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Increased 5'nucleotidase activity in the blood serum of brain tumor patients

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ABSTRACT

Extracellular nucleotides are degraded by several ecto-enzymes present on the cell membrane, and also by soluble enzymes in the bloodstream. Previous work has already found elevated 5'-nucleotidase activity in the serum of patients with head and neck cancer, but very few reports are available on the involvement of the nucleotidases in brain tumor development. The aim of this study was to evaluate the hydrolysis of adenine nucleotides in the blood serum of brain tumor patients. Blood samples were obtained from 15 patients between 17 and 76 years old with brain tumors, and from 25 healthy individuals (control). The pathological status of each tumor was confirmed by histology. Brain tumors included in this study were high-grade gliomas (glioblastoma and anaplastic astrocytoma) and low-grade gliomas (low-grade astrocytoma and meningioma). For the estimation of nucleotidase activity we used ATP (1.5mM), ADP (1.5mM) and AMP (2.0mM) as substrates. The products of enzymatic analysis were measured by spectrophotometer, and the amount of inorganic phosphate (Pi) liberated was determined by the method of malachite green. The results showed that, in comparison with controls, AMPase activity was significantly increased ($p<0.05$) in high-grade intracranial neoplasms, while there were no significant differences in ATPase and ADPase activities. In conclusion, our results indicate that 5'-NT activity may be useful for monitoring patients with high-grade brain tumors. We have reported the development of a reliable method to detect the activities of nucleotidases in the serum of glioma patients.

Palavras-chave
Tumor cerebral
Glioma
Nucleotídeos
5'nucleotidase

Aumento da atividade da 5'nucleotidase no soro de pacientes com tumor cerebral

RESUMO - Os nucleotídeos extracelulares são degradados por várias ectoenzimas que estão presentes na membrana celular e também por enzimas solúveis presentes na corrente sanguínea. Trabalhos anteriores já encontraram elevada atividade da 5'-nucleotidase no soro de pacientes com câncer de cabeça e pescoço, mas poucos relatos estão disponíveis sobre o envolvimento dos nucleotidases no desenvolvimento de tumor cerebral. O objetivo deste estudo foi avaliar a hidrólise de nucleotídeos da adenina no soro de pacientes com tumor cerebral. As amostras de sangue foram obtidas de 15 pacientes entre 17 e 76 anos com tumores cerebrais e de 25 indivíduos saudáveis (controle). O estado patológico de cada tumor foi confirmado por técnica histológica. Os tumores cerebrais incluídos neste estudo foram gliomas de alto grau (glioblastoma e astrocitoma anaplásico) e gliomas de baixo grau (astrocitoma de baixo grau e meningioma). Para a estimativa da atividade da nucleotidase foi utilizado como substratos: ATP (1,5mM), ADP (1,5mM) e AMP (2,0mM). Os produtos da análise enzimática foram medidos com um espectrofotômetro e a quantidade de fosfato inorgânico (Pi) liberado foi determinada pelo método de verde de malaquita. Os resultados mostraram que a atividade da AMPase foi significativamente aumentada ($p<0,05$) em neoplasias intracranianas de alto grau, enquanto que não houve diferenças significativas na atividade da ATPase e da ADPase, em comparação com os controles. Em conclusão, nossos resultados indicam que a atividade da 5'-nucleotidase pode ser útil

para monitorar pacientes com tumores cerebrais de alto grau. Também relatamos o desenvolvimento de um método confiável para detectar as atividades das nucleotidases no soro de pacientes com glioma.

1. Introduction

Nucleotides are important signaling molecules that mediate diverse biological effects (1). Nucleotides and nucleosides can act as trophic factors with the potential to regulate neural plasticity and development, affect proliferation and apoptosis of glial and brain capillary endothelial cells, and mediate the responses of the nervous system to pathological conditions (2,3).

Extracellular nucleotides such as adenosine 5' triphosphate (ATP), adenosine 5' diphosphate (ADP) and adenosine 5' monophosphate (AMP) are degraded by a variety of ectoenzymes, namely NTPDases, present on the extracellular side of the cell surface, and also by soluble enzymes in the bloodstream (4-5). These ecto-NTPDases hydrolyze nucleosides di- and triphosphate with different substrate preferences (6) and are involved in metabolism and control of extracellular nucleotide/nucleoside concentrations, which result in the modulation of purinergic signaling (7). Ecto-5'-nucleotidase hydrolyzes nucleotide monophosphates such as AMP to the respective nucleosides and is a key enzyme in the nucleotide degradation pathway (8-10). It helps to maintain strict control of adenosine levels, and its activity is most prominent in glia (11).

Takano et al. (12) showed that tumor progression in the central nervous system (CNS) can proceed by means of glutamate-induced neurotoxicity, which, mainly through the lysis of neurons, can lead to increased concentrations of extracellular ATP and other nucleotides around the borders of the growing tumors (13-14).

Extracellular ATP is a putative component of the proliferation-inducing autocrine/paracrine signaling pathway in tumors (15). ATP is a fast excitatory synaptic transmitter in both the CNS and the peripheral nervous system (PNS) (1). Studies have shown that ATP promotes association between neurons and glial cells and is directly involved in astrocytic calcium wave propagation (16), regulation of blood flow and hemostasis (17), and inflammatory reactions in the brain (18).

The purine adenosine is also an important neuromodulator with both excitatory and inhibitory actions within the CNS (19). Several studies describe the capacities of ATP and adenosine to stimulate proliferation in various cell types, including brain tumor cells (15, 18).

The most common brain tumors, gliomas, represent 50-60 % of these tumors and the median survival time for the patients is under one year (15). The causes of the recurrence seem to be mainly the high proliferation, invasiveness and resistance to radiation presented by these tumor cells (20). Radiation therapy combined with quimiotherapic agents show sensibility for the height-grade tumors, but chirurgic removal is the best option for patient improvement (although the recurrence rate is constant).

Therefore, measurement of the enzymatic hydrolysis of nucleotides in the blood serum is important and it can help in the diagnosis of cellular damage under pathological conditions (21). An increase in the activity of serum 5'- nucleotidase (5'NT) occurs mainly in hepatic disease (22) and in tumors associated with breast cancer (23), head and neck cancer (24) and ovarian carcinoma (25). Measurement of serum 5'-NT can be a useful tool to accompany the treatment and to detect the return of tumors, since a decrease in enzymatic activity occurs after responsive treatment, which suggests tumor inactivity (24). Previous data from our laboratory, demonstrated that rats that underwent temozolomide treatment had a significant decrease in blood serum hydrolysis of ATP and AMP when compared with the

glioma group (26). Therefore, the aim of this study is to evaluate the hydrolysis of adenine nucleotides ATP, ADP and AMP in the serum of brain tumor patients in order to monitoring the evolution of high-grade gliomas.

2. Material and methods

Sample collection

Blood samples were obtained from 15 brain tumor patients between 17 and 76 years of age. The pathological status of each tumor was confirmed by histology. A pathologist from the Pathology Service, Hospital São Lucas da PUCRS, performed the classification and malignancy grade for all the cases according to the WHO classification (27). Data collection was made between March 2008 and May 2010. Healthy controls were also studied during this period (n=25). Two groups were included in this study: high-grade gliomas -glioblastoma (n=8) and anaplastic astrocytoma (n=1) and low-grade gliomas - low-grade astrocytoma (n=3) and meningioma (n=3). Patients with hepatic disorders such as altered serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) or alkaline phosphatase activities, with metastasis, or who were using medications that could interfere in the enzymatic assay, were excluded from this study. The use of medications and the diagnostic of metastasis were obtained from patient medical records.

Pre-operative and recurrent tumor blood samples were obtained from the patients when they were examined. Blood was collected in tubes without anticoagulant. It was immediately centrifuged under refrigeration at 3000 x g for 10 min. Serum was carefully removed and separated from the cells. The serum samples obtained were stored at –4°C for up to 20 days and used to measure nucleotide hydrolysis. All the procedures were approved by the Ethical Committee of the PUCRS (nº 157/04).

Measurement of ATP, ADP and AMP hydrolysis

ATP and ADP hydrolysis were determined using the method previously described by Oses et al. (28). The reaction mixture containing ADP or ATP (1.5 mM), 450 mM Tris-HCl (pH 8.0), and 20 mM $CaCl_2$ was incubated with approximately 1.0 mg of serum protein at 37°C for 60 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 inorganic phosphate (Pi) liberated was measured by the method of malachite green (29). All samples were centrifuged at 5,000 x g for 5 minutes and the supernatant was used for the colorimetric assay. AMP hydrolysis was determined under the same conditions as for ATP and ADP, except that the substrate was added at 2.0 mM and pH 7.5. Enzyme activities were analyzed by B442-Micronal Spectrophotometer and expressed as μmol of Pi liberated/min/ per liter (U/L).

Statistical analysis

All experiments were carried out in triplicate. Data are presented as mean ± SD = standard deviation. Statistical analysis used a one-way nonparametric ANOVA test, followed by a Tukey-Kramer test. P values < 0.05 were taken to indicate statistical significance.

3. Results

In the present study we analyzed changes in the hydrolysis of the adenine nucleotides ATP, ADP and AMP in the blood serum of patients with brain tumors. Blood samples

were obtained from 15 patients with intracranial neoplasms. The various types of tumors included in this study were high-grade gliomas - glioblastoma (n=8) and anaplastic astrocytoma (n=1) - and low-grade gliomas - low-grade astrocytoma (n=3) and meningioma (n=3). Age- and sex-matched healthy individuals were also studied during this period as a control group (Table 1).

The 5'-nucleotidase activity in the serum of patients was shown in the Fig.1C, 5'-nucleotidase activity was significantly increased in the serum of patients with high grade tumors when compared with that of patients with low grade tumors and no tumors ($p < 0.05$).

ATPase and ADPase activities were decreased in most types of intracranial neoplasms when compared with AMPase, but the decreases remained statistically insignificant compared to the control. Healthy controls studied during this period (n=25) showed low ATPase (1.68 μmolPi/min/L ± 0.18), ADPase (1.70 μmolPi/min/L ± 0.21) and AMPase (4.04 μmolPi/min/L ± 0.20) activities. These data is in accordance to previous studies of our group (26, 30).

As mentioned before, the patients studied here did not present alteration in AST, ALT or alkaline phosphatase levels nor use medications that could interfere with the results.

Table 1. Clinical profile of patients with intracranial tumors.

Clinical condition		**WHO[27] grade**	**Male** N (Average age ± SD)	**Female** N (Average age ± SD)
Control (N= 25)		-	12 (42±19)	13 (34±16)
High Grade (N= 9)	Glioblastoma	4	6 (49±19)	2 (47±25)
	Anaplastic astrocytoma	3	1 (56)	-
Low Grade (N= 6)	Meningioma	2	3 (45±23)	-
	Low grade astrocytoma	2	2 (43±3)	1 (42)

SD = standard deviation

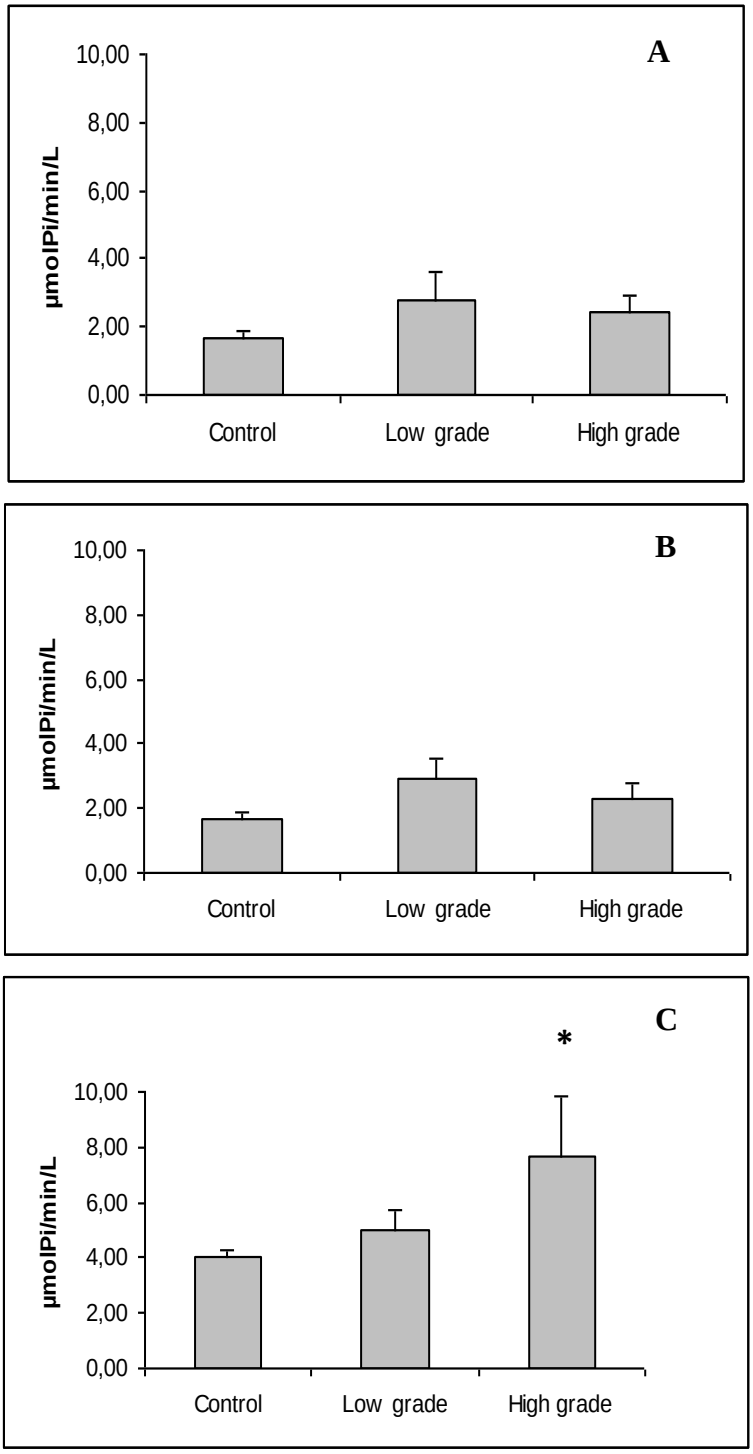

Figure 1. ATPase (A), ADPase (B) and AMPase (C) activities in blood serum of patients with brain tumors. All experiments were carried out in triplicate. Data are presented as mean ± SEM in units of µmolPi/min/L. * Significantly different from the control activity (one way nonparametric ANOVA test, followed by Tukey-Kramer test, $p < 0.05$).

4. Discussion

The present study was performed to investigate extracellular adenine nucleotide hydrolysis in the blood serum of patients with brain tumors. Most of the patients with high-grade gliomas were male, with average age of 49 years old. These results are in accordance with epidemiological data previously reported (31). These extracellular nucleosides can mediate several phenomena such as neural plasticity, proliferation and apoptosis (32). Data presented here demonstrate a significant increase in AMP hydrolysis in the serum (Fig. 1C) of patients with high grade tumors.

Changes in the activities of ectonucleotidases have been reported for several cancer cells, showing the important role for these enzymes in cancer (24, 33). *In vitro* studies in our laboratory demonstrated that cultured glioma cell lines (high grade glioma) exhibit low hydrolysis activity of ATP and ADP, but have highly active ecto-5'-nucleotidase enzyme (33), the same pattern of our *in vivo* study. Furthermore, nucleotidase-mediated hydrolysis is altered in the serum of patients with head and neck cancer (24), breast cancer (23), and ovarian carcinoma (25). Dao *et al*. (34) have demonstrated that serum 5'-NT levels decrease rapidly after mastectomy or therapy, indicating that tumor cells are a source of the enzyme.

Adenosine is an endogenous nucleoside that is present in the brain (35) and is an important neuromodulator with both excitatory and inhibitory actions within the CNS (19). After its extracellular release or formation, the adenosine spreads to the membrane of the cell, where it binds to specific receptors (1, 36). Adenosine interacts with the metabotropic P_1 receptors, which are divided into the four subtypes A_1, A_{2A}, A_{2B} and A_3, and activation of A_1 receptors, in particular, is associated with neuroprotection during hypoxic/ischemic events (2, 37, 38).

The increase in the AMPase activity found in the serum of patients with high-grade tumors can generate adenosine, which has protective functions (39). Patients with high-grade tumors exhibit, problematically, a more permeable blood-brain barrier, generating edema that can lead to patient death (40-41). In this way, cell elements changes can increase, liberating nucleotides and enzymes or other cells into the blood current, facilitating their detection.

5. Conclusion

Our method involves incubation of serum with adenosine 5' monophosphate (AMP) and measurement of either adenosine or inorganic phosphate, which is simple and inexpensive. Several assays have been developed using this approach. Our results indicate that 5 '-NT activity may be useful for monitoring patients with high-grade brain tumors.

We have reported in this paper the development of a reliable method to detect the activities of nucleotidases in the serum of patients. Although further studies are necessary to obtain more conclusive data, this study demonstrated that the hydrolysis of the adenine nucleotides can be an important indicator to follow the progression of brain tumors.

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Conflict of interest: The authors declare no conflict of interest.

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