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CELL DEATH INITIATED BY 3-DEAZAADENOSINE IN HL-60 CELLS IS APOPTOSIS AND IS PARTIALLY INHIBITED BY HOMOCYSTEINE

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Abstract—Cell death initiated by the adenosine analog 3-deazaadenosine (c^3Ado) was studied in human promyelocytic leukemia HL-60 cells. A rapid decrease in cell number was seen after 4-hr exposure to 50–100 μM c^3Ado. The dominating mode of cell death was apoptosis as demonstrated by condensation and fragmentation of the nucleus, formation of apoptotic bodies and endonucleolytic degradation of DNA. Four hour treatment with 100 μM c^3Ado resulted in a reduction of early S-phase cells, and appearance of cells with a lower DNA and protein content than that of the G_1 population. Whereas 25 and 50 μM c^3Ado only initiated apoptosis in S-phase cells, 75 and 100 μM c^3Ado also initiated apoptosis in G_1− and G_2 + M-phase cells, suggesting different mechanisms for cell death at different concentrations. Apoptosis initiated by 100 μM c^3Ado was completely inhibited by 1 mM $ZnCl_2$. Addition of homocysteine thiolactone (Hcy) partly inhibited cell death by c^3Ado. Light microscopic examination of cultures treated with 100 μM c^3Ado and 1 mM Hcy showed nuclear condensation and fragmentation consistent with the first stage in apoptosis, however, only a minor formation of apoptotic bodies took place in these cultures compared to that observed in cultures treated with 100 μM c^3Ado alone. The modifying action of Hcy on c^3Ado initiated apoptosis in HL-60 cells and this suggests that c^3Ado and 3-deazaadenosylhomocysteine (c^3AdoHcy) interact with different targets during initiation and progression of cell death in this cell line.

3-Deazaadenosine (c^3Ado§) is an adenosine analog with diverse biological effects including cytotoxicity [1–9]. c^3Ado is both an inhibitor and a substrate for *S*-adenosylhomocysteine (AdoHcy) hydrolase (EC 3.3.1.1.), and subsequent perturbations of transmethylation reactions are probably one part of its mechanism of action [10–12]. However, other modes of action for c^3Ado are reported [13, 14], and the mechanism by which c^3Ado causes cell death is still unknown.

In our previous studies of HL-60 cells treated with c^3Ado we observed loss of cells and extensive cell death at concentrations above 50 μM [3]. Recent observations from several laboratories indicate that human promyelocytic HL-60 cells respond to several cytotoxic drugs by apoptosis [15–18]. We therefore asked whether apoptosis could be one mode of HL-60 cell death initiated by c^3Ado.

Cells undergoing apoptosis show a set of coordinated structural changes that in the first stage are characterized by condensation and fragmentation of the nuclear chromatin. During the second stage of the process, there is fragmentation of both cytoplasm and nucleus, and the cell separates into small membrane-bound vesicles known as apoptotic bodies [19]. A characteristic biochemical event in apoptotis is activation of an endonuclease that cleaves DNA at internucleosomal linker sites [20]. Some studies have shown that cycling cells arrest in G_2 prior to apoptosis [15, 21]. However, other experimental data suggest that the apoptotic process is selective for cells that progress through S-phase [22].

In the present study we demonstrate that c^3Ado initiates apoptosis in HL-60 cells as evaluated by light microscopy, analyses of DNA fragmentation by agarose gel electrophoresis and flow cytometric analyses. The flow cytometric data indicate that the apoptotic process initiated by c^3Ado in this cell line is first triggered in S-phase cells. We also demonstrate that homocysteine thiolactone (Hcy), which usually potentiates c^3Ado effects, partially inhibits HL-60 cell apoptotis initiated by c^3Ado.

MATERIALS AND METHODS

Chemicals. c^3Ado was obtained from Southern Research Institute (Birmingham, AL, U.S.A.). Hcy, RNase and proteinase K were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Agarose was purchased from Pharmacia AB (Uppsala, Sweden) and 1 Kb ladder from Bethesda Research Laboratories (Rockville, MD, U.S.A.).

Cells. HL-60 cells were grown in RPMI 1640 supplemented with 10% heat inactivated horse serum in a fully humidified atmosphere of 5% CO_2 at 37°.

Experiments. Suspensions of cells were adjusted to concentrations of early logarithmic growth and

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§ Abbreviations: c^3Ado, 3-deazaadenosine; Hcy, homocysteine thiolactone; c^3AdoHcy, 3-deazaadenosylhomocysteine; AdoHcy, *S*-adenosylhomocysteine; c^3Ari, 3-deaza-(±)-aristeromycin; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; PI, propidium iodide.

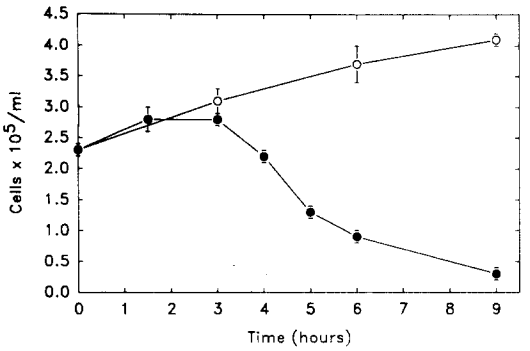

Fig. 1. Number of viable HL-60 cells in cultures treated with 100 μM c^3Ado for 1.5–9 hr. Exponentially growing cells were cultured with 100 μM c^3Ado (●) or medium (○) for 9 hr. Viability was determined as described in Materials and Methods.

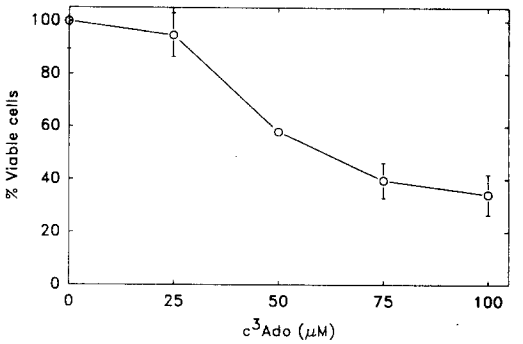

Fig. 2. Number of viable HL-60 cells in cultures treated with varying concentrations of c^3Ado for 6 hr. Exponentially growing cells were treated with different concentrations of c^3Ado as indicated for 6 hr. Results are expressed as per cent viable cells in the c^3Ado treated cultures compared to control cultures after 6 hr.

transferred to wells or culture flasks. The following day drugs dissolved in RPMI 1640 (10-fold of final concentration) were added, and cultures were harvested after 1.5, 3, 4, 5, 6 and 9 hr and processed for cell counting, light microscopic examination, flow cytometric or electrophoretic analysis.

Cell counts. Cell counts were determined by a hemocytometer. Since apoptotic cells exclude Trypan blue, only cells that excluded Trypan blue, and in addition had a smooth cytoplasmatic membrane and a diameter within the range of that of control cells, were judged as viable.

Light microscopic examination. Aliquots of 50 μL of cell suspension were spun in a cytocentrifuge. The cell preparations were then fixed in methanol and stained with May-Grunwald-Giemsa. Cell morphology was evaluated in a Zeiss Axiophot microscope with Plan Neofluar objectives. Photomicrographs were taken with Kodak type TMX 135 film.

Analysis of DNA fragmentation by agarose gel electrophoresis. DNA was extracted and separated according to standard procedures as described by Sambrook *et al.* [23]. Briefly, 10^6 cells were washed twice in phosphate-buffered saline (PBS) and resuspended in lysis buffer containing 5 mM Tris pH 8.0, 50 mM EDTA, 0.5% Triton X-100, 1% sodium dodecylsulfate. RNase was added to a final concentration of 40 μg/mL before the lysate was incubated for 2 hr at 37°. Proteinase K was added to a final concentration of 500 μg/mL, and the incubation continued for an additional 24–48 hr at 50°. The lysates were extracted twice in phenol/chloroform/isoamyl alcohol (25:24:1, by vol.) and once in chloroform/isoamyl alcohol (24:1, v/v). DNA was precipitated with 3 M potassium acetate and 100% ethanol, washed in 70% ethanol and resuspended in TE buffer (10 mM Tris pH 7.8, 1 mM EDTA). Nucleic acid content was determined spectrophotometrically by measuring absorbance at 260 nm, and approximately 2 μg of DNA from each sample were analysed. Electrophoresis was performed on 1.2% agarose gels containing 10 μg/mL ethidium bromide in TBE buffer (90 mM Tris pH 8, 90 mM boric acid, 2 mM EDTA). Gels were photographed under UV light with Polaroid type 55 film.

Measurements of cellular DNA and protein content by flow cytometry. DNA and protein staining were performed basically as described by Crissman *et al.* [24]. Briefly, cells were spun for 5 min at 200 *g*, washed in PBS, resuspended in saline, fixed in 70% ethanol and kept refrigerated until analysis. Prior to analysis, DNA and protein were stained by 5 μg/mL propidium iodide (PI) (Behring Diagnostics, La Jolla, CA, U.S.A.) and 0.37 μg/mL fluorescein isothiocyanate (FITC) isomer 1 (Janssen Chimica, B-2240 Geel, Belgium) in PBS containing 1 mM $MgCl_2$ and 100 μg/mL RNase for 20 min at room temperature. The smallest particles were excluded electronically. Red (PI) and green (FITC) fluorescence were measured on an Argus 100 Flowcytometer (Skatron, Lier, Norway). The percentage of cells in different cell cycle phases was estimated from the PI histograms using the Multicycle program (Phoenix Flow Systems, San Diego, CA, U.S.A.).

RESULTS

Cytotoxicity of c^3Ado

Figure 1 illustrates the number of viable HL-60 cells in cultures treated with 100 μM c^3Ado for 1.5–9 hr as assessed by cell counts. A striking drop in the number of viable cells was seen after 4 hr of incubation, and a reduction in the number of viable cells continued for 5 hr. To characterize the cytotoxic activity of c^3Ado further, cells were incubated with varying concentrations of c^3Ado for 6 hr (Fig. 2). A reduction in the number of viable cells was seen for concentrations above 25 μM.

Mode of cell death initiated by c^3Ado

The light microscopic features of the cell death initiated by 100 μM c^3Ado were typical of apoptosis [19, 25, 26] (Fig. 3). After 1.5 and 3 hr of incubation, cells with a slightly increased chromatin density, less prominent nucleoli, a greater variability in cell

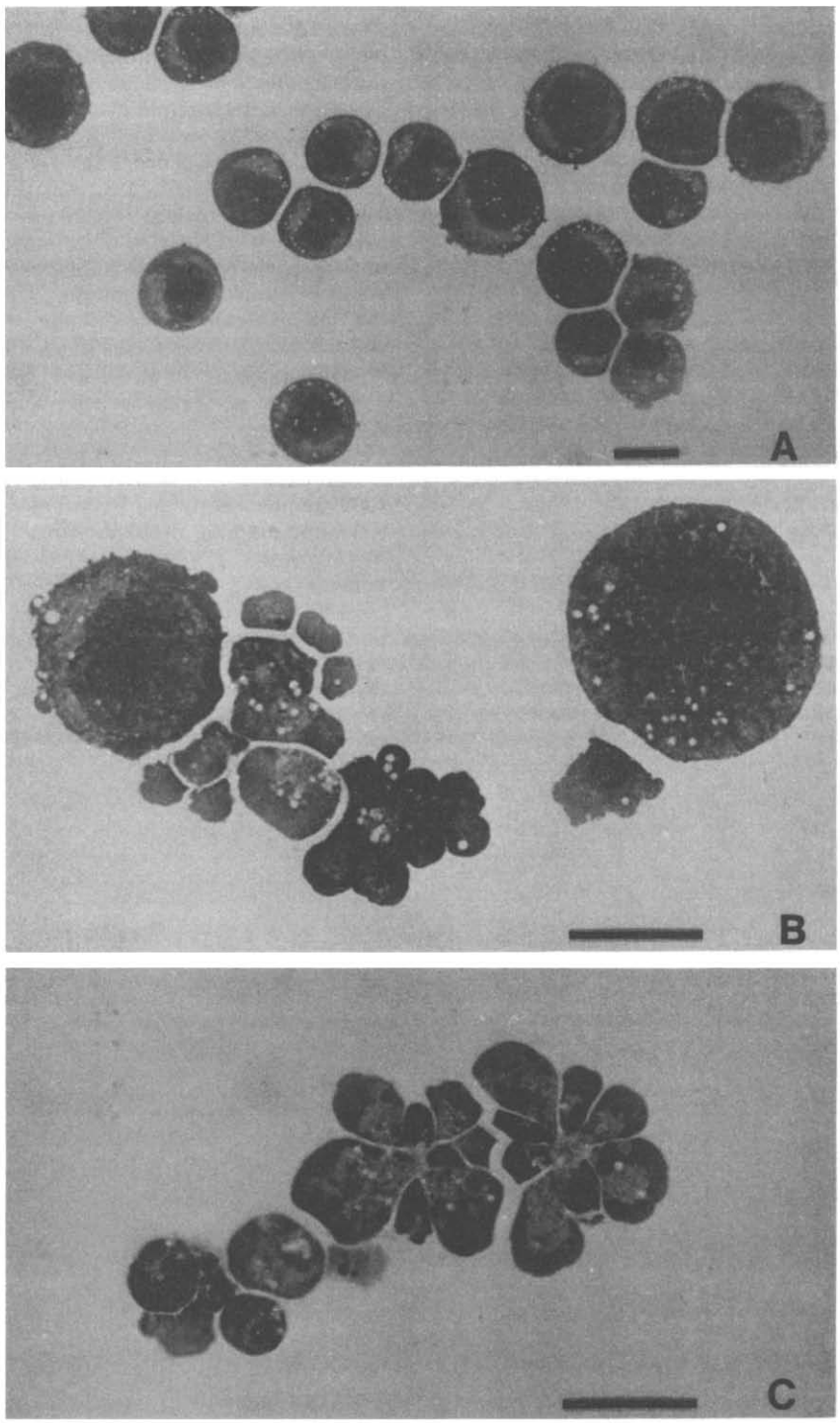

Fig. 3. Morphologic features of control HL-60 cells and cells cultured with 100 μM c^3Ado for up to 9 hr, illustrating typical apoptotic changes. Panel of exponentially growing HL-60 cells spun in cytocentrifuge, fixed in methanol and stained with May-Grunwald-Giemsa, with and without treatment of c^3Ado. (A) Untreated HL-60 cells with slightly varying size, with a rounded outer contour and no sign of cell death. Mitotic cells are seen in the lower right quadrant of the figure. (B and C) HL-60 cells treated with 100 μM c^3Ado. In panel B two intact cells to the far right and left. In addition several cytoplasmic fragments of different size and shape without and with condensed nuclear material consistent with apoptotic bodies. In panel C two apoptotic cells immediately prior to separation into apoptotic bodies. These cells exhibit severe blebbing of the cytoplasma membrane and nuclei completely transformed into several dense micronuclear spheres. To the lower left in panel C, a cluster of three to four apoptotic bodies with dense micronuclear spheres. Scale bar = 5 μm.

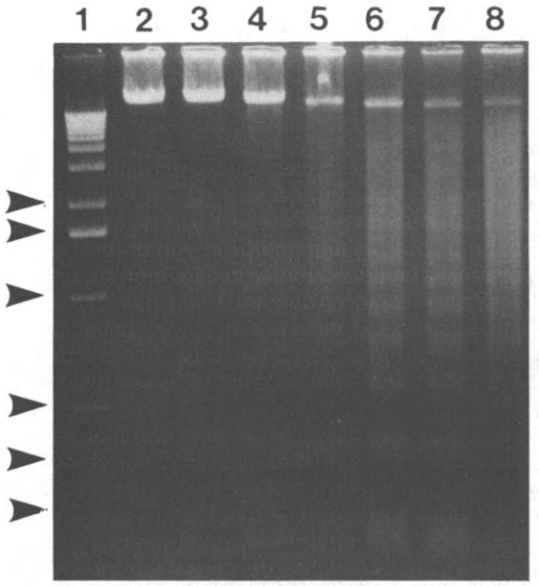

Fig. 4. DNA fragmentation following c³Ado treatment in HL-60 cells. Exponentially growing cells were treated with 100 μM c³Ado for 1.5, 3, 4, 5, 6 and 9 hr (lanes 3–8, respectively) and DNA was extracted and separated as described in Materials and Methods. Lane 2, control; lane 1, 1 Kb DNA ladder; Arrows, 2, 1.6, 1.0, 0.52, 0.35 and 0.22 kilobases from top to bottom.

diameter and a more smooth cell surface were observed. Nuclear fragmentation, cytoplasmatic blebs and small membrane-bound bodies were distinct after 4 hr of incubation. Typical apoptotic cells showed several cytoplasmatic blebs and micronuclear bodies of different size and color density. A pronounced increase in the number of apoptotic bodies, most of them containing micronuclear particles, became apparent after 5 hr of treatment. Lively mitotic activity, with both normal and atypical mitotic figures, was present in control cultures, and mitotic activity persisted through all experimental time intervals. In control cultures, we also observed a small number (less than 1%) of apoptotic cells as well as a few multinucleated giant cells.

Effects of c³Ado on DNA fragmentation

Extracted DNA from c³Ado treated cells was separated by agarose gel electrophoresis. Three and 4 hr after addition of 100 μM c³Ado, small amounts of fragmented DNA emerged on the gel (Fig. 4, lanes 4 and 5). After 5, 6 and 9 hr fragments consisting of multimers of approximately 200 base pairs were evident (Fig. 4, lanes 6, 7 and 8, respectively). These fragments correspond to the digestion products of an endonuclease.

DNA and protein content in HL-60 cells treated with c³Ado

Figure 5 illustrates the DNA and protein content in HL-60 cells treated with 100 μM c³Ado for 4

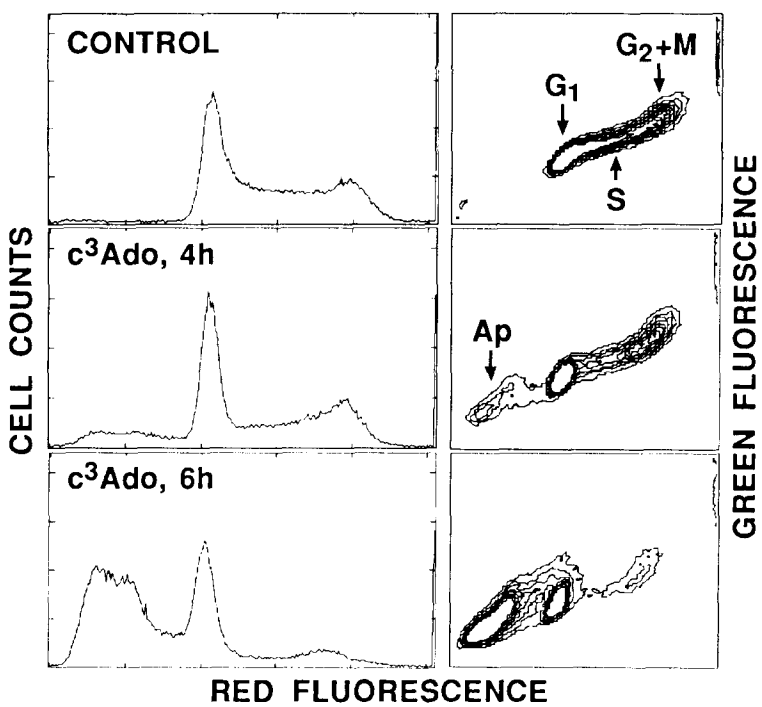

Fig. 5. DNA and protein content in HL-60 cells treated with 100 μM c³Ado. Exponentially growing cells were treated with 100 μM c³Ado for 0, 4 and 6 hr (upper, middle and lower panel, respectively) and processed for flow cytometric analysis as described in Materials and Methods. Right figures show DNA histograms, left figures show bivariate contour plots of red (DNA) and green (protein) fluorescence. Ap indicates apoptotic cells and apoptotic bodies.

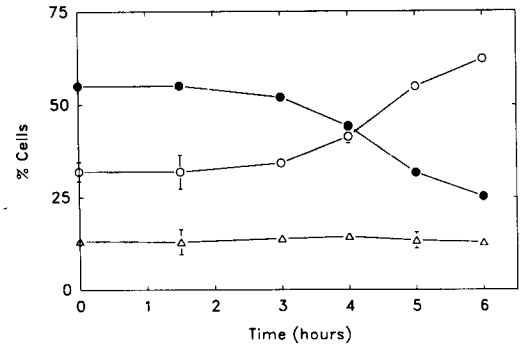

Fig. 6. Cell cycle distribution of HL-60 cells treated with 100 μM c³Ado. Exponentially growing cells were cultured with 100 μM c³Ado for 1.5–6 hr and processed for flow cytometric analysis as described in Materials and Methods. (○) G_1; (●) S; (△) G_2 + M. The percentage of cells in the different cell cycle phases was estimated from the DNA histograms using the Multicycle program.

Table 1. Cell cycle distribution of HL-60 cells treated with indicated concentrations of c³Ado for 6 hr

c³Ado (μM)	G_1	S	G_2 + M
None	32.3 ± 4.0	52.4 ± 1.7	15.3 ± 2.3
25	31.0 ± 1.1	42.9 ± 1.3	26.1 ± 0.6
50	51.6 ± 0.9	19.1 ± 0.9	29.4 ± 1.2
75	64.7 ± 1.5	20.7 ± 0.5	14.5 ± 1.1
100	65.0 ± 0.8	24.8 ± 0.7	10.2 ± 0.3

Values are means ± SD (N = 3).

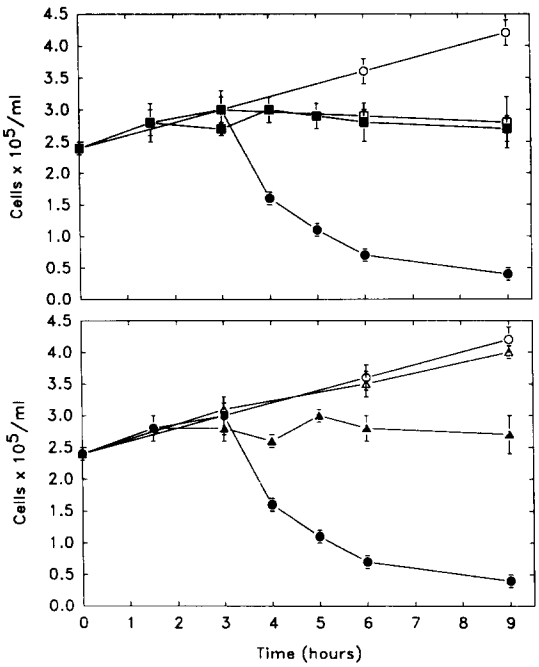

Fig. 7. Number of viable HL-60 cells in cultures treated with 100 μM c³Ado and 1 mM $ZnCl_2$ or 100 μM c³Ado and 1 mM Hcy. Exponentially growing cells were treated with 100 μM c³Ado and 1 mM $ZnCl_2$ (upper panel) or 1 mM Hcy (lower panel) for 9 hr. (○) control; (●) 100 μM c³Ado; (□) 1 mM $ZnCl_2$; (■) 100 μM c³Ado and 1 mM $ZnCl_2$; (△) 1 mM Hcy; (▲) 100 μM c³Ado and 1 mM Hcy. Viability was determined as described in Materials and Methods.

(middle panel) and 6 hr (lower panel). After 4 hr of incubation, we observed a reduction of early S-phase cells, and simultaneously particles with a lower DNA and protein content than that of the G_1 population became noticeable. These particles exhibited large variations in DNA and protein content, as well as forward angle light scatter (light scatter not shown). The percentage of cells in different cell cycle phases after 1.5–6 hr incubation with 100 μM c³Ado is presented in Fig. 6. After 4 hr the S-phase was decreased from 55.0 to 44.3%, whereas the G_1-phase was increased from 32.0 to 41.5%. A subsequent decrease in S-phase and increase in G_1-phase resulted in 62.3% G_1-phase cells and 25.2% S-phase cells after 6 hr. Due to very distorted histograms after 9 hr treatment with 100 μM c³Ado, estimations of cell cycle distribution in these cultures were not performed. The cell cycle distribution in control cultures did not change during the experimental time intervals (data not shown). To investigate the changes in cell cycle distribution initiated by c³Ado in further detail, cultures treated with different concentrations of c³Ado for 6 hr were analysed by flow cytometry. As shown in Table 1 all concentrations tested induced cell cycle perturbations. The lowest concentrations of c³Ado (25 and 50 μM) caused a drop in S-phase cells and a rise in G_2 + M-phase cells, whereas higher concentrations of c³Ado (75 and 100 μM) caused a drop in both S-phase and G_2 + M-phase cells. The higher percentage of G_1-phase cells in cultures treated with 75 and 100 μM c³Ado than that in cultures treated with 25 and 50 μM c³Ado, probably reflects the more selective loss of S-phase cells caused by 25 and 50 μM c³Ado.

Effects of $ZnCl_2$ and Hcy on c³Ado initiated cytotoxicity

The active endonuclease in apoptosis is considered as non-lysosomal, regulated by calcium and magnesium and inhibited by $ZnCl_2$ [27, 28]. Hcy has been shown to modify the effects of c³Ado in several studies [4, 6, 8, 12, 13]. To assess the effects of $ZnCl_2$ and Hcy on c³Ado initiated cell death, cell counts were performed in cultures treated with 100 μM c³Ado and 1 mM $ZnCl_2$ or 1 mM Hcy for 9 hr (Fig. 7). Both $ZnCl_2$ and Hcy inhibited c³Ado initiated cell death by cell counts. However, whereas the cell number in control cultures and cultures treated with 1 mM Hcy increased through the experimental time intervals, no increase in cell number was observed either in the cultures treated with $ZnCl_2$ alone, or in the cultures treated with c³Ado and $ZnCl_2$ or c³Ado and Hcy. To investigate the detailed effects of $ZnCl_2$ and Hcy on c³Ado cytotoxicity, cells treated with 100 μM c³Ado and 1 mM $ZnCl_2$ or 1 mM Hcy

Table 2. Cell cycle distributions of HL-60 cells treated with 100μM c³Ado in combination with 1 mM $ZnCl_2$ or 1 mM Hcy for 6 hr

Treatment	G_2	S	G_2 + M
None*	30.6 ± 3.1	53.3.± 2.0	16.1 ± 2.0
c³Ado*	61.9 ± 4.1	26.6 ± 2.8	11.4 ± 2.3
$ZnCl_2$	28.2 ± 1.6	53.9 ± 0.9	17.9 ± 1.8
c³Ado/$ZnCl_2$	29.8 ± 0.1	54.7 + 0.2	15.7 ± 0.7
Hcy	25.4 ± 3.2	55.7 ± 2.0	18.9 ± 3.2
c³Ado/Hcy	25.7 ± 2.5	40.2 ± 2.9	34.0 ± 1.0

Values are means ± SD (N = 3, *N = 6).

for 6 hr were processed for flow cytometric analysis and light microscopic examination. The cell cycle distributions of cells treated with c³Ado and $ZnCl_2$ or Hcy are presented in Table 2. Cultures treated with Hcy, $ZnCl_2$ or c³Ado and $ZnCl_2$ did not differ from control cultures in cell cycle distribution. Estimations of cell cycle distribution in cultures treated with c³Ado and Hcy revealed a lower percentage of S-phase cells and a higher percentage of G_2 + M-phase cells than that in control cultures. The number of particles with a sub-G_1 DNA content were highly reduced in cultures treated with c³Ado and Hcy compared to what observed in cultures treated with c³Ado alone. However, the coefficients of variation (CV) for the G_1 and the G_2 + M peaks in the DNA histograms of c³Ado and Hcy treated cells were higher than what obtained in other DNA histograms. Figure 8 shows typical morphological features of cells treated with 100 μM c³Ado and 1 mM $ZnCl_2$ (panel A) and 100 μM c³Ado and 1 mM Hcy (panel B) for 6 hr. No nuclear fragmentation was present in cultures with c³Ado and $ZnCl_2$. Cells treated with c³Ado and Hcy showed morphological changes consistent with the first stage in apoptosis, i.e. condensation and fragmentation of the nucleus, and subsequent formation of cells with micronuclear bodies of different size and color density. A lower number of apoptotic bodies was observed in c³Ado and Hcy treated cultures compared to the c³Ado treated cultures.

DISCUSSION

We have previously shown that c³Ado is cytotoxic to HL-60 cells at concentrations above 50 μM [3]. In the present study we confirm this finding and demonstrate that one mode of cell death initiated by c³Ado in this cell line is apoptosis. Apoptosis initiated by c³Ado was dose dependent, and even at the highest concentrations tested (75 and 100 μM), the mode of cell death was apoptosis and not necrosis [29].

The morphological appearance of apoptosis is well characterized with the cardinal features; condensation, nuclear fragmentation, blebbing of the cell surface and separation of the cell into apoptotic bodies [19, 25, 26]. In the present study, nuclear fragmentation, blebbing of the cell surface and apoptotic bodies were evident after 4 hr. These changes differed distinctly from the necrotic pattern of cell death initiated by 10 mM H_2O_2 (data not shown). In the present study we also observed a small number of apoptotic cells in control cultures. Such background apoptosis has been reported previously [18, 20]. One mode of cell death of terminally differentiated HL-60 cells is apoptosis [31], and spontaneous differentiation followed by terminal maturation and death may explain the appearance of a small number of apoptotic cells in control cultures.

DNA in apoptotic cells is degraded into multimers of approximately 200 base pair fragments, and an endonuclease which cleaves DNA in this pattern is found in isolated HL-60 nuclei from non-apoptotic cells [18]. In the present study, agarose gel electrophoresis of DNA from c³Ado treated cells showed small amounts of fragmented DNA after 3 and 4 hr of incubation, whereas a characteristic endonucleolytic ladder was distinct after 5 hr. These data indicate that c³Ado treatment results in endonuclease activation.

In the present study, cells with a DNA content lower than that of the G_1 population became apparent after 4 hr of treatment with 100 μM c³Ado, and at the same time, we observed a reduction of early S-phase cells. Prolonging of the treatment resulted in a further reduction of both S-phase cells and G_2 + M-phase cells, and a large increase in the number of nuclei with a sub-G_1 DNA content. Cells undergoing apoptosis show a decreased stainability with DNA specific fluorochromes compared to both cells undergoing necrosis and viable cells [32, 33]. The reduced stainability of apoptotic cells probably reflects partial loss of low molecular weight DNA secondary to DNA degradation. In addition, DNA is released to apoptotic bodies in the latter stage of apoptotis [32]. For these reasons cells with a lower DNA content than that of the G_1 population could represent both apoptotic cells and apoptotic bodies. The appearance of particles with a sub-G_1 DNA content in the present study corresponded to the appearance of apoptotic cells and apoptotic bodies observed by light microscopy. The broad distribution of DNA and protein content as well as forward angel light scatter of the sub G_1 particles observed in the present study, suggests that both apoptotic cells and apoptotic bodies are present among these particles, with apoptotic cells probably located closer to the viable cells than apoptotic bodies in the bivariate contour plot of red (DNA) and green (protein) fluorescence presented in Fig. 5. At higher concentrations (75 and 100 μM) of c³Ado, the DNA histograms indicated loss of both S and G_2 + M cells. However, by comparing the cell cycle data with the cell counts presented in Fig. 1, our results suggest that cells in all cell cycle phases are influenced by 100 μM c³Ado [34]. At lower concentrations of c³Ado (25 and 50 μM) cell loss was restricted to S-phase. Selective apoptosis of S-phase cells and concentration-dependent diversity of effects are reported for the topoisomerase I inhibitor camptothecin and the topoisomerase II inhibitors, teniposide and amsacrine [35]. Inhibition of DNA synthesis is reported for c³Ado in HL-60 cells [36], however, these data are somewhat difficult to interpret since

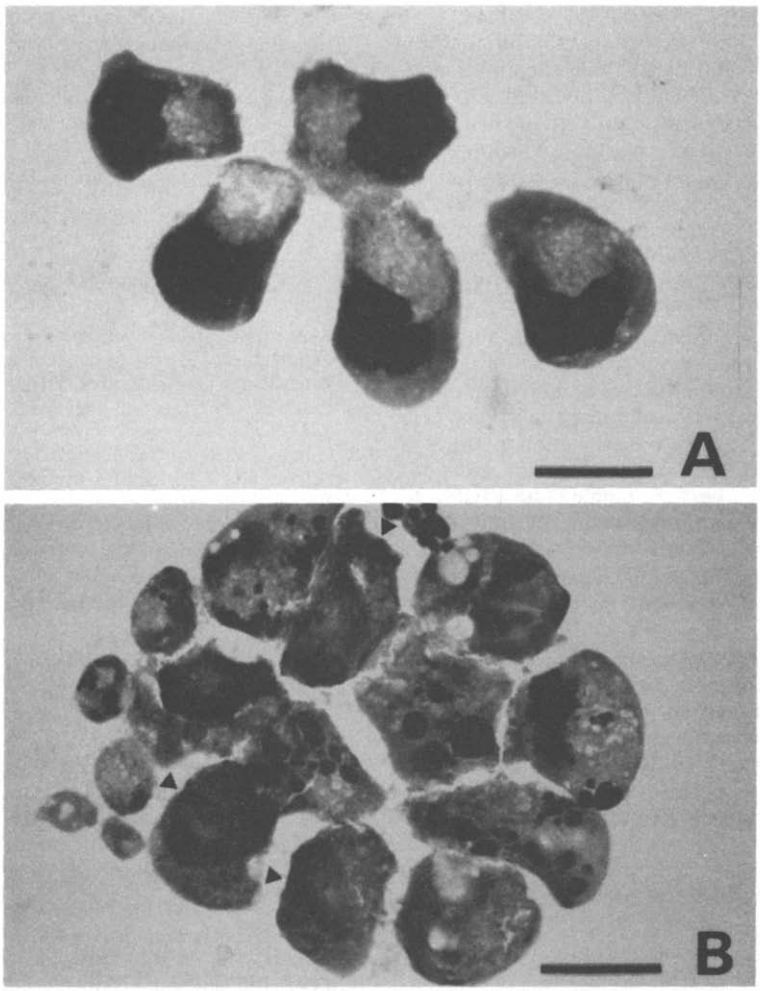

Fig. 8. Morphologic features of HL-60 cells cultured with 100 μM c^3Ado and 1 mM $ZnCl_2$ or 100 μM c^3Ado and 1 mM Hcy. Exponentially growing HL-60 cells spun in cytocentrifuge, fixed in methanol and stained with May-Grunwald-Giemsa. (A) The cells in this panel were treated with 100 μM c^3Ado and 1 mM $ZnCl_2$ for 6 hr. The cells show a strong condensation of the DNA with a pycnotic appearance of the nuclei which are eccentrically located within the cells. (B) The cells in this panel were treated with 100 μM c^3Ado and 1 mM Hcy for 6 hr. Some cells marked with (▲), have nuclei which are reasonably preserved with no distinct morphological evidence of apoptosis. Other cells demonstrate severe fragmentation and condensation of nuclear material, but the cell surface is reasonably preserved except for some cytoplasmic fragments seen to the left. This mode of morphological transmutation was in contrast to that observed without the addition of Hcy to the culture medium which resulted in a more pronounced formation of apoptotic bodies as demonstrated in Fig. 3, panel B and C. Scale bar = 5 μm.

inhibition of DNA synthesis and cytotoxicity occurs within the same concentration range. Taken together our flow cytometric data adds further support to the conclusion that the mode of cell death initiated by c^3Ado is apoptosis. The loss of S-phase cells observed at the lower concentrations of c^3Ado most likely suggests a selectivity of the apoptotic process for S-phase cells, however we can not exclude that this loss is partly due to a G_0/G_1-arrest [7]. Selective S-phase apoptosis may reflect a cell line specific phenomenon [37]. On the other hand, this observation may also provide a clue to the mechanism by which c^3Ado initiates apoptosis.

c^3Ado initiated apoptosis was completely inhibited by $ZnCl_2$ as evaluated by cell counts, light microscopy and flow cytometry. This strengthens the conclusion that the mode of cell death initiated by c^3Ado is apoptosis. Despite inhibition of DNA fragmentation by $ZnCl_2$, it is reported that thymocytes may undergo apoptotic death after glucocorticoid exposure [38]. Interestingly, we observed no growth in cultures treated with either 1 mM $ZnCl_2$ or 1 mM $ZnCl_2$ and 100 μM c^3Ado, and prolonged exposure (18 hr) to 1 mM $ZnCl_2$ resulted in cell death by necrosis in the present study.

In the present study, cell counts and the classic

viability test, Trypan blue exclusion, suggested that Hcy completely inhibited cell death initiated by c^3Ado. However, light microscopic examination of cells treated with c^3Ado and Hcy revealed cells with morphological changes consistent with the first stage in apoptosis. For these reasons it should be emphasized that Trypan blue exclusion is an insufficient viability criterion when cells undergo apoptosis.

c^3Ado may serve as both inhibitor and substrate for AdoHcy hydrolase, and residual AdoHcy hydrolase activity in cells treated with c^3Ado and Hcy probably catalyses the formation of 3-deazaadenosinehomocysteine (c^3AdoHcy) [10]. Both AdoHcy, which accumulates due to inhibition of the hydrolase, and c^3AdoHcy inhibit methyltransferase activity [2, 12]. The DNA methylation pattern is known to influence chromatin structure, and non-methylated DNA has a higher sensitivity to degradation by nucleases than methylated DNA [39]. One mechanism which may contribute to the rapid degradation of DNA in HL-60 cells treated with c^3Ado might therefore be perturbations of methylation reactions and subsequent hypomethylation of DNA. However, it has been difficult to demonstrate general hypomethylation of DNA in HL-60 cells exposed to c^3Ado and the more specific AdoHcy hydrolase inhibitor, 3-deaza-(±)-aristeromycin (c^3Ari) [40]. Our data do not exclude a possibility that perturbations or methylation reactions may play a role in HL-60 cell apoptosis, however, ongoing studies with c^3Ari do not support a general role of perturbations of transmethylation reactions in this mode of cell death in HL-60 cells.

It has recently been reported that microfilament-disrupting agents prevent the formation of apoptotic bodies in HL-60 cells undergoing apoptosis, suggesting a central role of actin assembly in this process [41]. However, it has also been demonstrated that one of these agents, cytochalasin B, by itself is able to initiate DNA degradation typical of apoptosis [42]. In the present study we observed extensive formation of apoptotic bodies in cultures with c^3Ado alone, whereas few apoptotic bodies were observed in cultures treated with c^3Ado and Hcy. c^3Ado has been shown to disorganize microfilaments in mouse macrophages [5], and to inhibit the contractile response in guinea-pig lung parenchyma [43, 44]. Our data do not exclude the possibility that perturbations of methylation reaction may explain the inhibitory effect of Hcy on formation of apoptotic bodies in c^3Ado initiated apoptosis [43]. However, at least two other mechanisms may interfere with microfilament function in cells treated with c^3Ado and Hcy. Firstly, c^3AdoHcy may inhibit microfilament function independently of its methyltransferase action [44]. Secondly, since the ability of c^3Ado to increase cyclic AMP levels is potentiated by Hcy [45], we can not exclude the possibiity that the inhibitory effect of c^3Ado and Hcy on microfilaments is mediated through changes in cyclic AMP levels.

In summary, we have shown that c^3Ado initiates apoptosis in HL-60 cells, and that the apoptotic process initiated by c^3Ado is initially triggered in S-phase cells. Apoptosis initiated by c^3Ado is completely inhibited by $ZnCl_2$, and partly inhibited by Hcy. The effects of Hcy on apoptosis initiated by c^3Ado is under further investigation in our laboratory.

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