THE ROLE OF OLIGOCHITOSANS IN AKT KINASE REGULATION

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Abstract

Among characteristic properties of cancers, there is their increased glycolytic activity.Contrary to normal cells, neoplastic cells use anaerobic glycolysis, even when a sufficient amount of oxygen is available. The intensity of the process is associated with a considerable demand for energy in the form of ATP.

Akt, which - acting through the mTOR pathway - activates the HIF-1 factor, which in turn activates hexokinase that participates in glucose phosphorylation, stimulates the transport of glucose to cells via increasing glucose transporters (GLUT) and activates lactate dehydrogenase (which transforms pyruvate to lactate). Chitosan, as well as products of its degradation - oligochitosans - contribute to inhibiting the activity of the Akt kinase, and thus contribute to inhibiting excessive glycolytic activity of Ehrlich ascites tumor (EAT) cells and to decreasing proliferation of these cells.

Key words: *M2 pyruvate kinase, Akt kinase – PKB, EAT cells proliferation, HIF-1 factor.*

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1. Introduction

A characteristic property of tumor cells is an increased glycolytic activity and by the same token, an increased demand for glucose, which is degraded to lactic acid. In the cytoplasm of normal cells, glucose is metabolized through glycolysis to pyruvate, which subsequently is subjected in the mitochondrion to oxidative decarboxylation to acetyl-CoA that is in turn consumed in the Krebs cycle and oxidative phosphorylation to carbon dioxide and water [1]. The amount of energy obtained under aerobic conditions from one mole of glucose in normal cells is fifteen times higher as compared to that obtained under the same conditions in tumor cells, what was for the first time determined by Warburg [2]. An increased energy demand in tumor cells makes the amount of oxidized glucose in these cells approximately 30 times higher as compared to normal cells [3].

Numerous authors indicate that a significant regulatory role in the altered metabolism of tumor cells is fulfilled by kinase Akt (serine/threonine protein kinase Akt) that is the principal signal transducer in the 3-kinase phosphatidylinositol pathway, as well as in growth and proliferation regulation [4-6].

In the majority of cell types, GLUT1 transporter is responsible for glucose transport; its increased expression is seen in tumor as compared to normal cells [2], and its increased expression is affected through the mediation of Akt, which activates kinase mTOR [6]. An increased expression of GLUT1 transporter is triggered in tumor cells mainly by the transcription factor HIF-1 (hypoxia-inducible factor -1), induced by hypoxia.

An increased glycolytic activity in tumor cells may be evoked by an increased activity of kinase Akt mediated by the HIF-1 factor that increases the expression of enzymes participating in the glycolysis process, especially the key enzymes participating in the said process [7,8].

The increase in kinase Akt contributes to increasing the glycolytic activity of tumor cells, what allows for meeting their energy needs through increased ATP synthesis (Fig.1), as well as in cell cycle and cell viability regulation [9,10] (Fig. 2a and 2b).

Akt contributes to increased cell proliferation through phosphorylation and inhibition of expression of inhibitors of cyclin-dependent kinases; among others, it phosphorylates protein p27 resulting in its capture in the cytoplasm and weakening its activity as a cell cycle inhibitor [11] and it may phosphorylate protein p21, also capturing it in the cytoplasm [12]. The contribution of Akt in proliferation stimulation occurs through mTORC1 activation, which activates cyclin D1 expression [13].

In numerous types of cancers, including mammary gland carcinoma, increased Akt expression occurs and for this reason, compounds that inhibit its activity may be potential anticancer agents. Such a compound that inhibits the activity of

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Akt seems to be chitosan and products of its degradation - oligochitosans. Preliminary results obtained while employing chitosan and oligochitosans point to a possible inhibition of Akt activity in tumor cells, while chitosan and oligochitosans do not evoke a similar effect in normal cells.

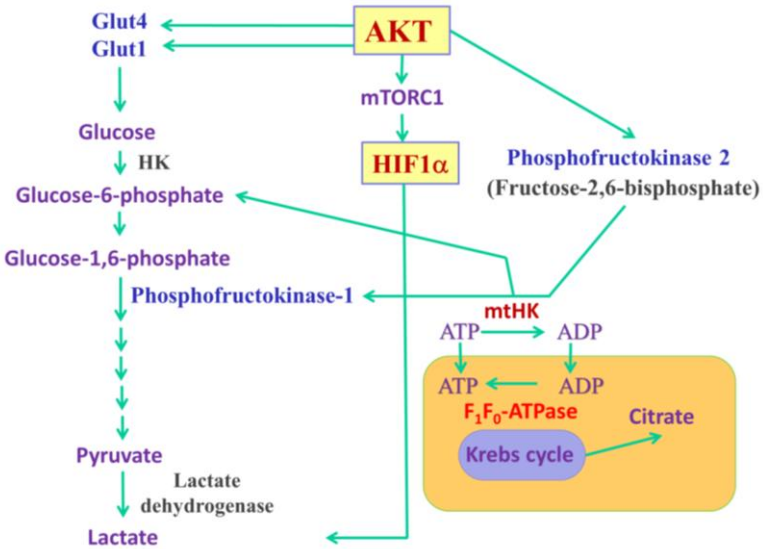

Fig. 1.

2. Materials and methods

2.1. Cell cultures

The studies were carried out on normal mouse mammary epithelial cell line CRL 1636 and Ehrlich ascites tumor (EAT) cells (American Type Culture Collection). Normal cells were cultured in the DME medium (Sigma Chemical Co.) (pH 7.4) supplemented with 10% fetal calf serum (FCS - Gibco), 1% L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 μg/ml insulin. EAT cells were suspended in the NCTC-135 (Sigma Chemical Co.) enriched with 10% fetal calf serum (FCS – Gibco), 1% L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin. The cell cultures were maintained at 37°C in a humidified atmosphere - the normal cells in 10% CO_2 and the neoplastic cells in 5% CO_2.

2.2. Reagents

A – chitosan (deacetylation degree – DD: 97.7%, Mv: 331 kDa) (no cytotoxicity after 48 h). Chitosan was degraded using the combined method employing physical (temperature, ultrasounds) and chemical factors (hydrogen peroxide).

B – oligochitosan, insoluble fraction – MKCh (V/CH/D/22-24) (deacetylation degree DD – 97.7%, Mv – 52 kDa, polymer content – 5.08%) (no cytotoxicity after 48 h) and C – oligochitosan (deacetylation degree DD – 97.7%, Mv – 1.28

kDa, polymer content – 4.0% (no cytotoxicity after 48 h) was provided by the Institute Biopolymers and Chemical Fibers in Łódź. The degree of oligomers deacetylation was measured by the potentiometric titration [14].

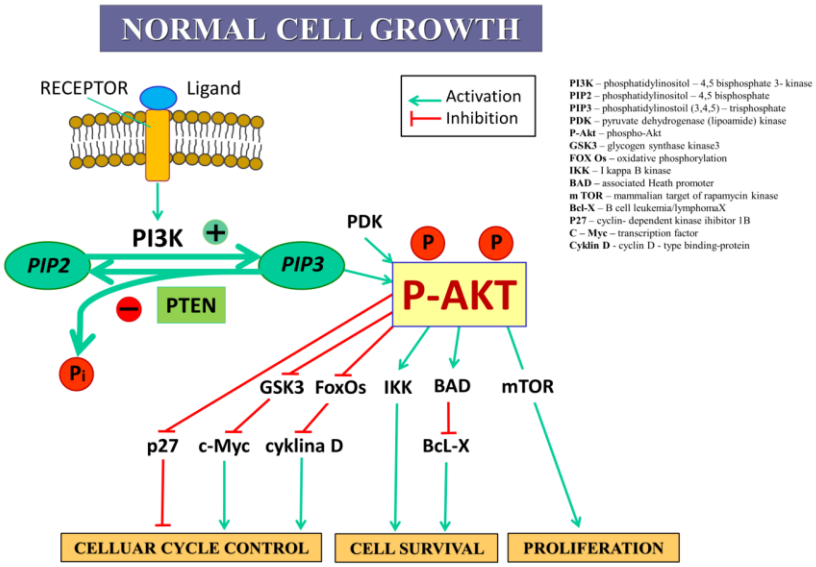

Fig. 2a.

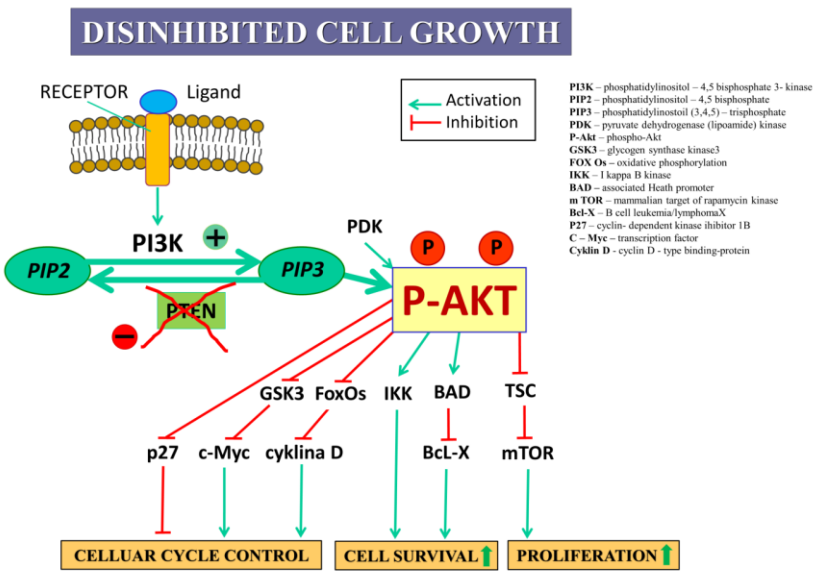

Fig. 2b.

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2.3. Detection of cytotoxicity

The cells were seeded in triplicates into 96-microwell plates at the density of 1-8 x 10^3 cells per well and incubated without or with different factors for 24 or 48 hours. Afterwards, the cells were mixed with the reaction mixture from the Cytotoxicity Detection Kit (LDH) (Roche Applied Science, Germany). The reaction was stopped with 1 M HCl. The colorimetric assay for the quantification of cell death was based on the measurement of lactate dehydrogenase activity released from the damaged cells into the supernatant. The absorbance of the colored product - formazane – was measured at 490 nm by an ELISA reader.

2.4. Incubation procedure

The stock solution of reagent (0.5%) was prepared in the medium in accordance with the type of cells – for CRL 1636 normal cells, in the DME medium, while EAT cells were suspended in the NCTC-135 before each experiment and stored at 37°C. The medium containing 0.05% reagent was replaced every 24 hours throughout the experiments. The cells were incubated for 72 hours.

2.5. Methods

Proliferation (Crystal Violet Test) and PCNA (Western blot analysis)
RNA extraction (QIAGEN); cDNA synthesis (Superscript II RNase H reverse transcriptase) PCR amplification DNA Taq – (QIAGEN)
Western blot - the first antibody: β-actin, c-myc, β-catenin, Akt, P-Akt, M2PK, PCNA (*Cell Signaling Technology)*. Goat anti-mouse or anti-rabbit immunoglobulins conjugated with alkaline phosphatase were used as a second antibody. The reaction was detected by the reduction of 4-nitroblue tetrazolium salt in the presence of 5-bromo-4-chloro-3-indolyl-phosphate in buffer Tris/HCl (pH 9.5) containing 0.05 M $MgCl_2$ and 1M NaCl – Roche).

3. Results and discussion

In the experiments employing chitosan and products of its degradation - oligochitosans, the authors observed inhibition of the M2 pyruvate kinase (PK) isoenzyme expression, as well as decreasing the degree of kinase Akt phosphorylation (a decrease in its activity) (Fig. 3 and 4).

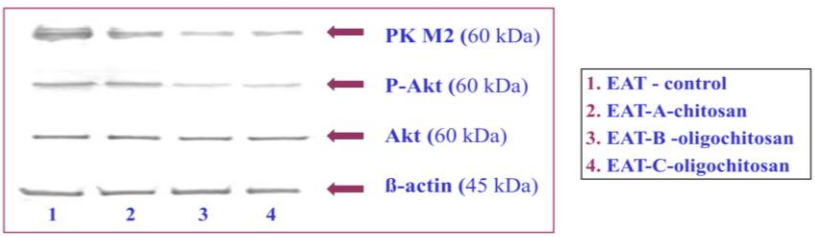

Fig. 3. Western blot showing decreased PK M2 and p-Akt protein in the studied cell lines treated with A – chitosan (deacetylation degree – DD: 97.7%) B – oligochitosan (DD: 97.7% - insoluble fraction) and C – oligochitosan (DD 97.7% - soluble fraction). All the experiments were repeated 3 times and data are expressed as mean ± SE*p,0.05.

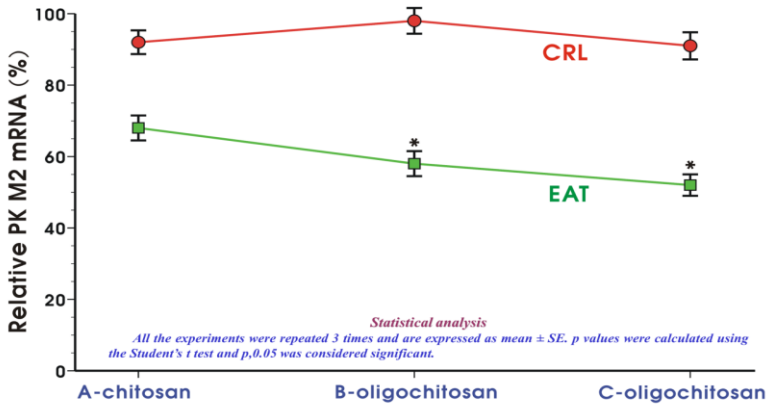

Fig. 4. Chitosan treatment decreases PKM2 mRNA in Ehrlich ascites tumor (EAT) cells by approximately two times. GAPDH was taken as an endogenous control and normalized to PKM2 mRNA. Real-time PCR (RQ-PCR) was performed using the 7500 Real-Time PCR System (Applied Biosystems, Foster City, USA) and TaqMan probes. Specific primers and probes for the PKM2 gene and the *ABL* control gene were purchased from TIB MOLBIOL (Poznań, Poland) and Applied Biosystems (Applied Biosystems, Cheshire, UK).

Inhibition of expression of the M2 PK isoenzyme, one of the key enzymes involved in the glycolysis process, may result from inhibition of the activity of HIF-1 factor, occurring indirectly via kinase Akt and directly via kinase mTOR. Kinase Akt is inhibited by PTEN protein (phosphatase and tensin homolog deleted on chromosome ten) encoded by the suppressor gene [15,16]. PTEN protein is a phosphatase which causes hydrolytic detachment of the phosphate residue 3' from IP3 (phosphatidylinositol 3,4,5-triphosphate). Mutations in the PTEN gene leading to synthesis of an abnormal molecule contribute to the loss of the ability to inhibit tumor cell proliferation, especially in case of mammary gland cancer [16]. On the contrary, stimulation of the PTEN gene expression may contribute to inhibiting the Akt signaling pathway and thus to inhibition of growth, viability, metabolism and proliferation of tumor cells.

mTOR kinase is also a specific sensor of the cellular ATP level [17]; inhibition of the activity of the key enzyme in the glycolysis process of the M2 PK isoenzyme that is directly responsible for ATP synthesis may contribute to disturbing the synthesis of proteins participating in tumor cells in controlling the cell cycle, metabolism and proliferation [18.19].

mTOR kinase functions as a catalytic subunit in two separate protein complexes – mTORC1 and mTORC2 [20]. mTORC1 complex is a combination of mTOR kinase, raptor protein (regulatory associated protein of mTOR) and mLST8/GβL protein (mammalian LST8/G-protein β-subunit like protein) [19,21]; the complex is susceptible to changes in ATP concentration [17]. The

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kinase activity of mTORC1 complex is regulated through mTOR kinase binding to raptor protein - with potent effects within the N-terminal domain and less potent effects within the C-terminal domain of mTOR kinase [19]. In case of a high ATP level in the cell, binding of raptor protein by the C-domain of mTOR kinase is attenuated, thus contributing to increasing the kinase activity of mTOR. On the other hand, a decrease of ATP level in the cell results in strengthening the mTOR-raptor binding within the C-terminal domain of mTOR kinase, what in turn decreases the kinase activity of mTOR [19].

4. Conclusion

Highly deacetylated chitosan, as well as its degradation products - oligochitosans - inhibit the glycolytic activity of mouse mammary gland carcinoma (EAT), in particular, they decrease the ATP level, what directly contributes to inhibiting the activity of kinase mTOR activity in these cells. No such effect has been observed in the reference normal cells.

Chitosan and oligochitosans inhibit kinase Akt phosphorylation in EAT cells; decreased Akt phosphorylation results in inhibition of proliferation of these cells. In normal cells exposed to chitosan and oligochitosans, no inhibition of proliferation has been noted.

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