

Epitopes on CA125 from cervical mucus and ascites fluid, and characterization of six new antibodies: Third Report from the ISOBM TD-1 workshop

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Abstract

CA125 antigen was collected from different sources basically to study if the source of ligand influenced epitope characterization. Ascites derived CA125 varied in size from about 190 kDa to about 2700 kDa. CA125 from cervical mucus was collected at two places. Treatment with ultrasound change its apparent size from more than 20 000 kDa to 700 kDa. Epitope mapping of antibodies was not grossly influenced by the size or source of CA125 used as target. However, low molecular weight CA125 i.e. ascites fractions CA 17/E, CA 17/F and CA 10/7 did show differences in certain assay combinations which probably can be explained by steric effects due to the smaller size compared with the most abundant forms of CA125 present in serum and other body fluids.

The specificity of 6 new monoclonal antibodies to CA125 was tested by cross-inhibition and immunometric assay combinations and compared to reference antibodies. One antibody X306 belonged to the OC125-like antibodies. Four antibodies X52, X75, X325 and VK8 were M11-like. The M11-like group can also be divided into subgroups. X75 and X325 showed strongest similarity to M11 and was named B1, whereas X52 was similar to K101 and called B2. VK8 is unique and was inhibited by all M11-like antibodies, but was itself a poor inhibitor of the other M11-like antibodies. This antibody therefore represent a separate B-group. The sixth antibody, 7C12, reacted with an epitope which was difficult to define. This antibody was inhibited by M11-like antibodies and OV197. However, used as an inhibitor 7C12 inhibited only itself. We grouped it as an OV197-like antibody, but clearly different from OV197.

The topography of epitopes was studied by analysing all antibody pairs in an immunoradiometric assay. These results confirmed the grouping of antibodies described above and is in accordance with previous findings that the highest signal is obtained using an OC125-like antibody or OV197 on the solid phase and a M11-like antibody as tracer.

The composition of the sample in terms of high and low molecular weight species of CA125 was measured with different responds depending on the antibody pair used. This might be one reason for discrepancies between assay results for CA125 using different assays.

Introduction

CA125, an ovarian cancer-associated antigen, is defined by the antibody OC125, which was produced by Bast et al.[2] using the ovarian cell line OVCA 433 as immunogen. After 20 years part of the gene was cloned in 2001 by Yin and Lloyd [3] [4]. It is probably one of the largest glycoprotein / mucins found in the body coded for by about 65 000 base pairs. CA125 has a cytoplasmic tail with a phosphorylation site, and the release of CA125 might be enzymatically regulated. Released CA125 contains about 60 repeat sequences consisting of 156 amino acids. The overall structure is well conserved, but very few of the repeats are identical to each other in exact amino acid composition. A disulfide bridged loop of 19 amino acids is present in all repeats. The N-terminal end contains a hairbrush structure which is heavily O-glycosylated. The deduced size would be 2-3 MDa for the protein part and with added carbohydrate this could further increase to 4-6 MDa[4].

The epitope specificity of 26 antibodies was studied in the first report from the International Society of Oncodevelopmental Biology and Medicine (ISOBM) Works Shop TD-1[5]. The application of 22 antibodies in immunohistochemistry was reported in the second report from the TD-1 workshop[6]These antibodies could be grouped as OC125-like, M11-like or OV197-like. The OC125-like antibodies was further subdivided into four subgroups A1-A4, whereas OV197 was alone in its group, and the M11-like antibodies was separated in two.

CA125 is found in body fluids in a variety of molecular weight forms. The largest species are found in normal abdominal fluid and cervical mucus [1,7]. The present publication therefore incorporates CA125 derived from these sources as well as ascites fluid. The results are in general very similar to those obtained in the first workshop, although with some interesting differences.

Materials and Methods

Monoclonal antibodies

The antibodies were submitted to the scientific coordinator at the Norwegian Radium Hospital, coded, and subjected to SDS PAGE electrophoresis under non reducing conditions using a Novex 4 - 12% gradient gel (Invitrogen, CA, USA). All antibodies were of high purity.

CA125 preparations

Cervical mucus (CM) was collected from women with stimulated ovarian cycles as part of *in vitro* fertilization in Groningen by H. W. A. de Bruijn and in Hiroshima by K. Shigemasa. Recombinant FSH was used for stimulation of ovulation and the cervical mucus was aspirated into 1 ml syringes just before the follicles were collected.

CM-1 Shigemasa's preparation was untreated mucus with added protease inhibitors (Complete from Boehringer/Roche Diagnostics, Mannheim, Germany), and stored frozen until sent to Oslo. The rubber like material was dissolved in an equal volume of 8 mol/L guanidine, 0.1 mol/L NaCl and 0.05 mol/L Tris, pH 7.0. Material not dissolved under these conditions was removed by centrifugation at 20 000 g for 10 min. The supernatant was distributed in 4 mol/L guanidine in the same buffer.

CM-2 De Bruijn's first preparation was treated with ultrasound. Samples with turbid material only were discarded. From 64 patients samples clear mucus were collected, pooled and stored frozen at -20°C. The pooled cervical mucus (19 g) was thawed and mixed with 8 mg EGTA and 2 tablets of protease inhibitor Complete. The pooled mucus was liquified by sonication on ice for 1 min in an MSE sonicator with microtip and the debris was removed by centrifugation at 2000 g for 10 min. The supernatant was sonicated and centrifuged.

CM-3 De Bruijn's second preparation was collected in the same manner as CM-2, but only a fraction of the material was sonicated.

CM-4 This represents CM-3 which was not sonicated. After shipment to Oslo 6 mol/L guanidine in 0.1 mol/L NaCl and 0.05 mol/L Tris, pH 7 was added, and insoluble material removed by centrifugation 20 000 g for 30 min. The sedimented material was suspended in 4 mol/L guanidine buffer and centrifuged to give CM-4A. The same procedure was repeated to produce CM-4B to F.

Abdominal fluid (AF) was collected by K. Shigemasa from patients undergoing sterilization and with no abdominal disease. Complete protease inhibitors was added as described above. The abdominal fluid preparation and CM-3 and CM-4 was not distributed to the participants, but used only in Oslo to clarify the differences between CM-1 and CM-2, see results.

OVCAR-3 culture fluid was used as a source of high molecular weight CA125, and purified by affinity chromatography using antibody VK8 as described before [8].

Ascites fluid from patients with ovarian cancer was collected in Moscow and purified at Xema-Medica Co, by Y. Lebedin. Each preparation was collected from a single patient and purified by gel filtration on Sephacryl S300 and ion exchange chromatography on Q-Sepharose Fast Flow.

CA10/7 Low molecular weight fraction collected between 100-200 kDa from gel filtration. Before gel filtration the starting material was treated by heating to 70°C for 15 min in acid buffer (0.1M sodium acetate, pH 5.0). This preparation was stored at +4C for longer than 1 year.

CA25C Low molecular weight fraction collected between 400-600 kDa from gel filtration. This preparation was stored at +4C for longer than 1 year.

CA17E and CA17F Low molecular weight fraction collected between 400 and 600 kDa from gel filtration and then applied to ion exchange chromatography on Q-Sepharose Fast Flow. CA17E was bound and eluted with salt, whereas CA17F represent the flow through fraction. This preparation was stored at +4C for longer than 1 year.

CA23 High molecular weight fraction collected as the void fraction after gel filtration on Sephacryl S-300. The ascites was preselected for high concentration of CA125. This preparation was stored frozen at -70C before use.

CA125-1 Ascites preparation from 10 patients collected in Oslo. Precipitated with ammonium sulfate and used in previous epitope studies [5].

Radioiodination of antibodies

All antibodies were radioiodinated with equimolar amount of ^{125}I using the indirect Iodo-Gen method with pre-coated tubes (Pierce, IL, USA).

Enzyme labelling of CA125

CA23 was labelled with Horse Radish Peroxidase (HRPO, Faizyme, Lansdowne, RSA) using the periodate method.

Coating of microtiter plates

Coating-1 Antibodies (100 μl of 10 $\mu\text{g}/\text{mL}$ in 0.05 mol/L carbonate buffer, pH 9.5) was added to microtiter plates (Enhanced Binding, Labsystems, Finland), and incubated at 4°C overnight. The plates were washed three times in wash-solution (saline with 0.1% Tween-20), and treated by the blocking solution overnight at 4°C (0.1 mol/L PBS containing 0.5 % casein, 0.05% phenol, 5% sucrose and 0.5% D-sorbitol, pH 7.2). Finally the plates were washed twice, dried, packed under vacuum into aluminium foil bags, and stored at room temperature up to three months.

Coating-2 The procedure was similar to Coating-1 with the following modifications. Coating buffer was 0.2 mol/L NaH_2PO_4 buffer pH 4.3. Microtiter plates was Nunc BreakApart plates. Blocking solution was 0.05 mol/L Tris, 6 % Sorbitol, 0.1 % BSA and 0.05 % NaN_3 pH 7.3.

Cross-inhibition assays

Cross-inhibition with radiolabelled antibodies This was done as Cross-Inhibition-1 in the first TD-1 study [5]. In short, CA125 was allowed to bind to microtiter wells coated with 1 $\mu\text{g}/\text{well}$ of either K93, K95 or K101 using Coating-2. Inhibiting antibodies (1 μg) was added 30 min before the radiolabelled antibody (10 ng). After further 60 min incubation wells were washed and bound radioactivity counted.

Cross-inhibition with HRPO-CA23

Fifty microliters of assay buffer (0.1 mol/L PBS, 0.1 % Tween 20, 0.1 % casein and 0.05 % phenol, pH 7.2) or diluted antibodies (10 $\mu\text{g}/\text{ml}$ in assay buffer) were incubated in antibody coated microtiter plates (Coating-1) followed by 50 μl of CA23-HRPO (1:500 in assay buffer). The microtiter plates were incubated for 45 min at room temperature on a shaking platform. After washing five times, 100 μl of TMB substrate solution (K-Blue, Neogen, KY) were added to each well. The enzymatic reaction was stopped after 15 min by addition of 5 % sulphuric acid, and the optical density at 450 nm was measured in microplate reader. All combinations of antibodies were run in duplicates. Percentages of inhibition were calculated by dividing average optical density in actual wells by average in control wells.

Immunometric assay combinations

These were tested as described for IRMA-1 in [5]. In short, microtiter plates were coated with sheep anti mouse Ig by Coating-2. Catcher antibodies were added (100 ng in 100 μl) incubated for 1 hr and washed twice. CA125 (200 U in 100 μl) was added and allowed to react for 1 hr followed by three washes. Finally radiolabelled antibodies (10 ng in 100 μl) were added and allowed to bind for 1 hr followed by three washing steps. In the two last steps the assay buffer was supplemented with 10 $\mu\text{g}/\text{ml}$ of mouse IgG1 and IgG2a to avoid binding of labelled antibodies to the sheep anti mouse Ig.

Gel filtration on Sephacryl S-1000

A Sephacryl S 1000 column (1.6 x 69 cm) was run at 13 ml/h in 4 mol/L guanidine, 0.05 mol/L Tris, 0.1 mol/L NaCl, 5 mmol/L EDTA, pH 7 at 4°C. Fractions of 2.4 ml were collected. Radio-labelled mouse IgM was added as an internal reference and 1 ml of each sample was applied to the column.

Radioimmunoprecipitation and SDS-PAGE

In vivo labeling of CA125 with ^{35}S -Met/Cys from OVCAR-3 cells, immunoprecipitation and analysis by electrophoresis was performed as described [9].

CA125 assay

Our in house assay for CA125 uses K93 as catcher and K101 as tracer antibody adopted to the AutoDELFI (PerkinElmer/ Wallac, Finland) as described in {Norum, Erikstein, et al. 2001 5886 /id} with the following modification. Instead of using directly coated K93 we now use streptavidin coated plates (PerkinElmer/Wallac) and biotinylated $\text{F}(\text{ab}')_2$ of K93. The assay buffer has been supplemented with 15 mg/L of heat-polymerized mouse IgG1 (MAK33, Roche Diagnostics). Both modification have been done to avoid interference from heterophilic antibodies [10].

Results

Characterization of CA125 preparations

It is known that CA125 exists in various molecular weight forms and that the largest CA125 is found in normal abdominal fluid, cervical mucus, and in secretion from certain cell lines [1,7,8]. We therefore collected high molecular weight CA125 from cervical mucus, abdominal fluid, and cell supernatant. The lower molecular weight forms of CA125 found in ascites was partly purified as described in methods. The intention was to see if epitopes were equally represented on the various preparations, and if different antibody combinations when used in immunometric assays measured the various forms of CA125 equally.

CA125 analysis of all preparation in the assay using antibody K93 as catcher and K101 as tracer gave dilution curves which was parallel to our ascites based standard.

Radioimmuprecipitation analysis of *in vivo* labelled CA125 antigen present in OVCAR-3 cell supernatant were analyzed by SDS-PAGE gave the same pattern for all tested antibodies, namely a major antigen band in the 3% stacking gel, and three faint bands of about 200 kDa in the 7% separation gel (Fig. 1). A major band at the interface between the stacking and separation gel was nonspecific as was is present also in the negative control.

This experiment clearly demonstrated that the antigen released from the medium from these cells is very large. The material purified from these cells (KOL1) showed diffuse staining in the separation gel, and three bands in the separation gel starting at the interface between the 5% stacking gel and the 7% separation gel. Thus the highest molecular weight forms present in the cell culture medium was partly lost during purification. Purified CA23 from ascites contained the same protein bands in the separation gel, and in addition many proteins of lower molecular size not related to CA125 (Fig. 2).

Gel filtration on Sephacryl S-1000 of CA125 from abdominal fluid and cervical mucus showed that abdominal fluid contained mainly CA125 of extremely high molecular weight, however, with tailing all the way down to species of about 200 kDa in size (Fig. 3). We did not have molecular weight markers which correspond to the main peak, but extrapolation from added radiolabelled IgM and IgG gave a major peak with relative size of 20 000 kDa. Note that the radiolabelled IgM was old and contained a major peak at 900 kDa, aggregates at V_0 , free iodine at V_i and a peak corresponding to 160 kDa (Fig. 3-5).

Cervical mucus CM1 contained an additional minor peak at about 2 000 kDa. However, cervical mucus CM2 showed a completely different elution pattern with the major peak at just under 900 kDa and only traces of activity corresponding to the major peak in CM1. The data discussed among the workshop participants (ISOBM meeting Munich 2000) suggested that the explanation might be that CM2 was treated by ultrasound to make it less viscous. A new round of cervical mucus collection proved this to be right. CM3 which was collected and treated with ultrasound had the same elution pattern as CM2. Exclusion of ultrasonic treatment, followed by extraction into guanidine, CM4B, gave the same elution pattern as with CM1. Insoluble material in CM4B was dissolved in guanidine several times (see methods), and gel filtration of the soluble material from each step showed that the peak at 2 000 kDa gradually disappeared. Renewed chromatography of the 20 000 kDa peak gave one peak with no tailing (Fig. 4).

Gel filtration of CA125 from ascites on Sephacryl S-1000 gave heterogenous elution profiles with CA23 and CA25C before and after the IgM peak, respectively. The relatively low molecular weight preparations CA17/E, CA17/F and CA10/7 eluted corresponding to the IgG peak (Fig. 5). The main data for each CA125 preparation have been summarized in Table 1.

Cross-Inhibition studies

Cross-inhibition with labelled CA125 (CA23-HRPO) was performed by one working group. In this experiment the test antibody was coated on microtiter plates and the competing antibody was added in **approximately 10x** molar excess before adding the enzyme labelled CA23. Inhibition of binding by the same antibody in solution should in usually be above 90 %. This is not true for the reference antibodies K93 and M11, indicating that the concentration of inhibitory antibody was lower than ideal for defining antibody groups (Table 2).

Cross-Inhibition using immunoextracted CA125 and analysis of free epitopes using radiolabelled antibodies was performed in the first workshop [5]. We found that catching with K93 or K101 could be used for analyzing the free epitopes for all antibodies. In this workshop one antibody, 7C12, did not bind to CA125 under these conditions. However, when using K95 as catcher the specificity of 7C12 could be analyzed. The binding of all antibodies was studied using these three antibodies as catchers and with cervical mucus, crude ascites and purified ascites fractions as source of CA125. A striking finding was

that certain antibody combinations prefer CA125 with relatively low molecular weight i.e. CA 17/E, CA 17/F, and CA 10/7. This was true for combinations with catchers K95 and K101, but almost absent for catcher K93 (Fig. 6).

The complete cross-inhibition experiment was performed with high molecular weight CA125 from cervical mucus (CM4A), a broad range of molecular weight forms in ascites (AS1), and low molecular weight forms purified from ascites (CA 17/E) as shown in Table 3. Inhibited binding of labelled antibody by the same antibody and by antibody grouped with each other was above 87%.

Assay combinations was studied using one representative of high molecular weight CA125 (cervical mucus CM4A) and one low molecular weight CA125 (CA 17/E) using directly coated antibodies as catchers and radio-labelled antibodies as tracers (Table 4). Antibodies previously grouped as OC125-like, M11-like and OV197-like did not form assay combinations with themselves or other antibodies in their own group. Antibody combinations which measured these preparations equally well was found when M11-like antibodies were used as tracers and OC125-like or OV197 were used as catchers (bold numbers in table 4).

Adsorption of CA125 to microtiter wells was studied after dilution of each sample to the same concentration. An unexpected finding was that ascites derived CA 10/7 showed no binding of any antibody, indicating that this species of CA125 (**treated by heating at low pH by purification**) did not bind to plastic, alternatively that all epitopes are hidden.

Discussion

Molecular forms of CA125

The broad elution pattern observed with all native CA125 samples do represent a mixture of molecular weight variants and is not due to interactions with the separation medium. Thus re chromatography of cervical mucus peak fraction resulted in a relatively homogenous elution pattern (Fig. 4). The same is true for CA125 purified from ascites. The explanation for the existence of multiple molecular weight forms can either be degradation of the original very large parent molecule, or assembly of multiple forms during synthesis. *In vivo* proteolysis certainly can degrade CA125 and studies of synthesis of CA125 in cell culture gave rise to a ladder-like structure over time suggesting a stepwise assembly of the final molecule [9].

CA125 preparations of different size do differ in their behaviour in assays, depending on the antibodies used. This was clearly demonstrated when cervical mucus and an low molecular weight ascites fraction (CA17/E) were compared in all assay combinations tested. Only catcher antibodies from the OC125-group, and OV197 combined with tracer antibodies of the M11-like type relatively similar results. This is an circular argument for one of the pairs, K93/K101, as they are used in the assay where the initial concentration was measured. Many assay combinations preferred the low molecular weight CA 17/E, whereas those combinations that gave better response with cervical mucus were few and generally gave low binding.

Specificity of antibodies tested

Cross-inhibition was done by two different approaches, but with similar outcome. Enzyme-labelled CA125 was allowed to bind to coated antibodies in the presence of excess soluble antibodies in the most direct test for cross-inhibition. The limitation of this experiment was available labelled material, and was therefore done only with CA 23 (table 2).

The alternative approach used reference antibodies coated on microtiter plates for immuno-extraction of CA125. Epitopes not engaged by the catcher antibody can then be studied using excess unlabelled antibody added before the radio-labelled antibody. The limitation of this procedure is the influence of catcher antibody on the epitope under investigation. This is indeed the case and in addition to catcher antibody K93 and K101 used in workshop 1 [5], we had to include K95 to analyse antibody 7C12 (table 3, Fig. 4). The advantage of this procedure is that many different CA125 preparation can be used, and we are not dependent on adsorptive properties of the antigen.

Grouping of reference antibodies by cross-inhibitions were similar when three CA125 preparations were used i.e. cervical mucus, ascites, and low molecular fractionated ascites. Also there is an systematic difference between the inhibition pattern between the two high molecular fractions (cervical mucus and ascites) versus the low molecular fraction (CA 17/E). This is most apparent for inhibition of antibodies belonging to the same epitope group as the catcher antibody i.e. inhibition by M11-like antibodies when analysing an antibody belonging to the OC125-like group. This could represent

displacement of low molecular fraction from the catcher antibody or a steric effect.

Of the new antibodies, one antibody X306 belonging to the OC125-like group. Its subgroup can not be defined as we did not have reference antibodies from OC125 subgroups A3 and A4.

Four antibodies X52, X75, X325 and VK8 were M11-like. The M11-like group can also be divided into subgroups. X75 and X325 showed strongest similarity to M11 and was named B1, whereas X52 was similar to K101 and called B2. VK8 was inhibited by all M11-like antibodies, but was itself a poor inhibitor of the other M11-like antibodies. This antibody therefore represent a separate B-group. Also the data from the first CA125 workshop are in agreement with the subgrouping of the M11 group, see Table 2-3 in [5].

One antibody 7C12 reacted with an epitope which was difficult to define. This antibody was inhibited by M11-like antibodies and OV197. However, used as an inhibitor 7C12 inhibited only itself. We grouped it as an OV197-like antibody, but clearly different from OV197.

Concluding remarks

The present study underlines the excessive size of CA125 as released from cells in culture and cells lining the abdominal cavity, and those found in uterine cervix. This molecule can be broken down by the shearing force of ultrasound or by proteolytic degradation. The tumor marker which we refer to as CA125 is thus a mixture of molecular weight forms of a parent molecule of immense size. It is rather surprising that this does not interfere seriously with the measurement of CA125 in immunometric assays as the standard used will never have the same composition of CA125 molecules as the sample. The reason can be that most samples are mostly composed of high molecular weight forms which behaves surprisingly similar in assays. In fact CA125 behaves like a molecule with few repeating epitopes as no antibody make an good assay with itself as both tracer and catcher. This means that the repeating epitopes, which we now know exists, must be unavailable when the molecule is measured in an immunometric assay. This contrast sharply to another molecule with repeating epitopes like MUC1 where antibodies form assays with themselves, and where it is difficult to find saturation concentrations of tracer antibodies. Only studies of the various repeats and their epitopes and a full insight into the structure of the assembled protein will explain the many mysteries of this interesting molecule. We will also have to await the studies on recombinant repeats to see how well our epitope mapping agrees with the true epitopes.

The new antibodies fits well into the pattern of epitope studied. It is interesting that a new unique specificity, 7C12, was found.

Acknowledgements

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