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Design of cyclic RKKH peptide-conjugated PEG liposomes targeting the integrin $\alpha_2\beta_1$ receptor

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ABSTRACT

Peptide conjugation to the surface of stealth liposomes has been studied for liposomal drug targeting to cells expressing specific receptors to provide a site-specific drug delivery. This study investigated the potential of peptide-conjugated liposomes for targeting cells expressing the human integrin $\alpha_2\beta_1$ receptor. A 12 amino acid head-to-tail cyclic peptide derived from the Jararhagin protein containing the Arg-Lys-Lys-His (RKKH)-specific binding site was conjugated to the distal ends of poly(ethylene glycol) (PEG) chains on PEGylated liposomes. Epithelial cells expressing the receptor showed increased cellular association and uptake of peptide-conjugated liposomes at 4 °C, compared to liposomes conjugated with a non-specific peptide. The interaction between cells and peptide-conjugated liposomes was significantly increased at 37 °C suggesting that a possible uptake mechanism might be energy-dependent endocytosis. In keratinocyte cell cultures, the ligand-conjugated liposomes loaded with the vitamin D_3 analogue calcipotriol induced transcription of the gene encoding the antimicrobial peptide cathelicidin, which is activated through the vitamin D_3 receptor upon binding of vitamin D_3 analogues. This suggests that the liposomes are internalized and that calcipotriol is delivered intracellularly and released in an active form. In conclusion, the 12 amino acid head-to-tail cyclic RKKH peptide seems promising for targeting of liposomes to the integrin $\alpha_2\beta_1$ receptor.

1. Introduction

Integrin receptors are transmembrane glycoproteins that mediate cell–cell interactions and anchor cells to the extracellular matrix. In man, 24 different integrin receptor subtypes have been identified, which have been further divided into four subgroups based on their ligand recognition pattern. Different integrin receptors play important and distinct roles for the progression of several diseases, e.g. psoriasis, and consequently, the expression of specific subtypes of integrin receptors is regulated with respect to cell type, differentiation status and diseased state (Call-Culbreath and Zutter, 2008). The tissue-specific expression of specific integrin receptors can be exploited for the site-specific delivery of drugs (Dunehoo et al., 2006). An example is the human integrin $\alpha_2\beta_1$ receptor that belongs to the collagen-binding integrin subfamily, and it is the only collagen I integrin receptor expressed on keratinocytes in the basal layer of the skin (Parks, 2007). A drug delivery system specifically binding to the integrin $\alpha_2\beta_1$ receptor is therefore an interesting vehicle for targeting of drugs to the lower epidermis.

The interaction between the integrin $\alpha_2\beta_1$ receptor and collagen I is mediated through the extracellular insert domain of the integrin α_2 subunit (α2I-domain) (Tuckwell et al., 1995), which can be inhibited by the Jararhagin protein isolated from the venom of *Bothrops Jararaca* (DeLuca et al., 1995; Ivaska et al., 1999). A small nine amino acid peptide has been derived from Jararhagin (Cys-Thr-Arg-Lys-Lys-His-Asp-Asn-Ala-Gln-Cys, C^{241}TRKKHDNAQ^{249}C) with an internal disulfide bond, also denoted (RKK9, Fig. 1). It

Abbreviations: α2 I-domain, extracellular insert domain of the integrin α_2 subunit; CAMP, cathelicidin antimicrobial peptide; DiD, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate; DMEM, Dulbecco's modified Eagle's medium; DSPC, 1,2-distearoyl-sn-glycero-3-phosphatidylcholine; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HEKa, adult human epidermal keratinocytes; HPLC, high pressure liquid chromatography; HRP, horse radish peroxidase; LMVs, large multilamellar vesicles; Mal-PEG_{2000}-DSPE, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide-poly(ethylene glycol)2000]; PDI, polydispersity index; PEG, poly(ethylene glycol); PEG_{2000}-DSPE, (carbonyl-methoxypoly(ethylene glycol)-2000)-1,2-distearoyl-sn-glycero-3-phosphoethanol-amine; SATA, N-succinimidyl-S-acetyl thiolacetate; SUVs, small unilamellar vesicles; UPLC, ultra performance liquid chromatography.

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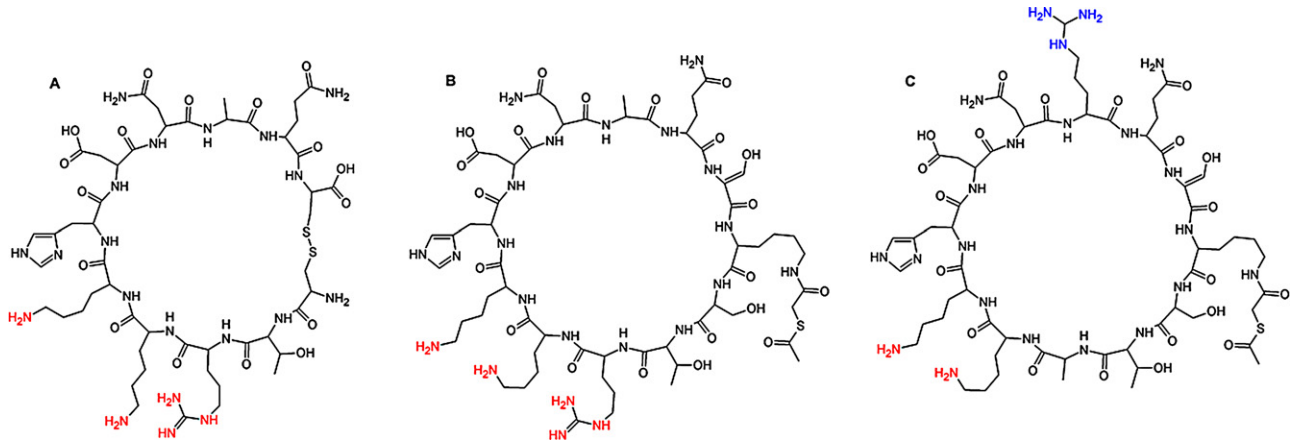

Fig. 1. Structure of peptides derived from the Jararhagin protein. (A) Cyclic CTRKKHDNAQC C1-C11 peptide (RKK9). (B) Head-to tail cyclized H-KHDNAQS-(SATA)KSTRK-OH peptide (RKK12). (C) Head-to tail cyclized H-KHDNRQS-(SATA)KSTAK-OH control peptide (AKK12). The binding domains are marked with red. For the AKK12 control peptide, an arginine was substituted for an alanine in the binding site. The opposite substitution was performed outside the binding domain to preserve the overall amino acid composition for both peptides (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

binds to the α2I-domain and inhibits the interaction with collagen I (Ivaska et al., 1999). The Arg-Lys-Lys-His (RKKH) part of the peptide is important for the interaction between the α2I-domain and the RKK9 peptide (Ivaska et al., 1999; Lambert et al., 2008). The specific ring structure of the peptide mimics the loop in Jararhagin, and it is essential for the α2I-domain recognition of collagen I (Ivaska et al., 1999). Isothermal titration calorimetry and nuclear magnetic resonance examinations of the interaction between RKK9 and the α2I-domain confirm that the peptide binds directly to the α2I-domain in close proximity to the metal ion-dependent adhesion site without introducing any major conformational changes in the α2I-domain (Lambert et al., 2008). The peptide is suggested to lock the α2I-domain in a closed conformation, and thereby inhibits the binding to collagen I (Lambert et al., 2008).

Liposomes are commonly applied drug delivery vehicles used to improve drug stability and to increase the therapeutic index of drugs by targeting of the liposomes to specific tissues (Musacchio and Torchilin, 2011). One way to increase liposomal drug targeting is by conjugation of receptor-specific ligands to the outer surface of liposomes that can facilitate a selective targeting to cells expressing specific receptors and increase the cellular uptake (Musacchio and Torchilin, 2011). Ligand targeting to integrin receptors has been studied intensively, e.g. applying the small cyclic peptide with the amino acid sequence Arg-Gly-Asp (RGD) for targeting of drug-loaded liposomes to integrin receptors from the RGD-binding subfamily including the integrin $\alpha_v\beta_3$ and $\alpha_v\beta_5$ receptors (Schiffelers et al., 2003; Xiong et al., 2005; Schiffelers and Storm, 2008). However, the improved therapeutic efficacy of integrin-targeted liposomes is not believed to be a result of an increased accumulation of liposomes at the target site, but is rather a consequence of enhanced cellular uptake of drug via binding of liposomes to internalizing surface integrin receptors (Schiffelers et al., 2003; Xiong et al., 2005).

Calcipotriol is a widely used drug for topical treatment of psoriasis (Su and Fang, 2008). It inhibits proliferation and stimulates differentiation of keratinocytes in the lower epidermis expressing the integrin $\alpha_2\beta_1$ receptor (Jensen et al., 1998; Watt, 2002). Calcipotriol is a vitamin D_3 analogue that binds to the vitamin D_3 receptor and activates the transcription of the gene encoding the cathelicidin antimicrobial peptide (*CAMP*) (Bury et al., 2001; Weber et al., 2005). In healthy human skin, low levels of cathelicidin are constitutively expressed by keratinocytes in the basal layer, but during psoriasis the expression of cathelicidin is upregulated, and topical treatment of psoriatic plaques with calcipotriol further arguments the transcription of cathelicidin (Peric et al., 2009).

This study aimed to examine the potential of ligand-conjugated liposomes intended for topical delivery of the antipsoriatic vitamin D analogue calcipotriol for targeting to kerationocytes expressing the human integrin $\alpha_2\beta_1$ receptor during psoriasis. A novel 12 amino acid peptide ligand derived from Jararhagin including the RKKH-binding site was coupled to the distal ends of poly(ethylene glycol) (PEG) chains on PEGylated liposomes for liposomal targeting to the integrin $\alpha_2\beta_1$ receptor. To evaluate the effect of the new peptide, the liposomes were labeled with fluorescence, and the cellular association and internalization were evaluated. In addition, calcipotriol was intercalated in the lipid bilayer of the liposomes (Knudsen et al., 2011). The biological effect of peptide-conjugated liposomes loaded with calcipotriol was evaluated.

2. Materials and methods

2.1. Materials

1,2-Distearoyl-sn-glycero-3-phosphatidylcholine (DSPC) (≥99% purity) and N-[carbonyl-methoxypoly(ethylene glycol)-2000]-1,2-distearoyl-sn-glycero-3-phosphoethanol-amine (PEG_{2000}-DSPE), sodium salt (≥98% purity), were obtained from Lipoid GmbH (Ludwigshafen, Germany). Sodium cholate (≥99% purity) was provided by Acros Organics (Geel, Belgium). 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide-poly(ethylene glycol)2000] (Mal-PEG_{2000}-DSPE), ammonium salt, was purchased from Avanti Polar Lipids (Alabaster, AL, US). Calcipotriol monohydrate (≥94% purity, Mw = 430.6 Da, log *P* = 4.9) was obtained from LEO Pharma A/S (Ballerup, Denmark). 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate (DiD) was provided by Molecular Probes (Eugene, OR, USA). All other general chemicals and reagents were obtained commercially at analytical grade.

2.2. Peptide synthesis

The head-to-tail cyclized peptides were synthesized with an N-succinimidyl-S-acetyl thiolacetate (SATA) group by JPT Peptide Technologies GmbH (Berlin, Germany) according to the manufacturer's regular procedures using solid phase synthesis with Fmoc-protected amino acids. The head-to-tail cyclized targeting peptide and the control peptide had the sequences H-KHDNAQS-(SATA)KSTRK-OH (denoted RKK12) and H-KHDNRQS-(SATA)KSTAK-OH (denoted AKK12), respectively (Fig. 1). Both