# An Apolipoprotein E-Derived Peptide Mediates Uptake of Sterically Stabilized Liposomes into Brain Capillary Endothelial Cells †

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# An Apolipoprotein E-Derived Peptide Mediates Uptake of Sterically Stabilized Liposomes into Brain Capillary Endothelial Cells†

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ABSTRACT: A promising strategy to solve the problems of insufficient membrane penetration of drugs and low target specificity is the localization of targeting and uptake-facilitating ligands on the surface of drug-carrier systems. This study investigated the role of a peptide derived from the LDL receptor (LDLr)-binding domain of apolipoprotein E (apoE) in initiating endocytosis in brain capillary endothelial cells. The highly cationic tandem dimer of apoE residues (141−150) was coupled covalently onto poly(ethylene glycol)-derivatized liposomes. Membrane binding and cellular uptake was monitored qualitatively by confocal-laser-scanning microscopy as well as quantitatively using a fluorescence assay. The peptide mediated an efficient, energy-dependent translocation of liposomes across the membrane of brain capillary endothelial cells. Liposomes without surface-located peptides displayed neither membrane accumulation nor cellular uptake. Low peptide affinity to LDLr and internalization of the complex into fibroblasts with up- and down-regulated receptor expression levels, as well as complex translocation into cells incubated with an antibody against the LDLr, pointed to a dominating role of an LDLr-independent transport route. Enzymatic digestion of heparan sulfate proteoglycan (HSPG) with heparinase I and addition of heparin and poly-L-lysin as competitors of HSPG and HSPG ligands, respectively, resulted in a significant loss in liposome internalization. The results suggested that HSPG played a major role in the apoE−peptide-mediated uptake of liposomes into endothelial cells of brain microvessels.

The passage of many substances from the circulating blood flow into brain tissues is limited by the blood−brain barrier (BBB). Thus, the treatment of various diseases by drug delivery to the brain is a particular challenge (*1*). A promising method for the delivery of compounds that are unable to pass the BBB by themselves is the use of transport vectors, which activate natural transport routes (*2*). The development of vectors arises from an understanding of the pathway of solute transport such as either carrier mediated, e.g., for nutrients, or receptor mediated, e.g., for peptides. Specific receptors on brain capillaries have been identified for transferrin (*3*, *4*), insulin (*5*), and insulin-like growth factors (*6*). So far, evidence for the significance of vector-mediated drug delivery to the brain is most convincing in the case of monoclonal antibodies to the transferrin or insulin receptor (*7*, *8*). Coupling of vectors to liposomes combines the advantages of targeting, incorporation of drugs at high concentration, reduced side effects, and circumvention of the multidrug efflux system (*9*). An additional advantage provide sterically stabilized liposomes equipped with poly(ethylene glycol) (PEG)[1] chains. They have a distinctly prolonged circulation time in the blood stream compared to conventional liposomes (*10*). The success of the strategy of vector-carrier coupling for brain targeting has been documented with immunoliposomes, which are transported across the BBB via receptor-mediated endocytosis (*11*, *12*) or the efficient uptake of cationized albumin-coupled liposomes by brain capillaries via adsorptive endocytosis (*13*).

Besides receptors for transferrin, insulin, or insulin-like growth factors, the occurrence of the low-density lipoprotein receptor (LDLr) on brain capillary endothelial cells has been demonstrated for several species (*14*−*16*). Furthermore, it

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[1] Abbreviations: apoE, apolipoprotein E; RBCEC, rat brain capillary endothelial cell; BSA, bovine serum albumine; CLSM, confocal-laser-scanning microscopy; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; Fmoc, *N*-(9-fluorenyl)methoxycarbonyl; HSPG, heparan sulfate proteoglycan; LDL, low-density lipoprotein; LPDS, lipoprotein-deficient serum; MALDI−TOF, matrix-assisted laser desorption ionization−time-of-flight; mal-PEG-PE, 1,2-stearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[maleiimide(poly(ethylene glycol)-2000)]; MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl)tetrazolium bromide; PBS, phosphate-buffered saline; PDS, plasma-derived bovine serum; PEG, poly(ethylene glycol); PEG-PE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(poly(ethylene glycol)-2000)]; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; Rhod-PE, Lissamine rhodamine B 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt; Tris, tris(hydroxymethyl)aminomethan.

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has been shown that cholesterol-rich low-density lipoproteins (LDLs) interact with the LDLr and are transported across the BBB via subsequent transcytosis, bypassing lysosomal degradation (*17*). The fact that the expression of LDLr is down-regulated in peripheral vessels (*16*) supports the idea of using the LDLr-mediated pathway for drug transport into the brain. An LDLr-related improved analgetic effect has been discussed for Dalargin-loaded nanoparticles coated with apolipoprotein E (apoE) (*18*). The observation that the effect was detectable only for the particle-adsorbed drug supported the suggestion that diffusion through a disturbed barrier is not involved but rather a specific transport process plays a role (*19*).

ApoE is a 34-kDa, 299 amino acid residue protein that mediates the endocytotic uptake and metabolism of triglyderide-rich lipoproteins through its high-affinity binding to the LDLr and members of its gene family (*20*). The amino-terminal domain contains the receptor-binding sequence, which was narrowed down to residues in the vicinity of 136−150 by examining apoE isoforms, engineered point mutations, and proteolytic fragments (*21*−*24*). Several other regions in the globular N-terminal domain (*25*) have been identified to distinctly influence the receptor affinity, and the critical role of the lipid-anchoring carboxyl-terminal apoE domain for receptor-binding activity is convincingly documented (*26*, *27*). Studies with synthetic peptides characterized the structural features of the LDLr-binding sequence of apoE (*28*−*33*). Whereas the sequence (141−155) is not recognized, its tandem dimer competes with LDL for receptor binding (*30*), and also the tandem dimer $(141-150)_2$ induces an endocytotic process in cultivated cortical neurons (*34*). For a peptide composed of the monomer (141−150) and a high-affinity lipid-associating domain, it was found that the enhanced internalization and degradation of LDL in fibroblasts was caused by an LDLr-independent but heparan sulfate proteoglycan (HSPG)-associated pathway (*35*).

Besides receptor-mediated endocytosis, receptor-independent processes may contribute to the transport of molecules across cell membranes. Adsorptive endocytosis is initiated by binding of polycationic substances to negative charges on the plasma membrane. The HSPGs are a heterogeneous group of highly negatively charged membrane-anchored proteins that are found almost ubiquitously on the cell surface and in intracellular granules of virtually all mammalian cells (*36*). The proteins avidly bind positively charged ligands and are rapidly internalized into cells, thus enabling endocytotic uptake of bound compounds. HSPG was found to be involved also in cellular uptake and turnover of lipoproteins, in part by enhancing the accessibility of lipoproteins to their receptors and lipases (*37*). The role of apoE in enhancing binding and internalization of lipoproteins and remnants via an HSPG-dependent pathway has been demonstrated for different cells (*38*−*40*). The presence of an anionic surface matrix at the BBB has been demonstrated before (*41*). Perlecan, an HSPG core protein, is expressed at mouse brain capillary endothelial cells and involved in the internalization of the basic fibroblast growth factor (*42*). The uptake of other positively charged peptides (*43*), peptide−drug chimeras (*44*), and liposomes coupled to cationized albumin (*13*) into brain capillary endothelial cells gave further evidence for the importance of HSPG as a mediator of adsorptive endocytosis at the BBB.

In the present study, we demonstrate that the tandem sequence $(141-150)_2$ of human ApoE covalently coupled to PEG-derivatized lipids mediates an efficient uptake of sterically stabilized liposomes into primary rat brain capillary endothelial cells (RBCECs) as monitored by confocal-laser-scanning microscopy (CLSM) and a fluorescence-based assay. Studies with human fibroblasts with up- and down-regulated LDLr expression and in the presence of an LDLr antibody, as well as under conditions influencing endocytotic transport processes, pointed to a dominating role of an LDLr-independent transport route. HSPG was identified as an efficient mediator for cytosolic liposome delivery.

## MATERIALS AND METHODS

*Materials*. Protected amino acids were purchased from Novabiochem, Switzerland. The lipids 1-palmitoyl-2-*sn*-glycero-phosphocholine (POPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(poly(ethylene glycol)-2000)] (PEG-PE), 1,2-stearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[maleimide(poly(ethylene glycol)-2000)], ammonium salt (mal-PEG-PE), and cholesterol were purchased from Avanti Polar Lipids Inc., U.S.A. Lissamine rhodamine B 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (Rhod-PE) was from Molecular Probes, The Netherlands. All tissue-culture media and reagents for cell biological experiments were from Biochrom AG, Germany. All other chemicals and reagents were obtained from Sigma, Germany, if not indicated otherwise.

*Peptide Synthesis and Characterization.* Peptides were synthesized on solid phase using an Applied Biosystems 433A automatic peptide synthesizer following the standard *N*-(9-fluorenyl)methoxycarbonyl (Fmoc) chemistry protocol (*45*). Peptides were either N-terminally acetylated or coupled to a mercaptopropionic acid following the Fmoc protocol. All peptides were purified by preparative reversed-phase high-performance liquid chromatography (RP-HPLC) using a Shimadzu LC-10AD system (Japan) operating at 220 nm to give final products more than 95% purity by HPLC analysis. The compounds were further characterized by matrix-assisted laser desorption−time-of-flight (MALDI−TOF) mass spectrometry (Voyager-DE STR, Perseptive Biosystems, U.S.A.).

*Preparation of Liposomes and apoE*−*Peptide Liposomes*. Appropriate amounts of POPC, cholesterol, PEG-PE, mal-PEG-PE, and Rhod-PE at a molar ratio of 64:30:2.5:2.5:1 (62:30:2.5:2.5:3 for uptake experiments using CLSM) were dissolved in a mixture of chloroform/methanol 2:1 (v/v). The solvent was removed using a rotary evaporator, and the lipid film was dried under vacuum overnight. After addition of buffer (10 mM Hepes and 140 mM NaCl at pH 7) and vortex mixing for 5 min, the liposome dispersion was frozen in liquid nitrogen and thawed in a 37 °C water bath for eight cycles. Liposome size was reduced by extrusion (Lipex Biomembranes Inc., Canada), 10 times through two stacked 0.1 $\mu$m pore size polycarbonate membranes and 10 times using 0.5 $\mu$m pore size polycarbonate membranes (Nuclepore).

The thiol-modified peptide was added to the liposome dispersion and incubated overnight at room temperature to form a thioether bond with mal-PEG-PE. Usually, the peptide was added to 2 mL of a 10 mM liposome dispersion to give

a final molar lipid/peptide ratio of 200. Unbound peptide was removed by size-exclusion chromatography using a PD-10 column (Pharmacia, Sweden) and Hepes buffer as the eluent.

*Characterization of Liposomes*. Liposome size was determined by dynamic light scattering (N4 Plus, Coulter Corporation). The amount of lipid in the liposome dispersion was determined by phosphorus analysis (*46*). Successful coupling of the thiol-modified peptide to mal-PEG-PE was confirmed by MALDI−TOF analysis (Voyager-DE STR, Perseptive Biosystems). The amount of liposome-bound peptide was evaluated by tryptophan fluorescence spectroscopy (excitation wavelength $\lambda_{ex} = 280$ nm) using a Jasco FP-6500 fluorescence spectrometer, Japan. Spectra were recorded between 300 and 450 nm, and the approximate peptide content was derived from the fluorescence intensity at the emission maximum $\lambda_{em} = 350$ nm using the fluorescence intensity of the peptide dissolved in buffer as the standard.

*Cells.* Primary RBCEMs were isolated and cultured as described (*47*). Briefly, brain capillary endothelial cells were isolated from the forebrains of 10-days-old Wistar rats. Meninges were removed, and the gray matter was minced and then digested with collagenase type-2 (1 mg/mL) in Dulbecco's modified Eagle's medium (DMEM) containing gentamycin (100 $\mu$g/mL, Gibco, Germany). The pellet was separated by centrifugation in DMEM containing 20% bovine serum albumin (BSA). The microvessels obtained in the pellet were digested further with collagenase Dispase containing (1 mg/mL) DMEM. Microvessel endothelial cells were separated on a Percoll gradient, collected, and washed twice in DMEM before plating in poly-L-lysine-covered plastic tissue-culture dishes. RBMECs were cultured in DMEM containing 20% plasma-derived bovine serum (PDS) (Firstlink, U.K.) and 1 ng/mL basic fibroblast growth factor (Boehringer Ingelheim, Germany) at 37 °C in a humidified atmosphere of 5% $CO_2$.

Normal human foreskin fibroblasts BaGL-3 were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) at 37 °C and under 10% $CO_2$ atmosphere.

*Preparation of $^{125}$I-Labeled LDL and Lipoprotein-Deficient Serum (LPDS).* LDL and LPDS were isolated from human plasma by sequential centrifugation according to ref *48*. Subsequent iodination of LDL was performed by the iodine monochloride method as described as well (*48*).

*Competition of LDL and apoE−Peptide for $^{125}$I-LDL-Binding to the LDLr.* The affinity of LDL and the apoE−peptide $(141-150)_2$ to the LDLr was determined in a competition assay with $^{125}$I-labeled human LDL using human primary fibroblasts (*49*). A total of 1 week before the experiment, the cells were plated at $1.4 \times 10^4$ cells/12 × 22 mm multiwell dishes. A total of 48 h before the experiment, the cells were exposed to DMEM containing 10% LPDS to permit up-regulation of LDLr expression (*50*). On day 7, cells were incubated at 4 °C for 3 h in medium containing 2 $\mu$g/mL $^{125}$I-labeled-LDL and 0−20 $\mu$g/mL peptide or human LDL. After washing, displacement of $^{125}$I-LDL by the apoE−peptide $(141-150)_2$ was assayed by $^{125}$I counting. The concentration of the competitor displacing 50% of bound $^{125}$I-LDL was determined from an exponential-decay curve-fitting model.

*Cell Viability Measurement by (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl)tetrazolium Bromide (MTT) Assay.* Cell viability was detected by the MTT method as described in ref *51*. A total of $10^4$ primary RBMECs per well were seeded 48 h prior to the experiment in a 96-well plate and allowed to grow to confluency. After the medium was removed, the cells were exposed to increasing concentrations of apoE−peptides or apoE−peptide liposomes in serum-free DMEM for 1 h ($T = 37$ °C). After washing, adding 180 $\mu$L fresh medium and 20 $\mu$L of MTT solution [5 mg/mL phosphate-buffered saline (PBS)] per well, and incubating the cells at 37 °C for 4 h, the medium was replaced by 100 $\mu$L of dimethylsulfoxide/well. Cell viability was quantified by measuring the optical density at 550 nm using a microplate reader (Tecan Safire, Switzerland). Absorption determined with untreated cells referred to 100% cell viability.

*Liposome Uptake Measurements by CLSM.* A total of $10^4$ rat primary RBCECs was plated on poly-L-lysine-coated coverslips (diameter 30 mm) positioned in 35 mm plastic culture dishes and cultured in serum-free medium. After the cells had reached semiconfluency as checked by microscopic inspection, the plates were washed twice with PBS at pH 7.4 and exposed to liposomes suspended in serum-free medium for 30 min at 4 °C or 37 °C. apoE−peptide and blank liposomes were exposed to the cells at a lipid concentration of 33 $\mu$M, corresponding to a rhodamine concentration of 1 $\mu$M. After washing 3 times with ice-cold PBS, fluorescent CLSM pictures were taken within 10 min using an LSM 510 inverted confocal-laser-scanning microscope (Zeiss, Germany), equipped with a helium−neon laser and a dichroitic mirror HFT 543 for wavelength selection. Excitation was performed at 543 nm, and emission was registered using a 560 nm cutoff filter LP560 in front of the detector.

*Binding and Uptake of Liposomes into Human Fibroblasts.* The cells were plated and cultured as described in *Competition of LDL and apoE−Peptide for $^{125}$I-LDL-Binding to the Receptor*. A total of 48 h before an experiment, the medium was either renewed to achieve receptor expression at normal level or changed to DMEM containing 10% LPDS to permit up-regulation of LDLr expression (*50*). For binding experiments, the cells were washed twice with PBS and incubated with liposomes suspended in HEPES-buffered serum-free DMEM (Gibco) for 3 h at 4 °C. The lipid concentration ranged between 1 and 100 $\mu$M. For liposome uptake, the cells were incubated for 2 h at 37 °C followed by a heparin wash (10 mg/mL) for about 1 h at 4 °C to remove the membrane-bound part (*48*). After washing for 3 times with both 0.2% BSA/PBS and PBS and subsequent solubilization of the cells with 0.5 mL of 0.1 N NaOH per well, bound and internalized rhodamine-labeled liposomes, respectively, were determined fluorimetrically using a multiwell plate reader (Tecan Safire, Switzerland) (excitation wavelength $\lambda_{ex} = 550$ nm, and emission wavelength $\lambda_{em} = 590$ nm).

To check nonspecific binding, the LDLr was blocked using a polyclonal anti-human LDLr antiserum (*52*). The antibody was added at a concentration of 20 $\mu$g/mL to the medium and allowed to bind for 1 h at 4 °C. Prior to the experiment, the amount of antibody needed for total blocking of the LDLr of cells with a normal LDLr expression level was determined in a $^{125}$I-LDL-binding assay (data not shown). After washing, the binding experiments were performed by incubating the

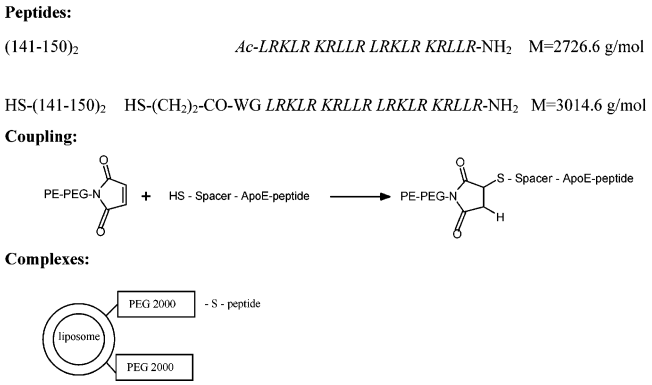


FIGURE 1: Sequence of apoE-derived peptides and preparation of apoE−peptide liposomes. Schematic diagram of coupling of the thiolated apoE−peptide to sterically stabilized liposomes containing mal-PEG-PE.

cells with apoE−peptide-covered and blank liposomes at a lipid concentration of 50 $\mu$M as described above.

The role of cell-surface HSPG in liposome binding and uptake was investigated by removal of HSPG with heparinase I (heparin lyase I from *Flavobacterium heparinum*) as previously reported (*33*, *35*). A stock solution was prepared by dissolving heparinase I in sterile 0.15 M NaCl. An aliquot of the solution was immediately added to the serum-free medium onto the cells to give a final heparinase concentration of up to 10 units/mL. After incubation at 37 °C for 2 h and washing, binding and uptake experiments at lipid concentrations up to 100 $\mu$M were performed as described above. Experiments in the presence of heparin and poly-L-lysine were performed to block HSPG-mediated liposome binding and uptake (*42*). The inhibition assays were performed by incubating the liposomes with cells as described above in the presence of one of the compounds. The final lipid concentration was 100 $\mu$M, the heparin concentration was 1 $\mu$g/mL, and the concentration of poly-L-lysine was 50 $\mu$M. To study the effect of energy depletion (*53*) on liposome binding and uptake, cells were incubated with 10 mM sodium azide and 25 mM 2-deoxyglucose in PBS at pH 7.4 for 1 h before the addition of apoE−peptide liposomes. All binding and uptake data were derived from two to four independent experiments and referred to the total protein mass of cells.

*Protein Determination*. The concentration of cell protein was determined using a Pierce Coomassie protein assay reagent kit (Perbio Science, Germany) and 96-well microplates. A total of 200 $\mu$L of reagent was added to a 20 $\mu$L aliquot of the cell suspension solubilized with 0.1 N NaOH (see above). Absorption was read at 595 nm on a Tecan Safire plate reader (Switzerland), and the protein concentrations were calculated on the basis of a BSA standard curve.

## RESULTS

*ApoE−Peptides.* The sequences of the used apoE-derived peptides are shown in Figure 1. The tandem repeat (141−150)$_2$ was N-terminally acetylated and C-terminally amidated. For covalent coupling the apoE−peptide was provided with a spacer sequence bearing a tryptophan residue for fluorescence monitoring and mercaptopropionic acid at the N terminus. The HPLC purity for all peptides was higher than 95%.

*Characterization of Blank Liposomes and apoE−Peptide Liposomes.* The liposomes prepared by extrusion (final pore sizes of 50 nm) had a mean diameter of 75 nm. The thiol-modified peptide was coupled via the maleimide function onto the distal end of PEG chains as shown in Figure 1. Successful coupling of peptide and lipid was confirmed by MALDI−TOF mass spectrometry giving a molecular mass distribution around 5800 g/mol. The result corresponds to the theoretical mass of mal-PEG-PE and bound peptide with a variation of 44 mass units, which is characteristic of the mass distribution of PEG chains (data not shown). Peptide coupling slightly increased the mean diameter of the liposomes to 78 nm, but the polydispersity index of about 0.1 was indicative of a narrow size distribution. Peptide conjugation was additionally monitored by tryptophan fluorescence. The comparison of the fluorescence intensities of vesicle-conjugated apoE−peptides and peptide standard solutions was used to determine the coupling efficiency (Figure 2). At a peptide/lipid ratio in the reaction mixture of 1:200 (mol/mol), the ratio of coupled peptide to lipid was 1:400. The blue shift in the emission maximum from 356 nm of apoE−peptide dissolved in tris(hydroxymethyl)aminomethan (Tris) buffer to 350 nm when coupled to liposomes is generally observed when tryptophan is transferred into a less polar environment or becomes motionally restricted (*54*). Because the surface of the PEG liposomes is rather polar, we associate the observed blue shift with motional constraints of the bound peptide. If it is assumed that 25 000 lipid molecules in the outer layer of a liposome with a diameter of 75 nm are accessible to the peptide and with a content of 2.5 mol % of reactive PEG maleimide lipid and a coupling efficiency of 50%, approximately 100 peptide molecules were located at the liposome surface.

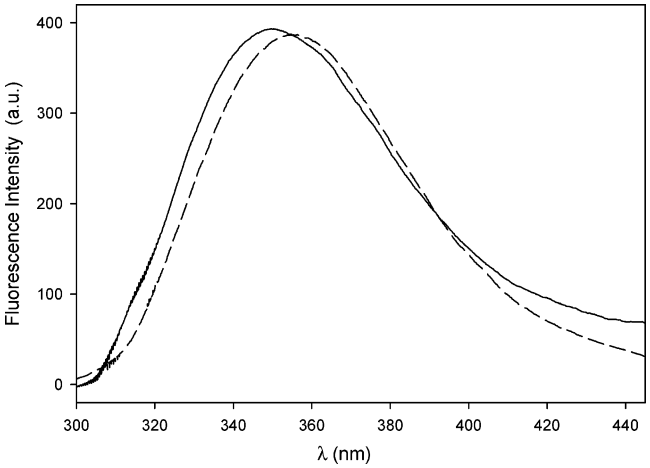


FIGURE 2: Fluorescence spectra of apoE−peptide liposomes at a lipid concentration of 0.53 mM (—) and of the tandem dimer peptide HS(141−150)$_2$ at a concentration of 1.25 $\mu$M (- - -). The fluorescence was excited at 280 nm.

*Competition of apoE−Peptide for $^{125}$I-LDL-Binding to the LDLr.* The binding abilities of the (141−150)$_2$ peptide and the natural ligand human LDL to the LDLr of human fibroblasts are compared in Figure 3. Fibroblasts are known for high LDLr expression (*50*). For the natural ligand LDL, 50% displacement of $^{125}$I-LDL ($IC_{50}$) was found at a concentration of 2.5 $\mu$g/mL, which corresponds to 5.2 nM. The binding constant of LDL has been reported to be 2.8 nM (*49*). Binding of the apoE−peptide (141−150)$_2$ was determined at a concentration of 7 $\mu$g/mL corresponding to

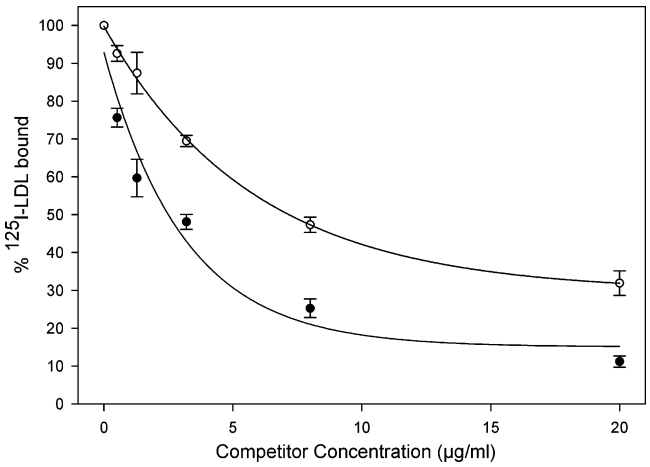


FIGURE 3: Ability of the tandem dimer peptide $(141-150)_2$ (○) and human LDL (●) to compete with $^{125}$I-labeled LDL for binding to the LDLr of fibroblasts. Cells were incubated with the ligands for 3 h at 4 °C in the presence of 2 $\mu$g/mL $^{125}$I-labeled LDL.

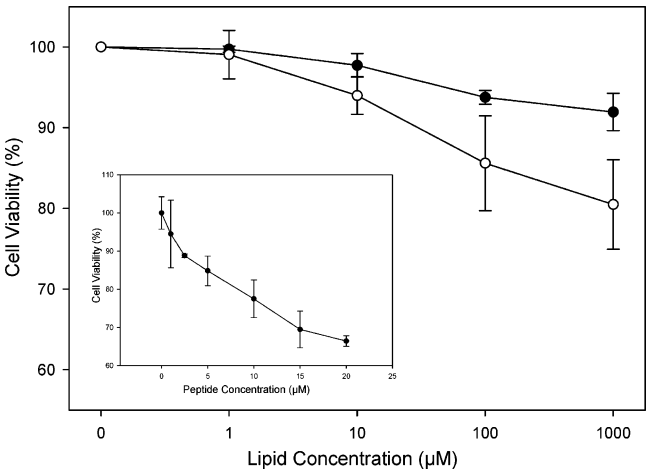


FIGURE 4: Cell viability of primary RBCECs. The toxic effect of blank liposomes (●), apoE−peptide liposomes (○), and HS$(141-150)_2$ (inset) was determined after cell incubation for 1 h at 37 °C using the MTT assay.

2.6 $\mu$M for 50% $^{125}$I-LDL displacement. The affinity of the tandem dimer compared to the natural ligand was 1000-fold reduced, a result that is in accordance with reports about comparable peptides (*28*).

*Toxicity*. Toxicity studies showed that the viability of primary RBCECs was dependent on peptide and liposome concentrations (Figure 4). Up to a concentration of 20 $\mu$M of the apoE−peptide HS$(141-150)_2$, cell viability was higher than 65% (inset of Figure 4). For comparison, 3.2 and 4 $\mu$M concentrations of the tandem dimers $(141-155)_2$ and $(141-149)_2$, respectively, reduced viability of interleukin 2-dependent T lymphocytes to 50% (*55*). Liposomes at 1 mM lipid concentration without coupled apoE−peptide reduced cell viability to 93%. Cell viability of 81% after peptide coupling suggested that the toxic effects of vesicles and vector peptide were additive. At 1 mM lipid concentration, the peptide concentration was about 2.5 $\mu$M and contributed about 12% to the cytotoxic effect (inset of Figure 4).

*Liposome Uptake−CLSM Studies.* ApoE−peptide liposomes were efficiently internalized into primary RBCECs (Figure 5). Cells treated with rhodamine-labeled apoE−peptide liposomes showed a strong vesicular intracellular fluorescence when incubated at 37 °C (Figure 5A). Treatment at 4 °C did not lead to any detectable fluorescence within the cells (data not shown). The temperature dependence and the vesicular distribution of the intracellular fluorescence pointed to an endocytotic uptake process by engulfing the liposomes into endocytotic vesicles. The uptake-mediating role of apoE−peptides was confirmed in experiments with blank liposomes. No intracellular rhodamine fluorescence was found at 37 °C (Figure 5B) nor at 4 °C.

*LDLr Binding and Uptake of apoE−Peptide Liposomes into Fibroblasts*. Low peptide affinity to the LDLr but efficient uptake of apoE−peptide liposomes into RBCECs initiated studies of the uptake mechanism. As shown in Figure 6, binding of apoE−peptide liposomes to human fibroblasts with normal and up-regulated LDLr expression did not show significant differences up to a lipid concentration of 100 $\mu$M. Up-regulation of LDLr expression under lipopoprotein-deficient culturing conditions is known to be very efficient (*49*, *50*). We observed a 25-fold increase in binding of $^{125}$I-LDL after receptor up-regulation (data not shown). Also, blocking the LDLr-binding site with an antibody had no influence on the interaction between apoE−peptide liposomes and the cells (Figure 7). Uptake experiments performed at 37 °C showed a pattern comparable to Figure 7 (not shown). Blank liposomes did not interact with fibroblasts. The observations correlated with the outcome of CLSM studies with primary RBCECs (Figure 5) and suggested that apoE−peptide liposomes do not interact specifically with the LDLr. Their membrane translocation might be dominated by an LDLr-independent pathway.

*Mechanism of Liposome Internalization*. To gain insight into the mode of liposome uptake, the effect of endocytosis inhibitors and the role of cell-surface HSPG were investigated. Treatment of cells with sodium azide, a cytochrome oxidase inhibitor, and 2-deoxyglucose, a glycolytic inhibitor, leads to depletion of ATP and subsequent inhibition of all energy-requiring internalization processes such as endocytosis and transcytosis (*53*). As shown in Table 1, binding and uptake of apoE−peptide liposomes under energy-depleting conditions was reduced to 30 and 15%, respectively. Furthermore, removal of the cell-surface HSPG by heparinase I treatment reduced binding and uptake of apoE−peptide liposomes to 50% (Table 1). Figure 8 illustrates that an increased heparinase concentration was concomitant with a reduction in apoE−peptide liposome binding, whereas blank liposomes did not interact with HSPG. In experiments with heparin as the competitor of HSPG and polycationic poly-L-lysine as the competitor of HSPG ligands, both binding and uptake of apoE−peptide liposomes were almost completely abolished (Table 1). These results suggested that the internalization of apoE−peptide-functionalized PEG liposomes may follow an HSPG-dependent pathway.

## DISCUSSION

Large and charged bioactive compounds cannot penetrate through cell membranes, and especially their transport through the tightly packed endothelial cell layer of the BBB requires active-transport mechanisms. Liposomes as carriers equipped with uptake facilitating and targeting ligands have been recognized to be an effective tool for the brain delivery of such compounds (*9*, *43*).

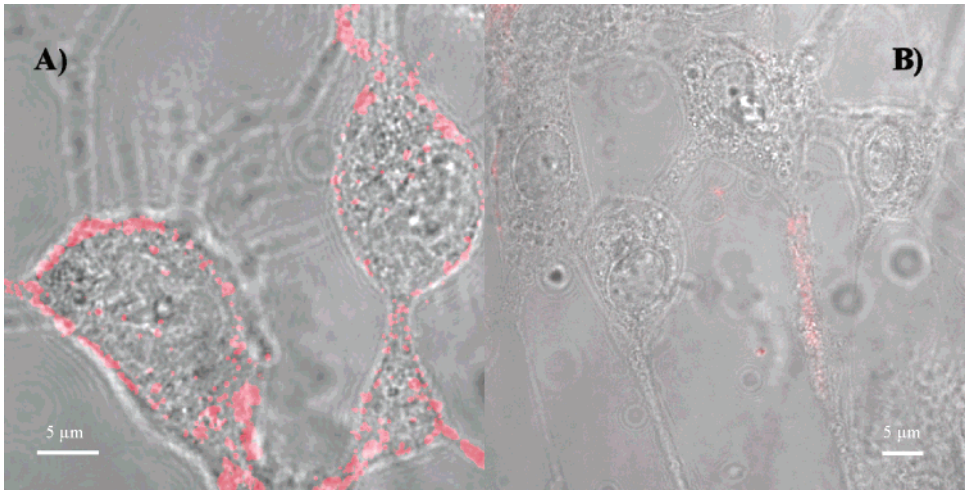


FIGURE 5: CLSM images of primary RBCECs exposed to apoE−peptide (A) and blank (B) liposomes. Liposome uptake at a lipid concentration of 33 $\mu$M was examined within 10 min after 30 min of incubation at 37 °C.

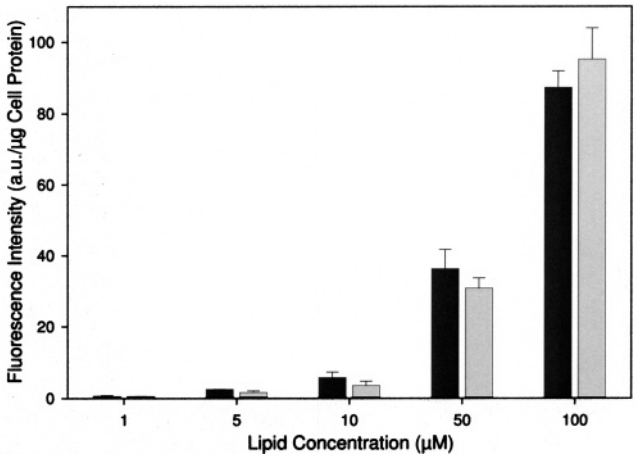


FIGURE 6: Binding of rhodamine-labeled apoE−peptide liposomes at primary human fibroblasts as a function of the lipid concentration. The cell-bound fluorescence at normal (gray bars) and up-regulated (black bars) LDLr expression levels was monitored after incubation for 3 h at 4 °C.

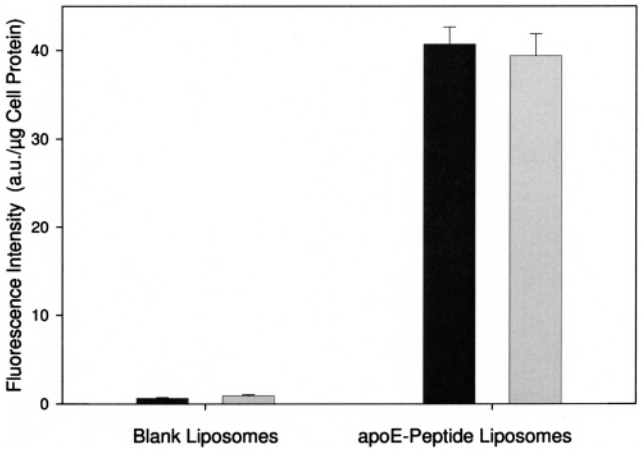


FIGURE 7: Binding of rhodamine-labeled apoE−peptide and blank liposomes at primary human fibroblasts with (gray bars) and without (black bars) blocking the LDLr. The LDLr was blocked by treating the cells before the experiment with a polyclonal anti-LDLr antiserum for 1 h at 4 °C. The fluorescence was monitored after cell incubation with liposomes at a lipid concentration of 50 $\mu$M for 3 h at 4 °C.

This study describes the preparation of a vector−carrier complex by covalent coupling of an apoE-derived peptide to sterically stabilized liposomes. An efficient peptide-mediated uptake of the complexes into primary rat brain capillary endothelial cells was observed, whereas no internalization of liposomes without coupled apoE−peptide could be found. The fluorescence-labeled apoE−peptide liposomes accumulated in distinct intracellular compartments, and the dependence of the temperature pointed to an endocytotic process. Independence of binding and uptake into human fibroblasts of the level of LDLr expression and receptor blocking by an antibody led to the suggestion that the uptake of apoE−peptide liposomes into cells was dominated by an LDLr-independent mechanism. Cell-surface HSPG was identified as the membrane component playing a major role in the uptake process into fibroblasts and was very likely also responsible for the effective internalization of apoE−peptide liposomens into brain microvessel endothelial cells.

Previous studies have shown that peptides encompassing the LDLr-binding domain of apoE compete with LDL for binding to the LDLr (*28*, *30*). Our studies showed that the tandem dimer of the sequence (141−150) is also able to recognize the LDLr. However, compared to the natural ligand LDL, its affinity was much reduced. The receptor-active structure of apoE is known (*26*). To be active, association of the apolipoprotein with phospholipids is required. Upon opening the N-terminal four-helix bundle, the binding region is exposed in a binding-competent helical conformation.

Table 1: Binding and Uptake of apoE−Peptide Liposomes into Human Fibroblasts[a]

| treatment | effect | binding (%) | uptake (%) |
|---|---|---|---|
| | | 100 | 100 |
| Na, $N_3$ untreated | energy depletion | 30 | 15 |
| 2-deoxyglucose | | | |
| heparinase I | removal of HSPG | 50 | 50 |
| heparin | competitor of HSPG | 3 | 3 |
| poly-L-lysine | competitor of HSPG ligands | 8 | 8 |

[a] Effect of various additives on apoE−peptide liposome binding and uptake into human fibroblasts. The cells were incubated with liposomes at a lipid concentration of 100 $\mu$M, and rhodamin fluorescence was registered as described in the Materials and Methods. The $NaN_3$ and 2-deoxyglucose concentrations were 10 and 25 mM, respectively, the heparinase I concentration was 5 units/mL, and the heparin and loly-L-lysine concentrations were 1 $\mu$g/mL and 50 $\mu$M, respectively.

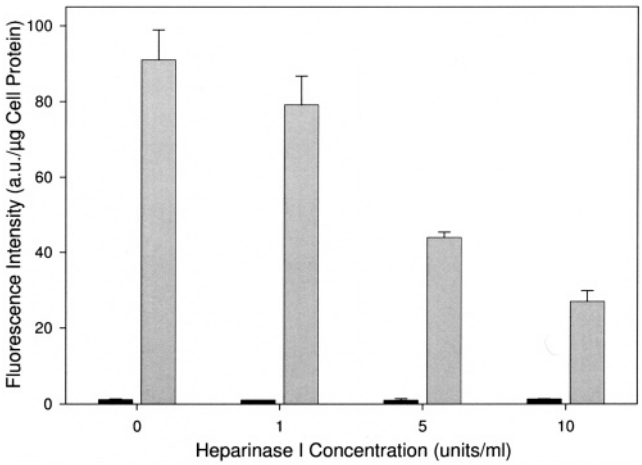


FIGURE 8: Binding of rhodamine-labeled liposomes to primary human fibroblasts as a function of the heparinase I concentration. The fluorescence of apoE−peptide (gray bars) and blank (black bars) liposomes bound to cells with an up-regulated LDLr expression level was monitored as function of HSPG removal after cell incubation with increasing concentrations of heparinase I for 2 h at 37 °C prior to a binding experiment. Liposomes at a lipid concentration of 50 $\mu$M were allowed to bind to the cells for 3 h at 4 °C.

Studies of apoE mutants and peptides derived from the LDLr-binding region confirmed that the LDLr affinity is highly dependent on the amino acid composition and the lipid-bound conformation (*28*, *31*, *32*). The apoE−peptide $(141-150)_2$ is a flexible chain in aqueous solution, and when coupled to PEG-modified lipids, it remains located in a rather hydrophilic environment as shown by the fluorescence properties of tryptophan. Furthermore, the vector peptide lacks the sequence 170−180, which was found to be important either by contributing one (arginine 172) or more residues for interaction with the LDLr or by stabilizing or aligning the receptor-binding region (*25*). Also the particle size (*56*) and the density of LDLr recognition sequences on the vesicle surface (*57*) have been found to be critical for LDLr binding and uptake.

Cell-surface HSPG plays an intriguing role in the cellular uptake of various ligands (*58*, *59*). The highly negatively charged cell-surface protein binds a large number of cationic molecules, catalyzes encounters between ligands and signaling receptors, and can be rapidly internalized, thus mediating adsorptive endocytosis of bound compounds. Our arginine- and lysine-rich apoE-derived vector peptide is highly cationic in nature. Furthermore, the sequence 141−150 includes the high-affinity binding side of apoE for heparin (*60*, *61*), a close structural homologue of HSPG. Thus, the tandem dimer $(141-150)_2$ will confer pronounced HSPG-affinity upon liposomes (*62*). Evidence for a HSPG-dependent uptake of apoE−peptide liposomes was obtained in experiments with heparinase-treated fibroblasts (*63*) and in the presence of heparin and poly-L-lysine, a high-affinity ligand for HSPG (*42*). Because of the broad distribution on mammalian cells, HSPG is expected to play a key role also in the internalization of apoE−peptide liposomes into the endothelial cells of brain microvessels. This suggestion is supported by reports on HSPG-mediated cellular uptake of the cationic TAT peptide (*64*), polycationic nonaarginine (*65*), and TAT-tagged liposomes (*66*) and the observation that cationic peptide−drug chimera (*4*) and liposomes coupled to cationized albumin (*13*) enter brain capillary endothelial cells via adsorptive endocytosis.

HSPG is also involved in the apoE-related clearance of remnant lipoproteins (*67*), and an apoE−peptide consisting of residues (141−150) and a model class A amphipathic helix has been shown to enhance internalization of LDL into cells via an HSPG-mediated pathway (*33*). The dependence of uptake upon charge distribution of the peptide led to the suggestion that also specific components might be involved (*35*). Most likely, specific interactions with the LDLr-related protein play a role (*20*). The apoE dimer peptide $(141-155)_2$ binds to the LDLr-related proteins (*28*), and binding of the sequences (130−149) and $(141-155)_2$ to fragments of the LDLr-related protein 1 has recently been reported (*68*). Also, the neurotoxcicity of apoE fragments is probably mediated by a HSPG/LDLr-related protein complex (*69*).

In summary, it was shown that an apoE−peptide can be covalently linked to liposomes to achieve uptake into brain capillary endothelial cells. However, localization of the tandem dimer peptide $(141-150)_2$ on the polar surface of PEG−liposomes was not sufficient to activate a specific LDLr-dependent transport route. Furthermore, the involvement of HSPG is expected to stimulate unspecific liposome transport into all HSPG-exposing cells. Nevertheless, the highly efficient uptake of apoE−peptide liposomes into brain capillary endothelial cells showed that vector peptide coupling to liposomal drug carriers might be a substitute for direct drug coupling to increase the amount of drug transported to the brain. Changes in the sequence, different anchoring and enhanced density of the vector peptide on the vesicle surface, or modifications in the composition and size of liposomes might influence an LDLr-dependent process. Whether quantitative differences in the complex uptake into different cells, indicative of a certain selectivity based on differences in the sulfation pattern of HSPG, exist and whether such differences provide a basis for cell-selective targeting is currently under investigation.

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