The Role of MAPK Pathway in Bone and Soft Tissue Tumors

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Abstract. *Aim: Expression of mitogen-activated protein kinase (MAPK) signaling and its role in cell proliferation of the bone malignancies, osteosarcoma (OS) and malignant fibrous histiocytoma (MFH) were investigated. Materials and Methods: Gene expression and protein levels of RAF1 and MEK1/2 in 6 human sarcoma cell lines and 7 surgically obtained OS specimens were assessed by RT-PCR and immuno-histochemistry, respectively. MEK inhibitor, U0126 [1,4-diamino-2,3-dicyano-1,4-bis (2-aminophynyltio) butadiene], was used for cell proliferation assays. Results: RAF1 and MEK 1/2 mRNA was detected in all cell lines and OS specimens. RAF1, MEK 1/2 and p-MEK protein was also expressed in the cells, as was MEK1/2 in OS specimens. Treatment with U0126 resulted in dose- and time-dependent inhibition of cell proliferation and suppression of p-ERK expression, opposite to promotion of p-MEK. Conclusion: U0126 blocks MAPK signaling and decreases cell proliferation in OS and MFH. Thus, selective MAPK inhibitors might be therapeutically advantageous in the treatment of bone and soft tissue sarcomas.*

Osteosarcoma (OS) is a malignant bone tumor that commonly affects adolescents and young adults, accounting for approximately 20% of primary bone malignancies in humans. Malignant fibrous histiocytoma (MFH) is the most common soft tissue sarcoma arising in late adult life. Advances in the treatment of both OS and MFH have led to multidisciplinary treatments that include surgery, chemotherapy and radiation therapy, all of which result in great improvement in the quality of life for patients with sarcomas (1, 2). However, current chemotherapeutic protocols for sarcomas demonstrate poorer results than for other malignancies, with the prognosis of sarcoma patients typically being local recurrence and metastasis.

Recently, molecular-targeting drugs have been developed for other human malignancies including cancer and hematopoietic malignancies (3-5). These drugs have selective inhibitory effects on the various factors (including growth factor receptors and intracellular signaling factors) involved in tumor proliferation, migration and metastasis (6). It has previously been reported that overexpression and abnormal activation of growth factors and intracellular signaling factors may be related to tumor proliferation, migration and metastasis in OS and MFH, as well as in other malignancies such as small cell lung cancer, gastrointestinal stromal tumors, pancreatic cancer, cholangiocellular carcinoma, and several types of leukemia (7-11).

The mitogen-activated protein kinase (MAPK) signaling pathway occurs downstream of RAS, which is an intracellular central signal transduction mediator also activated by growth factors (platelet-derived growth factor, vascular endothelial growth factor, *etc*.). This pathway consists of three kinases, including RAF1 (MAP kinase-kinase-kinase), MEK (MAP kinase-kinase), and ERK (extracellular signal-related kinase). This kinase cascade is closely related to tumor proliferation and metastasis in several types of cancer (12-14).

RAF1 is an essential serine/threonine kinase and is found in the first cascade from RAS. MEK is also a serine/threonine kinase and is found in the second cascade from Ras. Overexpression of RAF1 and MEK activates the MAPK signaling pathway. Clinical trials of RAF1 inhibitors have been studied in renal cancer and pancreatic cancer. Similarly, clinical trials of MEK inhibitors have also been studied in pancreatic cancer (15-17).

In this study, we establish whether inhibition of the MAPK pathway in OS and MFH also decreases cell proliferation and metastasis, as it does for other types of cancer. On the basis of these studies, we explore the relationship between the MAPK signaling pathway and cell proliferation in OS and MFH, and also show the effect of MAPK signaling pathway inhibitor. We believe that such inhibitors will be crucial components of new drugs for the treatment of OS and MFH in the future.

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Materials and Methods

Cell lines. Three human OS cell lines (KTHOS, MG63 and KHOS) and three human MFH cell lines (GBS-1, Nara-F and Nara-H) were used in this study (18-20). MG63 and KHOS were purchased from the American Type Culture Collection (Manassas, VA, USA). KTHOS cells were previously established by our co-author Dr. T. Hitora. GBS-1 was provided by Dr. H. Kanda (Department of Pathology, the Cancer Institute of the Japanese Foundation for Cancer Research, Tokyo, Japan). Nara-F and Nara-H were purchased from ScienStiff Co., Nara, Japan. All cell lines were grown in culture medium consisting of Dulbecco's modified Eagle's medium (DMEM) with glutamine (Sigma-Aldrich Co., St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Sigma Aldrich) and penicillin/streptomycin solution (Sigma Aldrich). All cell lines were routinely maintained at 37˚C in a humidified 5% CO_2 atmosphere.

Specimens. Seven OS specimens were used, originally diagnosed by pathologists based on histological data. All of the samples were obtained surgically at the Department of Orthopaedic Surgery, Kagawa University School of Medicine, between 2006 and 2009.

Reagents. U0126 was purchased from Promega. Stock solution was prepared in dimethylsulfoxide (DMSO) and diluted to the required concentrations in culture medium before use.

Messenger RNA expression of RAF1 and MEK1/2. Seven OS specimens and all of the OS and MFH cell lines mentioned above were used for the detection of the expression of the *RAF1* and *MEK1/2* genes. An RNeasy Mini Kit® (Qiagen Inc., Valencia, CA, USA) was used to purify total RNA. Reverse transcription of the RNA into cDNA was performed using the Reverse Transcription System (Promega, Madison, WI, USA), according to the manufacturer's instructions. *RAF1* and *MEK1/2* mRNA expressions were examined by reverse transcription (RT-) PCR. After PCR amplification, 8-ml aliquots of the PCR products were electrophoresed in a 2% agarose gel, followed by ethidium bromide staining.

Inhibitory effects of U0126 on cell proliferation. The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) assay with CellTiter 96® Aqueous One Solution Reagent (Promega) was used to determine cell proliferation. Cells were trypsinized and seeded at a density of approximately 5×10^3 cells/well in 96-well cell culture plates in 100 μl culture medium with 10% FBS. After 24 h, the medium was refreshed with 1% FBS containing U0126 (5, 20 and 50 μM). After 24, 48 and 72 h, the medium was removed and washed with phosphate-buffered saline (PBS), then refreshed with fresh medium containing MTS reagent (100 μl medium with 20 μl MTS reagent/well). The optical density was measured at 490 nm using an automatic microplate reader, SpectraMax Plus 384 (Molecular Devices, Sunnyvale, CA, USA) after 2 h of further incubation at 37˚C in a humidified atmosphere of 5% CO_2. The percentage viability of each well was calculated. At least three independent cultures were performed for each study.

Western blotting analysis. Cells were trypsinized and seeded at a density of approximately 1×10^6 cells/well in 6-well cell culture dishes in 3 ml of culture medium with 10% FBS. After 24 h, cells were treated for 1 h with 1% FBS containing 50 μM of U0126. Whole cell lysates were collected for protein content and were separated by SDS polyacrylamide gel electrophoresis under reducing conditions. Gels were electrophoretically transferred to PVDF membranes, the membranes were then blocked for 2 h, and incubated with 1:500 dilution of either primary monoclonal mouse anti-phospho-RAF1 antibody (Santa Cruz Biotechnology, Paso Robles, CA, USA), primary polyclonal rabbit anti-phospho-MEK1/2 antibody (Ser 218/Ser 222; Santa Cruz Biotechnology), or primary polyclonal rabbit anti-phospho-ERK 1/2 antibody (Chemicon International, Inc., Temecula, CA, USA) overnight at 4˚C. Secondary antibodies at 1:10,000 dilution included anti-mouse (for anti-phospho-RAF1) or anti-rabbit (for anti-phospho-MEK1/2 and anti-phospho-ERK 1/2) IgG HRP-linked whole antibody (GE Healthcare Bio-Sciences, Piscataway, NJ, USA). Bound antibodies were detected using the ECL Plus Western blotting detection system (GE Healthcare Bio-Sciences).

Immunohistochemical analysis. To determine MEK1/2 expression in the surgically obtained specimens, the specimens were fixed in 10% formalin and embedded in paraffin. Immunohistochemical staining was then performed using the avidin-biotin-complex (ABC) method. A monoclonal antibody for MEK1/2 was used at a dilution of 1:200 (Assay Designs, Ann Arbor, MI, USA). Briefly, sections were deparaffinized and incubated with 0.05% trypsin. The sections were then placed in H_2O_2 solution for 10 min to block endogenous peroxidase. After this, sections were incubated in normal goat serum at room temperature for 10 min, followed by a 1 h incubation with the primary antibodies for 1 h, then a biotinylated anti-mouse antibody for 10 min, and finally the avidin-biotin complex for 5 min. Chromogen substrate solution was used to detect the immunoreactivity, and the sections were counterstained with hematoxylin. For the negative control, normal immunoglobulin was used instead of the primary antibody.

Results

Messenger RNA expression. *RAF1* and *MEK 1/2* mRNAs were expressed in all six human sarcoma cell lines. Expression of *RAF1* and *MEK 1/2* was also detected in all seven OS specimens (Figure 1).

Inhibitory effect of U0126 on cell proliferation. U0126 showed a dose- and time-dependent inhibitory effect on all OS and MFH cell lines (Figure 2A, B). Treatment with 50 μM of U0126 after 48 h of stimulation inhibited cell proliferation by more than 50% in KTHOS, KHOS, GBS-1, Nara-F, and Nara-H cell lines, while 20 μM of U0126 inhibited cell proliferation by more than 50% in KHOS, Nara-F, and Nara-H after 72 h of stimulation. The inhibitory effect on MG63 was the weakest for all seven cell lines.

Western blotting analysis. Western blotting revealed that U0126 promoted the expression of p-MEK1/2 in KTHOS, MG63, and GBS-1 cells despite there being no remarkable changes in the expression of RAF1. However, U0126 suppressed the expression of p-ERK1/2 in all six cell lines (Figure 3).

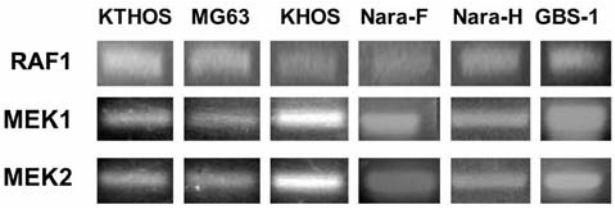

Figure 1. *Messenger RNA expression of RAF1 and MEK1/2 in cell lines. Messenger RNA was expressed in all OS and MFH cell lines.*

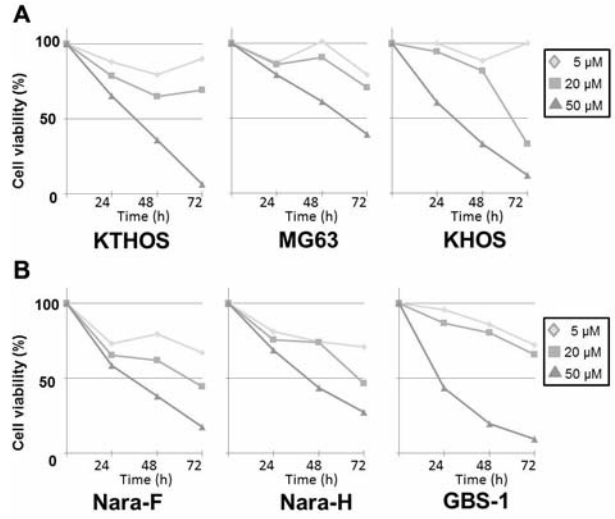

Figure 2. *Inhibitory effect of U0126 on cell proliferation. A: U0126 had dose- and time-dependent inhibitory effects on the cell proliferation of three OS cell lines, KTHOS, MG63 and KHOS. Significantly, proliferation with 50 μM U0126 had clear time-dependent curve in contrast with 5 μM and 20 μM U0126. B: U0126 had dose- and time-dependent inhibitory effects on cell proliferation of three MFH cell lines, Nara-H, Nara-F and GBS-1.*

Immunohistochemical findings. Immunohistochemical analysis of the surgically obtained specimens showed MEK1/2 immunoreactivity in all seven specimens. Figure 4 is an example of positive immunoreactivity of MEK1/2 in OS (Figure 4).

Discussion

The MAPK signaling pathway occurs downstream of the central signal transduction mediator, RAS (the downstream signaling molecule activated by growth factors such as PDGF, VEGF, *etc*.). Overexpression and activation of the MAPK pathway occurs in many human malignancies (liver, renal, pancreatic and esophageal cancer) (12-14, 21, 22) and is one of the most important cascades closely related to tumor proliferation and metastasis.

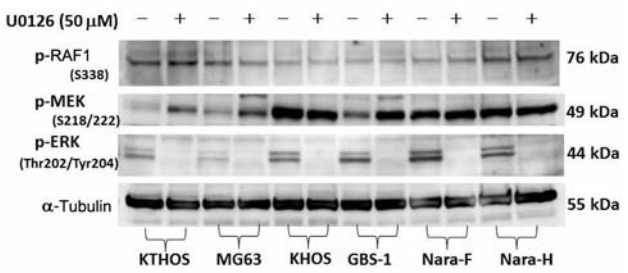

Figure 3. *Western blotting analysis. Phospho-RAF1, phospho-MEK 1/2 and phospho-ERK 1/2 were detected in all cell lines. Expression of phospho-MEK 1/2 was promoted in KTHOS, MG63 and GBS-1, but there was no promotion in KTHOS, Nara-F and Nara-H. In contrast with p-MEK, expression of ERK 1/2 was clearly suppressed in all cell lines treated for 1 hour with medium containing 50 μM U0126.*

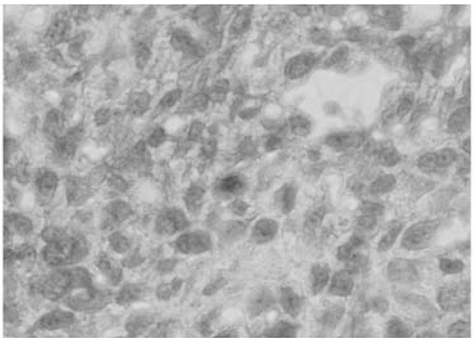

Figure 4. *Immunohistochemical findings. Example of a specimen from a 20-year-old patient with a diagnosis of osteosarcoma. The nuclei of the specimen were immunohistochemically positive for MEK 1/2.*

This pathway is transmitted by phosphorylation of three kinases, namely RAF1, MEK and ERK. RAF1 is an essential serine/threonine kinase as it links activated tyrosine kinases and RAS to MEK and MAPK (12,13).

Bone and soft tissue sarcomas, especially high-grade sarcomas such as OS and MFH, are clinically aggressive and have high-grade metastatic behavior to various organs. Thus, the control of metastasis is clinically important for survival. Although many high-dose chemotherapeutic protocols including doxorubicin, cisplatin and ifosfamide are used for human sarcomas, the results of these protocols have been poor. As such, these protocols do not improve the prognosis for patients who eventually develop local recurrence and metastasis (1, 2).

Recently, molecular-targeting drugs have been developed for human cancer and hematopoietic malignancies. Previous clinical studies on molecular-targeting drugs have shown remarkable antitumor effects on human malignancies including OS and MFH, as well as on other types of cancer. These drugs have selective inhibitory effects on the factors that relate to tumor proliferation, migration, and metastasis (23-28).

The MAPK signaling pathway is a very important target in molecular-targeting therapy, since it is specific for malignant tumors. The U.S. FDA recently approved the use of sorafenib, a selective RAF1 kinase inhibitor that targets the RAF/MEK/ERK pathway, for advanced renal cell carcinoma. Sorafenib is also under trial for lung cancer, cholangiocellular carcinoma, and esophageal cancer (12, 13).

U0126 is a selective MEK inhibitor that works by inhibiting the phosphorylation of ERK. This inhibitor displays inhibitory effects on several types of cancer, however the role of MAPK signaling and effects of the inhibitor on OS and MFH have not been demonstrated (15).

In this study, *RAF1* and *MEK* gene expression show the existence of the pathway in OS and MFH in the same way as in other types of cancer. The results of Western blotting show that the activation of the MAPK signaling pathway and the phosphorylation of ERK were regulated by U0126. The results show that the inhibitory effects of cell proliferation determined by MTS assay were caused by the decrease of phosphorylation of ERK. That is to say, MAPK signaling pathway has an important role for cell proliferation of OS and MFH, and the inhibition of the pathway has a critical effect on tumor cell survival. Furthermore, Ding *et al*. reported that in fibrosarcoma, the MAPK signaling pathway is involved in the promotion of growth and vascularization (14). This suggests that the MAPK signaling pathway has similar roles in various types of cancer, and inhibitors of MAPK signaling pathway offer the possibility of effective anticancer treatment for bone and soft tissue sarcomas. Moreover, our Western blotting results confirm the existence and importance of the MAPK signaling pathway in these cell lines. The selective MEK inhibitor U0126 reduced the phosphorylation of ERK and also reduced cell proliferation of both the OS and MFH cell lines. Thus, U0126 was able to block the MAPK signaling pathway and reduce cell proliferation in OS and MFH, as it does in other types of cancer.

In this study, from our immunohistochemical results, we confirm that the MAPK signaling pathway plays an important role in osteosarcoma cells and exists in sarcoma specimens. This highlights the existence and activation the pathway not only in OS and MFH cell lines but also in specimens. This indicates a role for the MAPK signaling pathway in the proliferation of sarcoma and an expectation for the effect of U0126.

We conclude that the proliferation of malignant bone and soft tissue tumors depends on the type of signaling pathway involved, and that inhibition of such pathways, especially the MAPK signaling pathway, results in antitumor activity. Although further studies are needed to explore the mechanisms of the signaling pathways in human OS and MFH, MAPK signaling appears to be extremely important not only for cancer in general, but also for malignant bone and soft tissue tumors, and is therefore a potential target for chemotherapeutic agents.

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