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Apoptosis by 2-chloro-2'-deoxy-adenosine and 2-chloro-adenosine in human peripheral blood mononuclear cells

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Abstract

Adenosine has profound effects on immune cells and has been implicated in the intrathymic apoptotic deletion of T-cells during development. In order to characterize adenosine effects on quiescent peripheral blood mononuclear cells (PBMC), we have evaluated the ability of the previously characterized adenosine receptor agonist 2-chloro-adenosine (2CA; Ceruti, Barbieri et al., 1997) and of the antineoplastic drug 2-chloro-2'-deoxy-adenosine (2CdA, cladribine) to trigger apoptosis of PBMC. Apoptosis was assessed by morphological changes, DNA fragmentation by agarose gel electrophoresis and appearance of hypodiploid DNA peak by flow cytometry. 2CA (10 μ M) and 2CdA (1 μ M) induced apoptosis in human PBMC, which are relatively insensitive to apoptosis. For both agents, the effect was concentration- and time-dependent, although 2CdA induced apoptosis more potently than 2CA. Evaluation of mitochondrial function in parallel samples using the mitochondrial membrane-potential-specific dye JC-1 showed that mitochondrial damage followed the same kinetics as apoptosis, hence an early damage of mitochondria is likely not responsible for adenosine-induced death of PBMC. The effect of 2CA was partially prevented by addition of dipyridamole (DP), a nucleoside transport inhibitor, hence some of the apoptotic effect of this nucleoside is, at least in part, due to intracellular action. Alternatively, DP did not affect 2CdA-induced apoptosis, suggesting that 2CdA may enter cells via a DP-insensitive transporter. 5-Iodotubercidin (5-Itu), a nucleoside kinase inhibitor, was also able to partially prevent the action of 2CA and was not able to affect 2CdA-induced apoptosis, suggesting a different role for phosphorylation in 2CA- vs 2CdA-induced apoptosis. To test the role of P1 receptors, agonists and antagonists selective at various P1 receptor subtypes were used. Data suggest that, for 2CA, apoptosis is partially sustained by activation of the A_{2A} receptor subtype, whereas no role is exerted by P1 receptors in 2CdA-dependent apoptosis. Moreover, in these cells, apoptosis could also be triggered through intense activation of the A₃ receptor via selective agonists such as 2-chloro-N⁶-(3-iodobenzyl)adenosine-5'-N-methyluronamide (CI-IB-MECA), but this mechanism plays no role in

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either 2CA- or 2CdA-induced apoptosis. On the whole, our results suggest that 2CA and 2CdA follow different pathways in inducing apoptosis of immune cells. Moreover, our data also suggest that there are at least three different ways by which adenosine derivatives may induce apoptosis of human PBMC: (i) through an A_{2A}-like extracellular membrane receptor; (ii) through entry of nucleosides into cells and direct activation of intracellular events involved in the apoptotic process; or (iii) through activation of the A₃ receptor.

1. Introduction

Adenosine is an endogenous modulator that acts by activating four different subtypes of extracellular receptors, the PI receptors, designated A₁, A_{2A}, A_{2B} and A₃, all of which, except the A₃ receptor, are antagonized by xanthine derivatives (the prototype being theophylline) (Fredholm et al., 1994, 1997). Adenosine has been implicated as a key regulator of neuro-endocrine-immune functions, being involved in central and peripheral neurotransmission (Ribeiro et al., 1996; Dunwiddie et al., 1996), hormonal regulation (Petit et al., 1996) as well as in modulation of immune cell function. In particular, in the immune system, adenosine can act as an immunosuppressor (Gorski and Grieb, 1994; Cheson, 1995; Carson et al., 1984) and inhibitor of TNF- α production (Prabhakar et al., 1995; Thiel and Chouker, 1995; Proctor et al., 1994) and has been implicated as a physiological signal in the apoptotic deletion of T-cells during intrathymic cell selection, a process which functions to prevent autoimmunity (Szondy, 1994, 1995). The mechanisms by which adenosine affects immune cells are not yet clearly understood; in particular, it is not completely elucidated to what degree induction of apoptosis is specifically mediated by the activation of extracellular receptors or is directly exerted intracellularly.

Adenosine-induced cell death is relatively well characterized in neural cells, where both receptor-mediated and receptor-independent mechanisms have been demonstrated (Abbracchio et al., 1997). In particular, we have previously characterized the apoptosis induced by a P1 receptor non-selective, metabolically stable adenosine analogue, 2-chloro-adenosine (2CA), on rat astroglial cells. We have shown that cell death by this agent may involve an atypical adenosine receptor, does not occur through activation of poly(ADP-ribose)polymerase or radical generation, and is not apparently due to early damage of the mitochondria (Abbracchio et al., 1995; Ceruti, Barbieri et al., 1997).

On this basis, in an attempt to characterize adenosine effects on human peripheral blood mononuclear cells (PBMC), in the present study we have focused our attention on the possible apoptotic effect of this adenosine analogue. We have compared the actions of 1CA with those exerted by another adenosine derivative, 2-chloro-2'-deoxy-adenosine (2CdA, cladribine), which has been previously shown to induce apoptosis of human lymphoid cells (Robertson et al., 1993; Seto et al., 1985; Wood, 1994) and that is currently used in the treatment of chronic lymphoid malignancies (e.g. chronic lymphocytic leukemia, hairy cell leukemia and cutaneous T-cell lymphoma) (Bryson and Sorkin, 1993; Beutler, 1992; Wood, 1994).

In order to elucidate the molecular mechanisms responsible for 2CA- and 2CdA-induced effects in PBMC and in particular, to assess whether entry of these compounds into cells is a

critical step in their functional actions, we have evaluated the possible modulation of their effects by the nucleoside transport inhibitor dipyridamole (DP) (Geyger and Fyda, 1991). Moreover, since, at least for 2CdA, the intracellular phosphorylation to 2-chloro-deoxy-ATP by an intracellular nucleoside kinase has been shown to represent a key step in its cytotoxic action (Seto et al., 1985; Beutler, 1992; Wood, 1994), we have also tested the effect of a nucleoside kinase inhibitor (5-iodotubercidin, 5-Itu) on both 2CA- and 2CdA-induced cell death. Finally, to assess the possible involvement of specific extracellular P1 receptors, we have evaluated the effects induced by agonists and antagonists selective for the various adenosine receptor subtypes.

2. Experimental procedures

2.1. Lymphocyte cultures

Peripheral blood from healthy volunteers was collected aseptically; mononuclear cells were separated by centrifugation in Lymphoprep gradient (Boyum, 1968), washed extensively with Hanks' medium and resuspended at a concentration of 10^6 cells/ml in RPMI 1640 medium supplemented by 10% heat-inactivated FCS, 2 mM L-glutamine, penicillin (100 units/ml) and streptomycin (100 μ g/ml). Cell suspensions were seeded into culture plates (Corning) and substances were added at the concentrations indicated in the legends to Figs and Tables. Cultures were incubated for 24, 48 and 72 h in a humidified atmosphere of 5% CO₂ in air at 37°C, with or without adenosine analogues. At each time point, cells were harvested and analysed by light microscopy, flow cytometry and gel electrophoresis.

2.2. Drugs and treatments

2CA, 2CdA, DP, adenosine (ADO), coformycin (CF) and propidium iodide (PI) were purchased from Sigma (St Louis, U.S.A.). Lymphoprep was obtained from Nycored Pharma AS (Oslo, Norway). RPMI 1640, Hanks' Balanced Salts Solution (HBSS), L-Glutamine and Penicillin-Streptomycin were from Gibco (Paisley, Scotland, U.K.) 2-chloro-N⁶-(3-iodobenzyl)adenosine-5'-N-methyluronamide (Cl-IB-MECA), DPCPX (1,3-dipropyl-8-cyclopentylxanthine) 5-Itu and XAC (8-[4-[[[(2-aminoethyl)amino]carbonyl]-methyl]oxy]phenyl]-1,3-dipropylxanthine) were obtained from Research Biochemicals Int. (Natick, MA, U.S.A.). SCH 58261 (5-amino-7-(phenylethyl)-2-(2-furyl)-pyrazolo.4,3-e]1,2,4-triazolo.1,5-c]pyrimidine) was a kind gift of Dr E. Ongini, Schering-Plough Research Institute, Milan, Italy. The adenosine uptake-inhibitor DP (10 μ M) and the nucleoside kinase inhibitor 5-Itu (100 nM) were added to cells 30 min before starting incubation with adenosine analogues.

2.3. Light microscopy

Cells were collected using a cytocentrifuge (Shandon, Cheshire, U.K.) at 500 rpm for 5 min and cell morphology was evaluated on May-Grunwald/Giemsa stained preparations by examination in a Axioscope (Zeiss, Germany). Apoptotic cells were identified using previously defined criteria (Kerr and Harmon, 1991).

2.4. DNA fragmentation and agarose gel electrophoresis

At 24, 48 and 72 h after treatment, 1×10^6 cells were centrifuged and pellets resuspended in lysis buffer (10 mM EDTA, 50 mM Tris, pH 8, 0.5% Sarkosyl, 0.5 mg/ml proteinase K). After 1 h incubation at 50°C the suspension was supplemented with 0.25 mg/ml heat-treated RNase A and further incubated in the water-bath at 50°C for 1 h. Crude extracts were then transferred to 70°C and added with loading buffer [10 mM EDTA, pH 8, containing 0.25% (w/v) bromophenol blue, 1% (w/v) low-melting agarose and 40% (w/v) sucrose]. Ethidium bromide was incorporated into the gel allowing DNA to be visualized by UV light. Electrophoresis was carried out overnight at 15 V on 2% (w/v) agarose gel.

2.5. Flow cytometry

2.5.1. Detection of apoptosis—2CA- and 2CdA-induced apoptosis was detected by reduced fluorescence of the PI—a DNA binding dye—in the apoptotic nuclei, as previously described (Barbieri et al., 1994). Briefly, the 200 g centrifuged cell pellet (1×10^6 cells) was gently resuspended in 1 ml hypotonic fluorochrome solution [PI 50 μ g/ml in 0.1% (w/v) sodium citrate plus 0.1% (v/v) Triton X-100 (Sigma) in bidistilled water]. Cells were analysed by FACS after a minimum of 30 min of incubation in this solution. Red fluorescence due to PI staining of DNA was recorded on a logarithmic scale. Debris were gated out based on light scatter measurements before the single parameter histograms and the contour graphs were drawn.

2.5.2. Evaluation of mitochondrial membrane potential—Mitochondrial membrane potential (Ψ) was evaluated by using the mitochondrial specific fluorescent probe 5,5',6,6',-tetrachloro-1,1',3,3-tetraethylbenzimidazole carbocyanine iodide (JC-1, Molecular Probes, Eugene, OR), as previously described (Cossarizza et al., 1993, 1994, 1995a). All these measurements were performed by utilizing a FACScan flow cytometer (Becton Dickinson, San José, CA, U.S.A.) equipped with a single 488 nm argon laser. A minimum of 10^4 cells per sample were analysed. Data were acquired in list mode and analysed with Lysis II software (Becton Dickinson).

2.6. Statistical analysis

Statistical analysis was performed by either one-way ANOVA followed by Scheffe's *F*-test for multiple comparison or two-way ANOVA utilizing the Bonferroni correction. A *P* value lower than 0.05 was considered statistically significant.

3. Results

3.1. Both 2CA and 2CdA induce alterations that are typical of apoptotic cells

The possible induction of apoptosis in 2CA- and 2CdA-treated cells was assessed using PI staining in flow cytometry. Both adenosine analogues induced a marked apoptosis of PBMNC. A typical flow cytometric analysis in a representative experiment is shown in Fig. 1. Both 2CA- and 2CdA-treated cells showed a marked reduction in the percentage of nuclei with diploid DNA content and a concomitant increase in the hypodiploid DNA peak, a clear sign of the presence of apoptotic bodies (Fig. 1C and 1E in comparison with 1A, control). For both analogues, the effect was concentration- and time-dependent (Fig. 2). The

minimum effective concentrations ($1\ \mu\text{M}$ for 2CdA and $10\ \mu\text{M}$ for 2CA) were chosen for all subsequent experiments.

Induction of apoptosis was confirmed by a morphological analysis of human PBMC treated with either $10\ \mu\text{M}$ 2CA or $1\ \mu\text{M}$ 2CdA. Alterations due to the induction of an apoptotic process were present (Fig. 3). Typical morphological signs of apoptosis (such as cytoplasmic condensation and compaction of chromatin) were evident in treated cells (indicated by arrows in Fig. 3B and 3C) vs control cultures, where the percentage of apoptotic cells was negligible (Fig. 3A). Such morphological changes were accompanied by fragmentation of DNA at the internucleosomal level resulting in a characteristic ladder on gel electrophoresis (Fig. 4). The DNA ladder was evident in both 2CA- and in 2CdA-treated cells and was practically absent in control untreated cells.

In order to rule out possible influences due to individual variability, $10\ \mu\text{M}$ 2CA or $1\ \mu\text{M}$ 2CdA were evaluated on PBMC obtained from 18 different individuals. As shown in Table 1, highly significant and time-dependent apoptosis was demonstrated for both adenosine analogues, with 2CdA being much more potent than 2CA in inducing apoptosis (maximal effect at 72 h: $81.2\pm1.9\%$ vs $45.8\pm2.4\%$ in 2CdA- and 2CA-treated PBMC, respectively; $P < 0.05$, Scheffe's *F*-test) at one-tenth the concentration ($1\ \mu\text{M}$ vs $10\ \mu\text{M}$). In control untreated PBMC, apoptosis never exceeded $7.9\pm0.9\%$.

We have also tested the ability of the natural nucleoside ADO to trigger apoptosis of human PBMC (Fig. 5). In the presence of $1\ \mu\text{M}$ CF, an inhibitor of the adenosine deaminase-dependent inactivation of the nucleoside, adenosine induced a time-dependent apoptosis of PBMC, although a $1\ \text{mM}$ concentration (i.e. a 100- and 1000-fold higher concentration with respect to 2CA and 2CdA, respectively), had to be used.

3.2. In 2CA- and 2CdA-induced cell death, mitochondrial damage is a consequence rather than a cause of apoptosis

Since mitochondria play a central role in the development of programmed cell death, cell loading with the mitochondrial-specific dye JC-1 was performed to obtain information about mitochondrial transmembrane potential (Ψ) in apoptotic cells in our experimental model. An increase of cells with depolarized mitochondria has been postulated to occur during induction of apoptosis (Cossarizza et al., 1995a,b; Richter et al., 1996). Representative examples of the cytofluorimetric analysis of JC-1-loaded cells in a typical experiment are shown in Fig. 1B, 1D and 1F. According to the technique we used, control untreated cells had a high fluorescence emission in both the FL1 (green) and FL2 (orange) ranges. 2CA- and 2CdA-treated cells showed a markedly decreased orange emission, which is indicative of an increased number of cells with depolarized mitochondria (such cells are those going from the middle of the quadrant to the lower right corner). Cells with depolarized mitochondria accounted for 14.6% of total cell number (Fig. 1B) in control untreated cells; a marked increase of this value was observed in cultures exposed to 2CA and to 2CdA (48.7% and 85.1%, respectively, Fig. 1D and 1F). The statistical analysis of this effect is reported in Table 2, with results obtained from six independent experiments. The increase of depolarized mitochondria was statistically significant after 48 h of exposure to the adenosine analogues and fully overlapped with the appearance of apoptotic nuclei in the same cultures.

These data apparently rule out an early involvement of mitochondrial damage in this type of apoptosis and suggest that, as already demonstrated for rat glial astrocytes (Ceruti, Barbieri et al., 1997), in adenosine-induced apoptosis, mitochondrial damage likely represents the consequence of a generalized derangement of cell organization.

3.3. The nucleoside transport inhibitor DP partially protects against 2CA- but not 2CdA-induced apoptosis

To test whether the uptake of 2CA and 2CdA is required for the induction of PBMC cell death, cells were incubated with 2CA and 2CdA in the presence of 10 μM (DP), a strong inhibitor of nucleoside transport. DP alone did not affect the viability of human PBMC (data not shown). In 2CA-treated cells, DP partially protected against apoptosis (which was decreased from $50.4 \pm 6.8\%$ to $23.0 \pm 3.7\%$ at 72 h of incubation, $P < 0.05$, Scheffe's F -test) (Fig. 6) suggesting that 2CA-induced cell death of human PBMC is partially due to a direct intracellular action consequent to membrane entry through the nucleoside transporter and partially by activation of extracellular adenosine receptors (see below). Entry of 2CA through the membrane nucleoside carrier is consistent with previous studies showing that this adenosine derivative is indeed a permeant for the nucleoside transporter in human cells, with an apparent K_m value of 23 μM (Jarvis et al., 1985). On the other hand, in contrast to results reported on thymocytes (Szondy, 1995), DP had no effect on 2CdA-induced cell death of human PBMC, raising the hypothesis that, in these cells, 2CdA toxicity is not mediated by entry through the DP-sensitive membrane transporter.

3.4. The nucleoside kinase inhibitor 5-Itu partially protects against 2CA- but not 2CdA-induced apoptosis

5-Itu significantly prevented the apoptotic action of 2CA both at 48 h and at 72 h in culture but was not able to affect 2CdA-induced apoptosis at any time-point (Fig. 7). These data suggest that enzymatic activation of 2CA by the nucleoside kinase is, at least in part, responsible for the apoptotic effect of this adenosine analogue, whereas 2CdA does not need to be phosphorylated intracellularly to exert its effects, in contrast to current hypotheses (Carson et al., 1980; Beutler, 1992; Bryson and Sorkin, 1993; Wood, 1994).

3.5. Protection of 2CA-, but not 2CdA-induced apoptosis, by selective adenosine receptor antagonists

To investigate if the apoptotic effect of adenosine analogues on human PBMC is, at least in part, dependent by the occupation of plasmamembrane adenosine receptors, the possible reversal of apoptosis by the A_1/A_{2A} receptor antagonist XAC was tested (Fig. 8). XAC alone did not affect the apoptosis of human PBMC at a concentration (1 μM) known to fully antagonize both receptor subtypes (Fredholm et al., 1994). No effect on 2CdA-induced apoptosis was observed, while a significant prevention was obtained in the case of 2CA ($27.92 \pm 1.7\%$ of apoptotic cells in the 2CA + XAC group vs $37.8 \pm 22.2\%$ after a 72 h exposure of PBMC to 2CA alone, $P < 0.05$, Scheffe's F -test), suggesting that xanthine-sensitive adenosine A_1 and A_{2A} receptors are, at least in part, involved in induction of apoptosis. On this basis, we have tested more selective A_1 and A_{2A} receptor antagonists on 2CA-induced apoptosis, in particular the A_1 receptor antagonist DPCPX (Fredholm et al., 1994) and the A_{2A} receptor antagonist SCH 58261 (Ongini et al., 1996). DPCPX did not

significantly affect apoptosis by 2CA (Fig. 9), while SCH 58261, utilized at a 50 nM concentration was able to partially protect 2CA-treated PBMC, in a similar way to XAC, by reducing the degree of apoptosis from $37.6 \pm 2.5\%$ to $27.5 \pm 1.8\%$ after a 72 h of incubation ($P < 0.05$ with respect to 2CA alone, Scheffe's F -test). No further protection was obtained by utilizing both DPCPX and SCH 58261 (data not shown); this, together with the observation that protection by SCH 58261 is equal to protection obtained with XAC (Fig. 9), suggests a specific involvement of A_{2A} receptors in this phenomenon.

3.6. An A_3 adenosine receptor-mediated apoptotic pathway is also present in human PBMC, but it does not contribute to 2CA- and 2CdA-induced effects

The adenosine A_3 receptor has been involved in induction of apoptosis of human HL-60 leukemia cells and U937 lymphoma cells (Kohno et al., 1996; Yao et al., 1997). In these cells, at concentrations $\geq 10 \mu M$, the selective A_3 receptor agonist Cl-IB-MECA produced apoptosis and increased the expression of the pro-apoptotic protein Bak, a member of the Bcl-2 family. To test whether the A_3 receptor can also modulate apoptosis in our experimental system, we have evaluated the ability of Cl-IB-MECA to trigger apoptosis of PBMC. Although not effective at low concentrations (1–100 nM), Cl-IB-MECA was able to induce a concentration-dependent and statistically significant apoptosis in the micromolar range (Fig. 10). This suggests the existence of an additional, A_3 receptor-mediated, apoptotic pathway in these cells. We hence asked whether apoptosis induced by 2CA and 2CdA (Figs 1–4) could involve activation of the A_3 adenosine receptor. To verify whether these two adenosine analogs can indeed interact with the human A_3 adenosine receptor, we have tested them on a transfected clone (A_3R -CHO) which expresses the human receptor at a relatively high level (Klotz et al., in press), in comparison with the corresponding A_3 receptor-lacking wild-type clone (WT-CHO). Exposure of both clones to 2CA and 2CdA resulted in very comparable reductions of cell number irrespectively of the presence of the A_3 receptor (Brambilla, R., Barbieri, D., Ceruti, S., Cattabeni, F., Klotz, K.-N., Lohse, M., Franceschi, C. Jacobson, K.A. and Abbracchio, M.P., data not shown). These results clearly suggest that 2CA and 2CdA do not significantly interact with the *human* A_3 adenosine receptor and hence that their effects on *human* PBMC are independent of the presence of this receptor subtype.

4. Discussion

We have previously characterized the apoptotic effects induced by the adenosine analogue 2CA on cells of the astroglial lineage (Abbracchio et al., 1995; Ceruti, Barbieri et al., 1997). The present study was undertaken with the aim of defining the actions of this analogue on PBMC, in comparison with another adenosine analogue, 2CdA (cladribine) which is already known to trigger apoptosis of lymphoid cells (Beutler, 1992; Bryson and Sorkin, 1993; Sasvari-Szekely et al., 1994; Wood, 1994). It is important to elucidate the mechanisms at the basis of the action of these compounds on immune cells, with particular reference to the possible role of extracellular P1 receptors. If extracellular receptors do play a role, novel therapeutic strategies may arise from the possibility of modulating the actions mediated by these receptors via specific pharmacological agents. Both adenosine analogues induced apoptosis of PBMC, as confirmed by both flow-cytometry and morphological evidence and

DNA fragmentation by agarose gel-electrophoresis. In a similar way to cells of the astroglial lineage (Ceruti, Barbieri et al., 1997), mitochondria do not seem to play a role in triggering apoptosis by adenosine analogues in these cells, as demonstrated by the fact that mitochondrial damage followed the same kinetics as the apoptotic process and therefore seems to represent a consequence rather than a cause of apoptosis. Similar to previous results with thymocytes (Szondy, 1995), 2CdA was more potent than 2CA in inducing apoptosis.

Our results also suggest that these two compounds utilize completely different apoptotic pathways.

In particular, apoptosis by 2CA was shown to involve both extracellular P1 receptors likely belonging to the A_{2A} subtype and a direct intracellular action following entry of the nucleoside into cells through the DP-sensitive membrane transporter. Moreover, phosphorylation to some other active metabolite (2-chloro-ATP?) may play a role in the apoptotic actions of this compound, as shown by the partial reversal of its effects by the kinase inhibitor 5-Itu. On the contrary, apoptosis by 2CdA was neither affected by DP nor by adenosine receptor antagonists, which rules out a role for P1 receptors and suggests that, if this nucleoside enters the cells, entry does not occur through the DP-sensitive transporter. This is exactly the opposite with respect to that previously observed on thymocytes, where DP significantly protected against 2CdA- but not against 2CA-induced apoptosis (Szondy, 1995). Such a difference in the sensitivity of these effects to the nucleoside transport inhibitor may reside in the different maturation/differentiation state of thymocytes with respect to mature lymphocytes.

Unlike a previous finding with pre-B leukemia cells (Gao et al., 1995), phosphorylation of 2CdA to 2CdATP may not play a key role in apoptosis of human quiescent PBMC, as suggested by the fact that the kinase inhibitor 5-Itu could not ameliorate apoptosis by 2CdA in our experimental system. Although the involvement of other 5-Itu-insensitive kinases cannot be ruled out, this may be due to differences in the metabolic activities of normal vs neoplastic lymphoid cells. Interestingly, we observed more or less the same effects on cells of the astroglial lineage, where toxicity by 2CdA could not be reverted upon pretreatment with 5-Itu, which could instead rescue cells from 2CA-induced death (Ceruti, S., Cattabeni, F., Malorni, W, and Abbracchio, MP, unpublished observations). Moreover, mechanisms other than phosphorylation of 2CdA have been previously suggested to contribute to the cytotoxic actions of this nucleoside. Irreversible inhibition of S-adenosyl-homocysteinase, with consequent perturbation of methylation processes and parallel reduction of enzyme-bound NAD⁺ (Abeles et al., 1980) have been suggested to play a role in 2CdA-induced apoptosis of human primary CNS lymphoma (Fabianowska-Majewska et al., 1995). These mechanisms were not specifically investigated in the present study but will be the subject of future investigations.

Our results also suggest that adenosine may utilize several different pathways in triggering apoptosis of PBMC. One pathway seems to involve the activation of extracellular A_{2A} adenosine receptors and accounts for approximately 30–40% of the apoptotic effects induced by 2CA. This result is in agreement with that previously found on chick ventricular

myocytes (Strickler et al., 1996). A second pathway involves the adenosine A₃ receptor which has indeed been previously reported to mediate apoptosis of other cell types, including both myeloid and lymphoid cells (Kohn et al., 1996; Yao et al., 1997). However, the results of studies performed on CHO cells transfected with the human A₃ receptor strongly argue against an involvement of this pathway in either 2CA- or 2CdA-induced apoptosis (Brambilla, R., Barbieri, D., Ceruti, S., Cattabeni, F., Klotz, K.-N., Lohse, M., Franceschi, C. Jacobson, K.A. and Abbracchio, M.P., unpublished observations). Activation of the A₃ receptor did not seem to be necessary for 2CA- and 2CdA-induced effects.

A third pathway seems to be directly activated intracellularly following entry of adenosine into cells; dissection of this pathway with different adenosine analogues such as 2CA or 2CdA suggests that multiple metabolic events may be responsible for initiation of the apoptotic process, not only phosphorylation by nucleoside kinase, but also some other as yet unidentified activation mechanisms.

On the whole, these results confirm that adenosine is a critical regulator of cell survival in cells of the immune system and reinforce the concept that pathological alterations of the 'adenosinergic' system may result in serious impairments of the immune system. For example, excessive accumulation of both adenosine and 2'-deoxy-adenosine in adenosine-deaminase immuno-deficiency syndrome results in a dramatic deletion of immune cells (Giblett et al., 1972; Cohen et al., 1978). Moreover, the present results also suggest that, in a similar way to cells of the neural lineage, modulation of apoptosis by adenosine seems to involve both receptor-independent and receptor-mediated mechanisms (Abbracchio et al., 1997). These latter may represent a novel target for the identification and development of new pharmacological agents, either receptor agonists or antagonists, that may prove useful, respectively, in either anti-tumor therapy or in reducing cell death in immuno-deficiency syndromes.

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Abbreviations:

ADO	adenosine
CF	coformycin
2CA	2-chloro-adenosine
2CdA	2-chloro-2'-deoxy-adenosine
CI-IB-MECA	2-chloro-N ⁶ -(3-iodobenzyl)adenosine-5'-N-methyluronamide
DP	dipyridamole

DPCPX	1,3-dipropyl-8-cyclopentylxanthine
5-Itu	5-iodotubercidin
JC-1	5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazol carbocyanine iodide
PBMC	peripheral blood mononuclear cells
PI	propidium iodide
SCH 58261	5-amino-7-(phenylethyl)-2-(2-furyl)-pyrazolo.4,3-e]1,2,4-triazolo.1,5-c]pyrimidine
XAC	8-[4-[[[(2-aminoethyl)amino]-carbonyl]methyl]oxy]phenyl]-1,3-dipropylxanthine

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Fig. 1.

Flow cytometric analysis of apoptosis and mitochondrial function in 2CA- and 2CdA-induced apoptosis of human PBMC. A and B: control untreated cells; C and D: cells treated with 10 μ M 2CA; E and F: cells treated with 1 μ M 2CdA. Left panels (A, C and E): cytofluorimetric analysis of human PBMC DNA content, as assessed by the PI-staining technique. Numbers indicate the percentage of cells with hypodiploid DNA content, i.e. apoptotic cells. Right panels (B, D and F): cytofluorimetric analysis of human PBMC mitochondrial function, as assessed with the mitochondrial-specific dye JC-1. Numbers

indicate the percentage of cells with depolarized mitochondria. Cells were analysed after 72 h of incubation in the absence (control) or presence of adenosine analogues. A representative experiment is shown in the figure; similar data were obtained in at least other 5 independent experiments (see also data in Table 1).

Fig. 2. Apoptosis of PBMC by 2CA and 2CdA is both concentration- and time-dependent. Cells were maintained for the indicated time-periods in the absence (C) or presence of either 2CA (upper panel) or 2CdA (lower panel) at the reported concentrations. Apoptosis was assessed by flow-cytometry (Fig. 1). The percentage of apoptotic cells is reported; data represent the mean $\pm$ S.E.M. of 3 experiments run in triplicate. * $P < 0.05$ with respect to corresponding control; # $P < 0.05$ with respect to corresponding control, 10 μ M 2CA and 1 μ M 2CA; $\wedge$ $P <$

0.05 with respect to corresponding control, 100 μM 2CA and 1 μM 2CA; two-way ANOVA (Bonferroni correction).

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Fig. 3. 2CA- and 2CdA-treated human PBMC show the morphological alterations typical of apoptosis. Morphological characteristics of control untreated cells (A) and PBMC exposed to either 10 μ M 2CA (B) or 1 μ M 2CdA (C) for 24 h of incubation. Treated cultures show classical features of apoptosis, such as condensed and fragmented state of the nuclear chromatin (arrows). Zeiss axioscope, magnification 1250 $\times$.

	C			2CdA			2CA		
T_0	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h

Fig. 4.

DNA fragmentation in 2CA- and 2CdA-treated human PBMC. Human PBMC (10^6 cells) were incubated in the absence (C) or presence of either $10 \mu\text{M}$ 2CA or $1 \mu\text{M}$ 2CdA for 24, 48 or 72 h. Crude extracts of cells underwent electrophoresis on 2% agarose gels to visualize DNA (see Experimental procedures for further details); T_0 : high molecular weight DNA from untreated PBMC, recovered immediately after lymphoprep separation. Results from a typical experiment are shown; similar data were obtained in 5 independent experiments.

Fig. 5.

Apoptosis of human PBMC by the endogenous nucleoside adenosine in the presence of the adenosine-deaminase inhibitor coformycin. Human PBMC were incubated for the indicated time periods in the absence (C) or presence of 1 mM ADO with or without the adenosine-deaminase inhibitor CF (1 μ M). Apoptosis was evaluated by flow cytometric analysis of PI-stained cell nuclei. CF alone had no significant effect on the % of apoptotic cells with respect to corresponding untreated controls (data not shown). Results from a typical experiment are shown.

Fig.6.

DP partially protects human PBMC against 2CA- but not 2CdA-induced apoptosis. Cells were incubated in the absence (C) or presence of either 10 μ M 2CA or 1 μ M 2CdA with or without 10 μ M DP, as indicated. After 24, 48 or 72 h, the percentage of apoptotic cells was quantified by flow cytometric analysis of PI-stained cell nuclei. Data represent the mean $\pm$ S.E.M. of determinations obtained from 4 separate experiments. # $P < 0.05$ with respect to corresponding control; * $P < 0.05$ with respect to 2CA alone; one-way ANOVA (Scheffe's F test). In parallel samples, DP alone had no effects on the % of apoptotic cells with respect to corresponding control cells.

Fig. 7.

5-Itu partially protects human PBMC against 2CA- but not 2CdA-induced apoptosis. Cells were incubated in the absence (C) or presence of either 10 μ M 2CA or 1 μ M 2CdA with or without 100 nM 5-Itu, as indicated. After 24, 48 or 72 h, the percentage of apoptotic cells was quantified by flow cytometric analysis of PI-stained cell nuclei. Data represent the mean $\pm$ S.E.M. of determinations obtained from 3 separate experiments. * P < 0.05 with respect to corresponding control; Λ P < 0.05 with respect to 2CA alone; one-way ANOVA (Scheffe's F -test). 5-Itu alone had no effects on the % of apoptotic cells with respect to corresponding control cells at any of the reported time points.

Fig. 8.

The A_1/A_{2A} antagonist XAC partially protects human PBMC against 2CA- but not 2CdA-induced apoptosis. Cells were incubated in the absence (C) or presence of either 10 μ M 2CA or 1 μ M 2CdA with or without 1 μ M XAC, as indicated. After 72 h, the percentage of apoptotic cells was quantified by flow cytometric analysis of PI-stained cell nuclei. Data represent the mean $\pm$ S.E.M. of determinations obtained from 6 separate experiments. * P < 0.05 with respect to control; # P < 0.05 with respect to control and 2CA alone; one-way ANOVA (Scheffe's F -test). In cells exposed to XAC alone, the % of apoptotic cells was not statistically different with respect to control (7.5 ± 1.4 vs 7.0 ± 1.1).

Fig. 9.

Partial involvement of the adenosine A_{2A} receptor in 2-CA-induced apoptosis of human PBMC. Cells were incubated in the absence (C) or presence of 10 μ M 2CA with or without the indicated adenosine receptor antagonists (either 1 μ M XAC, or 1 μ M DPCPX or 50 nM SCH 58261) as indicated. After 72 h, the percentage of apoptotic cells was quantified by flow cytometric analysis of PI-stained cell nuclei. Data represent the mean $\pm$ S.E.M. of determinations obtained in 4 separate experiments. XAC, DPCPX and SCH 58261 alone had no effect on the percentage of apoptotic cells (data not shown). * $P < 0.05$ with respect to

control; # $P < 0.05$ with respect to control and 2CA alone; one-way ANOVA (Scheffe's F -test).

Fig. 10.

Induction of apoptosis of human PBMC by activation of the adenosine A₃ receptor. Cells were incubated in the absence (C) or presence of CI-IB-MECA at the indicated concentrations. After 24, 48 or 72 h, the percentage of apoptotic cells was quantified by flow cytometric analysis of PI-stained cell nuclei. Data represent the mean ± S.E.M. of determinations obtained in 3 separate experiments. **P* < 0.05 with respect to corresponding control; one-way ANOVA (Scheffe's *F*-test).

Table 1

2CA- and 2CdA-induced apoptosis in human PBMC

Time (h)	Controls	2CA (10 μ M)	2CdA (1 μ M)
24	2.3 $\pm$ 0.3	8.2 $\pm$ 1.5	18.4 $\pm$ 3.3 *
48	6.2 $\pm$ 0.6	22.5 $\pm$ 1.7 *	57.7 $\pm$ 2.7 * [†]
72	7.9 $\pm$ 0.9	45.8 $\pm$ 2.4 *	81.2 $\pm$ 1.9 * [†]

Cells were incubated with 10 μ M 2CA or 1 μ M 2CdA for 24, 48 and 72 h. At the end of the incubation time percentage of apoptotic cells was assessed with cytofluorimetric analysis after PI-staining as described in Experimental procedures. Data represent mean $\pm$ S.E.M. of 18 independent experiments.

* $P < 0.05$ with respect to corresponding control;

[†] $P < 0.05$ with respect to corresponding 10 μ M 2CA, one-way ANOVA (Scheffe's F -test).

Table 2

Correlation between the extent of apoptosis and of mitochondrial depolarization in 2CA- and 2CdA-treated human PBMC

Time (h)		Controls	2CA (10 μ M)	2CdA (1 μ M)
24	% apoptotic cells	2.3 $\pm$ 0.3	8.6 $\pm$ 1.8	19.3 $\pm$ 3.9 *
	% cells with dep. mit.	4.8 $\pm$ 0.6	12.3 $\pm$ 1.1	23.8 $\pm$ 4.1 *
48	% apoptotic cells	6.8 $\pm$ 0.8	26.8 $\pm$ 3.5 **	59.9 $\pm$ 7.1 *
	% cells with dep. mit.	9.6 $\pm$ 1.6	20.3 $\pm$ 4.9	53.3 $\pm$ 9.4 *
72	% apoptotic cells	8.3 $\pm$ 2.0	52.0 $\pm$ 4.2 **	83.3 $\pm$ 2.4 *
	% cells with dep. mit.	12.3 $\pm$ 2.9	51.2 $\pm$ 3.9 **	74.4 $\pm$ 3.3 *

Cells were incubated with both adenosine analogues for different time-points. At the end of the incubation time cytofluorimetric analysis was performed to determinate percentage of apoptotic cells after PI-staining and percentage of cells with depolarized mitochondria (% cells with dep. mit.) after JC-1 staining as described in Experimental procedures. Data represent mean $\pm$ S.E.M. of six independent experiments.

* $P < 0.05$ with respect to corresponding control and 10 μ M 2CA;

** $P < 0.05$ with respect to corresponding control; one-way ANOVA, Scheffe's F -test