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Laboratory Investigation

Increased resistance of glioma cell lines to extracellular ATP cytotoxicity

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Summary

Glioblastomas are the most common form of primary tumors of the central nervous system (CNS) and despite treatment, patients with these tumors have a very poor prognosis. ATP and other nucleotides and nucleosides are very important signaling molecule in physiological and pathological conditions in the CNS. ATP is degraded very slowly by gliomas when compared to astrocytes, potentially resulting in the accumulation of extracellular ATP around gliomas. Cell lysis caused by excitotoxic death or by tumor resection may liberate intracellular ATP, a known mitotic factor for glioma cells. The aim of this study is to examine the effects on cytotoxicity induced by extracellular ATP in U138-MG human glioma cell line and C6 rat glioma cell line compared to hippocampal organotypic cell cultures. The cytotoxicity of ATP (0.1, 0.5, 5 mM) was measured using propidium iodide and LDH assays. Caspases assay was performed to identify apoptotic cell death. Results showed that the glioma cells present resistance to death induced by ATP when compared with a normal tissue. High ATP concentrations (5 mM) induced cell death after 24 h in organotypic cell cultures but not in glioma cell lines. Our data indicate that ATP released in these situations can induce cell death of the normal tissue surrounding the tumor, potentially opening space to the fast growth and invasion of the tumor.

Introduction

ATP is an important signaling molecule in the peripheral and central nervous system in physiological and pathological conditions [1] and its effect on cells is mediated by metabotropic (P2Y) and ionotropic (P2X) receptors [2,3]. Ectonucleotidases exert a strict regulatory control, which maintains the extracellular concentration of purines and pyrimidines at very low levels in physiological conditions [4]. However, in some pathological conditions, large amounts of intracellular ATP can be released from damaged cells [5,6], which is part of an important response mechanism to cell lyses as is the case for astrocyte responses to injury in CNS, in which several P2Y receptors are involved [7].

On the other hand, high concentrations of extracellular ATP can induce cell death in different cell types, including tymocites, hepatocytes, microglial and mieloid cells [8–12]. In some cell types this cell death is by necrosis and in other cells is mainly a caspase-dependent apoptosis mediated by the opening of the $P2X_7$ channel by concentrations of ATP in the mM range or by lower concentrations of the specific agonist benzoylbenzoylATP (BzATP) [8]. Studies *in vivo* described that ATP neurotoxicity in the rat striatum is induced by P2 receptors [13] more specifically the subtype $P2X_7$ [8,14].

In addition, several studies have shown the involvement of ATP in neuronal death. The extracellular ATP released from cerebellar granules neurons can cause excitotoxicity induced by high concentration of glutamate and apoptotic death induced by serum deprivation, which can be partially inhibited by P2 receptors antagonists [15,16]. In turn, in several neuronal populations excitotoxicity mediated by ionotropic glutamate receptors modulates the release of purines [17,18]. Moreover, in astrocytes cultures, ATP also stimulates calcium-dependent glutamate release [19].

Glioblastomas are the most common form of primary tumors of the brain [20] and despite treatment, patients with these tumors have a very poor prognosis [21]. Recently, studies from our laboratory have showed that ATP is degraded very slowly by gliomas when compared with astrocytes, potentially resulting in the accumulation of extracellular ATP around glioma tumors [22].

ATP can be liberated to the extracellular space by the excitotoxic death of the normal host cells and by the injury caused by tumor resection, which is the mainstay of initial therapy for gliomas [23]. Since in glioma cell lines nucleotides and nucleosides induce proliferation [24], and ATP can mediate death in dissociated primary cerebellar granule or striatal neurons and in hippocampal organotypic cultures [25], the aim of this study is to examine the degree of cytotoxicity induced by extracellular ATP on gliomas cell lines in comparison to normal tissue.

Materials and methods

Compounds

Propidium iodide, BzATP and ATP, and all other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Ac-DEVD-AMC (Ac-Asp-Glu-Val-Asp-MCA) was obtained from Peptide Institute.

Cell culture

The U138-MG human glioma cell line and C6 rat glioma cell line were obtained from American Type Culture Collection (Rockville, Maryland, USA). Cells were grown in culture flasks in Dulbecco's Modified Eagle's medium (DMEM) with 15 and 5% fetal calf serum (FCS) for U138 and C6 cell lines, respectively, and seeded in 24-well plates at densities of 5×10^3–1×10^4 cells/well in 500 µl medium per well. Cells were treated with ATP or BzATP for 24 h and death was measured as described below.

Organotypic cell cultures

Organotypic hippocampal slice cultures were prepared using the method described by Stoppini et al. [26]. Briefly, male Wistar rat pups (postnatal day 6–8) were decapitated, the brain removed and the hippocampi dissected out. Transverse sections of 400 µm were cut with Mcllwain tissue chopper and separated in Hank's balanced salt solution (HBSS). Six slices were placed on a Millicell-CM culture insert (Millipore®). The inserts were placed inside six well plates (Cell culture Cluster, Costar®), each well contained one insert and 1 ml of culture medium. The plates were then placed in an incubator at 36 °C, with a 5% CO_2 enriched atmosphere. The culture medium consisted of MEM (50%), horse serum (25%) and HBSS (25%). This basic medium was supplemented with (mM, final concentration): glucose 36; glutamine 2; HEPES 25 and $NaHCO_3$ 4. Fungizone 1% and garamicine 36 µl/100 ml were usually added to the medium. The pH was adjusted to 7.3 with NaOH and immediately after, the solution was filtered (Millex GS, Millipore®). Medium was changed every 3 days and experiments were normally performed after 14 days *in vitro*.

Treatment and propidium iodide assay

Experimental slices organotypic cultures and glioma cell lines were exposed to extracellular ATP (0.1, 0.5, 5 mM) and BzATP (0.1 mM) for different times up to 24 h in the simultaneous presence of propidium iodide (6.4 µM for gliomas and 7.5 µM for organotypic cultures). Control cultures were not treated with ATP or BzATP. Propidium iodide fluorescence was excited at 515–560 nm using an inverted microscope (Nikon Eclipse TE 300) fitted with a standard rhodamine filter. Images were captured using a digital camera connected to the microscope and analyzed using Scion Image Software.

Caspase assay

To measure caspase activity, organotypic slices and glioma cell lines (U138 and C6 cells) were washed in PBS mg/ml: Na_2HPO_4 0.0004, NaCl 0.008, KCl 0.0002, pH 7.4 and then lysed on iced PBS and Triton X-100 0.2%. The extract was centrifuged at $10{,}000 \times g$ for 5 min and supernatant was collected. For each reaction, 30 µg of the sample was incubated with a reaction buffer containing g/ml: sacarose 0.1, CHAPS 0.001, BSA 0.0001 and Hepes-NaOH 0.024, pH 7.5. The substrate, Ac-DEVD-AMC (Peptide Institute), had a final concentration of 0.02 mM. Caspases 3 and 7 cleaves the substrate between D and AMC, releasing the fluorescent AMC, which can be quantified by fluorimetry, using an excitation wavelength of 370 nm and an emission wavelength of 460 nm. All measurements were corrected for protein concentration. The fluorescence intensity was calibrated with standard concentrations of AMC, and the Caspase-3/7 activity calculated from the slope of the recorder trace and expressed in picomols per minute per mg of protein.

Lactate dehydrogenase (LDH) activity

Cell viability was evaluated by measuring the activity of lactate dehydrogenase (LDH, EC1.1.1.27) according to the procedure of Whitaker [27]. Briefly, after 24 h treatment the cell culture medium was collected and incubated with substrate mixture and LDH activity was determined by enzymatic colorimetric reaction. For additional comparison and to establish the limits of the method some cells were freeze–thawed (F/T) to obtain the control of 100% of cell death. Absorption was measured at 490 nm.

Statistical analysis

All experiments were carried out at least three times in triplicate or quadruplicate, and means ± SEM are presented. Data were analyzed by one-way analysis of variance – ANOVA, followed by Tukey–Kramer test and Dunnett test. P values <0.05 were taken to indicate statistical significance.

Results

In this study we investigated the effects of extracellular ATP in rat organotypic hippocampal slice cultures and glioma cell lines in different concentrations and times of treatment. High extracellular ATP concentrations induced an intensive cell death in organotypic slice cultures treated for 24 h. As shown in Figure 1, the propidium iodide incorporation was significantly higher in those cells treated with ATP at a concentration of 5 mM in comparison to control cells. The percentage of cell death was around 60% of the total area of the organotypic slices and the damage was prevalently observed in the CA1 area (data not shown).

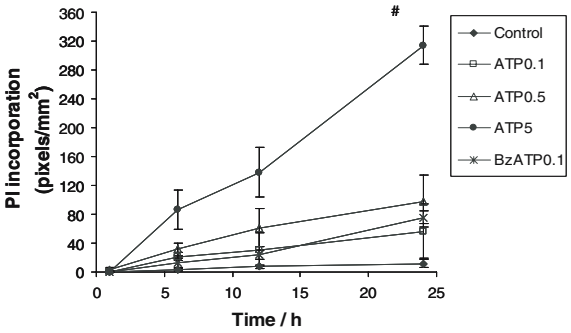

Figure 1. Effects of extracellular ATP on organotypic hippocampal slice cultures. Organotypic hippocampal cultures at 14 days were exposed to high extracellular ATP concentrations (0.1, 0.5, 5 mM) and BzATP (0.1 mM) for 24 h in the simultaneous presence of propidium iodide (7.5 μM). Cellular death was analyzed by PI incorporation that was visualized using a Nikon inverted microscope. Data represents the means ± SEM. $^{\#}P < 0.001$ as determined by ANOVA followed by Tukey–Kramer test.

It is well documented that ATP can induce cells death via apoptosis [9,28] and necrosis [29] and it seems that these effects can be mediated mainly *via* $P2X_7$ [5,11,14]. In order to examine if this receptor is involved in the death induced by ATP, we tested BzATP, a potent agonist of the $P2X_7$ receptor. BzATP, at a concentration of 0.1 mM, did not increase significantly propidium iodide incorporation in organotypic cultures after 24 h treatment (Figure 1).

In order to investigate whether glioma cells react differently to extracellular ATP, U138-MG human and C6 rat glioma cell lines were submitted to the same treatment as organotypic cultures. Figure 2 shows the time-course of propidium iodide incorporation of glioma cell lines in culture incubated with ATP (0.1–5 mM) and the analogue BzATP (0.1 mM). On the contrary to organotypic cultures, glioma cell lines presented a clear resistance to cell death induced by extracellular ATP. Both cell lines were resistant to the treatments for 24 h, with a cell death that did not surpass 20% when compared with control samples.

The resistance to cell death presented by glioma cell lines to extracellular ATP was confirmed by measuring the release of LDH activity to the extracellular medium. As observed in Figure 3, the treatments with ATP (0.1–5 mM) and BzATP (0.1 mM) did not increase the LDH activity in the culture supernatants of both glioma cell lines after 24 h of treatment in relation to control cells. In agreement with the propidium iodide incorporation (Figure 2) these results show that the exposure of glioma cells to extracellular ATP causes an extracellular release of cytosolic LDH, which is about 20% of the total release induced by freeze–thaw of the cells.

Considering that cysteine–aspartate proteases (caspases) activity is a hallmark of apoptotic cell death [30], the organotypic cultures and glioma cells were treated with the ATP (5 mM) and BzATP (0.1 mM) for 24 h and caspases 3 and 7 assay was performed. There was a significant increase in caspases 3/7 activities after the treatment with 5 mM ATP in hippocampal organotypic cultures and a smaller activation in cultures treated with the analogue BzATP (Figure 4). The treatment with the same concentrations of ATP and BzATP, in rat glioma cell line C6 and human glioma U138, did not activate significantly

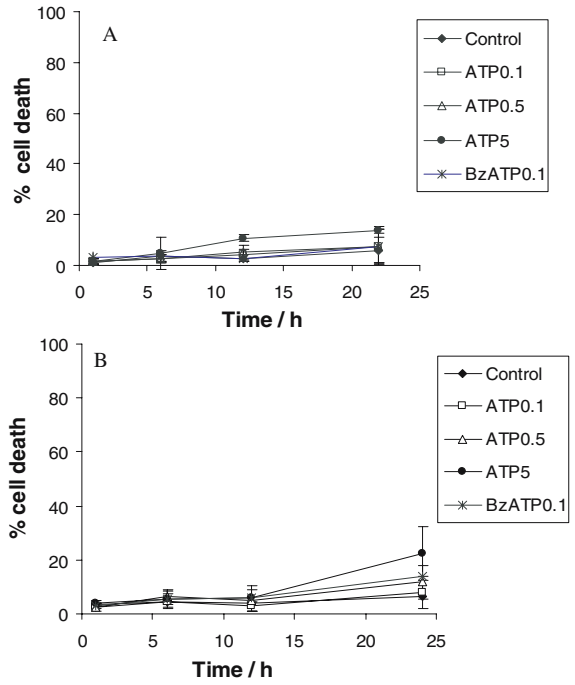

Figure 2. Effects of extracellular ATP on glioma cell lines. C6 rat cell line (A) and U138 human cell line (B) were treated as in Figure 1. Cell death is expressed by the percentage of positive cells for propidium iodide (6.4 μM) in relation to the total number of cells. Data represents the means ± SEM.

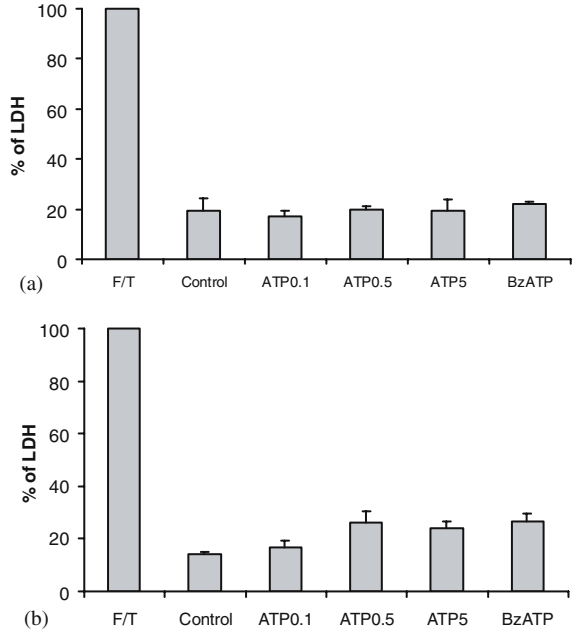

Figure 3. Lactic dehydrogenase (LDH) release. C6 (a) and U138 (b) cell death was quantified by measuring LDH release from damaged cells after 24 h treatment with ATP (at concentrations indicated) and Benzoyl-ATP (0.1 mM). For comparison, the cells were freeze–thawed (F/T) to obtain the control of 100% of cell death. Data represents means ± SEM of normalized optical densities of three independent experiments.

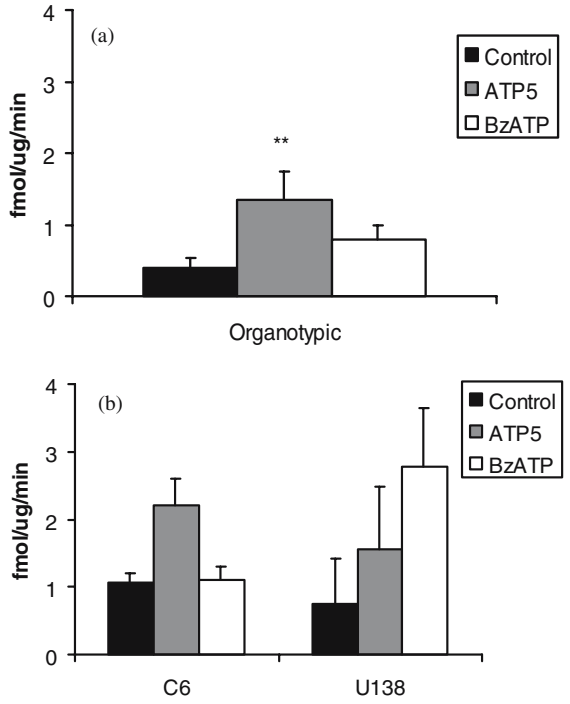

Figure 4. Caspases 3/7 activity in organotypic hippocampal slice cultures (a) and glioma cell lines (b). Cells were treated with ATP (5 mM) and BzATP (0.1 mM) for 24 h and caspases 3/7 activities were determined by fluorescence intensity examination. Data represents the means ± SEM. $^{**}P < 0.01$ as determined by ANOVA followed by Dunnett test.

caspases 3/7 (Figure 4), confirming the resistance to gliomas to extracellular ATP-induced apoptotic cell death.

Discussion

Here we have studied the effect of high extracellular ATP concentrations in two glioma cell lines (U138-MG and C6) and compared to rat organotypic hippocampal slice cultures. As detected by propidium iodide incorporation, glioma cells presented resistance to death induced by ATP at different concentrations, while exposure of organotypic cultures for 24 h to extracellular ATP induced cell death of around 60%, confirming, in this way, the sensitivity to ATP-evoked toxicity previously reported in normal neuronal tissue [25].

In the present work, we also observed that in organotypic cultures, the cytotoxic effect of $P2X_7$ analogue BzATP was smaller than that induced by ATP suggesting the involvement of receptors other than $P2X_7$ in the neuronal death evoked by extracellular ATP, as suggested by Amadio et al. [25].

The main finding of the present study is that the cell death induced by high concentrations of extracellular ATP found in organotypic cultures was not observed in the two gliomas cell lines (Figure 2). Solid tumor such as a glioma normally present clonal origin with an early loss or mutation of genes such as p53 and PTEN found in all cells of a tumor, with some variability due to its chromosomal instability [31,32]. Cell lines have similar mutations, and the process of growing the cell in culture probably adds some variability. This similarity makes cancer cell lines a good model for several aspects of cancer biology [33]. Therefore, the progression of a tumor is a process which involves the growth of one cell type, mimicked by the cell line, against a tissue, mimicked here by the organotypic culture, a widely used preparation which reproduces several pathophysiological properties of the neural tissue [25,26,31].

ATP is known to be released in the extracellular milieu of the CNS, both in physiological and pathological conditions [5,6,25,34]. The two main mechanisms that can liberate ATP to the extracellular space around the brain tumors are the excitotoxic death of the normal host cells, and the injury caused by the surgical tumor resection. Considering the internal concentration of ATP, which is from 5 to 10 mM in most cells [8], cell death potentially elevates the extracellular concentration of ATP to milimolar levels, similar to the concentration that induced cell death in organotypic cultures. We recently showed that different glioma cell lines have low rates of extracellular ATP hydrolysis [22] in contrary to astrocytes, and the astrocytic resistance to cell death induced by ATP could be due to, at least in part, its high rate of ATP hydrolysis in physiological conditions.

In the gliomas, because of its low rate of ATP hydrolysis, the resistance to ATP-induced death has to be at the level or downstream of purinergic receptors. One candidate of this downstream effect could be PTEN, which is deleted in a large fraction of gliomas, including the gliomas used in the present work, and which dephosphorylates phosphatidylinositol phosphates, reverting the effect of PI3K activation and leading to a suppression of the well established anti-apoptotic PI3K/Akt pathway [35]. A smaller activation of caspase activities seen in gliomas when compared to organotypic cultures indicates that the former presents resistant mechanism to ATP-induced apoptosis, not present in the latter.

Accordingly, it is well known that glutamate excitotoxicity is involved in numerous CNS disorders, and glutamate secreting glioma cells have a significant growth advantage [36,37]. Glutamate can induce ATP release from astrocytes [38] and in neuronal population excitotoxicity mediated by ionotropic glutamate receptors can modulate purine release [18], producing a positive feedback able to burst the tumor growth.

Considering that glioblastomas induce necrosis and consequent gliosis-like features [23] and that the current therapeutical strategies for gliomas are also strong gliosis inducers [23,39], it is reasonable to propose that the increased concentration of ATP in gliotic lesions could promote glioma proliferation and invasion by both inducing proliferation of the glioma cells [24] as well as opening space due to the death of healthy tissue that comes into contact with the glioma. A very important part of this process is the immunity of gliomas to the toxicity of ATP, as shown here. It is important to emphasize the positive feedback nature of this process, which could be responsible, at least in

part, for the fast growth and invasion usually presented by glioma tumors.

In conclusion, our study shows that glioma cells presented a clear resistance to death induced by cytotoxic concentrations of ATP when compared with normal brain tissue. Taken together with their inability to metabolize extracellular ATP, it seems probable that we identified a novel and important mechanism associated with malignance of these tumors. The inhibition of ATP release by gliomas or the blockade of ATP receptors will serve as an alternative strategy in the management of patients with malignant gliomas.

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