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RESEARCH

Biochemical and Morphological Differences Between CA125 Isolated from Healthy Women and Patients with Epithelial Ovarian Cancer from Tunisian Population

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Abstract Analysis of the structure of CA125 is essential for determining the physiological role of this significant tumor antigen. The objectives of this study were: (1) to identify the characteristics of the CA125 isolated from healthy and patient women with epithelial ovarian cancer; and (2) to determine the ferning structure of this antigen. The cancer-derived CA125 antigen (cCA125) purified by gel filtration and affinity chromatography (Concanavalin A) was run on SDS-PAGE and examined using light microscopy and compared with healthy-derived CA125 antigen (hCA125). Both purified antigen cCA125 and hCA125 showed a high molecular mass (> 2,000 kDa) with high mannose glycans. The ferning patterns related to cCA125 and hCA125 revealed distinct differences in the patterns of arborescence. The ferning morphology of cCA125 antigen was denser than that of hCA125 antigen making an obvious difference between cCA125 and hCA125, with respect to length, branching and distribution of crystals. The current study provides the first evidence for a potential functional link between CA125 and its structure which, in the light of a comparison between cCA125 and hCA125, might proof to be of significant biomedical importance in the future.

Keywords CA125 · Ovarian cancer · Tumor marker · Biochemical characterization · MUC16

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Introduction

The ovarian tumor marker CA125 has been reported to be associated with a mucin-like high-molecular-weight glycoprotein complex. This tumor marker CA125 is encoded by the gene MUC16 [1]. The discovery of CA125 resulted from an assay developed using a murine monoclonal antibody that reacts with an antigen found to be common to most non mucinous epithelial ovarian carcinomas [2]. The biochemical analysis of the CA125 antigen has produced conflicting data with evidence suggesting that it is a protein, a mucin, a carbohydrate and a membrane-associated glycoprotein [3]. The first biochemical analysis of the CA125 antigen, purified from human milk, suggested that it was a sialylsaccharide antigen carried on a mucin glycoprotein [4]. CA125 is an extremely complex molecule from both the proteomic and glycomic perspectives. The fact that CA125 is a heavily glycosylated and high-molecular-weight protein has been established by a number of studies, and CA125 purified by affinity chromatography

has suggested the presence of N- and O-linked carbohydrates, with oligosaccharides making up 24% of the total mass of the antigen [5]. Analysis of the structure of CA125 is essential for determining the physiological role of this significant tumor antigen. However, its very high molecular weight and mucinous nature poses major obstacles for performing structural characterization studies [6]. Principal among these is the fact that despite a significant and relatively recent increase in our understanding of its biology, there are still far more questions than answers about how its structure is changed during ovarian cancer. Information on the molecular nature of the CA125 antigen could help in understanding its biological role in normal and malignant tissues.

The objectives of this study were: (a) to identify the characteristics of the ovarian tumor-associated antigen CA125 isolated from healthy women and patients with epithelial ovarian cancer; and (b) to determine the ferning structure of this tumor marker.

Materials and Methods

Clinical Materials

Serum samples were obtained from six women with epithelial ovarian cancers, confirmed by histopathological examination of tissue at the time of original diagnosis. FIGO stages (International Federation of Gynecology and Obstetrics) at primary diagnosis in patients are as follow: 5 cases stage III and 1 case stage IV. For histological type, the serous type was the most frequent with 100% of cases. We noticed the presence of ascites in one patient. Four samples from Tunisian volunteers were included in this analysis. The median age was 56 years for patients and 44 years for controls. For one control, we choose a woman having a high CA125 level with gynecological pathology other than ovarian cancer. All subjects provided written informed consent according to the protocol submitted and approved by local and regional ethical committees. CA125 levels for each of these samples were assessed. All serum samples were aliquoted and stored at −70°C until assay.

CA125 Enzyme Immunoassay

Measurement of serum CA125 was done for each woman enrolled in this study. The CA125 values were quantitatively measured using a commercially available automated microparticle enzyme immunoassay method (Abbott AxSym system), following the manufacturer's recommendations. The cut-off value for "normal" in this system was <35 U/ml.

Purification of CA125 Antigen by Gel Filtration

Approximately 1 mL of serum was loaded onto a Sephadex S-1000 column (1×60 cm) pre-equilibrated with 50 mM sodium acetate buffer (pH 5). The column was calibrated with dextrin blue (20.10^3 kDa), Catalase (230 kDa), BSA (67 kDa), Ovalbumin (43 kDa) and vitamin B_{12} (1.350 kDa) as molecular mass standard. The column was eluted with 50 mM sodium acetate. 4 ml collected fractions were monitored for absorbance at 280 nm. CA125-immunoreactivity of each fraction (4 mL) was recorded by an immunoenzymatic assay using a monoclonal anti-CA125 (OC125). The immunoreactive fractions were pooled, concentrated by speed-vac and used for further characterization. The total protein in this peak was measured using the BioRad reagent.

Concanavalin A Chromatographic Affinity

Concentrated immunoreactive fractions of proteins were mixed with concanavalin A-sepharose resin [7]. A contact for 30 min between ConA and proteins was applied with moderate agitation of the eppendorf tube. The mixture was loaded onto ConA-Sepharose column (10 cm×1.2 cm from Pharmacia) pre-equilibrated with ConA buffer. The bound glycoproteins were eluted by washing with a step gradient (0- 1 M) of N-acetylglucosamine (NAG) and monitored for absorbance at 280 nm. Fractions from eluted peaks were pooled and the proteins were analyzed. CA125-immunoreactivity of each fraction was recorded by an immunoenzymatic assay

Protein Assay

The protein content of the samples was determined using the Biorad-Bradford assay with Optical Density reading at 595 nm and BSA used as standard (MM Bradford, 1976) [8].

Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis (SDS–PAGE)

To investigate the purity of the isolated CA125, samples were subjected to SDS-PAGE. Fractions of the dominant peak of CA125 were run on a SDS-PAGE gel. Electrophoresis under reducing conditions [9] was routinely conducted on 8% separating and 5% stacking gels. Coomassie Blue staining of gels was performed using protocols established in our laboratory.

Light Microscopy

CA125 antigen solution (10 μl) was allowed to dry on a glass surface at room temperature for 30 min. The sample

was deposited as a droplet spread out in all directions with a pipette tip. The preparations were examined microscopically and photographed. The ferning patterns of different preparations were qualitatively assessed by two independent investigators.

Results

1. Chromatographic Gel filtration analysis

Molecular weight and different isoforms of CA125 in the plasma of healthy women and patients with epithelial ovarian cancer were initially characterized by chromatographic gel filtration. Plasma of healthy women was injected onto Sephadex S-1000 gel filtration, the chromatographic profile illustrates more than one protein peaks at 280 nm (Fig. 1). When these fractions were tested by enzymatic immunoassay, one CA125-immunoreactive peak was detected in the very high molecular mass region (column void volume) of four patients' samples and four healthy women samples. For two patients' samples, two CA125-immunoreactive peaks were detected in the very high molecular mass (> 2,000 kDa). Representative elution profile from the S-1000 column relative to these two patients is shown in Fig. 1.

2. Chromatographic ConA affinity analysis

To determine if MUC16, derived from plasma of epithelial ovarian cancer and from plasma of healthy women, also expresses N-linked glycans, we loaded the CA125-immunoreactive pooled fractions on a lectin Concanavalin A. Eluted fractions were collected and the retained material was eluted using sequential washes containing increasing concentrations of N-acetylglucosamine (NAG). MUC16/CA125 was detected in all of the NAG fractions. This suggests that the glycans present in CA125 includes high mannose glycans.

3. Analysis of the isolated CA125 by SDS-PAGE

Electrophoretic analyses were initially employed to assess the purity of the isolated CA125 sample. Coomassie Blue staining indicated a band above the 200-kDa molecular mass marker (Fig. 2). In addition to the band suspected to correspond to CA125, some low molecular mass bands were also detected (Fig. 2). Such lower molecular mass bands are always present in CA125 preparations examined in other studies (6). In particular, a broad band at 40 kDa was detected in all samples, even in commercial antigen CA125. The sequencing of this peptide was performed and blasted against known databases (NCBI). The peptide sequence analysis and its molecular weight suggested that it corresponds to mesothelin.

4. Ferning patterns related to CA125

We examined the antigens CA125 purified in a saline buffered environment from these different plasmas (cancerous and non cancerous) and assessed their morphologies by light microscopy. Owing to the physical properties of mucins, they readily aggregate in solution through covalent and non-covalent associations. Under these experimental conditions, noticeable intra-sample variation related to the grouping of crystals was visible in the ferning patterns of CA125.

The ferning patterns related to CA125 isolated from plasma of epithelial ovarian cancer (cCA125) and CA125 isolated from plasma of healthy women (hCA125) are

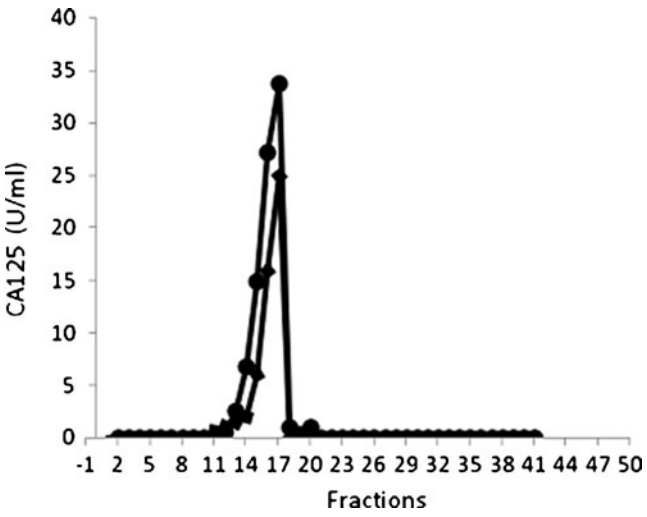

Fig. 1 Gel filtration separation of the CA 125 antigen isolated from healthy women (♦) and patients with epithelial ovarian cancer (●)

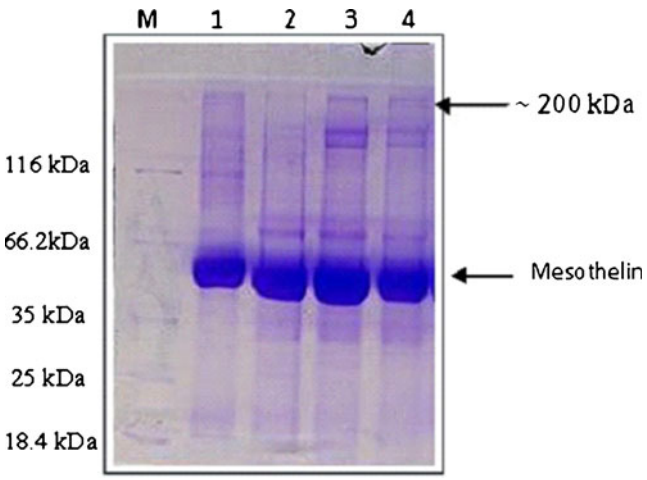

Fig. 2 SDS-PAGE of the commercial CA125 (*lane 1*), CA125 isolated from healthy women (*lane 2*) and patients with epithelial ovarian cancer (*lanes 3and 4*). M: The molecular weight standards (*fermentas SMO431*)

shown in Fig. 3. The results obtained for hCA125 antigen and cCA125 antigen revealed distinct differences in the patterns of arborescence. The extent of crystallization of cCA125 was extremely higher than that of hCA125 antigen. The spatial distribution of crystals was dispersed giving the shape of a star in both hCA125 and cCA125 but the ferning morphology of cCA125 antigen was denser than that of hCA125 antigen. This makes an obvious difference between cCA125 and hCA125, with respect to length, branching and distribution of crystals. In particular, some cCA125 show a spider shape in agreement with the structure of CA125 being more complex due to its concomitant interactions with other glycoproteins. Thus, a characteristic spatial organization relative to each antigen hCA125 and cCA125 is highlighted.

Discussion

CA125 is a tumor antigen which is the basis for a serum assay that is widely used in the monitoring of ovarian cancers [10]. Information on the molecular nature of the CA125 antigen could lead to improved assay methods as well as to an understanding of its physiological role in normal and malignant conditions. Molecular studies such as comparative genomic analysis for the gene MUC16 may provide a better understanding of the ovarian cancerogenesis [11]. In addition, elucidation of the role of the protein CA125 encoded by MUC16 may therefore supply new insights on molecular biology of ovarian cancer. Previous works on the biochemical structure of the CA125 antigen had resulted in confusing views of its

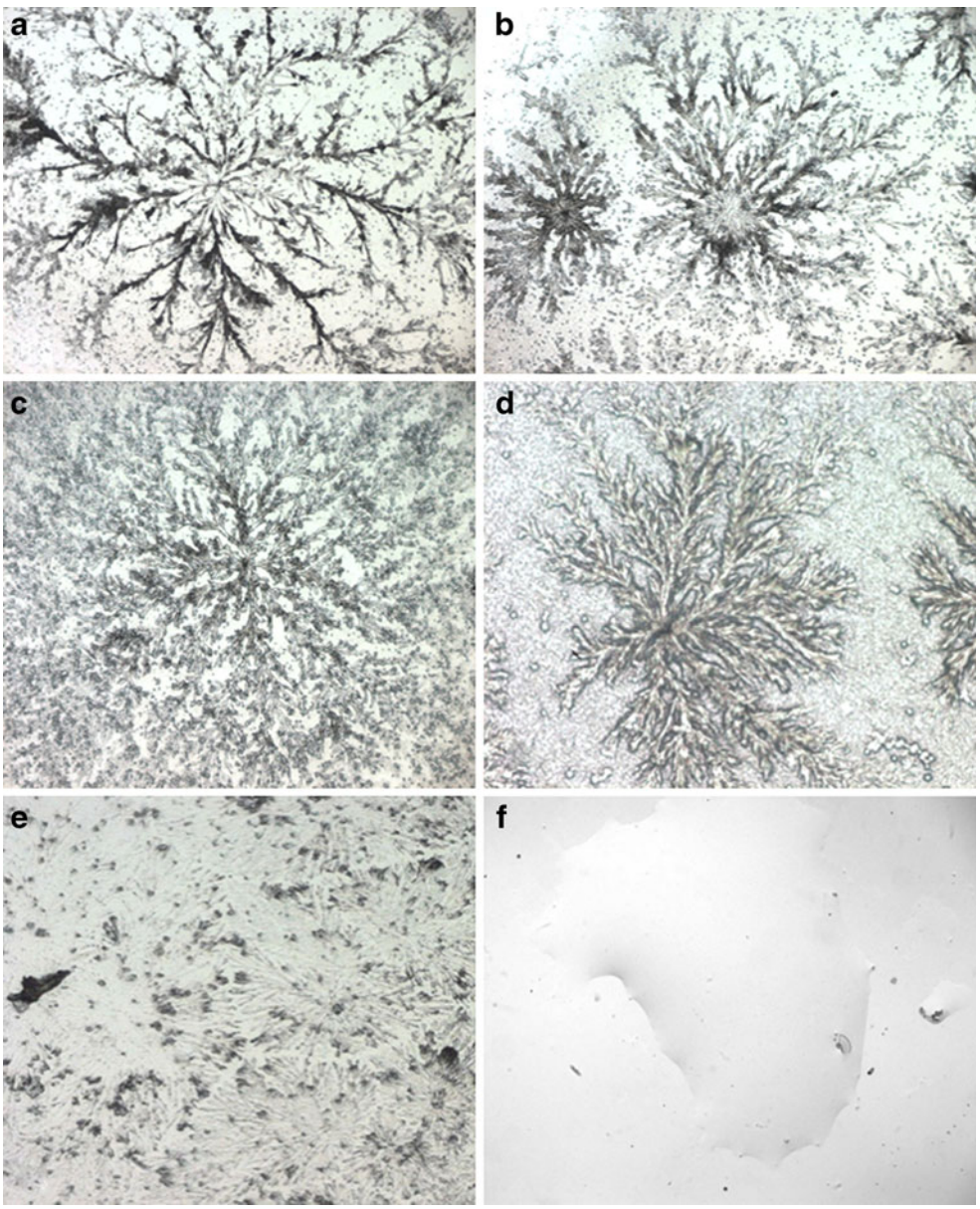

Fig. 3 Ferning morphology of CA125 antigen by light microscopy (X 100). **a**, **b**, **c** and **d**: Ferning morphologies of cCA125. **e** Ferning morphology of hCA125. **f** Sodium acetate buffer (*negative control*)

molecular structure and composition. While most of these studies agreed that CA125 is a high molecular weight glycoprotein, some other studies detected smaller reactive species [12]. Here we report on differences between CA125 isolated from plasma of epithelial ovarian cancer (cCA125) and CA125 isolated from plasma of healthy women (hCA125). In spite of the current study design that is relatively small, the discovery of some differences between ovarian cancer antigen CA125 derived from healthy women and patients with ovarian cancer is particularly interesting in that it gives more insight in the molecular biology of this disease. To the best of our knowledge, this is the first study that outlines such a comparison between CA125 derived from these two physiological conditions. Given the nature of the current work as a preliminary investigation, it will provide guidance for future CA125 biochemical studies either technically i.e. how to deal with such a complex mucinous protein or cognitively. Such data regarding aspect of CA125 are currently needed to better understand the causes of ovarian cancer.

Previously, Yu et al. employed sera from healthy women and sera from patients with ovarian cancer to determine how co-expression of different epitopes on complexes that express CA125 determinants might provide a more specific test for malignant disease. Through this study, they provide that macromolecular moieties containing CA125 determinants have a high molecular weight [13]. Using three different sources to isolate the antigen CA125, Davis et al. found that this antigen was shown, by gel electrophoresis, molecular size exclusion chromatography, and buoyant density ultracentrifugation to have a molecular weight of 200 000 to 1 million Daltons [14]. Our results are consistent with general observations on high molecular weight of CA125, but some differences in the profile of molecular forms were observed in comparison between non cancerous plasma-derived and cancer-derived CA125 antigen. According to Milutinovic et al. these differences could be related to the sources from which the antigen CA125 was purified [15].

In this study, we used the ConA-Sepharose based approach to partially assess on the structural properties of the CA125. The Con A lectin binds to high mannose type, hybrid type and biantennary complex type oligosaccharide chains [16]. Strong binding of this lectin ConA to CA125 was observed. The available literature data on the CA125 obtained using biophysical analysis, indicated that the N-linked glycans are predominantly composed of complex bisecting- and high mannose oligosaccharide chains. Taken together, the results obtained are in general agreement with previous observations on CA125-carbohydrate composition. CA125/MUC16 is known to express high amounts of O-glycans and also carries a significant proportion of N-linked oligosaccharides [17]. Besides, CA125 antigen from different biological sources was found to be heterogeneous in respect to the existence of multiple glycoforms, with O-linked glycan chains predominating. Distinct differences were found in carbohydrate composition between CA125 antigens isolated from amniotic fluid and OVCAR-3 cell line [18]. In this study no difference related to the N-linked glycans emerged between CA125 antigens isolated from plasmas of healthy women or plasmas of patients with epithelial ovarian cancer.

It has been found that the extraordinarily abundant *N*-glycans on CA125, presumably in the tandem repeat region, are required for binding to both glycosylated and non-glycosylated mesothelin. This observation might be of significant biomedical importance as mesothelin is a tumor marker involved in the regulation of cell proliferation and tumor progression [19]. In this context, the interaction between mesothelin and CA125 may facilitate the implantation and peri-toneal spread of tumors by cell adhesion [20]. The interaction observed does not appear to represent a simple protein–protein interaction but rather depends on additional aspects of specificity based on the cancerous environment that leads to specific ferning morphology of the antigen CA125.

In the present study we attempt to investigate purified hCA125 and cCA125 from human plasma and to assess their properties by light microscopy. CA125 antigen is a highly glycosylated molecule and its long chain glycans probably determine its molecular topology [21]. Our results indicated noticeable differences between hCA125 and cCA125 antigen, probably related to the carbohydrate composition of both N- and O-glycans [22], which might influence the observed ferning pattern. The ferning morphology of hCA125 antigen was less dense than that of cCA125 antigen being more complex due to its high degree of glycosylation and concomitant interactions with other glycoproteins.

Taking into consideration that the molecular structure is closely related to the function, we can suppose that the spider arborescence of cCA125 antigen is closely related to cancerous process. Whether this complex structure is a result of ovarian cancer or a cause remains unknown. However, the current study provides the first evidence for a potential functional link between CA125 and its structure which, in the light of a comparison between cCA125 and hCA125, might proof to be of significant biomedical importance in the future.

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