Dipalmitoleoyl-phosphatidylethanolamine Induces Apoptosis of NCI-H28 Malignant Mesothelioma Cells

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Abstract. Background/Aim: The phospholipid phosphatidylethanolamine regulates a wide range of cellular processes. The present study investigated the antitumor effect of 1,2-dipalmitoleoyl-sn-glycero-3-phosphoethanolamine (DPPE) on malignant pleural mesothelioma cells. Materials and Methods: Activities of protein phosphatases (PPs) such as PP1, PP2A, and protein tyrosine phosphatase IB (PTP1B) were assayed under cell-free conditions. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining, and western blotting were carried out on the human Met5A non-malignant mesothelial cell line and NCI-H28 malignant mesothelioma cell line. Results: DPPE significantly enhanced PP2A and PTP1B activities. DPPE tended to attenuate activity of extracellular signal-regulated kinase-1 (ERK1)/ERK2, with the greater efficacy for NCI-H28 cells than that for Met5A cells. DPPE reduced NCI-H28 cell viability in a concentration (1-100 μM)-dependent manner, while it had no effect on Met5A cell viability. DPPE markedly increased TUNEL-positive cells in the NCI-H28 cell line, but otherwise induced few TUNEL-positive cells in the Met5A cell line. Conclusion: The results of the present study clearly demonstrate that DPPE induces apoptosis of NCI-H28 malignant pleural mesothelioma cells. DPPE-induced enhancement of PP2A and PTP1B activities might at least in part contribute to the apoptotic effect of DPPE.

Malignant mesothelioma is an aggressive form of cancer that arises from mesothelial cells and may develop in the pleural space, pericardium, peritoneum, tunica vaginalis testis and ovarian epithelium (1). This type of cancer is often known to be resistant to chemotherapy. The median survival period of patients diagnosed with malignant mesothelioma is usually less than a year. Some factors have been associated with the development of malignant mesothelioma, which include asbestos, erionite, and simian virus 40 (SV40). Moreover, several genes are involved in the pathogenesis of this cancer: p16INK4A, p14ARF, and neurofibromatosis type 2 (NF2). The genes tumor protein 53 (TP53) and phosphatase and tensin homolog deleted from chromosome 10 (PTEN) are still under investigation for their role in the development of malignant mesothelioma. In spite of extensive and intensive studies, no beneficial therapy or drug for malignant mesothelioma has been established and challenges for such development are continuing.

Receptor tyrosine kinase (RTK) is implicated in the activation of extracellular signal-regulated kinase 1/2 (ERK1/2), a mitogen-activated protein (MAP) kinase (MAPK), as one of the major signaling pathways (Figure 1). When activated, RTK phosphorylates its own receptor or Src-homology and collagen homology-2 (SHC2). Phosphorylated SHC2 recruits the adaptor protein growth factor binding protein-2 (GRB2) and forms a complex with the exchanger factor son of sevenless (SOS); SHC2/GRB2/SOS, which activates the small G-protein RAS by exchanging GDP with GTP and activated RAS activates the effector RAF, a serine/threonine protein kinase. Activated RAF phosphorylates and activates MAP kinase kinase (MAPKK=MEK), which in turn, phosphorylate and activate MAPK (ERK1/2). ERK1/2 is recognized to promote cancer cell growth and protect cells from apoptosis. In this pathway, protein tyrosine phosphatase IB (PTP1B), a tyrosine phosphatase, de-phosphorylates phosphorylated RTK and SHC2; in other words, PTP1B down-regulates RTK signaling (Figure 1). Protein phosphatase-2A (PP2A), a serine/threonine phosphatase, de-phosphorylates phosphorylation of MAPKK, MAPK, and ERK1/2; in other words, PP2A inhibits ERK1/2 activation (Figure 1).

In our preliminary study, we have found that phosphatidylethanolamines (PEs) such as 1,2-diarachidonoyl-sn-glycero-3-phosphoethanolamine (DAPE), 1,2-dilinoleoyl-sn-
glycero-3-phosphoethanolamine (DLPE), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), and 1,2-dipalmitoleoyl-sn-glycero-3-phosphoethanolamine (DPPE) enhance PP2A and PTP1B activities, with the highest potential for DPPE. Then, we postulated that DPPE could exhibit an anti-tumor effect by suppressing MEK and ERK1/2 activities in association with PP2A and PTP1B activation. To address this hypothesis, the present study investigated the anti-tumor effect of DPPE on human malignant pleural mesothelioma cell lines.

Materials and Methods

Assay of protein phosphatase activities. Activities of protein phosphatases PP1, PP2A, and PTP1B under cell-free conditions were assayed as previously described (2). Human recombinant PP1 was purchased from New England BioLabs Inc. (Ipswich, MA, USA) and human recombinant PP2A from Millipore (Billerica, MA, USA). Human PTP1B was cloned into pGEX-6P-3 vector with a glutathione S-transferase (GST) tag at the NH2 terminus, and expressed in competent Escherichia coli BL21 (DE3), suitable for transformation and protein expression. GST-fusion PTP1B was affinity-purified using glutathione sepharose 4B (GE Healthcare, Piscataway, NJ, USA). Activity of each phosphatase was assayed by reacting with p-nitrophenyl phosphate (pNPP)(Sigma-Aldrich, St. Louis, MO, USA) as a substrate. PP1 (1 U/well), PP2A (0.2 U/well), or PTP1B (1 μg/well) was pre-incubated at 30˚C (for PP1) or 37˚C (for PP2A and PTP1B) for 30 min in a reaction medium [50 mM HEPES, 100 mM NaCl, 2 mM dithiothreitol, 0.01% (v/v) Brij-35, 1 mM MnCl2, pH 7.5 for PP1; 50 mM Tris-HCl, 0.1 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, pH 7.0 for PP2A; and 50 mM HEPES, 1 mM EDTA, 50 mM NaCl, 1 mM dithiothreitol, pH 7.2 for PTP1B] in the presence and absence of phosphatase inhibitors or DPPE. pNPP at a concentration of 5 mM for PP1, 0.5 mM for PP2A, and 10 mM for PTP1B was then added to the reaction medium followed by 60-min incubation, and the reaction was terminated by adding 0.1 N of NaOH. De-phosphorylated pNPP was quantified at an absorbance of 405 nm with a SpectraMax PLUS384 (Molecular Devices, Sunnyvale, CA, USA).

Cell culture. NCI-H28 cell, a human malignant pleural mesothelioma cell line, and Met5A, a human non-malignant mesothelial cell line, were purchased from the American Type Culture Collection (Manassas, VA, USA), and cultured by the method previously described (3). Briefly, cells were grown in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 0.003% (w/v) L-glutamine, penicillin (final concentration, 100 U/ml), and streptomycin (final concentration, 0.1 mg/ml), in a humidified atmosphere of 5% CO2 and 95% air at 37˚C.

Cell viability assay. Cell viability was evaluated by the method using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) as previously described (4).

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining. TUNEL staining was performed to detect in situ DNA fragmentation as a marker of apoptosis using an In Situ Apoptosis Detection Kit (Takara Bio, Otsu, Japan). Briefly, fixed and permeabilized cells were reacted with terminal deoxynucleotidyl transferase and fluorescein isothiocyanate (FITC)-deoxyuridine triphosphate for 90 min at 37˚C. FITC signals were visualized with a confocal scanning laser microscope (LSM 510; Carl Zeiss Co., Ltd., Oberkochen, Germany).

Western blotting. Cells were treated or not with DPPE, and then lysed in a lysis solution [150 mM NaCl, 20 mM Tris, 0.1% (v/v) Tween-20 and 0.1% (w/v) sodium dodecyl sulfate (SDS), pH 7.5] containing 1% (v/v) protease inhibitor cocktail and 1% (v/v) phosphatase inhibitor cocktail. The lysates were centrifuged at 3,000 rotation per minute (rpm) for 5 min at 4˚C. Proteins were separated by SDS polyacrylamide gel electrophoresis (PAGE) using a TGX gel (BioRad, Hercules, CA, USA) and then transferred to polyvinylidene difluoride (PVDF) membranes. Blotted membranes were blocked with TBS-T [150 mM NaCl, 0.1% (v/v) Tween-20 and 20 mM Tris, pH 7.5] containing 5% (w/v) bovine serum albumin and subsequently reacted with antibodies against phospho-MEK (pMEK) (Cell Signaling Technology, Inc., Danvers, MA, USA), MEK (Cell Signaling Technology), phospho-ERK1/2 (pERK1/2)(Santa Cruz Biotechnology), Santa Cruz, CA, USA), and ERK (Santa Cruz Biotechnology). After
washing, membranes were reacted with a horseradish peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG antibody. Immunoreactivity was detected with an ECL kit (Invitrogen, Carlsbad, CA, USA) and visualized using a chemiluminescence LAS-4000 mini detection system (GE Healthcare). Protein concentrations for each sample were determined with a BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA).

Statistical analysis. Statistical analysis was carried out using Dunnett’s test, and \( p<0.01 \) was regarded as significance.

Results

DPPE enhances PP2A and PTP1B activities. We initially examined the effects of DPPE (100 μM) on protein phosphatases. In the PP1 assay, calyculin A (20 nM), an inhibitor of PP1, clearly reduced PP1 activity (Figure 1A), confirming a reliable PP1 assay. No significant effect on PP1 activity was obtained with DPPE (Figure 2A).

In the PP2A assay, okadaic acid (2 nM), an inhibitor of PP2A, clearly reduced PP2A activity (Figure 2B), confirming a reliable PP2A assay. DPPE significantly enhanced PP2A activity, reaching nearly twice that of the control levels (Figure 2B).

In the PTP1B assay, sodium orthovanadate (Na₃VO₄) (1 μM), an inhibitor of PTP1B, clearly attenuated PTP1B activity (Figure 2C), confirming a reliable PTP1B assay. DPPE significantly enhanced PTP1B activity, reaching approximately 1.6 fold that of the control levels (Figure 2C). DPPE tends to attenuate ERK1/2 activity in NCI-H28 cells. If DPPE enhances PP2A and PTP1B activities, then the lipid should suppress activities of MEK and ERK1/2. To address whether DPPE affects protein phosphatase activities, we monitored phosphorylation of MEK and ERK1/2 in Met5A non-malignant mesothelial cells and NCI-H28 malignant mesothelioma cells.

DPPE (100 μM) reduced phosphorylation of MEK in a treatment time (10-60 min)-dependent manner in Met5A cells, but phosphorylation of ERK1/2 was little affected (Figure 3A). DPPE (100 μM) had little effect on phosphorylation of MEK, but reduced phosphorylation of ERK1/2 in a treatment time (10-60 min)-dependent manner in NCI-H28 cells (Figure 3B). The potential for the inhibitory effect of DPPE on phosphorylation of MEK and ERK1/2, however, was much lower than expected and not significant.

DPPE induces apoptosis of NCI-H28 cells. In the MTT assay, treatment with DPPE for 24-48 h reduced NCI-H28 cell viability in a concentration (1-100 μM)-dependent manner, but had no effect on Met5A cell viability (Figure 4). This indicates that DPPE induces cell death in NCI-H28 malignant mesothelioma cells, but not Met5A non-malignant mesothelial cells.

To determine whether the effect of DPPE was due to apoptosis, we finally carried out TUNEL staining. DPPE (30 and 100 μM) significantly increased the number of TUNEL-positive cells as compared with those for untreated control NCI-H28 cells (Figure 5). DPPE at a concentration of 100 μM significantly increased the number of TUNEL-positive Met5A cells, but to a much lesser extent than that for NCI-H28 cells (Figure 5). Taken together, these results indicate that DPPE induces apoptosis of NCI-H28 malignant mesothelioma cells rather than Met5A non-malignant mesothelial cells.
Discussion

The phospholipid PE is the most abundant lipid in the cytoplasmic layer of cellular membranes and PE is implicated in a wide range of cellular processes such as membrane fusion, cell cycle, autophagy, apoptosis, and cognitive function (5-9).

PE is produced through three main pathways: the cytidine 5'-diphosphate (CDP)-ethanolamine Kennedy pathway, mitochondrial phosphatidylserine (PS) decarboxylation pathway catalyzed by PS decarboxylase, and acylation of lysoPE catalyzed by lysophosphatidylethanolamine acyltransferase. The CDP-ethanolamine Kennedy pathway is the only route for de novo synthesis of PE (10).
Phosphorylation of ethanolamine by ethanolamine kinase is followed by the CTP:choline cytidylyltransferase 2 (PCyt2)-mediated production of CDP-ethanolamine, and PE production is catalyzed by CDP-ethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase. The analogous enzymes of the CDP-choline branch of the Kennedy pathway include choline kinase, PCyt1, and CDP-choline:1,2-diacylglycerol choline phosphotransferase. In the liver, PE is transformed into phosphatidylcholine (PC) catalyzed by PE N-methyltransferase. PE is also produced in mitochondria by PS decarboxylase-catalyzed decarboxylation of PS. Mammals do not synthesize PS de novo, and therefore, PS is produced by head-group exchange from PE catalyzed by PS synthase-2 or PC catalyzed by PS synthase-1. PE, on the other hand, is produced by lysoPE acyltransferase-catalyzed fatty acid esterification of lysoPE.
In the present study, DPPE, a PE, enhanced PP2A and PTP1B activities. To our knowledge, this is the first to show the new action of PE on protein phosphatases. How DPPE enhances PP2A and PTP1B activities, however, remains to be determined. Activation of ERK, a MAPK, leads to promotion of cell growth and proliferation not only of normal cells but also of cancer cells. Activation of ERK1/2 through a pathway along the RTK/(SHC2/GRB2/SOS)/RAS/RAF/MAPKK/MAPK axis is initiated by tyrosine phosphorylation of RTK and SHC2 (Figure 1). PTP1B dephosphorylates RTK, SHC2, MAPKK, and MAPK, thereby negatively-regulating RTK signaling (Figure 1). PP2A dephosphorylates and inactivates, MAPKK, and MAPK (Figure 1). Accordingly, DPPE, in order to enhance PP2A and PTP1B activities, should attenuate RTK signaling and inhibit ERK activation. Indeed, DPPE tended to reduce pMEK in Met5A cells and pERK1/2 in NCI-H28 cells at 60-min treatment, but the potential was much lower than expected.

Strikingly, DPPE clearly reduced cell viability and markedly increased TUNEL-positive NCI-H28 malignant mesothelioma cells. In contrast, DPPE had little effect on cell viability and the number of TUNEL-positive cells for Met5A non-malignant mesothelial cells. DPPE, thus, appears to preferentially induce apoptosis of NCI-H28 cells, but not of Met5A cells. This raises the possibility that DPPE could be developed as an anticancer drug for treatment of malignant mesothelioma.

**Conclusion**

The results of the present study demonstrate that DPPE preferentially induces apoptosis of NCI-H28 malignant mesothelioma cells rather than Met5A non-malignant mesothelial cells. DPPE enhanced PP2A and PTP1B activities in a cell-free system, and this action might, at least in part, contribute to DPPE-induced apoptosis of malignant mesothelioma cells.

**Conflicts of Interest**

None of the Authors have any potential conflict of interest.