Characterization of the Oligosaccharides Associated with the Human Ovarian Tumor Marker CA125

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Running Title: Sequencing of CA125 glycans
SUMMARY

CA125 is a mucin commonly employed as a diagnostic marker for epithelial ovarian cancer. Induction of humoral responses to CA125 leads to increased survival times in patients with this form of cancer, suggesting a potential role for this mucin in tumor progression. In this study, oligosaccharides linked to CA125 derived from the human ovarian tumor cell line OVCAR-3 were subjected to rigorous biophysical analysis. Sequencing of the O-glycans indicates the presence of both core type 1 and type 2 glycans. An unusual feature is the expression of branched core 1 antennae in the core type 2 glycans. CA125 is also N-glycosylated, expressing primarily high mannose and complex bisecting type N-linked glycans. High mannose type glycans include Man$_5$-Man$_9$GlcNAc$_2$. The predominant N-glycans are the biantennary, triantennary and tetraantennary bisecting type oligosaccharides. Remarkably, the N-glycosylation profiles of CA125 and envelope glycoprotein gp120 (derived from H9 lymphoblastoid cells chronically infected with HIV-1) are very similar. The CA125 associated N-glycans have also recently been implicated in crucial recognition events involved in both the innate and adaptive arms of the cell mediated immune response. CA125 may therefore induce specific immunomodulatory effects by employing its carbohydrate sequences as functional groups thereby promoting tumor progression. Immunotherapy directed against CA125 may attenuate these immunosuppressive effects, leading to the prolonged survival of patients with this extremely serious form of cancer.
INTRODUCTION

CA125 is a mucin first detected by Bast and colleagues using the monoclonal antibody OC125 (1,2). CA125 is also a significant tumor marker associated with many human cancers, but is most widely utilized for the diagnosis of epithelial ovarian cancer (3). A recent study indicates that the induction of anti-CA125 responses in ovarian cancer patients leads to their prolonged survival compared to untreated controls (4). Therefore CA125 could act as a targeting antigen to elicit antibody dependent cell mediated cytotoxicity against ovarian tumor cells. Another possibility is that CA125 plays some key physiological role that promotes tumor development in individuals with ovarian cancer. Its complete biochemical analysis is therefore crucial for defining its potential functional roles.

CA125 is an extremely complex molecule from both the proteomic and glycomic perspectives (5). Recent sequencing of its gene has yielded substantial information about the peptide backbone of this mucin (6,7). CA125 is composed of an N-terminal domain, a tandem repeat region, and a short cytoplasmic tail. The N-terminal domain of CA125 consists of 1637 amino acids. (8). The tandem repeat domain is made up of 40-60 repeats of 156 amino acids (7). The cytoplasmic tail of CA125 consists of 256 amino acids. Recently, an additional 10,431 amino acid extension of the amino terminal domain was reported (8). Thus the core protein sequence of CA125 could have a mass approaching 2.5 million Da (8).

CA125 is also highly enriched in serine and threonine residues, consistent with its recent designation as the MUC16 mucin (6). The carbohydrate content of CA125 based on its mass is estimated to be approximately 24-28% (5,9), with the majority being O-
linked glycans (7). This very high degree of glycosylation suggests that the average molecular weight of CA125 may be 3.5 million Da (8).

Complete structural analysis of the CA125 molecule also requires sequencing of its oligosaccharides and localization of its glycosylation sites. Lloyd and colleagues previously identified several O-linked glycans expressed on CA125 (9). However, the results obtained were based solely on indirect structural analysis. Moreover, this approach did not yield complete characterization of all the oligosaccharides linked to CA125.

Unambiguous sequencing of CA125-associated glycans requires the use of precise biophysical methods. We have characterized the glycans associated with CA125 using this very rigorous approach. The analysis detailed in this report reveals several notable structural features of the O-glycans associated with CA125. Surprisingly, the data also confirm robust N-glycosylation of CA125, supporting previous studies suggesting this possibility (5,10). This sequencing data, other more recent immunological findings, and the observed beneficial effects of vaccination strategies directed against CA125 suggest the distinct possibility that the carbohydrate sequences linked to this mucin may play a crucial role in promoting modulation of the immune response in patients with epithelial ovarian cancer.

EXPERIMENTAL PROCEDURES

Materials- The epithelial ovarian tumor cell line, OVCAR-3 was purchased from ATCC. Anti-CA125 monoclonal antibody, OC125, was the generous gift from Dr. Robert Bast of M.D. Anderson Hospital, Texas. Reagents for gel electrophoresis and Western blot analysis were purchased from BioRad. Fetal calf serum used for tissue culture was
obtained from Atlanta Biologicals and RPMI-1640 media was from Gibco. All other chemicals and reagents used in this study were purchased from Sigma unless otherwise stated.

Isolation of CA125 from OVCAR-3 cells- Isolation of CA125 was performed as described previously (11). Briefly, OVCAR-3 cells were cultured in 225 cm² tissue culture flasks using RPMI 1640 media containing 20% fetal calf serum till the cells were confluent. The culture media was removed and cells washed twice with serum free RPMI-1640 media that was devoid of phenol red. Cells were then cultured for 5 days in this media following which the culture media was removed and saved for extraction of CA125. Fresh serum free non-phenol red RPMI-1640 media was added to the tissue culture flasks and the process was repeated two more times.

The spent media was pooled and dialyzed through a 3,500 molecular weight cut-off membrane against water containing 0.02% sodium azide. Material retained in the dialysis bag was lyophilized and resuspended in a small volume of 10 mM ammonium bicarbonate. Total protein content of this material was determined by BCA (Pierce). 10 mg total protein in 500 µl of 10 mM ammonium bicarbonate buffer was loaded on a 1 X 45 cm Sephacryl-S500-HR size exclusion column. The column was eluted with 10 mM ammonium bicarbonate buffer and 0.8 ml fractions were collected. Fractions were monitored for absorbance at 280 nm. The two peaks obtained were pooled, lyophilized, and monitored for CA125 using Western blot analysis. CA125 isolated from different column runs was pooled and the amount of this tumor marker was determined by using a commercially available ELISA kit (Glycotech).
Electrophoretic and Western Blot Analysis of CA125- Electrophoresis of CA125 under denaturing conditions (SDS-PAGE) was routinely conducted on 5 or 7.5% separating and 3% or 5% stacking gels. Silver staining of the gels was conducted according to the suppliers (BioRad) specifications. Gels were stained with PAS reagent to detect glycoproteins. For Western blot analysis, CA125 separated on denaturing gels was transferred to nylon membranes using BioRad minigel apparatus. The nylon membranes were pre-incubated with BSA and then layered with OC125 for 1 h. A goat anti-mouse horseradish peroxidase labeled secondary antibody (Amersham) was used for detection of the CA125 bands using chemiluminescence reagents (Amersham).

To detect binding of CA125 to ConA, the mucin was transferred to nylon membranes as described above. After pre-incubation with BSA, the membranes were layered with 1 µg/ml horseradish peroxidase labeled ConA (E-Y Laboratories) in 0.1% BSA solution. After incubation for 1 h, the membrane was washed thoroughly and lectin binding to CA125 was detected by chemiluminescence.

Digestion of CA125 with PNGase F for Western blotting experiments- Lyophilized CA125 (50 µg) was dissolved in 50 mM ammonium bicarbonate (pH 8.4) containing 5 mM mercaptoethanol. 10 mU of PNGase F (EC 3.5.1.52, Roche Molecular Biochemicals) was added and the mixture was incubated overnight at 37°C. A parallel experiment was conducted under the same conditions except that PNGase F was omitted.

Reduction and carboxymethylation– Reduction and carboxymethylation was carried out as described (12). CA125 was reduced in 50 mM Tris-HCl buffer, pH 8.5, containing DTT in a 4-fold molar excess over the number of disulfide bridges. Reduction was
performed under nitrogen atmosphere at 37°C for 1 h. Carboxymethylation was carried out in iodoacetic acid (5-fold molar excess over DTT) and the reaction was allowed to proceed under a nitrogen atmosphere at 37°C for 1 h. Carboxymethylation was terminated by dialysis against 4 X 2.5 L of 50 mM ammonium bicarbonate, pH 8.5, at 4°C for 48 hours. After dialysis, the CA125 was lyophilized.

Tryptic Digestion— CA125 was incubated with trypsin (EC 3.4.21.4; Sigma) at a 50:1 ratio (w/w) in 50 mM ammonium bicarbonate, pH 8.5 for 5 h at 37°C. The digestion was terminated by placing in boiling water for 3 min, followed by lyophilization.

PNGase F digestion of tryptic glycopeptides— PNGaseF (EC 3.5.1.52, Roche Molecular Biochemicals) digestion was carried out in 50 mM ammonium bicarbonate, pH 8.5 for 16 h at 37°C with 3 U of enzyme. The reaction was terminated by lyophilization and the released N-glycans were separated from peptides and O-glycopeptides by Sep-Pak C18 (Waters Corp) as described (12).

Reductive Elimination— O-glycans were released by reductive elimination which was performed in 400 µl of sodium borohydride (1 mg/ml in 0.05 M sodium hydroxide) at 45°C for 16 hours. The reaction was terminated by dropwise addition of glacial acetic acid, followed by Dowex chromatography and borate removal (12).

Sequential Exoglycosidase Digestion— The released glycans were incubated with the following enzymes and conditions: neuraminidase (*Vibrio cholerae*, EC 3.2.1.18, Roche Molecular Biochemicals) 50 mU in 100 µl of 50 mM ammonium acetate buffer, pH 5.5; α–mannosidase (Jack Bean, EC 3.2.1.24, Glyko) 0.5 U in 100 µl of 50 mM ammonium acetate buffer, pH 4.6; β-galactosidase (bovine testes, EC 3.2.1.23, Calbiochem) 10 mU in 100 µl of 50 mM ammonium formate buffer, pH 4.6 and β-N-acetylhexosaminidase
(Jack bean, EC 3.2.1.30, Calbiochem) 0.2 U in 100 µl of 50 mM ammonium formate buffer, pH 4.6. All of the enzyme digestions were carried out at 37°C for 48 hours with a fresh aliquot of enzyme added after 24 hours. Each digestion was terminated by boiling for 3 min before lyophilization.

Endo-β-galactosidase Digestion— Endo-β-galactosidase (Escherichia freundii, EC.3.2.1.103, Calbiochem) digestion was carried out in 100 µl of 100 mM ammonium acetate, pH 5.5, 10 mU at 37°C for 48 hours with a fresh aliquot of enzyme added after 24 hours.

Periodate Cleavage— A solution of 40 mM sodium periodate in 100 mM ammonium acetate, pH 6.5, was prepared. Released and dried O-glycans were dissolved in 100 µl of this reagent. The reaction was allowed to proceed in the dark, at 0°C for 48 hours. The reaction was terminated by addition of 2-3 µl of ethylene glycol and incubation in the dark at room temperature 30 – 60 minutes. The products of the periodate cleavage were lyophilized and reduced with 200 µl of sodium borohydride (10 mg/ml) in 2 M ammonium hydroxide. The reaction was carried out at room temperature for 2 h and terminated by dropwise addition of glacial acetic acid. This sample was subjected to Dowex chromatography, borate removal, permethylation, and Sep-Pak clean-up using an acetonitrile gradient as described (12).

Methanolysis— The reagent was prepared by bubbling dry hydrochloric acid gas into methanol as described in a previous study (13). After cooling, 20 µl of this reagent was added to the permethylated sample. The reaction was performed at room temperature for 15 mins and terminated by drying under a nitrogen stream. The sample was then resuspended in methanol and an aliquot of 1 µl was removed for MALDI-TOF analysis.
The free hydroxyl groups were then deuteromethylated and subjected to linkage analysis.

**Chemical Derivatization for FAB-MS, GC-MS, MALDI-TOF and CAD MS/MS Analysis**–

Permethylation was performed using the sodium hydroxide procedure as described previously (12). Briefly, sodium hydroxide pellets were crushed with dimethyl sulfoxide to form a slurry. An aliquot of this slurry was added to dried glycans along with 1 ml of methyl iodide. The reaction was terminated by addition of water and permethylated glycans were recovered by chloroform extraction. The chloroform layer was washed several times with water to remove any impurities. Partially methylated alditol acetates were prepared from permethylated samples for GC-MS linkage analysis as described (14). Briefly, the permethylated glycans were hydrolyzed with 2 M trifluoroacetonic acid for 2 h at 121°C, reduced with 10 mg/ml sodium borodeuteride in 2 M aqueous ammonium hydroxide at room temperature for 2 h, and then acetylated with acetic anhydride at 100°C for 1 h.

**FAB-MS Analysis**– FAB mass spectra were acquired using a Fisons Instruments VG ZAB-2SE-2FPD mass spectrometer fitted with a cesium ion gun operated at 30 kV. The matrix used was monothioglycerol, and all samples were dissolved in methanol prior to loading. Data acquisition and processing was performed using VG Analytical Opus software.

**MALDI-TOF Analysis**– MALDI data were acquired using a Perseptive Biosystems Voyager-DE™ STR mass spectrometer in the Reflectron mode with delayed extraction. Permethylated sample was dissolved in 10 µl of methanol, and 1 µl of dissolved sample
was premixed with 1 µl of matrix (2,5-dihydrobenzolic acid) before loading onto a metal plate.

**GC-MS Analysis**– Linkage analysis of partially methylated alditol acetates was carried out on a Fisons Instruments MD 800 apparatus fitted with a RTX-5 fused silica capillary column (30 m x 0.32 mm internal diameter; Restek Corp.). The sample was dissolved in hexanes and injected onto the column at 65°C. The column was maintained at this temperature for 1 min and then heated to 290°C at a rate of 8°C per min.

**CAD ES-MS/MS Analysis**– CAD ES-MS/MS spectra were acquired using Q-TOF (Micromass, UK) and Q-STAR (Applied Biosystems, UK) instruments. The permethylated glycans were dissolved in methanol before loading into a spray capillary, coated with a thin layer of gold/palladium, inner diameter 2 µl (Protana, Denmark). A potential of 1.5 kV was applied to a nanoflow tip to produce a flow rate of 10-30 nl/min. The drying gas used was N₂ and the collision gas was argon, with the collision gas pressure maintained at 10⁻⁴ millibar. Collision energies varied depending on the size of the carbohydrate, typically between 30 and 90eV.

**RESULTS**

**Isolation of CA125 from OVCAR-3 cells**- An established two step isolation procedure was utilized to purify CA125 from the spent media of OVCAR-3 cells (11). In the first step, the media was dialyzed against a 3.5 kDa molecular weight cut-off membrane to remove low molecular weight impurities. The dialyzed material was lyophilized and separated on a Sephacryl-S-500-HR size exclusion column equilibrated in 10 mM ammonium bicarbonate. Fractions obtained from this column were monitored for absorbance at 260 nm and 280 nm. In a typical run, two peaks were obtained (Figure
CA125 was exclusively detected in the first peak when the fractions were analyzed by Western blot (data not shown). This peak was pooled, lyophilized, and used for further analysis. Total protein content in this sample was determined. Approximately, 2.5-3 million units of CA125 were detected per milligram of protein by ELISA assay. Similar values have been reported for highly enriched CA125 preparations isolated in other studies (6).

Electrophoretic and Western blot analyses were initially employed to determine the purity of the isolated CA125 sample. Silver stain analysis (Figure 2, panel A) indicated a broad band above the 200 kDa molecular weight marker. Some staining was also observed in the stacking gel as reported previously (9). These high molecular weight bands were easily detected by Western blot analysis using the anti-CA125 monoclonal antibody OC125 (Figure 2, panel B). Similar high molecular weight bands were also detected by PAS staining (Figure 2, panel C).

In addition to the band corresponding to CA125, some low molecular weight bands were also detected in the silver stained gel (Figure 2, panel A). Such lower molecular weight bands are always present in CA125 preparations examined in other studies (6). However, these lower molecular weight bands did not bind OC125 and were also not stained by PAS confirming that they are not glycosylated (Figure 2, panels B and C), and therefore would not interfere with oligosaccharide sequencing.

Confirmation that the high molecular weight band was CA125 was provided by proteomics analysis. ES-MS/MS analysis of an in-gel tryptic digest of this band gave data that corresponded to the peptide LTLLRPEK, which is a tandem repeat sequence that occurs 27 times in the CA125 sequence (7). In a complementary experiment,
MALDI-TOF analysis of a tryptic digest of a sample which had not been subjected to SDS-PAGE, gave molecular ions mapping to residues 2451-2479, 4637-4665, 5105-5133, 6651-6661, 6819-6847, 7287-7315, 8223-8251, 9938-9966, 10094-10122, 10862-10872, and 10406-10434 that have been shown to be present in CA125 (6). Peptides from proteins or glycoproteins other than CA125 were not detected in this analysis. The restricted PAS staining of the high molecular weight band within this preparation in combination with this proteomics data provides very strong evidence that the glycans analyzed in this study are covalently linked to CA125.

Structural Analysis Strategy- The overall structural strategy employed to characterize CA125 glycosylation is summarized in Scheme 1. Oligosaccharides were derivatized and recovered in 35% and 50% acetonitrile fractions from the C_{18} Sep-Pak cartridge prior to analysis by FAB-MS, MALDI-TOF, CAD MS/MS and linkage analysis. Data from these experiments were complemented by sequential exoglycosidase digestions, periodate oxidation and methanolysis experiments.

Analyses of PNGase F digests reveals abundant N-glycosylation of CA125– N-glycans were released from tryptic digests of reduced carboxymethylated CA125 by digestion with PNGase F. Figure 3 shows the MALDI data of the 50% acetonitrile fraction with the assignments given in Table 1. The MALDI data obtained from the 35% acetonitrile fraction (data not shown) are similar to the 50% fraction except that the signals are of lower abundance. The data indicate that CA125 is rich in N-glycans having compositions consistent with high mannose structures (Hex_{5-9}HexNAc_{2}), and complex type structures of compositions NeuAc_{0-1}Fuc_{0-2}Hex_{5-7}HexNAc_{4-7}. After α-mannosidase digestion, signals corresponding to high mannose structures disappeared and the
putative complex type structures were unaffected. Notable features of the data include: (i) complex type structures contribute to over 80% of the N-glycans with the remainder being high mannose; (ii) the most abundant complex type N-glycans have compositions consistent with mono-fucosylated bisected bi-antennary structures (m/z 2243, 2285, 2489, 2605 and 2850); (iii) the higher molecular weight components have compositions that are consistent with tri- and tetra-antennary structures; this result was confirmed by observing mass shifts after β-galactosidase digestion (data not shown); (iv) two minor components have compositions consistent with fucose being present both on the core and on one of the antennae (m/z 2664 and 2780); (v) the presence of a minor A-type fragment ion in the FAB spectrum (data not shown) at m/z 638 (FucHexHexNAc⁺) provides supportive evidence for fucosylated antennae; (vi) the level of sialylation is relatively low, with no components carrying more than a single sialic acid.

**CAD-ES-MS/MS defines sites of fucosylation** - Collisional activation nanoelectrospray tandem mass spectrometry (CAD-ES-MS/MS) of the major components observed in the MALDI experiment yielded fragment ions that allowed the assignment of the positions of fucose substitution. Data derived from Fuc₁Hex₅HexNAc₄, NeuAc₁Fuc₁Hex₅HexNAc₄ and Fuc₂Hex₅HexNAc₅ are shown in Figure 4, panels A, B and C, respectively. Assignments of key signals are given on the insets. The diagnostic signal for fucose being attached to the terminal GlcNAc of the core is m/z 474 (whereas antennae substituted with Fuc give a major fragment ion at m/z 660). The difucosylated molecule exhibits major signals at both these m/z values indicating that the majority of the glycans of this composition carry one Fuc on the core and one on the antennae. In contrast, the monofucosylated glycans show a major signal at m/z 474 and a minor
signal at m/z 660 indicating that these glycans are predominantly fucosylated on the core.

Linkage Analysis of N-glycans released by PNGase F – Linkage analysis data for the PNGase F released glycans and their desialylated counterparts are shown in Table II. These results are fully in agreement with high mannose and complex type structures being major constituents. Key features of these data are as follows: (i) the 3,4,6-linked Man confirms the presence of bisected glycans; (ii) the high abundance of 2-Man is consistent with the MALDI data which showed that biantennary structures are the most dominant N-glycans; (iii) the 3,4- and 4,6-linked GlcNAc are in accord with the CAD-ES-MS/MS data; and (iv) the presence of 3- and 6-linked Gal, which were both diminished after sialidase treatment, indicates that the sialic acids are attached at either the 3- or the 6-position of Gal.

Polylactosamine type antennae revealed by endo-β-galactosidase digestion – Digestion with endo-β-galactosidase was carried out to establish if any of the N-glycans contain polylactosamine chains whose existence should be revealed by new molecular ions in the low mass region corresponding to antennae fragments released by the enzyme. The permethylated products gave the data shown in Figure 5. The molecular ions at m/z 722, 896 and 1083 are consistent with the sequences Gal-GlcNAc-Gal, Gal-(Fuc)GlcNAc-Gal and NeuAc-Gal-GlcNAc-Gal, respectively, which are the predicted digestion of uncapped, fucosylated and sialylated polylactosamine chains, respectively. Disaccharides released from within the polylactosamine antennae give the signal at m/z 518 (GlcNAc-Gal). These data confirm the existence of polylactosamine chains. Evidence for the m/z 896 component being terminated by the Lewisx epitope (Galβ1-
4[Fucα1-3]GlcNAcβ1-), was obtained by mild methanolysis under conditions that are known to rapidly liberate fucose residues that are 3-linked to GlcNAc (15). This experiment afforded a new signal at m/z 708, concomitant with loss of m/z 896, consistent with methanolytic removal of Fuc (data not shown). Additional evidence for the Lewisx structure was provided by CAD-ES-MS/MS of m/z 896 (Figure 6) which yielded a major fragment ion at m/z 690 corresponding to removal of fucose, accompanied by loss of water, a fragmentation pathway which is diagnostic of 3-linked fucose (16). Corroborative evidence for this assignment was provided by the major signal at m/z 660 which corresponds to A-type cleavage to give [Gal(Fuc)GlcNAc+Na]+.

Sequential enzyme digestions define the cores of the polylactosaminyl glycans – To define the core structures that carry polylactosaminyl chains, N-glycans were sequentially digested with β-galactosidase, β-N-acetylhexosaminidase and endo-β-galactosidase. Under these conditions short sialylated or fucosylated antennae are unaffected, while their polylactosaminyl counterparts will be degraded by the endo-β-galactosidase. In addition, uncapped antennae with a single lacNAc backbone will be completely removed and uncapped antennae containing two lacNAc repeats will be shortened by a single lacNAc moiety. Aliquots were taken after each digestion, permethylated, and examined by MALDI-TOF after Sep-Pak purification. Partial MALDI data from the products of β-galactosidase plus β-N-acetylhexosaminidase and from the products of sequential treatment with all three enzymes are shown in Figure 7, panels A and B, respectively. Signals which show notable increases in abundance before and after endo-β-galactosidase are observed at m/z 2215 (Fuc2Hex4HexNAc4), m/z 2401 (NeuAcFucHex4HexNAc4), m/z 2460 (Fuc2Hex4HexNAc5) and m/z 2646
(NeuAcFucHex₄HexNAc₅). As indicated by the structural annotations in Figure 7, these data show that the glycans that carry the polylactosaminyl chains are core fucosylated bi- and/or tri-antennary glycans with a single short sialylated or Lewis\(^x\) antenna.

**Structural conclusions** – Taking into consideration the MALDI, FAB, linkage, CAD ES-MS/MS, methanolysis and exoglycosidase data, we conclude that the major N-glycans associated with CA125 have the structures shown in Figure 8.

**Additional evidence for expression of N-linked glycans on CA125** - Our sequencing data indicated that a substantial subset of the glycans present in CA125 include the Con A reactive high mannose and complex type biantennary N-glycans (17). To provide further evidence for the presence of such glycans on CA125, a Western blot was performed using HRP-labeled Con A lectin. Strong binding of this lectin was observed to the >200 kDa band corresponding to CA125 (data not shown). ConA-HRP did not bind to any of the low molecular weight proteins present in CA125 suggesting that the N-linked glycans detected in the FAB-MS analysis were likely not derived from any of these impurities.

**FAB and MALDI screening of CA125 O-glycans** - Permethylated O-glycans were analyzed by FAB and MALDI after Sep-Pak purification. The annotated MALDI spectrum of the 50% acetonitrile fraction is shown in Figure 9. The structures shown by the annotations are based on the combined data of the analyses described below. All assignments were corroborated by observing mass shifts after deuteromethylation (data not shown). From these data we conclude that (i) O-glycans containing up to at least fourteen sugar residues are present; (ii) the majority of O-glycans are sialylated and/or fucosylated and carry a maximum of two fucoses or two sialic acids or two fucoses and
two sialic acids; (iii) two major families are present with the first family being mono- and di-sialylated core type 1 (Galβ1-3GalNAc), while the members of the second family have compositions corresponding to core type 2 (Galβ1-3(GlcNAcβ1-6)GalNAc) with non-, mono- and di-fucosylation as well as sialylation; and (iv) an A-type fragment ion at m/z 638 (FucHexHexNAc+) in the FAB mass spectrum (data not shown) indicated the presence of the Lewis^x epitope in the O-glycans as well as the N-glycans (see above).

**Linkage analysis of the O-glycans** - The data for the linkage analysis are given in Table III. Notable features include: (i) both 3- and 3,6-linked GalNAcitol are observed consistent with the presence of core type 1 and 2 structures, respectively; (ii) the detection of 3-Gal and 6-Gal suggests that sialic acids are attached at both 3-linked and 6-linked position of galactose; and (iii) 3,4-GlcNAc and 2-Gal suggest the probable presence of Lewis^x and blood group H and/or Lewis^y respectively.

**Characterization of O-glycan structures by CAD ES-MS/MS Analyses**– Each of the components observed in the MALDI experiment were subjected to CAD-ES-MS/MS in order to assign sequences. Good quality fragment ion data were obtained on components up to 2000 Da allowing the sequence assignments shown in Figure 9. Corroborative data were obtained from similar analysis of a sample which was reductively eliminated using sodium borodeuteride instead of sodium borohydride which shifted the mass of each molecular ion by one mass unit. Representative data from three components of special interest are discussed below.

CAD MS/MS analysis of deutero-reduced Fuc1Hex3HexNAc2HexNAcitol (m/z 815^2+, Figure 10, panel A) unambiguously defines the presence of the Lewis^x epitope on the core 1 antenna of a core 2 structure (see assignments on inset). In contrast, the
difucosylated counterpart of this component (which gives the signal at m/z 1780 in the MALDI spectrum shown in Figure 9) yielded a complex set of fragment ions consistent with the presence of variously fucosylated antennae (data not shown) suggesting that the difucosylated component is a mixture of glycans carrying Lewis\(^y\) and blood group H antennae as well as Lewis\(^x\) antennae. The low abundance of m/z 1780 precluded further characterization. Finally, the CAD MS/MS spectrum of reduced Hex\(_4\)HexNAc\(_3\)HexNAcitol (m/z 953\(^{2+}\), Figure 10, panel B) confirms that extended core 1 antennae are a feature of CA125 O-glycans (see assignments on inset). Furthermore, the data also suggest that the core 1 arm is branched because the major fragment ion for terminal lacNac (m/z 486) is not accompanied by an ion at m/z 935 which would be predicted to occur if the two lacNac moieties were in a tandem repeat. To further investigate this unexpected finding the O-glycan sample was subjected to periodate degradation (see below).

*Periodate Cleavage Confirms Core Types and Antennae Backbones of O-glycans*—Reductively eliminated O-glycans were subjected to periodate oxidation, followed by reduction, permethylation, Sep-Pak clean up, and MALDI-TOF analysis. Under the relatively rigorous conditions used, a majority of the linear structures carrying vicinal hydroxyls (such as GalNAcitol) are cleaved. In addition, partial cleavage and subsequent hydrolytic loss of more resistant structures, such as terminal Fuc and Gal, also occurred. The partial MALDI spectrum of the 35% acetonitrile fraction (Figure 11) showed molecular ions corresponding to the two sets of products expected from cleavage of the GalNAcitol: one containing the C-1 to C-4 carbons (denoted C-4) attached to the 3-linked antenna, and the other containing the C-5 and C-6 carbons.
(denoted C-2) attached to the 6-linked antenna. Thus sequences containing the C-2 moiety represent the 6-arms of core type 2 components, whilst sequences containing the C-4 moiety correspond to the 3-arms of core types 1 and 2. The assignments are given in Table IV. Corroborative evidence for these assignments was provided by CAD-ES-MS/MS experiments. Representative data from \( m/z \) 807 (HexHexNAc\(_2\)-C2), \( m/z \) 835 (NeuAcHexHexNAc-C2), and \( m/z \) 936 (HexHexNAc\(_2\)-C4), are given in Figure 12 (see insets for assignments). From these spectra it is evident that core type 2 structures are abundant in CA125 but, as indicated by the experiments described earlier, many of these are extended on the 3-arm rather than the 6-arm. Importantly the data for the \( m/z \) 936 component (Figure 12, panel C) confirm that the core 1 arm is branched in a significant portion of the larger O-glycans.

**Structural conclusions** – Taking into consideration the MALDI, FAB, linkage, CAD ES-MS/MS and periodate data, we conclude that the major O-glycans found in CA125 have the structures shown in the annotations of Figure 9.

**DISCUSSION**

Analysis of the structure of CA125 is essential to determine the physiological role of this very significant tumor antigen. However, its very high molecular weight and mucinous nature pose major obstacles for performing structural characterization studies. Here we report the sequence of the glycans linked to CA125. To the best of our knowledge, this study outlines the first exhaustive analysis of CA125 glycans derived from epithelial ovarian tumor cells.

Lloyd and colleagues previously employed indirect approaches for characterizing the oligosaccharides associated with CA125 derived from OVCAR-3 cells (9). Due to the
relatively insensitive methods of detection that were employed, only a limited number of oligosaccharides were analyzed. We have circumvented these problems by using very sensitive and precise biophysical methods of carbohydrate structural analysis. This approach has provided unambiguous sequencing of the oligosaccharides linked to CA125.

Core 1 and core 2 type glycans are the major O-linked glycans expressed on CA125 based on the present results. Minor amounts of core type 2 glycans with up to two polylactosamine units were also detected. A small subset of the O-linked glycans are also decorated with Lewis\(^x\) antigens on their terminal ends. O-linked glycans carrying two fucose residues were also detected. However, since these difucosylated glycans were present in very low amounts, it was not possible to determine if these O-linked glycans carried two Lewis\(^x\) epitopes or one Lewis\(^y\) antigen or two blood group H epitopes. Lloyd and coworkers also reported the presence of both Lewis\(^x\) and Lewis\(^y\) antigens on CA125, but suggested that they were major components (9). This discrepancy cannot be readily explained, because CA125 analyzed in both studies was isolated from the OVCAR-3 cells using identical tissue culture conditions. However, more definitive methods of structural analysis were employed in the current study.

An unusual feature associated with CA125 O-linked glycans is the expression of branched core 1 structures. Only uromodulin, a pregnancy associated isoform of Tamm-Horsfall glycoprotein expresses this sequence (18). Like uromodulin, these branched core 1 structures linked to CA125 carry sialic acid and fucose. However, the branches of CA125 O-glycans appear to be either sialylated or fucosylated, in contrast to uromodulin which carries abundant sialyl Lewis\(^x\) moieties. The functional roles of these
unusual branched core 1 antenna in the core type 2 O-linked glycans have not been clearly established.

Conclusive evidence for the N-glycosylation of CA125 was also presented in the current study. These results are consistent with the existing literature. Previous compositional analyses indicate that CA125 from either OVCAR-3 cells or amniotic fluid contain mannose, albeit in much lower amounts than Gal, GlcNAc or GalNAc (9,19). However, significantly higher amounts of mannose have been reported to be associated with CA125 isolated from the ovarian tumor cell line OVCA 433 (5).

Clinical CA125 samples exhibit reduced binding to OC125 after treatment with PNGase F (10), an enzyme that specifically clips N-glycans. Treatment of CA125 with PNGase F resulted in a significant reduction in the molecular mass of this mucin and also a lower affinity for OC125 as detected by Western blot analysis (data not shown). The reduction in binding of OC125 to CA125 cannot be readily explained since this antibody recognizes a peptide epitope within the CA125 molecule (7). However, these observations clearly provide supportive evidence for the presence of N-linked glycans on CA125.

CA125 isolated from tumor tissues and the sera of ovarian cancer patients is also specifically retained during affinity chromatography on Concanavalin A-agarose in a carbohydrate dependent manner, consistent with the association of high mannose and biantennary complex N-linked glycans with this mucin (10). A prior study by Nagata and coworkers confirms that CA125 isolated from serum and ascites fluid of ovarian cancer patients also binds to E-PHA (10), consistent with the presence of biantennary and...
triantennary bisecting type glycans. Therefore both biophysical and lectin binding studies are consistent with these earlier findings.

A very recent study indicates that vaccination with the murine monoclonal anti-idiotypic antibody ACA125 elicits an immune response to CA125 in a subset of patients. Ovarian cancer patients that mediate potent anti-CA125 response show an increase in their survival time from $5.3 \pm 4.3$ months to $19.9 \pm 13.1$ months (4,20). This result suggests that CA125 could play a crucial role as a surface tumor antigen that facilitates antibody dependent cell mediated cytotoxicity.

Another distinct possibility is that CA125 could promote tumor progression by modulating the human immune response, perhaps by using its carbohydrate sequences as functional groups. The N-linked glycans associated with CA125 fall into two major classes: bisecting complex type and high mannose type oligosaccharides.

Recent data indicates that the specific cell surface lectin DC-SIGN interacts with ICAM-3 to enable screening of the MHC-peptide complexes required for subsequent formation of the immunological synapse (Reviewed in 21,22). DC-SIGN also acts as a novel HIV-1 attachment receptor that facilitates infection of T lymphocytes (23,24). More recent data indicates that DC-SIGN specifically recognizes both high mannose type N-glycans and Lewis$^y$/Lewis$^x$ terminated glycans (25,26). It is possible that CA125 could interfere with immunological synapse formation by binding to DC-SIGN via its high mannose type N-glycans. This possibility is under investigation.

Tumor survival and expansion likely rely upon overcoming potential cell mediated immune responses. It is well established that tumor cells often downregulate MHC class I molecules during the later stages of tumor development (27,28, Reviewed in 29). This
loss is thought to enable tumor cells to escape antigen driven lytic responses by class I restricted cytotoxic T lymphocytes, thereby allowing them to evade this adaptive arm of the immune cell mediated response. Epithelial ovarian tumor cells often lose their MHC class I molecules (30,31). However, this evasion should then make the tumor cells more susceptible to NK cells, which detect and kill MHC class I negative tumor cells (Reviewed in 32).

Bisecting type N-glycans are found on a great variety of different glycoproteins in the human. They are major sequences associated with human immunoglobulins, MHC class I molecules and glycophorin, a major erythrocyte membrane glycoprotein (33-35). Bisecting type glycans are also prominently expressed on human gametes (36) and neurons (37).

Bisecting type N-glycans may play a role in inducing suppression of NK cell mediated cytolytic responses when presented on cell surfaces. Electroinsertion of glycophorin, a glycoprotein expressing primarily bisecting type glycans, into the plasma membrane of K562 cells decreases their cytolysis by human NK cells (38). Similar insertion of glycophorin lacking its N-glycans does not mediate this protective effect (38). In addition, soluble forms of glycophorin also do not inhibit NK cell mediated responses in vivo.

K562 cells overexpressing N-acetylglucosaminyltransferase III, the enzyme that synthesizes the bisecting GlcNAc sequence, also exhibit greatly decreased sensitivity to NK cell mediated lysis (39). Increased expression of N-acetylglucosaminyltransferase III is also positively correlated with the increased metastatic potential of tumor cells (40,41).
An attractive hypothesis is that epithelial ovarian tumor cells downregulate their MHC class I molecules and upregulate CA125 expression on their cell surfaces in the late stages of ovarian cancer to escape responses mediated both by cytotoxic T lymphocytes and NK cells. CA125 or other glycoprotein secreted by ovarian tumors may not suppress NK cell mediated responses. However, transient expression of CA125 on the surfaces of ovarian tumor cells may enable them to evade NK cell mediated responses. This reasoning could explain why CA125 expression increases during tumor progression. This linkage is currently under investigation.

This type of subterfuge could also be linked to the reproductive imperative. NK cells constitute 70-80% of the maternal immune effector cells in the uterus during pregnancy (42). Localization studies indicate that CA125 is expressed in significant amounts by the human decidua and the amnion (43-45). Serum CA125 levels of women in their first trimester of pregnancy are elevated (43,45). It is therefore possible that similar to its potential tumor promoting activities, CA125 may also play a role in protecting the human embryo from the maternal immune response.

Human germ cells downregulate class I expression during their development, yielding gametes that lack MHC class I molecules (46,47). Human gametes also express substantial amounts of bisecting type glycans on their surfaces based on lectin binding studies (36). Therefore tumor cells may be employing the same strategy that gametes do to avoid potential immune responses.

In addition, several glycoconjugates expressed in the human male and female reproductive system or in other organs under the influence of pregnancy related hormones have been shown to suppress specific immune responses in vitro (Reviewed...
in 48). Many of the carbohydrate sequences linked to these glycoconjugates are also prevalently expressed on persistent human pathogens such as HIV-1, helminthic parasites, *Helicobacter pylori*, other human tumor cells (48). A hypothetical model linking pathological subterfuge to the suppression of immune responses essential to fulfill the reproductive imperative has been proposed (48,49).

The expression of many of the same carbohydrate sequences (Lewis\(^x\) sequences, bisecting type N-glycans, high mannose type N-glycans) previously implicated in immune suppression and reproduction, on CA125 is entirely consistent with this model. Another rather remarkable correlation in this respect is that the N-glycans associated with CA125 closely resemble the oligosaccharides attached to the viral coat glycoprotein gp120 synthesized in the HIV infected human T lymphoblastoid cell line H9 (50). That both CA125 and gp120 carry similar N-glycans may not be purely coincidental. Each could employ their oligosaccharides as functional groups to induce suppression of both innate and adaptive arms of the human immune response. By using this type of subterfuge, tumor cells and HIV-1 could in effect couple their survival to the human reproductive imperative.

In conclusion, we have characterized the oligosaccharides associated with CA125 derived from OVCAR-3 tumor cells. The results of lectin and antibody binding data suggest that similar carbohydrate sequences are expressed on CA125 from ovarian cancer patients. This data, together with the genomic and proteomic analysis reported earlier, will provide a solid basis for understanding the physiological role of CA125, defining new methods for detecting this tumor marker, and developing novel strategies that effectively combat epithelial ovarian cancer.
REFERENCES


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The abbreviations used include: CAD, collisionally activated decomposition; ConA, concanavalin A; DC-SIGN, dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin; DTT, dithiothreitol; E-PHA, erythroagglutinating phytohemagglutinin; ES, electrospray; ESI, electrospray ionization; FAB-MS, fast atom bombardment mass spectrometry; Fuc, fucose; Gal, galactose; GalNAc, N-acetylgalactosamine; GlcNAc, N-acetylglucosamine; GC, gas chromatography; Hex, hexose; HexNAc, N-acetylhexosamine; HRP, horseradish peroxidase; LacNAc, N-acetyllactosamine; MALDI-TOF, matrix assisted laser desorption ionization-time of flight mass spectrometry; Man, mannose; MS, mass spectrometry; NeuAc, N-acetylneuraminic acid; NK, natural killer; NP-40, nonidet P-40; PAS, periodic acid-Schiff's; PBS, phosphate buffered saline; PNGase F, peptide N-glycosidase F; and Q-TOF, quadrupole-orthogonal acceleration time of flight mass spectrometry.
FIGURE LEGENDS

FIGURE 1. Gel filtration separation of CA125. Dialyzed OVCAR-3 media was loaded on a column (1 X 45 cm) of Sephacryl S-500-HR and eluted with 10 mM ammonium bicarbonate buffer (pH 8.0). Fractions were monitored for absorbance at 280 nm (closed circles) and 260 nm (closed squares). CA125 containing fractions were identified by Western Blot analysis. The peak containing CA125 is indicated.

FIGURE 2. Electrophoretic and Western blot analysis of CA125. Purified CA125 (5-10 µg total protein) was separated by electrophoresis. The gels were stained with silver reagent (A) or with PAS reagent (B). Western blotting was performed using OC125 as the primary antibody (C). Molecular weights of markers are indicated for each panel. A single headed arrow indicates the interface between stacking and separating gel. The double-headed arrow indicates migration of the dye front.

FIGURE 3. MALDI-TOF mass spectrum of CA125 N-glycans. Glycans were released by PNGase F, permethylated and subjected to Sep-Pak clean-up. Data from the 50% acetonitrile fraction are shown. The signals are assigned in Table I.

FIGURE 4. CAD MS/MS spectra of the [M + 2Na]^{2+} molecular ions of (A) Fuc1Hex5HexNAc4, (B) NeuAc1Fuc1Hex5HexNAc4, (C) Fuc2Hex5HexNAc5. The major component in each case is shown in the cartoon. Additionally (A) and (B) contain a minor component which lacks the fucose on the core and instead carries a fucose on a lacNAc antenna, giving the fragment ion at m/z 660.

FIGURE 5. Partial FAB mass spectrum of products of endo-β-galactosidase digestion. Glycans were released from CA125 by PNGase F, digested with endo-β-
galactosidase, permethylated and subjected to Sep-Pak clean-up. Data from the 35% acetonitrile fraction are shown. The cartoons represent products of digestion. Non-annotated signals were present in samples which had not been treated with endo- β -galactosidase.

FIGURE 6. CAD MS/MS spectrum of the [M + Na]⁺ molecular ion of the m/z 896 product of endo- β -galactosidase digestion. Assignments are given on the cartoon structure.

FIGURE 7. MALDI-TOF mass spectra of glycosidase digests of CA125 N-glycans. Glycans were released by PNGase F, digested sequentially with β -galactosidase, β -N-acetylhexasaminidase and endo- β -galactosidase, permethylated and subjected to Sep-Pak clean-up. Data from the 50% acetonitrile fractions of (A) β -galactosidase plus β -N-acetylhexasaminidase, and (B) β -galactosidase plus β -N-acetylhexasaminidase plus endo- β -galactosidase are shown. Signals not present prior to digestion are annotated with cartoons.

FIGURE 8. Proposed structures of the major N-glycans present in CA125. As noted in the text and the legend to Fig 3, a minor portion of the mono-fucosylated glycans carry fucose on an antenna rather than the core. The polylactosamine-containing structures shown in the final cartoon were not rigorously characterized with respect to chain length. In addition the sialic acid and fucose residues are mutually exclusive.

FIGURE 9. MALDI-TOF mass spectrum of CA125 O-glycans. Glycans were released by reductive elimination, permethylated and subjected to Sep-Pak clean-up. Data from the 35% acetonitrile fraction are shown in the upper two panels and high mass data from the 50% acetonitrile fraction in the bottom panel. The structural assignments are derived from the combined data described in the text. For clarity, the cartoons show major components. The CAD-MS/MS and periodate experiments indicate that a minor portion of the components which are annotated with branched core 1 glycans are
unbranched and have linear oligolactosamine core 1 antennae. Compositions are given for ions of masses greater than m/z 2800 because the sequences of these glycans have not been determined.  ■, 3-GalNAcitol; ▲, 3,6-GalNAcitol; ●, terminal-Gal; ○, 3-Gal; □, 3,6-Gal; △, 4-GlcNAc; ◊, NeuAc; △, Fuc. NeuAc is attached to either 3- or 6-positions of Gal.

FIGURE 10. CAD MS/MS spectra of the [M + 2Na]^{2+} molecular ions of (A) Fuc_{1}Hex_{3}HexNAc_{2}HexNAcitol, (B) Hex_{4}HexNAc_{3}HexNAcitol. Sequence-informative fragment ions are shown on the structures.

FIGURE 11. Partial MALDI-TOF mass spectrum of periodate oxidized CA125 O-glycans. Glycans were released by reductive elimination, treated with periodate, permethylated and subjected to Sep-Pak clean-up. Data from the 35% acetonitrile fraction is shown. See Table IV for assignments.

FIGURE 12. CAD MS/MS spectra of the [M + Na]^{+} molecular ions at (A) m/z 807, (B) m/z 835, and (C) m/z 936. Sequence-informative fragment ions are shown on the structures. The non-reducing HexNAc residues are a result of periodate oxidation and subsequent hydrolytic loss of terminal Gal and Fuc residues in the original glycans.
TABLE I

Assignments of molecular ([M+Na\(^+\)]) ions observed in MALDI spectrum of permethylated N-glycans derived from the 50% acetonitrile fraction of CA125

<table>
<thead>
<tr>
<th>Signal (m/z)</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1579</td>
<td>Hex(_5)HexNAc(_2)</td>
</tr>
<tr>
<td>1783</td>
<td>Hex(_6)HexNAc(_2)</td>
</tr>
<tr>
<td>1987</td>
<td>Hex(_7)HexNAc(_2)</td>
</tr>
<tr>
<td>2069</td>
<td>Hex(_5)HexNAc(_4)</td>
</tr>
<tr>
<td>2191</td>
<td>Hex(_8)HexNAc(_2)</td>
</tr>
<tr>
<td>2243</td>
<td>FucHex(_5)HexNAc(_4)</td>
</tr>
<tr>
<td>2285</td>
<td>FucHex(_4)HexNAc(_5)</td>
</tr>
<tr>
<td>2315</td>
<td>Hex(_5)HexNAc(_5)</td>
</tr>
<tr>
<td>2489</td>
<td>FucHex(_5)HexNAc(_5)</td>
</tr>
<tr>
<td>2605</td>
<td>NeuAcFucHex(_5)HexNAc(_4)</td>
</tr>
<tr>
<td>2664</td>
<td>Fuc(_2)Hex(_5)HexNAc(_5)</td>
</tr>
<tr>
<td>2694</td>
<td>FucHex(_6)HexNAc(_5)</td>
</tr>
<tr>
<td>2780</td>
<td>NeuAcFuc(_2)Hex(_5)HexNAc(_4)</td>
</tr>
<tr>
<td>2850</td>
<td>NeuAcFucHex(_5)HexNAc(_5)</td>
</tr>
<tr>
<td>2938</td>
<td>FucHex(_6)HexNAc(_6)</td>
</tr>
<tr>
<td>3054</td>
<td>NeuAcFucHex(_6)HexNAc(_5)</td>
</tr>
<tr>
<td>3143</td>
<td>FucHex(_7)HexNAc(_6)</td>
</tr>
<tr>
<td>3300</td>
<td>NeuAcFucHex(_6)HexNAc(_6)</td>
</tr>
<tr>
<td>3388</td>
<td>FucHex(_7)HexNAc(_7)</td>
</tr>
<tr>
<td>3503</td>
<td>NeuAcFucHex(_7)HexNAc(_6)</td>
</tr>
</tbody>
</table>
TABLE II

**GC-MS analysis of partially methylated alditol acetates obtained from the PNGase F released N-glycans of CA125**

The 50% acetonitrile fraction after Sep-Pak purification of permethylated glycans was hydrolyzed, reduced, acetylated, and analyzed by GC-MS.

<table>
<thead>
<tr>
<th>Elution time (min)</th>
<th>Characteristic fragment ions</th>
<th>Assignment</th>
<th>Relative abundance</th>
</tr>
</thead>
<tbody>
<tr>
<td>17.12</td>
<td>102, 115, 118, 131, 162, 175</td>
<td>Terminal fucose</td>
<td>0.54</td>
</tr>
<tr>
<td>18.73</td>
<td>102, 118, 129, 145, 161, 205</td>
<td>Terminal mannose</td>
<td>0.47</td>
</tr>
<tr>
<td>19.03</td>
<td>102, 118, 129, 145, 161, 205</td>
<td>Terminal galactose</td>
<td>0.62</td>
</tr>
<tr>
<td>19.97</td>
<td>129, 130, 161, 190</td>
<td>2-linked mannose</td>
<td>1.00</td>
</tr>
<tr>
<td>20.15</td>
<td>118, 233</td>
<td>4-linked glucose</td>
<td>0.39</td>
</tr>
<tr>
<td>20.27</td>
<td>118, 129, 161, 234</td>
<td>3-linked galactose</td>
<td>0.35</td>
</tr>
<tr>
<td>20.84</td>
<td>99, 102, 118, 129, 162, 189, 233</td>
<td>6-linked galactose</td>
<td>0.47</td>
</tr>
<tr>
<td>21.22</td>
<td>130, 190, 233</td>
<td>2,4-linked mannose</td>
<td>0.26</td>
</tr>
<tr>
<td>21.65</td>
<td>129, 130, 189, 190</td>
<td>2,6-linked mannose</td>
<td>0.44</td>
</tr>
<tr>
<td>21.82</td>
<td>118, 129, 189, 234</td>
<td>3,6-linked mannose</td>
<td>0.61</td>
</tr>
<tr>
<td>22.29</td>
<td>118, 333</td>
<td>3,4,6-linked mannose</td>
<td>0.23</td>
</tr>
<tr>
<td>22.89</td>
<td>117, 143, 145, 159, 203, 205</td>
<td>Terminal GlcNAc</td>
<td>0.08</td>
</tr>
<tr>
<td>23.80</td>
<td>117, 159, 233</td>
<td>4-linked GlcNAc</td>
<td>0.71</td>
</tr>
<tr>
<td>24.72</td>
<td>117, 159, 346</td>
<td>3,4-linked GlcNAc</td>
<td>0.10</td>
</tr>
<tr>
<td>25.20</td>
<td>117, 159, 261</td>
<td>4,6-linked GlcNAc</td>
<td>0.15</td>
</tr>
</tbody>
</table>

*a* Increased abundance after treatment of N-glycans with *V.cholerae* sialidase

*b* Not observed after treatment of N-glycans with *V.cholerae* sialidase
TABLE III

**GC-MS analysis of partially methylated alditol acetates obtained from the reductively eliminated O-glycans of CA125**

The 50% acetonitrile fraction after Sep-Pak purification of permethylated glycans were hydrolyzed, reduced, acetylated, and analyzed by GC-MS

<table>
<thead>
<tr>
<th>Elution time (mins)</th>
<th>Characteristic fragment ions</th>
<th>Assignment</th>
<th>Relative abundance</th>
</tr>
</thead>
<tbody>
<tr>
<td>17.17</td>
<td>102, 115, 118, 131, 162, 175</td>
<td>Terminal fucose</td>
<td>0.01</td>
</tr>
<tr>
<td>18.72</td>
<td>102, 118, 129, 145, 161, 205</td>
<td>Terminal glucose</td>
<td>0.06</td>
</tr>
<tr>
<td>19.05</td>
<td>102, 118, 129, 145, 161, 205</td>
<td>Terminal galactose</td>
<td>1.00</td>
</tr>
<tr>
<td>20.28</td>
<td>129, 130, 161, 190</td>
<td>2-linked galactose</td>
<td>0.02&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>20.28</td>
<td>101, 118, 129, 161, 234</td>
<td>3-linked galactose</td>
<td>0.02&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>20.87</td>
<td>99, 102, 118, 129, 162, 189, 233</td>
<td>6-linked galactose</td>
<td>0.01</td>
</tr>
<tr>
<td>21.45</td>
<td>88, 130, 246, 290</td>
<td>3-linked GalNAcitol</td>
<td>0.07</td>
</tr>
<tr>
<td>22.08</td>
<td>118, 129, 189, 234</td>
<td>3,6-linked galactose</td>
<td>tr&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>23.40</td>
<td>88, 101, 130, 246, 318</td>
<td>3,6-linked GalNAcitol</td>
<td>0.05</td>
</tr>
<tr>
<td>23.85</td>
<td>117, 159, 233</td>
<td>4-linked GlcNAc</td>
<td>0.10</td>
</tr>
<tr>
<td>24.75</td>
<td>117, 159, 346</td>
<td>3,4-linked GlcNAc</td>
<td>0.01</td>
</tr>
</tbody>
</table>

<sup>a</sup> 3-Gal and 2-Gal are co-eluting therefore they have the same relative abundance

<sup>b</sup> tr = trace amount
TABLE IV

Assignments of molecular ([M+Na⁺]) ions observed in the MALDI spectrum for the products of O-glycans after periodate oxidation, reduction and permethylation

The denoted C4 contains C1 to C4 carbons attached to the 3-linked antenna whilst the denoted C2 contains C5 and C6 carbons attached to the 6-linked antenna. NeuAc residues lack C8 and C9 as a result of periodate cleavage.

<table>
<thead>
<tr>
<th>Signal (m/z)</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>562</td>
<td>HexHexNAc-C2</td>
</tr>
<tr>
<td>691</td>
<td>HexHexNAc-C4</td>
</tr>
<tr>
<td>719</td>
<td>NeuAcHex-C4</td>
</tr>
<tr>
<td>807</td>
<td>HexHexNAc₂-C2</td>
</tr>
<tr>
<td>835</td>
<td>NeuAcHexHexNAc-C2</td>
</tr>
<tr>
<td>895</td>
<td>Hex₂HexNAc-C4</td>
</tr>
<tr>
<td>936</td>
<td>HexHexNAc₂-C4</td>
</tr>
<tr>
<td>1140</td>
<td>Hex₂HexNAc₂-C4</td>
</tr>
<tr>
<td>1168</td>
<td>NeuAcHex₂HexNAc-C4</td>
</tr>
<tr>
<td>1385</td>
<td>Hex₂HexNAc₃-C4</td>
</tr>
</tbody>
</table>
CA125

↓

Reduction & carboxymethylation

↓

Trypsin digestion

↓

PNGase F Digestion / Sep-Pak Fractionation

Revised N-glycans

↓

Exoglycosidase & Endoglycosidase digestions

Permethylation

↓

ESI MS/MS Analysis

↓

FAB-MS & MALDI-TOF

↓

Linkage Analysis

↓

Permethylation

↓

FAB-MS & MALDI-TOF

↓

Linkage Analysis

Peptides and O-glycopeptides

↓

Reductive elimination

↓

Permethylation

↓

ESI MS/MS Analysis

↓

FAB-MS & MALDI-TOF

↓

Periodate oxidation & Reduction

↓

Permethylation

↓

FAB-MS & MALDI-TOF

↓

ESI MS/MS Analysis

SCHEME 1. Summary of overall experimental strategy employed to characterize CA125
Figure 1

[Graph showing the fraction number (1 ML) on the x-axis and O.D. on the y-axis. The graph includes two curves labeled CA 125.]
Figure 2

A

B

C

200 kDa
116 kDa
97.4 kDa
66.2 kDa
45 kDa
35.8 kDa

200 kDa
116 kDa
97.4 kDa
66.2 kDa
45 kDa
35.8 kDa

200 kDa
119 kDa
90 kDa
49 kDa
36 kDa
29 kDa
Figure 4A

- Man  - Gal  - GlcNAc  - Fuc
Figure 4C

- Man
- Gal
- GlcNAc
- Fuc

Mass spectrum and structural diagram showing various molecular weights and charges.
Figure 6

- Gal  - GlcNAc  - Fuc
Figure 7

A

B

-NeuAc •-Gal △-Fuc ○-Man □-GlcNAc
Figure 8

m/z 1579

m/z 1783

m/z 1987

m/z 2191

m/z 2069

m/z 2243

m/z 2285

m/z 2315

m/z 2489

m/z 2605

m/z 2664

-NeuAc  -Gal  -Fuc  -Man  -GlcNAc

m/z 2694

m/z 2780

m/z 2850

m/z 2938

m/z 3054

m/z 3143

m/z 3300

m/z 3388

m/z 3503

Downloaded from http://www.jbc.org/ on November 13, 2017
Figure 9

Mass (m/z)

Intensity

457.0 697.6 856.2 1096.8 1299.4 1600.0

100 80 60 40 20 0 10

534 747 895 983 1157 1345 1433

1518 1606 1677 1705 1780 1967 2056 2155 2244 2331

2417 2504 2604 2692 2866 2953 3055 3143 3318 3403
Figure 10B
Figure 11

[Graph showing mass spectra with peaks at various m/z values]
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