GSK-3 inhibitor inhibits cell proliferation and induces apoptosis in human osteosarcoma cells

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Abstract. Glycogen synthase kinase-3 (GSK-3) is a serine/threonine kinase that functions in numerous signaling pathways initiated by diverse stimuli. The functions of GSK-3 in cancer differ depending on cell type. In the present study, we examined the effects of a specific GSK-3 inhibitor on the regulation of osteosarcoma cell proliferation and apoptosis. Immunohistochemical analysis and real-time reverse transcription-polymerase chain reaction (RT-PCR) were performed to determine the expression pattern of GSK-3 in human osteosarcoma cells. We used the MTS assay, western blotting, measurement of single-stranded DNA and morphological analyses to study the effects of a GSK-3 inhibitor, SB216763 on osteosarcoma cell proliferation and survival. We detected an increase in mRNA expression of GSK-3 and aberrant nuclear accumulation of GSK-3 in the osteosarcoma cells. Pharmacological inhibition of GSK-3 led to a decrease in proliferation and survival of osteosarcoma cells. Inhibition of GSK-3 resulted in a decreased expression of Bcl-2 and a subsequent increase in osteosarcoma cell apoptosis via the mitochondrial pathway. The present study demonstrated that GSK-3 activity is critical for tumorigenicity and cell survival in osteosarcoma cells. Our findings suggest that GSK-3 is a potential therapeutic target for the treatment of human osteosarcoma.

Introduction

Osteosarcoma is the most frequently diagnosed primary malignant bone tumor in children and adolescents (1,2). The development of new surgical and screening technologies, in combination with neoadjuvant chemotherapy has led to great progress in osteosarcoma therapy; however, ~40-50% of adolescent patients eventually develop lung metastasis, and relapse is a common problem in ~80% of patients with

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metastasis at diagnosis (3). Current therapies typically lead to chemoresistance, and thus no significant change in the survival rate has been observed in recent decades. Therefore, new therapeutic strategies need to be developed. Molecular-targeting drugs, which have selective inhibitory effects on a wide variety of factors involved in tumor proliferation, migration and metastasis, including growth factor and intracellular signaling factors have recently been developed for other human malignancies (4).

Glycogen synthase kinase-3 (GSK-3) is a serine/threonine protein kinase that functions in numerous signaling pathways initiated by diverse stimuli. Two highly homologous isoforms of GSK-3, GSK-3α and GSK-3β, are found in mammalian cells (5). GSK-3 plays a role in a great number of cellular and physiological processes, including protein synthesis, glycogen metabolism, cell cycle division, apoptosis, cell fate determination and stem cell maintenance (6-9). The dysregulation of this protein is associated with a variety of disorders, including neurodegenerative diseases and cancers (5,8). It is primarily known for its role in the Akt and Wnt signaling pathway, where it acts as a negative regulator of the Wnt effector molecule β-catenin (7). GSK-3-mediated phosphorylation of β-catenin has also been shown to lead to ubiquitination and subsequently proteasomal degradation (10). GSK-3 suppresses the transcription of several protooncogenes, such as c-myc and cyclin D1, in a wide variety of tumors, and thus it was originally identified as a tumor suppressor; however, the functions of GSK-3 in cancer differ depending on cell type.

Recent research on numerous types of cancer, including prostate, colorectal, pancreatic, ovarian, renal cell carcinoma, multiple myeloma and leukemia, has shown that GSK-3 is involved in tumorigenesis, and that inhibition of the expression and activity of GSK-3 attenuates cell proliferation (11-19). The inhibition of GSK-3 has also been shown to induce apoptosis by suppressing the NF-κB signaling pathway, and various findings appear to suggest that GSK-3 plays a positive role in the regulation of NF-κB activity (13,19,20). Thus, the biological functions and contradictory roles of GSK-3 in apoptosis should be assessed in each type of tumor. A recent study showed that GSK-3 activity may promote osteosarcoma cell growth (21), but its molecular mechanisms when using specific GSK-3 inhibitor treatment have yet to be fully elucidated.

In the present study, with the aim of evaluating the effects of a specific GSK-3 inhibitor on human osteosarcoma cells,

we examined whether cell proliferation was inhibited and apoptosis was induced in human osteosarcoma cells by the GSK-3 inhibitor.

Materials and methods

Chemical reagents. SB216763, a highly specific GSK-3 inhibitor that does not significantly inhibit other kinases (22), was purchased from Sigma Chemical Co. (St. Louis, MO, USA), was dissolved in dimethylsulfoxide and stored at -20˚C. Anti-Akt and anti-phospho-Akt antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-mTOR and anti-phospho-mTOR antibodies were obtained from R&D Systems (Minneapolis, MN, USA). Anti-GSK-3β, anti-phospho-GSK-3β, anti-NF-κB, anti-Bcl-2, anti-cleaved caspase-9, anti-cleaved caspase-3, anti-cleaved PARP and ant-α-tubulin antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Cell lines and cell culture. In the present study, we used three human osteosarcoma cell lines (KTHOS, KHOS and MG63). The KTHOS cell line was established by Hitora *et al* (23), while KHOS and MG63 were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 100 U/ml penicillin (all from Sigma-Aldrich, St. Louis, MO, USA). All cells were routinely maintained at 37˚C in a humidified 5% CO_2 atmosphere, and cultures were harvested at mid-log phase.

Specimens. In the present study, we used a total of eight human bone and soft tissue tumor samples: three osteosarcomas, a schwannoma, an enchondroma, a lipoma, a giant cell tumor and a hemangioma. All sample specimens, which had originally been diagnosed by pathologists based on histological data, were surgically obtained between 2009 and 2015 from the Department of Orthopaedic Surgery, Kagawa University Hospital in accordance with institutional guidelines. Informed consent was obtained from all patients or their parents or guardians before the samples were collected. All procedures performed in the studies involving human participants were carried out in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Real-time reverse transcription-polymerase chain reaction (RT-PCR). All surgically obtained specimens were immediately stored at -80˚C until use. First, we evaluated mRNA expression of GSK-3 in the samples. Isogen (Nippon Gene, Tokyo, Japan) was used to extract total RNA from the cells, which was then reverse-transcribed into cDNA using high-capacity cDNA reverse transcription kits (Applied Biosystems, Foster City, CA, USA) for RT-PCR. Real-time quantitative PCR analysis was run on the Eco™ Real-Time PCR system (Illumina Inc., San Diego, CA, USA) using Power SYBR®-Green PCR Master Mix (Applied Biosystems). The primers for real-time PCR were synthesized and validated by Hokkaido System Science Co., Ltd. (Sapporo, Japan). The primers for

GSK-3 were as follows: forward, 5'-ACAGCAGCGTCAGAT GCTAA-3' and reverse, 5'-GGGACTGTTCAGGTGGAG-3'.

Immunohistochemical analysis. To determine GSK-3 expression, all surgically obtained specimens were fixed in 10% formalin, embedded in paraffin and coded. An osteosarcoma, a giant cell tumor, and a schwannoma specimen were chosen, and tissue slides were deparaffinized in xylene for two changes of 5 min each. Sections were hydrated gradually through a graded-alcohol series with 100, 95, 90, 80 and 70% ethanol solutions for 2 min in each solution, and samples were reactivated by the pressurization of sections in citric buffer (pH 7.0) for 10 min, and then treated with 3% hydrogen peroxide; non-specific sites were blocked with 3% bovine serum albumin. Next, samples were incubated for 60 min with the primary antibody for GSK-3 (code no. 12456; Cell Signaling Technology, Inc., Beverly, MA, USA). After rinsing in phosphate-buffered saline (PBS), samples were incubated with secondary anti-rabbit IgG antibody (code no. 424142; Nichirei Biosciences, Inc., Tokyo, Japan) for 30 min. The staining reaction was carried out with diaminobenzidine, and the sections were counterstained with hematoxylin.

In vitro proliferation assay. Cell proliferation was assessed using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS assay; Promega, Madison, WI, USA). Cells were trypsinized and seeded at a density of ~1x10⁴ cells/well in 96-well cell culture plates with 200 μl of culture medium containing 10% FBS and incubated for 48 h. Following the initial incubation, the growth medium was replaced with medium containing 10% FBS and SB216763 at concentrations of 0, 1, 5, 25 or 50 μM. After 24 and 48 h, the medium was removed, cells were washed with PBS and fresh medium containing MTS reagent was added to each well (100 μl medium plus 20 μl MTS regent/well). After 2 h of further incubation, an automatic microplate reader (SpectraMax® Plus 384 microplate reader; Molecular Devices, Sunnyvale, CA, USA) was used to measure the optical density of each well at 490 nm (absorbance is directly proportional to the number of living cells). The proliferation of cells in each well was calculated as a percent of the control, and a minimum of three independent experiments were performed for each assay.

Western blot analysis. We used the MG63 cell line in the present study. First, cells were trypsinized and seeded at a density of 6x10⁵ cells/well in 6-well cell culture plates with 2 ml of culture medium containing 10% FBS. After 48 h, the cells were treated with 10% FBS containing SB216763 at concentrations of 0, 1, 5, 10, 25 or 50 μM for 24 h. After treatment, the culture medium was replaced with lysis buffer (Cell Signaling Technology, Inc.), and cells were lysed on ice for 20 min. The cell lysates were then centrifuged at 15,000 x g (Tomy MTX-150; Tomy Seiko Co., Ltd., Fukuoka, Japan) at 4˚C for 30 min. The supernatant was then collected as the total cellular protein extract. A BCA protein assay kit (Pierce, Rockford, IL, USA) was used to determine protein concentrations, which were then standardized to bovine serum albumin. The total cellular protein samples were loaded onto sodium dodecyl sulfate (SDS) polyacrylamide gels (7.5, 10 or 12.5% commercial precast gel; Wako, Tokyo, Japan), and the proteins

were separated using SDS-polyacrylamide gel electrophoresis (PAGE) under reducing conditions, and electrophoretically transferred to nitrocellulose membranes (GE Healthcare Bio-Sciences, Piscataway, NJ, USA). Next, the membranes were blocked for 90 min in blocking buffer containing Tris-buffered saline (TBS-T) and EzBlock Chemi (Atto Co., Tokyo, Japan), and were then incubated overnight at 4°C with primary antibodies that were diluted in blocking buffer. Specific horseradish peroxidase (HRP)-conjugated secondary antibody (all used the same antibody: from rabbits) was used and incubations were performed overnight at 4°C with gentle agitation. The ECL Plus Western Blotting Detection System (GE Healthcare Bio-Sciences) and an LAS-1000 Plus image analyzer (Fuji Film Co., Tokyo, Japan) were used to detect the bound antibodies. Specific signals were quantified using densitometric analysis (ImageJ Software; NIH, Bethesda, MD, USA).

Measurement of single-stranded DNA. DNA in apoptotic cells is sensitive to formamide; therefore, a monoclonal antibody against single-stranded DNA using an ApoStrand™ ELISA apoptosis detection kit (Enzo Life Sciences, Plymouth Meeting, PA, USA) was used according to the manufacturer's instructions to detect denatured DNA. Cells were seeded into 96-well cell culture plates in culture medium containing 10% FBS. After 24 h, the medium was replaced with fresh medium containing 1% FBS and SB216763 at concentrations of 25 or 50 μM and the cells were then incubated for 24 h. Next, they were fixed, dried and attached to the plate surface, and then treated with formamide. Cells were then incubated with an antibody mixture for 30 min after non-specific binding sites were blocked. Peroxidase substrate was added to each well after washing, and absorbance was measured at 405 nm using an automatic microplate reader.

Fluorescence microscopic images of the Annexin V, ethidium homodimer III and Hoechst 33342 triple-staining assay for detection of apoptosis. Cells from the MG63 cell line were trypsinized and seeded at a density of ~1x10^6 cells/well on 25-mm circular coverslips in 2 ml of culture medium with 10% FBS. After 48 h, the cells were washed with PBS and treated with SB216763 at concentrations of 0, 5, 25 or 50 μM for 24 h. We then used Annexin V, ethidium homodimer III and Hoechst 33342 triple-staining assay to detect the apoptotic cells by using the PromoKine Apoptotic/Necrotic/ Healthy Cells Detection kit (PromoCell GmbH, Heidelberg, Germany), according to the manufacturer's recommendations. Stained cells were observed by an epifluorescence microscope (FSX100; Olympus Optical Co., Tokyo, Japan).

Statistical analysis. One-way or two-way analysis of variance (ANOVA) followed by post hoc analysis were used for the statistical evaluation of real-time PCR and measurement of single-stranded DNA. The significant difference of the cell proliferation assay curves was analyzed using log IC$_{50}$ statistically. All values are expressed as means ± standard deviation, and P<0.05 was considered to indicate a statistically significanct result using GraphPad Prism 5 (GraphPad, San Diego, CA, USA). All data were collected from at least three independent experiments for each group.
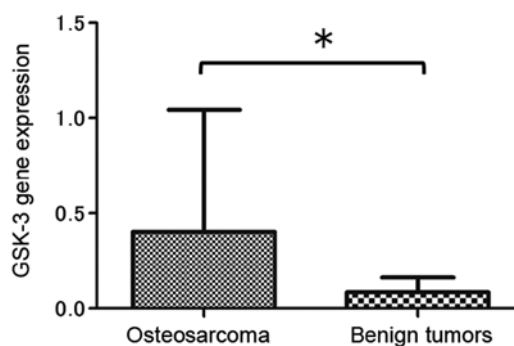

Figure 1. mRNA expression of GSK-3 in the surgically obtained specimens. Compared with that in the benign bone and soft tissue tumor samples, GSK-3 expression was increased in the osteosarcoma cells. Statistical significance by two-way ANOVA is shown (*P<0.05).

Results

Real-time PCR. mRNA expression of GSK-3 was evaluated in all surgically obtained specimens. Compared with that in the benign bone and soft tissue tumor samples, GSK-3 expression was increased in the osteosarcoma cells (Fig. 1); this indicated that a high level of GSK-3 expression is a feature of osteosarcoma.

Immunohistochemical analysis. Using immunohistochemical staining, we found aberrant nuclear accumulation of GSK-3 in the human osteosarcoma cells in comparison with the giant cell tumor and schwannoma specimens (Fig. 2).

SB216763 inhibits the cell proliferation of osteosarcoma cells. In our examination of the effect of SB216763 on the proliferation of osteosarcoma cells (KTHOS, KHOS and MG63), we found that SB216763 showed a dose- and time-dependent inhibitory effect on all osteosarcoma cell lines (Fig. 3). The IC$_{50}$ value at 48 h of SB216763 treatment was 11.75 μM in the KTHOS cells 36.24 μM in the KHOS cells and 26.68 μM in the MG63 cells.

Western blot analysis. MG63 cells, in which the IC$_{50}$ value for SB216763 was between those of KTHOS and KHOS cells, were used for western blot analysis. We found that SB216763 treatment resulted in a decrease in GSK-3β, phospho-GSK-3β, NF-κB and Bcl-2 levels in the MG63 cells (Fig. 4); Bcl-2 plays a major role in the suppression of apoptosis and is regulated by NF-κB. Next, to determine whether SB216763 induces caspase-dependent apoptosis in MG63 cells, we examined the effect of SB216763 on caspase activity. Western blot analysis indicated that treatment with SB216763 at concentrations ranging from 1 to 50 μM for 24 h resulted in the cleavage of poly(ADP-ribose) polymerase (PARP), as well as the activation of caspase-9 and caspase-3 in the MG63 cells in a dose-dependent manner (Fig. 4). These results suggested that SB216763 has the ability to induce apoptosis in a caspase-dependent manner in the MG63 cells.

We also examined the expression levels of Akt/mTOR signaling pathway-related proteins. Although phospho-Akt expression was increased in a dose-dependent manner, SB216763 treatment did not increase Akt, mTOR and
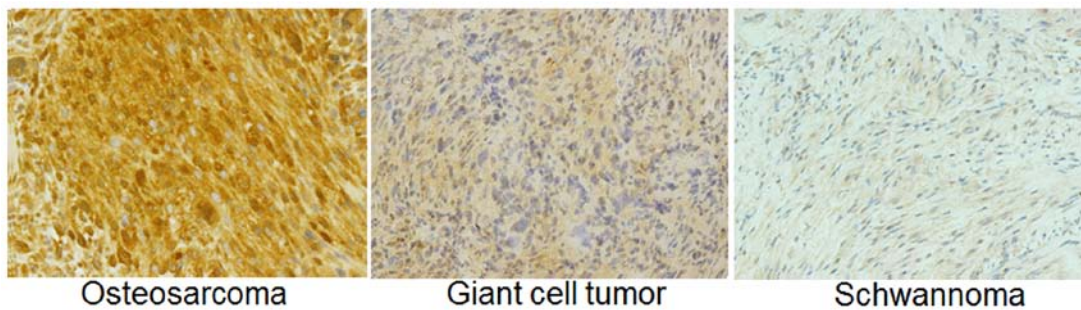
Figure 2. Immunohistochemical staining of GSK-3 expression in osteosarcoma cells, giant cell tumor cells, and schwannoma cells. Aberrant nuclear accumulation of GSK-3 in human osteosarcoma cells were increased in comparison with giant cell tumor and schwannoma.
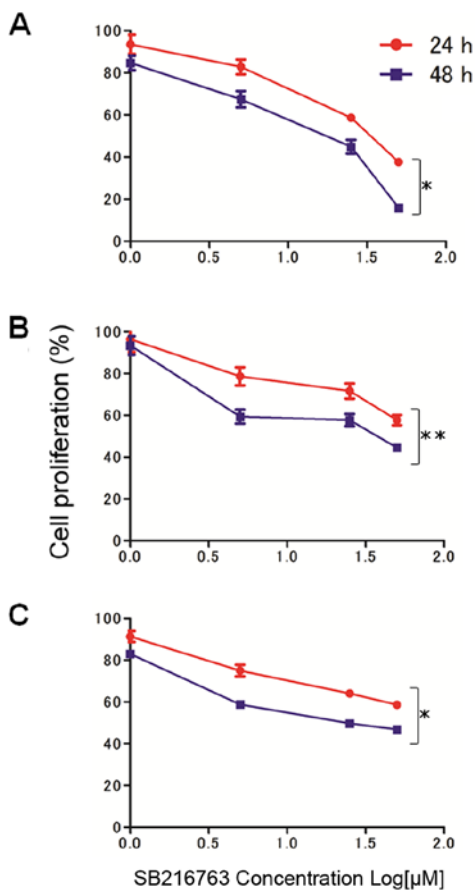

Figure 3. Cell proliferation assay for investigating the effect of SB216763 on the proliferation of osteosarcoma cells: (A) KTHOS, (B) KHOS and (C) MG63. SB216763 showed a dose- and time-dependent inhibitory effect on all osteosarcoma cell lines. Statistical significance were analyzed by log IC_{50} (*P<0.005, **P<0.001).

phospho-mTOR levels in the MG63 cells (Fig. 4). These findings indicate that GSK-3 is involved in MG63 cell proliferation via Akt/mTOR pathway-independent mechanisms.

Measurement of single-stranded DNA. For the determination of cellular apoptosis, we used a single-stranded DNA ELISA assay to examine whether SB216763 increases the number of apoptosis-induced cells. SB216763 treatment resulted in the induction of cellular apoptosis in all osteosarcoma cell lines. The high-dose treatment increased apoptosis in the KTHOS

cells more than the low-dose treatment. In contrast, no statistically significant differences were observed between the 25- and 50-μM doses in the other two cell lines (Fig. 5).

Fluorescence microscopy images. We then used Annexin V, ethidium homodimer III and Hoechst 33342 triple-staining assay to detect apoptotic cells. Hoechst 33342-positive cells (blue) were live cells, and Annexin V-FITC (green) is a marker for early apoptosis and ethidium homodimer III (red) is a marker for late apoptosis and necrosis. We observed several Annexin V-FITC-positive cells (early stage of apoptosis) following SB216763 treatment in a dose-dependent manner in the MG63 cells (Fig. 6).

Discussion

Osteosarcoma is a tumor characterized by genetic alterations in the signaling pathways involved in growth and development. Although a number of anticancer drugs, including methotrexate, cisplatin, doxorubicin, etoposide and cyclophosphamide are commonly used in combination to treat patients with osteosarcoma (24), a substantial proportion of these patients develop drug resistance and subsequently die due to disease progression. Therefore, for the improved management of patients with osteosarcoma, it is extremely important to identify the molecular mechanisms of this malignancy.

Although GSK-3, a pluripotent serine/threonine kinase with a large number of intracellular target proteins, has traditionally been recognized as a tumor suppressor inactivated in a variety of tumors, such as oral (25), skin (26) and larynx cancer (27), the functions of GSK-3 in cancer differ depending on cell type. These differing functions create the potential for GSK-3 to exert apparently cell- and context-dependent pro-apoptotic and anti-apoptotic effects (28). In the present study, GSK-3 was identified as a positive regulator of osteosarcoma cell survival and proliferation, and SB216763 was shown to inhibit the proliferation of human osteosarcoma cells via the induction of apoptosis.

In addition, mRNA expression of GSK-3 was found to be increased in osteosarcoma cells, and immunohistochemical analysis revealed aberrant nuclear accumulation of GSK-3 in human osteosarcoma cells. These findings are supported by those of previous studies that found nuclear overexpression of GSK-3 in pancreatic cancer, renal cell carcinoma and leukemia (18,29,30).
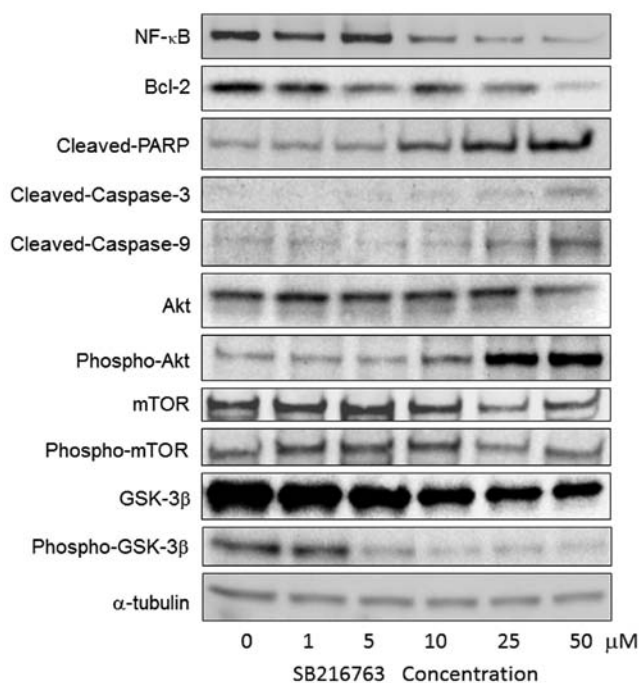
Figure 4. Western blot analysis. SB216763 treatment resulted in a decrease in GSK-3β, phospho-GSK-3β, NF-κB and Bcl-2 levels in the MG63 cells and resulted in the cleavage of PARP as well as the activation of caspase-9 and caspase-3 in a dose-dependent manner. Phospho-Akt expression was increased in a dose-dependent manner, while Akt, mTOR and phospho-mTOR expression were not increased in the MG63 cells following treatment with SB216763.

Figure 6. Fluorescent staining for detection of apoptosis. Apoptotic cells were detected by Annexin V, ethidium homodimer III and Hoechst 33342 triple-staining assay. Cells were stained blue (Hoechst 33342, living cells); green (Annexin V, early apoptotic cells); and red (ethidium homodimer III, late apoptotic cells). We observed several Annexin V-FITC-positive cells (early stage of apoptosis) following SB216763 treatment in a dose-dependent manner in the MG63 cells.
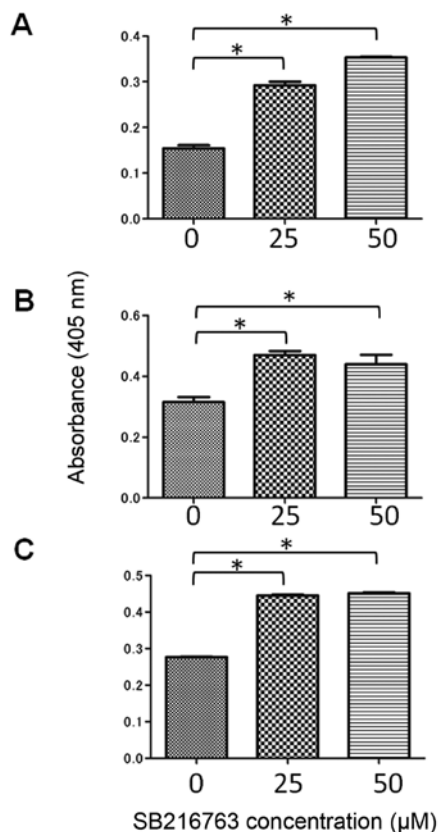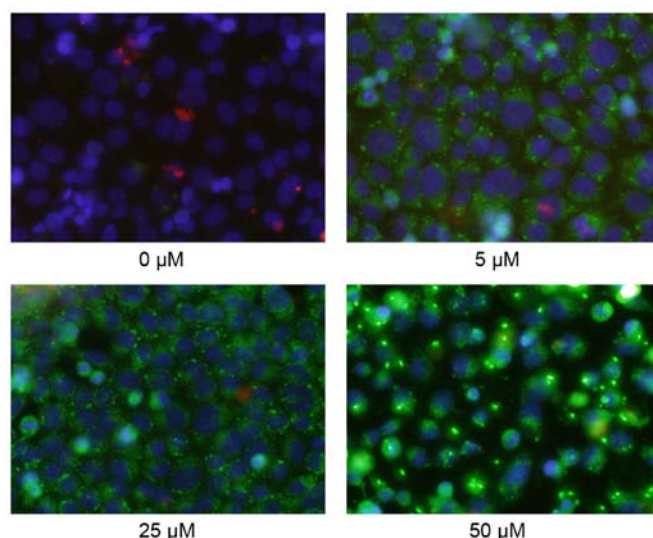

Figure 5. Measurement of single-stranded DNA in the KTHOS, KHOS and MG63 cells. (A) KTHOS, (B) KHOS and (C) MG63. SB216763 treatment resulted in the induction of cellular apoptosis in all osteosarcoma cell lines. Statistical significance by one-way ANOVA is shown (*P<0.05).

SB216763 was also shown to inhibit the proliferation of human osteosarcoma cells in a time- and dose-dependent manner using an MTS assay. SB216763 treatment suppressed GSK-3 and phospho-GSK-3 expression and led to a decrease in NF-κB and Bcl-2 in MG63 cells based on western blot analysis. Bcl-2, the transcriptional target of NF-κB, participates in the regulation of cell apoptosis (31). The NF-κB family of transcription factors is involved in the activation of a wide range of genes associated with inflammation, differentiation, tumorigenesis, metastasis, embryonic development and apoptosis (32-34). These genes are activated in response to extracellular stimuli such as inflammatory cytokines and growth factors. In addition, a previous study has shown that Bcl-2 family proteins are important in the regulation of apoptosis (35). In the present study, SB216763 inhibited osteosarcoma cell proliferation by downregulating the expression of GSK-3/NF-κB signaling pathway proteins.

Based on results from single-stranded DNA and fluorescence microscopy analysis, SB216763 treatment induced apoptosis in all osteosarcoma cell lines. Apoptosis plays an important role in cell growth and tissue development, and can be induced by a variety of signals either inside or outside of the cell (36,37). A key mechanism of anticancer therapy is the induction of apoptosis, which is regulated by various factors and signaling pathways, such as the endoplasmic reticulum, the mitochondrial and the death ligand pathway (38), in target cells. The mitochondrial pathway is activated in response to the activation of Bcl-2, and in turn, activates caspase-9 and caspase-3 in the downstream signaling pathways of Bcl-2 (39). Caspase-9 is a key member of the cysteine-aspartic acid protease family, and cleaved caspase-9 subsequently processes other caspase members, including caspase-3, to initiate a caspase cascade, which leads to apoptosis (40). Caspase-3 is responsible for the proteolytic cleavage of many key proteins, such as the nuclear

enzyme PARP (41), and is thus a critical executioner of apoptosis. PARP, which helps maintain cell viability, is one of the primary cleavage targets of caspase-3, and cleavage of PARP facilitates cellular disassembly and serves as a marker for cells undergoing apoptosis (42). Based on the results of the western blot analysis, which we performed in order to detect the expression levels of apoptosis-associated proteins, SB216763 treatment was shown to result in a dose-dependent increase in the expression levels of cleaved caspase-9, cleaved caspase-3 and cleaved PARP protein in the MG63 cells. These results suggest that SB216763 treatment suppresses the expression of the anti-apoptotic protein Bcl-2, and induces apoptosis in osteosarcoma cells via the mitochondrial pathway.

In the present study, SB216763 treatment was shown to result in a dose-dependent increase in phospho-Akt based on western blot analysis. Akt inhibits apoptosis through the phosphorylation and inactivation of several targets, including caspase-9, and thus promotes cell survival. These results suggest that feedback from the induction of apoptosis and GSK-3 inhibition increases the expression of phospho-Akt; however, SB216763 treatment did not appear to lead to increases in mTOR and phospho-mTOR in the MG63 cells. Thus, it appears that GSK-3 is involved in osteosarcoma cell proliferation via Akt/mTOR pathway-independent mechanisms. Therefore, the control of these signaling pathways is only one of many GSK-3 functions. These findings are supported by those in previous studies reporting that deregulated expression and activity of GSK3 in colorectal and prostate cancer contribute to cancer cell survival and proliferation in a manner unrelated to Akt/mTOR signaling activation (11,17). SB216763 inhibits GSK-3 in an ATP competitive manner (21). It is suggested that SB216763 increases phospho-Akt which is a molecule of the upper reaches of GSK-3 and suppresses the downstream signal transmission by clearly inhibiting GSK-3.

In conclusion, the findings from the present study appear to provide evidence of a positive association between GSK-3 activity and tumorigenicity, and that GSK-3 is crucial for the survival of osteosarcoma cells. In addition, SB216763 appeared to inhibit the proliferation of osteosarcoma cells in a dose- and time-dependent manner. The inhibition of GSK-3 resulted in the induction of caspase-dependent apoptosis through a decrease in NF-κB and Bcl-2 expression in osteosarcoma cells. Based on these results, GSK-3 may be a potential novel therapeutic target for the treatment of human osteosarcoma.

Acknowledgements

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