Abstract

Osteosarcoma (OS) is the most common primary malignancy of the skeletal system with a high grade of malignancy. OS represents less than 1% of all newly diagnosed cancers in adults and 3-5% in children. After leukemias and lymphomas, it is the most common primary cancer in adolescents. The phenotypic risk factors for developing OS are associated with physiological development and are both tall stature and high birth weight. In turn, genetic disorders (mutations) that are common in OS mainly involve tumor suppressor genes, such as *TP53*, *RB1*, *PTEN*, and *IDH*. In about 60% of OS cases, treatment has beneficial effects, whereas in about 40% of patients the cancer is highly metastatic, resistant to chemotherapy, and poses a worse prognosis. Therefore, new therapeutic agents that could improve the prognosis of patients with this cancer are still being sought.

Alpha-ketoglutarate (AKG) appears to be a promising compound. It is an intermediate metabolite in the Krebs cycle. This energy donor plays a key role in the energy metabolism in animal cells. AKG has both metabolic and non-metabolic functions in the organism. Its non-metabolic functions are related to the regulation of epigenetic processes and cellular signaling. AKG has the ability to regulate the activity of the HIF transcription factor, which is responsible for the development and progression of tumors. In addition, AKG affects the activity of enzymes involved in the epigenetic modification of chromatin. It is suggested that the increase in the amount of intracellular AKG may have an anticancer effect.

The aim of this study was to assess the anticancer activity of exogenous AKG against human osteosarcoma using two cell lines of this tumor: Saos-2 and HOS.

The effect of AKG on the viability of normal cells (human fibroblast cell line HSF and human osteoblast cell line hFOB 1.19) was evaluated with the LDH assay. Anti-proliferative activity of AKG against Saos-2 and HOS cells was assessed with the MTT and BrdU methods. The effect of AKG on progression of the cell cycle was examined by means of flow cytometry and PI/RNAase staining. Its ability to induce apoptosis or/and necrosis in both OS cell lines was evaluated by annexin V-FITC/PI double labelling and cytometric analysis. The effect of AKG on the level of cell cycle-associated proteins (cyclin D1 and p21 Waf1/Cip1) was determined by ELISA. Moreover, the AKG ability to activate caspase-3 (evaluated by flow cytometry), and caspases -8 and -9 (by Western blot and flow cytometry) as well as the effect on the expression of proteins associated with the intrinsic pathway of apoptosis, i.e. Bax and Bcl-2 (Western blot), were investigated. To examine the mechanism of AKG activity in OS cells, the AKG ability to modulate the phosphorylation

level of MAP kinases (JNK, ERK1/2, p38) and Akt kinase (by ELISA assay) in Saos-2 cells was investigated. The influence of AKG on production of TGF-β (growth factor for OS cells and cytokine stimulating cell migration, invasiveness, and metastasis) was also determined by ELISA. Moreover, the effect of AKG on production of pro-angiogenic cytokine VEGF by OS cells was investigated (by ELISA). The effect of AKG on osteosarcoma cell migration was assessed by a scratch wound healing assay, whereas invasiveness was evaluated by a basement membrane extract (BME) cell invasion assay.

The results showed that AKG exhibited low toxicity against normal cells. AKG inhibited proliferation of Saos-2 and HOS cells in a concentration dependent manner. This compound blocked the cell cycle progression at the G_1 stage in both OS lines, decreased the expression of cyclin D1 in HOS cells, and increased the level of expression of the cyclin-dependent kinase inhibitor p21 Waf1/Cip1 in Saos-2 cells. In addition, AKG induced apoptosis in osteosarcoma cells by activation of caspase-9, -8, and -3, increasing the expression of the pro-apoptotic protein Bax, and decreasing the level of the anti-apoptotic Bcl-2 protein. AKG increased JNK phosphorylation and inhibited ERK1/2 and Akt activation in the Saos-2 cells. AKG has also been shown to induce apoptosis in Saos-2 cells by activating JNK kinase, as a specific inhibitor of this kinase (SP600125) completely inhibited AKG-induced kinase activation and partially reduced AKG-induced apoptosis in Saos-2 cells. In addition, AKG decreased production of TGF-β and VEGF and inhibited cell migration and invasiveness of both osteosarcoma lines.

The *in vitro* results obtained in this study indicate the anticancer potential of AKG against osteosarcoma cells. AKG activates JNK and can thus induce apoptosis in OS cells. Moreover, the reduction of ERK1/2 activity by AKG may inhibit the progression of the cell cycle and cancer cell proliferation. In addition, the AKG-induced reduction of ERK1/2 and Akt activity may contribute to induction of apoptosis, inhibition of OS cell migration and invasion, and inhibition of angiogenesis. The study results suggest that AKG can inhibit both osteosarcoma growth and metastasis.