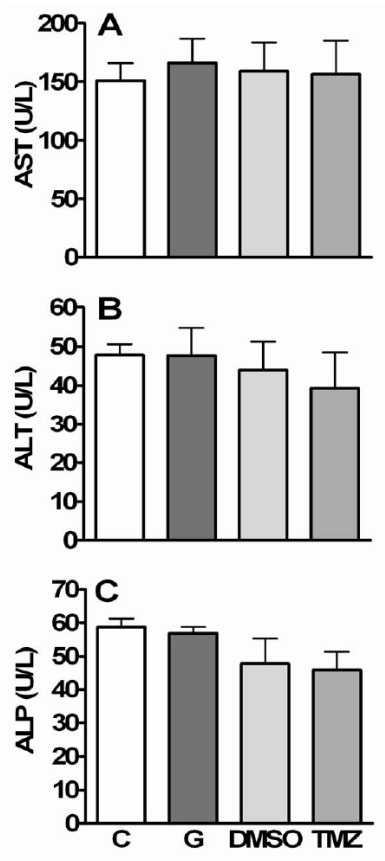

Figure 2 - AST (A), ALT (B) and ALP (C) enzymatic activities in the serum of rats Evaluation of alanine aminotransaminase (ALT), aspartate aminotransaminase (AST), and alkaline phosphatase (ALP) in the rat blood serum. C = control (n=5); G = Glioma (induction of glioma, n=8), DMSO = negative control (temozolomide vehicle alone) (n=4); TMZ = temozolomide treated rats (n=4).

for the first time, that the development of glioma in rats increased the serum ATPase and AMPase activities. Moreover, TMZ treatment of rats with implanted glioma decreased the serum ATPase and AMPase activities.

It is important to observe that the nucleotide hydrolysis was measured in the blood serum of rats with implanted gliomas and not in tumor cells. Therefore, the decrease in the serum ATP and AMP hydrolysis is probably a consequence of the diminished malignant characteristics, among those the vascular reactions and inflammatory process, caused by TMZ treatment on the gliomas *in vivo*.

The implantation of malignant cells into animal brain tissue closely resembles the mechanisms of tumor growth and has advantages over other models. For example, the presence of inflammatory and vascular reactions is observed, similar to the human pathological situation.[1] In this study, the injection of C6 cells intracerebrally into Wistar rats led to the development of tumors with several characteristics of malignant gliomas and treatment with temozolomide reduced these malignant characteristics, such as the number of cells, inflammation, and angiogenesis (Table 1).

The mechanism involved in glioma cell growth is not completely understood. However, our group has shown that the purinergic system is involved in this process.[8-11] We demonstrated that glioma cells present an almost undetectable ATP-hydrolysis activity, while they have a significantly higher AMP-degrading activity when compared to normal astrocytes.[9] Additionally, our group has demonstrated that prolonging confluence and culture times led to an increase in the expression and in the specific activity of ecto-5′-NT/CD73 by the glioma cells. This event could be related to an increased ability to infiltrate the brain parenchyma and to form the tumor microsatellites, which constitute the main cause of glioma recurrence.[30-31] Furthermore, ecto-5′-NT/CD73 was described as a proliferative factor involved in cell growth and invasiveness in numerous tumors.[32-35] In fact, the overexpression of ecto-5′-NT/CD73 in breast cancer cell lines increases cellular adhesion, migration, and invasiveness[36] and melanoma cell lines of a high-malignance grade also present an elevated expression of ecto-5′-NT/CD73.[33] Finally, CD73 siRNA in MB-MDA-231 cells led to *in vivo* and *in vitro* growth suppression, as well as the inhibition of invasion and migration.[37] All together, these observations suggest a central role of ecto-5′-NT/CD73 in glioma cell line growth.

Here, we show that animals with cerebral tumor presented a significant increase in ATP and AMP hydrolysis in blood serum in comparison to the respective control groups. It may be speculated that the increase in nucleotide hydrolysis observed in the glioma group could lead to the formation of elevated levels of adenosine, an endogenous anti-inflammatory, in the blood circulation as a compensatory mechanism in response to the pro-inflammatory stimulus. In this respect, various studies have shown that the agonists of A_{2A} receptors are involved in the blockade of inflammatory cascades, acting as anti-inflammatory agents and the activation of these receptors improves survival in mouse models of sepsis.[38-39] The increased enzymatic activity in blood serum found in the present study could be explained by the altered permeability of the blood-brain barrier (BBB), already described in rat C6 glioma models.[40] One of the main problems in patients with glioma is the increase in BBB

permeability, leading to edema formation and death.[41] Therefore, the high changes in fluid and cell elements between the blood and cerebrospinal fluid facilitate the transfer of enzymes released by the tumor or adjacent cells to the blood stream.

The treatment with TMZ decreased the serum nucleotide hydrolysis (ATP and AMP) activity *in vivo* (Figure 1). Several hypotheses may be raised to explain these *in vivo* decreases. The decrease of enzyme activities could be related to other cell types present around the tumor. Many reports have demonstrated that C6 cells preferentially grow around blood vessels, demonstrating their high affinity for the endothelial basement membrane.[42-43] An important characteristic of the glioblastomas derived from C6 cells in Wistar rats is a significant degree of invasion, characterized by a diffuse infiltrating border, in which individual cells invade the surrounding brain tissue.[44-45] The preferred route of migration was shown to be along neuronal fibers and through the perivascular space with processes attached to the endothelial basement membrane, instead of attachment to astroglial end-feet.[23,43,46-47] Since TMZ acts on tumors and high proliferation cells, the decrease of endothelial cells around the tumor, a potential source of the nucleotidases in the blood serum, could be involved in the TMZ-decreased ATPase and AMPase activities. Accordingly, measurement of the ATP enzymatic hydrolysis rate was described as an auxiliary tool in the unspecific diagnosis of cellular damage.[24] However, the decrease in serum AMP hydrolysis is perhaps a direct consequence of the reduction in C6 cell number, since ecto-5′-NT/CD73 may be released into the serum from the primary tumor or local metastasis. This situation has been described for patients with breast cancer, head and neck cancer or ovary carcinoma.[13,47-49]

Another surprising finding is that DMSO, used only as a vehicle for the drug, was able to inhibit the ATP and AMP hydrolysis activities when compared with the glioma group. This seems to be an important finding, since DMSO is already known to cross the blood–brain barrier and to have potent anti-inflammatory effects.[50-51]

In conclusion, the decrease in the serum ATP and AMP hydrolysis is probably a consequence of the diminished malignant characteristics, among those the vascular reactions and inflammatory process caused by temozolomide treatment on the gliomas *in vivo*. Although caution must be exerted in translating the data from laboratory animals to humans, the nucleotidase activities are being tested in human blood samples from healthy donors and patients to aid in the accompaniment of treatment of glioma recurrence. Furthermore, the identification of these soluble enzymes may contribute to our understanding of the regulation of the purinergic signaling in the blood of patients with brain tumors.

Acknowledgments

This study was supported by the Brazilian funding agencies Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). The authors thank MD Fabiana Viola for her support.

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