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200 kDa glycoprotein isolated from breast cancer tissue: a putative ligand for galectin-1

ABSTRACT

Background: *Interactions between lectins, carbohydrate-binding proteins, and glycoconjugates are important for the organisation of the cell and its response to regulatory factors. This study was aimed at isolation and characterization of the ligand for galectin-1, as a part of complex network of mutually interactive proteins expressed in breast cancer tissue.*

Materials and methods: *Soluble protein extract of human breast cancer tissue was purified by affinity chromatography on gal-1-Sepharose column. The molecular mass of isolated material was determined by gel filtration and its glycosylation was examined by means of lectin-affinity chromatography and a solid phase binding assay using plant lectins and specific antibodies.*

Results: *A protein of molecular mass of 200 kDa was isolated from breast cancer tissue on the affinity column with immobilized gal-1. Ricinus communis agglutinin I, wheat germ agglutinin and Datura stramonium agglutinin were found to bind most effectively to purified galectin-1 binding protein. Accordingly, this protein could be characterized as a glycoprotein with the predominance of galactose- and N-acetylglucosamine-containing structures. 200 kDa protein displaying ligand properties, was recognized by monoclonal antibodies specific for MUC1 mucin. This suggested that its oligosaccharide structures may be expressed on MUC1 peptide backbone.*

Conclusion: *The binding of 200 kDa breast cancer tissue glycoprotein, having mucin-like protein backbone, by gal-1, presupposes their possible interactions in vivo. It focuses the attention to their importance for the regulation of cell activity in health and disease.*

Key words: *Galectin-1; Breast cancer; Binding protein; Affinity chromatography; MUC1*

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INTRODUCTION

Lectins are multifunctional proteins, characterized by carbohydrate-binding activity, different from that of enzymes or antibodies (1). They are ubiquitously expressed in plant and animal kingdoms and together with complementary glycoconjugates constitute an evolutionary ancient system important for the regulation of the basic biological processes (2,3). Lectins act in terms of recognition and binding of distinct oligosaccharide chains of different cellular glycoproteins, glycolipids or proteoglycans (4). Thus, the protein-carbohydrate interactions are essential for cell-cell and cell-extracellular matrix communications (5-7). They lead to various physiological effects concerned with immune defense, infection, fertilization, leukocyte trafficking, metastasis, etc (8).

Carbohydrate recognition is also a critical event in the elaboration of tumorigenic and metastatic phenotypes influencing different homo- or heterotypic adhesive interactions (9,10). For instance, it is shown that carbohydrate-binding proteins that were found to be expressed on the surface of endothelial cells participate in tumor cell-endothelial cell adhesion relevant for blood borne organ colonization in metastasis (11). Generally, neoplastic transformation is accompanied with changes in the level of lectin expressions and in their cellular localization, as well as with the striking alterations in glycoproteins composition of tumor tissue (12-14).

Galectins comprise a distinct group of closely related lectins which have been isolated from a number of animal tissues including normal as well as malignant tissues of human origin (15,16). They shared two basic properties: affinity for beta galactosides and extensive aminoacid homology in carbohydrate-binding site (17). So far, ten different mammalian galectins are reported among which galectin-1 and galectin-3 are the most common and are studied most extensively. Galectin expressions were found to be associated with some aspects of malignancy (18, 19). Their concentrations can change in a variety of neoplastic cells and their surface expression correlates with metastatic potential of some cells (20, 21). For instance, transfection of normal human fibroblasts with functional gal-3 resulted in aquisition of macrophage-independent growth and morphological transformation *in vitro* (22). Gal-1 overexpressed cells loose the contact inhibition (23), and preincubation of metastatic cells with monoclonal anti-lectin antibodies abolished their ability to form lung tumor colonies (24). Therefore, the investigations of lectin-glycoprotein interactions in tumor tissues are not only of fundamental importance but also of practical clinical interest.

There are data indicating that different carbohydrate-binding proteins are expressed in both normal and breast cancer tissue (25, 26). In this study we used gal-1 as a tool to identify its complementary endogenous ligand in soluble protein extract of breast cancer tissue. Characterization of purified gal-1 binding protein was achieved by means of plant lectins and monoclonal antibodies. The results obtained indicated 200 kDa glycoprotein as gal-1 ligand in human breast cancer tissue.

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MATERIALS AND METHODS

Materials

Bovine serum albumin (BSA), GlcNAc (N-acetylglucosamine) and molecular mass markers were purchased from Sigma (St. Louis, USA). Peroxidase type VI from horseradish (HRPO), RCA I (*Ricinus communis* agglutinin I), lactose, galactose (Gal), mannose (Man), glucose (Glc) and GalNAc (N-acetylgalactosamine) were from ICN (Biochemicals, Cleveland, Ohio, USA), and 3,3-5,5-tetramethylbenzidine-TMB from ICN (ImmunoBiologicals, Lisle,USA). Sepharose 4B and Sephadex G-25 were from Pharmacia (Uppsala, Sweden). Lectins: ConA (lectin from *Canavalia ensiformis*), WGA (wheat germ agglutinin), PHA (*Phaseolus vulgaris* agglutinin), PSA (*Pisum sativum* agglutinin), SNA I (*Sambucus nigra* agglutinin I), PNA (*Arachys hypogea* agglutinin) and AAA (*Artocarpus altilis* agglutinin) were isolated in this laboratory according to well established protocols and conjugated with HRPO by the method of Nakane et al., 1974 (27). The following antibodies were used: a mouse monoclonal anti-CA 15-3 antibody (clone M411148) against human CA15-3 antigen (Fitzgerald Industries International, Inc.Concord, USA) and a mouse monoclonal anti-carcinoembrionic antigen (CEA) antibody (clone 5905) (Medix, Kauinainen, Finland). Polyclonal anti-bovine submaxillary mucin (BSM) antibody was prepared in the house. Microtiter plates were from NUNC (Denmark). All other chemicals were reagent grade.

Preparation of human breast cancer tissue extract

Breast cancer tissue (obtained by the courtesy of Institute of Oncology, Sremska Kamenica) was stored at -80°C until use. It was minced and homogenized in 0.1 M TRIS-HCl buffer, pH 7.4. Soluble proteins were separated by centrifugation for 25 minutes at 14000 rpm and the extract obtained was used for affinity purification.

Affinity chromatography

Galectin-1 (previously referred to as CBP 14, carbohydrate-binding protein 14) was isolated from rat liver nuclei on anti-gal-1 IgG-Sepharose 4B column as described by Čuperlović et al.,1995 (28). Soluble extract of human breast cancer tissue was applied on a gal-1-Sepharose 4B column equilibrated with 20 mM phosphate buffer, pH 7.2, containing 4mM EDTA and 2 mM beta mercaptoethanol (EDTA-MEPBS). Non-bound proteins were washed out with the same buffer until the absorbance base line was reached. Bound proteins were first eluted with 20 mM EDTA-MEPBS buffer, pH 7.2 supplemented with 100 mM lactose. Batch elution with total 30 mL (5 column volume) was performed. After that, the column was eluted with additional 30 mL of 0.1 M glycine-HCl buffer, pH 3.0, to remove tightly bound fraction. Both, lactose- and low pH eluates were pooled, concentrated on Amicon PM<10 and dialyzed against 20 mM EDTA-MEPBS, pH 7.2. The materials obtained were used for further characterization.

Iodination procedure

The isolated material (10 μg) was iodinated with 0.5 mCi of ^{125}I (Institute of Isotopes Co., Ltd., Budapest, Hungary) using chloramine T method as described by Greenwood et al.,1963 (29). The labelled protein was separated from free iodine on Sephadex G-25 column (20 mL bed volume), equilibrated with 0.1 M phosphate buffered saline, pH 7,2, containing 0.05% bovine serum albumin.

Gel filtration

Affinity purified gal-1 binding protein labelled with ^{125}I (400μL; 600000 cpm) was passed through a Sepharose 4B column (bed volume 20 mL) equilibrated and eluted with 0.1 M phosphate buffered saline, pH 7,2, containing 0.05% bovine serum albumin. Radioactivity (cpm) of collected fractions (each 0.7 mL) was counted on a gamma counter (ICN, Isomedix 4/600). The column was previously calibrated with labelled standard proteins: human tireoglobulin (hTg), 330 kDa; carcinoembryonic antigen (CEA), 200 kDa; mouse immunoglobulin G (mIgG), 160 kDa; bovine serum albumin (BSA) 67 kDa.

Lectin affinity chromatography

For lectin affinity cromatography, the following immobilized plant lectins were used: Con A, SNA I, AAA, PSA, PHA, DSA, and PNA. The lectins were coupled to Sepharose 4B using CNBr method, according to the manufacturers instructions. The common chromatographic scheme was applied to all columns. Affinity purified gal-1 binding protein labelled with ^{125}I (400μL; 600000 cpm) was loaded on each of the columns and the non-bound proteins were eluted with 0.1 M PBS pH 7.2, except for Con A-column which was eluted with 0.1M acetate buffer pH 6.0. The specific elution was achieved by addition of 0.1M mannose (for Con A- and PSA-column), 0.1 M lactose (for SNA- and PNA- column), 0.1 M galactose (for AAA- and PNA-column) and 0.1 M N-acetylglucosamine (for DSA-column). Finally, all columns were eluted with 0.1 M glycine-HCl buffer, pH 3.0. The fractions of 1.5 mL were collected and the radioactivity of each was recorded.

Solid phase binding assay

Serial dilutions (0.05 mL) of affinity purified gal-1 binding protein were adsorbed on microtitre plates (NUNC, Denmark) in 50 mM carbonate buffer, pH 9.3, overnight, at 4°C by physical adsorption. After blocking the nonspecific binding with 1% BSA (two hours at 4°C), wells were washed three times with 0.1M PBS, pH 7.2 (0,1 % Tween-20). The corresponding dilutions (0.05 mL) of lectins (Con A, PHA, RCA I, WGA, SNA I, PNA, PSA) or anti-CA 15-3 antibody (clone M411148) conjugated with HRPO, were added to each well and incubated at room temperature for three hours or overnight at 4°C, respectively. Plates were washed three times in TBS-Tween, and bound conjugates were detected by adding the substrate 0.01% H_2O_2 (0.05 mL) and chromogen TMB (0.05mL). The reaction was stopped after 15 minutes using 0.1M H_2SO_4 (0.05 mL) and the optical density read at 450 nm in ELISA reader. All probes were done in duplicate. Specificity of binding was controlled by inhibition with specific sugar at a concentration of 0.1M.

Radiolabelled 200 kDa protein (200000 cpm) in 0.1 M PBS-1%BSA pH 7.2 (150-300 ul) was added to antibody-coated tubes, and the incubation proceeded overnight, with constant shaking, at room temperature. The antibody-coated tubes from CIS-bio international (anti-CA 15-3 antibody, clone 115D8) and polystyrene tubes coated with monoclonal anti-carcinoembryonic antigen (CEA) antibodies and polyclonal anti-bovine submaxillary mucin antibodies (INEP-Zemun) were used. After the incubation time, the tubes were washed with 0.1M PBS-0.05% Tween 20, pH 7.2, three times (2mL), and the bound radioactivity was measured in gamma counter (ICN, Isomedic 4/600 HE). All probes were done in duplicate.

RESULTS

Isolation of gal-1 binding protein

The soluble protein extract, prepared by homogenization of breast cancer tissue in the buffer of neutral pH, had concentration of 0.4 mg/mL. It was used as a starting material for purification of galectin-1 binding proteins by affinity chromatography. After removal of non-bound proteins by washing the gal-1-Sepharose column with equilibraton buffer, the bound material was released by 0.1 M lactose solution which was followed by addition of glycine-HCl pH 3.0 buffer. Most of the bound material was found in the lactose-elutable fraction and the average yield was 25μg/mg of total loaded proteins. Due to its limited quantity available for further analysis, it was iodinated. This enabled us its easier detection during different experimental procedures.

The composition of gal-1 binding fraction was analysed by gel filtration on Sepharose 4B

column (Figure 1.). The elution pattern,

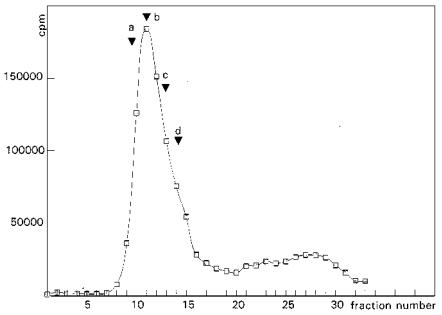

Figure 1. Gel filtration of gal-1 binding protein isolated from breast cancer tissue. Affinity purified protein was iodinated and loaded on Sepharose 4B column. The eluent was monitored by measuring radioactivity of the fractions. Each fraction was 0.7 mL. The arrows indicate the position of molecular mass standards: a) hTg, 330 kDa; b) CEA, 200 kDa; c) mIgG, 160 kDa; d) BSA, 67 kDa.

obtained by measuring the radioactivity of collected fractions, indicated one protein peak which corresponded to molecular mass of 200 kDa.

Glycosylation of gal-1-binding protein

Glycosylation of isolated gal-1 binding protein was examined by lectin affinity chromatography using various immobilized plant carbohydrate-binding proteins. The summary of lectin reactivities with 200 kDa protein is given in Table 1. The common thing for all chromato-

Table 1. Summary of the lectin-affinity chromatography of gal-1 binding protein isolated from breast cancer tissue

	SNA	Con A	PHA	DSA	PNA	AAA	PSA
neutral buffer	96.4*	80.8	96.4	87.0	97.4	96.4	93.3
eluent 1	2.6	10.2	2.4	9.4	1.4	2.3	4.1
eluent 2	1.0	9.0	1.2	3.6	1.2	1.3	2.6

* The total radoactivity of non-bound fraction (eluted with neutral buffer) and bound fractions (eluted with eluent 1 and eluent 2) was calculated and expressed as % of total radioactivity loaded on the column. Eluent 1 (0.1 M sugar solution); eluent 2 (0.1 M glycine-HCl buffer, pH 3.0)

graphic protocols was that the same quantity of iodinated protein was loaded on each of the columns. Non-bound fraction was washed with neutral buffer whose composition was adapted to specific requirements of any of lectins used. Part of bound radiolabelled protein was eluted with 0.1 M solution of specific sugar, (eluent 1), followed by 0.1 M glycine-HCl buffer, pH 3.0 (eluent 2). The total radioactivity of each fraction was calculated and expressed as % of total radioactivity applied on the column.

Under the applied experimental condition, none of the lectins showed striking reactivity with tested sample. SNA (specificity for Neu5Acα2,6Gal), PHA (specificity for Galβ1,4GlcNAcα1,2Man), PSA (specificity for α-Man > α-Glc > αGlcNAc), AAA (specificity for Galβ1,3GalNAc) and PNA (specificity for Galβ1,3GalNAc > GalNH2 > Gal) showed low reactivities separated in sugar-elutable (1.4%-4.1% of total radioactivity applied) and 0.1 M glycine-HCl buffer-elutable fraction (1.0%-2.6% of total radioactivity applied). Con A (specificity for Manα1,2Manα1,2Man > Manα1,2Man > α-Man > α-Glc > α-GlcNAc) and DSA (specificityfor GlcNAcβ1, 4GlcNAcβ1, 4GlcNAcβ1, 4GlcNAc > GlcNAcβ1, 4GlcNAcβ1, 4GlcNAc > GlcNAcβ1, 4GlcNAc > GlcNAc) bounded 200 kDa protein stronger than other lectins (13%- 20% of total radioactivity applied), giving characteristic elution profiles. Both non-bound (Figure 2, a) and bound fraction (Figure 2, b,c) eluted from the Con A-column, as broad peaks. Non-bound

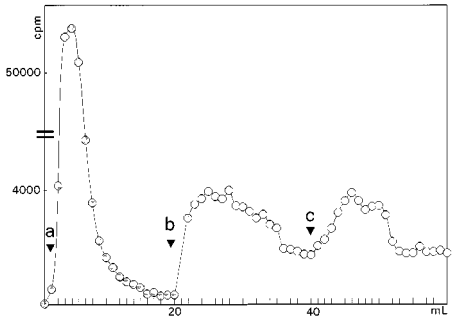

Figure 2. Affinity chromatography on Con A-Sepharose. The isolated 200 kDa protein was iodinated and allowed to react with plant lectin, immobilized on Se2pharose. The non-bound and bound fraction were monitored by measuring the radioactivity. The representative elution profile from the affinity column was shown. a) elution with 0.1 M acetate bufer pH 6.0; b) elution with 0.1 M mannose; c) elution with 0.1 M glycine-HCl buffer pH 3.0.

fraction was retarded in its elution from DSA-column (Figure 3, a), whereas the bound fractions released by 0.1 M N-acetylglucosamine (Figure 3, b) and 0.1 M glycine-HCl pH 3.0

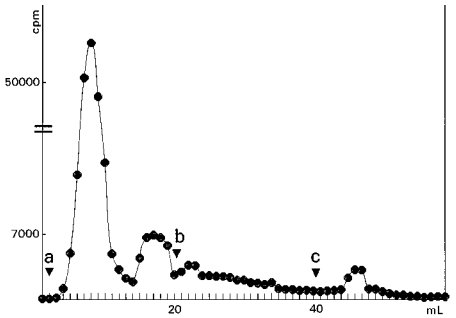

Figure 3. Affinity chromatography on DSA-Sepharose. The chromatography proceeded as described for Con A-Sepharose. The representative elution profiles from the affinity column was shown. a) elution with 0.1 M PBS pH 7.2; b) elution with 0.1 M N-acetylglucosamine; c) elution with 0.1 M glycine-HCl buffer pH 3.0.

(Figure 3, c) were eluted as sharp peaks.

Carbohydrate composition of 200 kDa protein was also characterized by means of a solid phase binding assay. In this assay, the examined sample was immobilized on the microtitre plates and then allowed to react with different lectin-peroxidase conjugates (Figure 4). The results obtained with the same lectins in two dif-

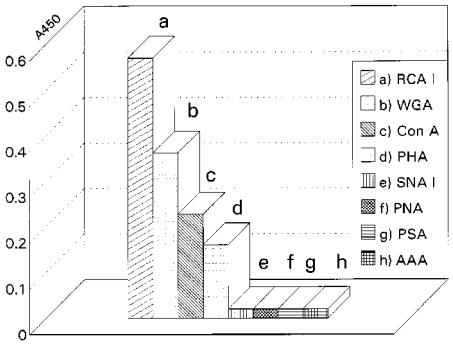

Figure 4. Glycosylation of gal-1 binding protein isolated from breast cancer tissue. The examined sample was adsorbed on microtitre plates and incubated with plant lectins labelled with peroxidase. The following carbohydrate-binding proteins were used: a) RCA I (Ricinus communis agglutinin I); b) WGA (wheat germ agglutinin); c) ConA (Canavalia ensiformis agglutinin); d) PHA (Phaseolus vulgaris agglutinin); e) SNA I (Sambucus nigra agglutinin I); f) PNA (Arachys hypogea agglutinin); g) PSA (Pisum sativum agglutinin); h) AAA (Artocarpus altilis agglutinin). Absorbance at 450 nm was recorded as a measure of bound conjugates.

ferent systems were in accordance with each other. In addition, the lectins used in this assay: RCA I (specificity for Gal > GalNAc; Galβ1, 4GlcNAcβ1-R) and WGA (specificity for GlcNAcβ1,4GlcNAcβ1,4GlcNAc > GlcNAcβ1,4GlcNAc > GlcNAc) were shown to react strongly with 200 kDa protein.

Immunodetection of antigen carrying gal-1-binding sugar structures

The isolated 200 kDa protein was probed with antibodies specific for: human MUC1

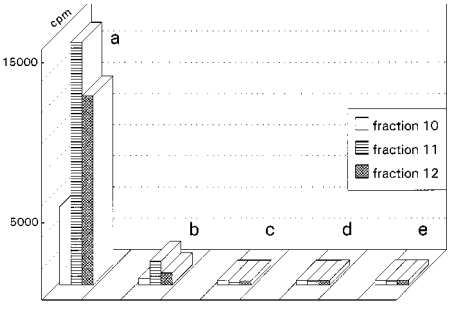

Figure 5. Immunoreactivity of isolated 200 kDa protein. The assay was performed on antibody-coated tubes. Reactions were estimated by total bound radioactivity. The iodinated 200 kDa protein (Figure 1, fractions 10, 11, 12) was tested with: a) monoclonal anti-CA 15-3 ; b) monoclonal anti-carcinoembryonic antigen (CEA); c) polyclonal anti-bovine submaxillary mucin (BSM); d) monoclonal anti-tireostimulating hormon (TSH); e) monoclonal anti-prolactin (PRL).

mucin, human carcinoembryonic antigen and bovine submaxillary mucin, as known ligands for gal-1 (Figure 5). Moderate recognition of radiolabelled 200 kDa protein by monoclonal anti-MUC1 (clone 115D8) was detected. Similar recognition was also detected using another distinct monoclonal antibody (monoclonal anti-CA 15-3 antibody) against the same antigen, MUC1 (data not shown). Anti -CEA antibodies gave very low reaction and anti-BSM antibodies gave no reaction with examined sample. Irrelevant monoclonal antibodies: anti-TSH and anti-PRL antibodies, used as controls, also gave no detectable reactions.

DISCUSSION

The identification of endogenous ligands for lectins is a relevant step towards the elucidation of their possible biological role. So far, different galectin-binding proteins have been demonstrated by histochemical localization and biochemical characterization in a variety of cells and tissues (30-35). In this study, human breast cancer tissue was used as a source in chromatography-based isolation of gal-1 binding proteins. The results obtained pointed out to a 200 kDa protein, as specifically recognized. The ability of lactose to displace this protein from affinity matrix indicated that carbohydrate-dependent, not protein-protein or hydrophobic interactions, were responsible for its binding to gal-1.

For an analysis of the oligosaccharide determinants of 200 kDa protein, two techniques were applied: lectin-affinity chromatography and solid phase binding assay. A panel of plant lectins was tested in each of them. They were chosen according to their specificities, encompassing the many types of carbohydrate structures in either N- or O-linked oligosaccharide chains. The corresponding lectins were allowed to react with examined sample, in solution or immobilized. These procedures were combined in order to overcome the differences which could originate from the methods applied (36). RCA I, WGA, and DSA bound most effectively to isolated 200 kDa protein. According to the carbohydrate determinants required for high affinity binding by these lectins, the presence of galactose and N-acetylgalactosamine at terminal (or as sialilated units) positions, can be presumed on breast cancer gal-1 binding protein. RCA I binds to Galβ1,4GlcNAcβ1-R (37). WGA and DSA binds GlcNAc, but WGA can also bind sialic acid (37). 200 kDa protein was not recognized by SNA, which specifically bind sialyl α2,6 galactose (38), indicating that sialic acid, if present, is bound by α2,3 linkage. All these results were in agreement with the carbohydrate specificity of galectins. Thus, it was reported that sialization by the α2,3 linkage had little influence on the binding affinity of galectins, whereas sialization of the terminal galactose residue by an α2,6 linkage led to a drastic decrease (39, 40). Galβ1,4GlcNAc and Galβ1,3GlcNAc as well as the blood group H structure or its analogues were reported as recognition unit for gal-1 (41, 42). These structures, such as for instance in form of poly-N-acetyllactosamine chains can occur on different glycoconjugates: glycoproteins, glycolipids and proteoglycans (43).

Judging by the immunological detection of 200 kDa protein with different monoclonal antibodies assigned as CA 15-3 antibodies (clone115D8, CIS-bio international; clone M11148; Fitzgerald Industries) specific for MUC1 mucin, the gal-1-binding sugar structures may be expressed on mucin peptide backbone. Mucin in gastrointestinal tract has already been reported as ligand for gal-1 (44). As regards the soluble MUC1 antigen, the epitope recognized by 115D8 antibody was characterized as sialic acid-dependent, but not as sensitive to periodate oxidation (45, 46). This antibody could detect MUC1 mucin in breast tissue regardless of its periodate treatment, whereas, in small intestine and colon this was possible only after periodate oxidation (47).

Mucins are characterized by the predominance of O-glycosydic linkage (8). It is known that both WGA and RCA, which were found to react with 200 kDa protein, can be used to identify O-linked GlcNAc before or after the addition of a terminal b1,4 linked galactose to the O-linked GlcNAc (48, 49). However, the reaction of 200 kD protein with Con A, which is specific for three-mannosyl core of N-linked glycans, was also evident (37). This reaction can occur due to the abundance of GlcNAc residues in the examined sample, causing increased binding by Con A. For instance, similar Con A-binding was reported for type III mucin (50).

In malignant breast tissue, MUC1 mucin is expressed in less glycosylated form (51). Such tumor-specific form of MUC1 mucin was localized immunoenzymatically, on the membrane of the alveolar cells (52). The elevation of its concentration in the circulation is connected with increased malignancy, and it serves as a useful tumor marker (51). Generally, malignant phenotype is characterized by ectopic expression, abnormal distribution and incomplete glycosylation of mucins as well as other form of glycoconjugates (53). For instance, the analysis of protein extract of human breast cancer indicated a considerably higher incidence of glycoproteins in the molecular mass range of 210-280 kDa than in benign samples, and fucosylation was also increased (54). Also, in primary and metastatic breast carcinomas, lectin binding sites were stained with Con A, RCA I and WGA more intensely than in normal breast, in mastopathy or in fibroadenomas (55).

CONCLUSION

Thus, the binding of 200 kDa breast cancer tissue glycoprotein, having mucin-like protein backbone, by gal-1, presupposes possible interactions *in vivo*. Galectins are involved in the processes of the cell migration, attachment and spreading, in the organization of extracellular matrix and they can also act as growth modulators (56-59). Together with their endogenous ligands, they can contribute to biological evolution of tumor, causing invasive or infiltrative properties of cancer cells (60-62). It is known that the prevention of tumor cells to interact with each other or with their microenviroment, can suppress tumor growth and metastasis (63, 64) The significance of the alterations in this kind of reactions has been evaluated in clinical trials for inflammatory diseases, transplantation and cardiovascular diseases (65-67). This speaks in favour of further investigations of lectins and their ligands, as a base for developing strategies for their clinical applicability. They hold promise as target molecules in diagnosis and treatment of breast cancer i.e. cancer in general.

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THE FIRST ANOUNCEMENT

Academy of Studenica: Application of MRS in Clinical Oncology

5th Inter University Scientific meeting:
Academy of Studenica
MR spectroscopy in Clinical Oncology

We would like to extend to you our sincere invitation to take part of:

5th Inter University Scientific meeting:
Academy of Studenica
MR SPECTROSCOPY IN CLINICAL ONCOLOGY

that is organized by the Institute of Oncology Sremska Kamenica, Novi Sad in cooperation with Academy of Studenica.
The Meeting is going to take place in Congress hall of the Institute of Oncology Sremska Kamenica, Novi Sad, May 26, 2000 at 9am.

The main topics of the Meeting are:

- Basic principles of MR spectroscopy
- MR spectroscopy in differential diagnosis of tumors
- MR spectroscopy in response of tumors to therapy
- In vitro MR spectroscopy

The aim of the Meeting is to inform our medical doctors about state-of-the-art in the field of MR spectroscopy and possibilities of application in clinical oncology.

The lecturers are world's leading scientists in the field of MR spectroscopy.

We would like to ask you kindly to inform us about your presence in these scientific Meeting until April 20, 2000.If you are interested in presenting your research results, please send us the abstracts until April 20, 2000 and in extenso papers in English language until May 1, 2000.
Abstracts and full papers are going to be published in Annals of the Academy of the Studenica. Please prepare your papers according to instructions for the authors: http://www.onk.ns.ac.yu
Official language of the Meeting is English.

Trip to Fruška Gora monasteries and to Sremski Karlovci is organized for all participants.

Yours faithfully,
Prof. dr sci med Vladimir Vit. Baltić

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