Short Communication

Effects of Adenosine on the Growth of Murine G:5:113 Fibrosarcoma Cells *in Vitro*

(G:5:113 fibrosarcoma cell line / cell growth / adenosine / dipyridamole)

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Abstract. It has been observed that adenosine suppresses the growth of G:5:113 murine fibrosarcoma cells *in vitro* with EC_{50} of 178 mM. Changes in the cell cycle including decreased percentage of cells in S-phase, increased portion of cells in G_0/G_1-phase, as well as prolonged generation time were found to be responsible for the growth suppression. Dipyridamole, a drug inhibiting the cellular uptake of adenosine, enhanced the growth suppression induced with adenosine in concentrations of 100 and 200 μM. It follows from these results that the action of adenosine on the G:5:113 cells is extracellular, mediated by adenosine receptors. Elevation of extracellular adenosine might serve potentially as an anticancer therapeutic agent.

Adenosine is an endogenous nucleoside, which has been demonstrated to modulate many biological functions including cell proliferation and death. Generally, adenosine is regarded to be a regulatory metabolite and an endogenous activator of defence mechanisms at various levels of biological systems. The effects of adenosine on a number of tumour and non-tumour cells have attracted the attention of many investigators, especially from the point of view of testing the potential antitumour effects of adenosine. In their studies, mostly inhibitory and/or proapoptotic effects of adenosine have been found (e.g., Tanaka et al., 1994 (experiments on leukaemia cells); Colquhoun and Newsholme, 1997 (leukaemia cells, breast tumour cells and lymphocytes); Fishman et al., 2000 (lymphoma cells); Brown et al., 2000 (normal and transformed keratinocytes); Schneider et al., 2001 (leukaemia cells); Dubey et al., 2001 (cardiac fibroblasts); Schrier et al., 2001 (neuroblastoma cells); Nucciarelli et al., 2003 (prostate and bladder carcinoma cells)). However, in some cell lines, stimulating action of adenosine on cell proliferation was observed (e.g., Lelievre et al., 1998a, b (various colonic adenocarcinoma cells); Dubey et al., 2002 (arterial endothelial cells)). Two different basic mechanisms have been found for the effects of adenosine on the functional status of cells. In some cell systems, adenosine acts predominantly through specific G protein-coupled adenosine receptors (for review see Abbracchio and Burnstock, 1998; Jacobson et al., 1998). This mechanism has been considered to be responsible for the adenosine action in studies by Colquhoun and Newsholme (1997), Lelievre et al. (1998a, b), Fishman et al. (2000), Schneider et al. (2001), and Dubey et al. (2001, 2002). The other mechanism consists in cellular uptake of adenosine and its intracellular action. The latter mechanism was found to act in studies by Brown et al. (2000), Schrier et al. (2001), and Nucciarelli et al. (2003). Dipyridamole prevents cellular uptake of adenosine (Thorn and Jarvis, 1996). It is generally accepted that the results of modulation of adenosine effects by dipyridamole can be taken as evidence of either receptor or intracellular adenosine action: in the presence of dipyridamole the intracellular action of adenosine can be ruled out. Thus, dipyridamole has been employed in this direction e.g. in studies by Brown et al. (2000), Schrier et al. (2001), and Nucciarelli et al. (2003) and in our experiments testing haematopoietic effects of adenosine (e.g., Pospíšil et al., 2001; Hofer et al., 2002).

Fibrosarcoma represents a tumour type that is often resistant to current non-surgical therapeutical procedures. The aim of the experiments presented here was to evaluate contingent modulatory effects of adenosine on the growth of the G:5:113 murine fibrosarcoma cell line and, by combining adenosine with dipyridamole, to contribute to the understanding of the mechanisms of these effects.

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Abbreviation: EC_{50} – effective concentration mediating 50% maximal response.

Material and Methods

G:5:113 cells, kindly provided by Dr. Margaret L. Kripke (University of Texas, M.D. Anderson Cancer Center, Houston, TX) (Donawho and Kripke, 1991) were cultivated at 37°C in 5% CO_2 atmosphere using RPMI 1640 medium (Sebak, Aidenbach, Germany) supplemented with 10% heat-inactivated foetal calf serum (FCS) (PAN Systems, Nürnberg, Germany), 100 µg/ml streptomycin (Gibco BRL, Paisley, Great Britain), 100 IU/ml penicillin (Gibco BRL) and 0.1 mg/ml gentamicin (PAN Systems) (more details in Hoferová et al., 2002, and Hoferová et al., in press) and taken for experiments during the exponential growth phase. The cells were harvested by trypsinization, resuspended in cultivation medium and seeded onto a dish at a density of 1.6 x 10^4 cells per cm^2. The drugs tested were added immediatelly after cell seeding. Adenosine (Sigma, St. Louis, MO) was employed at concentrations ranging from 0.5 µM to 500 µM. In experiments in which combined action of adenosine and dipyridamole was evaluated, adenosine in concentrations of 100, 200, and 300 µM and dipyridamole (Sigma) in concentrations of 5, 10, and 20 µM were used either individually or in combinations. All examinations were performed 48 h after adding the drugs. The cell number was determined using a Coulter counter (model ZM, Coulter Electronics, Luton, UK). The cell viability was evaluated microscopically by the 0.15% eosin exclusion assay as the percentage of viable (unstained) cells per 100 cells counted. The analysis of the cell cycle was performed by a FACScan flow-cytometer (Becton Dickinson, San Jose, CA) using methods described in detail elsewhere (Hoferová et al., 2002; Hoferová et al., in press). For estimation of the percentage of cells in each phase of the cell cycle (G_0/G_1, S and G_2/M) and the subG_0/G_1 population used for detection of apoptosis, histogram analyses of the relative DNA content were carried out with ModFit LT 2.0 software (VERITY, Sotware House, Inc., Topsham, ME). Cell generation time was calculated according to formulas $r = (3.32 \log X_n/X_0)/(t_n - t_0)$ and $g = 1/r$, where r is the multiplication rate or number of generations per unit time; X_n is the cell number per unit volume in the final population; X_0 means the cell number per unit volume in the inoculum; t_n is the time of the evaluation of the final population; t_0 is the time of the inoculation; and g is the doubling time of the population (Hayflick and Moorhead, 1961). All data are reported as means ± S.E.M. of two separate experiments, each performed in triplicate. For evaluation of inhibitory effects of adenosine on cell numbers, the EC_{50} value (effective concentration mediating 50% maximal response) were calculated using the formula $EC_{50} = (ln(2)/S)^{2/3}$, where S is the slope factor and $ln(2)$ is the natural logarithm 2. S was calculated from the formula $Y = Y_0 \, x \, exp(-S \, x \, X^{1.5})$, where Y is the number of viable cells at the agonist concentration X, and Y_0 is the number of viable cells at zero concentration. The equation was fit to raw data using CSS Statistica v.3.0 (Tulsa, OK). For statistical treatment of the data, the t-test with Holm's correction for multiple comparisons was used. The significance level was set at $P < 0.05$.

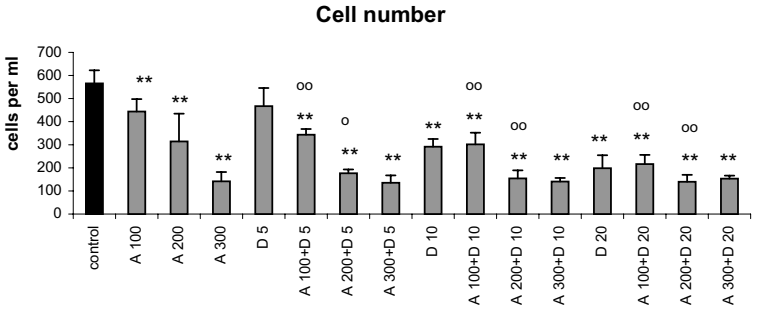

Fig. 1. Effect of adenosine and dipyridamole as single agents or in combination on the growth of G:5:113 cells after 48-h treatment concerning the cell number. A – adenosine; D – dipyridamole. The numbers at A and D denote concentrations (µM) of adenosine and dipyridamole tested. Statistical significance: *, ** - $P < 0.05$, $P < 0.01$, respectively, in comparison with controls; °, °° - $P < 0.05$, $P < 0.01$, respectively, in comparison with cultures treated only with the respective concentration of adenosine alone.

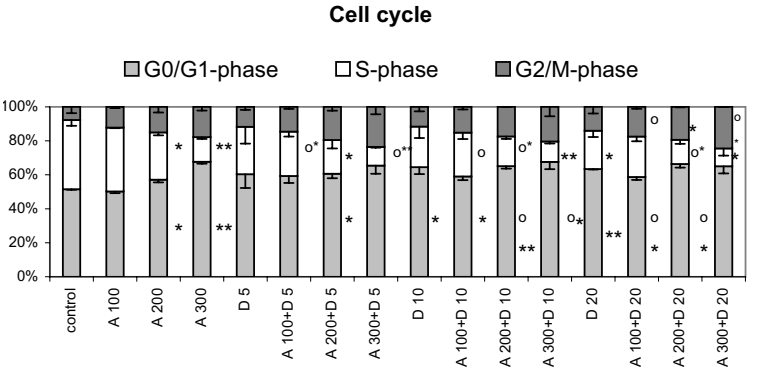

Fig. 2. Effect of adenosine and dipyridamole as single agents or in combination on the growth of G:5:113 cells after 48-h treatment concerning the cell cycle parameters. A – adenosine; D – dipyridamole. The numbers at A and D denote concentrations (mM) of adenosine and dipyridamole tested. Statistical significance: *, ** - $P < 0.05$, $P < 0.01$, respectively, in comparison with controls; °, °° - $P < 0.05$, $P < 0.01$, respectively, in comparison with cultures treated only with the respective concentration of adenosine alone.

Table 1. Generation times (hours) calculated for G:5:113 cells exposed to adenosine and dipyridamole alone or in combination

Adenosine (μM)	Dipyridamole (μM)			
	0	5	10	20
0	17.0	18.2	22.0	26.4
100	18.5	20.5	21.6	25.3
200	21.3	28.2	30.5	32.4
300	32.2	33.1	32.3	30.6

Values of generation times were calculated as mentioned in Material and Methods.

Results and Discussion

Low concentrations of adenosine (0.5 to 50 μM) did not significantly influence any of the studied parameters of the G:5:113 cell population (results not shown). The results in experiments with adenosine concentrations of 100, 200, and 300 μM are shown in Figs. 1 and 2. As shown, adenosine induced a gradual and significant decline of the cell number with EC_{50} of 178 ± 3 μM (Fig. 1). Since a rather high viability of the cells (96 to 88%) was observed at adenosine concentrations of 100 to 300 μM (results not shown), changes in the cell cycle were hypothesized to be responsible for the observed decrease in the cell number. This assumption was substantiated by flow-cytometric analysis (Fig. 2). A decrease in the percentage of the cells in S-phase and an increase in the proportion of the cells in G_0/G_1-phase was found. Higher concentrations of adenosine resulted in a rapid decline in cell viability (e.g., 41% of viable cells at the concentration of 500 μM – results not shown) and were not used in combined treatment with dipyridamole. The flow-cytometric analysis showed that neither of the treatments used in this study induced any apoptotic effects (results not shown). The calculated generation times for G:5:113 cells under the concentrations of 100, 200 and 300 μM of adenosine were 18.5, 21.3, and 32.2 h, respectively, as compared with 17.0 h in control cultures (Table 1).

The purpose of treatment of G:5:113 cells with dipyridamole alone or with the combination of adenosine and dipyridamole was to ascertain whether the observed effects of adenosine on the growth of the cells are produced by extracellular (receptor) action or whether adenosine evokes the effects intracellularly, after entering the cells. As shown in Fig. 1, dipyridamole alone in concentrations of 5, 10, and 20 μM decreased the cell number in a concentration-dependent and significant manner. If dipyridamole was added to the cultures concommitantly with adenosine in concentrations of 100 or 200 μM, it significantly enhanced the suppression of cell growth in comparison with cultures treated only with adenosine. When dipyridamole in any concentration was combined with 300 μM of adenosine, no significant changes in the cell number were observed in comparison with those after treatment with adenosine alone. A suitable approach for illustrating these effects is their expression in the values of cell generation time. Dipyridamole alone induced generation times of 18.2, 22.0, and 26.4 h in concentrations of 5, 10, and 20 μM, respectively (Table 1). Modulations of the growth effects when combining adenosine with dipyridamole were found to be dependent on the adenosine concentration. In all combinations up to adenosine concentration of 200 μM, generation times were prolonged as compared with those observed after treatment with adenosine alone. For example, generation times after adding 200 μM adenosine were prolonged from 21.3 h to 32.4 h by 20 μM dipyridamole (Table 1). However, different effects were observed when combining 300 μM adenosine with dipyridamole. Inexpressive changes of the generation times compared to adenosine alone (32.2 h) occurred, namely 33.1, 32.3, and 30.6 h for combinations with dipyridamole concentrations of 5, 10, and 20 μM, respectively (Table 1). Taken together, the evidence that dipyridamole enhances the suppressive effects of adenosine on cell growth suggests the extracellular, receptor-mediated action of adenosine, if added at concentrations of 100 and 200 μM. The effects of dipyridamole alone can be mediated by the elevation of extracellular adenosine as well. It was shown that growing tumours secrete adenosine in rather high concentrations into insterstitial fluid (Blay et al., 1997). Because dipyridamole prevents not only adenosine uptake by cells but also its extracellular degradation (Klabunde, 1983), thus induced increased extracellular concentrations of endogenous adenosine can act *per se* and, in addition, potentiate the effects of exogenously supplied adenosine. The reason for the loss of ability of dipyridamole to enhance the effects of adenosine given at the concentration of 300 μM is not clear. Hypothetically, these effects can be based on the attainment of the saturation level of extracellular receptor sites responsible for the adenosine action and/or on the activation of the contraregulatory receptor mechanisms under higher adenosine concentrations.

Under all treatments, the surviving cells retained their normal morphology resembling spindle-shaped fibroblasts (not shown).

It can be concluded that adenosine is able to suppress G:5:113 fibrosarcoma cell growth through extracellular, receptor-mediated action. The suppression of cell growth is accompanied by the impairment of cell cycle progression. These results, thus, class G:5:113 cells to the cell lines responding to adenosine by growth inhibition evoked by extracellular, receptor-mediated interaction of adenosine with the cell. In this sense the presented results are analogical e.g. to those of Dubey et al. (2001) on cardiac fibroblasts, Fishman et al. (2000) demonstrating similar effects of adenosine on rat lymphoma cells, or Colquhoun and Newsholme (1997)

on human leukaemic cells, human breast tumour cells, and lymphocytes. The lack of apoptotic effects of adenosine under our experimental conditions can be due to the relatively low concentration of adenosine used. As shown by Tanaka et al. (1994) and Schneider et al. (2001) using human leukaemic cells, apoptosis could be induced by high adenosine concentrations of 1000 μM. In experiments of these authors apoptotic effects of adenosine were prevented by dipyridamole in concentrations similar to those used in our experiments (10 μM). Thus, it can be inferred that only high concentrations of adenosine probably induce apoptosis by facilitating diffusion of adenosine into cells and intracellularly exerting cytotoxic effects like, e.g., nucleoside imbalance with pyrimidine starvation (Kim et al., 1998). Concerning the adenosine receptors which could act through a cytostatic pathway, as shown in our experiments, the A_3 adenosine receptor subtype seems to be the most probable candidate (Fishman et al., 2000; Hoferová et al., unpublished observations). Clarifying these questions could lead to novel anticancer therapies utilizing the mechanisms of adenosine receptor signalling.

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