EFFECT OF OLIGOCHITOSANS ON EXPRESSION OF INOS GENE IN EHRLICH ASCITES TUMOUR *IN VITRO*

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Abstract
Oligochitosans obtained through degradation of macromolecules of chitosan with a high degree of deacetylation turned out to be biologically active, contributing to an increase of nitric oxide levels in Ehrlich ascites tumor (EAT) cells through inducing expression of the isoform of inducible nitric oxide synthase (iNOS) gene. An increase of NO levels in EAT cells in the presence of the investigated oligochitosans might contribute to nitrosylation of L-cysteine – an allosteric effector of the M2 isoenzyme of pyruvate kinase (PK), which switches the PK kinase activity, responsible for ATP synthesis, to the histone kinase activity that may participate in histone H1 phosphorylation. Lack of the histone activity of the PK M2 isoenzyme may contribute to decreased histone H1 phosphorylation and thus inhibit EAT cells proliferation.

Key words: *chitosan oligomers, iNOS (inducible nitric oxide synthase), nitric oxide, histone H1.*

1. Introduction

Nitric oxide is a free radical that is generated in animals from L-arginine in a reaction catalyzed by nitric oxide synthase (NOS). The enzyme has four isoforms: (e-) endothelial, (n-) neuronal, (mit-) mitochondrial and (i-) induced [1, 2]. All the NOS isoforms were detected in tumor cells, and iNOS expression is correlated with malignancy grade and the degree of proliferation in breast cancer cells in humans [3, 4]. NO may affect zinc ion removal from zinc finger domain as well as proteins constituting transcription factors and thus lead both to inhibition and activation of gene expression depending on the promoter structure and types of transcription factors [5, 6]. Hmadcha et al. demonstrated that both endogenously generated and exogenously supplied NO may activate DNA methyltransferases. Methylation of the CpG sequences within a specified gene, in which DNA methyltransferases participate may result in silencing such genes [7]. Activation of DNA methyltransferases by NO most likely occurs via nitrosylation of cistern residues [8]. The reaction of NO with the sulfhydryl group from free cysteine and cysteine residuals of polypeptide chains contributes to formation of S-nitrosocysteine, which plays a significant role in cell functioning [9].

Tumor cells are characterized by an increased glycolytic activity evoked by such factors as increased expression of genes coding for glycolytic enzymes, including the pyruvate kinase (PK) M2 isoenzyme [10]. Pyruvate kinase (EC 2.7.1.40) is a regulatory glycolytic enzyme that is directly associated with ATP synthesis reaction. The activity of the enzyme was demonstrated both in cytoplasm and in nucleoplasm of tumor cells [11]. In tumor cells, there is the PK M2 isoenzyme, which exhibits regulatory activity that is different from that demonstrated by the M1 isoenzyme originating from normal mouse mammary epithelial cells (CRL 1636). In the presence of L-cysteine, the PK M2 isoenzyme exhibits the histone H1 kinase activity, while in the presence of L-S-nitrosocysteine, it shows the ATP synthesis-associated phosphotransferase activity [12]. Previous studies demonstrated a lower level of nitric oxide in Ehrlich ascites tumor cells (EAT) as compared to CRL 1636 cells in the presence of microcrystalline chitosan and products of its degradations, i.e. oligochitosans; an increase of NO levels was observed in EAT cells simultaneously with inhibition of glycolytic activity of tumor cells [13,14]. An increase in NO synthesis in tumor cells as the effect of microcrystalline chitosan and oligochitosans points to a possibility of L-cysteine nitrosylation and in consequence lack of histone H1 phosphorylation by the PK M2 isoenzyme.

The objective of the paper was to elucidate the mechanism of increased NO levels in EAT cells in the presence of the investigated chitosan preparations, as well as the role of NO in inhibiting their proliferation through L-cysteine nitrosylation.

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 peni-

cillin, 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

The oligomer A produced from chitosan using the thermal-mechanical degradation method (Mn 886 Da) (no cytotoxicity after 72 h), and the oligomer B produced from chitosan by the physic-chemical degradation method (Mn 900 Da) (no cytotoxicity after 72 h) were provided by the Institute of Biopolymers and Chemical Fibers in Łódź.

N (G)-nitro-L-arginine methyl ester (L-NAME) was provided by Sigma-Aldrich.

2.2. Incubation procedure

The stock solution of the reagent (A and B 0.5%) was prepared in the medium in accordance with the type of cells – for the CRL 1636 normal cells, in the DME medium, while the EAT cells were suspended in the NCTC-135 before each experiment and stored at 37 °C. The reagent-containing medium (final concentration - A and B 0.05%) was replaced every 24 hours throughout the experiments. The cells were incubated for 72 hours.

To determine nitric oxide levels, the EAT cells and CRL 1640 cells were rinsed twice in a saline solution, suspended in the Krebs-Ringer phosphate buffer (pH 7.4) containing 30 mM Tris and 10 mM glucose and incubated in a water bath (37^{o}C) without and with the studied compounds (final concentration - A and B 0.05%) for 1h.

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. 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; 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 suspended in 1× 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 solution 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 pellets were resuspended in ~70 ml (2/3X PCV) of Extraction Buffer containing the DTT and protease inhibitor cocktail. The tubes were mounted on a vortex mixer at medium to high speed for 15-30 minutes and centrifuged for 5 minutes at 20,000 - 21,000 × *g*. The supernatant with the 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, the expression of iNOS and H1-P were determined.

2.2.3. Western blot analysis

The cells were lysed in the 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: **iNOS** (ABCAM 1:1000), **H1-P** (ABCAM - 1.5 ug/ml) and β-actin (Sigma) were used to detect the indicated proteins. Bands were visualized using the alkaline phosphate- coupled secondary anti-mouse or anti-rabbit antibody (Sigma). Finally, immunoreactions were visualized by NBT/BCIP staining (Roche).

2.2.4. Protein concentration levels were determined using the method of Lowry et al. [16].

2.2.5. Production of chitosan oligomers

The oligomer A was produced as a result of thermal-mechanical degradation of microcrystalline chitosan gel (DD 97.7%) with the polymer content of approximately 1.0 weight percent in 1000 g and pH = 6.0 - 6.5; the gel was thermally degraded with superheated steam in a SMS ASVE steam autoclave at 121 °C for 3 hours and subsequently subjected to mechanical degradation using an IKA Ultra -Turrax type T 50 homogenizer with a G 45 F probe for 20 minutes at 38 °C to 71 °C at mixer rotation of 7600 - 8800 r.p.m. Following degradation and cooling, the gel solution was diluted to 0.7% and coagulated using 0.06% sodium hydroxide solution until the pH value in the preparation reached 7.5 - 7.7. The resultant chitosan suspension was filtered through batiste fabric using a Büchner filter funnel in order to separate water-soluble degradation products (oligomers) from the insoluble fraction of partially degraded chitosan. Selected chitosan oligomers were condensed in a rotary evaporator at 45 °C and 0.1 MPa until the concentration of amino sugars in the sample reached 0.5%.

The oligomer B was obtained as a result of physico-chemical degradation of microcrystalline chitosan gel (DD 97.7%) with the polymer content of approximately 1.0 weight percent in 1000 g and pH = 6.0 - 6.55; the gel was thermally degraded with superheated steam in a SMS ASVE steam autoclave at 121 °C for 25 minutes. Subsequently, following cooling, the gel solution was diluted to 0.7% weight percent. Water employed in gel cooling was enriched with hydrogen peroxide. The amount of hydrogen peroxide added (calculated for 100%) in relation to the amount of polymer contained in 100 g gel preparation was 0.05 : 1 Subsequently, the gel was coagulated using 0.06% sodium hydroxide solution until the pH value in the preparation reached 7.5 - 7.8. The resultant suspension of microcrystalline chitosan was filtered through batiste fabric using a Büchner filter funnel in order to separate water-soluble degradation products (oligomers) from the insoluble fraction of par-

tially degraded chitosan. Selected chitosan oligomers were condensed in a rotary evaporator at 45 °C and 0.1 MPa until the concentration of amino sugars in the sample reached 0.5%.

Based on the GPC/SEC analysis of water-soluble degradation products, it may be surmised that the obtained fractions constitute a mixture of monomeric and oligomeric fractions. The monomeric fraction predominantly consists of glucosamine and to a small degree of N-acetylglucosamine. On the other hand, the oligomeric fraction constitutes a mixture of oligomers that differ with respect to their degree of polymerization and chemical structure.

2.2.6. Nitric oxide was determined in the tumor and normal cells following the reaction of the investigated compounds with the Griss reagent [17]. The cells were centrifuged (1000 × *g*, 5 min at 4 °C), and then suspended in 20 mM Tris-HCl buffer (pH 7.4) and homogenized twice for 20 seconds in a Turrax homogenizer at 6,000 r.p.m. at 4 °C. The homogenates of the EAT and CRL 1640 cells were centrifuged (10,000 × *g*, 5 min at 4 °C), and nitric oxide and protein concentration were determined in the resultant homogenates.

3. Results and discussion

In the presence of microcrystalline chitosan [17] and oligochitosans A and B, the Ehrlich ascites tumor (EAT) cells showed an increase of nitric oxide levels to the values approximating these observed in normal cells. In the presence of L-NAME – a nitric oxide synthase inhibitor – there was noted a significant decrease in the level of nitric oxide to 50% of the control value, with the drop noted both in the tumor and normal cells (***Table 1***).

Such a significant increase of nitric oxide level might be evoked by inducing expression of the isoform of inducible nitric oxide synthase (iNOS) gene in the presence of the investigated oligochitosans (***Figure 1***).

Nitric oxide participates not only in gene expression regulation, especially those the transcription factors of which have the structure of zinc fingers [5], but it also participates in the process of nitrosylation of cysteine, glutathione and proteins [6, 9].

Nitrosylation of cysteine to L-S-nitrosocysteine seems to be of particular importance for regulation of activity of the PK M2 isoenzyme in nucleoplasm. The formation of L-S-

Table 1. *Nitric oxide production in Ehrlich ascites tumor cells (EAT) and normal mouse mammary epithelial cells (CRL 1640) incubated for 1 h in the Krebs-Ringer buffer (pH 7.4; 37 °C) enriched with 10 mM glucose with and without oligochitosan A and B (final concentration of 0.05%) and L-NAME (final concentration 50 nM);* [a] $P < 0.001$, [b] $0.001 < P < 0.01$.

Cells	EAT		CRL 1640	
Preparation	X (± SD) n = 10 (nmol/mg of protein)	% of control	X (± SD) n = 10 (nmol/mg of protein)	% of control
Control	40.1 (5.1)	100.0	75.4 (6.9)	100.0
+ A	65.8[a] (7.0)	164.1	85.1[b] (5.9)	112.9
+ B	63.1[a] (5.8)	157.4	87.3[b] (5.1)	115.8
+ L-NANE	21.8[a] (2.9)	54.4	36.1[a] (2.8)	47.9

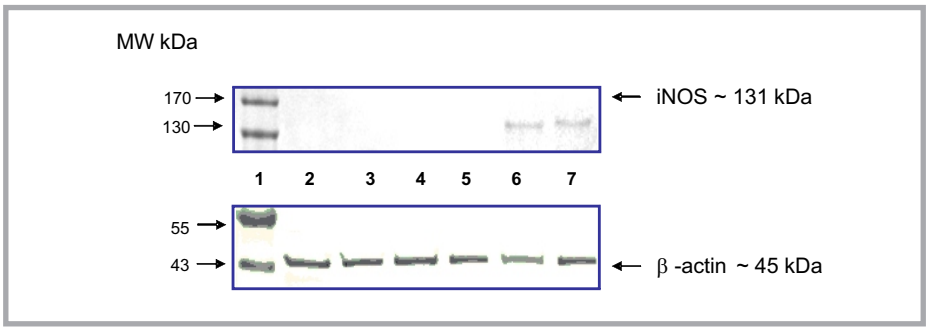

Figure 1. *Western blot analysis: The lower line shows the expression of the house-keeping gene β-actin in the same cells. Equivalent amounts of protein cell lysates – cytosol fraction – were separated by electrophoresis (PAGE-SDS, 10% gel) and probed by Western blotting with anti-iNOS antibodies.*

1. *Standard*
2. *CRL 1636- DMEM 10% PCS - control*
3. *CRL 1636 - DMEM 10% PCS + 0.05% oligochitosan A*
4. *CRL 1636 - DMEM 10% PCS + 0.05% oligochitosan B*
5. *Ehrlich - NTCT 135 10% PCS - control*
6. *Ehrlich - NTCT 135 10% PCS + 0.05% oligochitosan A*
7. *Ehrlich - NTCT 135 10% PCS + 0.05% oligochitosan B*

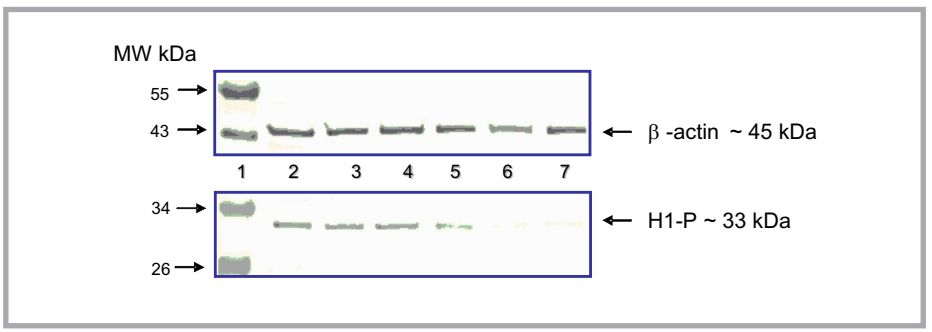

Figure 2. *Western blot analysis: The upper line shows the expression of the house-keeping gene p-actin in the same cells. Equivalent amounts of protein cell lysates - nuclear fraction -were separated by electrophoresis (PAGE-SDS, 10% gel) and probed by Western blotting with anti-H1-P antibodies.*

1. *Standard*
2. *CRL 1636- DMEM 10%FCS - control*
3. *CRL 1636 - DMEM 10% PCS + 0.05% oligochitosan A*
4. *CRL 1636 - DMEM 10% PCS + 0.05% oligochitosan B*
5. *Ehrlich - NTCT135 10% PCS - control*
6. *Ehrlich - NTCT135 10% PCS + 0.05% oligochitosan A*
7. *Ehrlich - NTCT135 10% PCS + 0.05% oligochitosan B*

nitrosocysteine contributes to lack of the histone H1 kinase activity [12]. Its absence may contribute to a decrease in histone H1 phosphorylation. Non-phosphorylated histone H1 is a potent inhibitor of replication and in consequence may be a contributing factor in EAT cell proliferation [19].

The electrophoretic analysis of the nucleoplasmic fraction employing monoclonal antibodies specific for phosphorylated histone H1 (H-1-P) demonstrated decreased histone H1 phosphorylation in the nucleoplasm of the EAT tumor cells incubated in the presence of chitosan oligomers A and B; no such effect was noted in the nucleoplasm of normal cells (***Figure 2***).

4. Conclusions

Oligochitosans of a very similar nuclear mass that have been obtained using two different methods suggest that their biological activity does not depend on the method of their production.

The oligochitosans employed in the present studies have appeared to be effective in inducing the iNOS gene expression, what contributed to an increase in NO level in the EAT cells and in consequence to nitrosylation of L-cysteine and thus to decreased histone H1 phosphorylation.

5. References

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