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ANALYSIS OF THE PROTEIN AND GLYCAN PARTS OF CA125 ANTIGEN FROM HUMAN AMNIOTIC FLUID

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Abstract – CA125 antigen is a mucin-type molecule with a complex protein backbone and oligosaccharide chain structure. In this study, we characterized CA125 antigen from human amniotic fluid by gel filtration, ion-exchange chromatography, peptide mass fingerprinting and lectin-binding assays**.** The obtained results indicate CA125 to be structurally heterogeneous, existing in different glycoisoforms with subtle differences in the profile of molecular forms in comparison to placental tissue-derived and cancer-derived CA125 antigen. The complexity of CA125 structure suggests that it can act as a multifunctional molecule. Further investigation is therefore needed in order for the biological meaning of the tissue-specific structural forms to be comprehended fully.

Key words: CA125 antigen, glycosylation, lectin-binding, amniotic fluid

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INTRODUCTION

CA125 antigen was first identified from affinity of the OC125 monoclonal antibody obtained by somatic hybridization of spleen cells from mice immunized with the ovarian carcinoma cell line OVCA433 (Bast *et al*., 1981). After successful cloning of part of its gene, CA125 was recognized as a mucin molecule and classified as MUC16 (Yin and Lloyd, 2001). Its molecular architecture is very complex, in terms of both the protein backbone and the oligosaccharide side chains, and is still mostly uncharted. Based on the gene structure, it is suggested that CA125 antigen is a transmembrane protein, comprising an extracellular domain, a transmembrane region, and an intracellular domain (O'Brien et al*.,* 2001; 2002). The extracellular domain harbors the N-terminal region containing over 10000 amino acids and the tandem repeat region characteristic of mucin primary structure. This is a site of intensive N- and O-glycosylation (Davis et al*.,* 1986; Kui Wong et al*.,* 2003). CA125 antigen may have 7-60 tandem repeats, each 156 amino acids long, containing the SEA domains (Maeda et al*.,* 2004). The intracellular region is short and carries several tyrosine residues (O'Brien et al*.,* 2001).

CA125 is primarily recognized as a serum marker of epithelial ovarian cancer, but is known to be expressed in many adult and embryonic tissues, in both normal physiological and pathological conditions (Nouwen et al*.,* 1987; Zurawski et al*.,* 1988; Hardardotir et al*.,* 1990; Montz, 1992). As well as in tissues, CA125 is found in serum, breast milk, cyst fluid, ascites, cervical and uterine secretions, seminal plasma, WISH cell culture medium and amniotic fluid (Hanish et al., 1985; De Bruijn et al., 1986; O'Brien et al., 1986).

CA125 levels in amniotic fluid of normal pregnancies are approximately 300-600 times higher than in the maternal serum at the same gestational age and the levels increase with the duration of pregnancy (Spenser et al*.,* 1997). Amniotic fluid mostly consists of water (98.7%), sodium chloride, sodium phosphate, proteins, glucose and fetal epithelial cells. It is produced by secretion from the epithelial cells forming the amnion sac and partly by filtration of allantoic fluid through the amnion wall. The amniotic epithelium is generally thought to be the major source of CA125 antigen; therefore this form of the antigen can therefore be considered as fetal in origin (Takeshima et al., 1993).

The available published data mostly concern cancer-related CA125 antigen and little is known about the anti-

gen of fetal origin. We previously characterized CA125 antigen from human first trimester placenta and found it to be heterogeneous, as several molecular and glyco-isoforms exist, and different from the cancer-derived antigen (J a n k o v i ć and T a p u š k o v i ć, 2005). In the present study, we characterized CA125 antigen purified from human amniotic fluid in order to gain further insight into structural aspects of the antigen of fetal origin as an initial step towards elucidation of its obscure biological role.

The protein part of amniotic fluid CA125 antigen was characterized by gel filtration, ion-exchange chromatography, and peptide mass fingerprinting. The glycan component of the antigen was characterized by lectin-binding assay with a panel of plant lectins of different carbohydrate specificities.

MATERIALS AND METHODS

Materials

Amniotic fluid samples (n = 13) were obtained by amniocentesis originally performed for prenatal diagnosis.

Mouse monoclonal anti-CA125 antibodies: clone X325 (M11-like) and clone X306 (OC125-like) were from HyTest (PharmaCity, Turku, Finland). Sephadex G-75, Sepharose 4B and DEAE Sephadex A-50 were purchased from Pharmacia Fine Chemicals AB (Uppsala, Sweden). Biotinylated plant lectins and avidin/biotinylated horseradish peroxidase (HRPO) were from Vector Laboratories (Burlingame, USA). Anti-H2 antibody (Acris, Hiddenhausen, Germany) was conjugated with HRPO by the method of N a k a n e et al., 1974. Radioiodine (^{125}I) was from the Institute of Isotopes Co., Ltd., Budapest, Hungary. Bovine serum albumin (BSA) and urea hydrogen peroxide were from Sigma (St. Louis, USA). Tetramethylbenzidine (TMB) and HRPO were from ICN Biomedicals, Cleveland, USA. Sulfuric acid was from Lachema (Neratovitze, Czech Republic). Polystyrene microplates were from NUNC (Rosklide, Denmark). All other chemicals were reagent grade.

Ion-exchange chromatography

Ion-exchange chromatography of amniotic fluid was performed on a DEAE Sephadex A-50 column (20 mL bed volume) equilibrated with 50 mM Tris–HCl buffer, pH 7.6. The column was washed free of protein with the same buffer and the bound material was eluted batchwise using: 50 mM NaCl, 100 mM NaCl, 200 mM NaCl, 1 M NaCl, and 3 M NaCl in 50 mM Tris–HCl buffer, pH 7.6. Fractions (2 mL) were collected and CA125-immunoreactivity was determined as described above.

Gel filtration

CA125 antigen from pooled amniotic fluid samples was separated by gel filtration on a Sepharose 4B column (bed volume 36 mL), equilibrated, and eluted with 0.1 M phosphate buffered saline, pH 7.2 (PBS). The optical density at 280 nm of each fraction (1 mL) was measured using a double-beam spectrophotometer (CE594 CECIL, Cambridge, UK). CA125-immunoreactivity of each fraction (0.1 mL) was recorded by a solid phase assay using monoclonal anti-CA125 (clone X-325) for capture and ^{125}I-radiolabeled monoclonal anti-CA125 (clone X-306) as the tracer (0.1 mL) after overnight incubation at room temperature. After rinsing the tubes with water (3x1 mL), the bound radioactivity (cpm) was measured on an Isomedix 4/6000 gamma counter (ICN Biochemicals, Cleveland, OH, USA). The immunoreactive fractions were pooled, concentrated by ultrafiltration, dialyzed, and used for further characterization.

Iodination

The anti-CA125 antibody (clone X306) was labeled with ^{125}I, using the chloramine T method as described by G r e e n w o o d et al. (1963). The labeled CA125 was separated from free iodine on a Sephadex G-75 column (10 mL bed volume) equilibrated with 0.1M PBS, pH 7.2, containing 0.05% BSA.

Matrix-assisted laser desorption/ionization – "time of flight" mass spectrometry (MALDI-TOF MS)

The purified CA125 antigen was resolved on SDS-PAGE under reducing conditions (L a e m m l i, 1970) and further processed ProteomeFactory AG (Berlin, Germany). The protein band was subjected to "in gel" trypsin digestion and the resulting mixture of protein fragments was extracted from the gel, desalted, concentrated, and further analyzed by MALDI-TOF MS using a Voyager STR-DE (PE Biosystems, USA) mass spectrometer programmed in the reflecting mode.

Analysis of MS data

The mass spectrum of CA125 antigen from human

amniotic fluid was analyzed using FindMod and FindPept softwares, available at www.expasy.org/tools. The data for CA125 antigen from the OVCAR-3 carcinoma cell line deposited in the SWISS-Prot/TrEMBL database (entry no. Q96RK2/Q8WX17) was used as the template. FindPept software identifies peptides based on experimental MS data, taking into account the possibility of chemical modification, posttranslational modification, unspecific proteolysis, and enzyme autolysis. FindMod software permits the prediction of possible posttranslational modification, based on the differences between experimental and theoretical MS data.

Lectin-binding assay

Serial dilutions of CA125 antigen in 0.1 M carbonate buffer, pH 9.2, were prepared and 0.1 mL of each dilution was immobilized on polystyrene microplates overnight at 4°C. The plate was rinsed five times with 0.3 mL of 0.1 PBS, pH 7.2, followed by blocking of non-specific binding using 0.3 mL of 0.5% BSA in 0.1 M PBS, pH 7.2, for 2 h at room temperature. The plate was rinsed five times with 0.3 mL of 0.1M PBS, pH 7.2, and then 0.3 mL of biotinylated lectin or anti-H2-HRPO solution was added and the plates were incubated for 1 h at room temperature. The following biotinylated plant lectins were used: wheat germ agglutinin (WGA), *Ulex europaeus* agglutinin (UEA), *Lens culinaris* agglutinin (LCA), *Glycine max* agglutinin (SBA) and *Vicia villosa* agglutinin (VVA). The concentration of the lectins was 1 μg/mL and of the antibody 5 μg/mL. The unbound lectin conjugate was rinsed as described previously, 0.1 mL of avidin/biotinylated horseradish peroxidase solution was added, and the plates were incubated for 30 min at room temperature. After rinsing, the bound material was detected by adding substrate solution containing 0.01 M tetramethylbenzidine (TMB) and 0.01% H_2O_2. After 15 min of incubation, the reaction was stopped with 0.1 mL of 2 M sulfuric acid and the optical density at 450 nm was measured using an LKB 50-60-006 Micro Plate Reader (LKB, Austria).

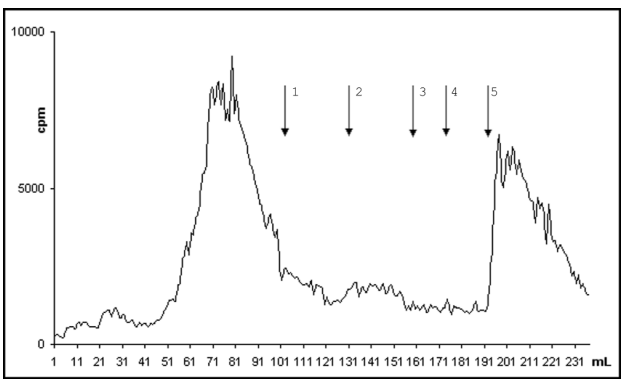

Fig. 1. Ion-exchange chromatography of human amniotic fluid on a DEAE Sephadex A-50 column. The elution was monitored by measuring CA125-immunoreactivity (cpm) in each fraction (2 mL). Arrows indicate the start of elution with a discontinuous gradient of NaCl: 1) 50 mM; 2) 100 mM; 3) 200 mM; 4) 1 M; 5) 3 M.

RESULTS

Molecular forms of CA125 antigen from human amniotic fluid

Ion-exchange chromatography of amniotic fluid on a DEAE Sephadex A-50 column revealed the presence of two distinct CA125 isoforms: one eluted with 50 mM Tris–HCl, pH 7.4, and the other with 3 M NaCl in 50 mM Tris–HCl, pH 7.4 (Fig. 1). Gel filtration indicated that both isoforms had high molecular mass (Fig. 2). Thus, immunodetection of CA125 antigen resolved on a Sepharose 4B column revealed the presence of one broad peak overlapping one of the protein peaks and eluting in the column void volume, suggesting a molecular mass exceeding 660 kDa.

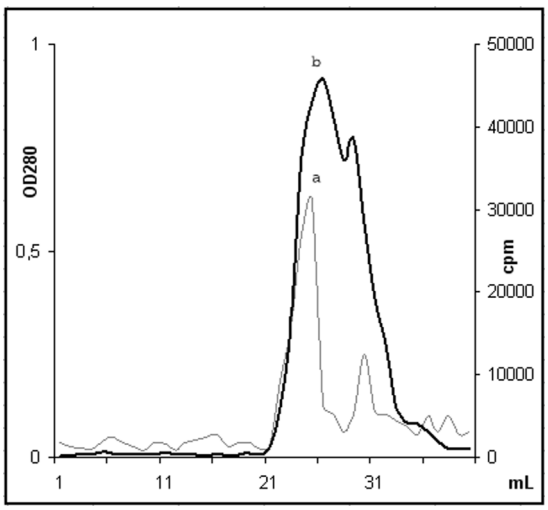

Fig. 2. Gel filtration of human amniotic fluid on a Sepharose 4B column. The elution was monitored by measuring: optical density at 280 nm (a) and CA125-immunoreactivity (b) in each fraction (1 mL).

Protein part of CA125 antigen from human amniotic fluid

The CA125-immunoreactive fractions obtained

from several separate gel filtration runs were pooled, concentrated by ultrafiltration, and used for mass spectrometric analysis. Tryptic digestion of the purified antigen produced peptide fragments of varying masses (from 308.95 to 1110.52 ppm). The experimentally obtained monoisotopic masses (user mass) were compared to that of virtually digested CA125 antigen from a human carcinoma cell line (DB mass) using FindPep and FindMod software (Fig. 3). The resulting summary of database searches indicated 46 peptides mapping throughout the entire sequence of the template, *i.e.,* the cancer-derived CA125 sequence deposited in the protein databank. One of the identified peptides corresponded to an amino acid sequence conserved in the tandem repeat region typical of mucin structure. When posttranslational modifications were taken into account, even more peptides were identified using FindMod software (results not shown). Of all the masses obtained through MALDI-TOF, four were found to result from enzyme autolysis and 74 corresponded to human keratin, a commonly identified contaminant (data not shown).

Matching peptides for specific cleavage:

User mass	DB mass	Δmass (daltons)	peptide	position	modifications	missed cleavages
472.210	472.277	0.066	(R)VLDPK/(S)	4012-4015		0
472.210	472.277	0.066	(R)VLDPK/(S)	4326-4329		0
472.210	472.277	0.066	(R)VLDPK/(S)	4483-4486		0
472.210	472.277	0.066	(R)VLDPK/(S)	4639-4642		0
494.260	494.297	0.037	(R)SLFK/(S)	6161-6164		0
544.980	545.315	0.335	(R)VRVDR/(V)	6889-6892		1
547.090	547.283	0.193	(K)DREK/(I)	341-344		1
548.750	548.304	-0.446	(R)VNSSIK/(S)	6851-6855		0
548.750	548.315	-0.434	(R)VTGSRK/(F)	3956-3960		1
571.430	571.393	-0.037	(R)VVIALR/(S)	6466-6470		0
572.330	572.315	-0.014	(R)SWPNR/(H)	6868-6872		0
576.460	576.321	-0.138	(R)VRTGSR/(K)	3955-3959		1
587.030	587.410	0.379	(R)VRKKK/(E)	6968-6971		2
590.220	590.289	0.069	(K)DGAATR/(V)	5873-5878		0
590.220	590.326	0.105	(R)SSLGAR/(Y)	6455-6460		0
599.090	599.261	0.170	(R)VYTGCR/(V)	6461-6465		0
600.920	601.403	0.483	(R)VLSLR/(S)	4773-4777		0
604.980	605.304	0.324	(K)FSHSK/(S)	3215-3219		0
609.030	609.339	0.309	(R)VLYWK/(L)	5899-5902		0
615.340	615.419	0.078	(R)VLTLLR/(S)	4304-4308		0
615.340	615.419	0.078	(R)VLTLLR/(S)	4461-4465		0
630.160	630.321	0.160	(K)SPGWDR/(E)	4330-4335		0
630.160	630.321	0.160	(K)SPGWDR/(E)	4487-4492		0
642.940	643.352	0.412	(K)SPGLNR/(E)	4016-4021		0
642.940	643.352	0.412	(K)SPGLNR/(E)	4174-4179		0
642.940	643.352	0.412	(K)SPGLNR/(E)	4643-4648		0
643.920	644.336	0.416	(K)SPGLDR/(E)	5891-5896		0
644.670	644.336	-0.333	(K)SPGLDR/(E)	5891-5896		0
644.670	644.336	-0.333	(K)SPGLDR/(E)	6047-6052		0
646.960	647.311	0.350	(K)NGAETR/(V)	6474-6479		0
660.920	661.363	0.442	(K)DKAATR/(V)	5561-5566		1
670.340	670.388	0.048	(K)IPGLDR/(Q)	5735-5740		0
681.470	681.379	-0.090	(R)VHPGSRK/(F)	3799-3804		1
713.740	713.405	-0.334	(K)RPGLDR/(E)	4955-4960		0
735.560	735.425	-0.135	(K)YTTTTALK/(T)	2187-2193		0
736.370	736.366	-0.004	(K)YTTDMLR/(T)	307-312		0
737.070	737.358	0.287	(K)YNATER/(E)	3805-3810		0
745.920	745.445	-0.474	(R)VLSTSPIK/(T)	771-777		0
770.290	770.415	0.125	(K)YGLHTGGTK/(R)	2169-2176		0
770.290	770.488	0.198	(R)VGTIRPVK/(G)	2150-2156		0
787.090	787.467	0.377	(R)VSTPLTR/(K)	1059-1065		0
795.560	795.348	-0.212	(R)VETSSSER/(I)	126-132		0
846.130	846.468	0.337	(R)VTEALSLGR/(T)	449-456		0
943.950	943.557	-0.392	(R)VLTSLRPEK/(D)	4929-4936		0
943.950	944.447	0.497	(R)VSPENPSWK/(S)	1709-1716		0

Fig. 3. Comparison of MS-data for CA125 antigen from human amniotic fluid and cancer-derived CA125 antigen using FindPept software. The sequence in the tandem repeat region is highlighted. DB - database; peptide – amino acid sequence; position – amino acid number.

Glycan part of CA125 antigen from human amniotic fluid

The glycan part of CA125 antigen was assessed from the binding profiles of lectins with different carbohydrate specificities. Thus, in solid phase assay, the examined sample was immobilized and then allowed to react with corresponding lectin conjugates (Fig. 4). Among the lectins tested, VVA (specificity for terminal GalNAc) and SBA (specificity for GalNAcα1-Ser/Thr and/or GalNAcGalβ1,3GalNAcα1-Ser/Thr) exhibited the strongest binding to amniotic fluid CA125 antigen. WGA (specificity for GlcNAc and its β1,4 oligomers) also reacted

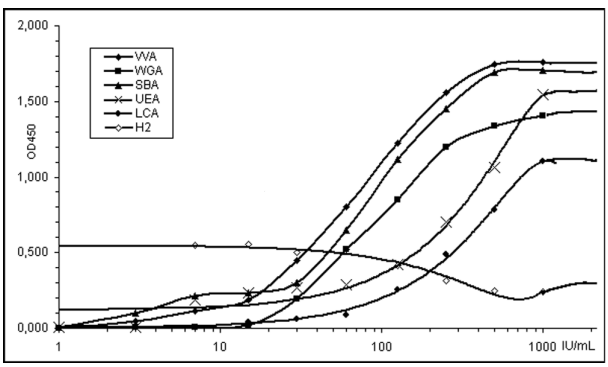

Fig. 4. Lectin-reactivity to CA125 antigen from human amniotic fluid. CA125 antigen was adsorbed on a solid phase and incubated with plant lectins of different carbohydrate specificity or carbohydrate-directed monoclonal antibody, as described in Material and Methods. The absorbance at 450 nm was recorded as a measure of bound conjugates.

strongly. However, the binding curve slope for LCA (specificity for Fucα1,6Man) and UEA (specificity for Fuc α1,2) indicated substantially lower reactivity. In addition, anti H2 antibody, recognizing fucose α1,2 bound to terminal Gal epitopes, showed no reaction with the tested CA125 antigen.

DISCUSSION

Analysis of CA125 antigen from human amniotic fluid indicated the existence of different glycoisoforms, on the basis of specificities of both the protein and the glycan part, which is in general agreement with its known extreme structural heterogeneity. Thus, separation by ion-exchange chromatography showed the existence of two distinct isoforms with immunoreactive epitopes residing on a high molecular mass fraction (> 660 kDa). Our previous investigations of fetal CA125 antigen origin isolated from first trimester placental extract showed the presence of these immunoreactive epitopes on both high and low (300–100 kDa) molecular mass fractions (Janković and Tapušković, 2005). A difference was also seen in the abundance of particular isoforms. Thus, the CA125 antigen isoform eluted with 3 M NaCl was predominant in amniotic fluid but present in relatively small amounts in placental CA125 antigen.

It is known that CA125 antigen can vary widely in molecular mass, depending on the biological source of the antigen and the physiological condition in which it is expressed (Kobayashi et al., 1992). The largest molecular mass forms may be up to 4 MDa and are found in cervical mucus and normal abdominal fluid, while forms with molecular mass as low as 76 and 162 kDa can be detected in ovarian tissue (De Bruijn et al., 1986; Nagata et al., 1991; Nustad et al., 1998, 2002). The variations in molecular mass may also be due to continuous deglycosylaton in body fluids (Konishi et al., 1994), as well as to alternative splicing resulting in a variable number of tandem repeats (McLemore and Aouizerat, 2005). The results of proteomic analysis of CA125 antigen from amniotic fluid employed in this study identified one sequence corresponding to the tandem repeat region, which is consistent with the proposed mucin structure of CA125 antigen.

Concerning the glycan part, the available published data claim that the oligosaccharide content of CA125 antigen exceeds 28%. The predominance of O-glycans, mostly of core type 1 and 2, is suggested, but high mannose and bisecting biantennary N-glycans are also present (Davis et al., 1986; Kui Wong et al., 2003). However, only a few studies employed a systematic approach to glycosylation analysis. In this study, we characterized the glycan part of CA125 antigen from amniotic fluid using plant lectins with different carbohydrate-binding specificities. The level and type of O- and N-glycosylation (relating particularly to fucosylation) was assessed from the corresponding lectin-binding profiles.

VVA has high affinity for the simplest O-glycan, the Tn-antigen, *i.e.,* GalNAcα-Ser/Thr, but it can also bind terminal GalNAc in other glycans (Goldstein and Poretz, 1986). SBA binds with high affinity to GalNAcα1-Ser/Thr and/or GalNAcGalβ1,3GalNAcα1-Ser/Thr (core type 2) (Goldstein and Poretz, 1986). WGA exhibits very complex specificity, binding with high affinity to GlcNAc and its β1,4 oligomers and various mucin-type molecules (Madiyalakan et al., 1996; Wright, 1990). The strong reaction of these lectins with CA125 antigen confirms previous observations indicating the predominance of O-glycans (Kui Wong et al., 2003). In addition, the obtained results imply that CA125 antigen from amniotic fluid can be fucosylated at both core and outer positions, exhibiting a very similar pattern of fucosylation to that observed for placental CA125 antigen (Jankovića and Tapušković, 2005). Fucosylation was assessed using LCA, which exclusively binds the fucosylated trimannosyl core of N-glycan (Fucα1,6Man); UEA, which recognizes fucose α1,2 bound in the outer positions of oligosaccharide chains (Cummings, 1994, 1997; Goldstein and Poretz, 1986); and the anti-H2 antibody, which specifically recognizes fucose α1,2 bound to terminal Gal (Karsten et al., 1993).

In summary, our results are in agreement with general observations on CA125 glycosylation, but subtle differences in the profile of molecular forms were observed in comparison to placental tissue-derived and cancer-derived CA125 antigen. They could be related to the examined source, *i.e.,* the fact that we characterized soluble antigen forms. The mechanism of CA125 secretion is not known with certainty. Both the possibility of proteolysis within an SEA domain leading to the release of the N-terminal portion of the antigen (Boshell et al., 1992) and that of alternative splicing of CA125 mRNA removing the transmembrane domain of mature mRNA (McLemore and Aouizerat, 2005) have been proposed. Intrinsic protease activity of the antigen

(K o n i s h i et al., 1994; F e n d r i c k et al., 1997) preceded by phosphorylation at either or both Ser or Thr (N a g a t a et al., 1991; K o n i s h i et al., 1994; O ' B r i e n et al., 1998) also seems to be important for secretion.

The biological role of CA125 is still elusive. The complexity of its structure suggests that it can act as a multifunctional molecule, *i.e*., both the protein and the glycan parts may be involved in different kinds of interactions. Since this antigen is expressed in a variety of tissues and physiological conditions, further investigation is needed before the meaning of tissue-specific structural forms can be fully comprehended.

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АНАЛИЗА ПРОТЕИНСКОГ И ГЛИКАНСКОГ ДЕЛА CA125 АНТИГЕНА ИЗ ХУМАНЕ АМНИОНСКЕ ТЕЧНОСТИ

БОЈАНА МИЛУТИНОВИЋ и МИРОСЛАВА ЈАНКОВИЋ

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CA125 антиген је муцински тип молекула, са веома комплексном структуром како у погледу протеинског ланца тако и олигосахаридне компоненте. У овом раду смо окарактерисали протеинску и гликанску компоненту CA125 антигена из хумане амнионске течности, користећи гел филтрацију, јоноизмењивачку хроматографију, методу пептидних отисака и тест везивања лектина. Добијени резултати су указали на структурну хетерогеност испитаног антигена тј. на постојање више гликоизоформи, чији се профил разликује од профила гликоформи CA125 антигена плацентног и карциномског порекла. Сложена структура CA125 антигена указује да он може деловати као мултифункционални молекул, а даља истраживања на овом пољу помоћи ће у расветљавању биолошког смисла постојања ткивно-специфичних структурних форми.