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Overexpression of phospholipase D suppresses taxotere-induced cell death in stomach cancer cells

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Abstract

Phospholipase D (PLD) catalyzes the hydrolysis of phosphatidylcholine to generate phosphatidic acid (PA) and choline. There are at least two PLD isozymes, PLD1 and PLD2. Genetic and pharmacological approaches implicate both PLD isozymes in a diverse range of cellular processes, including receptor signaling, membrane transport control, and actin cytoskeleton reorganization. Several recent studies reported that PLD has a role in signaling pathways that oppose apoptosis and promote cell survival in cancer. In this study, we examined the role of PLD in taxotere-induced apoptosis in stomach cell lines; normal stomach (NSC) and stomach cancer cells (SNU 484). Taxotere treatment resulted in increase of PLD activity. To confirm the role of PLD in taxotere-induced apoptosis, PLDs were transfected into SNU 484 cells. Overexpression of PLD isozymes resulted in inhibition of taxotere-induced apoptotic cell death, evidenced by decreased degradation of chromosomal DNA, and increased cell viability. Concurrently, Bcl-2 expression was upregulated, and taxotere-induced activation of procaspase 3 was inhibited after PLD's transfection. However, when PLD was selectively inhibited by specific siRNA-PLD1 or -PLD2, taxotere-induced apoptosis was exacerbated in SNU 484 cells. On top of this, PA—the product of PLDs, also resulted in upregulation of Bcl-2 in SNU 484. Although PA-induced Bcl-2 expression was blocked by mepacrine, an inhibitor of phospholipase A_2 (PLA_2), increased Bcl-2 expression by PA was not abrogated by propranolol, an inhibitor of PA phospholyhydrolase (PAP). Taken together, PLD1 and PLD2 are closely related with Bcl-2 expression together with PLA_2, but not with PAP, during taxotere-induced apoptosis in SNU 484 cells.

Keywords: Phospholipase D; Apoptosis; Bcl-2; Phosphatidic acid; SNU 484 cell

1. Introduction

Stomach cancer is the second most common cancer in the world [1]. More than 50% of patients develop metastatic cancer, which for the majority is beyond cure and, despite many advances that have been achieved in the management of gastric cancer over the past 15 years, patient prognosis remains very poor. The need for the development of more effective therapies that are likely to further improve survival time cannot be overemphasized. Recently, a number of newer compounds in this tumor type have been intensively studied. These mainly include taxotere (taxanes), topoisomerase I inhibitors and oral fluoropyrimidines. The therapeutic value of taxotere-based chemotherapy of gastric cancer is currently being widely investigated in several randomized trials as a single agent and in combination with other active agents [2].

In the present study, normal stomach cell lines (NSC), and poorly differentiated stomach cancer cell lines (SNU 484 [3]) were used for investigation of taxotere-induced apoptosis in vitro. Taxotere induces a number of other molecular events. For example, taxotere can induce apoptosis by disorganization of microtubule structure. The effect of this antimicrotubule

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action is believed to result in the inactivation of Bcl-2, one of the most potent inhibitors of apoptosis, induced by a wide variety of stimuli [4], through phosphorylation [5]. However the critical mechanism has not been well characterized yet. In addition to this signaling, Maestre and colleagues reported that phosphatidylcholine (PC)-derived phosphatidic acid (PA) by phospholipase D (PLD) and diacylglycerol (DAG) by phospholipase C (PLC) were involved in the signaling pathways activated by taxotere [6]. However, in the absence of a PLD-specific inhibitor, they could not well define whether PLD was involved in taxotere-induced apoptosis in human myeloblastic cell line U937. Recently, we also demonstrated that over-expressed PLD2 possibly enables the cells to survive against Fas-induced apoptosis by increasing Bcl-2 and Bcl-xL expression in A20 murine B lymphoma cells [7].

PLD is a ubiquitous enzyme which catalyzes the hydrolysis of PC to PA and choline. PLD has two isoforms, PLD1 and PLD2, and they differ in the mechanism of activation and subcellular localization [8,9]. PLD metabolism is implicated in various physiological processes [10–12], including membrane trafficking, cytoskeletal reorganization, cell proliferation [13], differentiation, survival, and apoptosis [14]. In numerous human cancers, both expression and activity of PLD are aberrantly increased [15,16]. PA can be generated by two pathways; first, the hydrolysis of PC by PLD [17], and second, from diacylglycerol (DAG) by the action of diacylglycerol kinase (DGK) [17]. Recently, it has been reported that PLD plays an anti-apoptotic role in many cells and tissues, such as PC 12 cells, human neutrophils, v-Src-transformed rat fibroblasts, and MDA-MB-231 breast cancer cells [15,18–21]. Furthermore, PLD activity is important to gastric cancer promotion in vivo [22].

Collectively, these observations suggest that PLD plays a critical role in taxotere-induced apoptosis in gastric cancer cells, and they also indicate that PLD or molecules involved in PLD signaling could possibly be valuable targets for therapeutic intervention in cancers, where a substantial fraction of tumor cells apparently have elevated PLD activity. Here, we show that PLD and its product PA have anti-apoptotic activity through elevation and retention of Bcl-2 expression against taxotere-induced apoptosis.

2. Materials and methods

2.1. Materials

Taxotere® (taxane; docetaxel) was obtained from Aventis Pharmaceuticals (Collegeville, PA, USA) and was prepared as 100 mM stock solution in absolute ethanol. The monoclonal anti-caspase 3 antibody, monoclonal anti-Bcl-2 and polyclonal anti-cleaved-PARP were obtained from Cell Signaling Technology (Beverly, MA, USA). Polyclonal anti-PLDs were kindly donated by Dr. Do-Sik Min (Pusan National University, Korea), and HRP-labeled anti-mouse IgG, HRP-labeled anti-rabbit IgG and PVDF membrane were purchased from Bio-Rad Laboratories (Hercules, CA, USA). MTT was purchased from Duchefa (Haarlem, The Netherlands). Acridine orange, propranolol, and mepacrine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Phosphatidylethanol (Pet) standard and PA obtained from Avanti Polar Lipids (Alabaster, AL, USA) were dissolved in chloroform. [^{3}H] palmitic acid was from Amersham Pharmacia Biotech (Buckinghamshire, England).

2.2. Cell culture

Poorly differentiated stomach cancer cells, SNU 484 cells, obtained from Seoul National Cell Bank were cultured at 37 °C in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Normal stomach cells (NSC; originated from an autoimmune deficient fetus; ATCC CRL7407) were cultured at 37 °C in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells grown at 37 °C in 5% CO_2 atmosphere were then washed with RPMI 1640 or DMEM containing 0.1% (w/v) bovine serum albumin (BSA), 100 U/ml penicillin and 100 μg/ml streptomycin (serum-free medium) and incubated in the serum-free medium for 18 h at 37 °C before stimulation with agonists.

2.3. PLD's retroviral vector construction and virus production

To construct retroviral vectors, the rat PLD1 cDNA (rPLD1) and human PLD2 cDNA (hPLD2) were amplified with the following primers; rPLD1, 5′-CGGTGGCGTTTGTGGGTGGG-3′ (forward) and 5′-GTCCTTGAAGA-CAAAGTTGC-3′ (reverse); hPLD2, 5′-ATGACGGCGACCCCTG-3′ (forward) and 5′-CTATGTCCACACTTCTA-3′ (reverse). High fidelity taq polymerase (Gibco/BRL, USA) was used with 30 cycles of amplification, including denaturation for 30 s at 94 °C, annealing for 1 min at 55 °C, and extension for 3 min at 68 °C. The PLD PCR products were cloned into the pGEM T-Easy TA cloning vector, digested with *Not*I restriction enzymes and cloned into corresponding sites in the IRES-BsdEGFP-CL retroviral vector (PLDs-BsdEGFP-CLBC3). PLDs-BsdEGFP-CLB3 plasmids were then introduced into 293GPG retrovirus packaging cells [23] by transient transfection with Lipofectamine (Gibco/BRL, USA). After 72 h, the supernatants were harvested and stored at −70 °C until use.

2.4. Stable transfection of PLDs

Stomach cells were plated on 6-well plates, and the filtered retroviral supernatants of high-titer cloned packaging cell lines were added to the plates for 4 h in the presence of polybrene (1 μg/ml), and the plate was incubated for 2 days. Successful transfection was confirmed by fluorescence microscopy, and transfected cells were selected by adding 10 μg of blasticidin into the culture medium to make stable cell lines. After forming single colonies in the presence of blasticidin, all the cells were pooled to make a stable cell mixture, and they were then maintained in the presence of 3 μg/ml blasticidin, which was withdrawn 1 week before experiments. The expression of PLDs in stably transfected cells was confirmed by both western blot/RT-PCR and PLD activity assay.

2.5. RT-PCR

Total RNA was isolated from SNU 484 cells, which were stably transfected with PLDs, using the Trizol reagent (Life Technologies, Inc., Rockville, MD, USA) according to the manufacturer's instructions. For the reverse transcriptase reaction, 5 μg of total RNA were mixed with oligo $(dT)_{16}$ primer and Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega, USA), and the mixture was incubated for 60 min at 42 °C. The transcribed products were mixed with each primer set and Taq DNA polymerase (Takara Shuzo, Japan), and then amplified according to the following amplification profiles: for rPLD1, 25 cycles including denaturation for 30 s at 94 °C, annealing for 1 min at 62 °C, and extension for 1 min at 72 °C ; for hPLD2, 30 cycles including denaturation for 30 s at 94 °C, annealing for 1 min at 60 °C, and extension for 1 min at 72 °C; for β-actin, 30 cycles including denaturation for 1 min at 95 °C, annealing for 1 min at 62 °C, and extension for 1.5 min at 72 °C. The primers used to amplify rPLD1 were 5′-ACTCTGTCCAAAGTTAACATGTCACTG-3′ (forward) and 5′-GGCTTTGTACTTGAGCAGCTCTCT-3′ (reverse). For hPLD2, the primers were 5′-TCCATCCAGGCCATTCTGCAC-3′ (forward) and 5′-CGTTGC-TCTCAGCCATGTCAAG-3′ (reverse). The primers for β-actin were 5′-AACACCCCAGCCATGTACG-3′ and 5′-ATGTCACGCACGATTTCCC-3′ (reverse). The PCR products were analyzed by 1% agarose-gel electrophoresis and ethidium bromide staining. The products of constitutively expressed β-actin

mRNA served as a control. All of the products were assayed in the linear range of the RT-PCR amplification process.

2.6. DNA fragmentation assay

After treatment with 10 μM taxotere for 24 h and 48 h, cells were collected and solubilized by vigorous vortexing in TE9S buffer (0.5 M EDTA, 10 mM NaCl, 10 mM Tris–HCl, pH 9, 1% (w/v) SDS) containing proteinase K (1 mg/ml). This was followed by 3 h of incubation at 50 °C and DNA extraction with phenol-chloroform. After incubation with RNase A (1 mg/ml) for 1 h at 20~22 °C, DNA samples were analyzed with conventional electrophoresis in 1.2% (w/v) agarose gels. The gels were visualized by a gel documentation system (Bio-Rad, USA).

2.7. PLD activity assay

PLD activity was determined as previously described, by measuring [^{3}H] phosphatidyl ethanol (PEt) produced via PLD-catalyzed transphosphatidylation in [^{3}H] palmitic acid-labeled cells [24]. Briefly, stomach cells (0.4×10^6 cells/well) cultured on 6-well plates were metabolically labeled with 1 μCi/ml of [^{3}H] palmitic acid in the serum-free medium for 18 h. The cells were then treated with 1% (v/v) ethanol for 15 min before stimulation with taxotere. After stimulation, the cells were quickly washed with ice-cold phosphate-buffered saline (PBS) and suspended in 0.5 ml of ice-cold methanol. Lipids were extracted according to the method of Bligh and Dyer [25], and PEt was separated from other phospholipids by TLC on silica gel 60 Å plates, using a solvent system of ethyl acetate/isooctane/acetic acid/water (110/50/20/100, v/v). The regions corresponding to the authentic PEt bands were identified with 0.02% (w/v) primulin in 80% (v/v) acetone and then scraped. Radioactivity was counted by a scintillation counter.

2.8. Western blot analysis

Serum-starved cells on 100-mm dishes (0.4×10^6 cells/ml) were incubated with 10 μM taxotere for indicated times, scraped in PBS, and harvested by microcentrifugation. The cells were then resuspended in 0.5 ml of lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NAF, 1 mM Na_3Vo_4, 1 mM PMSF, 1% Triton X-100, 0.5% NP-40, 10 μg/ml aprotinin, 10 μg/ml leupeptin) on ice and disrupted by sonication. Cell lysates were centrifuged for 10 min at 10,000 ×*g* at 4 °C. Proteins (40 μg of protein/lane) from control or treated cells were resolved by SDS-PAGE and transferred to nitrocellulose membranes using a Bio-Rad semi-dry transfer system. The membranes were blocked for 1 h with 5% (v/v) non-fat milk in TTBS (tris-buffered saline containing 0.1% Tween 20) and then incubated with diluted specific antibody (1 μg/ml) for an another hour. Unbound primary antibodies were removed by three washes (5 min each) with TTBS. The membranes were then incubated with horseradish peroxidase-coupled goat anti-rabbit or anti-mouse IgG (diluted 1:1000 with TTBS; KPL, Gaithersburg, MD, USA) for 1 h, followed by three washes (5 min each) in TTBS. Visualization of signals was achieved by using chemiluminescence (ECL reagent, Amersham Biosciences) according to the manufacturer's protocol. Protein concentrations were routinely determined by the Bradford procedure [26] with Bio-Rad dye reagent and BSA as a standard.

2.9. Detection of apoptosis nuclei

Stomach cells treated with 1 μM taxotere for 24 h and 48 h were stained with 100 ng/ml acridine orange (Sigma, USA) [27] and were immediately observed with a fluorescence microscope using filters for green fluorescence (Diaphot 300 attached with epifluoresecence: Nikon, Japan).

2.10. Measurement of phosphatidylserine (PS) exposure

PS exposure was measured by the binding of annexin V-fluorescein isothiocyanate (FITC) using a protocol outlined in the TACS apoptosis detection kit (Trevigen, The Netherlands). Cells (1×10^6) were first harvested and washed with PBS. They were then incubated for 15 min with annexin V-FITC and propidium iodide (PI). The cells (1×10^4) were subsequently analyzed with a flow cytometer (Becton Dickinson, NJ, USA). The combination of annexin V-FITC and PI allows differentiation between early apoptotic cells (annexin V-FITC-positive), late apoptotic and/or necrotic cells (annexin V-FITC- and PI-positive), and viable cells (unstained).

2.11. Cell viability

The viability assay was performed as previously described [28]. MTT was dissolved in PBS at 5 mg/ml. The stock solution was filtered through a Millipore filter (pore size, 0.22 μm) and added to the culture medium at a dilution of 1:10. The plates were incubated at 37 °C for 4 h. The dark brown formazan crystals formed after reduction of tetrazolium by the mitochondria of living cells were dissolved in dimethyl sulfoxide, and the optical densities of the samples were read at 570 nM.

2.12. Small interference RNA (siRNA) synthesis and transfection

A human PLD1 sequence (from 1454 to 1474; AAGGUGGGACGACAAUGAGCA) and a human PLD2 sequence that is completely conserved in mouse, rat and human species (from 434 to 452; GACACAAAGUCUUGAUGAG) were chosen for PLD1 and PLD2 RNAi targeting, respectively. This siRNA for PLD1 and PLD2 were purchased from Ambion (Austin, TX, USA). A BLAST search of all siRNA sequences revealed no significant homology to any other sequences in the database. The siRNA duplex were transiently transfected into SNU 484 cells (5×10^4 cells/well; 6-well plate) using the Lipofectin reagent. Forty-eight hours after transfection, the cells were harvested for RT-PCR or western blotting. A negative control was carried out with Neg-siRNA#2 (from Ambion), which is a 19-bp scrambled sequence with 3′ dT overhangs (sequence not disclosed by Ambion) certified not to have significant homology to any known gene sequences from mouse, rat, or human species and causing no significant changes in gene expression of transfected cells after 48 h at the same concentration as in the siRNA experiment.

2.13. Statistical analysis

All experiments were performed in triplicate, and results are expressed as mean value±SD. The significance of differences was assessed by an unpaired *t*-test.

3. Results

3.1. Apoptosis of stomach cancer cells (SNU 484 cells) induced by taxotere

We observed significant death of SNU 484 cells after treatment with taxotere (1 μM) for 24 h. Thus, after treatment of the cells with 1 μM taxotere for 12 h, 24 h, and 48 h, apoptotic nuclei were analyzed with fluorescence staining by acridine orange, and cell viability by the MTT assay. Our results showed that 1 μM taxotere induced significant apoptotic condensation of apoptotic nuclei, which are pyknotic fragmented, and those apoptotic nuclei were more prominent in SNU 484 cells than in NSC (Fig. 1A). As shown in Fig. 1B, taxotere induced apoptosis in SNU 484 cells more than NSC, showing that less than 40% of SNU 484 cells were viable after treatment with 1 μM taxotere for 48 h, whereas over 70% of NSC were alive. We also analyzed stomach cell apoptosis with flow cytometry and the recordings of the fluorescence histograms obtained by annexin V and PI. The fluorescence distribution of stomach cells indicated a single cell population before taxotere stimulation. After 24 h or 48 h of culture in the presence of 1 μM taxotere,

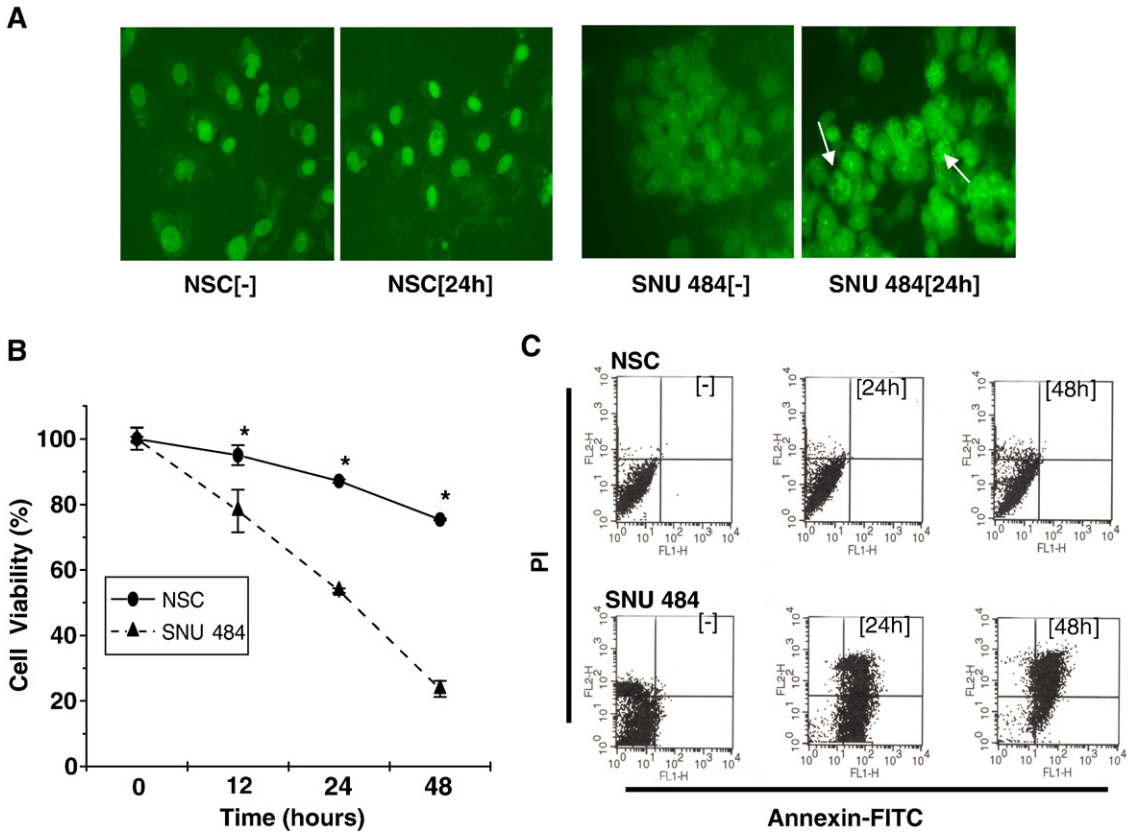

Fig. 1. Apoptosis induced by taxotere in SNU 484 stomach cancer cells. Panel A, fluorescence staining of apoptotic nuclei using acridine orange. Serum-starved cells were incubated with 1 μM taxotere for 24 h. Cells were stained with acridine orange to check the fluorescence staining of apoptotic nuclei in stomach cells. White solid arrows indicate apoptotic cells. Panel B, cell viability by MTT assay. Serum-starved cells on 96-well plates (1×10^4 cells/well) were incubated with 1 μM taxotere (IC_{50}) for the indicated times. After treatment, cell viability was checked by MTT assay as described under Materials and methods. Each point represents the mean of triplicate analyses with SD. *Differences between NSC and SNU 484 ($p<0.05$) were evaluated by the Student's *t*-test. Panel C, double staining of cells with FITC-labeled annexin V and PI. Stomach cells were treated with 1 μM taxotere for 24 h or 48 h. The cells were stained with PI and FITC-conjugated annexin V. Stained cells were analyzed by FACScan flow cytometry. A minimum of 10,000 events for each sample was collected in list mode.

however, two fluorescent populations were observed in SNU 484 cells, but not in NSC (Fig. 1C). These results indicate that normal and cancer cells have different signal pathways against taxotere treatment.

3.2. Effect of taxotere on Bcl-2 and PLD expression level in stomach cells

Expression levels of Bcl-2 and PLD's two isoforms (PLD1 and PLD2) with or without taxotere treatment were investigated in cultured stomach cells. As shown in Fig. 2, Bcl-2 protein concomitantly decreased over time when SNU 484 were treated with taxotere, but slightly decreased in NSC. Additionally, PLD1 expression level in NSC was much higher than that of SNU 484 cells. Moreover, while taxotere treatment could not suppress PLD1 expression in NSC, PLD1 expression in SNU 484 cells was slightly decreased by taxotere treatment. Apoptosis indicating proteins, such as caspase 3 and cleaved-PARP, were also investigated. It is known that taxotere induces apoptosis in several types of cells via caspase 3 activation, and that caspase 3 is synthesized as a 32-kDa inactive precursor that has to be proteolytically cleaved to become a mature enzyme which is composed of 17 and 12 kDa subunits [29,30]. Cleavage and activation of caspase 3 were observed by the disappearance of the 32 kDa procaspase protein band upon induction of apoptosis with taxotere in SNU 484 cells. The cleaved-PARP increased over time after taxotere treatment in SNU 484 cells, indicating an increasing rate of apoptotic cell death (Fig. 2). From these results, it seems that a low expression level of PLDs should exacerbate the taxotere-induced apoptosis signal pathway in SNU 484 cells relative to NSC; moreover, elevated PLD activity confers a survival signal in MCF7 cells [31].

3.3. Effect of PLD on taxotere-induced cell death in stomach cells

To elucidate the involvement of PLDs in taxotere-induced apoptosis, we examined whether taxotere activated PLD. We labeled the lipid pool with [^{3}H] palmitic acid and used ethanol for transphosphatidylation to measure PLD activities in cells in the presence or absence of taxotere. As shown in Fig. 3A (a and b), treatment of stomach cells with 1 μM taxotere resulted in a time-dependent increase of PLD activity

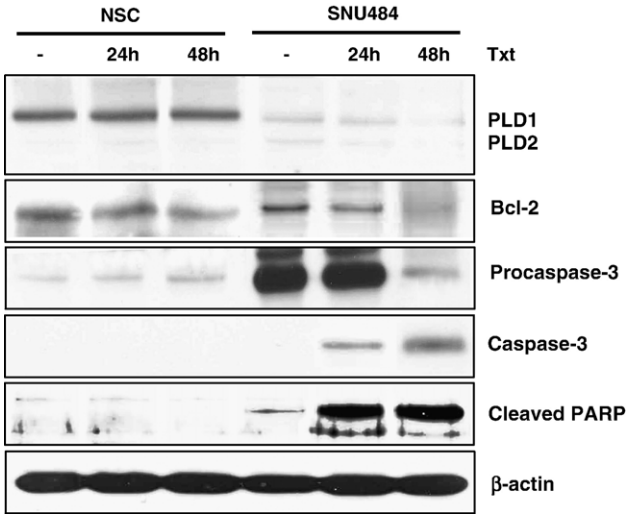

Fig. 2. Expression patterns of molecules involved in taxotere signaling. Stomach cells were cultured for 24 h or 48 h in the presence of 1 μM taxotere after starvation for 18 h. Cells after the treatment were analyzed with 8~15% SDS-PAGE and subsequently by western blotting as described in the Materials and methods. Figures show protein expression levels of PLD, Bcl-2, caspase 3, and cleaved-PARP before and after taxotere treatment in NSC and SNU 484 for 24 h and 48 h, respectively. All experiments were repeated three times with consistent results.

up to 30 min and also taxotere treatment showed dose-dependent activation of PLD up to 1 μM, respectively. It is noteworthy that basal PLD activity of NSC was about 2-fold higher than that of SNU 484 cells. However, the PLD activity of NSC was not much higher relative to PLD1 protein expression in NSC relatively. It can be explained that unlike PLD2, PLD1 has a lower basal activity [9]. In this experiment, PLD activity was blocked by primary alcohol in NSC and SNU 484 to elucidate the role of PLD in taxotere-induced cell death; this assay has widely been used to both demonstrate PLD activity and ascertain the PLD requirement by blocking the production of PA, the product of PLD, by preferential utilization of primary alcohols over H_2O in the hydrolysis of phosphatidylcholine [32]. As negative controls to eliminate alcohol cytotoxicity, secondary and tertiary alcohols can also be used, because they have a bulky structure that prevents their residue from being utilized in the reaction. As shown in Fig. 3B, the primary alcohol, 1-butanol, inhibited taxotere-induced PLD activity, but 2-butanol did not show any inhibition. When SNU 484 cells were treated with 1 μM taxotere in the absence of alcohols, about 50% of the cells died within 24 h. However, over 80% of the SNU 484 cells died when PLD activity was blocked by pretreating the cells with 1-butanol, but not with 2-butanol (Fig. 3C). Although not as exacerbated as SNU 484, the apoptosis rate of NSC had the same pattern as SNU 484 when PLD activity was blocked by 1-butanol, but not 2-butanol. This result indicated that not only was PLD activity successfully inhibited by 1-butanol without alcohol toxicity, but also PLD is strongly involved in the taxotere-induced apoptosis in that it potentially acts as an apoptosis suppressor.

3.4. Overexpression of either PLD1 or PLD2 inhibits taxotere-induced apoptosis in cultured SNU 484 cells

We hypothesized that PLD may confer an anti-apoptotic effect on signal transduction of taxotere-induced cell death. Therefore, to examine this possibility, we established SNU 484 cells stably transfected with PLD1 or PLD2, and the expression of PLD-GFP fusion proteins was first confirmed by observing bright fluorescence in the transfected cells under a fluorescence microscope (data not shown). The cells were then selected with blasticidin (10 μg/ml) for 2 weeks, and the expression and activation of PLD-GFP fusion proteins were further confirmed by using RT-PCR, western blot and PLD activity (Figs. 4A, B, and C). While the control cells, harboring only GFP, underwent significant apoptosis after treatment with 1 μM taxotere for 48 h, the apoptosis rate was significantly decreased in the cells that overexpressed either PLD1 or PLD2, which was detected under a microscope (Fig. 4D), by MTT assay (Fig. 4E) and by DNA fragmentation assay (Fig. 4F). As shown in Fig. 4G, taxotere significantly reduced the PLD activity in the GFP control cells below the basal activity; however, the cells that were stably transfected with PLD1 or PLD2 displayed significantly high activity than the control cells in the presence of taxotere. These results suggested that the attenuation of taxotere-induced apoptosis was clearly PLD activity-dependent. Several studies have suggested that antimicrotubule agents, such as taxotere or paclitaxel, are known to promote apoptosis in cancer cells and to be involved in the regulation of apoptosis by Bcl-2 family proteins [33–36], and we also reported that PLD2 possibly survived against Fas-induced apoptosis by increasing Bcl-2 and Bcl-xL expression in A20 murine B lymphoma cells [7]. Therefore, as shown in the middle panel of Fig. 4B, Bcl-2 expression was upregulated when PLD1 or PLD2 was overexpressed. Altogether, these results suggest that PLD1 and PLD2 are directly or indirectly related with Bcl-2 expression.

3.5. Expression of PLD suppresses taxotere-induced cell death through Bcl-2 regulation

To address the roles of PLDs played in taxotere-induced apoptosis and Bcl-2 expression in cultured stomach cells, we examined the expression of PLDs, Bcl-2, and apoptosis marker proteins including caspase 3 and cleaved-PARP by western blot analysis in overexpressed PLD1 or PLD2 in SNU 484. Interestingly, taxotere-induced cell death was inhibited by overexpression of PLD1 or PLD2 in SNU 484 cells, evidenced by the inhibition of caspase 3 activation and decreased cleaved-PARP (Fig. 5A). When western blot analysis, resulting in the second line of Fig. 5A, was converted into a comprehensible diagram, the overexpression of PLD1 or PLD2 attenuated the falling rate of Bcl-2 expression by taxotere treatment (Fig. 5B). These findings suggest that increased PLD activity suppresses taxotere-induced apoptosis, possibly through increasing or maintaining anti-apoptotic Bcl-2 protein.

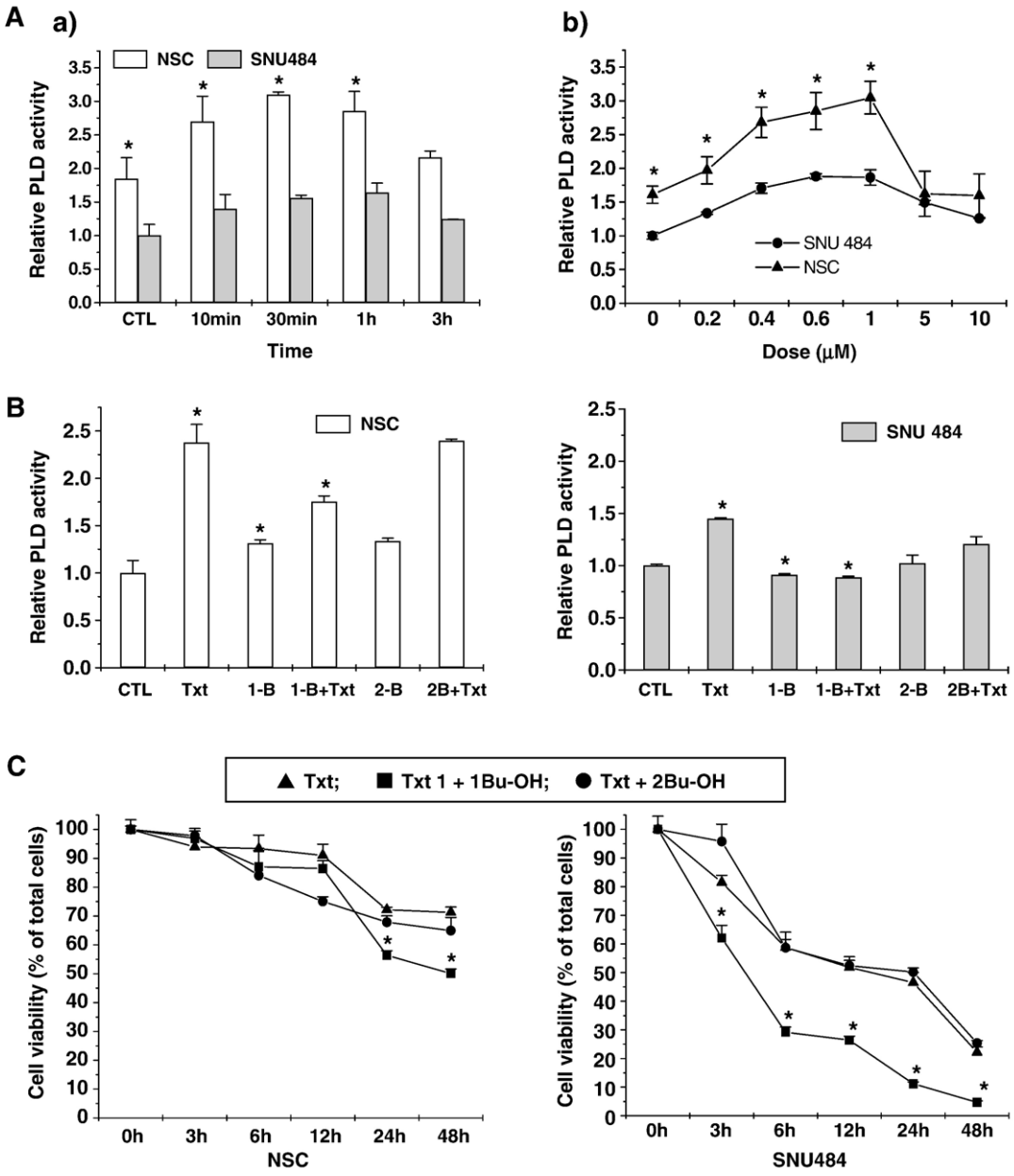

Fig. 3. Effect of PLD on taxotere-induced cell death in stomach cells. Panel A, time- or dose-dependent activation of PLD with taxotere treatment. Cells (0.4×10^6) grown in 6-well dishes were labeled with 1 μCi/ml of [^{3}H] palmitic acid for 18 h in serum-free medium prior to stimulation with taxotere. To determine PLD activity by transphosphatidylation, 1% (v/v) ethanol was added 15 min prior to 1 μM taxotere treatment, for the indicated times (a) or at the indicated doses for 30 min (b), * $p<0.05$, compared with the control value of SNU 484 cells by the Student's *t*-test. Panel B, effect of 1-butanol on PLD activity. Cells (0.4×10^6) grown in 6-well dishes were labeled with 1 μCi/ml of [^{3}H] palmitic acid for 18 h in serum-free medium. One % (v/v) ethanol was added for 15 min and then pretreated with 0.3% 1-butanol or 0.3% 2-butanol for 30 min before taxotere treatment. After stimulation, lipids were extracted, and [^{3}H] PEt bands were separated by TLC, as described under Materials and methods. Radioactivity associated with [^{3}H] PEt bands was counted, using a scintillation counter, and the relative ratio of [^{3}H] PEt counts to the total count of [^{3}H] palmitic acid-labeled lipid was calculated as PLD activity. 1-B, 1-butanol; 2-B, 2-butanol; Txt, Taxotere. Panel C, effect of 1-butanol on taxotere-induced apoptosis. Cells were seeded at 1×10^4 cells/96-well plate and treated with 1 μM taxotere for the indicated time periods either in the presence or absence of 0.3% 1-butanol after starvation for 18 h. After stimulation, MTT assay was performed, as described in the Materials and methods. Data shown are means±SD from three separate experiments, each performed in triplicate. *Differs from Txt and Txt 2-butanol in NSC and SNU 484 ($p<0.05$) by Student's *t*-test.

3.6. Bcl-2 expression was inhibited by blocking of PLD1 or PLD2 using small interfering PLD 1 or PLD2 RNA

We examined whether alteration in the level of PLD1 or PLD2 expression affects Bcl-2 expression by using siRNA PLD1 or PLD2. SNU 484 cells were transfected with PLD1 or PLD2 siRNA. Scrambled siRNA was transfected as a control. As shown in Fig. 6A and B, treatment of PLD1 or PLD2 siRNA efficiently decreased both the mRNA and protein levels of PLD1 or PLD2, respectively. The siRNA PLD1 or PLD2 treatment also decreased Bcl-2 expression in SNU 484 cells, resulting in increased cell death induced by taxotere treatment

compared to scrambled control siRNA (Fig. 6C). This result supported the hypothesis that both PLD1 and PLD2 expressions are completely related to Bcl-2 expression in SNU 484 cells.

3.7. PA is involved in upregulation of Bcl-2 expression

In general, PLD functions through the generation of PA which is a functional and enzymatic product of PLD. Therefore, we investigated whether PA has an effect on Bcl-2 expression. As shown in Fig. 7A, when we treated SNU 484 cells with PA, the expression of Bcl-2 increased in both a time- and dose-dependent manner. However, PA treatment did not change the expression of the pro-apoptotic Bcl-2 protein family such as Bad (data not shown). It is well known that PA can be degraded into DAG by PAP [37] or converted to LPA by PLA_2 [17]. Therefore, to address which molecule is involved in PLD

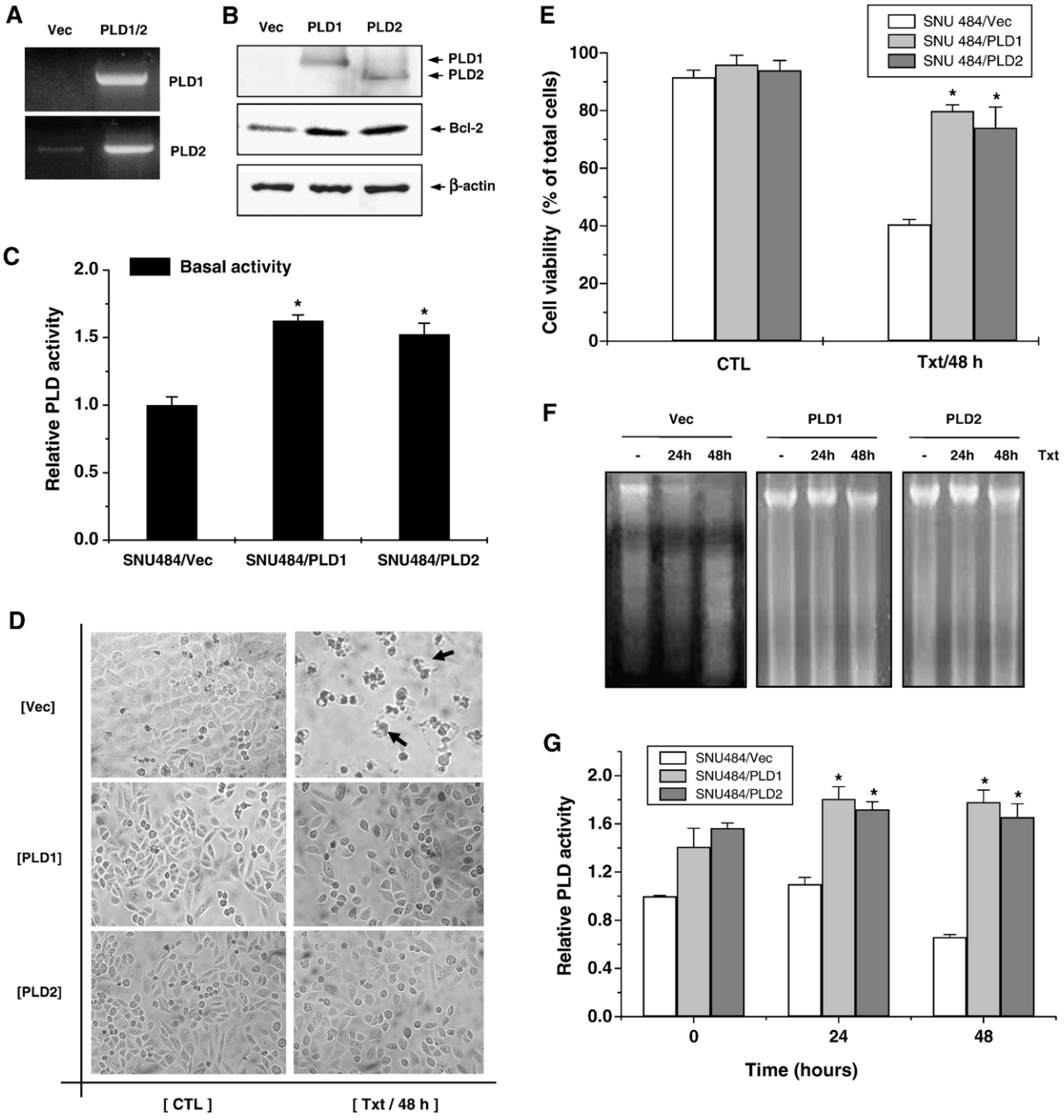

Fig. 4. Overexpression of either PLD1 or PLD2 in SNU 484 cells increased Bcl-2 expression resulting in significant inhibition of apoptosis induced by taxotere. Panels A and B, mRNA and protein levels of PLDs and protein level of Bcl-2 were increased in cells that are stably transfected with PLD1 or PLD2. Panel C shows that the total basal PLD activities were increased in both PLD1 and PLD2 transfected cells. Microscopic images (panel D), and DNA fragmentation (panel F) experiments also show that overexpression of either PLD1 or PLD2 attenuated taxotere-induced cell death. As shown in panels D and E, apoptotic cell death was decreased notably in PLD-transfected cells compared to the GFP-transfected control cells. Black arrows in panel D indicate apoptotic cells. Panel G represents PLD activities in stably transfected cells with PLDs during taxotere treatment. The asterisk indicates a significant difference, compared to the GFP vector control cells ($p<0.05$) by the Student's *t*-test.

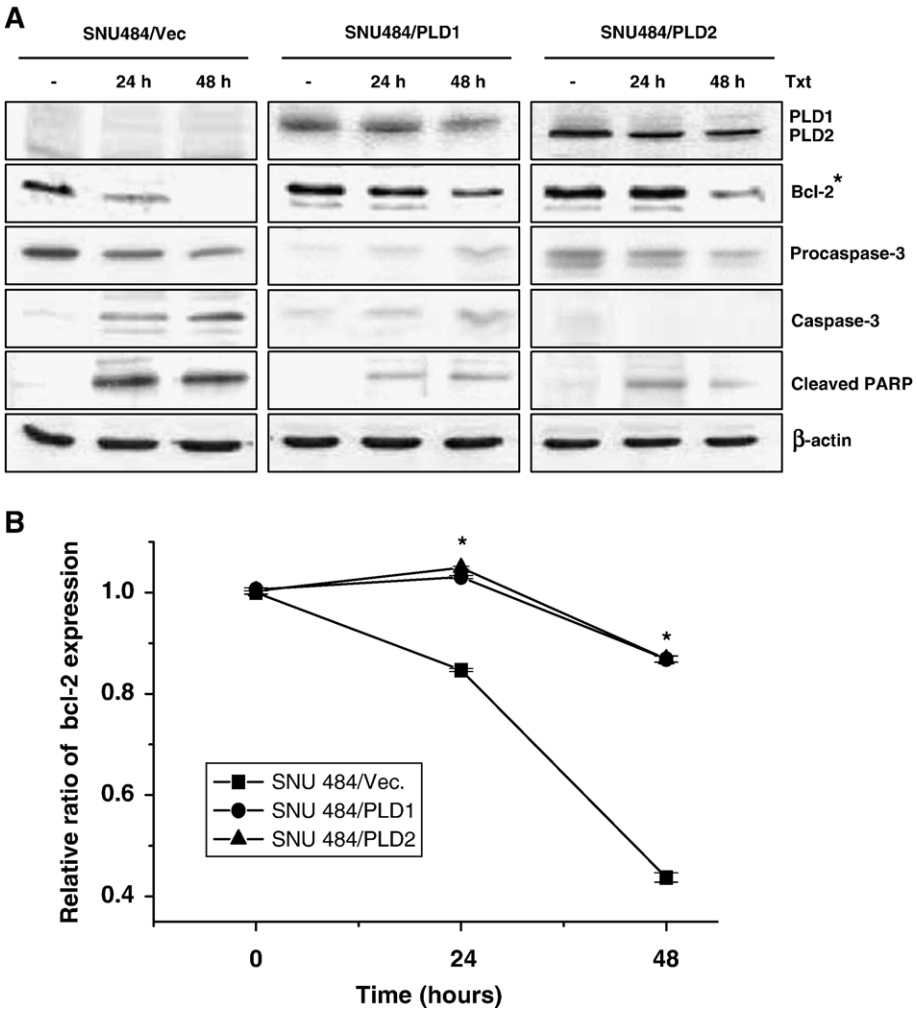

Fig. 5. Expression of PLD suppresses taxotere-induced cell death through Bcl-2 regulation. Stomach cells were cultured for 24 h or 48 h in the presence of 1 μM taxotere after starvation for 18 h. Cells were analyzed after the treatment with 8~12% SDS-PAGE and subsequently by western blotting, using anti-PLD antibody, anti-Bcl-2 antibody, anti-caspase 3 antibody, and PARP antibody as described in the Materials and methods. Panel A, stably transfected SNU 484 cells harboring viral GFP-PLD1 or viral GFP-PLD2 were characterized using western blots for PLDs, Bcl-2, caspase 3, and cleaved-PARP after taxotere treatment for 24 h and 48 h. Panel B shows the relative Bcl-2 protein expression level in stably transfected SNU 484 cells after treatment with taxotere for 24 h or 48 h. The intensity of the Bcl-2 bands (the asterisk in panel A indicates Bcl-2) obtained by western blotting was quantified by densitometry (panel B) and normalized with β-actin. All experiments were repeated three times with consistent results. The asterisk indicates a significant difference, compared to the GFP vector control cells ($p<0.05$) by the Student's *t*-test.

signaling, SNU 484 cells were cotreated with taxotere (1 μM) along with or without PA (20 μM) after pretreatment of propranolol (50 μM), a well known PAP inhibitor, or mepacrine (100 μM), a PLA_2 inhibitor. As shown in Fig. 7B, propranolol did not affect Bcl-2 expression and cell viability (data not shown). However, mepacrine treatment blocked the PA-induced expression of Bcl-2, demonstrating that both PLD and PLA_2 play an important role in controlling the expression of Bcl-2. Taken together, all results indicate that PA generated by PLD1 or PLD2 upregulates the expression of anti-apoptotic Bcl-2 protein via PLA_2 pathway in stomach cancer cells.

4. Discussion

Taxotere is a widely used antineoplastic agent that has demonstrated efficacy, both alone and in combination with other chemotherapy agents, in a wide range of tumor types. In randomized studies, taxotere-containing therapy has conferred survival advantages in patients of advanced stomach cancer [38], hormone-refractory prostate cancer [39,40] and locally advanced squamous cell carcinoma of the head and neck [38], and as first- [41,42] and second-line treatment [43,44] of advanced non-small cell lung cancer.

Taxotere induces apoptosis through phosphorylation of Bcl-2 in several cell types [5]. Previous studies have demonstrated that Bcl-2 phosphorylation can be specifically induced by drugs like taxotere that affect microtubule depolymerization or disorganization of microtubule structure. The effect of this antimicrotubule action is believed to result in the inactivation of microtubule assembly. However, the mechanisms involved remain largely undefined. This effect is not seen by DNA-damaging agents [5,45,46]. As this study shows, Bcl-2 expression was reduced by taxotere treatment in SNU 484, but not in NSC. Previous studies reported that Bcl-2 family

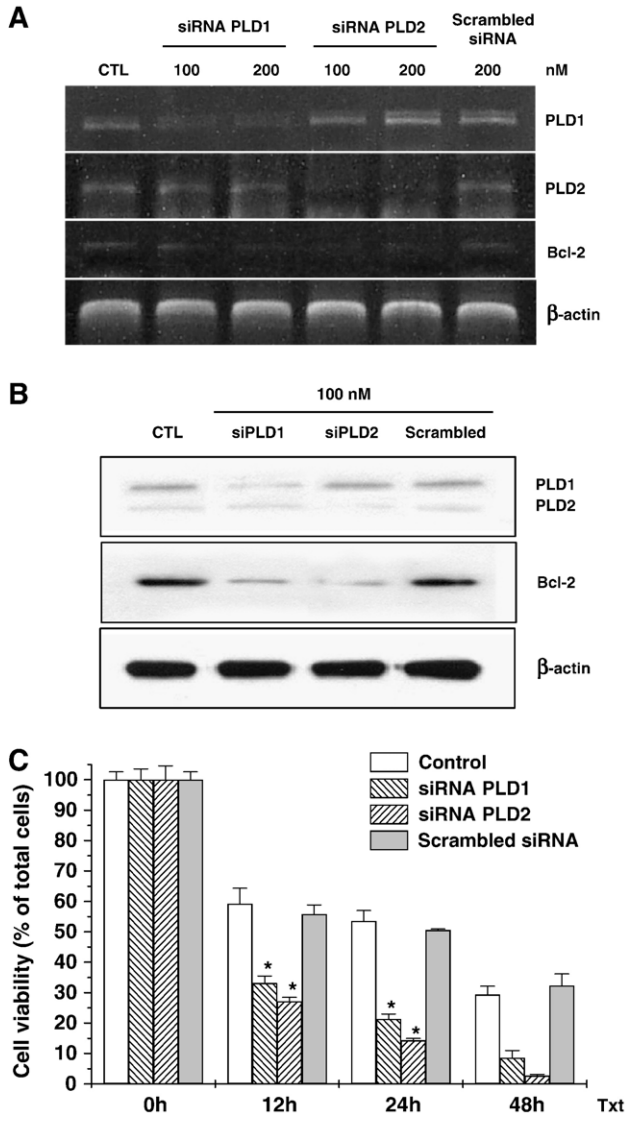

Fig. 6. Effects of PLD1 or PLD2 inhibition using siRNA on expression of Bcl-2 in SNU 484 cells. The Bcl-2 expression was decreased when PLD1 or PLD2 siRNA was transfected. Panel A, RT-PCR results of PLD1, PLD2, and Bcl-2 after inhibition of PLD1 or PLD2 expression using 100 nM and 200 nM of siRNA PLD1 or siRNA PLD2 as described in the Materials and methods. Panel B, western blot results of PLD1, PLD2, and Bcl-2 after inhibition of PLD1 or PLD2 expression by 100 nM siRNA PLD1 or PLD2. Panel C, cell viability by MTT assay. Serum-starved cells on 96-well plates (1×10^4 cells/well) were incubated with 1 μM taxotere (IC_{50}) for the indicated times after siRNA PLD1 or siRNA PLD2 transfection with 100 nM concentration. After treatment, cell viability was checked by MTT assay as described in the Materials and methods. Each point represents the mean of triplicate analyses with SD. *The difference between the control (including scrambled siRNA) and siRNA PLD1 or PLD2 was compared by the Student's *t*-test ($p<0.05$).

members are crucial regulators of apoptosis. The Bcl-2 family includes both anti-apoptotic molecules such as Bcl-2 and Bcl-xL, as well as pro-apoptotic molecules such as Bax, Bak, Bid, and Bad. Bcl-2 and Bcl-xL inhibit apoptosis in many cell types such as neurons [47,48]. Among these proteins, Bcl-2 plays an important role in microtubule-related apoptotic cell death. It is an integral membrane protein localized mainly on the outer mitochondrial membranes and endoplasmic reticulum. All activities of the mitochondria in an apoptotic signal can be blocked by Bcl-2 [49–51]. Therefore, we investigated Bcl-2 family proteins described above, but they were not changed in response to taxotere in SNU 484 cells, except Bcl-2 (data not shown). These results suggest that taxotere-induced apoptosis mainly occurs through Bcl-2 regulation in stomach cancer cells.

Very recently, according to one of our reports, Bcl-2 protein was directly increased by PLD2 overexpression in A20 cells [7]. Several studies have also suggested that PLD could play an anti-apoptotic role in apoptosis induced by various cytotoxic effectors including ceramide, actinomycin D, glutamate and Fas/CD95 agonist [19,52–54]. In mammalian cells, PLD has been implicated in a broad range of physiological cell responses, including long-term responses such as proliferation, differentiation, immune responses, and apoptosis [12,20]. Like Bcl-2 regulated by taxotere, the implication of PLD activation in the cellular response to DNA damage has not been documented [55]. However, the relationship between PLD and Bcl-2 expression in taxotere-induced apoptosis is not yet fully understood, and both molecules may be involved in the same signaling pathway triggered by taxotere in stomach cancer cells.

In the present study, we focused our efforts on this relationship between PLDs and the Bcl-2 expression level with taxotere treatment in SNU 484 cells. According to previous reports, many pro-apoptotic signals including a taxotere-inducing signal converge at the mitochondria and change

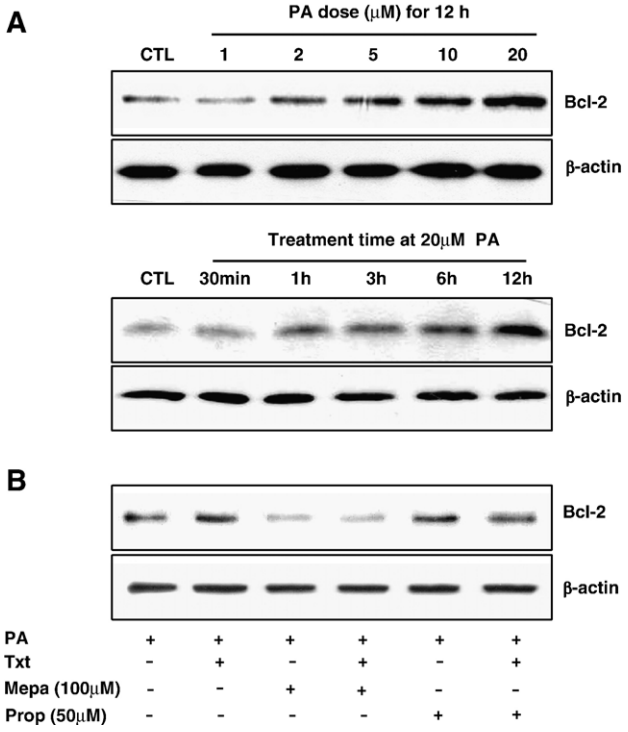

Fig. 7. Effects of PA on expression of Bcl-2 in SNU 484 cells. Panel A shows that PA increased Bcl-2 expression in both a dose- and time-dependent manner. Panel B shows the changes of Bcl-2 expression in the cells that were cultured in the presence of 20 μM PA and/or 1 μM taxotere for 12 h after pretreatment with propranolol or mepacrine in the indicated concentration for 30 min. All experiments were repeated three times with consistent results.

mitochondrial membrane permeability, resulting in the release of several mitochondrial proteins, such as cytochrome c, into the cytoplasm, which triggers the major event of mitochondria-mediated apoptosis. Consequently, caspase 3 is activated by apoptosomes composed of caspase 9, apoptotic protease activating factor, and dATP [56]. This cytosolic cytochrome c is necessary for the initiation of the apoptotic program (incidentally, apoptosis will be inhibited by Bcl-2 through inhibition of mitochondrial loss of cytochrome c.). Finally, cytochrome c released into cytosol leads to DNA fragmentation through activation of iCAD and cleavage of PARP [49,51]. At first, the apoptotic response of stomach cells to taxotere was examined, and the apoptosis was verified by three different approaches, including DNA fragmentation, direct observation of apoptotic morphology, and FACs analysis, and the results led us to conclude that normal and stomach cancer cells have completely different apoptotic responses to taxotere. Following the result, PLD activity, PLD1 expression, and Bcl-2 expression were higher in NSC than in SNU 484. Increments of caspase 3 and cleaved-PARP expression indicate that cell death induced by taxotere occurred through the apoptosis pathway. Consequently, we investigated whether PLD activity is involved in taxotere-induced apoptosis by the blocking of PLD activity or overexpression of PLD1 and PLD2. We found that 1 μM taxotere can induce 80% cell death within 48 h after treatment. Interestingly, this apoptosis rate was exaggerated by the inhibition of PLD in SNU 484 activity using primary alcohols, 1-butanol, and normal cells also showed a slightly increasing apoptosis rate when PLD activity was blocked. In contrast, when either PLD1 or PLD2 were overexpressed, the apoptotic effect of taxotere was attenuated in SNU 484 cells. Moreover, shown in the middle panel of Fig. 3B, one of the critical instances anti-apoptotic protein Bcl-2 expression was upregulated without any stimulation beside overexpression of either PLD1 or PLD2. On the other hand, Bcl-2 expression and cell viability were significantly decreased when PLD1 or PLD2 was inhibited by their corresponding siRNA (Fig. 6). These results strongly suggest that PLD activity has an important role in taxotere-induced apoptosis, and provide evidence of a close relationship between the activation of PLD and the expression of Bcl-2 in stomach cells.

In general, PLD functions through the generation of PA, which is a functional and enzymatic product of PLD. It is well known that PA is a key signaling molecule in cell physiology. However, it has been a great challenge to clarify how PA produced by PLDs allows cells to survive. After treatment with PA in SNU 484 cells, the expression of Bcl-2 was increased in both a dose- and time-dependent manner, but PA did not also change the expression of other members of the Bcl-2 family. PA is converted to DAG by PAP, and PA is hydrolyzed by PLA_2 to AA and LPA. In our data, pretreatment of SNU 484 cells with propranolol had no effect on PA-induced Bcl-2 expression, while pretreatment of mepacrine (PLA_2 inhibitor) significantly suppressed PA-induced Bcl-2 expression, suggesting that PLA_2 is involved in the increased expression of anti-apoptotic Bcl-2 proteins during taxotere-induced apoptosis in SNU 484 cells.

Taken together, stomach cancer cells could contain different entities that have different abilities of survival against an anti-cancer agent—taxotere in this case. However, the key molecules that determine this progression are unknown. Moreover, dose-limiting toxicity with the approved regimens such as severe neutropenia is the major problem during chemotherapy. Investigation of molecules that are closely linked with survival mechanisms will be beneficial to development of a treatment strategy for overcoming the dose limit. The data in the present study indicate that by increasing Bcl-2 proteins, PLD1 or PLD2 acts as a survival enzyme during taxotere-induced apoptosis in SNU 484 cells. These results may also explain the higher susceptibility of SNU 484 cells than NSC to taxotere-induced cell death. Our studies seem to suggest that PLD1, PLD2, and their product, PA, should be targets for new anti-cancer drug development which can assist the taxotere therapeutic effect by enhancing taxotere-sensitivity of cancer cells.

Acknowledgements

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