INHIBITION OF EHRLICH ASCITES TUMOUR (EAT) CELLS PROLIFERATION THROUGH CHITOSAN-MEDIATED REGULATION OF ACTIVITY OF THE AKT PATHWAY

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Abstract
Isoenzyme M2 pyruvate kinase, which is a marker of cancer transformation, can take both tetramer (cytosol) and dimer (nucleus) forms. The former is responsible for ATP synthesis, and the latter demonstrates histone H1 kinase activity. Regulation of the expression of pyruvate kinase through which Akt controls the expression of genes involved in Ehrlich ascites tumour (EAT) cell proliferation, migration and death, also involves cross-talk with the other signalling pathways, transcription factors and co-regulatory proteins such as β-catenin and c-Myc. Treatment of EAT cells with chitosans significantly reduced their proliferation (by 45-60%), expression of nuclear β-catenin, c-Myc as well as cell migration. After 48–72 hours of treatment of the cell with oligochitosans, lower levels of p-Akt were detected. Simultaneously, decreased expression of isoenzyme M2 PK protein levels was observed. The dimeric form (nucleus) can participate in H1 histone phosphorylation, which contributes to increased EAT cell proliferation.

Key words: *M2 pyruvate kinase, chitosan, EAT cells proliferation, Akt kinase - PKB.*

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1. Introduction

One characteristic trait in the metabolism of cancer cells is increased glycolytic activity in comparison to healthy cells, which is expressed as the increased production of lactic acid in aerobic environments [1]. Glucose degradation in cancer cells (depending upon the type of neoplasm) is 20 - 30 times greater than that seen in healthy cells [2].

According to many authors, this effect is associated with participation of Akt kinase (the serine/threonine protein kinase or protein kinase B–PKB). Increased glycolytic activity is possible due to the quickened transport of glucose to cells. Expression of the glucose transporter Glut1 is significantly increased in comparison to healthy cells [3]. Increased expression of Glut1 is connected with the participation of the Akt1 isoform, indirectly activating the kinase mTOR signalling pathway (mammalian target of rapamycin) [4].

Glucose that has been transported into the cell undergoes phosphorylation in the reaction of ATP catalysed by hexokinase I and II, which are located on the external mitochondrial membrane side, activated by Akt1 [5]. Some authors suggest that Akt contributes through pathway activation with the participation of mTOR kinase to increase expression of the transcription factor HIF-1 (hypoxia-inducible factor-1) [6, 7].

Factor HIF-1 causes increased expression of enzymes in the glycolysis process, including the isoenzyme pyruvate kinase (PK) M2 [8,9], which is directly associated with energy production in the form of ATP.

Depending upon the tissue type, four isoforms exist: L, R, M1 and M2. Isoenzyme L-PK is found in the liver, kidneys and intestines [10-12]. Isoenzyme R-PK exists in erythrocytes and presents similar regulatory features as the isoenzyme L-PK [13]. The R- and L-PK isoenzymes are encoded by the same gene and are expressed under the control of different tissue-specific promoters [14]. Isoenzymes M1-PK and M2-PK are products of the same M-gene, thus developing from the differentiation process of scaling exons [18,19]. The terminal domain present in the isoenzyme M2-PK is responsible for disassociation to the dimeric form or association to the tetrameric form [20-23]. Isoenzymes M1-PK and M2-PK show different regulatory features [24]. Pyruvate kinase type L-, K-, M1- and M2-, existing in the tetrameric form, are responsible for energy synthesis in the form of ATP; however, pyruvate kinase type M2 existing in the dimeric form possesses kinase histone H1 activity, which is associated with regulation of neoplastic cell proliferation [25]. The ratio of tetrameric and dimeric forms of the isoenzyme M2-PK alters depending on the concentration of metabolites or oncoproteins [24].

In the presence of high levels of the glycolysis process intermediate fructose 1,6-P2 or L-serine, association of the dimeric form to the tetrameric form is noted; however, a decrease of the fructose 1,6-P2 level leads to disassociation of the tetrameric form to the dimeric form [19, 20, 26].

Our aim was to present the influence of chitosan with various degrees of deacetylation (various charges) on activation of kinase Akt, regulating the expression of pivotal enzymes of glycolysis-pyruvate kinase M2, taking part in regulation of neoplastic cell proliferation.

2. Materials and methods

2.1. Cell cultures

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

2.2. Reagents

A – chitosan (deacetylation degree – DD - 97.7%, Mv - 331 kDa) (no cytotoxicity after 48 h), B – chitosan (DD - 81%, Mv: 255 kDa) (no cytotoxicity after 48 h) and C – chitosan (DD - 68.9%, Mv - 356 kDa) (no cytotoxicity after 48 h) was provided by the Institute Biopolymers and Chemical Fibres in Łódź. The degree of deacetylation oligomers was measured by the potentiometric titration [27].

2.3. Detection of cytotoxicity

The cells were seeded in triplicate into 96-microwell plates at a density of 1 - 8 × 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 coloured product, formazan, was measured at 490 nm by ELISA.

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, this was DME medium, while EAT cells were suspended in 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

- We studied the effect of oligochitosans on cell migration [Boyden's chamber - BD BioCoat™ Matrigel™ Invasion Chamber-Falcon].
- 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 Signalling Technology)*. Goat anti-mouse or anti-rabbit immunoglobulins conjugated with alkaline phosphatase were used as a secondary 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).

- Translocation of beta-catenin was observed using the CelLytic NuCLEAR Extraction Kit (SIGMA).

3. Results and discussion

Regulation of the expression of pyruvate kinase through which Akt controls the expression of genes involved in EAT cell proliferation, migration and death also involves cross-talk with the other signalling pathways, transcription factors and co-regulatory proteins such as β-catenin and c-Myc. Treatment of EAT cells with chitosans significantly reduced their proliferation (by 45-60%) - PCNA, expression of nuclear β-catenin, and c-Myc (***Figure 1***) well as cell migration (***Figure 2***). After 48–72 hours of treatment of the cell with chitosans, lower levels of p-Akt were detected (***Figure 3***). Simultaneously, decreased expression of isoenzyme M2 PK protein levels was observed (***Figure 3***).

The study employed RQ-PCR, targeting mRNA of M2 PK expression levels (***Figure 4***).

β-catenin is the central protein of the WNT/β-catenin pathway in tumour cells [28]. Phosphorylation of the above protein is necessary for its recognition by ubiquitin ligase, which is responsible for β-catenin degradation in lysosomes. The absence of β-catenin phosphorylation allows its transport to the nucleus, where it forms complexes with transcription

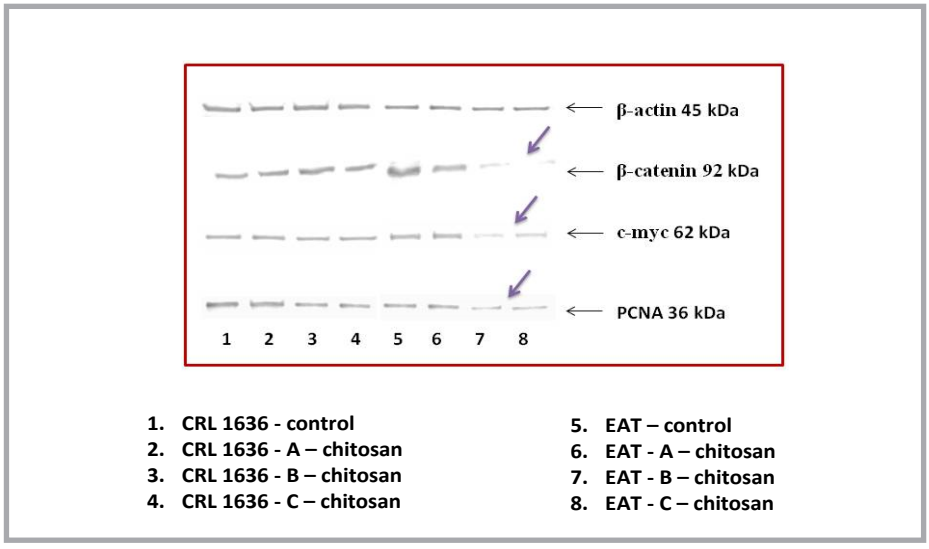


Figure 1. *Expression of β-catenin, c-Myc, and PCNA in Ehrlich ascites tumour (EAT) cells and normal mouse mammary gland epithelial cells (CRL 1636) at the protein level. The upper lanes show the levels of the housekeeping gene β-actin under the same culture conditions.*

MIGRATION – Boyden Chamber/Matrigel

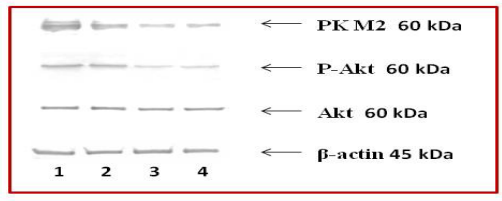
Cell invation after 48hrs treatment with
A – chitosan (deacetylation degree – DD: 97.7%)
B – chitosan (DD: 81%) and C – chitosan (DD 68,9)

Factor \ Cell line	CRL 1636	EAT
Control	5-8	35
A – chitosan	no change	29
B – chitosan	5-6	18
C – chitosan	6	18

Figure 2. *Cell invasion assays through Matrigel coated Boyden chamber are presented in a table which quantifies the extent of cell invasion.*

factors, thus contributing to induction of the expression of genes participating in regulation of the cell cycle, apoptosis, proliferation and tumour progression (among others, the increased expression of c-myc and cyclin D) [29, 30]. Thus, inhibition of β-catenin expression mediated by chitosan may constitute a factor that induces the apoptosis and proliferation of tumour cells.

The *PTEN* gene (phosphatase and tensin homolog deleted on chromosome ten) belongs to a family of suppressor genes that regulate the cell cycle; the protein encoded by this gene is a phosphatase, which is involved in the dephosphorylation of phosphatidylinositol (3,4,5)-triphosphate (PIP3). Mutations in the *PTEN* gene lead to the synthesis of an abnormal protein that does not prevent cell division, which causes excessive proliferation of cells in mammary gland carcinoma [31, 32]. Increased expression of the normal *PTEN* gene under the influence of chitosan might contribute to the increased dephosphorylation of PIP3, and the consequent decrease of the level of p-Akt, which inhibits mTOR kinase (mammalian target of rapamycin kinase) and tumour cell proliferation.



1. EAT – control
2. EAT - A – chitosan
3. EAT - B – chitosan
4. EAT - C – chitosan

Figure 3. *Western blot showing decreased PKM2 and p-Akt protein in the cell lines studied on treatment with A – chitosan (deacetylation degree – DD: 97.7%) B – chitosan (DD: 81%) and C – chitosan (DD 68.9%). All experiments were repeated 3 times and data are expressed as mean ± SE*p<0.05.*

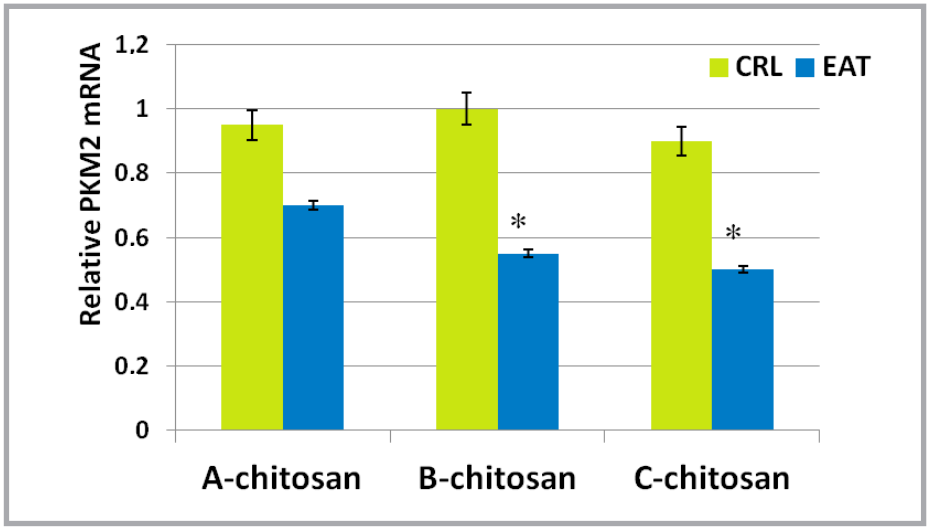


Figure 4. *Chitosan treatment decreases PKM2 mRNA in Ehrlich ascites tumour (EAT) cells by approximately two-fold. GAPDH was taken as an endogenous control and normalised to PKM2 mRNA.*

Real-time PCR (RT-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).

Statistical analysis. All experiments were repeated 3 times and are expressed as mean ± SE. p values were calculated using student's t test and $p < 0.05$ was considered significant.

Decreasing the PK M2 gene expression in the presence of chitosan may also point to the decreased phosphorylation of histone H1 through the activity of histone H1 kinase evoked by PK M2; less phosphorylated histone H1 is a potent inhibitor of cell proliferation.

4. Conclusion

Chitosan blocks the cell membrane receptor EAT, taking part in the inhibition of kinase Akt activity and consequently inhibiting expression of the pyruvate kinase M2 isoenzyme.

Inhibition of kinase Akt activity leads to the inhibition of mTOR kinase, which in turn decreases expression of the β-catenin and c-Myc genes, leading to EAT cell proliferation [33, 34]. Such effects in the presence of chitosan were not observed in healthy cells.

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