SB365 induces apoptosis and suppresses proliferation of glioblastoma cells

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Abstract:
CONTEXT: Glioblastoma is a malignant brain tumor with limited treatment modalities due to its nature. SB365, Pulsatilla saponin D, is known to induce apoptosis and inhibit the growth of many cancer cells.
AIM: We elucidated the anticancer effects of SB365 in glioblastoma cells.
METHODS: We examined the antiproliferative activity of SB365 in human glioblastoma cell lines. Apoptosis was evaluated using the Hoechst assay, TUNEL assay, DAPI nuclear staining, and Western blotting analysis. To test the antimetastatic capacity of SB365, cell migration assay was conducted, and hypoxia-inducible factor-1 alpha (HIF-1α) expression and vascular endothelial growth factor (VEGF) level were determined under hypoxic conditions.
STATICAL ANALYSIS: Significance of the results was confirmed by a one-way analysis of variance analysis.
RESULTS: SB365 treatment suppressed the growth of glioblastoma cells and resulted in apoptotic morphological features such as nuclear condensation and fragmentation, enhancing the expression of cleaved poly (ADP-ribose) polymerase and caspase-3. It also significantly delayed cell migration and decreased the HIF-1α expression and VEGF secretion.
CONCLUSION: Our findings thus demonstrate that SB365 induced apoptosis and delayed the growth and migration of human glioblastoma cells. It is considered that SB365 would be a promising therapeutic option for glioblastoma.
Keywords: Apoptosis, glioblastoma, hypoxia-inducible factor-1 alpha, SB365, vascular endothelial growth factor
tumor, clinical trials have targeted the VEGF signaling pathway.\textsuperscript{[7]}

Traditional natural compounds can provide interesting therapeutic alternatives. Over the past few decades, numerous traditional natural compounds have been reported to induce apoptosis of cancer cells in preclinical studies.\textsuperscript{[8]} Among them, Pulsatilla koreana, a hairy, tufted, perennial herb, is used as a traditional Korean herbal medicine.\textsuperscript{[9]} It contains several medically active constituents including saponin D (hereafter called SB365) which has antitumor activities with antiangiogenesis.\textsuperscript{[10]}

Therefore, we studied the anticancer activity of SB365 in glioblastoma cells.

**Methods**

**Cell proliferation assay**

Human glioblastoma cells (U87MG, A172, and T98G) were cultured in DMEM (Dulbecco’s Modified Eagle’s Medium) containing 10% fetal bovine serum and 1% antibiotics (Invitrogen, Carlsbad, CA, USA). The cells were seeded and then incubated overnight, and SB365 was added for 72 h at 37°C. 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in fresh media was incubated for 4 h, followed by dimethyl sulfoxide (DMSO) addition. The optical density of each well was then read at 540 nm. The reduction in cell viability was expressed as percentage compared with DMSO alone.

**Assessment of apoptotic morphology**

The adherent cells were fixed with paraformaldehyde after SB365 treatment, followed by staining with Hoechst 33258 at 37°C for 30 min. They were observed under a fluorescence microscopy at ×400 magnification.

**DAPI and TUNEL staining assay**

Cells were seeded into a cover glass and treated with 10 µM SB365 for 24 h, and fixed in paraformaldehyde, and stained with 4',6-diamidino-2-phenylindole (DAPI) (2 µg/ml). The cells were observed for fluorescent nuclear fragmentations, and TUNEL staining was performed.

**Wound migration assay**

The cells were seeded, and a linear wound was made at 90% confluency using a 200-µl pipette tip. After washing with Dulbecco’s Phosphate-Buffered Saline (DPBS), the monolayer was treated with SB365 for 24 h. They were washed and fixed in methanol, and the migrated cells were measured by magnifying ×40 under a phase-contrast microscope.

**ELISA assay**

To determine the VEGF proteins secreted into the media, the plates were coated with anti-VEGF antibody and were incubated with 1% bovine serum albumin. Conditioned media were incubated for 2 h with biotinylated anti-VEGF antibody, and the plates were embedded with horseradish peroxidase (HRP)-conjugated streptavidin for 30 min. After adding 2N H\textsubscript{2}SO\textsubscript{4}, the absorbance was determined by a microplate reader at 450 nm.

**Western blotting**

The cells were lysed using Radioimmunoprecipitation assay buffer (RIPA), and the proteins were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and incubated with the primary antibodies such as HIF-1, cleaved poly (ADP-ribose) polymerase (PARP), cleaved caspase-3, and actin. After the incubation with subsequent secondary antibodies, the bands were detected using the enhanced chemiluminescence plus system (Amersham Biosciences).
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Figure 2: Effect of SB365 on apoptosis in glioblastoma cells. (a and b) The induction of apoptosis by SB365 treatment was examined by DAPI and TUNEL staining, followed by imaging at ×400 magnification. Arrows indicate morphological features of apoptotic cells, such as fragmented and condensed nuclei in SB365-treated cells. (c) The expression of cleaved caspase-3 and cleaved poly (ADP-ribose) polymerase was determined by Western blotting in SB365-treated cells at the indicated doses for 72 h.

Figure 3: Apoptotic morphology after SB365 treatment in glioblastoma cells. Cells were stained with the Hoechst 33342 DNA-binding fluorescent dye after SB365 treatment for 72 h. In morphological analysis, SB365-induced apoptosis recognized as nuclear chromatin condensation and fragmentation in U87MG and A172 glioblastoma cells. Data are represented as the mean ± standard deviation from three independent experiments ×400 magnification. *P < 0.05 and ***P < 0.001 versus control group.
Statistical methods
SPSS software (version 10.0: SPSS, Chicago, IL, USA) for Windows was applied for statistical analysis, and all bar graphs were expressed as the means and standard deviation.

Results

Cell growth
After glioblastoma cells (U87MG, A172, and T98G) were exposed to indicated concentrations of SB365, the anticancer effect of SB365 was determined with MTT assay. SB365 inhibited the cell growth dose dependently in glioblastoma cells [Figure 1].

Apoptosis of glioblastoma cells
In DAPI staining, SB365-treated cells showed apoptosis-typical changes including fragmented and condensed nuclei [Figure 2a]. The phenomena were confirmed by TUNEL staining for DNA fragmentation [Figure 2b]. Moreover, Western blotting analysis showed the increase of cleaved PARP and cleaved caspase-3 in a dose-dependent manner [Figure 2c]. From staining with Hoechst 33258 under fluorescence microscope, control cells showed round nuclei, whereas apoptotic cells were increased after SB365 treatment, displaying perinuclear apoptotic bodies and bright nuclear condensation [Figure 3].

Migration and invasion of glioblastoma cells
To assess the antimetastatic property of SB365, migration assay was performed using glioblastoma cells. U87MG and A172 cells were exposed to various SB365 doses for 24 h. As a result, the control group showed high migration to the wound area, whereas SB365 significantly suppressed cell migration [Figure 4].

Hypoxia-inducible factor-1 alpha and vascular endothelial growth factor expression
When the A172 glioblastoma cells were treated with SB365 in hypoxia-mimicking condition, the upregulated HIF-1α expression was blocked in the glioblastoma cells [Figure 5a]. In the ELISA study to measure VEGF secretion, SB365 suppressed the...
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Increased VEGF secretion in a dose-dependent manner [Figure 5b].

Discussion

Despite advances in treatment modalities, glioblastoma still remains an incurable disease. Due to the nature of glioblastoma, angiogenesis has emerged as a major target for drug development against glioblastoma over the past decade. Bevacizumab got accelerated approval for recurrent glioblastoma in the United States, and its combination with irinotecan showed effective outcomes in a clinical study.\[11\] Other anti-VEGF inhibitors have also shown promising outcomes in preclinical studies.\[12\] However, in a recent REGAL trial, an oral pan-VEGF RTK inhibitor did not improve survival outcomes in recurrent glioblastoma patients compared to lomustine.\[7\] The CENTRIC EORTC 26071–22072 study also reported that the combination of cilengitide and temozolomide did not prolong survival outcomes in newly diagnosed glioblastoma patients.\[13\] Other randomized Phase II clinical trials have also shown that anti-VEGF agents did not show any clinical benefits.\[14,15\] Therefore, further studies are needed to overcome the limitations of current treatment modalities.

Natural products have several advantages as anticancer agents. They have relatively tolerable side effects and synergistic effects with cytotoxic chemotherapy.\[16\] Particularly, natural products such as curcumin and ginger have shown promising anticancer effects in various cancer cell lines.\[17,18\] In the past, the dissonance of medicines from natural compounds was a time-consuming procedure. However, finding active compounds from plants has now been accelerated by modern techniques. Therefore, great efforts are underway to discover active natural compounds in order to treat cancer.\[17\]

SB365 has been shown to exhibit its antitumor effects through apoptosis and antiangiogenesis in hepatocellular carcinoma, pancreatic cancer, and colon cancer.\[19–21\] In this study, the growth of glioblastoma cells was suppressed by SB365 treatment by 40%–70%. Our results also show that apoptosis, evident by Hoechst staining, DAPI staining, and TUNEL assay, was observed in SB365-treated glioblastoma cells.\[22\] In addition, apoptosis by SB365 was reconfirmed by the increase in cleaved PARP and caspase-3 levels.

Hypoxia-induced necrosis and neovascularization are also major pathognomonic features of glioblastoma. The hypoxic condition in glioblastoma activates the expression of genes such as HIFs and VEGF, which promote more aggressive phenotypes such as tumor growth, angiogenesis, migration, and metastasis.\[23,24\] In particular, inhibition of HIF-1\(\alpha\) may induce apoptosis and prevent tumor progression.\[24\] Here, SB365 inhibited the migration of glioblastoma cells and the HIF-1\(\alpha\) expression and VEGF secretion, which are clearly akin to previous literatures that SB365 reduced angiogenesis.\[19–21\]

Our study has some limitations. First, it was conducted in vitro in cell lines, and further, in vivo study is necessary to confirm these results. However, to our knowledge, this is the first report, indicating that SB365 has an anticancer effect in glioblastoma cells.

Conclusion

SB365 demonstrates anticancer activity against glioblastoma cells by apoptosis and antiangiogenesis suppressing cell growth. These findings indicate that SB365 could be a good natural candidate for the therapy of brain tumors.

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