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Authors: Yasugi, Etsuko, Uemura, Isao, Kumagai, Tsukasa, Nishikawa, Yoshihisa, Yasugi, Sadao, et al.

Source: Zoological Science, 19(1) : 7-13

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.19.7

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ZOOLOGICL SCIENCE **19**: 7–13 (2002) © 2002 Zoological Society of Japan

Disruption of Mitochondria Is an Early Event during Dolichyl Monophosphate-induced Apoptosis in U937 Cells

Etsuko Yasugi[1※*], Isao Uemura[2※], Tsukasa Kumagai[3], Yoshihisa Nishikawa[3], Sadao Yasugi[2] and Akira Yuo[1]

[1]*Department of Hematology, Research Institute, International Medical Center of Japan, 1-21-1, Toyama, Shinjuku-ku, Tokyo 162-8655, Japan*
[2]*Department of Biological Science, Graduate School of Science, Tokyo Metropolitan University, 1-1, Minamiosawa, Hachioji, Tokyo192-0397, Japan*
[3]*Department of Life Chemistry, School of Engineering, Tokai University, 1117 Kitakaname, Hiratsuka, Kanagawa 259-1292, Japan*

ABSTRACT—Dolichyl monophosphate (Dol-P) is involved in the attachment of carbohydrate chains to proteins in the formation of N-linked glycoprotein. We found that this compound induces apoptosis in human leukemia U937 cells. During this apoptotic execution, the increase of plasma membrane fluidity (5–20 min), reduction in mitochondrial transmembrane potential ($\Delta\psi m$) and translocation of apoptosis-inducing factor (1–3 hr), caspase-3-like protease activation (2–4 hr), chromatin condensation and DNA ladder formation (3–4 hr) were observed successively. In this study, we examined mitochondrial morphological changes by electron microscopy and $\Delta\psi m$ by JC-1 from immediately after treatment of Dol-P. After 5 min of treatment, we observed clearly that mitochondrial cristae began to be disrupted ultrastructurally and almost all the cristae were disintegrated after 1 hr of treatment. The $\Delta\psi m$ of Dol-P treated cells was reduced to 34% as compared with that of control cells immediately after treatment and was quartered within 1 hr. The reduction in $\Delta\psi m$ was not inhibited by cyclosporin A, N-acetyl-L-cysteine and vitamin E. These results indicate that mitochondrial disruption is one of the first triggering events of Dol-P-induced apoptosis.

Key words: apoptosis, mitochondria, dolichyl monophosphate, electron microscopy, human leukemia cells

INTRODUCTION

Apoptosis, or programmed cell death, is a fundamental feature of naturally occurring cell death in animal and plant cells. This is an indispensable process during normal development, maintenance of homeostasis and integrity, interaction with environment, and regulation of immune system (Ameisen, 1996; Vaux and Korsmeyer, 1999; Meier *et al*., 2000). The blocking of programmed cell death may be important in cancer development. Conversely, the inducing apoptosis in tumor cells is one of the useful tools for cancer therapy (Evan and Littlewood, 1998). The onset of apoptosis leads to cell shrinkage, membrane blebbing, chromatin compaction and DNA ladder formation (Green, 2000; Hengartner, 2000).

Recently, mitochondria have been shown to play a key intermediary role in apoptosis through the release of cytochrome c, Smac/DIABLO, apoptosis-inducing factor (AIF), endonuclease G and several procaspases. These multiple proteins might be necessary for cells to determine whether they undergo cellular death or not (Green and Reed, 1998; Chai *et al*., 2000; Hengartner, 2000, 2001; Goyal, 2001; Li *et al*., 2001). Among them, cytochrome c is thought to evoke caspase cascade activation and to generate DNA fragmentation (Adrain and Martin, 2001). The other liberated substance, AIF, in mitochondrial intermembrane space, 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 (Susin *et al*., 2000; Daugas *et al*., 2000). Thus, these released proteins from mitochondria are key regulators of apoptotic process.

In many apoptotic pathways, the mitochondrial inner transmembrane potential ($\Delta\Psi m$) collapses, indicating the opening of a large conductance channel known as the mitochondrial permeability transition (PT) pore. The reduction in $\Delta\Psi m$ induces liberation of cytochrome c from mitochondrial intermembrane space to cytosol. Although these data

* Corresponding author: Tel. +81-3-3202-7181;
FAX. +81-3-3202-7364.
E-mail: yasugi@ri.imcj.go.jp
※ These two authors contributed equally to the present study.

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clearly demonstrate the importance of mitochondria in the apoptosis, their ultrastructural changes during the early phase of apoptosis have not been completely elucidated.

Dolichyl monophosphate (Dol-P) is a long-chain polyisoprenoid which containing 17–21 isoprene units with phosphate group at α-terminal end (Hemming, 1992). The biological activity of Dol-P is involved in the attachment of carbohydrate chains to proteins in the formation of N-linked glycoprotein and Dol-P-mannose serves as a mannosyl donor to glycosyl phosphoinositol anchor protein (Leloir, 1977; Orlean, 1997). We found that this compound induces apoptosis in rat glioma C6 cells and human monoblastic leukemia U937 cells (Yasugi *et al*., 1995; Dohi *et al*., 1996; Fujimoto *et al*., 2000). In Dol-P treated U937 cells, increase of plasma membrane fluidity (5–20 min) (Fujimoto *et al*., 1999), reduction in Δψm (1 hr) and translocation of apoptosis-inducing factor (3 hr) (Yasugi *et al*., 2000), increase of caspase-3-like protease activity (2–4 hr) (Yokoyama *et al*., 1997; Yasugi *et al*., 1998), chromatin condensation and DNA ladder formation (3–4 hr) were observed successively (Yasugi *et al*., 1995). From the effects of specific inhibitors of caspases, we speculate at least two redundant parallel pathways may lead to chromatin processing during Dol-P-induced apoptosis. One of these pathways involves caspases and leads to oligonucleosomal DNA fragmentation. The other pathway, which is caspase-independent, involves AIF and leads to peripheral chromatin condensation (Yasugi *et al*., 2000).

In this study, to examine further the importance of mitochondrial changes during early phase of apoptosis induced by Dol-P, we investigated morphologically and biochemically when and how mitochondria start to be disrupted after treatment with Dol-P in U937 cells. By electron microscopic observation, mitochondrial cristae began to disappear after 30 sec of treatment although mitochondrial outer and inner membranes were conserved. The Δψm examined by JC-1of Dol-P treated cells was reduced to 34% compared that of control cells even at 30 sec after treatment and quartered within 1 hr. From these results, we concluded that Dol-P-mediated death of U937 cells involves mitochondrial disruption and this mitochondrial morphological change may be a trigger of apoptotic pathway.

MATERIALS AND METHODS

Reagents

Dolichyl monophosphate (diammonium salt) was kindly synthesized by Dr. I. Yamatsu (Serendip Research Institute, Tokyo, Japan). Dol-P was dissolved in solvent, ethanol-dodecane (98:2, by volume), according to Ji *et al*. (1995). This reagent was added to culture medium at a final concentration of 6 μM. The concentration was calculated based on the average molecular weight because this compound is mixture of 17–21 isoprene units. JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazole carbon cyanine iodide) was obtained from Molecular Probes Inc. (Eugene, Oregon, USA). Cyclosporin A and N-acetyl-L-cysteine were purchased from Sigma-Aldrich Japan (Tokyo, Japan). α-Tocopherol (vitamin E) was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Cell culture

The human monoblastic 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% CO_2. 400 μl of cell suspension (1.25×10^6 cells/ml) in RPMI 1640 medium containing 10% FCS was placed to each well of 24-well plate. Dol-P was added to the culture medium and mixed well with the medium, with shaking. The cells were incubated for indicated periods and harvested for experiments. To examine the effect of inhibitors, cyclosporin A (1 or 10 μM) and N-acetyl-L-cysteine (10 mM), the cells were treated with inhibitor for 2 hr prior to Dol-P treatment. In the case of vitamin E (0.5 mM), the cells were preincubated for 24 hr with vitamin E and were washed with RPMI 1640 with 10% FCS, according to Yano *et al*. (2000).

Measurement of mitochondrial transmembrane potential (Δψm)

To measure Δψm, JC-1 was used. Dol-P-treated cells were washed with phosphate buffered saline (PBS), and were stained with JC-1 at the final concentration of 10 μg/ml for 15 min at 37°C. The cells stained with JC-1 were used for observation with fluorescent microscope (Olympus BX-50, Olympus, Tokyo, Japan). The fluorescent strength of JC-1 stained cells was measured by Fluor Imager 595 (Amersham Life Science, Tokyo, Japan) using 96-well-plate and was calculated as percentage of that of control cells.

Ultrastructural analysis

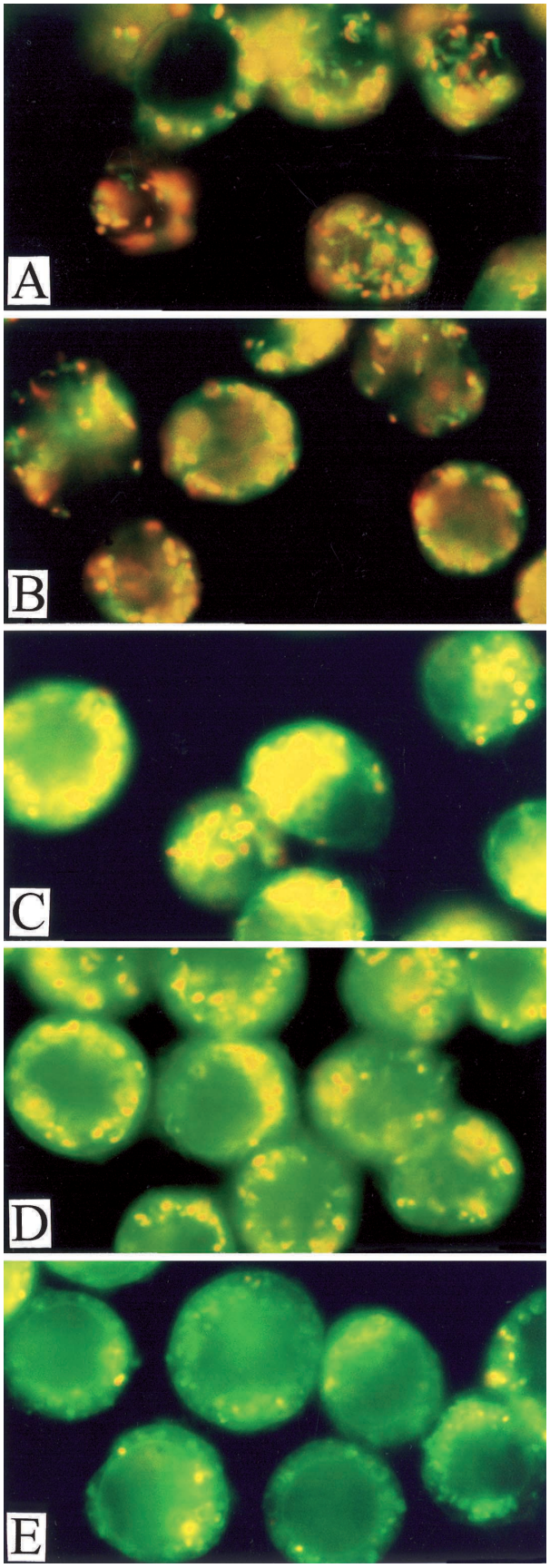
The cells were fixed with 1% glutaraldehyde in PBS for 24 hr at 4°C, post-fixed with 2% osmium tetraoxide in PBS for 1 hr at 4°C, and were then block-stained with 4% aqueous uranium acetate for 1 hr at room temperature. After dehydration in ethanol, the cells were embedded under the Spurr's recipe modified by Kushida (1980). Ultrathin sections were cut on a Reichert Ultracut S (Leica, Germany), then stained with lead citrate for 2 min. Sections were examined with an electron microscope (JEOL JEM1010, JEOL Ltd. Tokyo, Japan) at 80 kV. Images were taken with the Imaging Plate system (PIX system 20, JEOL Ltd.) and stored as computer files, then printed out using Canon BJF870 (Canon Inc. Tokyo, Japan)

RESULTS

Reduction in mitochondrial transmembrane potential

The loss of ΔΨm has been observed in U937 cells 1 hr after treatment of Dol-P (Yasugi *et al*, 2000). To investigate reduction in ΔΨm during early step of apoptosis induced by Dol-P, we used fluorescence of the potential-sensitive dye, JC-1. The JC-1 which is a mitochondria-specific dye shows high sensitivity for Δψm change and forms aggregates (590 nm) at the normal membrane potential. A loss in ΔΨm can be monitored as an increase in JC-1 monomers (527 nm) (Cossarizza *et al*, 1993). As shown in Fig 1A, JC-1 was avidly accumulated in intact cells where it displayed a bright red and yellow fluorescence indicating a high potentials. The solvent of Dol-P had no effect on Δψm of U937 cells (Fig. 1B). In contrast, JC-1 was poorly accumulated in Dol-P treated cells which displayed only a weak yellow or slight green fluorescence indicating low membrane potential (Fig. 1C, D, E). This tendency of Δψm decrease occurred in a time-dependent manner, so that cells treated for a longer period showed decreased JC-1 accumulation. Next, to study more quantitatively the decrease in the membrane potential, the fluorescent strength of JC-1 stained cells was measured

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by Fluor Imager 595 and calculated as percentages to the value obtained with normal cells (Fig.2). The $\Delta\psi m$ of Dol-P treated cells was reduced to 34% compared that of control cells even at 30 sec after treatment and fell to quartered within 1 hr. These results indicate that reduction in mitochondrial $\Delta\psi m$ occurred immediately after treatment of Dol-P and continued for 60 min. Antioxidants, such as N-acetyl-L-cysteine and vitamin E, did not show any inhibitory effect on reduction in $\Delta\psi m$, suggesting that Dol-P-induced apoptosis was not generated by reactive oxygen species (data not shown). Cyclosporin A, inhibitor of PT, had no effect on decrease of $\Delta\psi m$ as well (data not shown). Recently, we reported that increase of plasma membrane fluidity was observed at 5–20 min after Dol-P treatment in U937 cells (Fujimoto *et al.*, 1999). The reduction in $\Delta\psi m$ in cytoplasm was observed simultaneously with increase of plasma membrane fluidity. Presumably, treatment with Dol-P triggered mitochondrial membrane perturbation and resulted in the reduction in $\Delta\psi m$.

Morphological changes of mitochondria induced by Dol-P

We investigated morphological changes in Dol-P

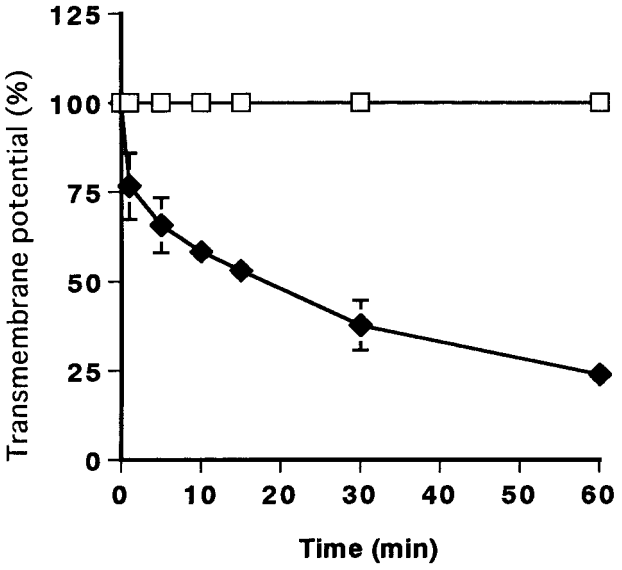
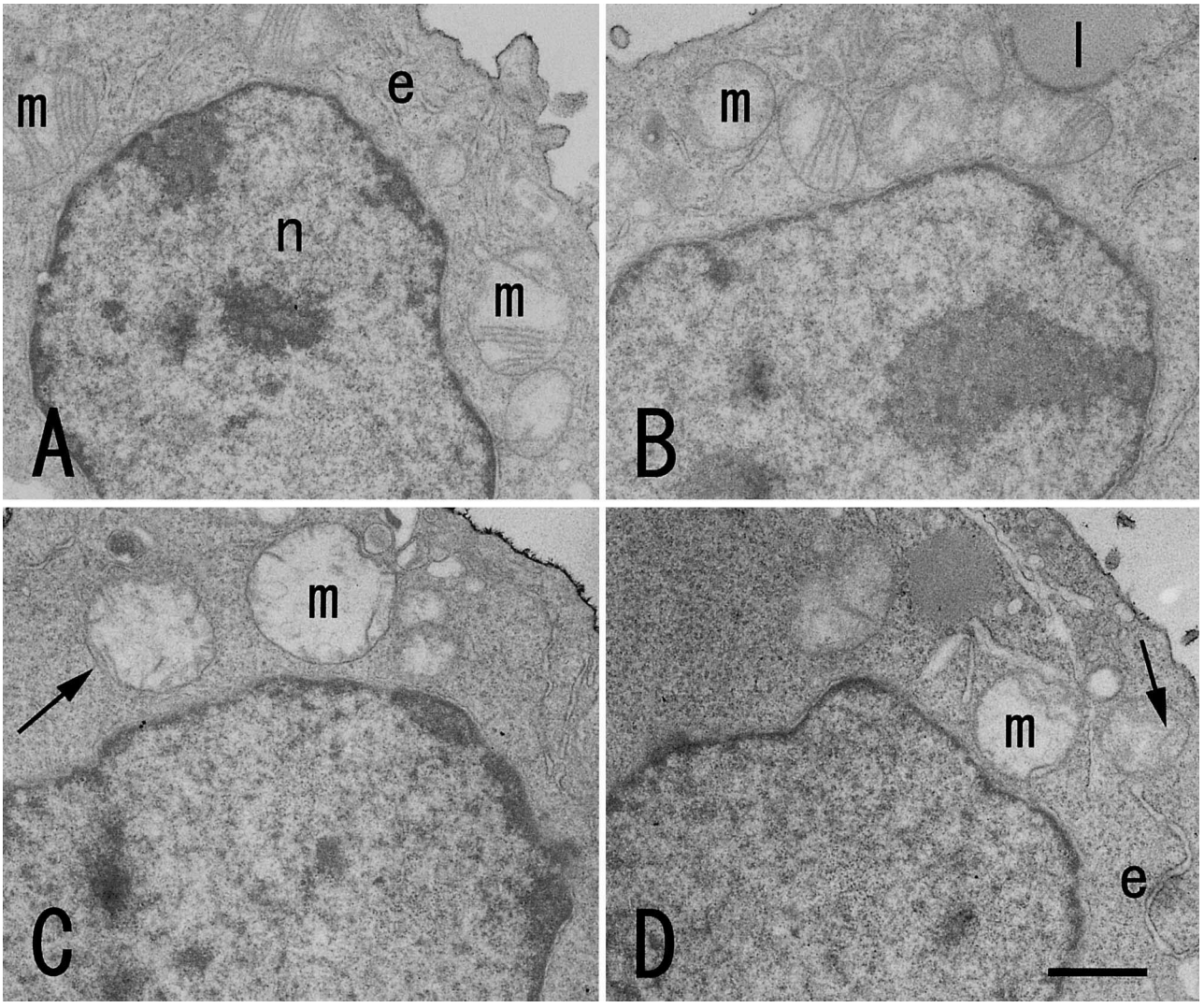


Fig. 2. Reduction in mitochondrial transmembrane potential ($\Delta\psi m$) of U937 cells treated with solvent (-□-) or Dol-P (6μM) (-◆-) for indicated time and stained with JC-1. The fluorescent strength of stained cells was measured by Fluor Imager 595 and calculated as percent to that of intact cells. Values are means ± SE.

Fig. 1. Mitochondrial transmembrane potential of U937 cells treated with Dol-P (6μM), stained with JC-1 and observed with fluorescent microscope. Red or yellow spots (aggregated form of JC-1) in cytoplasm show mitochondria with higher membrane potential and weak green fluorescence represents monomer of JC-1 at sites of low membrane potential. A, intact cells. B, cells treated with solvent for 15 min. C, cells treated with Dol-P for 30 sec. D, cells treated with Dol-P for 5min. E, cells treated with Dol-P for 15min × 1,200.

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treated cells by electron microscope from just after the onset of treatment to 2 hr (Fig. 3). The control cells and cells treated with the solvent showed normal morphology of U937cells: the nucleus was rather large and there were many mitochondria with well developed cristae. The endoplasmic reticulum and some lipid droplets were also present (Fig. 3A, B). Just after the treatment of cells with Dol-P, there occurred morphological changes in the structure of mitochondria. The cristae disoriented in most of the mitochondria although some still remained (Fig, 3C, arrow). Also the shape of the mitochondria became round. Other organelles such as the nucleus, endoplasmic reticulum and lipid droplets were almost intact.

Drastic changes in morphology were observed if the treatment with Dol-P was prolonged for more than 5 min. Cells treated for 5 min (Fig. 3D) showed mitochondria in which cristae were almost perfectly disintegrated. Moreover, the inner spaces of mitochondria came to be filled with somewhat electron-dense materials (Fig. 3D, arrow). Treatment with Dol-P for 5 min did not affect severely the morphology of other cellular components. In cells treated for more than 10 min, we could not find mitochondrial cristae (Fig. 3E, F, G, H). It should be noted that the mitochondrial membranes still maintained their double-layered structure (Fig. 3F, arrow), indicating that Dol-P treatment affected primarily the structure of the cristae. Cells treated for more than 10 min also possessed many vesicles with unit membrane which seemed to be swellings of endoplasmic reticulum (Fig. 3E, G, H, arrowheads). In accordance with this, we could not observe intact endoplasmic reticulum in these cells.

In U937 cells treated with Dol-P for longer than 1 hr, the mitochondria were always filled with electron-dense materials and there were no cristae at all (Fig.3G). At 2 hr after the onset of treatment, we could no longer detect normal mitochondria and it was sometimes difficult to judge whether mitochondria-like structures are in fact mitochondria (Fig.3H). Small vesicles increased in number and condensed around nucleus (Fig.3H). Thereafter, cells underwent typical apoptotic changes with nuclear fragmentation (Yasugi *et al*., 1995).

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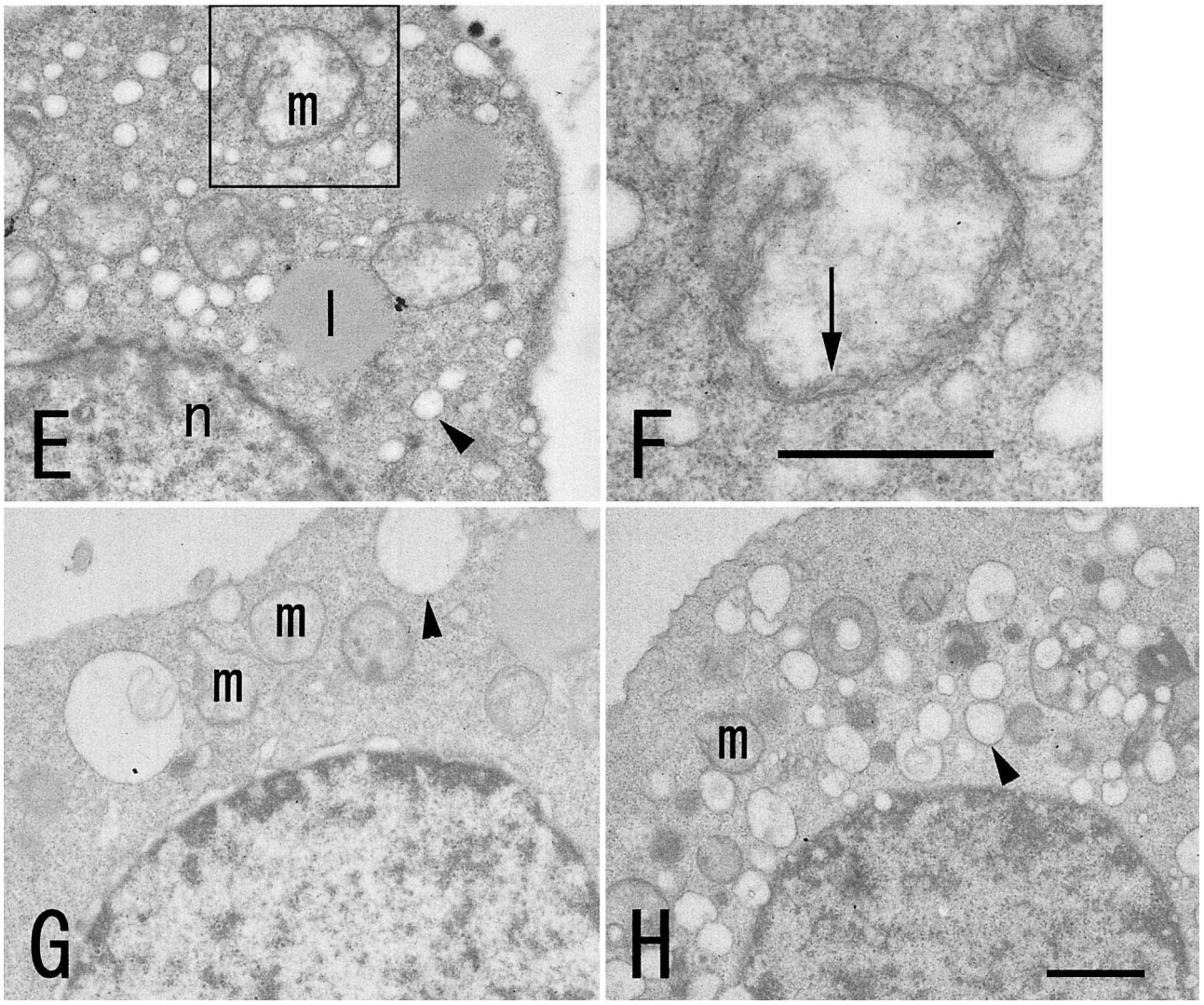


Fig. 3. Electron microscopic observations of U937 cells treated with Dol-P. A, intact cell. B, cell treated with solvent for 2 hr. C, cell treated with Dol-P for 30 sec. Disruption of mitochondrial cristae is seen (arrow). D, cell treated with Dol-P for 5 min. Disruption of mitochondrial cristae is seen (arrow). E, cell treated with Dol-P for 10 min. Many small vesicles appeared (arrowhead). F, higher magnification of inset in E. Mitochondrial inner and outer membranes are clearly shown (arrow). G, cell treated with Dol-P for 1 hr. Arrowhead indicates vesicle. H, cell treated with Dol-P for 2 hr. Arrowhead indicates vesicle. e, endoplasmic reticulum; m, mitochondrion; n, nucleus; l, lipid droplet. Bar, 1μm. All figures except F are the same magnification.

DISCUSSION

We have demonstrated that mitochondrial $\Delta\psi m$ decreases 1 hr after the treatment of cells with Dol-P (Yasugi *et al*., 2000). In this study we examined the early step of mitochondrial changes during Dol-P-induced apoptosis in U937 cells, and showed the reduction in $\Delta\psi m$ immediately after treatment of Dol-P in JC-1 stained cells. Lemasters *et al*. (1998) reported that mitochondrial PT in cell death is a common mechanism in necrosis, apoptosis and autophagy. When the mitochondrial PT involves only a few mitochondria, autophagy is stimulated, whereas when many or all mitochondria are involved, the mitochondrial PT begin to promote apoptosis or necrosis, successively. Susin *et al*. (1998), who reviewed mitochondrial functions during apoptosis, mentioned the same results and concluded that cell death inducers cause a $\Delta\psi m$ reduction which precedes signs of apoptosis or cytolysis. Although some experimental evidence supports these views, other studies suggested that PT-pore opening is a secondary, caspase-dependent event that is often preceded by cytochrome C release (Adrain and Martin, 2001). Our results clearly show that mitochondrial PT-pore opening is a first step of Dol-P induced apoptosis in cytoplasm, since the activation of caspase occur 2 hr after Dol-P treatment (Yasugi *et al*., 1998).

During the pre-mitochondrial induction phase, different pro-apoptotic signal transduction pathways are activated. Generally, genotoxic stress involves generation of reactive oxygen species (Susin *et al*., 1998). Then, we treated cells with free radical scavengers, N-acetyl-L-cysteine and vita-

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min E, before Dol-P was added. Both inhibitors, however, did not exert apparent inhibitory effect on reduction in $\Delta\psi m$. Mitochondrial PT-pore inhibitor, cyclosporin A, also had no effect. These findings strongly suggest that Dol-P was incorporated directly into mitochondrial membrane and caused membrane perturbation.

Next, we observed ultrastructural changes of mitochondria in Dol-P-treated cells by electron microscopy. The disruption of mitochondrial cristae was clearly seen immediately after treatment of Dol-P. Frey and Mannella (2000) reported the structure of mitochondria during apoptosis and proposed two mechanisms by which cytochrome c could exit mitochondria. One is outer membrane rupture following matrix swelling that results in opening of channel in the inner membrane, and the other is that the matrix did not swell but was very condensed, with no visible ruptures of the outer membrane. During ceramide-dependent cell death in neuronally differentiated PC12 cells, enlargement of the endoplasmic reticulum was the first ultrastructural alteration, followed by mitochondrial swelling, in which rupture of the outer mitochondrial membrane and unfolding of the inner membrane were frequently seen (Muriel *et al*., 2000). Apoptogenic ganglioside GD3 directly induces the mitochondrial PT and ensuing swelling (Scorrano *et al*., 1999; Kristal *et al*. 1999). In our results, mitochondria did not swell although only cristae were disoriented and disintegrated even in cells treated only for 5 min with Dol-P. It is to be noted that outer and inner membranes remained unaffected when cristae were disintegrated (Fig. 3F). The example of disorganization of cristae without disruption of double-layered membrane was reported by Ghadially (1975). Other morphological alterations of cells, such as appearance of small vesicles which seemed to be swollen endoplasmic reticulum, were observed much later. Thus the first sign toward apoptotic pathway in Dol-P-treated cells is a disruption of mitochondrial cristae.

Although we do not know at present whether such morphological changes are related to the reduction in $\Delta\psi m$ of mitochondria, it is highly conceivable that they are in fact intimately coupled. Thus, the present work, together with our previous results (Yasugi *et al*., 1995; Fujimoto *et al*., 1999; Yasugi *et al*., 2000), suggests that lipid-mediated cell death might involve perturbation of plasma membrane, followed by the disruption of mitochondria and endoplasmic reticulum. These changes of membrane structure and function seem to generate successive apoptotic cascade.

ACKNOWLEDGMENT

We thank Dr. I. Yamatsu (Serendip Research Institute, Tokyo, Japan) for providing Dol-P.

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(Received October 3, 2001 / Accepted November 3, 2001)

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