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Biochimica et Biophysica Acta 1613 (2003) 115–121
BIOCHIMICA ET BIOPHYSICA ACTA
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Targeting of antibody conjugated, phosphatidylserine-containing liposomes to vascular cell adhesion molecule 1 for controlled thrombogenesis

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Received 10 March 2003; received in revised form 1 May 2003; accepted 9 May 2003

Abstract

Phosphatidylserine (PS) membrane exposure plays an important role in blood coagulation, and the development of a liposome formulation containing PS may be of potential therapeutic utility if they can be designed to achieve tumor selective thrombosis. The objective of this study was to develop proof-of-principle data for a thrombogenic PS liposome targeted to vascular cell adhesion molecule 1 (VCAM-1) via the attachment of an anti-VCAM-1 monoclonal antibody (Ab). We have evaluated binding of the anti-VCAM-1 Ab-conjugated PS liposomes to VCAM-1 using two in vitro models, as well as assessing the ability of these liposomes to catalyze blood coagulation reactions. Binding of the Ab-conjugated PS liposomes containing 2 or 14 mol% 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[poly(ethylene glycol) 2000] (DSPE-PEG_{2000}) to interleukin 1α stimulated human umbilical vein endothelial cells was 8- and 16-fold higher than those without conjugated Ab, respectively, based on the percentage relative increase in cell associated lipid for these liposomes. Binding to VCAM-1-coated ELISA plates produced similar results. The VCAM-1-bound Ab-conjugated PS liposomes were capable of catalyzing blood coagulation reactions upon the exposure of the thrombogenic PS membrane surface. This control of PS surface exposure was achieved using exchangeable PEG-derivatized phosphatidylethanolamines (PE-PEG), with 97% of clotting activity recovered after PE-PEG exchanged out. Our results demonstrate the potential for considering further development of procoagulant liposomes that selectively target thrombogenesis in tumor vasculature.

Keywords: Liposome; Poly(ethylene glycol); Phosphatidylserine; Steric stabilization; Vascular cell adhesion molecule-1; Vascular targeting

1. Introduction

There is growing interest in the treatment of solid tumors based on vascular targeting agents that are designed to selectively damage tumor vasculature. One approach to achieve this goal has been to induce tumor selective thrombosis that leads to shutdown of the blood supply to tumor cells [1,2]. This strategy has several advantages: (1) tumor vascular endothelial cells are freely accessible to agents that are injected intravenously; (2) tumor endothelial cells are believed to be genetically stable and thus more predictable in terms of response to therapy when compared to heterogeneous tumor cell populations; (3) occlusion of multiple tumor vessels has the potential to produce an amplified tumor cell killing effect since each tumor vessel feeds many tumor cells; and (4) the strategy can be applied to different solid tumor types since tumor blood vessels have similar

Abbreviations: PEG, poly(ethylene glycol); PEG-lipids, poly(ethylene glycol)-lipid conjugates; PE-PEG, poly(ethylene glycol)-derivatized phosphatidylethanolamine; MPS, mononuclear phagocytic system; PS, phosphatidylserine; DOPS, 1,2-dioleoyl-*sn*-glycero-3-[phospho-L-serine]; PC, phosphatidylcholine; SM, sphingomyelin; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; DSPE-PEG_{2000}, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamone-*N*-[poly(ethylene glycol) average MW 2000]; DSPE-PEG_{2000}-MAL, poly(ethylene glycol)-α-distearoyl phosphatidylethanolamine,-ϖ-maleinmide; Chol, cholesterol; CHE, cholesterylhexadecyl ether; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphatidylethanolamine; BSA, bovine serum albumin; DTT, dithiothreitol; MAL, maleimide; HUVEC, human umbilical vein endothelial cells; DIC, disseminated intravascular coagulation; HBS, HEPES buffered saline; IL-1α, interleukin 1α; LUV, large unilamellar vesicles; MLV, multilamellar vesicles; PBS, phosphate-buffered saline; SPDP, *N*-succinimidyl-3-(2-pyridyldithio) propionate; VCAM-1, vascular cell adhesion molecule 1

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0005-2736/03/$ - see front matter © 2003 Published by Elsevier B.V.
doi:10.1016/S0005-2736(03)00142-1

morphological and biochemical properties [3]. With the well-known propensity of phosphatidylserine (PS) containing membranes to catalyze blood coagulation reactions [4], the potential to develop a liposome-based vascular targeting agent that contains PS for the induction of tumor selective thrombosis is intriguing. In this regard, such PS-containing liposome would likely have to exhibit unique attributes in order to limit liposome recognition and thrombogenesis in the central blood compartment while permitting binding and the PS-mediated thrombogenesis once at the tumor site.

During the development of procoagulant PS-containing liposomes, we have overcome several formulation challenges. First, we were able to protect and extend the circulation longevity of PS-containing liposomes that would otherwise be eliminated very rapidly by the mononuclear phagocytic system (MPS). Although a surface density of 5 to 7 mol% PEG_{2000} is widely known to be an effective steric barrier against plasma protein binding and cellular interactions for neutral liposomes composed of PC, SM and Chol [6,7], our findings demonstrated that >10 mol% DSPE-PEG_{2000} is required to effectively protect PS-containing liposomes from the high-affinity, PS-mediated plasma protein interactions that occur in circulation [5]. This protection of the thrombogenic PS liposome surface is also required to prevent disseminated intravascular coagulation. However, to be of therapeutic value, the thrombogenic activity of PS must be revealed following binding to an appropriate vascular target. Once within the tumor vasculature, for example, the well-protected PS liposome surface must transform to one that is reactive toward the blood clotting factors to trigger thrombosis. This transformation can be controlled by selecting PEG modified lipids that desorb from the liposomes, thus exposing the PS membrane surface. In this regard, we have demonstrated that the membrane surface reactivity of PS-containing liposomes toward blood clotting factors can be controlled in a time-dependent fashion by using exchangeable PEG-lipids of various acyl chain lengths [8].

In the development of targeted thrombogenic liposomes for tumor targeting, the PS-containing liposomes must also selectively accumulate and localize within the tumor vasculature. Furthermore, this must be accomplished without significantly altering the controlled surface exposure and reactivity of the PS liposomes. One way to achieve this selectivity is to exploit altered features of the vascular endothelium within tumors. In solid tumors, the vasculature is often marked by antigens that are overexpressed and/or selectively expressed in the endothelial lining [9,10]. Vascular cell adhesion molecule 1 (VCAM-1) is one such antigen, and is present on the vascular endothelium of several human malignant tumors including neuroblastoma [11], renal carcinoma [12], non-small-cell lung carcinoma [13], Hogkin's disease [11], angiosarcoma [14] and colon carcinoma [15]. In normal human tissues, a few vessels in the thyroid, thymus and kidney display weak and patchy VCAM-1 expression [14]. Thus, VCAM-1 would serve as an appropriate candidate for targeting thrombogenic PS liposomes.

Before assessing the efficacy and toxicity of the VCAM-1-targeted thrombogenic PS liposomes in animal models, it was first necessary to demonstrate that these PS liposomes would retain the surface reactivity toward the blood coagulation proteins after binding to the desired target cell. The in vitro studies presented here were undertaken to establish that target bound thrombogenic PS liposomes remain functional with respect to procoagulant activity. In this model system, a monoclonal antibody (Ab) conjugated to the thrombogenic PS liposomes was used for targeting the liposomes to VCAM-1 expressing endothelial cells. Our results contribute not only to the design of sophisticated, multifunctional liposome carriers for targeted drug delivery, but more importantly, to the development of potential lipid-based therapeutics that target the tumor vasculature for cancer therapy.

2. Materials and methods

2.1. Materials

All lipids were purchased from Avanti Polar Lipids (Alabaster, AL) except for DSPE-PEG_{2000}-MAL, which was from Shearwater Polymers Inc. (Huntsville, AL). The [^{3}H]CHE was obtained from NEN Life Sciences Products (Oakville, ON, Canada). Cholesterol, DTT, ellagic acid and Sephadex G-50 size exclusion gel were purchased from Sigma. Recombinant human IL-1α was purchased from R&D Systems. The hybridoma cell line VIII-6G10 (produces mouse IgG_1 against human VCAM-1) was obtained from the American Tissue Culture Collection (Manassas, VA), and was sent to Antibody Solutions (Palo Alto, CA) for production in bioreactors with purification. Mouse anti-human VCAM-1 IgG_1 (clone 1.G11B1), labeled with FITC, was obtained from Calbiochem. Bio-Gel A 15-m size exclusion gel was obtained from Bio-Rad (Mississauga, ON, Canada). *N*-succinimidyl-3-(2-pyridyldithio) propionate (SPDP) was purchased from Pierce.

2.2. Preparation of various liposomes

Large unilamellar vesicles were prepared according to established procedures [16–18]. Lipids were dissolved in chloroform and subsequently dried under a stream of nitrogen gas. The resulting lipid film was placed under high vacuum for a minimum of 2 h, and was hydrated in HBS at 65 °C. The resulting preparation was frozen and thawed five times prior to extrusion 10 times through stacked 0.1 μm polycarbonate filters (Nucleopore Co., Canada) at 65 °C with an extruder (Northern Lipids Inc., Vancouver, BC, Canada). The resulting mean LUV diameter was 100–120 nm as determined by quasi-elastic light scattering using the Nicomp submicron particle size model 370/270. MLV, used

as a membrane sink to promote PEG-lipid exchange, were prepared with Egg PC/Chol (55:45 mole ratio), as described elsewhere [8].

2.3. Conjugation of thiolated anti-VCAM-1 Ab to liposomes

The anti-VCAM-1 Ab and the irrelevant Ab (humanized monoclonal Ab, Herceptin®, which contains a human framework region with complimentary determining region of a murine anti-p185 HER2 Ab that binds to HER2) were conjugated to the liposomes according to established procedures [19,20]. The Ab was first modified with SPDP (1 nmol/μl) in a 1:5 mole ratio for 25 min at room temperature, and was subsequently passed down a Sephadex G-50 column equilibrated with sodium acetate buffer (pH 4.5). Fractions containing the Ab were pooled and were added to DTT powder to give a final DTT concentration of 25 mM. This mixture was incubated at room temperature for 25 min. The thiolated Ab was then isolated by size exclusion chromatography with a Sephadex G-50 column equilibrated with HBS, and was immediately added to liposomes containing 1 mol% DSPE-PEG_{2000}-MAL (10 mM final lipid concentration). The conjugation reaction was carried out at room temperature for 18 h. The mixture was then passed down a Bio-Gel A 15-m column equilibrated with HBS to isolate the Ab-conjugated liposomes. The concentration of the Ab-conjugated liposomes was determined by liquid scintillation counting from aliquots mixed with 5.0 ml Pico-fluor 15 scintillation fluid (Packard Biosciences, The Netherlands), and counted with a Packard scintillation counter model 1900 TR. The amount of Ab conjugated to liposomes was determined using the Pierce Micro BCA protein assay in the presence of 0.5% Triton X-100.

2.4. Liposome binding to surface bound VCAM-1

This assay was based on a previously described procedure [21]. Briefly, recombinant human VCAM-1 (1 μg/well, from R&D Systems) was coated onto Maxisorp 96-well plate (Nalge Nunc International, Rochester, NY) overnight at 4 °C in coating buffer (1.59 g Na_2CO_3/2.93 g $NaHCO_3$ to 1 L, pH 9.6). The plate was then blocked with 10% fetal bovine serum for 1 h at 37 °C. Liposomes were then added to the wells, and were incubated for 2 h at 37 °C. The wells were washed three times with phosphate-buffered saline (PBS), followed by the addition of 5% Triton X-100. After a 15-min incubation at 37 °C, the content from each well was transferred to a scintillation vial with three rinses of PBS, and the radioactivity was determined by liquid scintillation counting.

2.5. Induction and assessment of VCAM-1 expression on human umbilical vein endothelial cells (HUVEC) by IL-1α

HUVEC were purchased from Clonetics, and were cultured in EGM-2 medium supplemented with BulletKit (Clonetics). Cells in the 3rd to 5th passages were used, and all studies were completed with cells at ~70% confluence. HUVEC were stimulated with 6.25 ng/ml IL-1α for 4 h at 37 °C. At the end of incubation, HUVEC were washed with PBS/0.1% BSA twice, and were dislodged with PBS/2.5 mM EDTA for 5 min. Cells (2×10^5/sample) were pelleted and resuspended in 0.1 ml PBS/10% human serum containing the FITC-labeled mouse anti-VCAM-1 Ab and incubated on ice for 30 min. Cells were then washed twice with ice-cold PBS/0.1% BSA, and resuspended in 0.4 ml ice-cold PBS/0.1% BSA/propidium iodide for flow cytometry analysis using an EPICS Elite ESP flow cytometer (Beckman-Coulter, Miami, FL) equipped with an Enterprise 621 laser (Coherrent, Santa Clara, CA) set to measure fluorescence of FITC. The flow cytometer scale was calibrated with Quantum 24 or 25 beads (Flow Cytometry Standards, San Juan, Puerto Rico), and 10^4 events were collected for each sample. Data were analyzed with the ExpoMFA software. A previously described protocol was followed to quantify the number of Ab binding sites, using Simply Cellular beads (Flow Cytometry Standards) as a standard to determine the fluorescence intensity per Ab [22].

2.6. Liposome binding to IL-1α stimulated HUVEC

HUVEC were stimulated with IL-1α as described above. Subsequently, 1×10^6 cells were placed in polypropylene tubes, and [^{3}H]CHE-labeled liposomes were added to achieve a final liposomal lipid concentration of 2 mM. Following an incubation at 4 °C for 4 h, the cells were centrifuged at $300 \times g$ for 5 min, and washed three times with PBS. Cells were solubilized with 0.9% Triton X-100 in PBS, and the contents were transferred to a scintillation vial for liquid scintillation counting.

2.7. In vitro clotting time assay

This assay was based on a procedure previously utilized for assessing procoagulant activity of PS-containing liposomes [5,8]. Briefly, Maxisorp Nunc-Immuno tubes were coated with VCAM-1 (5 μg/tube) as described in Section 2.4 and pre-blocked with 10% serum. Liposomes (250 nmol in 0.5 ml HBS) were added and incubated for 2 h at 37 °C. The tubes were then washed three times with PBS. Human citrated plasma (125 μl), 10^{-5} M ellagic acid (125 μl) and HBS (125 μl) were then added to the tubes, which were then incubated for 2 min at 37 °C. Calcium (8.75 mM, final concentration) was added to initiate the clotting reaction, and the mixture (0.5 ml total) was gently shaken. The time at which the mixture turned into a viscous gel was recorded, and noted as the time required for the clotting reaction to be completed. As indicated, MLV were added to the tubes and incubated for 15 min, prior to the addition of the plasma mixture. The amount of lipid bound to the tube was assayed as described above.

3. Results and discussion

3.1. Conjugation of anti-VCAM-1 Ab to PS-containing liposomes

The anti-VCAM-1 Ab was conjugated to PS-containing liposomes based on a well-established procedure [19,20]. The coupling efficiencies for PS liposomes containing 2 mol% DSPE-PEG_{2000} were between 55% and 61%, which is comparable to other procedures that employed a thioether linkage under similar reaction conditions [19,20,23,24]. Increasing the DSPE-PEG_{2000} level to 14 mol% decreased the coupling efficiencies. When the initial Ab to lipid ratio was 45 μg Ab/μmol lipid, the coupling efficiency was 51%, but when the initial ratio increased to 60 μg Ab/μmol lipid, the measured levels of coupled Ab did not increase and therefore the efficiency of the reaction was less than 40%. Reduced efficiency is possibly due to the decreased flexibility of the PEG spacer when elevated levels of DSPE-PEG_{2000} are present. An initial ratio of 45 μg Ab/μmol lipid produced similar final Ab to lipid ratios for PS liposomes containing 2 or 14 mol% DSPE-PEG_{2000} (23–27 μg Ab/μmol lipid). Based on these results, this ratio was used for subsequent conjugation reactions to produce liposomes that had similar Ab to lipid ratios for the evaluation of target binding.

3.2. Binding of anti-VCAM-1 Ab-conjugated PS liposomes to target VCAM-1

Binding of the Ab-conjugated PS liposomes to VCAM-1 was first evaluated by an ELISA plate-based assay. Binding trends were comparable for liposomes containing 2 or 14 mol% DSPE-PEG_{2000} with lipid concentrations up to 0.5 mM (Fig. 1). The amount of lipid recovered for the Ab-conjugated PS liposomes with 2 mol% DSPE-PEG_{2000} reached maximum binding levels of 3.2 nmol per well, representing a binding efficiency of ~1.6%. The amount of binding for liposomes prepared with 14 mol% DSPE-PEG_{2000} with added concentrations of >1 mM were less than that observed for the formulation prepared with 2 mol% DSPE-PEG_{2000}. The amounts of lipid recovered for control Ab- and no Ab-conjugated PS liposomes with 2 mol% DSPE-PEG_{2000} at 2.0 mM lipid were 6- and 11-fold lower than the value observed for anti-VCAM-1 Ab-conjugated PS liposomes. For those liposomes prepared with 14 mol% DSPE-PEG_{2000}, the amount of lipid recovered was 9- to 20-fold higher than the indicated controls. The in vitro binding assay described above indicated that in a cell-free system, specificity could be achieved for the anti-VCAM-1 Ab conjugated liposomes, and that nonspecific binding due to the presence of PS was not substantially higher than that observed for liposomes that lack PS.

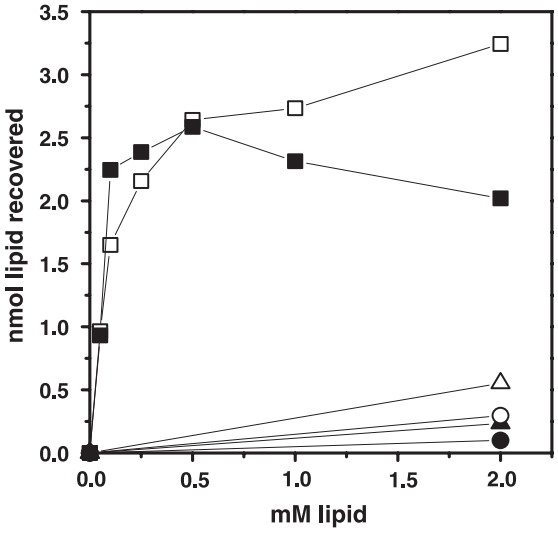

Fig. 1. Binding of anti-VCAM-1 Ab-conjugated PS liposomes (squares), irrelevant Ab-conjugated PS liposomes (triangles) and PS liposomes without Ab (circles) containing 2 mol% (open symbols) or 14 mol% (solid symbols) DSPE-PEG_{2000} to human VCAM-1 coated ELISA plates. Liposomes (100 μl) at various concentrations were added to the ELISA plates pre-blocked with 10% fetal bovine serum, and were incubated at 37 °C for 2 h. Data points represent the average of two experiments.

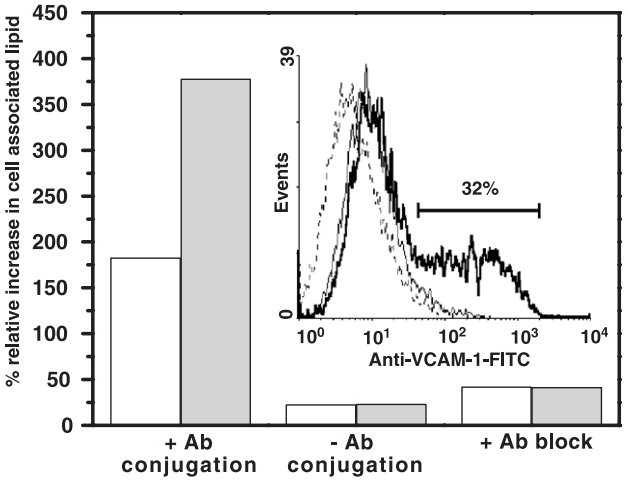

Fig. 2. Binding of PS liposomes with or without Ab conjugation containing 2 mol% (white bar) or 14 mol% (gray bar) DSPE-PEG_{2000} to HUVEC at 4 °C for 4 h. HUVEC after stimulated with IL-1α were also blocked with free anti-VCAM-1 Ab for 1 h before incubation with Ab-conjugated PS liposomes containing 2 or 14 mol% DSPE-PEG_{2000}. The percent relative increase in cell associated lipid was calculated as follows: % relative increase=$(L_{stim} - L_{unstim})/L_{unstim} \times 100\%$, where L_{stim} represents the amount of lipid per 10^6 cells for IL-1α stimulated HUVEC and L_{unstim} represents the amount of lipid per 10^6 cells for unstimulated HUVEC. Two independent experiments were done for each group with data points representing averages. Evaluation of VCAM-1 expression on HUVEC by flow cytometry post IL-1α stimulation at 37 °C for 4 h is shown as inset. Dotted line, unstimulated HUVEC stained with IgG_1-FITC; thin solid line, unstimulated HUVEC stained with anti-VCAM-1-FITC; thick solid line, stimulated HUVEC stained with anti-VCAM-1-FITC.

The binding of liposomes to VCAM-1 was also evaluated using HUVEC stimulated with 6.25 ng/ml IL-1α (Fig. 2). Flow cytometric analysis indicated that approximately 32% of the HUVEC were stained positive with an FITC-labeled anti-VCAM-1 Ab, and it was estimated that these cells have

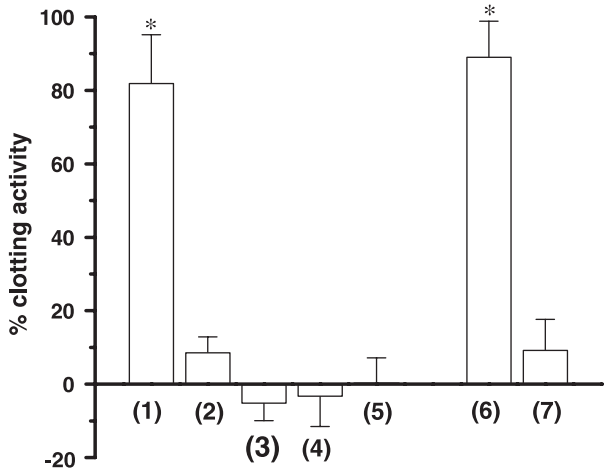

Fig. 3. Clotting activity of Ab-conjugated PS liposomes post binding. Various liposomes were allowed to bind to VCAM-1-coated ELISA tubes, and the clotting activities were then assayed. The percent clotting activity was calculated as follows: $\%=(t_{blank}-t_{test})/(t_{blank}-t_{PS})\times 100\%$, where t_{blank}, t_{PS} and t_{test} represent the clotting times for HBS, 10% PS liposomes in solution and test liposomes, respectively. The various liposomes (250 nmol in 0.5 ml HBS) tested following addition to the VCAM-1 coated ELISA tubes include: (1) PS 10%/PEG 2%/VCAM-1 Ab, (2) PS 10%/PEG 14%/VCAM-1 Ab, (3) PS 10%/PEG 2%, (4) PS 10%/PEG 14%, (5) PS 10%, all with DSPC/Chol as bulk lipids. All tubes were washed extensively prior to addition of plasma. As controls, PS 10%/PEG 2%/VCAM-1 Ab (6) and PS 10%/PEG 14%/VCAM-1 Ab (7) were added in same amounts to the plasma to demonstrate the clotting activity of these liposomes free in solution. Triplicates were done for each group, and the error bars represent S.D. * denotes that the percentage clotting activities for (1) and (6) are statistically different from the other groups ($P<0.01$), while (1) and (6) are not statistically different from each other, as analyzed with one-way ANOVA and Newman–Keuls test.

1×10^6 antigen binding sites per cell. It has been suggested that efficient targeting to cells is obtained when the target molecule is expressed in the range of 10^5–10^6 copies per cell [25]. Although not all of the cells exhibited this level of expression in this study, it can be argued that VCAM-1 is a good target candidate for the thrombogenic PS liposomes since localized thrombus formation within the tumor vasculature will not require binding to all endothelial cells.

Stimulated HUVEC were incubated with the pegylated PS liposomes with or without conjugated anti-VCAM-1 Ab. In the presence of the conjugated Ab, 4.8 and 4.2 nmol lipid were associated with 1×10^6 stimulated HUVEC for PS liposomes containing 2 and 14 mol% DSPE-PEG_{2000}, respectively. By comparing binding to HUVEC that were stimulated with IL-1α to those obtained with HUVEC that were not exposed to IL-1α (no VCAM-1 expression), the relative increases in cell-associated lipid for PS liposomes containing 2 and 14 mol% DSPE-PEG_{2000} were calculated to be 180% and 380%, respectively. The binding efficiency of the anti-VCAM-1 Ab-conjugated PS liposome to IL-1α-treated HUVEC was comparable to other studies, which targeted liposomes to cell adhesion molecules such as ICAM-1 and E-selectin [26–29]. Although the presence of 14 mol% DSPE-PEG_{2000} compromised the coupling efficiency of the Ab, this level of PEG-lipid did not substantially interfere with the binding of the Ab-conjugated PS liposomes to VCAM-1. In fact, the data shown in Fig. 2 suggest that the Ab-conjugated PS liposomes with 14 mol% DSPE-PEG_{2000} (gray bars) bound more efficiently than the Ab-conjugated PS liposomes with 2 mol% DSPE-PEG_{2000}. To demonstrate the specificity of the Ab-conjugated pegylated PS liposome for the target VCAM-1, IL-1α-treated HUVEC were pre-incubated with free anti-VCAM-1 Ab prior to the addition of liposomes. Under these conditions, binding values were reduced to those observed with liposomes which did not have a conjugated Ab.

3.3. Assessment of the clotting activity of Ab-conjugated PS liposomes post binding

In designing a thrombogenic PS liposome formulation that is targeted to the tumor vasculature for triggering tumor specific thrombosis, it is important for the PS liposomes to maintain thrombogenicity after binding. We evaluated the clotting activity of the bound Ab-conjugated PS liposomes in ELISA tubes, where the lipid concentration used was one that would result in maximum binding. As shown in Fig. 3, all PS liposomes without Ab conjugation (Fig. 3, bars 3–5), which exhibited negligible binding to the surface-bound

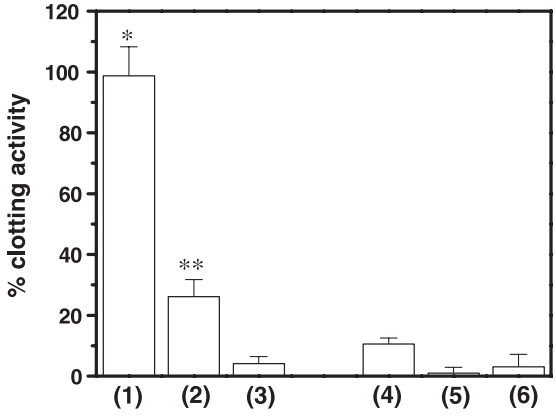

Fig. 4. Restoration of clotting activity upon the exchange of DMPE-PEG_{2000} out from the bound Ab-conjugated PS liposomes post binding. The nonexchangeable DSPE-PEG_{2000} was included as a control. The test conditions were described in Fig. 3 and the liposomes tested include: (1) PS 10%/DMPE-PEG 9%/VCAM-1 Ab+MLV incubation, (2) PS 10%/DMPE-PEG 9%/VCAM-1 Ab−MLV incubation, (3) PS 10%/DMPE-PEG10%+MLV incubation, (4) PS 10%/DSPE-PEG 14%/VCAM-1 Ab+MLV incubation, (5) PS 10%/DSPE-PEG 14%/VCAM-1 Ab−MLV incubation, (6) PS 10%/DSPE-PEG 15%+MLV incubation. All liposomes had DSPC/Chol as bulk lipids. The MLV (as egg PC/Chol) were used as a sink to capture the PEG-lipid that exchanged out of the PS liposomes, and were added in 100-fold molar excess (0.6 μmol in 0.5 ml) HBS. Triplicates were done for each liposome, with error bars representing S.D. * and ** denote that (1) and (2) are statistically different from the other groups, respectively, as analyzed with one-way ANOVA and Newman–Keuls test with $P<0.01$.

VCAM-1, provided very low clotting activities. This is due to the fact that unbound liposomes were washed from the tubes prior to initiating the coagulation reactions. The amount of lipid recovered for Ab-conjugated PS liposomes containing 2 and 14 mol% DSPE-PEG_{2000} were comparable, which were 6.6 and 5.0 nmol per tube, respectively. When these amounts of the specified liposomes were added directly to the clotting assay (i.e., liposomes were not bound to a surface), the clotting activities measured were comparable to those measured with the bound liposomes (compare bars 1 and 2 to bars 6 and 7 in Fig. 3). Importantly, only the Ab-conjugated PS liposomes containing 2 mol% DSPE-PEG_{2000} (Fig. 3, bar 1) exhibited clotting activity (80%) when bound to the ELISA tubes. Tubes with bound Ab-conjugated PS liposomes prepared with 14 mol% DSPE-PEG_{2000} (Fig. 3, bar 2) gave $<10\%$ clotting activity. These results are consistent with our previous studies, which demonstrated that elevated levels of a non-exchangeable PEG-lipid inhibits PS clotting activity [5].

3.4. Controllable surface thrombogenicity of VCAM-1 bound PS liposomes using exchangeable PEG-lipids

We have utilized the exchangeable DMPE-PEG_{2000} to demonstrate that loss of PEG from the PS liposomes is crucial to the promotion of thrombi formation. The amount of Ab conjugated to the DMPE-PEG 2000 containing PS liposomes was 20 μg Ab/μmol lipid, which was comparable to that of DSPE-PEG_{2000} containing PS liposomes. The clotting activities of the Ab-conjugated PS liposomes prepared with maximum levels of PEG-lipid incorporation (9 mol% for DMPE-PEG_{2000} and 14 mol% for DSPE-PEG_{2000}) were measured after binding and incubation with multilamellar vesicles (MLVs) to serve as a "sink" for the PEG-lipids (Fig. 4) [8]. Both anti-VCAM-1 Ab-conjugated PS liposomes containing 9 mol% DMPE-PEG_{2000} or 14 mol% DSPE-PEG_{2000} exhibited similar levels of bound lipid following an incubation with the VCAM-1 coated ELISA tubes, with 5.0 and 4.8 nmol lipid bound per tube, respectively. The level of bound liposomal lipids was also comparable under conditions where MLV were added, indicating that MLV did not displace the Ab-conjugated PS liposomes from the binding sites. The Ab-conjugated PS liposomes protected by DMPE-PEG_{2000} recovered approximately 97% clotting activity upon incubation with MLV (Fig. 4, bar 1), compared to only 25% clotting activity observed from those same liposomes but in the absence of an MLV incubation (Fig. 4, bar 2). The Ab-conjugated PS liposomes protected by the nonexchangeable DSPE-PEG_{2000} exhibited $<10\%$ clotting activity under the same conditions regardless of whether the ELISA tubes were incubated with or without MLV (Fig. 4, bars 4 and 5). This clearly demonstrates that surface bound anti-VCAM-1 Ab-conjugated PS liposomes can promote thrombus formation and that this activity can be regulated through the use of exchangeable PEG-lipids.

4. Conclusion

The results of the in vitro studies presented here provide proof-of-concept evidence that the VCAM-1 targeted thrombogenic PS liposomes maintain their surface reactivity toward the blood coagulation factors, and that initiation of coagulation activity can be controlled through the use of exchangeable PEG-lipids. The ultimate therapeutic utility of this liposomal formulation will depend on the ability to selectively localize the triggered blood coagulation response in tumor blood vessels without undesirable thrombosis in healthy tissues. Given that cancer can induce an inflammatory response with production of various cytokines, which in turn, can induce VCAM-1 expression in vascular beds other than that of the tumor, the specificity of anti-VCAM-1 Ab-conjugated PS liposomes for the tumor vascular endothelium will need to be carefully evaluated in relevant preclinical models. We are currently establishing an appropriate tumor bearing mouse model to evaluate the in vivo efficacy and safety of these thrombogenic PS liposomes. In summary, our in vitro results have demonstrated the functional requirements of a liposome-based PS formulation for the induction of tumor selective thrombosis, and have provided supportive evidence for continued development of such formulations for therapeutic applications in vivo.

Acknowledgements

We are grateful to Jean Heggie, Norma Hudon and Margaret Kliman-Depa for their assistance in blood collection, and Wieslawa Dragowska for her expertise in flow cytometry. This research project is supported by a research grant from the Canadian Institute of Health Research. G.N.C.C. is supported by a graduate fellowship from the University of British Columbia.

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