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Research paper

VCAM-1 directed target-sensitive liposomes carrying CCR2 antagonists bind to activated endothelium and reduce adhesion and transmigration of monocytes

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ABSTRACT

Chemokines are critically involved in the development of chronic inflammatory-associated diseases such as atherosclerosis. We hypothesized that targeted delivery of compounds to the surface of activated endothelial cells (EC) interferes with chemokine/receptor interaction and thereby efficiently blocks inflammation. We developed PEGylated target-sensitive liposomes (TSL) encapsulating a CCR2 antagonist (Teijin compound 1) coupled with a specific peptide recognized by endothelial VCAM-1 (Vp-TSL-Tj). TSL were characterized for size (by dynamic light scattering), the amount of peptide coupled at the liposomal surface and Teijin release (by HPLC). We report that Vp-TSL-Tj binds specifically to activated EC *in vitro* and *in situ*, release the entrapped Teijin and prevent the transmigration of monocytes through activated EC. This is the first evidence that nanocarriers which transport and release chemokine inhibitors at specific pathological sites can reduce chemokine-dependent inflammatory processes.

1. Introduction

Atherosclerotic plaque formation is an inflammatory process that is associated with expression of a specific repertoire of chemokines and cell adhesion molecules by endothelial cells (EC) [1]. Both factors contribute to leukocyte recruitment to the developing atheroma. Chemokines are small, highly specialized polypeptides that, among others, function as modulators of cellular traffic through their interactions with G protein-coupled receptors at a spatially defined site [2]. Chemokines bind to glycosaminoglycans (GAGs) present on the EC membrane and guide leukocyte entry into the vessel wall. Based on the findings of the key importance of chemokines in the development of atherosclerosis, a pharmacological interference at the level of chemokines may open new avenues in the prevention and treatment of this disorder and its complications. Thus, functional manipulation of the chemokine system using chemokine antagonists (CA) or chemokine receptor antagonists (CRA) constitutes an important therapeutic option to prevent the accumulation of inflammatory immune cells that drives atherogenesis. However, systemic administration of chemokine antagonists or interventions in the function of one or more chemokines have potentially a high risk of side effects, such as impaired host defence against pathogens. Experimental and clinical testing of potential inhibitors of chemokines and their receptors for the treatment of

Abbreviations: CCR2, CC chemokine receptor 2; EC, endothelial cells; DOPE, 1, 2-dioleoyl-sn-glycero-3-phosphoethanolamine; DOPA, 1,2-dioleoyl-sn-glycero-3-phosphatidic acid; PEG-DSPE, 1,2-distearoyl-sn-glycero-3-phosphoetanolamine-N-[poly(ethylene glycol)-2000]; Mal-PEG-DSPE, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide (polyethylene glycol)-2000]; rh VCAM-1, recombinant human vascular cell adhesion molecule-1 (VCAM-1) Fc chimera; TNF-α, tumour necrosis factor-α; TSL, target sensitive liposomes; Vp-TSL, VCAM-1 recognizing peptide coupled to target sensitive liposomes; Vp-TSL-Tj, VCAM-1 recognizing peptide coupled to target sensitive liposomes encapsulating Teijin; TSL-Tj, target sensitive liposomes encapsulating Teijin without VCAM-1 recognizing peptide coupled; Vp-CL, VCAM-1 recognizing peptide coupled to conventional liposomes; Vp-CL-Tj, VCAM-1 recognizing peptide coupled to conventional liposomes encapsulating Teijin.

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inflammatory diseases revealed detrimental immunological effects [3]. Therefore, there is a need for the development of new and innovative therapeutic approaches or strategies to manipulate selectively specific chemokine(s) function in a particular pathophysiological context with few or no adverse effects on the immune system. We hypothesized that the local targeted delivery of CA or CRA at specific sites utilizing appropriately designed carriers may constitute a novel and improved strategy to hold back or interrupt a confined inflammatory process.

Despite progress in the field of targeted carrier technologies, different approaches require a design of carriers with specific properties with respect to tissue targeting. To interfere with the chemokine axis within the local microenvironment at the EC surface, the carrier system employed should have the potential to (i) efficiently entrap drugs (CA or CRA), (ii) target sequences of specific receptors on the activated endothelial surface, and (iii) have a specific release mechanism for the drug cargo at the site of adhesion prior to cellular uptake. In this respect, liposomes appear most promising thanked to their tuneable properties in drug loading and release characteristics as well as their manifold coupling technologies for specific homing devices.

Vascular targeting of liposomes was previously applied in inflammatory-related diseases, usually combined with targeting EC adhesion receptors [4–8]. The shortcoming of this approach is that the liposomes were predominantly taken up by EC, an effect entirely inadequate when inhibition of chemokine/chemokine receptor interaction at the cell's surface is sought after [9–13]. To circumvent this problem, we revisited an old principle of target-triggered release of drugs from target-sensitive liposomes (TSL) initially described by Huang and colleagues [14–17]. These carriers have a specific phospholipid bilayer composition, which upon target recognition and binding exhibit destabilization that is followed immediately by cargo release. Taking advantage of these properties, we hypothesized that these type of liposomes modified accurately for our aim, could be employed successfully in the chemokine targeting approaches. Here we demonstrate that the principle of TSL combined with PEGylation leads to particles that characteristically have long storage and circulation time while maintaining the trigger-release properties. We provide evidence that PEGylated TSL coupled with a recognizing peptide directed against vascular cell adhesion molecule-1 (VCAM-1) and encapsulating an antagonist to CCR2 namely Teijin compound 1 (Vp-TSL-Tj) bind specifically to activated EC in culture and inhibit the ensuing adhesion and transendothelial migration of monocytes. In ApoE-deficient mice, Vp-TSL target specifically aortic plaque endothelial VCAM-1 and Teijin compound 1 reduces the mouse monocyte/macrophage cell line (RAW 264.7) adhesion/infiltration into the aorta. This is a novel and promising approach to target initial inflammatory processes as common critical events in numerous major diseases, including atherosclerosis.

2. Materials and methods

2.1. Reagents

Reagents were obtained from following sources: 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-sn-glycero-3-phosphatidic acid (DOPA), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide (polyethylene glycol)-2000] (Mal-PEG-DSPE), 1,2-distearoyl-sn-glycero-3-phosphoetanolamine-N-[poly(ethylene glycol)-2000] (PEG-DSPE), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt) (Rhodamine-PE) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) from Avanti Polar Lipids (Alabaster, AL/USA), mouse anti-human VCAM-1, irrelevant mouse IgG1 and recombinant human (rh) VCAM-1 Fc chimera from R&D Systems and Teijin compound 1 from Tocris Bioscience (Biomedica Medizinprodukte Romania SRL), Dulbecco's modified Eagle's medium (DMEM), foetal calf serum (FCS) from Gibco BRL (Antisel Selidis Ro SRL), cell culture plates from Corning (New York, NY/USA), black 96-well plates from Nalge NUNC International (Rochester, USA), peptide with high affinity for VCAM-1 (VHPKQHRGGSKGC) was synthesized by GeneCust (Dudelange, Luxembourg), Amicon centrifugal filter columns with a cutoff of 100 kDa from Millipore (Merck Romania SRL), transmigration chambers from Costar Europe Ltd. (Badhoevedorp, The Netherlands), tris (2-carboxyethyl) phosphine (TCEP), phospholipid determination, bicinchoninic acid protein assay kit, as well as all other chemicals were from Sigma–Aldrich (Redox Lab Supplies Com S.R.L.).

2.2. Preparation of VCAM-1 directed, PEG-stabilized target-sensitive liposomes (Vp-TSL)

2.2.1. Preparation of TSL

PEG-stabilized TSL were prepared by a mixture of DOPE, DOPA and a functionalized phospholipid anchor (Mal-PEG-DSPE) used to covalently couple the peptide with affinity for VCAM-1 to the liposome surface. As control, non-sensitive, conventional liposomes (CL) were prepared using POPC and cholesterol instead of DOPE and DOPA (POPC:Chol:Mal-PEG-DSPE:DSPE-PEG at a molar ratio 60:35:2:3 mol%). Unilamellar liposomes with different composition and lipid ratio (as indicated in Supplementary Table 1) were obtained by extruding multilamellar vesicles through polycarbonate membranes as previously described [18]. Briefly, the mixture of phospholipids in chloroform was dried in a rotary evaporator under reduced pressure. The resulted lipid film was hydrated with a solution containing the substance to be encapsulated into liposomes, i.e. phosphate buffered saline (PBS), calcein or CCR2 antagonist, so as to reach the final lipid concentration of 10 mM. The resulting multilamellar vesicles, obtained by thorough mixing the aqueous solutions of lipids, were extruded 10 times through a 100 nm polycarbonate membrane using a Mini-Extruder (Avanti Polar Lipids, Alabaster, AL/USA).

To fluorescently label the phospholipid bilayers of liposomes, 1 mol% Rhodamine-PE was added as an ethanol solution subsequent to liposomes preparation.

For content release studies, the liposomes were prepared by hydration of the phospholipid mixture with a solution of 100 mM calcein or CCR2 antagonist (Teijin compound 1). Removal of non-encapsulated calcein or CCR2 antagonist was accomplished by gel filtration on a Sephadex G-50 column using as an eluting solution the following buffer: 10 mM Na_2HPO_4, 10 mM NaH_2PO_4, 2 mM EDTA and 30 mM NaCl, pH: 6.7 (coupling buffer).

2.2.2. Coupling of VCAM-1 recognizing peptide to the surface of TSL

A VCAM-1 binding peptide with the amino acids sequence VHPKQHRGGSKGC (described by Kelly et al. [19]) has been coupled to the maleimide group at the distal end of PEG by sulfhydryl-maleimide as previously described [20]. Before coupling, the peptide was reduced with tris (2-carboxyethyl) phosphine (TCEP) (2 h at room temperature) that breaks the disulphide bond between peptides and activates the sulfhydryl group. The excess TCEP was removed by dialysis (using cellulose ester membrane with a cut-off of 500–1000 Da) against coupling buffer, overnight at 4 °C. Then, an aliquot of the solution of the VCAM-1 recognizing peptide in coupling buffer was added at a concentration of 10 μg peptide/μmol total lipid to maleimide functionalized PEG-DSPE containing liposomes and incubated overnight at room temperature under shaking. To saturate non-reacted maleimide, 1 mM L-cystein was added for 30 min at room temperature and