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Analysis of SM4 sulfatide as a P-selectin ligand using model membranes ☆

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ABSTRACT

Carcinoma tumor cells express highly glycosylated mucins acting as ligands for selectin adhesion receptors and thus facilitating the metastatic process. Recently, a sulfated galactocerebroside SM4 was detected as solely P-selectin ligand on MC-38 colon carcinoma cells. Here we characterize the functionality of SM4 as selectin ligand using model membrane approaches. SM4 was found concentrated in lipid rafts of MC-38 cells indicating a local clustering that may increase the avidity of P-selectin recognition. To confirm this, SM4 was incorporated at various concentrations into POPC model membranes and lateral clustering was analyzed by fluorescence microscopy and found to be comparable to glycolipids carrying the sLe^x epitope. SM4 containing liposomes were used as cell models, binding to immobilized P-selectin. Quartz crystal microbalance data confirmed SM4/P-selectin liposome binding that was inhibited dose-dependently by heparin. Comparable binding characteristics of SM4 and sLe^x liposomes underscore the similarity of these epitopes. Thus, clustering of SM4 on tumor cells is a principle for binding P-selectin.

1. Introduction

The major cause of death by cancer patient is the spread of cancer cells — metastasis. The underlying mechanisms of haematogenous metastasis are highly complex and, although in the focus of research interests, not yet completely understood [1].

Adhesion receptors, i.e. selectins are critically involved in tumor cell metastasis. Selectins are carbohydrate binding proteins that are expressed by the endothelium (E- and P-selectin), platelets (P-selectin) and leukocytes (L-selectin) and play an important role in leukocyte trafficking in inflammation [2,3]. However, selectins are able to mediate various interactions between tumor cells that entered the blood system with leukocytes or platelets. Platelet-mediated tumor cell emboli formation is primarily mediated by P-selectin and leads to escape of tumor cells from the host immune response [4]. In addition, the formation of microemboli of tumor cells and blood components can lead to physical arrest in the microvasculature.

Selectin ligands are sialylated and fucosylated mucins, often based on the tetrasaccharide epitopes sialyl Lewis^X (sLe^x) or sialyl Lewis^a. Carcinoma mucins have been identified on the surface of different tumors cell lines and functionally been related to the malignant progression [5–8].

Elevated expression of sulfatides in various colorectal carcinomas and ovarian carcinomas were also correlated with poor prognosis [9,10]. Sulfatides are a class of sulfated glycosylceramides that possess a binding capacity to several physiological components, including the matrix substrates laminin and vitronectin. This binding ability has been related to modulate the metastatic potential of renal cell and hepatocellular carcinomas [11,12]. Sulfatides were also found to be recognized by P- and L-selectins [13,14]. Aruffo et al. showed that soluble sulfatides could inhibit binding of myeloid cells to P-selectin [13]. This inhibition was explained by the existence of two non-identical, but overlapping binding sites in the P-selectin molecule; one for sulfatides and the other for sLe^x-related structures [15]. Analysis of synthetic sulfatide analogs displayed that the binding of L-selectin to sulfatides is dependent on the position of the sulfate, and 3-sulfated–galactosylceramide, SM4, was the best ligand [14]. Cell surface SM4 sulfatides were shown to mediate platelet activation and their aggregation through P-selectin [16].

Recently, a direct evidence for the role of sulfatides as selectin ligands that contributes to metastasis was presented in a mouse model of experimental metastasis [17]. Garcia et al. identified the sulfated galactosylceramide SM4 as the only ligand for P-selectin on MC-38 colon carcinoma cells. The enzymatic removal of sulfation from the cell surface of MC-38 cells led to a significant decrease of P-selectin binding and resulted in attenuation of metastasis [17]. These data directly link SM4 sulfatide expression with the progression of metastasis. Nevertheless, several functional aspects of sufatides as cellular ligands of selectin-mediated cell adhesion remain to be elucidated. Binding affinity of

Abbreviations: DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholin; DiO, 3,3′-dioctadecyloxacarbocyanineperchlorate; LMWH, low molecular weight heparins; POPC, 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphocholin; (sLe^x), sialyl Lewis^X; (QCM), quartz crystal microbalance; SM4, 3-sulfated–galactosylceramide; UFH, unfractionated heparin.

☆ Dedicated to Prof. Alfred Blume on the occasion on his 65th birthday.

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P-selectin to SM4 sulfatide has not been determined, although it is expected to be comparably low as that of the sLe^x epitope [18]. The low affinity of sLe^x epitopes can be covered by lateral clustering, the resulting avidity increase was shown to be a basis of selectin-mediated cell binding *in vitro* [19]. Although a recent study demonstrated the formation of artificial lipid rafts by sulfogalactoglycerolipids and -ceramides in supported bilayer systems [20], no data exist on the lateral distribution of sulfatides in tumor cell membranes.

Several randomized-controlled clinical trials confirmed that the application of low molecular weight heparins (LMWH) display a survival benefit in cancer patients [21–23]. Heparin affects the metastatic progression, and this activity is not only related to a prevention of thrombosis. Several animal experiments confirmed that P- and L-selectin inhibition by heparin is one of the major activities contributing to the attenuation of metastasis [24–26].

Using a biosensor technique, we evaluated the kinetic constants of heparin and LMWHs binding to P- and L-selectin [27,28]. A high affinity binding of heparin to P- and L-selectin was found and referred to the slow off-rate of heparin dissociating from the receptors. Nevertheless, the ability of heparin to interfere with sulfatide-mediated selectin binding remains to be defined.

To obtain further insights into the role of SM4 sulfatide as a ligand for P-selectin-mediated metastasis, we introduced a model membrane system in combination with a high sensitive acoustic biosensor. We provide evidence that SM4 is arranged in lipid rafts on MC-38 colon carcinoma cells suggesting a role of SM4 clusters for increased avidity. This was simulated by incorporating different amounts of sulfatides into model membranes, analyzing the clustered appearance by confocal laser scanning microscopy and related to P-selectin binding. Binding of SM4-sulfatide liposomes to immobilized P-selectin was monitored with a biosensor and the potential of heparin to inhibit this interaction was analyzed.

2. Materials and methods

2.1. Materials

Recombinant mouse P-selectin-Fc chimera was prepared in CHO cells as described [29]. Cyanuric chloride, 3,3′-dioctadecyloxacarbocyanineperchlorate (DiO), 1-hexadecanethiol, hydrogen peroxide, monochlordimethyloctadecyl-silane, SM-4-sulfatide (HSO_3-3-Galβ-1-Cer), sulfuric acid, triton X-100, tris × HCl, and ethanol were purchased from Sigma, Deisenhofen, Germany. 6-mercaptohexan-1-ol was purchased from Fluka, Neu-Ulm, Germany, and chloroform was from Riedel de-Haen, Seelze, Germany.

1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphocholin (POPC), and 1,2-distearoyl-*sn*-glycero-3-phosphocholin (DSPC) were from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). The sLe^x-based dialkylglycerolipid sLe^x-TEG_{N2} was synthesized by Dr. Christian Gege (University Konstanz) as described [19].

Liquemin N 5,000® was from Hoffman-La-Roche AG, Grenzach-Wyhlen, Germany, (Ch.-B. F002611, 5000 IU, 0.5 mL). Nadroparin calcium (Fraxiparine®) was purchased from GlaxoSmithKline GmbH & Co. KG, Munich, Germany (Ch.-B. 53283; 1900 IU/0.2 mL; average MW, 4.5 kD).

All salts and buffers were of analytical grade.

2.2. Cell lines

MC-38 mouse colon carcinoma cells as well as LS180 cells were cultured as described previously [29,30].

2.3. Lipid raft isolation and TLC immune-overlay

All steps of the lipid rafts preparation were accomplished at 4 °C. MC-38 cells were grown to 90% confluency, washed with PBS, and once with TNE buffer (50 mM Tris–Cl, pH 7.0, 140 mM NaCl, and 5 mM EDTA). Cells were scraped in TNE buffer and centrifuged for 5 min., 4000 rpm. The pellet was resuspended in 250 µL TNE buffer supplemented with protease inhibitor cocktail. Cells were homogenized by 25 strokes through a 25-G needle. Triton X-100 was added to 198 µL of cell homogenate to reach a final concentration of 1% and mixed for 30 min. The preparation was mixed with 440 µL of OptiPrep (60% stock solution) and 600 µL of the mixture were overlayed with 1.2 mL 30% OptiPrep diluted in 2 × TNE. Upon the addition of TNE, the samples were spun down at 200,000 g for 150 min at 4 °C. The upper floating fraction containing the lipid rafts was analyzed for the presence of Caveolin by Western Blot. Lipids were extracted from the floating fraction by methanol/chloroform. The extracted lipids were dried down and resuspended in methanol prior to loading on HPTLC. Lipid fraction was separated on HPTLC followed by P-selectin detection as described previously [17].

2.4. Preparation of model membranes for microscopic and biosensor investigations

Glass slides (diameter 18 mm and thickness 0.2 mm) were used as model membrane supports in confocal laser scanning microscopy. The slides were ultrasonificated in a mixture of conc. H_2SO_4:H_2O_2 (1:3) for 15 min at 80 °C. After rinsing ten times with aqua dest., the density of silanol groups on the surface was increased by a second cleaning protocol using NH_3:H_2O_2:H_2O (1:1:5) before rinsing fifteen times with aqua dest. The glass slides were dried at 70 °C and then incubated in a solution of monochlordimethyloctadecyl-silane for 10 min at 70 °C to form a covalently bound hydrophobic supporting layer. After rinsing with chloroform and aqua dest., the glass slides were dried under a stream of air.

Quartz crystals were supplied by ifak (Barleben, Germany) and cleaned three times in a mixture of conc. H_2SO_4:H_2O_2 (1:3) for 3 min. The quartz crystals were placed in a chloroform solution of hexadecanthiol ($C_{16}H_{33}SH$, 10 mM) for 12 h to form a self assembled monolayer. They were rinsed briefly with ethanol and dried under air stream.

Solutions of POPC or DSPC resp., in chloroform (10 mM) were mixed with aliquots of SM4-sulfatide or sLe^x-TEG_{N2}, resp. in chloroform (0.1 mM) to obtain yields between 0.5 mol% and 20 mol% of the glycolipids. For microscopic experiments, the samples were labeled with 1 mol% DiO. Ligand containing films were pre-formed on the air–water interface of a Langmuir trough. The films were characterized by the ∏/A isotherms, and 95% of the maximum compressibility was chosen as transfer pressure. Hydrophobized glass slides or quartz crystals were moved vertically through the lipid layer on the Langmuir trough with a transfer speed of 0.5 mm min^{-1} to complete the bilayer.

Freshly prepared supported bilayers were immediately used for laser scanning microscopy (Carl Zeiss LSM 710, Germany; ex. 487 nm and em. 501 nm) and QCM experiments (2.6.).

2.5. Liposome preparation

Liposomes were prepared by hydration technique. Chloroform solutions of POPC and SM4-sulfatide or sLe^x-TEG_{N2}, resp. were mixed in a round bottom flask to obtain the indicated ligand concentration. Chloroform was removed using a rotary evaporator at 200 mbar and 40 °C forming a dry lipid film. The film was hydrated with 1 mL PBS buffer (pH 7.4) to reach a final lipid concentration of 1 mM. Buffer and lipids were shaken over night on an orbital shaker. The resulting multilamellar liposomes were extruded six times (extruder Lipex, Vancouver, Canada) through a 100 nm polycarbonate membrane (Whatman Nuclepore polycarbonate membrane, Richmond, USA). Particle size was detected by PCS (Malvern Autosizer) and found to be about 100 nm.

2.6. QCM measurements

QCM measurements were performed with a LiquiLab21 quartz crystal microbalance (ifak e.V., Barleben, Germany). Measurement chambers were made of polycarbonate and had a chamber volume of 100 µL. Flow conditions and transport of the test substances to the sensor surface were assured by a peristaltic pump.

AT-cut quartz crystals (10 MHz resonance frequency, 14 mm diameter) were prepared as described in Section 2.4. Alternatively, recombinant mouse P-selectin–Fc chimera was immobilized on the sensor surface as described [28]. Quartz crystals were inserted into the measurement chambers and rinsed with PBS buffer containing Ca^{2+} (1 mM) and Mg^{2+} (1 mM). The volume flow rate was 270 µL min^{-1} corresponding with a shear slightly below that in postcapillary venules. Equilibration of the quartz crystal oscillation was achieved after 30 min under flow conditions. The indicated concentrations of selectin ligand containing liposomes with or without heparin derivatives were added under flow conditions. Frequency changes were monitored in real time using a connected PC device.

2.7. Statistical analysis

Data are represented as means of at least three independent experiments ± standard deviation (S.D.). Statistical comparisons were performed with the unpaired Student's *t*-test. *Indicates significance at $p \leq 0.05$ and **indicates significance at $p \leq 0.01$.

3. Results

3.1. SM4 is localized in lipid rafts in MC-38 cell membranes

SM4 sulfatide was recently identified as the only native P-selectin ligand on MC-38 colon carcinoma cells that mediated metastasis in a P-selectin dependent manner [17]. Although P-selectin binding to sulfatides has been described previously, the functional aspects of this interaction on cell adhesion and metastasis remain to be defined. To study the efficiency of P-selectin binding on the cellular level, we tested the binding of MC-38 cells, carrying SM4 sulfatides, and compared them with LS180 cells, carriers of sLe^x structures on cell surface mucins known to be recognized by selectins. Both cell lines bound efficiently to P-selectin coated surfaces, but the binding of MC-38 cells was inhibited by lower heparin concentrations than the binding of LS180 cells (Fig. 1). This observation alone cannot be interpreted by weaker binding of P-selectin to SM4 sulfatide compared to sLe^x-based sialomucins, because structural arrangement and concentration of both types of ligands are unknown thus not comparable.

Sialic acid-containing glycolipids, as well as sulfoglycolipids are concentrated on cell surface membranes in lipid rafts [31]. To determine whether SM4 sulfatide on MC-38 cells is located in the lipid rafts, we isolated lipid rafts from MC-38 cells. The purity of lipid raft fractions was confirmed by the detection of caveolin (data not shown). The extraction of lipid from the lipid rafts was further analyzed by HPTLC. Due to the limited amount of extracted lipids from the rafts by this procedure, the orcinol staining displayed only a very weak band at a similar level of the standard bovine SM4 sulfatide (Fig. 2A). To confirm the presence of SM4 in this fraction, an immune-overlay with the P-selectin chimera was performed (Fig. 2B). A distinct band was identified by P-selectin in the lipid raft fraction. A small mobility difference of the SM4 sulfatide isolated from MC-38 cells compared to the standard bovine SM4 sulfatide was previously confirmed by mass spectrometry [17]. This observation indicates that SM4 sulfatide recognized by P-selectin on the cell surface of MC-38 cells is presented in lipid raft membrane domains.

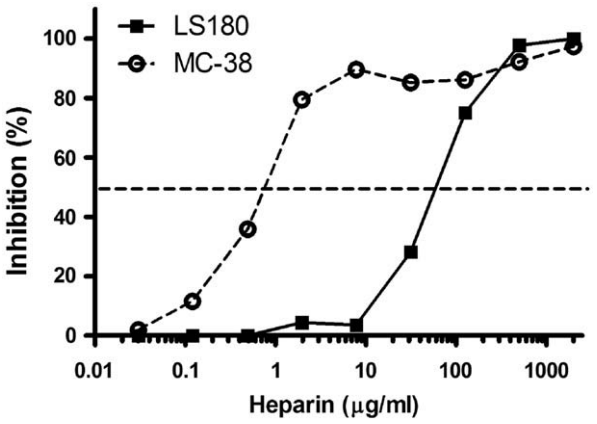

Fig. 1. Heparin inhibition of P-selectin binding to tumor cells with various selectin ligands. ELISA plates were coated with mouse P-selectin to which calcein AM labeled LS180 human or MC-38 mouse carcinoma cells were added in the presence of serial dilutions of unfractionated heparin at concentrations ranging from 0.03 to 2000 µg/mL.

3.2. Artificial rafts of SM4 in model membranes

To evaluate the SM4 ligand function for P-selectin in an appropriate model, SM4 was incorporated into model membranes. Lateral separation of SM4 as artificial rafts in the lipid matrix was aspired and detected by confocal fluorescence microscopy for SM4 concentrations between 2 and 20%. Either DSPC as a rigid matrix was chosen, or POPC referring to the fluidity of biological membranes. SM4 has a strong separation tendency forming clusters at all concentrations used in the matrices (exemplarily shown for 10 and 20% SM4; Fig. 3A–D). The number of clusters represented by the dark spots increases with respect to SM4 concentration in DSPC, accompanied by a slight increase in cluster size (diameters around 2 µm at 10% SM4, and 2–5 µm at 20% SM4 in DSPC,

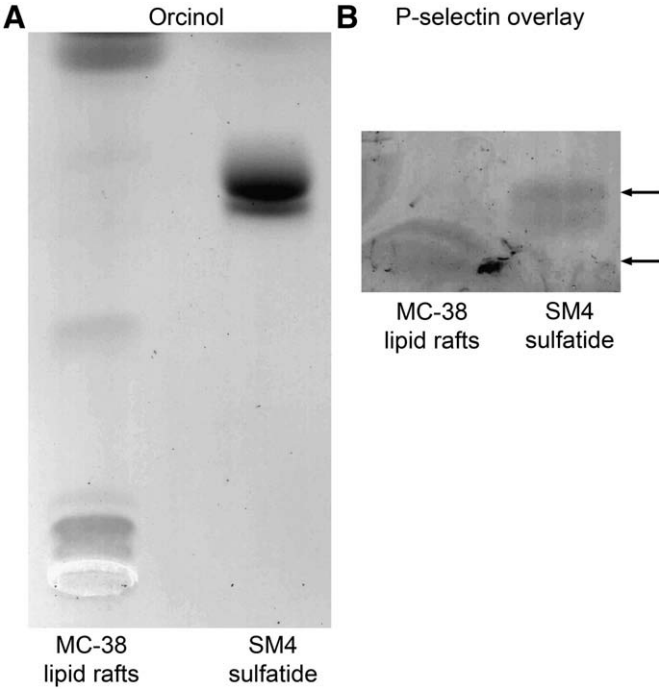

Fig. 2. SM4 sulfatide detected in the lipid raft fraction of MC-38 cells. A) Lipids extracted from the lipid raft fraction of MC-38 cells were separated by HPTLC and glycolipids were visualized by orcinol–sulfuric acid staining. B) P-selectin overlay of separated lipids. SM4 sulfatide was used as a control. Arrows indicate bovine SM4 sulfatide staining and the murine MC-38 derived SM4 sulfatide with a small shift as described previously [17].

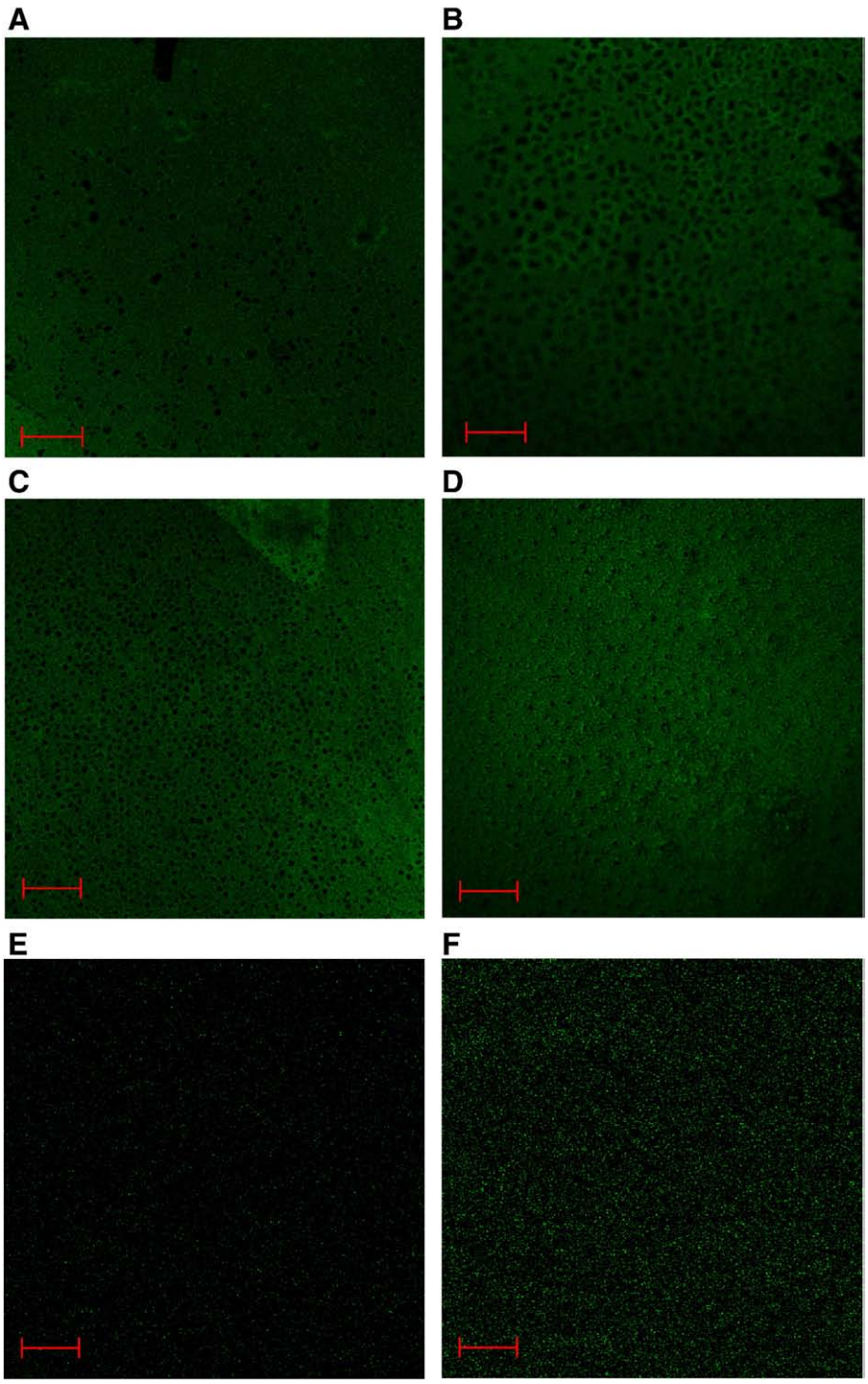

Fig. 3. Lateral separation of SM4 or sLe^x lipid from different matrices creating artificial membrane rafts. The incorporation of 10 mol% or 20 mol% SM4 in a DSPC matrix (A and B) or in a POPC matrix (C and D) resulted in a local clustering of SM4 indicated as dark spots, the dimension of the clusters deviate slightly within the different approaches. For comparison, the sLe^x lipid was incorporated at 10 mol% (E) and 20 mol% (F) into POPC, bars represent 20 μm.

Fig. 3A,B). In the POPC matrix, the clusters display a more uniform size in a dimension of 1–2 μm (Fig. 3C,D). As a special feature in D, the dye accumulated at the phase borders. The uniform size of clusters in POPC and their relatively size independence on SM4 concentration appeared as a more sufficient model system to simulate the natural conditions in the cell membrane than in DSPC.

For comparison, a dialkylglycero-glycolipid carrying the tetrasaccharide sLe^x was also incorporated into POPC. In contrast to SM4, the fluorescence dye accumulated in the sLe^x clusters indicating bright spots of sLe^x lipids in the dark POPC matrix (Fig. 3E,F). The sLe^x clusters at both concentrations were slightly below 1 μm in diameter and thus, provide a sufficient basis for comparing both SM4 and sLe^x lipids for binding experiments. Amounts of 10% or 20% of binding epitopes in membranes are much higher than physiologically relevant. However, with respect to the model membrane approach focusing on avidity increasing effects of clusters in binding to

P-selectin, the use of these relative high concentrations appeared arguable.

The functionality of the glycolipid clusters for P-selectin binding was investigated by biosensor measurements using a quartz crystal microbalance (QCM). Model membranes of POPC with 0.5% to 20% of SM4 were transferred to the sensor surface and interaction with soluble P-selectin was evaluated referring to the decrease in oscillation frequency of the sensor. Whereas P-selectin induced a drop of 10 Hz by binding to the pure POPC membrane, already 0.5% of SM4 caused a frequency decrease by 22 Hz. Further increase in SM4 concentrations in the POPC membrane up to 5% induced a non-linear drop in frequency (up to 32 Hz) reaching a saturation above 5% SM4 in the membrane. These findings were interpreted with respect to the appearance of clusters and their functional activity. Since evaluation of binding kinetics is based on a 1:1 binding model, not assumed in a clustered presentation, no further kinetic binding calculations were performed.

3.3. Binding of SM4 containing cell models to P-selectin and inhibition by heparin

To simulate the SM4 ligand function in MC-38 cells interacting with endothelial or platelet selectins, SM4 was incorporated at different concentrations into liposomal membranes and liposomal binding to P-selectin, immobilized at the sensor surface was analyzed by QCM. For comparison, sLe^x liposomes were identically applied at a final concentration of 10 μM. While pure POPC liposomes displayed hardly any binding to immobilized P-selectin, increasing concentrations of both, SM4 or sLe^x lipid in the liposomes increased the sensor interaction, as illustrated exemplarily for 10% of ligands in Fig. 4.

Heparin possesses antimetastatic activities that are at least in part explained by the inhibition of P-selectin binding to carcinoma mucins on tumor cells [29]. To investigate whether the SM4 binding to P-selectin is also inhibited by heparin, increasing concentrations of the unfractionated heparin (UFH) Liquemin N were added in the low micromolar and mean nanomolar ranges. As illustrated in Fig. 5A, heparin inhibited liposome binding to P-selectin in a concentration dependent manner. Interestingly, the highest concentration of heparin (column 6) did not display the best inhibition, which should be referred to the detected mass effect of P-selectin bound heparin itself. Even at the lowest UFH concentration a dominant inhibition of liposome binding to P-selectin was evident. The binding of sLe^x liposomes to P-selectin showed comparable behavior (Fig. 5B) albeit the binding was not affected at the lowest heparin concentrations (columns 2 and 3). Similar effects on P-selectin binding to SM4 and sLe^x liposomes were observed with the use of a low molecular weight heparin (LMWH; Nadroparin) (Fig. 6A,B). Nevertheless, the LMWH tends to show a slightly lower inhibition of SM4-liposome binding to P-selectin. However, the data confirm the P-selectin dependency of SM4 liposome binding and the inhibition by both fractionated and unfractionated heparin.

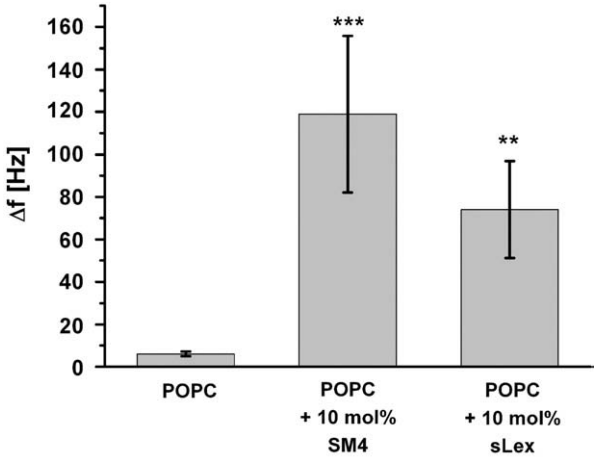

Fig. 4. Biosensor detection of liposome binding onto an immobilized P-selectin layer, binding correlates with the change in frequency. Whereas pure POPC liposomes did not bind, the incorporation of 10 mol% SM4 or 10 mol% sLe^x lipid into the liposomes induced a significant P-selectin binding.

4. Discussion

Sialomucins as selectin ligands, i.e. the P-selectin ligand PSGL-1 were regarded as ideal structures for obtaining sufficient binding affinities by presenting the selectin binding epitopes in a specific manner. The single carbohydrate epitopes, such as the tetrasaccharide sLe^x displays only low affinities in the micro or millimolar range [32]. PSGL-1 possesses core-2 structured carbohydrates as a mode of local epitope accumulation [33]. Although these structures are important for binding, they are not alone sufficient to mediate the high affinity of PSGL-1 to P-selectin [34]. Nevertheless, the local concentration of carbohydrate binding epitopes is regarded as a principle to increase selectin affinities [35]. Referring to this, previous model membrane experiments provided evidence that a lateral clustering of sLe^x moieties as artificial rafts provides sufficient binding avidity for binding and rolling of selectin-expressing cells [19,36]. Similar studies on sulfatides are not known.

Here we provide evidence that SM4 is localized in MC-38 membrane rafts. Thus it can be expected that this local concentration of SM4 creates sufficient binding avidity for mediating cell binding to P-selectin comparable to the capacity of sialomucin ligands, as illustrated in Fig. 1. The recent observation that MC-38 cells binds to platelets through P-selectin-SM4 sulfatide axis and thereby promote metastasis strongly support the functionality of SM4 sulfatide. The model membrane approaches of the present study confirm this hypothesis.

Earlier it was shown that sulfatides were efficiently recognized by P- and L-selectin [13,15]. Whether sulfatides may contribute to metastasis through selectin-mediated interaction was not studied until recently. The inhibition of selectin-mediated cell binding by the addition of sulfatides was reported [13,14,37] and the present data refer to the postulation that clustering of SM4 sulfatides enhanced avidity of P-selectin recognition.

The incorporation of SM4 into POPC model membranes results in SM4 clusters, which can be regarded as artificial rafts for simulating the conditions in the MC-38 cell membrane. Since DiO tends to accumulate in the liquid expanded lipid phase, the distribution of the fluorescent lipid implies a higher packing density of the SM4 clusters compared to the sLe^x clusters in the POPC matrix, although the dimensions of both are comparable. The QCM binding studies confirmed the accessibility of SM4 in the membranes for P-selectin binding, nevertheless, this approach does not allow the calculation of kinetic binding constants of individual monovalent SM4/P-selectin binding.

The incorporation of SM4 into liposomal membranes and their use as cell models is a valuable option to simulate the tumor cell binding to P-selectin being expressed either by platelets or the endothelium. A comparable SM4 clustering in the liposomal membranes and in the Langmuir monolayers (Fig. 3) was assumed and supported by microscopic investigation of surface-fused SM4 liposomes labelled with DiO. The biosensor data confirmed that SM4 clusters are able to mediate a particle binding under shear flow conditions. The SM4 patches displayed a comparable capacity to the sLe^x lipid to act as a P-selectin ligand. Since several studies report on the multimerization of sLe^x as a way to increase selectin binding avidity [19,35,38], our findings provide evidence that the same mechanism can also be

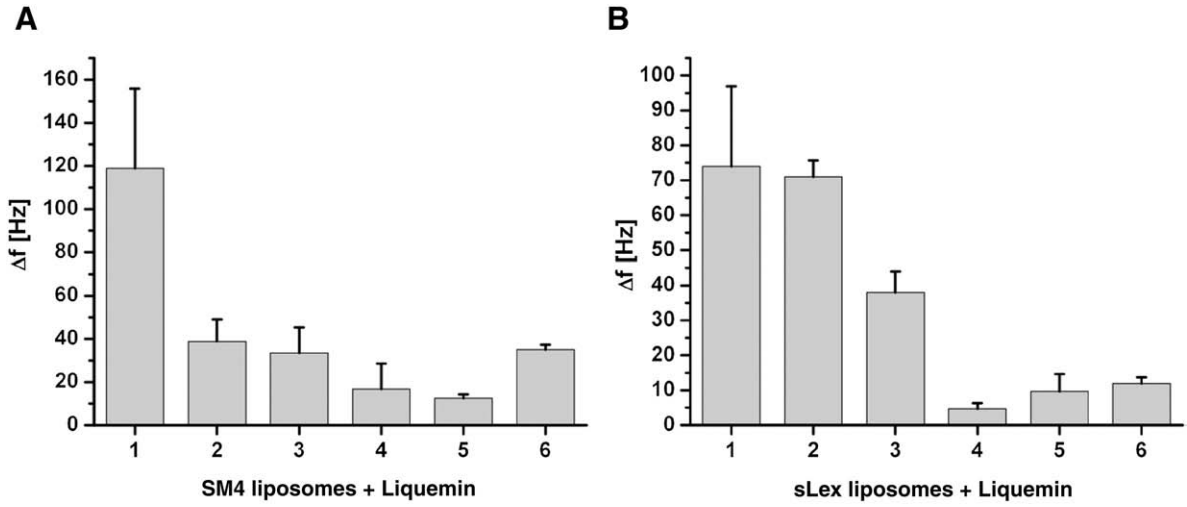

Fig. 5. Inhibition of the P-selectin binding of liposomes containing 10 mol% of SM4 (A-1) or 10 mol% of sLe^x lipid (B-1) by different concentrations of UFH (Liquemin), detected by QCM. Liquemin was applied at the following concentrations: 2 — 6.7×10^{-8} M; 3 — 2.2×10^{-7} M; 4 — 1.1×10^{-6} M; 5 — 3.3×10^{-6} M; and 6 — 5.6×10^{-6} M.

applied to SM4 sulfatide. With respect to the frequency drop of the P-selectin covered sensors, indicating the intensity of liposome interaction, a slightly stronger effect of SM4 sulfatide than of sLe^x could have been observed under identical liposomal conditions, referring to a less closely sensor coverage by the latter.

Heparin is an attractive P-selectin inhibitor possessing affinities in the high nanomolar range [28]. P- and L-selectin binding of heparin were related to its antimetastatic activity, i.e. inhibiting the interaction of tumor cell sialomucins with platelet P-selectin or L-selectin from leukocytes. Since sulfatides and sLe^x related ligands do not share the same binding site in the P-selectin molecule [15], we aimed to compare the effectivity of heparin as an inhibitor of P-selectin binding to these ligands. Here we show that heparin inhibits both SM4 sulfatide and sLe^x structures to a similar extent, thus confirming its potential to inhibit P-selectin-mediated interaction to both types of ligands. In accordance with our recent data on heparin affinity to P-selectin [28], both UFH and LMWH were able to block the liposome binding in the low micromolar range, and UFH displayed a slightly higher activity than LMWH. Inhibition of SM4 liposome binding was slightly more effective at the lowest UFH concentration than the inhibition of sLe^x liposomes. Nevertheless, the strong differences in binding and heparin inhibition of SM4 and sLe^x-based sialomucin on the cellular level (Fig. 1) were not reflected. This indicates that in contrast to our model membrane approach providing identical SM4- and sLe^x patches for binding, the presentation of sialomucins on LS180 cells is different from the model membranes. However, these data refer to the excellent inhibitory capacity of heparin to inhibit different selectin ligands and support the evidence for antimetastatic effects of heparin to be caused mainly by its selectin inhibitory activity.

Collectively, the presented model membrane studies confirm that SM4 in a clustered arrangement is a functional P-selectin ligand to mediate cell binding in the course of metastasis of the MC-38 cells. Applying a liposomal tumor cell model, selectin ligand mediated binding can be analyzed with high sensitivity using a biosensor technique, such as QCM. The SM4/P-selectin binding is sensitive to heparin interference, which should be confirmed in further *in vivo* studies of metastasis.

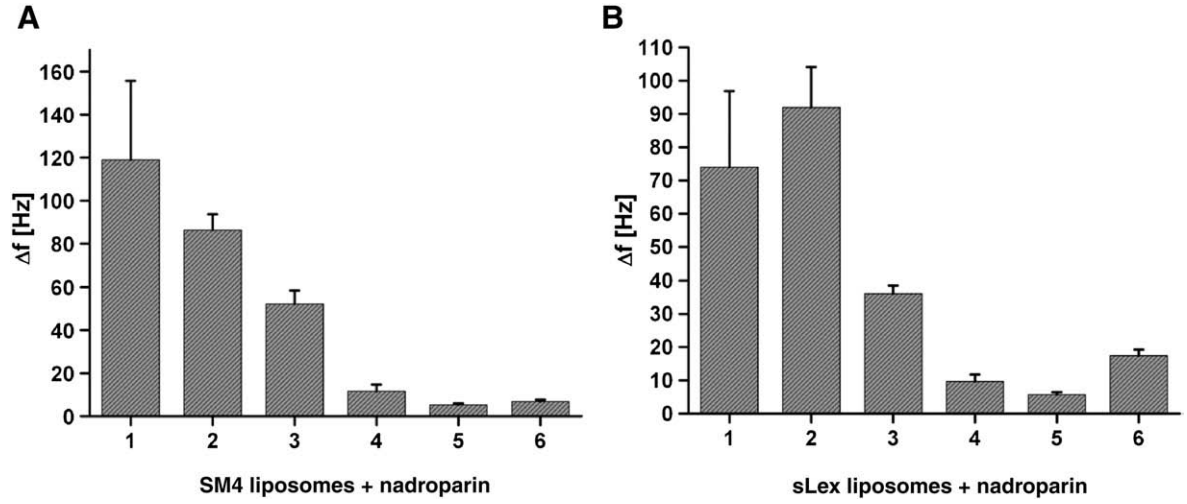

Fig. 6. Application of the LMWH nadroparin to inhibit the P-selectin binding of liposomes containing 10 mol% of SM4 (A-1) or 10 mol% of sLe^x lipid (B-1). Nadroparin concentrations were: 2 — 7.4×10^{-8} M; 3 — 7.4×10^{-7} M; 4 — 3.7×10^{-6} M; 5 –1.1×10^{-6} M; and 6 — 1.8×10^{-5} M.

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