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Targeting of immunoliposomes to endothelial cells using a single-chain Fv fragment directed against human endoglin (CD105)

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Abstract

We generated immunoliposomes targeting proliferating endothelial cells by chemically coupling a single-chain Fv fragment (scFv A5) directed against human endoglin to the liposomal surface. For this purpose, we introduced an additional cysteine residue at the C-terminus of the scFv fragment. This scFv′ fragment was expressed in soluble form in bacteria and allowed for a site-directed coupling to sulfhydryl-reactive lipids incorporated into the lipid bilayer. The immunoliposomes (ILA5) showed rapid and strong binding to human endoglin-expressing endothelial cells (HUVEC, HDMEC), while no binding was observed with various endoglin-negative cell lines and blood lymphocytes. In vitro, ILA5 were stable for several hours in serum- or plasma-containing medium. Incubation of endothelial cells with ILA5 at 37 °C led to increased binding and internalisation of the liposomes as evidenced by a perinuclear accumulation. In vitro, doxorubicin-loaded ILA5 showed an increased cytotoxicity towards endothelial cells compared to untargeted liposomes and free doxorubicin. Since the vasculature of tumours is easily accessible to drug carrier systems, the described endothelial cell-specific immunoliposomes may be useful for the development of efficacious and safe vascular targeting agents in cancer therapy.

Keywords: Immunoliposome; Single-chain Fv; Vascular targeting; Endothelial cell; Doxorubicin

1. Introduction

Liposomes are versatile carrier systems for the delivery of cytotoxic drugs in tumour therapy [1]. Liposomes have the advantage to protect the encapsulated drug from degradation and elimination and to increase its serum residence time. The incorporation of tumour-specific ligands into the liposome surface allows active targeting to tumour cells [2]. However, active targeting of tumour cells requires passive extravasation of the liposomes from the circulation in order to reach the tumour cells [3]. Furthermore, recent studies have shown that binding of targeted liposomes to tumour cells is not sufficient to elicit a strong anti-tumour effect but requires internalisation of the liposomes upon binding to the cell surface [4,5].

Novel approaches aim at targeting the tumour vasculature rather than the tumour cells itself [6–8]. These vascular targeting agents (VTAs) bypass the need for extravasation from the bloodstream as endothelial cells are directly accessible to circulating VTAs. Furthermore, the destruction of a few endothelial cells affects a large number of tumour cells relying on their supply. Since tumour-endothelial target structures are expressed in all solid tumours, such VTAs are broadly applicable.

Antibodies are versatile ligands for the generation of vascular targeting agents and various antibodies have been identified which recognise structures associated with angiogenesis and neovascularisation [9]. Immunoliposomes displaying scFv fragments recognising the ED-B isoform of fibronectin expressed in the stroma and on endothelial cells of the tumour vasculature already demonstrated targeting to tumours and the induction of an anti-tumour response [10].

Endoglin (CD105), which is an accessory protein of the transforming growth factor β receptor complex, has become

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an attractive molecule for the targeting of the tumour vasculature [11]. Up-regulation of endoglin on proliferating endothelial cells is associated with tumour neovascularisation [12,13]. Elevated levels of endoglin were detected in the vasculature of different tumours, e.g. prostate cancer, breast cancer, and melanoma [14–16]. Furthermore, endoglin expression was described to be a superior indicator of intratumoural microvascular density (IMVD) compared to other markers such as CD34 [13,17–19]. The application of anti-endoglin immuno-conjugates already demonstrated that targeting of cytotoxic agents (toxins, radionuclides) to endoglin can lead to a long-lasting anti-tumour response [20–22].

We have recently isolated anti-endoglin scFvs from synthetic antibody phage libraries [23,24]. These scFv molecules showed specific binding to human endothelial cells. In the present study we used one of these anti-endoglin scFv (scFv A5) to produce immunoliposomes targeting endoglin-expressing cells. For this purpose, we engineered scFv molecules displaying an additional C-terminal cysteine residue for site-specific coupling to maleimide-containing liposomes. We could show that these immunoliposomes exhibit specific and efficient binding to proliferating endothelial cells and improved cytotoxicity in vitro.

2. Materials and methods

2.1. Materials

Lipids were purchased from Avanti (USA) or Nektar (USA). Anti-His-tag antibodies were purchased from Santa Cruz (USA). HUVECs and HDMECs were obtained from Promocell (Germany) and maintained in EGM-2 medium. Fluorescein-maleimide was purchased from Pierce. Bafilomycin A1 was purchased from Sigma (Germany) and carboxyfluorescein (CF) from Fluka (Germany).

2.2. Cloning of scFv-HC and scFv-CH

ScFv-HC were generated by PCR amplification of scFv genes cloned into vector pAB1 [25] with primers LMB3 (5′-CAG GAA ACA GCT ATG ACC-3′) and HisCysForNot (5′-TAG TGC GGC CGC TTA GCA TGC TCC GCC ATG GTG ATG GTG ATG ATG CGC ACG TTT GAT TTC CAG TTT GGT-3′) and cloning of the resulting product as *Sfi*I and *Not*I fragment into pAB1. ScFv-CH was cloned accordingly with primers LMB3 and CysHisForNot (5′-TAG TGC GGC GCG TTA ATG GTG ATG GTG ATG ATG TGC TCC GCA ACC CGC ACG TTT GAT TTC CAG TTT GGT-3′).

2.3. ScFv′ expression and purification

ScFv′ molecules were purified by immobilised metal-affinity chromatography [25]. One to four litres of 2 × TY, 100 μg/ml ampicillin, 0.1% glucose were inoculated with 10 ml/l of an overnight culture of recombinant antibody-expressing bacteria and grown at 37 °C to an OD_{600} of 0.8. Protein expression was induced by adding 1 mM IPTG and cells were grown for an additional 3 h at 23 °C. Cells were harvested by centrifugation and resuspended in 30 mM Tris–HCl pH 8, 1 mM EDTA, 20% sucrose. After 15 min, $MgSO_4$ was added to a final concentration of 5 mM and cells were centrifuged at 8000 rpm for 15 min. Supernatant was dialysed against IMAC buffer (50 mM sodium phosphate buffer pH 7.5, 500 mM NaCl) containing 20 mM imidazole (IMAC loading buffer) and loaded onto a Ni-NTA column (Qiagen) equilibrated with loading buffer. After washing with IMAC buffer containing 35 mM imidazole (wash buffer), scFv were eluted with IMAC buffer containing 100 mM imidazole (elution buffer). Peak fractions were dialysed overnight against PBS. Proteins were analysed by SDS-PAGE under reducing or non-reducing conditions and stained either with Coomassie brilliant blue or immunoblotted with an anti-His-tag antibody.

2.4. Fluorescein-coupling to scFvs′ fragments

ScFv′ fragments (35 μg) were reduced by incubation with 10 mM cysteamine in PBS, 5 mM EDTA for 1 h at 30 °C. Cysteamine was removed by gel filtration on a Sephadex G25 column (Pharmacia). Fluorescein-maleimide was added at a 25 M excess and incubated by rotating overnight at 4 °C. Labelled proteins were separated from free fluorescein-maleimide by gel filtration on a Sephadex G25 column. Protein fractions were analysed for fluorescence using a victor^2 photometer (Wallac).

2.5. Preparation of liposomes

Liposomes consisting of neutral phospholipids, cholesterol, and functionalised coupling lipid (MPB-PE, MCC-PE) at a molar ratio of 6:3:1 were produced from lipid films hydrated with PBS. The final concentration was 10 μmol lipid/ml buffer. The hydration was performed in a water bath at 34 °C for 30 min. The resulting multilamellar vesicles were extruded (LiposoFast Extruder) 21 times through a polycarbonate filter (Armatis) with a pore size of 50 nm. The liposome size was determined by dynamic light scattering using a Malvern Autosizer. For fluorescence microscopy and FACS analysis, rhodamine-DPPE (Avanti) was incorporated at a concentration of 0.3 mol%. CF was encapsulatd into liposomes by adding 10 mM CF to the hydration buffer. Unincorporated CF was removed by gel filtration as described for TCEP.

2.6. Coupling of scFv-HC to liposomes

scFv-His-Cys (2 nmol, 50 μg) were reduced using 2 mM Bond-Breaker TCEP (Pierce) under argon atmosphere for 1 h at 37 °C. After gel filtration using a Sephadex G25 column, 0.5 to 2 nmol reduced scFv-HC was incubated with preformed maleimide-containing liposomes (1 μmol lipid) under

argon atmosphere overnight at room temperature. Unreacted maleimide groups were inactivated by incubation with 0.5 mM cysteine for 15 min at room temperature. Non-conjugated scFv-HC was removed by gel chromatography using a Sepharose 4B column (Pharmacia).

2.7. FACS analysis of immunoliposomes

Cells were detached from cell culture dishes using 0.02% EDTA (Sigma). Cells (150 000 to 200 000) were incubated with liposomes (50 nmol lipid) under the indicated assay conditions. After washing with 1% BSA in PBS, cells were resuspended in 400 μl PBS and analysed by flow cytometry (FACSCalibur, Becton Dickinson).

2.8. Internalisation studies

Cells grown on coverslips were washed twice with PBS and were incubated with rhodamine-labelled liposomes (50 nmol lipid) in the presence of culture medium for 2 h at 4 or 37 °C, respectively. After washing with cold PBS, cells were fixed using 3.7% formaldehyde in PBS and mounted with Mowiol. Cell staining was analysed by fluorescence microscopy. For further studies, cells were preincubated for 30 min at 37 °C with bafilomycin A1 at a concentration of 300 nM before adding rhodamine-labelled ILA5 or ILA5 containing encapsulated CF. After incubation for 1 h, cells were washed extensively and incubated further for 1 to 20 h at 37 °C.

2.9. Remote loading of liposomes with doxorubicin and cytotoxicity assays

Doxorubicin was loaded into uncoupled liposomes applying a transmembrane pH-gradient. First, 200 μl of the liposome suspension (50 μmol lipid/ml) was diluted with 200 μl phosphate buffer (pH 7.4, 10 mM). To achieve the pH gradient (intraliposomal media: pH 4, extraliposomal media: pH 7.5), NaOH (2 M) was added to the extraliposomal media. This suspension was heated in a water bath to 60 °C for 5 min. Then the doxorubicin–HCl solution was added (molar ratio dox to lipids 0.2:1) and the volume was filled up to 1 ml with phosphate buffer to obtain a final lipid concentration of 10 μmol/ml. The loading process was performed for 15 min at 60 °C and for 15 min by RT. To remove unencapsulated doxorubicin, the liposomal suspension was applied to Viva-Spins (2 ml, VivaScience) and centrifuged for 20 min at 2400 rpm (1200 × *g*). The encapsulated doxorubicin content was measured with a spectrophotometer at 490 nm. ScFv′ fragments were coupled to dox-loaded liposomes as described above. Cells (HUVEC, HDMEC, HEK293) were incubated with free or liposomal doxorubicin for 2 h at 37 °C in medium at final drug concentration between 10 nM and 10 μM. Cells were then washed and incubated for additional 48 h. Cytotoxicity was determined by crystal violet staining and stained cells were quantified photometrically at 540 nm.

2.10. Pharmacokinetics

Liposomes labeled with ^{3}H-cholesterol-oleoylether (^{3}H-COE) were injected into the tail vein of nude mice and blood samples were taken at the indicated time points. For comparison, we calculated the 50% values, i.e. the time after which 50% of the injected dose is eliminated from the blood stream. The initial half-life ($t_{1/2}\alpha$) was calculated using the values between 3 and 20 min and the terminal half-life ($t_{1/2}\beta$) was calculated using the values between 60 and 360 min.

3. Results

3.1. Generation of scFv′ fragments

Single-chain Fv A5 directed against the extracellular region of human endoglin [24] was genetically modified to expose a cysteine residue at the C-terminus for coupling to maleimide-containing liposomes. Two configurations were chosen with the cysteine either in front or following the hexahistidyl sequence used for purification. ScFv A5-HC contained the cysteine at the C-terminus preceded by the hexahistidyl sequence while scFv A5-CH contained the cysteine in front of the hexahistidyl sequence (Fig. 1A and D). Both molecules were purified from periplasmic preparations of induced bacterial cultures in soluble form. Yields of scFv A5-HC and scFv A5-CH were around 0.5 mg per litre of culture, similar to yields obtained for scFv A5. SDS-PAGE analysis revealed that the scFv fragments were present as a mixture of monomeric and dimeric proteins (Fig. 1B). As judged from Coomassie-stained gels, approximately 50–60% of the proteins were present as monomers. Although both scFv molecules possess an identical molecular weight, scFv A5-CH had a slightly increased mobility compared to scFv A5-HC, indicating some conformational differences of the C-terminal region. Both antibody molecules recognised endoglin in ELISA (not shown). Reactivity of scFv A5-HC and scFv A5-CH with maleimide groups was demonstrated by coupling fluorescein-maleimide to the scFv constructs reduced under mild conditions prior to coupling (Fig. 1C). In these experiments, coupling of fluorescein-maleimide to scFv A5-HC was approximately twice as efficient as coupling to scFv A5-CH. Thus, all further experiments were performed with scFv A5-HC.

3.2. Coupling of scFv A5-HC to malemide-containing liposomes

ScFv A5-HC was coupled to neutral liposome formulations containing maleimide-phosphatidylethanolamine (Mal-PE) as a coupling lipid either in the form of MCC (maleimido-methyl-cyclohexane-carboxamide)-PE or MPB (malemido-phenyl-butyramide)-PE. Although MCC-PE was described to be more stable in aqueous solutions, we did not

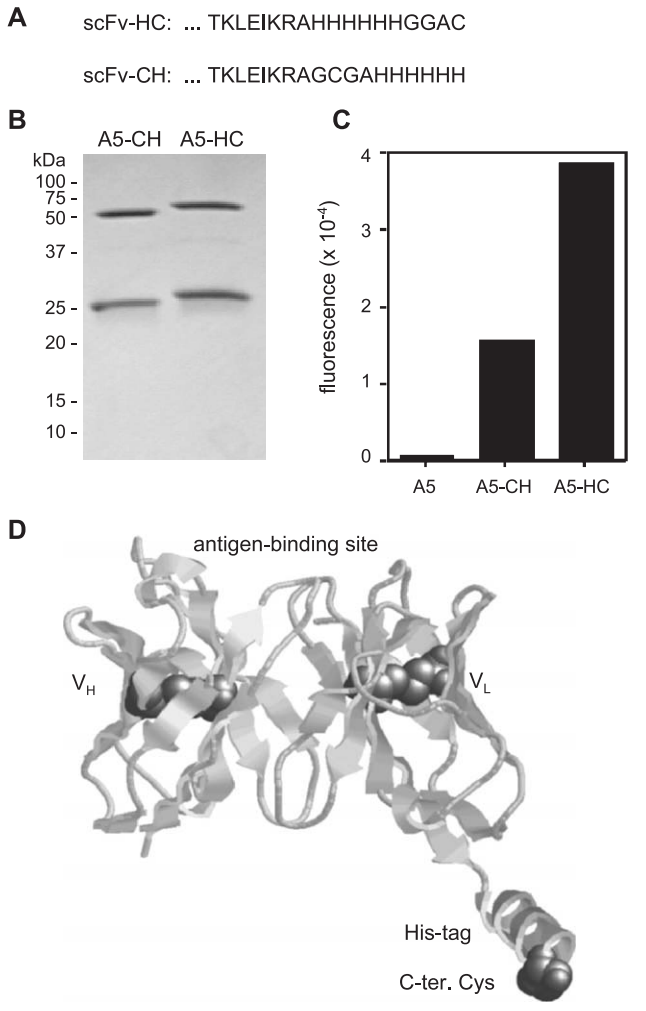

Fig. 1. (A) Structure of the C-terminus of scFv A5-HC and scFv A5-CH containing an engineering cysteine residue either in front or after the hexahistidyl-tag. The V_L sequence is underlined. (B) Purified scFv A5-CH and scFv A5-HC contain a mixture of monomeric and dimeric molecules as shown by SDS-PAGE analysis of non-reduced samples. (C) Coupling of fluorescein-maleimide to scFv A5-CH or scFv A5-HC reduced with TCEP prior to conjugation. (D) Three-dimensional model of a scFv-HC molecule. The C-terminal extension of scFv-HC was modelled using the program Swiss-PdbViewer [43] and was visualised with the program RasMac 2.6 (Roger Sayle, Glaxo Wellcome, UK). Cysteines are shown space-filled. The Linker connecting the V_H and the V_L domain is not shown.

observe differences in coupling efficiency in our experiments using freshly prepared liposomes for coupling. Coupling efficiency was in the range of 10–20% as judged by Coomassie staining or immunoblotting experiments of liposomes before and after separation of uncoupled scFv by gel filtration. The average size of the A5 immunoliposomes (ILA5) was 77 ± 4 nm, identical to unconjugated liposomes.

3.3. Specific binding of ILA5 to endoglin-expressing endothelial cells

ILA5 produced by incubating 1 nmol scFv′ with 1 μmol lipid showed strong binding to human microvascular endothelial cells (HDMEC) or human umbilical vein endothelial cells (HUVEC), while no binding was observed with various endoglin-negative cell lines (Fig. 2). As controls, we included unconjugated liposomes. In addition, liposomes coupled with an irrelevant scFv′ (scFv M5.2; [24]) showed only weak background binding to all cells tested (Fig. 3A). Specificity of binding was further confirmed by competition experiments with soluble scFv A5 or M5.2. In this experiment, scFv A5 inhibited binding of ILA5 in a dose-dependent manner while control scFv M5.2 showed only marginal inhibition at the highest concentration used (Fig. 3B). Further control experiments with scFv A5-HC incubated with liposomes lacking Mal-PE did not lead to any binding demonstrating that covalent coupling of scFv A5-HC to the liposome surface is essential (not shown).

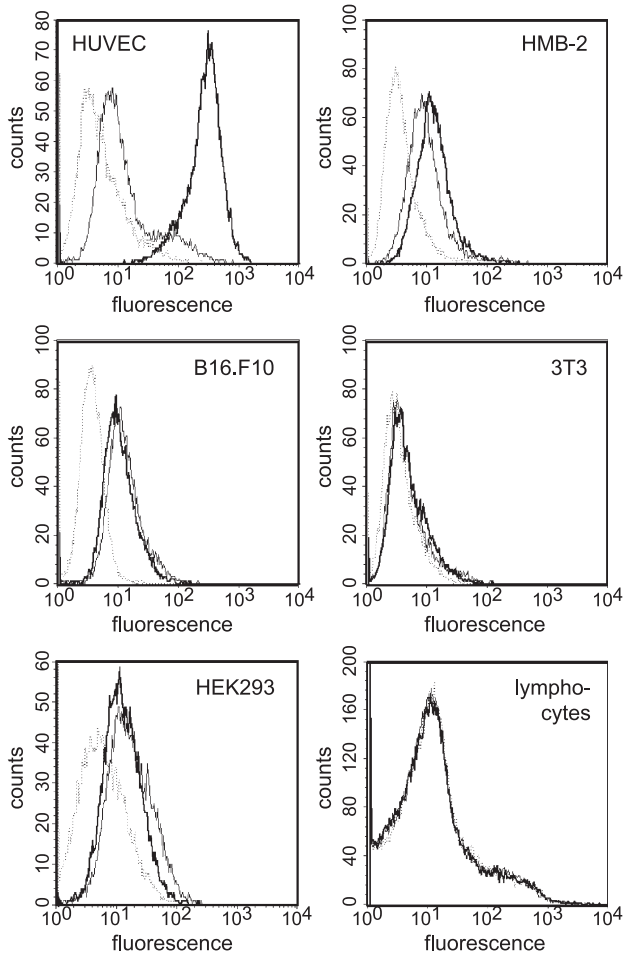

Fig. 2. Binding analysis of A5 immunoliposomes (ILA5) to endothelial cells (HDMECs), human melanoma cell line HBM-2, murine melanoma cell line B16.F10, mouse fibroblasts (NIH 3T3), human embryonic kidney cells (HEK 293), or human lymphocytes. Strong binding is observed with HDMECs expressing high levels of endoglin, while no or only very weak binding is observed for the other cells and cell lines. Doted line = cells alone, thin line = unconjugated liposomes, bold line = ILA5.

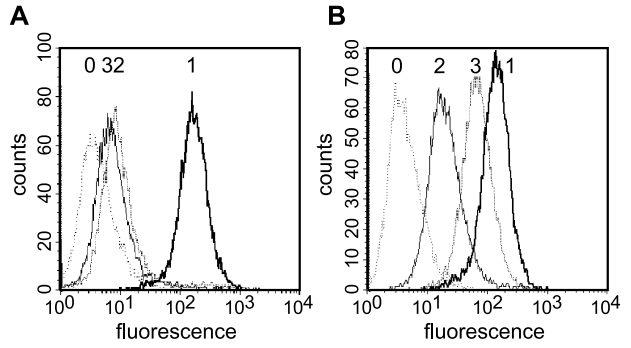

Fig. 3. Specificity of binding of ILA5 to HDMECs. (A) Experiments with unconjugated liposomes (2) or liposomes conjugated with an irrelevant scFv (3) demonstrated specific and strong binding of ILA5 (1) to HDMECs (0 = cells alone). (B) Competition experiments of binding of ILA5 to HDMECs (1) with excess amounts (50 μg/ml) of scFv A5 (2) or an irrelevant scFv (3) at the same concentration (0 = cells alone).

3.4. Binding kinetics and stability of ILA5 in vitro

No differences in fluorescence intensity were observed using scFv A5-HC between 0.5 and 2 nmol per coupling reaction (1 μmol lipid) corresponding to approximately 6–25 scFv molecules per liposome (based on a 20% coupling efficiency) (not shown). This indicates that coupling of a few scFv molecules is sufficient for efficient binding to the cell surface. Increased binding to HDMECs was seen with increasing lipid concentrations (not shown). A time course experiment showed that binding of ILA5 to HDMEC reached a maximum already after 15–30 min (Fig. 4A). Binding experiments in the presence of FCS or human plasma up to 50% did not show any reduction of binding to HDMEC (Fig. 4B and C). A time course experiment incubating ILA5 with 50% human plasma at 37 °C before assaying for binding to HDMEC demonstrated stability of ILA5 for several hours. A reduced binding became evident only after 6 h of preincubation and was further reduced after 24 h (Fig. 4D). Compared to incubation at 4 °C, binding of ILA5 to HDMEC was increased at 37 °C (Fig. 5A).

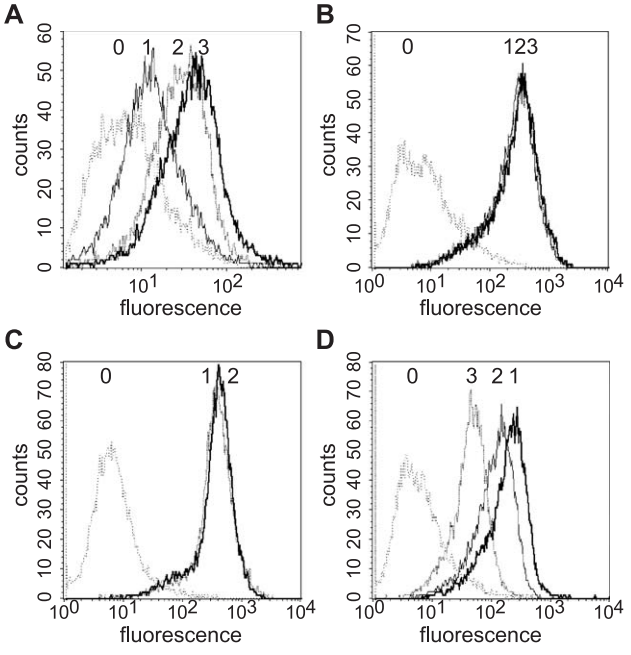

Fig. 4. (A) time course experiment of binding of ILA5 to HDMECs. Cells were incubated with ILA5 for 0 min (1), 15 min (2) or 60 min (3) before analysing for binding by FACS (0 = cells alone). (B) Binding of ILA5 to HDMECs in the presence of 2% FCS (1), 10% FCS (2), or 50% FCS (3) (0 = cells alone). (C) Binding of ILA5 to HUVECs in the presence (2) or absence (1) of 50% human plasma (0 = cells alone). (D) Stability of ILA5 in human plasma at 37 °C. Liposomes were incubated with human plasma for 0 h (1), 6 h (2), or 24 h (3) before analysing for binding by FACS (0 = cells alone).

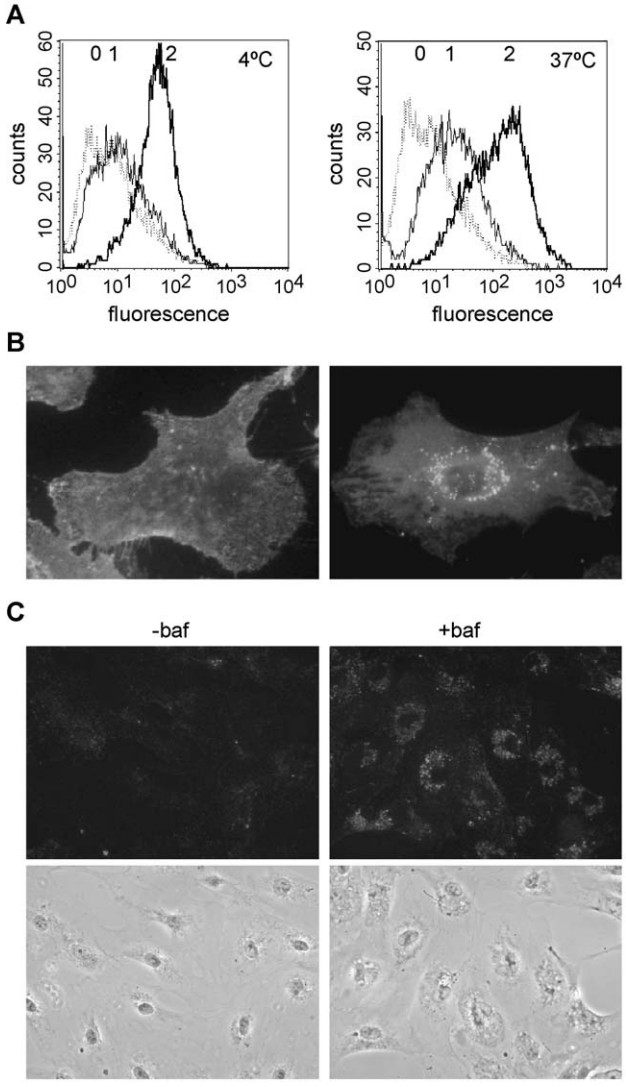

Fig. 5. (A) Comparison of binding of rhodamine-labelled ILA5 (2) to HDMECs at 4 or 37 °C (0 = cells alone, 1 = unconjugated liposomes). (B) Fluorescence microscopy analysis of HDMECs incubated with rhodamine-labelled ILA5 at 4 °C (left image) or at 37 °C (right image) for 2 h. Incubation at 37 °C led to a perinuclear accumulation of fluorescent particles, which is not found with cells incubated at 4 °C. (C) Fluorescence microscopy analysis of HUVECs incubated with rhodamine-labelled ILA5 for 20 h in the presence or absence of 300 nM bafilomycin.

3.5. Internalisation of ILA5

Incubation of endothelial cells with rhodamine-labelled ILA5 at 37 °C led to internalisation of the liposomes as visualised by a perinuclear accumulation of fluorescence after incubation for 2 h at 37 °C (Fig. 5B). No such staining was seen after incubation at 4 °C. Intracellular accumulation of rhodamine-labelled ILA5 was still visible after 2 to 4 h of incubation in the presence of bafilomycin A1, an inhibitor of vacuolar-type H^+-ATPases involved in acidification of endosomes. After prolonged incubation (20 h) cells treated with bafilomycin showed an increased vesicular accumulation of the fluorescent dye compared to untreated cells, which showed a weaker and more homogenous distribution (Fig. 5C). These results were confirmed using ILA5 containing encapsulated CF (not shown), indicating that endosomal trafficking of liposomal lipids and encapsulated compounds is blocked by bafilomycin.

3.6. In vitro cytotoxicity of doxorubicin-A5 immunoliposomes

Fluorescence microscopy of HUVECs incubated for 2 h with doxorubicin-loaded ILA5 or untargeted liposomes showed an increased intracellular and nuclear fluorescence, indicating increased uptake of doxorubicin by ILA5 (Fig. 6). This was also reflected by an increased cytotoxicity of doxorubicin-loaded ILA5 towards endothelial cells (HUVEC, HDMEC) (Table 1). In this assay, cells were incubated for 2 h with liposomes and for additional 48 h after removing unbound liposomes. These assay conditions were chosen to minimise possible cytotoxic effects of doxorubicin released from the liposomes by leakage. No cytotoxic effects were observed after incubation of HEK293 included as a non-targeted cell type. In addition, untargeted doxorubicin-loaded liposomes or unloaded ILA5 showed no cytotoxicity within the analysed concentration range (Table 1). In these assays doxorubicin-loaded ILA5 were approximately 3- to 10-fold more potent in killing endothelial cells than free doxorubicin.

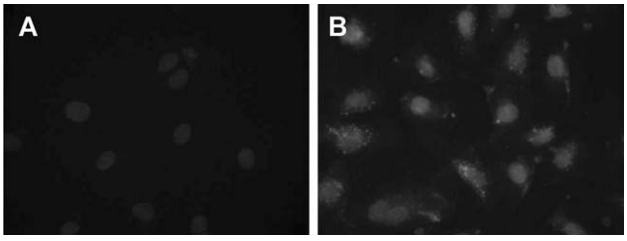

Fig. 6. Fluorescence microscopy of HUVECs incubated for 2 h with doxorubicin-loaded untargeted liposomes (A) or doxorubicin-loaded ILA5 (B). This experiment showed an increased intracellular and nuclear fluorescence after incubation with doxorubicin-loaded ILA5.

Table 1
Cytotoxicity of free or encapsulated doxorubicin on various cell types after 2 h of incubation with drug and analysed after incubation for additional 48 h without drug (IC_{50} values are indicated in μM)

Formulation	HUVEC	HDMEC	HEK 293
Free dox	3	1.4	0.4
ILA5 dox	1.2	0.1	>10
Liposomal dox	>3	>10	>10
ILA5	>10	>10	>10

3.7. Pharmacokinetics of A5 immunoliposomes

Analysis of the blood circulation time of ^{3}H-labelled ILA5 or unconjugated liposomes containing the same amount of coupling lipid showed rapid clearance of these liposomes from the circulation with 50% values < 3 min. In contrast, the same liposomal formulation without coupling lipid had 50% values of approximately 1.5 h ($t_{1/2}\alpha = 45$ min, $t_{1/2}\beta = 9$ h), indicating that Mal-PE is responsible for the drastically reduced circulation time.

4. Discussion

Using a genetically engineered scFv′ fragment exposing an additional cysteine residue at its C-terminus, we were able to generate immunoliposomes targeting endoglin-expressing endothelial cells. The application of scFv′ fragments allows for a site-directed coupling via a single reactive thiol group. Since the C-terminus of a scFv molecule is located opposite the antigen-binding site. this allows unhindered binding to the antigen (Fig. 1D). In addition, it avoids aggregate formation which is often observed using coupling through reactive groups such as ε-amino groups of lysines exposed in scFv molecules at multiple sites.

The scFv A5-HC fragment could be purified in soluble form from the periplasm of bacterial cultures. Thus, the presence of an additional cysteine residue does not lead to the formation of insoluble aggregates as observed previously for a murine scFv′ expressed in bacteria [26]. This might be attributable to the codon-optimised scFv sequence and the isolation of scFv A5 from a phage display library [24]. Due to the oxidising milieu in the periplasm, a substantial amount of the scFv′ fragment was found to be present as disulfide-linked dimers. This dimer formation was also observed for various other scFv′ fragments expressed in prokaryotic or eukaryotic expression systems [4,10,27]. A recent study with an anti-erbB2 scFv′ molecule used for the generation of PEGylated immunoliposomes showed that only 10% of the purified antibody molecules had a free thiol group [4]. Hence, as observed in our study, coupling to liposomes containing functionalised lipids required reduction of the antibody molecules under mild conditions. Coupling of scFv A5-HC to preformed liposomes yielded a coupling efficiency of approximately 10–20%. Similar values were also described for a scFv′ molecule directed

against the fibronectin ED-B domain which was coupled to maleimide-PEG liposomes [10]. In addition, similar values were found in a comparative analysis for MPB-PE and other maleimide-containing lipids with a short and nonpolar spacer demonstrating the influence of spacer length and polarity on coupling efficiency [28]. Thus, the observed low coupling efficiency to MPB-PE or MCC-PE may be attributable to the close distance of the maleimide group to the liposome surface resulting in an insufficient presentation to reactive ligands.

A5 immunoliposomes showed strong and specific binding to endoglin-expressing endothelial cells, identical in binding activity to scFv A5 [24]. Coupling of 6–25 scFv A5 molecules to liposomes confered efficient binding. In another study, Park et al. [29] found that PEGylated liposomes containing 30–40 anti-HER2 scFv molecules per liposome showed optimal binding to erbB2-expressing tumour cells. In contrast, strongest internalisation was already observed at a density of 25–30 scFv molecules per liposomes [4]. These studies demonstrate that coupling of only a few scFv molecules is sufficient to mediate strong binding and internalisation into target cells.

Our studies using bafilomycin as inhibitor of endosome acidification and endosome–lysosome fusion [30] showed an increased granular accumulation within the cells in the presence of bafilomycin, which was most prominent after prolonged incubation. This finding is in accordance with an uptake of the liposomes by endocytosis and processing in the lysosomal compartment [31,32]. An intracellular delivery of encapsulated compounds and release into the cytoplasm was further confirmed with ILA5-Dox liposomes. However, we cannot exclude at present that other mechanisms, e.g. plasma membrane fusion, macropinocytosis or external release, contribute to uptake of encapsulated compounds or lipids into the cells [33,34].

Binding of ILA5 to endothelial cells was stable under physiological conditions and only incubation of A5 immunoliposomes in human plasma at 37 °C for a prolonged period of time reduced the binding activity. This finding indicates that the binding activity of the scFv molecules is little affected by the presence of plasma components. However, our preliminary data show that the in vivo pharmacokinetic properties of the liposomes are drastically influenced by the coupling lipids. This finding further demonstrates the negative influence of the functionalised lipids and the coupling chemistry on the in vivo behaviour of the liposomes [35–37]. These limitations might be overcome by the use of PEGylated liposomes, a reduced number of coupling lipids or alternative approaches such as post-insertion methods [4,38]. In preliminary experiments we could already show that PEGylated A5 immunoliposomes also exhibit strong binding activity for endothelial cells (not shown). Further studies with PEGylated A5 immunoliposomes have to demonstrate the beneficial effects on pharmacokinetics.

Compared to murine IgG molecules or fragments thereof, human scFv molecules should improve the in vivo efficacy of immunoliposomes due to the lack of Fc-mediated clearance and an absent or reduced neutralising immune response [39,40], although a neutralising anti-idiotypic immune response cannot be ruled out. Currently, there is no data available on the behaviour of immunoliposomes composed of human scFv fragments upon repeated injection into immunocompetent animals or in clinical settings.

A5 immunoliposomes will allow for the targeting of tumour endothelial cells expressing endoglin. Several studies have established that endoglin is up-regulated in the vasculature of solid tumours and a versatile marker for tumour angiogenesis and intratumoural microvascular density [11–17]. Importantly, binding of A5 immunoliposomes resulted in internalisation of the carrier systems. This finding is in accordance with studies applying FITC-labelled anti-endoglin antibodies, which described internalisation into mouse endothelial cells [21]. Thus, A5 immunoliposomes should be useful to deliver therapeutic drugs into tumour endothelial cells as already demonstrated in our in vitro experiments. The species specificity of scFv A5 [24], however, puts obstacles on the in vivo analysis in animal models of A5 immunoliposomes as human tumour xenografts are supported by the host vasculature. One possibility is the use of a chimaeric human/mouse skin model in which human skin is transplanted onto immunodeficient mice [41]. Tumours grown in the human skin transplant contain vessels composed of a mixture of murine and human endothelial cells. Such a tumour model was already employed to analyse anti-tumour effects of anti-human endoglin monoclonal antibodies, which cross-react only very weakly with murine endothelial cells [42]. Alternatively, endoglin knock-out knock-in animals could be used to analyse endoglin-targeting antibodies and immunoliposomes.

In summary, we applied an engineered recombinant human antibody fragment for the generation of immunoliposomes for the targeting of endoglin-expressing proliferating endothelial cells. Since the vasculature of tumours is easily accessible to drug carrier systems, endoglin-targeting immunoliposomes may be useful for the development of efficacious and safe vascular targeting agents in cancer therapy.

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