The 2-chlorodeoxyadenosine-induced cell death signalling pathway in human thymocytes is different from that induced by 2-chloroadenosine

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2-Chloroadenosine induced DNA fragmentation and cell death in human thymocytes primarily by Ca^{2+} -dependent mechanisms. Incubation of human thymocytes with 2-chlorodeoxyadenosine (5–1000 nM) also induced cell death (apoptosis) which was dependent on macromolecule synthesis and involved activation of an endonuclease which was inhibited by Zn^{2+} . The effect of 2-chlorodeoxyadenosine was prevented by addition of dipyrindamole, a strong nucleoside transport inhibitor, or of deoxycytidine, previously shown to compete for uptake by deoxycytidine kinase. 2-Chlorodeoxyadenosine-induced

apoptosis did not involve increases in the cytosolic Ca^{2+} concentration, but required the presence of intracellular Ca^{2+} . It was not inhibited by activators of protein kinase C previously shown to inhibit Ca^{2+} -dependent cell death. Addition of 2-chlorodeoxyadenosine induced an increase in the amount of p53 in human thymocytes, while 2-chloroadenosine had no effect. These data suggest that 2-chloroadenosine and 2-chlorodeoxyadenosine induce cell death in human thymocytes via different signalling pathways.

INTRODUCTION

Apoptosis is a genetically programmed mechanism of cell death which is often characterized biochemically by internucleosomal DNA cleavage [1]. In the thymus it is a critically important component of the intrathymic cell selection process which functions to prevent autoimmunity. Several stimuli have been reported to induce apoptosis in thymocytes, including an antibody against the CD3/T-cell receptor complex [2], glucocorticoids [3], ATP [4] and various purine nucleosides [5]. We have shown recently [6] that 2-chloroadenosine (2-CA), a receptor site analogue of adenosine, triggers apoptosis in human thymocytes primarily by inducing a sustained elevation in the cytosolic Ca^{2+} concentration. Although the K_m of adenosine kinase for 2-CA is very high and the V_{max} is very low [7], we could not exclude the possibility that during long-term incubation some of the 2-CA is taken up and converted to 2-chlorodeoxyadenosine (2-dCA) or its metabolites, with the compounds formed being responsible for the observed cell death.

2-CdA is a congener of deoxyadenosine that is resistant to adenosine deaminase. It was developed as an immunosuppressive and anti-neoplastic agent based on the observation that elevated plasma deoxyadenosine is lymphocytotoxic in congenital deficiencies of adenosine deaminase [8]. 2-CdA was reported to induce programmed cell death in peripheral resting lymphocytes [9] by an unknown mechanism. Suggestions were made that the accumulated chloro-dATP formed from 2-CdA induced DNA strand breaks which activated poly(ADP-ribosylation) and led to exhaustion of the NAD^+ pool [10]. The present studies, which characterize 2-CdA-induced cell death in human thymocytes, were undertaken to determine whether 2-CdA or 2-chloro-dATP formation could be responsible for the cytotoxic effect of 2-CA. The data presented in this paper suggest that 2-CA and 2-CdA induce cell death in human thymocytes via different signalling pathways.

MATERIALS AND METHODS

Cell preparation and culture

Thymus glands were obtained from patients of less than 2 years old that were undergoing corrective cardiac surgery. Thymocyte suspensions were prepared by mincing the glands in RPMI 1640 medium (Gibco Ltd., Paisley, Scotland, U.K.) supplemented with 2% foetal calf serum (FCS) (Gibco), 2 mM glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin. Thymocytes were washed three times and diluted to a final concentration of 10^7 cells/ml before incubation at 37 °C in a humidified incubator under an atmosphere of 5% CO_2 and 95% air. Cell death was measured by Trypan Blue uptake. A total of 95–98% of cells routinely excluded Trypan Blue after the isolation procedures.

Quantification of DNA fragmentation

Thymocytes were incubated in 24-well plates with various agents. After 1 day, 0.8 ml of cell suspension was lysed by addition of 0.7 ml of ice-cold lysis buffer containing 0.5% (v/v) Triton X-100, 10 mM Tris and 20 mM EDTA, pH 8.0, before centrifugation for 15 min at 13000 g in a microcentrifuge. The DNA in the supernatant (DNA fragments) and pellets (intact chromatin) was precipitated with equal amounts of 10% trichloroacetic acid, resuspended in 5% trichloroacetic acid and quantified using the diphenylamine reagent [11]. Alternatively, the cells were lysed as described; the 13000 g supernatants were then precipitated overnight in ethanol containing 0.15 mM NaCl. The pellets were redissolved in a buffer containing 10 mM Tris, 1 mM EDTA, pH 8.0, treated with RNase, sequentially extracted with equal volumes of phenol and chloroform/3-methylbutan-1-ol (24:1, v/v) and precipitated in ethanol before electrophoresis for 3 h at 60 V in a 1.8% agarose gel. DNA fragments were then visualized by UV light after staining the gel with ethidium bromide.

Measurement of cytosolic Ca^{2+} concentration

Thymocytes (10^7 cells/ml) were incubated in the presence of 5 μM fura-2/AM in RPMI 1640 medium supplemented with 2% FCS for 30 min at room temperature, washed free of extracellular fura-2 with Krebs–Henseleit medium supplemented with 20 mM Hepes, 5.5 mM glucose, 1.3 mM CaCl_2 and 2% BSA, pH 7.2, and incubated for another 15 min to allow the non-hydrolysed fura-2 to diffuse from the cells. 2-CdA was either added to this medium and the changes in the cytosolic Ca^{2+} concentration were determined by taking samples at different time points, or added directly to the cuvette to determine the immediate Ca^{2+} changes. Before measuring the cytosolic Ca^{2+} concentration, cells were resuspended in the same medium supplemented with 0.2% BSA. Fluorescence intensity was determined on a Sigma ZFP 22 dual-wavelength spectrofluorimeter equipped with a thermostatically controlled cuvette holder to maintain the temperature at 30 °C in order to minimize dye leakage from the cells [12]. Changes in the fluorescence intensity were monitored by recording changes in the fluorescence ratio, as previously described [13].

Western blot analysis of p53 expression

At various time points of culture, 10^7 cells were collected, washed with PBS and lysed in Laemmli buffer [14]. The proteins were separated on SDS/7.5%-polyacrylamide gels and transferred to poly(vinylidene difluoride) membranes (Millipore). The membrane was blocked and probed with a specific monoclonal antibody (PAb 1801) against p53. p53 was detected by using a horseradish peroxidase-labelled second antibody and the Amersham ECL system.

Materials

2-CdA was a gift from Tanya Spasokukotskaja (Department of Biochemistry, Karolinska Institutet, Stockholm, Sweden), while the specific monoclonal antibody against p53 was a gift from László Székely (Department of Tumor Biology, Karolinska Institutet). Fura-2/AM, RNase A and T_1 were purchased from Boehringer (Mannheim, Germany); 2-CA, cycloheximide, deoxycytidine, dipyrindamole, actinomycin D, phorbol dibutyrate (PdBu), nicotinamide, 3-aminobenzamide and quin-2/AM were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other reagents used were obtained from commercial sources and were of analytical grade.

RESULTS

2-CdA induces DNA fragmentation in human thymocytes that is characteristic of apoptotic cells

2-CdA induced a dose-dependent increase in DNA fragmentation and loss of cell viability in human thymocytes (Table 1). Compared with 2-CA, much lower concentrations of 2-CdA were required to induce cell death. Whereas in the presence of 2-CA there was no significant increase in DNA cleavage up to 1 μM , and the maximum DNA fragmentation was observed at about 40 μM [6], with 2-CdA significant increases were observed with concentrations below 1 μM . This finding is in good agreement with the previously reported EC_{50} of 0.43 μM in FM3A cells [15].

Endonuclease activation in apoptotic cells results in the production of oligosome-length DNA fragments ('DNA ladder') which can be distinguished from the random cleavage observed in cells undergoing necrosis [16]. The chromatin cleavage pattern

Table 1 Effect of increasing concentrations of 2-CdA on DNA fragmentation and cell viability of human thymocytes after 24 h in culture

Thymocytes (10^7 cells/ml) were incubated for 24 h in the presence of the indicated compounds. The amount of fragmented DNA and the cell viability were determined as described in the Materials and methods section. Data represent means $\pm$ S.D. of three determinations.

Compound	Concentration (μM)	Fragmented DNA (%)	Viability (%)
None	—	21.7 $\pm$ 2.1	85 $\pm$ 2
2-CdA	0.005	27.1 $\pm$ 0.4	79 $\pm$ 1
	0.010	30.9 $\pm$ 0.1	75 $\pm$ 1
	0.1	40.6 $\pm$ 0.9	65 $\pm$ 2
	1.0	41.2 $\pm$ 0.9	62 $\pm$ 1
	5.0	42.5 $\pm$ 0.3	61 $\pm$ 2
	10.0	44.4 $\pm$ 2.5	60 $\pm$ 1
	20.0	47.9 $\pm$ 2.4	58 $\pm$ 2
2-CA	40.0	43.7 $\pm$ 1.3	65 $\pm$ 3

Figure 1 Electrophoretic fractionation of DNA extracted from cultured human thymocytes

Thymocytes were cultured with or without 20 μM 2-CdA for 24 h. The DNA was extracted, electrophoresed on a 1.8% agarose gel as described in the Materials and methods section and visualized after staining with ethidium bromide. Lane 1, DNA from 2-CdA-treated thymocytes; lane 2, DNA from 2-CdA-treated thymocytes in the presence of 1 mM deoxycytidine; lane 3, DNA from untreated thymocytes.

elicited by 2-CdA (Figure 1) was indistinguishable from that observed in 2-CA-treated thymocytes undergoing programmed cell death [6], suggesting that the same endonuclease activity was involved.

DNA fragmentation induced by glucocorticoids has been reported to be inhibited by Zn^{2+} ions [17], and we have found that zinc also inhibits 2-CA-induced DNA fragmentation [6]. In the present study, zinc at a 0.5 mM inhibited DNA cleavage in the 2-CdA-treated thymocytes (Table 2).

DNA fragmentation induced by 2-CdA requires macromolecule synthesis

2-CA-induced DNA fragmentation and cell killing were found to require macromolecule synthesis. 2-CdA-induced DNA fragmentation was also inhibited to below control levels by actinomycin D at a concentration of 10 $\mu\text{g}/\text{ml}$, and by cycloheximide

Table 2 Effect of various compounds on DNA fragmentation induced by 2-CdA in human thymocytes

Thymocytes (10^7 cells/ml) were incubated for 24 h in the presence of the indicated compounds. The amount of fragmented DNA and the cell viability (values in parentheses; %) were determined as described in the Materials and methods section. Data represent means $\pm$ S.D. of three determinations. * Differs significantly from the 2-CdA-treated control as determined by Student's paired *t* test; $P < 0.05$.

Compound added	DNA fragmentation (%)	
	—CdA	+ 2-CdA (10 nM)
No addition	17.5 $\pm$ 1.2 (94)	31.1 $\pm$ 2.3 (75)
ZnSO ₄ (0.5 mM)	14.1 $\pm$ 0.5 (81)	12.1 $\pm$ 0.2* (80)
Actinomycin D (10 μ g/ml)	10.5 $\pm$ 1.7 (89)	13.5 $\pm$ 2.1* (88)
Cycloheximide (50 μ g/ml)	15.9 $\pm$ 2.8 (96)	16.4 $\pm$ 1.2* (95)
Dipyridamole (50 μ M)	17.1 $\pm$ 1.0 (94)	18.5 $\pm$ 0.7* (92)
Deoxycytidine (1 mM)	17.9 $\pm$ 0.8 (92)	20.4 $\pm$ 1.1* (87)
3-Aminobenzamide (5 mM)	15.5 $\pm$ 2.1 (92)	28.9 $\pm$ 3.3 (80)
Nicotinamide (5 mM)	19.1 $\pm$ 3.8 (93)	33.0 $\pm$ 2.3 (89)
Quin-2 (100 μ M)	10.5 $\pm$ 0.7 (97)	12.2 $\pm$ 1.4* (94)
PdBu (100 ng/ml)	10.7 $\pm$ 1.4 (97)	30.7 $\pm$ 1.1 (76)

at a concentration of 50 μ g/ml (Table 2). A decrease in DNA fragmentation was accompanied by an increase in cell viability, as judged by the Trypan Blue exclusion test. Both compounds also inhibited the spontaneous DNA fragmentation observed in the control samples, suggesting that ongoing transcription and protein synthesis were required for the induction of DNA cleavage.

2-CdA must be metabolized to induce cell death in human thymocytes

It was previously shown [18] that the lymphocytotoxic effect of 2-CdA requires its phosphorylation and is inhibited by deoxycytidine. To test whether the uptake of 2-CdA is also required for the induction of thymocyte cell death, thymocytes were incubated with 50 μ M dipyridamole, a strong nucleoside transport inhibitor [19], or 1 mM deoxycytidine before addition of 2-CdA (Table 2). Dipyridamole or deoxycytidine alone did not affect the viability of human thymocytes, but significantly reduced the DNA fragmentation and cell death induced by 2-CdA (Figure 1). On the other hand, dipyridamole had no effect on 2-CA-induced cell death (results not shown), in accordance with our previous observation [6] that 2-CA induces cell death in human thymocytes by activating cell surface adenosine receptors.

2-CdA-induced cell death is not inhibited by inhibitors of poly(ADP-ribosylation)

Activation of poly(ADP-ribosylation), a process which utilizes cellular NAD⁺, is closely related to the repair of DNA strand breaks [20]. Carson and co-workers [18] demonstrated that 2-CdA induced DNA breaks in resting human lymphocytes and led to cell death by exhausting the NAD⁺ pool. They have shown

Figure 2 p53 expression in 2-CA- and 2-CdA-treated human thymocytes at various time points

Human thymocytes were isolated and cultured either in the absence or in the presence of 40 μ M 2-CA or 20 μ M 2-CdA. Samples of 1×10^7 cells were collected at the indicated time points, washed in PBS, lysed in Laemmli buffer and tested for p53 expression by Western blot analysis as described in the Materials and methods section.

that inhibitors of poly(ADP-ribosylation) or addition of nicotinamide, a precursor of NAD⁺, can reduce lymphoid cell death. In human thymocytes, however, 3-aminobenzamide, an inhibitor of poly(ADP-ribosylation), or nicotinamide had no effect on the DNA fragmentation induced by 2-CdA (Table 2), although both compounds increased cell viability. Our findings suggest that exhaustion of the NAD⁺ pool may have a role in the mechanism of cell death induced by 2-CdA, but cannot be a determining factor, since endonuclease activation was not prevented by the addition of nicotinamide.

2-CdA-induced cell death requires Ca²⁺

It was previously shown that an increase in the cytosolic Ca²⁺ concentration precedes DNA fragmentation in thymocytes exposed to a number of different stimuli [2,4,21,22], including 2-CA [6]. To determine whether a similar Ca²⁺ increase also preceded 2-CdA-induced DNA fragmentation, thymocytes were loaded with the Ca²⁺ probe fura-2 and then incubated with 2-CdA. Incubation with 2-CdA did not change the cytosolic Ca²⁺ concentration significantly during the 2 h period examined (results not shown). On the other hand, chelation of intracellular Ca²⁺ using another Ca²⁺ dye, quin-2/AM, did block 2-CdA-stimulated DNA fragmentation (Table 2). This suggested that, although Ca²⁺ did not participate in the signalling pathway of 2-CdA-induced cell death, the process required the presence of intracellular Ca²⁺.

Protein kinase C activation does not interfere with 2-CdA-induced cell death

Previous work has shown that activators of protein kinase C can inhibit the Ca²⁺-stimulated pathway of endonuclease activation in thymocytes [23], including 2-CA-induced cell death [6]. To test whether protein kinase C activation can also interfere with 2-CdA-induced cell death, PdBu was added at the beginning of the culture. As shown in Table 2, the phorbol ester failed to inhibit 2-CdA-induced DNA fragmentation and cell death.

2-CdA induces sustained expression of p53

Compounds which cause DNA strand breakage in thymocytes were shown to induce and require the p53 tumour suppressor protein in the initiation of the apoptotic program [24,25]. Since 2-CdA was shown to induce DNA strand breaks [18], it was interesting to see whether it affected the expression of p53 in human thymocytes. As shown in Figure 2, p53 expression was slightly increased at 3 h both in the control and 2-CA-treated thymocytes. However, after 6 h, when the first increase in the amount of DNA fragmentation was detected (results not shown),

this increase had already disappeared. On the other hand, 2-CdA induced a significant and sustained increase in p53 expression.

DISCUSSION

The present study shows that human thymocytes exposed to 2-CdA undergo programmed cell death. Cell death induced by 2-CdA, similar to that triggered by 2-CA, required active protein and RNA synthesis and involved activation of an endonuclease which resulted in DNA fragmentation. Zinc, which was shown to inhibit endonuclease activation induced by glucocorticosteroid hormones [17] or by 2-CA [6] in thymocytes, also inhibited 2-CdA-induced DNA fragmentation in the present study. These observations suggest that a similar programme of cell death was initiated which is regulated by newly synthesized proteins and involves activation of a common endonuclease.

Although it was previously demonstrated that a sustained increase in the cytosolic Ca^{2+} concentration is required for the induction of programmed cell death by various stimuli in thymocytes, 2-CdA-induced cell death did not seem to be dependent on a sustained elevation of cytosolic Ca^{2+} . On the other hand, the processes involved in this apoptotic programme did require the presence of Ca^{2+} , perhaps because of the Ca^{2+} -dependence of some of its key enzymes (endonuclease [16], transglutaminase [26] or calpain [27]). We do not know the exact mechanism by which 2-CdA induces cell death in human thymocytes, but the present data suggest that 2-CdA has to be taken up and further metabolized, the p53 level is increased, and exhaustion of the NAD^+ pool may play an additional role.

In thymocytes a complex signalling system determines whether a cell enters the cell death programme or is positively selected and differentiates into a functionally mature T-cell. Although the mechanism of positive selection is not fully understood and the extracellular signal(s) involved are not yet known, there is increasing evidence that the activation of protein kinase C is involved in this process [23]. In this context it is worthy of note that activation of protein kinase C by PdBu did not prevent 2-CdA-induced cell death. Since addition of 2-CdA mimics the metabolic situation observed in adenosine deaminase-deficient patients characterized by severe combined immunodeficiency syndrome, our observation suggests that the altered deoxy-adenosine metabolism triggers a specific cell death pathway which cannot be controlled by the signals of positive selection.

Our data also demonstrate that 2-CA and 2-CdA induce cell death via different signalling pathways in human thymocytes. 2-CA-induced cell death does not require the uptake of the nucleoside, is dependent on increases in the cytosolic Ca^{2+} concentration, does not involve significant p53 induction and is inhibited by activators of protein kinase C. The 2-CdA-induced pathway requires the phosphorylation of the nucleoside, is independent of increases in cytosolic Ca^{2+} , induces p53 and cannot be prevented by addition of protein kinase C activators.

Although the possibility existed that 2-CA mediates its cytotoxic effects on human thymocytes via 2-CdA metabolites, our data provide evidence for a 2-CA-induced, 2-CdA-independent apoptotic pathway.

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