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Isolation and characterization of galectin-1 binding proteins from human placenta

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Galectin-1 binding proteins were isolated from human placenta by affinity chromatography on a column with immobilized endogenous lectin. The molecular masses of the isolated proteins of 170, 67 and 56 kDa were estimated by gel filtration and SDS-PAGE. These proteins were characterized as galactose-containing glycoproteins, based on their reactivity with *Ricinus communis* agglutinin. In addition, sialylated-lacto-*N*-fucopentaose II was detected in the 170 kDa protein, using anti CA 19-9 monoclonal antibodies. The interaction of the isolated proteins with human placental galectin-1 was investigated by a solid phase binding assay using asialofetuin as the glycoprotein ligand. The 67 kDa and 56 kDa proteins were found to inhibit galectin-1 binding of asialofetuin, whereas the 170 kDa protein had the opposite effect. It caused an increase in the binding of asialofetuin, suggesting a positive cooperative binding.

Keywords: galectin-1, human placenta, affinity chromatography, RCA I, CA19-9.

Galectins comprise a family of animal, soluble, metal-independent, carbohydrate-binding proteins, widely distributed from invertebrates to higher animals.[1,2] They posses two basic properties: affinity for beta galactoside and extensive aminoacid sequence homology.[3,4] According to their primary structure, galectins are subdivided into proto, chimera, and tandem-repeat types.[2] Galectin-1 belongs to the proto type, composed of only one single lectin domain with a carbohydrate-recognition domain, and occurring as a homodimer.[2,5] Galectin-1 has been isolated from a number of mammalian tissues and the corresponding gene, LGALS1, is located at the q12–13 region of chromosome 12 in man.[3]

Lectins belonging to the galectin family are relatively abundant in placental tissue. A human beta galactoside binding lectin, HP14, was isolated from term placenta.[6] It was identified as galectin-1[7] according to its activity and determined aminoacid sequence. HP14 has also been isolated from first trimester placental tissue and amniotic fluid,[8] and its activity and concentration were found to change during gestation.[9] Using polyclonal antibodies against galectin-1, HP14 was localized in villous (fetal) mesenchyme,[10,11] while transient expression in cell columns,

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depending of the stage of differentiation, was also observed.[12] In first trimester pregnancy placenta, galectin-1 was localized in the syncytiotrophoblast and cytotrophoblast of the middle and distal cell column, and it was absent from fully invasive, interstitially migrating cytotrophoblast.[13]

Although the biological function of galectins is not clearly understood, conservation of their primary structure suggests that they might be responsible for basic processes common to all living organisms.[14] It is thought that galectin-1 could play a modulating role during trophoblast invasion by interaction with glycoconjugates of the extracellular matrix, influencing cell attachment, detachment and adhesion.[15–17] In addition, galectin-1 might affect growth[18,19] and some data suggest a possible immunomodulatory role.[20]

As a way to find clues about the possible intracellular role of galectin-1, galectin-1 interacting proteins in the soluble fraction of placental tissue were examined. They were separated by affinity chromatography with endogenous lectin, CBP 14, as the ligand. CBP 14, a carbohydrate-binding protein, (*Mr* 14 kDa) was previously isolated from rat liver nuclei and characterized according to its specificity for beta galactoside and cross-reactivity with anti-galectin-1 antibodies.[21] A panel of plant lectins and monoclonal antibodies towards well-defined carbohydrate antigens was used to examine the glycosylation of affinity purified gal-1 binding proteins. Two groups of placental proteins, differing in molecular mass and glycoprotein composition, were identified. They were found to have a different influence on the carbohydrate binding ability of human placental gal-1.

EXPERIMENTAL

Asialofetuin (ASF) from fetal calf serum type III and molecular mass standards for electrophoresis were purchased from Sigma (St. Louis, USA). Peroxidase type VI from horseradich (HRPO), RCA I (*Ricinus communis* agglutinin I), bovine serum albumin (BSA) and lactose were from ICN (Biochemicals, Cleveland, Ohio, USA) and 3,3-5,5 tetramethylbenzidine - TMB from ICN (Immunobiologicals, Lisle). Sepharose 4B, Sephadex G-200 and molecular mass standards for gel filtration were from Pharmacia (Uppsala, Sweden). Lectins (PHA-*Phaseolus vulgaris* agglutinin; WGA-wheat germ agglutinin; Con A-*Canavalia ensiformis* agglutinin; PSA-*Pisum sativum* agglutinin; PNA-peanut agglutinin; SNA I-*Sambucus nigra* agglutinin) were prepared in house, according to established procedures and conjugated with HRPO by the method of Nakane *et al.*[22] A mouse monoclonal anti CA 19-9 antibody (clone M602208a) against affinity pure human CA 19-9 antigen was purchased from Fitzgerald Industries International, Inc. (Concord, USA). The concentration of CA 19-9 and CA 15-3 were estimated using ELSA CA 19-9 and ELSA 15-3 kits (CIS-bio international, France). Microtiter plates were from NUNC (Denmark). All other chemicals were reagent grade.

Preparation of placental extract

Human first trimester placentas, from patients undergoing elective termination of pregnancy at 6–12 weeks, were used. Tissue was collected in cold PBS, brought to the laboratory within 60 minutes and washed free of blood. The placental tissue was homogenized in 0.1M PBS (pH 7.2) and then centrifuged at 105000 · *g* for 60 min. The supernatant, containing the cytosol fraction, was used for affinity purification.

Affinity chromatography

CBP 14 was isolated from rat liver nuclei on asialofetuin-Sepharose 4B and anti-HP14 IgG-Sepharose 4B column as described by Čuperlović *et al.*[21] The soluble extract of first trimester

placental tissue was loaded onto a CBP 14-Sepharose 4B column equilibrated with 20 mM phosphate buffer (pH 7.2) containing 4 mM EDTA and 2 mM 2-mercaptoethanol (EDTA-MEPBS). Non-bound proteins were washed out with the same buffer until the absorbance base line was reached. Bound proteins were eluted with 20 mM EDTA-MEPBS buffer (pH 7.2) supplemented with 100 mM lactose. The absorbance at 280 nm was recorded and fractions (5 mL) were collected. Fractions containing bound proteins were pooled, concentrated on Amicon PM <10 and dialyzed against 20 mM EDTA-MEPBS (pH 7.2) to remove the excess lactose. The material obtained was used for further characterization.

Gel filtration

Affinity purified proteins (500 μL) were separated on a Sephadex G-200 column (bed volume 20 mL), equilibrated and eluted with 20 mM EDTA-MEPBS (pH 7.2). The absorbance at 280 nm of each 0.5 mL fraction was recorded. The column was calibrated with molecular mass standards (Pharmacia, Uppsala, Sweden): immunoglobulin G 150; bovine serum albumin 67; ovalbumin 43; chymotrypsinogen 25 and ribonuclease 13.7 kDa.

SDS-PAGE

Molecular masses were determined by SDS-PAGE in 12.5% separating gel with a 3.75% stacking gel, under reducing conditions, according to Laemmli.[23] After electrophoresis, the gels were stained with silver nitrate according to Wedrychowski *et al.*[24] The gel was calibrated with molecular mass standards (Sigma, St. Louis, USA): BSA 66; ovalbumin 45; pepsin 34.7; trypsinogen 24; beta-lactoglobulin 18.4 and lysozyme 14.3 kDa.

Solid phase binding assay

a) Galectin-1 (HP14) was isolated from first trimester placenta as described by Hirabayashi and Kasai.[6] Serial dilutions of galectin-1 (40– 0.07 μg; 100 μL) were physically adsorbed on microtitre plates (NUNC, Denmark) in 50 mM carbonate buffer (pH 9.3), overnight at 4 °C. After blocking the nonspecific binding with 1% BSA, the plates were washed three times with PBS-0.1% Tween-20. The gal-1 binding proteins separated by gel filtration (50 μL) and asialofetuin-HRPO (1:1000; 50 μL) were combined and added to each well in duplicate. Incubation proceeded for three hours at room temperature. The wells were washed with PBS-0.1% Tween-20 and bound asialofetuin was detected by adding the substrate solution containing TMB and 0.01% H_2O_2. The reaction was stopped with 2 M H_2SO_4 (100 μL) and the absorbance was read at 450 nm in an ELISA reader.

b) Glycosylation of the affinity purified binding proteins were tested by a solid phase binding assay following the forementioned procedure. Corresponding conjugates (50 μL) of RCA I (1:2000), PHA (1:1000), WGA (1:1000) or of monoclonal anti-CA 19-9 antibodies (1:1000) were allowed to react with the immobilized samples for three hours at room temperature (plant lectin conjugates) or overnight at 4 °C (monoclonal antibodies). After washing out non-bound conjugates with PBS-0.1% Tween-20, the substrate solution was added and the procedure continued as described. The specificity of binding was controlled by inhibition with specific sugar at a concentration of 0.1 M.

RESULTS

Isolation of galectin-1 binding proteins

The cytosol extract of first trimester pregnancy placenta, prepared by homogenization of tissue in a buffer of neutral pH, was used as a source of soluble galectin ligands. When it was applied to CBP 14-Sepharose, the bound fraction eluted as a single peak, after addition of 100 mM lactose as the specific sugar. The average yield was 20 μg/mg of loaded proteins. After dialysis and concentration, the affinity purified material was analyzed by gel filtration on Sephadex G-200 (Fig. 1). Three

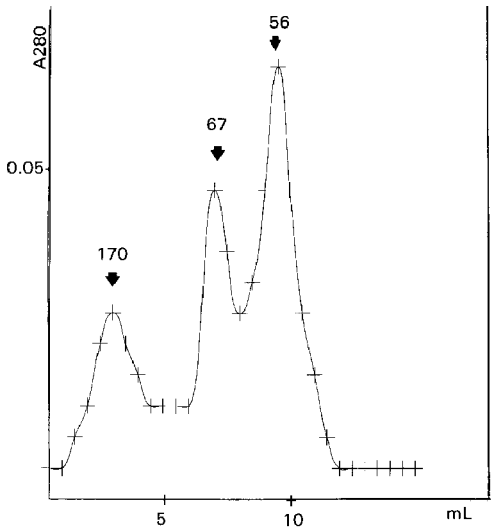

Fig. 1. Sephadex G-200 gel filtration of placental gal-1 binding proteins. Affinity purified proteins were loaded on the column and the eluent was monitored at 280 nm. Each fraction was 0.5 mL. The numbers indicate the molecular mass of the corresponding protein peaks.

protein peaks, corresponding to native molecular masses of 170, 67 and 56 kDa, were obtained, and they are referred to as gal-1 binding proteins. Under reducing and denaturating conditions, the same eluate was resolved on 12.5% PAGE into three bands, having molecular masses of 84, 67 and 56 kDa (Fig. 2). This indicated that the 67 kDa and 56 kDa fractions were monomers and the 170 kDa a dimer with a 84 kDa subunit.

Fig. 2. Electrophoretical separation of placental gal-1 binding proteins. Affinity purified proteins were subjected to 12.5% SDS-PAGE under reducing conditions. Proteins were visualized by silver staining. The numbers indicate the molecular mass of the corresponding protein bands.

Interaction of human placental gal-1 and galectin-1 binding proteins

Galectin-1 binding proteins were tested for their reactivity with human placental gal-1, as homologous galectin isolated from the same biological source. It was immobilized on a solid phase and incubated with labelled asialofetuin in the presence of affinity purified proteins (corresponding to fractions: 5, 13, 18 in Fig. 1). The binding of asialofetuin to galectin-1 as a function of lectin concentration is presented in Fig. 3A. The carbohydrate-binding activity of gal-1 was affected differently by the addition of isolated placental proteins. As shown in Fig. 3B, in the presence of the 170 kDa protein, gal-1 binding of asialofetuin was increased

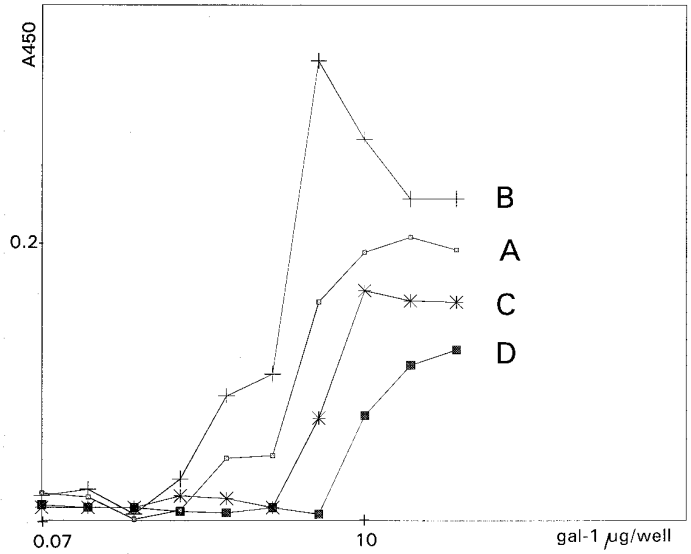

Fig. 3. Effects of placental gal-1 binding proteins on carbohydrate binding activity of gal-1. Assay conditions for binding of asialofetuin to gal-1 were as described in the experimental section. A) Binding of ASF-HRPO to gal-1 (control); B) binding of ASF-HRPO to gal-1 in the presence of fraction 5 (170 kDa), C) binding of ASF-HRPO to gal-1 in the presence of fraction 13 (67 kDa); D) binding of ASF-HRPo to gal-1 in the presence of fraction 18 (56 kDa).

over the tested concentration range. However, the lower molecular mass proteins had the opposite effect *i.e.,* an inhibitory effect. The placental proteins of 67 kDa (Fig. 3C) and 56 kDa (Fig. 3D) reduced the binding of the glycoprotein ligand, asialofetuin. This inhibition implied competition between asialofetuin and gal-1 binding proteins, confirming interactions *via* carbohydrate recognition.

Glycosylation of galectin-1 binding proteins

The carbohydrate moieties of placental proteins were probed with plant lectins of different specificities. In the solid phase binding assay, each fraction obtained by gel filtration of the affinity purified proteins was adsorbed on plastic and allowed to react with the corresponding carbohydrate-binding proteins. Among the lectins tested, RCA I (specificity for b-galactose) exhibited the strongest binding to the

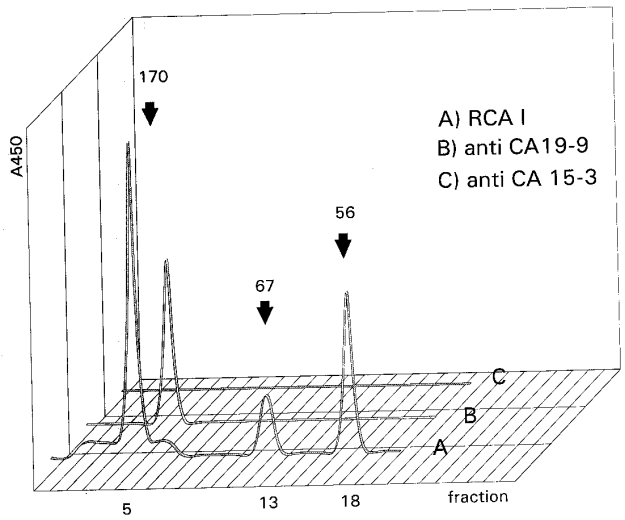

Fig. 4. Lectin reactivity with placental gal-1 binding proteins. Affinity purified gal-1 binding proteins separated by gel filtration were adsorbed on a solid phase and incubated with plant lectins of different carbohydrate specificity or carbohydrate directed monoclonal antibodies, as described in the experimental section. The absorbance at 450 nm, as a measure of bound lectin-peroxidase conjugate, was recorded. A) Reactivity with RCA I; B) reactivity with anti CA19-9 antibodies; C) reactivity with anti CA 15-3 antibodies. The numbers indicate the molecular masses.

examined proteins. It recognized the 170 kDa and 56 kDa proteins and also reacted less with the 67 kDa protein (Fig. 4A). PHA (complex specificity for galactose) and WGA (specificity for GlcNAc and sialic acid) weakly recognized the 170 kDa whereas the other tested lectins, ConA (specificity for a-methyl mannopyranoside), SNA (specificity for NeuAca(2,6)GalNAc) and PNA (specificity for Galb(1,3)GalNAc), did not bind to any of the isolated proteins (data not shown).

In addition to lectin-binding, the isolated gal-1 binding proteins were tested for reaction with monoclonal antibodies towards mucin-associated antigens. Anti CA 19-9 (sialyl-Lewis^a) and anti CA 15-3 (MUC-1 apomucin peptide) were used. The results obtained indicated that anti CA 19-9 recognized the 170 kDa protein but not the 67 kDa and 56 kDa proteins (Fig. 4B). Monoclonal anti CA 15-3, however, showed no reaction with any of the isolated placental protein (Fig. 4C). Also, using commercially available kits for quantitation of the examined antigens, it was confirmed that the total isolated proteins contained 26 IU/mL CA 19-9 but no CA 15-3 antigen.

DISCUSSION

Oda and Kasai[25] were the first to demonstrate that chicken galectin-1 specifically binds to polylactosamine glycoconjugates. In subsequent studies, a number of endogenous lactosamine-containing glycoproteins have been reported as putative ligands for galectins from various sources. It was found that galectin-1 can bind

integrin a7b1 on skeletal muscle,[26] lactosamine -containing glycoprotein on olfactory neurons,[27] human brain glycoprotein[28,29] and act as a receptor for ganglioside GM1.[30] Its interactions with intestinal mucin,[31] CEA,[32] lysozyme-associated membrane proteins[32,33] and core 2–O–glycan on thymocytes and thymoblastoid cells[34] were also shown.

Using gal-1 from frog *Rana catesbeiana* eggs, placental fibronectin and laminin were identified as galectin ligands on the cell surface and extracellular matrix.[35] In this study, first trimester placental tissue was examined for the presence of soluble, galectin-1-interacting glycoproteins. Their putative interactions were based on data about the cytoplasmatic localization of galectin-1 and the heterogenous distribution of polylactosamine, which is also a structural unit of a different form of cellular glycoconjugates.[36]

Three proteins having molecular masses of 170, 67 and 56 kDa were isolated on the CBP 14-Sepharose column. The results of their biochemical characterisation and glycosylation analysis pointed to two distinct groups of putative endogenous ligands for gal-1 in placenta: two low molecular mass 67 kDa and 56 kDa, galactose-rich, RCA I- binding proteins and a high molecular mass, CA 19-9 antigen-containing 170 kDa protein. Examination of their interactions with human galectin-1 showed that they have a negative (67 kDa and 56 kDa) and a positive (170 kDa) cooperativity with asialofetuin binding to galectin-1. So far, a positive coopertivity has been reported as a characteristic for the binding of gal-3, but not for gal-1.[37] The property of cooperative binding might be significant in view of the proposed role of galectins in cross-linking activities inside, as well as outside cells. Previous studies of the binding characteristics of gal-1 indicated that there may be a slight increase in binding affinity when di- and tri- lactose are present in the same ligand molecule.[38] Hence, the observed differences in reactivity of isolated placental proteins and homologous human placental gal-1 might be due to differences in their glycosylation.

The observed strong reaction of the isolated proteins with RCAI is in agreement with its similarity to gal-1 in terms of carbohydrate specificity and its biinding characteristics.[39] RCA I binds to galactose and galectins exhibit carbohydrate-binding ability for lactose, Gal b1,4 GlcNAc and Gal b1,3 GlcNAc *i.e.*, to the poly-*N*-acetyllactosamine structure at terminal or internal positions in the oligosaccharide chains.[40,41] In addition to its RCA I reactivity, the 170 kDa gal-1 binding placental protein was found to react with WGA and PHA, suggesting the presence of sialic acid and polylactosamine in the complex oligosaccharides structures. In accordance with this, the CA 19-9 structure was detected in the 170 kDa protein. The carbohydrate determinant[42] called CA 19-9 is sialylated-lacto-*N*-fucopentaose II, Neu5Ac(a2-3)Gal(b1-3)(Fuc(a1-4)GlcNAc. The finding of CA 19-9, a sialyl derivate of human Lewis^a blood group antigen, as a structural part of the isolated protein is not contradictory to the specificity of gal-1. Poly-*N*-acetlyllactosamine serves as the backbone of A, B, H, O, I/i and Lewis^a and Lewis^x antigenic determinants, which are created by substitutions of the b-galactosides.[43] It is known that sialization of

the a2,3 linkage has little influence on the binding affinity of galectins, whereas sialization of the terminal galactose residue by an a2,6 linkage leads to a drastic decrease.[40,41] Furthermore, probing with anti CA 15-3 antibodies, which recognize the less glycosylated form of MUC-1,[44,45] gave no reaction with the examined samples. Thus, substitutions are possible that influence interactions with particular galectins in different manners. This might be of general importance for the regulation of galectin-1 activity.

The reaction *in vitro* between human placental gal-1 and isolated glycoproteins originating from the same source presuppose possible interactions *in vivo*. It has been reported that CA 19-9 containing glycoproteins are mainly located in the cytosolic fraction of decidual cells and amnionic epithelial cells[46] and an elevation of its level can be found in women during pregnancy, as well as during the menstrual cycle.[47] The CA 19-9 determinant is normally expresed in some individuals in certain tissues, but is also found as a tumor mucin associated carbohydrate antigen.[48] Mucin core protein is coded by specific genes (Muc1-Muc8) and act as a carrier of specific carbohydrate-structures.[49] It is known that the female reproductive tract (placenta, endometrium, cervix) exibits strong expression of mucin mRNA which are categorized as tracheal Muc8.[50] Depending on the type, it may be localized in term placental villi and fetal endothelium, syncytiotrophoblast and cytotrophoblast.[51] Mucin in the gastrointestinal tract has been already reported as a ligand for gal-1.[31] The elucidation of the structure of the carrier proteins of CA 19-9 antigen, isolated as gal-1 placental ligand in relation to its possible similarity or identity to known ligands and in relation to similarity in activity of trophoblast and cancer cells could have relevance beyond reproductive biology.

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И З В О Д

ИЗОЛОВАЊЕ И КАРАКТЕРИЗАЦИЈА ХУМАНИХ ПЛАЦЕНТНИХ ПРОТЕИНА КОЈИ ВЕЗУЈУ ГАЛЕКТИН-1

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Протеини који везују галектин-1 су изоловани из хумане плаценте афинитетном хроматографијом на колони са имобилисаним ендогеним лектином. Гел филтрацијом и електрофорезом одређене су њихове молекулске масе од 170, 67 и 56 kDa. Ови протеини су окарактерисани као гликопротеини који садрже галактозу, на основу реактивности са *Ricinus communis* I аглутинином. Поред тога, код протеина молекулске масе 170 kDa детектована је сијалил-лакто-*N*-фукопентоза, коришћењем моноклонских анти CA 19-9 антитела. Интеракција изолованих протеина и хуманог плацентног галектина-1 је испитивана у тесту везивања на чврстој фази, коришћењем асијалофетуина као гликопротеинског лиганда. Нађено је да протеини молекулске масе 67 kDa и 56 kDa инхибирају

везивање асијалофетуина за галектин-1, док је протеин молекулске масе 170 kDa имао супротан ефекат. Он је повећавао везивање асијалофетуина што је указало на позитивну кооперативност у везивању.

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