

Involvement of apoptosis-inducing factor during dolichyl monophosphate-induced apoptosis in U937 cells

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Abstract Dolichyl monophosphate (Dol-P) has been found to induce apoptosis in human leukemia U937 cells. During this apoptotic execution, the increase of plasma membrane fluidity (5–20 min), caspase-3-like protease activation (2–4 h), chromatin condensation and DNA ladder formation (3–4 h) were observed successively. Here, we report that reduction in mitochondrial transmembrane potential and translocation of apoptosis-inducing factor (AIF) are early events (1–3 h) in the apoptotic process induced by Dol-P in U937 cells. The AIF was concentrated around nuclei and partly translocated to the nuclei, which was confirmed by immunocytochemistry using specific anti-AIF antibody. Both caspase-8 and caspase-3 inhibitors blocked only DNA fragmentation but not mitochondrial processes, AIF migration and chromatin condensation. These results indicate that mitochondrial changes are an early step in the apoptosis induced by Dol-P and AIF is one of the important factors which induce chromatin condensation in nuclei. © 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Apoptosis-inducing factor; Dolichyl monophosphate; Apoptosis; Mitochondrion; Caspase

1. Introduction

Apoptosis is associated with a number of distinct morphological changes including cell shrinkage, chromatin degradation, cytoplasmic and nuclear condensation and blebbing. Numerous biochemical alterations are included in these morphological alterations of the cells during apoptotic cell death.

Recently, mitochondria have been shown to undergo a number of profound changes early within the apoptotic program and appear to play a central role in apoptosis in a number of systems [1,2]. A reduction in mitochondrial transmembrane potential ($\Delta\Psi_m$) is thought to be mediated by the opening of the mitochondrial permeability transition pore which includes a polyprotein complex, such as voltage-dependent anion channel and adenine nucleotide translocator [3,4]. The $\Delta\Psi_m$ is regulated by the Bcl-2 family of proteins, whose

members may act as anti- or pro-apoptotic [5]. The reduction of $\Delta\Psi_m$ induces liberation of apoptosis-inducing factor (AIF) and release of cytochrome *c* from the mitochondrial intermembrane space to the cytosol [6–8]. The released AIF travels to and accumulates in the nuclei, and activates chromatin condensation at the nuclear periphery and cleavage of the chromosomal DNA into large scale fragmentation. This factor does not act through downstream caspases [6,7]. The other liberated substance, cytochrome *c* has been implicated in the activation of the caspase cascade, such as caspase-9 and caspase-3 [9]. Both two factors, AIF and cytochrome *c* play an important role in apoptotic cell death.

Caspase proteinases drive apoptotic signaling and execution by cleaving critical cellular proteins solely after aspartate residues [10]. Caspases exist as latent zymogens, but apoptotic death stimuli activate the initiator caspases, such as, caspase-8 and caspase-9. Once activated, initiator caspases in turn activate the executioner caspases, caspases-3, -6, and -7. The active executioners promote apoptosis by cleaving cellular substances that induce the morphological and biochemical features of apoptosis. Caspase-8 is recruited to a death-inducing signaling complex when death receptors such as Fas or the tumor necrosis factor receptor are oligomerized after binding of specific ligands. Active caspase-8 can act either directly on downstream effector caspases or indirectly on mitochondria. Caspase-9, which is activated by binding to cytochrome *c* and Apaf-1, cleaves and activates procaspase-3. The executioner caspase, caspase-3 plays a central role in apoptotic DNA fragmentation by inactivating DFF45/ICAD, thereby releasing active DFF40/CAD [11].

Dolichyl monophosphate (Dol-P) is involved in the attachment of carbohydrate chains to proteins in the formation of *N*-linked glycoprotein [12]. We found that this compound induces apoptosis in rat glioma C6 cells and human monoblastic leukemia U937 cells [13,14]. In Dol-P-treated U937 cells, increase of membrane fluidity (5–20 min), increase of caspase-3-like protease activity (2–4 h) and DNA ladder formation (3–4 h) were observed successively [13,15–17]. In this study, we examined whether mitochondrial function is related to the apoptosis induced by Dol-P in U937 cells. We also investigated whether mitochondrial change is upstream in caspase cascade using specific caspase inhibitors of caspase-8 or -3. The cells treated with Dol-P undergo to apoptosis accompanied by loss of $\Delta\Psi_m$. This mitochondrial disruption in these cells is observed after 1 h of treatment. AIF gathered around nuclear periphery (3 h) and chromatin condensation followed.

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These processes were carried out independently against caspase-3 or -8 inhibitor. We conclude that Dol-P-mediated death of U937 cells involves mitochondrial disruption including AIF migration and that this mitochondrial change occurs in early step of apoptotic pathway before caspase activation. We also suggest that AIF might have a direct effect on nuclei without caspase activation, triggering chromatin condensation.

2. Materials and methods

2.1. Reagents

Dol-P (diammonium salt) was kindly synthesized by Dr. I. Yamatsu (Serendip Research Institute, Tokyo, Japan). Dol-P was dissolved in ethanol-dodecane (98:2, by volume), according to Ji et al. [18]. This reagent was added to the culture medium at a final concentration of 6 μ M. The concentration was calculated based on the average molecular weight because this compound is a mixture with 17–21 isoprene units. A rabbit anti-AIF polyclonal antibody was generated against amino acids 151–200 of AIF [6]. Rhodamine-labeled anti-rabbit IgG antibody was from Chemicon International Inc. (Temecula, CA, USA). Rhodamine 123 and JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl benzimidazole carbon cyanine iodide) were obtained from Molecular Probes Inc. (Eugene, OR, USA). Hoechst 33342 was purchased from Sigma Chemical Co. (St. Louis, MO, USA). The tetrapeptide caspase-3 inhibitor, acetyl-Asp-Glu-Val-Asp-aldehyde (DEVD-CHO) and caspase-8 inhibitor, acetyl-Ile-Glu-Thr-Asp-aldehyde (IETD-CHO) were obtained from Peptide Institute, Inc. (Osaka, Japan). RNase was purchased from Wako Pure Chemical Co. (Osaka, Japan).

2.2. Cell culture

The human promonocytic leukemia cell line U937 was grown in RPMI 1640 medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS) (JRH Biosciences, Lenexa, KS, USA) at 37°C in an atmosphere of 5% CO₂. 400 μ l of cell suspension (1.25 \times 10⁶ cells/ml) in RPMI 1640 medium containing 10% FCS was placed to each well of a 24-well plate. Dol-P (6 μ M) was added to the culture medium and mixed well with the medium, with shaking. The cells were incubated for 1 or 3 h and harvested for experiments. To examine the effect of caspase inhibitors, the cells were treated with inhibitor for 2 h prior to Dol-P treatment.

2.3. Morphological evaluation of cells and nuclei

Cell morphology was observed by light microscope (Olympus BX-50, Tokyo, Japan) with Nomarski differentiated interference contrast. For detection of chromatin condensation, cells were stained with Hoechst 33342 at final concentration of 4 μ g/ml in phosphate-buffered saline (PBS) and examined using fluorescent microscope (Olympus BX-50).

2.4. Detection of fragmented DNA

Fragmented, apoptotic nuclei were recognized by their sub-diploid DNA content. Briefly, cells were washed with PBS, fixed with 80% ice-cold ethanol and stocked at -20°C. For analysis of DNA, fixed cells were treated with RNase (100 μ g/ml) for 30 min at 37°C and stained with 50 μ g/ml of propidium iodide. Cell cycle analysis was performed by Becton Dickinson FACS Calibur (Nippon Becton Dickinson Co., Tokyo, Japan) and the number of cells in the area corresponding to the sub-G1 region was calculated using the CELLQuest program (Nippon Becton Dickinson Co.).

2.5. Measurement of mitochondrial transmembrane potential ($\Delta\Psi_m$)

To measure $\Delta\Psi_m$, rhodamine 123 and JC-1 were used. Dol-P-treated cells were washed with PBS, and were stained with rhodamine 123 or JC-1 at the same final concentration of 5 μ g/ml for 30 min at 37°C. Samples stained with rhodamine 123 were subjected to flowcytometry and those stained with JC-1 were used for observation of fluorescent microscopy (Olympus BX-50). Flowcytometry was operated by Becton Dickinson FACS Calibur with an excitation wavelength of 488 nm. The emission wavelength was detected through the FL1 channel. Data were acquired and analyzed with CELLQuest software.

2.6. Analysis of intracellular localization of AIF by immunocytochemistry

Cells were harvested, rinsed once with PBS. Cells were fixed with 4% paraformaldehyde containing with 0.19% picric acid in PBS for 1 h at room temperature and were permeabilized with 0.1% sodium dodecyl sulfate. The permeabilized cells were blocked with 10% FCS in PBS. The cells were dropped and mounted on a slide glass coated with 3-aminopropyltriethoxysilane (Matsunami Glass Ind., Ltd., Osaka, Japan). The slide glass was incubated with anti-AIF antibody diluted 1:250 in PBS containing 0.5% bovine serum albumin (BSA) for 40 min at 37°C in a humidified chamber. Excess antibody binding was removed by washing the slide glass with PBS. The secondary antibody, rhodamine-labeled anti-rabbit IgG antibody, was added and incubated for 30 min at 37°C. After washing with PBS, photo-labeled cells were analyzed using a fluorescent microscope (Olympus BX-50) and a laser scanning confocal microscope (Olympus FV500).

3. Results and discussion

3.1. Reduction in mitochondrial transmembrane potential

The loss of the mitochondrial transmembrane potential ($\Delta\Psi_m$) has been identified as common early events occurring in apoptosis induced by a variety of stimuli [19,20]. To investigate whether a reduction in $\Delta\Psi_m$ might relate to apoptosis induced by Dol-P, $\Delta\Psi_m$ was monitored by fluorescence of the potential-sensitive dye, rhodamine 123, by flow cytometry. In control cells treated for 3 h with solvent (0.8% ethanol:dodecane (98:2)), $\Delta\Psi_m$ showed high potential (Fig. 1A). However, after 1 h of incubation with Dol-P (6 μ M), rhodamine 123-stained cells were separated to two populations by fluorescent strength and 21% of cells showed a drastic reduction of the $\Delta\Psi_m$ (Fig. 1B). After 3 h of treatment, the low $\Delta\Psi_m$ group of cells increased to 50% (Fig. 1C). In order to examine the involvement of caspases during this reduction in $\Delta\Psi_m$, cells were pretreated for 2 h with caspase inhibitors before adding Dol-P. Both inhibitors of caspase-3 and -8 (400 μ M) had no effect on the reduction of $\Delta\Psi_m$ (Fig. 1D,E).

Salvioli et al. reported that JC-1, cationic lipophilic dye, is a reliable probe for analyzing $\Delta\Psi_m$ with flow cytometry, while the rhodamine 123 shows a low sensitivity in U937 cells [21]. Then, we attempted to use JC-1 to monitor $\Delta\Psi_m$ with fluorocytometry and flow cytometry. JC-1 is a mitochondrial specific dye and forms aggregates (590 nm) at the normal membrane potential. A loss in $\Delta\Psi_m$ can be monitored as an increase in JC-1 monomers (527 nm) [22]. As shown in Fig. 1F, JC-1 was avidly accumulated in sensitive control cells where it displayed a bright fluorescence as higher potentials. In contrast, JC-1 was poorly accumulated in Dol-P-treated cells which displayed only a slight fluorescence as low membrane potential (Fig. 1G). The flow cytometric data of JC-1 staining cells after treatment of Dol-P were almost the same as those of rhodamine 123 staining cells (data not shown).

Recently, our group reported that increase of plasma membrane fluidity was observed 5–20 min after Dol-P treatment in U937 cells [15]. The reduction of $\Delta\Psi_m$ (1–3 h) is the second step in the apoptotic pathway and it is independent of caspase-3 or caspase-8 activation. Presumably, treatment with Dol-P triggered mitochondrial membrane perturbation and resulted in the reduction of $\Delta\Psi_m$.

3.2. Translocation of AIF in Dol-P-treated cells

Susin et al. reported that the reduction of $\Delta\Psi_m$ in mitochondria causes the release of AIF from the intermembrane space to the cytosol followed by its translocation to the nu-

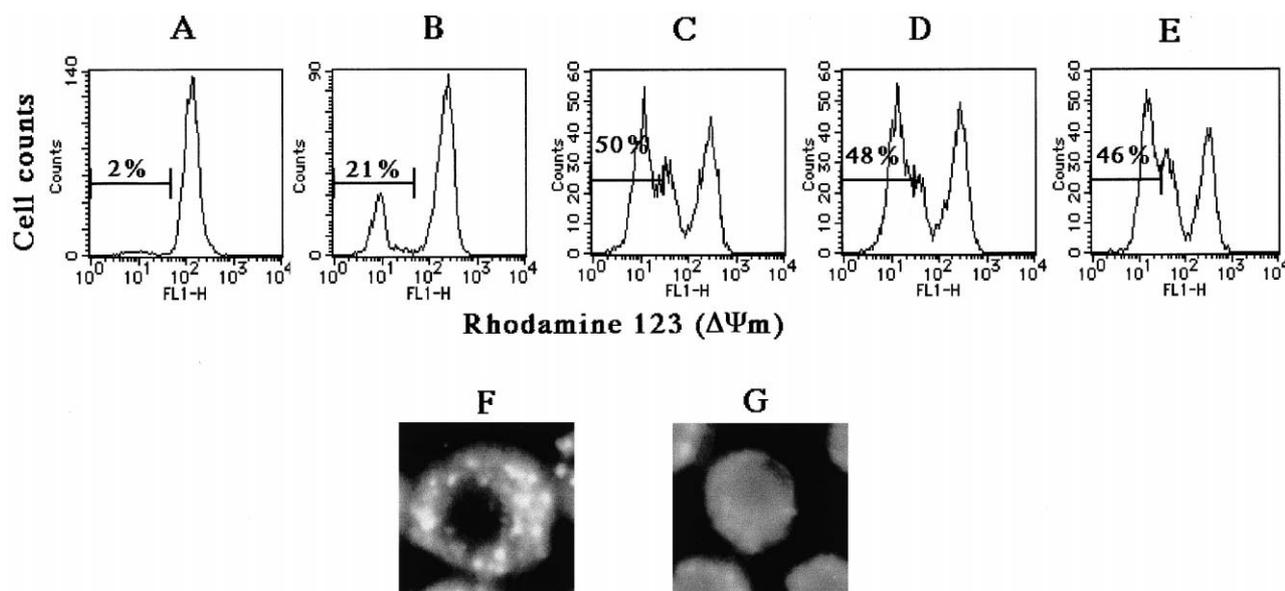


Fig. 1. Decrease of the mitochondrial membrane potential. U937 cells were stained with rhodamine 123 and $\Delta\Psi_m$ was monitored by flow cytometry. Cells treated with solvent (ethanol:dodecane=98:2) for 3 h (A), Dol-P (6 μ M) for 1 h (B) and Dol-P (6 μ M) for 3 h (C). The effects of caspase-3 and -8 inhibitors on mitochondrial membrane potential during Dol-P-induced apoptosis are shown in (D) (caspase-3 inhibitor, 400 μ M) and (E) (caspase-8 inhibitor, 400 μ M), respectively. The mitochondrial membrane potential was visualized by JC-1 staining. Cells were stained with JC-1 and observed by fluorescent microscope. The bright spots in the cytoplasm are mitochondria with higher membrane potential and weak fluorescence exists as a monomer of JC-1 at low membrane potential. U937 cells were treated with solvent for 1 h (F) or Dol-P for 1 h (G).

cleus [6]. Then, we examined the cellular localization of AIF in U937 cells during Dol-P-induced apoptosis by immunofluorescent detection (Fig. 2). In control cells after 3 h of treatment with solvent, AIF displayed cytoplasmic staining, which is in agreement with its localization in mitochondria (Fig. 2A). After 3 h of apoptosis induction by Dol-P, apoptotic cells exhibited mostly condense AIF staining with bright spots in the perinuclear region (Fig. 2B). It suggests that mitochondria became clustered around the nuclei. This phenomenon has recently been proposed as early characteristic event during the occurrence of apoptosis [23–25]. In Fig. 2B, strong bright AIF immunostaining in the nuclei was also observed in a part of the apoptotic cells. However, the percentage of cells manifesting mitochondrion-nuclear AIF translocation was very low, less than 5%. These results were confirmed by confocal microscopy (data not shown). The inhibitors of caspase-3 and -8 did not affect perinuclear clustering of mitochondria and the AIF immunostaining around the nuclei (Fig. 2C,D). The effect of caspase inhibitors on AIF translocation to nuclei could not be evaluated because the number of AIF-translocated nuclei were not frequent even among caspase inhibitor-untreated cells. These data indicate apparently that AIF was concentrated around the nuclei and partly translocated to the nuclei in U937 cells during the apoptotic pathway induced by Dol-P. The AIF immunostaining at peripheral nuclei was independent of caspase-3 or -8 activation.

3.3. Morphological changes of nuclei and DNA fragmentation induced by Dol-P

Previously, we demonstrated that caspase-3 inhibitor blocked DNA ladder formation in U937 cells treated with dihydroprenyl monophosphate [16,17]. In this study, we investigated the effects of caspase-3 or -8 inhibitor on nuclear morphological changes in Dol-P-treated cells (Fig. 3). In Fig. 3B, nuclei staining with Hoechst 33342 of Dol-P treated cells

(3 h) showed apparent chromatin fragmentations. When U937 cells were pretreated with caspase-3 or -8 inhibitor followed by Dol-P treatment, deformation of nuclei and chromatin condensation but not chromatin fragmentation were observed (Fig. 3C,D). These results suggest that some apoptotic signal can induce chromatin condensation without caspase-3 or -8 activation in U937 cells. Susin et al. reported that AIF injection into cytoplasm-induced peripheral condensation of chromatin and microinjection of anti-AIF antibodies blocked this

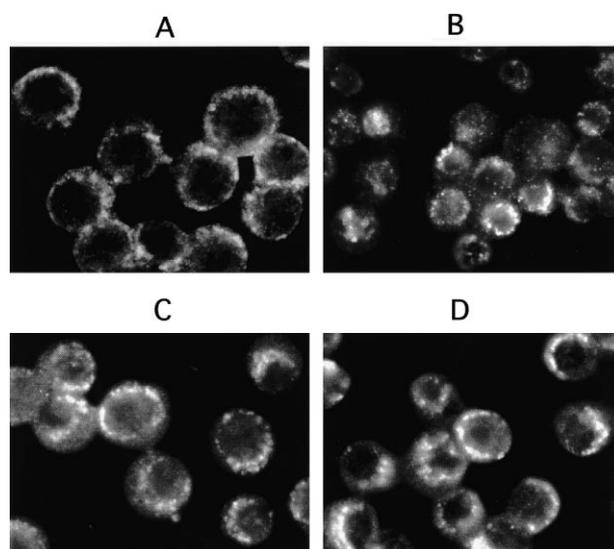


Fig. 2. Immunofluorescent detection of AIF in U937 cells treated with Dol-P. Cells were treated with solvent (ethanol:dodecane=98:2) for 3 h (A), Dol-P (6 μ M) for 3 h (B), caspase-3 inhibitor (400 μ M) and Dol-P (6 μ M) for 3 h (C), caspase-8 inhibitor (400 μ M) and Dol-P (6 μ M) for 3 h (D). The cells were stained with rabbit anti-AIF antibody which were detected with rhodamine-conjugated anti-rabbit IgG antibody.

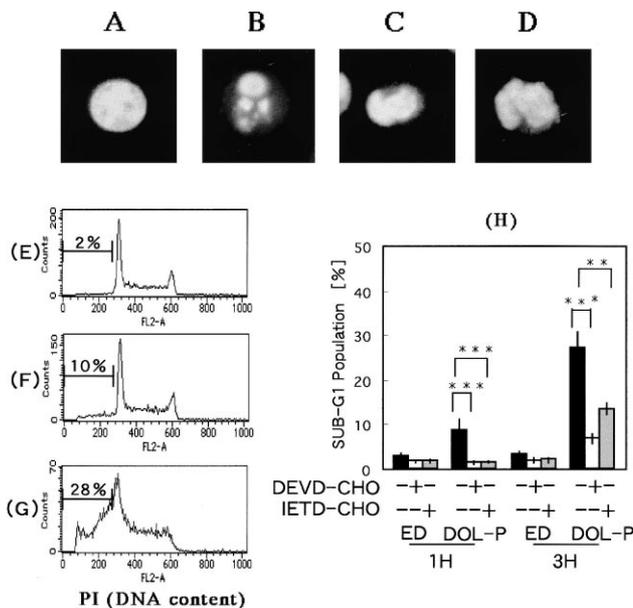


Fig. 3. Effects of caspase-3 or caspase-8 inhibitor on nuclear morphology and DNA fragmentation. U937 cells were stained with Hoechst 33342 and observed by fluorescent microscope. Cells were treated with solvent (ethanol:dodecane=98:2) for 3 h (A), Dol-P (6 μ M) for 3 h (B), caspase-3 inhibitor (400 μ M) and Dol-P (6 μ M) for 3 h (C), caspase-8 inhibitor (400 μ M) and Dol-P (6 μ M) for 3 h (D). Fragmented DNA (sub-G₁ population) were stained with propidium iodide and analyzed by flow cytometry. U937 cells were treated with solvent for 3 h (E), Dol-P (6 μ M) for 1 h (F), Dol-P (6 μ M) for 3 h (G). Effects of caspase-3 inhibitor, DEVD-CHO (400 μ M) or caspase-8 inhibitor, IETD-CHO (400 μ M) on the sub-G₁ population induced by Dol-P (H). Student's *t*-test was used for statistical analysis. ***P* < 0.01, ****P* < 0.001

condensation [6]. They also indicated that AIF is independent of the caspase cascade. Thus, chromatin condensation induced by Dol-P in the presence of caspase-3 or -8 inhibitor might depend on AIF stimuli.

Dol-P induced DNA ladder formation in U937 cells confirmed by agarose gel electrophoresis [13]. In this experiment, we analyzed DNA fragmentation by FACS analysis for calculating the percentage of the sub-G₁ population. Dol-P induced increment of sub-G₁ population of U937 cells in a time-dependent manner, such as 10% of the sub-G₁ population after 1 h and 28% after 3 h of treatment (Fig. 3E–G). This DNA fragmentation was significantly inhibited by both caspase-3 and -8 inhibitors (Fig. 3H). The inhibition of DNA fragmentation by caspase-3 inhibitor was more prominent than that by caspase-8 inhibitor. Wang and Nagata's groups independently purified nuclease DFF40/CAD which is activated by caspase-3 and results in DNA fragmentation [26,27]. Healthy cells contain a heterodimer comprised of DFF40/CAD and DFF45/ICAD. Caspase-3 activates the DFF40/CAD by cleaving the inhibitor DFF45/ICAD at two sites. The activated CAD/DFF40 provides internucleosomal DNA degradation. Therefore, DNA fragmentation induced by Dol-P might depend on the activation of CAD/DFF40 and this is downstream of the caspase cascade.

Conclusively, these results indicate that apoptosis induced by Dol-P stimulates a decline in $\Delta\Psi_m$, AIF condensation at the peripheral nuclei and translocation of AIF to the nuclei. This occurs not downstream of caspase-8 and -3 in U937 cells. However, the translocation of AIF to the nuclei was observed

in a part of apoptosis-induced cells, but not in all cells. On the contrary, nuclear condensation occurred in the most of Dol-P treated cells. These findings suggest that undetectable levels of AIF translocated into nuclei effectively induce chromatin condensation of U937 cells. Alternatively, it might be considered that other factors than existence of AIF in cytosol or mitochondria induce chromatin condensation and work independently of the caspase cascade.

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