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The role of glycolipids in mediating cell adhesion: a flow chamber study[1]

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Abstract

Selectins constitute a family of proteins that mediate leukocyte tethering and rolling along the vascular endothelium by recognizing various carbohydrate ligands in response to inflammation. To test the hypothesis that multivalent binding of selectins to their ligands is the molecular basis for achieving sufficient binding forces, we have performed this flow chamber study. Selectin-containing Chinese hamster ovarial cells (CHO-E) bind and roll along a support-fixed phospholipid membrane containing a defined concentration of a synthetic Sialyl Lewis^x (sLe^x) glycolipid ligand. Ligands are either homogeneously distributed, or arranged in defined lateral clusters, as illustrated here for the first time. The lateral glycolipid clusters which appear as recognition motifs are essential for mediating cell rolling. Furthermore, the transition from firm cell adhesion to cell rolling depends on the site density of ligands. Rolling velocity shows little dependence on shear forces within a broad range. As we found out that cells do not roll along the model membranes with homogeneous ligand distribution, our results therefore support the hypothesis of multivalent binding events. Since these investigations suggest that lipid-anchored sLe^x, functionally embedded in a lipid matrix, can mediate cell rolling, this study demonstrates the relationship between dynamic glycolipid binding to selectins with the hypothesis of multivalency of binding for the first time. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Selectin; Cell adhesion; Glycolipid; Sialyl Lewis^x; Cell rolling; Planar bilayer, supported

1. Introduction

The receptor-mediated recruitment of leukocytes to sites of injury, or infection is essential for the development of an appropriate immune response. Leukocyte rolling along endothelial cells under the shear force in postcapillary venules represent the first step in a sequence of adhesive interactions that lead to firm attachment and subsequent emigration through the venular wall [1]. The selectins, a family of three adhesion molecules, are thought to mediate rolling by binding carbohydrate presenting ligands

Abbreviations: AFM, atomic force microscopy; CHO-E, Chinese hamster ovarial cells (containing E-selectin); CLA, cutaneous lymphocyte associated antigen; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; FITC, fluorescein-5-isothiocyanate; NBD-PC, 1-palmitoyl-2-[12-[(7-nitro-2-1,3 benzoxadiazol-4-yl)amido]-dodecanoyl]-*sn*-glycero-3-phosphocholine; NBD-PE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl); PBS, phosphate-buffered saline; POPC, 1-palmitoyl, 2-oleoyl-*sn*-glycero-3-phosphocholine; sLe^x, sialyl Lewis^x

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[1] This paper is dedicated to Professor H.W. Meyer (University of Jena) on the occasion of his 65th birthday.

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with rapid association and dissociation rates constants [2]. E-selectin and P-selectin can be expressed on endothelial cells by inflammatory signals, and both bind to carbohydrate ligands on subsets of leukocytes [3]. L-selectin, which is constitutively expressed on leukocytes and shed after activation, binds inducible ligands on the endothelium [4]. All selectins share a highly conserved N-terminal lectin domain that can interact with sialylated and fucosylated polylactosaminoglycans presented by mucin-type glycoproteins, where sLe^x epitopes (NeuAcα2–3Galβ1–4(Fucα1–3)GlcNAc) are of key importance. Although numerous studies performed under static conditions have confirmed fundamental findings about selectin binding characteristics and structure–activity relations of their binding epitopes [5–7], they do not provide information on how ligands interact with selectins under dynamic conditions.

Springer and coworkers have performed various studies to investigate the role of dynamic parameters and the contribution of the individual selectins in the rolling process [8,9]. Therefore, they immobilized defined amounts of E-selectin or P-selectin into planar membranes or onto plastic and analyzed the rolling interaction of neutrophils under defined shear flow conditions. They could show that tethering, rolling velocity and shear force resistance of neutrophils are regulated by the kinetics of bond formation and dissociation, which are different for each of the individual selectins [10].

These studies had been focused on modifying selectins for the characterization of their binding properties. Several questions concerning the carbohydrate ligands remained unanswered. Whereas selectins bind the identified single carbohydrate epitopes with low affinity, they bind, however, with much higher affinity to the sialomucins [11]. Multiple protein–carbohydrate interactions are therefore thought to be the reason for a higher binding efficiency mediated by a special molecular arrangement of binding epitopes [12]. Various groups prepared synthetic oligomeric sLe^x derivatives, which could only in part support this hypothesis of slowly increased affinities [13,14]. Furthermore, the role of the carbohydrate presenting anchor group is not fully elucidated. Contrary to the natural occurring glycoproteins, various binding studies have been performed with glycolipids, which were disorderedly immobilized onto solid surfaces. But in our opinion, these surfaces cannot adequately simulate conditions at the membrane surface.

We have performed this study to investigate the molecular features of ligands such as structure, density and lateral clustering, which are fundamental for the rolling process. Therefore we incorporated various concentrations of glycolipid ligands into planar support-fixed phospholipid membranes to get a well defined model membrane, which could simulate the conditions at cell surfaces. By using the Langmuir–Blodgett technique, glycolipids can be organized as defined clusters in the matrix, and may serve thus as recognition determinants. We wanted to illuminate if lateral clusters are the basis for the discussed higher binding affinity of sialomucins by multivalent binding. Therefore, the model membrane was assembled into a flow chamber to interact with selectin-containing cells under defined shear flow conditions. Using transparent support materials, direct detection of cell binding events with an inverted confocal laser scanning microscope was made possible.

To examine whether carbohydrates presented by lipid anchor groups in a defined membrane are able to mediate cell rolling, we choose a synthetic derivative where the sLe^x moiety is directly linked to a ceramide anchor (NeuAcα2–3Galβ1–4(Fucα1–3)GlcNAc-ceramide). By modifying the density and the appearance of clustered lateral arrangement of this ligand in the phospholipid matrix, the role of multiple ligand–receptor bonds should be detectable.

We were able to show that sLe^x ceramide can act as ligand which mediates rolling or firm adhesion in a concentration-dependent manner, when laterally organized in clusters. The absolute ligand concentration was further reduced, when ligand clusters were diluted by non-reactive lipids. When the glycolipid was homogeneously distributed into a phospholipid matrix, cells adhered selectin-induced, but did not roll upon diluting the ligand density.

Thus, our results support the hypothesis of multivalent ligand receptor interactions as prerequisite for rolling and higher binding constants.

2. Materials and methods

2.1. Materials

1,2-Distearoyl-*sn*–glycero-3-phosphocholine (DSPC), 1-palmitoyl, 2-oleoyl-*sn*–glycero-3-phosphocholine (POPC) were purchased from Sigma (Deisenhofen, Germany). The purity of these lipids was analyzed by HPTLC and HPLC and regarded as greater than 99%. POPC was frequently analyzed during this study and did not show any oxidative degradation products. 1,2-Dipalmitoyl-*sn*–glycero-3-phosphoethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-PE) and 1-palmitoyl-2-[12-[(7-nitro-2-1,3 benzoxadiazol-4-yl)amido]dodecanoyl]-*sn*–glycero-3-phosphocholine (NBD-PC) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). The glycolipid was synthesized as previously published [15] and used with a purity of greater than 99%. The substances were used without further purification.

2.2. Cell cultivation

Mouse E-selectin-transfected CHO-cells (CHO-E cells) were a generous gift of P. Vasalli. Cells were grown in MEM-α media containing 10% fetal calf serum, 2 mM L-glutamine and 100 nM penicillin/streptomycin. Flasks seeded with 5×10^4 CHO-E cells were incubated at 37°C in 5% CO_2 for 3 or 4 days until cells were grown nearly confluent. After trypsinization for 3 min with 2 ml 0.25% trypsin in EDTA, cells were transferred to slowly rotating plastic tubes. Cells stayed in suspension for up to 4 h. Within this time, rolling experiments had to be performed in the flow chamber.

2.3. Immunolabeling and embedding for electron microscopy

Cells (approximately 1.8×10^7) transferred and washed in PBS were fixed with 2.5% formaldehyde in PBS for 5 min at room temperature and washed with PBS, afterwards with PBS containing 50 mM glycine (blocking free aldehyde groups) and finally with PBS containing 0.5% bovine serum albumin and 0.2% gelatine (PBG). Cells were incubated overnight at 4°C with specific anti-E-selectin antibody from rat. After six times washing with PBG, cells were incubated for 1 h with 12 nm gold-labeled secondary antibody (anti-rat IgI from goat; Dianova) and afterwards carefully washed with PBG and PBS (all centrifugation steps at 1200 rpm = $120\times g$).

For embedding, the cells were postfixed for 2 h with 4% formaldehyde/0.5% glutaraldehyde, then washed in PBS and suspended in 1.25% agar. Small cell–agar pieces were conventionally dehydrated in acetone and embedded in Durcupan (Fluka, Neu-Ulm, Germany), hardened at 65 and 100°C. Ultrathin sections were stained with UO_2 acetate and Pb citrate.

2.4. Film balance and fluorescence film balance technique

A film balance system of type R and K (Riegler and Kirstein, Germany) was used with MilliQ water as subphase. The lipids were dissolved in freshly distilled chloroform/methanol (2:1, v/v) at 40–60 mM. After spreading the lipid solution at the water surface, an equilibrium period of 10 min followed. Then, the film was compressed at 25°C with a compression speed of 0.01 $nm^2\ min^{-1}\ molecule^{-1}$.

To simultaneously measure the isotherms and domain growth, the trough was integrated into a fluorescence microscope (Olympus, Japan) with a motor driven xy stage, using the dye NBD-PC at 0.5 mol%, that preferentially partitioned into disordered liquid-expanded (LE) phases.

2.5. Preparation of supported planar bilayers

Supported planar bilayers were prepared using the Langmuir–Blodgett technique. Microscope slides (glass, diameter of 18 mm, thickness of 0.2 mm) were used as transparent supports. Slides were first cleaned to achieve a highly homogeneous surface. Therefore, slides were treated with concentrated H_2SO_4/H_2O_2 mixture (7/3) at 80°C for 30 min under ultrasonic conditions, and were then rinsed with ultrapure water for 30 min. To increase the density of silanole groups at the surface, a cleaning procedure with $NH_3/H_2O_2/H_2O$ (1/1/5) followed, before finally rinsing with ultrapure water and drying the slides. The first step in forming a supported bilayer is the covalent binding of monochlordimethyloctadecyl-si-

lane (Sigma, Deisenhofen, Germany) at 50°C for 30 min to produce a first monolayer at the slide. The bilayer was completed by transferring the preformed lipid film at the Langmuir trough.

The lipid mixtures were transferred at a lateral pressure of 38 mN/m and a speed of 0.5 mm/min to hydrophobic substrates as a X-type monolayer. The transfer ratios were between 0.95 and 1. Freshly prepared supported bilayers were immediately used for experiments in the flow chamber.

2.6. Atomic force microscopy

The Langmuir–Blodgett films for AFM were transferred at an applied surface pressure of 38 mN/m to a hydrophobized silicon wafer (Wacker, München, Germany, rectangular 15×7 mm, thickness 2 mm) as a hydrophobic X-type monolayer. The samples were examined within 6 h of preparation. AFM measurements were performed with a Nanoscope IIIa (Digital Instruments, Santa Barbara, USA) under full hydrated conditions in a special water cell (Digital Instruments, Santa Barbara, USA). The contact modus AFM was used with commercial Si_3N_4 cantilevers with a nominal force constant of 0.12 N/m.

2.7. Laminar flow experiments

The parallel plate flow chamber used in these studies has been described in detail in our previous investigations [16]. The flow apparatus was mounted onto an inverted fluorescent microscope Axiovert 135 of a Laser Scanning Microscope (LSM 410 invert, Carl Zeiss, Germany).

Adhesion experiments were performed at 25°C in a temperature-controlled manner to maintain the lateral structure of the model membrane. MEM-α medium (Section 2.2) was used as flow medium at shear rates between 200 and 1000 s^{-1} powered by hydrostatic pressure. For the flow experiments, 10^6 fluorescently labeled CHO-E cells in 100 μl medium were injected into the streaming medium without dilution. Either, cell adhesion or rolling was analyzed immediately, or, the flow was stopped for 5 min to allow cells to interact with the supported membrane. After this time, flow with the desired shear force was continued and adhesion behavior of cells was monitored by a sequence of images taken each 2 s. To characterize the cell movement, 50–150 cells within an area of 630×630 μm were analyzed throughout 20 s. Only those cells observed to directly contact the membrane in absence of prior contacts with adherent cells were counted and analyzed. The experiments for the presented data were repeated four times under similar conditions.

3. Results and discussion

3.1. Detection of selectins at the CHO-E cell surface

The aim of the present study was to characterize the influence of molecular features of synthetic selectin ligands on cell adhesion or cell rolling. In order to focus on ligand effects more distinctly, constant conditions on receptor site had to be employed. Therefore we choose CHO-E cells that constitutively express high levels of E-selectin without the need of further activation. Despite the fact that CHO-E cells are widely used for various selectin-experiments, we started to illustrate the selectin density and distribution at the cell surface by immunogold labeling to get an impression of surface topology and for a better comparison of our results with previous binding

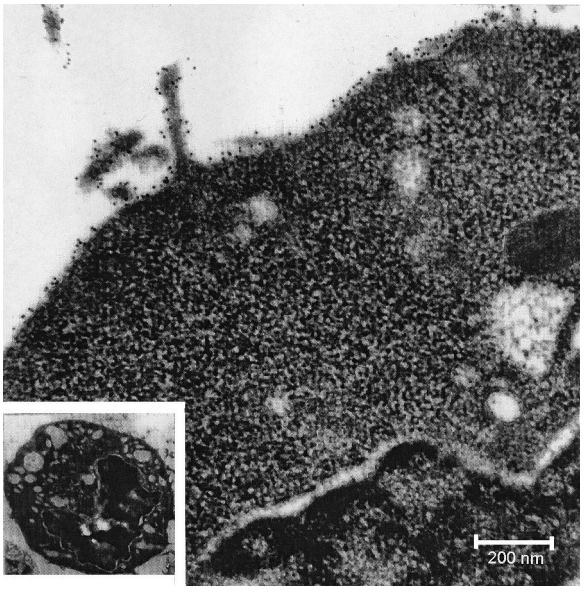

Fig. 1. Immunogold labeling (12 nm gold) of E-selectins (black points) at the surface of a CHO-E cell.

studies using leukocytes. The electron microscope picture in Fig. 1 demonstrates a plain cell surface with a homogeneously distributed high level of E-selectin. This appearance of selectins remains constant at the cell surface upon activating the cell with reagents, such as TNFα, IL1α or -β (data not shown). Therefore, CHO-E cells appear as excellent models in our investigations to focus on ligand behavior.

3.2. Preparation and characterization of the supported planar model membranes

The naturally occurring selectin ligands are mucin-like glycoproteins which present their carbohydrate side chains in a special molecular arrangement, which has been hypothesized to be responsible for higher affinity [12]. We introduce sLe^x ceramide as a glycolipid ligand with a common lipid anchor in our model membranes. By using the Langmuir–Blodgett technique, the glycolipids will be embedded in a phospholipid matrix similar to the conditions at the cell membrane surface. Furthermore, according to temperature, lateral pressure and the kind of phospholipid in the membrane, glycolipids can either be homogeneously distributed in the matrix or be arranged in lateral local clusters. Since such clusters display concentrated multiple copies of a binding epitope, their influence on cell binding could be checked to support the multivalent binding hypothesis in comparison to the membranes with homogeneous ligand distribution.

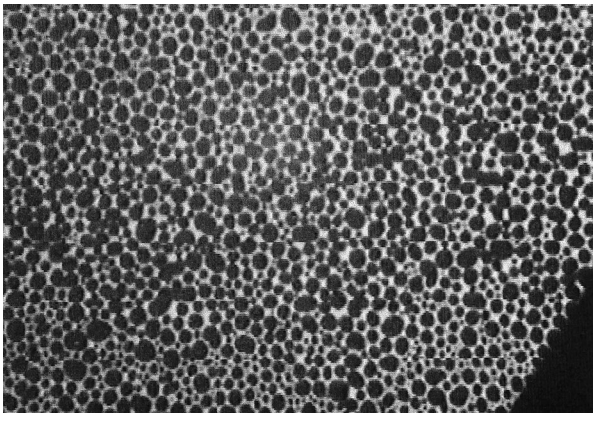

Fig. 2. Fluorescence microscope image of a sLe^x glycolipid-containing DSPC monolayer at air water interface. The picture shows fluorescently marked distribution of sLe^x lipid (bright areas) in the DSPC matrix (dark) at a Langmuir trough at lateral pressure of 41 mN/m. Diagonal: 110 μm.

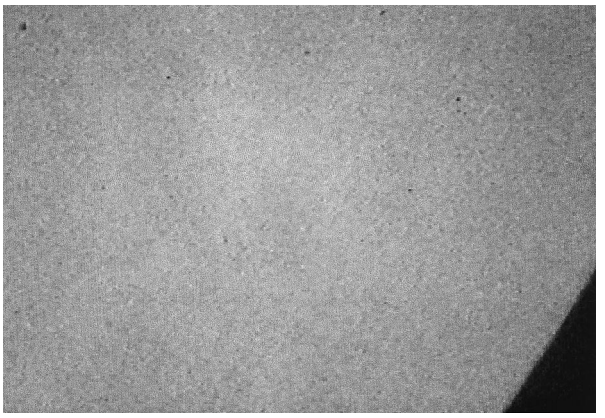

Fig. 3. Fluorescence microscope image of a sLe^x glycolipid-containing POPC monolayer at air/water interface. The sLe^x lipid is homogeneously distributed in the POPC matrix at a Langmuir trough at lateral pressure of 41 mN/m. Diagonal: 110 μm.

We started our work establishing optimal experimental conditions for the preparation of highly reproducible membranes. Therefore we first analyzed the lipid monolayers at the air/water interface, which should thereupon be transferred to the solid support. We found optimal conditions for glycolipid-induced DSPC-clustering using ceramide concentrations below 10 mol%. The appearance of sLe^x-enriched clusters is illustrated by fluorescence microscopy for the preparation with 10% as shown in Fig. 2.

In comparison, we prepared monolayers with homogeneous ceramide distribution. Since clustering had been the result of a phase separation of the matrix lipid and the coexistence of fluid expanded and crystalline areas and subsequent accumulation of the ceramide in one of these phases, we chose POPC as matrix lipid to avoid phase transitions and therefore cluster generation in our experiments. As illustrated in Fig. 3, the sLe^x ceramide is homogeneously distributed in the POPC matrix, when employed below 10 mol%.

The retention of the membrane structures after transfer to the glass surface is shown in Fig. 4. Whereas the POPC membrane shows a homogeneous appearance (not shown), glycolipid clusters (0.025%) are detectable in a DSPC matrix. Since clusters of those low ligand concentrations could not be detected with fluorescently labeled lipids, the structure

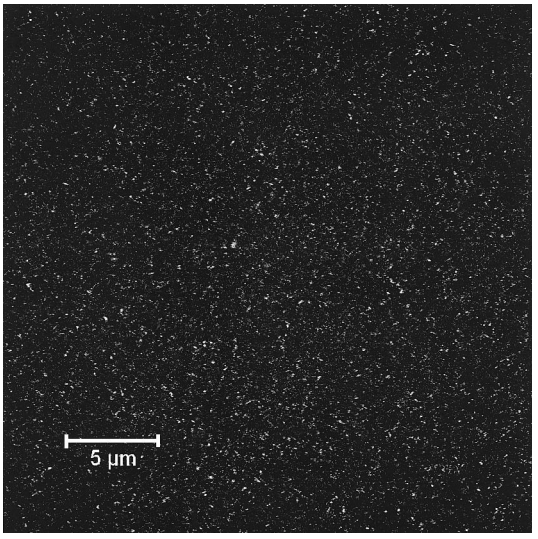

Fig. 4. Laser scanning microscope image of transferred sLe^x glycolipid clusters in a DSPC matrix. The model membrane was treated with FITC-conjugated rat anti-human CLA mAbs, that specifically bind to sLe^x moieties. CLA, cutaneous lymphocyte-associated antigen; a carbohydrate domain shared by sialyl-Lewis^x and sialyl-Lewis^a antigens.

was proved by binding FITC-conjugated anti-sLe^x antibodies to the clusters in the membrane.

Furthermore, using Atomic Force Microscopy of transferred films we could analyze the geometry of clusters and the influence of glycolipid reduction on cluster formation in the membrane. Analyzing different DSPC membranes with varying glycolipid amounts, we could see that a glycolipid reduction leads to a decrease in the number of clusters with obviously no changes in their size (diameter of about 20 nm corresponding to about 500 sLe^x molecules). An example of an AFM picture of a membrane with 1% sLe^x is shown in Fig. 5. Furthermore, this picture illustrates that in membranes with higher glycolipid concentrations (above 0.5%), clusters tend to organize in greater aggregates consisting of about 10–20 single clusters.

In analogy to natural membranes, supported planar bilayers show lateral lipid diffusion [17]. To guarantee that the transferred structure is preserved during cell binding studies, rather than changed by diffusion processes, we analyzed the time range of the lateral diffusion. Therefore, we employed a modified fluorescence recovery after photobleaching (FRAP) technique, where the time of diffusion of fluorescence markers into a previously bleached membrane area of defined size is analyzed. The experimental data of a POPC matrix in Fig. 6 show, that the lipid diffusion into an area of 30×30 µm took more than 120 min for total recovery. Therefore, we conclude that the freshly prepared and analyzed model membranes maintain their clustered structure when used within 30 min.

3.3. Influence of clustered ligand concentration on cell adhesion and rolling

The amount of selectin-binding epitopes in the natural sialomucins is low and their number is not well characterized [18–20]. On the other hand, the number of sLe^x lipids at neutrophil surfaces is estimated, but it has not been proved whether all these glycolipids are involved in selectin binding. Therefore, we started our cell adhesion experiments analyzing the influence of ligand concentration in the clustered ligand membranes on cell behavior. The cells were allowed to interact with the model membrane under static conditions for 5 min and the num-

Table 1
Characterization of the cell adhesion or rolling behavior in dependence on the ligand concentration in a DSPC matrix at a shear rate of 200/s

Ligand concentration (%)	Remaining bound cell fraction (%)	Rolling cell fraction (%)	Detached cell fraction (%)
10	91.7	0	8.3
1	92.4	0	7.6
0.1	90.1	0	9.9
0.05	27.4	61.2	11.4
0.015	9.3	74.6	16.1
0.01	5.0	77.2	17.8
0.005	4.7	0	95.3

Data represent means of at least 4 measurements.

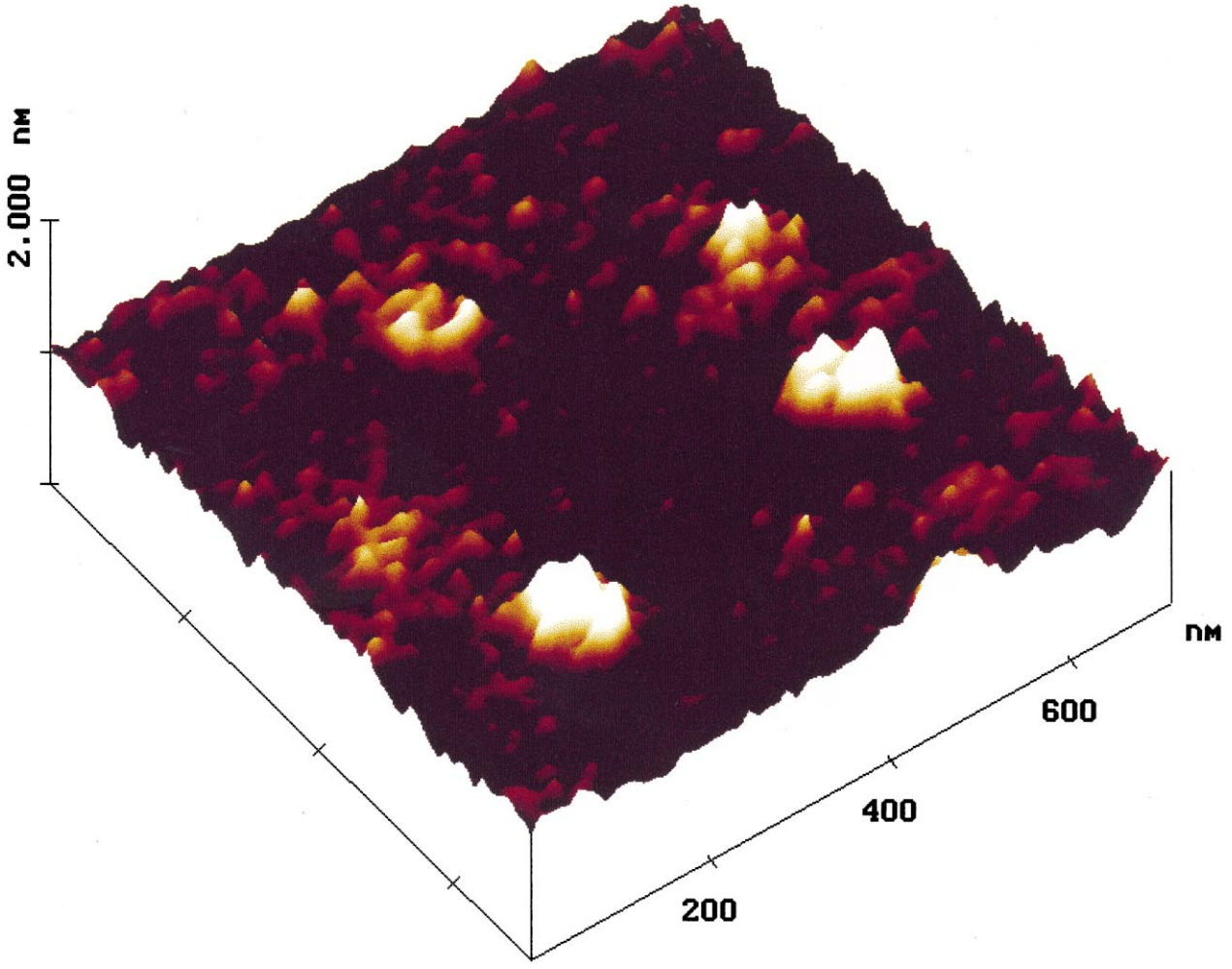

Fig. 5. Atomic force microscopic image of a DSPC membrane containing 1% sLe^x ceramide. The glycolipid clusters appear as bright elevations.

ber of sedimented cells was counted. After that time, the cell adhesion and rolling was analyzed at a shear rate of 200 s^{-1} and illustrated by a sequence of microscopic pictures within the first 60 s. In this way, the fraction of cells which remained bound, the number of rolling cells and their velocity could be detected. At ligand concentrations between 10 and 0.05%, a fraction of approximately 90% of cells stayed adhered in the shear flow and cell rolling did not occur. Further reducing the ligand concentration in the membrane resulted in the appearance of a great rolling cell fraction between 60 and 80% under the same conditions. Despite the different ligand concentrations between 0.05 and 0.01%, there were hardly any differences in number of rolling cells as described in Table 1 and their velocity. At glycolipid concentrations below 0.01% in the membrane, a small fraction of cells roll with higher velocity, whereas further reduction of ligand density abolished cell–membrane interactions. The pure DSPC matrix shows no interaction with the selectin-containing cells under flow conditions, as control experiments. The cell interaction with the ceramides could be blocked totally by an E-selectin antibody, or by the addition of EDTA, which suggests that rolling is a selectin-dependent process.

To further adapt these measurements to physiological conditions, we repeated these experiments without preincubating the cells at the membrane under static conditions. In general, we could detect similar cell behavior in response to ligand concentration, but the absolute number of interacting cells at the mem-

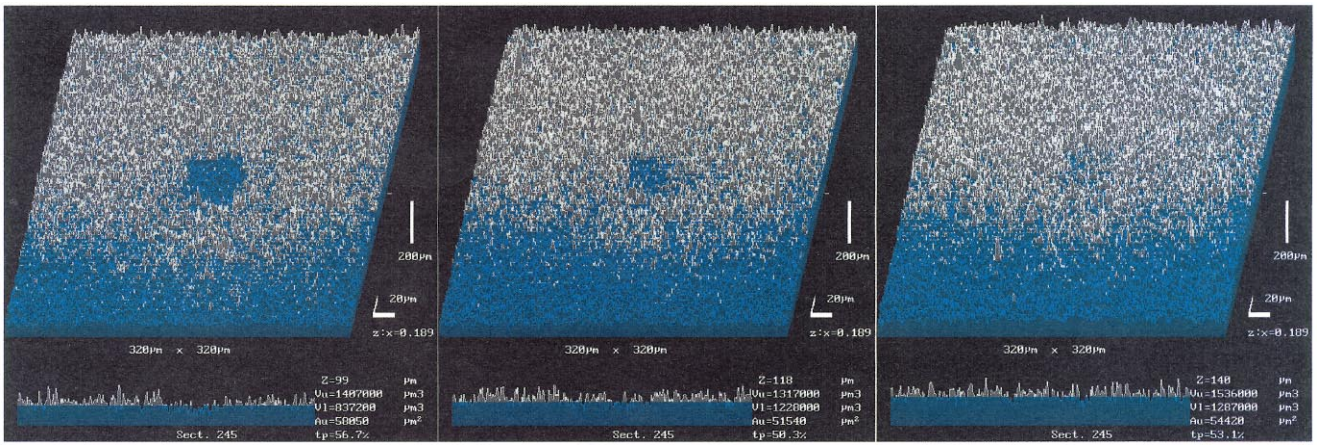

Fig. 6. Laser scanning microscope image of a transferred POPC monolayer in topographic version (NBD-PE-labeled). Pictures are taken after 0, 60, 120 min (from left). The lipid diffusion into an area of 30×30 µm is recovered totally after more than 120 min.

brane was reduced to about one-third (about 30 cells per analyzed area versus about 100 cells after settling). This should be explained with the geometric conditions of our flow chamber. The model device should offer because of its greater dimensions worse opportunities for an initial cell-membrane contact compared to postcapillary venules. Furthermore, the surface of the CHO-E cells, which has no microvilli presentation of selectins, will also contribute to the reduced interaction. Nevertheless, these results demonstrate, in principle, the functionality of our glycolipid-based model system under these more physiological conditions. In order to get more cell adhesion data allowing a better statistical evaluation, we continued our further studies with such experiments, where cells can interact with the membrane before rinsing.

From this point, we could conclude that functional organized sLe^x glycolipids can mediate cell rolling, implying that the ability to mediate rolling does not depend on sialomucin structures. The interplay between firm cell adhesion and rolling movement is ligand density controlled, whereas only a very small ligand fraction is necessary for mediating rolling.

Our study gives an impression about the ratio of ligand number and area which is necessary for mediating cell rolling. The ligand concentrations of 0.05 or 0.01% correspond to site densities of about 800 to about 20/µm^{2,} respectively, assuming an area of about 60 Å^2 for sLe^x tetrasaccharide headgroup and 45 Å^2 for DSPC at a transfer pressure of 40 mN/m. The range of site densities of sLe^x capable to mediate rolling is below the amount of sLe^x at leukocytes, which is thought to be about 50 000/µm^2 [21]. Assuming an idealized round cell shape of CHO-E cells (Fig. 1) with a diameter of 10 µm and a selectin density of about 1000/µm^2, these data correspond to ligand/receptor densities of about 4:5 or 1:5, respectively, in the contact zone. Therefore, glycolipid clustering is essential to enable cell-membrane interactions by drastically increasing the local ligand/receptor ratio.

Contrary to our glycolipid, where the tetrasaccharide is closely connected to the hydrophobic moiety, naturally occurring sLe^x lipids seem to be more complex, which leads to higher affinity and tethering capacity. Our results show that glycolipids, which have recently been discussed as physiological selectin ligands [22,23], can act as functional ligands, mediating rolling in our in vitro investigations, when arranged in clustered forms.

3.4. Cell rolling at various shear force conditions

To examine the dynamic characteristics of rolling process, we performed further experiments with a constant ligand concentration of 0.025% in the DSPC matrix at different shear force conditions. Therefore, after sedimentation of cells within the first 5 min, shear rate was increased in steps every 10 s until a shear rate of 1000 s^{-1} was reached. Up to a shear rate of 50 s^{-1} the cells adhered firmly and rolling could not be observed, representing the critical shear threshold for initiating rolling. Between shear

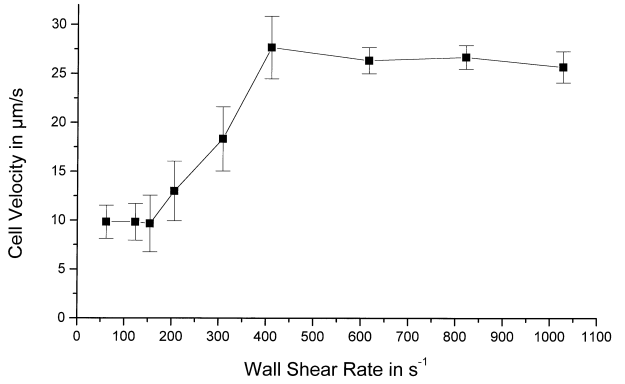

Fig. 7. Velocity of the rolling fraction of CHO-E cells above a DSPC matrix containing 0.025% sLe^x, in dependence on shear stress.

rates of 50 and 170 s^{-1} cells roll slowly without dependence on shear rate. Increasing shear levels up to 450 s^{-1} rolling velocity increased proportionally to shear stress and reached a near-plateau at about 27 µm/s with further increase in shear force, as illustrated in Fig. 7. These findings totally agree with previous results of neutrophil rolling on immobilized selectins [8]. This balanced rolling behavior upon changed shear force conditions was hypothesized to be a special feature of selectins contrary to other receptor proteins [10], whereas the molecular basis of this phenomenon is still not clear.

Considering the fraction of rolling cells at different shear rates, it is evident that above the shear threshold of 50 s^{-1} an equal cell fraction of about 75% is rolling similarly to the experiments with changed ligand concentrations. Accordingly, the fraction of cells that remain bound is nearly constant at a very low level at different shear rates above the threshold.

Despite the individual characteristics, in general, all cells of the rolling fraction rolled with relative similar velocities at the corresponding shear rates. Actually, cells rolled with jerky motion. They repeatedly rolled a distance and than interrupted rolling by brief pauses which should be attributed to a movement along the ligand clusters.

3.5. Cell adhesion at membranes with homogeneous ligand distribution

In order to investigate the importance of ligand clusters for cell interactions we repeated the experiments under modified conditions. Employing the POPC membranes with the homogeneous ligand distribution, differences in the cell adhesion or rolling should be evident compared to the DSPC results. Similar to the DSPC experiments we reduced the ligand concentration starting from 10 mol% and analyzed the cell behavior. The results (shown in Fig. 8) suggest that analogous to the previous results, cells strictly adhered at the membrane, at ligand concentrations above 0.05%. This cell adhesion could be inhibited by a blocking E-selectin antibody which demonstrates the specific nature of interactions. However, a small fraction of cells (about five cells corresponding to 5% of total adhered fraction) remain bound after blocking. Experiments with lower ligand concentrations show only the adhesion of a similar small cell fraction (about five cells) adhered both at these membranes and at a pure POPC matrix. This should be attributed to unspecific interactions at the membrane surface, because this cell binding could not be blocked with E-selectin antibodies. Contrary to the DSPC matrix, a certain ligand concentration which mediates both specific cell binding and cell rolling could not be achieved in POPC matrices. The remaining cells show only a slow movement with descending velocity of about 2 µm/s at higher shear rates which should not be defined as cell rolling.

Since CHO-E cells have a strong tendency to attach at surfaces within a short time, this may be the reason for the unspecific attachment of some cells. One can assume that the liquid expanded POPC

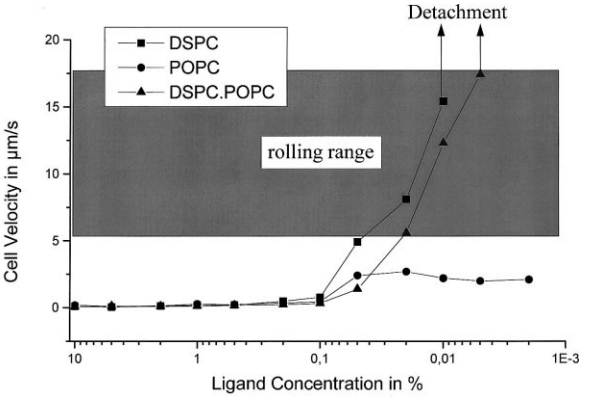

Fig. 8. Dependence of cell rolling velocity on ligand concentration. Rolling velocity was detected at a shear rate of 200 s^{-1} at decreasing ligand concentrations in dependence on various phospholipid matrices. Velocities below 5 µm/s has not been defined as rolling movement.

membrane gives better opportunities for the cells to spread on it than the crystalline DSPC.

The biophysical basis for the differences in cell adhesion at DSPC and POPC matrices are likely to be complex, requiring consideration of differences in phase separations, microdomain formations and phase transitions. Because this goes beyond the scope of this study, it should be described separately. Nevertheless, we conclude that the appearance of clustered glycolipids in this study is a prerequisite for mediating rolling.

3.6. Studies at membranes with mixed POPC/glycolipid clusters

To further support this thesis and to verify that the inability to mediate cell rolling is not a POPC effect for itself, we further modified the lipid matrix. In order to maintain ceramide clusters in combination with POPC, fluid expanded POPC clusters, that contain the similar fluid glycolipid molecules, were prepared in a crystalline DSPC matrix. This should lead to glycolipid-containing clusters, which have a lower glycolipid concentration per area compared to the clusters in the pure DSPC experiments. We interpreted this fact from confocal laser scanning microscope pictures of these membranes (not shown), which showed greater, but less fluorescent, glycolipid clusters opposite to Fig. 4. To maintain an overall ceramide concentration of 0.025%, POPC mixed with 0.5% ceramide was incorporated into a DSPC matrix with 5% in order to create clusters. If the assumption of this experiment is true, the resulting rolling behavior should be similar as described for DSPC in Table 1.

We could see that this membrane was able to mediate cell rolling under the described conditions. It was evident that cells with a average velocity of 10 μm/s rolled slower than at the DSPC membrane with an equal glycolipid amount. The results demonstrate that this form of glycolipid clustering is practicable and that POPC does not abolish cell rolling in general.

This slower cell movement should be an indicator for a more effective cell binding compared to the similar glycolipid concentration in the case of the pure DSPC matrix. If one assumes that in the mixed DSPC/POPC matrix the glycolipids are more diluted within the clusters because of their greater areas, one should conclude that the absolute amount of glycolipids in these membranes could be decreased without loss of binding ability. To try this out, we further reduced the ceramide concentration in the membrane to 0.005 and 0.002%, concentrations which are not able to mediate rolling in the pure DSPC matrix.

Surprisingly, we could see that cells rolled normally along these membranes as in the case of higher glycolipid densities in the DSPC membrane as demonstrated in Fig. 8. We interpret these results as follows: despite the fact that a clustered arrangement of glycolipids in the membrane is necessary to induce rolling, the glycolipid density within the clusters can be modified. As shown here for a distinct concentration range, the mixed clusters of POPC and glycolipid are as effective as the higher local glycolipid concentration within the clusters in the DSPC membrane. The range of glycolipid tolerance mediating rolling can thus be shifted to lower concentrations without loss of functionality.

Leaving out this tolerance range with lower concentrations ($<0.002\%$), no cell membrane interactions could be detected again. Furthermore, contrary to the pure POPC membrane the amount of attached cells is very low, which proves that adhesive POPC effects are not involved in the described cell behavior.

Summarizing, these results support the hypothesis that a lateral clustered arrangement of glycolipid ligands is necessary for mediating cell rolling. Furthermore, it could be shown that ligand clusters, once developed, retain their ability to induce rolling upon diluting their glycolipid concentration within an appropriate concentration range.

4. Conclusions

In this study, we focused on the molecular features of glycolipid ligands for selectins in the cell rolling process. Therefore we introduced an in vitro model, where selectin-containing cells adhere or roll along a support fixed, ligand-containing membrane in a simulated shear flow of vasculature. These model membranes, which were created using the Langmuir–Blodgett technique, allow the incorporation of several concentrations of sLe^x ceramide in either defined lateral clusters, or homogeneously distributed in the

matrix lipid. This was the basis to investigate the importance of multivalent ligand–receptor interactions.

In membranes with glycolipid clusters, the transitions from firm attachment to rolling to detachment is glycolipid density-controlled. Since membranes with homogeneous ligand distributions have been shown to be unable to mediate rolling, lateral ligand clustering appears as an essential prerequisite for rolling. Furthermore, we could show that the required ligand concentration can further be reduced upon diluting the glycolipid concentration within the clusters without loss of functionality. These results clearly support the hypothesis of multivalent selectin–carbohydrate interactions for getting sufficient binding strength.

Since this study demonstrates that carbohydrates presented by a lipid carrier rather than by a polypeptide backbone are able to mediate rolling, it represents the first report which combines dynamic glycolipid binding studies with the multivalent binding hypothesis.

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