Association of Sialyl-Lewis\textsuperscript{a} and Sialyl-Lewis\textsuperscript{x} with MUC-1 Apomucin in a Pancreatic Cancer Cell Line\textsuperscript{1}

Jenny J. L. Ho,\textsuperscript{2} Bader Siddiki, and Young S. Kim

Gastrointestinal Research Laboratory (151M2), Veterans Affairs Medical Center and Department of Medicine, University of California, San Francisco, California

ABSTRACT

We have shown previously that the mucins of the human pancreatic cancer cell line, SW1990, have both sialyl-Lewis\textsuperscript{a} and sialyl-Lewis\textsuperscript{x} carbohydrate ligands that are implicated in tumor cell metastasis. In the present study, we undertook to identify the protein core of these mucins. SW1990 mucins that carry sialyl-Lewis\textsuperscript{a} and sialyl-Lewis\textsuperscript{x} bound to the MUC1 peptide-specific mAb 139H2. Removal of most of the sialic acids from SW1990 mucins by neuraminidase greatly enhanced binding of two other MUC1 peptide specific antibodies, HMFG-2 and SM-3. After removal of sialic acids, most of the mucins rich in sialyl-Lewis\textsuperscript{a} and sialyl-Lewis\textsuperscript{x} oligosaccharides no longer bound to a DEAE-cellulose column at pH 8.0. These results indicate that at least part of the sialyl-Lewis\textsuperscript{a} and sialyl-Lewis\textsuperscript{x} in SW1990 cells is associated with the MUC1 polypeptide. Moreover, sialic acids play an important role in determining the net negative charge of sialyl-Lewis\textsuperscript{a} and sialyl-Lewis\textsuperscript{x} rich mucins and in obscuring MUC1 peptide regions.

INTRODUCTION

Sialylated oligosaccharides of mucins inhibit the homotypic cellular aggregation of pancreatic cancer cells and their adhesion to extracellular matrix proteins (1). Thus, sialic acids play an important role in certain biological characteristics of pancreatic cancer mucins that are implicated in cellular invasiveness, differentiation, and tumorigenicity. In addition, the expression of specific sialylated carbohydrate structures is higher in mucins from pancreatic tumors than it is from normal tissues (2). One of these is sialyl-Lewis\textsuperscript{a} (sLex).\textsuperscript{3} Its expression is correlated with increased metastatic potential of tumor cells (3) and poor patient survival (4). Sialyl-Lewis\textsuperscript{x} (sLea), a related structure, is present in both normal and malignant pancreatic tissues. However, its level is frequently elevated in the circulation of pancreatic cancer patients. It is the basis of the CA19-9 blood test for pancreatic cancer (5). Cell surface sLea and sLex are both implicated in tumor cell binding to the endothelial cell adhesion molecule E-selectin and in cellular extravasation during metastasis (6). The human pancreatic cancer cell line, SW1990, which has a high rate of liver metastases in the splenic injection model (7), produces mucins that have high levels of both sLea and sLex (8). Purified SW1990 mucins, as well as pancreatic cancer sera rich in the sialylated Lewis antigens, inhibit SW1990 cell binding to E-selectin (9).

In SW1990 cells, the mucin polypeptide core to which the sLea and sLex carbohydrate antigens are attached has not been identified previously. At least seven different apomucins have now been identified in human tissues (10). Although the MUC1 apomucin is the major type in the pancreas (11–13), other mucin core polypeptides occur in pancreatic cancer cells (11, 14, 15). In this study we identify the protein core of sLea and sLex mucins in SW1990 cells and examine some of the characteristics of the carbohydrates of these mucins.

MATERIALS AND METHODS

Culture of SW1990 Cells. The cancer cell line SW1990 was derived from a human pancreatic ductal adenocarcinoma (16). SW1990 cells were cultured in DMEM 21 supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 \mu g/ml streptomycin. When cells were confluent they were scraped and homogenized in PBS (1 mm phenylmethylsulfonyl fluoride) by sonication for 20 s. Cytosol was obtained by centrifugation at 100,000 \times g for 1 h. Cells were radiolabeled by culturing them for 24 h in medium that contained 10 \mu C/ml of tritiated glucosamine (specific radioactivity, 40 Ci/mmol; ICN Radiochemicals, Irvine, CA).

Buoyant Density. Cytosol samples containing 0.54 g/ml of CsCl were centrifuged in a Beckman SW41 rotor for 48 h at 150,000 \times g. The densities of the fractions (1.5 ml) were determined gravimetrically.

Antibodies. The rabbit polyclonal antibody anti-SWB was generated by immunization with mucins that had been purified from SW1990 xenografts grown in athymic nude mice and that were fully deglycosylated (17). mAb DF3 was a gift from Dr. Donald Kufe (Dana Farber Cancer Institute, Boston, MA; IgG, ascites; Refs. 18, 19). mAbs HMFG-2 and SM-3 were gifts from Dr. Joyce Taylor-Papadimitriou (Imperial Cancer Research Fund, London, UK; IgG, media; Ref. 20). mAbs 139H2 and 115D8 were gifts from Dr. John Hilkens (The Netherlands Cancer Institute, Amsterdam, the Netherlands; IgGs; Refs. 21, 22). The 19-9 hybridoma was obtained from the American Type Culture Collection (IgG, anti-sLex; Ref. 23). mAb SNH3 was a gift from Drs. S. I. Hakomori and A. Singhal (Biomembrane Institute, Seattle, WA; IgM, anti-sLex, ascites; Ref. 18). mAb HMFG-2 and SM-3 were gifts from Dr. John Hilkens (IgM, ascites; Ref. 19).

Purified mAbs against Lea and Lex antigens were gifts from Dr. R. Murray Ratcliffe (Chemibond, Ltd., Edmonton, Alberta, Canada).

SDS-PAGE and Western Blotting. The procedures have already been described (8). Samples were separated with a 4% stacking and 7% resolving gel. The following dilutions of antibodies were used: DF3 (1:500 of ascitic fluid), HMFG-2 (1:10 of medium), 139H2 (1:500 of ascitic fluid), SNH3 and 19-9 (1:100 of ascitic fluid), and anti-SWB (1:50 of serum). The molecular weight standards (prestained; Bio-Rad, Hercules, CA) were: myosin, M, 194,000; β-galactosidase, M, 116,000; bovine serum albumin, M, 85,000; and ovalbumin, M, 49,000.

Purification of Antibodies. Anti-SWB (rabbit polyclonal anti-sera) and 19-9 and 139H2 mAbs (IgG, ascitic fluids) were purified by affinity chromatography on protein A (Econo-Pac Protein A kit; Bio-Rad).

Peroxidase Conjugation of 19-9 Antibody. Purified 19-9 antibody was conjugated to horseradish peroxidase by periodate oxidation (24).

Lectin Binding and Immunoassays. Two types of assays were performed (17): (a) purified capture antibody (polyclonal anti-SWB or monoclonal 139H2, sandwich assays), or (b) antigen was adsorbed directly to the plate (direct assays). Optimal concentrations of all antibodies or lectins were determined by serial dilution. Soluble antigens or purified antibodies (in PBS) were adsorbed overnight to enzyme immunoassay/RIA microtiter plates (Costar, Cambridge, MA) at 4°C. After aspiration of the solutions the wells were blocked with 100 μl of 1% BSA (in PBS) for 1 h. In the sandwich assays the subsequent incubation was with 50 μl of antigen (in PBS) followed by an incubation with 50 μl of first antibody (in 1% BSA-PBS). In the direct assays the subsequent incubation was with first antibody or peroxidase conjugated lectin (in 1% BSA-PBS). Peroxidase conjugated lectins from wheat germ (WGA) and peanut (PNA) were obtained from Sigma (St. Louis, MO).

Quantitation of the first antibody was accomplished by incubation with 50 μl of peroxidase-conjugated second antibody (in 1% BSA-PBS). When peroxi-

Received 3/13/95; accepted 6/7/95.

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1 This study was supported in part by the Veterans Affairs Medical Research Service and USPS Grant CA 24321 from the National Cancer Institute.

2 To whom requests for reprints should be addressed, at Veterans Administration Medical Center, University of California, 4150 Clement Street (151M2), San Francisco, CA 94121.

3 The abbreviations used are: sLex, Sialyl-Lewis\textsuperscript{a}; sLea, Sialyl-Lewis\textsuperscript{x}; WGA, wheat germ agglutinin; PNA, peanut agglutinin.
SIALYLATED-LEWIS ANTIGENS AND MUC1

Fig. 1. Buoyant density profiles of MUC1 and sLea antigens from SW1990 cell homogenates. SW1990 cytosol was fractionated according to buoyant density by CsCl gradient centrifugation. Top panels, Western blots after SDS-PAGE. Arrowheads, boundary between the 4% stacking gel and the 7% resolving gel. Bottom panels, ELISAs of the corresponding CsCl fractions. Density of each fraction was determined gravimetrically. First column (a-SWB/HMFG-2): A, HMFG-2; D, a-SWB. The apparent molecular weights of the two bands are 160,000 and 210,000. Second column (139H2): D, 139H2. Third column (DF3/sLea): D, sLea; A, DF3. High molecular weight immunoreactivity, >400,000. O, buoyant density.

RESULTS

Buoyant Density and SDS-PAGE Profiles of Mucins in SW1990 Cytosol. Intracellular mucins of SW1990 cells can be divided into two main types by their buoyant densities. The low buoyant density (1.2 g/ml) fraction contained two low molecular weight proteins (Mr >400,000 and 210,000) that have MUC1 peptide epitopes accessible to peroxidase-conjugated 19-9 was the first antibody, no second antibody was necessary. The peroxidase-conjugated secondary antibodies were goat anti-mouse IgM (µ specific, for SPan-1 and SNH3 Sigma), goat anti-rabbit IgG (Fc specific, for DF3, HMFG-2, 139H2, and SM-3 Sigma). The substrate solution was 100 µL of 2 mM 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) in 0.1 M citrate buffer (pH 4.0) containing 0.05% H2O2. Absorbance was determined at 414 nm with a Titertek Multiskan microtiter plate reader (ICN Biomedical, Huntsville, AL).

Ion Exchange Chromatography. Unfractionated cytosol or high buoyant density antigens (1.39–1.50 g/ml) were applied to 2-ml columns (1.1 X 3.2 cm) of DEAE-cellulose [10 mM Tris (pH 8)]. Six 1-ml fractions were collected for each concentration of NaCl in 10 mM Tris (0, 50, 100, 150, 200, 250, 300, and 1000 mM).

Treatment with Neuraminidase. Soluble antigens or antigens adsorbed to microtiter plates were treated with Clostridium perfringens neuraminidase [Type X (200 milliunits/ml PBS); Sigma] for 1 h at 37°C.

Immunoprecipitation. The method has been described previously (6). Briefly, radiolabeled cells were homogenized by sonication in PBS (0.1% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride) for 20 s. Asctic fluid of 19-9 (1:50) was used. Antigens were eluted from protein A cells by incubation with 3 M potassium thiocyanate for 1 h at 37°C and centrifugation for 5 min at 12,800 x g. β elimination was performed on immunoprecipitated sLea antigen as described previously (25). sLea antigen was treated with 0.05 N NaOH and 1 µM NaBH4 at 45°C for 48 h. Radioactive samples were counted in a Beckman LS 5801 counter after mixing with Ecolite (ICN Radiochemicals).

Column Chromatography. High density antigens (1.39–1.50 g/ml) were applied to 0.8 × 18-cm columns of Sephacryl S-400. The fractions were 0.5 ml.

Association of sLea and sLex with MUC1 Apomucin and Effect of Neuraminidase on Charge of sLea/sLex Antigens. sLea and sLex both bound to either purified anti-SWB or 139H2 antibodies when they were used as capture antibodies in sandwich immunoassays (Fig. 2). As a control, the SPan-1 carbohydrate epitope, reported previously to be associated with MUC1 apomucin in SW1990 cells (17), was also captured by either anti-SWB or 139H2. sLea antigen from high buoyant density fractions eluted principally

Fig. 2. Sandwich immunoassays with either polyclonal a-SWB or monoclonal 139H2 anti-sera as capture antibody. SW1990 cytosol was fractionated by buoyant density in a CsCl gradient. The reporter antibody is indicated at the top right corner of each box. a-SWB alone without capture antibody; ↓, α-SWB as the capture anti-sera; □, 139H2 as capture antibody; and ○, no capture antibody.
A, sLea antigen; B, Lea antigen present in the same fractions without neuraminidase treatment; C, total Lea antigen, the same fractions treated with 200 milliunits/ml of Clostridium perfringens neuraminidase for 1 h at 37°C after chromatography to desialylate sLea antigen; and D, total Lea antigen, antigen treated with neuraminidase before chromatography.

Effect of Neuraminidase on Reactivities of MUC1 mAbs with Cytosol Mucins. When unfractionated cytosol was separated by ion-exchange chromatography (Fig. 4) much of the anti-SWB and HMFG-2 antigens did not bind to DEAE. Part of the 139H2 antigen eluted with 150 and 200 mM NaCl (Fig. 4, large arrowheads), while part was eluted at lower concentrations of NaCl. All of the DF3, sLea, and sLex antigens bound to DEAE and was eluted with 150 and 200 mM. Thus, the elution patterns of MUC1 antigens from DEAE are apparently related to their buoyant density and SDS-PAGE patterns as follows: (a) the smaller antigens detectable in low buoyant density fractions that react well with MUC1 peptide-specific anti-sera do not bind well to DEAE at pH 8; and (b) the large antigens that are present in high buoyant density fractions and react best with anti-sera specific for carbohydrates are negatively charged at pH 8.0. Partial removal of sialic acid greatly increased HMFG-2 immunoreactivity with those fractions that were eluted from DEAE by 150 and 200 mM NaCl (Fig. 4). Little change in HMFG-2 immunoreactivity was observed with fractions that did not bind to the column.

When cytosol was fractionated by buoyant density, the greatest effect of neuraminidase on HMFG-2 reactivity was seen with the high-density fractions (Fig. 5). Neuraminidase also increased anti-SWB and SM-3 reactivity with these fractions. In contrast, reactivities of 115D8, DF3, 19-9 (Fig. 6, sLea), and SNH3 (Fig. 5, sLex) were reduced by neuraminidase. High buoyant density fractions contained most of the radioactivity that is immunoprecipitable by 19-9 antibody (sLea) from cells radiolabeled with tritiated glucosamine and most of the WGA and PNA lectin-binding activities (Fig. 6). Neuraminidase reduced WGA binding (specific for N-glcNAc or sialic acid), whereas it increased the reactivity of PNA (specific for gal-galNAc).

When high density fractions of cytosol were separated by column chromatography on Sephacryl S-400 (Fig. 7), both HMFG-2 and SM-3 antibodies reacted principally with void volume fractions. Reactivity was higher after fractions were treated with neuraminidase before assay (Fig. 7A). sLea and sLex antigens were also present mainly in the void volume fractions (Fig. 7B). Metabolically radiolabeled sLea antigen immunoprecipitated by the 19-9 antibody from high buoyant density fractions of cytosol of cells eluted mainly in the void volume fractions (Fig. 7C). After β elimination, most of the radioactivity eluted in the included volume.

Fig. 4. Separation of SW1990 cytosol by ion exchange chromatography on DEAE-cellulose. Ion exchange chromatography of unfractionated cytosol was performed as described in Fig. 3. Immunoreactivity was determined by ELISA. HMFG-2 reactivity was also tested after treatment of the fractions with 200 milliunits/ml of Clostridium perfringens neuraminidase for 1 h at 37°C (plus neuraminidase).

with 150 mM and 200 mM NaCl (large arrowheads, Fig. 3A). sLea from unfractionated cytosol had a similar elution pattern (Fig. 4). Because the 19-9 antibody does not react with desialylated Lea, an anti-Lea antibody was used to examine the effect of desialylation on the elution pattern of the sLea antigen. As with sLea, Lea antigen in untreated samples bound to DEAE at pH 8.0 and eluted principally with 150 and 200 mM NaCl (Fig. 3B). When fractions were treated with neuraminidase after their elution, there was an increase in total Lea antigen because sLea was converted to Lea (Fig. 3C). If high density antigen was treated with neuraminidase first, before chromatography, most of the total Lea antigen no longer bound to DEAE (Fig. 3D). Similar results were obtained with sLex/Lex (results not shown).

Fig. 5. Effect of neuraminidase on the expression of MUC1 peptide and carbohydrates by intracellular mucins. Cytosol was fractionated according to buoyant density by CsCl centrifugation. Density was determined gravimetrically. Immunoreactivity was determined by ELISA. Δ, no neuraminidase; Δ, after incubation with 200 milliunits/ml of Clostridium perfringens neuraminidase for 1 h at 37°C.
DISCUSSION

In SW1990 human pancreatic cancer cells the sialylated oligosaccharide structures sLea and sLex, ligands that are involved in binding to the E-selectin, are O-glycosylated to mucinous glycoproteins (9). In the present study we have shown that at least some of the SW1990 sLea and sLex are associated with the MUC1 polypeptide because mucins containing these structures bind to the MUC1 directed mAb 139H2 and polyclonal anti-SWB. sLea is also associated with MUC1 in colon (26) and gallbladder (27). However, it is also associated with MUC3 in gallbladder (27) and with a small mucin distinct from MUC1 in colon (26, 28) and salivary glands (28). sLex is associated with MUC1 (29) and MUC2 (30) in colonic cancer tissues. In the present study we have also shown that the sLea and sLex mucins of SW1990 cells lose much of their net negative charge after desialylation. The adhesiveness of SW1990 cells to each other and to extracellular matrix proteins is reduced by cell surface sialic acids and mucin-associated oligosaccharides (1). The negative charge of sLea and sLex mucins may contribute to this inhibition.

Studies with breast (31) and pancreatic (32) cell lysates have shown that glycosylation of the MUC1 tandem repeat occurs on the threonines in positions 1 and 5 of Fig. 8 but not in position 3. Thus, the threonine in the epitopes of HMFG-2/SM-3 (Fig. 8, site 3) is not glycosylated, yet in our studies this region is not readily accessible to the antibodies. In a previous report (17), we showed that partial deglycosylation of secreted SW1990 mucins by a combined treatment of neuraminidase, periodate oxidation, and β elimination revealed the glycosylated, yet in our studies this region is not readily accessible to the antibodies. In a previous report (17), we showed that partial deglycosylation of secreted SW1990 mucins by a combined treatment of neuraminidase, periodate oxidation, and β elimination revealed the HMFG-2 and SM-3 epitopes (Fig. 8). In the present study we showed that it was not necessary to remove the entire oligosaccharide. Removal of the sialic acids was sufficient. The epitope of DF3 (18) includes part of the HMFG-2 and SM-3 epitopes, TR and TRP, respectively. Unlike HMFG-2 and SM-3, the reactivity of DF3 antibody seems to be slightly reduced by removal of sialic acids. It is not known if the sialic acids that enhance DF3 binding are on the same oligosaccharides as those that reduce binding by HMFG-2/SM-3. Alternatively, the action of sialic acid may be a general effect on the conformation of the entire mucin molecule and not specific to a particular oligosaccharide.

Patients with breast (33), ovarian (34), and pancreatic (35, 36) cancer produce cytotoxic T cells and antibodies (37, 38) that recognize peptide regions of MUC1. In breast mucins the epitope recognized by the SM-3 is normally masked but becomes more exposed in tumor cells (39, 40). These observations suggest that vaccines, based on the MUC1 peptide core, may be a possibility (38). However, the enhancing effect of neuraminidase in the present study suggests that access to the MUC1 protein core is still somewhat restricted by sialic acids on neighboring oligosaccharides. The protein core of surface MUC1 mucins in colon (41) and breast cancer cells (40, 41) is also shielded by sialic acids. Sialylation of MUC1 mucins occurs during a series of cycling events in which mucins are brought to the cell surface and then recycled back into the cell (42). Little is known about what regulates the extent to which MUC1 mucins are sialylated. It has been reported that circulating MUC1 mucins in patients with ovarian cancer are not as highly sialylated as mucins from normal tissues (43). A better understanding of the role of sialic acids in determining mucin structure may improve the effectiveness of vaccines directed against the MUC1 peptide.

In summary, this study has shown that in SW1990 mucins: (a) both sLea and sLex carbohydrate structures are present on MUC1; (b) sialic acids contribute significantly to the net negative charge of sLea and sLex rich mucins; and (c) the peptide binding sites of two MUC1
antibodies, HMF-G-2 and SM-3, are blocked by sialic acids on neighboring oligosaccharides.

ACKNOWLEDGMENTS

We thank Sandra Cheng for her assistance in the preparation of this manuscript.

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