Mgr Aleksandra Żurek
Zakład Wirusologii i Immunologii
Instytut Mikrobiologii i Biotechnologii,
Wydział Biologii i Biotechnologii
Uniwersytet Marii Curie-Skłodowskiej w Lublinie

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The influence of alpha-ketoglutarate on osteogenesis in osteoblast models *in vitro*

SUMMARY:

Bone tissue, the hardest tissue in the human body, owes its mechanical properties to the presence of mineralized bone matrix produced by osteogenic cells during their maturation and differentiation. Despite their seemingly constant structure, intense metabolic processes continually occur in bones in order to maintain a proper mass and structure of bone tissue. During the remodeling process, exchange of old or damaged bone to a new one takes place, and it is based on the balanced resorptive activity of osteoclastic cells and the osteogenic activity of osteoblastic cells. With aging of the organism, the metabolic balance of bone tissue is disturbed and bone resorption starts to dominate over osteogenesis, which leads to bone loss, often resulting in the development of bone diseases such as osteoporosis. In the treatment of diseases involving bone loss, the most often used drugs are those with anti-resorptive activity, which inhibit resorption performed by osteoclasts. Nevertheless, in such diseases, it is also important to stimulate the osteogenic activity of osteoblasts to rebuild bones weakened by excessive bone resorption. However, the number of commercially available drugs with osteogenic activity is substantially lower than the number of antiresorptive agents, and what is very important, both groups of medicines often exhibit side effects. Therefore, it is necessary to search for new substances that stimulate bone formation and are safe for the patient.

Alpha-ketoglutarate (AKG) appears to be a compound with potential for use in the treatment of metabolic bone diseases. This metabolite of the Krebs cycle is characterized by pleiotropic activity, which is widely described in the literature. As a donor of energy in the process of cellular respiration, AKG plays an important role in energy metabolism of animal cells and, as a precursor of amino acids, it contributes to the maintenance the protein balance. This metabolite is also a co-substrate of 2-oxoglutarate-dependent dioxygenases (2-OGDDs) - enzymes involved in destabilization of the transcriptional factor HIF-1 (responsible for the development and progression of cancer), also playing a major role in the biosynthesis of type I

collagen - the main protein of bone extracellular matrix. As a ligand for GPR80/99, AKG can also function as a signal molecule and regulate cellular processes connected with growth and differentiation. Exogenous AKG in a form of various salts can be administered as a dietary supplement, because they are well tolerated by the body. It has been shown that supplementation with AKG contributed to complementation of the shortage of proteins in the states of increased proteolysis. This metabolite also showed a protective effect on cells subjected to oxidative stress, as well as immunomodulatory and anti-cancer activity.

In numerous *in vivo* studies, the positive effect on bone mineral density and bone strength was observed after supplementation by AKG. Despite these reports, the mechanism of AKG action on bone tissue is still unknown. It is also unclear whether the anabolic effect of AKG on bone involves direct stimulation of osteogenesis by this compound, given the lack of *in vitro* studies on the effect of exogenous AKG on osteoblasts.

The aim of this study was to evaluate the direct impact of alpha-ketoglutarate (in a form of alpha-ketoglutaric acid disodium salt dihydrate, NaAKG) on the maturation and differentiation of three osteoblast cell lines, which are commonly used as models for the study of osteogenesis *in vitro*, and to determine the impact of NaAKG on the mineralization of bone matrix produced by osteoblasts.

In the experiments, the following cell lines were used: hFOB 1.19 (human normal preosteoblasts), MC3T3-E1 (mouse normal preosteoblasts), and Saos-2 (human cancer osteoblasts). In the first stage of the study, the effect of a wide range of NaAKG concentrations on osteoblast proliferation was investigated by MTT and BrdU methods. Secondly, the effect of this compound on cell viability was examined using NR and LDH assays. Based on the results of the cell viability assays, non-toxic NaAKG concentrations to all three osteoblast cell lines were selected and used in further studies on cell differentiation and mineralization of extracellular matrix produced by osteoblasts. By the qRT-PCR method, the influence of NaAKG on the expression of genes encoding protein markers of the early stage (*ALPL, COL1A1, IBSP*) and late stage (*SPP1, BGLAP*) of osteoblast differentiation was investigated. The level of production of those markers in cells was further examined after the incubation of osteoblasts with NaAKG. ALP activity was determined by the colorimetric method and *in situ* staining, the cellular amount of collagen type I was measured by Western blotting, and protein levels of BSP II, OPN, and OCN were determined by the ELISA assay. The level of extracellular matrix mineralization was determined by Alizarin Red S staining. Additionally, the ability of NaAKG to induce apoptosis and necrosis in Saos-2 cells (Hoechst 33342 and propidium iodide staining) and the influence of NaAKG on the migration of Saos-2 cells

(scratch assay) were investigated. In the last stage of the study, the level of mRNA (by the qRT-PCR method) encoding the GPR80/99 protein (the receptor for which AKG is a ligand) as well as the presence of the GPR80/99 protein (by Western blotting and immunofluorescence staining) were investigated in osteoblast cell lines. Also, the functionality of the receptor after its stimulation by alpha-ketoglutaric acid or NaAKG (by the IP-One ELISA assay) was tested.

After carrying out the above experiments, the following results were obtained:

- NaAKG at concentrations up to 10 mM did not affect the proliferation of cell lines hFOB 1.19, MC3T3-E1, and Saos-2, while at a concentration in the range of 25 - 200 mM, it inhibited their proliferation.
- NaAKG at concentrations below 50 mM was non-toxic to the tested osteoblast cell lines; therefore, concentrations of 5, 10, and 25 mM NaAKG were selected for the research on cell differentiation.
- NaAKG increased gene expression and production of the protein markers of early and late stage of osteoblast differentiation as well as the mineralization of bone matrix in normal osteoblast cell lines: hFOB 1.19 and MC3T3-E1.
- NaAKG increased gene expression of early and late differentiation markers, but decreased their protein level and inhibited mineralization of bone matrix produced by cancer cell line Saos-2.
- NaAKG induced apoptosis in Saos-2 cells.
- NaAKG significantly inhibited the migration of Saos-2 cells.
- The presence of GPR80/99, for which AKG is a ligand, was detected in cell lines hFOB 1.19, MC3T3-E1, and Saos-2. The presence of this protein in osteoblasts has not been described before.
- It was shown that GPR80/99 is a functional receptor in the tested osteoblast cell lines and is activated by alpha-ketoglutaric acid, but is not activated by the NaAKG in the range of the concentrations tested.

The assessment of the alpha-ketoglutarate impact on the osteogenesis *in vitro* contributed to extension of the knowledge about the effects of this compound on bone tissue, and it is also a complement to the results obtained in numerous independent *in vivo* studies. Based on the results of the research carried out in this study, it can be concluded that alpha-ketoglutarate exhibits the potential to be used in the prevention or/and therapy of bone diseases

such as osteoporosis and for the chemoprevention or/and treatment of bone cancer - osteosarcoma.