Journal of Cellular Biochemistry 100:349–361 (2007)

Generation of Reactive Oxygen Species Is an Early Event in Dolichyl Phosphate-Induced Apoptosis

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Abstract The mechanism of induction of apoptosis by dolichyl phosphate (Dol-P) was investigated in U937 cells. Studies using isolated mitochondria revealed that the respiratory complex II activity was almost completely inhibited by 20 μg/ml of Dol-P but not by the same concentration of dolichol. Activities of complex I and III were also inhibited by Dol-P, but nearly 50% of activity still remained at 20 μg/ml. Dol-P induced release of cytochrome-*c* from the isolated mitochondria. Fluorometric microtiter plate assay revealed that generation of reactive oxygen species (ROS) increased in a time-dependent manner. Flow cytometric analysis also indicated that Dol-P caused loss of mitochondrial membrane potential ($\Delta\psi_m$) and increased ROS generation. The addition of the antioxidant pyrrolidine dithiocarbamate (PDTC) significantly inhibited Dol-P-induced ROS generation and activation of caspase-3. A specific inhibitor of respiratory complex II, thenoyltrifluoroacetone (TTFA), increased ROS generation, potentially mimicking the consequence of inhibition of electron flow at complex II by Dol-P in U937 cells. Electron microscopy revealed that mitochondria became swollen and spherical in shape by the treatment with Dol-P. Neither the tyrosine kinase inhibitor k252a nor mitogen activated protein kinase/extracellular signal-regulated kinase kinase (MEK) inhibitors PD98059 and U0126 inhibited the Dol-P-induced apoptosis. Together, these results suggest that the direct disruption of mitochondrial respiratory complexes and the consequent ROS generation play a critical role in the initiation of Dol-P-induced apoptosis. J. Cell. Biochem. 100: 349–361, 2007.

Key words: dolichyl phosphate; apoptosis; reactive oxygen species; electron transport chain; pyrrolidine dithiocarbamate; caspase-3; mitochondria

Recent investigations have revealed that apoptosis is regulated by at least two signal transduction pathways [Wajant, 2002; Sprick and Walczak, 2004]. The first pathway proceeds through the binding of ligands to death receptors-like tumor necrosis factor receptor (TNF)-1 and Fas/CD95/APO-1 on the cell surface. In this pathway, caspase-8 is activated by a signaling complex that includes the cytoplasmic tail of the receptors and caspase-8 [Ashkenazi and Dixit, 1998]. The second pathway is characterized by the convergence of the signals on (into) the mitochondrion and the participation of Bcl-2 family proteins. The release of cytochrome-*c* into the cytosol causes the activation of caspase-9, which eventually leads to a direct activation of executor caspase-3 [Li et al., 1997]. Although the precise mechanism remains unknown, an increase in generation of reactive oxygen species (ROS) has been shown to induce

Abbreviations used: COX, cytochrome-*c* oxidase; Dol-P, dolichyl phosphate; CM-H_2DCFDA, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate; JC-1, 5, 5′, 6,6′-tetrachloro-1, 1′, 3, 3′-tetraethylbenzimidazolylcarbocyanine iodide; MAP, mitogen-activated protein; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; 1-methoxy PMS, 1-methoxy-5-methylphenazium methylsulfate; 3-NP, 3-nitropropionic acid; PDTC, pyrrolidine dithiocarbamate; PTP, permeability transition pore; TNF, tumor necrosis factor; TTFA, thenoyltrifluoroacetone; ROS, reactive oxygen species.

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Received 29 July 2005; Accepted 9 May 2006

DOI 10.1002/jcb.21024

cytochrome-*c* release from mitochondria [Kirkland and Franklin, 2001]. Mitochondria are the richest source of ROS in the cell, converting 1–2% of reduced oxygen into superoxide [Richter et al., 1995]. ROS have been suggested to regulate the process involved in the initiation of the apoptotic signaling cascade. Inhibition of the mitochondrial electron transport chain by compounds such as rotenone and ceramide leads to release of ROS and is an early event in many forms of apoptosis [Quillet-Mary et al., 1997; Li et al., 2003].

Dolichyl phosphate (Dol-P) plays an important role in yeast and mammalian cells as a glycosyl carrier lipid in the biosynthesis of glycosylphosphatidylinositol anchors, protein C- and O-mannosylation, and protein N-glycosylation. The availability of Dol-P has been regarded as a regulating factor in the generation of glycoproteins [Burda and Aebi, 1999; Schenk et al., 2001]. We previously demonstrated that Dol-P induced typical apoptotic changes in human monocytic leukemia U937 cells and other cell lines, including caspase-3 activation, nuclear fragmentation, and internucleosomal cleavage of genomic DNA in several hours [Yasugi et al., 1995, 1998; Yokoyama et al., 1997]. Treatment with swainsonine and tunicamycin, specific inhibitors of N-linked glycosylation, could not block Dol-P-induced apoptosis, suggesting that N-linked glycoprotein might not be associated with Dol-P-induced apoptosis [Yasugi et al., 1995].

Oxygen was initially believed to be converted to superoxide anion by regularly operating complex I or III [Raha and Robinson, 2000]. Recently, several reports indicate that ROS are produced in significant amounts at complex II [McLennan and Degli, 2000]. Furthermore, mutation of succinate dehydrogenase cytochrome-b accompanying a loss of ubiquinone reductase activity has been demonstrated to cause an increase in ROS generation [Ishii et al., 1998; Senoo-Matsuda et al., 2001]. Huntington's disease with neuronal cell death is also characterized by inhibition of complex II and increase in ROS generation [Rosenstock et al., 2004]. The inhibition of complex II by inhibitors such as thenoyltrifluoroacetone (TTFA) [Albayrak et al., 2003; Mehta and Shaha, 2004] and 3-nitropropionic acid (3-NP) [Rosenstock et al., 2004] increases generation of ROS to trigger the apoptotic signals. The transient inhibition of complex II by overexpression of cybL, a subunit of complex II, causes the elevation of ROS level and apoptosis [Albayrak et al., 2003].

In this report, we demonstrate that Dol-P leads to several mitochondrial dysfunctions. These include morphological changes of mitochondria, decreases in mitochondrial membrane potential ($\Delta\psi_m$), inhibitory effects on mitochondrial respiratory enzyme complexes (I–III), release of cytochrome-*c* from isolated mitochondria. We also show that Dol-P enhances the generation of ROS, and pyrrolidine dithiocarbamate (PDTC) effectively inhibits the ROS generation and apoptosis. Results described here suggest that Dol-P affects mitochondrial electron transport, thereby increasing ROS generation, which is an indispensable event for the initiation of apoptotic signaling. These findings should aid in the understanding of the precise mechanisms of mitochondria-dependent apoptotic cascades.

MATERIALS AND METHODS

Reagents

Dol-P, dolichol, decylubiquinone (DB), cytochrome-*c*, and PDTC were purchased from Sigma-Aldrich (St. Louis, MO). 2′,7′-Dichlorodihydrofluorescein diacetate (H_2DCFDA), 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA), and 5, 5′, 6,6′-tetrachloro-1, 1′, 3, 3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) were from Molecular Probes (Eugene, OR). Mouse monoclonal anti-cytochrome-*c* antibody, goat anti-cytochrome-*c* oxidase III (COX III) antibody, horseradish peroxidase-labeled goat anti-$F(ab')_2$ fragment to mouse IgG(H+L) antibody, and horseradish peroxidase-labeled donkey anti-goat IgG antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). Tetrazolium salt WST-1, 1-methoxy-5-methylphenazium methylsulfate (1-methoxy PMS), herbimycin A, K252a, U0126, and PD98059 were from Wako Pure Chemical Industries (Osaka, Japan). Recombinant human TNF-α was kindly provided by Dainippon Sumitomo Pharma (Osaka, Japan).

Cell Culture and Induction of Apoptosis

Human monocytic leukemia U937 cells were maintained in RPMI 1640 supplemented with 10% heat inactivated fetal calf serum at 37°C and 5% CO_2. Cell death was induced by

treatment with Dol-P dissolved in ethanol/dodecane (98:2, v/v), according to Ji et al. [1995]. The concentration of ethanol/dodecane was less than 1% in the culture medium. Control cultures received the same amount of ethanol/dodecane as the treated cultures, and the concentration did not affect cell viability.

Preparation of Mitochondria

Mitochondria were isolated from U937 cells as described previously [Trounce et al., 1996]. In brief, U937 cells (2×10^7) were collected, washed three times with 20 mM HEPES pH 7.4 containing 300 mM mannitol, 1 mM EDTA, and 0.1 mM PMSF and homogenized in the same buffer with 40 strokes of a glass Dounce homogenizer on ice. The supernatant from centrifugation at 600*g* for 5 min was centrifuged at 8,000*g* for 20 min. The supernatant containing microsomes was carefully removed, and the precipitate was washed with the same buffer by centrifugation at 8,000*g* for 20 min. The mitochondrial fraction was dissolved in the same buffer and stored at -80°C until use. The purity of mitochondria was checked by measuring of the activity of a cytosolic enzyme, lactate dehydrogenase. Measurements of lactate dehydrogenase were performed using a kit (Sigma-Aldrich) according to the instruction provided by the manufacturer. Based on Bostrom et al. [2004], mitochondria with lactate dehydrogenase activity less than 5% were used for present experiments.

Assay of Respiratory Complexes

We examined the effect of Dol-P or dolichol on each activity of respiratory complex. Enzyme activities of complexes I to IV of the respiratory chain were measured according to the methods described before [Cornelissen et al., 1995; Trounce et al., 1996; Cardoso et al., 1999; Okubo et al., 2004]. The indicated concentration of Dol-P or dolichol was added to the reaction mixture at the beginning of the preincubation. Each reaction was performed at 30°C. For the assay of complexes I, II, III, and IV, a Shimadzu Spectrophotometer Type UV-300 was used, and the activity was expressed as nmol/mg protein/min.

In the case of complex I activity, mitochondrial fraction (20 μg of protein) was incubated in 50 mM Tris-HCl buffer pH 7.4 containing 1 mM EDTA, 250 mM sucrose, 2 mM KCN, 10 μM DB, and 0.6 mM *n*-dodecyl-β-D-maltoside in the absence or presence of Dol-P or dolichol. After a 1-min preincubation, reactions were initiated by the addition of 50 μM NADH. Rotenone (5 μg/ml) sensitive activity was regarded as complex I activity. Oxidation of NADH was monitored at 340 nm minus 380 nm.

In the case of complex II activity, mitochondrial fraction (25 μg) was incubated in 50 mM potassium phosphate buffer pH 7.4 supplemented with 20 mM succinate, 2 mM KCN, 2 μg/ml antimycin A, 4 μg/ml rotenone, 0.6 mM *n*-dodecyl-β-D-maltoside, and 50 μM 2, 6-dichlorophenolindophenol in the absence or presence of Dol-P or dolichol. After a 1-min equilibration period, the reaction was started by the addition of 50 μM DB to the assay mixture, and the decrease in absorbance at 600 nm minus 750 nm was monitored. TTFA (0.5 mM)-sensitive activity was regarded as complex II activity.

Complex III donates electrons from reduced DB (ubiquinol) to cytochrome-*c*, leading to the reduction of cytochrome-*c*. The activity of complex III was assayed using 10 μg of mitochondrial fraction. Incubation was carried out in 25 mM potassium phosphate buffer pH 7.2, 1 mM EDTA, 2.5 mM $MgCl_2$, 0.6 mM *n*-dodecyl-β-D-maltoside, 2 mM KCN, and 50 μM cytochrome-*c* for 1 min in the absence or presence of Dol-P or dolichol, and the reaction was started by the addition of 50 μM ubiquinol. The activity was estimated from the linear absorbance increase at 550 nm minus 540 nm. Ubiquinol was obtained by reducing DB with sodium dithionite [Cornelissen et al., 1995]. Antimycin A (5 μg/ml)-sensitive activity was regarded as the complex III activity.

Complex IV activity was measured by evaluating the oxidation of cytochrome-*c* as a decrease in the absorbance at 550 nm. The reaction mixture contained 25 mM potassium phosphate buffer pH 7.4, 1 mM *n*-dodecyl-β-D-maltoside, and 20 μM reduced cytochrome-*c* in the absence or presence of Dol-P or dolichol. After a 1-min preincubation, the reaction was started by the addition of 48 μg of mitochondria. The linear absorbance decrease was monitored at 550 nm minus 540 nm. Reduced cytochrome-*c* was prepared as described previously [Trounce et al., 1996] and separated from ascorbate by column chromatography on a 20-ml Sephadex G-25 column. KCN

(2 mM)-sensitive activity was regarded as the complex IV activity.

ROS Detection

H_2DCFDA was used for ROS capture. Assays were performed using 24-well tissue culture plates, according to the method of Wan et al. [1993]. Briefly, cells (2×10^5) were suspended in Hank's balanced salt solution and labeled with 100 μM H_2DCFDA for 1 h. They were then incubated with or without 20 μg/ml Dol-P at 37°C for various periods of time. The fluorescence was monitored with excitation at 485 nm and emission at 530 nm using CytoFluor II fluorescence reader (PE Biosystems Japan, Chiba, Japan). The increase in the fluorescence was used to measure the chemical process of H_2O_2 generation.

Flow cytometry using CM-H_2DCFDA was also conducted to confirm ROS generation. CM-H_2DCFDA is a chloromethyl derivative of H_2DCFDA, which exhibits much better retention in live cells. CM-H_2DCFDA working solution was added directly to the medium to reach 5 μM and then incubated at 37°C for 20 min. Approximately 10,000 U937 cells were aspirated into the flow cytometer (Epics XL MCL, Beckman Coulter, Fullerton, CA) and examined for fluorescence on the channel for DCF. The data were analyzed with WinMDI software v.2.8.

Caspase-3 Activity

Caspase-3 activity was assayed as described previously [Yokoyama et al., 1997]. Activity was expressed as AMC cleaved/min/mg protein.

Detection of $\Delta\psi_m$

$\Delta\psi_m$ was estimated using JC-1 as described previously [Okubo et al., 2004]. In brief, cells (2×10^6) were labeled with 5 μg/ml of JC-1 for 20 min at room temperature, and $\Delta\psi_m$ was analyzed using a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA). The filter at the FL1 photomultiplier was 530 nm and that at FL2 was 590 nm. A minimum of 10,000 events per sample was acquired and then analyzed with CELL Quest software (Becton Dickinson). The number of cells that lost $\Delta\psi_m$ was estimated by calculating the changes in percentage expression in the lower right quadrant, which represents cells having both reduced fluorescence at 590 nm and increased fluorescence at 530 nm.

Immunoblotting

Immunoblotting was performed as described previously [Yokoyama et al., 1997]. Freshly isolated mitochondria (1 mg/ml) were suspended in 300 mM mannitol, 20 mM HEPES pH 7.4, 0.1 % BSA, and 10 mM KH_2PO_4 and incubated in the absence or presence of Dol-P at 37°C. The reaction mixture was centrifuged at 10,000*g* for 10 min at 4°C to separate the supernatant fractions from the mitochondrial pellets. The pellets were resuspended in the same volume of 300 mM mannitol, 20 mM HEPES pH 7.4, and 0.1 % BSA and 10 mM KH_2PO_4. Five microliters of the pellets and the supernatant fractions were separated on 14% SDS–polyacrylamide gels under reducing conditions, then transferred to PVDF membrane (Hybond-P, Amersham Biosciences, Amersham Place, England). Anti-cytochrome-*c* antibody and anti-COX III antibody were used at a dilution of 1/1,000. After reaction with the horseradish peroxidase-labeled secondary antibodies, products were visualized using the ECL system (Amersham Biosciences). Quantitative densitometry was performed using Quantity One software (Bio-Rad).

Electron Microscopy

Cells were fixed by treatment with Karnovsky reagent (0.1 M phosphate buffer pH 7.2 and 2% glutaraldehyde solution) for 1 h at 4°C. Further fixation was carried out for 1 h with potassium ferricyanide in 1% osmium tetroxide. The specimen was stained with 2% aqueous uranyl acetate for 2 h and washed with water. Dehydration was performed with acetone, and cells were embedded in a mixture of epoxy resin and acetone. Samples were examined using a Hitachi H7100 TEM microscope at 75 kV. Photographs were taken at magnifications of 3,200 and 24,000-fold.

DNA Fragmentation Assay

Cells were inoculated in 96-well plates. DNA fragmentation was evaluated by examination of cytoplasmic histone-associated DNA fragments (mononucleosomes and oligonucleosomes) using a Cell Death Detection ELISAPlus kit (Roche Diagnostics GmbH) according to the manufacturer's instructions.

WST-1 Assay

U937 cells were inoculated at 3×10^4 cells in 100 μl of medium containing 0.5% or 10% FCS and treated with different concentrations of herbimycin A. After 30 min, the activity to reduce WST-1 in the presence of 1-methoxy PMS was determined according to the manufacturer's instructions. The absorbance was detected at 450 nm by use of an MPR-A4 microplate reader (Tosoh, Tokyo, Japan).

Statistical Analysis

The results shown derive from experiments repeated three or four times, unless otherwise indicated. Results are expressed as mean ± SD. Statistical significance was analyzed using the two-tailed Student's *t*-test. A *P*-value of 0.05 was adopted as the criterion for statistical significance.

RESULTS

Effects of Dol-P on $\Delta\psi_m$

Previously our data showed that the caspase-3 activity increased with increase of Dol-P concentration from 2.5 to 7.5 μg/ml. Induction of activity was maximum at the concentration of 20 μg/ml after treatment for 3 h [Yokoyama et al., 1997]. Disruption of $\Delta\psi_m$ has been recognized to be implicated in apoptosis [Bernardi et al., 2001]. We examined the effect of Dol-P on $\Delta\psi_m$ to clarify the pathway involved in Dol-P-induced apoptosis. U937 cells were treated with 20 μg/ml Dol-P for different times and then loaded with JC-1. Signals in the lower right quadrant increased from 0% to 23% after 30 min of treatment with Dol-P, concomitantly with their decrease in the upper left quadrant from 85.8% to 24.0% (Fig. 1A,B). The increase in signals in the lower right quadrant, which implied an increase in JC-1 monomers, indicated the disruption of $\Delta\psi_m$. When the cells were treated with Dol-P for 1 h, signals in the lower right quadrant further increased to about 70% (Fig. 1D) and the levels continued up to 2 h (Fig. 1F). Signals did not change after incubation for 2 h without Dol-P (Fig. 1A–C).

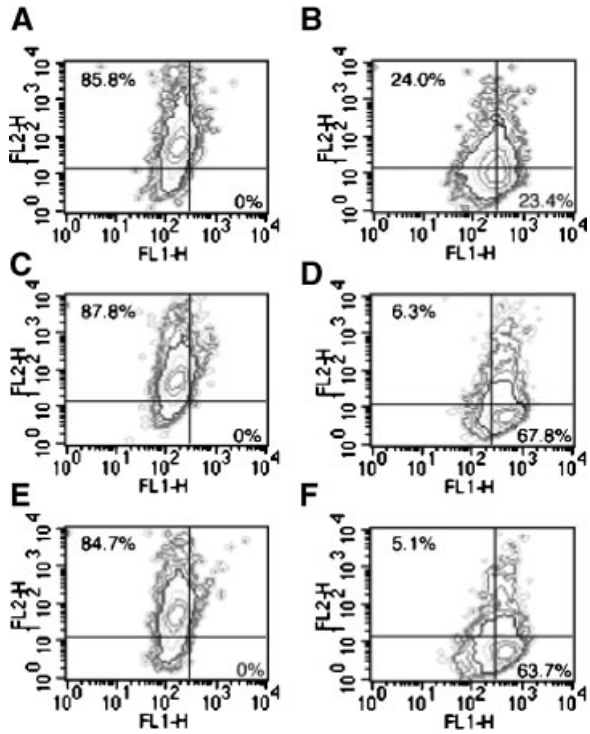

Fig. 1. Dol-P-induced loss of $\Delta\psi_m$ in U937 cells. Cells were treated with (**B**, **D**, **F**) or without (**A**, **C**, **E**) 20 μg/ml Dol-P for 30 min (A, B), 1 h (C, D), or 2 h (E, F), and $\Delta\psi_m$ was assessed by FACScan analysis of cells stained with JC-1. The JC-1 fluorescence was measured by flow cytometry at both 530 nm and 590 nm. A representative of four experiments is shown, with a minimum of 10,000 events per sample.

Effects of Dol-P on Respiratory Complexes

The disruption of $\Delta\psi_m$ is a biological reaction consequent to the inhibition of respiratory complexes I, II, and III. To determine whether respiratory complexes are affected by Dol-P, the activities of enzyme complexes I through IV of the respiratory chain were measured. Exposure of isolated mitochondria to increasing concentrations of Dol-P caused a progressive inhibition of complex I and II activities (Fig. 2A,B). The IC_{50} values for complex I and II were approximately 18.0 μg/ml and 6.6 μg/ml, respectively, indicating that complex II was more sensitive to inactivation by Dol-P. Complex III activity was inhibited in a dose-dependent manner but showed different inhibition kinetics. While more than 40% of complex III inhibition was observed at the low concentration of Dol-P (2 μg/ml), complete complex III inhibition was not achieved with up to 40 μg/ml Dol-P (Fig. 2C). In the presence of 20 μg/ml Dol-P, complex II activity was almost completely inhibited, but about 50% of complex I and III activities still remained (Fig. 2A–C). Thus complex II was more severely inhibited by Dol-P than the other respiratory chain enzyme complexes. In

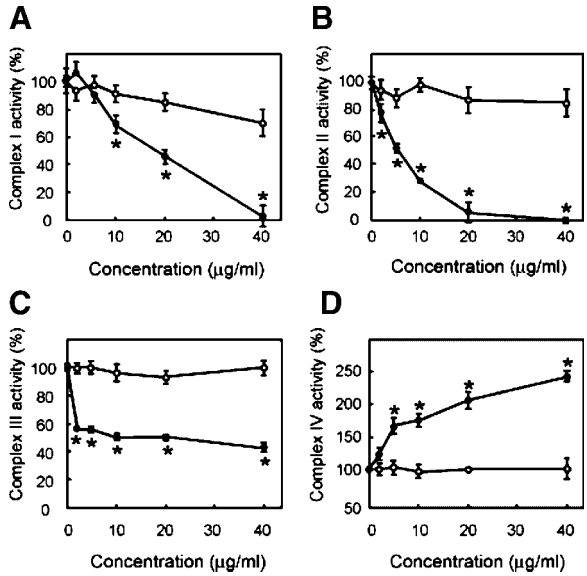

Fig. 2. Effects of Dol-P and dolichol on the activities of mitochondrial respiratory complexes. Isolated mitochondria were treated with different concentrations of Dol-P (●) or dolichol (○) for 1 min as described in Materials and Methods. Then, activity of complex I (**A**), II (**B**), III (**C**), or IV (**D**) in the absence or presence of Dol-P or dolichol was examined as described in Materials and Methods. Rotenone, TTFA, antimycin A, or KCN sensitive activity was regarded as complex I, II, III, or IV, respectively, and taken as 100%. Specific activities in control (vehicle only-treated) were as follows: complex I, 12.3 ± 0.8; II, 87.1 ± 6.8; III, 798.3 ± 12.8; IV, 87.1 ± 8.8 nmol/min/mg protein. The values are the mean ± SD (n = 3), some of which are within the size of symbols. *indicates $P < 0.01$ compared with controls.

contrast, complex IV activity was stimulated in mitochondria treated with Dol-P as the concentration of Dol-P increased (Fig. 2D). Previously, we showed that DNA fragmentation was not induced by treatment with dolichol (8 μg/ml) for 4 h, whereas the same concentration of Dol-P induced DNA fragmentation [Yasugi et al., 1998], suggesting that dolichol has a much lower ability to induce apoptosis. Indeed, the activities of complexes II, III, and IV were not affected by dolichol at concentrations up to 40 μg/ml (Fig. 2B–D). Complex I activity was attenuated by less than 30% at the dolichol concentration of 40 μg/ml (Fig. 2A).

Inhibitory Effects of PDTC on Dol-P-Induced ROS Generation and Apoptosis

ROS in Dol-P-treated U937 cells were assayed with the H_2DCFDA fluorescent probe. Figure 3A shows the increase in the fluorescence intensity of 20 μg/ml Dol-P-treated cells in a time-dependent manner, reflecting generation of ROS in U937 cells treated with Dol-P. The increase in ROS was detected within 15 min and continued up to 180 min. When the fluorescent probe CM-H_2DCFDA was used in place of H_2DCFDA, treatment with 20 μg/ml Dol-P for 30 min increased DCF fluorescence (Fig. 3B). PDTC, an effective oxygen radical scavenger inhibited Dol-P-induced caspase-3 activation in a dose-dependent manner. PDTC at 50 and 100 μM inhibited the caspase-3 activation by approximately 49% and 92%, respectively (Fig. 3C). To investigate whether the inhibition of mitochondrial respiratory complex II really causes ROS generation, we examined the effect of TTFA, a commonly used specific inhibitor of complex II, on the generation of ROS using flow cytometry. As shown in Figure 3D, treatment of cells with 25 μM TTFA for 30 min caused an apparent ROS generation detected as an increase in cellular DCF fluorescence. PDTC suppressed ROS generation by both Dol-P and TTFA (Fig. 3B,D).

Release of Cytochrome-*c* From Isolated Mitochondria

We next determined whether cytochrome-*c*, a key protein in apoptosis, is released from mitochondria by the inhibition of activity of mitochondrial respiratory complexes and ROS generation with Dol-P. The amount of cytochrome-*c* in the supernatant obtained by centrifugation of the mitochondrial suspension treated with or without Dol-P was evaluated by Western blot analysis. As shown in Figure 4, Western blots and densitometric analysis of cytochrome-*c* demonstrated that immunoreactivity was strongly positive in the supernatant fraction obtained from mitochondrial suspension treated with Dol-P for 1 h ($P < 0.01$ vs. control), and further increased after 2 h. In contrast, cytochrome-*c* immunoreactivity was undetectable in the supernatant fractions in controls for up to 2 h. In mitochondrial fractions (pellets), the amounts of cytochrome-*c* and COX III were not significantly affected by the treatment with Dol-P. COX III protein, an integral component of the inner membrane, was not detected in the supernatant fractions obtained from mitochondrial suspension treated with or without Dol-P for up to 2 h (data not shown).

Morphology of the Cells

Most of the mitochondria became swollen and spherical in shape after 2 h of treatment with

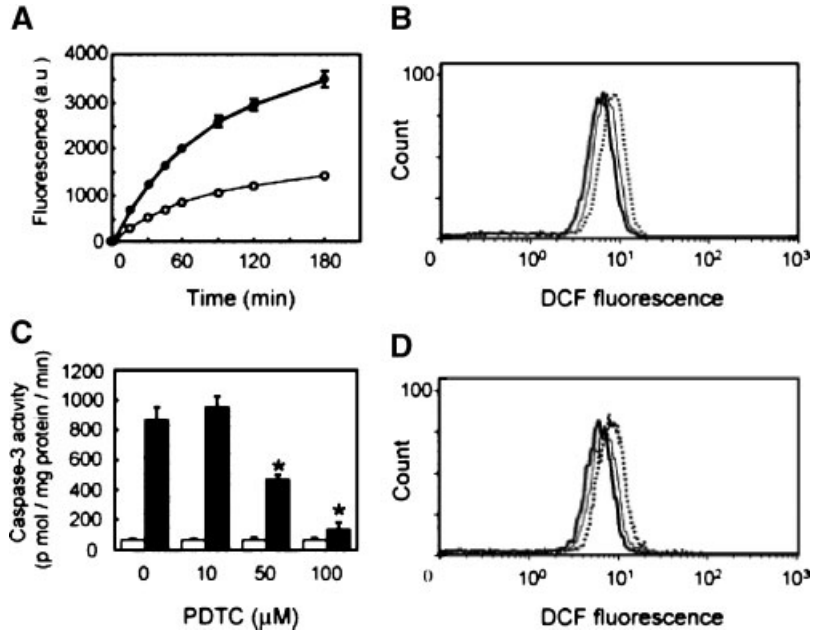

Fig. 3. Effects of PDTC on Dol-P-induced caspase-3 activation and ROS generation. **A**: Dol-P-induced ROS generation. U937 cells were labeled with H_2DCFDA for 1 h and treated with (●) or without (○) 20 μg/ml Dol-P for various periods. ROS was determined as described in Materials and Methods. The values are the mean ± SD (n = 4–5), some of which are within the size of symbols. a.u., arbitrary unit. **B**: Effects of PDTC on Dol-P-induced ROS generation. U937 cells were treated with 20 μg/ml Dol-P and/or 10 μM PDTC for 30 min. Cellular ROS generation was estimated by flow cytometry. ———control, ... 20 μg/ml Dol-P, —— 20 μg/ml Dol-P + 10 μM PDTC. **C**: Effects of PDTC on Dol-P-induced caspase-3 activity. U937 cells were treated with 20 μg/ml Dol-P and/or PDTC at the indicated concentration for 2.5 h. Cells were lysed and the caspase-3 activity was measured as described in Materials and Methods. Values are mean ± SD (n = 3–5). Closed bars, Dol-P-treated; Open bars, control. $^*P < 0.01$ as compared to cells treated with Dol-P alone. **D**: ROS generation in TTFA-treated cells. U937 cells were treated with 25 μM TTFA and/or 10 μM PDTC for 30 min. Cellular ROS generation was estimated by flow cytometry. ———control, ...25 μM TTFA, ——25 μM TTFA + 10 μM PDTC. For B and D, a representative of four experiments is shown, with a minimum of 10,000 events per sample.

Dol-P. Many cristae disappeared, and crista-like structures were clearly detached from the inner membrane (Fig. 5C,D). In cells incubated with vehicle alone, the cristae of all mitochondria exhibited the normal transverse orientation (Fig. 5A,B). No change of morphology was observed in cells treated with dolichol (data not shown).

Participation of Mitogen-Activated Protein (MAP) Kinase

Dohi et al. [1996] suggested that activation of p42 MAP kinase is implicated in the apoptotic signal caused by Dol-P in U937 cells as a result of experiments using herbimycin A, a tyrosine kinase inhibitor. We examined the effect of herbimycin A on Dol-P-induced apoptosis. U937 cells were treated for 3 h with Dol-P at the concentration of 5 μg/ml, which activates caspase-3 to about 70% of maximum activity [Yokoyama et al., 1997], in the absence or presence of herbimycin A. As shown in Figure 6A, 50 μM herbimycin A caused DNA fragmentation, and simultaneous treatment with Dol-P and herbimycin A enhanced DNA fragmentation. We also tested the effect of herbimycin A on the reduction of WST-1 (Fig. 6B). The color development due to the reduction of WST-1 was completely inhibited at 50 μM herbimycin A after only 30 min of incubation. WST-1 assay is known to partly reflect dehydrogenase activity of respiratory complex II. We examined the effect of herbimycin A on respiratory complex II using isolated mitochondria (Fig. 6C). Herbimycin A inhibited respiratory complex II activity in a dose-dependent manner.

We further examined the effect of a kinase inhibitor K252a on the induction of caspase-3 activation by Dol-P (5 μg/ml). K252a (2–10 nM) did not inhibit the induction by Dol-P, whereas 2 nM K252a significantly inhibited the induction of caspase-3 activity by TNF-α. Similar results were obtained when Dol-P was added after cells were pretreated with 2 nM K252a for 24 h (data not shown). K252a at the

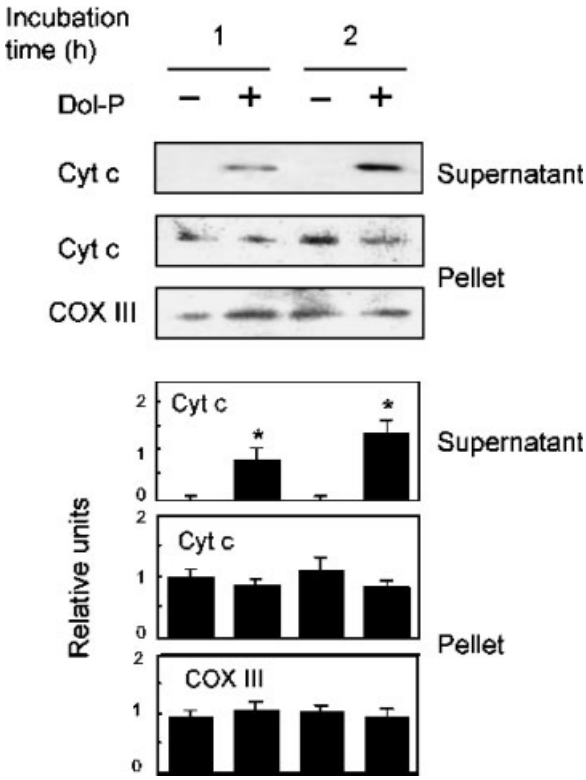

Fig. 4. Release of cytochrome-*c* from mitochondria treated with Dol-P. Mitochondria isolated from U937 cells were incubated for 1 or 2 h at 37°C with 20 μg/ml Dol-P. After centrifugation, mitochondrial pellets and supernatant fractions were separated by SDS–PAGE, and their respective contents of cytochrome-*c* (Cyt-*c*) and COX III analyzed by Western blotting. **Top**: Western blot of cytochrome-*c* and COX III. **Bottom**: The relative amounts of cytochrome-*c* and COX III were quantified by densitometric analysis and expressed as integrated optical density. The blot shown is representative of three experiments with similar outcomes. $^*P < 0.01$ as compared to the controls treated without Dol-P at each time.

concentration of 10 nM was cytotoxic in the presence of TNF-α as shown by the increase in caspase-3 activation (Table I).

We also measured activation of caspase-3 induced by Dol-P (5 μg/ml) in the absence or presence of two different MAP/extracellular signal-regulated kinase kinase (MEK) inhibitors, PD98059 (5–50 μM) and U0126 (2–10 μM) (Table I). These inhibitors did not affect caspase-3 activation by Dol-P. Similar results were obtained when Dol-P was added after cells were pretreated with PD98059 or U0126 for 24 h (data not shown). On the other hand, caspase-3 activity induced by TNF-α decreased in a dose-dependent manner in the presence of either PD98059 (10–50 μM) or U0126 (2–10 μM) (Table I). Treatment with PD98059 (50 μM) or U0126 (5 or 10 μM) did not inhibit DNA fragmentation induced by Dol-P (data not shown).

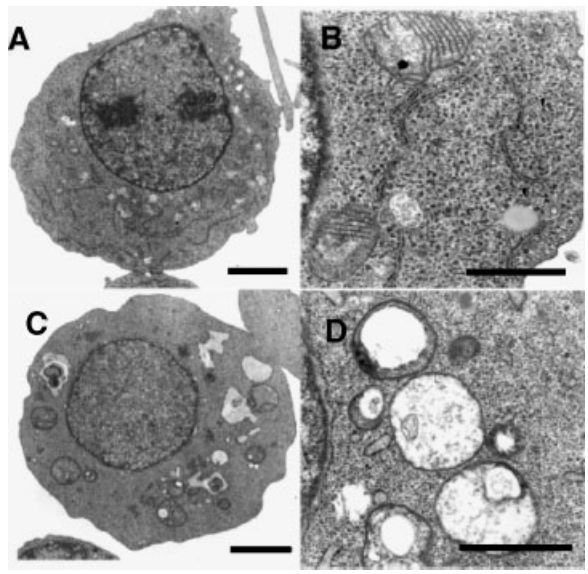

Fig. 5. Electron microscopic analysis of U937 cells treated with Dol-P. U937 cells were treated with (**C**, **D**) or without (**A**, **B**) 20 μg/ml Dol-P for 2 h. Bar = 1 μm.

DISCUSSION

In our previous articles [Yasugi et al., 1995, 1998; Yokoyama et al., 1997], we have shown that Dol-P caused typical apoptotic disruption of cells involving nuclear and DNA fragmentation and the activation of caspase-3. However, the mechanisms by which Dol-P induces apoptosis remain obscure. Mitochondria are a potential source and target of ROS in cells treated with a variety of proapoptotic agents [Simon et al., 2000]. It is generally accepted that elevation of cellular ROS can promote apoptosis [Kelso et al., 2001]. Several respiratory chain inhibitors, such as the complex I inhibitors rotenone and 1-methyl-4-phenylpyridinium, and the complex III inhibitor antimycin A, have been shown to induce the elevation of levels of mitochondrial respiratory chain-derived ROS and lead to apoptotic cell death [Hasegawa et al., 1990; Li et al., 2003; Chen and Yan, 2005]. In the present study, we show that Dol-P inhibited the activities of mitochondrial respiratory complexes and increased ROS generation.

We demonstrated that $\Delta\psi_m$ decreased in Dol-P-treated U937 cells at an early stage (Fig. 1), which is consistent with the previous report by Yasugi et al. [2000]. Caspase-3 activity was

undetectable until after 1 h of treatment [Yokoyama et al., 1997; Yasugi et al., 1998], indicating that the loss of $\Delta\psi_m$ occurred prior to the activation of caspase-3 in U937 cells treated with Dol-P. Because $\Delta\psi_m$ loss is generated by the pumping of protons out across the inner mitochondrial membrane at the mitochondrial complexes (I, III, and IV), it is likely that the decrease in complex activity could result in a decrease in $\Delta\psi_m$.

In order to assess whether Dol-P induces changes in the mitochondrial function of U937 cells, the effects of Dol-P on the activities of respiratory complexes I–IV were estimated using isolated mitochondria. Dol-P was found to affect complexes I, II, and III, as shown in Figure 2. The range of Dol-P concentration capable of inhibiting activities of respiratory complexes I–III is similar to that previously reported for induction of apoptosis by Dol-P. Dolichol, which has a much lower ability to induce apoptosis [Yasugi et al., 1995, 1998], had little inhibitory effect on the activities of complexes I–IV at concentrations up to 20 μg/ml (Fig. 2), suggesting that the inhibition of respiratory complexes is associated with the apoptotic signal in Dol-P-treated cells. Complex IV activity was enhanced by the addition of Dol-P, although the mechanisms of action remain unknown. It is possible that the generated ROS at least in part enhanced cytochrome-*c* oxidation. Dol-P itself did not oxidize reduced cytochrome-*c* in the absence of the mitochondrial fraction (data not shown).

Because Dol-P inhibited respiratory complexes, we determined whether it could modulate generation of ROS in U937 cells (Fig. 3A–C). Dol-P increased the generation of ROS in a time-dependent manner, as determined by 24-well plate assay and flow cytometry. The generation of ROS by Dol-P occurred earlier (<1 h) than the activation of caspase-3, which is undetectable

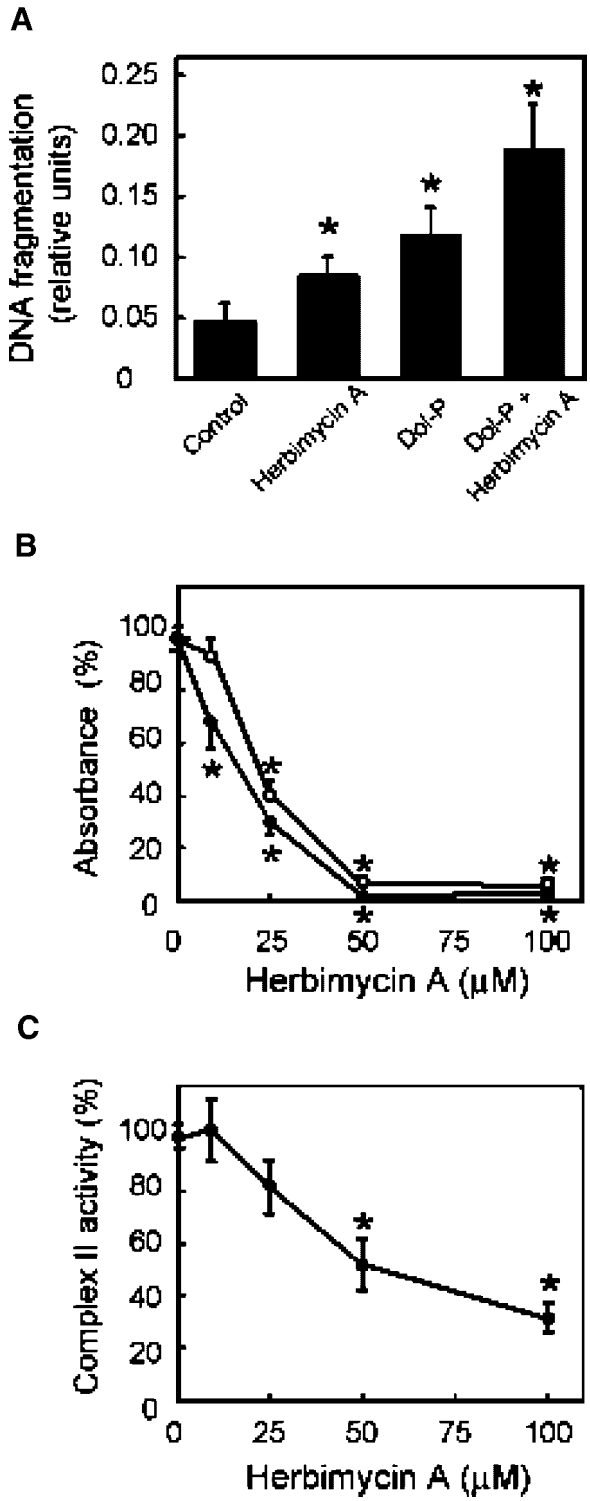

Fig. 6. Cytotoxicity of herbimycin A in U937 cells. **A**: Effect of herbimycin A on DNA fragmentation in the presence or absence of Dol-P. U937 cells were pretreated with or without 50 μM herbimycin A for 2 h, then Dol-P (5 μg/ml) was added. Apoptosis was estimated by a DNA fragmentation ELISA 3 h after Dol-P treatment. Values are mean ± SD (n = 4–5). **B**: Inhibitory effects of herbimycin A on the reduction of WST-1. U937 cells were cultured for 24 h in RPMI 1640 containing FCS at the concentration of 10% (●) or 0.5% (○), then treated with the indicated concentration of herbimycin A for 30 min. Reduction of WST-1 was then determined spectrophotometrically. Absorbance is expressed as a percentage of controls (vehicle-treated). **C**: Inhibitory effects of herbimycin A on respiratory complex II activity. Isolated mitochondria were treated with various concentrations of herbimycin A for 1 min. Complex II activity was measured as described in Materials and Methods. Specific activity of complex II in the control was 75.6 ± 5.3 nmol/min/mg protein. Values are mean ± SD (n = 4–5). For each panel, * indicates $P < 0.01$ compared with appropriate controls.

TABLE I. Effects of PD98059, U0126, and K252a on Activation of Caspase-3 Activity in U937 Cells

	Caspase-3 activity (%)	
	Dol-P-induced	TNF-α-induced
Control	100.0 ± 8.1	100.0 ± 4.2
PD98059		
5 μM	113.4 ± 11.8	95.9 ± 11.7
10 μM	100.7 ± 0.7	84.8 ± 1.5*
50 μM	110.3 ± 9.6	65.1 ± 2.3*
U0126		
2 μM	98.4 ± 0.2	81.7 ± 2.8*
5 μM	103.2 ± 8.8	65.1 ± 3.9*
10 μM	92.4 ± 3.8	57.8 ± 2.9*
K252a		
2 nM	116.7 ± 0.2	84.7 ± 2.7*
10 nM	110.7 ± 2.5	116.3 ± 8.5

Cells were incubated at 37°C in either control medium or medium supplemented with the indicated inhibitors for 2 h. Then Dol-P (5 μg/ml) or TNF-α (0.5 ng/ml) was added. Caspase-3 activity was determined 2.5 h after Dol-P treatment or 24 h after TNF-α treatment, and expressed as a percentage of the appropriate control under each experimental condition. Specific activity induced in the control by Dol-P and TNF-α was 606.8 ± 49.2 and 490.6 ± 20.6 pmol/min/mg protein, respectively. Treatment of U937 cells with PD98059, U0126, or K252a alone had no effect on caspase-3 activity. Values are mean ± SD (n = 4–5).
*$P < 0.01$ as compared to cells treated with TNF-α alone.

until 1 h [Yokoyama et al., 1997]. The antioxidant PDTC inhibited Dol-P-enhanced ROS generation and apoptosis as determined by caspase-3 activation. These results suggest the enrollment of ROS in Dol-P-induced apoptosis. Increased mitochondrial formation of ROS has been shown to trigger the intrinsic apoptotic pathway by (1) increasing the permeability of the outer mitochondrial membrane through the opening of the permeability transition pore (PTP), probably due to the oxidation of the glutathione pool and the formation of critical protein dithiols [Chernyak and Bernardi, 1996; Chernyak, 1997; Crompton, 1999; Halestrap et al., 2004]; and (2) inducing dissociation of cytochrome-*c* from cardiolipin on the inner mitochondrial membrane [Imai and Nakagawa, 2003; Zhao et al., 2004]. Cytochrome-*c* moves through the open PTP from the intermembrane space into the cytoplasm [Martinou et al., 2000], where it forms apoptosome and then activates caspase-9, which in turn activates caspase-3 [Li et al., 1997]. Kroemer et al. [1998] reported that induction of the opening of PTP may be involved in disruption of the mitochondrial membrane potential and release of cytochrome-*c* from the intermembrane space of mitochondria to the cytosol. In our study, (1) cytochrome-*c* was released from Dol-P-treated isolated mitochondria (Fig. 4) and (2) loss of $\Delta\psi_m$ occurred in Dol-P-treated cells (Fig. 1), which may result not only from the inhibition of respiratory complexes but also from the PTP opening. It is probable that the ROS generated by Dol-P contribute to the PTP opening, the release of cytochrome-*c* from the intermembrane space of the mitochondria and the caspase activation. Proapoptotic and antiapoptotic proteins such as Bax/Bak and Bcl-2/xL participate in mitochondrial membrane permeabilization, causing the release of cytochrome-*c* in apoptotic signals [Kim et al., 2006]. The mRNA or protein levels of Bcl-2, Bax, and Bid were unchanged after 2 h of incubation of the cells with Dol-P (data not shown).

Mitochondrion-derived ROS could be modified when the mitochondrial respiratory chain is interrupted under pathological conditions or by respiratory chain inhibitors [Turrens, 1997]. In addition to the commonly accepted view that the inhibition of complex I or III causes ROS generation [Hasegawa et al., 1990; Li et al., 2003; Chen and Yan, 2005], it has recently been shown that the inhibition of complex II could also cause ROS generation [Ishii et al., 1998; Senoo-Matsuda et al., 2001; Albayrak et al., 2003; Mehta and Shaha, 2004; Rosenstock et al., 2004]. These reports suggest that impaired electron transport in the enzyme may result in ROS formation. Suppression of complex II by the drug TTFA resulted in generation of H_2O_2 and superoxide, leading to decrease in $\Delta\psi_m$ and apoptosis in Leishmania donovani promastigotes [Mehta and Shaha, 2004]. This is consistent with the report by Albayrak et al. [2003] that TTFA elevated generation of ROS and induced apoptosis in human HeLa cells. In our studies, TTFA was used to support the correlation between the inhibition of complex II and generation of ROS in U937 cells. Generation of ROS occurred in TTFA-treated U937 cells, which was reversed by the addition of PDTC (Fig. 3D), suggesting the possibility that inhibition of electron flow at complex II could result in ROS generation in U937 cells.

An increasing number of reports support the notion that a transient inhibition of complex II causes apoptotic cell death. For example, inhibitors of complex II such as TTFA and 3-NP [Albayrak et al., 2003; Brito et al., 2003;

Rosenstock et al., 2004; Mehta and Shaha, 2004] have been shown to induce apoptosis in several cell lines. Induction of cell death in the cybL-deficient cells by expression of cybL, a subunit of the complex II, is also consistent with a transient inhibition of complex II. Inhibition of complex II was stated to lead to blockage of the electron flow of the mitochondrial respiratory chain, the generation of oxygen radicals, and the induction of apoptosis. We found that Dol-P inhibited complex II activity effectively in a dose-dependent manner. Sipos et al. [2003] noted that ROS formation was not detected until complex III was inhibited up to $71.4 \pm 4\%$ in isolated nerve terminals. In our data, more than 40% of complex III activity still remained even at high concentration of Dol-P. We assume that a certain amount of ROS generated in Dol-P-treated U937 cells may be attributable to the inhibition of electron transport at complex II, though the possibility that the inhibition of complex I or III may lead to the generation of ROS cannot be ruled out. The ROS-generating activities of cytosolic enzymes such as NADP(H) dehydrogenase/oxidase, xanthine dehydrogenase/oxidase, and cyclooxygenase are normally thought to be low. However, the possibility that these enzymes are activated by Dol-P to produce ROS remains to be clarified.

Dol-P caused severe degeneration of the fine structure of mitochondria. Swollen mitochondria lacking the fine structure of cristae were observed in Dol-P-treated cells, such changes being typical of mitochondrial structure after treatment of cells with Dol-P (Fig. 5). Inhibition of complex I by chloromethyltetramethylrosamine caused PT pore opening with concomitant depolarization and swelling of mitochondria [Scorrano et al., 1999]. According to Martinou et al. [2000], the most common mode of cytochrome-*c* release is following outer mitochondrial membrane rupture as a result of mitochondrial matrix swelling. It is conceivable that Dol-P inhibited respiratory complexes and attenuated $\Delta\psi_m$, causing the disruption of the mitochondrial structure and the release of cytochrome-*c* from mitochondria.

MAP kinase has been shown to be involved in the "cascade" of Dol-P-induced apoptosis through experiments using a tyrosine kinase inhibitor, herbimycin A [Dohi et al., 1996]. It is not known, however, whether cross-talk occurs between the Dol-P-induced mitochondria-dependent signal and the MAP kinase cascade. In our data, the tyrosine kinase inhibitor K252a and the MEK inhibitors PD98059 and U0126 significantly inhibited TNF-α-induced caspase-3 activity, whereas none of these three inhibitors decreased the Dol-P-induced caspase-3 activity (Table I). Herbimycin A (50 μM) itself caused DNA fragmentation and did not attenuate DNA fragmentation in the presence of Dol-P (Fig. 6A). At the concentration of less than 50 μM, herbimycin A also did not attenuate DNA fragmentation or caspase-3 activation in the presence of Dol-P (data not shown). These results suggest that MAP kinase may not participate in the process of apoptosis induced by Dol-P. Dohi et al. employed herbimycin A at a relatively high concentration as an inhibitor of signal transduction. We show that herbimycin A alone at concentrations of more than 50 μM completely inhibited the color development in WST-1 assay in U937 cells (Fig. 6B) and also inhibited succinate ubiquinone reductase activity of respiratory complex II (Fig. 6C). The WST-1 assay is the method of choice for detection of mitochondrial dehydrogenase activities [Toimela and Tahti, 2004] such as succinate dehydrogenase activity, which is a partial activity of succinate ubiquinone reductase of complex II, as well as for detection of cell proliferation [Miyadera et al., 2003]. It is likely that our results of WST-1 assay reflect the inhibitory effects of herbimycin A at the high concentration on the electron transfer from succinate to WST-1 via 1-methoxy PMS, because no decrease of cell number was found after 30 min of treatment of U937 cells with herbimycin A (data not shown). No evidence was found to suggest that herbimycin A protected cells from Dol-P-induced apoptosis.

A role of p53 in mediating apoptosis was ruled out by the fact that U937 cells express a non-functional mutant p53 [Naujokat et al., 2000]. We confirmed that 43 bp of exon 5 of the p53 gene is deleted at the C-terminal (730-772) in the U937 cells that we used (data not shown).

The mechanism by which Dol-P inhibits activities of respiratory complexes I, II, and III is not known. As described in the Results, dolichol has a much lower ability to induce apoptosis [Yasugi et al., 1995, 1998] and had little inhibitory effect on the activities of complexes I, II, III, or IV at the same concentrations as Dol-P (Fig. 2). These facts may exclude the possibility that the lipid moiety plays an important role in the inhibition.

This study shows that Dol-P causes apoptosis in p53-defective leukemia U937 cells as a consequence of generation of ROS accompanying a decrease in the $\Delta\psi_m$. To our knowledge, this is the first demonstration that Dol-P inhibits the activity of mitochondrial respiratory complexes. These results suggest that the leakage of electrons and subsequent generation of ROS due to the uncoupling of electron flow of the respiratory complexes may play an important role in this form of apoptosis.

ACKNOWLEDGMENTS

We thank Ms. Junko Ikemoto, Department of Transfusion Medicine, Hyogo College of Medicine, for her kind support in the monitoring of ROS generation using flow cytometry. We also thank Dr. Mitsutaka Yoshida, Division of Electron Microscopy, Central Laboratory of Medical Science, Juntendo University for his help with electron microscopy.

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