THE EFFECT OF CHITOSAN ON THE SYNTHESIS OF L-S-NITROSOCYSTEINE THAT PARTICIPATES IN THE REGULATION OF THE M2 PYRUVATE KINASE ISOENZYME ACTIVITY ASSOCIATED WITH EHRLICH ASCITES CELLS PROLIFERATION *IN VITRO*

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Abstract
L-S-nitrosocysteine formation in EAT tumor cells and normal CRL-1636 cells incubated with microcrystalline chitosan was confirmed by RP-HPLC. The metabolite was identified based on UV-VIS spectra. The formation of L-S-nitrosocysteine in EAT tumor cells contributes to decreasing the level of L-cysteine in these cells. L-cysteine as an effector of the bifunctional M2 isoenzyme of pyruvate kinase (PK) initiates its histone kinase activity, which is responsible for histone H1 phosphorylation. A decrease of L-cysteine level in EAT tumor cells contributes to lack of histone H1 phosphorylation by the M2 PK isoenzyme and by the same token to inhibition of EAT cell proliferation.

Key words: *microcrystalline chitosan, L-S-nitrosocysteine, pyruvate kinase M2 isoenzyme, L-cysteine, inhibition of EAT cell proliferation.*

1. Introduction

As a polycation, chitosan is a polymer of β-D-2-desozy-2-aminoglucose and a product of partial hydrolysis and deacetylation of chitin [1]. Chitosan demonstrates a series of unique properties, such as lack of toxicity [2], immunostimulatory effects [3], biodegradability [4], antitumor activity [5, 6], as well as capability of binding to both proteins and nucleic acids [7, 8]. In view of its large molecular mass, chitosan is not capable of crossing cell membranes; as a polycation, it may only react with the cell membranes of tumors cells, which have a reduced membrane charge as compared to the charge of normal cells. Acting upon the cell membrane surface of T24 human bladder carcinoma cell line [9] and Ehrlich ascites tumor cells (EAT) [10], microcrystalline chitosan resulted in a decrease in their elasticity, what was the effect of reorganization of their cytoskeleton.

In addition to a lower membrane surface charge as compared to normal cells, tumor cells are also characterized by an increased glycolytic activity, triggered by such factors as increased expression of genes that encode regulatory enzymes involved in the process of glycolysis, including the M2 isoenzyme of pyruvate kinase (PK) [11].

In tumor cells with their increased proliferation, there occurs a switch of oxygen metabolism to glycolytic metabolism. This occurs due to the necessity of maintaining a high reduction potential in these rapidly dividing and intensely DNA, RNA and proteins-synthesizing cells [12].

Earlier experiments demonstrated inhibition of glycolytic activity expressed by lactate production and ATP level by microcrystalline chitosan in EAT cells [13] as well as in T24 bladder carcinoma cells [9].

Pyruvate kinase (EC 2.7.1.40) (PK) is one of the three regulatory enzymes involved in the process of glycolysis and is directly associated with ATP synthesis. The activity of the enzyme has been demonstrated in both the cytoplasm and nucleoplasm of tumor cells [14, 15]. In tumors cells, the M2 PK enzyme is present, the activity of which is regulated in a different way as compared to the M1 isoenzyme from mouse mammary gland epithelium cells [16].

Tumor cells exhibit a lower level of nitric oxide synthesis in comparison to normal cells. In tumor cells, all three isoforms of nitric oxide synthase (iNOS, eNOS, nNOS) participate in NO synthesis. The most common form is the cytoplasmic iNOS and the expression of this isoform is correlated with the grade of malignancy and proliferation of human breast carcinoma [17, 18]. A low level of NO that approaches physiological values inhibits programmed cells death [17], while a high NO level induces cell apoptosis [19]. An increase of NO synthesis in EAT cells as the effect of microcrystalline chitosan [20] indicates a possibility of L-cysteine nitrosylation and L-S-nitrosocysteine formation [21, 22]. The reaction, in which L-cysteine is bound, results in its being deactivated as an allosteric effector that switches the kinase activity of the M2 PK enzyme to histone kinase activity, what leads to lack of phosphorylation of H1 histone by the M2 PK isoenzyme. Lack of H1 histone phosphorylation may be a mechanism of inhibiting excessive proliferation of tumor cells [14].

The objective of the present paper was to demonstrate the formation of L-S-nitrosocysteine in EAT cells as the effect of microcrystalline chitosan, L-S-nitrosocysteine being a metabolite that plays a significant role in regulating the activity of the bifunctional M2 PK isoenzyme in tumor cells.

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 neoplastic cells in 5% CO_2.

2.1.1. Reagents

Microcrystalline chitosan (molecular weight – 320 kDa, polymer content – 2.1%, deacetylation degree – 98.8%) (no cytotoxicity after 72 h) was provided by the Institute of Chemical Fibres in Łódź. The molecular mass of chitosan was determined employing the viscosimetric method and the degree of deacetylation was measured by the potentiometric titration [23]. L-cysteine (Sigma-Aldrich). L-S-nitrosocysteine was obtained in the reaction of L-cysteine with sodium nitrate (II) (Sigma-Aldrich) [24, 25]

2.2. 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.

2.2.1. Detection of cytotoxicity

The cells were seeded in triplicates 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. 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.

2.2.2. The L-S-nitrosocysteine level in Ehrlich ascites tumor cells and in normal CRL-1636 cells was determined by RP-HPLC (reversed-phase high performance liquid chromatography). The LC system (Shimadzu Corporation Kyoto Japan) consisted of two solvent deliv-

ery module LC 10 AT vp and DGU-14 A degasser, a CTO-10 ASvp column oven, a SIL-10 ADVp autosampler, a SPD-M 10 Avp Diodearray detector. The CLASS-VP 7.2.1. was used for data collection and processing. The samples were separated at 20 °C on a Phenomenex Luna C_{18} (4 × 2.0 nm i.d.) guard column.

The supernatants of tumor and normal cells were used in derivatization. Derivatization consists in preparation of nitrophenyl derivatives, including N-DNP-GSNO. Samples to be derivatized contained: 100 µl of supernatant, 20 µl of N-methyl-L-Lysine (internal standard), 40 µl of 10% PCA/1 mM BPDS, 96 µl of 2 M KOH-2,4 M KHCO3and 200 µl of 1% DNFB. Following 24-hour derivatization at ambient temperature in dark, the samples were acidified by addition of 35 µl 70% PCA and centrifuged at 5600 g for 2 min. The resulting supernatants were filtered through a PTFE-Supelco filter and a 90-minute procedure of separation of the analyzed compounds was carried out. The chromatographs were analyzed and the separated compounds were identified according to their retention times compared to sample solutions.

Samples were eluted [26] with a mobile phase consisting of solvent A (water/0.1% trifluoroacetic acid – TFA) and solvent B (acetonitrile/ 0.1% TFA). After injection the samples were eluted with 20% B followed with a 35 min linear gradient to 50% B and followed with a 20 min linear gradient to 55% B, then a 15 min linear gradient to 100% B and a 5 min isocratic period. The column was then re-equilibrated to the initial conditions for 15 min. All HPLC solvents were filtered through a 0.20 µm nylon filter. Analyses of 20 µl of samples were performed at a flowrate of 1.0 ml/min with UV-VIS detection at 365 nm.

2.2.3. Isolation of nuclei from normal and tumor cells

The Ehrlich ascites tumor cells and reference cells were homogenized in a glass Potter-Elvehjm homogenizer with A buffer (containing 0.25 M saccharose, 3 mM $CaCl_2$, 10 mM Tris) (pH = 7.4), and subsequently centrifuged for 10 min. at 4 °C and 1000 g. (nuclear sediments were washed twice in A buffer). The nuclear sediments from the investigated cells (at the ratio of 1:9) were then suspended in B buffer (containing 2.2 M saccharose, 3 mM $CaCl_2$, 10 mM Tris) (pH = 7.4) and centrifuged for 60 min. at 4 °C and 40 000 g. The pellets obtained were washed twice in buffer A and centrifuged at 1000 × g for 5 min. Purified nuclei were lysed according to Bloom and Anderson [27].

2.2.4. Pyruvate kinase activity determination

The activity of pyruvate kinase was determined by the spectrophotometric method according to Bucher and Pfleiderer [28].

2.2.5. Protein concentration levels were determined using the method of Lowry et al. [29].

3. Results and discussion

Using the method of reverse phase high-performance liquid chromatography (RP-HPLC), the authors confirmed the formation of L-S-nitrosocysteine in EAT tumors cells and in normal CRL-1636 cells. L-S-nitrosocysteine may be formed in the reaction of L-cysteine with nitric oxide (Equation 1).

$$\text{L-CSH} + \text{NO} \rightarrow \text{L-CSNO} + \text{H}^+ \quad (1)$$

Nitric oxide is formed directly from L-arginine in the reaction catalyzed by nitric oxide synthase (NOS). The compound is characterized by a short half-life; in order to affect a tumor cell, it must be synthesized in the cell itself. The most common form of the enzyme responsible for nitric oxide synthesis is cytoplasmic iNOS and the expression of this form correlates with malignancy grade and proliferation of human breast cancer [17, 18].

Previous studies demonstrated that the level of nitric oxide in EAT tumor cells was lower as compared to reference CRL 1636 cells. In the presence of microcrystalline chitosan, EAT cells demonstrated an almost twofold increase in nitric oxide level, while in normal CRL 1636 cells exposed to chitosan, the increase was less significant [15]. An increase of the level of nitric oxide in EAT cells may contribute to an increased nitrosylation of L-cysteine and by the same token, to an increased synthesis of L-S-nitrosocysteine.

As it follows from the comparison of chromatograms (***Figure 1.A*** and ***1.C***, as well as ***Figure 2.A*** and ***2.C***), both in EAT tumor cells and in normal CRL 1636 cells in the presence of microcrystalline chitosan there occurs an intensified synthesis of L-S-nitrosocysteine, what most assuredly contributes to decreasing the level of L-cysteine in these cells.

Table 1. *The activity of pyruvate kinase (PK) in cytoplasm and nucleoplasm of EAT tumor cells (M2 PK isoenzyme) and normal CRL 1636 cells (M1 PK isoenzyme) incubated in the presence and absence of microcrystalline chitosan (final concentration of 0.05%) for 72 h at 37^{0}C, and the effect of 1 mM L-S-nitrosocysteine (final concentration) and 1 mM of L-cysteine (final concentration) on the PK activity of the studied effectors.*

Cells	Pyruvate kinase activity, mU/mg of protein / % of control			
	control	+ chitosan	+ L-CSNO	+ L-CSH
EAT (PK M2)	X ± SD (n=12)			
- cytosol:	7350 ± 260	80	100	53
- nucleus:	2850 ± 160	72	100	58
CRL 1636 (PK M1)				
- cytosol:	4350 ± 230	100	100	100
- nucleus:	2110 ± 130	100	100	100

In contrast to normal CRL 1636 cells incubated with microcrystalline chitosan, EAT cells showed a decreased activity of the M2 PK isoenzyme as the effect of microcrystalline chitosan, seen both in cytoplasm and nucleoplasm; the decrease of the activity of the M2 PK isoenzyme correlated – as it had been previously demonstrated – with inhibition of the M2 PK gene expression in EAT cells incubated in the presence of microcrystalline chitosan [30].

Of the investigated effectors, only L-cysteine did result in a twofold decrease of the M2 PK isoenzyme activity in cytoplasm and nucleoplasm of tumor cells, not affecting, however, the activity of the M1 PK isoenzyme in normal cells.

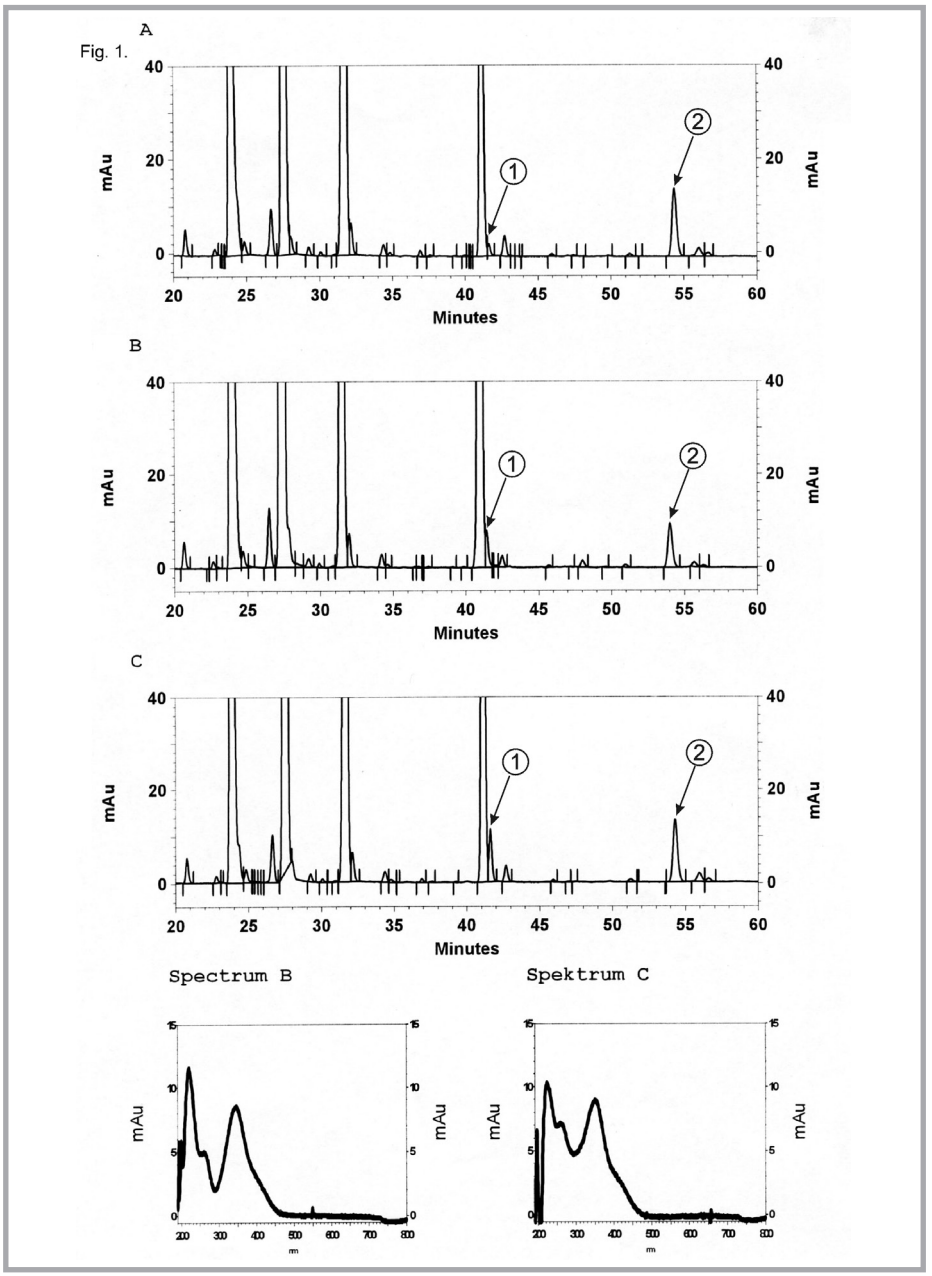

Figure 1. *Chromatograms of (A) EAT lysate with N^4-methyllysine, (B) EAT lysate with 1 mM L-S-nitrosocysteine and N^4-methyllysine, (C) EAT (incubated for 72 hours with microcrystalline chitosan) lysate with N^4-methyllysine. Peaks: 1 = N-DNP-L-S-nitrosocysteine, 2 = N,N'-di-DNP-N^4-methyllysine. Spectrum B and C in the range of 200-800 nm obtained by a diode detector for peak 1 of chromatograms B and C (maximum absorbance for L-S-nitrosocysteine – 350 nm).*

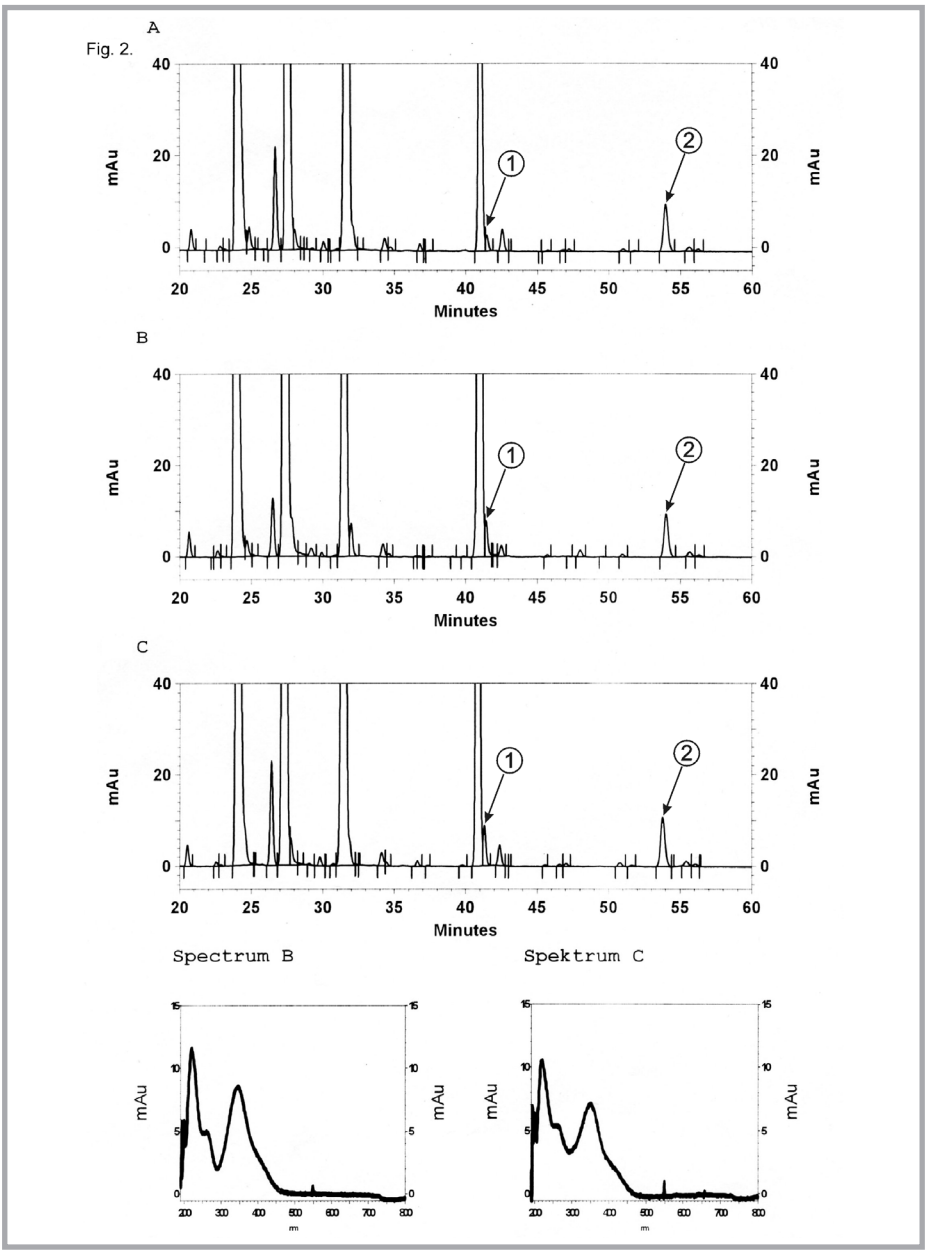

Figure 2. *Chromatograms of (A) CRL 1636 lysate with N^4-methyllysine, (B) CRL 1636 lysate with 1 mM L-S-nitrosocysteine and N^4-methyllysine, (C) CRL 1636 (incubated for 72 hours with microcrystalline chitosan) lysate with N^4-methyllysine. Peaks: 1 = N-DNP-L-S-nitrosocysteine, 2 = N,N'-di-DNP- N^4-methyllysine. Spectrum B and C in the range of 200-800 nm obtained by a diode detector for peak 1 of chromatograms B and C (maximum absorbance for L-S-nitrosocysteine – 350 nm).*

L-cysteine is an effector for the bifunctional M2 PK isoenzyme, in the presence of which there occurs a change of the kinase activity responsible for transferring the phosphoryl residue from 2-fosfoenolopyruvate (2-PEP) to ADP occurring with pyruvate and ATP formation, to the kinase activity of H1 histone, which is responsible for transferring the phosphoryl residue from 2-PEP to H1 histone occurring with formation of pyruvate and phosphorylated H1 histone [**16**]. An increased synthesis of nitric oxide that leads to an increased nitrosylation of L-cysteine and, by the same token, an increased synthesis of L-S-nitrosocysteine, contributes to a significant decrease of L-cysteine level in EAT cells, what results in lack of H1 histone phosphorylation by the M2 PK isoenzyme. H1 histone is a potent inhibitor of cell proliferation. The observed effect of inhibition of EAT cell proliferation in the presence of microcrystalline chitosan may be partially explained by inhibition of the histone kinase activity of the M2 PK isoenzyme in consequence of a significant decrease of L-cysteine level in these cells.

4. Conclusions

In the presence of microcrystalline chitosan, Ehrlich ascites tumor cells demonstrated an increased synthesis of nitrous oxide and formation of L-S-nitrosocysteine.

Nitrosylation of L-cysteine to L-S-nitrosocysteine is a process, which excludes this signaling particle from participation in initiation of H1 histone phosphorylation and may contribute to inhibition of H1 histone phosphorylation and in consequence lead to inhibition of excessive tumor cell division.

5. References

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