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Immunoliposomes That Target Endothelium In Vitro Are Dependent on Lipid Raft Formation

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Abstract: Lipid rafts are plasma membrane microdomains rich in cholesterol, sphingolipids, and cell surface receptors. Recent studies demonstrated the upregulation and localization of two receptors, intercellular cell adhesion molecule-1 (ICAM, CD54) and endothelial leukocyte adhesion molecule-1 (E-selectin, CD64E), within lipid raft microdomains of inflamed or injured endothelial cells (ECs). We hypothesized that the localization of ICAM and E-selectin within lipid rafts may be essential for drug delivery vehicles labeled with antibodies against ICAM (aICAM) and E-selectin (aE-selectin). To eliminate localization of cell surface receptors, ECs were treated with a cholesterol depleting drug, methyl-β-cyclodextrin. We also tested if antibody mobility and the ratio of aICAM to aE-selectin on immunoliposomes influenced binding to lipid-raft-depleted cells. Liposomes were prepared from either 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine (DOPC, $C_{18:1}$, $T_m = -20$ °C) or 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC, $C_{16:0}$, $T_m = 42$ °C) which are in the liquid crystalline and gel phase at 37 °C, respectively. Mobility and the aICAM:aE-selectin ratio influenced cellular binding only when lipid rafts form. In the absence of lipid rafts, cellular binding of both DOPC and DPPC immunoliposomes was reduced to the nonspecific binding level. These results, which were obtained under static conditions, suggest that the presence of lipid rafts in ECs is critical for targeted drug delivery.

Keywords: Lipid rafts; endocytosis; cell adhesion molecule; immunoliposome; targeting; membrane fluidity; endothelial cells; ICAM; E-selectin; DOPC; DPPC

Introduction

Targeting the vascular system has been proposed as a therapeutic strategy to treat cancer,[1] inflammation,[2] and ischemia.[2] Increased expression of specific adhesion molecules has been exploited to facilitate the delivery of therapeutic agents to inflamed endothelial cells (ECs). Drug delivery systems modified with antibodies against ICAM,[3,4] E-selectin,[5] and vascular cell adhesion molecule-1[6] (VCAM) have shown selectivity for ECs activated with cytokines (i.e., interleukin-1α (IL-1α)), which mimics the inflammatory response in vitro that results in the upregulation of adhesion molecules.

Transiently upregulated cell adhesion molecules on ECs have been shown to enhance binding specificity and cellular uptake of drug delivery vehicles when presenting multiple targeting ligands.[7] Microbubble contrast agents conjugated with dual targeting antibodies against VCAM and P-selectin adhered almost twice as effectively as single-targeting

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(1) Heath, V. L.; Bicknell, R. Anticancer strategies involving the vasculature. *Nat. Rev. Clin. Oncol.* **2009**, *6*, 395–404.

(2) Simone, E.; Ding, B. S.; Muzykantov, V. Targeted delivery of therapeutics to endothelium. *Cell Tissue Res.* **2009**, *335*, 283–300.

(3) Villanueva, F. S.; Jankowski, R. J.; Klibanov, S.; Pina, M. L.; Alber, S. M.; Watkins, S. C.; Brandenburger, G. H.; Wagner, W. R. Microbubbles targeted to intercellular adhesion molecule-1 bind to activated coronary artery endothelial cells. *Circulation* **1998**, *98*, 1–5.

(4) Muro, S.; Wiewrodt, R.; Thomas, A.; Koniaris, L.; Albelda, S. M.; Muzykantov, V. R.; Koval, M. A novel endocytic pathway induced by clustering endothelial ICAM-1 or PECAM-1. *J. Cell Sci.* **2003**, *116*, 1599–1609.

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microbubbles.[8] Likewise, the adhesion of microspheres bearing both aICAM and sialyl Lewis^x, a selectin ligand, showed a concentration dependence of both ligands.[9,10]

Recently, we have established that the antibody ratio, surface density, and mobility are factors that influence the cellular binding of immunoliposomes.[11] Our findings suggested that mobility of antibodies on the liposome surface at an optimal antibody ratio and density allows for the synergistic binding of aICAM and aE-selectin. However, these conclusions were based on one set of conditions that did not account for the fact that the cell surface is not a static display of molecules.

Changes in the presentation of adhesion molecules at the cell surface may alter cellular binding. E-selectin and ICAM have been reported to cluster following their engagement in a cytokine or shear-induced response.[12] Clustering of ICAM and E-selectin at the lipid surface is required for subsequent cell signaling and gene expression.[13,14]

Growing evidence has indicated that ICAM and E-selectin are incorporated into detergent-insoluble membrane domains,

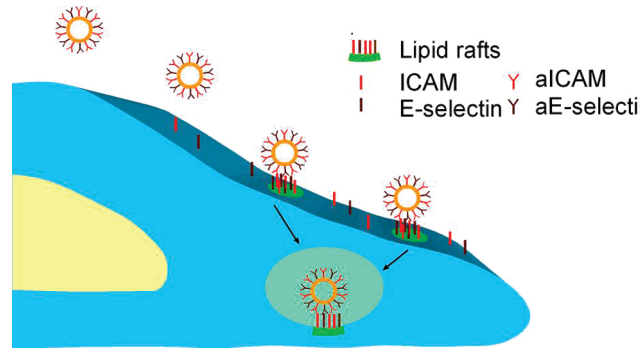

Figure 1. Schematic of ICAM and E-selectin localizing in lipid rafts on ECs. ICAM and E-selectin exist both in lipid-raft microdomains and throughout the plasma membrane. EC binding and endocytosis of immunoliposomes conjugated with aICAM and aE-selectin occur only when aICAM and aE-selectin bind to the receptors colocalized in the lipid-raft microdomains.

consistent with their association with lipid rafts.[12,15] Lipid rafts are specialized areas in the plasma membrane that regulate signal transduction by concentrating cell surface receptors.[13,16,17] Compartmentalization of signaling molecules within rafts aids the formation of large molecular complexes upon receptor binding. Clustered rafts then become associated with cytoskeletal molecules, which provide a mechanism for regulation of Src family kinases.[17,18] ICAM and E-selectin clustering within lipid rafts have dual roles as adhesion receptors for leukocytes and also function as signaling receptors in ECs.

In this study, we proposed to target endothelial cells using cell adhesion molecules pooled in lipid raft microdomains. We investigated the role of lipid rafts on the cellular binding of immunoliposomes bearing two distinct antibodies (aICAM and aE-selectin) (Figure 1). Immunoliposomes were engineered by altering two properties: (1) antibody mobility and (2) aICAM:aE-selectin ratio. The mobility was altered using liposome formulations composed of lipids with different chain length and degree of saturation. DPPC is saturated; it has a C16 acyl chain that packs tightly and forms a gel below its transition temperature, $T_m = 42$ °C. DOPC is unsaturated, having a cis double bond ($\Delta 9$) in each C18 chain. DOPC lipids are mobile above $T_m = -20$ °C. Treatment of ECs with the cholesterol-depleting drug methyl-β-cyclodextrin (MCD) was used to delocalize raft-associated molecules such as ICAM and E-selectin.

(5) Spragg, D. D.; Alford, D. R.; Greferath, R.; Larsen, C. E.; Lee, K.-D.; Gurtner, G. C.; Cybulsky, M. I.; Tosi, P. F.; Nicolau, C.; Gimbrone, M. A., Jr. Immunotargeting of liposomes to activated vascular endothelial cells: A strategy for site-selective delivery in the cardiovascular system. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 8795–8800.
(6) Voinea, M.; Manduteanu, I.; Dragomir, E.; Capraru, M.; Simionescu, M. Immunoliposomes directed towards VCAM-1 interact specifically with activated endothelial cells - A potential tool for specific drug delivery. *Pharm. Res.* **2005**, *22* (11), 1906–1917.
(7) Laginha, K.; Mumbengegwi, D.; Allen, T. Liposomes targeted via two different antibodies: Assay, B-cell binding and cytotoxity. *Biochim. Biophys. Acta* **2005**, *1711*, 25–32.
(8) Ferrante, E. A.; Pickard, J. E.; Rychak, J.; Klibanov, A.; Ley, K. Dual targeting improves microbubble contrast agent adhesion to VCAM-1 and P-selectin under flow. *J. Controlled Release* **2009**, *140* (2), 100–107.
(9) Eniola, A. O.; Hammer, D. A. In vitro characterization of leukocyte mimetic for targeting therapeutics to the endothelium using two receptors. *Biomaterials* **2005**, *26*, 7136–7144.
(10) Eniola, A. O.; Willcox, J. P.; Hammer, D. A. Interplay between rolling and firm adhesion elucidated with a cell-free system engineered with two distinct receptor-ligand pairs. *Biophys. J.* **2003**, *85*, 2720–2731.
(11) Gunawan, R. C.; Auguste, D. T. The role of antibody synergy and membrane fluidity in the vascular targeting of immunoliposomes. *Biomaterials* **2010**, *31*, 900–907.
(12) Tilghman, R. W.; Hoover, R. L. E-selectin and ICAM-1 are incorporated into detergent-insoluble membrane domains following clustering in endothelial cells. *FEBS Lett.* **2002**, *525*, 83–87.
(13) Kiely, J.-M.; Hu, Y.; Garcia-Cardena, G.; Gimbrone, M. A., Jr. Lipid raft localization of cell surface E-selectin is required for ligation-induced activation of phospholipase Cg. *J. Immunol.* **2003**, *171*, 3216–3224.
(14) Wang, Q.; Doerschuk, C. M. The signaling pathways induced by neutrophil-endothelial cell adhesion. *Antioxid. Redox Signaling* **2002**, *4*, 39–47.
(15) Amos, C.; Romero, I. A.; Schultze, C.; Rousell, J.; Pearson, J. D.; Greenwood, J.; Adamson, P. Cross-linking of brain endothelial intercellular adhesion molecule (ICAM)-1 induces association of ICAM-1 with detergent-insoluble cytoskeletal fraction. *Arterioscler. Thromb. Vasc. Biol.* **2001**, *21*, 810–816.
(16) Anderson, R. G. The caveolae membrane system. *Annu. Rev. Biochem.* **1998**, *67*, 199–225.
(17) Simons, K.; Toomre, D. Lipid rafts and signal transduction. *Nat. Rev. Mol. Cell Biol.* **2000**, *1*, 31–41.
(18) Tilghman, R. W.; Hoover, R. L. The Src-cortactin pathway is required for clustering of E-selectin and ICAM-1 in endothelial cells. *FASEB J.* **2002**, *16*, 1257–1259.

Materials and Methods

Materials. 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-dodecanoyl (*N*-dod-PE), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) were purchased from Avanti Polar Lipids (Alabaster, AL). IL-1α, mouse anti-human ICAM (CD54), mouse anti-human E-selectin (CD62E) monoclonal antibodies (mAbs), and IgG_1 isotype (mAb) were procured from R&D Systems (Minneapolis, MN). *N*-Ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide (EDC), *N*-hydroxysuccinimide (NHS), bovine serum albumin (BSA), rhodamine-B isothiocyanate-conjugated dextran (10 kDa MW), ammonium molybdate, MCD, and ascorbic acid were obtained from Sigma (St. Louis, MO). Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse, tetramethyl rhodamine isothiocyanate (TRITC)-conjugated sheep anti-mouse, and rabbit anti-sheep secondary antibodies were obtained from Abcam (Cambridge, MA). Sheep anti-human E-selectin polyclonal antibodies were purchased from Leinco Technologies (St. Louis, MO). Dulbecco's phosphate buffered saline (PBS) and 0.25% trypsin/2.6 mM ethylenediaminetetraacetic acid (EDTA) were obtained from Invitrogen (Carlsbad, CA).

Liposome Preparation. Unilamellar liposomes were prepared by the extrusion method as reported previously.[11] DPPC and DOPC liposomes were studied due to their wide use in the drug delivery and surface rheology literature. In short, a mixture of DOPC:*N*-dod-PE or DPPC:*N*-dod-PE (95:5 mol %) was dissolved in a mixture of DMSO:EtOH (7:3 v/v) after being dried in a rotary evaporator under reduced pressure. Lipid mixtures (0.3 mL) were resuspended with 3 mL of PBS (pH 7.4) containing rhodamine-conjugated dextran (1 mg/mL) and agitated at 650 rpm to give a final concentration of 50 mM lipid. The temperature of the DPPC mixture was increased to 50 °C during extrusion. To remove excess dextran, dextran-encapsulated liposomes were dialyzed in PBS using a Slide-A-Lyzer dialysis cassette (MWCO 20 kDa, Pierce Biotechnology, Inc., Rockford, IL) for 24 h at room temperature (RT). Liposome size and zeta potential were determined by dynamic light scattering on a ZetaPALS analyzer (Brookhaven Instruments, Corp., Holtsville, NY) in PBS (pH 7.4).

A phosphate assay was used to determine the concentration of lipid in solution as previously described.[19] A diluted liposome sample was added to 0.2 mL of sulfuric acid (10% v/v) at 200 °C for 1 h, followed by addition of 50 mL of hydrogen peroxide (30% v/v) and further heating at 200 °C for 40 min. Samples were then cooled down to RT. 480 mL of deionized water and 0.5 mL of color reagent (0.5% w/v ammonium molybdate, 2% w/v ascorbic acid) were added to each sample followed by heating at 45 °C for 20 min. The color intensities of the samples were measured at 820 nm using a Spectramax Plus 384 spectrophotometer (Molecular Devices, Sunnyvale, CA).

Preparation of Antibody-Labeled Liposomes and Microspheres. Antibodies were conjugated to liposomes via the *N*-dod-PE anchor. Although PEG tethers are typically incorporated in drug delivery vehicles to enhance binding,[20] this study utilizes short C12 tethers to emphasize the effect of lateral diffusion of the antibodies. EDC (2 mg) and NHS (3 mg) were mixed with 1 mmol of lipid (liposomes) in PBS (pH 7.4) and incubated for 6 h at RT. A Zeba Spin desalting column (MWCO 7 kDa, Pierce Biotechnology, Inc.) was used to remove excess EDC and NHS. IgG_1 isotype (100 mol %, nonspecific liposome) or mixtures of aICAM:aE-selectin (1:0, 0:1, 2:1, 1:1, and 1:2 molar ratios) were added to EDC-modified liposomes at a molar ratio of 1:1000 antibody:phospholipid and incubated overnight at RT. Unconjugated antibodies were removed using a Sepharose CL-4B column (fractionation range: 60−20,000 kDa, Sigma).

The antibody density of aICAM and aE-selectin on immunoliposomes was quantified. Liposomes are not detected by flow cytometry because of their small size. Therefore, 2 μm borosilicate beads (Duke Scientific, Palo Alto, CA) were encapsulated within PC:*N*-dod-PE (95:5) liposomes by agitating small unilamellar liposomes with microbeads in PBS for 6 h. Microbeads were rinsed 3 times with PBS through centrifugation−suspension cycles to separate free liposomes. Conjugation of different ratios of aICAM:aE-selectin and control IgG_1 (nonspecific binding) to microbead encapsulating liposomes was performed using EDC/NHS chemistry. After free primary antibodies were separated, microbeads were conjugated with FITC-conjugated goat anti-mouse (aE-selectin binding) and TRITC-conjugated rabbit anti-sheep (aICAM binding) secondary antibodies (10 mL, 10 ng/mL) overnight at 4 °C. Free secondary antibodies were removed using suspension−spin cycles. The density of aE-selectin and aICAM conjugated to microbead encapsulating immunoliposomes was determined with reference to Quantum Simply Cellular microbeads (Bangs Laboratory, Inc., Fishers, IN), which have defined numbers of antibody binding sites per bead.

Cell Culture. Human umbilical vein endothelial cells (ECs) were cultured in endothelial growth medium-2 (EGM-2) media with supplements (Lonza, Allendale, NJ). Cells were maintained at 37 °C in a humidified incubator with 5% CO_2 and used for experiments at passages 2−5.

Densities of ICAM and E-Selectin on ECs. ICAM and E-selectin expression by ECs was evaluated by flow cytometry after a 6 h incubation with IL-1α (5 ng/mL). Quantification of the density of molecules on the surface was determined with Quantum Simply Cellular microbeads, using the protocol as provided by the manufacturer. After ECs were activated with IL-1α for 6 h, 10^6 cells were collected from a 6-well plate, spun down, and resuspended with ice cold 1% BSA in PBS. ECs were rinsed 3 times through suspension−spin cycles and blocked with 1% BSA in PBS

(19) Itoh, Y. H.; Itoh, T.; Kaneko, H. Modified Bartlett assay for microscale lipid phosphorus analysis. *Anal. Biochem.* **1986**, *154*, 200–204.

(20) Harding, J. A.; Engbers, C. M.; Newman, M. S.; Goldstein, N. I.; Zalipsky, S. Immunogenicity and pharmacokinetic attributes of poly(ethylene glycol)-grafted immunoliposomes. *Biochim. Biophys. Acta* **1997**, *1327*, 181–192.

for 30 min in an ice bath. ECs were incubated with mouse anti-human ICAM and sheep anti-human E-selectin mAbs (1 mL, 10 ng/mL) for 30 min in an ice bath. After rinsing with 1% BSA in PBS 3 times to remove free mAbs, ECs were stained with FITC-conjugated goat anti-mouse and TRITC-conjugated rabbit anti-sheep secondary antibodies for 30 min in an ice bath. ECs were finally rinsed with 1% BSA in PBS 3 times, resuspended in PBS, and followed by flow cytometry analysis.

Immunoliposome Binding by ECs. Liposome binding by ECs was analyzed using flow cytometry as previously described.[21−23] ECs were seeded in 6-well plates (3×10^5cells/well) and allowed to adhere overnight. After activation with IL-1α for 6 h, ECs were incubated overnight at 37 °C with (1) dextran-loaded nonspecific (IgG_1) liposomes; and (2) dextran-loaded immunoliposomes conjugated with different ratios of aICAM/aE-selectin mAb. The concentration used was 1 mmol lipid/10^6 cells.

Treated ECs were washed with PBS, harvested using trypsin/EDTA solution, and collected in a polystyrene culture tube. Cells were washed with PBS three times. Immunoliposomes were not detected by fluorescence in the trypsin solution used to remove ECs (see Supporting Information, Figure SI1). Binding data were acquired using an LSRII flow cytometer (BD Immunocytometry Systems, San Jose, CA) and analyzed with WEASEL software developed by WEHI (Parkville, Australia). The fold-over isotype value, which was calculated by dividing the mean fluorescence intensity for immunoliposomes conjugated with aICAM:aE-selectin by that of the isotype-conjugated liposomes, was used to normalize the data. The significant difference in liposome uptake from different samples was evaluated using a two-tailed Student's *t* test. A *P* value less than 0.05 was considered statistically significant.

The ECs used in this study are large venous endothelial cells present in the umbilical cord of newborns. Therefore, the data presented should not be automatically extended to the endothelium in adults. Kline et al. have compared ECs from the umbilical vein to the human vena saphena and the femoral artery.[24] They and others[5,25] have found ECs to be a relevant model for the study of adhesion molecules. In vitro results should be cautiously extrapolated to in vivo conditions. ECs are used here due to their commercial availability and reproducibility.

Lipid Raft Delocalization. To disrupt lipid rafts on the cell surface, ECs were treated with 5 mM MCD for 1 h after activation with IL-1α for 6 h. After the initial 1 h incubation with MCD, ECs were treated with DOPC:*N*-dod-PE and DPPC:*N*-dod-PE (1 mmol of lipid/10^6 cells) bearing different ratios of aICAM:aE-selectin in the presence of MCD and IL-1α overnight at 37 °C.

To eliminate endocytotic events, ECs were activated with IL-1α for 6 h, incubated with MCD for 1 h at 37 °C, and then placed at 4 °C for 1 h before they were incubated with DOPC:*N*-dod-PE and DPPC:*N*-dod-PE immunoliposomes overnight at 4 °C. The following day, ECs were harvested as previously described.

MCD has been widely used as a cholesterol depleting agent; however cytotoxic effects may be observed above 5 mM MCD.[24] In this study, ECs were viable after exposure to 5 mM MCD for 12 h (see Supporting Information, Table SI1).

ICAM and E-Selectin Blocking. ECs were treated with IL-1α (5 ng/mL) for 6 h. To remove lipid rafts, ECs were incubated with MCD (5 mM) for 1 h. Blocking was performed by incubating with aICAM and aE-selectin individually or in a mixture (10 mg/mL) after removal of IL-1α for 1 h at 37 °C. ECs were then treated with DOPC:*N*-dod-PE and DPPC:*N*-dod-PE immunoliposomes (1 mmol of lipid/10^6 cells) labeled with an aICAM:aE-selectin molar ratio of 1:1 overnight at 37 °C. Flow cytometric analysis of immunoliposome binding was performed immediately after ECs were harvested.

Results

Characterization of Immunoliposomes. Liposomes were prepared with 5 mol % *N*-dod-PE and either 95 mol % DOPC or DPPC. Conjugation of aICAM and aE-selectin to *N*-dod-PE lipids was assessed by changes in the liposome diameter, the surface charge, and the antibody density. Liposome diameters less than 250 nm were prepared to mimic vehicles used for systemic administration. The diameters of unconjugated DOPC:*N*-dod-PE and DPPC:*N*-dod-PE liposomes were 205 ± 5 nm and 201 ± 4 nm, respectively, as determined from dynamic light scattering. After conjugation with aICAM and aE-selectin, the diameters increased to 223 ± 3 nm for DOPC and 219 ± 7 nm for DPPC. The diameters were similar for all immunoliposome formulations. The antibody density and aICAM:aE-selectin ratio were determined using standardized microbeads (Table 1). The average antibody density was 1746 ± 60 molecules/mm^2 or 123 ± 5 molecules/liposome. The surface charge (1.1 ± 0.5 mV at pH 7.4) of both DOPC and DPPC immunoliposomes were

(21) Marty, C.; Meylan, C.; Schott, H.; Ballmer-Hofer, K.; Schwendener, R. A. Enhanced heparan sulfate proteoglycan-mediated uptake of cell-penetrating peptide-modified liposomes. *Cell Mol. Life. Sci.* **2004**, *61*, 1785–1794.

(22) Curtsinger, J. C.; Deeths, M. J.; Pease, P.; Mescher, M. F. Artificial cell surface constructs for studying receptor-ligand contributions to lymphocyte activation. *J. Immunol. Methods* **1997**, *209*, 47–57.

(23) Kerby, M. B.; Urban, J. C.; Mouallem, L.; Tripathi, A. Circulating IgSF Proteins Inhibit Adhesion of Antibody Targeted Microspheres to Endothelial Inflammatory Ligands. *Appl. Biochem. Biotechnol.* **2009**, *159* (1), 208–220.

(24) Kline, M. A.; O'Connor Butler, E. S.; Hinzey, A.; Sliman, S.; Kotha, S. R.; Marsh, C. B.; Uppu, R. M.; Parinandi, N. L. A simple method for effective and safe removal of membrane cholesterol from lipid rafts in vascular endothelial cells: implications in oxidant-mediated lipid signaling. *Methods Mol. Biol.* **2010**, *610*, 201–211.

(25) Muro, S.; Gajewski, C.; Koval, M.; Muzykantov, V. R. ICAM-1 recycling in endothelial cells: a novel pathway for sustained intracellular delivery and prolonged effects of drugs. *Blood* **2005**, *105*, 650–658.

Table 1. Antibody Density and Zeta Potential of DPPC:*N*-dod-PE Immunoliposomes

ratio aICAM:aE-selectin	aICAM (molecules/mm^2)	aE-selectin (molecules/mm^2)	zeta potential (mV)
1:0	1786 ± 87		1.1 ± 0.4
2:1	1252 ± 123	554 ± 39	0.8 ± 0.5
1:1	832 ± 85	836 ± 30	1.6 ± 0.6
1:2	586 ± 93	1111 ± 155	0.7 ± 0.3
0:1		1773 ± 115	1.2 ± 0.4

Table 2. IL-1α Stimulated Expression of ICAM and E-Selectin on ECs (molecules/cell)

	not activated	activated without MCD	activated with MCD
ICAM	11300 ± 2800	207000 ± 29000	197000 ± 12000
E-selectin	32500 ± 680	179000 ± 14000	174000 ± 14000

similar at all tested ratios (Table 1). The surface density of 5 mol % *N*-dod-PE was used to conjugate antibodies since there was no change in cellular binding between 5 and 10 mol % *N*-dod-PE (data not shown).

Disruption of Lipid Rafts Decreased Cellular Uptake of Immunoliposomes. IL-1α has been shown to upregulate the expression of ICAM and E-selectin on ECs.[26] Upregulation of ICAM and E-selectin on ECs in response to treatment with IL-1α and MCD is reported in Table 2. Incubation with bare liposomes did not change EC expression of ICAM and E-selectin. The density of ICAM increased by 15-fold on activated ECs while the expression of E-selectin increased by 5-fold. Cellular binding of immunoliposomes on ECs was studied after a 6 h activation with IL-1α, which had maximal expression of ICAM and E-selectin.[11]

Cellular binding of immunoliposomes was measured by flow cytometry as a function of time (see Supporting Information, Figure SI2). Liposome binding increased with incubation time. After a 1 h incubation, ECs bound 1:1 aICAM:aE-selectin DOPC liposomes 30% more than MCD treated ECs. Differences in uptake were more pronounced for longer periods. Subsequent studies were performed at 12 h to exacerbate differences in binding.

Immunoliposome binding was measured using flow cytometry and analyzed as a function of the aICAM:aE-selectin ratio (Figure 2). To determine whether cellular uptake requires ICAM and E-selectin localization in cholesterol-enriched microdomains, ECs were treated with the cholesterol depleting drug MCD. This treatment diminishes membrane cholesterol and delocalizes raft-associated molecules.[27,28] The binding level of DPPC immunoliposomes by ECs in the absence of MCD initially increased with decreasing aICAM:aE-selectin molar ratio, eventually reaching a maximal level at 1:1 aICAM:aE-selectin (Figure 2, $P < 0.05$). DPPC immunoliposome binding was approximately 2-, 2.6-, and 2.3-fold for 2:1, 1:1, and 1:2 (aICAM:aE-selectin, mol:mol), respectively. Treatment with MCD reduced DPPC immunoliposome binding to levels similar to nonspecific binding (1.2-fold).

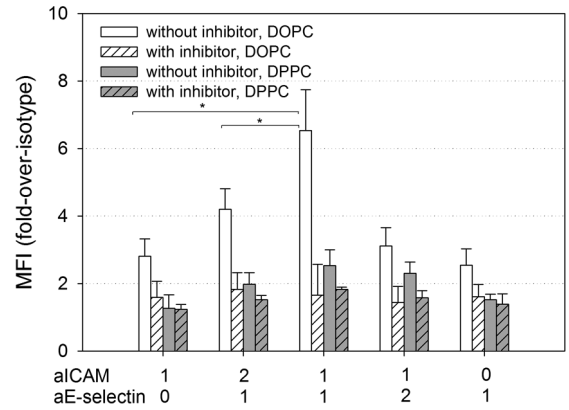

Figure 2. Uptake of immunoliposomes by ECs at 37 °C in the presence and absence of lipid rafts. Activated ECs (IL-1α for 6 h) were treated with either rhodamine-labeled dextran encapsulated in DOPC:*N*-dod-PE (95:5 mol:mol) or DPPC:*N*-dod-PE (95:5 mol:mol). ECs were treated with or without the lipid raft inhibitor, MCD (5 mM for 1 h). Flow cytometry was used to determine the cellular uptake of DOPC or DPPC immunoliposomes conjugated with various ratios of aICAM:aE-selectin. Results are presented relative to uptake by IgG_1-labeled liposomes (*$P < 0.05$ as measured by the *t* test).

The binding level of DPPC immunoliposomes was compared to DOPC immunoliposomes to determine if the increase in mobility affected cellular binding in the absence of lipid rafts on the cell surface (Figure 2). As previously observed,[11] binding of mobile DOPC liposomes was higher than that of more rigid DPPC immunoliposomes at 37 °C when cholesterol was present on the plasma membrane. Maximal binding of immunoliposomes bearing 1:1 (aICAM:aE-selectin, mol:mol) for DOPC immunoliposomes was 2.6-fold higher than DPPC immunoliposomes. In the absence of lipid rafts, binding of DOPC immunoliposomes, at all aICAM:aE-selectin ratios, was equivalent to nonspecific binding ($P > 0.05$), similar to all DPPC immunoliposome formulations.

(26) Springer, T. A. Traffic signals on endothelium for lymphocyte recirculation and leukocyte emigration. *Annu. Rev. Physiol.* **1995**, *57*, 827–872.

(27) Green, J. M.; Zhelesnyak, A.; Chung, J.; Lindberg, F. P.; Sarfati, M.; Frazier, W. A.; Brown, E. J. Role of cholesterol in formation and function of a signaling complex involving avb3, integrin-associated protein (CD47), and heterotrimeric G proteins. *J. Cell Biol.* **1999**, *146*, 673–682.

(28) Harder, T.; Scheiffele, P.; Verkade, P.; Simons, K. Lipid domain structure of the plasma membrane revealed by patching of membrane components. *J. Cell Biol.* **1998**, *141*, 929–942.

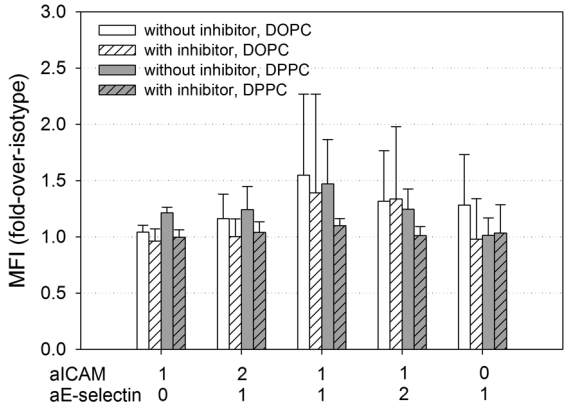

Figure 3. Immunoliposome binding to ECs at 4 °C in the presence and absence of lipid rafts. Activated ECs (IL-1α for 6 h) were treated with either rhodamine-labeled dextran encapsulated in DOPC:*N*-dod-PE (95:5 mol:mol) or DPPC:*N*-dod-PE (95:5 mol:mol). ECs were treated with or without the lipid raft inhibitor, MCD (5 mM for 1 h). Flow cytometry was used to determine the cellular uptake of DOPC or DPPC immunoliposomes conjugated with various ratios of aICAM:aE-selectin. Results are presented relative to uptake by IgG_1-labeled liposomes.

To reduce the diffusivity of the plasma membrane and arrest endocytosis, immunoliposome binding and uptake by ECs was measured at 4 °C. In comparison to 37 °C, the fluorescent signal decreased by 4.3- and 1.6-fold for 1:1 aICAM:aE-selectin DOPC and DPPC liposomes, respectively (Figure 3). In the absence of lipid raft formation, the fluorescent signal at 37 °C was reduced by 1.3- and 1.6-fold for 1:1 aICAM:aE-selectin DOPC and DPPC liposomes, respectively. Endocytosis was a function of lipid raft formation and antibody mobility.

Immunoliposome Binding Is Mediated via Cell Adhesion Molecules Colocalized within Lipid Rafts. To determine if immunoliposome binding was mediated by ICAM and E-selectin localized in lipid raft microdomains, ECs were pretreated with aICAM and aE-selectin to block the activity of ICAM and E-selectin. Blocking was performed with either aICAM or aE-selectin individually or with a combination of aICAM and aE-selectin. Binding of DOPC and DPPC immunoliposomes conjugated with 1:1 aICAM:aE-selectin (mol:mol) were reduced when ICAM and E-selectin were blocked in the absence of MCD (Figure 4). The DOPC binding level was reduced by 6.5-fold while DPPC binding was reduced by 2-fold for all combinations of aICAM and aE-selectin blocking. Blocking with both aICAM and aE-selectin did not significantly reduce the binding level in comparison to the individual blocking of aICAM and aE-selectin ($P > 0.05$). When ECs were treated with MCD, the binding level was similarly decreased with the lowest binding occurring when both aICAM and aE-selectin were blocked ($P > 0.05$).

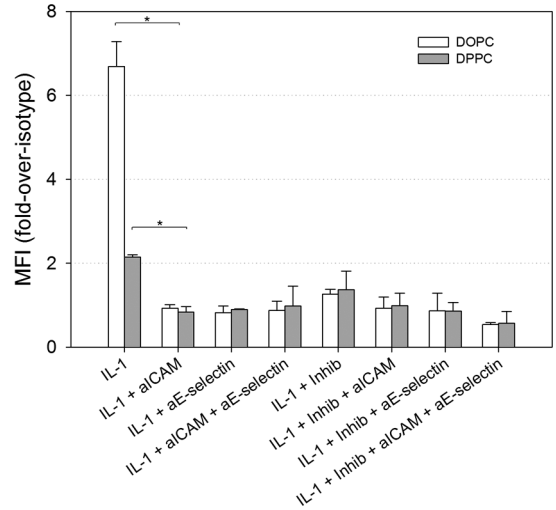

Figure 4. Binding of immunoliposomes to ECs in the presence of the lipid raft inhibitor and after blocking ICAM and E-selectin receptors. Activated ECs (IL-1α for 6 h) were blocked with aICAM and aE-selectin (10 mg/mL for 1 h) after treatment with MCD (5 mM for 1 h). Flow cytometry was used to measure the cellular binding level of DOPC:*N*-dod-PE (95:5 mol:mol) and DPPC:*N*-dod-PE (95:5 mol:mol) conjugated with 1:1 aICAM:aE-selectin (*$P < 0.05$ as measured by the *t* test).

Discussion

Cell adhesion molecules have been a primary target either alone or in combination to increase delivery of molecules or vehicles to ECs. We have recently shown that enhanced binding requires both an optimal aICAM:aE-selectin ratio and antibody mobility.[11] Rearrangement of antibody-conjugated lipids on the liposome surface allows for cooperative binding of immunoliposomes to ECs. However, the organization of aICAM and aE-selectin on the liposome surface remains unknown. In this study, we have investigated how the localization of ICAM and E-selectin within lipid raft microdomains influences binding.

Lipid rafts are rich in highly ordered saturated lipids and cholesterol that are laterally mobile in the plane of a more fluid disordered bilayer of largely unsaturated lipids.[29] Lipid rafts maintain ICAM and E-selectin as clusters[12,13,15] and restrict lateral diffusion within the plasma membrane, creating concentrated microdomains. Depletion of cholesterol from the cell surface resulted in the disruption of lipid rafts and dispersion of ICAM and E-selectin as previously reported.[12,13]

Our data demonstrated that disruption of lipid rafts using the cholesterol-depleting drug MCD significantly reduced immunoliposome binding. We reported that antibody mobility increased binding of aICAM:aE-selectin immunoliposomes with ratio dependence.[11] Binding of DPPC and DOPC

(29) Parton, R. G.; Richards, A. A. Lipid rafts and caveolae as portals for endocytosis: new insights and common mechanisms. *Traffic* **2003**, *4*, 724–738.

immunoliposomes was highest at an equimolar aICAM:aE-selectin ratio but was reduced almost to the basal level of nonspecific binding upon disruption of lipid rafts. Neither antibody ratio nor antibody mobility improved cellular binding in the absence of lipid rafts. This finding suggests that binding of aE-selectin and aICAM immunoliposomes occurred mostly within the lipid raft microdomains.

Immunoliposomes bearing immobile antibodies had greater binding than when lipid rafts were disrupted. Thus, homogeneously dispersed antibodies across the liposome surface fared better than a random presentation of ICAM and E-selectin on the cell surface. The presentation of antibodies on delivery vehicles is as important as the arrangement of cell adhesion molecules within lipid rafts on the cell surface.

Altering the antibody ratio did not significantly increase cellular binding of both DOPC and DPPC immunoliposomes at 4 °C. Endocytosis was arrested at 4 °C,[30,31] which reduced immunoliposome uptake within cells. Liposome binding was not affected by antibody mobility at 4 °C. Muro et al. observed that uptake of aICAM targeting nanocarriers at 4 °C decreased by 20%−25% relative to 37 °C; the surface accessible ICAM on ECs remained high at 4 °C.[25] Depleting cholesterol from the plasma membrane did not reduce DOPC or DPPC immunoliposome binding ($P > 0.05$) at 4 °C. Thus, disrupting lipid rafts had a similar effect to arresting endocytosis and immobilizing lipids at 4 °C.

Our data suggested that the endocytotic mechanism of dual targeting immunoliposomes was lipid-raft and clathrin-mediated. ECs have been reported to internalize vehicles that target ICAM and E-selectin by CAM-[25] and clathrin-mediated endocytosis,[32] respectively. Clustering of E-selectin within lipid rafts and clathrin-coated pits has been reported.[33] In addition, clustering of ICAM was necessary for internalization.[4] In this study, we measured a reduction in binding and uptake of aICAM:aE-selectin labeled liposomes upon treatment with MCD (Figure 2). Treatment with MCD has been reported to inhibit lipid raft formation[24] and impair clathrin- and caveolae-mediated endocytosis.[34] Further study is required to delineate between lipid raft and clathrin-mediated endocytosis.

The effect of disrupting lipid rafts on immunoliposome binding was assessed by blocking ICAM and E-selectin in the presence or absence of MCD. Blocking ECs with free aICAM and aE-selectin molecules prior to incubation with DOPC and DPPC immunoliposomes showed a significant reduction in binding. The reduction in binding was not dependent on the presence of lipid rafts. Binding was the most diminished when ECs were blocked with both aICAM and aE-selectin and treated with MCD. Disruption of lipid rafts (dispersion of ICAM and E-selectin across the cell surface) was equivalent to blocking one or both antibodies; blocking and cholesterol-depletion hindered antibody synergy and drastically reduced cooperative binding.

Translation of these binding experiments to in vivo conditions requires consideration of the impact under flow. We have observed a 3-fold enhancement in EC binding of 1:1 aICAM:aE-selectin DOPC immunoliposomes at 1 dyn/cm^2 relative to DPPC immunoliposomes (see Supporting Information Figure SI3). This suggests that the rearrangement of surface molecules may be significant in vivo.

Conclusions

Targeted delivery of immunoliposomes presenting aICAM:aE-selectin in lipid raft microdomains to activated ECs was investigated. Immunoliposomes labeled with aICAM:aE-selectin bound ECs primarily within lipid raft microdomains. Disruption of lipid rafts and blocking a priori with aICAM and aE-selectin prevented binding of DOPC and DPPC immunoliposomes. Blocking and cholesterol-depletion hindered antibody synergy and drastically reduced cooperative binding. Understanding the organization of molecules on the cellular membrane and their role in cellular binding may enable the design of drug delivery vehicles that distribute predominantly within the target site.

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Supporting Information Available: Figures depicting detachment of immunoliposomes from EC surface in the presence of trypsin, sequential course of immunoliposome uptake by ECs at 37 °C in the presence and absence of lipid rafts, and uptake of immunoliposomes by ECs at 37 °C under flow conditions and table of data for cytotoxicity of ECs in the presence of MCD. This material is available free of charge via the Internet at http://pubs.acs.org.

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(30) Goldenthal, K. L.; Pastan, I.; Willingham, M. C. Initial steps in receptor-mediated endocytosis. The influence of temperature on the shape and distribution of plasma membrane clathrin-coated pits in cultured mammalian cells. *Exp. Cell Res.* **1984**, *152*, 558–564.

(31) Silverstein, S. C.; Steinman, R. M.; Cohn, Z. A. Endocytosis. *Annu. Rev. Biochem.* **1977**, *46*, 669–722.

(32) Everts, M.; Koning, G. A.; Kok, R. J.; Asgeirsdóttir, S. A.; Vestweber, D.; Meijer, D. K. F.; Storm, G.; Molema, G. In vitro cellular handling and in vivo targeting of E-selectin-directed immunoconjugates and immunoliposomes used for drug delivery to inflamed endothelium. *Pharm. Res.* **2003**, *20*, 64–72.

(33) Setiadi, H.; McEver, R. P. Clustering endothelial E-selectin in clathrin-coated pits and lipid rafts enhances leukocyte adhesion under flow. *Blood* **2008**, *111*, 1989–1998.

(34) Rodal, S. K.; Skretting, G.; Garred, O.; Vilhardt, F.; van Deurs, B.; Sandvig, K. Extraction of cholesterol with methyl-beta-cyclodextrin perturbs formation of clathrin-coated endocytic vesicles. *Mol. Biol. Cell* **1999**, *10*, 961–974.