Inhibitors of poly(ADP-ribose) polymerase suppress nuclear fragmentation and apoptotic-body formation during apoptosis in HL-60 cells

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Abstract The effects of 3-aminobenzamide (3ABm) and benzamide (BAm), known specific inhibitors of poly(ADP-ribose) polymerase (PARP), on actinomycin D (Act D)-induced apoptosis in HL-60 cells were examined. These inhibitors had no appreciable effect on apoptotic DNA fragmentation, chromatin condensation or PARP restriction cleavage, but clearly inhibited morphological changes, especially nuclear fragmentation and apoptotic-body formation, in a dose-dependent manner. These results suggest that the synthesis of ADP-ribose polymers is not essential for the progression of apoptotic DNA fragmentation and chromatin condensation, but is required in the processes leading to nuclear fragmentation and the subsequent apoptotic-body formation during apoptosis in HL-60 cells.

Key words: Apoptosis; Poly(ADP-ribosyl)ation; Cell morphology; DNA fragmentation; HL-60 cell

1. Introduction

Apoptosis is gene-directed active cell death caused by a variety of physiological and pathological stimuli [1–4]. Cell death by apoptosis is characterized by specific morphological patterns including the disappearance of microvilli, cell shrinkage, chromatin condensation, nuclear collapse and cellular fragmentation into apoptotic-bodies [5–7]. In most cases, these morphological changes are accompanied by internucleosomal DNA fragmentation [7–10]. Although many molecules participating in apoptosis have recently been reported, the molecular mechanisms by which these morphological changes are regulated remain unknown.

The poly(ADP-ribosyl)ation of chromosomal proteins is a post-translational modification that occurs in eukaryotic cells [11–13]; its physiological roles are suggested to include the regulation of DNA replication, repair and transcription through modulation of the chromatin structure [14–17]. Recently, a poly(ADP-ribose) synthetic enzyme, PARP, has been shown to be activated during the early stage of apoptosis in many cell types [18–24]. Furthermore, PARP is proteolytically digested to 85 and 30 kDa fragments by caspase family during apoptosis [24–26]. Thus, the importance of poly(ADP-ribosyl)ation in apoptosis has recently become a focus of research. However, the role of poly(ADP-ribosyl)ation during apoptosis remains unclear.

To address the significance of poly(ADP-ribosyl)ation in apoptosis, we examined the effects of PARP inhibitors on apoptosis in HL-60 cells. The results show that specific inhibitors of PARP suppress nuclear fragmentation and apoptotic-body formation without affecting chromatin condensation or DNA fragmentation. Our findings suggest that poly(ADP-ribosyl)ation plays an important role in late stage morphological changes that occur during apoptosis in HL-60 cells.

2. Materials and methods

2.1. Materials

3-Aminobenzamide (3ABm), benzamide (BAm), 3-amonobenzoic acid (3BAc), benzoic acid (BAc), actinomycin D (Act D), BSA fraction V, RNase A and Proteinase K were purchased from Sigma. Glutaraldehyde was from Seikagaku Kougyou. Hoechst 33258 was from Hoechst Japan. Immobilon P^{SQ} was from Millipore. All other reagents were of analytical grade. The polyclonal antibody against human PARP was a gift of Dr. Miwa M. and Dr. Uchida K. (University of Tsukuba, Japan).

2.2. Cell culture and induction of apoptosis

The human promyelocytic leukemia cell line HL-60 was maintained in suspension culture in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (Bocknek Ltd.). HL-60 cells were precultured for 1 h in the presence or absence of various concentrations of 3ABm or BAm. Apoptosis was induced by the addition of 1 μg/ml Act D to the culture medium.

2.3. Assay of DNA fragmentation

1×10^6 Cells were collected by centrifugation at $400 \times g$ and lysed in lysis buffer (50 mM Tris-HCl (pH 7.8), 10 mM EDTA, 0.5% (w/v) sodium *N*-lauroyl sarcosinate). The DNA was prepared by successive treatments with 0.5 mg/ml RNase A for 20 min and 0.5 mg/ml proteinase K for 30 min at 50°C. The resulting DNA preparation was subjected to electrophoresis in 2% agarose gels. DNA fragmentation, visualized by ethidium bromide staining, was examined in photographs taken under UV-illumination [32].

2.4. Western blot analysis

Total proteins from 2×10^5 cells were separated by SDS-PAGE and transferred onto PVDF membrane. Blots were blocked in TBST (20 mM Tris-HCl (pH 8.0), 400 mM NaCl, 0.05% (w/v) Triton X100) containing 2.5% BSA for 1 h and probed with the anti-PARP antibody. After washing with TBST, antibody retained on the membrane was detected using peroxidase-conjugated anti-guinea pig IgG (Jackson Immuno Research) and Proto Blot Western detection kit (Promega).

2.5. Microscopic analyses

Cells were collected by centrifugation at $400 \times g$ and fixed with 1% glutaraldehyde in PBS(−) at room temperature for 10 min. After washing with PBS(−), the cells were stained with 1 mM Hoechst 33258 in PBS(−) and observed under fluorescence microscopy. Transmission electron microscopic analysis was performed as described previously [27,28]. The frequency of apoptotic cells was determined by counting the blebbing cells under phase-contrast microscopy.

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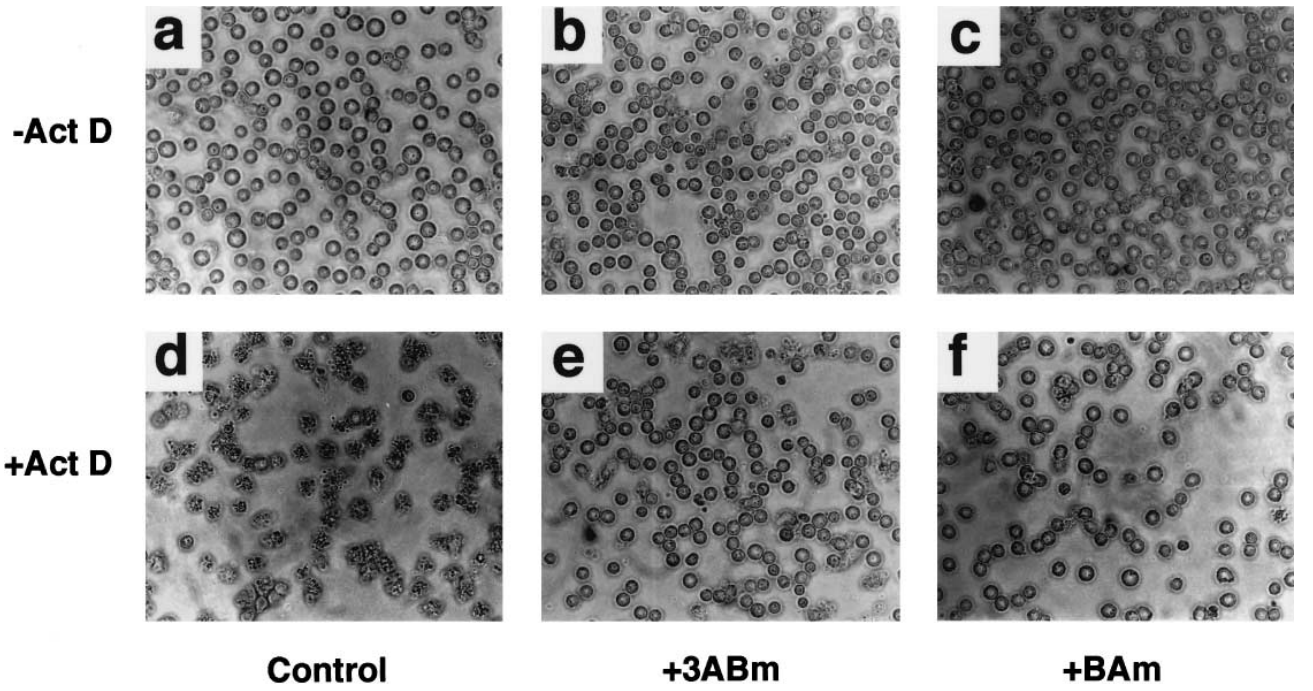

Fig. 1. Phase-contrast microscopic analysis of the morphology of HL-60 cells treated with Act D for 6 h in the presence or absence of 3ABm or BAm. Control (a), 1 mM 3ABm (b), 1 mM BAm (c), 1 μg/ml Act D (d), 1 mM 3ABm and 1 μg/ml Act D (e), 1 mM BAm and 1 μg/ml Act D (f). Magnification: ×400.

3. Results

Treatment with Act D (1 μg/ml) caused typical apoptotic morphological changes in HL-60 cells. Shrunken cells began to appear after 3 h. By 6 h, about 90% of cells had turned into membrane-bound small particles, so-called apoptotic-bodies (Fig. 1dFig. 4c,g). As illustrated in Fig. 4c,g, the characteristics of apoptosis, such as chromatin condensation, nuclear fragmentation and the disappearance of cell surface microvilli, were clearly observed. We next examined whether these morphological changes were followed by nuclear DNA fragmentation and PARP cleavage. As shown in Fig. 2d,g (upper panel), internucleosomal DNA cleavage and proteolysis of PARP occurred in a time-dependent manner. These results show that Act D induces typical apoptosis in HL-60 cells.

To address the importance of poly(ADP-ribosyl)ation in apoptosis, we examined the effects of the PARP inhibitors 3ABm and BAm on apoptosis in HL-60 cells. Neither 3ABm nor BAm (1 mM) alone had any essential cytotoxic effect on HL-60 cells. Neither morphological abnormalities (Fig. 1b,cFig. 4b,f) nor DNA fragmentation (Fig. 2b,c) was observed and cell viability also remained at control levels (data not shown). Next, we induced apoptosis in the presence of PARP inhibitors. HL-60 cells were pretreated with 3ABm or BAm (1 mM) for 1 h and apoptosis was induced by the addition of Act D (1 μg/ml). Microscopic analysis revealed that the cell shrinkage and apoptotic-body formation, which were observed in apoptosis caused by Act D alone, were strongly inhibited by 3ABm or BAm (Fig. 1e,f). As shown in Fig. 3a, the suppression of apoptotic-body formation by PARP inhibitors was achieved in a dose-dependent manner. Half-maximal inhibition by BAm and 3ABm was obtained at 140 μM and 200 μM, respectively (Fig. 3a). In contrast, 3ABc and BAc, non-inhibitory analogues of 3ABm and BAm, respectively, had no effects on apoptotic-body formation (Fig. 3a), suggesting that morphological suppression by PARP inhibitors is not due to non-specific effects but to the inhibition of PARP.

In contrast, apoptotic DNA fragmentation occurred normally in the presence of 3ABm or BAm (1mM) (Fig. 2e,f). The extent of DNA laddering was the same as that induced by Act D alone (Fig. 2d). Western blot analysis using the anti-PARP polyclonal antibody revealed that proteolytic cleavage of PARP was not also affected by 1 mM 3ABm (Fig. 2g, lower panel). The presence of up to 10 mM 3ABm had no effect on DNA fragmentation (Fig. 3b, upper panel). Although a slight inhibition was observed at higher BAm concentrations (Fig. 3b, lower panel), this was thought to be an artificial effect of BAm, because the concentration required for the inhibition of DNA fragmentation was significantly higher than that required for PARP inhibition [29].

To examine morphological suppression by PARP inhibitors in greater detail, we further examined the cells by fluorescence and electron microscopies. Fig. 4 clearly shows the suppression of nuclear fragmentation and apoptotic-body formation by 3ABm. However, PARP inhibitors could not prevent other apoptotic morphological changes. Fig. 4d,h show the failure of 3ABm to suppress chromatin condensation around the nuclear envelope or the disappearance of microvilli during apoptosis in HL-60 cells. The same results were obtained with BAm, but not 3ABc or BAc (data not shown).

4. Discussion

Since inhibitors of RNA or protein synthesis can prevent or delay the induction of apoptosis, the processes are thought to be under the regulation of suicide-genes and to require the new synthesis of proteins encoded by these genes [30–32].

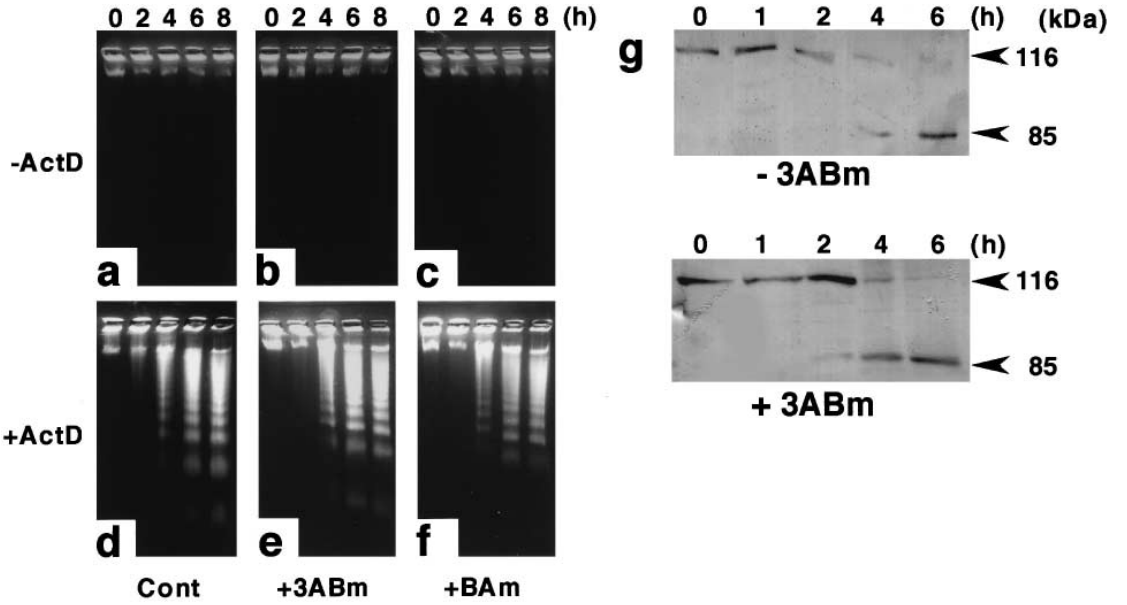

Fig. 2. Analyses of DNA and PARP cleavages in HL-60 cells treated with Act D in the presence or absence of PARP inhibitors. Apoptosis was induced by 1 μg/ml Act D in the absence or presence of 1 mM 3ABm. HL-60 cells were harvested at the indicated times after the induction of apoptosis and subjected to agarose gel electrophoresis (a–f) and Western blot analyses (g) as described in Section 2. Control (a), 1 mM 3ABm (b), 1 mM BAm (c), 1 μg/ml Act D (d,g, upper panel), 1 mM 3ABm and 1 μg/ml Act D (e,g, lower panel), 1 mM BAm and 1 μg/ml Act D (f). The 116 and 85 kDa bands represent intact and cleaved PARP, respectively.

However, these inhibitors are also known to induce or enhance apoptosis in some cell types [33–35]. In this study, we show that an RNA synthesis inhibitor, Act D, by itself induces typical apoptosis in HL-60 cells. This is consistent with previous reports [18,33,34] and suggests the existence of a new gene-expression-independent pathway(s) for apoptosis in this system.

By using this system, we examined the role of poly(ADP-ribosyl)ation in the process(es) of apoptosis. Our data of agarose gel electrophoresis clearly showed PARP inhibitors 3ABm or BAm to have no appreciable effect on apoptotic DNA fragmentation. The progression of DNA fragmentation occurred normally regardless of the presence of the PARP inhibitors. Furthermore, we saw no changes in the DNA ladder patterns in the presence of 3ABm up to 10 mM. These results suggest that the activation of PARP, poly(ADP-ribose) formation, is not essential in the cleavage of genomic DNA to nucleosomal units.

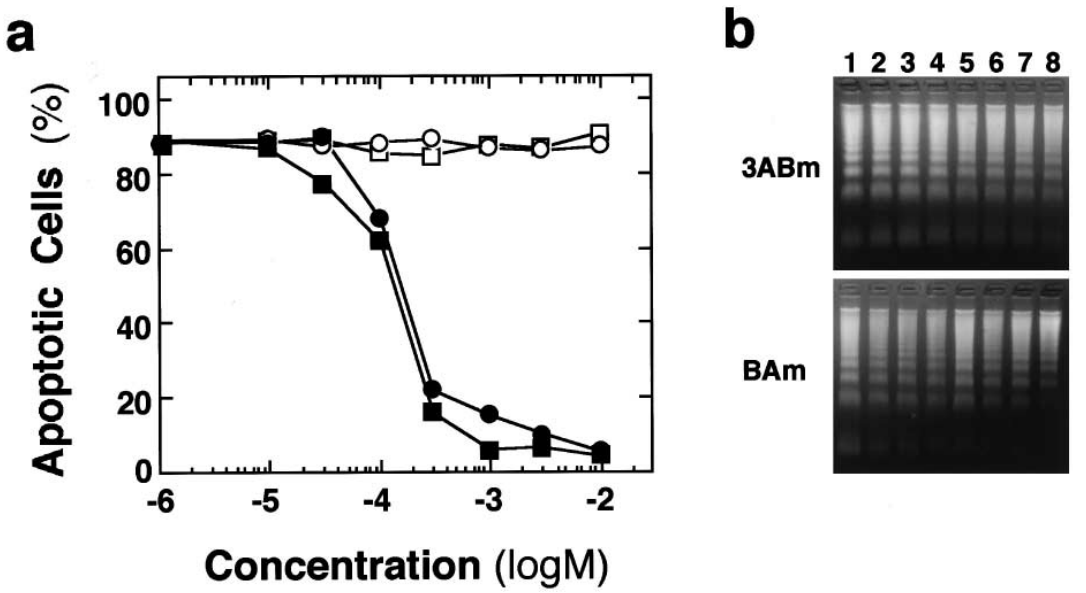

Fig. 3. Dose effects of PARP inhibitors on apoptotic-body formation (a) and DNA fragmentation (b) during Act D-induced apoptosis of HL-60 cells. (a) Apoptosis was induced by 1 μg/ml Act D in the presence of increasing concentrations of 3ABm (-●-), BAm (-■-), 3-ABc (-○-) or BAc (-□-). Apoptotic cells (apoptotic-body formation) were counted under phase-contrast microscopy after 6 h of treatment. Values are the averages of three separate experiments. (b) DNA fragmentation in the presence of 3ABm (upper panels) or BAm (lower panels) at concentrations of 0, 0.01, 0.03, 0.1, 0.3, 1, 3 or 10 mM (lanes 1–8, respectively) was analyzed by 2% agarose gel electrophoresis.

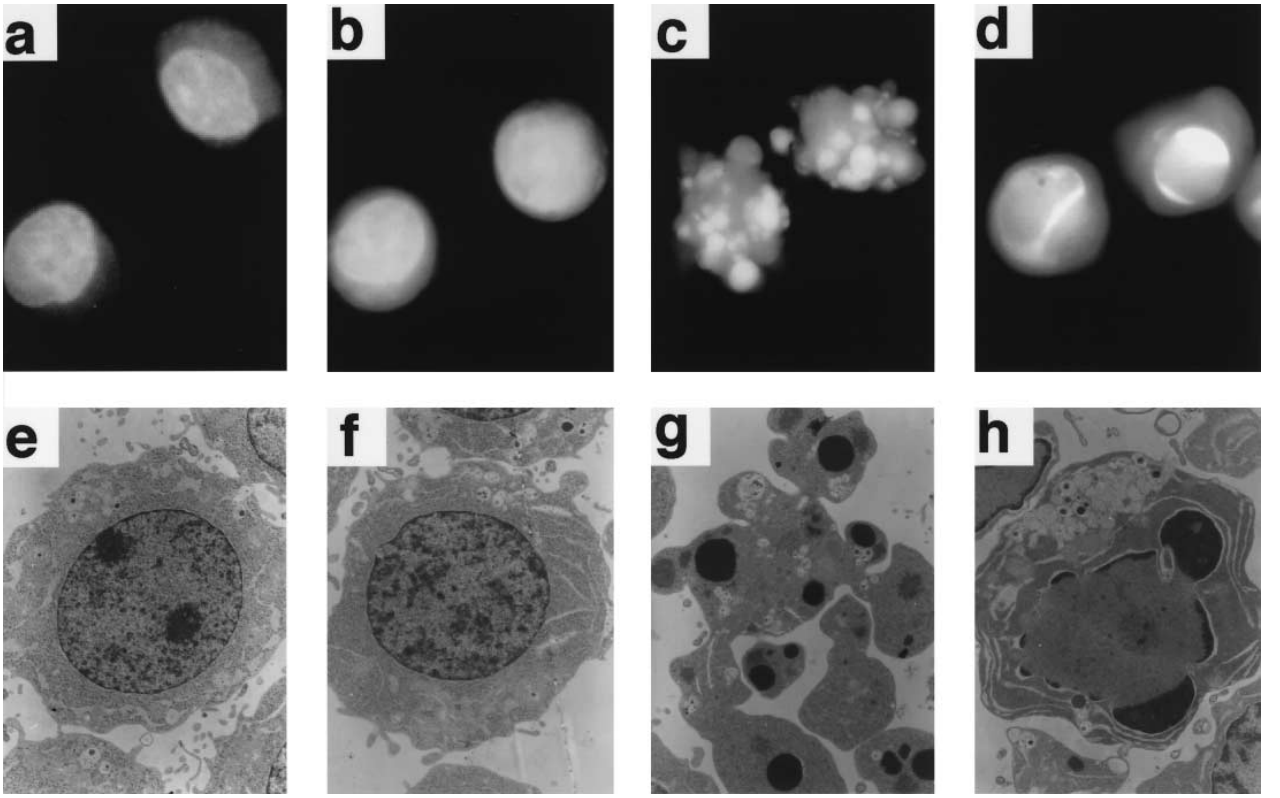

Fig. 4. Fluorescence (a–d) and transmission electron (e–h) microscopic analyses of HL-60 cells treated with Act D for 6 h in the presence or absence of 3ABm. Control (a,e), 1 mM 3ABm (b,f), 1 μg/ml Act D (c,g), 1 mM 3ABm and 1 μg/ml Act D (d,h). Magnifications: ×1000 (fluorescence microscopy); ×2500 (transmission electron microscopy).

In contrast, microscopic analyses revealed that some apoptotic morphological changes in HL-60 cells were prevented by PARP inhibitors. Importantly, nuclear fragmentation and subsequent apoptotic-body formation were strongly suppressed, although the disappearance of microvilli and chromatin condensation were not inhibited by PARP inhibitors. These results suggest that poly(ADP-ribosyl)ation of specific acceptor proteins and/or poly(ADP-ribose) metabolism through synthesis by PARP and hydrolysis by poly(ADP-ribose) glycohydrolase (PARG) [36–38] are required for nuclear collapse and apoptotic-body formation during the progression of apoptotic morphological changes (Fig. 5). The same results were obtained using camptothecin, adriamycin or cychrohexiimide as apoptosis inducers, indicating that the morphological suppression is not restricted to Act D-induced apoptosis.

The involvement of poly(ADP-ribosyl)ation in the apoptotic morphological changes seems to be contradictory to the proteolysis of PARP during apoptosis. The restriction

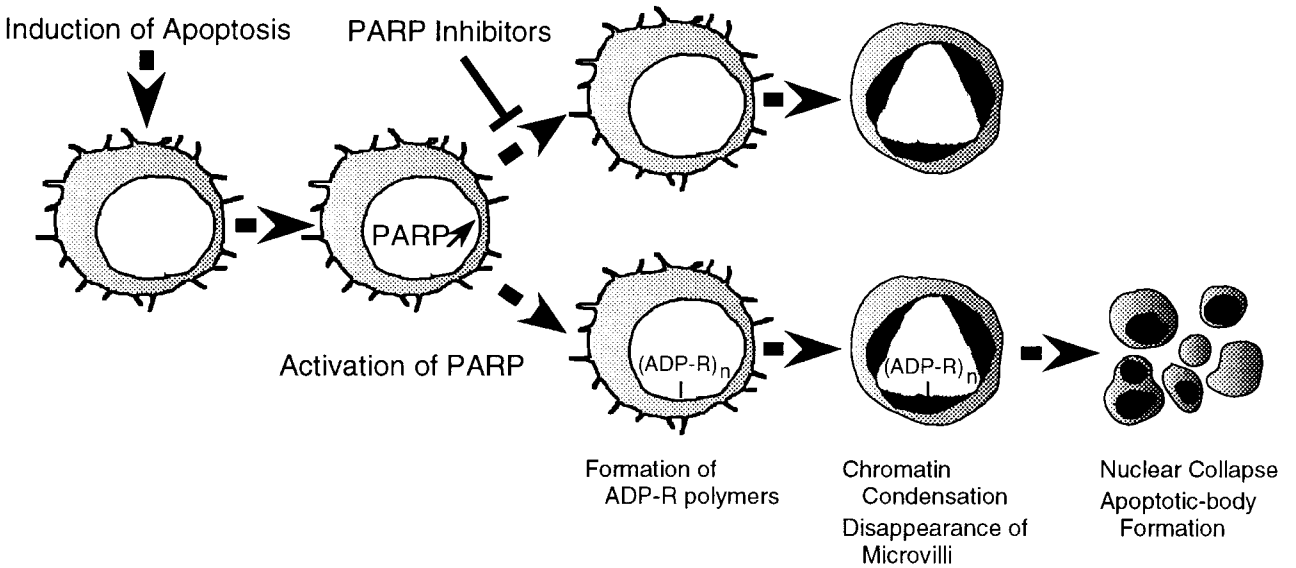

Fig. 5. Schematic illustration of the progression of apoptotic morphological changes in the presence or absence of PARP inhibitors.

cleavage of PARP is actually observed in our experimental system regardless of the existence of 3ABm. Previous studies have shown that PARP activation after induction of apoptosis precedes the commitment of cell death [18–24], and that proteolysis of PARP by caspase family occurs after the cells pass on the point of no-return [24–26]. Fig. 2g shows that PARP remains intact until 2 h after induction of apoptosis and begins to be proteolyzed as death by apoptosis goes on. Therefore, the morphological suppression by PARP inhibitors is thought to be due to the inhibition of PARP activation during the initial phase of apoptosis. That is, transient activation of PARP is considered to be required for the morphological changes occurred in the latter stage. This explanation is supported by the observation that PARP inhibitors could no longer suppress the morphological changes when they were added after the onset of PARP cleavage (data not shown).

The effects of PARP inhibitors, including 3ABm and BAm, on apoptosis have previously been examined [21–23,39–43]. In these papers, PARP inhibitors have shown to work inhibitory [21–23,39–41] or stimulatory [41–43] on apoptosis and it depends on cell types, culture conditions and apoptosis inducers. These facts indicate the involvement of poly(ADP-ribosyl)ation in multiple points of the pathways leading to and executing apoptosis.

In this report, we show that PARP inhibitors suppress nuclear fragmentation and apoptotic-body formation without affecting DNA fragmentation during the apoptosis of HL-60 cells. At present, the reason why the inhibition of poly(ADP-ribosyl)ation results in this suppression is unknown. Elucidation of the molecular mechanism(s) by which poly(ADP-ribosyl)ation is involved in the morphological changes of apoptosis is the next target of study.

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