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P-selectin mediates metastatic progression through binding to sulfatides on tumor cells

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Hematogenous carcinoma metastasis is associated with tumor cell emboli formation, which is now known to be facilitated by selectins. P-selectin-mediated interactions of platelets with cancer cells are based mostly on mucin- and glycosaminoglycan-type selectin ligands. We previously showed that mouse colon carcinoma cells (MC-38) carry P-selectin ligands of nonmucin origin, which were not identified. Here we show that P-selectin ligands recognized on MC-38 cells are sulfated glycolipids, thereby facilitating experimental metastasis in a syngeneic mouse model. Metabolic inhibition of sulfation by incubation of cells with sodium chlorate almost completely abrogated P-selectin binding. Metabolic labeling of MC-38 cells with ^{35}S sulfate revealed only a single band as detected by high-performance thin layer chromatography analysis of a total lipid extract. Matrix-assisted laser desorption/ionization tandem time-of-flight/time-of-flight analysis (MALDI-TOF-TOF) analysis of the purified sulfate-containing lipid fraction identified the selectin ligand to be a sulfated galactosylceramide SM4 (HSO_3-3Galβ-1Cer). Modulation of glycolipid biosynthesis in MC-38 cells altered P-selectin binding, thereby confirming sulfoglycolipids to be major P-selectin ligands. In addition, P-selectin was also found to recognize lactosylceramide sulfate SM3 (HSO_3-3Galβ-4Glcβ-1Cer) and gangliotriaosylceramide sulfate SM2 [GalNAcβ-4(HSO_3-3)Galβ-4Glcβ-1Cer] in human hepatoma cells. Finally, the enzymatic removal of sulfation from the cell surface of MC-38 cells resulted in decreased P-selectin binding and led to attenuation of metastasis. Thus, SM4 sulfatide serves as a native ligand for P-selectin contributing to cell–cell interactions and to facilitation of metastasis.

Key words: carbohydrate sulfation/glycolipids/MALDI-TOF/metastasis/selectin

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Introduction

A common phenotypic change associated with malignancy is the dramatic alteration of cellular glycosylation. Differences in the expression of glycoproteins and glycosphingolipids were predominantly defined by monoclonal antibodies (Hakomori 1996; Kim and Varki 1997; Kannagi et al. 2004). Structural identification of glycolipid antigens revealed that not all glycans are uniquely "tumor-specific", yet are predominantly present on tumors. Significant correlations between aberrant glycosylation of primary tumors and clinical prognosis have stimulated interest into identification of their biological function (Irimura et al. 1993; Kim and Varki 1997; Kannagi et al. 2004). Tumor cell carbohydrates were shown to participate in cell–cell interactions, and glycosphingolipids in particular were identified as potential effectors of signal transduction (Hakomori 1985; Kannagi et al. 2004).

Sulfatides are a class of sulfate-containing glycosylceramides distributed in various tissues, including the brain, kidney, and gastrointestinal tract (Natomi et al. 1993; Ishizuka 1997). Biosynthesis of sulfatides requires transfer of sulfate to the glycolipid moiety, which is catalyzed by a unique cerebroside sulfotransferase (CST) (Honke et al. 1997; Hirahara et al. 2000). The physiological function of sulfated glycosylceramides has been investigated in mice lacking the CST gene, which caused a complete absence of sulfatides (Honke et al. 2002; Ishibashi et al. 2002). The lack of sulfatides resulted in neurological disorders and arrest of spermatogenesis. Several studies provided evidence that sulfatides expressed on cell surfaces of different cells exert biological functions through mediating interactions with various proteins, such as laminin, thrombospondin, amphoterin, selectins, galectin, and hepatocyte growth factor (Roberts et al. 1985; Aruffo et al. 1991; Suzuki et al. 1993; Kobayashi et al. 1994; Shikata et al. 1999; Rouhiainen et al. 2000; Merten and Thiagarajan 2001; Ideo et al. 2005). Some of these proteins are adhesion molecules that are involved in cell–cell and cell–extracellular matrix interactions. In this context, selectins and laminin were shown to be involved in metastasis (Roberts et al. 1985; Aruffo et al. 1991; Kobayashi et al. 1994; Shikata et al. 1999).

Selectins are vascular adhesion molecules involved in leukocyte trafficking, inflammation, thrombosis, autoimmunity, and cancer (Varki 1994; Kansas 1996). The selectin family (P-, E-, and L-selectin) recognizes sialyl Lewis$^{x/a}$-containing structures, which are normally found on mucin-type glycoproteins of leukocytes and endothelium (Dennis and Laferte 1987; Nakamori et al. 1993; Hakomori 1996; Kannagi 1997). The observed correlation between sialyl Lewis$^{x/a}$ expression and a poor prognosis due to tumor progression and metastasis (Nakamori et al. 1993; Hakomori 1996) was further validated

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in different mouse models, where E-, P-, and recently also L-selectin were associated with metastatic progression (Biancone et al. 1996; Kim et al. 1998; Borsig et al. 2001, 2002; Ludwig et al. 2004). In addition to sialylated and fucosylated mucin structures, P- and L-selectin are able to efficiently bind sulfatides (Aruffo et al. 1991; Suzuki et al. 1993; Bajorath et al. 1994; Ishizuka 1997). L-selectin mediation of monocyte infiltration in renal interstitial inflammation was shown to be facilitated by sulfatides, thus demonstrating sulfatides to be endogenous ligands of L-selectin (Shikata et al. 1999). Human neutrophils could be activated by sulfatides through L-selectin binding (Laudanna et al. 1994). Additionally, P-selectin was shown to bind efficiently sulfatides on the plasma membranes of granulocytes and human tumor cells in vitro (Aruffo et al. 1991).

Elevated levels of sulfatides were detected in gastric, hepatocellular, and renal cell carcinomas (Hiraiwa et al. 1990; Kobayashi et al. 1994; Morichika et al. 1996), which was shown to be due to upregulation of CST (Morichika et al. 1996; Ishizuka 1997; Honke et al. 1998; Zhong Wu et al. 2004). Significantly higher expression of sulfatides in colorectal carcinomas and ovarian carcinomas was correlated with poor prognosis (Morichika et al. 1996; Makhlouf et al. 2004). Sulfatides were shown to mediate carcinoma adhesion to laminin and vitronectin, thereby modulating metastatic potential of renal cell and hepatocellular carcinomas (Kobayashi et al. 1994; Zhong Wu et al. 2004). Alteration of glycolipid expression, including expression of sulfatides, led to specific reduction of laminin-mediated attachment and migration (Inokuchi et al. 1990). Although sulfatide-mediated interactions of carcinomas with selectins were shown in vitro, their potential to facilitate metastasis had not been investigated.

Carcinoma-associated P-selectin ligands are mostly sialylated, fucosylated glycans on tumor cell mucins (Byrd and Bresalier 2004). Previously, we suggested the possibility that sulfoglycosylceramides could be involved in selectin-mediated metastasis (Borsig et al. 2002). Here we validate sulfatides as functional P-selectin ligands and evaluate their potential to facilitate metastasis of mouse carcinoma cells.

Results

Mouse colon carcinoma cell line MC-38 contains sulfated selectin ligands

Previously, we showed that MC-38 cells possess ligands for L- and P-selectin (Borsig et al. 2002). However, the nature of the ligands had not been fully determined. To identify the functional selectin ligands, MC-38 cells were stained with recombinant selectin chimeras after treatment with different carbohydrate-degrading enzymes and analyzed by flow cytometry. Initial analysis has shown that P-selectin ligands on MC-38 cells are not mucins (Borsig et al. 2002). Therefore, we checked for other potential selectin ligands, glycosaminoglycans, and sulfatides (Varki 1997). Treatment of carcinoma cells with a mixture of glycosaminoglycan-hydrolyzing enzymes (heparinase and chondroitinase) did not affect binding of P-selectin, but partially decreased recognition by L-selectin (Figure 1A). The removal of sulfate groups by arylsulfatase treatment resulted in a clear reduction of P-selectin binding, as well as a reduction of L-selectin binding, although to a lesser extent (Figure 1A). Although L-selectin binding to MC-38 cells was calcium-dependent, the binding of P-selectin was only partially affected by the absence of calcium (Figure 1A) (Borsig et al. 2002). These data suggested that L-selectin ligands on MC-38 cells are represented partially by glycosaminoglycans, which was in agreement with the EDTA sensitivity (Koenig et al. 1998). Earlier we have shown that L-selectin binds mucin-type ligands also (Borsig et al. 2002). Meanwhile, P-selectin recognized a unique type of ligands in a calcium-independent manner. Together with the requirement for sulfation, but independent of glycosaminoglycans, the nature of the P-selectin ligand appeared to be sulfoglycolipids (Needham and Schnaar 1993; Koenig et al. 1998).

To confirm that MC-38 cells interact also with P-selectin-expressing cells, we tested the adherence of these cells to

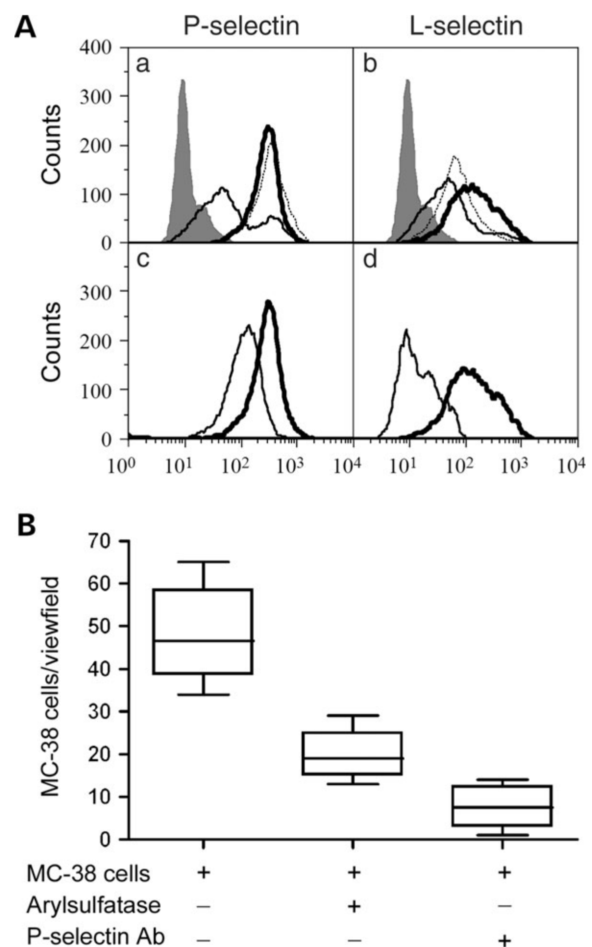

Fig. 1. Characterization of the nature of selectin ligands on MC-38 cell surfaces. (**A**) MC-38 cells were stained with mouse selectins and analyzed by flow cytometry. P-selectin [(a) and (c)] and L-selectin [(b) and (d)] stained untreated cells are represented by bold solid lines. Filled areas [(a) and (b)] represent controls stained with the secondary antibody Ab only. The dashed line represents selectin-stained cells after heparinase–chondroitinase treatment, whereas the thin line represents selectin-stained cells after arylsulfatase treatment. Thin line [(c) and (d)] represents selectin staining in the presence of 30 mM EDTA. (**B**) Adhesion of MC-38 cells to immobilized platelets. Arylsulfatase-treated or sham-treated MC-38 cells were incubated on slides with activated platelets for 20 min under low shear. Adherent cells were counted.

activated platelets. Slides with immobilized activated platelets were incubated with MC-38 cells while shaking on an orbital shaker (Figure 1B). Tumor cells adhered readily to activated platelets, and this adherence was blocked by function-blocking anti-P-selectin antibody. Arylsulfatase treatment also led to reduction of adherence, although to a lesser extent. The incomplete inhibition of tumor cell–platelet interactions by removal of sulfation corresponded to incomplete reduction of P-selectin binding to MC-38 cell (Figure 1A). Finally, there was no adherence of MC-38 cells to nonactivated platelets (data not shown). Thus cell-surface ligands of MC-38 cells enable the adherence to platelets in a P-selectin-dependent manner.

P-selectin binds to ligands in lipid fractions of MC-38 cells

To check whether P-selectin recognizes sulfated glycolipids as functional ligands on MC-38 cell surfaces, total lipid extract from these cells was prepared. Isolated lipids were separated by Folch partitioning into a lower phase (LP lipids), consisting of neutral glycolipids smaller than tetrasaccharides, together with phospholipids and sulfated glycolipids (sulfatides), and an upper phase (UP lipids) containing mostly gangliosides. Lipid fractions were coated on ELISA plates and analyzed by P-selectin chimera binding (Figure 2A). Sulfated galactosylceramide [HSO_3-3Galβ-1Cer (SM4)], a recognized P-selectin ligand, was used as a control (Aruffo et al. 1991). Although P-selectin bound to LP lipids, there was no recognition of UP lipids (data not shown). The extent of P-selectin binding to LP lipids was comparable with the recognition of SM4. The LP lipids from Folch partition contain sulfatides and phospholipids, which are known potential P-selectin ligands (Malhotra et al. 1996). To confirm that sulfation is a prerequisite for P-selectin recognition, LP lipids were treated with arylsulfatase prior to P-selectin chimera binding. Desulfation of the LP lipids reduced binding of P-selectin by more than 55% compared with about a 45% reduction of binding to SM4 (Figure 2A). P-selectin binding to LP lipids was only partially affected by the presence of EDTA, which was similar to P-selectin recognition of SM4. Recognition of the LP lipids was not affected by neuraminidase treatment, confirming that the P-selectin binding is independent of sialic acid (Borsig et al. 2002).

To identify P-selectin ligands in LP and UP lipid fractions, lipid samples were separated by high-performance thin layer chromatography (HPTLC) (Figure 2B and C). The large amount of phospholipids in the LP lipid fraction was hydrolyzed by treatment with phospholipase C (PLC) prior to separation on TLC plates. Visualization of glycolipids by orcinol–sulfuric staining revealed the presence of a broad panel of glycolipids. The majority of glycolipids in the LP phase (lane 2) were migrating slower than the sulfatide SM4 (lane 1). Developing conditions for HPTLC were chosen so that the best resolution of short oligosaccharide-chain glycolipids and sulfatides would be achieved. Thus, an accumulation of gangliosides with minimal migration was visible in the UP lipid fraction (lane 3). Despite the large variety of lipids in each fraction, P-selectin bound only to two distinct lipid bands in the LP fraction of MC-38 cells (Figure 2C). There was no binding to lipids in the UP fraction, suggesting no recognition of sialic acid-containing gangliosides (Borsig et al. 2002). As a positive control, SM4 was used, and a

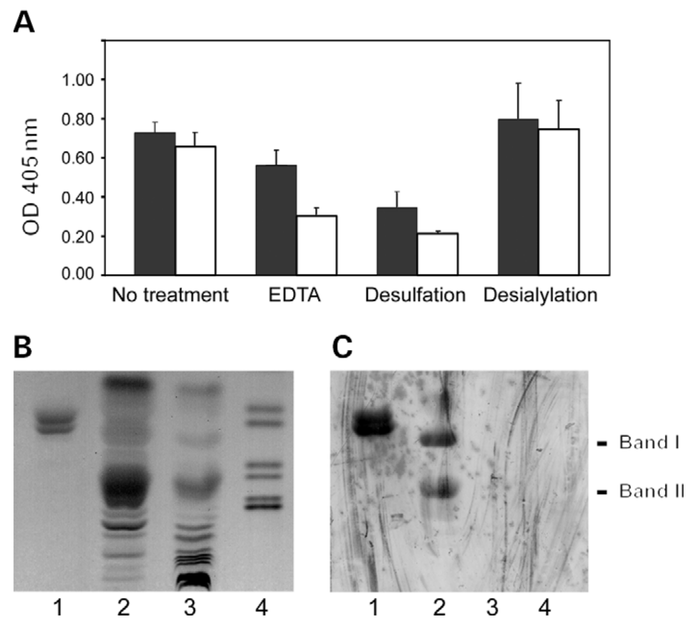

Fig. 2. Lipid extracts of MC-38 cells contain P-selectin ligands. (**A**) ELISA plates were coated with LP lipids isolated by Folch partitioning (white bars) and with bovine sulfatide-SM4 (black bars) as a control. "No treatment" represents P-selectin binding to extracted lipids. "EDTA" represents P-selectin binding to lipid fraction in the presence of 30 mM EDTA. The third and fourth sets show P-selectin recognition of lipids after enzymatic treatment, desulfation, or desialylation, respectively. Data shown are mean $\pm$ SD of triplicates. The difference between nontreated lipids and arylsulfatase-treated lipids (both MC-38 extracts and SM4 sulfatide) were statistically significant; as determined by one-way ANOVA followed by a pairwise t test, $P < 0.001$. [(**B**) and (**C**)] MC-38 lipid extracts were separated by HPTLC and developed in chloroform:methanol:0.2%$CaCl_2$ (60:35:7). Loading of samples: SM4 (lane 1); LP lipids from MC-38 cells (lane 2); UP lipids of MC-38 cells (lane 3), and a neutral ganglioside marker (lane 4). (B) Glycolipids visualized by orcinol–sulfuric acid staining. (C) P-selectin overlay of separated lipid ligands.

neutral glycolipid marker served as a negative control (lanes 1 and 4). The lipids recognized by P-selectin corresponded to a band with high mobility that migrated close to the SM4 sulfatide (Band I), whereas the more diffuse Band II migrated with slower mobility. When the P-selectin overlay of a TLC plate was prepared in the presence of EDTA, no visible reduction of binding was observed (data not shown). To test whether the Band I corresponds to sulfated-lactosylceramide [HSO_3-3Galβ-4Glcβ-1Cer (SM3)], we analyzed cell extracts of HepG2 cells, which are known to contain SM3 sulfatide (Spitalnik et al. 1989). P-selectin was found to bind the SM3 sulfatide, but this lipid band did not correspond to Band I of MC-38 cells (see Supplementary Material).

Sulfation determines P-selectin ligands on MC-38 cells

To confirm the presence of sulfate groups in the MC-38 lipid bands recognized by P-selectin, cells were metabolically labeled with sodium ^{35}sulfate, and glycolipids were isolated. Labeled lipid extracts were separated by HPTLC, and the location of the radioactive signal on the plate was determined by film exposure. Sulfate incorporation was detected in the region of Band I as a double band (Figure 3A). The appearance of a double band by metabolic labeling is typical for glycolipids, which indicates a difference in hydroxylation of

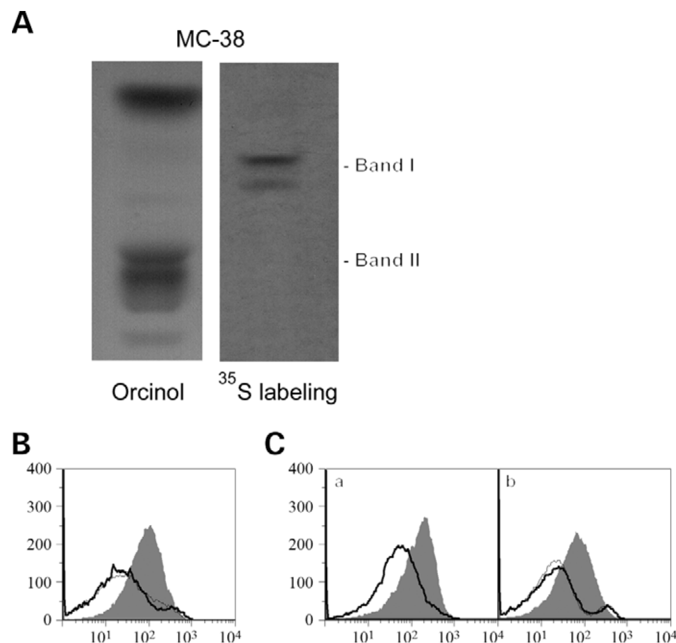

Fig. 3. P-selectin ligand recognition requires sulfation. (**A**) MC-38 cells were metabolically labeled with ^{35}S sodium sulfate as described in the Materials and methods section. Lipid extracts were separated by HPTLC, and the incorporation of the radio-labeled sulfate was detected by exposure to X-ray film with a subsequent visualization by orcinol–sulfuric acid staining. (**B**) MC-38 cells were incubated in the presence of 100 mM sodium chlorate for 72 h before staining with P-selectin and flow cytometry analysis. Bold line represents cells treated with sodium chlorate; shaded area represents cells not treated; thin line represents cells treated with arylsulfatase only. (**C**) P-selectin staining of MC-38 cells. (a) MC-38 cells were treated with arylsulfatase (bold line) and compared with sham-treated cells. (b) Arylsulfatase-treated cells were further incubated for 72 h in the presence of sodium chlorate (thin line). Bold line represents sham-treated cells (a), which were incubated in sodium chlorate. Shaded area represents sham-treated cells incubated in normal medium as a control.

their acyl chains. However, there was no signal detected in the region of Band II, indicating that these lipids are not sulfate-containing structures. Indeed, MS/MS analysis of these fractions only detected a series of glycerophosphoinositides in this fraction, in contrast to the same P-selectin-binding band from HepG2 cells, where SM2a and SM3 with a short ceramide chain were found, next to the same glycerophosphoinositides (see Supplementary Material). The visualization of glycolipids by orcinol revealed a typical glycolipid pattern as seen before (Figure 2B), whereas P-selectin staining confirmed the ^{35}S sulfate-labeled band to contain selectin ligands (data not shown).

The requirement of sulfation for P-selectin ligand recognition was further confirmed by an inhibition of sulfate metabolism. MC-38 cells were incubated in the presence of sodium chlorate, a known inhibitor of PAPS (3′-phosphoadenosine 5′-phosphosulfate) formation, which is the sulfate donor (Ishizuka 1997). P-selectin binding to sodium chlorate-treated cells was clearly reduced, as detected by flow cytometry (Figure 3B). The extent of P-selectin binding reduction was comparable with arylsulfatase treatment. To confirm that the reduced P-selectin binding to MC-38 after arylsulfatase treatment eliminates the same selectin ligands as achieved by sodium chlorate treatment, MC-38 cells were treated with arylsulfatase, followed by sodium chlorate incubation (Figure 3C). The sequential treatment of MC-38 cell did not show any further decrease of P-selectin binding, thereby indicating that both treatments targeted the same ligands. Similarly, the binding of P-selectin to HepG2 cells was reduced by sodium chlorate treatment (see Supplementary Material). All data taken together, we concluded that the Band I P-selectin ligand on MC-38 cells is a sulfated glycolipid.

MALDI-TOF/TOF analysis identified SM4 as P-selectin ligand on MC-38 cells

To characterize the glycolipid recognized by P-selectin on MC-38 cells (Band I in Figure 3A), the LP lipids were treated with PLC and/or were saponified prior to purification on a DEAE-sepharose column. The charged glycolipid fraction was collected and separated by HPTLC. P-selectin immuno-overlay of the plate confirmed the presence of a P-selectin ligand (Figure 4A). The purified samples were analyzed by mass spectrometry (MS) as described in the *Materials and methods* section (Figure 4B–D). In nonsaponified samples, we detected two families of compounds: one family represented the sulfatide SM4, with the heterogeneity contributed by the lipid part (chain length differences, presence of hydroxylation, and saturation levels of the fatty acid). The slight difference in mobility of Band I, in comparison with SM4 standard as observed by HPTLC (Figure 2C), is possibly due to the total lipid load in the LP lipid–TLC lane. Moreover, a higher fraction of the SM4-ceramide in MC38 cells is hydroxylated compared with the standard SM4, which typically reduces the mobility under the TLC conditions used here. The other family of compounds was identified as cardiolipin (CL) by MS/MS analysis (data not shown). CL has been reported to be virtually exclusively confined to mitochondria, with the exception of apoptotic cells, where it can be found at the plasma membrane (Sorice et al. 2004). Therefore, it is probably an unlikely candidate for the P-selectin ligand on the surface of cancer cells. Nevertheless, we confirmed in vitro by repeating the overlay experiment with purified CL from a commercial source that CL is indeed a P-selectin ligand (our unpublished results). At this point, we cannot exclude that CL is also a biological ligand for P-selectin. Upon saponification of the sample, the CL peaks disappeared from the spectrum as expected, whereas the SM4 peaks remained with unaltered distribution. The P-selectin binding signal in the saponified fraction (Figure 4A, lane 3) was rather weak, yet clearly present. These findings indicate that SM4 is a P-selectin ligand in MC-38 cells.

MC-38 cell surface P-selectin ligands are SM4 sulfatides

The identification of SM4 sulfatide in MC-38 cell lipid extracts, together with P-selectin binding to intact cells treated with hydrolytic enzymes (Figure 1), strongly suggested that SM4 sulfatide is the primary P-selectin ligand. To provide further evidence that SM4 sulfatide is indeed the cell surface ligand of P-selectin, we analyzed MC-38 cells with altered glycolipid biosynthesis. We have shown that an inhibition of sulfate metabolism led to a decrease of P-selectin binding (Figure 3B). When exogenous SM4 sulfatide was added to untreated cells, no alteration of P-selectin binding was observed (Figure 5A). However, the addition of

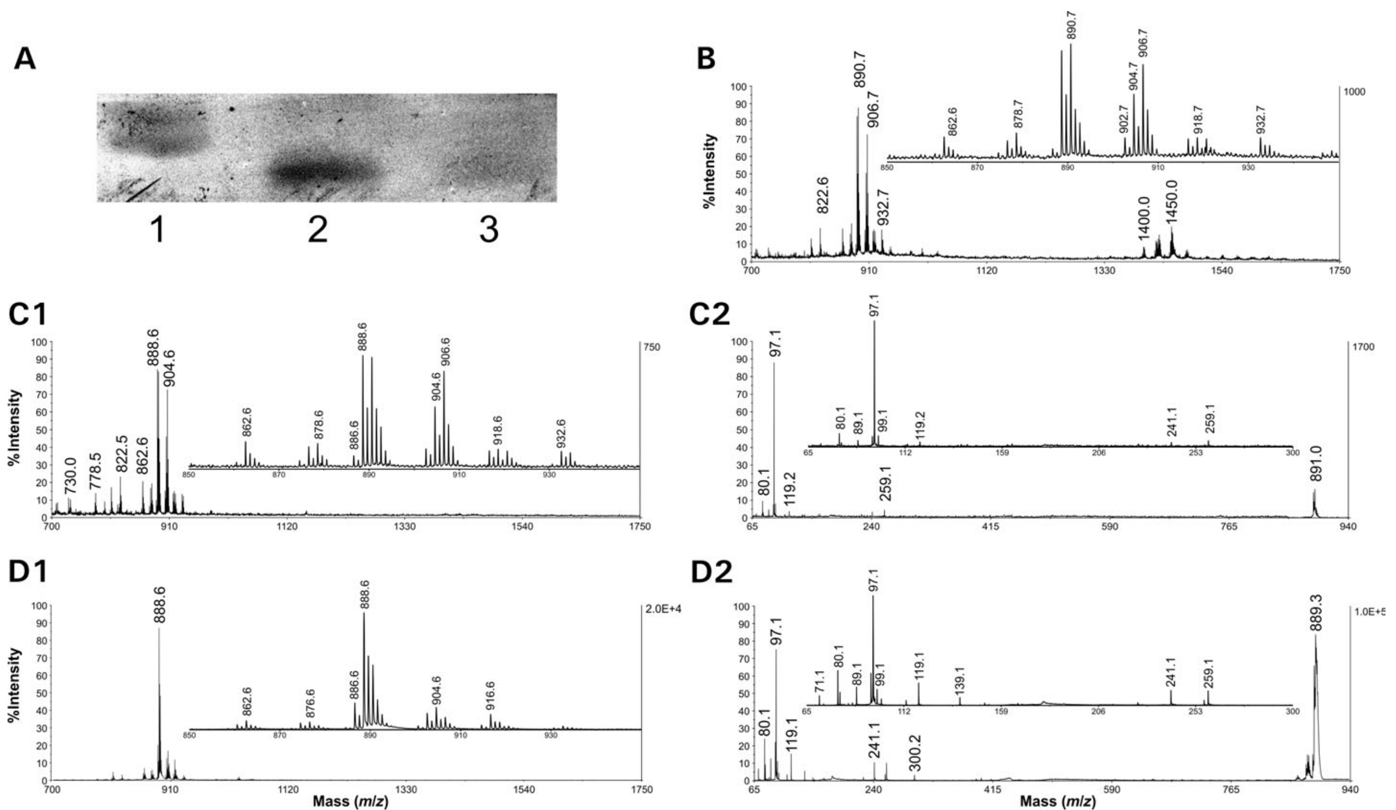

Fig. 4. MALDI TOF and TOF/TOF mass spectrometry analysis of the MC-38 P-selectin ligand. The specific band recognized by P-selectin was extracted from the TLC plate and prepared for mass spectrometry analysis. (**A**) Immuno-overlay of the TLC-separated lipids with P-selectin. MC-38 lipid extracts from Folch partitionings' LP treated with PLC (lane 2) or saponified (lane 3) and purified on a DEAE column; SM4 sulfatide standard (lane 1). (**B**) Negative reflectron-mode profile of Band I recognized by P-selectin in MC-38 lipid extract treated with PLC. There are two families of peaks: a first group around 890 m/z, corresponding to SM4, and a second group around 1400–1475 m/z, corresponding to CL. Insert corresponds to SM4 region. (**C1**) Negative reflectron-mode profile of Band I recognized by P-selectin in MC-38 lipid extracts saponified with ammonia as described in the Materials and methods section. Insert corresponds to SM4 region. CL is undetectable upon this saponification procedure. (**C2**) MS/MS negative ion mode profile of m/z 890.6. The fragment at 80 m/z represents the sulfate-specific radical anion $[\cdot SO_3]^-$, whereas the fragment at 97.1 m/z represents $[HSO_4]^-$. Together, this ion fragment pair is diagnostic for sulfated compounds under these conditions. The peaks at 241.1 and 259.1 m/z correspond to the B_1- and C_1- fragments, respectively, resulting from cleavage of the glycosidic bond which links the hexose-sulfate moiety to the ceramide. Insert corresponds to the region 65–300 m/z. (**D1**) Negative reflectron-mode profile of SM4 standard after being purified by TLC as described in the Materials and methods section. Insert corresponds to SM4 region. A different pattern of lipid heterogeneity of the SM4 spectra from MC-38 cells versus the standard could be observed. (**D2**) MS/MS negative ion mode profile of m/z 888.5 in the standard. Insert corresponds to the region 65–300 m/z. Note the virtual identicalness of the fragment ion pattern between the standard SM4 and the compound in (**C2**), further confirming the identity of this compound as SM4.

exogenous SM4 sulfatide to sodium chlorate-treated cells almost completely recovered P-selectin binding (Figure 5A). This observation indicated that SM4 sulfatide can reconstitute selectin ligands on MC-38 cells to the same level and binding specificity. To confirm the glycolipid nature of P-selectin ligands, MC-38 cells were incubated in the presence of PPMP, an inhibitor of glucosylceramide synthase. This treatment blocked synthesis of gangliosides and complex sulfatides, only allowing the biosynthesis of galactose-linked glycolipids, where the SM4 sulfatide is the predominant product (Ishizuka 1997). The overall reduction of gangliosides could be detected by orcinol staining of total lipid extracts from equal number of cells, either PPMP-treated or control cells (Figure 5B). The glucosylceramide biosynthesis inhibitor treatment resulted in an enhanced binding of P-selectin to cells (Figure 5A). This increase in P-selectin binding was also observed in the absence of calcium, which was in agreement with the partial calcium independence of P-selectin recognition of a pure SM4 sulfatide (Figure 2A). Finally, trypsin treatment of MC-38 cells did not affect P-selectin binding, whereas a reduction in PSGL-1 epitopes in a parallel treatment of HL-60 cells was observed (data not shown). Taken together, these results provide evidence that SM4 is the bona fide P-selectin sulfoglycolipid ligand on MC-38 cells.

Removal of cell surface sulfation attenuates metastasis

To ascertain whether SM4 sulfatide is the native P-selectin ligand facilitating metastasis, we injected C57Bl/6J mice with MC-38GFP carcinoma cells previously treated with arylsulfatase. Lung sections from mice euthanized 30 min after tumor cell injection were evaluated for platelet-MC-38 interactions (Figure 6A and B). Arylsulfatase treatment reduced the platelet-thrombi formation around tumor cells. Furthermore, platelet-tumor cell emboli formation of MC-38 cells is comparable with previously observed interactions of platelets with human colon carcinoma cells, which carry carcinoma mucins only (Kim et al. 1998; Borsig et al. 2001).

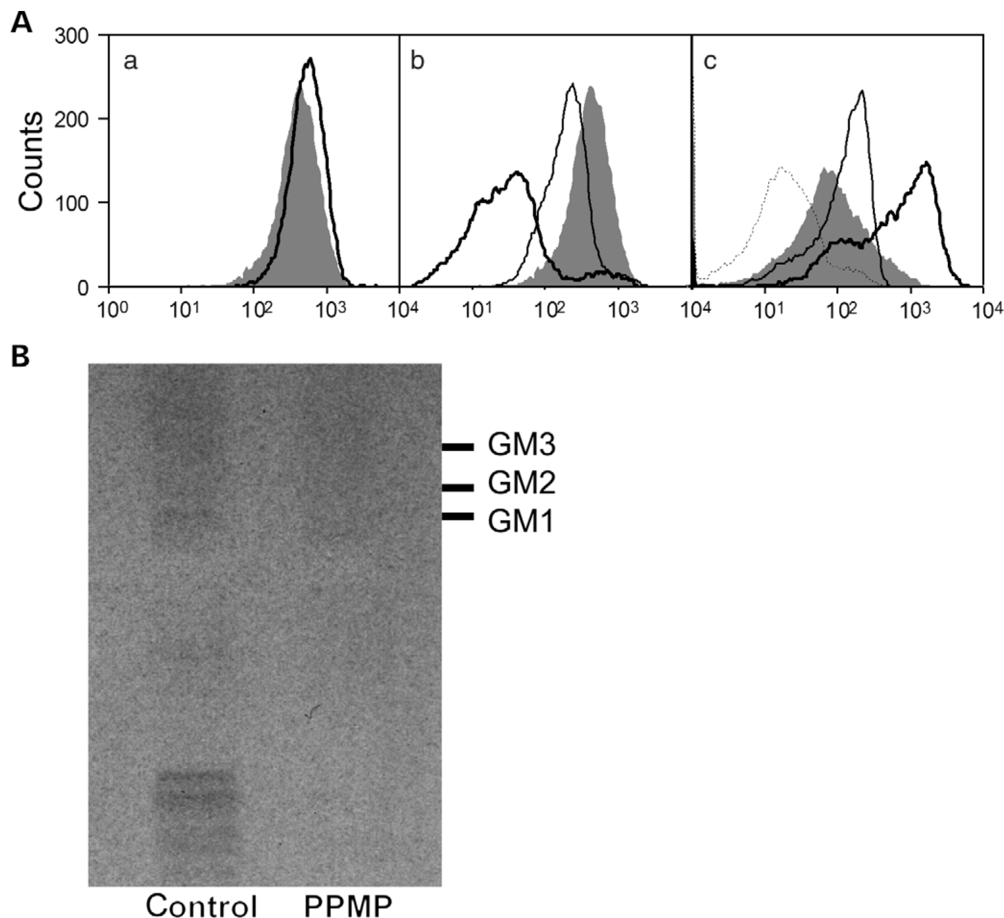

Fig. 5. Modulation of glycolipid biosynthesis affects P-selectin binding. (**A**) MC-38 cells were stained with mouse P-selectin and analyzed by flow cytometry. (a) MC-38 cells exposed to exogenous SM4 sulfatide did not alter P-selectin binding to cells (bold line). Filled area represents control cells without sulfatide addition. (b) To sodium chlorate-treated MC-38 cells (bold line) was exogenously added SM4 sulfatide and the cells were stained with P-selectin (thin line). Filled area represents binding of P-selectin to untreated cells. (c) MC-38 cells were incubated in the presence of PPMP for 72 h and stained with P-selectin (bold line). P-selectin binding was only partially affected when incubated with 30 mM EDTA (thin line). Control cells, without glucosylceramide synthase inhibitor, were stained with P-selectin without EDTA (filled areas) or in the presence of EDTA (dotted line). (**B**) Total lipid extracts from cells treated with PPMP (PPMP) or untreated (control) were separated by HPTLC and stained by orcinol. Equal amount of cells has been extracted and loaded on TLC plate. Reduction in monosialogangliosides as well as highly charged glycolipid could be observed.

Thus, sulfatides on tumor cell surfaces are functional ligands for platelets in vivo. When mice were euthanized 4 weeks later, the extent of metastasis was evaluated (Figure 6C and D). The lungs of mice injected with sham-treated carcinoma cells were almost completely displaced by tumors, as observed previously (Borsig et al. 2002). In contrast, mice injected with arylsulfatase-treated carcinoma cells showed virtually tumor-free lungs. Of the 10 mice, 8 had no or only one visible metastatic foci (Figure 6C). The dramatic reduction of metastasis was further confirmed by fluorescence measurement of the lung homogenate (Figure 6D). To exclude any possibility that the arylsulfatase treatment impaired behavior of cells (Kobayashi et al. 1994; Uemura et al. 2003), these were analyzed thoroughly in vitro. There was no difference in viability, proliferation, adhesion, or morphology of the treated cells when compared with sham-treated cells (data not shown). Heparinase–chondroitinase-treated cells behaved as the control cells and exhibited no difference in proliferation, adherence, or morphology (data not shown). Thus it is unlikely that the arylsulfatase treatment affects glycosaminoglycans primarily. Arylsulfatase, but not heparinase–chondroitinase, treatment of MC-38 cells also reduced P-selectin-mediated platelet aggregation as observed on lung sections at different time points after intravenous injection. Furthermore, the number of tumor cells trapped in the lung vasculature was lower in mice injected with arylsulfatase treated cells. This agrees with the previously shown decrease in tumor cells seeding to the lungs when selectin ligands on tumor cells were eliminated (Borsig et al. 2001). The elimination of sulfation on MC-38GFP cells was associated with a reduced platelet aggregation and attenuation of metastasis, confirming the facilitating role of P-selectin in this process.

Discussion

Sulfatides have been detected in various tissues, including the brain, kidney, and gastrointestinal tract (Ishizuka 1997). A systematic analysis of glycosphingolipids in human gastrointestinal tract has shown the presence of sulfatides, SM4 in particular, in normal mucosa (Natomi et al. 1993). The preferential enrichment of sulfatides in the gastric mucosa

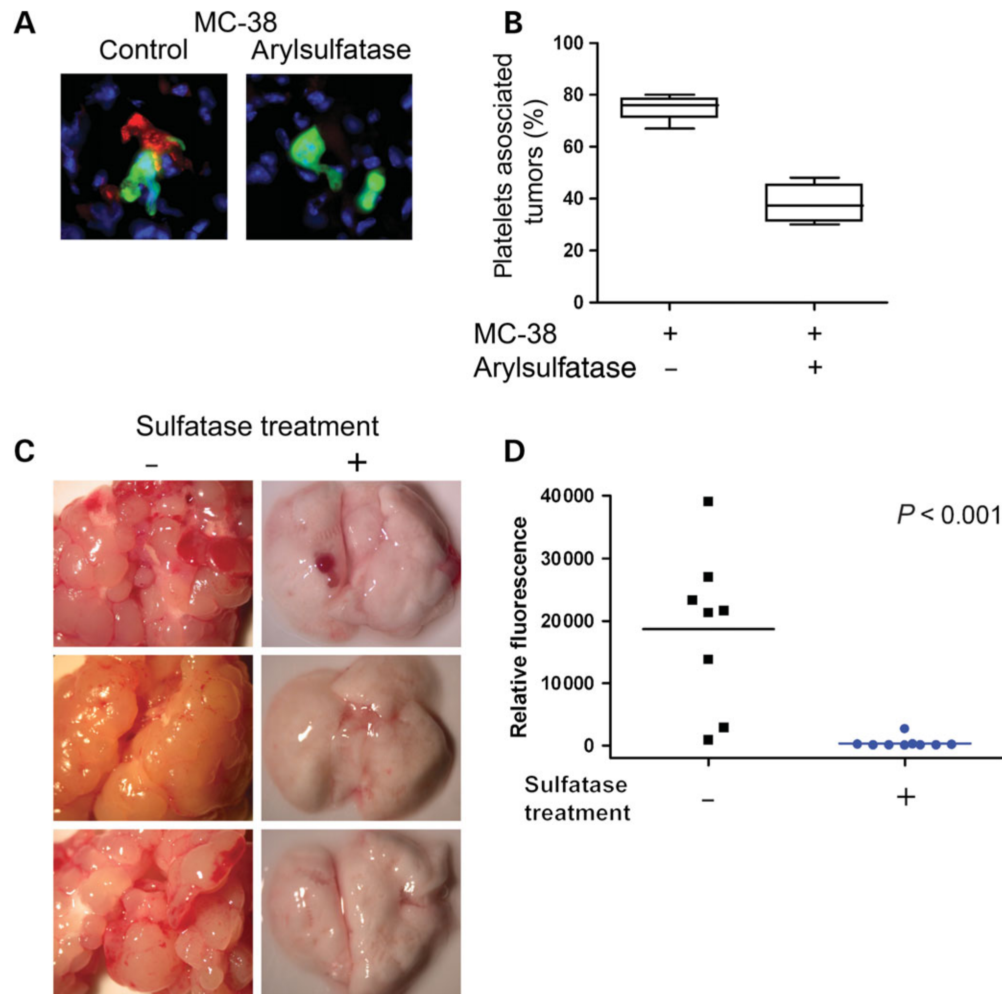

Fig. 6. Elimination of cell surface sulfation attenuates metastasis. Mice were injected intravenously with 3–4 × 10^5 MC-38GFP cells either treated with arylsulfatase or sham treated. (**A**) Platelet interactions with injected cells were evaluated in lungs from mice terminated 30 min after injection. Representative images untreated-control tumor cells (green) with associated platelets (red), which were largely diminished when MC-38 cells were treated by arylsulfatase prior to injection. Blue are stained nuclei. (**B**) Quantification of platelet–tumor cell interactions from three independent experiments. [(**C**) and (**D**)] Mice were euthanized after 28 days, lungs were dissected and photographed, and GFP fluorescence in the lung homogenate was quantified. The number of animals studied was 8–9 in each group. (C) Representative examples of dissected lungs from mice injected with arysulfatase treated (+) and sham treated (−) MC-38GFP cells. (D) The extent of metastasis was measured by GFP fluorescence analysis of lung homogenate. Statistical significance was determined by the Student *t* test.

strongly indicates its possible involvement in mucosal protection. Sulfatides are also increasingly expressed in several human cancer tissues, including the lung (Miyake et al. 1992), colon (Siddiqui et al. 1978; Osawa et al. 1997), kidney (Sakakibara et al. 1989), liver (Hiraiwa et al. 1990), and ovary (Kiguchi et al. 1992). Even though the pathological correlation of sulfatides with cancer in these tissues remains unclear, their enhanced expression was correlated with poor metastatic prognosis (Morichika et al. 1996; Makhlouf et al. 2004). Here we provide one possible mechanism for how sulfatides might contribute to metastasis in a syngeneic mouse model. First, SM4 sulfatide was shown to be virtually the sole P-selectin ligand on colon carcinoma cells (MC-38), as determined by MALDI-TOF/TOF analysis of purified glycolipids. Secondly, P-selectin-mediated interactions with MC-38 cells through SM4 sulfatide were shown to facilitate metastasis, thus identifying SM4 sulfatide to be a functional native ligand in this process. Previously, we and others have shown that the absence of P-selectin led to attenuation of metastasis (Kim et al. 1998; Borsig et al. 2001; Ludwig et al. 2004). Here we show that enzymatic removal of sulfation from tumor cells prior to intravenous injection strongly attenuates metastasis. This observation is in agreement with previous findings that any interference in P-selectin-mediated interactions between tumor cells and platelets and/or endothelium, achieved either by removal of ligands from tumor cells or by temporal inhibition by heparin, resulted in attenuation of metastasis, indicating their function in early steps of metastasis (Borsig et al. 2001, 2002; Fuster et al. 2003; Ludwig et al. 2004). Therefore, sulfatides on tumor cells could serve as ligands for P-selectin-mediated interactions, thereby contributing to metastatic spread.

Sulfatides and glycosaminoglycans (e.g. heparin and chondroitin sulfate) represent other nonsialylated ligands for selectins, which are expressed on several carcinomas and also on human granulocytes (Aruffo et al. 1991; Nelson et al. 1993; Suzuki et al. 1993; Kawashima et al. 2000). L-selectin was shown to bind not only SM4 sulfatide, but also

sulfated lactosylceramide SM3 and sulfated tri- and tetra-glycoceramides GalNAcβ-4(HSO$_3$-3)Galβ-4Glcβ-1Cer (SM2) and SB1a, respectively (Suzuki et al. 1993). Conversely, P-selectin was demonstrated to bind SM4 sulfatide only (Aruffo et al. 1991). Here we show that P-selectin is also able to recognize SM3, and SM2 sulfatides, which were found in HepG2 cells (Supplementary Material). This observation indicates that a variety of sulfatides can be recognized by P-selectin. Whether sulfatides expressed on variety of human carcinomas contribute to metastatic spread through selectin-mediated interaction requires further studies. Interestingly, sulfatides were found to facilitate P-selectin-mediated platelet adhesion and aggregation (Merten and Thiagarajan 2001). The recent finding that sulfatides could lead to platelet activation and their aggregation with leukocytes suggest this mechanism to play an important role in hemostasis and thrombosis (Merten et al. 2005). In this context, our finding that murine colon carcinoma cells carrying sulfatides can effectively induce P-selectin-mediated platelet aggregation raises the possibility for the involvement of these interactions also during hematogenous metastasis.

The negatively charged phospholipid CL is normally confined to the mitochondrial inner membrane (Schlame et al. 2000). In addition, CL was also detected on the cell surface of apoptotic cells after redistribution from mitochondria (Sorice et al. 2004). Modulation of the internal CL pool by palmitate was associated with apoptosis of breast cancer cells (Hardy et al. 2003). We have shown that CL can be purified from membranes of MC-38 cells and is recognized by P-selectin. By HPTLC separation, CL was co-migrating with SM4 sulfatide and could be eliminated by saponification only. Interestingly, significant amounts of CL were found in MC-38 cell extracts, although CL usually comprises a minor component of the total lipid extracts in other cell lines (our unpublished results). Previously, L-selectin was shown to bind CL in calcium-independent manner (Malhotra et al. 1996). We have confirmed that P-selectin can bind CL. Whether the enhanced presence of CL in MC-38 has any biological relevance for P-selectin recognition remains to be elucidated. Nevertheless, MC-38 cells after sulfatase treatment or inhibition of sulfate metabolism by sodium chlorate showed significantly reduced P-selectin binding, strongly suggesting that the main ligand for P-selectin-mediated metastasis is a sulfoglycolipid.

The association of enhanced sulfatide expression on cancer cells with metastasis has not been fully clarified. Human renal-cell carcinoma and hepatocellular carcinoma cells require cell surface expression of sulfoglycolipids for attachment to laminin (Kobayashi et al. 1994; Zhong Wu et al. 2004). Incorporation of externally supplemented SM4 sulfatide increased haptotactic migration of cancer cells on laminin, which was associated with accelerated lung colonization (Kobayashi et al. 1994). The presence of sulfatides on cancer cells was correlated with the expression of ceramide sulfotransferase gene (CST) (Honke et al. 1998) and associated with metastatic potential of human hepatocellular carcinoma cells (Zhong Wu et al. 2004). Expression of particular sulfatides was also found to be dependent on the availability of precursor glycolipids (Kobayashi et al. 1994; Zhong Wu et al. 2004). Meanwhile, the presence of SM3 sulfatide was linked to upregulation of CST gene expression, which also mediated cell adhesion to vitronectin and $\alpha V\beta 3$ integrins (Honke et al. 1998; Zhong Wu et al. 2004). On the contrary, expression of SM3 sulfatide induced by CST transfection in murine Lewis lung carcinoma cells led to suppression of cell adhesion to laminin and $\beta 1$ integrin and, subsequently, metastasis (Kabayama et al. 2001; Uemura et al. 2003). The discrepancy between these results may be due to the clonal nature of the Lewis lung carcinoma cells and the subsequent differences in their signal transduction (Kabayama et al. 2001; Uemura et al. 2003). Recently, sulfatides were also found to be potential native ligands for Galectin-4, a galactose-binding lectin (Ideo et al. 2005). Our findings propose that SM4 sulfatide is contributing to metastasis through P-selectin-mediated interactions with platelets and/or endothelium. The extent of metastasis observed with MC-38GFP cells devoid of sulfation, and thereby P-selectin ligands (Figure 6), was even less than that observed in P-selectin-deficient mice (Borsig et al. 2002). Thus it is likely that sulfatides may contribute to metastasis by more than one mechanism. Further studies are needed to elucidate how P- and/or L-selectin binding to sulfatides affects metastasis.

Materials and methods

Cell lines and reagents

Mouse colon carcinoma cell line MC-38 (Borsig et al. 2002) was grown in DMEM with high-glucose (10% fetal calf serum [FCS]) medium (Invitrogen, Carlsbad, CA). For experimental metastasis studies, we used cells stably expressing GFP (MC-38GFP) (Borsig et al. 2002). All reagents were from Sigma (St. Louis, MO) unless otherwise stated.

Flow cytometry analysis of MC-38 cells

Cells were grown to 90% confluency, detached by incubating in PBS, containing 2 mM EDTA, for 5 min at 37 °C, and washed three times with the cold Hank balanced salt solution (HBSS). After blocking with HBSS containing 1% BSA (HBSS/BSA), cells were incubated with mouse P- or L-selectin chimeras, containing the Fc region of human IgG (Borsig et al. 2002). Prior to addition to tumor cells, selectin chimeras were preincubated with a biotinylated goat-antihuman IgG Ab (1:200) for 1 h at RT in HBSS/BSA. After incubating tumor cells with preconjugated selectin chimeras for 1 h at 4 °C, Streptavidin-PE-Cy5 (Becton Dickinson, Mountain View, CA) was added and incubated for 20 min. Cells were washed with HBSS/BSA and analyzed by flow cytometry. As controls, cells were stained with P-selectin chimera in the presence of 5 and 30 mM EDTA, respectively (Borsig et al. 2002). In some cases, tumor cells were enzymatically treated prior to incubation with selectin chimeras. Glycosaminoglycans were removed by treatment with a mixture of heparinase II and chondroitinase ABC in HBSS for 1 h at 37 °C (Borsig et al. 2002). Sulfate groups were eliminated by arylsulfatase H-5 treatment in HBSS buffer for 1 h at 37 °C.

MC-38 cells adhesion to immobilized platelets

Mouse platelets were isolated and labeled with Calcein AM (Invitrogen, Carlsbad, CA), as previously described (Kim et al. 1998). Glass chamber slides (LabTek, Nunc, Rochester,

NY) were pretreated with 4% solution of 3-aminopropyltriethoxysilane (Buttrum et al. 1993). Isolated platelets were added to the chamber slides and spun at 200*g* for 10 min. Slides were blocked by PBS/BSA for 10 min. Platelets were activated with thrombin (1 U/mL) for 10 min. Even coating of the slide by platelets was confirmed by fluorescence microscopy. Slides coated with platelets were loaded with 20 000 MC-38 cells/well and incubated for 20 min on Orbital shaker (Heidolph Instruments, Schwabach, Germany) at 70 rpm (Koenig et al. 1998). In some cases, P-selectin expressed of platelets was blocked by an addition of 6 μg of function-blocking P-selectin antibody (BD Pharmingen). After incubation, nonattached cells were aspirated, and slides were washed once with PBS. Attached MC-38 cells were counted at the microscope 12 view fields by 100× magnification.

Lipid extraction from MC-38 and HepG2 cells

Semiconfluent cells were detached with PBS containing 2 mM EDTA and washed three times with HBSS. Cells were resuspended in water and homogenized by sonicating three times for 1 min. Lipids were extracted with 10 volumes of chloroform/methanol (1:1) for 4 h at RT while shaking. Supernatants containing lipids were separated by centrifugation, and the cell pellet was re-extracted with chloroform/methanol (1:1) overnight. Pooled lipid-rich supernatants were separated by Folch partition (Schnaar 1994), where two phases were obtained: the UP containing less hydrophobic lipids and the LP containing more hydrophobic lipids. The UP was dialyzed against water. The LP was treated with PLC in 50 mM potassium phosphate buffer, pH 7.4 (Sonnenburg et al. 2002), and incubated at 37 °C for 3 h while vigorously shaking, followed by dialysis against water and drying down. The sugar content of the lipid extract was measured by a phenol–sulfuric acid colorimetric assay (Dubois et al. 1956). Briefly, an aliquot of the lipid preparation or various concentrations of glucose were added to a glass vial and brought to 200 μL with water. Phenol was added (to 5% final concentration) together with 1 mL of concentrated sulfuric acid. Probes were vigorously mixed and the absorbance was measured at 490 nm. Sugar content of the lipid extract was calculated against a standard curve obtained from the various glucose solutions.

ELISA analysis of lipids from MC-38 cells

Lipids, along with bovine sulfatide (SM4) as a control, were resuspended in methanol and used for coating of the ELISA plates by evaporating the methanol (Borsig et al. 2002). The wells were washed with HBSS and blocked with 0.5% BSA in HBSS for 1 h at RT. Mouse selectin chimeras were preincubated with goat-antihuman IgG Ab conjugated with alkaline phosphatase (1:100) for 1 h at RT. Preincubated selectins were added into the wells and incubated for 2 h at RT in the absence or presence of 30 mM EDTA, followed by three washes with HBSS. After a final wash, the *p*-nitrophenyl phosphate substrate was added and allowed to develop for 20 min at RT, after which absorbance was measured at 405 nm. Some lipid samples were enzymatically treated prior to coating on ELISA plates (Hiraiwa et al. 1990). Briefly, aliquots of lipid fractions were placed in glass vials and dried down under a N_2 stream. Samples for arylsulfatase H-5 treatment were resuspended in 50 mM acetate buffer, pH 5.0, whereas those for neuraminidase treatment were treated in 50 mM citrate buffer, pH 5.0. After treating for 90 min at 37 °C, lipids were dried down and resuspended in methanol.

High-performance thin layer chromatography (HPTLC)

Fifteen microgram of glucose equivalents of our sample were applied to a preactivated silica plate (MERCK, Darmstadt, Germany). Plates were developed in a TLC tank pre-equilibrated with chloroform/methanol/0.25% $CaCl_2$ (50:40:10) or with chloroform/methanol/0.2%$CaCl_2$ (60:35:7). SM4 and a neutral ganglioside mix (Matreya) were used as markers. Glycolipids were visualized by the orcinol–sulfuric acid reagent.

TLC immuno-overlay

Samples were applied to TLC plates and developed as described in *HPTLC*. Dried plates were plastified in a solution of Hexane/2% polyisobutylmethacrylate (ALDRICH) in chloroform (84:16) (Schnaar 1994). Air-dried plates were incubated with a blocking solution, HBSS-containing 5% BSA, for 2 h at RT, followed by incubation with P-selectin chimera (Borsig et al. 2002) in HBSS/BSA overnight at 4 °C. Plates were washed three times with HBSS/BSA and incubated with a secondary antibody conjugated with horseradish peroxidase for 1 h at RT. After three washes with HBSS, plates were developed with 400 μg/mL *o*-phenylendiamine in 50 mM citrate–phosphate buffer, pH 5.0, with 0.12% hydrogen peroxide and dried (Sonnenburg et al. 2002).

Metabolic ^{35}S labeling of lipids

MC-38 cells were grown in T175 flasks until 80% confluent. The complete medium was changed for a low-sulfate medium containing Met (7.5 mg/L) and Cys (50 mg/L) supplemented with 10% dialyzed FCS. After sulfate-starving for 3 h, cells were labeled with 0.5 mCi of sodium $[H^{35}SO_4]^-$ in the low-sulfate medium for 16 h (Tooze 2003). Labeled cells were washed with PBS and detached with PBS/2 mM EDTA. Total lipids were extracted from the cells as follows: the cell pellet was resuspended in five volumes of H_2O and vortexed and bath-sonicated followed by an addition of 10 volumes of chloroform/methanol (1:1). Lipids were extracted for 3–4 h at RT while shaking vigorously. Samples were centrifuged at 4 °C to pellet the precipitates. The supernatant was transferred into a new tube and stored at 4 °C. The pellet was re-extracted overnight in a similar volume of chloroform/methanol (1:1). Both lipid extractions were pooled and dried down. Samples were resuspended in methanol, loaded on a TLC plate, and developed as described in *HPTLC*. Lipids, which incorporated sulfate, were revealed by exposure to X-ray film (Kodak) for 15 days. The TLC plate was stained with orcinol–sulfuric reagent.

Purification of sulfoglycolipids through anion exchange chromatography (DEAE–sepharose) and preparative HPTLC

To hydrolyze triacylglycerides, glycerophospholipids, and sphingolipids, the LP was saponified with ammonia solution according to an adopted protocol (Ferguson 1993). The samples were dried down and resuspended in a solution containing a final concentration of 17.5% ammonia in methanol.

After incubation for 6 h at 50 °C, the preparation was dried down in a SpeedVac centrifuge (Sovant Instruments, Hicksville, NY) and washed twice with 50% methanol. Separation of negatively charged lipids from neutral lipids was performed as described previously (Schnaar 1994). Briefly, PLC-treated or saponified lower-phase lipid extract was dissolved in solution A, chloroform/methanol/water (30:60:8 v/v). The DEAE-sepharose (Amersham Biosciences) was changed into acetate form by mixing the dry resin with solution B, chloroform/methanol/0.8 M aqueous sodium acetate (30:60:8 v/v). After washing the column several times with solution A, the samples were loaded and the flow through was discarded. The negatively charged lipids were eluted with several bed volumes of solution B. The eluate was dialyzed and samples were separated by HPTLC. The areas corresponding to lipids recognized by P-selectin were scratched and collected into a glass tube. To recover the lipids from silica, samples were resuspended in methanol, mixed, and spun down. The supernatant, containing the lipids, was transferred into a new tube and dried down.

Modulation of cellular sulfatides

MC-38 cells and HepG2 cells were incubated in regular medium containing 100 mM sodium chlorate for 72 h (Xia et al. 2003). Cells were detached by 5 mM EDTA/PBS treatment, and the extent of P-selectin binding was analyzed by flow cytometry. When the incorporation of externally added SM4 sulfatide was evaluated, cells were resuspended in HBSS containing 1% BSA at a concentration of 2×10^6 cells/mL and were incubated for 5 min with 10 μM SM4 sulfatide at 37 °C (Kobayashi et al. 1994). Cells were immediately put on ice, washed with ice-cold HBSS buffer, and incubated with P-selectin. For the inhibition of glucosylceramide synthase, tumor cells were cultured in the presence of 15 μM of D,1-threo-1-phenyl-2-hexadecanoylamino-3-morpholino-propanol (PPMP; Matreya, Pleasant Gap, PA) (Burdick et al. 2003; Dimitroff et al. 2004). After 72 h cultivation in the presence of the inhibitor, cells were stained with P-selectin and analyzed by flow cytometry.

Mass spectrometry

For MALDI-TOF and TOF/TOF mass spectrometry, preparative TLC was performed as described in *HPTLC*. Upon solvent development, the aluminum-backed plate was cut through the lane of interest. One-half was used for the detection of the regions of interest using P-selectin immuno-overlay, upon which both halves of the plate were re-aligned, and the silica present in narrow bands on the nonstained half of the plate was scraped off and carefully collected. After having experienced significant losses of analytes on plastics and chromatographic materials, we found that simple extraction of the silica with 100% methanol yielded preparations that were sufficiently pure for direct analysis via MALDI-TOF MS, although minimizing the chance for material loss during sample workup. We used 192-spot polished stainless steel targets for the ABI 4700 Proteomics Analyser. Small droplets of the methanol extracts were applied onto the target spot under a continuous stream of cold air to allow instantaneous drying. We repeated this sample application procedure until a whitish film became visible on the dried target spot (applying more sample results in decreased spectral quality). We used a preparation of 20 mg/mL 6-aza-2-thiothymine (ATT; Fluka, Buchs, CH) in 70% MeOH containing 10 mM NH_4-citrate for the matrix. One microliter of this matrix preparation was spotted onto the sample-loaded target spots and pipetted up and down to provide for proper sample–matrix mixing. The drops were then left to dry. ATT was chosen at the matrix by comparing this matrix for the analysis of the SM4 standard with several other preparations that have been reported in the literature to be useful for polar lipids (2,5-dihydrozybenzoic acid, 2,5-dihydroxybenzoic acid/5-methoxysalicylic acid co-matrix, 3-hydroxypicolinic acid/6-hydroxypicolinic acid co-matrix, α-hydroxy-4-cyano-cinnamic acid). The ATT matrix is exceptionally "clean" in the low-m/z region, which is important for the analysis of lipids, and is sufficiently "cold" to allow for intact ionization of sulfated, mono- and di-sialylated, and phosphorylated lipids. All the reported analyses were performed in the negative ion reflectron mode of the instrument, tuned for an optimal sensitivity/resolution ratio for this matrix. Calibration in the 900–4000-m/z range was performed using a standard peptide mixture (ABI) in the same ATT matrix as used for the lipid analytes. The spectra were obtained by the summation of at least 50 subspectra, each averaged over 50 laser shots. TOF/TOF MS/MS analysis was carried out with air at 5.10E–6 Torr as the collision gas, and the spectra were obtained by the summation of 100 subspectra, each averaged over 50 laser shots. The instrument was optimized to select the parent ion ± 3–4 Da, which is the narrowest available limit with this ion selector.

Methodological note: a mass spectrometry method to distinguish sulfation from phosphorylation

Sulfation and phosphorylation of a certain class of biomolecules (in this case, polar lipids) can be very difficult to distinguish at trace levels. In the analytical situation we faced here, sulfoglycolipids were present in a mixture with phospholipids and, most problematically, with phosphoinositides. To obtain positive evidence for sulfation versus phosphorylation, we made use of the fact that TOF/TOF fragmentation follows the behavior of higher energy single collision-induced fragmentation as is typical for triple quadrupole and magnetic sector instruments, rather than the behavior of low-energy multiple collision-induced fragmentation as is observed in most of the modern analyzers (ion traps). In high-energy collision-induced dissociation (CID), sulfated compounds yield the sulfite radical anion $[\cdot SO_3]^-$ at m/z 80 and $[HSO_4]^-$ at m/z 97. Phosphorylated compounds yield $[PO_3]^-$ at m/z 79 and $[H_2PO_4]^-$ at m/z 97 (Wilm et al. 1996). We report here that MALDI-TOF/TOF yields these diagnostic high-energy CID ions very reliably and at sufficiently high intensities to be potentially useful also in phospho- versus sulfo-proteomics. We suspect that the extremely short time between CID and detection in the TOF/TOF geometry contributes to the radical anion being robustly detected. Because Ser/Thr sulfation has recently been described in a wide range of organisms and because the distinction between Ser/Thr sulfation and phosphorylation cannot be made using the most common phosphoproteomics methodology, the TOF/TOF analyzer will be extremely useful for this purpose.

Experimental metastasis assays

Mice (strain C57Bl/6J) were injected with $3{-}4 \times 10^5$ MC-38GFP syngeneic adenocarcinoma tumor cells, which were either arylsulfatase or mock treated. After 4 weeks, mice were euthanized, a picture of dissected lungs was taken, and lungs were further processed for quantitation of metastasis by detection of GFP fluorescence (Borsig et al. 2002).

Supplementary material

Supplementary data are available at *Glycobiology* online (http://glycob.oxfordjournals.org/).

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Conflict of interest statement

None declared.

Abbreviations

ATT, 6-aza-2-thiothymine; CID, collision-induced dissociation; CL, cardiolipin; CST, cerebroside sulfotransferase; FCS, fetal calf serum; HBSS, Hank balanced salt solution; HPTLC, high-performance thin layer chromatography; LP, lower phase; MALDI TOF/TOF, matrix-assisted laser desorption/ionization time-of-flight/time-of-flight analysis; MS, mass spectrometry; PLC, phospholipase C; SM2, sulfated triganglioside GalNAcβ-4(HSO_3-3)Galβ-4Glcβ-1Cer; SM3, sulfo-lactosylceramide HSO_3-3Galβ-4Glcβ-1Cer; SM4, sulfated galactosylceramide/sulfatide HSO_3-3Galβ-1Cer; UP, upper phase.

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