ROLE OF OLIGOCHITOSANS IN REGULATION OF CELLULAR ACTIVITY AND LOCATION OF PYRUVATE KINASE (PK) ISOENZYME M2 THAT AFFECTS PROLIFERATION OF EHRLICH ASCITES TUMOR CELLS (EAT)

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Abstract
The pyruvate kinase isoenzyme M2 originating from the nucleoplasm and cytoplasm of tumor cells, with its highest affinity to the 2-phosphoenolpyruvate (2-PEP) and sensitivity to L-cysteine, contributes to an increased generation of energy as ATP, necessary for tumor cell proliferation. In the presence of L-cysteine, the isoenzyme M2 PK demonstrates the activity of histone kinase, transferring the phosphoryl group from 2-PEP to the ε-amine residue of the H1 histone lysine. Oligochitosans induce expression of the inducible nitric oxide synthase gene (iNOS), what results in an increased synthesis of nitric oxide, which reacts with L-cysteine and produces L-S-nitrosocysteine. Lack of L-cysteine contributes to inhibition of kinase activity of the H1 histone, an M2 PK isoenzyme. Decreased phosphorylation of the H1 histone contributes to inhibition of EAT cell proliferation. No effect on proliferation of normal cells that include the PK M1 isoenzyme has been observed in the presence of oligochitosans.

Key words: *oligochitosans, isoenzyme M1 and M2 pyruvate kinase, nucleus, ε-methyl-L-lysyne.*

1. Introduction

The organization of chromatin in the cell nucleus is of a high importance in regulation of replication [**1**] and most of all transcription processes [**2, 3**]. The structure of chromatin is modified through processes that depend on ATP hydrolysis, nucleosome shifting and changes in histones-DNA interactions [**4, 5**], acetylation and methylation of core histones [***6, 7***]. The H1 histone is a strongly basic protein; it occupies a different place in the nucleosome structure than core histones [**1**]. Such a location of the H1 histone points to its specific role in stabilizing the nucleosome structure, what renders access to DNA more difficult for other proteins and thus plays a significant role in inhibiting gene expression. The H1 histone strongly inhibits acetylation of core histones [**8**] and affects nucleosome-shifting factors [**9**]; such processes are important in gene expression activation. The molecules of the H1 histone in the nucleus are present in the state of dynamic equilibrium between free and nucleosome-bound forms [**10**]. This points to its participation in maintaining a specific balance between various organizational states of chromatin organization [**11**].

An increasing role in cellular cycle regulation is attributed to glycosylation processes that consist in binding single b-N-acetyl-D-glucosamine [GlcNAc] residues to serine or threonine residues by the O-glycosidic bond of nuclear proteins that participate in the process. At present, more than 200 nuclear proteins have been identified, in which the presence of O-GlcNAc residues has been confirmed [**12, 13**]. Also transcription factors are subject to such a modification, what may affect their stability, intracellular location, activity and interactions with other proteins belonging to transcription complexes or DNA [**14 - 22**]. In case of certain proteins, e.g. class II polymerase, both O-glycosylation and phosphorylation may occur within their C-terminal domains [**22**]; thus, these opposed modifications affect regulations of RNA II polymerase-transcribed genes.

Among natural polymers, chitin [poly-(2-deoxy-2-acetylamineglucose)] is one of the most common glycoaminoglycans. Chitin is present in shells of marine crustaceans and insects, in fungal tissues and in cellular walls of microorganisms [**23**]. A product of chitin deacetylation and depolymerization, chitosan – [poly-(2-deoxy-2-aminoglucose)], is characterized by its solubility in aqueous solutions of organic acids, such as acetic, formic, lactic, pyruvic and oxalic acid. Depending on a technologic process, depolymerization and deacetylation of chitosan allows for obtaining various forms of chitosan, and its degradation using the thermal-mechanical method results in oligochitosans production. In animals, oligochitosans may be produced by chitosan hydrolysis with participation of lysozyme. In view of amine groups being present in chitosan molecules, they may form hybrid systems with proteins or nucleic acids [**24**].

The objective of the present investigations was to demonstrate the role of oligochitosans (potential donors of glucosamine and N-acetylglucosamine) in affecting the location and activity of the pyruvate kinase M2 isoenzyme – an enzyme that is a marker of neoplastic transformation, as well as to determine its role in inhibition of proliferation of Ehrlich ascites tumor cells and propose a possible mechanism of its activity.

2. Material 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 and 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 neoplastic cells in 5% CO_2.

2.1.1. Reagents

A – oligomer (deacetylation degree – DD: 97.7%) (no cytotoxicity after 72 h) and B – oligomer (DD: 70.3%) (no cytotoxicity after 72 h) were provided by the Institute of Biopolymers and Chemical Fibres in Łódź. The degree of deacetylation of the oligomers was measured by the potentiometric titration [**25**]. C – L-S-nitrosocysteine (L-CSNO) was obtained in the reaction of L-cysteine with sodium nitrate (II) (Sigma-Aldrich) [**26, 27**]. D – L-cysteine (L-Cys), L-lysyne (L-Lys), α-methyl-L-lysyne (α-methyl-L-Lys), ε-methyl-L-lysyne (ε-methyl-L-Lys) and ADP (Sigma-Aldrich).

2.2. Incubation procedure

The stock solution of reagent (A and B 0.5%, C and D 10 mM) 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 (final concentration - A and B 0.05%, C and D 1 mM) reagent was replaced every 24 hours throughout the experiments. The cells were incubated for 72 hours.

2.2.1. Detection of cytotoxicity

The cells were seeded in triplicate into 96-microwell plates at the density of 1 - 8 × 10^3 cells per well and incubated without or with different factors for 24, 48 or 72 hours. Subsequently, 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.

2.2.2. Preparation of nuclear and cytoplasmic cell lysates

Nuclear and cytoplasmic extracts were prepared using the CellLytic-NuCLEAR-Extraction Kit (Sigma) according to the manufacturer's protocol and subsequently, equal amounts of protein were used for immunoblot analysis. The procedure for the nuclear protein extraction method is to allow cells to swell with hypotonic buffer. The cells are then disrupted, the cytoplasmic fraction is removed, and the nuclear proteins are released from the nuclei by a high salt buffer [**15**]. Adherent cells from 70 - 90% confluent monolayer culture (CRL 1636.) and cells in suspension (Ehrlich ascites tumor cells) were used. The cells were

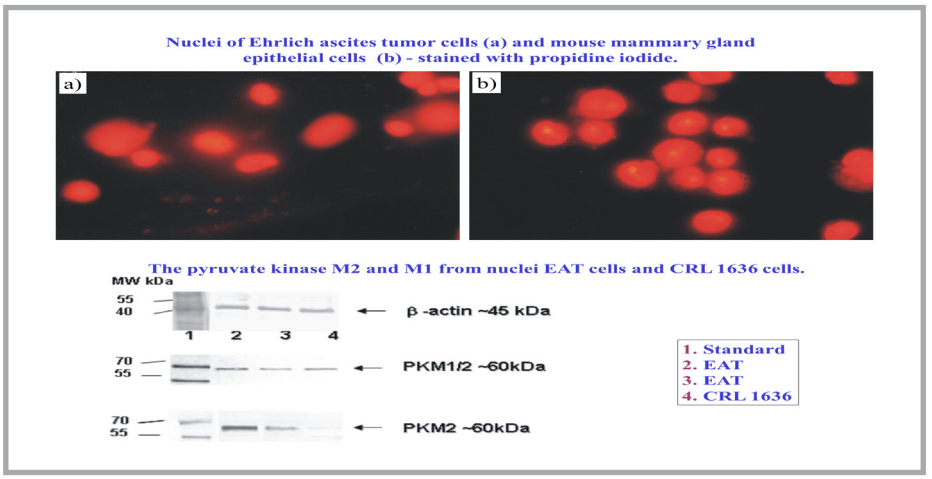


Figure 1. *The pyruvate kinase M2 and M1 from nuclei EAT cells and CRL 1636 cells.*

suspended in 1X Lysis Buffer (including DTT and protease inhibitors). The packed cells were incubated in the lysis buffer on ice for 15 minutes. To the swollen cells in lysis buffer, IGEPAL CA-630 dissolved to a final concentration of 0.6% (6 ml per 100 ml of mixture) was added and vortexed vigorously for 10 seconds. The cells were immediately centrifuged for 30 seconds at 10,000 - 11,000 × *g*. The supernatant with the cytoplasmic fraction was transferred to a fresh tube and stored at -20 °C. The crude nuclei pellet was resuspended in ~70 ml (2/3X PCV) of Extraction Buffer containing DTT and protease inhibitor cocktail. The tubes were mounted on a vortex mixer at a medium to high speed for 15 - 30 minutes and centrifuged for 5 minutes at 20,000 - 21,000 × *g*. The supernatant with nucleic fraction was transferred to a clean, chilled tube, snap-frozen in aliquots with liquid nitrogen and stored at –70 °C. In the resultant fraction, PK M1 and M2 were identified and their activity was determined.

2.2.3. Western blot analysis

The cells were lysed in sample buffer (0.0625 M Tris/HCl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol). The protein concentration was determined by Qnant-iT Protein Assay Kit (INVITROGEN). Cell lysates containing equal amounts of protein were separated on 10% SDS-PAGE gels, and subsequently transferred onto a PVDF membrane. Antibodies against PK M1, PK M2 and β-actin (Sigma) were used to detect the indicated proteins. Bands were visualized using alkaline phosphate-coupled secondary anti-mouse or anti-rabbit antibody (Sigma). Finally, immunoreactions were visualized by NBT/BCIP staining (Roche) see ***Figure 1***.

2.2.4. Protein concentration levels were determined using the method of Lowry et al. [**28**].

2.2.5. Pyruvate kinase activity determination

The activity of pyruvate kinase was determined by the spectrophotometric method according to Bucher and Pfleiderer [**29**].

3. Results and discussion

A feature that distinguishes tumor cells from normal cells is an increased glycolytic activity triggered by their containing various pyruvate kinase (PK) (EC 2.7.1.40) isoenzymes. The M1 and M2 isoenzymes are products of the same gene, but they are produced in the process of alternative exon splicing [**30**]. In the cytoplasm of both normal and tumor cells, pyruvate kinase is associated with glycolysis.

The PK M1 and M2 isoenzymes in the cytoplasm of normal and tumor cells act as catalysts in a reaction associated with ATP production. On the other hand, modification of these proteins through O-glycosylation with participation of N-acetyl-D-glucosamine allows for their shifting to the nucleus. The presence of the PK M1 and M2 isoenzymes in nuclei of normal and tumor cells has been confirmed by the Western blot technique employing PK M1/M2-specific and PK M2-specific antibodies (***Figure 1***).

In some of its properties, the M2 isoenzyme originating from tumor cells resembles the M1 isoenzyme (***Table 1***), yet is demonstrates different sensitivity to certain effectors and ability to be present in a tetrameric form, with a high affinity to 2-phosphoenolpyruvate (2-PEP), just as the other isoenzymes, and also in a dimeric form, with a low affinity to 2-PEP [**31**]. These two molecular forms of the M2 PK isoenzyme seem to fulfill diversified functions, depending on their location within the cell.

Figure 2 presents the effect of various effectors on PK M2 dissociation and association. The tetrameric form (showing a high affinity to 2-phosphoenolopyruvate – (2-PEP)) demonstrates kinase activity associated with ATP synthesis, while the dimeric form (with a low affinity to 2-PEP) may be associated with the activity of histone kinase of the H1 histone.

Table 1. *Molecular and kinetic characteristics of the pyruvate kinase isoenzyme [31].*

Molecular and kinetic characteristics of the pyruvate kinase isoenzymes

	M1-PK	M2-PK
tissue distribution	brain, muscle	adipocytes, pancreatic islet, retina, lung, all proliferating cells, such as normal proliferating cells, embryonic cells, adult stem cells, tumor cells
encoding gene	PKM	PKM
number of amino acids	531	531
molecular weight of single subunit	58 kDa	58 kDa
kinetic characteristics	– tetrameric form with high affinity to PEP	– tetrameric form with high affinity to PEP and – dimeric form with low affinity to PEP
regulation	– not allosterically regulated, – not phosphorylated – not influenced by diet	regulated by – metabolic intermediates, such as fructose 1,6-P2, several amino acids and fatty acids – phosphorylation – oncoprotein and peptide binding

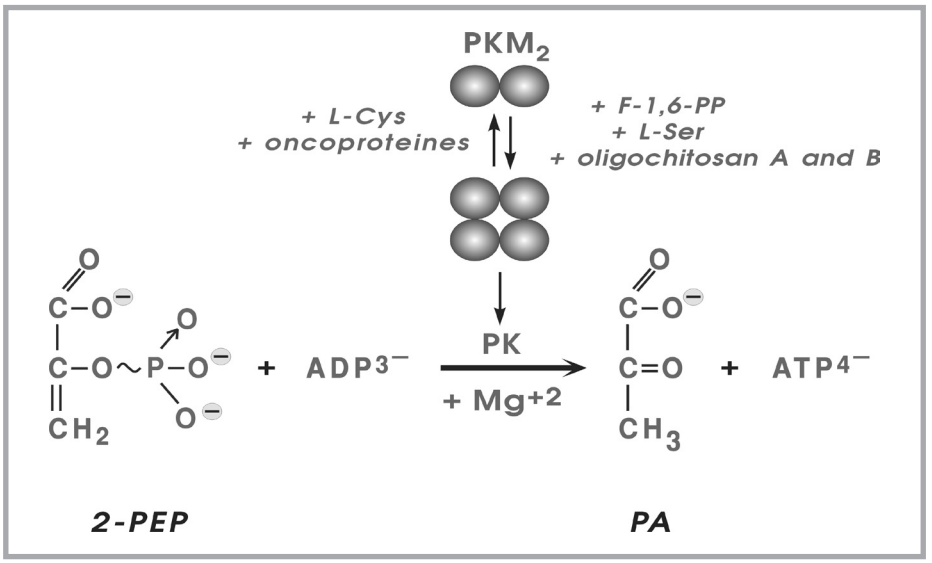


Figure 2. *The effect of various effectors (L-cysteine, L-Cys; onkoprotein[31], fructose-1,6-biphosphate, F-1,6-PP[31], L-serine, L-Ser [31] and oligochitosans A and B on PK M2 dissociation and association. The tetrameric form (showing a high affinity to 2-PEP, 2-phosphoenolopyruvate; PA, pyruvate) demonstrates kinase activity associated with ATP synthesis, while the dimeric form (with a low affinity to 2-PEP) may be associated with the activity of histone kinase of the H1 histone.*

Increased glycolytic activity of tumor cells is related to the cells switching from oxidative to anaerobic metabolism, what in a way allows them for compensating for energy production as ATP. Also the intermediate products in the process of glycolysis are employed as precursors to synthesize structural elements of the cell. This suggests that in the proliferating tumor cells, it is the ratio of the tetrameric to dimeric form in the cytoplasm that decides whether glucose is degraded to lactate in the process of energy production or else whether some compounds produced through glycolysis are employed in the processes of structural compounds synthesis.

On the other hand, the role of the M2 PK isoenzyme in the nucleoplasm of tumor cells seems to be associated with regulation of cell proliferation.

L-cysteine inhibits kinase activity responsible for ATP synthesis of the M2 PK isoenzyme only - a reaction initiated by ADP. On the other hand, L-lysine and a-methyl-L-lysine initiate their phosphorylation with participation of 2-PEP in absence of ADP, what suggests a possibility of phosphorylation of both L-lysine and α-methyl-L-lysine in the e-amine group. This type of phosphorylation may be supported by the lack of histone kinase activity when e-methyl-L-lysine is employed. The present results point to a possibility of the H1 histone phosphorylation in the presence of L-cysteine with participation of the PK M2 isoenzyme in nucleoplasm, what may increase tumor cell proliferation.

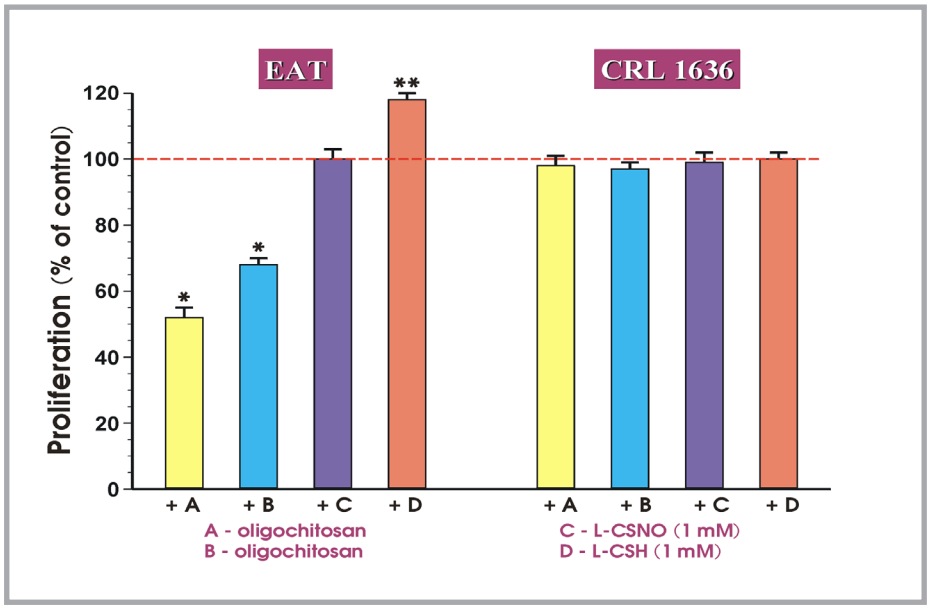
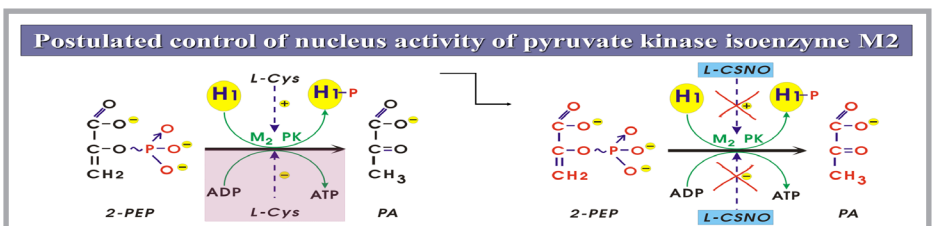


Figure 3. *The effect of oligomers A and B (final concentration 0.05%), L-S-nitrosocysteine (C) (final concentration 1 mM) and L-cysteine (D) (final concentration 1 mM) incubated for 72 h in 37 °C on the proliferation of EAT and CRL 1636 cells. * P < 0.001, ** P < 0.05 (Student's t-test) vs. the control.*

Table 2. *The influence of effectors on the activity of the pyruvate kinase isoenzyme M2 and M1 from the nuclei of EAT and CRL 1636 cells. The final concentration of amino-acid was 0.05 mM. Results are expressed as a percentage of the control activity(EAT PK M2 – 3010 ± 190 mU/mg protein n = 10, CRL 1636 PK M1 – 1990 ± 145 mU/mg protein n = 10).*



The influence of various effectors on activity of the pyruvate kinase isoenzyme M2 and M1 from nucleus EAT and CRL 1636

Effector	Start reaction	% of control activity PK	
		PK M2	PK M1
—	ADP	100	100
L-Cys	ADP	58	100
L-Lys	ADP	72	100
—	L-Lys	52	no activity
α-methyl-L-Lys	ADP	78	100
—	α-methyl-L-Lys	60	no activity
ε-methyl-L-Lys	ADP	100	100
—	ε-methyl-L-Lys	no activity	no activity
L-CSNO	ADP	100	100
—	L-CSNO	no activity	no activity

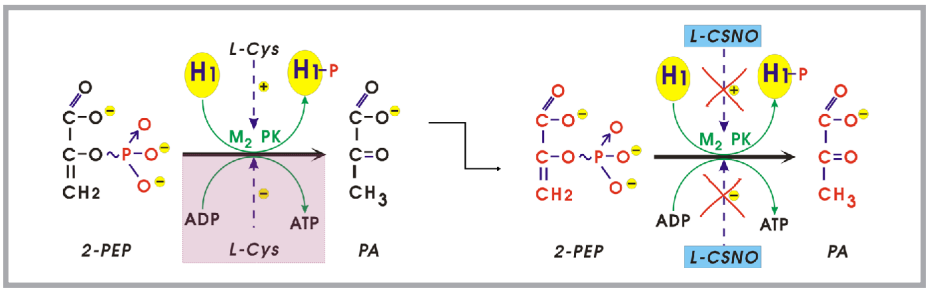


Figure 4. *Postulated control of nucleus activity of M2 pyruvate kinase (M2 PK) by L-cysteine (L-Cys) and L-S-nitrosocysteine (L-CSNO). 2-PEP, 2-phosphoenolpyruvate; PA, pyruvate; H1, histone H1; H1-P, phosphohistone H1; (+) activation; (–) inhibition; P, refers to phosphate groups.*

Microcrystalline chitosan and products of its degradation – oligochitosans – contribute to an increase of nitric oxide in tumor cells [**32**], what results in synthesis of L-S-nitrosocysteine in a reaction with L-cysteine [**33**]. Lack of L-cysteine inhibits the activity of the M2 PK histone kinase and in consequence does not effect the H1 histone phosphorylation [**32**]. Non-phosphorylated H1 histone is a potent inhibitor of cell proliferation [**34**] see ***Figure 4***.

4. Conclusions

- Oligochitosans, as donors of glucosamine and N-acetylglucosamine, may contribute – through their participation in protein glycosylation – to formation of β-O-N-acetylglucosamine derivatives, what allows for directing these proteins (including the PK M1 and M2 isoenzymes) to the nucleus and for regulating both replication and transcription processes.
- Oligochitosans contribute to an increase of nitrogen oxide production in EAT cells; nitrogen oxide reacts with L-cysteine and contributes to deactivation of the PK M2 histone H1 kinase activity, what renders impossible phosphorylation of the H1 histone, which is the most potent inhibitor of cell proliferation.

5. References

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