1. Introduction

Neoplastic cells are characterized by morphological and biochemical properties that vary from the features observed in normal cells. Tumor cells demonstrate an increased glycolytic activity [1], a higher reduction potential expressed by a high NADPH level [2], an intensified synthesis of DNA, RNA and proteins [2, 3], changes in the cytoskeleton (4) and in cellular membrane structure [5]. The differences in the glycolytic activity between tumor and normal cells are triggered by many factors, including the fact that these two types of cells contain different isoenzymes of pyruvate kinase (PK) (EC 2.7.1.40). The L - isoenzyme is the dominant form in hepatic cells [6], the M₁ - isoenzyme predominates in muscle and brain cells, while the M₂ - isoenzyme is most abundantly represented in tumor cells [7 - 9]. These three PK isoenzymes have separate amino acid composition and constitute the products of various genes (9). The M₁ and M₂ - isoenzymes are produced by the same gene, but they are formed by alternative exon mergeing [10]. Some properties of the M₂ - isoenzyme originating from a tumor, resemble the M₁ - isoenzyme in view of its hyperbolic kinetics, lack of activation by fructoso-1,6-biphosphate and low sensitivity to the inhibitory effect of ATP [11].

In addition to other factors, the increased glycolysis rate in tumor cells results from overexpression of genes that encode enzymes responsible for glucose metabolism [12], including the key enzyme in the glycolytic process, i.e. the pyruvate kinase M₂ - isoenzyme, directly associated with ATP synthesis both in the cytoplasm and in the nucleoplasm [13].
The PK M₂ - isoenzyme is one from among the four regulatory enzymes, active in the process of glycolysis, the very process being directly related to ATP synthesis. The presence of the enzyme in the nuclei is associated with the process of glycolysis occurring in the nucleoplasm [14]. The thus formed ATP provides a supplementary source of energy for intensified transcription and translation. The regulation of glycolysis in the nucleoplasm of tumor cells depends on the interaction between the PK M₂ - isoenzyme with DNA or the H₁ histone [15].

A factor that restricts the glycolytic activity of tumor cells, which is expressed by a depressed lactate production and a decreased ATP level [16], as well as by an inhibition of neoplastic cell proliferation [17] has been found in microcrystalline chitosan. For this reason, the present investigations have aimed at determining the effect of microcrystalline chitosan on the expression of selected genes involved in glycolysis in Ehrlich ascites tumor and normal mammary epithelial cells in mice.

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, 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% CO₂ and neoplastic cells in 5% CO₂.

2.2. Reagents

Microcrystalline chitosan (A) (molecular weight – 299 kDa, polymer content – 1.95%, deacetylation degree – 97.7%) was provide by the Institute of Chemical Fibers in Łódź, (no cytotoxicity after 72 h).

Chitosan-Fit (B) (chitosan lactate, molecular weight –171 kDa, polymer content – 2.35%, deacetylation degree – 78% ) was provide by Gumitex Poli-Farm in Łowicz (no cytotoxicity after 72 h).

The molecular mass of chitosan was determined employing the viscosimetric method and the degree of deacetylation was measured by the potentiometric titration [18].

The cells were treated by Microcrystalline Chitosan and Chitosan-Fit at the final solution concentration of 0.05% in medium from 1 to 3 days.

2.3. Incubation procedure

The stock solution of chitosan (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
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The cells were suspended in the NCTC-135 before each experiment and stored at 37°C. The medium containing 0.05% reagents was replaced every 24 hours throughout the experiments. The cells were incubated for 24 hrs or for 72 hours, and then the mRNA levels were measured using the RT-PCR methods.

2.4. 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, 48 or 72 hours. Afterwards, the cells were mixed with the reaction mixture from the Cytotoxicity Detection Kit (Boehringer-Roche). The reaction was stopped with 1M HCl. The colorimetric assay for the quantification of cell death was based on the measurement of lactate dehydrogenase (LDH) 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.

2.5. Analysis of cell proliferation
The cell proliferation was examined by the colorimetric ELISA BrdU assay (Roche, Mannheim, Germany) at 24, 48 and 72 hours of culture in 96-well plates according to the manufacturer’s instructions. The absorbance was measured using a microtiter plate reader at 450 nm wavelength.

2.6. RNA extraction
Following 24 and 72 h incubation with 0.05% Microcrystalline Chitosan and Chitosan-Fit or vehicle alone, the cells were harvested and immediately processed to isolate the total RNA. The total RNA was isolated from the cells and purified using the RNasy Mini Kit from QIAGEN (Germany). The yield and purity of the isolated RNA were checked by a UV spectrophotometric measurement and by electrophoresis in 1.2% agarose gel followed by routine ethidium bromide staining. RNA preparations characterized by A_{260}/A_{280} not less than 1.8 (≥ 1.8) and showing only 18S (1/3) and 28S (2/3) rRNA bands were used for further analysis. The isolated RNA samples were stored in -80°C until used for the RT-PCR analysis.

2.7. cDNA synthesis
RNA samples were reverse transcribed in a final volume of 20 µl reaction mixture containing: 1 x reverse transcriptase buffer – 0.5 mM each dNTP, 3mM MgCl₂, 75mM KCl, and 50mM Tris-HCl (pH 8.3); 10 units of RNase inhibitor (Promega, USA); 10 mM DTT; 50 units of Superscript II RNase H reverse transcriptase (QIAGEN, Germany); 1 µM oligo-dT primer (Sigma) and 1µg total RNA. Prior to addition to the reaction mixture, the RNA samples were incubated at 65 °C for 5 min and cooled at 4 °C for 2 min. cDNA synthesis was carried out at 37 °C for 50 min, and reverse transcriptase was inactivated by heating the reaction mixture at 93 °C for 5 min.

2.8. Polymerase chain reaction
The amplification of cDNA samples was run in a 20 µl reaction volume that contained the following: 1 µl of synthesized cDNA; 1 µM of each of gene-specific primer pair (Oligo
Company, Poland); 0.5 U Taq DNA polymerase in PCR reaction, 1 x buffer supplemented with 1.5 mM MgCl\textsubscript{2} and 0.2 mM of each dNTPs (QIAGEN). The temperature profile of amplification consisted of activation of Taq polymerase at 95 °C for 4 min., denaturation of cDNA at 95 °C for 60 s, elongation at 72 °C for 1 min for the following 30 cycles, and was finished by an extension step for 10 minutes. The amplifications were done as single tube PCR in a Thermal Cycler PTC 200 (MJ Research Inc.) (Table 1). The PCR products were analyzed by 2% agarose gel electrophoresis in the TAE buffer (40 mM Tris-acetate, 2 mM EDTA, pH 8.5) and visualized by ethidium bromide staining.

The PCR products were separated on 2% agarose gel (Figure 2 a, and 2 b).

### Table 1.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences for amplification of gene transcripts</th>
<th>Product</th>
</tr>
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<tbody>
<tr>
<td>M2-PK</td>
<td>5' ccatctacactgagcttattgc 3' 5' tctagtgacagcagtcgc 3'</td>
<td>431 bp</td>
</tr>
<tr>
<td>M1-PK</td>
<td>5' agctcgtgaggctgagcggc 3' 5' cacatcccctccttggaagaagct 3'</td>
<td>382 bp</td>
</tr>
<tr>
<td>L-PK</td>
<td>5' acctctgcttttcttttctctgact 3' 5' tggaaagactcgtgctgtct 3'</td>
<td>322 bp</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5' ccatggagaaggctcgg 3' 5' caaagttgtcatggatgacc 3'</td>
<td>180 bp</td>
</tr>
</tbody>
</table>

### 3. Results and discussion

The effect of chitosan on the expression of M\textsubscript{1}-PK, M\textsubscript{2}-PK, L-PK on mRNA level was studied on two cell lines CRL 1636 and EAT. Routinely, incubation with chitosan was continued from 24 to 72 hrs. No cytotoxic effect of applied concentration of chitosan A and B [0.1%] on any of studied cell lines was observed. The cell lines reacted differently is response to chitosan treatment.

Microcrystalline chitosan, as well as chitosan lactate inhibited Ehrlich ascites tumor cell proliferation (Figure 1). The inhibition of EAT cell proliferation in the presence of microcrystalline chitosan after 72 h amounted to 56% of the control value and the effect was stronger than that observed when chitosan lactate was employed. The inhibitory effect exerted on EAT cell proliferation after 72 h was three times stronger in the presence of microcrystalline chitosan as compared to chitosan lactate.

The results were analysed statistically using Student’s t-test.

The chitosan-induced inhibition of EAT cell proliferation may result from the inhibition of the glycolytic activity in tumor cells by chitosan, which is expressed by a decreased lactate production and a depressed ATP level (16, 18). ATP is indispensable for DNA and RNA synthesis in the process of cell proliferation.
Contrary to tumor cells, normal cells demonstrated no cellular proliferation inhibition as the effect of the investigated chitosan preparations. After 24 hours, chitosan lactate showed a slight stimulatory effect on normal cell proliferation (Figure 1.).

In the investigations of the expression of selected genes of glycolytic enzymes – glyceraldehydephosphate dehydrogenase (GAPDH), as well as the pyruvate kinase M1, M2 and L - isoenzymes in tumor and normal cells studies employing the RT-PCR method in the presence of the tested chitosan preparations, noted were differences in expression solely in the EAT cells and involved the expression of the PK M2 isoenzyme gene (Figure 2a and 2b).

A decrease in the ATP level in EAT cells incubated in the presence of chitosan may have been caused by a depressed activity of the PK M2 - isoenzyme resulting from a decreased expression of the gene that encodes the isoenzyme. Tumor cells are characterized by a higher degree of plasticity as compared to normal cells, what results from different organization of their cytoskeletons (4), as well as a lower charge of the cellular membrane (5). Chitosan reacts with a negatively charged cellular membrane surface, resulting in their decreased plasticity, which is associated with reorganization of their cytoskeletons (20). Changes in cytoskeletal organization may cause the association of cytosol-soluble enzymes involved in glycolysis and the cytoskeletal proteins, which in turn may trigger changes in their activity. Pyruvate kinase forms reversible bonds with cytoskeletal proteins; the latter have specific domain for such bonding, i.e. tubulin (21). PK may also bond phospholipids contained in cellular membranes and subcellular structures, what also leads to changes in its activity (22-25). However, the inhibition of the PK M2 - isoenzyme gene expression in EAT cells in the presence of chitosan seems to play a decisive role in depressing the

**Figure 1.** The effect of microcrystalline chitosan and lactate chitosan on the proliferation of EAT and normal mouse mammary epithelial cells after 24, 48 and 72 h incubation at 37 °C; a \( p<0.001 \); b \( 0.001<p<0.01 \); c \( 0.02<p<0.05 \).
The effect of highly deacetylated microcrystalline chitosan on the pyruvate kinase activity of the isoenzyme that is directly associated with ATP production. The problem requires further investigations.

4. References

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