Targeting of skeletal muscle *in vitro* using biotinylated immunoliposomes

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In the present study, a non-covalent (biotin–streptavidin) coupling procedure for the preparation of pegylated immunoliposomes is presented, which simplifies the attachment of targeting vectors to sterically stabilized liposomes. A biotinylated poly(ethylene glycol) (PEG)-phospholipid [bio-PEG-distearoylphosphatidylethanolamine (DSPE)] was used as a linker between a streptavidin-conjugated monoclonal antibody (mAb) (i.e. the OX26 mAb raised against the rat transferrin receptor) and 150 nm liposomes. OX26–streptavidin had a biotin binding capacity of two to three biotin molecules per OX26–streptavidin conjugate. Immunostaining experiments with the OX26 mAb followed by fluorescent confocal microscopy revealed immunofluorescence labelling of the transferrin receptor on skeletal muscle, as well as in L6 cells,

a continuous cell line derived from rat skeletal muscle. Uptake experiments with L6 cells using the OX26 mAb, fluorescence-labelled OX26–streptavidin or fluorescent OX26-immunoliposomes demonstrated cellular uptake and accumulation within an intracellular compartment of the OX26 mAb and its conjugates. Cellular uptake of OX26 conjugates was sensitive to competition with free OX26 antibody. In summary, these studies describe the design of biotinylated immunoliposomes as a universal drug transport vector and their potential for targeting of the transferrin receptor of skeletal muscle.

Key words: biotin, drug targeting, immunoliposome, skeletal muscle, streptavidin, transferrin receptor.

INTRODUCTION

Site-specific delivery of drugs to diseased cells can lead to increased therapeutic effects and to a significant reduction of toxicity. Drug targeting by antibody-conjugated liposomes or immunoliposomes [1] represents a technology that has been applied to the targeting of specific sites of drug action, such as the brain [2], lung [3], cancer cells [4–9], HIV-infected cells [10–12] or cells of the immune system [13]. Site-specific targeting is mediated by the high-affinity binding of monoclonal antibodies (mAbs), i.e. the targeting vector, to their specific antigens. The efficacy of the method therefore depends first on the target specificity of the vector, and secondly on cellular uptake and intracellular delivery of the liposomal load. In the present study, the OX26 murine mAb raised against the rat transferrin receptor [14] has been used as a vector. This antibody has been shown to bind to an extracellular epitope on the receptor, at a site removed from the transferrin-binding site [15]. In cultured cells such as the RG2 rat glioma cell line, receptor-mediated endocytosis and thus intracellular accumulation of the OX26 mAb, as well as that of OX26-conjugated liposomes, has been observed [16].

In vivo studies have shown that conventional (immuno)-liposomes are rapidly removed from the circulation by cells of the reticuloendothelial system, i.e. tissue-resident phagocytes present in a number of organs, particularly the spleen and the liver [17,18]. This interaction is not observed with sterically stabilized liposomes, which are prepared by insertion of monosialoganglioside (GM₁) [19]- or poly(ethylene glycol) (PEG) [20]-derivatized lipids within the lipid bilayer of conventional liposomes. Such liposomes coated with inert polymers show a substantial improvement in their blood circulation half-life (in humans, of the order of days, as opposed to minutes for conventional liposomes [21]).

Sterically stabilized immunoliposomes are often prepared by coupling of antibodies to the distal ends of PEG chains [2,5,22].

Using the PEG chains as a linker between the liposome and antibody leads to an enhanced antibody–antigen binding, since the antibody is not shielded by the steric barrier activity of PEG. Several covalent coupling methods have been developed for attaching (derivatized) antibodies at the PEG terminus. They make use of functionalized PEG-lipids, with a chemically reactive end-group such as hydrazide, *N*-3'-(pyridyldithio)propionate [23], maleimide [2], succinyl [24], *p*-nitrophenylcarbonyl [25] or cyanuric chloride [26]. The drawback of these chemical coupling strategies consists of the difficulty in obtaining reproducible and high coupling efficiencies [26]. Slow hydrolysis of the reactive PEG derivative may, for example, compete with antibody binding. This may result in loss of antibody, and makes it necessary to determine in separate experiments the amount of bound antibody for each batch of immunoliposomes.

In the present study, a new coupling procedure for the preparation of pegylated immunoliposomes is presented. A biotinylated PEG-phospholipid [bio-PEG-distearoylphosphatidylethanolamine (DSPE)] is used for a non-covalent (biotin–streptavidin) method of attachment of the OX26 mAb to sterically stabilized liposomes. The biotin is thereby coupled with the PEG terminus, which allows an optimal target recognition of the bound mAb. Coupling of a streptavidin-conjugated antibody is simple, rapid and highly reproducible. Streptavidin-conjugated OX26 mAb was used, since streptavidin has a much lower isoelectric point (pI 5–6) as compared with the highly basic pI of 10 for avidin. In addition, streptavidin is not a glycoprotein, which reduces its potential for binding to carbohydrate receptors [27]. Both factors reduce the amount of non-specific binding, and thereby the systemic clearance *in vivo* [28].

The transferrin receptor mediates endocytosis of iron bound to the plasma glycoprotein transferrin. Transferrin receptors are expressed by a variety of cells, but their expression levels differ greatly [29]. As a consequence, receptor mapping studies using

Abbreviations used: DSPC, distearoylphosphatidylcholine; FCS, fetal-calf serum; mAb, monoclonal antibody; MBS, *m*-maleimidobenzoyl-*N*-hydroxy-succinimide ester; PEG, poly(ethylene glycol); PEG-DSPE, PEG-derivatized distearoylphosphatidylethanolamine; bio-PEG-DSPE, biotinylated PEG-DSPE.

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different antibodies against the human transferrin receptor have demonstrated binding to different organs and tissues in cynomolgus monkeys [30]. Interestingly, skeletal muscle was among tissues from which the highest percentages of the injected doses of antibody were recovered [30]. We therefore decided to explore whether the OX26 mAb raised against the rat transferrin receptor would bind to rat skeletal muscle, and whether this antibody could subsequently serve as a targeting vector in a novel design of biotinylated immunoliposomes.

MATERIALS AND METHODS

Materials

Cholesterol and distearoylphosphatidylcholine (DSPC) were from Sigma (St Louis, MI, U.S.A.). PEG-DSPE was purchased from Avanti Polar Lipids (Alabaster, AL, U.S.A.). For conjugation of the OX26 mAb to the liposome, a biotin-derivatized linker lipid was used, which consists of a bifunctional 2000 Da PEG that contains a lipid at one end (DSPE) and a biotin molecule at the other end. Bio-PEG-DSPE was custom-synthesized by Shearwater Polymers (Huntsville, AL, U.S.A.). 5(6)-Carboxyfluorescein was purchased from Acros Organics (Geel, Belgium); Sephadex G-75 and Sephacryl S-200 High Resolution were from Amersham Pharmacia Biotech (Uppsala, Sweden). Cy2-conjugated secondary antibody was obtained from Jackson Laboratories (West Grove, PA, U.S.A.). Unspecific mouse IgG_{2a} isotype mAb was from Sigma. [³H]Biotin was obtained from Amersham Pharmacia Biotech. The IgG_{2a} anti-(rat transferrin receptor) OX26 mAb [14] was harvested from cell-culture supernatants of the OX26 hybridoma cell line, and was purified by Protein G–Sephadex affinity chromatography, as described previously [28]. The OX26 mAb was thiolated using 2-iminothiolane (Traut's reagent; Sigma) as described previously [2] by the conversion of one primary amine per mAb, and then linked to streptavidin (Sigma) by activation of the latter with *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) (Pierce, Rockford, IL, U.S.A.). All other chemicals were of analytical grade and were obtained from commercial sources.

Synthesis of streptavidin-conjugated OX26 mAb

The OX26 mAb (1.65 mg; 11.2 nmol) was thiolated using a 20:1 molar excess of 2-iminothiolane (Traut's reagent) as described previously [2]. With this procedure, a single thiol-reactive group is introduced into the OX26 mAb. In parallel with the antibody thiolation, streptavidin (2 mg, 33 nmol) was dissolved in 200 μ l of 0.1 M PBS (100 mM phosphate/150 mM sodium chloride, pH 7.4) and activated by using a 20:1 molar excess of MBS [27]. After incubation for 1 h at 20 °C with gentle shaking, the reaction mixture was purified by Sephadex G-75 gel-filtration chromatography and UV detection at 280 nm. The thiolated antibody was conjugated to the activated streptavidin at a molar ratio of 1:3 (OX26:streptavidin) by incubation overnight at 20 °C with gentle shaking. The reaction products were purified by Sephacryl S-200 gel-filtration chromatography and OX26–streptavidin-containing samples were detected by measuring absorbance at 280 nm. The protein concentration was quantified using the BCA (bicinchoninic acid) protein assay (Pierce) with BSA as the reference. OX26–streptavidin was stored in 0.01 M PBS (10 mM phosphate, 150 mM sodium chloride, pH 7.4) at –20 °C.

Biotin binding assay

Binding of [³H]biotin to the neutral streptavidin–OX26 conjugate was determined by an ultrafiltration method [31] using Centri-

free[®] MPS micropartition devices (Millipore, Bedford, MA, U.S.A.) containing a low-adsorptive hydrophilic membrane with a 30 kDa exclusion. The binding measurements were performed in a final volume of 1 ml of 10 mM PBS containing 0.1 μ Ci of [³H]biotin and unlabelled biotin at concentrations ranging from 16 to 500 nM. The assay was initiated by the addition of neutral OX26–streptavidin to a final concentration of 32 nM. Samples were incubated for 15 min at 37 °C and unbound biotin was separated from the bound fraction by centrifugation (for 10 min at 1250 g, followed by two wash steps). The percentage of unbound biotin in the filtrates was determined by liquid scintillation counting using a Packard Beta-counter (Downers Grove, IL, U.S.A.).

Preparation of fluorescent biotinylated OX26–immunoliposomes

DSPC (5.2 μ mol), cholesterol (4.5 μ mol), PEG-DSPE (0.3 μ mol) and linker lipid (bio-PEG-DSPE; 0.015 μ mol) were dissolved in chloroform. A lipid film was prepared by vacuum evaporation using a Rotavapor (Büchi, Switzerland). Dried lipid films were hydrated at 40 °C in 0.01 M PBS, such that a final lipid concentration of 10 mM was achieved. For the preparation of carboxyfluorescein-filled immunoliposomes, the dried lipids were hydrated in 0.01 M PBS containing 0.1 mM 5(6)-carboxyfluorescein. Lipids were subjected to five freeze–thaw cycles, followed by extrusion (5 times) at 20 °C through a 100 nm-pore-size polycarbonate membrane employing an extruder (Avestin, Ottawa, Canada). Extrusion was repeated 9 times using a 50 nm polycarbonate membrane. Mean vesicle diameters were 150 nm, as determined by dynamic light scattering using a Zetasizer 4 Particle Analyzer (Malvern Instruments, Worcestershire, U.K.).

Buffer was exchanged for 10 mM PBS by applying the liposome suspension to a 1.6 cm $\times$ 20 cm Sephadex G-75 column. Aliquots of column eluates were analysed by on-line absorbance measurements at 280 nm. Fractions containing fluorescent biotinylated liposomes were used immediately for conjugation with streptavidin-linked OX26 mAb by addition of antibody (using a molar ratio of bio-PEG-DSPE:OX26–streptavidin of 1:1). Excess biotin-binding sites on the OX26–streptavidin vector were blocked, before coupling to the biotinylated liposomes, using free biotin (in a molar ratio of OX26–streptavidin:biotin of 1:1) in order to minimize cross-linking and thus precipitation of immunoliposomes. The average number of bio-PEG-DSPE molecules, and thus mAb bound per liposome, was calculated to be 30, assuming that one 100 nm liposome contains 100 000 phospholipid molecules [23].

Cell cultures

Early passages of L6 cells (A.T.C.C. cat. no. CRL-1458) [32] were obtained from A.T.C.C. (Manassas, VA, U.S.A.) and were grown using Dulbecco's modified Eagle's medium supplemented with 4 mM L-glutamine, 1.0 mM sodium pyruvate, 4.5 g/l glucose, 1.5 g/l sodium bicarbonate, 10 % (v/v) heat-inactivated FCS (fetal calf serum), 100 μ g/ml streptomycin and 100 units/ml penicillin G (all obtained from Gibco BRL, Paisley, Renfrewshire, Scotland, U.K.).

Immunohistochemistry

For immunohistochemistry of skeletal rat muscle tissue, fresh rat muscle tissue fragments were transferred to plastic moulds (Tissue-Tek Cryomould, Miles, Torrance, CA, U.S.A.), embedded in Tissue-Tek OCT[™] reagent and frozen immediately in a mixture of propan-2-ol and dry ice. Samples were kept at –70 °C until

Figure 1 Preparation of OX26 immunoliposomes by coupling of streptavidin-conjugated OX26 mAbs to biotinylated PEG liposomes (A), and chemical structure of bio-PEG-DSPE, the biotinylated linker phospholipid (B)

There are approx. 5500 strands of PEG of molecular mass 2000 Da attached to the liposome surface, of which approximately 30 carry a biotin group at their distal end. SA, streptavidin.

use. Sections (6 μm) of the frozen OCT-embedded tissue samples were made using a HM560 cryostat (Microm, Volketswil, Switzerland), mounted on uncoated coverslips, dried for 24 h at 20 °C, fixed with ice-cold 4% (w/v) paraformaldehyde for 20 min and washed with PBS. All subsequent incubations were carried out using PBS containing 3% FCS (fetal calf serum) to reduce unspecific binding. Sections were incubated with mAb OX26 (125 $\mu\text{g}/\text{ml}$) for 30 min at 20 °C, washed with PBS containing 3% FCS and incubated with rabbit Cy2 secondary anti-mouse antibody (Jackson Laboratories) for 1 h (1:200 dilutions of antibody). Fluorophor protector (FluorSafe Reagent, Calbiochem, San Diego, CA, U.S.A.) was added to the sections, and slides were sealed and then analysed by confocal microscopy using a Zeiss LSM 510 confocal microscope. Control experiments performed in parallel included incubations with secondary antibody only. Alternatively, the primary OX26 mAb was replaced with an unspecific mouse monoclonal IgG_{2a} isotype control antibody.

Immunocytochemistry and uptake experiments

Cell surface expression of the transferrin receptor, as well as intracellular accumulation of the OX26 mAb by L6 cells, was visualized by immunostaining experiments: L6 cells were grown on chamber slides (Nalge Nunc, Naperville, IL, U.S.A.). In order to visualize surface expression of the transferrin receptor, L6 cells were incubated on ice for 30 min with the OX26 mAb (100 $\mu\text{g}/\text{ml}$). Cellular uptake and intracellular accumulation of the OX26 mAb was visualized by incubations for 30 min at 37 °C under cell-culture conditions with OX26 mAb (30 $\mu\text{g}/\text{ml}$). Cells were washed and fixed for 20 min with ice-cold 4% (w/v) paraformaldehyde, permeabilized by incubation for 5 min at 20 °C with 0.5% (v/v) Triton X-100 in PBS, transferred for 15 min at 20 °C to PBS containing 3% FCS and incubated with rabbit Cy2 secondary anti-mouse antibody (Jackson Laboratories) for 1 h [33]. All incubations with antibody were carried out using PBS containing 3% FCS to reduce unspecific binding. Cells were mounted in FluorSafe Reagent (Calbiochem) and analysed by confocal microscopy (using a Leica TCS NT confocal microscope). Control experiments performed in parallel did include incubations with secondary antibody only, or the use of an unspecific mouse monoclonal IgG_{2a} isotype control antibody.

Uptake experiments with OX26-conjugates (i.e. OX26 conjugated to streptavidin or OX26-immunoliposomes) were performed with L6 cells grown on chamber slides (Nalge Nunc). Fluorescent OX26-immunoliposomes (carrying 70 $\mu\text{g}/\text{ml}$ of bound

OX26 mAb) or biotin–fluorescein bound to OX26–streptavidin (70 $\mu\text{g}/\text{ml}$) in 10 mM PBS were diluted with one volume of cell-culture medium. Incubations with L6 cells were performed for 1 h at 37 °C. Competition experiments were carried out in the presence of 200 $\mu\text{g}/\text{ml}$ OX26 after a 15 min pre-incubation of the cells with the free OX26. Cells were washed 4 times with ice-cold 10 mM PBS and fixed (20 min at 4 °C) using 200 μl of 4% (w/v) paraformaldehyde. Cells were washed and mounted using FluorSafe Reagent (Calbiochem). Slides were sealed and analysed by confocal fluorescence microscopy (using a Leica TCS NT confocal microscope).

Microscopy

Tissue sections and L6 cells, as shown in Figure 5(B), were analysed with a Zeiss LSM 510 inverted scanning confocal microscope using 20 $\times$ and 40 $\times$ Zeiss Plan-Neofluor lenses with a numerical aperture of 0.5 and 1.3 respectively. A 488 nm argon laser was used in combination with fluorescein band-pass filters. Optical sections of 5.3 μm (muscle tissue) or 1.4 μm (L6 cells) were acquired and processed on a silicon graphics workstation (Silicon Graphics, Mountain View, CA, U.S.A.) using Zeiss software.

Immunofluorescence images of L6 cells were recorded with a Leica TCS NT confocal microscope (Leica) using 25 $\times$ and 40 $\times$ Plan Fluotar or 63 $\times$ Plan Apochromat oil-immersion objectives with numerical apertures of 0.75, 1.0 and 1.32 respectively. Optical sections of 0.7–1 μm were analysed using the Imaris software (Bitplane AG, Zurich, Switzerland) and processed using Photoshop software (Adobe Systems, Mountain View, CA, U.S.A.).

RESULTS AND DISCUSSION

In the present study, a novel design of biotinylated immunoliposomes is presented, where a streptavidin-conjugated targeting vector (i.e. the OX26 mAb raised against the rat transferrin receptor) is attached to the surface of the liposome using a PEG spacer (Figure 1A). Biotin was thereby coupled to the tip of the pegylated phospholipid PEG-DSPE (Figure 1B), and subsequently incorporated into sterically stabilized liposomes (PEG liposomes). A biotinylated PEG liposome carries approximately 5500 strands of 2000 Da PEG and 30 strands of the linker lipid biotin-PEG-DSPE, and has a measured particulate size of 150 nm. The use of PEG as a flexible tether allows the biotin, and thereby the attached OX26 mAb, to extend away from the liposome surface,

Figure 2 Column chromatography separation and analysis of OX26-streptavidin using a Sephacryl S-200 packed column

(A) Elution profile by fluorescence detection (excitation wavelength of 485 nm; emission wavelength of 535 nm) of OX26-streptavidin (I) labelled with an excess of fluorescein-labelled biotin (II). (B) Chromatographic separation after synthesis of OX26-streptavidin (210 kDa; I) and unconjugated OX26 (150 kDa; II). (C) Elution profile of a set of molecular-mass markers: thyroglobulin (of 670 kDa; void volume; I), γ -globulin (of 158 kDa; II), ovalbumin (of 44 kDa; III) and myoglobin (of 17 kDa; IV). OD, absorbance.

which minimizes steric hindrance by unconjugated PEG moieties. Previous studies [34] have indeed demonstrated that biotinylation of PEG liposomes does not affect their *in vivo* pharmacokinetics in control rats, but promotes their accumulation in subcutaneous *Staphylococcus aureus* abscesses in the rabbit. Furthermore, direct measurement of the ligand-receptor interaction potential using biotinylated PEG liposomes and streptavidin on a supported lipid bilayer indicated a markedly extended range of the interaction [35].

Biotinylated PEG liposomes can be used for the non-covalent conjugation of any avidin- or streptavidin-conjugated ligand. Such ligands could, for example, include genetically engineered single-chain antibody-streptavidin fusion proteins, such as the OX26 single chain Fv antibody-streptavidin fusion protein [36], which can be produced on a large scale in prokaryotic or eukaryotic expression systems [37]. In the present study, streptavidin was

Figure 3 Concentration-dependent binding of [³H]biotin to a streptavidin-OX26 conjugate

Unbound [³H]biotin was separated from streptavidin-OX26 by ultrafiltration using a filter membrane with a molecular-mass cut-off of 30 kDa. Saturation of total [³H]biotin binding in the presence of 32 nM streptavidin-OX26 was observed at biotin concentrations of 64–100 nM.

chemically combined with the hybridoma-generated OX26 mAb. MBS was used as a hetero-bifunctional cross-linker reactive for primary amines in streptavidin and a single thiol group introduced into the OX26 mAb by thiolation with Traut's reagent (2-iminothiolane). This procedure has been shown previously to retain both the antigen affinity of the OX26 mAb [2] and the biotin-binding site of streptavidin [38]. The OX26-streptavidin conjugate was purified and analysed by gel-filtration chromatography (Figure 2). The conjugate was identified by a shift in molecular mass as compared with unconjugated OX26 mAb, and its ability to bind the fluorescent label biotin-fluorescein (Figure 2A). A separation was achieved between the cross-linked precipitates OX26-streptavidin (Figure 2B, peak I) and unbound OX26 mAb (Figure 2B, peak II), and this was confirmed by SDS/PAGE analysis (results not shown). The approximate yield of streptavidin-conjugated OX26 recovered after purification was 40%. The binding capacity of the OX26-streptavidin conjugate for [³H]biotin was determined by an ultrafiltration method (Figure 3). There are normally four biotin-binding sites on the tetrameric streptavidin [27]. Analysis of the biotin binding curve indicates that biotin binding is only partially impaired as a consequence of the coupling of streptavidin with the OX26 mAb. Owing to the extremely high affinity of biotin binding to streptavidin, with a K_d approximating 10^{-15} M [27], the affinity constant of the OX26-streptavidin conjugate in itself cannot be determined. However, using 32 nM OX26-streptavidin conjugate, saturation of biotin binding is observed between 64 and 100 nM biotin, indicating the presence of two to three biotin-binding sites on each OX26-streptavidin conjugate. The presence of multiple biotin-binding sites on each OX26-streptavidin conjugate may potentially lead to cross-linking of biotinylated liposomes, and thus to the formation of liposomal aggregates. To minimize this risk and to block excess biotin-binding sites, free biotin was added to OX26-streptavidin in a molar ratio of 1:1 prior to coupling of the OX26-streptavidin conjugate to biotinylated liposomes.

The question arises of whether targeting of skeletal muscle could be achieved using OX26-streptavidin as a vector directed against the transferrin receptor. The transferrin receptor is expressed by different organs, such as the liver, spleen, general vascular endothelium and the blood-brain barrier. In skeletal muscle, transferrin has been shown to have an important role in muscle

Figure 4 Analysis of muscle tissue sections by confocal microscopy using the OX26 mAb (125 $\mu\text{g/ml}$) and a secondary Cy2-labelled polyclonal antibody

Immunostaining of rat muscle tissue which is cryo-sectioned (A) orthogonal or (B) parallel to the muscle fibres. The inset to (B) shows the control experiment (incubation with secondary antibody only). Scale bars represent 100 μm .

growth, i.e. the process of myoblast and satellite cell proliferation, their fusion to myotubes and their further differentiation into muscle fibres [39]. Studies with, for example, primary cultures of chick muscle cells [40] have clearly demonstrated high expression levels of functional transferrin receptors regardless of the state of growth or differentiation of these cells. We confirmed, by immunostaining experiments using the OX26 mAb, the presence of the transferrin receptor in rat skeletal muscle tissue (Figure 4). Tissue sections perpendicular to the muscle fibres (Figure 4A) showed an intense immunostaining at the periphery of the myofibres (which have a diameter of 50–100 μm), as well as at the periphery of the enclosed myofibrils. The staining of the myofibre surface can also be seen in longitudinal sections (Figure 4B). Little or no fluorescent signal was observed in control experiments using secondary antibody only (Figure 4B, inset) or an unspecific mouse IgG_{2a} isotype control antibody (results not shown). Incubation of L6 cells (a cell line derived from rat skeletal muscle) with OX26 mAb at 37 °C followed by visualization of the OX26 mAb using a fluorescent secondary antibody (Figures 5A and 5B) reveals a fluorescent signal on the cell surface as well as a particulate intracellular staining pattern. This observation suggests that the OX26 mAb is internalized by L6 cells under cell-culture conditions, and subsequently accumulates within the endosomal compartment of these cells. At 4 °C, binding of OX26 to the cell surface only was observed (Figure 5C) because of inhibition of receptor-mediated endocytosis at low temperature.

Figure 5 Immunocytochemistry and uptake experiments using the OX26 mAb and L6 cells

(A) Cellular uptake of the OX26 mAb (100 $\mu\text{g/ml}$) by L6 cells at 37 °C and visualization of internalized antibody using a Cy2-labelled secondary antibody: overlay between a phase contrast image of L6 cells and the corresponding fluorescence image. The inset shows the negative control, using an unspecific IgG_{2a} isotype antibody. (B) Magnification of a single L6 cell (incubation with 30 $\mu\text{g/ml}$ OX26 at 37 °C) reveals a particulate intracellular staining pattern. The inset shows the control (secondary antibody only). (C) Immunolabelling of surface receptors by incubation of L6 cells with OX26 mAb (100 $\mu\text{g/ml}$) at 4 °C. The inset shows the control (unspecific IgG_{2a} isotype antibody). Scale bars represent 20 μm .

Figure 6 Cellular uptake of fluorescence-labelled OX26-streptavidin (OX26-streptavidin biotin-fluorescein) and fluorescent OX26 immunoliposomes by cultured L6 muscle cells

Cells were fixed after 1 h incubation and analysed by confocal microscopy. The concentration of OX26 was always 70 $\mu\text{g/ml}$. (A) Incubation with OX26-streptavidin biotin-fluorescein; (B) incubation with OX26 immunoliposomes loaded with carboxyfluorescein; (C) competition of cellular uptake of OX26 immunoliposomes in the presence of 200 $\mu\text{g/ml}$ free OX26; (CONTROL) background fluorescence of L6 cells. Scale bars represent 50 μm .

Our findings are in line with previous reports on expression levels of the transferrin receptor in L6 cells, which indicated a receptor density of approximately 200 000 receptors per L6 myocyte [41]. We conclude from these experiments that the L6 cell line can be considered to be a representative cell-culture model of skeletal muscle that may be used to study transferrin-receptor-mediated transport processes *in vitro*.

Uptake experiments in L6 cells (Figure 6) demonstrated that the functionality of the OX26 mAb is retained after chemical modification and coupling to streptavidin. Incubation of OX26-streptavidin, which was fluorescence-labelled using biotin-fluorescein, resulted in a strong intracellular accumulation of fluorescence in L6 cells (Figure 6A). Similar results were obtained using carboxyfluorescein-loaded OX26 immunoliposomes

(Figure 6B). The observed effects were mediated specifically by the OX26 mAb, since competitive inhibition of cellular uptake was observed in the presence of free OX26 mAb (Figure 6C). The percentage of positive cells in these experiments was estimated to be over 90%. We conclude that biotinylated OX26 immunoliposomes, despite their big particulate size of 150 nm, are transported across the plasma membrane of L6 muscle cells by means of receptor-mediated endocytosis.

In the present project, a novel design of biotinylated immunoliposomes was developed and validated. Using the streptavidin-conjugated OX26 mAb directed against the rat transferrin receptor, targeting of immunoliposomes to L6 skeletal muscle cells *in vitro* followed by intracellular accumulation was achieved. The question arises of whether such an immunoliposome design could be used for drug targeting to skeletal muscle *in vivo*. Previous studies have demonstrated that a similar design of immunoliposomes led to favourable *in vivo* pharmacokinetic properties, such as minimal unspecific tissue binding [2,16]. Tissue distribution of the immunoliposomes was mediated by the OX26 mAb, with selective accumulation in brain tissue. However, receptor mapping studies in the cynomolgus monkey using mAbs against the human transferrin receptor showed that the distribution of such antibodies was not confined to brain tissue, but that a significant percentage of the injected dose did accumulate in other tissues expressing the transferrin receptor, such as skeletal muscle [30]. Interestingly, the overall pattern of tissue distribution was quite different for the two mAbs which were tested. This may be a consequence of binding of the antibodies to different structural motifs on the transferrin receptor. These results, together with our findings, suggest that *in vivo* drug targeting to skeletal muscle may be achieved by targeting of the transferrin receptor. The tissue specificity will thereby depend on the used anti-(transferrin receptor) antibody. Biotinylated PEG liposomes in combination with a streptavidin-conjugated vector to the transferrin receptor thus form a versatile platform to refine an immunoliposome-based delivery system *in vivo* for the targeting of drugs to skeletal muscle.

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