THE ROLE OF CHITOSAN IN AKT KINASE REGULATION ACTIVITY

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Abstract

A decrease in migration of tumor cells incubated with the investigated chitosan preparations was correlated with a decreased activity of MMP-2 and MMP-9 metalloproteinases, what significantly affected inhibition of tumor cell proliferation.
In the investigations of the effects of various chitosan preparations on expression of PCNA, Akt and β-catenin in the normal human 184A1 cells and in breast carcinoma MCF7 cells evaluated at the protein level, significant differences in inhibition of expression of selected genes were noted in the tumor cells.
Similarly as in the case of human cells, in mouse cells, the differences in expression of the investigated genes involved solely the Ehrlich carcinoma cells. In the presence of the investigated chitosan preparations, there was observed inhibition of expression of the N-cadherin, β-catenin, Akt and PCNA genes. In case of p21 protein, its level increased, similarly as in the human breast carcinoma cells, what may also be related to phosphorylation of the protein, its capture by the cytosol and prolonging its half-life as compared to the non-phosphorylated form.
In case of the normal human 181A1 cells and mouse CRL 1636 cells, no significant alterations were noted in expression of the investigated genes in presence of the employed chitosan preparations.

Key words: *Akt kinase (PKB), cells migration, metalloproteinases, M2 pyruvate kinase.*

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1. Introduction

Protein kinase B (PKB) (Akt) is responsible for phosphorylation of numerous proteins associated with regulation of fundamental cellular processes, such as metabolism, transcription and proliferation [1]. Disturbances in kinase Akt activation are noted among others in numerous tumors, where increased expression is observed; in particular, this is seen in mammary gland cancer [2,3]. An increased activity of kinase Akt in tumors may result from increased expression of the phosphatidylinositol 3-kinase (PI3K) gene, as well as from loss of activity of PTEN (phosphatase and tensin homolog deleted on chromosome ten). An increased kinase Akt activity is frequently correlated with tumor progression [4].

A characteristic property of tumor cells is an increased glycolytic activity manifested by increased glucose metabolism, which is 20-30-fold higher as compared to normal cells, increased lactate production and higher levels of ATP that is an energy supplier for numerous metabolic processes, such as synthesis of nucleic acids and proteins in cells with intensified proliferation [3,5]. Increased synthesis of proteins specific for neoplastic cells may be a consequence of activation of the mTOR (mammalian target of rapamycin) pathway by kinase Akt; by phosphorylation of numerous proteins, mTOR regulates the protein synthesis rate and the activity of elongation factor that directly participates in protein biosynthesis [6-8].

An increased glycolytic activity of tumor cells is triggered by increased expression of glycolysis regulatory enzymes, including the pyruvate kinase M2 isoenzyme (PKM2) [9,10]. The PKM2 isoenzyme that is a marker of neoplastic transformation has the C-terminal domain which is responsible for association of dimeric forms or dissociation of the tetrameric form [11]. The tetrameric form, produced at a high level of fructoso-1,6-P2 [12], is responsible in the cytoplasm for ATP synthesis, while the dimeric form, produced at a low level of fructoso-1,6-P2 and capable of penetrating into the nucleoplasm [13], may participate in regulation of kinase Akt activity and thus in regulation of the mTOR signaling pathway activity that is directly associated with tumor cell proliferation [14].

Chitosan, similarly as oligochitosans, inhibits excessive glycolytic activity of tumor cells and thus may contribute to inhibiting kinase Akt activity, indirectly to inhibiting the mTOR signaling pathway and in consequence to inhibiting excessive proliferation of tumor cells.

The objective of the present paper was to determine a possible mechanism of inhibiting Akt kinase activity by chitosan preparations affecting a decreased expression of the Akt kinase gene in both human and mouse breast tumor cells.

2. Materials and methods

2.1. Cell cultures

Studies were carried out on human breast cell lines: normal 184A1and cancer MCF-7 [American Type Culture Collection]. The MCF-7 cells were cultured in MEM medium supplemented with 10% fetal calf serum (FCS), 1% L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, insulin, amino acids. 184A1 breast epithelial cells were cultured in MEGM (Lonza, Clonetics), serum free supplemented with 0.005 mg/ml transferin and 1ng/ml cholera toxin (Gibco). Cell cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2.

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, insoluble fraction – MKCh (V/CH/D/22-24) (deacetylation degree – DD: 97.7%, Mv: 326 kDa , polymer content –2.52%) (no cytotoxicity after 48 h),
B – chitosan, insoluble fraction – MKCh (Chito Clear hqg 95) (deacetylation degree DD – 81.0%, Mv – 293 kDa, polymer content – 2.78%) (no cytotoxicity after 48 h).
Chitosan was provided by the Institute Biopolymers and Chemical Fibers in Łódź. The degree of oligomers deacetylation was measured by the potentiometric titration [15].
C – chitosan lactate + nanoparticles (low concentrating), (Chitosan Fit C) (deacetylation degree – DD: 78%, Mv: 171 kDa , polymer content –2.35%) (no cytotoxicity after 48 h),
D– chitosan lactate + nanoparticles (high concentrating), (Chitosan Fit C) (deacetylation degree – DD: 78%, Mv: 171 kDa , polymer content –2.35%) (no cytotoxicity after 48 h),
C and D was provided by the Poli-Farm Company o.o. in Łowicz.

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, formazan, 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 48 hours.

2.5. Methods

2.5.1. Western blot

Total cellular protein was isolated from the cells according to the procedure described previously [16]. The Western blot analysis was performed on equal amounts of each protein samples. The identification of proteins was performed with respective antibodies: monoclonal mouse antibody against: β-actin (Sigma), PCNA, AKT, p21, N-cadherin, beta-catenin (Cell Signaling). Total protein loading was determined by probing the membranes for β-actin. Bands were visualized using alkaline phosphate-coupled secondary anti-mouse or anti-rabbit antibody (Sigma). Finally, immunoreactions were visualized by NBT/BCIP staining (Roche). After incubation with the secondary antibody, the immune-complexes were visualized by the enhanced chemiluminescence detection system

2.5.2. Cell migration and invasion assay

Cell migration and invasion assays were performed using conventional Boyden chamber transfer methods according to the manufacturer's protocol (BD BioCoat-Tumor Invasion System No. 354166). Quantitation of invading cell was achieved by post-invasion cell labeling with Calcein and measurement the fluorescence of invading cells**.**

2.5.3. Zymography

Gelatinolytic activities of metalloproteinases MMP-2 and MMP-9 were evaluated from the conditioned medium. The proteins were separated on 10% polyacrylamide gel containing 0.1% gelatin (Sigma) in nonreducing conditions. After electrophoresis, the gel was washed two times for 30 min in 2.5% TritonX-100. After 48 h incubation at 37°C in buffer (50 mM Tris pH 7.5; 10 mM CaCl2; 0.15 mM NaCl), the gel was stained with 1% Coomasie blue R250 for 1h and its excess was washed out with methanol (50%)/acetic acid (10%) solvent. Gelatinolytic activity was observed as clear areas in the gel.

3. Results and discussion

Metalloproteinases (MMPs) are proteolytic enzymes that participate in degradation of the extracellular matrix [17,18], thus affecting the growth, migration and invasion of tumor cells [19]. The activity of MMPs depends predominantly on gene expression that is regulated by numerous cytokines and growth factors, and on stabilization or destabilization of their mRNA, post-translation modification, rate of their secretion from the cell or their interaction with tissue inhibitors of matrix metalloproteinases (TIMP). A function of MMP-2 and MMP-9 is degradation of collagen IV, the main component of the basement membranes, including the vascular basement membrane, what is associated with cell migration [20,21].
The normal human 184A1 cells and tumor MCF7 cells incubated for 48 h in presence and absence of the investigated chitosan preparations demonstrated variable migration (Table 1), with the highest decrease in case of the MCF7 tumor cells incubated in presence of D, C and B preparations. The preparations demonstrated a similar degree of deacetylation, and by the same token charge, what may be of significance in interactions between chitosan particles and cell membrane receptor. D and C chitosans differed in nanoparticle concentration; a higher concentration in D preparation proved to be more effective in inhibiting tumor cell migration.

Table 1. Cell invasion assays through Martigel coated Bowden chamber (quantification of the extent of cell invasion**).**

Cell line **Factor**	**184A1**	**MCF7**
Control	5-8	87
A– chitosan	no change	65
B– chitosan	5-6	18
C- chitosan	4	14
D- chitosan	4	12

MIGRATION – Boyden Chamber/Matrigel
Cell invasion after 48 hrs treatment with chitosans

A decrease in migration of tumor cells incubated with the investigated chitosan preparations was correlated with a decreased activity of MMP-2 and MMP-9 (Fig. 1.). Metalloproteinases participate in degradation of the surface membrane receptors and may also activate or inactivate some cytokines, chemokines and growth factors, thus taking part in signal transduction to the cells. By activating TGF-β, MMP-2 and MMP-9 stimulate proliferation, what increases invasiveness of tumor cells [22]. One may conclude that regulation of MMP-2 and MMP-9 activity may exert a significant effect on inhibition of tumor cell proliferation.

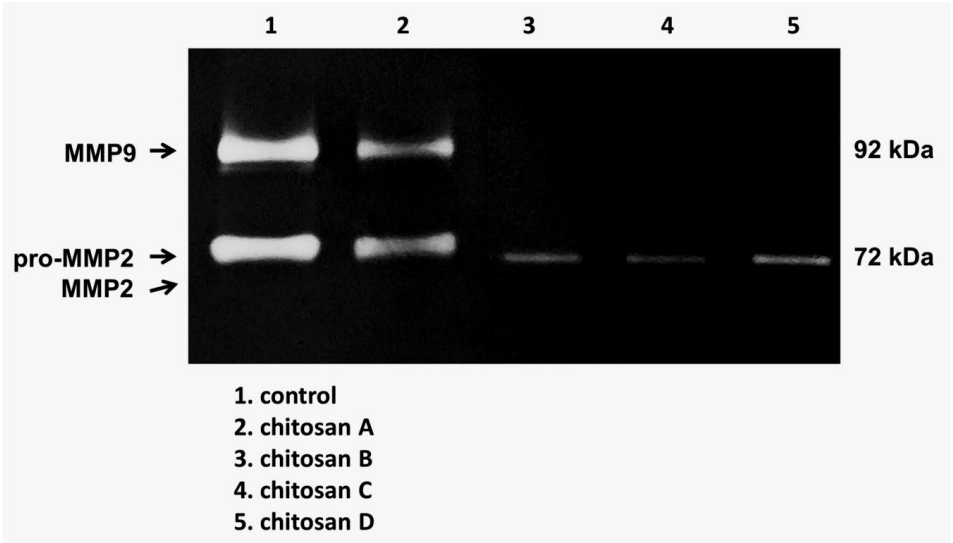


Figure 1. Gelatinolytic activities of metalloproteinases MMP-2 and MMP-9 in human cancer MCF7 cell line**.**

A significant role in regulation of tumor cell metabolism and proliferation is ascribed to the kinase Akt, the amino-acid sequences of which in human and mouse cells demonstrate in 95% the same amino-acid composition [23]. Increased expression and activity of kinase Akt was observed in numerous tumors, including breast carcinoma [2]. A characteristic property of tumor cell metabolism is an increased glycolytic activity [11,12], which is among others manifested as an increased expression of glycolysis regulatory enzymes, including the pyruvate kinase M2 isoenzyme [13]. An increased glycolytic activity in tumor as compared to normal cells results from the increase in ATP levels, necessary for metabolic processes [5], and from an increased activity of kinase Akt that stimulates expression of proteins involved in Akt signaling, including that of hypoxia-inducible factor-1 (HIF-1) [8]. An increased glycolytic activity in tumor cells contributes to an increase of ATP level and a decrease of fructose-1,6-bisphosphate level, what facilitates the pyruvate kinase M2 isoenzyme assuming the dimeric form [13], which is capable of penetrating the nucleus and thus might participate in kinase Akt phosphorylation, resulting in increasing its activity.

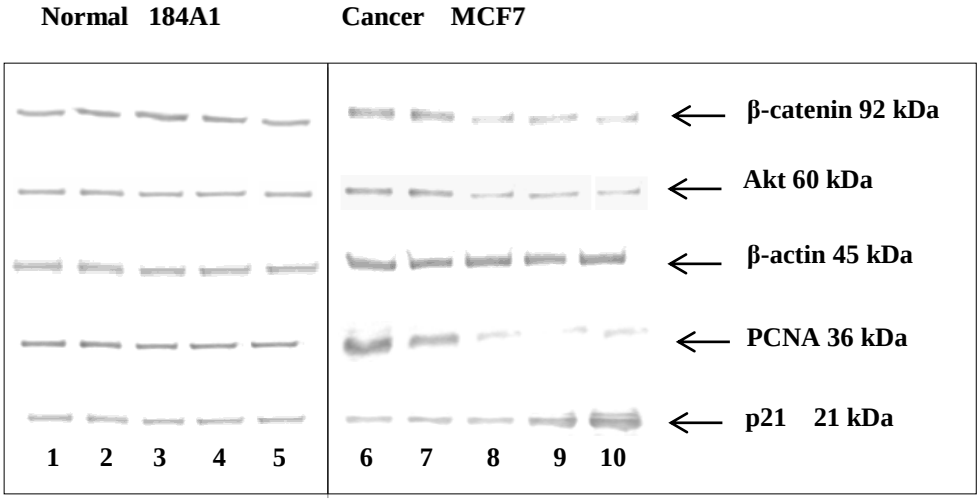
Kinase Akt increases cell proliferation by protein p21 phosphorylation, what leads to capturing the protein in the cytoplasm, preventing its penetration to the nucleus [24]. Kinase Akt also contributes to increased metastasis of tumor cells through its effect exerted on translocation of β-catenin to the nucleus, what stimulates expression of specified integrins as well as changes in expression of surface markers, e.g. E-cadherin, the decreased level of which contributes to weakening cell adhesion. This leads to formation of an invasive phenotype of tumor cells of epithelial origin [2,25].

A significant role in formation of the invasive phenotype of tumor cells is played by MMP-2 [26] and MMP-9 [27]. The role of Akt in tumor cell motility also results from its effect on action cytoskeleton organization [28].

Proliferating cell nuclear antigen (PCNA) is a non-histone nuclear protein. PCNA is a marker of cell proliferation and constitutes an index of proliferation intensity in various types of cancer. PCNA is a factor that collaborates with delta-type DNA polymerase not only in DNA replication processes, but also in DNA repair [29,30]. PCNA may be employed as a marker in assessing neoplastic potential of breast carcinoma cells [31].

In the study assessing the effect of various chitosan preparations on expression of selected genes in the human normal 184A1 and breast carcinoma MCF7 cells evaluated at the protein level, significant differences in inhibiting expression of the selected genes were observed in tumor cells (Fig.2.a.). The B, C and D chitosan preparations proved to be the most active in inhibiting PCNA [32], Akt and β-catenin gene expression.

In case of protein p21, there was observed an increase in its level, what may be related to its increased phosphorylation and in consequence to accumulation of the protein in the cytoplasm. In case of normal cells, no significant alterations resulting from the effect of the employed chitosan preparations were noted in expression of the investigated genes.



1. control
2. chitosan A
3. chitosan B
4. chitosan C
5. chitosan D

6. control
7. chitosan A
8. chitosan B
9. chitosan C
10. chitosan D

Figure. 2.a. Expression of β-catenin, Akt, PCNA and p21 in normal 184A1 and cancer MCF7 in human cell line at the protein level. The upper lanes show the levels of the housekeeping gene β-aktin under the same culture conditions

Similarly as in the case of human cells, in mouse cells, the differences in expression of the investigated genes involved solely the Ehrlich carcinoma cells (Fig.2.b.). In the presence of the investigated chitosan preparations, there was observed inhibition of expression of the N-cadherin, β-catenin, Akt and PCNA genes. In case of p21 protein, its level increased, similarly as in the human breast carcinoma cells, what may also be related to phosphorylation of the protein, its capture by the cytosol and prolonging its half-life as compared to the non-phosphorylated form.

Also in case of the normal mouse CRL 1636 cells, no significant alterations were noted in expression of the investigated genes as the effect of the employed chitosan preparations.

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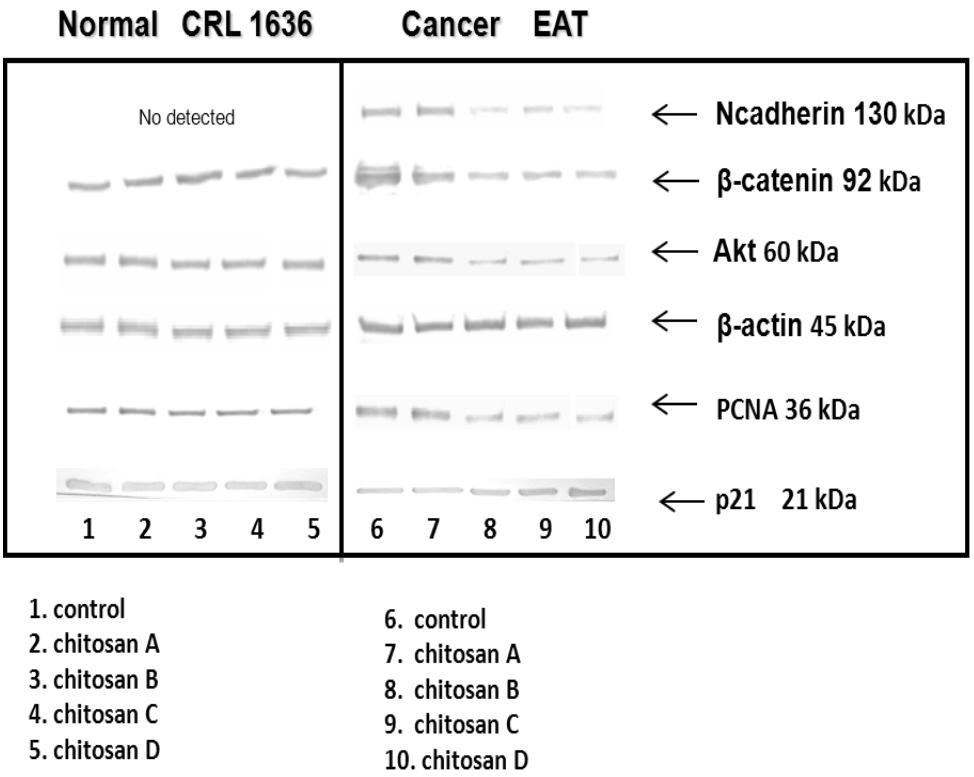

Figure 2.b. Expression of N-cadherin, β-catenin, Akt, PCNA and p21 in normal CRL 1636 and in Ehrlich ascites tumour (EAT) cell line at the protein level. The upper lanes show the levels of the housekeeping gene β-aktin under the same culture conditions.

As it follows from the present investigations, it is suggested that employing specific inhibitors of the Akt signaling pathway may be of a high importance in cancer therapy.

4. Conclusion

1. The increased activity of Akt kinase in mammary gland tumor cells is inhibited by chitosan, which contributes to decreasing the overexpression of the Akt kinase genes, both in human and in murine cells.
2. Akt kinase activates mTOR kinase that directly affects the expression of the HIF-1 (hypoxia-inducible factor-1) transcription factor, which directly affects the glycolysis process enzymes; thus, a decrease in Akt kinase activity will contribute to inhibition of glycolytic metabolism of tumor cells.
3. The present studies employing potential inhibitors of Akt kinase may provide the foundations for developing new therapeutic methods associated with treatment of some neoplastic diseases.

4. Chitosan with a higher-concentration of added nanoparticles seems to be an effective inhibitor of both human and mouse breast tumor cell proliferation.

5. Acknowledgements

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