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# Caspase Inhibition and $N^6$-Benzyladenosine-Induced Apoptosis in HL-60 Cells

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**Abstract** As an extension of our recently published work (Mlejnek and Kuglík [2000] J. Cell. Biochem. 77:6–17), the role of caspases in $N^6$-benzylaminopurine riboside (BAPR)-induced apotosis in HL-60 cells was evaluated in this study. Here, BAPR-induced apoptosis was accompanied by activation of caspase-3 and caspase-9. However, when these caspases were selectively inhibited, the progression of BAPR-induced apoptosis was not markedly affected. Besides that, activation of caspase-3 and caspase-9 was found to be rather late event in apoptotic process. These results suggested that other caspases might be critically implicated. Indeed, pan-specific caspase inhibitor, Z-VAD-FMK, completely prevented DNA cleavage and apoptotic bodies formation. However, Z-VAD-FMK failed to prevent cell death and it was incapable to fully counteract the main apoptotic hallmark-chromatin condensation. Finally, our data indicate that cellular decision between apoptosis and necrosis is made upon the availability of both caspase proteases and intracellular ATP. J. Cell. Biochem. 83: 678–689, 2001. 

**Key words:** caspase-3; caspase-9; Ac-LEHD-CMK; Z-DEVD-FMK; Z-VAD-FMK; cell death; $N^6$-benzylaaminopurine riboside; nuclear apotosis

Artificial adenosine derivatives are very potent inducers of apoptosis in many cell types [reviewed by Franceschi et al., 1996]. Some of them, including flurabidine (9-β-D-arabinofuranosyl-2-fluoroadenine), clarabidine (2-chlorodeoxyadenosine), and 2′-deoxycoformycin, are successfully applied in cancer therapy [Johnson et al., 1996; Juliusson et al., 1996]. The effectiveness of such cancer chemotherapy depends largely on the ability of anticancer drugs to trigger apoptosis in malignant cells. Although, it is well documented that cytotoxicity of artificial adenosine derivatives is related to intracellular accumulation of their 5′-triphosphate metabolites [Lin et al., 1988; Cottam et al., 1993], the mechanism by which 5′-triphosphates could participate or eventually trigger apoptotic cell death program, has remained unknown for a long time. Only recently, it was demonstrated that stereotypical changes accompanying apoptosis reflect complex biochemical events in which central role was attributed to activation caspase proteases [reviewed by Cohen, 1997].

Caspases are a family of mammalian cysteine proteases that exhibit significant homology to gene *ced-3* coding for a protein that is required for developmental cell death in the nematode *Caenorhabiditis elegans* [Horvitz et al., 1994]. A distinctive feature of all caspases is the absolute requirement of aspartic acid residue in target sequence of their substrates. Caspases can be subdivided into three groups according to their substrate and inhibitor profile. Group I, comprising caspase 1, 4, and 5, possess WEHD sequence within their preferred substrates (ICE subfamily). Group II, comprising caspase 2, 3, and 7, prefer an invariant aspartate at the start of tetrapeptide DEHD/DEVD that they cleave. These caspases are often referred to as executioners of apoptosis since they cleave nuclear scaffold proteins, cytoskeletal proteins, proteins that participate in DNA repair [e.g., poly(ADP-ribose)polymerase], and many other important cellular proteins. Caspase-3 has been reported to be the most frequently activated caspase protease in apoptotic cells, indicating its crucial

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role in the cell death process. In some cell systems, caspase-3 activity is essential for chromatin condensation and for DNA degradation but not for other apoptotic hallmarks. Group III, comprising caspase 6, 8, 9, and 10, cleaves and activates many pro-caspases and other "apoptotic" substrates which indicates that they probably work as upstream activators of caspase pathway. Precisely the choice which caspase family members are activated seems to be stimulus dependent and tissue-specific for a given stimulus [Cohen, 1997; Faleiro et al., 1997; Woo et al., 1998].

Currently three pathways that lead to activation of caspases are well characterized; the mitochondria-mediated pathway, the cell surface death receptor pathway, and granzyme B-mediated pathway. In the mitochondria-mediated pathway, apoptotic program seems to be initiated by the release of cytochrome *c* from mitochondria to cytosol. In cytosol, cytochrome *c* binds to Apaf-1 protein (apoptosis protein-activating factor) and, in the presence of ATP/dATP, activates procaspase-9. This molecular event triggers activation of caspase cascade that leads to a rapid and irreversible proteolysis resulting in stereotypical morphological and biochemical changes-apoptosis [reviewed by Budihardjo et al., 1999]. Genini et al. [2000] recently demonstrated that 5′-triphosphates of many derivatives of adenosine, that are already being clinically used (such as Ara-ATP, 9-fluoro-9-beta-D-arabinofuranosyladenine 5′-triphosphate, 2CdATP, and 9-beta-D-arabinofuranosylguanine 5′-triphosphate), can co-operate with cytochrome *c* and Apaf-1 to trigger a caspase activation in a cell-free system.

The activation of cell surface death receptor pathway is initiated by the engagement of cell surface death receptors with their specific ligands such as Fas/CD95 ligand or tumor necrosis factor (TNF). This event initiates the activation of downstream caspases by first turning on caspase-8 [reviewed by Budihardjo et al., 1999].

In the third pathway, directly caspase-3, a major executing caspase, becomes activated by granzyme B released by T lymphocytes into the target cell [Darmon et al., 1995].

In this study, the role of caspases in $N^6$-benzylaminopurine riboside-induced apoptosis was investigated. We observed that in HL-60 cells, the process of nuclear apoptosis consists of at least two components with different sensitivity to caspase inhibitors. While the first component (chromatin condensation) seems to be only partially caspase-dependent, the second one (formation of apoptotic bodies and DNA cleavage) seems to be fully dependent on caspase activity. Importantly, although they become active, caspase-3 and caspase-9 are not critical for apoptosis to proceed in HL-60 cells.

## MATERIALS AND METHODS

### Cell Culture and Cell Treatment

HL-60 cells were cultured as described elsewhere [Mlejnek and Kuglík, 2000]. Treatment with $N^6$-benzyladenosine = $N^6$-benzylaminopurine riboside (BAPR) was done according to Mlejnek and Kuglík [2000].

Pan-specific caspase inhibitor, Z-VAD-FMK, and specific caspase-3 inhibitor, Z-DEVD-FMK, purchased from Calbiochem, were dissolved in DMSO. Specific caspase-9 inhibitor, Ac-LEHD-CMK, purchased from Bachem was dissolved in DMSO. To achieve maximum inhibitory effect caspase inhibitors were applied at least 1 h prior the cell treatment. The final concentration of DMSO in culture medium was approximately 0.1%.

### Plasma Membrane Permeability Analysis

Differential staining using fluorescein diacetate (FDA) and propidium iodide (PI) was done as described elsewhere [Mlejnek and Kolman, 1999].

### Morphological Analysis of Apoptosis

Cells were fixed and stained with Hoechst 33342 as described previously [Mlejnek and Kuglík, 2000]. Nuclear morphology was examined using fluorescence microscope.

### DNA Integrity Measurement

DNA fragmentation to nucleosomal level was assessed using standard agarose gel electrophoresis [Mlejnek and Kuglík, 2000].

### Caspase Activity Measurement

Cells were washed twice with PBS and then lysed in caspase extraction buffer [50 mM HEPES/NaOH, pH 7.4, 1 mM EDTA, 0.2% Chaps, 5 mM dithiothreitol, and proteinase inhibitor cocktail (Roche)] for 15 min at 4°C. Lysates were centrifuged for 30 min at 30,000*g* at 4°C. Clarified lysates were either used

immediately or stored at −80°C until measurement. The assay was carried out in 1-ml tubes, aliquots of lysates (80–100 μg of total protein) were mixed with 1 ml of caspase assay buffer (25 mM PIPES/KOH, 2 mM EGTA, 2 mM $MgCl_2$, and 5 mM DTT, pH 7.3 and 6.6 for caspase-3 and caspase-9, respectively) and reactions were initiated by addition of specific substrate (100 μM). After 30 min incubation at 37°C fluorescence was monitored at appropriate excitation and emission wavelengths. Caspase-1-like activity was measured with substrate Ac-YVAD-AMC (caspase class I substrate) and caspase-3-like protease activity was measured using Ac-DEVD-AMC (caspase class II substrate) at excitation and emission wavelengths of 380 and 445 nm, respectively. Caspase-9-like activity was measured with substrate Ac-LEHD-AFC at excitation and emission wavelengths of 400 and 500 nm, respectively.

### Statistical Analysis

Data are reported as the mean ± SD. Statistical significance of differences was determined by Student's *t*-test. The *P* values less than 0.05 were considered significant.

## RESULTS

In our previous study we have described the effects of $N^6$-benzylaminopurine-riboside (BAPR) on human promyelocytic HL-60 cells. We have demonstrated that BAPR is a new and very potent inductor of apoptosis as documented by chromatin condensation, formation of apoptotic bodies, and cleavage of DNA to nucleosomal fragments. Moreover, BAPR-induced apoptosis proceeded relatively fast and synchronously with respect to changes in nuclear morphology [Mlejnek and Kuglík, 2000]. Here, hydrolysis of caspase-1 (caspase class I) substrate, Ac-YVAD-AMC, and caspase-3 (caspase class II) substrate, Ac-DEVD-AMC, were determined in lysates of BAPR-treated cells, in order to investigate whether BAPR-induced apoptosis is associated with caspase activation. As shown in Figures 1a,b, while only negligible hydrolysis of Ac-YVAD-AMC was observed upon the treatment with BAPR, Ac-DEVD-AMC was extensively hydrolyzed under the same conditions. Besides caspase-3, also caspase-9 became active as clear from the elevated hydrolysis of Ac-LEHD-AFC, a specific substrate of caspase-9 (Fig. 1c). Together, these findings suggested the involvement of caspase-3 and caspase-9 in BAPR-induced apoptosis.

To approach the possible role of caspase proteases in BAPR-induced apoptosis, three different caspase inhibitors were employed, Z-DEVD-FMK as the selective inhibitor of caspase-3 (DEVDase activity), Ac-LEHD-CMK as the selective inhibitor of caspase-9 (LEHDase activity), and Z-VAD-FMK as the pan-specific caspase inhibitor. As expected, activities of both caspase-3 and caspase-9 were completely inhibited by Z-VAD-FMK. Similarly, Ac-LEHD-CMK completely inhibited caspase-9 and diminished also the activity of caspase-3. However, Z-DEVD-FMK completely inhibited not only caspase-3 activity but negatively influenced also the activity of caspase-9 (Fig. 2). Thus, these results could not support hypothesized simple hierarchical relationship between caspase-9 and caspase-3.

The integrity of plasma membrane, nuclear morphology, and the integrity of DNA were all assessed in BAPR-treated HL-60 cells in order to determine whether caspase inhibition could affect BAPR-induced cell death. Combined staining using FDA and PI indicated that both Z-DEVD-FMK and Ac-LEHD-CMK could substantially reduce the number of dying cells 24 h after the treatment (Fig. 3). However, only slight difference in the number of dying cell was observed 48 h after the treatment (Fig. 3). Hoechst staining revealed that neither caspase-3 nor caspase-9 inhibitors substantially reduced the number of apoptotic cells at the same observation period (Fig. 4). Therefore, it was evident that both inhibitors only retarded disruption of plasma membrane in dying cells. None of the specific caspase inhibitors, Z-DEVD-FMK and Ac-LEHD-CMK, thoroughly prevented manifestation of nuclear hallmarks of apoptosis, such as chromatin condensation and formation of apoptotic bodies (Figs. 4, 5c, and d), but only slightly retarded apoptotic process (not shown).

Although Z-VAD-FMK was unable to protect cells from dying, and exhibited only weak protective effect on plasma membrane integrity (Figs. 3 and 4), it completely blocked the formation of apoptotic bodies in BAPR-treated cells (Figs. 4, 5e, and f). This pan-specific inhibitor also prevented cytoplasmatic changes that are typical for apoptosis, such as membrane blebbing and cell shrinkage (not shown). However, Z-VAD-FMK failed to fully prevent

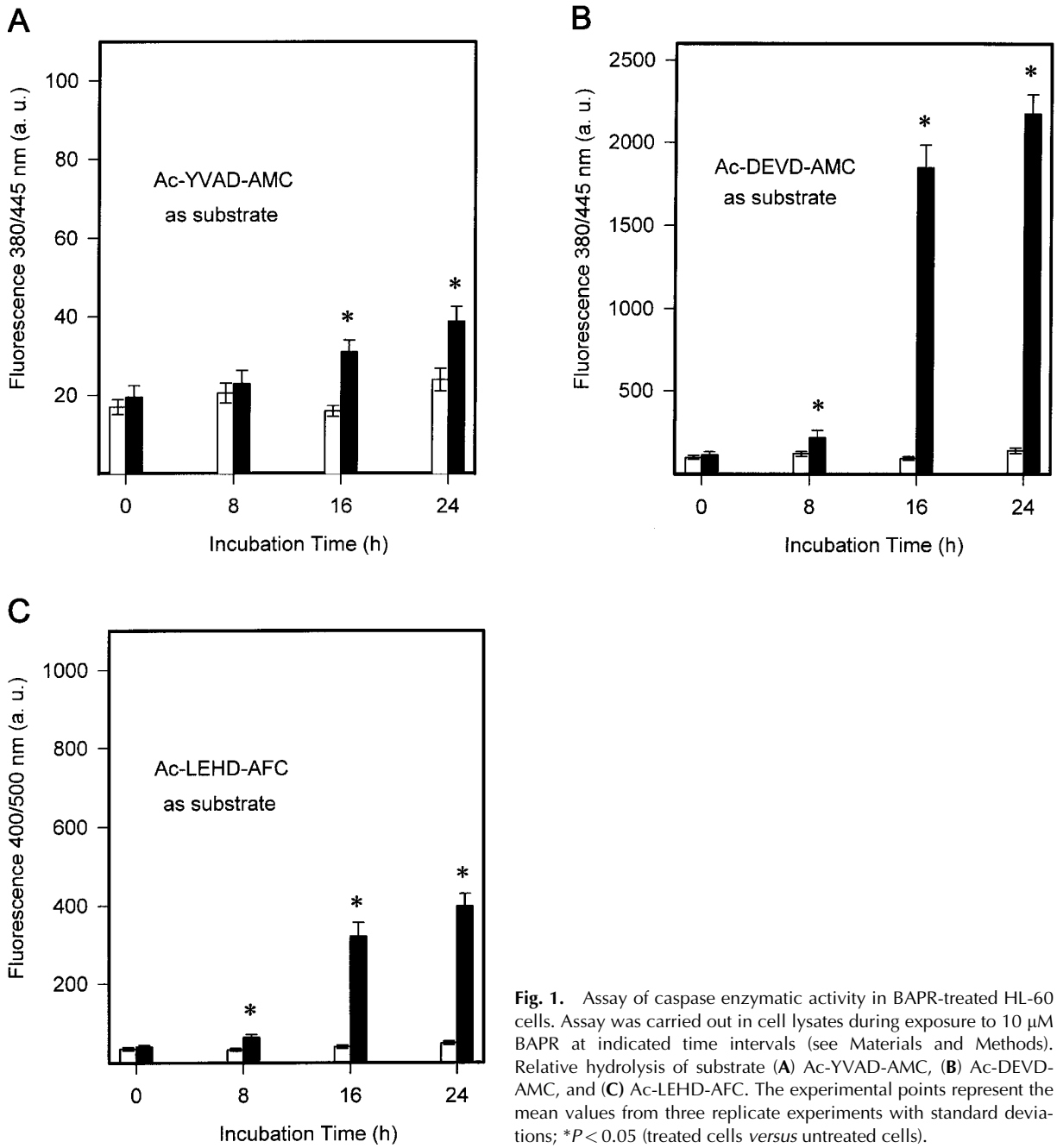


**Fig. 1.** Assay of caspase enzymatic activity in BAPR-treated HL-60 cells. Assay was carried out in cell lysates during exposure to 10 μM BAPR at indicated time intervals (see Materials and Methods). Relative hydrolysis of substrate (**A**) Ac-YVAD-AMC, (**B**) Ac-DEVD-AMC, and (**C**) Ac-LEHD-AFC. The experimental points represent the mean values from three replicate experiments with standard deviations; $*P < 0.05$ (treated cells *versus* untreated cells).

chromatin condensation in BAPR-treated cells even when applied in concentration of 50 μM. Therefore, necrosis-like nuclear morphology was observed only in approximately 50% of dying cells (Fig. 5f). Remaining 50% of nuclei in dying cells formed small mono-spherical ("collapsed") nuclei with dense chromatin (Fig. 5e). These results suggested that chromatin condensation is the only process occurring during apoptosis that could not be fully abolished by inhibiting caspases. Increasing the concentrations of BAPR in the presence of Z-VAD-FMK led to proportional increase in the number of cells with necrotic-like nuclear morphology (Fig. 6). Thus, 50 and 100 μM BAPR combined with 50 μM Z-VAD-FMK induced cell death with almost 100% necrotic-like nuclear morphology (Fig. 6). It is important to mention that these supra-lethal BAPR concentrations induced 100% apoptosis in the absence Z-VAD-FMK

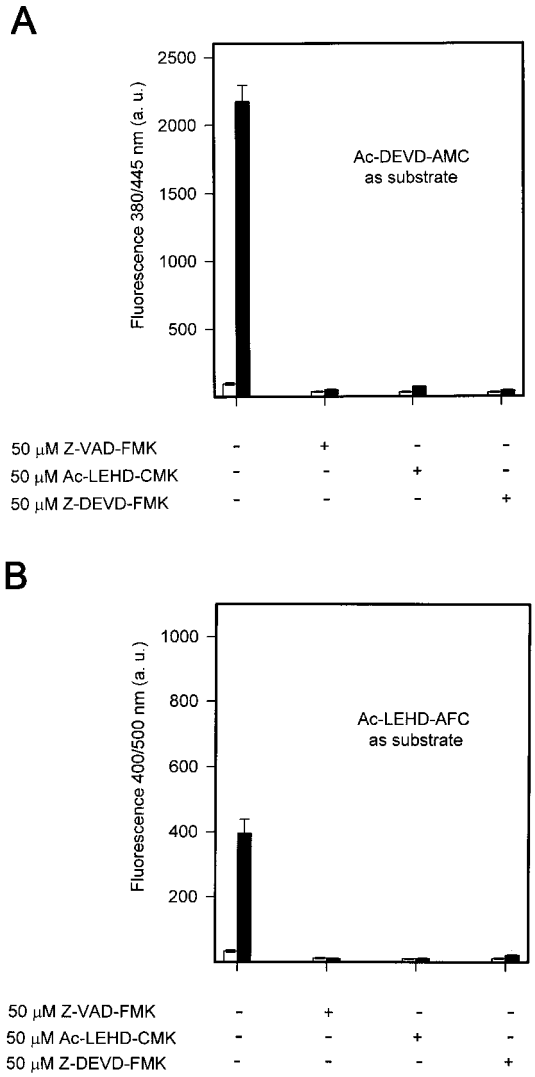


**Fig. 2.** Effect of caspase inhibitors on hydrolysis of (**A**) caspase-3 substrate and (**B**) caspase-9 substrate. Assay was determined in lysates prepared from cells that were treated with 10 μM BAPR with or without caspase inhibitor, as indicated. Treated cells (black columns), control cells (plain columns). The experimental points represent the mean values from three replicate experiments with standard deviations.

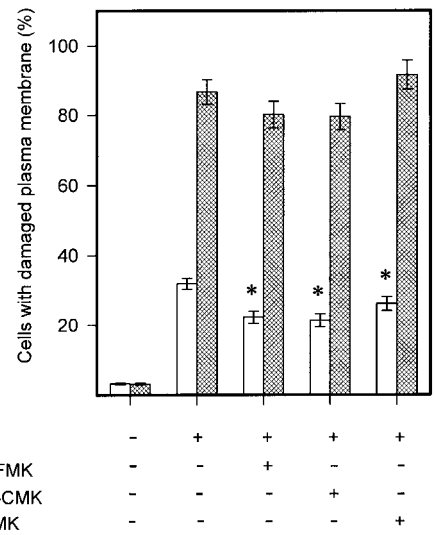


**Fig. 3.** Effect of caspase inhibitors on plasma membrane integrity. Cells were treated with 10 μM BAPR in the presence or absence of caspase inhibitor, as indicated. Cells were incubated prior staining with FDA and PI for 24 h (plain columns) and 48 h (diamond dashed columns). Number of cells with damaged plasma membrane (= red cells) was evaluated using fluorescence microscopy. At least 250–300 cells were examined in one experiment. The experimental points represent mean values from three replicate experiments with standard deviations; $^*P < 0.05$ (BAPR-treated cells without inhibitor *versus* BAPR-treated cells with inhibitor at the same observation period).

[Mlejnek and Kuglík, 2000]. Such increase in occurrence of necrotic-like nuclear morphology might be related to the availability of intracellular ATP. This hypothesis stems from our previous finding that the supra-lethal BAPR concentrations caused rapid depletion of intracellular ATP [Mlejnek and Kuglík, 2000].

Analyzing DNA integrity revealed that neither Z-DEVD-FMK nor Ac-LEHD-CMK was able to fully prevent nucleosomal DNA cleavage (Fig. 7). Still, detailed timely analysis have shown that both inhibitors slightly slowed down the cleavage of DNA (not shown). In contrast, no fragmentation of DNA took place upon the treatment with Z-VAD-FMK (Fig. 7). Therefore, it was evident that Z-VAD-FMK prevented DNA cleavage in both populations of morphologically different nuclei.

Detailed kinetic analysis of caspase-3 and caspase-9 activities in BAPR-treated cells was accomplished in order to elucidate whether caspase-9 activation precedes the activation of caspase-3. As it is obvious from Figure 8, the results did not provide evidence for such hierarchy in the activation of caspases. To decide whether caspase activation is an early or rather late event, the dynamics of changes in chromatin structure and of caspase activity were determined in BAPR-treated cells and they were compared with each other. The concentration of 50 μM was used, since high BAPR concentrations offer better results due to perfect synchrony of changes in chromatin morphology [Mlejnek and Kuglík, 2000]. The activation of both caspase-3 and caspase-9 was associated with final rather than early stages of apoptotic chromatin (Fig. 9). Similar results were obtained also for lower concentrations of

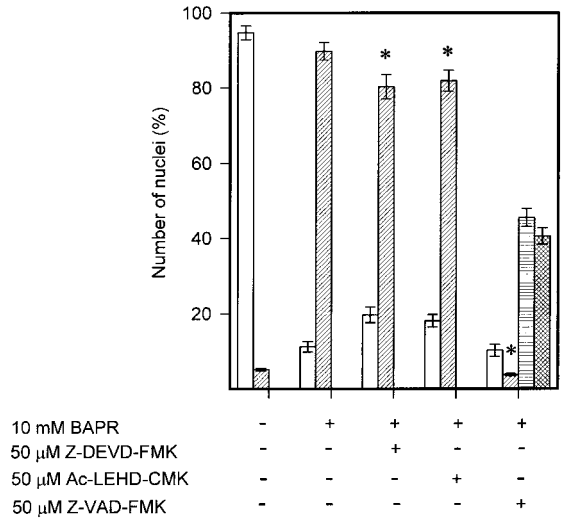


**Fig. 4.** Effect of caspase inhibitors on nuclear morphology. Cells were treated with 10 μM BAPR in the presence or absence of caspase inhibitor, as indicated. After the 24-h incubation, cells were stained with Hoechst and nuclear morphology was examined using fluorescence microscopy. Normal nuclear morphology (plain columns), nuclei fragmented in apoptotic bodies (angel dashed columns), small condensed nuclei (horizontal dashed columns), and necrotic nuclei (diamond dashed columns). Untreated cells were taken as control. At least 250–300 cells were examined in one experiment. The experimental points represent mean values from three replicate experiments with standard deviations; $*P < 0.05$ (apoptotic cells induced by BAPR without inhibitor *versus* apoptotic cells induced by BAPR with inhibitor).

BAPR (not shown). Therefore, caspase-9 and caspase-3 activation seems to be the late event in BAPR-induced apoptosis. Although such finding was rather surprising, it offered consistent explanation for the failure of caspase-specific inhibitors to influence apoptotic process in BAPR-induced apoptosis.

## DISCUSSION

Detailed measurement of caspase activity using specific caspase substrates was performed in order to identify the members of caspase protease family that are involved in BAPR-induced apoptosis. Fluorometry-based assay of caspase activity revealed that BAPR-induced apoptosis was accompanied by marked increase in caspase-3 (DEVDase) as well as caspase-9 (LEHDase) activity (Fig. 1b,c). We assume that the negligible but significant increase in hydrolysis of Ac-YVAD-AMC could be attributed to some kind of non-specific hydrolysis rather than to caspase-1 (YVADase) activity (Fig. 1a). These results indicate that BAPR-induced apoptosis most likely belongs to a large group of apoptotic systems with distinct caspase-3 and caspase-9 activation.

To specifically address the role of caspase-3 and caspase-9 in BAPR-induced apoptosis the effect of three different caspase inhibitors, Z-VAD-FMK, Z-DEVD-FMK, and Ac-LEHD-CMK on cell survival, chromatin morphology, and DNA integrity was studied. The data document that neither pan-specific caspase inhibitor Z-VAD-FMK nor specific inhibitors of caspase-3 and caspase-9, Z-DEVD-FMK, and Ac-LEHD-CMK, respectively, are able to prevent BAPR-induced cell death (Figs. 3 and 4). An observable weak protective effect exhibited by caspase inhibitors was most likely mainly due to the kinetic retardation of plasma membrane disruption (Figs. 3 and 4). Therefore, it seems that caspase inhibition does not prevent the signaling phase of apoptosis induced by BAPR. This observation is very much consistent with findings of other authors [e.g., Hirsch et al., 1997]. Furthermore, comparison of two different staining methods (Hoechst *versus* FDA/PI) indicated that discrimination of living and dead cells based only upon the plasma membrane integrity might not be quite reliable criterion for apoptosis to occur. It is due to the fact that only cells in advanced stages of apoptosis are detectable by the combined staining using FDA and PI (Figs. 3 and 4).

Notably, manifestation of nuclear apoptotic hallmarks including chromatin condensation, apoptotic bodies formation, and DNA cleavage was substantially affected by Z-VAD-FMK (Figs. 4, 5e,f, and 7). This effect was found to be markedly dependent on the concentration of BAPR applied. At sub-lethal concentrations of BAPR, Z-VAD-FMK completely inhibited DNA cleavage (Fig. 7) as well as formation of apoptotic bodies but it did not fully prevent the condensation of chromatin. This resulted in the occurrence of two morphologically distinct populations of nuclei (Fig. 5e,f). The first population, in which the condensation of chromatin was prevented by Z-VAD-FMK, was typical by the necrotic-like nuclear morphology (Fig. 5f). Such nuclear morphology was observable in about 50% of dying cells. Owing to the fact that DNA of those cells was intact (Fig. 7) and their cytoplasm underwent changes that correspond to necrosis (not shown), these cells can be considered as the "true" necrotic. The

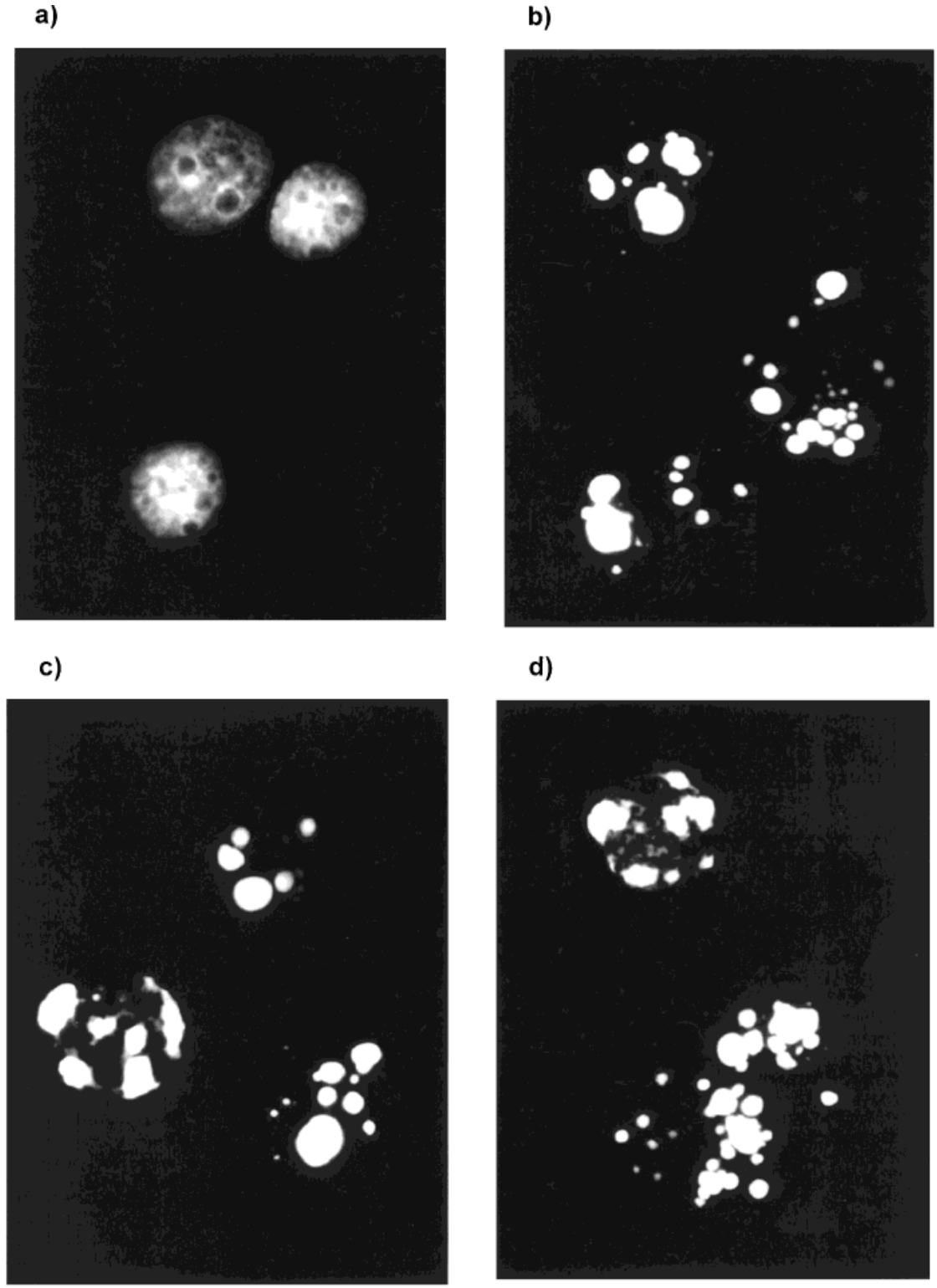


**Fig. 5.** Effect of caspase inhibitors on chromatin morphology in BAPR treated HL-60 cells. Cells were treated with 10 μM BAPR in the presence or absence of caspase inhibitor, as indicated. Cells were further incubated for 24 h prior to staining with Hoechst 33342, as described in Material and Methods, and processed for fluorescence microscopy. Cell nuclei of untreated cells (**a**), cells cultured in the presence of 10 μM BAPR (**b**), 10 μM BAPR+50 μM Z-DEVD-FMK (**c**), 10 μM BAPR+50 μM Ac-LEHD-CMK (**d**), 10 μM BAPR+50 μM Z-VAD-FMK (small condensed) (**e**), and 10 μM BAPR+50 μM Z-VAD-FMK (necrotic/swollen) (**f**).

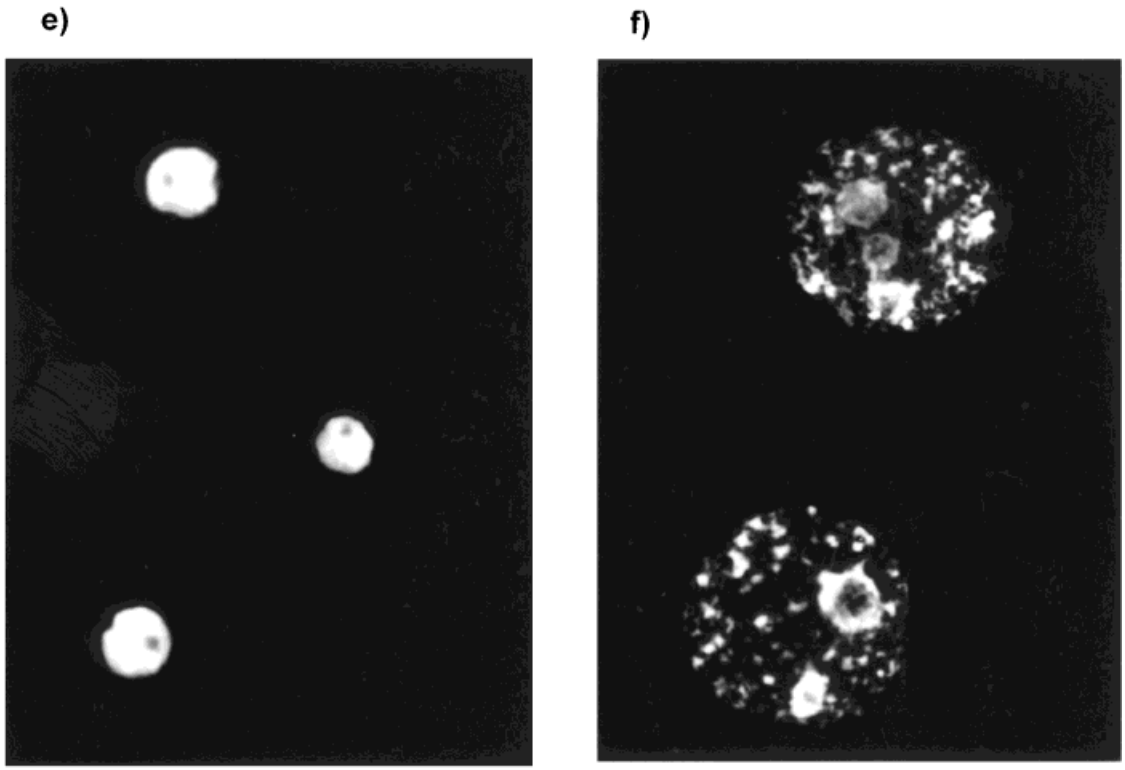


**Fig. 5.** *(Continued)*

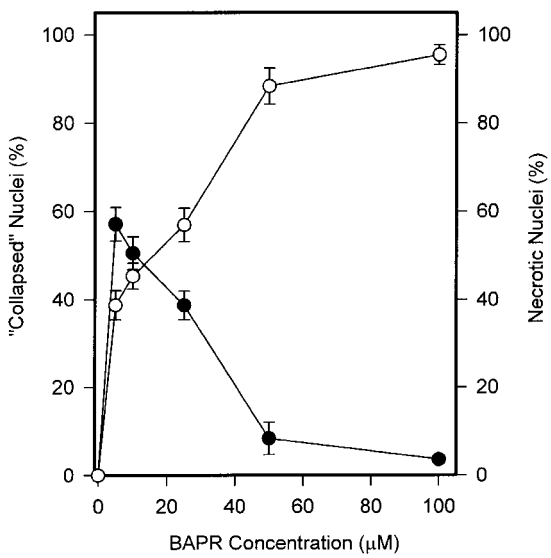


**Fig. 6.** Effect of BAPR concentration on nuclear morphology in the presence of Z-VAD-FMK. Cells were treated with 50 μM Z-VAD-FMK in the presence of given BAPR concentration. Cells were further incubated for 24 h prior to staining with Hoechst 33342 and nuclear morphology was examined using fluorescence microscopy (see Material and Methods). Small ''collapsed'' nuclei, left axis (filled circles), necrotic nuclei, right axis (open circles). 100% = Σ of dead cells i.e., cells with apoptotic + necrotic + small ''collapsed'' nuclei. The experimental points represent mean values from three replicate experiments with standard deviations; at least 250–300 cells were examined for each dose in one experiment.

second population of cells, in which chromatin condensation proceeded even in the presence of Z-VAD-FMK, were characterized by the occurrence of small mono-spherical ("collapsed") nuclei with dense chromatin (Fig. 5e). Such altered chromatin condensation appeared in about 50% of dying cells. While nuclear DNA of cells with collapsed nuclei was obviously intact (Fig. 7) even at the level of high molecular weight fragments (not shown), their cytoplasm underwent changes that correspond to necrosis (not shown). Still, the chromatin of such cells was condensed (Fig. 5e). Taken these phenomena together, the mode of cell death in those cells cannot be considered as "true" necrotic. This conclusion is based on the classical definition of apoptosis published by Kerr and Harman [1991], where chromatin condensation is one of the crucial apoptotic hallmarks. Rising concentrations of BAPR have led to the proportional increase in occurrence of nuclei with necrotic-like morphology (Fig. 6). Z-VAD-FMK completely prevented manifestation of nuclear apoptosis including chromatin condensation only upon the application of supra-lethal concentrations of BAPR (50 and 100 μM), which normally still induced apoptosis [see Mlejnek and Kuglík, 2000]. In this case dead cells fully

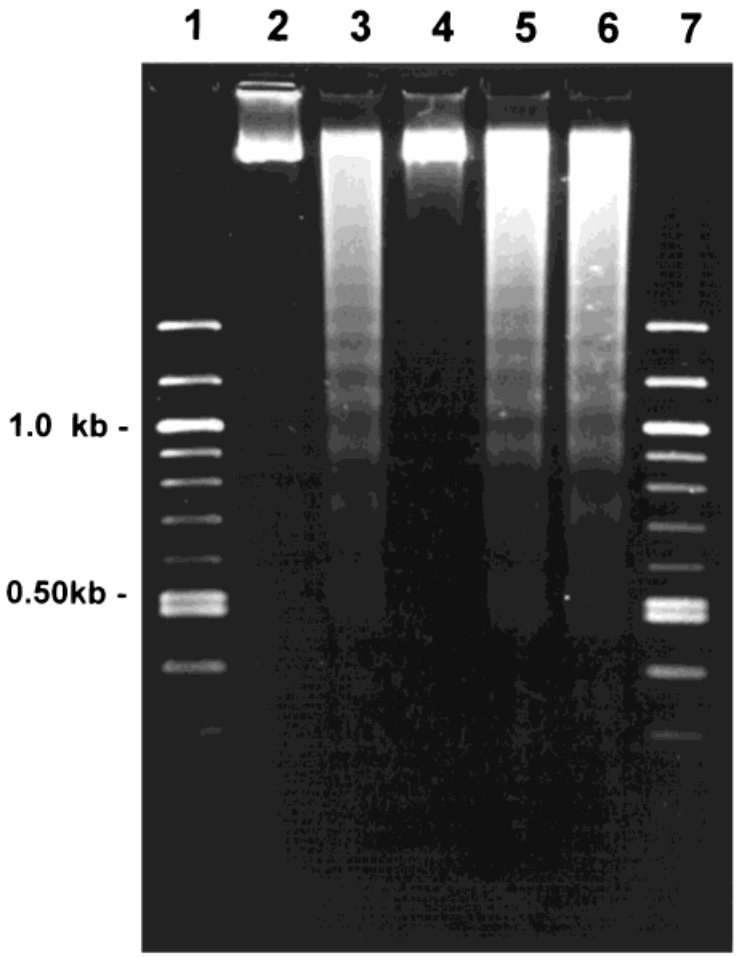


**Fig. 7.** Effect of caspase inhibitors on DNA fragmentation in BAPR-treated HL-60 cells. DNA was extracted 24 h after cell treatment with 10 μM BAPR (**lane 3**), 10 μM BAPR + 50 μM Z-VAD-FMK (**lane 4**), 10 μM BAPR + 50 μM Z-DEVD-FMK (**lane 5**), and 10 μM BAPR + 50 μM Ac-LEHD-CMK (**lane 6**). Untreated cells were taken as a control (**lane 2**), molecular weight markers (**lane 1** and **7**).

matched the criteria for the necrotic cell death mode (Figs. 5f and 6). Our results indicate that, at least in BAPR-treated HL-60 cells, the nuclear apoptosis involves two processes with different sensitivity to caspase inhibition. The first, process of chromatin condensation is only partially sensitive to caspase inhibition and thus only indirectly or partially caspase-dependent. This conclusion is in a good agreement with recent observation that early events in apoptotic process that precede caspase activation, such as translocation of apoptosis inducing factor (AIF) from mitochondria to nucleus, are responsible for chromatin condensation [Lorenzo et al., 1999]. The second, process of formation of apoptotic bodies and of DNA cleavage is sensitive to caspase inhibition and thus fully caspase-dependent. We assume that the process of chromatin condensation is dependent not only on the availability of caspase activity but also on availability of ATP. This conclusion stems from the findings that i) BAPR induces depletion of intracellular ATP, ii) the rate of ATP depletion is proportional to the BAPR concentration applied [Mlejnek and Kuglík, 2000], and iii) Z-VAD-FMK completely prevents chromatin condensation only after application of supra-lethal BAPR concentrations. Indeed, chromatin condensation, in contrast to apoptotic bodies formation and DNA cleavage, seems to be energy requiring event in the process of nuclear apoptosis [Kass et al., 1996, Mlejnek and Kuglík, 2000]. Thus, our experimental system reflects the well-known fact that the boundary between apoptosis and necrosis is determined mainly by the intracellular level of ATP [reviewed by Tsujimoto, 1997]. Our results do not seem to be in conflict with the concept published by Hirsch et al. [1997] who suggested that the availability of apoptogenic proteases determine the choice between the two death modalities, apoptosis

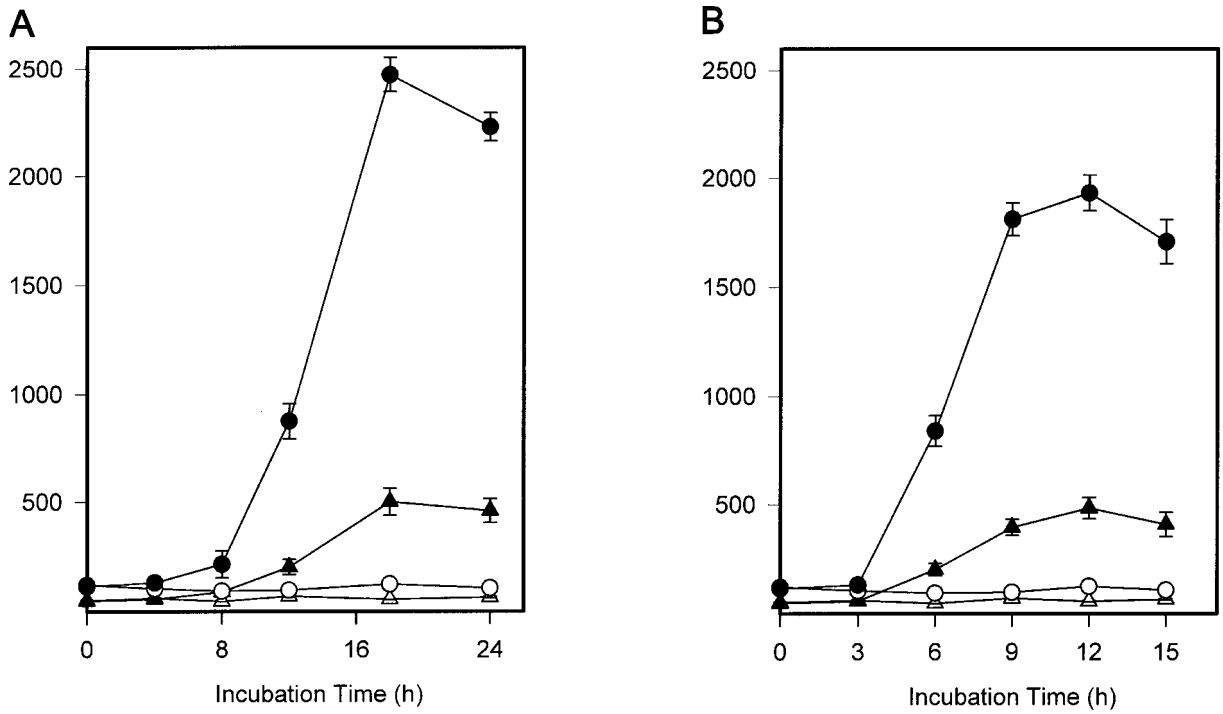


**Fig. 8.** Time-course of caspase activity during exposure of 10 μM (**A**) and 50 μM (**B**) BAPR. Relative hydrolysis of caspase-3 substrate Ac-DEVD-AMC in treated (filled circles) and untreated (open circles) cells. Relative hydrolysis of caspase-9 substrate Ac-LEHD-AFC (filled triangles) and untreated (open triangles) cells. The experimental points represent the mean values from three replicate experiments with standard deviations.

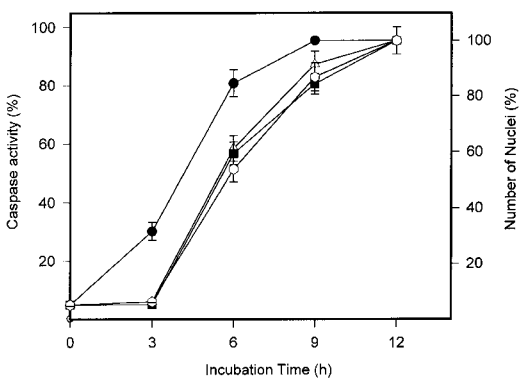


**Fig. 9.** Time course of changes in chromatin morphology and caspase activity. Examination of nuclear morphology and caspase activity assessment were done during the cell treatment with 50 μM BAPR in aliquots at indicated time intervals. Caspase activity, left axis (open symbols), caspase-3 (triangles), caspase-9 (hexagons). For simplicity, the maximum value of each caspase activity was normalized to 100%. Nuclei with apoptotic chromatin, right axis (filled symbols); apoptotic chromatin, all stages including early (circles), apoptotic chromatin, only late stages = apoptotic bodies (squares). The experimental points represent the mean values from three replicate experiments with standard deviations.

and necrosis. Indeed, it appears that the boundary between apoptosis and necrosis is controlled by both caspase proteases and intracellular ATP (Figs. 4–7).

The exclusive properties of "collapsed" nuclei, small size, and no hypoploid DNA content (i.e., intact DNA), predetermine the flow cytometry as the most advantageous and convenient technique for their qualitative as well as quantitative analysis (not shown). The effect of Z-VAD-FMK on nuclear morphology in dying cells mentioned above has not been described yet in relation to apoptosis. Premature chromosome condensation (PCC) is the only phenomenon in cellular biology that involves occurrence of nuclear morphology similar to that described in this study [e.g., Schlegel and Pardee, 1986]. However, whether this similarity is just accidental or has some functional significance remains to be elucidated.

As mentioned above, the role of caspase proteases in the processes of apoptotic bodies formation and DNA cleavage appears to be indispensable since Z-VAD-FMK completely prevents both events (Figs. 4, 5e,f, and 6). Indeed,

according to currently accepted hypothesis, which is largely derived from *in vitro* studies, caspase-9 appears to be one of the key regulatory enzymes that activates many other downstream caspase proteases including caspase-3 [reviewed by Budihardjo et al., 1999]. In contrast, caspase-3 was suggested to be the most important effector caspase being responsible for proteolytic cell disassembly [Faleiro et al., 1997, Woo et al., 1998]. Moreover, direct involvement of caspase-3 in activation of deoxyribonuclease responsible for nucleosomal DNA cleavage was recently identified [Enari et al., 1998]. However, our results could not confirm an essential role of caspase-3 and/or caspase-9 in BAPR-induced apoptosis in HL-60 cells. No hierarchy between caspases-9 and caspase-3 was documented in experiments using their specific inhibitors (Fig. 2). Similarly, detailed kinetic analysis of caspase-9 and caspase-3 activities did not provide any clue on whether activation of caspase-9 precedes that of caspase-3 or *vice versa* (Fig. 8). Such results are not in any conflict with recent data obtained by other authors and only reflect the complexity of pathways that are responsible for activation of caspase proteases. Indeed, recent data suggest that effector caspase maturation is followed by activation of other caspases including some that were previously believed to function as initiators [reviewed by Thornbery, 1999]. Such amplification loop was previously suggested to operate in the process of caspase-9 activation. In this scenario, caspase-9 may be activated by caspase-3 since pro-caspase-9 contains a target motif similar to that found in poly(ADP-ribose)-polymerase [reviewed by Cohen, 1997]. Therefore, we believe that such relationship between caspase-9 and caspase-3 fits well our findings (Figs. 2 and 8).

It was also shown here that irreversible inhibition of caspase-3 activity by Z-DEVD-FMK and/or Ac-LEHD-CMK failed to prevent manifestation of apoptotic hallmarks (Figs. 4, 5, and 7). Consistently with this finding, we have observed that activation of both caspase-9 and caspase-3 is rather late event which occurs in timely correlation with nucleosomal DNA cleavage [Mlejnek and Kuglík, 2000] during the final stages of apoptotic chromatin (Fig. 9). In other words, during BAPR-induced apoptosis in HL-60 cells caspase-3 and caspase-9 become active well after the cellular changes that they are supposed to induce. Still, even these findings are not in major conflict with data on the role of caspase-3 in apoptotic process that were recently published by others. Taken together, it is evident that caspase-3 is not required for all apoptosis-related processes to take place. Experiments using caspase-3 knockout mice revealed that while most somatic cells undergo apoptosis normally, cells of the neural tissue fail to undergo apoptosis during development [reviewed by Porter and Janicke, 1999]. Importantly, a large number of experiments employed pan-specific caspase inhibitor Z-VAD-FMK to inhibit caspase-3 thus providing only indirect evidence for the involvement of caspase-3 in apoptotic process. Early experiments with caspase-3 specific inhibitor clearly indicated that inhibition of caspase-3 activity does not really affect the progression of apoptotic process [e.g., Slee et al., 1996]. The most recent data suggest at least two mechanisms that may explain such failure. First, caspase-3 is not the only effector caspase but also other caspases, at least caspase-2, -6, and -7, play such role in regulating apoptotic process. Second, blocking certain caspase(s) results in activation of alternate caspases ensuring that the apoptosis process will be completed [e.g., Zheng et al., 2000]. Correspondingly to such hypothetical mechanisms, our preliminary data show that activation of caspase-2 and -6 accompanies BAPR-induced apoptosis in HL-60 cells (not shown).

In conclusion, our data unambiguously confirmed that caspase proteases are in fact responsible for stereotypical cell disassembly. However, these are not caspase-9 and/or caspase-3 that are indispensable for BAPR-induced apoptosis to occur. Moreover, it is not only availability of caspases but also the availability of intracellular ATP that decides between entering apoptotic and/or necrotic cell death program.

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