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Activation of Mitogen Activated Protein Kinase in Dolichyl Phosphate-Induced Apoptosis in U937 Cells

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Exogenous dolichyl phosphate (Dol-P) induced apoptosis in the human monoblastic leukemia cell line U937 within 4 hours. Phosphorylation of p42 mitogen-activated protein kinase (MAP kinase) increased prior to DNA fragmentation. MAP kinase activation occurred within 5 min, and the maximum response was observed at 30 min. Inhibition of tyrosine phosphorylation of MAP kinase by herbimycin A resulted in complete inhibition of DNA fragmentation and partial inhibition of cell death. These results suggested that Dol-P-induced apoptosis is mediated by the MAP kinase cascade.

Dolichol and dolichyl phosphate (Dol-P) are long-chain polyisoprenoids that function as intermediates in protein glycosylation (1). Recent investigations in our laboratory demonstrated that dolichyl phosphate is a potent inducer of apoptosis in rat glioma C6 cells (2). To investigate the mechanism of induction of apoptosis, we examined the activation of mitogen activated protein kinases (MAP kinases). These kinases are important intermediates in signal transduction pathways that are initiated by many types of cell surface receptors and mediate both mitogenic, differentiation and stress responses in various cell types. MAP kinases are activated by dual tyrosine and threonine phosphorylation. Here, we demonstrate that the Dol-P induced apoptosis in U937 cells was associated with activation of MAP kinase, and inhibition of tyrosine phosphorylation of MAP by herbimycin A resulted in inhibition of DNA fragmentation.

MATERIALS AND METHODS

Materials. Dol-P was provided from the Tsukuba Research Laboratories of Eisai Company. Herbimycin A was purchased form Wako Chemicals (Tokyo, Japan). Dol-P was dissolved in ethanol/ dodecane (98:2), and herbimycin A was dissolved in demethylsulfoxide. Anti phospho-MAP kinase antibody and anti ERK2 antibody were purchased from New England Biolabs, Inc. (Beverly, MA, USA) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), respectively. Cell Counting Kit, which contains sulfonated tetrazolium salt that produces a highly water soluble formazan dye by living cells (WST reagent), was purchased from Dojindo (Kumamoto, Japan).

Cell lines and culture. The human monoblastic leukemia cell line U937 was grown in RPMI 1640 supplemented with 10 % fetal calf serum (FCS). Cells were cultured in RPMI 1640 supplemented with 0.5% FCS for 24h before each experiment. Treatment with Dol-P and herbimycin A was done in the presence of 0.5 % FCS.

Analysis of DNA fragmentation. 5×10^5 cells/well were placed in each well of a 24-well plate in 0.4 ml RPMI 1640 containing 0.5 % FCS. After incubation with reagents, cells were pelleted by centrifugation. One hundred μl of lysis buffer containing 10 mM Tris-HCl (pH 7.4), 10 mM EDTA and 0.5 % Triton X-100 was added to the pellet and the preparation was left at 4°C for 20 min. After centrifugation at 10,000 $\times$ g for 10 min at 4°C, the supernatant was incubated at 37°C with 40 μg of RNase A (Sigma) for 30 min, followed by additional incubation for 30 min with 40 μg of pronase K (Wako). Then fragments of DNA was precipitated with 20 μl of 5 M NaCl and 120 μl of 2-propanol and left overnight at -20°C. After centrifugation for 15 min, DNA was analyzed using 1.7 % agarose gel electrophoresis.

Detection of activated MAP kinase. 1×10^7 cells were treated with reagents in 0.4 ml RPMI1640 containing 0.5

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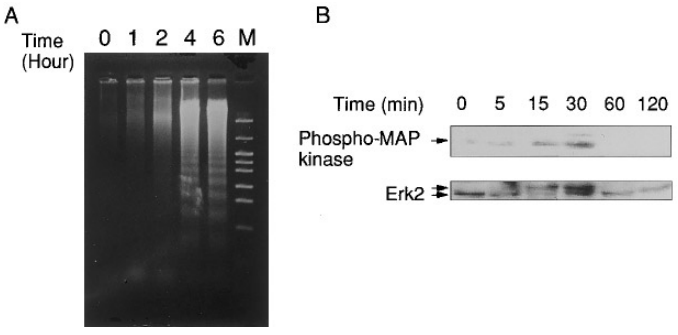

FIG. 1. Time course of Dol-P-induced DNA fragmentation and activation of MAP kinase in U937 cells. (A) Cells were treated with 8 μg/ml of Dol-P, and DNA fragmentation was analyzed. Lane M, PHY markers. (B) Cells were treated with 8 μg/ml of Dol-P for the indicated time, and cellular lysate was analyzed by SDS-PAGE and immunoblotting with anti phospho-MAP kinase antibody and anti ERK2 antibody.

% FCS. After the indicated time, the cells were pelleted, frozen in liquid nitrogen, and extracted with 30 μl of buffer containing 50 mM HEPES pH 7.4, 1% Triton X-100, 2 mM Na_3VO_3, 100 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. After leaving for 1h in ice and centrifugation at 10,000 $\times$ g for 10 min, the supernatant was denatured by heating at 95°C for 5 min with 30 μl of 2 time-concentrated Laemmli's sample buffer (3), and 25 μl/lane was applied on 10 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by electrotransfer onto a PVDF membrane. The membrane was blocked overnight with 10 mM Tris buffered saline, pH 8.0 containing 3 % milk and 1 % bovine serum albumin , then incubated with an antibody. The bound antibody was detected with a biotinylated secondary antibody and the avidin-biotin complex (Vector, Burlingame), using ECL kits (Amersham, Buckinghamshire, England). The chemiluminescent reaction on the membrane was analyzed using a luminescense analyzing system equipped with a CCD camera and quantified with a densitometry system (ATTO, Tokyo, Japan).

Analysis of cell viability. 1×10^4 cells/well were placed in a 96 well-plate in 0.1 ml RPMI1640 containing 0.5 % FCS, incubated with reagents for 4 h, then 10 μl of WST reagent of Cell Counting Kit was added. After 2h-incubation in a CO_2 incubator, absorbance at 450 nm was measured. Cell viability was determined by percentage absorbance of non-treated cells.

RESULTS

Figure 1A shows that DNA fragmentation in U937 cells began at 2 h and was prominent at 4 h after adding Dol-P to the culture medium. Figure 1B shows that phosphorylated MAP kinase was detected at 5 min, reached maximum in 30 min, and disappeared in 60 min. As phosphorylation of MAP kinase could also be determined by the mobility shift in SDS-PAGE, the result was confirmed by the appearance of two bands stained with anti ERK2 antibody at 15 and 30 min. The MAP kinase activation occurred prior to DNA fragmentation observed at 2 h as shown in Fig. 1A. Figure 2 shows that the DNA fragmentation was inhibited in the presence of by herbimycin A in a dose-dependent manner. For complete inhibition of DNA fragmentation 40 μM or more of herbimycin A was required. Figure 3 shows that 40 μM herbimycin A, blocked MAP kinase phosphorylation, and Fig. 4 shows that the Dol-P-induced cytotoxicity was inhibited by herbimycin A, in a dose-dependent manner. Herbimycin A itself showed no cytotoxicity at the concentrations used.

DISCUSSION

We earlier reported that Dol-P induced apoptosis in a rat glioma cell (2). Other polyprenylalcohols structurally related to Dol-P, such as geranylgeraniol (4) and farnesol (5) were also reported to induce apoptosis in human leukemic cell lines. The mechanism of this effect remained to be determined. The objective of our study was to determine the signal pathway which transduces the Dol-P-induced apoptosis. We obtained evidence that Dol-P induced

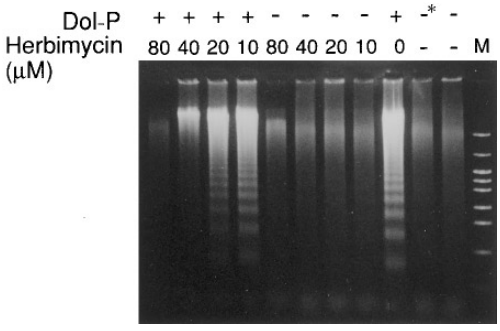

FIG. 2. Inhibition of Dol-P-induced DNA fragmentation by herbimycin A in U937 cells. Cells were treated for 4 h with various concentrations of herbimycin A with/without 8 μg/ml of Dol-P, and DNA fragmentation was analyzed. Lane M, PHY markers; lane*, cells were incubated with 3.3 μl of ethanol:dodecane (98:2) and 1.6 μl of demethylsulfoxide vehicle.

phosphorylation of the 42-kDa protein detected by anti-phospho MAP kinase antibody and anti ERK2 antibody which recognizes 42 kDa protein of MAP kinase. MAP kinase activation was time-dependent and occurred prior to DNA fragmentation, as shown in Figs. 1A and 1B. There was not significant phosphorylation of 44 kDa MAP kinase when the anti ERK1 antibody was used (data not shown). To assess the relation between MAP kinase activation and DNA fragmentaion, we examined the effect of herbimycin A, which is an inhibitor of tyrosine phosphorylation of protein. As shown in Figs. 2 and 3, herbimycin A inhibited DNA fragmentation, in a dose-dependent manner and with a concomitant inhibition of the MAP kinase phosphorylation shown in Fig. 3. These results indicate that MAP kinase pathway is significantly associated with the apoptosis in U937 cells. It has been reported that MAP kinase is activated in cases of cell proliferation and differentiation. Seimiya *et al.* observed MAP kinase activation in phorbol ester-induced differentiation of U937 cells (6). We speculate that the Dol-P-induced apoptosis is not related to cell proliferation or to the cell cycle in U937 cells. Cell cycle arrest in culture with FCS-depleted medium or treatment with hydroxyurea or bisbenzimide fluorochrome did not prevent Dol-P-induced DNA fragmentation (Dohi *et al.*, unpublished data). In HL60, a human myeloid cell line HL-60, the MAP kinase pathway is also activated by exogenous ceramide (7) or ceramide related signaling processes (8). The

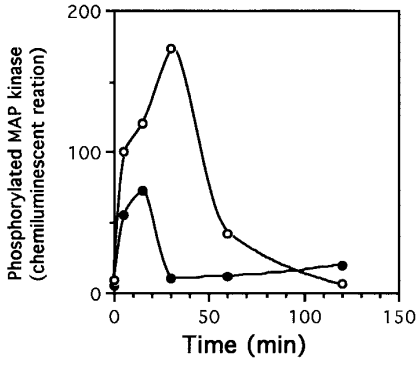

FIG. 3. Inhibition of Dol-P-induced MAP kinase activation by herbimycin A. U937 cells were treated with 8 μg/ml of Dol-P with (●) or without (○) 40 μM herbimycin A. Phosphorylated MAP kinase was detected by SDS-PAGE and immunoblotting with anti phospho-MAP kinase antibody as in Fig. 1B and quantified with densitometry. Representative data from three independent experiments are shown.

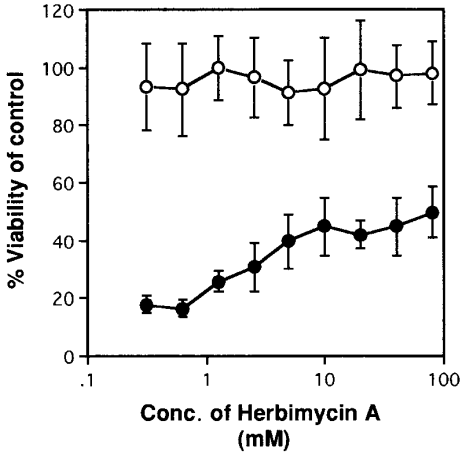

FIG. 4. Inhibition of Dol-P induced cell death with herbimycin A. U937 cells were incubated for 4 h with various concentrations of herbimycin A with (●) or without (○) 8 μg/ml of Dol-P. Cell viability was determined using Cell Counting Kits as described under Materials and Methods.

ceramide-related signaling pathway is reported to be involved in the apoptosis of HL-60 cells (9). Ceramide was found to be a potent inducer of apoptosis in U937 cells (10). Thus, the MAP kinase cascade is probably involved in the apoptosis induced by ceramide. In view of the activation of MAP kinase, the apoptotic signal by Dol-P may share the ceramide pathway. To examine the relation of ceramide pathway and the Dol-P-induced apoptosis, an analysis of sphingomyelin metabolism in Dol-P treated cells is now under investigation. Ji *et al.* reported that the DNA fragmentation induced by TNFα or ceramide in U937 cells was inhibited by herbimycin A (11). Lee *et al.* reported that herbimycin A inhibited dexamethasone-induced DNA fragmentation in rat thymocytes (12). Although they did not discuss the MAP kinase phosphorylation, all these and our results do indicate that tyrosine phosphorylation affected by herbimycin A is commonly involved in apoptosis in U937 cells induced by dexamethasone, TNFα, ceramide and Dol-P.

Figure 4 shows that herbimycin A itself had no cytotoxic effects and inhibited the Dol-P-induced cell death. However, the concentration of herbimycin A, high enough to block DNA fragmentation completely, as shown in Fig 2 only partially prevented cell death. In some other cell lines Dol-P was cytotoxic without DNA fragmentation (Dohi *et al.*, unpublished data). Apoptotic cells and non-apoptotic dead cells among Dol-P-treated U937 cells can not be differentiated using an ordinary microscope. The mechanism of Dol-P-induced cytotoxicity may not be a simple signal transduction, and it may depend on cell types. Further investigation on biological effect of Dol-P is required.

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