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Effect of sialyl Lewis X-glycoliposomes on the inhibition of E-selectin-mediated tumour cell adhesion in vitro

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Abstract

The aim of this study was to evaluate the potential of different types of sialyl Lewis X-conjugated liposomes as competitive inhibitors for tumour cell adhesion to endothelial E-selectin.

Sterically stabilised liposomes with the sLe^X ligand at the terminal end of the polyethyleneglycol (PEG) chain, as well as vesicles that had the ligand embedded within the PEG-layer, were compared to ligand-bearing liposomes without sterical stabilisation.

First, 14 different tumour cell lines were characterised for their expression of sialyl Lewis X and/or A. Tumour cell adhesion was characterised in three static assays in vitro using: (i) immobilised E-selectin, (ii) CHO cells, transfected to express E-selectin and (iii) human umbilical vein endothelial cells (HUVEC).

Sterically stabilised liposomes with the ligand at the terminal end of the polyethylene chain were the most effective inhibitors in all three assays and inhibited the adhesion of HT29 colon- and Lewis lung (LL) carcinoma cells by about 60–80%. The binding was not affected by a PEG-coating of the liposomes. Sterical stabilisation, on the other hand, completely prevented macrophage uptake (J774 cell line) independently of the presence of the ligand, while plain liposomes were taken up in an amount of 5.4 nmol liposomal lipids/10^6 macrophages.

Keywords: Adhesion; E-selectin; Sialyl $Lewis^X$; Sterically stabilised glycoliposome

1. Introduction

Blood-borne metastasis is a complex multi-step process that includes, among others, the essential extravasation of circulating tumour cells from the blood system, followed by the invasion into the organ at the site of metastasis. These steps involve interactions of different homotypic and heterotypic cell–cell adhesion molecules expressed on circulating tumour cells and/or on blood vessel cells.

A possible concept for the prevention of metastasis is to target the circulating tumour cells and to block the ligands at their surface, which are necessary to adhere to the endothelial surface. Conversely, the competitive inhibition of receptors at the endothelial site would also be possible.

In this context, E-selectin plays a crucial role [1,2]. This membrane protein is exclusively expressed on activated endothelial cells and is involved in the first step of the adhesion cascade taking place on the endothelial cell surface in adhesion processes of leukocytes [3] and metastatic tumour cells [4,5]. E-selectin recognises specifically the tetrasaccharides sialyl $Lewis^X$ (sLe^X) and A (sLe^A), which are expressed either protein- or lipid-bound on the surface of different tumour cells and of leukocytes [6–8].

The inhibition of the E-selectin-mediated adhesion is discussed as a possibility to prevent or (at least) to reduce

Abbreviations: CH, cholesterol; DCP, dicetylphosphate; DSPE, distearylglycerophosphatidylethanolamine; EDTA, ethylenediamine tetraacetate; HPTS, 1-hydroxypyrene-3,6,8-trisulfonic acid; HUVEC, endothelial cells from human umbilical vein; LUVET, large unilamellar vesicles made by extrusion technique; MLV, multilamellar vesicles; MPS, mononuclear phagocyte system; PBS, phosphate-buffered saline solution; PC, egg phosphatidylcholine; PE, phosphatidylethanolamine; PEG, polyethyleneglycol; PEG_{2000}DSPE, *N*-(*O*-methoxy-polyethylene glycyl)-1,2-distearyl-*s*,*n*-glycero-3-phosphatidylethanolamine; RT, room temperature; S.D., standard deviation; sLe^A, sialyl $Lewis^A$; sLe^X, sialyl $Lewis^X$

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metastatic tumour spread, especially under certain conditions of high risk, e.g. during tumour surgery, when the number of circulating tumour cells may be increased, but has been investigated so far by only a few groups [9–14].

If the endothelial membrane is used as a target, for example to transport a pro-drug [9,13] or to deliver antisense oligonucleotides [15] to the endothelium, several different opportunities can also be exploited to prevent or to reduce tumour cell adhesion. This includes blocking of selectins [11], as well as the suppression of integrin [16,17] or VCAM-1 expression [15]. The inhibition of tumour neovascularisation [18] has also been used for that purpose [8].

Useful tools for a competitive blockade of the endothelial E-selectin, and thus for an inhibition of the initial step of tumour cell adhesion, are specific antibodies [16], peptides [11] and liposomes equipped with specific ligands [11,15,19,20]. For this purpose, carbohydrates like sLe^X, a tetrasaccharide that selectively recognises the carbohydrate binding region of E-selectin, can be conjugated to the liposomal surface to obtain glycoliposomes with inhibitory potential [11,14,15,19]. Liposomes, compared to the free ligand, have the advantage of forming ligand clusters on their surface. This enables the glycoliposomes to a multivalent-ligand adhesion, which finally promotes the binding efficacy strongly between a vesicle and the receptor cluster on the cell surface [21].

In continuing our previous studies [21,22], we evaluated in this study different types of sLe^X-liposomes as inhibitors of E-selectin-mediated tumour cell adhesion in different static assays in vitro. The results of this study demonstrated a strong inhibition effect on the binding of different cell lines in vitro, especially for sterically stabilised liposomes with the ligand at the distal end of the polyethyleneglycol (PEG) chain.

2. Materials and methods

2.1. Materials

Egg phosphatidylcholine (PC) was obtained from Lipoid GmbH (Ludwigshafen, Germany), DMPE from Sigma (Taufkirchen, Germany) and EDTA from Serva (Heidelberg, Germany). sLe^X-DMPE was synthesised as described previously [21], whereas sLe^X-PEG_{2000}DSPE, was custom-synthesised by SYNTHESOME mbH (Munich, Germany). 3-[*N*-methoxy-(polyethylene-$glycol_{2000}$)]-phosphatidylethanol-amine (PEG-DSPE) was purchased from Sygena LTD (Liestal, Switzerland). HPTS, 1-hydroxypyrene-3,6,8-trisulfonic acid, was obtained from Molecular Probes (Eugene, OR, USA), whereas ^{51}Cr-sodium chromate was a product of Amersham Pharmacia (Buckinghamshire, England). The pCDM8 expression vector containing the hE-selectin cDNA, the pSV2-*neo* vector with the gene for neomycin phosphotransferase, recombinant human E-selectin, the anti-human E-selectin monoclonal antibody BBA2 as well as the FITC-conjugated anti-hE-selectin antibody BBA21 were received from R&D Systems GmbH (Wiesbaden, Germany). The antibiotic G418 was a product of Invitrogen (Paisley, UK). The anti-human sLe^A mouse monoclonal antibody KM231 as well as anti-human sLe^X mouse monoclonal antibody KM93 were obtained from Calbiochem (La Jolla CA, USA), the FITC-conjugated mouse anti-h sLe^X monoclonal antibody 2H5 from PharMingen Europe and the anti-mouse IgG specific Cy™2-antibody were purchased from Dianova (Hamburg, Germany).

Media, sera and other chemicals for cell culturing were purchased from Life Technologies (Karlsruhe, Germany).

2.2. Preparation and characterisation of liposomes

The different compositions of liposomes are shown in Table 1. Vesicles were prepared as follows: first, appropriate volumes of stock solutions of indicated components in CH_2Cl_2/MeOH (7:3) were mixed, the solvent evaporated to produce a thin lipid film which was then dispersed in PBS buffer (156 mM; including 6.8 mM Ca^{2+} and Mg^{2+}; pH 7.4, Life Technology, #14040) to obtain a concentration of total lipid between 7 and 9 mg/ml. The suspension was shaken for 12 h at room temperature to prepare multilamellar vesicles (MLV). Large unilamellar liposomes (LUVET) were generated from the MLV by repeated extrusion through polycarbonate filters of 100 nm pore size using a LiposoFast™ Basic System (Avestin, Inc., Ottawa, Canada). Vesicle size was determined by dynamic light scattering using a Coulter Counter N4 MD (Coulter Electronics Inc., Hialeah, USA). Liposomes were used if the vesicles had an unimodal diameter between 100 and 120 nm with a polydispersity index of 0.20 or lower.

The final lipid concentration of the liposomal suspension was determined by phosphatidylcholine quantification using HPTLC as described previously [23].

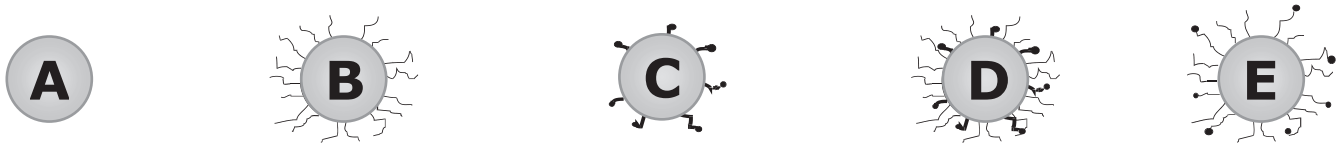
2.3. Transfection of E-selectin cDNA in CHO cells and isolation of a stable CHO^{transf} cell line

Chinese hamster ovary (CHO) cells were grown at 37 °C with 5% CO_2 in Dulbecco's modified Eagle medium (DMEM), supplemented with 10% foetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, and 1 mM sodium pyruvate. Cells (5×10^5) were reseeded on 60-mm plates 1 day before transfection.

The TransFast Transfection Reagent was used to transfect CHO cells with a full-length 2.7 kb hE-selectin cDNA cloned in pCDM8 expression vector. A pSV2-*neo* vector containing the gene for neomycin phosphotransferase, which confers resistance to the antibiotic G418 was cotransfected. After 48 h of transfection, cells were trypsinised and replated in medium containing G418. About 2 weeks later, individual colonies were isolated and grown again in the presence of G418.

Table 1
Composition of liposomes used for inhibition of tumour cell adhesion

Symbolic structure of liposomes[1]:



No	Type	Molar composition[2]				
		PC	DMPE	DSPE-PEG	DMPE-sLe^X	DSPE-PEG-sLe^X
A	Plain liposomes	93	7			
B	PEG-liposomes	93	3.5	3.5		
C	sLe^X-liposomes	93	3.5		3.5	
D	Combination of B and C	93		3.5	3.5	
E	sLe^X-PEG-liposomes	93	3.5			3.5
1	sLe^X-ligand:	PEG-chain:		PEG-sLe^X-ligand:		
2	Given in mol %					

Finally, E-selectin-expressing cells were selected by FACS sorting: FITC-conjugated anti-hE-selectin antibody BBA21, diluted 1:20, was added to 10^7 cells and incubated for 1 h at 4 °C. Unbound antibody was removed by washing three times with PBS containing 1% FCS. CHO cells with a fluorescence intensity comparable to that of activated HUVECs, were sorted out by FACS analysis, collected in 500 μl fractions and grown in DMEM in a CO_2 incubator at 37 °C for 2 weeks. FACS sorting was repeated three times. E-selectin expression was proven by immune fluorescence microscopy using the BBA21 antibody.

2.4. Cell culture and radiolabelling

CHO^{transf} and HT29 colon carcinoma cells were grown in DMEM, whereas all other tumour cells used in this study (Table 3) were kept in RPMI media. All the media were supplemented with 10% FCS.

HUVECs were isolated from human umbilical veins by treatment with α-chymotrypsin (0.2% w/v) as reported previously [24]. They were used within four passages.

Tumour cells were radiolabelled by incubating the cells for 4 h at 37 °C with 1–1.5 MBq/ml ^{51}Cr-sodium chromate, washed three times by centrifugation (10 min, 1400 × *g*) and resuspended in phosphate buffered saline containing 2 mM Ca^{2+} (Ca-PBS).

2.5. Characterisation of tumour cell ligand expression by immune fluorescence

Tumour cells were fixed on glass slides with 5% formalin in PBS for 5 min at room temperature, washed and incubated with 50 μl of monoclonal antibodies KM 231 (anti-sLe^X) or 2H5, a FITC-labelled anti-sLe^X antibody (PharMingen International), diluted 1:50 (PBS/0.3% BSA) at room temperature for 1 h.

If KM231 was used, cells were then incubated with 50 μl of fluorescently labelled secondary antibodies Cy™2 for 1 h at 4 °C to visualise anti-mouse IgG. Cells were finally covered with Aqua-Poly/Mount (Polysciences Inc., Warrington, USA).

Immune microscopy was performed and micrographs taken with a Axiophot photo microscope (Zeiss), using the Axio Vision 3.1 software.

2.6. Cellular uptake of liposomes by J774 macrophages

These experiments were done according to Ref. [25] as described recently [26]. Briefly: 2×10^4 J774 mouse macrophages (DMSZ, Hannover, Germany) were seeded per well in a 96-well FluoroNunc™ microtiter plate (Nunc A/S, Roskilde, Denmark). After 8 h, cells were incubated at 37 °C for 1, 3, 6 and 21 h with plain liposomes A, sterically stabilised liposomes B and sLe^X-bearing liposomes E, all containing 30 mM HPTS, and diluted with RPMI/10% FCS to a final concentration of 50 μM. Cells were additionally incubated for control purposes with the liposomes at 4 °C for 4 h to determine the non-specific binding of vesicles and also co-incubated with a solution of HPTS in medium (100 mM) for 24 h to characterise the fluorescence properties of HPTS in the low pH compartment.

The amount of cell associated HPTS was determined from a standard curve of free HPTS in PBS buffer (15–1500 pmol/ml), pH 7.4.

Differentiation between general uptake and internalised marker was calculated using the equation $m_{intern} = R_{exp}/R_{pin} \times 100$ with m_{intern}, cellular internalised liposomal HPTS in percentage of total uptake; R_{pin}, fluorescence ratio (450:415 nm) in cells after uptake of free HPTS; and R_{exp}, fluorescence ratio (450:415 nm) determined for liposomal HPTS which was taken up by the cells.

The HPTS amount was finally used to calculate the concentration of liposomal OPP found associated with cells, exploring the ratio of HPTS/OPP (mmol/mol) for each individual liposomal preparation used. These results were corrected for non-specific uptake.

2.7. Tumour cell adhesion assays

2.7.1. Adhesion to immobilised E-selectin

Forty microliters of recombinant human E-selectin (5 μg/ml Ca^{2+}-PBS) were immobilised per well on 96-well microtiter plates overnight at room temperature (RT). After a single washing step with 50 μl of Ca^{2+} containing TRIS buffer (Ca-TRIS), the non-specific binding sites were blocked with BSA/Ca-Tris (2%) for 2 h at RT and finally washed again two times with 50 μl/well Ca-TRIS.

Then, 10 μl with 10^5 radiolabelled tumour cells were added to each well for determination of adhesion rates.

2.7.2. Adhesion to endothelial cells and to transfected CHO cells

HUVEC or CHO^{transf} target cells were grown in a 96-well microtiter plate (Nunc) until near confluence (HUVEC: $2-3 \times 10^4$ cells/well; CHO^{transf}: 3.5×10^4 cells/well). HUVECs were additionally co-incubated with 0.5 ng TNFα/well for 4 h/37 °C to stimulate E-selectin expression. Only results of such experiments were taken into account in which an at least 4-fold higher binding of tumour cells to activated HUVEC than to non-activated target cells were obtained. Wild-type CHO cells (wt CHO) were used as control for adhesion to CHO^{transf}.

After washing the cells twice with Ca-Tris buffer, tumour cell binding was determined as described for the E-selectin assay above.

As control for specificity, the binding of tumour cells to non-stimulated HUVEC or to wt CHO cells was additionally determined, and subtracted to eliminate the non-specific binding.

2.7.3. Inhibition of adhesion

Adhesion of tumour cells was followed as described above, but before addition of radiolabelled tumour cells, the inhibitors EDTA (10 mM) for non-specific blocking of binding, monoclonal antibody BBA2, specific for E-selectin (11–33 ng/well), or liposomes with or without 3.5 nmol sLe^X-ligand were added to each well with immobilised E-selectin, HUVEC or CHO cells. After tumour cell addition, the total volume was equalised with medium to 40 μl/well.

After 1 h of incubation at RT, unbound cells were washed away two times with 50 μl Ca-TRIS, before remaining adherent cells were lysed by addition of 50 μl 1 N NaOH/well. Finally, radioactivity of each well was measured with a COBRA II γ-Counter to quantify bound tumour cells. The inhibition of tumour cell adhesion is expressed in percent in comparison to control cells treated similarly but without any addition of inhibitor.

2.8. Statistics

Each in vitro experiment was done in triplicate. The results represent the mean ± S.D. of at least three independently performed experiments. Statistical analysis was performed with the unpaired Student's *t*-test. Differences were considered to be significant at $P < 0.05$.

3. Results

3.1. Characterisation of liposomes

The individual compositions for vesicles used in this study are summarised in Table 1. Five different liposome types were used in this study: plain liposome A and sterically stabilised liposome B were prepared without sLe^X ligand, whereas liposomes C, D, and E contained 3.5 mol% ligand conjugated to a phosphatidylethanolamine (PE)-anchor. Liposomes C and D contained sLe^X-DSS-DMPE (M: 1671 g/mol, Fig. 1), which ensured that sLe^X was closely located to the liposomal surface because of the disuccinimidyl suberate (DSS) spacer. Liposome D was similar to liposome C but was prepared with additional PEG-DSPE to obtain sterically stabilised liposomes with the ligand embedded within the PEG-layer, and finally liposome E, with sLe^X-PEG-DSPE (M: ~ 3590 g/mol), containing the ligand at the distant end of the long PEG spacer (Fig. 1).

3.2. Uptake of liposomes by macrophages in vitro

J774 cells were co-incubated with HPTS-containing liposomes A, B and E for different times and cellular uptake was followed to prove if the presence of the sLe^X ligand at the polyethylene chain of liposome E enhances macrophage uptake.

Fluorescence measurements of liposomes with HPTS allows to discriminate between total amount of vesicles taken up by macrophages if the concentration-dependent fluorescence at 414 nm is used, while the pH-dependent fluorescence at 450 nm (both at 510 nm emission wavelength) can be exploited to calculate the amount of internalised liposomal HPTS in the low pH compartment of the cell [25].

It was found that both sterically stabilised liposomes B and E independently on the presence of the sLex-ligand were not taken up by J774 macrophages (Table 2), whereas plain liposome A were taken up in a time-dependent manner (Fig. 2). The amount of macrophage-associated lipid from these liposomes was 5.40 ± 0.86 nmol/10^6 cells after 21 h, 50.5% of this was internalised.

3.3. Tumour cell characterisation

To select suitable tumour cell lines for inhibition experiments, 14 colon, breast, lung, liver and skin tumour cell

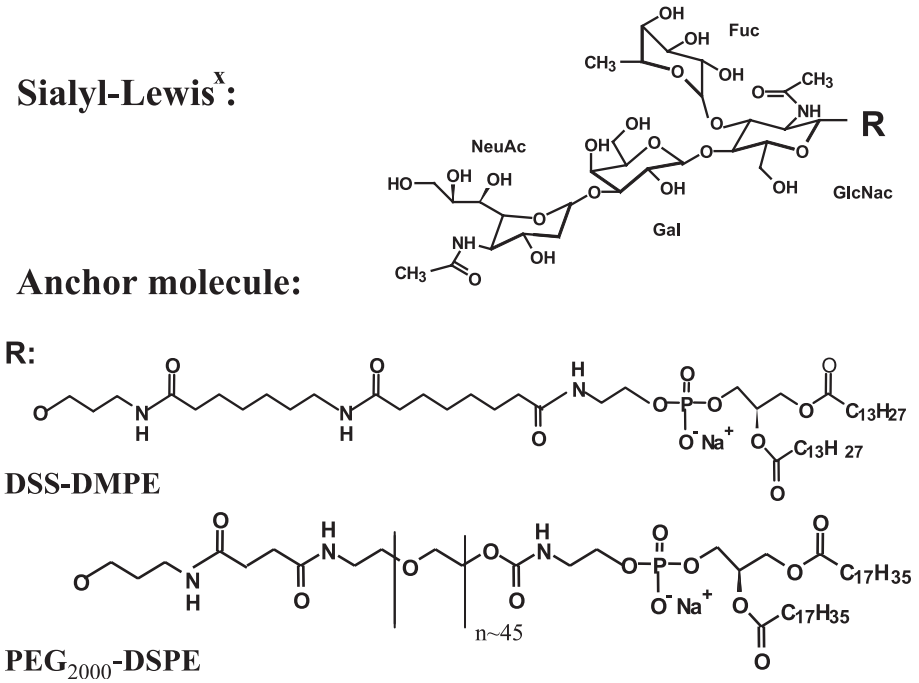


Fig. 1. Molecular structure of the ligand sialyl-Lewis^X and the anchor lipid phosphatidylethanolamine. The sLe^X (Neu5Acα2 → 3Galβ1 → 4(Fucα1 → 3)GlcNac)-ligand and the PE-anchor was connected covalently using the DSS spacer and the PEG-chain spacer.

lines were characterised by immune fluorescence for their sLe^X- and sLe^A-equipment, using specific monoclonal antibodies to recognise these ligands on the tumour cell surface.

The results for ligand-expressing cells and additionally for Lewis lung carcinoma cells (LL) are summarised in Table 3. Tumour cell lines were divided into cell lines with weak, medium and strong presence of sLe^X- and sLe^A ligand (Fig. 3). The colon carcinoma cell line HCT 116, and the breast cancer cell lines MCF 7, MT1, T47D or MDA-MB 435, all of human origin, had neither sLe^X nor sLe^A ligands. For mouse cell lines B16-BL6 (melanoma), and LL, as well as C26 (colon carcinoma), no ligand could be detected with the specific antibodies.

Table 2
Amount of liposomal lipid bound by J774 macrophages after incubation with liposomes

Time (h)	Lipid (nmol/10^{6}cells) after incubation with liposomes		
	A	B	E
0	2.65 ± 0.57	2.81 ± 0.55	3.04 ± 0.68
1	3.21 ± 0.20	1.95 ± 0.92	2.52 ± 1.00
3	3.60 ± 0.22	1.62 ± 0.90	2.17 ± 1.08
6	4.62 ± 1.04	2.12 ± 1.00	2.07 ± 0.71
21	8.05 ± 0.86	2.34 ± 0.80	2.18 ± 0.41

2×10^4 J744 mouse macrophages/well were incubated with HPTS containing liposomes A, B and E diluted with RPMI medium/10% FCS to a final concentration of 100 μM for indicated times at 37 °C. Fluorescence of HPTS was determined at wavelength F_{EX} 450 nm with F_{EM} at 510 nm and was used to calculate the amount of total cell associated liposomal lipid as described in Materials and methods. It was finally normalised to the uptake of 10^6 cells.

3.4. Tumour cell adhesion

The adhesion of selected tumour cells was determined in three assays, representing different E-selectin patterns: to

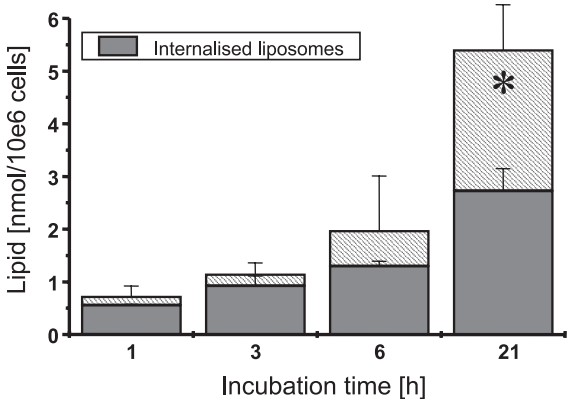


Fig. 2. Total and internal uptake of unmodified liposome A by J774 macrophages. Liposomes for macrophage uptake were prepared by substitution of 40 mol% of PC by an equivalent molar amount of cholesterol and resuspension of the lipid film with a 30 mM HPTS/PBS solution, pH 7.4; 2×10^4 J744 mouse macrophages/well were incubated with HPTS containing liposome A, diluted with RPMI medium (without FCS) to a final concentration of 100 μM for indicated times at 37 °C. Fluorescence of HPTS was determined at wavelengths F_{EX} 414 and 450 nm with F_{EM} at 510 nm and was used to calculate the amount of cell associated liposomal lipid as described in Materials and methods. It was finally normalised to the uptake of 10^6 cells. The complete bars show the total uptake of liposomal lipid and, in addition, the internalised amount of liposomal lipid (grey parts). All data are corrected for non-specific uptake. (*) Significantly different to uptake at each other time point ($P < 0.05$).

Table 3
Characterisation of sLe^X- and sLe^A-ligand expression and adhesion behaviour of selected tumour cells to E-selectin targets in vitro

Cell lines		Ligand equipment[a,b]		Binding of tumour cells[c] to		
Tumour type	Name	sLe^X	sLe^A	E-Selectin	HUVEC	CHO^{transf}
Colon	HT29	◆◆◆	◆◆◆	28.4 ± 8.1	6.7 ± 2.1	30.4 ± 6.1
	LS174T	◆◆	◆◆◆	19.6 ± 5.6	15.5 ± 3.5	n.d.
	C26	▼	▼	39.9 ± 7.0	12.5 ± 5.9	45.2 ± 3.6
	SW480	▼	◆◆	13.4 ± 8.6	1.6 ± 0.3	n.d.
Breast	MT3	◆◆◆	◆◆◆	35.9 ± 12.1	8.9 ± 3.6	44.7 ± 6.8
Hepatoma	HepG2	◆◆◆	◆	28.7 ± 7.4	16.7 ± 2.9	36.5 ± 13.9
Lung	LL	▼	▼	2.9 ± 1.2	17.2 ± 6.1	5.9 ± 2.4

n.d.: not determined.

[a] Ligand equipment was determined by fluorescence microscopy after treatment of cells with specific antibodies as described in Materials and methods. $s\text{-}Le^{A \text{ or } X}$ presence: (▼) no ligand; (◆) weak; (◆◆) medium; (◆◆◆) strong.

[b] Additional cell lines investigated but were found without any $s\text{-}Le^{A \text{ or } X}$ ligand expression were: HT116 (colon carcinoma), MCF7, MT1, T47D, MDA-MB 435 (breast cancer), B16BL6 (melanoma).

[c] ^{51}Cr-labelled tumour cells (10^5/well) were added to immobilised E-selectin, to endothelial cells or CHO cells for 1 h at RT. Unbound cells were washed away and the binding was quantified by radioactivity measurements as described in Materials and methods. Given is the radioactivity of bound cells in comparison to that of added cells in percentage mean ± S.D.

immobilised E-selectin, to transfected CHO cells (cells that permanently express E-selectin), and to E-selectin on activated endothelial cells (Table 3).

The adhesion was dependent on the ligand expression and, because of this, also dependent on the tumour cells. We found adherence of tumour cells with high ligand expression

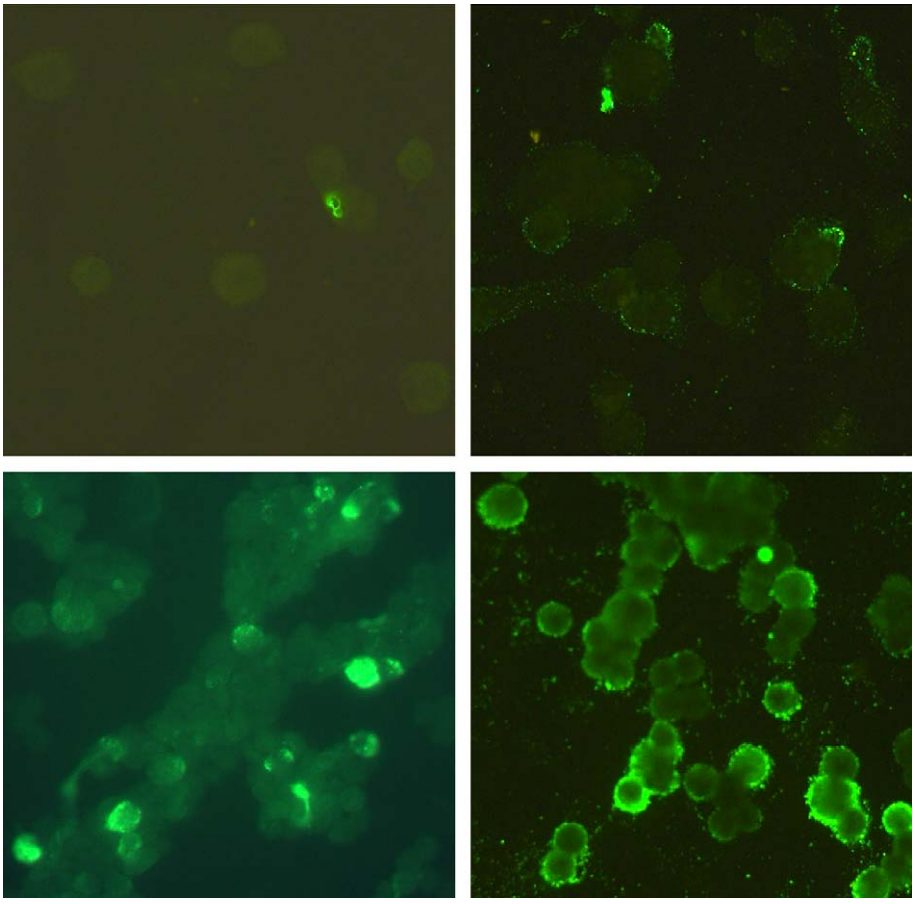


Fig. 3. Immune fluorescence of sLe^X ligand expression of selected tumour cell lines. Cells were fluorescently labelled as described in Materials and methods using the FITC-labelled monoclonal anti-sLe^X antibody 2H5 (PharMingen International), diluted 1:50. All micrographs were taken with 40-fold magnification and with identical sensitivity settings. Top/left: B16BL6 melanoma cells, representing no ligand presence; top/right: C26 colon carcinoma cells, weak ligand presence; bottom/left: LS174T colon carcinoma cells, medium ligand presence; bottom/right: MT3 breast carcinoma cells, strong ligand presence.

to be between 30% and 45% in the E selectin and CHO^{transf} assay, whereas adhesion was generally lower in the HUVEC assay (9–17%). The data obtained in the HUVEC assay were more heterogeneous compared to those from other systems and did not show a correlation between ligand expression and cell adhesion.

HT29 colon carcinoma cells with high binding potential in the E-selectin and the CHO assay and LL carcinoma cells with weak/no ligand expression and low adhesion were selected for the following inhibition experiments.

Cell adhesion was further determined for control reasons by co-incubating the cells with EDTA to remove all Ca^{2+} ions, which are necessary for E-selectin binding. This completely prevents the adhesion of all tumour cells used in the E-selectin assay (see Table 3). Results for LL and HT29 carcinoma cells are shown in Table 4. Negative values resulted from experiments where the (non-specific) adhesion in the control experiments was higher than the adhesion after co-incubation with inhibitor.

In addition, the specific receptor function of E-selectin was blocked using recombinant monoclonal antibody BBA2, which is known to bind specifically to the lectin domain of E-selectin. Remaining adherence between 14% and 69% for HT29 and LL cells after co-incubation with the inhibitors suggests that additional components are likely to be involved in mediating the adhesion between tumour and target cells, especially to CHO^{transf} cells.

3.5. Inhibition of tumour cell adhesion in vitro

The inhibitory effect of glycoliposomes was determined in all three adhesion assays and the results for HT29 colon carcinoma (Fig. 4, upper part) and for LL cells (Fig. 4, lower part) are shown. The inhibition experiments were always performed with liposomes containing 3.5 nmol sLe^X-ligand per well (8.75×10^{-5} M).

Table 4
E-selectin-mediated adhesion for LL and HT29 carcinoma cells after co-incubation with mAB BBA2 or EDTA

Adhesion (% of control)				
Assay	BBA2		EDTA	
Cells	LL	HT29	LL	HT29
E-Selectin	14.4 ± 15.2	28.6 ± 6.9	− 7.8 ± 14.4	1.1 ± 1.4
CHO^{transf}	46.7 ± 19.8	68.8 ± 14.5	26.8 ± 14.6	3.5 ± 1.2
HUVEC	28.2 ± 25.3	48.9 ± 37.6	− 19.4 ± 45.5	− 25.8 ± 36.1

^{51}Cr-sodium chromate labelled tumour cells were added to immobilised hu E-selectin, CHO^{transf} or HUVEC cells in the presence of mAB BBA2 or EDTA and the remaining tumour cell adhesion was determined by measurement of radioactivity of bound cells as described in Materials and methods.

Adhesion (A) was calculated as follows: A (%)=[$R_x - R_{unspec}$]/[$R_{tot} - R_{unspec}$] × 100; R_x radioactivity of test cells, R_{unspec}: radioactivity of non-specific bound cells, R_{tot}: radioactivity of cells incubated without inhibitor.

Results represent the mean adhesion ± S.D. after co-incubation with the inhibitor in comparison to untreated tumour cells for four to five independently performed experiments, each done in triplicate.

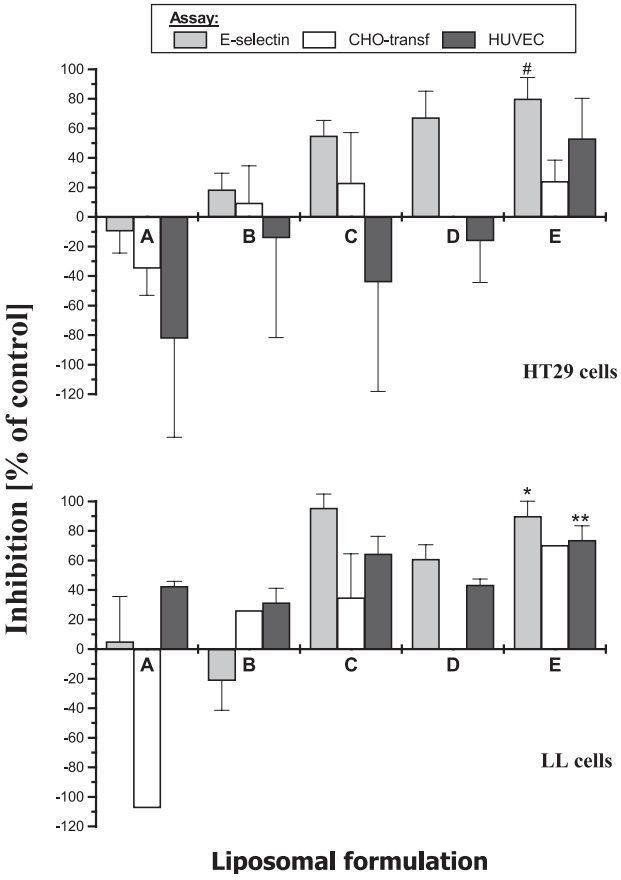


Fig. 4. Inhibition of tumour cell adhesion by sLe^X ligand-bearing liposomes. The targets were co-incubated with the inhibitors before 1×10^5 Cr^{51}-labelled tumour cells/well were added in each assay. Adhesion was quantified after 1 h at RT by scintillation measurements as described in Materials and methods. Given is the inhibition (positive values) or stimulation (negative values) of adhesion in percentage ± S.D. compared to control (no inhibition, 0%) for HT29 colon carcinoma cells (upper panel) and LL carcinoma cells (lower panel). Each bar indicates the mean of at least three to four independently performed experiments each done in triplicate. HT29 colon carcinoma cells: (#) significantly different to PEG-liposomes (B) and sLex-liposomes (C) in the E-selectin assay. LL-carcinoma cells: (*) significantly different to PEG-liposomes (B) in the E-selectin assay, (**) significantly different to PEG-liposomes (B) in the HUVEC assay.

Ligand-free liposomes A and B had no significant influence on the adhesion of HT29 or LL cells; values varied around zero. In some cases, adhesion was rather stimulated. SLex-PEG liposome E inhibited the adhesion of both cell lines in all three assays used up to 80%. A similar or only slightly lower inhibition was also obtained with ligand-bearing but not sterically stabilised liposome C and liposome D containing the ligand within the PEG-layer.

It is further obvious that the strongest inhibition was obtained in the assay with immobilised E-selectin. Interestingly, focussing on the adhesion of HT29 cells to HUVECs, which is the best mimicking model for interaction of human tumour cells with vascular endothelial cells, only liposome E lead to an inhibition of binding while all other liposomes rather stimulate adhesion.

4. Discussion

The aim of this study was to directly compare for the first time glycoliposomes that differ in the accessibility of the sLex ligand and in sterical stabilisation for their potency to inhibit competitively the E-selectin-mediated tumour cell adhesion in vitro.

It was already shown earlier that liposomes are suitable vesicles to block the adhesion process [11,21,22]. Liposomes have the advantage of an especially high flexibility of the liposomal bilayer. Because of this, ligands at their surface are able to form clusters and to fulfil in this way the requirement for a multivalent ligand interaction [21].

To obtain an efficient binding between the liposomes and E-selectin at the endothelial site, the tetrasaccharide sLe^X was chosen as a well-known ligand for this receptor [27]. Glycoliposomes with this ligand, on the other hand, also competitively prevent the binding of other ligands for E-selectin at the same time, for example of sLe^A, which can additionally be present at the membranes of some tumour cells.

In a first experiment, we quantified the effect of sterical stabilisation on cellular uptake by macrophages in the absence and presence of the ligand to make sure that the uptake of investigated ligand decorated liposomes by the monocyte/phagocytic system (MPS) will be reduced or prevented in future animal experiments. It is well known that liposomes which are covered by PEG chains (called sterically stabilised or PEG-liposomes) have a prolonged circulation in the bloodstream because of the ''stealth'' effect and the resulting decrease in the uptake by the MPS [28,29]. Liposomes with a modified surface like immuno- or glycoliposomes, but without a sterical stabilisation, are very likely to be taken up by the MPS [30,31]. In addition, the presence of a ligand together with a PEG cover can also enhance uptake [32,33]. Thus, the uptake of liposome E was quantified in a time dependent way in comparison to liposome A (unmodified surface) and liposome B (sterically stabilised without ligand). Only liposome A was taken up and internalised by J774 cells in vitro in a time-dependent way (Fig. 2), whereas sterically stabilised liposomes were not (Table 2), indicating that liposome E is suitable for in vivo applications.

In the next step, we characterised the $sLe^{X/A}$ ligand expression of different human and murine tumour cell lines. Based on this, cells which differently expressed the ligands were selected for quantification of adhesion. Therefore, three static assays were used. Adhesion of tumour cells to, first, immobilised E-selectin, second, to CHO^{transf} cells, transfected to express permanently E-selectin, and third, to the endothelial cells HUVEC, was determined.

Most of the investigated tumour cell lines (like HT29, SW480, MT3 and HepG2) adhered similarly in all three assays, showing a strong adhesion in the E-selectin and CHO assay and a weaker binding in the HUVEC assay. The low adhesion to endothelial cells may be caused by the lower number of cells used in this assay because of the larger size of the HUVEC cells. In contrast to this, LL cells were found to adhere to a lower degree in the E-selectin and CHO assay, but to a higher degree in the HUVEC assay. This suggests especially for LL cells, that not only the interaction between sLe^X and sLe^A with E-selectin determines the adhesion to HUVEC cells. The involvement of other ligand/receptor combinations between tumour and target cells could be responsible for the increase observed for LL cell adhesion. This was confirmed by quantification of the specific adhesion to E-selectin using the inhibitor BBA2, a monoclonal antibody which specifically recognises human E-selectin, and EDTA, which removes Ca^{2+}, a cation necessary for E-selectin binding (Table 4). The binding to immobilised E-selectin could be reduced to 14–29% under standard conditions and completely if the concentration of the antibody was increased (data not shown). The remaining binding of tumour cells to CHO- and HUVEC cells of 28–69% after co-incubation with the BBA2 antibody suggests the partial involvement of additional binding sites like integrins or mucins [33–35] in a cell line-dependent way in the interaction between tumour cells and the endothelial surface.

The inhibition of tumour cell adhesion by the different liposomes was performed with selected tumour cell lines in all three assays in vitro and results are shown for HT29 colon carcinoma cells as well as for LL carcinoma cells (Fig. 4).

As expected, glycoliposome C and sLe^X-PEG-liposome E had in general the highest inhibitory potential. The inhibition was dependent on the concentration of the liposomes, the adhesion assay and on the cell line used. Liposome E inhibited the adhesion of tumour cells to E-selectin up to 80% (or up to 40–60% in the HUVEC assay). This demonstrated that the PEG layer had no restrictive influence on the binding ability of the terminal sLe^X group, or could even enhance the inhibition compared to vesicles C probably because of the high flexibility of this ligand construct.

In contrast, glycoliposome D with the sLe^X ligand embedded within the PEG layer, had a lower inhibition potency, suggesting that the accessibility of the ligand was reduced by the PEG layer.

While liposome E had a strong inhibitory effect on the adhesion of both cell lines in the HUVEC assay, liposomes A–D seem to cause an increase in HT29 cell adhesion in this assay, but related to the strong variation between the different experiments, this conclusion still has to be proved. If there is an enhancement of adhesion, an (non-specific) attraction between the colon carcinoma and the endothelial cells could be responsible for this, which is not related to E-selectin. All ligand-bearing liposomes used in other assays inhibited HT29 cell adhesion, and in addition, all LL cell binding in any assay.

To summarise our results, it was found that sLe^X-equipped liposomes are an interesting tool for the inhibition

of the adhesion between tumour cells and targets with E-selectin as receptor. Based on the systematic investigation of different liposomal preparations concerning their adhesion inhibition potency, this study revealed that sterically stabilised liposomes with the sLe^X ligand at the distal end of a PEG chain (sLe^X-PEG-liposome E) had the most powerful inhibitory potency in static adhesion assays in vitro. Additionally taking into account that these liposomal formulations are not taken up by macrophages, in vivo experiments are warranted with these liposomes and will be performed.

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