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Affinity manipulation of surface-conjugated RGD peptide to modulate binding of liposomes to activated platelets

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Abstract

Platelet adhesion, activation and fibrinogen-mediated aggregation are primary events in vascular thrombosis and occlusion. An injectable delivery system that can carry thrombolytics selectively to the sites of active platelet aggregation has immense potential in minimally invasive targeted therapy of vascular occlusion. To this end we are studying liposomes surface-modified by fibrinogen-mimetic RGD motifs that can selectively target and bind integrin GPIIb–IIIa on activated platelets. Here we report liposome surface-modification with a conformationally constrained high affinity cyclic RGD motif to modulate the GPIIb–IIIa-binding capability of the liposomes. Such affinity enhancement is important for practical in vivo applications to compete with native fibrinogen towards binding GPIIb–IIIa. The platelet-binding of RGD-modified liposomes was studied by fluorescence and scanning electron microscopy, and flow cytometry, *in vitro*. Binding of RGD-modified liposomes was also tested *in vivo* in a rat carotid injury model and analyzed *ex vivo* by fluorescence microscopy. The results from all experiments show that cyclic RGD-liposomes bind activated platelets significantly higher compared to linear RGD-liposomes. Hence, the results establish the feasibility of modulating the platelet-targeting and binding ability of vascularly targeted liposomes by manipulating the affinity of surface-modifying ligands.

Keywords: Cyclic RGD; Liposomes; Peptide modification; Platelet-targeting

1. Introduction

Site-targeted drug delivery holds significant promise in the treatment of vascular injury-associated thrombotic and occlusive events caused by cardiovascular diseases (e.g. atherosclerosis) or interventional procedures (e.g. angioplasty and stenting) [1–3]. Current strategies for site-specific delivery are focused primarily on the local administration of therapeutic agents via trans-catheter [4] or drug eluting stent techniques [5,6]. Such techniques are usually expensive [7] and may suffer from limitations of early drug washout, reduced control on drug release kinetics and recurrence of occlusive events at sub-optimal levels of drug concentration leading to dysregulated healing and late-stage thrombosis [8,9]. Nanoparticles, surface-modified with 'homing' motifs for targeting to and localizing at thrombotic sites, may provide an effective alternative for cardiovascular site-specific drug delivery [10–13]. Such bio-engineered nanocontainers present the advantage of protecting encapsulants from plasma interactions, thereby improving the payload circulation half-life and release profiles. In this respect, nanoscale unilamellar liposomes, with their biocompatibility, their relatively high drug encapsulation efficacy and their capability of cell-selective binding by virtue of surface-conjugated targeting motif, are attractive candidates.

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Considering the mechanistic criteria and the spatio-temporal patterns of cellular and molecular distribution in thrombosis, integrin GPIIb–IIIa ($\alpha_{IIb}\beta_3$,) on platelets is an attractive candidate for liposome targeting [14–16]. Ligand interactions with GPIIb–IIIa have been reported extensively, particularly the arginine-glycine-aspartate (RGD) motifs located in each of fibrinogen's two Aα chains, and the sequence, HHLGGAKQ AGDV, located within fibrinogen's γ chains [14,16]. Based upon these mechanisms, previously, platelets themselves [17] and fibrinogen coated lipid vesicles [18] have been explored as cardiovascular drug delivery vehicles. We have previously demonstrated the feasibility of a moderate affinity linear RGD peptide as the liposome surface-modification motif for targeted delivery specifically to activated platelets [19]. For practical *in vivo* applications, the liposomes need to have a sufficiently high binding ability to GPIIb–IIIa, in order to compete with native ligands (e.g. fibrinogen). The liposome binding ability depends upon the specificity and affinity of the surface-modifying ligands. For peptidic ligands, specificity and affinity have been found to be dictated by the conformational constraint of the peptide sequence at the apex of a solvent exposed loop [20]. Consequently, conformationally constrained cyclic RGD peptides have shown a higher binding affinity to receptors [21,22]. Several cyclic RGD structures having much higher specificity and affinity (compared to linear RGD structures) for GPIIb–IIIa, have been reported [23]. Following this rationale, we postulated that modification of liposome surface with GPIIb–IIIa-specific cyclic RGD motifs will result in enhancement of platelet affinity, when compared with linear RGD (lRGD) motifs. The cyclic RGD peptide, CNPRGDY(OEt)RC (terminal cysteines (C) cyclized through disulfide), has been reported to have high affinity and selectivity for GPIIb–IIIa ($\alpha_{IIb}\beta_3$,), compared with other RGD-recognizing receptors like $\alpha_V\beta_3$, $\alpha_V\beta_5$ and $\alpha_5\beta_1$ [23]. Hence this cyclic RGD peptide (cRGD) was developed by solid phase synthesis and conjugated to lipid for incorporation into liposomes, such that the peptide ligands stay displayed on the liposome surface. Fig. 1 shows a schematic model of liposomes surface-modified by fibrinogen-mimetic cRGD peptide for active platelet-selective thrombus-targeted delivery. Liposomes surface-modified with moderate affinity linear RGD and non-specific linear RGE motifs were used as comparison controls. The interaction of the surface-modified liposomes with activated platelets was studied *in vitro* by microscopy and flow cytometry and *ex vivo* by microscopy.

2. Patients and methods

2.1. Reagents and supplies

All amino acid derivatives and peptide synthesis reagents were purchased from Anaspec Inc. All lipids were purchased from Avanti Polar Lipids and NOF America Corp. For fluorescence studies 1-palmitoyl-2-[12-[(7-nitro-2-1,3-benzodiazol-4-yl)amino]dodecanoyl]-*sn*-glycero-3-phosphocholine (PDPC-NBD, emission 534 nm, green fluorescence) was purchased from Avanti Polar Lipids and, AlexaFluor-546-tagged human fibrinogen (AlexaFluor-546-Fg, emission 573 nm, red-orange fluorescence) was purchased from Invitrogen and stored at −20 °C prior to use. Cholesterol, bovine serum albumin (BSA), phosphate buffered saline (PBS) and sodium citrate were obtained from Sigma Aldrich. Mass spectrometry and microscopy supplies were used from stock at respective facilities at Case Western Reserve University.

2.2. Development of peptides and peptide–lipid conjugates

An 11-residue linear RGD-containing peptide (GSSSGRGDSPA, lRGD), a 9-residue linear precursor of cRGD peptide [CNPRGDY(OEt)RC] and an 11-residue RGE-containing negative control peptide (GSSSGRGESPA, lRGE) were synthesized using standard FMoc chemistry by solid phase peptide synthesizer (ABI433A, Applied Biosystems) [24]. For cRGD synthesis, the terminal cysteine residues of the linear precursor were cyclized by a disulfide bond using ferricyanide-mediated oxidation process [25]. All peptides were purified by HPLC and chemical purity was confirmed by mass spectrometry (MALDI-TOF). Peptide–lipid conjugates were prepared following methods described by Wu et al. [26]. For this, peptides, while still on the resin, were reacted through their N-terminal, to an *N*-hydroxysuccinimide (NHS)-activated polyethylene glycol carboxyester derivative of distearoylphosphatidylethanolamine (DSPE-PEG-COO-NHS, from NOF America Corporation) and the resultant lipid–peptide conjugates were then cleaved from the resin, purified by dialysis and characterized by mass spectrometry. For lipid–cRGD conjugate, the linear peptide precursor was conjugated to lipid first and the cyclization via disulphide bond was performed subsequently.

2.3. Platelet affinity of free peptides

Affinity of free lRGD and cRGD peptides to bind activated platelets was characterized by 'half maximal Inhibitory Concentration' (IC_{50}) value, which, in our assays, was the concentration of peptide required to inhibit fibrinogen-mediated platelet aggregation in platelet-rich plasma (PRP) by 50%, in an aggregometry (Bio/Data, PAP-4) set-up. PRP was prepared from citrated human whole blood by centrifugation at 800 rpm for 15 min at 25 °C. Aliquots (450 μl) of PRP were warmed to 37 °C for 2 min, incubated with various concentrations of lRGD and cRGD peptides in the presence of agonist adenosine di-phosphate (ADP, 10 μM) and the maximal aggregation percentage was determined while stirring for 15 min. Consequently, from the maximal aggregation percentage and comparison to aggregation without peptides, inhibition percentage was determined.

2.4. Preparation of peptide-modified liposomes

All liposomes were prepared by reverse-phase evaporation followed by extrusion through nanoporous (100 nm) Nuclepore polycarbonate membrane, as described previously for our lRGD-liposomes [19]. In all formulations, the final peptide–lipid conjugate content was kept at 1 mol%, which is lower than our previously reported lRGD-liposome formulations with 5 mol% peptide–lipid conjugate. The rationale was that if liposome surface-modification with a higher affinity peptide (e.g. cRGD) has an enhancing effect on platelet-binding, the enhancement will be more sensitive and discernible at low peptide concentrations. PDPC-NBD was incorporated at 1 mol% in the formulations as a fluorescent probe. Liposome size and stability were characterized and monitored by dynamic light scattering using a Model 90Plus Brookhaven Instruments Corp Particle Size Analyzer for 30 days from the day of liposome preparation.

2.5. Microscopy analysis of liposome binding to platelets in vitro

Monolayers of platelets were adsorbed from human platelet suspension onto collagen-III-coated glass coverslips following similar methods as described for our lRGD-liposome studies [19]. Three μl of ADP (10 μM) in 120 μl PBS was added onto the platelet-adhered coverslips to ensure sufficient activation of adhered platelets. The presence of surface-adsorbed platelets was confirmed by staining with fluorescein isothiocyanate (FITC)-tagged anti-GPIIb–IIIa monoclonal antibody (FITC-anti-CD41a mAb, from BD BioSciences) and observing with a Nikon Diaphot epifluorescence microscope. The 'activated' state of the adherent platelets was confirmed by scanning electron