Purine Nucleoside-Dependent Inhibition of Cellular Proliferation in 1321N1 Human Astrocytoma Cells

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ABSTRACT

We examined the effects of purines and the pyrimidine UTP on cellular proliferation in the human astrocytoma cell line 1321N1. Treatment of cultured cells with 100 μM ATP or 2-chloroadenosine (2-CA) resulted in significant reductions in cell numbers after 2 days, whereas adenosine (ADO) exhibited a slower time course of inhibition of cell growth. Treatment with 100 μM UTP had no effect on cell numbers. 2-Chloroadenosine but neither ATP nor ADO resulted in an increase in cell death rates. A significant portion of the inhibitory response to ATP, ADO, or 2-CA was sensitive to the purine nucleoside transport inhibitor S-(p-nitrobenzyl)-6-thioguanosine, suggesting that uptake into cells was required for the inhibitory response. At least the majority of the observed responses to purines was not mediated by P1 (adenosine) receptors, because effects of ATP, ADO, or 2-CA were not affected by treatment of cells with the P1 receptor antagonist 8-(p-sulfophenyl)-theophylline. The absence of any known P2 (nucleotide) receptors in 1321N1 cells, coupled with the failure of the relatively stable ATP analog adenosine 5’-O-(3-thiotriphosphate) to alter cell growth rates, suggests that ATP acts indirectly to inhibit proliferation via one or more metabolic products. Although intracellular effects of purine nucleosides should be taken into account in future studies using 1321N1 cells, our findings also suggest 1321N1 cells as an excellent model for intracellular actions of nucleosides.

Extracellular nucleosides and nucleotides are known to communicate with cells in two ways: 1) by binding to extracellularly oriented receptors located on the plasma membrane (both nucleosides and nucleotides), and 2) by being transported through specific transporters into the cytosol (only nucleosides). Nucleotides such as ATP and UTP interact primarily with the P2 family, which is broken down into the two-transmembrane domain multimeric P2X and the seven-transmembrane domain P2Y subfamilies (Ralevic and Burnstock, 1998). Nucleosides such as adenosine (ADO) interact primarily with the G protein-coupled P1 receptors, which are subdivided into the A1, A2 (A2A and A2B), and A3 subtypes (Klotz, 2000). Adenosine is known to be taken up into target cells via a specific uptake mechanism, which has been shown to be sensitive to inhibition by agents such as dipyridamole and S-(p-nitrobenzyl)-6-thioguanosine (NBGTG) (Pearson et al., 1978).

A number of studies have now reported effects of extracellular purines, including ATP and ADO on the regulation of cell cycle, proliferation, and apoptosis in cells of both tumor (Abbracchio et al., 1995; Ceruti et al., 2000; Fishman et al., 2000; Ohana et al., 2001) and nontumor (Apasov et al., 1995; Dawicki et al., 1997; Rounds et al., 1998; Peyot et al., 2000) origin. In the majority of cases ADO has been reported to inhibit cellular proliferation, although there are reports that ADO at low concentrations can stimulate proliferation in some cell types (Ohana et al., 2001); in several examples where ADO inhibits proliferation it appears to induce an increase in the rate of apoptosis (Dawicki et al., 1997; Rounds et al., 1998; Peyot et al., 2000), although there is a recent report of ADO protecting leukemia 2H3 mast cells from apoptosis (Gao et al., 2001). In experiments performed on human astrocytoma (ADF) (Ceruti et al., 2000), murine myoblastic (Ceruti et al., 2000), and bovine and human pulmonary artery endothelial (Dawicki et al., 1997; Rounds et al., 1998) cells, the effects of either ADO or its analogs were shown to be dependent not upon interaction with P1 receptors, but rather upon uptake into the cells; in the studies by Rounds and colleagues, the effects of ATP were reported to be indirect via its breakdown to ADO.

The human astrocytoma cell line 1321N1 is a useful tool in purine and pyrimidine receptor expression studies because it appears to lack expression of any nucleotide (P2) receptors, and expresses at most one subtype (A1; Ohana et al., 2001); in several examples of purine nucleosides should be taken into account in future studies using 1321N1 cells, our findings also suggest 1321N1 cells as an excellent model for intracellular actions of nucleosides.

Extracellular nucleosides and nucleotides are known to communicate with cells in two ways: 1) by binding to extracellularly oriented receptors located on the plasma membrane (both nucleosides and nucleotides), and 2) by being transported through specific transporters into the cytosol (only nucleosides). Nucleotides such as ATP and UTP interact primarily with the P2 family, which is broken down into the two-transmembrane domain multimeric P2X and the seven-transmembrane domain P2Y subfamilies (Ralevic and Burnstock, 1998). Nucleosides such as adenosine (ADO) interact primarily with the G protein-coupled P1 receptors, which are subdivided into the A1, A2 (A2A and A2B), and A3 subtypes (Klotz, 2000). Adenosine is known to be taken up into target cells via a specific uptake mechanism, which has been shown to be sensitive to inhibition by agents such as dipyridamole and S-(p-nitrobenzyl)-6-thioguanosine (NBGTG) (Pearson et al., 1978).

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ABBREVIATIONS: ADO, adenosine; NBGTG, S-(p-nitrobenzyl)-6-thioguanosine; ADF, human astrocytoma; 8SPT, 8-(p-sulfophenyl)-theophylline; 2-CA, 2-chloroadenosine; FBS, fetal bovine serum; ATP-γ-S, adenosine 5’-O-(3-thiotriphosphate).
While attempting to treat P2 receptor-transfected 1321N1 cells with purine nucleotides over the course of several days, we noticed significant reductions in cell numbers during the course of the experiments; control experiments demonstrated a similar effect in cells that had not been transfected with nucleotide receptors. We have now studied the effects and mechanisms of several purines and the pyrimidine UTP on 1321N1 cells, and find that, despite the presence of the P1 receptor antagonist 8-(p-sulfophenyl)-theophylline (8SPT) and the absence of any endogenous nucleotide receptors, ADO has a significant inhibitory effect on cell proliferation, whereas the ADO analog 2-chloroadenosine (2-CA) actually causes cell death. ATP appears to act indirectly to inhibit cell proliferation, and in all cases uptake of nucleoside appears to mediate the majority of the inhibitory response.

Materials and Methods

Cell Culture. Unless otherwise stated, reagents were obtained from Sigma Chemical (St. Louis, MO). Human 1321N1 astrocytoma cells were grown to confluency in Dulbecco’s modified Eagle’s medium (low glucose, with l-glutamine, 110 mg/ml sodium pyruvate, and pyridoxine hydrochloride; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Mediatech, Herndon, VA), penicillin (100 U/ml), streptomycin (100 μg/ml), Fungizone (5 μg/ml; Invitrogen), and gentamycin (150 μg/ml) at 37°C in a water-saturated atmosphere of 95% O2, 5% CO2. Subculturing was accomplished by a 2-min 0.5-g trypsin/0.2-g EDTA digest followed by centrifugation and resuspension in culture medium.

Cell Proliferation Assays. 1321N1 cells were harvested by trypsin digest and resuspended in culture medium containing 10% FBS and antibiotics. Approximately 250,000 cells were added to 1 ml of culture medium in each well of 24-well tissue culture plates (Corning Costar, Cambridge, MA); 24 h after initial seeding, 10% FBS culture medium was replaced with medium containing 2% FBS. After 24 h, test reagents or appropriate volumes of vehicle (phosphate-buffered saline or dimethyl sulfoxide for experiments using NBGTG) were added, and plates were cultured for various time intervals; reagents and culture media were replenished every 24 h. At the appropriate time points, cells were harvested by trypsin-EDTA digest, resuspended in 1 ml of culture medium, and counted by hemocytometry. Each test (or its paired control) was performed in triplicate for each time point.

Data Analysis and Statistical Treatments. Cell counts were evaluated statistically by means of one-way analysis of variance followed by Tukey’s multiple-comparison test (Figs. 4-6), or by Student’s two-tailed, paired t test (Fig. 1). Differences were considered significant when P values were less than 0.05.

Results

We determined in pilot experiments that the minimum concentration of FBS required to support growth in 1321N1 cells was 2% (v/v), and this concentration of FBS was therefore used for 24 h before and during all cell culture experiments. After 1321N1 cells were treated with purines or UTP for 5 days, the number of cells remaining in culture wells was determined (Fig. 1). Compared with control wells receiving only phosphate-buffered saline, treatment with ATP, ADO, or 2-CA at 100 μM resulted in statistically significant reductions in cell numbers, whereas treatment with UTP or the relatively nonhydrolyzable ATP analog adenosine 5’-O-(3-thiotriphosphate) (ATP-S) produced no change in cell numbers. The reductions in cell numbers seen in response to ATP, ADO, or 2-CA were concentration (Fig. 2) and time (Fig. 3) dependent; EC50 values for ATP, ADO, and 2-CA were estimated from the curve fits in Fig. 2 to be 21, 18, and 43 μM, respectively.

To test whether the responses to ATP, ADO, or 2-CA were due to activation of P1 receptors, cells were incubated with 8SPT for 1 h before and continuously during treatment with near-maximal concentrations of purine; 8SPT has been shown to antagonize all three subtypes of P1 receptors (A1, A2, and A3) in humans with approximately equal potency (K<sub>i</sub> of ~5 μM; J. Linden, personal communication). As shown in Fig. 4, 8SPT at either 30 or 100 μM had no effect on its own upon 1321N1 cell growth rates; 8SPT also had no effect upon the ability of ATP, ADO, or 2-CA at concentrations as high as 100 μM to inhibit cell growth. No effect of 8SPT was seen upon the time course of inhibition of cell growth by ATP, ADO, or 2-CA (data not shown). These results suggest that at least the majority of the ATP, ADO, and 2-CA inhibition of cell growth was mediated by the majority of the ATP, ADO, and 2-CA inhibition of cell growth.
1321N1 cell growth is via a P1 receptor-independent mechanism.

Pretreatment with and inclusion of NBTG at a concentration known to be sufficient to block purine nucleoside transport was used to determine the role of uptake in the response to ATP, ADO, and 2-CA. Treatment with NBTG completely reversed the inhibitory effect of ADO on 1321N1 cell growth (Fig. 5, top), and there was no statistically significant difference between the change in cell numbers in control wells and that which occurred in wells treated with both ADO and NBTG. NBTG also reversed the inhibitory effect of 2-CA (Fig. 5, bottom) in a statistically significant manner; although not statistically different from one another, the means for control and 2-CA + NBTG values suggest the presence of an NBTG-insensitive component of the response to 2-CA. Also present in the data within Fig. 5 is our finding of a cytotoxic effect of 2-CA on 1321N1 cells; because the data in Figs. 5 and 6 represent changes in cell numbers between the start and finish of experiments, the negative values seen in response to 2-CA reflect an actual reduction in cell numbers over time, and not simply inhibition of cellular growth rates. That cell death did in fact occur in response to 2-CA (and not in response to either ATP or ADO) was confirmed by trypan blue exclusion viability assays (data not shown).

When NBTG was included in experiments along with ATP during treatment of 1321N1 cells, a statistically significant difference was observed between mean changes in cell numbers in wells treated with ATP and NBTG and those treated with ATP alone (Fig. 6, top). The response to ATP was not, however, reversed completely by NBTG treatment, because a statistically significant difference between control and ATP + NBTG values remained after the 5-day treatments, suggesting that a portion of the response to ATP was not dependent upon cellular uptake. Finally (and consistent with the results presented in Fig. 1), use of the relatively stable ATP analog ATP$_{γ}$S resulted in no significant reduction of change.

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**Fig. 3.** Time course of purine effects in 1321N1 cells. 1321N1 cells were cultured with the agents shown for various lengths of time, after which cells were counted by hemocytometry. Results are presented as percentage of appropriate time-paired control values and were determined from triplicate assays performed on at least three different cell culture groups; error bars are S.E.M.

**Fig. 4.** Lack of effect of P1 antagonism. Pretreatment with and inclusion of the P1 receptor antagonist 8SPT had no effect on the ability of ADO, ATP, or 2-CA to inhibit growth rates in 1321N1 cells; 8SPT had no effect on its own on growth rates. Results are expressed as percentage of paired control values, and are means ± S.E.M. from triplicate assays performed on three different groups of cultured cells; bars of the same shading were found to be not significantly different from one another.

**Fig. 5.** Adenosine uptake blockade reverses growth inhibition by adenosine. Inclusion of the purine nucleoside uptake blocker NBTG (10 μM) during the course of treatments of 1321N1 cells completely reversed the inhibitory effect of ADO (100 μM) on cell growth rates (top), and partially reversed the effect of the ADO analog 2-CA (100 μM, bottom), although the latter effect was not statistically significant. Data are presented as means of the percentage change in original cell counts. Asterisks denote a mean that is statistically different (P < 0.01) from both the control and the NBTG + ADO means, whereas the diamonds denote a statistically significant difference between 2-CA and control means only (P < 0.001). Values for n are indicated in parentheses.
in cell numbers in 1321N1 cells, and inclusion of NBGT along with ATP-γS had no effect compared with control values; these data suggest that the effects of ATP may in fact be in response to a product of ATP metabolism.

Discussion

The results from our study may be summarized as follows. 1) Treatment of 1321N1 cells with ATP, ADO, or 2-CA significantly decreased cell numbers after 5 days; treatment with UTP had no such effect. For 2-CA, not only were cell numbers decreased but also cell death rates were significantly increased; we are not yet able to state whether this increase in cellular death is due to a necrotic or apoptotic effect of 2-CA. 2) At least a portion of the nucleoside effects was dependent upon uptake into the cells, because NBGT had a significant effect upon the ability of both ADO and 2-CA to reduce cell numbers. The majority of the remaining portion of the nucleoside effects (those which were not reversed by NBGT treatment) does not appear to be mediated by P1 receptors, because inclusion of the nonselective P1 antagonist 8SPT had no effect upon any responses measured. 3) The effects of ATP appeared to be mediated by one or more breakdown products of ATP, because treatment of cells with the relatively nonhydrolyzable ATP analog ATP-γS resulted in no significant reduction in cell numbers compared with controls; additionally, the absence of any known P2 receptors in these cells makes it unlikely that ATP is interacting with these receptors to elicit a response. The fact that NBGT significantly (although incompletely) reversed the effect of ATP on cell numbers suggests that ATP is affecting proliferation rates via its breakdown to and the subsequent uptake of ADO.

That purines such as ADO might be useful either endogenously or exogenously in the control of cell growth has been suggested by several groups (Abbracchio et al., 1995; Dawicki et al., 1997; Gao et al., 2001); there have also been reports that ADO might preferentially inhibit the growth of tumor cells over nontumor cells (Fishman et al., 2000; Ohana et al., 2001). In our studies on the astrocytoma-derived cell line 1321N1, the naturally occurring purines ADO and ATP acted to inhibit the rate of cellular proliferation; again, ATP appeared to act indirectly via a product of its metabolism. The ADO analog 2-CA also inhibited cellular proliferation; unlike ADO and ATP, 2-CA treatments were found to result in an increase in cell death rates, and it may be that 2-CA inhibited cellular proliferation simply as a result of its cytotoxicity. We do not know why 2-CA alone had this cytotoxic effect, particularly because it too can be taken up by cells via an uptake inhibitor-sensitive transporter to act intracellularly (Ceruti et al., 2000). The effects of both 2-CA and ADO were significantly reversed by pretreatment of cells with NBGT, a finding that differs somewhat from that of Ceruti et al. (2000), who found that uptake inhibition blocked the apoptotic effect of 2-CA but not ADO in ADF human astrocytoma cells. The most likely explanation for this discrepancy is that we find no evidence for a cytotoxic effect of ADO in 1321N1 cells: the effect that is reversed by treatment of these cells with NBGT is inhibition of cell growth and not cell death. How it is that ADO induces apoptosis in ADF cells by an uptake-independent mechanism (Ceruti et al., 2000) but inhibits 1321N1 cell proliferation by an uptake-dependent (NBGT-sensitive) mechanism remains unknown.

At least a portion of the responses in 1321N1 cells to ATP, ADO, or 2-CA appeared to be insensitive to NBGT, despite the fact that it was used at a relatively high concentration. Given the known ability of 8SPT to block all three subtypes of P1 receptors in humans, and the lack of effect of 8SPT on any purine-dependent inhibition of cell growth rates, it is unlikely that the NBGT-insensitive response to purines is due to activation of ADO receptors. It may be that heterogeneity exists in purine nucleoside transporters (with some being relatively insensitive to uptake blockers), or that the effects of NBGT were not consistent throughout the experiment: NBGT was added to cell media at the same time as purines were added, and it is possible that its effects (inhibition of nucleoside transport) occurred with a different time course than those of the nucleosides themselves. Nonetheless, both the cytotoxic effects of 2-CA and the growth inhibition by ATP and ADO had statistically significant NBGT-sensitive components, suggesting that 2-CA and ADO have different fates once taken up into 1321N1 cells.

Our findings support very well the findings of Rounds and colleagues, who found uptake-dependent purine inhibition of pulmonary artery endothelial cell growth (Dawicki et al., 1997; Rounds et al., 1998); this work, as well as the finding by Peyot et al. (2000) of an apoptotic effect of ADO in arterial smooth muscle cells, is evidence that inhibition of cell growth by purine nucleosides is not limited to cells derived from smooth muscle.
tumors. The advantage that 1321N1 cells may have over other cell types or lines for studies of intracellular actions of purine nucleosides is that although most other cells express a variety of purine and/or pyrimidine receptors (including endothelial cells; Yang et al., 1996), 1321N1 cells appear to express at most a single subtype of P1 receptor, which mediates a relatively weak response by intracellular second messengers (Hughes and Harden, 1986; R. Nicholas, personal communication), and they express no nucleotide receptors at all. Thus, although the intracellular effects of purine nucleosides should be taken into account in future studies with 1321N1 cells as an expression system for purine receptors, our work also suggests that these cells might be an excellent model for the elucidation of the specific mechanisms associated with these effects.

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References


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