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ARTICLES

Induction of Apoptosis in HL-60 Cells by N^6-Benzyladenosine

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Abstract Treatment of HL-60 cells with micromolar concentrations of N^6-benzyladenosine (N^6-benzylaminopurine riboside [BAPR]) led to the occurrence of apoptosis in a concentration-dependent manner. Incubation period as short as 2 h in the presence of BAPR was sufficient for triggering irreversible changes leading to apoptosis even after the transfer of cells to the standard medium (without BAPR). Cell death induced by BAPR proceeded rapidly and in a very synchronous fashion. Detailed study of temporal changes in a chromatin structure and DNA integrity showed that the movement of chromatin toward the nuclear periphery is the fundamental event within dying cells. We demonstrated that this rearrangement of chromatin is irreversible and it takes place without apparent DNA degradation. The extensive DNA cleavage seems to be a rather late event, as it was observed in cells that exhibited a typical apoptotic morphology (apoptotic bodies). On the basis of temporal changes in the ATP level within dying cells, it is concluded that ATP is essential for the movement of chromatin toward the nuclear envelope but not for the subsequent chromatin condensation leading to the formation of apoptotic bodies. DNA fragmentation also seems to be ATP independent. BAPR interfered with neither pyrimidine nor purine biosynthesis, as none of the tested bases and the corresponding nucleosides prevented or reduced apoptosis in BAPR-treated cells. Adenosine was the only exception that substantially reduced the effect of BAPR. Since transport of exogenous adenosine into cells was essential to manifest its protective effect, we assume that adenosine is a competitive inhibitor of adenosine kinase and thus reduces intracellular phosphorylation of BAPR. Indeed, 4-amino-3-iodo-1(β-D-ribofuranosyl)pyrazolo[3,4-d]pyrimidine, a potent inhibitor of adenosine kinase, fully prevented BAPR-induced apoptosis. These results suggest that cytotoxic effect of BAPR is related to its activation by phosphorylation within cells, rather than to its interaction with extracellular adenosine receptors. J. Cell. Biochem. 77:6–17, 2000.

Key words: apoptosis; N^6-benzyladenosine; HL-60 cells

Generally, cell death has two quite distinct mechanisms: apoptosis and necrosis. Apoptosis is a mode of cell death that occurs under physiological conditions; it is often found during the cell differentiation and development, normal cell turnover and tissue homeostasis, embryogenesis, and it is particularly important in development of the immune system. Apoptosis also occurs as a response to cell injury induced by a spectrum of physical and chemical agents [reviewed by Cotter et al., 1990; Ellis et al., 1991; Kerr et al., 1991; Stewart, 1994]. On the contrary, necrosis occurs when cells are exposed to extreme conditions, such as hyperthermia, overdose of cytotoxic agents [reviewed by Wyllie et al., 1980; Cotter et al., 1990; Darzynkiewicz et al., 1992].

Extracellular purines were identified as effective modulators of the cell growth and inductors of differentiation in numerous cell types [Rathbone et al., 1992; Neary et al., 1996]. Recent evidence suggests that trophic effects of purines are mediated through activation of membrane-bound purinoceptors [reviewed by Franceschi et al., 1996; Neary et al., 1996]. Purines take part in signal transduction of many pathological processes including cell death.

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In addition to naturally occurring purines such as adenosine [Kizaki et al., 1990; Tanaka et al., 1994], ATP [Franceschi et al., 1996], deoxyadenosine, and deoxyguanosine [Kizaki et al., 1990] many artificial derivatives of adenosine can induce apoptosis [Lin et al., 1988; Kohno et al., 1996]. In general, two distinct cytotoxic mechanisms are considered: (1) intracellular, purines may become cytotoxic after their intracellular phosphorylation [Lin et al., 1988; Cottam et al., 1993], (2) extracellular, cell death can be mediated via extracellular adenosine receptors [e.g., Kohno et al., 1996].

In the present study, the effects of N^6-benzyladenosine on cultured HL-60 cells were investigated. We demonstrated that this compound is a new and very potent inductor of apoptosis with typical morphological and biochemical hallmarks. Moreover, BAPR-induced apoptosis proceeded fast and in a very synchronous fashion. The observed effect of BAPR did not seem to be mediated by extracellular adenosine receptors since its transport into a cell with subsequent phosphorylation is essential for the manifestation of its cytotoxicity.

MATERIALS AND METHODS

Cell Culture

A suspension of human promyelocytic HL-60 cells was cultured in RPMI-1640 medium supplemented with a 10% fetal calf serum (FCS) and antibiotics in 5% CO_2 atmosphere at 37°C. Cells were maintained at density from 1×10^5 to 6×10^5 cells per ml. HL-60 cells were obtained from ECACC.

Cell Treatment

N^6-Benzyladenosine (N^6-benzylaminopurine riboside [BAPR]) (Sigma Chemical Co.) was dissolved in 0.075 M HCl to obtain a stock solution 50–500 times higher than that of the cell treatment concentration. Cells were seeded and cultured in a standard medium for 24–30 h; BAPR was then added and the cells incubated further for a given time interval. A slight decrease in pH attributable to the addition of the highest amount of BAPR was adjusted with a $NaHCO_3$ solution.

4-Amino-3-iodo-1β -D-ribofuranosylpyrazolo [3,4-d]pyrimidine (AIRPP), a gift of Dr. Cottam, was dissolved in warm water.

3-Aminobenzamide (3-ABA) purchased from Sigma was dissolved in dimethylsulfoxide (DMSO). The final concentration of DMSO in culture medium were less than 0.25%.

Dipyridamole (DP) purchased from Sigma was dissolved in methanol. The final concentration of methanol in culture medium was less than 0.1%.

Morphological Analysis of Apoptosis

Cells were harvested, washed in phosphate-buffered saline (PBS), and fixed in an ice-cold methanol + acetic acid (3:1) fixation mixture. Aliquots of cells were spread on glass slides and stained with Hoechst 33342 (2 μg/ml) in PBS. The cell nuclei morphology was examined by a fluorescence microscope Olympus BX60. The images were captured by a monochrome b/w CCD camera (COHU 4910) and processed by an image analysis system LUCIA M (Laboratory Imaging s.r.o., Prague). The pictures were printed on a Mitsubishi CP-D1E dye-sublimation printer.

DNA Fragmentation Assay

Aliquots of cells were washed in PBS and lysed by the addition of DNA lysis buffer (50 mM Tris/HCl, pH 8.0, 10 mM EDTA, 0.5% sodium lauroyl sarcosine, and 0.5 mg/ml proteinase K) at 37°C overnight. Samples were treated further with DNase-free RNase A (0.2 mg/ml) at 37°C for 3 h. The resulting extracts were loaded onto 1.8% agarose gel containing ethidium bromide (0.5 μg/ml). Electrophoresis was carried out at 25 V (constant) at room temperature for 12 h. After washing in distilled water (30 min), the gel was viewed with an ultraviolet (UV) transilluminator and was photographed.

DNA Analysis Using Pulse-Field Gel Electrophoresis

Aliquots of cells were washed in PBS and then embedded in LMP agarose as described previously [Den Dunnen et al., 1993]. The agarose-embedded samples were incubated in a lysis buffer (1% sodium lauroyl sarcosine, 0.5M EDTA, pH = 8.0, and 0.5 mg/ml proteinase K) at 37°C for 6 h, with gentle agitation. The lysis buffer was replaced by a fresh one and the incubation proceeded at 50°C for another 12 h. Samples were either rinsed with the TE buffer before PFGE or transferred into sterile EDTA solution (0.5M pH=8.0) for storage at 4°C. Samples were loaded onto 1.0% agarose gel and

run with a linear ramp of pulse at 2.5–30 s for 20 h at 180 V at 15°C in 0.5 TBE buffer. After electrophoresis, the gel was stained with ethidium bromide (0.3 µg/ml; 60–90 min) and then washed in distilled water for 30 min. The gel was viewed with a UV transilluminator and was photographed.

ATP Determination Using HPLC

About 2×10^6 cells were collected by centrifugation (3 min/2,500 rpm) at room temperature. The cell pellet was extracted by ice-cold perchloric acid (5%) for 20 min at 4°C. Cell extract was neutralized by addition of 2 M $K_{2C}O_3$. Aliquots of extract were loaded on a SEPARON SGX C18 column (5 µm, 150 × 4 mm); ATP was eluted under isocratic conditions: mobile phase, 0.1 M NaH_2PO_4, pH 6.4; 5 mM tetrabutylammonium phosphate + 10% acetonitrile, flow rate, 0.4 ml/min. Quantitation was done using an external standard.

RESULTS

HL-60 cells responded to the treatment with various concentrations of BAPR by a concentration-dependent decrease in cell density (Fig. 1). Hoechst staining revealed that 12 h after the drug addition the number of apoptotic nuclei progressively increased with increasing concentration of BAPR (Fig. 2). The analysis of DNA integrity in BAPR treated cells is illustrated in Figure 3. The appearance of the fragments was equivalent to approximately 200 bp and multiples, as detected by standard gel electrophoresis, also indicated that cells die by apoptosis (Fig. 3). The lowest concentration of BAPR that elicited apoptosis in 7–9% of cells in 24 h was as low as 1.0 µM (not shown); concentrations of >25 µM were effective in approximately 100% of cells.

A more detailed investigation of temporal changes in chromatin morphology showed that formation of apoptotic bodies proceeded rapidly and in a synchronous fashion for the lethal concentration of BAPR, i.e., 50 and 100 µM, respectively. Most apoptotic bodies were observed at 3–6 h after the addition of lethal concentrations of BAPR. The application of lower concentrations, however, resulted in cell death that occurred less synchronously (Fig. 4). Temporal changes in chromatin structure with those in the DNA integrity in response to 50 µM BAPR are compared in Figures 5–7, as an example, as there were no substantial differences among lethal BAPR concentrations applied. The

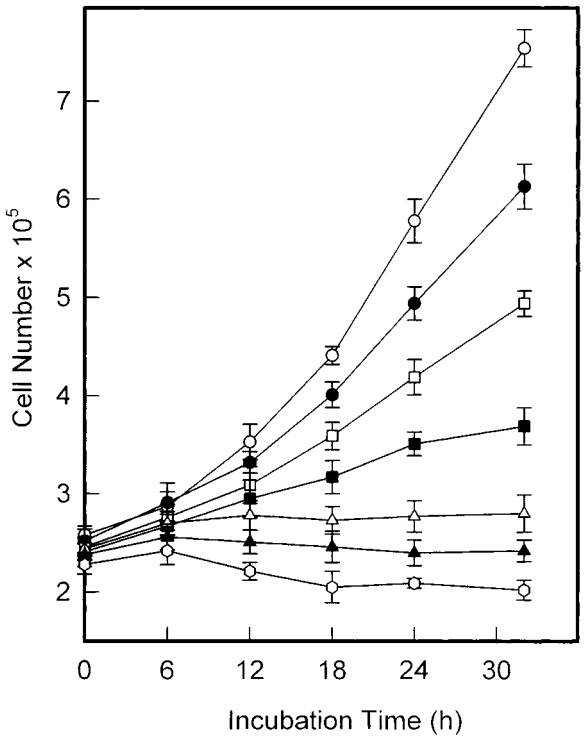

Fig. 1. Effect of benzylaminopurine riboside (BAPR) on proliferation of HL-60 cells. Exponentially growing cells were treated with 2 µM (filled circle), 5 µM (open square), 10 µM (filled square), 25 µM (open triangle), 50 µM (filled triangle), and 100 µM (open hexagon) BAPR. Cells cultured without BAPR were taken as a control (open circle). At the indicated time, the cell density was measured using electronic particle counter. The experimental points represent the mean values from three replicate experiments with standard deviations.

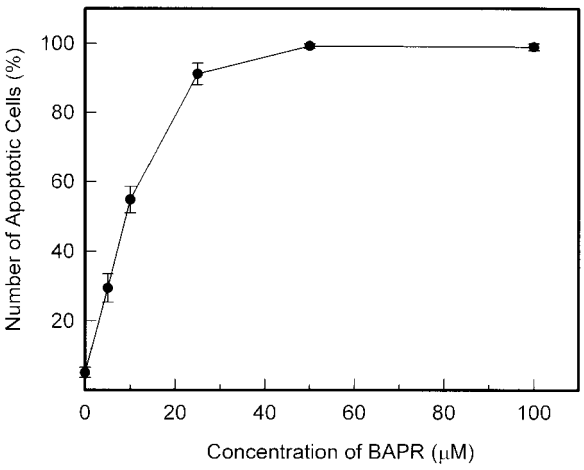

Fig. 2. Concentration-dependent induction of apoptosis in HL-60 cells by benzylaminopurine riboside (BAPR). Cells were incubated for 12 h in the presence of given concentrations of BAPR and then stained with Hoechst 33342, as described under Material and Methods. The frequency of apoptotic nuclei was examined microscopically in at least 500 cells. Only cells that contained apoptotic bodies were counted. The experimental points represent mean values from three replicate experiments with standard deviations.

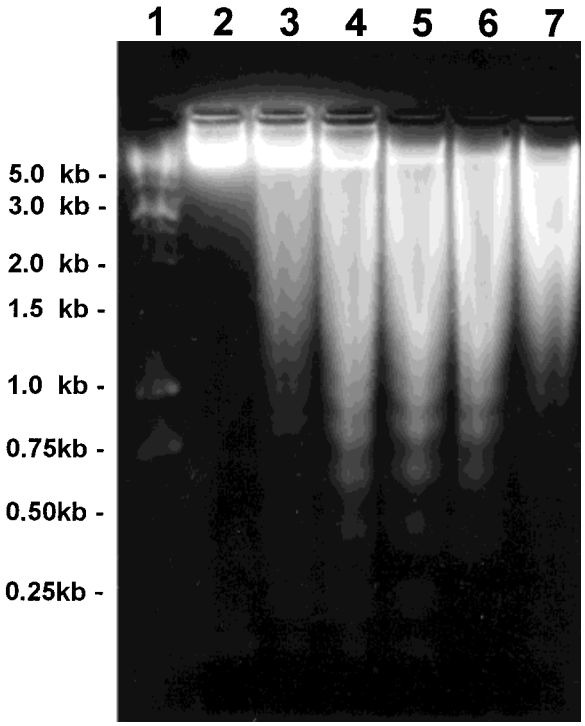

Fig. 3. Dose-dependent effects of benzylaminopurine riboside (BAPR) on DNA fragmentation in HL-60 cells. DNA was extracted from cells incubated for 12 h with various concentrations of BAPR: 5 μM (**lane 3**), 10 μM (**lane 4**), 25 μM (**lane 5**), 50 μM (**lane 6**), and 100 μM (**lane 7**). Untreated cells were taken as a control (**lane 2**), molecular weight markers (**lane 1**).

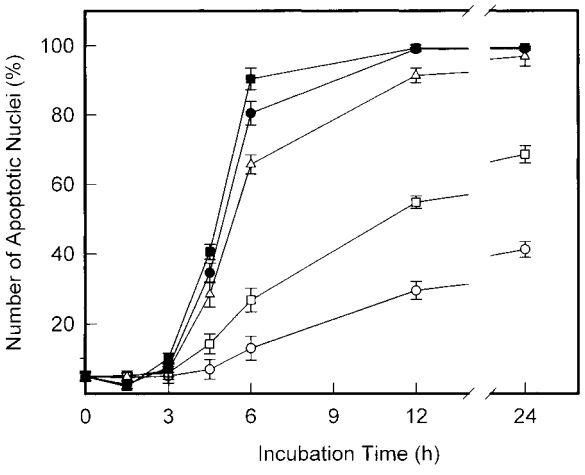

Fig. 4. Time course of apoptotic bodies occurrence in HL-60 cells treated with benzylaminopurine riboside (BAPR). Cells were incubated for given time intervals in the presence of 5 μM (open circle), 10 μM (open square), 25 μM (open triangle), 50 μM (filled circle), and 100 μM (filled square) BAPR and then stained with Hoechst 33342, as described under Material and Methods. The frequency of apoptotic nuclei was microscopically examined in at least 500 cells. Only cells that contained apoptotic bodies were counted. The experimental points represent the mean values from three replicate experiments with standard deviations.

first detectable change within dying cells was the migration of chromatin toward nuclear surface resulting in appearance of sponge-like structure (Fig. 5b). This first stage of apoptotic chromatin occurred 1.5–2 h after the BAPR addition and was not accompanied by detectable DNA cleavage (Figs. 6, 7). The subsequent chromatin condensation was characterized by the formation of a number of small irregular patches of dense chromatin mutually connected by protuberances surrounding the whole nucleus envelope (Fig. 5c). This second stage was observed from about 3–4 h after the BAPR application, accompanied by a limited fragmentation of DNA into 50-kbp fragments (Fig. 6). Further chromatin condensation led to the enlargement of condensed patches as well as to the reduction in their number. The protuberances were also less visible (Fig. 5d). The third stage, that of preapoptotic bodies, was observed from about 4–6 h after the drug addition. Only this relatively late morphological pattern was evidently connected to the extensive DNA degradation into 50-kbp and nucleosomal size fragments (Figs. 6, 7). The following stage (fourth) was then characterized by the regular circular shape of broken chromatin without connecting protuberances: apoptotic bodies. The formation of apoptotic bodies appeared to be the final stage, as no subsequent morphological development was observed (Fig. 5e–g). However, an intensive degradation of DNA continued within the apoptotic bodies (Figs. 6, 7).

A detailed temporal analysis of DNA integrity, assessed by simultaneous PFGE and standard agarose gel electrophoresis, showed that chromatin movement toward the nuclear periphery did not reflect apoptotic pattern of DNA cleavage (Figs. 5–7). A distinctive DNA degradation into HMW fragments corresponding to 50 kbp was not observed in the first 3 h of BAPR application (Fig. 6). No previous or simultaneous 300-kbp fragments were detected (Fig. 6). The results further indicated that DNA cleavage into 50-kbp fragments occurred before the internucleosomal cleavage (Figs. 6, 7). Fragments of nucleosomal size were observed about 4–5 h after BAPR addition (not shown). It is evident that intensive DNA cleavage continued even at 12–24 h after the drug application (Figs. 6, 7). While the discrete fragmentation of genomic DNA into 50-kbp fragments remained preserved until 24 h after the addition of BAPR (Fig. 6), the oligonucleosomal size DNA appeared to be further degraded and formed a

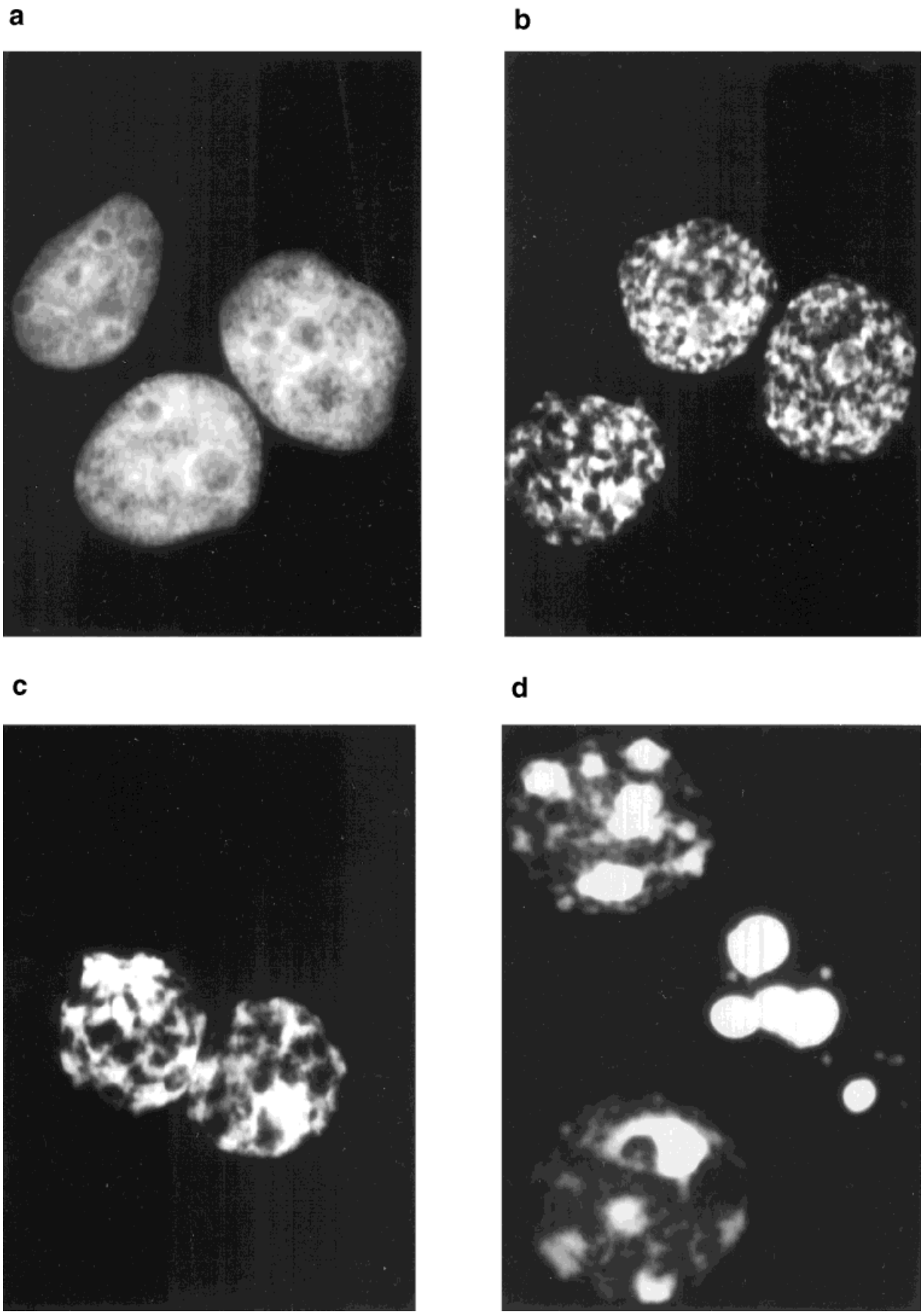

Fig. 5. Temporal changes in morphological features of chromatin in BAPR-treated HL-60 cells. Benzylaminopurine riboside (BAPR) was added to the exponentially growing HL-60 cells at concentration of 50 μM. At given time intervals cells were stained with Hoechst 33342, as described under Materials and Methods, and processed for fluorescence microscopy. Cell nuclei of untreated cells (**a**), nuclei of cells cultured for 1.5 h (**b**), 3 h (**c**), 4.5 h (**d**), 6 h (**e**), 12 h (**f**), and 24 h (**g**) in the presence of 50 μM BAPR.

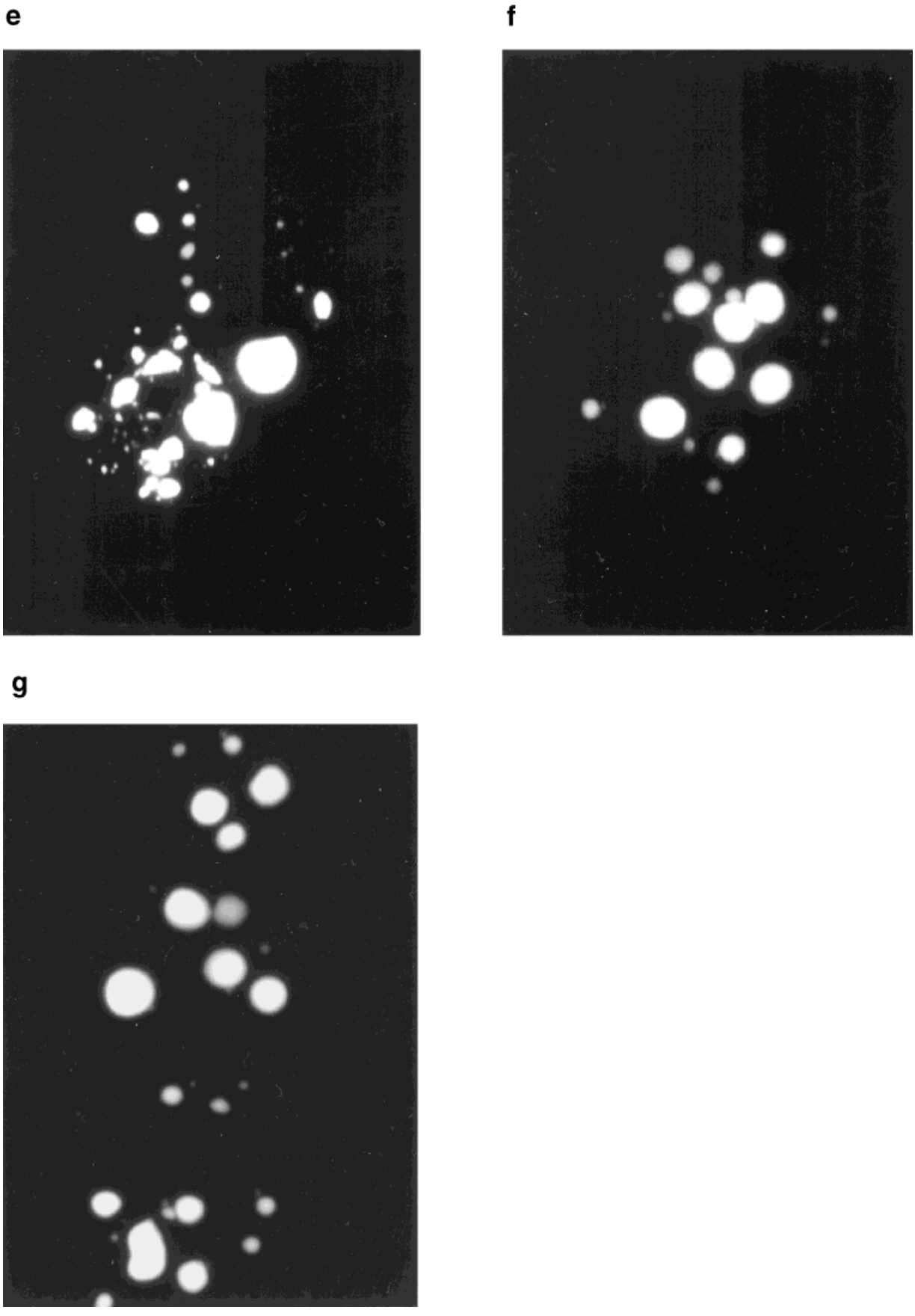

Figure 5. (Continued.)

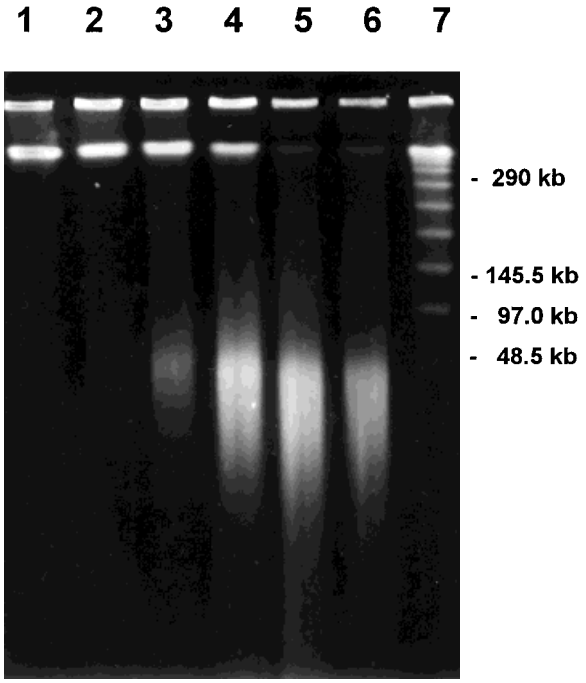

Fig. 6. Analysis of DNA degradation during apoptosis in HL-60 cells using a pulsed-field gel electrophoresis. Time course of DNA fragmentation in cells treated for 1.5, 3, 6, 12, and 24 h with 50 μM benzylaminopurine riboside (BAPR) (**lanes 2–6**), untreated cells (**lane 1**). Molecular weight standard (**lane 7**).

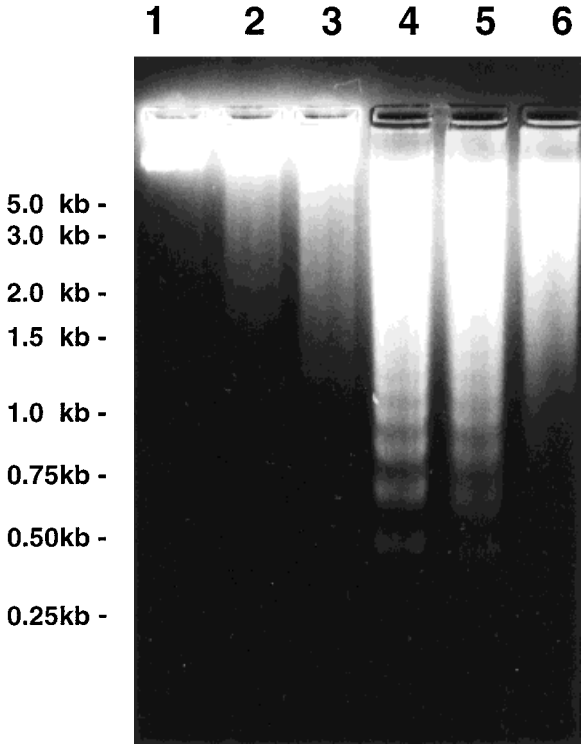

Fig. 7. Time course of internucleosomal cleavage of DNA from HL-60 cells treated by benzylaminopurine riboside (BAPR), using conventional gel electrophoresis. DNA was extracted 1.5, 3, 6, 12, and 24 h after the addition of 50 μM BAPR (lanes 2–6). Untreated cells were taken as a control (**lane 1**).

"smeared ladder" (Fig. 7). Degradation of oligonucleosomal DNA seemed to be proportional to the concentration of BAPR applied (Fig. 3).

We tried to prevent BAPR-induced apoptosis by transferring the cells into a fresh incubation medium in order to identify what changes within dying cells are irreversible. We found that 1.5–2 h of HL-60 cell incubation in the presence of 50 μM BAPR was sufficient for the full development of apoptosis during their subsequent incubation in the standard medium, i.e., without BAPR (Fig. 8). Irreversible changes within dying cells appeared to be related to the chromatin movement toward the nuclear envelope, rather than to DNA cleavage (Figs. 5b, 6, 7).

The intracellular level of ATP was strongly affected by BAPR. While low concentrations of BAPR induced a slow decrease in ATP content that was preceded by a transient increase, higher concentrations caused a rapid depletion of ATP (Fig. 9). Comparison of temporal changes in the ATP level with those of the development of apoptotic hallmarks showed that both apoptotic bodies formation (Figs. 4, 5) and extensive DNA cleavage (Figs. 6, 7) take place after ATP

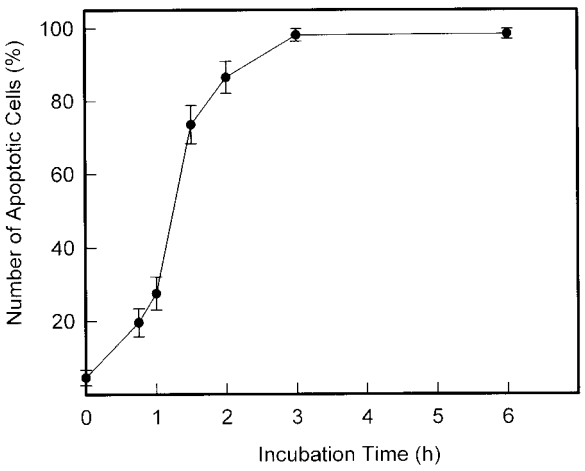

Fig. 8. Prevention of apoptosis by benzylaminopurine riboside (BAPR) withdrawal. 50 μM BAPR was added to the exponentially growing HL-60 cells. Cells were incubated in the presence of BAPR for a given time interval, washed, and then transferred into a fresh medium without BAPR. Cells were stained with Hoechst 33342 24 h later and frequency of apoptotic nuclei was determined microscopically. The experimental points represent the mean values from three replicate experiments with standard deviations. At least 500 cells were examined in each experiment.

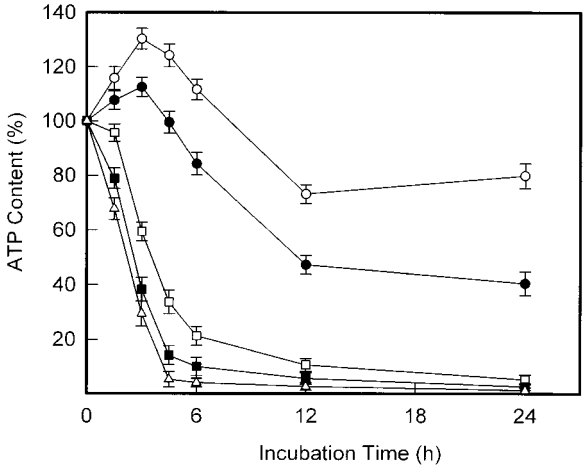

Fig. 9. Effect of benzylaminopurine riboside (BAPR) on intracellular level of ATP in HL-60 cells. Exponentially growing cells were treated with 5 μM (open circle), 10 μM (filled circle), 25 μM (open square), 50 μM (filled square), and 100 μM (open triangle) BAPR. The intracellular level of ATP was measured at the indicated times, as described under Materials and Methods. Each point represents the ratio of ATP in treated cells compared with that of nontreated cells at the time indicated. ATP level in untreated cells was 758 pmol/10^6 cells. The experimental points represent mean values from three replicate experiments with standard deviations.

depletion (Fig. 9). The only event that occurred at relatively high level of ATP (75–80% of its control level) was the partial condensation of chromatin and its migration toward the nuclear envelope, i.e., formation of the sponge-like structure (Figs. 5b, 9). These results indicated that only the first event—chromatin condensation at the nuclear periphery envelope—was likely ATP dependent, while the subsequent chromatin condensation as well as DNA cleavage did not require ATP. Moreover, the residual amount of ATP seemed to modulate the extent of nucleosomal DNA degradation; the lower residual ATP level the more intensive degradation of nucleosomal DNA (Figs. 3, 9).

The change in ATP level was one of the earliest observable events in BAPR-treated cells (Figs. 4–7, 9), indicating possible interference of the drug with ATP turnover within cells. Therefore, we tested whether poly(ADP-ribosyl)ation, a potential common mechanism for cytotoxic drug action accompanied by rapid NAD and ATP depletion is related to the cell killing. However, this explanation does not seem to be probable since even 10 mM 3-ABA, an inhibitor of poly(ADP-ribosa)polymerase, failed to either prevent or reduce BAPR-induced cell death (not shown).

To test the possibility that the toxic effects of BAPR are the result of some type of imbalance in pyrimidine or purine biosynthesis, different bases and corresponding nucleosides were examined for their ability to prevent the effect of BAPR. It seemed that BAPR does not interfere with either pyrimidines or purines biosynthesis, as all of them failed to prevent apoptosis (Table I). The adenosine was only exception that reduced the cytotoxic effects of BAPR in a concentration-dependent manner (Fig. 10); 0.3 mM adenosine substantially reduced the effect of BAPR (Table I). Dipyridamole (DP), an adenosine transport inhibitor, was also found ineffective in prevention of BAPR-induced cell death

TABLE I. Effects of the Different Pyrimidine and Purine Bases and the Corresponding Nucleosides on BAPR-Induced Cell Death

Base (mM)	BAPR (μM)	No. of apoptotic cells (%)
—	—	5.3 ± 2.3
—	10	54.8 ± 3.6
Uridine 1.0	—	4.5 ± 1.9
Uridine 1.0	10	56.0 ± 2.9
Uracil 1.0	—	6.1 ± 1.6
Uracil 1.0	10	58.7 ± 3.8
Cytidine 1.0	—	5.7 ± 2.1
Cytidine 1.0	10	59.2 ± 3.0
Cytosine 1.0	—	4.9 ± 2.3
Cytosine 1.0	10	59.9 ± 3.3
Thymidine 1.0	—	5.9 ± 2.6
Thymidine 1.0	10	56.6 ± 3.7
Thymine 1.0	—	6.0 ± 1.8
Thymine 1.0	10	59.2 ± 4.1
Guanosine 1.0	—	6.1 ± 2.6
Guanosine 1.0	10	56.1 ± 3.1
Guanine 1.0	—	5.8 ± 2.2
Guanine 1.0	10	5.1 ± 1.9
Inosine 1.0	—	5.6 ± 3.0
Inosine 1.0	10	58.8 ± 4.1
Hypoxanthine 1.0	—	6.0 ± 2.6
Hypoxanthine 1.0	10	55.7 ± 3.7
Adenosine 0.3	—	9.1 ± 2.3
Adenosine 0.3	10	23.3 ± 2.8
Adenine 1.0	—	4.5 ± 2.2
Adenine 1.0	10	50.5 ± 2.3

BAPR, benzylaminopurine riboside. Exponentially growing cells were treated with indicated substances for 12 h and then stained with Hoechst 33342, as described in Materials and Methods. The frequency of apoptotic nuclei was examined microscopically in at least 500 cells. Only cells that contained apoptotic bodies were counted. Data shown represent mean values from three replicate experiments with standard deviations.

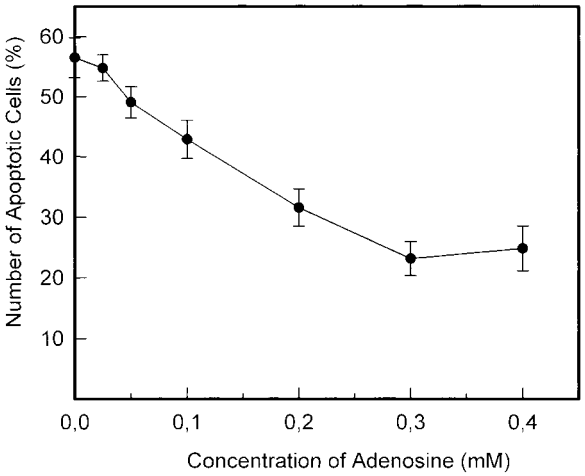

Fig. 10. Prevention of benzylaminopurine riboside (BAPR)-induced apoptosis by adenosine. Cells were incubated in the presence of 10 μM BAPR together with various concentration of adenosine. After 12–16 h, cells were stained with Hoechst 33342 and the frequency of apoptotic nuclei determined microscopically. The experimental points represent the mean values from three replicate experiments with standard deviations. At least 500 cells were examined in each experiment.

TABLE II. Reversal of the Protective Effect of Adenosine on BAPR-Induced Cell Death by Dipyridamole

Dipyridamole (μM)	BAPR (μM)	Adenosine (mM)	No. of apoptotic cells (%)
—	—	—	5.4 ± 2.1
10	—	—	6.5 ± 2.2
—	10	—	55.4 ± 3.1
—	—	0.3	9.1 ± 2.3
10	10	—	60.1 ± 4.2
—	10	0.3	23.2 ± 2.8
10	10	0.3	62.0 ± 4.1

BAPR, benzylaminopurine riboside. Exponentially growing cells were treated with indicated substances for 12 h and then stained with Hoechst 33342, as described in Materials and Methods. The frequency of apoptotic nuclei was examined microscopically in at least 500 cells. Only cells that contained apoptotic bodies were counted. Data shown represent mean values from three replicate experiments with standard deviations.

TABLE III. Effect of 4-Amino-3-iodo-1-(β-D-ribofuranosyl)pyrazolo·[3,4-d]pyrimidine (AIRPP) on BAPR-Induced Cell Death

AIRP (μM)	BAPR (μM)	No. of apoptotic cells (%)
—	—	5.4 ± 2.1
1.0	—	6.3 ± 2.0
—	10	56.4 ± 3.6
1.0	10	4.8 ± 2.3

BAPR, benzylaminopurine riboside. Exponentially growing cells were treated with indicated substances for 12 h and then stained with Hoechst 33342, as described in Materials and Methods. The frequency of apoptotic nuclei was examined microscopically in at least 500 cells. Only cells that contained apoptotic bodies were counted. Data shown represent mean values from three replicate experiments with standard deviations.

(Table II). However, DP fully reversed the protective effect of adenosine (Table II). Thus, only intracellular adenosine was able to abolish cytotoxic effects of BAPR. 4-Amino-3-iodo-1(β -D-ribofuranosyl)pyrazolo[3,4-d]-pyrimidine (AIRPP), a novel and selective inhibitor of adenosine kinase [Cottam et al., 1993] was used to test whether BAPR is cytotoxic per se or is activated by phosphorylation. We found that 1 μM AIRPP was able to fully reverse BAPR-induced apoptosis in HL-60 cells (Table III). These results indicated that both transport of BAPR into cells as well as its phosphorylation are necessary for manifestation of cytotoxic effects of BAPR.

DISCUSSION

Treatment of HL-60 cells with BAPR resulted in retardation of the cell proliferation as well as in the loss of cell viability in a concentration-dependent manner (Figs. 1, 2). Morphological analysis of nuclei stained with Hoechst 33342 indicated that BAPR-treated cells show a typical apoptotic morphology, i.e., occurrence of condensed chromatin and apoptotic bodies (Fig. 2). DNA extracted from these cells was found to be fragmented into integer multiples of approximately 200 bp (Fig. 3).

Apoptosis induced either by physiological stimuli or by the majority of cytotoxic agents occurs in an asynchronous fashion when only a limited number of cells die. These inherent properties of apoptosis complicate its study. In contrast, apoptosis induced by lethal concentrations of BAPR offers unique opportunity to study the cell death that occurs in very synchronous fashion. Moreover, the apoptotic process in this experimental system proceeds relatively quickly, as approximately 90% of the cells die within 6 h after the drug addition (Figs. 4, 5). The synchronization of cell death enabled us to distinguish at least three different morphological stages that preceded apoptotic body formation (Fig. 5b–d). The described sequence of morphological

changes within dying HL-60 cells does not seem to be restricted to this particular experimental system since similar morphological stages we observed in starving HL-60 cells as well as in starving human skin fibroblasts (unpublished results).

Until now, most investigators assumed that the condensation of chromatin reflects an endonucleolytic cleavage of DNA [reviewed by Wyllie et al., 1980; Compton, 1992]. However, several recent reports have revised this dogma [Cohen et al., 1992; Zahra et al., 1992; Oberhammer et al., 1993a,b; Kass et al., 1996]. Owing to the perfect synchrony in morphological changes in dying cells we were able to re-examine the relationship between DNA integrity and morphological features also in our experimental system. Our results indicated that the principal apoptotic hallmark—chromatin condensation—does not reflect the DNA fragmentation (cf. Figs. 5–7). The first and principal change in dying cells is the morphological one characterized by a partial condensation of chromatin and its migration toward the nuclear envelope, i.e., formation of the "sponge-like" structure (Fig. 5b). Chromatin migration toward the nuclear envelope seems to play a crucial role in the apoptotic process since the changes that led to its manifestation are irreversible and determine cells to death (Fig. 8). We assume that the initial movement of the chromatin toward nuclear periphery is an energy dependent process, as it took place at a relatively high level of ATP (Figs. 5b, 9) and in synchronous fashion (more than 80% of cells exhibited identical morphology of chromatin (not shown). A similar conclusion was made by Kass et al. [1996], who demonstrated on in vitro system that the presence of ATP is necessary for the chromatin movement to the nuclear envelope as well as for the apoptotic body formation. On the contrary, we observed that the late morphological changes—gradual condensation of chromatin at nuclear periphery—occur evidently after the ATP depletion. In addition, these late morphological changes are less synchronous than the early ones (Figs. 5b,c,d,e, 9). Therefore we assume that the final events of the chromatin condensation are energy independent and might be controlled by thermodynamic laws. This conclusion appears to be in contrast to the conclusion reported by Kass et al. [1996]. However, they did not distinguish different stages of chromatin condensation during the process of apoptotic body formation and considered the whole process as one event.

Detailed temporal analysis of DNA integrity demonstrated that DNA cleavage seemed to be a rather late event in dying cells (Figs. 5–7) and started approximately 3 h after addition of the drug, i.e., in cells with condensed chromatin. The onset of DNA degradation indicated an occurrence of 50-kbp fragments observed before nucleosomal fragments (Figs. 6, 7). Although we carried out these experiments many times (at least 5 times), we never observed 300-kbp fragments. The appearance of high-molecular weight (HMW) fragments (300-kbp size) is likely related to the gradual degradation of a specific and complex units of folded chromatin, rosettes, according to the model suggested by Filipski et al. [1990]. However, the interpretation of 300-kbp fragments occurrence is not consistent. Some investigators consider their appearance as an indicator of a synchronous engagement of apoptosis [e.g., Oberhammer et al., 1993a]. The others assume that these fragments arise from either the drug interaction with topoisomerase II [Filipski et al., 1990] or from a DNA damage induced by the drug [Oberhammer et al., 1993b]. On the contrary, there is general agreement that occurrence of 50-kbp fragments can be taken as a reliable indicator of endonucleolytic activity during apoptosis [Cohen et al., 1992; Oberhammer et al., 1993a,b; Walker et al., 1994]. Therefore, the absence of fragments 300 kbp and higher might indicate that BAPR did not induce the separate DNA breaks except for those related to apoptosis. Extensive DNA cleavage into 50-kbp and nucleosomal fragments appeared approximately 5–6 h after the drug addition, i.e., in cells that exhibited distinct apoptotic morphology (Figs. 5–7). More than 50% of DNA is degraded into fragments of 50 kbp and lower at 3–6 h, as estimated by densitometric evaluation of PFGE gel images (not shown). While discrete DNA cleavage into 50-kbp fragments continued even 12–24 h after BAPR application, oligonucleosomal DNA fragments gradually disappeared as a result of further degradation (Figs. 6, 7). The intensity of oligonucleosomal DNA degradation is evidently related to the concentration of BAPR applied; the oligonucleosomal DNA usually fully diminished 12 h after the application of 50 and 100 μM BAPR (Fig. 3).

The DNA cleavage into HMW and oligonucleosomal fragments seems to be energy indepen-

dent, as it takes place in cells with depleted ATP (Figs. 6, 7, 9). This finding is in a good agreement with the observations of other authors [Compton, 1992; Kass et al., 1996]. However, ATP level somehow modulates the intensity of nucleosomal fragment degradation. Compton [1992] showed that DNA within isolated nuclei treated by nucleases is fully degraded in the absence of ATP, while DNA degradation stopped at the nucleosomal level in the presence of ATP. Our results indicated similar causal relationship between the ATP level and an intensity of oligonucleosomal DNA degradation: the lower level of ATP, the more extensive degradation of nucleosomal DNA fragments was observed (Figs. 3, 9). Such intensive cleavage of oligonucleosomal DNA is rather unusual because oligonucleosomal DNA degradation proceeds relatively very slowly in other experimental systems with different mammalian cells. Starving HL-60 cells can be given as an example because nucleosomal DNA could be detected even 10 days after cell death (unpublished results). However, full degradation of oligonucleosomal DNA seems to be physiological because it takes place not only in isolated nuclei [Compton, 1992], but also in intact cells (our case).

Nevertheless, molecular mechanism by which ATP modulates the degree of DNA degradation is unknown. It must be noted that the application of strong inhibitors of the ATP synthesis that block production of ATP from both glycolysis and oxidative phosphorylation results in a prevention of DNA cleavage or in the apoptosis itself, respectively. It is likely, that the lack of ATP prevents triggering of the energy-requiring step before DNA cleavage [Eguchi et al., 1997; Kaufmann, 1989].

Owing to the fact that BAPR is a derivative of adenosine, and it undoubtedly shares some of its molecular properties, we compared cytotoxic effects of BAPR with that of adenosine. Although adenosine interferes with pyrimidine biosynthesis [e.g., Ishii et al., 1973], the cytotoxic effects of BAPR cannot be explained by this mechanism, as none of the used pyrimidines could prevent or reduce cell death (Table I). Tanaka and coworkers recently reported that, adenosine induced apoptosis in HL-60 cells is initiated by an accumulation of DNA double-strand breaks with subsequent formation of apoptotic bodies. Their explanation was supported by the finding of a marked enhancement of endogenous poly(ADP-ribosyl)ation activity in cells detected at relatively early stage of apoptosis [Tanaka et al., 1994]. Poly(ADP-ribosyl)ation, a known cellular response to DNA strand breaks, causes depletion of NAD and ATP, and consequently cell death. Therefore, inhibitors of poly(ADP-ribosyl)polymerase such as 3-ABA are able to protect cells from NAD and ATP depletion, and thus from apoptosis. Because 3-ABA failed to reduce BAPR-induced cell death (not shown) we assume that BAPR does not induce DNA strand breaks except those related to apoptosis. Moreover, in our experimental system, morphological changes evidently preceded DNA degradation (cf. Figs. 5–7). In addition, the fact that BAPR does not induce G2/M arrest in HL-60 cells further supports conclusion mentioned above as most of DNA damaging agents induces distinct G2/M arrest (unpublished results). On the basis of these findings, we assume that the mechanism of BAPR cytotoxicity differs from that of adenosine.

Purines, except adenosine, also failed to prevent apoptosis in BAPR-treated cells (Table I). Adenosine reduced the BAPR-induced apoptosis in a dose- dependent manner (Fig.10). However, we were unable to reach the full protection, since adenosine at concentrations exceeding 0.4 mM becomes cytotoxic itself (not shown). Dipyridamole (DP), an adenosine transport inhibitor, was also found to be ineffective in prevention of apoptosis in our experimental system (Table II). However, a protective effect of adenosine was reversed by DP (Table II). The exclusive protective effect of adenosine might indicate the antagonism between BAPR and adenosine rather than an interference of BAPR with the purine biosynthesis. Cell death induced by adenosine derivatives can be due either to the activation of extracellular adenosine receptors or to an intracellular mechanism of action [e.g., Franceschi et al., 1996]. Alteration of adenosine derivatives by phosphorylation seemed to be the most common intracellular mechanism of transformation of nontoxic compounds into toxic ones [Lin et al., 1988; Cottam et al., 1993]. The finding that adenosine can counteract the effect of BAPR only at the intracellular level (Table II) and in a concentration-dependent manner (Fig. 10), together with the fact that adenosine might act as a competitive inhibitor of adenosine kinase [Cottam et al., 1993], speaks for the latter mechanism. To prove

this hypothesis, a novel adenosine kinase inhibitor, AIRPP, was used to test whether phosphorylation of BAPR within cells is necessary for its activation. As 1 μM AIRPP fully reversed the cytotoxic effect of BAPR (Table III), it is evident that intracellular phosphorylation is necessary for the manifestation of BAPR cytotoxicity. Nevertheless, the causal connection between phosphorylated BAPR nucleotide(s) and apoptosis triggering remains to be elucidated.

N⁶-Benzylaminopurin-riboside (BAPR) seems to be a novel and very potent inductor of apoptosis in HL-60 human promyelocytic leukemia cells. Unique properties of BAPR-induced apoptosis, synchronous onset and course of apoptotic process, provide us with the opportunity to use this system as a new in vitro model for the study of apoptosis. Moreover, the short incubation period in the presence of BAPR necessary for apoptosis induction could be of potential therapeutic value in the treatment of leukemia.

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