SB365, *Pulsatilla* saponin D suppresses proliferation and induces apoptosis of pancreatic cancer cells

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Abstract. *Pulsatilla koreana* has been used as a traditional medicine for the treatment of various diseases. The purpose of this study was to determine whether SB365, *Pulsatilla* saponin D isolated from the root of *Pulsatilla koreana* inhibits the progression of pancreatic cancer. We found that SB365 strongly suppressed the growth and proliferation of 5 human pancreatic cancer cell lines (MIAPaCa-2, BXPC-3, PANC-1, AsPC-1 and HPAC). The apoptotic effect of SB365 was demonstrated by increased levels of cleaved caspase-3 and decreased Bcl-2 expression via mitochondrial membrane potential, as well as elevated numbers of terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling (TUNEL)-positive apoptotic cells. SB365 was also found to exert an anti-angiogenic effect by decreasing the expression of HIF-1α and VEGF, major factors of angiogenesis, which was confirmed by the suppression of tumor sphere formation of pancreatic cancer cells. An *in vivo* mouse xenograft study showed that SB365 significantly inhibited tumor growth through the induction of apoptosis and inhibition of angiogenesis with strong anticancer activity. Therefore, SB365 is a good candidate as a natural product for use in the treatment of pancreatic cancer.

Introduction

Pancreatic cancer is one of the most aggressive and lethal forms of cancer with an overall 5-year survival rate of less than 5%. It is currently the fourth cause of death from cancer in the Western world. Despite this fact, there has been little improvement in its prognosis over the past 20 years (1). The poor prognosis of pancreatic cancer can be attributed to the lack of early diagnosis, the aggressive biological behavior of the tumor and the insensitivity to most therapies including chemotherapy, radiotherapy and immunotherapy (2). Moreover, few patients have the chance to undergo radical surgery because of its poor diagnosis and those patients with unresectable pancreatic cancer usually survive for no more than 3-5 months, despite systemic treatment (3). Currently, gemcitabine remains the most effectvie agent available for the treatment of advanced pancreatic cancer, but the disease rapidly develops resistance to the drug. Thus, novel and efficacious strategies including low toxic agents that can overcome either the effect of gemcitabine or chemoresistance to drugs are urgently needed for the treatment for pancreatic cancer.

Non-toxic phytochemicals offer promising options for effective chemotherapeutic strategies for various types of cancer. Some studies support the concept that the consumption of bioactive dietary phytochemicals reduces the risk of pancreatic cancer (4,5). Additionally, a number of studies have shown that various herbal extracts and compounds possess antitumor activities (6). One of these is *Pulsatilla koreana* which belongs to the family Ranunculaceae. The roots of this plant have been widely used in traditional medicine for the treatment of various diseases, including malaria and amoebic dysentery (7). This compound has also been reported to possess anti-inflammatory and anti-parasitic effects (8). This plant includes various effective components such as saponins, ranunculin, anemonin, protoanemonin and triterpenes (9). Specifically, there are 17 saponins in *Pulsatilla koreana*, among which, saponin D has been reported to demonstrate cytotoxicity against lung cancer cells (10,11). Thus, *Pulsatilla* saponin D (hereafter designated as SB365) was selected from many types of saponins isolated from *Pulsatilla koreana* for further investigation. In the present study, we assessed the chemotherapeutic effect of SB365 in pancreatic cancer cells and conducted *in vivo* tumor xenograft studies. Our results showed that treatment with SB365 resulted in inhibition of cell growth/proliferation, angiogenesis, and induction of apoptosis in pancreatic cancer.

Materials and methods

Cells and materials. Human pancreatic cancer cell lines PANC-1, MIAPaCa-2, BXPC-3, AsPC-1 and HPAC were

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purchased from the American Type Culture Collection (AATC, Manassas, VA, USA). PANC-1 and MIAPaCa-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM). BXPC-3 and AsPC-1 cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium, and HPAC cells were cultured in DMEM-F12 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. FBS and all other agents used in the cell culture studies were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). The cultures were maintained at 37˚C in a CO_2 incubator with a controlled humidified atmosphere composed of 95% air and 5% CO_2. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and proteinase K were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Preparation of SB365. SB365 was isolated from the roots of *Pulsatilla koreana*, as previously described by Kim *et al* (11). Briefly, the powdered roots (50 g) were extracted 3 times using 50% aqueous ethanol (500 ml), and the residue was then suspended in 300 ml of acetone and centrifuged. The precipitate was suspended in water and filtered to remove the insoluble portion. Next the fraction was chromatographed using a Sephadex LH-20 column (200 g, 60x4 cm) and solid phase high performance liquid chromatography (HPLC; solid phase; RP-C18, 250x10 mm, mobile phase; MeOH:H_2O (82:20) as the mobile phase, 210 nm, 1 ml/min) to yield SB365, a saponin D. To generate a more exact analysis of the purified SB365, we also used mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy.

Measurement of cell proliferation. Cell viability was measured using the MTT assay. Briefly, PANC-1, BXPC-3, MIAPaCa-2, AsPC-1 and HPAC pancreatic cancer cells were plated at a density of 1-3x10^4 cells/well in 96-well plates and then incubated for 48 h. The media were then removed, and the cells were treated with either dimethyl sulfoxide (DMSO), as a negative control, or various concentrations of SB365. The final concentration of DMSO in the media was ≤0.1% (v/v). After the cells were incubated for 48 h, 20 μl of MTT solution (2 mg/ml) was added to each well, and the cells were incubated for another 4 h at 37˚C. The formed formazan crystals were dissolved in DMSO (200 μl/well) with constant shaking for 5 min. The plate was then read on a microplate reader at 540 nm. Three replicate wells were used for each analysis. The median inhibitory concentration (IC_{50}, defined as the drug concentration at which cell growth was inhibited by 50%) was assessed using the resulting dose-response curves.

Immunofluorescence microscopy. Pancreatic cancer cells (PANC-1 and BXPC-3) were plated on 18-mm cover glasses in DMEM or RPMI-1640 medium and incubated for 24 h so that ~70% confluence was reached. The cells were then incubated in the presence or absence of 10 μM SB365, washed twice with PBS, and fixed in an acetone:ethanol solution (1:2) for 10 min at -20˚C. Next, cells were blocked in 1.5% horse serum in PBS for 30 min at room temperature, and then incubated overnight at 4˚C with the primary antibody (1:50) in a humidified chamber. After washing twice with PBS, the cells were incubated with mouse fluorescein-labeled secondary antibody (1:100; Dianova, Hanburg, Germany) for 1 h at 37˚C. The cells were also stained with 4,6-diamidino-2-phenylindole (DAPI) to visualize the nuclei. The slides were then washed twice with PBS and covered with DABCO (Sigma-Aldrich) before being viewed with a confocal laser scanning microscope (Olympus, Tokyo, Japan).

DAPI staining and terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling (TUNEL) assay. PANC-1 pancreatic cancer cells were plated onto 18-mm cover glasses in DMEM medium and grown to ~70% confluence for 24 h. The cells were then treated with SB365 at a dose of 10 μM for 24 h. They were fixed in ice-cold 1% paraformaldehyde, washed with PBS and then stained with 2 μg/ml of DAPI for 20 min at 37˚C. The stained cells were examined for fluorescence of nuclear fragmentation. TUNEL was performed using the TUNEL kit (Millipore, Billerica, MA, USA).

Measurement of mitochondrial membrane potential. BXPC-3 pancreatic cancer cells were plated on 18-mm cover glasses in RPMI-1640 medium and incubated for 24 h so that ~70% confluence was reached. The cells were then incubated in the presence or absence of SB365 (10 μM) for 6 h after which they were incubated with 5 μM JC-1 fluorescence dye for 20 min in a CO_2 incubator. The slides were then washed twice with PBS and covered with DABCO before being viewed with a confocal laser scanning microscope.

Analysis of cytochrome c localization. BXPC-3 cells were plated on 18-mm cover glasses in RPMI-1640 medium and incubated for 24 h so that ~70% confluence was reached. The cells were then treated with SB365 (10 μM) for 6 h and washed twice with PBS, and fixed in an acetic acid:ethanol solution (1:2) for 5 min at room temperature. Cells were incubated overnight at 4˚C with cytochrome *c* antibody (1:20; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). After washing twice with PBS, the cells were incubated with mouse fluorescein-labeled secondary antibody (1:50) containing 100 nM of a mitochondrion-specific dye (MitoTracker® Red FM; Molecular Probes Inc., Eugene, OR, USA) for 45 min at 37˚C. The cells were also stained with DAPI to visualize the nuclei. The slides were then washed twice with PBS and covered with DABCO before viewing with a confocal laser scanning microscope.

Western blotting. BXPC-3 cells were washed 3 times with ice-cold PBS before lysis using a buffer containing 1% Triton X-100, 1% Nonidet P-40, and the following protease and phosphatase inhibitors: aprotinin (10 mg/ml), leupeptin (10 mg/ml) (ICN Biomedicals, Asse-Relegem, Belgium), phenylmethylsulfonyl fluoride (1.72 mM), NaF (100 mM), $NaVO_3$ (500 mM) and $Na_4P_2O_7$ (500 mg/ml) (Sigma-Aldrich). Equal amounts of protein were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. The protein transfer was then checked using Ponceau S staining solution (Sigma-Aldrich). Immunostaining of the blots was performed using the primary antibodies, followed by the secondary antibody-conjugated to horseradish peroxidase with detection using enhanced chemiluminescence reagent (Amersham Biosciences, Piscataway, NJ, USA). Primary antibodies were

purchased as follows: Bcl-2 (Santa Cruz Biotechnology Inc.), β-actin (Abcam, Cambridge, UK), cleaved caspase-3 (Cell Signaling Technology, Inc., Beverly, MA, USA). The secondary antibodies were purchased from Amersham Biosciences. The bands were visualized with the ECL Plus system (Amersham Biosciences).

Tumor sphere assay. PANC-1 cells were seeded in a 24-well low adhesion plate (Corning Inc., Lowell, MA, USA) at a density of $1x10^4$ cells/well and an initial volume of 500 ml as described by Dontu *et al* (12). Spheres were grown in DMEM medium (Gibco-BRL, Carlsbad, CA, USA) supplemented with vitamin B27, 20 ng/ml FGF, and 20 ng/ml epidermal growth factor (EGF; R&D Systems, Minneapolis, MN, USA) and 4 μg/ml heparin. Samples were subsequently treated with 10 μM SB365. EGF and FGF were replenished every 3 days. Aside from the addition of heparin, cells were agitated daily to minimize clumping. Spheres were counted and photographed after 14 days of continuous growth factor and supplemental exposure. Spheres were monitored under the microscope daily to ensure that they were derived from single cells and that they did not become confluent during the experiment.

Tumor xenograft study. Male athymic BALB/c nude mice were obtained from Orient Bio. Animal Inc. (Seoul, Korea). Animal care and all experimental procedures were conducted in accordance with the approval and guidelines of the INHA Institutional Animal Care and Use Committee (INHA IACUC) of the Medical School of Inha University (approval ID: 090518-5). The animals were fed standard rat chow and tap water *ad libitum*, and maintained under a 12 h dark/light cycle at 21˚C. Male nude mice (6 weeks old, weighing 22-26 g) were randomly divided into 2 groups (control and SB365 30 mg/kg). PANC-1 cells were harvested and mixed with PBS (200 μl/mouse) and then inoculated into one flank of each nude mouse ($5x10^6$ of PANC-1 cells). When the tumors had reached a volume of ~50-100 mm^3, mice were given an intraperitoneal injection of SB365 (30 mg/kg, treatment group) or the vehicle (200 μl PBS, control group) for 37 days. The tumor dimensions were measured twice a week using a digital calliper and the tumor volume (V) was calculated using the formula: $V = \text{length} \times \text{width}^2 \times 0.5$. At the end of the experiment, mice were sacrificed, and tumors were excised and weighed.

Immunohistochemistry. After being blocked with normal goat serum (Vector Laboratories, Burlingame, CA, USA) for 1 h, frozen tissue sections were incubated for 1 h at room temperature in dilutions of 1:100 of PCNA, cleaved caspase-3, VEGF and CD34 antibodies. The sections were visualized by an avidin-biotin peroxidase complex solution using an ABC kit (Vector Laboratories). The sections were washed in PBS and developed with a diaminobenzidine tetrahydrochloride substrate for 15 min and then counterstained with hematoxylin.

Statistical analysis. Data are expressed as the means ± SD. Statistical analysis was performed using ANOVA. A $P \leq 0.05$ was considered to indicate a statistically significant result. Statistical calculations were performed using SPSS software for Windows operating system (version 10.0; SPSS, Inc., Chicago, IL, USA).

Results

Effect of SB365 on the proliferation of human pancreatic cancer cells. To evaluate the anticancer properties of SB365, we first compared the cell growth of 5 pancreatic cancer cell lines (MIAPaCa-2, BXPC-3, PANC-1, AsPC-1 and HPAC cell lines) that had been treated with SB365. As shown in Fig. 1A, the cells were exposed to various concentrations (0, 1, 2, 5, 10 and 20 μM) of SB365 for 48 h. The results revealed that SB365 treatment inhibited cell growth in a dose-dependent manner. SB365 induced a reduction in cell growth, starting at a dose of 1 μM in pancreatic cancer cells and strongly inhibited up to 80% of cell growth at doses of 5 and 10 μM. To confirm the effect of SB365 on cell growth and proliferation, we identified expression of Ki-67, a cell proliferation marker using a fluorescent imaging system. As shown in Fig. 1B, SB365 significantly decreased the number of Ki-67-positive cells, when compared with the control.

Effects of SB365 on the apoptotic cell death of pancreatic cancer cells. To identify the pro-apoptotic effect of SB365 on pancreatic cancer cells, we first conducted DAPI staining and TUNEL assay. As shown in Fig. 2A, the cells treated with 10 μM of SB365 showed morphological features of apoptotic cells, such as bright nuclear condensation and perinuclear apoptotic bodies, upon DAPI staining. SB365-induced apoptosis was confirmed by detection of DNA fragmentation using TUNEL staining. To identify involvement of SB365 in changes in the mitochondrial membrane potential ($\Delta\Psi_m$), which are correlated with intrinsic apoptosis, we also performed JC-1 staining. As shown in Fig. 2B, control cells showed heterogeneous staining of the cytoplasm with both red and green fluorescence coexisting in the same cells. Consistent with mitochondrial localization, red fluorescence (corresponding to high mitochondrial membrane potential) was primarily found in granular structures distributed throughout the cytoplasm. Treatment of PANC-1 and BXPC-3 cells with SB365 decreased the red fluorescence and led to occurrence of frequent clusters of mitochondria. Treatment of SB365 induced marked changes in $\Delta\Psi_m$ evidenced by the disappearance of red fluorescence or the increase in green fluorescence in most cells. In addition, we observed that SB365 led to increased cytochrome *c* release along with a concomitant decrease in the-co-localization of cytochrome *c* and mitochondria (Fig. 2C). Furthermore, SB365 increased the protein expression levels of cleaved caspase-3 and decreased Bcl-2 expression in BXPC-3 pancreatic cancer cells (Fig. 2D). Collectively, these results indicate that SB365 induced apoptosis of pancreatic cancer cells.

Effects of SB365 on the expression of HIF-1α/VEGF and tumor sphere formation of pancreatic cancer cells. Considering the importance of HIF-1α and VEGF in hypoxia, we examined the effects of SB365 on the expression pattern of HIF-1α and VEGF in BXPC-3 cells. BXPC-3 pancreatic cancer cells were treated with SB365 (10 μM) under mimicked hypoxia induced by treatment with 100 μM $CoCl_2$ for 24 h. As shown in Fig. 3A, the HIF-1α and VEGF expression increased under hypoxic condition, whereas SB365 treatment inhibited the hypoxia-induced HIF-1α and VEGF expression. Moreover, to test for the ability of pancreatic cancer cells to grow as tumor spheres in culture, PANC-1 cells were plated on ultra-low

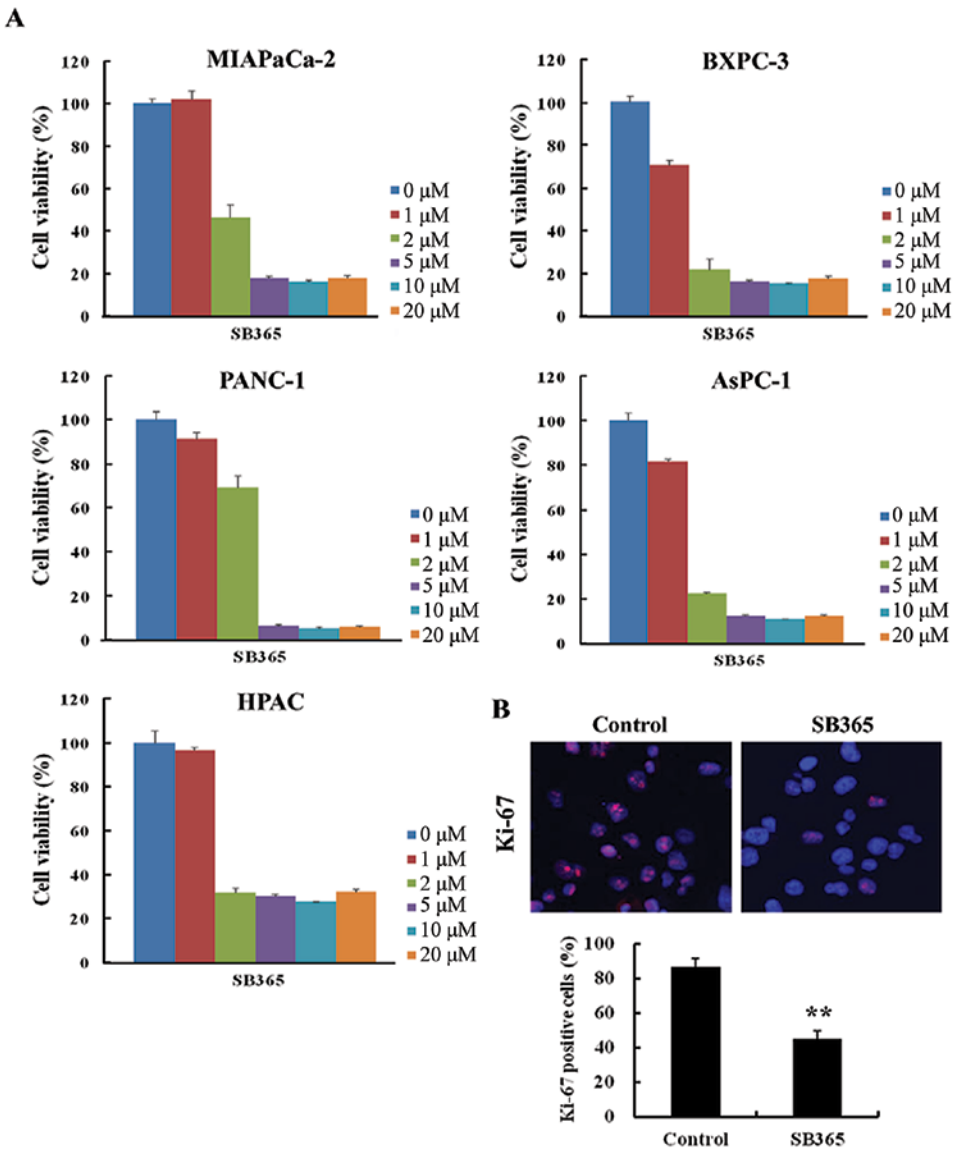

Figure 1. Effect of SB365 on the proliferation of human pancreatic cancer cells. (A) Cytotoxic effects of SB365. Viability of MIAPaCa-2, BXPC-3, PANC-1, AsPC-1 and HPAC pancreatic cancer cells was measured by MTT assay. Pancreatic cancer cells were seeded in 96-well culture plates. After incubation for 24 h, the cells were treated with various concentrations of SB365 or with 0.1% dimethyl sulfoxide (DMSO) as a negative control. After incubation for 48 h, the cells were subjected to the MTT assay. (B) Effect of SB365 on proliferation of PANC-1 cells was measured by Ki-67 staining, which was photographed at x200 magnification. Results are expressed as percent cell proliferation relative to the proliferation of the control. Data are represented as the means ± SD from triplicate wells. **P<0.01 vs. control.

attachment plates in defined medium containing EGF, FGF and B27. After 2 weeks, tumor spheres consisting of ~50-100 cells were observed, ~50% of which had symmetrical ball-like structures (Fig. 3B). However, treatment of SB365 inhibited the formation of tumor spheres of pancreatic cancer cells in a dose-dependent manner.

Inhibition of pancreatic tumor growth by SB365 in a mouse xenograft model. Strong anticancer efficacy of SB365 led us to investigate the *in vivo* efficacy of SB365 against xenografts of PANC-1 pancreatic cancer cells in nude mice. As shown in Fig. 4A, SB365 suppressed tumor growth at a dose of 30 mg/kg for 37 days compared with the control group. SB365 administration resulted in a significant reduction in tumor volume (55%) in nude mice. In addition, there was no difference in the body weight in the mouse groups treated with SB365 when compared with the control group, showing that SB365 exhibited little toxicity to mice at the curative dose (Fig. 4B). Consistent with this observation, the weight of tumors isolated from the SB365-treated mouse groups decreased significantly by 51% in the SB365 30 mg/kg group, compared with the control (Fig. 4C, P<0.05). Taken together, our results demonstrated that SB365 had strong antitumor efficacy against pancreatic cancer xenograft models.

Effects of SB365 on proliferation, angiogenesis and apoptosis in a xenograft model. Based on histopathological analysis using hematoxylin and eosin (H&E) staining, we observed a greater degree of tumor apoptosis and necrosis in the SB365-treated mouse group when compared to the control groups.

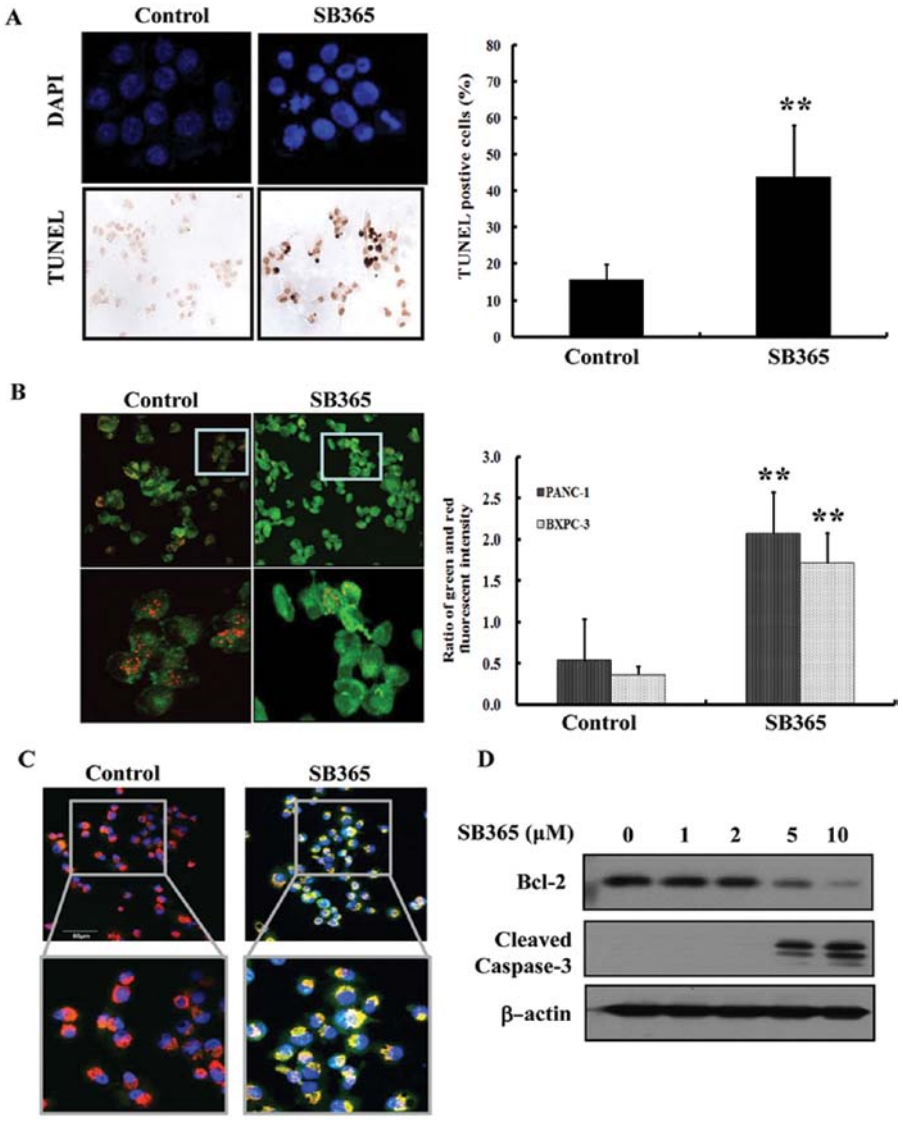

Figure 2. Effect of SB365 on the apoptosis of pancreatic cancer cells. (A) Induction of apoptosis in PANC-1 cells by SB365 (10 μM) was determined by 4,6-diamidino-2-phenylindole (DAPI) and terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling (TUNEL) staining, which was photographed at x400 magnification. Data are expressed as the means ± SD from the 3 experiments. **P<0.01 vs. control. (B) PANC-1 and BXPC-3 cells were treated with SB365 (10 μM) for 6 h, and the mitochondrial membrane potential was determined by JC-1 staining and analyzed using an LS 55 luminescence spectrometer, as well as photographed at x400 and x800 magnification (PANC-1 cells). The results (green/red ratio) are expressed as the percentage of cells treated with SB365. Data are expressed as the means ± SD of triplicate wells. **P<0.01 vs. control. (C) After treatment with SB365 (10 μM) for 6 h, BXPC-3 cells were stained with anti-cytochrome *c* antibody, MitoTracker and DAPI. The immunostained cells were analyzed under an Olympus confocal laser scanning microscope at x400 and x800 magnification (D) PANC-1 cells were treated with SB365 (0, 1, 2, 5 and 10 μM) for 48 h. Cell lysates were prepared and subjected to western blotting for Bcl-2, cleaved caspase-3 and β-actin.

Furthermore, SB365 decreased the expression of PCNA, a cell proliferation marker, as well as both VEGF and CD34 in the SB365-treated group compared with the control group. In addition, the apoptotic effect of SB365 on pancreatic cancer tumor tissues was identified by expression of cleaved caspase-3 and DNA fragments by TUNEL assay. These results suggest that SB365 had an anti-angiogenic effect on pancreatic cancer xenografts (Fig. 5).

Discussion

Owing to the advanced stage of the disease and resistance to cytotoxic chemotherapeutic agents and radiation therapy, pancreatic cancer remains one of the most intractable human malignancies. In recent years, bioactive components from plant origin have obtained considerable attention as promising options for the development of effective strategies for the prevention or treatment of cancer. Accordingly, the discovery of active medicinal compounds from herbal/natural sources has provided alternative treatment choices for patients (13). Therefore, we isolated SB365, saponin D from *Pulsatilla koreana* and conducted a comprehensive analysis of the effect of SB365 on pancreatic cancer using both *in vivo* and *in vitro* models. In the present study, we report that SB365 significantly reduced cell growth and proliferation while inducing apoptosis in pancreatic cancer cells. This is the first report validating the anticancer properties of SB365 in both pancreatic cancer cells and a tumor xenograft animal model.

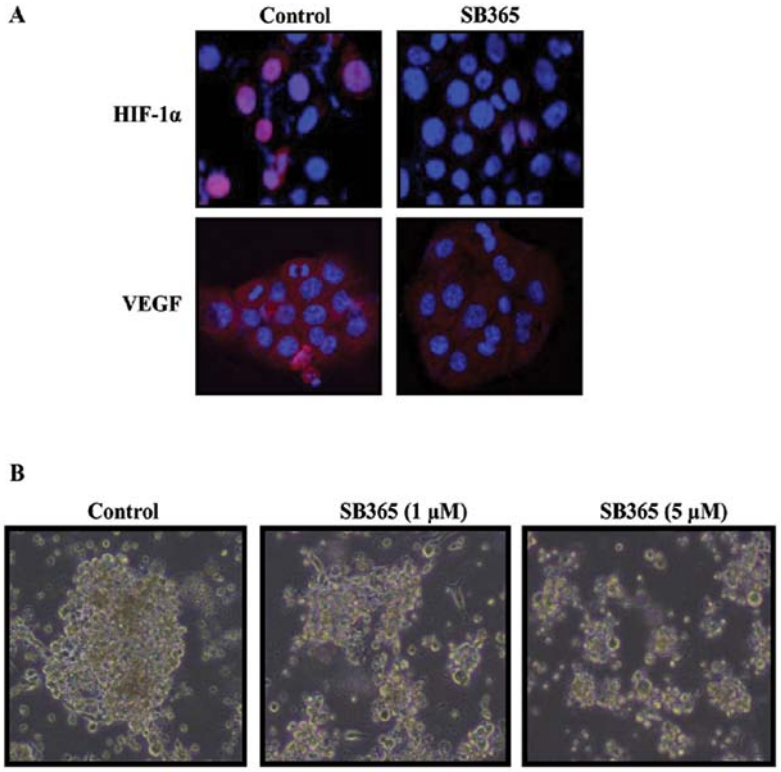

Figure 3. Effect of SB365 on the angiogenesis of pancreatic cancer cells. (A) Immunofluorescence imaging showing the expression of HIF-1α and VEGF in hypoxic BXPC-3 cells (hypoxia induced with 100 μM $CoCl_2$) treated with SB365 (10 μM). Magnification, x800. (B) Typical PANC-1 cell tumor spheres generated from single cells after 14 days. Bright field micrographs showing tumor sphere formation in PANC-1 cells after treatment with SB365 (1 and 10 μM). Magnification x400.

Loss of cell proliferation and/or induction of apoptosis are two major mechanisms by which cancer cells are killed by chemotherapeutic agents (14). In this study, SB365 inhibited 50-80% of the growth of pancreatic cancer cells (MIAPaCa-1, BXPC-3, PANC-1, AsPC-1 and HPAC cells) at concentrations of 2-10 μM, and the level of Ki-67-positive cells was also significantly decreased by SB365 treatment, indicating that SB365 has anti-proliferative effect. These results are in line with those of previous studies, in which many types of saponins were found to inhibit cell proliferation in various types of cancer cells (15-18).

While the effect of SB365 on proliferation likely contributes to its anticancer activity, it appears that pancreatic cancer cells are more sensitive to apoptosis induced by SB365. In fact, the IC_{50} values reflect the overall change in cell viability, which includes changes in both proliferation and apoptosis. Indeed, SB365 showed low IC_{50} values (0.8-2 μM) in 5 pancreatic cancer cell lines. Therefore, we investigated whether SB365 had an apoptotic effect in pancreatic cancer. In our study, we observed that SB365 has the capacity to induce pancreatic cancer cells to undergo apoptosis. DNA fragmentation and nuclear chromatin condensation were demonstrated in pancreatic cancer cells treated with SB365. Moreover, the observation of caspase-3 activation and decreased Bcl-2 confirms the promotion of apoptosis by SB365. In addition, changes in the mitochondrial membrane potential are induced to create pores of mitochondria, which dissipates the transmembrane potential and leads to the release of cytochrome *c* into the cytoplasm (19,20), finally resulting in the induction of apoptosis. Therefore, to identify the effect of SB365 on mitochondrial membrane potential, we conducted JC-1 and cytochrome *c* immunofluorescence staining. SB365 induced significant changes in mitochondrial membrane potential ($\Delta\Psi_m$) and increased cytochrome *c* release along with a concomitant increase in co-localization of cytochrome *c* with mitochondria. Collectively, these results indicate that SB365 induced an apoptotic effect by mitochondrial-mediated apoptotic molecules against pancreatic cancer cells.

Angiogenesis is a process that plays an important role in the growth and metastasis of solid tumors (21). The inhibition of angiogenic pathways is also an alternative for targeting cancer cell proliferation. In this process, VEGF is a potent inducer of angiogenesis and HIF-1α is the major regulator of VEGF transcriptional activation (22,23). In the present study, SB365 obviously decreased the expression of HIF-1α and VEGF under hypoxia, showing that SB365 inhibited hypoxia-induced angiogenesis in pancreatic cancer. In our previous study, SB365 inhibited not only tube formation and migration of HUVECs but also the formation of neovessels in Matrigel plug (24). Based on these findings, we expected that the anti-angiogenic capacity of SB365 would accompany inhibition of metastasis, which is associated with tumor formation and migration. Indeed, bursts of angiogenic activity can be sufficient to initiate sustained tumor angiogenesis and metastasis (25). To grow beyond a small sphere, tumor cells must be able to invade across a barrier known as the basement membrane and into local tissue. At this point, they can also produce angiogenic factors such as VEGF that recruit

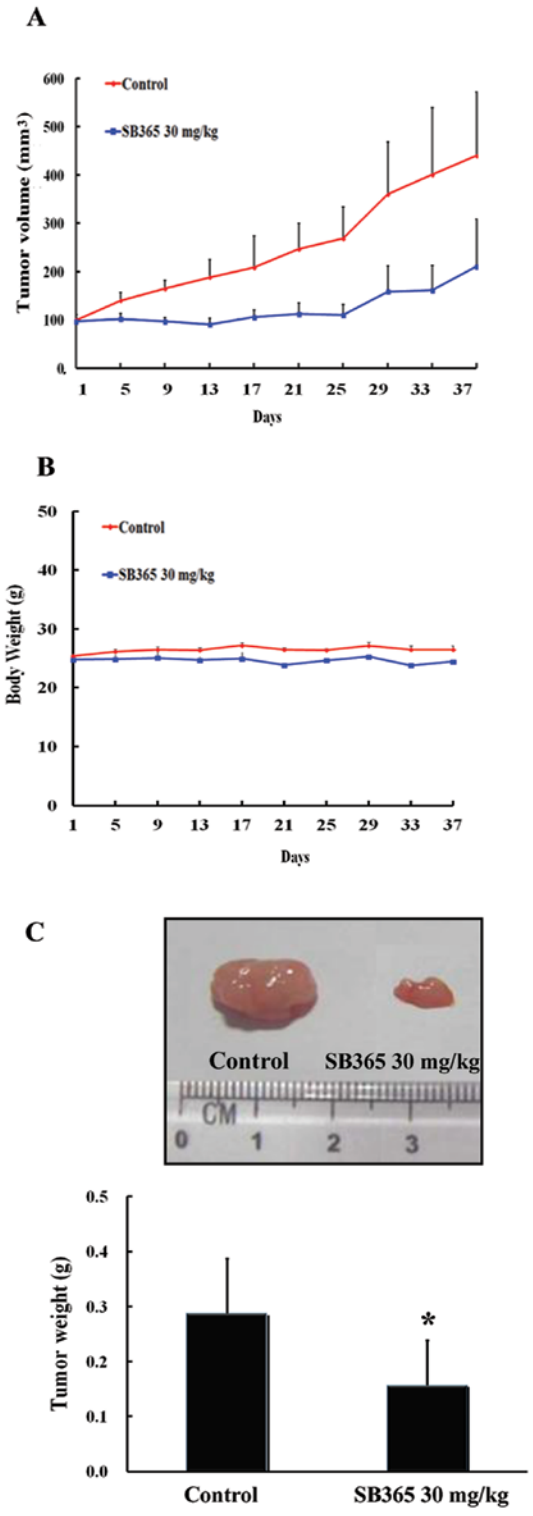

Figure 4. *In vivo* effect of SB365 in the pancreatic cancer mouse xenograft model. (A) Tumor growth of PANC-1 xenografts in nude mice. After the tumors reached 50-100 mm^3 in size, the mice received an intraperitoneal administration of SB365 (30 mg/kg) 3 times a week for 37 days. (B) Average body weight of nude mice. (C) Tumor size and weight in PANC-1 mouse xenografts. Data are represented as the means ± SD (n=6). **P<0.01 vs. control.

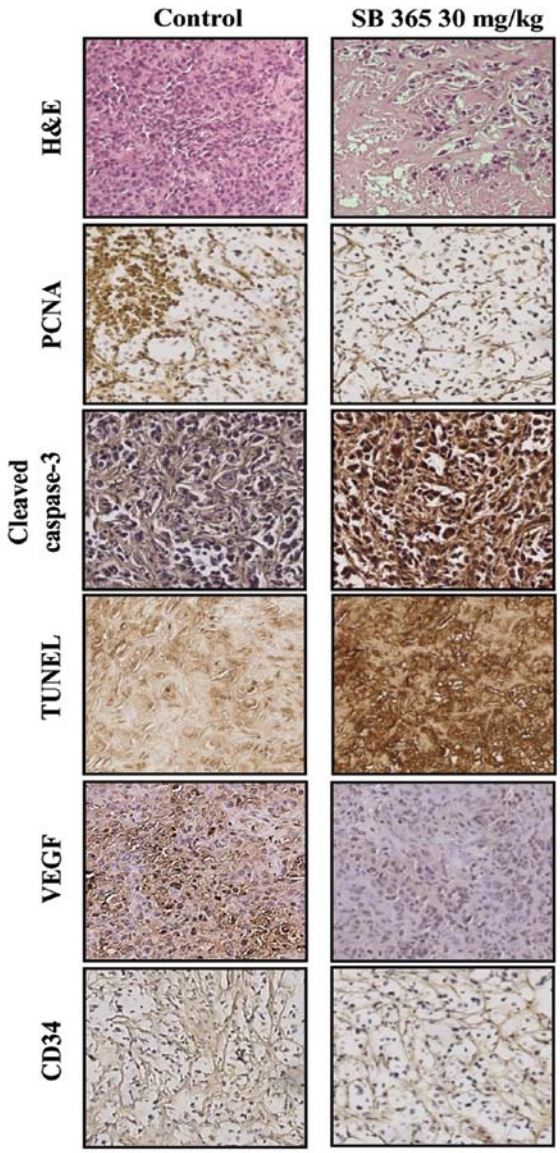

Figure 5. *In vivo* effect of SB365 on the proliferation, apoptosis and angiogenesis of PANC-1 xenografts. Tumors were excised and processed for immunostaining for PCNA, cleaved caspase-3, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL), VEGF and CD34 including hematoxylin and eosin (H&E) staining. Magnification, x400.

new blood vessel formation (26). Moreover, when tumor cells are allowed to grow to a large size, these new vessels support tumor metastasis (27). Based on this knowledge, we evaluated whether SB365 inhibits pancreatic tumor sphere formation. Importantly, we found that SB365 obviously inhibited tumor sphere forming in a population of pancreatic cancer cells with tumor sphere-forming ability. These findings indicate that SB365 demonstrated potent anti-angiogenic effects along with the potential for inhibition of metastasis.

Most importantly, our *in vitro* results were corroborated *in vivo* in a pancreatic cancer xenograft model. Specifically, SB365 inhibited tumor growth in the mouse xenograft models. These data are in accordance with the decreased proliferation and angiogenesis documented by PCNA, and CD34 and VEGF immunostaining, respectively. Additionally, SB365 induced apoptosis as indicated by increased cleaved caspase-3 and TUNEL staining in tumor tissues. These *in vivo* results are similar to our *in vitro* study, which further suggests that SB365 may be an applicable approach to enhance the anti-tumor activity against pancreatic cancer.

In conclusion, the present study demonstrated that SB365 potentiated the anticancer effect against pancreatic cancer by inducing apoptosis as well as inhibiting cell growth/proliferation and angiogenesis. Based on our findings, SB365 is a potential therapeutic agent against pancreatic cancer. However, further investigation to explore possible clinical uses of SB365 as a natural compound is needed in terms of therapeutic strategy.

Acknowledgements

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