GSK-3 Inhibitor Inhibits Cell Proliferation And Induces Apoptosis In Human Osteosarcoma Cells

Hideki Nishimura.
Kagawa University School of Medicine, Kita-gun, Japan.

Disclosures:  H. Nishimura: None.

Introduction: Osteosarcoma (OS) is the most common primary malignant bone tumor and is characterized by an extremely aggressive clinical course with rapid metastatic development. Due to the development of multidisciplinary treatment protocols, long-term local control of OS and patient survival rates have improved significantly. However, a considerable number of OS patients can develop drug resistance and die because of disease progression. Therefore, new therapeutic strategies need to be evaluated to improve the OS survival rate.

Glycogen synthase kinase-3 (GSK-3) is a serine/threonine kinase that functions in numerous signaling pathways initiated by diverse stimuli. Originally studied for its role in glycogen metabolism and insulin action, GSK-3 has subsequently been shown to have central functions in many cellular and physiological processes including transcription, cell cycle division, apoptosis, cell fate determination and stem cell maintenance. Its dysregulation is implicated in many disorders, such as neurodegenerative diseases and cancers. However, the function of GSK-3 in cancer can differ depending on cell type. One of the most well-known substrates of GSK-3, β-catenin, is an important regulator of the Wnt-β-catenin signaling pathway.

Here we examined the effect of a specific GSK-3 inhibitor on the regulation of OS cells proliferation and apoptosis.

Methods: Cell lines and reagent;
Three human osteosarcoma cell lines (KTHOS, KHOS and MG63) were used in this study. All cell lines were grown in culture medium consisting of Dulbecco’s Modified Eagle Medium (DMEM; Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich). The cell lines were routinely maintained at 37°C in a humidified 5% CO2 atmosphere. SB216763 (Sigma-Aldrich) was used for GSK-3 inhibitor. SB216763 are highly specific GSK-3 inhibitors which does not significantly inhibit other kinases.

In vitro proliferation assay
Cell proliferation was determined by CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA). Cells were trypsinized and seeded at a density of approximately 1 × 104 cells/well in 96-well cell culture plates with 200 µl culture medium containing 10% FBS and incubated for 48h. Following this initial incubation, the growth medium was replaced with medium containing 10% FBS and SB216763 at a concentration of 0, 1, 5, 25, or 50µM. After 24h and 48h, the medium was removed, the cells were washed with phosphate-buffered saline, fresh medium containing MTS reagent was added to each well. The optical density was measured at 490 nm with an automatic microplate reader after 2 h of further incubation following the addition of the MTS reagent. Absorbency is directly proportional to the number of living cells.

Real-time reverse transcription-polymerase chain reaction (RT-PCR);
Total RNA was extracted from the cells by using ISOGEN (Nippon Gene, Tokyo, Japan). RNA was then reverse-transcribed into cDNA by using High Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA, USA) for RT-PCR. The real-time quantitative PCR analysis was performed on an EcoTM Real-Time PCR System (Illumina, Inc., San Diego, CA, USA) by using Power SYBR® Green PCR Master Mix (Applied Biosystems). The primers (GSK-3) for real-time PCR were synthesized and validated by Hokkaido System Science Co., Ltd (Hokkaido, Japan).

Western blotting analysis;
For the western blotting analysis, cells were collected, and protein concentration was determined by the BCA assay. Equal amounts of protein were separated by SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were then blocked with Ez block chemi (ATTO Corporationan, Tokyo) and incubated by primary antibodies. Cleaved-PARP, Caspase-3, Caspase-9 and Cleaved-Caspase-9 (Cell Signaling Technology, Beverly, MA) were used as primary antibodies for detection of apoptosis. Then, The membranes were incubated by secondary antibodies and the blots were probed with the ECL system.

Apoptosis Detection;
For the assessment of apoptosis, the ApoStrand ELISA Apoptosis Detection Kit (Biomol, Plymouth Meeting, PA) which detects single-stranded DNA was used. Cells were seeded in 96-well cell culture plates in culture medium with 10% FBS. After 24h, the medium was refreshed with 1% FBS containing SB216763 in the indicated concentrations. After 24h, the medium was removed and the cells were fixed for 30 min and assayed according to the manufacturer's instructions.

Morphological analysis;
Cells were seeded and treated with GSK-3 inhibitor for 24h, apoptotic cells were evaluated. Cells were then incubated with Annexin-V-FITC and PI using Annexin-V- Fluos Staining Kit (Roche Applied Science, Penzberg, Germany) for 15 min in a dark room according to the manufacturer’s recommendations. Stained cells were observed under a fluorescence microscope.

**Results:** GSK-3 inhibitor inhibited cell proliferation of osteosarcoma cell lines;
SB216763 showed a dose- and time-dependent inhibitory effect on all cell lines. SB216763 inhibited cell proliferation of all cell lines at a concentration of 50M to less than 50% of control cell viability for 48h (Figure 1).

Real-time PCR;
mRNA expression of GSK-3 was evaluated in OS cells and other benign tumor cells. The expression of GSK-3 in OS cells was increased compared with benign tumor cells.

Western blotting;
Western blotting analysis revealed that Cleaved-PARP, Caspase-3, Caspase-9 and Cleaved-Caspase-9 protein expression levels were increased dose-dependent by SB216763 in MG63 cells. These proteins are particular marker for apoptosis (Figure 2).

Apoptosis Detection;
Apoptotic cells was detected dose-dependently by SB216763 in all cell lines.

Fluorescent microscopy images;
We used Annexin-V and PI to detect apoptotic cells. Annexin-V is a marker for early apoptosis, and PI is a marker for late apoptosis and necrosis. Annexin-V positive cells were increased by SB216763 treatment in all osteosarcoma cell lines (Figure 3).
**Discussion:** In this study, we demonstrated that GSK-3 activity is positively associated with clonogenicity and tumorigenicity and is critical for cell survival in osteosarcoma. We found that GSK-3 inhibitor inhibited the proliferation of osteosarcoma cell lines in a dose- and time-dependent manner. GSK-3 inhibitor induced apoptosis to all osteosarcoma cell lines. Fluorescent staining as well as Annexin V/PI analysis both showed a strong apoptosis-inducing effect of the compound. These results indicate that the anti-tumor activity of GSK-3 inhibitor in osteosarcoma cells is due to a G2/M arrest and apoptosis. GSK-3 signaling pathway is closely related to Wnt and Hedgehog pathway. So those pathways may affect the antitumor effect and the apoptosis induction. We conclude that therapeutic targeting of the GSK-3 pathways may be a promising strategy to enhance the therapeutic activity of anticancer drugs against osteosarcoma.

**Significance:** This study is very important because of evaluating the role of GSK-3 signaling pathway in human osteosarcoma cells. Therapeutic targeting of the GSK-3 pathways may be a promising strategy to enhance the therapeutic activity of anticancer drugs against osteosarcoma.