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Adenosine Contributes to the Amine Oxidase-Mediated Spermine Cytotoxicity to Human Myelogenous Leukemia K562 Cells

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The effects of adenosine, aminophylline, dipyridamole and salbutamol on the amine oxidase-mediated spermine cytotoxicity to KS62 human myelogenous leukemia cells without Ph-chromosome, spontaneously enriched with mildly adherent cells, were studied. In the absence of spermine, adenosine expressed very mild inhibitory action on K562 cell survival, while in combination with the polyamine an almost additive increase in spermine-FBS cytotoxicity was observed. Aminophylline and salbutamol attenuated both spermine-FBS and spermine-FBS-adenosine suppression of cell survival and viability when equimolar concentrations of these agents and the adenosine were applied. Pre-treatment of the cells with higher adenosine levels, in the presence of either aminophylline or dipyridamole, or salbutamol, was associated with decreased K562 cell survival, with the appearance of morphological changes in 10-20% of cells. Additional spermine-FBS cytotoxic effect was not observed in cells pre-treated with adenosine-aminophylline, or adenosine-salbutamole, but morphological changes in 10-20% of cells, even in the presence of spermine, was observed again. Dipyridamole alone suppressed very weakly K562 cell survival. In cells pretreated with dipyridamole, in the presence of spermine-FBS, an additive decrease in cell survival was observed. Pre-treatment of cells with dipyridamole and adenosine in presence of spermine-FBS did not result in a decrease of cell survival compared to the one obtained in dipyridamole-spermine-FBS treated cells.

Key Words: K562 cells, Adenosine, Salbutamol, Aminophylline, Dipyridamole, Spermine cytotoxicity, Amine oxidases

Besides their role in the processes of replication, transcription and translation, polyamines are involved in the regulation of cell proliferation (1). A biosynthesis of these widely distributed organic cations was significantly increased during cell proliferation, as judged by the activity of ornithine decarboxylase, a key enzyme in their biosynthesis (2, 3). Increased levels of polyamines have been detected in tissues known to fail immunological host rejection such as embryonic (4) and malignant ones (5). Although indispensable for normal growth and proliferation, extrinsic polyamines inhibit cell proliferation and express cytotoxicity to several cell lines (6-9). These cytotoxic effects, mediated by copper-dependent amine oxidases (AO), are suppressed by aminoguanidine (7, 10). It was recently shown that terbutaline, a potent β_2-adrenoceptor agonist, prevented the amine oxidase-mediated spermine cytotoxicity, while epinephrine, another β_2-adrenoceptor agonist did not influence spermine cytotoxicity, but acted itself as a cytotoxic agent to K562 cells (7). Some other agents acting through the accumulation of intracellular cAMP *e. g.* salbutamol, a β_2-adrenoceptor agonist and aminophylline, an inhibitor of cyclic nucleotide phosphodiesterases (8), also an inhibited cytotoxic amine oxidase-mediated spermine effects. In addition, aminophylline, in low concentrations, acts as an antagonist of A2 subtype of adenosine receptors (11). Since the mechanism(s) underlying modulations of amine oxidase-mediated spermine cytotoxicity with different pharmacologically active compounds are far from being completely understood, we examined the effects of high concentrations of the natural nucleoside, adenosine alone, or in combination with aminophylline or dipyridamole or salbutamol, on spermine cytotoxicity to K562 human myelogenous leukemia cells in the presence of foetal bovine serum (FBS) as a constituent of the cell culture medium and the source of amine oxidases.

Cell line, medium and chemicals. Experiments were done on K562 human myelogenous leukemia cell line with lymphoblast-like morphology, without Ph chromosome, spontaneously enriched with mildly adherent cell-Some experiments were done on other kind of K562 cells grown completely as a suspension culture RPMI 1640 cell culture medium and FBS were Gibco products. Adenosine was obtained from Schwartz Bioresearch Inc. (Mount Vernon, NY, U.S.A.), aminophylline was a product from Jugoremedija (Yugoslavia) and salbutamol from Sigma (St. Luis, MO, U.S.A.). Spermine tetrachloride was from Calbiochem (San Diego, CA, U.S.A.); dipyridamole was from Behring, Germany.

MMT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) was obtained from Sigma. It was prepared as a 5 mg/ml stock in phosphate-buffered saline, and stored for no more than one week in the dark at 4°C. Before use it was filtered through a 0.22 μm filter pore.

Cell culture. Human myelogenous leukemia K562 cells were grown as a suspension culture in nutrient medium RPMI 1640 supplemented with L-glutamine (3 mM), streptomycin and garamycin (100 μg/mL, each), with 10% heat inactivated foetal bovine serum (FBS). The cells were maintained at 37°C in 5% CO_2 and humidified air atmosphere by twice weekly subculture.

Treatment of K562 cells. K562 cells were treated in quadruplicate in 24 flatbottom wells (Costar) at cell density $4x10^4$ cell/mL. Spermine was applied upon the transfer of the cells into a fresh medium. It was dissolved in 154 mM NaCl solution before the onset of experiments and diluted with the complete culture medium to the desired concentration.

Freshly prepared solutions of salbutamol or aminophylline (final conc. 1.0 mM, each) were added to cell cultures and their effects on survival (N/Nc) and on viability (V%) were examined 24 hrs later. When combined action of either these agents and spermine was studied, the polyamine (final conc. 30, or 35, or 50 μM) was introduced into the cell cultures 40 min upon addition of salbutamol or aminophylline. In a series of experiments the cells were pre-treated with varying adenosine concentrations (0.5, 1.0, or 2.0 mM) for 15 min and than the above agents were added by the aforementioned schedule. A nucleoside transport inhibitor dipyridamole in final concentration 10 μM was added into the cell culture 40 min. before adenosine and 55 min. before spermine.

Counting of viable K562 cells. Cell viability was assessed by trypan blue dye exclusion test. Number of viable and dead cells was counted using Fuchs-Rosenthal chamber, and a Carl Zeiss, Jena, microscope. Viability (V) was expressed as the number of viable cells per 100 cells of either treated or control culture. Cell survival (N/Nc, %) was expressed as a number of survived cells exposed to either of the agents applied, or their combinations, in 1 mL of cell suspension per number of survivors in 1 mL of control culture x 100.

To get more information on modulation of spermine-FBS effect on K562 cells, an additional method for determining cell survival was performed.

MTT assays. The assay was carried out in flat-bottomed 96-wells (Costar). Cells were plated at approx. $9x10^3$ cells per well, in final volume of 0.1 mL of nutritient medium. Cell treatment was almost the same as when the assay was done in 24 flat-bottom wells. Samples were maintained at 37°C in humidified atmosphere of 5% CO_2 in air for 44 hours.

After incubation for 44 hours, 20 μl of MTT was added in each well. The plates were further incubated in humidified atmosphere at 37°C, for 4 h. Then 100 μl of 10% SDS in 0.01 N HCl (12) was added in each sample. After overnight incubation absorption of a sample with cells was measured at 570 nm versus blank (nutritient medium + applied agents in their corresponding final concentrations). Cell survival, A/Ac (%), was expressed as the ratio of the absorbance of cells treated with the investigated agent(s) A, divided by absorbance of a control sample (of cells grown in a nutritient medium only) Ac, x100.

Results

The influence of adenosine on amine oxidase-mediated spermine cytotoxicity to human myelogenous leukemia cells is presented in Table I.

As seen, adenosine (0.5-2 mM) contributes to the spermine-FBS cytotoxicity, and decreases cell survival by approximately 25%, but did not affect cell viability in the absence of the polyamine, as judged by trypan blue dye exclusion.

Salbutamol (1 mM) inhibited spermine-FBS suppression of both cell survival and viability. However, pretreatment of the cells with up to 2 mM adenosine partially abolished such a protective salbutamol action against spermine-FBS cytotoxicity, as shown in Table II.

Pre-treatment of the cells with adenosine for 15 min and further exposure to aminophylline for 40 min followed by spermine did not affect cell survival and viability comparing to the cultures exposed to spermine

Table I - The effect of different adenosine concentrations on spermine-FBS cytotoxicity to K562 human myelogenous leukemia cells

Treatment	N/Nc (%)	V (%)
Control	100±8	99±1
FBS + 0.5 mM adenosine	63±3	97±2
FBS + 1.0 mM adenosine	76±8	98±2
FBS + 2.0 mM adenosine	76±8	98±2
FBS + spermine	26±7	35±6
FBS + 0.5 mM adenosine + spermine	8±4	12±4
FBS + 1.0 mM adenosine + spermine	7±2	10±3
FBS + 2.0 mM adenosine + spermine	0.5±0.5	1±1

Adenosine was added upon the transfer of the cells into a fresh RPMI 1640 medium supplemented with 10% FBS, or when applied with spermine (final concentration 30 μM) 15 min before the addition of polyamine. Effects on cell survival and onviability were determined 24 h later by trypan blue exclusion method. The results are means ± CV quadruplicate.

Table II - The effect of adenosine on salbutamol-induced protection of K562 cells against spermine-FBS cytotoxicity

Treatment	N/Nc (%)	V (%)
Control	100±8	99±1
FBS + spermine	26±7	35±6
FBS + salbutamol + spermine	85±10	98±2
FBS + O.5mM adenosine + salbutamol + spermine	80±10	97±1
FBS + I.OmM adenosine + salbutamol + spermine	80±6	99±1
FBS + 2.0mM adenosine + salbutamol + spermine	59±8	82±5

Adenosine was added upon the transfer of the cells into a fresh RPMI 1640 medium supplemented with 10% FBS, or when applied with spermine (final concentration 30 μM) 55 min before the polyamine, but 15 min before addition of salbutamol (1 mM). Effects on cell survival and on viability were determined 24 h later by trypan blue exclusion method. The results are means ± CV quadruplicate.

and aminophylline, i. e. adenosine did not affect protective aminophylline effect against spermine-FBS cytotoxicity after 24 h (Table III). This protective aminophylline action in adenosine pretreated cultures was gradually decreasing during the 72 hour-period (data not shown).

However, some morphological changes, seen as droplet shaped cells with a cytoplasmatic protrusion(s), were observed in the cultures exposed to aminophylline and spermine (5-8% of cells). Approximately 10-20% of cells pretreated with adenosine and further treated with aminophylline or dipyridamole or salbutamol, in the presence as well as in the absence of spermine exhibit the asymmetric mitosis, that develops dwarf-cell-like structures (13), or uneven small cellular particles, apoptic bodies, that maintained their ability to exclude trypan blue dye, of the same pattern as it was observed by Tanaka et al. (14) during their investigation of the adenosine effect on HL-60 cell lines. (In other type of K562 cells grown completely as suspension culture, neither cytoplasmatic protrusions in cells exposed to aminophylline and spermine nor dwarf cell-like structures, when cells were pretreated with adenosine and further treated with aminophylline or salbutamol or dipyridamole, were observed.)

Some examinations of cell survival were done by MTT test. Data on the survival of K562 cells treated with adenosine, or spermine, or aminophylline alone,

Table III - The influence of adenosine on aminophylline-related inhibition of spermine-FBS cytotoxicity to K562 cells.

Treatment	N/Nc (%)	V (%)
Control	100±15	99±1 1
FBS + spermine	12±4	25±6
FBS + adenosine + spermine	0.5±0.5	1±1
FBS + aminophylline + spermine	46±8	98±1
FBS + adenosine + aminophylline + spermine	42±6	96±2

Adenosine (final conc. 2.0 mM) was added upon the transfer of the cells into a fresh RPMI 1640 medium supplemented with 10% FBS, or when applied with spermine (final concentration 35 μM) 55 min before the addition of polyamine, while in experiments with aminophylline, adenosine was applied 15 min before aminophylline (1 mM) i. e. 40 min before the polyamine. Effects on cell survival and on viability were determined 24 h later by trypan blue exclusion method. The results are means ± CV quadruplicate.

or in their combinations, 44 hrs after the agents action, using MTT test, are presented on Table IV and V. An inhibitory action of adenosine on K562 cell survival, that depends on adenosine concentration, could be observed. Pretreatment of K562 cells with adenosine and further treated with spermine-FBS led to the almost additive decrease cell survival. (It must be emphasized that the same pretreatment of other kind of K562 cells grown completely as suspension culture, with adenosine and further treated with spermine-FBS did not show any decrease in cell survival compared to that obtained after the action of spermine-FBS). Target K562 cell, treated with adenosine-aminophylline, showed a decrease in cell survival compared to that of cells treated with adenosine only. Survival of K562 cells pretreated cells with adenosine-aminophylline in the presence as well as in the absence of spermine, was the same. This survival was lower than one observed in culture treated with adenosine-spermine or aminophylline spermine, due to mutual adenosine-aminophylline action on K562 cells. This means that aminophylline, even in the presence of adenosine, maintained its ability to attenuate spermine-FBS suppression of cell survival.

The effects of nucleoside transport inhibitor, and cGMP-dependent phosphodiesterase inhibitor dipyridamole on adenosine, adenosine-spermine-FBS as well as on spermine-FBS mediated suppression of K562 cell survival are shown in Table VI. It could be seen that dipyridamole alone mildly decreased survival of K562 cells. This decrease in K562 cells survival was more pronounced in the presence of added spermine-FBS. Further, a mild decrease in cell survival induced by adenosine was much more pronounced (together

Table IV - Effects of aminophylline or adenosine or their combinations on spermine-FBS mediated suppression of K562 cell survival A/Ac (%)

Treatment	A/Ac
Control	100
Spermine 47 μM	56*
Spermine 94 μM	27
Spermine 187 μM	7
Spermine 547 μM	6
Adenosine 0.7 μM	78
Adenosine 1 mM	61
Adenosine 2 mM	40
Adenosine 0.7 mM + Spermine 47 μM	54
Adenosine 1 mM + Spermine 47 μM	39
Adenosine 2 mM + Spermine 47 μM	20
Aminophylline 1 mM	48
Aminophylline 1 mM + Adenosine 1 mM	26
Aminophylline 1 mM, Adenosine 1 mM, Spermine 47 μM	25
Aminophylline 1 mM + Spermine 47 μM	45
Aminophylline 1 mM + Spermine 94 μM	43

Adenosine was added upon the transfer of the cells into a fresh RPMI 1640 medium supplemented with 10% F13S, or when applied with spermine, 55 min before the addition of polyamine, while in experiments with aminophylline, adenosine was applied 15 min before aminophylline, i. e. 55 min before the polyamine. Effects on cell survival were determined 44 h later by MTT test. The results are means of triplicates x100. Relative error was less than 6%.
** It must be noted that, due to a lower spermine-FBS / target cell ratio, the spermine-F13S cytotoxic action was observed at higher concentration of spermine when experiments were done in 96 micro wells, compared with experiments done in 24 well-plates.*

Table V - Effects of aminophylline or adenosine or their combinations on spermine-FBS mediated suppression of K562 cell survival A/Ac (%)

Treatment	A/Ac
Control	100
Spermine 50 μM	67*
Adenosine 1 mM	79
Adenosine 2 mM	70
Adenosine 1 mM + Spermine 50 μM	58
Adenosine 2 mM + Spermine 50 μM	51
Aminophylline 0.5 mM	66
Aminophylline 0.5 mM + Adenosine 1 mM	41
Aminophylline 0.5 mM, Adenosine 1 mM, Spermine 50 μM	41
Aminophylline 0.5 mM + Adenosine 2 mM	36
Aminophylline 0.5 mM + Adenosine 2 mM, Spermine 50 μM	37
Aminophylline 0.5 mM + Spermine 50 μM	68

Adenosine was added upon the transfer of the cells into a fresh RPMI 1640 medium supplemented with 10% FBS, or when applied with spermine, 55 min before the addition of polyamine, while in experiments with aminophylline, adenosine was applied 15 min before aminophylline, i. e. 55 min before the polyamine. Effects on cell survival were determined 44 h later by MTT test. The results are means of triplicates x 100. Relative error was less than 6%.
** It must be noted that, due to a lower spermine-FBS / target cell ratio, the spermine-FBS cytotoxic action was observed at higher concentration of spermine when experiments were done in 96 micro wells, compared with experiments done in 24 well-plates.*

Table VI - Effects of adenosine on dipyridamole-related protection from spermine-FBS mediated suppression of K562 cell survival A/Ac(%)

Treatment	A/Ac(%)
Control	100
Adenosine 1 mM	93
Adenosine 2 mM	82
Dipyridamole 10 μM	88
Adenosine 1 mM + dipyridamole 10 μM	72
Adenosine 2 mM + dipyridamole 10 μM	63
Spermine 50 μM	59*
Spermine 50 μM + dipyridamole 10 μM	43
Adenosine 1 mM + spermine 50 μM + dipyridamole 10 μM	44
Adenosine 2 mM + spermine 50 μM + dipyridamole 10 μM	43
Adenosine 1 mM + spermine 50 μM	61
Adenosine 2 mM + spermine 50 μM	48

Adenosine was added upon the transfer of the cells into a fresh RPMI 1640 medium supplemented with 10% FBS, or when applied with spermine, 15 min before the addition of polyamine, while in experiments with dipyridamole, adenosine was applied 40 min after dipyridamole. Effects on cell survival were determined 44 h later by MTT test. The results are means of triplicates x100. Relative error was less than 6%.
** It must be noted that, due to a lower spermine-FBS / target cell ratio, the spermine-FBS cytotoxic action was observed at higher concentration of spermine when experiments were done in 96 micro wells, compared with experiments done in 24 well-plates.*

with the appearance of apoptic bodies) in adherent-type of K562 cells pretreated with dipyridamole. Survival of K562 cells pretreated with dipyridamole-adenosine, in the presence of spermine, was lower than that of the cells treated only with adenosine-dipyridamole and almost the same as of culture treated by dipyridamole-spermine.

Discussion

Although many publications have been dedicated to the problems concerning the manifold effects of polyamines on different cell lines, tissues and organs, the precise mechanism(s) of their action at molecular level is still far from being completely elucidated. The same holds true for the action of many modulators involved in the expression of different polyamine activities. In this light, we have found of interest to examine possible modulations of amine oxidase-mediated spermine cytotoxicity to human myelogenous leukemia cell line employing four compounds from different chemical and pharmacological categories. Our recent studies demonstrated that the agents known to cause the accumulation of intracellular cAMP through their action on beta-2 adrenergic receptors (terbutalin and salbutamol), or inhibiting cAMP dependent phosphodiesterases (aminophylline), inhibited spermine-FBS cytotoxicity toward K562 cells (7, 8).

The data obtained in this work showed that nucleoside transport inhibitor, and preferentially cGMP-dependent phosphodiesterase inhibitor, dipyridamole, enhanced almost additively amine-oxidase mediated spermine-FBS suppression of K562 cell survival. This finding points to the possible importance of the ratio of intracellular cGMP / cAMP in the control of cell survival in the presence of spermine-FBS.

The results of the present work showed that natu-

rally occurring nucleoside adenosine contributed to the cytotoxic amine oxidase-mediated spermine effect to K562 cells inducing, even alone, a mild suppression of cell survival. This effect of adenosine alone, was more pronounced by the additional action of aminophylline. Similar effect of adenosine was reported earlier by Szondy (15), on human thymocytes. She showed that adenosine acts via extracellular adenosine receptors, by Ca2+ mediated mechanism, and agents that elevated intracellular cAMP (forskolin and 3-isobutyl-1-methylxantine, IBMX) potentiated DNA fragmentation in the cells. Since adenosine could act through its binding to the extracellular, specific plasma membrane receptors Al, A2, A3, or intracellular P-site (16, 17, 18), it was of interest to find out which of the main adenosine subtypes is involved in adenosine suppression of K562 cell survival as well as to the elevation of cytotoxic spermine-FBS action. Our data demonstrated that, spermine-FBS suppression of cell survival was abrogated by the agents known to induce an increase in intracellular cAMP level. The K562 cell survival was the same in cells pretreated with adenosine-aminophylline, both in the presence and in the absence of spermine. This indicates that adenosine did not disturb metabolic processes induced by aminophylline to protect cells from death induced by spermine-FBS, although some mutual action of adenosine and aminophylline was observed. The same holds true for the adenosine action on salbutamole activated protection of K562 cell from spermine-FBS action. This means that when adenosine extracellular action is coupled with the metabolic processes induced by higher intracellular cAMP, independently of spermine-FBS, the apoptosis in K562 cells is activated. Similar effect of adenosine analog 2-Cl-deoxyadenosine in the presence of dipyridamole was observed earlier on human thymocytes (19). It is noteworthy to mention that Hoskin et al. (20) reported the same effect of adenosine alone or in combination with Al, or A2 blocker, or in combination with dipyridamole on MHC-unresticted killing of mouse P815 mastocytoma target cells by activated, via antiCD3+mAB, killer cells. This effect of adenosine was deemed to be mediated through nonAl/A2, non P adenosine receptors. The effects of adenosine and its combinations with naturally occurring polyamines in vivo could be very intriguing. Having in mind that thymus tissue (21) and lymphocytes (22) contained high levels of polyamines, it would be of great significance to learn whether an excess of adenosine characterizing hereditary immunodeficiency associated with the lack of adenosine deaminase, in the presence of polyamines, leads to a decreased number of T and B lymphocytes in such patients. Mandler et al. (23) observed an abnormal adenosine induced immunosuppression in patients with systemic lupus erythematosus, a disease characterized also by an increased polyamine level in the circulation (24). So, the question arises: is the modulated (through signals that regulates the level of intracellular cAMP in target cells), AO-mediated, spermine effect in the presence of adenosine, at least partially, responsible for the diversity of the afore mentioned biological phenomena?

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