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Adenosine acts as an inhibitor of lymphoma cell growth: a major role for the A3 adenosine receptor

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Abstract

In this study, we demonstrated several mechanisms exploring the inhibitory effect of low-dose adenosine on lymphoma cell growth. Adenosine, a purine nucleoside present in plasma and other extracellular fluids, acts as a regulatory molecule, by binding to G-protein associated cell-surface receptors, A1, A2 and A3. Recently we showed that low-dose adenosine released by muscle cells, inhibits tumour cell growth and thus attributes to the rarity of muscle metastases. In the present work, a cytostatic effect of adenosine on the proliferation of the Nb2-11C rat lymphoma cell line was demonstrated. This effect was mediated through the induction of cell cycle arrest in the G0/G1 phase and by decreasing the telomeric signal in these cells. Adenosine was found to exert its antiproliferative effect mainly through binding to its A3 receptor. The cytostatic anticancer activity, mediated through the A3 adenosine receptor, turns it into a potential target for the development of anticancer therapies.

Keywords: Adenosine; Lymphoma cells; Adenosine receptors; Cell cycle; Telomeric DNA

1. Introduction

Adenosine, a ubiquitous nucleoside, is released into the extracellular environment from metabolically active or stressed cells. It is known to act as an important regulatory molecule through its binding to specific G-protein-associated A1, A2 and A3 cell surface receptors [1–3]. The interaction of adenosine with its receptors initiates signal transduction pathways, including the adenylate cyclase effector system, which utilises cAMP as a second messenger. While the A1 and A3 receptors, coupled with Gi proteins, inhibit adenylate cyclase and lead to a decreased intracellular cAMP, the A2 receptors, coupled to G proteins activate adenylate cyclase, thereby increasing cAMP levels [4]. It has been demonstrated that adenosine is capable of inducing proliferation in a wide range of normal cell types [5–7] whilst inhibiting the growth of some tumour cell lines [8–10].

Since specific surface receptors for adenosine are found in nearly all cells, almost every organ system is regulated by its release. Adenosine induces a cardioprotective effect by regulating the electrophysiological properties of the heart, acts as a neuroprotective agent through the sedation and suppression of the release of neurotransmitters and regulates renin production and vascular tone in the kidney [11–14]. In the immune system, adenosine exerts various effects, including anti-inflammatory activity through the inhibition of cytokine production, inhibition of platelet aggregation, induction of erythropoietin production and modulation of lymphocyte function [10,15–18].

Recently we demonstrated that low-dose adenosine and other small molecules released by muscle cells, inhibit tumour cell growth and thus attribute to the rarity of muscle metastases [19,20]. This finding led us to explore further the molecular mechanisms involved in the inhibition of tumour cell growth by adenosine.

The aim of this study was to investigate the effect of adenosine on lymphoma cell growth and to explore the adenosine receptor through which this activity is mediated.

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2. Materials and methods

2.1. Drugs

All drugs were purchased from Sigma Chemical Co. St Louis, MO, USA excluding the A2 and A3 adenosine agonists and the A3 adenosine antagonist which were ordered from RBI Massachusetts, USA.

Adenosine was dissolved in water and kept as a stock solution at a concentration of 1 mg/ml. Dilutions in RPMI medium were carried out and final concentrations of 5, 10, 25 and 50 μM were used. The following antagonists to adenosine receptors were used: 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), an adenosine A1 receptor antagonist; 3,7-dimethyl-1-propargyl-xanthine (DMPX), an A2 receptor antagonist; 9-chloro-2-(2-furanyl)-5-[(phenylacetyl)amino] [1,2,4,] -triazolo[1,5-c] quinazoline (MRS-1220), an adenosine A3 receptor antagonist.

Three selective adenosine agonists were used: 2-chloro-N6-cyclopentyladenosine (CCPA), an A1 receptor agonist; N^6-[2-(3,5-Dimethoxyphenyl)-2-(2-methylphenyl)-ethyladenosine (DMPA), an A2 receptor agonist and 1-Deoxy-1-[6-[[(3-iodophenyl)methyl]amino]-9H-purine-9-yl]-N-methyl-β-D-ribofuranuronamide (IB-MECA), an A3 receptor agonist.

2.2. Cell proliferation assays

2.2.1. Cell count assay

The effect of adenosine on the proliferation of rat Nb2-11C lymphoma cells was monitored by cell counting. 1.2×10^5 cells/ml were cultured in 24-well plates in 1 ml RPMI medium containing 5% fetal bovine serum (FBS, Biological Industries, Beit Haemek, Israel) and various concentrations of adenosine were added. The cultures were incubated at 37°C at 5% CO_2 and cells were counted after 48 h in a Coulter Counter. Cell viability was evaluated by staining with trypan blue in a Neubauer Camera.

2.2.2. [^{3}H]-Thymidine incorporation assay

The rat Nb2-11C lymphoma cell line was used in all the experiments [21]. Cells (1.2×10^4/well) were incubated in triplicate with RPMI medium containing 5% FBS in 96-well microtitre plates for 48 h. To explore the specific receptor through which adenosine exerts its activity, adenosine antagonists (DPCPX, DMPX and MRS-1220) at concentrations of 0.1, 0.05 and 0.001 μM, were introduced to cultures of cells in the absence and presence of 25 μM adenosine. Agonists to the A1, A2 and A3 adenosine receptors (CCPA, DMPA and IB-MECA, respectively), at concentrations of 0.1, 0.05, 0.01 and 0.005 μM were added to Nb2-11C cultures in the absence of adenosine. During the last 18 h of incubation, each well was pulsed with 1 μCi [^{3}H]-thymidine. Cells were harvested and the [^{3}H]-thymidine uptake was determined in an LKB liquid scintillation counter (LKB, Piscataway, NJ, USA). Each sample was analysed in triplicate.

Results are expressed as per cent of cell proliferation inhibition calculated according to the following:

$$\%\ \text{of inhibition} = 100 - \frac{A \times 100}{B}$$

where A = cell count of sample; B = cell count of control. According to this calculation control values will be 0% of inhibition.

2.3. Cell cycle analysis

Flow cytometric analysis of the cell cycle of Nb2-11C cells was carried out by propidium iodide staining according to Krishan [22]. Cells, at a concentration of 1.2×10^5/ml, were cultured in RPMI medium supplemented with 5% FBS in the presence of adenosine at a concentration of 25 μM for 24 h at 37°C in a CO_2 incubator. Cells cultured in RPMI supplemented with 5% FBS served as control. At the end of the incubation period, the cells were washed three times with PBS and resuspended in staining buffer containing 0.1% bovine serum albumin (BSA), 50 μg/ml propidium iodide, 0.1% Triton X-100 and 1 mg/ml RNase (boiled for 10 min). Samples were examined after 30 min of staining on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA). Results are expressed as per cent of control (each phase of the cell cycle in the sample is compared with the relative phase in the control).

2.4. Detection of apoptosis by acridine orange staining

Acridine orange staining was performed according to Hare and Bahler [23]. Cytospin preparations were made directly on slides from Nb2-11C cells treated with 50 and 25 μM adenosine. The slides were fixed with 100% ethanol for 10 min. Acridine orange at 1.2 μg/ml was dissolved in citrate/EDTA buffer (0.13 M Na_2HPO_4, 0.35 M citric acid and 1 μM Na_2EDTA, pH 6.5) and applied on the slides for 30 min. Cells (100 per sample) were examined and counted under a fluorescence microscope (Olympus BH-2). Results were expressed as mean±S.D. apoptotic cells in the sample.

2.5. Telomeric signal analysis

Nb2-11C cells were incubated in the presence of 25 μM of adenosine for 48 h. Thirty min prior to harvest the cells were treated with colcemid (final concentration, 0.04 μg/ml, GIBCO BRL). At the end of the incubation period, the culture content was centrifuged and the cell pellet was treated with hypotonic solution (KCl, 0.06

M) for 20 min, and then fixed in a mixture of acetic acid and methanol (1:3 v/v). Slides were prepared following the standard air-drying procedure [24].

Cytological preparations from the control as well as treated cultures were used for fluorescence *in situ* hybridisation (FISH) experiments to evaluate the amount of telomeric signals in cells.

For FISH analysis, the cytological preparations were hybridised with a biotin-labelled all human telomeric DNA probe according to the manufacturer's protocol (Oncor, Inc., Gaithersburg, MD, USA) with slight modifications [25]. All slides were coded and analysed using a fluorescence microscope (Nikon, Mellville, NY, USA) equipped with FITC and PI filters (Chroa Technology Corp., Brattleboro, VT, USA). A minimum of 200 interphase nuclei were analysed.

The per cent telomeric area in the interphase nuclei in FISH preparations was quantified by using a software package (Metaview Imaging system version 3.6a, Universal Imaging Co., Westchester, PA, USA). From each sample, at least 50 interphase nuclei were quantified and the mean value of per cent telomeric area compared with the total nuclear area was calculated. The values of the treated groups were compared with those of control after decoding of the slides.

2.6. Statistical analysis

Each experiment was performed five times. Statistical analysis of data was carried out using the Student *t*-test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Adenosine exerts a cytostatic antiproliferative effect on Nb2-11C lymphoma cells

When added to Nb2-11C lymphoma cell cultures, adenosine produced a dose-dependent inhibitory effect on cell count (Table 1) and [^{3}H]-thymidine incorporation (Fig. 1). The maximal inhibitory effect was at a concentration of 50 μM adenosine. Part of this inhibitory activity may be attributed to the apoptosis exerted by this high adenosine concentration since a 12.8%±2.2 increase in the number of apoptotic cells, observed by acridine orange staining and 15% decrease compared with the control in the viability of the cells was revealed by trypan blue staining. However, at a lower adenosine concentration of 25 μM, an inhibition of 32%±2.7 in cell proliferation was observed, which was not followed by apoptosis. At this adenosine concentration (25 μM), cell cycle analysis revealed a shift of the cell population from the S and G2 phases to G0/G1 (Fig. 2). These results demonstrated that low-dose adenosine inhibits lymphoma cell growth through a cytostatic rather than an apoptotic pathway.

Table 1
Effect of various concentrations of adenosine on the growth of Nb2-11C lymphoma cells as measured by cell count and viability[a]

	Cell number×10^5/ml	% of viability[b]
Control	2.72±0.18	90±6.3
Adenosine 50 μM	1.63±0.12	75±5.7
Adenosine 25 μM	1.96±0.17	86±7.1
Adenosine 10 μM	2.12±0.19	91±9.5
Adenosine 5 μM	2.31±0.15	88±8.3

[a] A dose-dependent inhibition of lymphoma cell growth was observed with a statistically significant decline in cell viability only at a concentration of 50 μM of adenosine.

[b] Assessed by trypan blue exclusion assay.

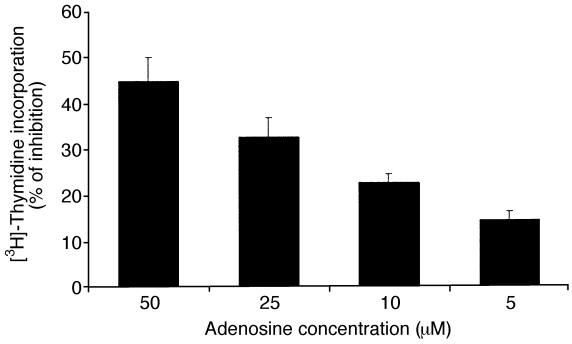

Fig. 1. Adenosine, at different concentrations, inhibits Nb2-11C cell proliferation, measured by [^{3}H]-thymidine incorporation assay.

To evaluate the effect of adenosine on the telomeric DNA of the Nb2-11C cell line, the cells were treated with 25 μM adenosine for 48 h. Quantitation of the telomeric signals in adenosine-treated cytological preparations showed a significant ($P < 0.01$) reduction in the per cent telomeric area compared with the control

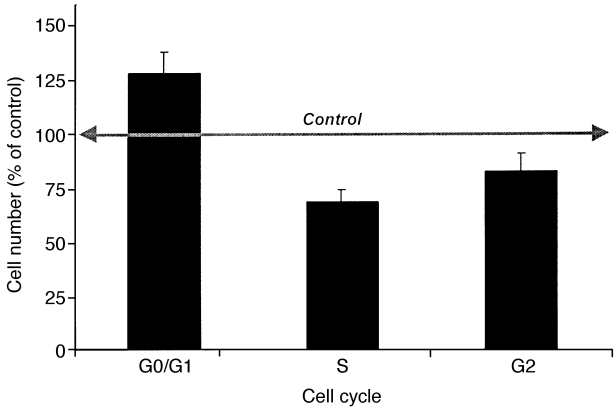

Fig. 2. Adenosine induces a cytostatic effect on lymphoma cell growth. Flow cytometric analysis of the cell cycle status of Nb2-11C lymphoma cells following incubation with 25 μM adenosine. Distribution of the cells at the different phases of the cell cycle is specified. An increase in the number of cells in the Go/G1 phase is seen.

(0.96±0.09 versus 1.45±0.09, respectively), as demonstrated in Fig. 3.

3.2. The inhibitory effect of adenosine is mediated through purinergic receptors

To find the adenosine receptor responsible for the inhibitory effect, a pharmacological profile based on the cell response to adenosine receptor antagonists and agonists was carried out. Three adenosine receptor antagonists were used: DPCPX, A1 antagonist; DMPX, A2 antagonist; MRS-1220, A3 receptor antagonist. There was no difference between the results obtained with the various concentrations of the antagonists. Therefore, the following data represent results using a concentration of 0.1 μM of each antagonist.

The antagonists themselves, in the absence of adenosine, had no inhibitory effect on lymphoma cell proliferation. In the presence of adenosine, DPCPX (αA1) did not have any effect on cell growth. DMPX (αA2) decreased, whilst MRS-1220 (αA3) abolished the inhibitory effect of adenosine on Nb2-11C cell proliferation ($P < 0.05$, $P < 0.001$, respectively) (Fig. 4). These findings indicate that the A3 receptor has a prominent role in the inhibition of cell proliferation by adenosine and a minor effect can be attributed to the A2 receptor.

To confirm these results, the agonists CCPA, DMPA and IB-MECA at various concentrations were added to a culture of Nb2-11C cells. At concentrations lower than 0.1 μM, only IB-MECA exerted an inhibitory effect, whilst the other two agonists were not active. Using 0.1 μM of each agonist, a mirror image to that obtained with the antagonists was seen (Fig. 5). CCPA failed to inhibit cell proliferation, indicating that the A1 receptor is not involved in this activity. However, IB-MECA mimicked the effect of adenosine and induced a statistically significant inhibitory effect on tumour cell growth ($P < 0.001$). DMPA had a less inhibitory effect ($P < 0.03$). A dose-dependent inhibitory effect of IB-MECA on Nb2-11C cell proliferation was observed (Fig. 6). IB-MECA or DMPA (at all concentrations that were examined) did not exert an apoptotic effect (as examined by acridine orange staining) on the Nb2-11C cells.

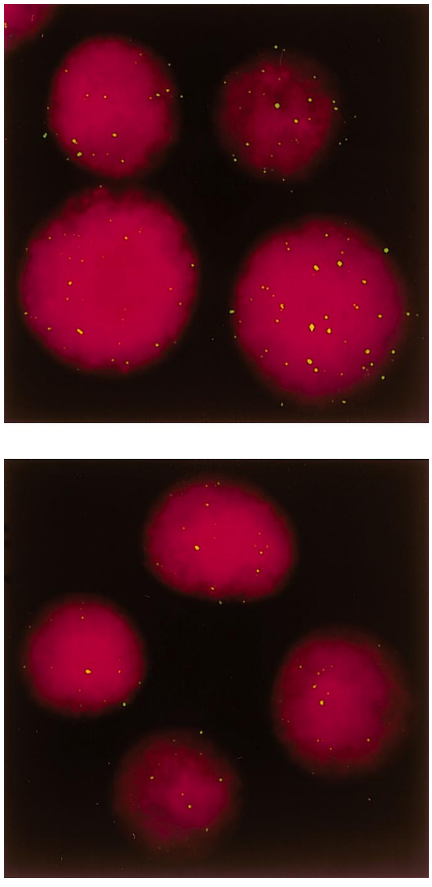

Fig. 3. Adenosine reduces the telomeric signal in Nb2-11C lymphoma cells. FISH preparations with telomeric DNA in Nb2-11C lymphoma cells treated with 25 μM adenosine. Note more signals in the upper panel (control) compared with the lower panel (treated).

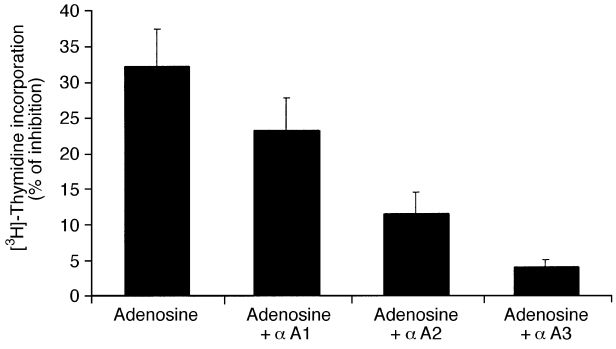

Fig. 4. Effect of adenosine receptor antagonists on Nb2-11C cell proliferation in the presence of adenosine (25 μM). Cell proliferation was measured by [3H]-thymidine incorporation assay.

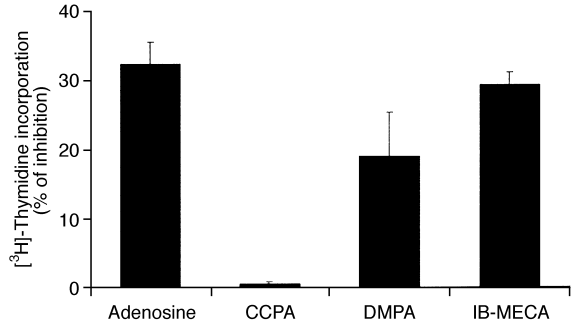

Fig. 5. Effect of CCPA, DMPA and IB-MECA (adenosine A1, A2 and A3 receptor agonists, respectively) on the proliferation of Nb2-11C lymphoma cells. Cell proliferation was measured by [3H]-thymidine incorporation assay.

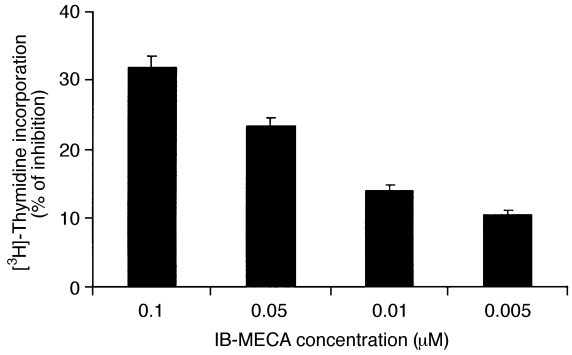

Fig. 6. Dose-dependent inhibitory effect of IB-MECA (A3 adenosine receptor agonist) on the proliferation of Nb2-11C cells. Cell proliferation was measured by [^{3}H]-thymidine incorporation assay.

4. Discussion

The results of the present study show that extracellular adenosine, interacting with specific cell surface receptors, exerted a dose-dependent cytostatic effect on Nb2-11C lymphoma cells *in vitro*.

Adenosine induced cell proliferation inhibition as was observed by [^{3}H]-thymidine incorporation and cell count assay. The inhibitory activity of adenosine on lymphoma cell growth was reversed by MRS-1220 and partially by DMPX (adenosine A3 and A2 receptor antagonists, respectively). Moreover, adenosine could be mimicked by IB-MECA and DMPA (adenosine A3 and A2 receptor agonists, respectively). Both antagonists reversed the inhibitory effect that was exerted by adenosine, although MRS-1220 induced a more pronounced inhibitory effect. This effect could be explained by either a different affinity of the antagonists to the receptors, or the presence of more A3 adenosine receptors on the cell surface of the Nb2-11C cells. Support for the latter, is given by the results with the agonists, showing a dose-dependent inhibitory effect by IB-MECA alone. Thus, it can be concluded that the inhibitory effect of adenosine on Nb2-11C cells was mediated through the A3, and to a lesser extent the A2, cell surface receptors.

An increase in cAMP level has been shown to inhibit the proliferation of various tumour cell lines including the Nb2-11C cells [21,26–29]. Since both the A2 and A3 receptors were found to be involved in the inhibition of lymphoma cell growth, and each is responsible for either increasing or decreasing cAMP level, we hypothesised that tumour proliferation inhibition is mediated through different signal transduction pathways.

Adenosine (5–25 μM) exerted a dose-dependent inhibitory effect on lymphoma cell growth, while at a higher dose (50 μM), apoptosis was observed. At lower doses, adenosine induced cell cycle arrest in the G0/G1, with a decreased number of cells in the S and G2 phases. Moreover, a lower telomeric signal was noticed following incubation with low-dose adenosine. Telomeres are repeated DNA sequences that guard the ends of chromosomes, serving as a checkpoint for cell cycle progression and regulate cell senescense and apoptosis. We have demonstrated earlier that amplification of telomeres is related to the invasive and metastatic potential of tumour cells whilst a decreased telomeric signal correlates well with cell cycle arrest and cell death [30,31].

The inhibition in [^{3}H]-thymidine incorporation, the decrease in cell count, the cell cycle arrest in the G0/G1 phase and the reduction of the telomeric signal suggests that adenosine acts as a cytostatic agent, thus preventing lymphoma cell growth.

The effect of high-dose (100–200 μM) adenosine on tumour cell growth has been shown to increase the rate of cell death rather than reduce the rate of cell division [32,33]. High-dose adenosine induced apoptosis in leukaemia HL-60, lymphoma U-937, GH3 tumour pituitary cell lines and in chronic lymphocytic leukaemia cells [10,33–35]. The active transport of adenosine into cells through the nucleoside transporters was found to be responsible for the apoptotic effect. Cell surface adenosine receptors were not involved in this activity, since neither the antagonists nor the agonists were able to reverse or mimic, respectively, the apoptotic effect of adenosine. However, agents that inhibit adenosine uptake through adenosine transporters, such as dipyridamole, completely blocked its inhibitory effect. In contrast, the cytostatic effect obtained in this study using low-dose adenosine, resulted from the activation of adenosine cell surface receptors, mainly A3. Moreover, we showed that IB-MECA or DMPA, at low concentrations, inhibited cell proliferation, but not through an apoptotic pathway, thus acting similarly to adenosine.

Yao and colleagues [36] demonstrated that apoptosis of HL-60 and U-937 cells was induced by high concentrations of either antagonists or agonists to the A3 adenosine receptor mediated via the A3 receptor. In contrast, in this report, in the present study we did not observe an apoptotic effect in the low range of concentrations used, neither by adenosine nor by its A3 receptor agonist IB-MECA.

Taken together, it can be concluded that low-dose adenosine or its A3 receptor agonist, led to lymphoma cell proliferation inhibition through a cytostatic pathway. This mechanism is different from that of high adenosine concentrations, or its A3 receptor agonist/antagonist, which are known to induce apoptotic cell death.

A3 adenosine receptor activation has been found to mediate important cytoprotective functions, i.e. a cerebroprotective activity following chronic administration of IB-MECA to gerbils with cerebral ischaemia [37]; a cardioprotective activity during prolonged simulated ischaemia through its capability to rescue injured

myocytes [38]; an anti-inflammatory effect [39]. Thus, through the A3 adenosine receptor both cytoprotection and cytostatic antitumour activities are mediated. The A3 adenosine receptor becomes a potential target for the development of anticancer therapies.

Acknowledgements

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