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Endoglin based *in vivo* near-infrared fluorescence imaging of tumor models in mice using activatable liposomes

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ABSTRACT

Background: Endoglin (CD105) is overexpressed on tumor cells and tumor vasculatures, making it a potential target for diagnostic imaging and therapy of different neoplasms. Therefore, studies on nanocarrier systems designed for endoglin-directed diagnostic and drug delivery purposes would expose the feasibility of targeting endoglin with therapeutics.

Methods: Liposomes carrying high concentrations of a near-infrared fluorescent dye in the aqueous interior were prepared by the lipid film hydration and extrusion procedure, then conjugated to single chain antibody fragments either selective for murine endoglin (termed mEnd-IL) or directed towards human endoglin (termed hEnd-IL). A combination of Dynamic Light Scattering, electron microscopy, cell binding and uptake assays, confocal microscopy and *in vivo* fluorescence imaging of mice bearing xenografted human breast cancer and human fibrosarcoma models were implemented to elucidate the potentials of the liposomes.

Results: The mEnd-IL and hEnd-IL were highly selective for the respective murine- and human endoglin expressing cells *in vitro* and *in vivo*. Hence, the hEnd-IL bound distinctly to the tumor cells and enabled suitable fluorescence imaging of the tumors, whereas the mEnd-IL bound the tumor vasculature, but also to the liver, kidney and lung vasculature of mice.

Conclusions: The work highlights key differences between targeting vascular (murine) and neoplastic (human) endoglin in animal studies, and suggests that the hEnd-IL can serve as a delivery system that targets human endoglin overexpressed in pathological conditions.

General significance: The endoglin-targeting liposomes presented herewith represent strategic tools for the future implementation of endoglin-directed neoplastic and anti-angiogenic therapies.

1. Introduction

The heterogeneity of tumors and the poor therapeutic outcome seen between patients with the same tumor entity demands constant search for suitable approaches to target tumors for drug delivery purposes. Generally, the growth and survival of tumors is highly dependent on vascular supply. Hence, the tumor vessels which are derived from normal tissue vasculatures acquire several properties as adaptation to the demands of the tumors. The endothelial cells of tumor vessels show a 20–2000 fold higher turnover than those of normal tissues [1]. Therefore, several molecules overexpressed or involved in tumor angiogenesis, such as the vascular endothelial growth factor receptor (VEGFR) [2], integrins [3,4], endothelial specific molecule (ESM)-1 [5], endosialin [6] and endoglin [7] are potential targets for anti-angiogenic therapy, and could serve as targets for drug delivery *via* nanocarriers. Endoglin (also known as CD105) for example, is a type I homodimeric transmembrane glycoprotein which is expressed at low levels on the vasculature of normal tissues, but overexpressed on the vessels of many neoplasia, implicating a potential role as target for diagnosis and anti-angiogenic therapy [7–10]. As a component of the transforming growth factor (TGF)-β receptor complex [11], it plays vital roles in the neovascularization of tumors and other diseases [9,12]. Thus, the benefits of targeting endoglin could be enormous, regarding its impact in different pathological conditions, including cancer growth, preeclampsia and hereditary hemorrhagic telangiectasia [13].

In the underlying work, we therefore sought to elucidate the

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feasibility of targeting endoglin with liposomal nanocarriers, and to pinpoint organs and tissues in which endoglin-based targeting of tumors for diagnostic and drug delivery purposes is possible. Liposomes are nanocarriers with excellent biocompatibility and high drug payload properties [14] which are approved for clinical delivery of therapeutic drugs in cancers and other disorders [15]. Furthermore, liposomes are quite flexible and can be modified for use in studies on disease pathogenesis, drug delivery and therapeutic efficacy, or encapsulated with contrast agents for diagnostic and therapy monitoring purposes. Hence, we designed endoglin-targeting liposomes suitable for the validation of endoglin as potential tumor marker. To be able to monitor the liposomes in preclinical animal studies, the liposomes were encapsulated with high concentrations of the near-infrared fluorescent (NIRF) dye, DY-676-COOH, which undergoes intrinsic quenching at high concentrations. Thus, the intact liposomes were NIRF-quenched, making NIR fluorescence detection only possible after degradation of the liposomes within target cells, which leads to the release and activation of the dye. Besides the quenched NIRF DY676-COOH, the liposomes were labeled with a non-quenched green fluorescent phospholipid embedded in their lipid bilayer. This enabled tracking of intact liposomes in cells/tumors before their degradation and activation of the NIRF dye. Previous reports demonstrated that the quenched, non-targeted liposome termed LipQ is taken up predominantly by phagocytosis [16], but when coupled with targeting ligands, undergo lesser phagocytic uptake, but rather a reliable target-selective uptake both *in vitro* and *in vivo* in tumor models [17,18]. Hence, we envisioned that the fate of the quenched liposomes when targeted with ligands such as those specific for endoglin, should provide reliable evidence on the targetability of endoglin for *in vivo* delivery purposes. Therefore, we investigated the fate of the encapsulated DY-676-COOH after liposomal conjugation to single chain antibody fragments directed either towards murine endoglin (termed mEnd-IL) in order to detect the vascular endoglin derived from host mice, or towards human endoglin (termed hEnd-IL), so as to detect neoplastic endoglin of human xenografted tumor models in mice. A demarcation between targets of the two antibody fragments is feasible, because of the low amino acid sequence homology seen between human and murine endoglin, hence negligible antibody cross-reactivity [19]. Accordingly, the mEnd-IL and hEnd-IL were implemented to validate the selectivity of targeting neoplastic (human) and vascular (murine) endoglin in the *in vivo* setup in mice models.

The results demonstrate that mEnd-IL and hEnd-IL distinctly detect murine endoglin and human endoglin expressing cells respectively. This was evident *in vitro* and also *in vivo* in human xenografted breast and fibrosarcoma tumor models in mice. Whereas, the hEnd-IL enabled macroscopic optical imaging of the tumors with high fluorescence intensities hence high tumor to background ratios, the mEnd-IL accumulated in the endothelia of the liver, kidneys and lungs of mice, and revealed lower tumor signals. Confocal microscopy of the animal tissues and tumors pinpointed the mEnd-IL based fluorescence in the endothelia of the tumors and the afore-mentioned organs, whereas hEnd-IL accumulated predominantly in the tumor cells. Taken together, the mEnd-IL and hEnd-IL reported herein could serve as tools for the future implementation of endoglin directed neoplastic and anti-angiogenic therapies.

2. Materials and methods

2.1. Phospholipids and other materials for liposome preparation

Except otherwise indicated, all the phospholipids needed for liposome preparation were acquired from Lipoid GmbH (Germany) and Avanti Polar Lipids (USA). These included egg phosphatidylcholine (EPC), cholesterol (chol), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000] (ammonium salt) (mPEG2000-DSPE), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-3400] (ammonium salt) (MalPEG3400-DSPE) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (ammonium salt) (NBD-DOPE). Tris(hydroxymethyl)-aminomethane (Tris) and 4-(1,1,3,3-Tetramethylbutyl)phenyl-polyethylene glycol (Triton-×100) were purchased from Sigma (Taufkirchen, Germany), whereas the near infrared fluorescent dye DY-676-COOH was from DYOMICs GmbH (Jena, Germany).

2.2. Preparation and physicochemical characterization of liposomes

Quenched liposomes were prepared by the film hydration method whereby a lipid film of the composition EPC:Chol:mPEG2000-DSPE at a molar ratio of 6.5:3:0.5 and 0.3 mol% of the lipophilic marker NBD-DOPE were hydrated with a 4.3 mM concentration of the near-infrared fluorescent (NIRF) dye, DY-676-COOH (excitation/emission: 674 nm/699 nm) dissolved in 10 mM Tris pH 7.4. Elaborate details on the preparation and characterization of the fluorescence quenched and hence activatable liposomes were reported earlier [16–18]. The high concentrations of the NIRF dye, DY-676-COOH encapsulated in the aqueous interior of the liposomes grants fluorescence quenching, whereas the green fluorescent phospholipid NBD-DOPE (excitation/emission: 480 nm/530 nm) embedded in the lipid bilayer enables detection of intact liposomes in cells prior to release and activation of the NIRF dye.

2.3. Preparation of antibody fragments

Plasmid DNA encoding the single chain antibody fragments (scFv) acquired by phage display and reported in detail previously as scFv-mE12 (specific for murine endoglin) [20] or as scFv-A5 (for human endoglin) [21] were propagated into human embryonal kidney (HEK293T) cells using Lipofectamine™ 2000 (Invitrogen). Stable clones were selected by treatment with 500 μg/ml Zeocin (Invitrogen), expanded in RPMI medium containing 5% fetal calf serum then further cultured in Opti-MEM® medium to enable expression of the secreted proteins. The secreted proteins were then purified from the culture supernatant by immobilized metal affinity chromatography (IMAC) as reported previously [22]. The purity of the single chain antibody fragments before and after conjugation to micelles was validated by polyacrylamide gel electrophoresis.

2.4. Conjugation of antibody fragments to preformed quenched liposomes

The quenched liposomes were post-inserted with murine or human endoglin single chain variable antibody fragments (scFv)-conjugated micelles ($MalPEG_{3400}$-scFv) at 50 °C for 60 min as described earlier [18]. The number of scFv molecules inserted in the liposomes was estimated based on literature [23,24] and micellar lipid titration protocols (Supplementary data S1). The resulting endoglin targeting, quenched liposomes were termed mEnd-IL (murine) and hEnd-IL (human), respectively. Murine and human endoglin share very low amino acid sequence homology, hence no antibody cross reactivity is expected. To maintain similar properties between the targeted and control liposomes, the control LipQ was got by fusing LipQ* with empty micelles generated after cysteine reduction of $MalPEG_{3400}$-DSPE. The dye content, lipid concentration, size, zeta potential and morphology of the liposomal vesicles were validated by physicochemical methods, dynamic light scattering and electron microscopy as described in detail elsewhere [18].

2.5. Validation of the liposomal quenching and activation in vitro

The fluorescence-quenching and activation properties of the liposomal formulations were validated *in vitro*, by measuring the absorption (Ultrospec 4000 photometer) and fluorescence emission (Jasco FP6200 spectrofluorometer) before and after triggering liposomal membrane damage by freezing at −80 °C. For each assay, 100 nmol (final lipids) in