Immunocytochemical and Pharmacological Characterisation of P2-purinoceptor-mediated Cell Growth and Death in PC-3 Hormone Refractory Prostate Cancer Cells

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Abstract. Background: Extracellular nucleotides (e.g. adenosine 5'-triphosphate, ATP) influence biological processes via purinergic receptors. We characterised the P2-purinoceptors in human hormone refractory prostate cancer (HRPC) cells (PC-3 cells). Results: 1. Immunofluorescent staining demonstrated P2X3, P2X4, P2X5, P2X7 and P2Y2 receptors. 2. ATP inhibited cell growth by up to 91% after 72h. Pharmacological characterisation indicated a P2X-purinoceptor-mediated response. 3. Comparable maximum growth inhibition was seen after either a single addition of 1mM or daily addition of 100mM ATP. ATP concentrations ([ATP]) returned to baseline levels within 24h if the initial [ATP] was ≤100 μM, while [ATP] remained high for 72h if a single concentration of 1mM was used. 4. ATP 1mM significantly (p<0.001) increased the proportion of cells undergoing apoptosis from 0.27% (± 0.04%) to 5.28% (± 0.77%). Conclusion: Threshold concentrations of ATP inhibited HRPC cell growth in vitro via the activation of P2X-purinoceptors. The role of nucleotides in the treatment of HRPC requires further investigation.

The prostate gland is vital for normal male reproductive function, but is responsible for much morbidity and mortality, particularly in later life. Adenocarcinoma of the prostate is the second most common malignancy in European and North American men. More than 100,000 new cases and 35,000 deaths per annum are reported in the European Union (1). About 20% of patients present with metastatic disease and approximately 50% of patients initially treated for localised disease ultimately fail treatment (2). Relapse with hormone refractory prostate cancer (HRPC) has a poor prognosis with a mean survival time between nine and twelve months. At present there is no effective treatment for HRPC and the search for new therapies continues.

The extracellular signalling molecule, adenosine 5'-triphosphate (ATP) mediates a variety of biological functions including synaptic neurotransmission, smooth muscle contraction or relaxation, and exocrine or endocrine secretion (3). There is now increasing awareness that ATP, acting through specific receptors (P2-purinoceptors) can have trophic actions on a variety of cells including induction of cell proliferation, differentiation, migration and death (4).

Fang et al. first demonstrated that ATP can inhibit the growth of prostate cancer cell lines in vitro (5). Using pharmacological characterisation with various extracellular nucleotides, they concluded that this response was likely to be mediated by P2-purinoceptors. Further studies by Janssens and Boeynaems found that this ATP-induced growth inhibition may, in part, be due to reduced proliferation, although this effect was delayed and not seen until after 4 days of ATP treatment (6). Qualitative assessment also implied the induction of apoptosis in ATP-treated cells.

*Both R.C. Calvert and M. Shabbir made an equal contribution to the experimental and written aspects of this study.

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The P2-purinoceptor family is subdivided into P2X receptors, which are ligand-gated ion channels (P2X<sub>1,7</sub>), and P2Y receptors which are coupled to G-proteins (P2Y<sub>1,2,4,6,11,12,13,14</sub>) (7). Using reverse transcriptase-polymerase chain reaction (RT-PCR), Janssens and Boeyeamae found RNA for P2X<sub>4</sub>, P2X<sub>5</sub> and P2X<sub>7</sub> purinoceptors in the PC-3 cell line. Using Northern blotting, they also found P2Y<sub>2</sub>, P2Y<sub>6</sub> and P2Y<sub>11</sub> purinoceptor RNA in the same cell line.

In our study, we used immunocytochemical techniques to investigate which of these purinoceptor RNAs were expressed as receptor proteins. Using various purinergic agonists and, for the first time, antagonists, we undertook the pharmacological characterisation of these receptor subtypes, to assess which were functionally involved in growth inhibition. Using cell death detection kits (TUNEL), we quantitatively assessed the contribution of apoptosis to the observed growth inhibition. We also assessed the breakdown of ATP in culture medium using luciferin-luciferase, to help further ascertain its mechanism of action and optimal method of administration.

Materials and Methods

Cell culture. The HRPC cell-line, PC-3, was obtained from the European Collection of Cell Cultures (Salisbury, Wiltz, UK). Cells were grown in Minimum Essential Medium supplemented with 7.5% foetal calf serum (GIBCO-BRL) and 0.5% gentamycin and 0.5% ampicillin (Sigma, Dorset, UK) in a humidified atmosphere containing 5% CO<sub>2</sub> at 37°C.

Immunocytochemistry. Immunocytochemistry was performed on PC-3 cell cultures using a Cy-3 red fluorescent stain as follows: Cells were grown on permnox chamber slides. Cells were fixed for 10 min in 4% formaldehyde in 0.1M phosphate-buffered saline (PBS). After three washes with PBS, the slides were incubated for 40 min at room temperature in 10% normal horse serum (NHS) in PBS + 0.05% mercaptoethanol (Sigma, Poole, UK). Next, the slides were incubated overnight in primary antibody diluted 1:200 in 10% NHS in PBS + mercaptoethanol at room temperature. The following day, the slides were washed and incubated for 1 h at room temperature in donkey anti-rabbit immunoglobulin Cy-3 (Jackson Immunoresearch, Luton, UK) diluted 1:300 in 1% NHS in PBS/mercaptoethanol. Following further washing, the slides were mounted under coverslips in a glycerol mountant (Citifluor Ltd, London, UK) and viewed using a Zeiss Axiosplan (Germany) microscope with a dark field linked to a Leica DC200 digital camera (Switzerland).

Primary antibodies against P2X<sub>1,7</sub> and P2Y<sub>1,2,4</sub> receptors were used. The anti-P2X antibodies were obtained through collaboration with Roche Bioscience (Palo Alto, CA, USA) and prepared according to methods described by Lee et al. (8). Antibodies to P2Y<sub>1,2,4</sub> were obtained from Alomone Labs (Jerusalem, Israel). Controls were performed by omitting the primary antibody and also by pre-absorbing the primary antibody using the corresponding cognate peptides.

Measuring the effect of P2-agonists and antagonists on cell growth. PC-3 cells were seeded at a density of 50,000 cells/ml in 24-well multi-well plates. After 24h, the number of viable cells in control wells was recorded using the haemocytometer method, counting trypan blue excluding cells. ATP in increasing concentrations (10 nM, 100 nM, 1 µM, 10 µM, 100 µM, 1 mM and 5 mM: n=6 for each concentration) was added to different wells. At 96h from seeding (i.e. after 72h of incubation with ATP), cell viability was again assessed using the haemocytometer and trypan blue exclusion. The effect of a single and daily addition of ATP was also compared. Experiments were repeated in the presence of the following antagonists (n=6 per antagonist): 30 µM 8-thiophenyltheophylline (P1-receptor antagonist), 100 µM suramin and 100 mM PPADS (non-specific P2-receptor antagonists). Experiments were also repeated using the agonists adenosine 5'-diphosphate (ADP), uridine triphosphate (UTP), uridine diphosphate (UDP) and α,β-methylene adenosine 5'-triphosphate (αβmATP) in place of ATP (n=6 per agonist).

Measuring the ATP breakdown in the cell culture medium. Cells were incubated with ATP 10 µM, 100 µM, 1 mM and control (n=6 per concentration); 15 min after the application of ATP, and daily thereafter, 50 ml samples of medium were pipetted off each well taking care not to remove or disturb any cells. The samples were immediately frozen at minus 20°C to allow all samples to be analysed in one batch. The ATP concentration was assessed using the luciferin-luciferase assay. Samples and ATP standards were pipetted into white, non-phosphorescent 96-well plates. Plates were analysed in a luminometer (Luminex 750, Anthos Labtec, Salzburg, Austria) after addition of 100 µl luciferin-luciferase reagent (ATP monitoring reagent, Bio-Orbit, Turku, Finland) into each well. The ATP concentrations were calculated from a calibration curve constructed from ATP standards. The detection limit was approximately 5 fmol per sample.

Measuring ATP-mediated apoptosis in PC-3 prostate cancer cells. PC-3 cells were grown on coverslips in wells and incubated with 1mM ATP or control (n=5 for each). After a further 72 h, the cells were assessed using the in situ cell death detection kit stain (TUNEL, Roche) applied to the coverslips according to the manufacturer's protocol. This immunofluorescence marker detects apoptosis by staining cells with DNA strand breaks. Cells were then examined using microscopy to determine the extent of apoptosis and to compare the extent of apoptosis in the two groups. Cells were then photographed and the proportion of cells with positive staining was counted in 5 representative high-powered fields on each coverslip.

Statistical analysis. Cumulative response curves were plotted and compared using a 2-way analysis of variance (2-way ANOVA). The mean proportions of apoptotic cells in the ATP and control groups were compared with a Student's t-test. The hypotheses were rejected if p<0.05. Prism V2.0 (GraphPad Software Inc) statistical software was used for the calculations.

Results

Immunofluorescent localisation. The PC-3 prostate cancer cells showed immunoreactivity for P2X<sub>3,4,5,7</sub> and P2Y<sub>2</sub> whereas P2X<sub>1,2,6</sub> and P2Y<sub>1</sub> and 4 receptors were not detected. Negative controls, either omitting or pre-absorbing the primary antibody, substantially reduced the intensity of staining (Figure 1).
The effects of P2-agonists and antagonists on prostate cancer cell growth. At 24h the number of viable cells had increased to 60,000 cells/ml in control wells. After a further 72h, the cell numbers in control wells increased to 206,000 cells/ml (i.e. the cell doubling-time was approximately 36h). This increase in cell numbers was inhibited by 88% (±2%) by daily application of 100 mM ATP (Figure 2a). This growth inhibition was concentration-dependent. Single addition of 100 mM ATP inhibited cell growth by only 45% (±1%) (see Figure 2b). However, the single addition of 1mM ATP inhibited cell growth by 91% (±1%). The addition of the P1 antagonist 8-thiophenyltheophylline had no effect on ATP-mediated growth inhibition. The addition of the P2 antagonists PPADS (p=0.01) and suramin (p=0.02) partially inhibited the anti-neoplastic action of ATP (see Figure 2c). The P2Y agonists UDP and UTP had no significant anti-neoplastic effect (Figure 2c).

ATP breakdown in the culture medium. Figure 3 demonstrates the concentration of ATP in the cell culture medium at daily intervals following the application of ATP. The baseline concentration of ATP was 60 nM. At 15 mins the ATP concentration was not significantly different to the anticipated dose added at time=0. After 24h, the concentration of ATP was no different to baseline except in the wells which had 1 mM ATP added initially. In these wells, the concentration of ATP remained above 100 μM at 48h and above 10 μM at 72h.

Induction of apoptosis in PC-3 prostate cancer cells by ATP. The mean proportion of control cells that exhibited positive staining for apoptosis was 0.27% (±0.04%). After incubation with 1mM ATP, the mean proportion of cells which exhibited positive staining for apoptosis was 5.28% (±0.77%) (Table 1). This difference was highly significant (Student's t-test p<0.001).

Discussion

At present, the only treatment option for prostate cancer once it escapes hormonal control is palliative care. Previous studies have already implied an anti-neoplastic action for ATP in PC-3 hormone refractory prostate cancer cells acting via P2-purinoceptors (5). RT-PCR and Northern blotting has identified the presence of different P2-purinoceptor subtype mRNA (P2X4, P2X7, P2X7 and P2Y2, P2Y6, P2Y11) (6). The aim of this study was to help further define the role of purinoceptors in prostate cancer and develop the potential use of ATP as a novel therapeutic agent for the treatment of advanced hormone refractory disease.

Using immunocytochemical techniques, we found that PC-3 cells express P2X3, P2X4, P2X5, P2X7 and P2Y2 receptor proteins. Using pharmacological characterisation, we were able to assess which of these receptors were functionally active in the inhibition of cell growth.

The P1-agonist 8-thiophenyltheophylline had no effect on the ATP-induced response, showing that ATP was not acting via its breakdown product, adenosine. However, the P2-receptor antagonists, PPADS and suramin, reversed ATP-mediated growth inhibition, confirming both a key role for P2-purinoceptors and excluding the involvement of the P2X4 receptor in the control of cell growth, since these antagonists are ineffective at this receptor (9). The P2X3 and P2X7 receptor agonist, αβmeATP, had no significant effect on cancer cell growth, excluding any functional role for these receptor subtypes. The P2Y agonists UTP (P2Y2, P2Y4) and UDP (P2Y6) exerted no anti-neoplastic effect, excluding any role for these receptor subtypes in the observed growth inhibition. These findings suggest the anti-neoplastic action of ATP is mediated via P2X5, P2X7 and/or P2Y11 receptor subtypes. Further pharmacological characterisation is difficult at present due to the paucity of effective selective agonists and antagonists for these receptor subtypes.

Throughout this study the term "growth" has been used to represent the increase in number of viable cells during the period of the study. A change in overall viable cell number is dependent on three factors:
1. Cell proliferation
2. Cell death due to apoptosis
3. Cell differentiation (leading to inhibition of cell proliferation)

Chemotherapy for advanced prostate cancer has not been as effective as expected, partly due to the low fraction of cells in the tumour undergoing active proliferation. The ideal chemotherapeutic agent should therefore have significant pro-apoptotic activity as well as being anti-proliferative. ATP has previously been shown to inhibit the proliferation of PC-3 cells in vitro, although this was noted only after 4 days of treatment (6). Using the TUNEL in situ cell death detection kit in this study, we were able to quantify ATP-induced apoptosis. We found a significant increase in apoptosis after 72h (% apoptotic cells 0.27% ± 0.04% control vs. 5.28% ± 0.77% after incubation with 1mM ATP). This method of apoptosis detection is likely to give an underestimate due to the reduced adherence of dying cells to the coverslip, resulting in some apoptotic cells being lost during the staining procedure.

Although P2Y receptors have been shown to play an important role in mediating the proliferating effects of ATP in other cell types (10,11), we found no role in the observed anti-neoplastic response in PC-3 prostate cancer cells. P2X receptors are largely viewed as mediators of short-term, fast cell-cell communication (3). However, recent studies have demonstrated an apoptotic role for P2X7 receptors (12,13) and potential differentiating roles for P2X4 receptors (14,15) in cancer.
for P2X₅ receptors (11,14), suggesting that P2X receptors could also mediate trophic effects. Ryten et al. demonstrated that the activation of P2X₅ receptors mediated the stimulation of cell differentiation markers and thereby inhibited proliferation in skeletal muscle cells (15). This study has identified the presence of functionally active P2X₅ and P2X₇ receptor subtypes, which may inhibit the growth of hormone refractory prostate cancer cells via a combination of apoptosis induction and increased cellular differentiation resulting in reduction of proliferation. The assessment of differentiation using cellular markers would help define the contribution of this process to the overall reduction in viable cell number.

The final aspect of this study was to assess the bioavailability and best method of administrating ATP. We found that the effect of ATP (100 μM) was enhanced when added daily to the cell culture rather than as a single dose. However, if a single high concentration of ATP was used (1 mM), the cytotoxic effect was equivalent to adding ATP 100 μM daily for 3 days. Analysis of ATP breakdown showed that when ATP was added at a concentration of 100 mM or less, the concentration of ATP in the culture medium had reached background levels before 24h. When 1 mM of ATP was added, the ATP concentration in the medium was maintained well above background concentrations for at least 72h and this was above the EC₅₀ for growth inhibition for more than 48h. These
Figure 2. a) The effect of the daily addition of 100 μM ATP on the growth of PC-3 prostate cancer cells (2-way ANOVA p<0.001).

b) The daily addition of 100 μM ATP inhibits the PC-3 prostate cancer cell growth more than a single addition of 100 mM, but the single addition of 1 mM ATP produces the maximum effect.

c) ATP-mediated growth inhibition of PC-3 prostate cancer cells is not affected by the P1-receptor antagonist thiophenylethylamine (SPT), but reversed by the P2-receptor agonists PPADS and suramin. Uridine triphosphate (UTP) and α,β-methylene adenosine 5'-triphosphate (α,βmP) had little effect. All concentrations were all 100 μM.
The results suggest that the ATP concentration needs to achieve a minimal threshold level, which must be sustained for a short time (<24h) in order to successfully trigger growth inhibition. It is not clear whether ATP breakdown is inhibited at higher concentrations or whether there is increased release of ATP, either due to cell death or due to ATP-induced ATP release from the PC-3 cells, comparable to that reported in red blood cells (16) and endothelial cells (17). Rapaport and Fontain demonstrated ATP-induced ATP release from erythrocytes in mouse tumour models, which enhanced the observed ATP-mediated anticancer effect (18). Calculation of a ‘triggering’ dose, which may lead to further endogenous ATP-induced ATP release, may hold the key to the best method of administration of this compound in the treatment of cancer.

In this preliminary study, we immunocytochemically and pharmacologically characterised the P2-purinoceptor subtypes in the PC-3 hormone refractory prostate cancer cell line. We demonstrated that ATP induces cell death by apoptosis and that this is likely to be mediated by P2X receptor subtypes. Furthermore, analysis of ATP breakdown in culture medium provides evidence of a threshold concentration of ATP which must be achieved in order to cause maximal cell death and also raises the possibility of endogenous ATP-induced ATP release. Further exploitation of this finding may help clarify the best method of ATP administration. Intravenous infusions of ATP have already been tested in patients with advanced malignancy. A randomised control trial demonstrated that ATP has beneficial effects on weight, muscle strength and quality of life in patients with advanced non-small cell lung cancer (19).

The results of our study help further define the role of ATP and P2-purinoceptors in the control of cell growth in HRPC. The identification and characterisation of pharmacological targets is a vital process in the development of new treatments in cancer therapy. With further clarification of the purinergic control of prostate cancer cell growth, the potential for developing a successful therapeutic agent for HRPC will be one step closer.

References


