Multiple Hepatic Receptors Cooperate to Eliminate Secretory Mucins Aberrantly Entering the Bloodstream: Are Circulating Cancer Mucins the "Tip of the Iceberg"?

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Abstract

Hollow organs lined by columnar epithelial cells normally secrete mucins and their proteolytic fragments vectorially into the lumen. These heterogeneously O-glycosylated molecules are known to aberrantly enter the bloodstream in the setting of epithelial carcinomas and possibly during injury or inflammation. We have recently shown that carcinoma mucin fragments can trigger the rapid formation of platelet-rich microthrombi *in vivo*. Thus, mechanisms to clear such aberrantly secreted mucins must exist. Indeed, we found that i.v. injected carcinoma mucin fragments had an ~1 minute half-life in mice, which was primarily due to rapid clearance by hepatic reticuloendothelial cells. Inhibition of known glycan-recognizing hepatic clearance receptors showed involvement of multiple partially overlapping clearance systems. Studies of genetically deficient mice and incomplete competition between different mucins confirmed this result. Thus, multiple hepatic clearance receptors cooperate to eliminate secretory mucins entering the circulation, limiting potential pathology. This may also explain why mucin-type clustered O-glycosylation is rare on plasma proteins. Notably, small subsets of injected carcinoma mucins remained unrecognized by clearance systems, had a much longer half-life, and carried highly sialylated O-glycans. Similar circulating mucins were found in tumor-bearing mice despite lack of saturation of hepatic clearance mechanisms. Thus, circulating cancer mucins currently used as clinical diagnostic markers likely represent only the clearance-resistant "tip of the iceberg." Such aberrantly circulating mucins could play pathologic roles not only in cancer but also during injury or inflammation of hollow organs and in liver disease. (Cancer Res 2006; 66(4): 2433-41)

Introduction

Mucosal epithelial cells lining hollow organs and glands produce and vectorially deliver membrane-bound and secretory mucins toward the lumen, where they function in lubrication and physical protection, and in specific interactions with pathogenic and commensal microbes. Mucins are glycoproteins defined by the presence of abundant O-linked oligosaccharides (O-glycans) clustered on a protein backbone. At least 13 mucin genes have been identified (1) that encode multiple mucin types (MUC), including membrane-bound molecules (*MUC1, MUC3A, MUC3B, MUC4, MUC12, MUC13,* and *MUC17*), secreted gel-forming ones (*MUC2, MUC5AC, MUC5B,* and *MUC6*), and others (*MUC7, MUC8, MUC9, MUC11,* and *MUC15, MUC16*; refs. 2–5). O-glycans actually constitute much of the weight of mucins. Variation in O-glycan monosaccharide content results in extensive molecular heterogeneity within a mucin (6), between different mucins (4, 5), between mixtures of mucins at different anatomic locations (7–10), and under different physiologic and pathologic states (11–15).

Occasional scattered O-glycans occur on some plasma proteins, e.g., IgA and erythropoeitin. However, true mucins with clustered O-glycans are rare in the vascular system, being mostly limited to high endothelial venules of lymphoid organs, or the surface of blood cells (16–22). Such vascular mucins often constitute ligands for the selectin family of adhesion molecules, coordinating interactions among leukocytes, endothelium, and activated platelets (reviewed in ref. 23).

Aberrant O-glycosylation is particularly prominent in epithelial cancers (4, 5, 11, 24), a feature that has been termed "glycodynamics" (15). Soluble heterogeneously O-glycosylated mucins can also aberrantly enter the bloodstream after such malignant transformation of mucosal epithelia. This exception to epithelial mucins being "outside" the body provides diagnostic biomarkers for detection and monitoring of cancer. For example, the CA19-9 antibody detects sialyl Lewis A (Neu5Acα2-3Galβ1-3[Fucα1-4]GlcNAc) presented on a circulating high-molecular-weight mucin, providing an assay for diagnosis and monitoring of stomach, colon, and pancreatic cancer (25); and O-glycans on circulating MUC16 recognized by antibody CA-125 provides for diagnosis and monitoring of ovarian cancers (26). "False-positives" in such assays arise in acute and chronic pancreatitis (27–29), acute cholangitis (30), cystic fibrosis (31), bacterial pneumonia (32), endometriosis (33), benign ovarian cysts, the first trimester of pregnancy, and pelvic inflammatory disease (33–36). Thus, although such biomarker assays have focused on cancer detection, epithelial mucins can also apparently "spill" into the circulation in a broad range of pathologies involving hollow organs and/or glands, including injury or inflammation.

We have recently reported that i.v. injection of carcinoma mucin fragments into mice triggers formation of extensive platelet- and leukocyte-rich microthrombi—a pathologic response that is thrombin independent but L-selectin and P-selectin dependent, and is heparin sensitive (37). This provided a potential pathophysiologic basis for the frequent association of "Trousseau syndrome" with mucinous adenocarcinomas. We next determined the fate of i.v. injected carcinoma mucins, presuming that they would be mostly trapped in leukocyte/platelet-rich microthrombi (37). Surprisingly, we found that the great majority of injected molecules are rapidly

cleared by the liver, with only a small fraction involved in forming microthrombi. We here elucidate the physiologic mechanisms for such mucin clearance, show that a distinct fraction of mucins is resistant to rapid clearance, and conclude that serum mucin marker tests currently used as diagnostic tests in cancer likely detect only this resistant "tip of the iceberg." Although the clearance mechanisms for a few specific glycoproteins has been previously described, there is, to our knowledge, no prior report of this type of "complex" mechanism by which an entire class of glycoproteins is efficiently cleared by a combination of overlapping receptors.

Materials and Methods

Cells and mice. Human colonic adenocarcinoma LS180 cells (ATCC CL 187; American Type Culture Collection, Manassas, VA) were cultured in αMEM medium containing 10% fetal bovine serum. Xenograft mucin-producing human tumors were generated by s.c. injecting 2×10^6 LS180 cells into the flanks of immunodeficient RAG2 null mice (129S6/SvEvTac-Rag2^{tm1}; Taconic Farms, Germantown, NY) to produce tumors as described (37). Hepatic lectin-2 null mice [HL-2; also called asialoglycoprotein receptor (ASGPR); ref. 38] and macrophage galactose (Gal) lectin-1 (MGL-1) null mice (39) have been previously described. P-selectin/L-selectin doubly deficient (PL-sel null) mice and wild-type C57Bl/6J mice were from The Jackson Laboratory (Bar Harbor, ME). All mouse studies were approved by the University of California San Diego Animal Subjects Committee.

Purification of soluble mucin fragments. LS180 human colon carcinoma mucins were prepared from xenografts grown in RAG2 null mice as described (37). Porcine submaxillary mucin (PSM) and ovine submaxillary mucin (OSM) were isolated from whole tissues as described (40). Bovine submaxillary mucin (BSM) was purchased from Sigma Chemical Company (St. Louis, MO) and porcine gastric mucin (PGM) was purchased from MP Biomedicals, Inc. (Aurora, OH). To generate concentrated solutions of nonviscous, high-molecular-weight, large-core mucin fragments, PSM, OSM, BSM, and PGM were exhaustively digested with 1% trypsin protease (Life Technologies, Gaithersburg, MD), 1% (w/w) PBS, and 0.01% sodium azide at 37°C overnight (41). Following digestion, the reactions were boiled for 5 minutes to inactivate residual enzyme activity and then subjected to size exclusion chromatography to collect the surviving large core mucin fragments as described (37). Eluted fractions were monitored by phenol sulfuric acid detection of neutral sugars (42). In each case, a large peak near the void volume >200 kDa was collected, dialyzed (10-12,000 MW cutoff) against 800 volumes of water at 4°C, lyophilized, and resuspended in sterile PBS (20 mg/mL) before use *in vivo*.

In vivo tracer studies. Mucin fragments were ^{3}H-labeled using NaB[^{3}H]$_4$, either in their sialic acid (Sia) residues after mild periodate oxidation of Sia side chains (43) or after Gal oxidase treatment of existing terminal Gal residues (44). The former method of ^{3}H-labeling causes a subtle change of one to two carbon atoms in the Sia residues but does not result in desialylation. This material was primarily used as a control to ensure that Sia recognition was not involved in clearance. The latter method introduces a label but also restores the original Gal back to its native state. Most of the tracer studies were done with this native material. Aliquots of radiolabeled mucin (1×10^6 cpm) dissolved in tissue culture–grade PBS (Invitrogen, Grand Island, NY) were injected into the lateral tail veins of mice (100 μL/mouse) anesthetized with isoflurane (VEDCO, St. Joseph, MO) mixed with or without a saturating concentration (typically ~2 mg/mouse) of unlabeled mucin or various inhibitors of hepatic clearance receptors. The following inhibitors were obtained from the sources as indicated: bovine serum albumin (BSA), ovalbumin, hyaluronic acid, chondroitin sulfate B (dermatan sulfate), keratan sulfate, fucoidan, and fetuin from Sigma Chemical; unfractionated heparin from Fujisawa (Deerfield, IL); methyl-2-acetamido-2-deoxy-α-D-galactopyranoside (methyl-α-GalNAc) from Calbiochem (San Diego, CA); and 3′-sulfo-Lewis X-PAA from GlycoTech (Rockville, MD). Asialofetuin was prepared by desialylation of fetuin as described (45). 3-Sulfo-Gal-cerebroside (sulfatide; Matreya, Pleasant Gap, PA) was sonicated in 1% BSA/PBS to generate

micelles; hyaluronic acid (20 mg/mL in PBS; Sigma Chemical) was sonicated to reduce viscosity. Low-density lipoprotein (LDL; 10 mL solution of ~155 mg/mL; ICN, Costa Mesa, CA) was treated with 10 μmol/L Cu_2SO_4 at 37°C for 6 hours to generate oxidized LDL (OxLDL; ref. 46), or treated with acetic anhydride (an initial addition of 1 mL saturated acetic anhydride followed by sequential additions of 50 μL every 5 minutes for 30 minutes, then a final addition of 200 μL with vigorous stirring at 4°C for 30 minutes) to generate acetylated LDL (AcLDL; ref. 47). Both reactions were stopped by dialysis (12-14,000 MW cutoff) against 800 volumes of PBS with 2 mmol/L EDTA at 4°C.

To calculate the percentage radioactivity remaining in plasma, whole blood was collected at various time points by tail bleed into dry EDTA tubes or by cardiac puncture into 1:10 (v/v) 3.8% sodium citrate. Small aliquots of plasma [obtained after centrifugation of blood at 1,200 relative centrifugal force (rcf) for 8 minutes at room temperature] were counted, and total cpm in circulation was calculated. The total blood volumes per mouse were assumed to be 5.5% lean body weight with a hematocrit of 44%.[1]

To calculate the percentage radioactivity recovered in various organs (lungs, liver, kidneys, spleen, heart, and brain), tissues were removed, briefly rinsed in saline, and then digested (1 mg/mL proteinase K, 50 mmol/L Tris, 100 mmol/L NaCl, 1% Triton, and 0.01% sodium azide) overnight at 55°C. Homogenates were centrifuged 10,000 rcf for 8 minutes at room temperature. Small aliquots of supernatant were counted to avoid quenching by endogenous chromophores and the total numbers were corrected accordingly. The tissues collected (plasma, liver, kidneys, spleen, lungs, heart, and brain) typically accounted for >90% recovery of injected material.

Histology. Mucin fragments were labeled with FITC at their amino groups using Fluoro Tag FITC Conjugation kit (Sigma Chemical). For histologic studies, 50 μg FITC-labeled mucin in 100 μL PBS was i.v. injected. Acetone-fixed cryosections (5 μmol/L) were labeled with purified rabbit anti-FITC (Zymed, South San Francisco, CA; PBS, 2% BSA, 0.02% NaN$_3$, 4°C overnight) and FITC-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) to amplify the signal of the FITC-labeled mucin fragments. Other antibodies used were purified rat anti-mouse F4/80 (Serotec, Inc., Raleigh, NC); CD31, Mac-1, and CD41 (PharMingen, San Diego, CA); and AffiniPure Cy^3-conjugated donkey anti-rat IgG (H+L; Jackson ImmunoResearch Laboratories). Tissue sections were mounted with Vectashield mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Inc., Burlingame, CA).

Mucin characterization. Profiles of O-linked glycans released from different mucin preparations were studied by matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) at the University of California San Diego Glycotechnology Core Facility. Briefly, O-glycans were released from mucins by reductive β-elimination using 1 mol/L sodium borohydride in 0.5 mol/L sodium hydroxide for 14 to 18 hours at 4°C. The reaction was neutralized on ice by stepwise additions of 5 μL glacial acetic acid to pH 6. The mixture of O-glycan alditols was desalted using an OnGuard II H column (Dionex Corp., Sunnyvale, CA) equilibrated with 5% acetic acid and then lyophilized. Excess borate methyl esters were removed from the O-glycans by coevaporating the borates with 10% acetic acid in methanol under a stream of nitrogen. Thoroughly dried samples were permethylated in 100 μL of 200 mg/mL NaOH/anyhydrous DMSO with 50 μL methyl iodide, and the reaction was quenched by addition of 200 μL of 1% acetic acid. The permethylated O-glycans were collected by organic extraction using chloroform, dried down, and brought up in water. The permethylated O-glycans were purified by Sep-Pak C18 column using aqueous acetonitrile stepwise elutions (0%, 15%, 35%, 50%, and 75% acetonitrile). Elution of O-glycans was observed only in the 50% acetonitrile eluate by UV absorption at 206 nm. The permethylated O-glycans were then analyzed by MALDI-TOF MS using 2,5-dihydroxybenzoic acid as the matrix. Mass accuracy was calibrated with dialyzed Maltrin (maltodextrin; Grain Processing Corporation, Muscatine, IA) containing fragments of glucose homopolymers.

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[1] Monica Justice. Clinical hematology parameters. Accession number MPD:132. Mouse Phenome database web site, The Jackson Laboratory. See http://www.jax.org/phenome.
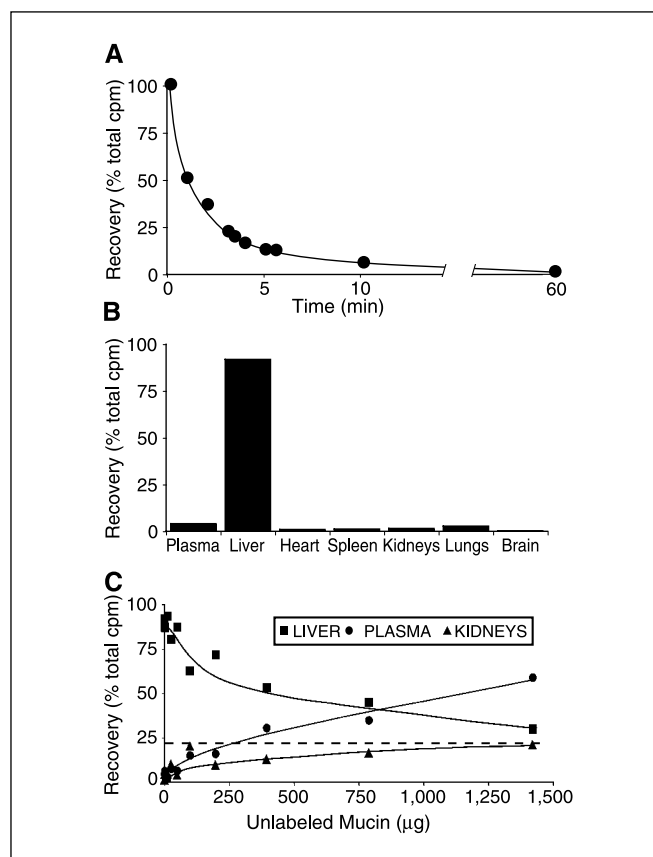
Analysis of resistant subsets of radioactive tracer mucins. Whole blood was collected by cardiac puncture into 50 μg/mL hirudin (Refludan; Aventis Pharmaceuticals, Inc., Bridgewater, NJ) at $t = 15$ minutes or $t = 60$ minutes from donor mice that had been injected with high levels of tracer mucin. Plasma prepared from these samples was then split for use in plasma clearance studies in a recipient mouse or for use in high-pH anion exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) of O-glycans.

Measurement of persistently circulating carcinoma mucins in tumor-bearing mice. Plasma was prepared from whole blood obtained by cardiac puncture into hirudin. Sialyl-Lewis X-PAA (GlycoTech; 200 ng/well) was coated onto ELISA plates overnight at 4°C in ELISA coating buffer [50 mmol/L sodium carbonate/bicarbonate (pH 9.5)]. Soluble recombinant murine E-selectin/human IgG Fc chimera probes were precomplexed with goat antihuman IgG antibody (Bio-Rad Laboratories, Inc., Hercules, CA) in ELISA buffer [20 mmol/L HEPES, 125 mmol/L NaCl, 2 mmol/L CaCl$_2$, 2 mmol/L MgCl$_2$, 1% BSA, 0.1% NaN$_3$ (pH 7.4)] for 1 hour at room temperature while rocking. A mixture of 90 μL probe solution and 10 μL mouse plasma was exposed to the coated wells overnight at 4°C. Following washing, binding of the probe was measured by secondary antibody conjugated to alkaline phosphatase, reacted in *pnp*-phosphate and detected by absorbance at 405 nm. One unit of inhibitory activity is arbitrarily defined as inhibition of selectin binding obtained with 1 μg of purified mucin after subtracting background inhibition seen with normal mouse serum.

O-Glycan analysis of plasma from tumor-bearing mice. To characterize mucins recovered from the circulation of mice, citrated plasma samples were subjected to profiling of O-linked glycans by high-performance liquid chromatography (HPLC) on a Dionex column at the University of California San Diego Glycotechnology Core Facility. Briefly, O-glycans were released from circulating mucins (including the background level of plasma protein O-glycans) by reductive elimination by adding 2 mol/L sodium borohydride in 1 mol/L sodium hydroxide (1:1 v/v) at 4°C overnight. The reaction was neutralized, desalted, and depleted of excess borates as describe above. Thoroughly dried samples were dissolved in 200 μL water and analyzed by HPAEC-PAD. Plasma samples from LS180 tumor-bearing mice were pooled ($n = 5$ mice that had elevated E-selectin plasma inhibition ELISA measurements). Preliminary comparisons of plasma versus purified LS180 mucins revealed six peaks that were unique to the mucins, thus carcinoma mucins could be identified among the background of peaks from plasma.

Results

Carcinoma mucins are rapidly removed from the circulation by the liver. Based on our prior work (37), our working hypothesis was that the i.v. injected ³H-labeled carcinoma mucins would be mostly bound by P-selectin– and L-selectin–expressing cells, forming microthrombi within the vasculature. Instead, we were surprised to find that the liver rapidly cleared most of the label from the circulation (Fig. 1A and B, range 81-95%; $n = 6$), and the circulating $t_{1/2}$ was only ~1 minute. Attempted saturation of hepatic uptake by coinjecting increasing amounts of nonradiolabeled mucins with the tracer gave a curvilinear response, leveling off in the 250 to 500 μg range (Fig. 1C). Corresponding increases in circulating plasma levels occurred. However, higher amounts of unlabeled mucins (up to 1.5 mg) still gave ~25% recovery of the radioactive tracer in the liver. P- and L-selectin blockade by heparin had no effect (Table 1; detailed data not shown). This was confirmed using P- and L-selectin double-deficient mice in which no significant reduction in clearance or liver accumulation was seen (Table 1, detailed data not shown). Thus, although blood cells in transit through the liver can bear L- and P-selectins, they have no major contribution to sequestering mucins in the liver (i.e., intrasinusoidal microthrombi do not simply "trap" mucins in the liver).

Figure 1. Carcinoma mucins are rapidly removed from the blood circulation by the liver. Typical results following i.v. injection of 1×10^6 cpm [³H]Sia-labeled carcinoma mucins in 0.1 mL PBS into the tail vein. *A*, blood samples were collected at the indicated times and the percentage of starting radiolabel in the plasma obtained by cardiac puncture was determined. *B*, tissue samples were obtained 10 minutes after injection, homogenized by enzymatic digestion, and the amount of radiolabel was determined. *C*, saturation of hepatic clearance by coinjection of increasing amounts of nonlabeled mucin mixed with the radiolabeled mucins. A clearance curve is shown for plasma (●), with corresponding uptake curves for liver (■) and kidneys (▲). *Dotted line*, background level in the liver resulting from plasma pooling in hepatic sinusoids.

An alternative explanation for the residual 25% in the liver after saturation of clearance is simply blood trapping within venous sinusoidal spaces of the liver (48). We determined the volume of this trapped blood by measuring liver extract hemoglobin levels compared with those of whole blood obtained by cardiac puncture. Correcting for the total volume of liver homogenates and assuming a total blood volume of 5.5% lean body weight with a 44% hematocrit (see Materials and Methods), we calculated that the isolated liver contains ~25% of the total blood volume, a number consistent with other reports (48). Thus, the leveling-off of the saturation curve with injections of unlabeled mucin is due to entrapment of plasma-associated mucin tracer inside the sinusoids and the hepatic clearance system for mucins is actually saturated with ~1 mg mucin per mouse at least at a 10-minute time point.

Mucins are sequestered in the liver by reticuloendothelial cells. To determine which cell types in the liver were involved in the uptake, we did histologic studies following i.v. injection of carcinoma mucins labeled at their amino termini with FITC. The FITC label colocalized with hepatic sinusoidal macrophages (Kupffer cells, F4/80-positive) and endothelium (CD31-positive), and to a very small degree with hepatocytes (CD31-negative

Table 1. Multiple hepatic clearance systems eliminate carcinoma mucins from the circulation

Inhibitor and/or genetically altered mouse	Percentage recovery of [³H]Gal mucins (%)		Inhibition of liver uptake*
	Plasma	Liver	Percent (range)
None	5	92	0
BSA	4	94	0 (0)
FET	5	92	0 (0-3)
ASF	14	78	20 (13-27)
GalNAcα-*O*-Me	14	79	20 (19-20)
ASF/GalNAcα-*O*-Me	11	78	20 (15-27)
MHL-2 null	16	81	16 (7-27)
MHL-2 null + ASF	20	72	29 (25-36)
MHL-2 null + GalNAcα-*O*-Me	12	73	36 (36)
MGL-1 null	9	83	10 (6-15)
MGL-1 null + ASF	20	67	27 (27)
MGL-1 null + GalNAcα-*O*-Me	8	88	4 (4)
OvA	12	82	15 (11-17)
MHL-2 null + OvA	11	73	36 (15-44)
FCN	16	73	21 (15-26)
3′-Sulfo-LeX-PAA	8	87	7 (7)
Sulfatides (3-sulfo-Gal-ceramides)	10	85	11 (9-11)
OxLDL + AcLDL	18	71	19 (12-25)
HA	10	85	8 (0-22)
HEP	5	92	0 (0)
P-/L-Selectin null	5	89	4 (0-7)
ASF/OvA/HA/FCN	49	40	76 (64-87)

NOTE: [³H]Gal-labeled LS180 carcinoma mucins were coinjected with ∼2 mg of the indicated clearance system inhibitor and/or into genetically modified mice (*n* = 3-6 mice per group). At 10 minutes following injection, the amount of radiolabel was determined in blood plasma and liver. For more details, see expanded table in Supplementary Table S1.

Abbreviations: FET, fetuin; ASF, asialofetuin; OvA, ovalbumin; FCN, fucoidan; HA, hyaluronan; HEP, heparin.

*Inhibition of liver uptake was corrected for entrapment of ∼25% of plasma-associated tracer within liver sinusoidal spaces (see Materials and Methods).

sialylated fetuin (three N-glycans and three O-glycans per molecule, containing α2-3-Sia and α2-6-Sia; ref. 49) did not compete for mucin uptake. Furthermore, labeled mucins with truncated Sia side chains had a similar clearance rate compared with those that did not (hepatic uptake at t = 10 minutes of [³H]Sia-labeled mucins 92% versus [³H]Gal-labeled mucins 95%; data not shown). Most further studies were done with the [³H]Gal-labeled mucins in which the labeled Gal is maintained in its original native state.

Clearance by Gal-recognizing receptors. Hepatic clearance of macromolecules and microbes bearing terminal Gal/GalNAc residues can be potentially mediated by a somewhat overlapping set of receptors expressed by hepatocytes [ASGPR, also known as mouse hepatocyte lectin (MHL)-1 and MHL-2] and macrophages (Kupffer cell Gal/GalNAc lectins, MGL-1, and MGL-2 in the mouse). Both types of receptors form homo-oligomer/hetero-oligomer, permitting recognition of a broad range of glycans containing terminal βGal, βGalNAc, or αGalNAc (39, 50). The ASGPR was also recently reported to recognize Siaα2-6GalNAcβ1-2Man (51).

Incomplete sialylation of some of the mucin glycans can result in exposure of various terminal monosaccharides, particularly Gal and GalNAc. Mucin tracer uptake was reduced by ∼16% in ASGPR null mice and by ∼20% after coinjection of 2 mg asialofetuin into wild-type mice. Controls showed that 2 mg asialofetuin (but not intact sialylated fetuin) efficiently blocked hepatic uptake of radiolabeled asialofetuin (data not shown). GalNAc-α-*O*-methyl glycoside (in either free monosaccharide form or as a cluster of approximately eight monosaccharides conjugated to polyacrylamide), which mimics the Core GalNAc-Ser/Thr linkage of O-glycans (Tn antigen) reduced uptake by 20%. This likely reflects inhibition of MGL-2 receptors, indicating an overlapping involvement of Kupffer cell MGLs with the hepatocyte ASGPR. Blockade of all Gal/GalNAc receptors (i.e., including

Figure 2. Mucins are sequestered in the liver by hepatic reticuloendothelial cells. Carcinoma mucins were FITC labeled at their amino termini and were i.v. injected. Frozen sections of livers following i.v. injection of 50 μg FITC-labeled carcinoma mucins in 0.1 mL PBS into the tail vein. Detection of FITC-mucins was visually enhanced using rabbit anti-FITC antibody followed by goat anti-rabbit IgG-FITC. *Arrows,* examples of areas of maximal green fluorescence. Tissue sections were counterstained with DAPI to visualize nuclei (*blue*). Colocalization is shown at 1 and 30 minutes following injection of FITC-mucins, with red fluorescent Kupffer cells (F4/80-Cy3; *A* and *C*) and endothelium (CD31-Cy3; *B* and *D*). Mucins are bound to the cell surface of Kupffer cells and endothelial cells at 1 and 30 minutes are localized intracellularly in these same cell types.

parenchymal cells identified by nuclear DAPI labeling). No colocalization was observed with monocytes and granulocytes (Mac-1 positive), or with platelets (CD41-positive). Labeled mucins associated with the lumenal face of Kupffer cells and endothelial cells at 1-minute postinjection (Fig. 2*A* and *B*) and were compartmentalized inside the same cell types at 30 minutes (Fig. 2*C* and *D*).
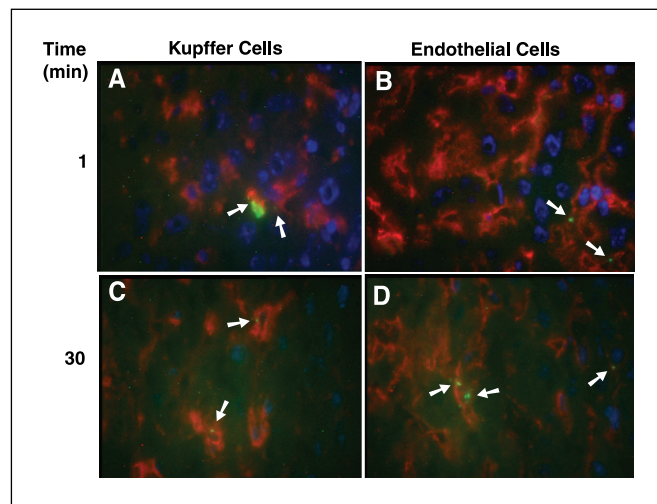
Hepatic uptake is mediated by multiple clearance receptors. To identify receptors responsible for eliminating mucins from the bloodstream, previously known hepatic clearance systems were inhibited with appropriate reagents and/or by using genetically altered mice (all results are summarized in Table 1; details are presented in Supplementary Table S1). BSA was an ineffective competitor and was used as a routine control. Uptake is also likely not dependent on Sia because this is present in high amounts on circulating plasma proteins. In keeping with this, intact fully

hepatocyte and Kupffer cell receptors) by injection of asialofetuin into ASGPR null animals reduced uptake by ~ 29%. Thus, a significant fraction of the carcinoma mucins (but not the majority) can be cleared by a combination of these Gal/GalNAc receptors.

Clearance by mannose/GalNAc-4-SO₄ receptors. The Man/GalNAc-4-SO₄ receptor is a multifunctional phagocytic/endocytic receptor (reviewed in ref. 52). When expressed as a monomer by liver macrophages, the Man/GalNAc-4-SO₄-R recognizes glycoconjugates terminating in Man, GlcNAc, and Fuc by way of C-type lectin carbohydrate recognition domains. In addition to their dense array of O-glycans, mucins also carry some N-glycans containing Man residues (4). MUC1 was also recently reported to be recognized by the Man/GalNAc-4-SO₄-R expressed on dendritic cells (53). Inhibition of the hepatic Man/GalNAc-4-SO₄-R with 2 mg ovalbumin (54), which bears high-mannose- and hybrid-type N-glycans, reduced uptake by ~ 15%, indicating a minor role of the Man/GalNAc-4-SO₄-R on either Kupffer cells or endothelial cells. Ovalbumin injections in ASGPR null mice showed no added inhibition, indicating that ovalbumin likely blocked only Man/GalNAc-4-SO₄ receptors.

Liver sinusoidal endothelial cells predominantly express dimeric forms of the Man/GalNAc-4-SO₄ receptor, which have inactive C-type lectin carbohydrate recognition domains, but instead contain functional terminal cysteine-rich domains (cys-MR) with "R-type" carbohydrate recognition domains that recognize certain sulfate esters (55). Possible natural ligands include glycoprotein hormones, microbes, sulfated glycolipids, and glycosaminoglycans that bear various sulfate residues (56). More detailed analyses show that Man/GalNAc-4-SO₄-R recognizes 3-O-sulfo-Gal and 4-O-sulfo-GalNAc on N-glycans, 3-SO₄-Lewis X and 3-SO₄-LeA (but not 6-SO₄-Lewis X or 6-SO₄-LeA), as well as polyanionic ligands such as acetyl-LDL, poly(I), fucoidan, and lipopolysaccharide (57).

Two types of sulfate esters characteristic of mucin O-glycans are 3-O-sulfo-Gal and 6-O-sulfo-GlcNAc. Inhibition of the Man/GalNAc-4-SO₄-R with sulfatides (3-sulfo-Gal-ceramides) or 3′-sulfo-Lewis X-PAA had limited effects, reducing uptake by 7% and 11%, respectively (but see also the following section). These results suggest that hepatic mucin uptake is not primarily mediated by the Man/GalNAc-4-SO₄-R on endothelial cells. Furthermore, lack of inhibition by Keratan Sulfate (a repeating sequence of [(6-O-sulfo)Galβ1-3(6-O-sulfo)GlcNAc]$_n$) suggests that uptake is not mediated by a novel receptor that recognizes 6-O-sulfo-GlcNAc residues on carcinoma mucins.

Clearance by scavenger receptors. The family of scavenger receptors (SR) expressed in the liver include endothelial cell and Kupffer cell SR-AI and SR-AII, hepatocyte SR-BI (CD36-related; ref. 58), and Kupffer cell SR-D (CD68, macrosialin). Although all SRs recognize acetylated proteins and lipoproteins and/or oxidized lipoproteins, SR-AI/II and SR-D also recognize some polyanionic compounds (59, 60), including chondroitin sulfate A and C (but not D), poly(I) and polyguanylic acid, fucoidan, lipopolysaccharide, and carcinoma mucins (61).

The tracer mucins used here contained ~ 17% Sia (w/w total carbohydrate; ref. 37), the majority of which were Neu5Ac (80%) and Neu5Gc (10%) along with smaller amounts of mono-O-acetylated and di-O-acetylated forms (by HPLC-DMB analysis; data not shown). SRs could potentially recognize such acyl moieties of Sia and/or the polyanionic charge of mucins. Broad inhibition of SRs with OxLDL plus AcLDL reduced uptake by ~ 19% (Table 1).

Specific inhibition of SR-AI/II and SR-D with coinjection of 3′-sulfo-Lewis X and sulfatides (3-O-sulfo-Gal-ceramide; ref. 59) suggested that uptake of ~ 10% of mucins is mediated by SR-AI/II and/or SR-D (potential overlap with the Man/GalNAc-4-SO₄-R; see above). Subtracting this value of SR-AI/II and/or SR-D inhibition from total SR inhibition (i.e., including SR-BI) suggests that SR-BI contributes ~ 9% (59). Overall, we conclude that SRs contributed to ~ 19% clearance of the tracer mucins, divided among SR-AI/II and/or SR-D (~ 10%) and SR-BI (~ 9%).

Clearance by the hyaluronan receptor. The hyaluronic acid receptor for endocytosis expressed by hepatic sinusoidal endothelial cells mediates a very active constitutive clearance system that recognizes hyaluronan (HA) [(Galβ1-4GlcA)$_n$] and chondroitin sulfate [4- and/or 6-sulfated (GalNAcβ1-4GlcA/IdoA)$_n$; ref. 62]. Inhibition of the hyaluronic acid receptor for endocytosis with 2 mg hyaluronan (63) reduced uptake of mucins by ~ 8%. Because heparin and keratan sulfate had no effect, the effect of hyaluronan is not simply due to the fact that it is a polyanion.
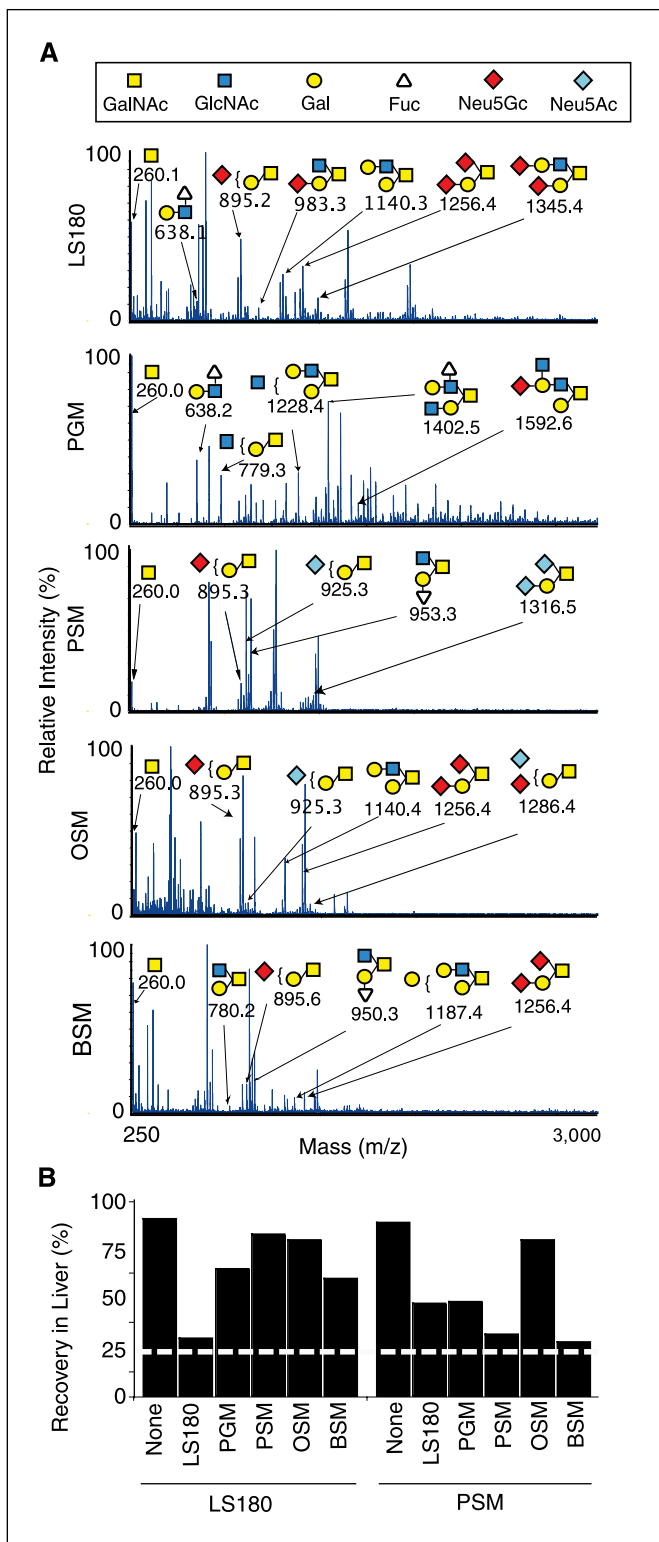
Fucoidan, which inhibits multiple receptors, including the Man/GalNAc-4-SO₄-R (55, 64), SR-AI/II and SR-D (65), and P-selectin and L-selectin (66, 67), reduced uptake by ~ 21%. Finally, a mixture of competitors (asialofetuin/ovalbumin/hyaluronan/fucoidan) that blocked most of the potential clearance systems, including ASGPR, MGL-2, MR, Man/GalNAc-4-SO₄, SRAI/II, SRD and HR, blocked ~ 76% of uptake (Table 1). This essentially reflects complete inhibition as hepatic uptake is inversely proportional to plasma levels, and plasma-phase tracer mucins entrapped inside liver sinusoidal spaces can represent ~ 25% of total blood plasma (see above).

Different mucins target unique profiles of receptors. Mucins produced at different anatomic locations or by different species have distinctive O-glycan profiles. To ask if such differences determine the relative use of different clearance receptors, we purified mucin fragments from various sources and tissues, verified their distinctive O-glycan profiles, and did cross-competition studies. MALDI-TOF MS spectra of the O-glycans from the different mucin preparations show extensive heterogeneity and a unique profile for each one (Fig. 3A). Each profile includes several previously described O-glycan structures. Among the preparations characterized, PGM showed the most diverse array of O-glycans, ranging from large sialylated blood group antigens [i.e., sialyl Lewis X; Siaα2-3Galβ1-4[Fucα1-3]GlcNAc; near 2,500 m/z] to the monomeric core structure Tn antigen (GalNAcα; near 250 m/z). In contrast, PSM showed the simplest profile, with only a few types of O-glycans consisting of three or four sugar residues. The human colon carcinoma mucins (LS180), BSM, and OSM showed intermediate profile complexities. Importantly, none of the five mucins had equivalent profiles.

Given extensive structural heterogeneity among these mucins, we asked how well one mucin blocked the uptake of another (Fig. 3B). The LS180 carcinoma mucin and PSM showed nonreciprocal cross-competition; LS180 could block the uptake of PSM but PSM was able to only partially block uptake of LS180 mucin. Of the other mucins studied, only BSM significantly affected the uptake of PSM (~ 60% inhibition). These two mucins also happen to have the most closely matching profiles of O-glycans (see Fig. 3A). Thus, the uptake of mucins is likely mediated by multiple receptors participating to varying degrees for a given source and type of mucin.
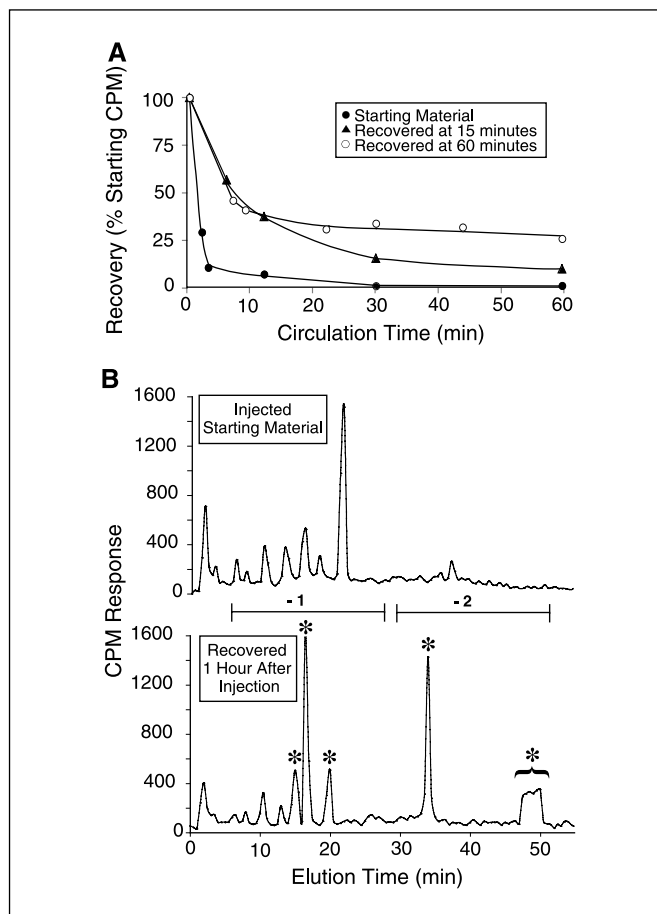
Small subsets of mucins are resistant to hepatic clearance. We next considered if heterogeneity within a given mucin

Figure 3. Selectivity of hepatic clearance mechanism for soluble mucins. Mucin preparations obtained from various tissues and species were structurally characterized before comparing their hepatic uptake properties. *A,* MALDI-TOF MS of permethylated O-glycans released from xenografted LS180 human colonic carcinomas (LS180), PGM, PSM, OSM, and BSM. Selected peaks are annotated with most probable structures; however, various isomeric structures are also possible. *B,* mucins from different sources (2 mg/mouse) were tested for their ability to cross-compete uptake of tritiated forms of either LS180 carcinoma mucins or PSM (label in the Sia side chains). *Dotted line,* background level expected due to blood pooling in liver sinusoids.

preparation was reflected in clearance kinetics. Initial results had indicated that ∼10% of carcinoma mucins were resistant to immediate clearance from the blood circulation (see Fig. 1*B*) and at 60 minutes postinjection, ∼2% still circulated. To differentiate this small, "resistant subset" from the rapidly cleared "starting material," plasma samples were obtained from tracer mucin–injected mice at various time points and injected back into a second mouse to follow clearance of these resistant subsets (Fig. 4*A*). As a control, similar volumes of normal plasma were added to the "starting material" tracer mucin, injected, and shown to have a clearance curve similar to that of injected tracer mucin alone ($t_{1/2}$ = 1 minute). Circulating tracer mucins from a donor mouse at 15-minute postinjection had a 10-fold enhanced plasma half-life in a recipient mouse ($t_{1/2}$ = 10 minutes). Circulating mucins obtained at 60 minutes postinjection also had a 10-fold enhanced plasma half-life ($t_{1/2}$ = 10 minutes); however, after ∼10 minutes postinjection, the clearance curve rapidly stabilized at ∼30% not eliminated and remained stable for up to 1 hour. These data show that the removal

Figure 4. Subsets of soluble carcinoma mucins are resistant to clearance. *A,* clearance curves of mice receiving a single bolus injection of 200 μL hirudin-anticoagulated mouse plasma added to radiolabeled LS180 mucin fragments (●); 200 μL plasma obtained from a donor mouse 15 minutes following i.v. injection of radiolabeled mucins and reinjected into another mouse (▲); and 200 μL plasma obtained from a donor mouse 1 hour following i.v. injection of radiolabeled mucins and reinjected into another mouse (○). *B,* O-glycan profiles obtained by HPAE chromatography coupled with radioactive detection of elution fractions. Spectra of normal mouse plasma added to tracer mucin labeled in Sia side chains and plasma obtained 1 hour following i.v. injection of tracer mucin. *, peaks unique to the 1-hour time point. *Brackets,* expected elution times for mono- (−1) and di- (−2) sialylated glycans.
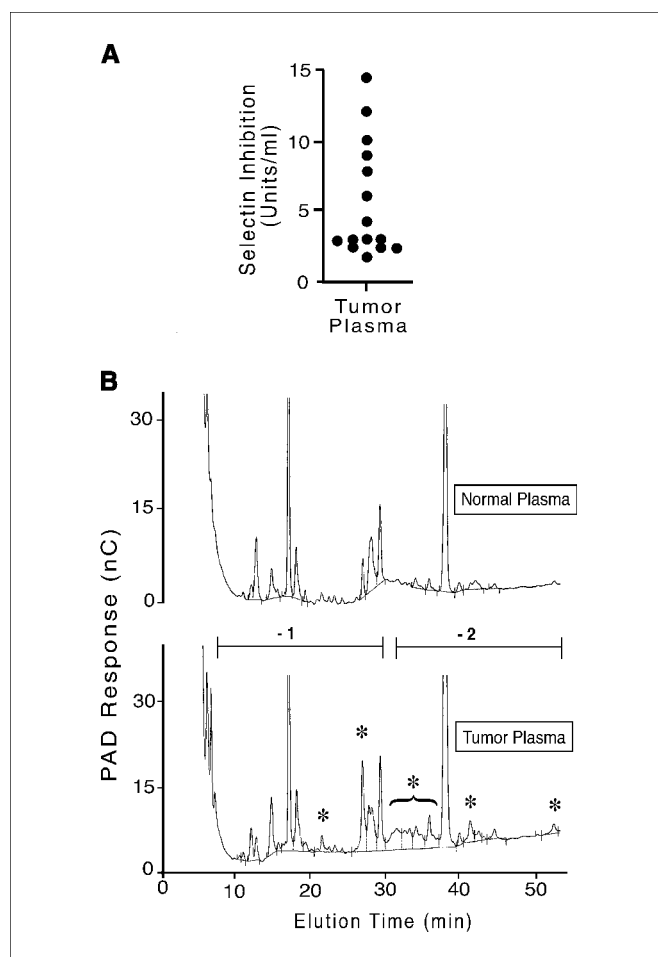
of soluble mucins from the bloodstream is likely dependent on structural differences among the glycans attached to the mucins. Thus, the kinetics of clearance is a function of the heterogeneity present in a single source of mucins.

Clearance-resistant mucin subsets are enriched in heavily sialylated O-glycans. Plasma samples were obtained at various time points from mice injected with the tracer mucins labeled in the Sia residues, and profiles of the sialylated O-glycans determined by HPLC analysis (Fig. 4*B*). Compared with the profile in the starting material (plasma added to the radiolabeled mucin fragments), 60 minutes of circulation in the mouse effectively enriched a distinct subset, containing more of the larger, more heavily sialylated structures. These data also assure that the small subset of surviving label is indeed in mucin glycans and not in some trace contaminant. This is consistent with the notion that terminal sialylation shields underlying glycans that would otherwise be recognized by various clearance systems. Thus, a small subset of total tumor mucins is not recognized by the different hepatic clearance systems and has a much longer circulating half-life.

Circulating mucins in tumor-bearing mice are also structurally distinct, long-lived subsets. We had earlier shown features of Trousseau syndrome (platelet-rich microthrombi in tissues and microangiopathic hemolytic anemia) in mice carrying mucin-producing LS180 tumors (37). Mice carrying such xenografted tumors (which were the original source for the carcinoma mucins used in these studies) were now screened for the presence of naturally circulating tumor mucins using an ELISA-based assay similar to the commercially available tests used to monitor cancers in humans. The assay used a recombinant soluble E-selectin-IgFc chimeric probe binding to a stationary sialyl Lewis X–polyacrylamide target antigen. Plasma samples from multiple tumor-bearing mice were anticoagulated with hirudin and mixed with the soluble selectin probe. Inhibition of binding above that seen with normal plasma indicated circulating mucins that competed for E-selectin binding to the target sialyl Lewis X antigen (Fig. 5*A*). A standard curve of normal mouse plasma added to a titration of mucin fragments indicated a sensitivity of the assay to be in the low μg/mL range. All tumor-bearing mouse plasma samples studied showed elevated levels of circulating mucin inhibitor, with a median value of ∼5 μg/mL (range = 2-14 μg/mL, *n* = 16 mice). No significant correlation was noted between tumor size and concentration of circulating mucin (range of xenograft tumor sizes 0.1-2 g; data not shown). The concentrations of circulating mucin detected in the mice were similar to those frequently observed in humans (68), which occasionally are even >100 μg/mL (69).

To show that the inhibitory activity of tumor plasma is indeed in mucins, as opposed to sialylated glycolipids that can also be released into the bloodstream by tumors (70), we analyzed tumor versus normal plasma for the profile of O-glycans (Fig. 5*B*). Tumor-specific O-glycan peaks were present at levels significantly above the background pattern of normal plasma, consistent with the low concentration of circulating mucins detected by the inhibition ELISA method.

Hepatic clearance systems are not saturated in tumor-bearing mice. Finally, we asked whether these naturally circulating tumor mucins had saturated some of the hepatic clearance receptors identified in Table 1. In fact, hepatic uptake of the i.v. injected tracer mucins purified from the same xenografted LS180 tumors was not different from that observed in normal mice (92% and 95% at 10 minutes postinjection, respectively). Because these

Figure 5. Evidence for persistently circulating carcinoma mucins in tumor-bearing mice. *A,* plasma was prepared from whole blood obtained by cardiac puncture into hirudin. Plasma samples from normal versus LS180 tumor-bearing mice were assayed for competitive inhibition of binding between a soluble E-selectin:IgG chimeric probe and a stationary phase sialyl-Lewis X target. Binding was measured by secondary antibody conjugated to alkaline phosphatase, reacted in pNPP, and detected by absorbance at 405 nm. Inhibitory activity of tumor-bearing mouse plasma (*n* = 15) above that seen with normal mouse plasma (*n* = 40). *B,* profiles of O-glycans obtained by HPAE chromatography coupled with PAD detection of glycans. O-glycans present in plasma from normal mice and from LS180 xenograft tumor-bearing mice, showing circulating tumor mucins. *, peaks above the background of normal plasma O-glycans. *Brackets,* expected elution times for O-glycans carrying negative charges of one (−1) or two (−2) Sia residues.

naturally circulating tumor mucins do not compete for hepatic uptake of the tracer mucins, they evidently represent a distinct subset of total tumor mucins entering the bloodstream that remained resistant to hepatic clearance.

Discussion

We and others have presented extensive evidence that aberrant mucin expression by carcinomas affects their progression and spread (4, 5, 11, 15, 24, 71). In our own work, we have shown that selectin-mucin interactions facilitate hematogenous metastasis (71-74) and that soluble circulating carcinoma mucins can account for many of the classic features of Trousseau syndrome, a hyperthrombotic state associated with mucin-producing carcinomas (37). In pursuing the latter observation, we asked about the fate of soluble carcinoma mucins that enter the circulation. To our

surprise, we found that the circulating half-life of the bulk of such mucins is only ~ 1 minute, and that the great majority is cleared by liver rather than being trapped within disseminated microthrombi containing selectin-expressing platelets and leukocytes.

We further show here that multiple hepatic clearance systems contribute to varying extents to this newly discovered process that clears soluble epithelial mucins that aberrantly enter the circulation. Receptors collaborating in the clearance of human colon carcinoma mucins included the hepatocyte ASGPR (HL-I/II), Kuppfer cell Gal-Rs (MGL-1 and 2) and Man/GalNAc-4-SO$_4$-R, endothelial cell Man/GalNAc-4-SO$_4$-R, hyaluronic acid receptor for endocytosis, and SRs (SRAI/II, SRD, and SRBI). Such a "complex" high-capacity clearance mechanism actually makes physiologic sense, ensuring that secretory mucins from any hollow organs or glands that happen to enter the circulation are cleared before they cause pathologies, such as those involving platelet and leukocyte aggregation. Indeed, mucins purified from different anatomic locations were all rapidly cleared by the liver, but none completely competed clearance of another, again indicating that each source of mucins engages a variable set of clearance receptors.

Mice carrying mucin-producing tumor xenografts had elevated levels of circulating tumor mucins. However, these did not compete for uptake of injected tracer mucins, indicating that they represent clearance-resistant subsets. Naturally circulating tumor mucins in cancer patients are therefore likely composed of such structurally distinct minor subsets that survive clearance (i.e., they represent the "tip of the iceberg," an analogy adopted to indicate ~ 10% persisting in plasma and ~ 90% sequestered in the liver). Future half-life studies should determine whether the very high levels of circulating mucins seen in some cancer patients is due to voluminous spillage of epithelial mucins that saturate hepatic clearance systems or due to enrichment of the resistant subsets over time. Regardless, this system is an important molecular mechanism regulating the clearance kinetics of serum tumor markers (75) that are used by clinicians to diagnose and monitor cancer in humans. It also seems very likely that this is a mechanism to clear traces of normal gastrointestinal tract mucins that may be entering the systemic circulation in the healthy state and/or during injury or inflammation. Studies comparing the levels of mucins in portal blood versus systemic blood would be one approach to test this hypothesis. Future studies might also determine if the integrity of the mucin clearance system is altered in patients with liver dysfunction. Elevated levels of the serum CA19-9 mucin antigen are in fact associated with hepatocellular disease and liver cirrhosis, although the liver is not a mucin-producing tissue. Given the observation that mucin serum markers can be detected at low levels in healthy subjects, the elevated levels seen in liver disease may simply represent failure of the mucin clearance system to remove the low levels of naturally "spilt" mucins. This may also provide a potential explanation for the link between liver disease and "false positive" laboratory tumor marker results (76), as well as some of the unexplained coagulopathies seen in chronic liver disease (77).

Additional studies should also determine if long half-lives of the "resistant" subset of the mucins correlate with selectin-binding activity. Based on our findings, such mucins would be expected to be relatively abundant in sialylated and/or sulfated blood group antigens. Consequently, the popularly used serum marker tests that detect this family of epitopes (CA19-9, CA125, CA15.5, etc) might also provide information on thrombogenic risk (e.g., pathologic selectin-mucin interactions) in patients suspected of harboring mucin-producing tumors and in a broad range of mucosal pathologies. It is also possible that there are actually three broad subsets of such mucins originating from carcinomas. The first large subset is cleared rapidly by the liver. The second smaller subset may be the one that interacts with selectins, triggering Trousseau syndrome. A final small subset may then be biologically inert material that is neither cleared nor "trapped" within microthrombi but continues to circulate, providing the tumor markers used by clinicians. Further studies are needed to determine what exactly are the structural features that differentiate these three proposed classes of mucins.

Finally, our data might provide an explanation for why true mucin-like clustered O-glycan patterns are a rare feature inside the vasculature, limited to only a few examples of restricted expression of selectin ligands, and why blood plasma glycoproteins are dominated by N-glycans whereas those with O-glycans are rare, especially as clusters of O-glycans.

Acknowledgments

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