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Characterization of cancer associated mucin type O-glycans using the exchange sialylation properties of mammalian sialyltransferase ST3Gal-II

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Abstract

Our previous studies suggest that the α2,3sialylated T-antigen (NeuAcα2,3Galβ1,3GalNac-) and associated glycan structures are likely to be elevated during cancer. An easy and reliable strategy to label mucinous glycans that contain such carbohydrates can enable the identification of novel glycoproteins that are cancer associated. To this end, the present study demonstrates that the exchange sialylation property of mammalian ST3Gal-II can facilitate the labeling of mucin glycoproteins in cancer cells, tumor specimens and glycoproteins in cancer sera. Results show that: i) the radiolabeled mucin glycoproteins of each of the cancer cell lines studied (T47D, MCF7, LS180, LNCaP, $SKOV_3$, HL60, DU4475 and $HepG_2$) is distinct either in terms of the specific glycans presented or their relative distribution. While some cell lines like T47D had only one single sialylated O-glycan, other like LS180 and DU4475 contained a complex mixture of mucinous carbohydrates. ii) [^{14}C]sialyl labeling of primary tumor cells identified a 25–35 kDa mucin glycoprotein unique to pancreatic tumor. Labeled glycoproteins for other cancers had higher molecular weight. iii) Studies of [^{14}C] sialylated human sera showed larger mucin glycopeptides and 2fold larger mucin-type chains in human serum compared to [^{14}C]sialyl labeled glycans of fetuin. Overall, the exchange sialylation property of ST3Gal-II provides an efficient avenue to identify mucinous proteins for applications in glycoproteomics and cancer research.

Keywords

Cancer-glycoproteins; 3′sialyl T-hapten; sialylation; radiosialyl tagging; lectin affinity binding; Tn epitope clustering

Cancer causes changes in a number of cellular processes that promote proliferation and tumorigenesis. Among these, cancer dramatically alters the cellular glycosylation machinery[1]. This results in an altered pattern of cell surface glycan expression during disease. Tumor associated antigens are also commonly found as sialylated mucin-type glycans[2]. In this regard, mucins are high molecular weight glycoproteins containing varying numbers of serine and threonine-rich tandem repeat regions. The O-glycan chains associated with these Ser/Thr units of mucins constitute 50–80% of its mass[3]. These glycans confer

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characteristic biochemical and biophysical properties to the mucins[4,5]. The number of tandem repeats have an effect on O-glycosylation and this may be associated with increased susceptibility to certain human diseases[6–12]. For example, the disialyl core NeuAcα2,3Galβ1,3(NeuAcα2,6)GalNAcα-O-Thr of a mucin type glycoprotein is a prototypic platelet aggregating factor on cancer cells that influences tumor metastasis[13].

The identification of proteins bearing mucin-type O-linked glycans remains elusive due to the complex nature of their biosynthesis and the lack of tools[14] comparable to those used to study N-linked glycosylation[15–18]. The large molecular size of mucins and the diversity of their carbohydrate structures on such molecules also contribute to difficulties in investigating the molecular features of mucins. These limitations highlight the importance of developing newer approaches for the proteomic analysis of mucin-type O-linked glycans. During the synthesis of O-glycans, the first step involves the attachment of GalNAc in α-linkage to hydroxyl groups of Ser/Thr by a family of ppGalNActransferases. This enzyme family has ~20 members, that display distinct substrate specificities and tissue distibutions[19, 20]. Since O-glycosylation is a post-protein folding event, only Ser/Thr residues that are exposed on the protein surface such as coil, turn and linker regions are expected to be glycosylated. The exact nature of the O-glycan formed is also a result of additional biosynthetic pathways and enzymes localized in the Golgi, especially glycosyltransferases and sulfotransferases[21, 22]. Among the glycosyltransferases, we have determined a dominant activity for α2,3 sialyltransferase towards Galβ1,3GalNAcα in various tumor tissues and cancer cell lines[23, 24]. Further α1,2-L-fucosyltransferase and Gal: 3-0-sulfotransferase acting on Galβ1,3GalNAcα are not widely expressed by cancer cells[23–25].

Since NeuAcα2,3Galβ1,3GalNAcα type structures could be dominant structures associated with cancerous mucins, it would be attractive to develop methods to identify such entities. In this context, we report here that the exchange sialylation properties of the mammalian sialyltransferase ST3Gal-II can aid the identification of such glycans. In previous studies, we have demonstrated that this enzyme in addition to its direct sialylation activity towards Galβ1,3GalNAcα[26] can also catalyze the formation of CMP-NeuAc from 5′CMP in presence of a donor containing the NeuAcα2,3Galβ1,3GalNAc-unit[27]. This reaction, which proceeds in a direction opposite to the normal sialylation reaction, is termed ‘reverse sialylation’. In addition to this, we also reported that ST3Gal-II is capable of exchanging sialyl residues between CMP-NeuAc and NeuAcα2,3Galβ1,3GalNAc-unit of mucin glycoproteins[28]. This reaction process is termed ‘exchange sialylation’. In the present study, we demonstrate that the utilization of the exchange sialylation catalytic properties of ST3Gal-II enables the study of mucin type glycoproteins present in cancer cells, tumor tissues and cancer sera. While the present study demonstrates the labeling and preliminary characterization of primary mucinous cancer specimens, further studies on these radiosialyl labeled glycoproteins in the future would be able to determine the precise structure of these labeled glycans that are associated with cancer.

MATERIALS AND METHODS

Cancer cell lines

Cancer cell lines T47D, MCF-7, DU4475 (breast), LS180 (colon), LNCaP (prostate), SKOV3 (ovarian), HL60 (leukemic) and HepG2 (hepatic) were cultured as recommended by ATCC (Manassas, VA)[25]. All cell samples were homogenized with 0.1 M Tris-Maleate pH 7.2 containing 2% Triton X-100 using a Dounce glass, hand-operated homogenizer. The homogenate was centrifuged at 16,000g for 1h at 4°C. The cell extracts (1ml each) was incubated separately at 37°C for 24 h in 0.1M Na Cacodylate pH 6.0, 10μCi CMP-[9-^{3}H] NeuAc and 100 mU of ST3Gal-II (reaction volume 1.4 ml). After incubation, the reaction

mixtures were dialyzed in the cold room against 2L deionized distilled water with 4 changes for 72h, lyophilized to dryness, weighed and then picked up in 1.0 ml water. These [9-^{3}H] sialyl labeled cell extracts were used in biochemical studies.

Tumor Specimens

A total of 10 tumor specimens from 10 different donors (pancreatic cancer:3; breast cancer: 1; colon cancer:1; ovarian cancer:2 and prostate cancer:3) obtained during surgery at the Roswell Park Cancer Institute, were frozen within 1h of collection at −70°C. Tissues were homogenized at 4°C with 4 volumes of 0.1 M Tris-Maleate pH 7.2, 0.1% NaN_3 using Kinematica. After adjusting the TritonX-100 concentration to 2%, these homogenates were mixed in the cold room for 1h using Speci-Mix (Thermolyne) and then centrifuged at 20,000g for 1h at 4°C. The clear fat-free supernatant was stored frozen at −20°C until use. The tumor extracts (0.1ml each) were incubated separately at 37°C for 20h in 0.1M Na cacodylate pH 6.0, 0.2 μCi CMP-[^{14}C] NeuAc and 25mU ST3Gal II (reaction volume 0.16mL). After incubation, the reaction mixture was diluted with 1.0 mL water and dialyzed against water as described for the cell extracts.

In some cases, aliquots of these preparations were subjected to SDS-PAGE. SDS-PAGE was carried out using 4–20% polyacrylamide gradient gels. Following transfer to nitrocellulose membrane, radioactive glycoprotein bands were visualized using autoradiography.

Serum Samples

Serum from each donor was examined initially for the incorporation of [^{14}C] NeuAc into mucin glycoproteins; 20μL aliquots of sera were incubated separately with 0.04μCi CMP-[^{14}C] NeuAc and 2 mU of cloned ST3Gal-II for 20h at 37°C. These incubated samples were diluted to 1 mL with water and then dialyzed against water (2L) with several changes in the cold room for 72 h. These dialyzed preparations contained significant amount of [^{14}C] radioactivity in the range of 2–6 × 10^4. Based on these results a large scale study was undertaken as follows. Normal sera 0.6mL (a mixture of 0.15mL each from 4 donors) was incubated at 37°C for 22h in 0.1M Na cacodylate pH 6.0, 0.07 μCi CMP-[^{14}C] NeuAc and 100mU ST3Gal-II (reaction volume 0.75mL). Ovarian cancer sera 1.8mL (a mixture of 0.36mL each from 5 donors) and pancreatic cancer sera 1.8mL (a mixture of 0.36mL each from 5 donors) were incubated separately 37°C for 22h in 0.1M Na cacodylate pH 6.0, 0.2 μCi CMP-[^{14}C] NeuAc and 300mU ST3Gal-II (reaction volume 2.3mL). These samples were fractionated separately on Biogel P6 column (Fine Mesh; 1.0×116.0 cm). Fractions containing radioactivity excluded as first peak were pooled, lyophilized to dryness, weighed and dissolved in water to a concentration of 20mg per mL. In each case 0.7 mL of [^{14}C] sialyl glycoprotein preparation (14mg) was subjected to exhaustive pronase digestion and 0.5 mL (10mg) was treated with alkaline borohydride.

Preparation of [^{14}C] labeled sialyl fetuin

[^{14}C] labeled sialyl fetuin was prepared by incubating 30mg fetuin (Sigma catalog F3004) with 1.5μCi CMP-[^{14}C]NeuAc and 100mU ST3Gal-II in 0.8ml volume of 0.2M Na cacodylate pH 6.0 for 20h at 37°C. The reaction mixture was separated using Biogel-P2 (1.0×116.0 cm) chromatography. The first radioactive peak emerging at the void volume contained radiolabeled fetuin. This was lyophilized to dryness and used in the studies.

Proteolytic treatment and separation of glycopeptides

Pronase-digestion of [9-^{3}H] sialyl mucin glycoproteins from cell extracts present in 0.2mL dialyzed sialyl extract was carried out in 1.0 ml of 0.1M Tris-HCl pH 7.0, 1 mM $CaCl_2$, 1% ethanol and 0.1% NaN_3 containing 10 mg Pronase CB (EMD-Chemicals) at 37 °C for 24h.

After the treatment, the samples were kept frozen at −20°C before fractionation on Biogel P6 column. In the case of serum samples, 0.7mL of the three extensively dialyzed serum samples containing [^{14}C] sialyl mucin glycoproteins were subjected to pronase (20mg) digestion as described above (reaction volume 1.4mL). After incubation at 37°C for 24h, 20mg pronase was again added and the incubation continued for another 24h.

Release of O-glycans from protein backbone and their separation

Mild alkaline borohydride treatment of [9-^{3}H]/[^{14}C] labeled sialyl mucin glycoproteins present in 0.2mL dialyzed cell extract and 0.5mL dialyzed serum was performed in Teflon lined screw-capped test tubes using 1.0M Na borohydride in 0.1 N NaOH in a total volume of 1.0 ml. Samples were incubated at 45°C for 24h, excess borohydride was destroyed by adding drops of acetic acid carefully, and storing frozen samples at −20°C before fractionation on Biogel P6 column. As anticipated, TLC of radio sialyl fetuin after this treatment showed one major component representing NeuAcα2,3Galβ1,3GalNAcα-ol and one minor component NeuAcα2, 3Galβ1,3(NeuAcα2,6)GalNAcα-ol.

Column chromatography

The following chromatography methods were applied for product separation: a) Biogel-P6 column (Fine Mesh; 1.0×116.0 cm) chromatography was carried out with 0.1 M pyridine acetate (pH5.4) as the eluent. Void volume of this column is 30mL; b) Lectin-agarose affinity chromatography using columns of 7ml bed volume of WGA- and VVL-agarose (Vector Lab, Burlingame, CA) under conditions recommended by supplier[26, 27]. Fractions of 1 mL were collected. After binding was allowed to occur till fraction 15 in all cases, the bound product was eluted. Product from WGA-agarose was eluted with 0.5 M GlcNAc and from VVL-agarose with 0.2 M GalNAc. c) Thin layer chromatography using Silica gel GHLF (250μm scored 20X20cm; Analtech Newark DE) was also used for further product separation[26, 27]. TLC was carried out on Silica gel GHLF (250μm scored 20X20cm; Analtech Newark DE). The solvent system 1-propanol/NH_4OH/H_2O (12/2/5 v/v) was used. The [9-^{3}H] sialyl products were located by scraping 0.5cm width segments of silica gel and soaking in 2.0ml water in vials followed by liquid scintillation counting. The [^{14}C] sialyl products were located by phosphorimaging.

RESULTS

Characterization of [9-^{3}H] sialylated mucin-type glycoproteins from human cancer cell lines

The exchange sialylation reaction was applied to radiolabel mucin type glycoproteins from human cancer cells and the results are summarized in Table 1. The radiosialyl mucin glycoproteins were separated using WGA (Figure 1A) as well as VVL (Figure 1B) affinity chromatography. WGA binds terminal GlcNAc residues of glycans and also glycoproteins via sialyl residues. Using synthetic mucin core-2 compounds[26], we have noted that WGA binds to mucin core-2 [9-^{3}H]NeuAcα2,3Galβ1,3(GlcNAcβ1,6)GalNAcα- and its derivatives containing substituents on GlcNAc such as 6-O-Sulfo, β1,4 linked Gal or both and β1,4 linked (3-O-Sulfo)Gal. It does not bind to [9-^{3}H]NeuAcα2,3 or α2,6Galβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-. VVL on the other hand binds the Tn epitope (GalNAcα-O-Ser/Thr) of mucinous glycoproteins.

WGA-agarose affinity chromatography of [9-^{3}H] sialylated glycoproteins of cell lysates indicated that breast cancer cell line T47D contained exclusively WGA binding mucin glycoproteins (Fig 1Aa). The other breast cancer cell line MCF-7 and the colon cancer cell line LS180 contained mostly WGA binding mucin glycoproteins (Fig 1Ab, 1Ac), though a fraction of the product did not bind WGA. The prostate cancer cell line LNCaP contained

almost equal amounts of WGA binding and non-binding mucin glycoproteins (Fig 1Ad). The ovarian cancer cell line SKOV3, the hepatic cancer cell line HepG2 and the leukemia cancer cell line HL60 contained about 60% WGA binding mucin glycoproteins (Fig 1Ae–1Ag). Overall, the WGA binding properties of T47D, MCF7 and LS180 glycoproteins were different from that of the other cell lines.

VVL-agarose affinity chromatography of [9-^{3}H] sialylated glycoproteins obtained from these same cell lysates indicated that T47D contained about 70% VVL binding mucin glycoproteins (Fig 1Ba) followed by MCF-7 and LS180, which contained about 40% VVL binding mucin glycoproteins (Fig 1Bb, 1Bc). LNCaP, SKOV3, HL60 and HepG2 contained more than 90% VVL non-binding mucin glycoproteins (Fig 1Bd–1Bg). The results demonstrate additional differences in the expression of the GalNAcα-O-Ser/Thr (Tn–epitope) in the cancer cells including the possible existence of clustered Tn epitopes inT47D, MCF-7 and LS180.

Analysis of [9-^{3}H] sialylated glycopeptides and glycans

Besides evaluating full glycoproteins, additional analysis of individual mucinous glycans can be performed following pronase digestion of peptide backbone and alkaline borhydride treatment to release O-glycans.

Upon pronase digestion, as seen, T47D gave rise to one excluded and another major glycopeptide fraction that was included in the column (Fig 2Aa). LS180, on the other hand, contained mostly Biogel P6 excluded mucin glycopeptides (Fig 2Ab). The other cell lines gave rise to a similiar Biogel P6 pattern consisting of 2–3 component glycopeptide fractions (Sup Fig 1). The distinction between the glycans of LS180 and T47D was further revealed by resolving various fractions from the Biogel P6 column using WGA- and VVL- affinity chromatography (Fig. 2B). As seen, most of the high molecular weight fraction from T47D (fraction A) bound WGA-agarose (Fig 2Ba) but not VVL-agarose (Fig 2Bd). On the other hand, the glyopeptide fraction B from T47D displayed equal binding to WGA and VVL (Fig 2Bb, 2Be). The fraction of LS180 that was excluded in the Biogel P6 column (fraction A) behaved similar to fraction A of T47D in that it contained mostly WGA-agarose binding material (Fig 2Bc) whereas VVL-agarose binding material was only 20% (Fig 2Bf).

Mild alkaline borhydride treatment of [9-^{3}H] sialylated mucin-type glycoproteins of cell lysates followed by Biogel P6 column chromatography showed that T47D, MCF-7, LS180 and also HepG2, contained similar O-glycan components, one major and one minor component except for an excluded fraction in the case of LS180 (Fig 3Aa, 3Ab, 3Ac; Sup Fig 2D). LNCaP, SKOV3 and HL60 gave rise to 3–4 components (Sup Fig 2A–2C). Thin layer chromatography of the Biogel P6 fractions obtained from alkaline borohydride treated [9-^{3}H] sialylated glycoproteins of cell lysates indicated that T47D and MCF-7 contained one distinct major component whereas LS180 contained three distinct major components (Fig 3B). The glycan from T47D and MCF7 that eluted at 60–65mL moved similarly on the TLC plate as the major tritium labeled sialylated O-glycan of fetuin (NeuAcα2,3Galβ1,3GalNAc-ol) which also eluted at 60–65mL on the Biogel P6 column.

While detailed structural information is not revealed in this study, the data from Fig. 1–3 demonstrate that each of the cancer cell lines examined has a distinct pattern of O-linked glycosylation, either in terms of the identity of the underlying glycan or its relative abundance.

[^{14}C] radiolabeling of mucin type carbohydrate chains using ST3Gal II

C-14 labeling of mucins is preferred when carbohydrate complexity increases since autoradiography is more straightforward using C-14 compounds compared to tritiated

molecules. We demonstrate this by analyzing the mucin type glycoproteins from cell lysates of the breast cancer cell line DU4475 and colon cancer cell line LS180. Both glycoproteins were labelled using CMP [^{14}C] NeuAc and ST3Gal-II. Bovine fetal protein fetuin was used as a standard during chromatography analysis. Radiolabeled samples were subjected to mild alkaline borohydride treatment and then fractionated on Biogel P6 column (Fig 4A–4C). The chromatogram of DU44745 (Fig 4B) and LS180 (Fig 4C) are more complex as compared to T47D (Fig. 3Aa). In this regard, DU4475 and LS180 exhibited larger mucin carbohydrate chains compared to T47D. Fractions separated by Biogel P6 were further subjected to TLC followed by autoradiography (Fig. 4D). While fetuin (Fig 4D Lane 4) contains one dominant glycan (NeuAcα2,3Galβ1,3GalNAc-ol), both DU4475 (Lanes 1–3) and LS180 (Lanes 5–7) contained multiple O-glycans with different mobilities. In this regard, DU4475 and LS180 are known to express Gal:3-O-sulfotransferase that act on mucin core 2 LacNAc type 2 chains [23,25]. Thus, complex mucin type structures are anticipated in these cells.

SDS-PAGE analysis of ^{14}C sialyl mucin glycoproteins from tumor specimens

In order to demonstrate the utility of this methodology with primary human samples, we radiolabeled using CMP[^{14}C]-NeuAc and ST3Gal-II, the mucin glycoproteins present in three pancreatic, one breast, one colon, two ovarian and three prostate tumor specimens. Labeled glycoproteins isolated from these tumor specimen were subjected to SDS-PAGE. Each tumor was observed to exhibit radiolabled mucins with distinct molecular mass. The three specimens of pancreatic tumor (Sup Fig. 3 Lanes 1–3) exhibited two distinct bands in the molecular weight range 25–35KD. Higher molecular mass glycoproteins are seen in colon and ovarian tumors (Sup Fig. 3 Lanes 5,6). Taken together, the data demonstrate the ability to label mucins on primary tissue using the exchange sialylation reaction.

Mucin type glycoproteins in human serum

The exchange sialylation properties of ST3Gal-II was utilized for [^{14}C]sialyl labeling of mucin glycoproteins present in pooled mixtures of four normal, five ovarian cancer and five pancreatic cancer sera. The [^{14}C]sialyl labeled mucin glycoproteins were isolated by Biogel P6 chromatography. In parallel, [^{14}C]sialyl fetuin was prepared from fetuin through exchange sialylation by ST3Gal-II. Exhaustive pronase digestion (Sup Fig. 4A–4D) as well as mild alkaline borohydride (Sup Fig. 4E–4H) products were separated using Biogel P6 column chromatography. In the case of pronase digested samples, the major glycopeptides from sera elute at 35–45 mL (Sup Fig. 4A–4C) whereas the two glycopeptide fractions from feuin elute at 45–50 mL and 55–60mL (Sup Fig. 4D). These results indicate that the sialylated mucin glycopeptides from serum are larger than fetuin mucin glycopeptides. The distribution of radioactivity in Biogel P6 fractions after alkaline borohydride treatment (Sup Fig. 4E–4H) expressed as percent is as follows: fractions 40–60 mL and 61–70 mL: 39.0 & 61.0 in ovarian sera; 36.7 & 63.3 in pancreatic sera, 39.4 and 60.6 in normal sera and fetuin 17.4 & 82.6. Even though the majority of glycans are small in the human sera as well as in fetuin, the proportion of large mucin chains (fractions 40–60) are considerably larger (36.7–39.4% vs 17.4%; more than two- fold) in human sera compared to fetuin. The studies in Sup Fig. 4 demonstrate not only the utility of exchange sialylation in identifying mucin glycoproteins in serum but also open up the research area for further efforts for identifying distinct cancer associated antigen in patient serum.

DISCUSSION

Among the various posttranslational modification reactions of proteins, glycosylation is the most abundant. Glycosylation can alter the charge, conformation and stability of proteins, and induce heterogenous profiles as a consequence of the production of variable glycoforms. Cancer associated glycans are often found on a class of proteins called mucins[29], which are

large, highly O-glycosylated proteins involved in the protection and control of signalling at epithelial surface. Mucins themselves exert profound tumor-promoting effects. This is in part mediated through interactions with their glycan moities[4, 30, 31]. The following sections summarize our experimental finding in the context of existing knowledge regarding various cancers.

Ovarian Cancer

CA125 is a mucin commonly employed as a diagnostic marker for epithelial ovarian cancer[32]. CA125 is highly enriched in serine and threonine residues and recently designated as MUC16 mucin[33]. Its carbohydrate content based on its mass is estimated to be 24–28% with the majority being O-linked glycans[34–36]. Core 1 and core 2 type glycans are the major O-linked glycans expressed on CA125[37]. The ovarian cancer cell line $SKOV_3$ examined in this study has not been shown to express CA125 but it does express MUC1 and MUC2[38]. These mucins are apparently the source of large O-glycans obtained in the present study as evident from the Biogel P6 chromatography elution profiles of pronase digested fractions (Sup Fig. 1C) and alkaline borohydride released glycans (Sup Fig. 2B).

Leukemic cells

P-selectin glycoprotein ligand (PSGL-1) is a disulfide-bonded homodimeric mucin-type glycoprotein on leukocytes that interacts with both P- and E-selectin. A majority of the O-glycans in PSGL-1 are disialylated or neutral forms of core 2 tetrasaccharide[39]. A comparison of PSGL-1 and CD43 expressed by the leukemia cell line HL60 cells indicates they are differently O-glycosylated[39]. CD43 lacks the fucosylated glycans found on PSGL-1 and is enriched with the non-fucosylated, disialylated core 2 tetrasaccharide. The present study showed that [9-^{3}H] sialyl mucin glycoproteins in the HL60 cell lysate when subjected to pronase digestion gave large size glycopeptides fractions (Sup Fig. 1D) and upon alkaline borohydride treatment gave rise to large size O-glycans as evident from the elution profile obtained on Biogel P6 chromatography (Sup Fig. 2C).

Breast Cancer

Cancer associated mucins show antigenic differences from normal mucins. Analysis of the O-glycans attached to the mucin produced by the normal lactating breast and by breast cancer cell lines has shown that the oligosaccharides attached to the normal mucin are core2 based structures whereas short core-1 based structures dominated in the cancer associated mucin[40]. The composition as well as the number of O-glycans added to MUC1 was found altered in breast cancer[40]. A two-fold reduction in the actual number of O-glycans attached to MUC1 was observed in normal cells as compared with T47D cells[41]. Further MUC1 purified from the serum of an advanced breast cancer patient contained 83% mostly sialylated core1 glycans and 17% core2 glycans[42]. MUC6 has been detected in breast carcinoma[43]. Freire et al[44] showed that MUC6 expressed by MCF-7 breast cancer cells was aberrantly glycosylated because it contained the Tn antigen. They demonstrated the feasibility of MUC6-Tn glycoconjugates as an attractive target to be used in cancer immunotherapy[45]. In the present study [9-^{3}H]sialylated glycoproteins of cell lysates through the enzymatic exchange sialylation showed the predominance of clustered Tn epitopes in breast cancer cell lines T47D and MCF-7 as evident from VVL-agarose affinity chromatography of the mucin glycoproteins before (Fig. 1Ba, 1Bb) as well as VVL-agarose chromatography after pronase digestion (Fig 2Be). These results are consistent with a recent study demonstrating that the GSTA region of the MUC1 tandem repeat contains a highly immunodominant epitope when presented with immature short O-glycans such as GalNAcα-O-Ser/Thr (Tn) and NeuAcα2,6 GalNAc α-O-Ser/Thr (STn); the cancer specific expression of this glycopeptide epitope makes it a prime candidate for immunodiagnostic and therapeutic measures[46]. The present study further showed that the breast cancer cell line

DU4475 expresses complex, large mucin carbohydrate chains as evident from pronase digestion (Fig. 4B) and TLC of Biogel P6 fractions obtained alkaline borohydride treatment (Fig 4D, Lanes 2,3). We found earlier that DU4475 expresses Gal:3-O-sulfotransferase specific for mucin core2 LacNAc type 2 chains[25, 27].

Colon cancer

Expression of MUC1 in colorectal carcinomas was found to be associated with tumor progression[47, 48]. As anticipated, in the present study the colon cancer cell line LS180 has been shown to synthesize complex sialylated mucin carbohydrate structures (Fig 2Ab and Fig 3Ac). LS180 is known from our earlier study[25, 27] to express Gal:3-O-sulfotransferase specific for mucin core 2 LacNAc type 2 chains. The present study has shown further that LS180 synthesizes Tn epitopes in clusters on the mucin polypeptide chain (Fig 1Bc and Fig 3B Lanes 3,4; Fig 4C and 4D Lanes 5, 6).

Pancreatic cancer

Inflamation has been shown to be involved in pancreatic cancer development and progression[49, 50] and could provide the conditions necessary to stimulate changes in the glycosyltransferases and other factors that produce cancer associated glycans. Over expression of MUC 1 in advanced pancreatic cancer[51] and MUC1-based immunotherapeutic treatment strategies have been reported[52]. Human pancreatic ribonuclease (RNase1) has a molecular weight of 31 kDa[53]. High levels of RNase 1 have been detected in the sera of most patients with pancreatic adenocarcinoma. RNase1 contains three N-glycosylation sites occupied[54] and two unoccupied O-glycosylation sites Asn-XX-Ser/Thr[55]. It is interesting to note that the present study identified in the three pancreatic tumor specimens examined, two [^{14}C] sialyl mucin glycoproteins in the range of 25–35 kDa (Sup Fig. 3) suggesting their similarity to pancreatic RNase which might have acquired sialylated O-glycan chains.

Prostate cancer

MUC1 is a tumor associated antigen that is highly related to tumor progression in prostate cancer patients[56]. A disialyl core type 1 linked to Thr was identified in MUC1 tandem repeat[57]. Mono and disialyl core-type 1 O-linked carbohydrate chains were identified in haptoglobin of prostate cancer sera[58]. In the present study, alkaline borohydride treated [9-^{3}H]sialyl mucin glycoprotein of the prostate cancer cell line LNCaP gave rise to two distinct peaks A and B (Sup Fig 2A), the structure of B being NeuAcα2,3Galβ1,3GalNAcOH.

Human sera

The molecular size of [^{14}C] sialyl mucin glycopeptides from human sera was quite larger than that of [^{14}C] sialyl fetuin glycopeptides (Sup Fig. 4A–4D). This would suggest that [^{14}C] sialyl O-glycan chains are either large or in cluster or in very close proximity to N-linked carbohydrates in the polypeptide chain not cleavable by pronase, in contrast to fetuin carbohydrate chains. In support of these findings, we found the large size [^{14}C] sialyl mucin type chains resulting from alkaline borohydride treatment as 38% from sera as opposed to 17% from fetuin (Sup Fig. 4E–4H).

CONCLUSION

Overall, the present study examined cancer cell lines, tumor specimens and sera by utilizing the exchange sialylation catalytic properties of ST3Gal-II for radiosialyl labeling and showed distinct differences in mucin glycoproteins as well as the mucin glycopeptide species arising upon pronase digestion from various cancer cell lines. Further, it indicated

not only differences in the distribution of mucin carbohydrate chains but also the different structures of sialylated mucin carbohydrate chains among the various human cancer cell lines studied. The data from mild alkaline borohydride treatment were also supportive of the above contention. SDS-PAGE of [^{14}C]sialylated glycoproteins in the extracts of various tumor specimens further showed unique differential pattern of mucin glycoproteins in various cancers. The mucin glycopeptides as well as the mucin-type carbohydrate chains arising from human cancer sera were found to be distinctively different both qualitatively and quantitatively from that of fetuin. In essence the specific sialyl labeling technique by exploiting the exchange sialylation capability of ST3Gal-II would be very valuable for studying mucin type structures in health and diseases. Hence, ST3Gal-II could serve as a versatile tool in glycoproteomics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CMP	Cytidine 5′ monophosphate
NeuAc	N-acetylneuraminic acid (sialic acid)
PSGL-1	P-selectin glycoprotein ligand-1
T	Galβ1,3GalNAcα-O-Ser/Thr
Tn	GalNAcα-O-Ser/Thr
VVL	Vicia villosa lectin
WGA	Wheat germ agglutinin
RM	Reaction Mixture
SDS	Sodium dodecyl sulfate
TLC	Thin layer chromatography
PAGE	Polyacrylamide gel electrophoresis
KDa	Kilo Daltons

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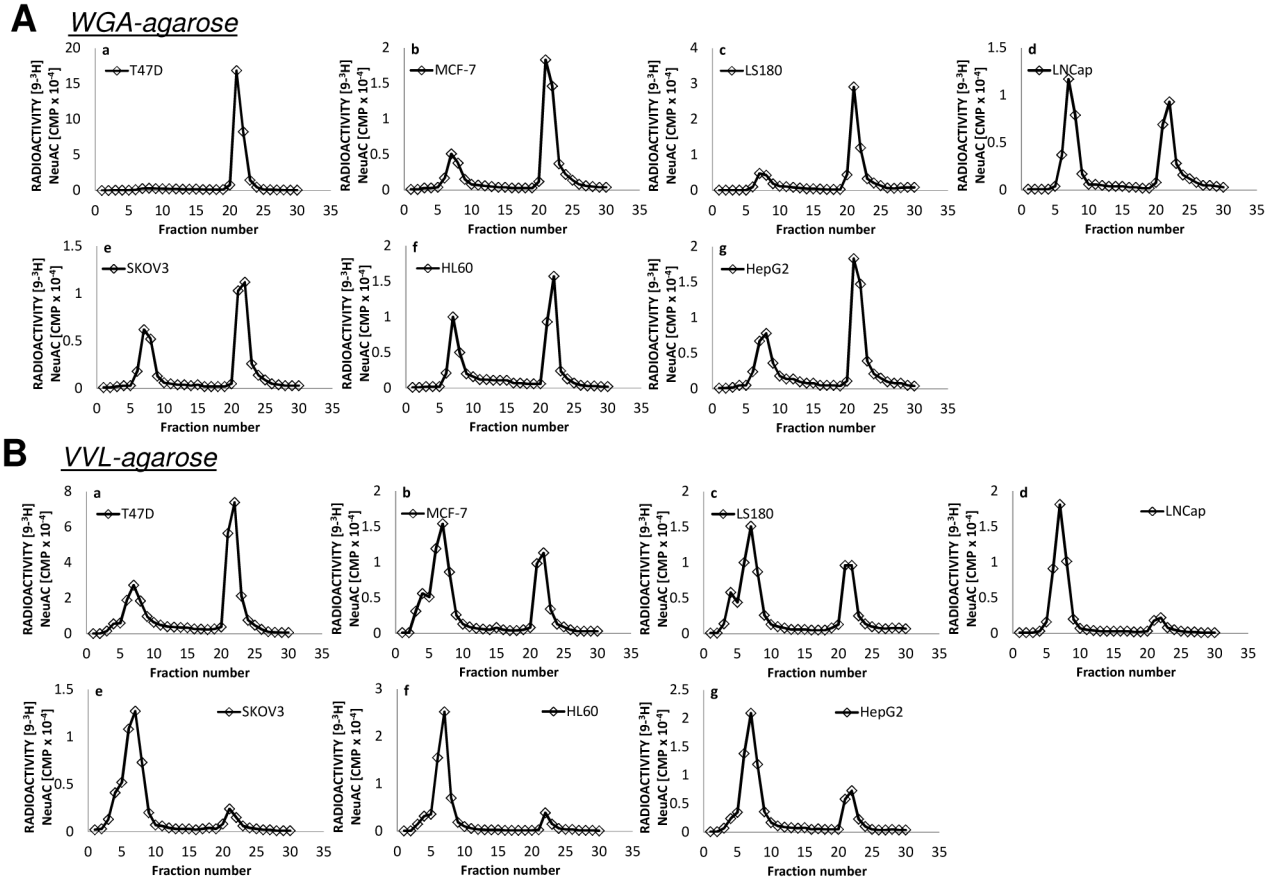


Fig 1. Lectin affinity chromatography of intact proteins

Lectin-agarose affinity chromatography of [9-^{3}H] sialylated glycoproteins of lysates from human cancer cell lines **a**: T47D; **b**: MCF-7; **c**: LS180; **d**: LNCaP; **e**: SKOV3; **f**: HL60; **g**: HepG2. A column of 7ml bed volume of WGA-agarose (top half of figure, **A**) or VVL-agarose (bottom half of figure, **B**) was employed using 10mM Hepes pH7.5 containing 0.1mM $CaCl_2$, 0.01mM $MnCl_2$ and 0.1% NaN_3 as the running buffer. An aliquot (either 50 or 100 μL) of [9-^{3}H] sialyl dialyzed cell extract preparation was diluted to 1 mL with the running buffer and applied to the affinity column. Fractions of 1.0ml were collected. After fraction 15, the bound material was eluted with 0.5M GlcNAc or 0.2M GalNAc in the same buffer.

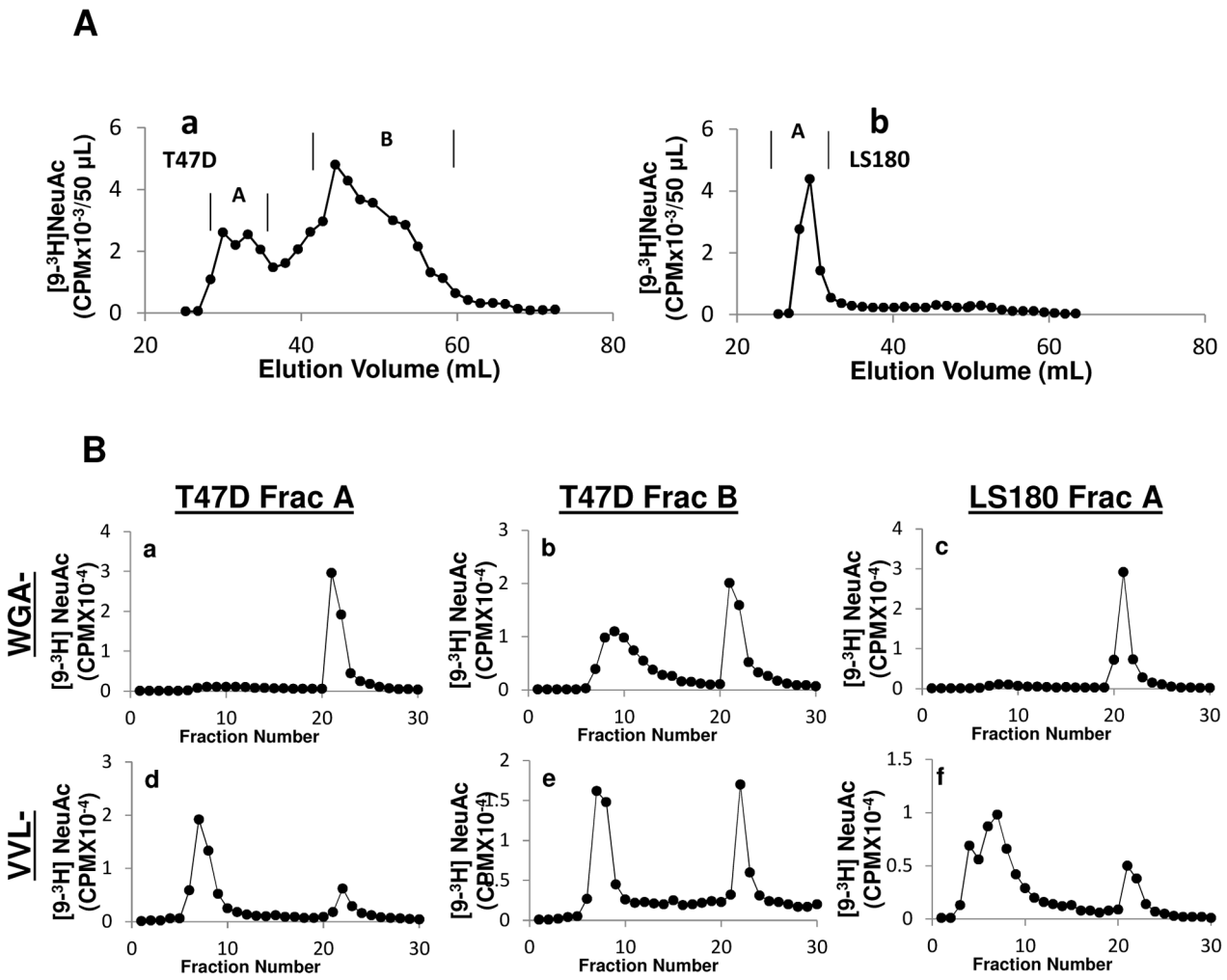
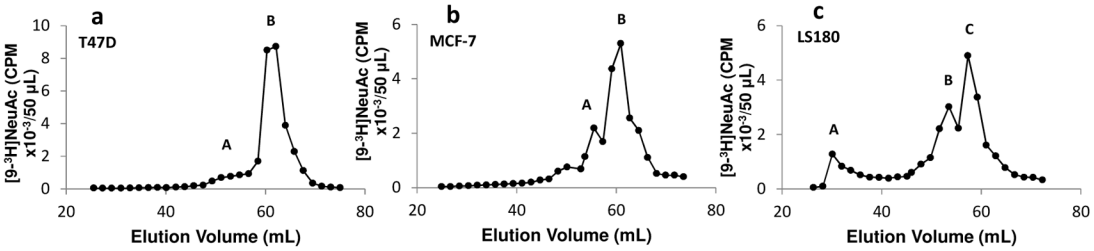


Fig 2. Analysis of pronase-digested fragments

A. Fractionation of pronase-digested [9-^{3}H] sialylated glycoproteins from human cancer cell lines (T47D in subpanel **a** and LS180 in subpanel **b**) using Biogel P6 size exclusion chromatography. Here, [9-^{3}H] sialyl mucin glycoproteins from cell extracts were digested in 1.0 ml of 0.1M Tris-HCl pH 7.0, 1 mM $CaCl_2$, 1% ethanol and 0.1% NaN_3 containing 10 mg Pronase CB (EMD-Chemicals) at 37 °C for 24h. A Biogel P6 column (Fine Mesh; 1.0×116.0 cm) was used with 0.1 M pyridine acetate (pH5.4) as the eluent at room temperature. **B.** Fractions containing radioactivity in panel A peaks were pooled, lyophilized to dryness and dissolved in a small volume of water. Lectin affinity chromatography of these fractions was performed as described in Fig 1. Subpanels **a–c**: WGA-agarose chromatography and subpanels **d–f**: VVL-agarose chromatography. Both fractions A and B for T47D and fraction A of LS180 were resolved as indicated in figures.

A



B

	T47D.B	MCF-7.B	LS180.B	LS180.C	Fetuin
25	318	271	202	211	106
24	214	251	164	336	117
23	200	221	202	249	117
22	261	206	204	145	82
21	425	164	233	209	100
20	339	179	243	193	212
19	1073	300	397	227	263
18	881	537	677	788	580
17	12052	1016	602	1401	5673
16	10545	4772	615	6397	2427
15	1139	3869	615	6799	774
14	1254	1774	1144	4185	429
13	920	803	2599	2021	421
12	777	592	2290	1395	137
11	433	397	3507	1316	160
10	359	322	2238	1221	106
9	682	325	1527	714	129
8	457	310	1286	622	107
7	435	311	1418	565	137
6	475	270	1224	403	132
5	643	335	1440	390	123
4	583	346	1144	389	138
3	359	480	897	756	139
2	312	486	796	726	161
1	217	291	781	330	130

Fig 3. Mild alkaline borohydride treatment of glycoproteins

A: Mild alkaline borohydride treatment of [9-^{3}H] labeled sialyl mucin glycoproteins was performed in Teflon lined screw-capped test tubes using 1.0M Na borohydride in 0.1 N NaOH in a total volume of 1.0 ml. Samples were incubated at 45°C for 24h, excess borohydride was destroyed by adding drops of acetic acid carefully, and product was fractionated on Biogel P6 column as described in Fig 2 A. Results are presented for T47D (subpanel **a**), MCF-7 (subpanel **b**) and LS180 (subpanel **c**). **B:** Thin layer chromatography (TLC) analysis of Biogel P6 fractions obtained in panel A [T47D fraction B; MCF-7 fraction B; LS180 fractions B, C] and also the major O-glycan of fetuin. TLC was carried out on Silica gel GHLF (250µm scored 20X20cm; Analtech Newark DE) with 1-propanol/ NH_4OH/H_2O (12/2/5 v/v) as solvent. The [9-^{3}H] sialyl products were located by scraping 0.5cm width segments of silica gel and soaking in 2.0ml water in vials followed by liquid scintillation counting.

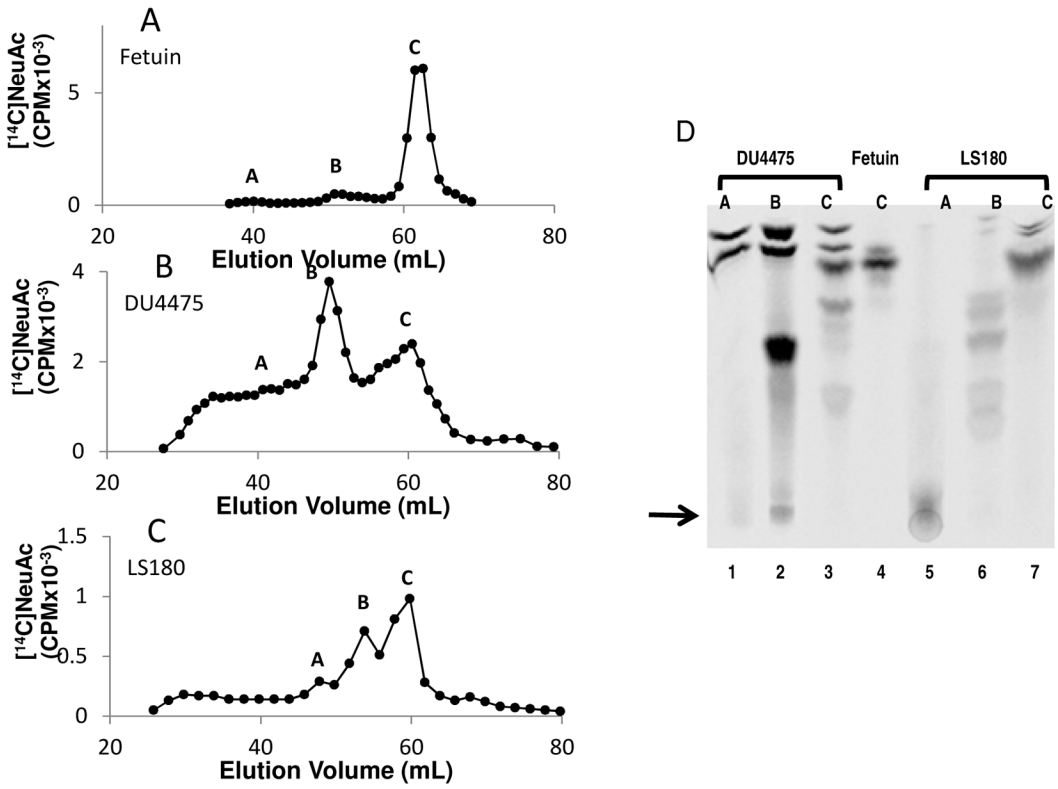


Fig 4. Analysis of C-14 labeled glycans

[^{14}C] sialyl mucin glycoproteins from various cells were subjected to mild alkaline borohydride treatment as described in Fig 3. Biogel P6 column chromatographic patterns are presented for **A:** [^{14}C] sialyl Fetuin, **B:** [^{14}C] sialyl DU4475 mucin glycoproteins and **C:** [^{14}C] sialyl LS180 mucin glycoproteins. **D**. Thin layer chromatography of fractions obtained from panels A–C are provided for [^{14}C] sialyl DU4475 Biogel P6 fractions A, B and C (lanes 1, 2, 3 respectively), [^{14}C] sialyl Fetuin O-glycan (lane 4) and [^{14}C] sialyl LS180 Biogel P6 fractions A, B and C (lanes 5, 6, 7 respectively). The arrow indicates the origin of the TLC. Mobile phase moves from bottom to top. The [^{14}C] sialyl products were located by phosphorimaging of TLC plates.

TABLE 1

Characteristics of [9-^{3}H]sialylated glycoproteins present in human cancer cell lysates

	Incorporation of [9-^{3}H]NeuAc/mg wt*	**[9-^{3}H]sialyl Glycoproteins binding**		**Biogel P6 [9-^{3} H]Sialyl fractions from pronase digestion**	
Cancer cell line	CPM x 10^{-4}	WGA- Agarose %	VVL- Agarose %	P6 excluded	P6 included
T47D (breast)	20.54	100	70	Minor WB 95% VB 5%	Major WB 40% VB 40%
MCF (breast)	4.75	>90	40	Major	Major
LS180 (colon)	8.31	>90	40	Major WB 95% VB 20%	negligible
$SKOV_3$ (ovary)	8.48	60	<10	Major	Major
Hep G_2 (hepatic)	3.35	60	<10	Major	Major
HL60 (leukemia)	3.10	60	<10	Major	Major
LNCap (prostate)	8.89	50	<10	Major	Major

*Extensively dialyzed and then lyophilized material from [9-^{3}H]sialylated cancer cell lysates

WB: WGA- agarose binding and VB: VVL-agarose binding