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SCIENCE DIRECT®
European Journal of Pharmaceutical Sciences 25 (2005) 299–305
EUROPEAN JOURNAL OF PHARMACEUTICAL SCIENCES
www.elsevier.com/locate/ejps

Targeting liposomes with protein drugs to the blood–brain barrier in vitro

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Received 16 June 2004; received in revised form 1 March 2005; accepted 8 March 2005
Available online 18 April 2005

Abstract

In this study, we aim to target pegylated liposomes loaded with horseradish peroxidase (HRP) and tagged with transferrin (Tf) to the BBB in vitro. Liposomes were prepared with the post-insertion technique: micelles of polyethylene glycol (PEG) and PEG-Tf were inserted into pre-formed liposomes containing HRP. Tf was measured indirectly by measuring iron via atomic absorption spectroscopy. All liposomes were around 100 nm in diameter, contained 5–13 μg HRP per μmol phospholipid and 63–74 Tf molecules per liposome (lipo Tf) or no Tf (lipo C).

Brain capillary endothelial cells (BCEC) were incubated with liposomes at 4 °C (to determine binding) or at 37 °C (to determine association, i.e. binding + endocytosis) and the HRP activity, rather than the HRP amount was determined in cell lysates. Association of lipo Tf was two- to three-fold higher than association of lipo C. Surprisingly, the binding of lipo Tf at 4 °C was four-fold higher than the association of at 37 °C. Most likely this high binding and low endocytosis is explained by intracellular degradation of endocytosed HRP.

In conclusion, we have shown targeting of liposomes loaded with protein or peptide drugs to the BCEC and more specifically to the lysosomes. This is an advantage for the treatment of lysosomal storage disease. However, drug targeting to other intracellular targets also results in intracellular degradation of the drug. Our experiments suggest that liposomes release some of their content within the BBB, making targeting of liposomes to the TfR on BCEC an attractive approach for brain drug delivery.

Keywords: Transferrin receptor; Horseradish peroxidase; Blood–brain barrier; Endosomes; Lysosomes

1. Introduction

The central nervous system (CNS) is protected by the blood–brain barrier (BBB). This barrier is located at the interface between blood and brain and its primary function is to maintain homeostasis in the brain. Unique features, such as tight junctions, low vesicular transport and high metabolic activity accomplish this barrier function (Rubin and Staddon, 1999). Drug delivery to the brain is limited due to the BBB. Only small molecules (molecular weight lower than 600 Da) can pass the BBB paracellularly or transcellularly, depending on their lipophilicity (Pardridge, 1999). However, high molecular weight drugs, such as (recombinant) proteins, peptides or DNA, do not cross the BBB (Pardridge, 2002).

Over the years, many drug targeting and delivery strategies have been explored. Drug delivery strategies to the brain involve chimeric proteins, in which the protein drug is covalently linked to a transport vector (Pardridge, 1998). For the delivery of antisense drugs, encapsulation of the antisense in targeted liposomal drug carriers has been applied (Pardridge, 1998; Shi et al., 2001). In our previous work, we have shown

Abbreviations: BBB, blood–brain barrier; BCEC, brain capillary endothelial cells; EPC-35, egg-phosphatidyl choline, partially saturated; HBS, HEPES buffered saline; HRP, horseradish peroxidase; PEG, polyethylene glycol (MW 2000); PBS, phosphate buffered saline; p.i., polydispersity index; PL, phospholipid; TL, total lipid; SATA, *N*-succinimidyl-*S*-acetylthioacetate; TCEP, Tris(2-carboxyethyl)phosphine hydrochloride; Tf, transferrin; TfR, transferrin receptor; TMB, 3,3′,5′5-tetramethylbenzidine

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doi:10.1016/j.ejps.2005.03.008

that drug targeting to the transferrin receptor (TfR) with conjugates of transferrin (Tf) and horseradish peroxidase (HRP) shows accumulation of Tf-HRP in brain capillary endothelial cells (BCEC) in vitro (Visser et al., 2004b). We used the endogenous ligand Tf, as it provides an opportunity to study the mechanisms behind receptor-mediated endocytosis. Furthermore, we use bovine BCEC, since our in vitro BBB model is established with these cells (Gaillard et al., 2001). For in vivo drug targeting to the TfR the antibody OX26 is often used, since this has no competition from endogenous Tf (examples in Pardridge, 1998; Shi et al., 2001). However, the antibody OX26 against the rat TfR does not bind to the bovine TfR.

In this study, we extend our investigations to deliver proteins to the BBB. Therefore, we have incorporated HRP in Tf-tagged pegylated liposomes. By using liposomes the amount of drug molecules per targeting vector (i.e. Tf) can be increased. HRP was chosen as a model compound, since it does not cross the BBB (Broadwell, 1989; Banks and Broadwell, 1994) and is readily quantified. Liposomes were prepared according to the post-insertion technique (Iden and Allen, 2001; Moreira et al., 2002), in which liposomes containing HRP were incubated with micelles of polyethylene glycol (PEG) and PEG-Tf. Tf was tagged to the distal end of the PEG chain via a maleimide-thiol coupling. Primary BCEC were incubated with liposomes at 4 °C (to determine binding) or at 37 °C (to determine association, i.e. a combination of binding and endocytosis). Incubation was performed based on adding equal HRP concentrations in liposomal form, to be able to compare non-tagged liposomes with Tf-tagged liposomes. From these studies with Tf-tagged liposomes and from our previous research on Tf-HRP conjugates (Visser et al., 2004b), we are able to indicate differences between route and rate of uptake of Tf-tagged liposomes and Tf-protein conjugates. In this respect, we postulate some conclusions about the differences in intracellular trafficking and fate of HRP.

2. Materials and methods

2.1. Materials

Culture flasks were obtained from Greiner (Alphen a/d Rijn, the Netherlands) and 96 wells plates from Corning Costar (Cambridge, MA, USA). PBS, DMEM, supplements and fetal calf serum were purchased from BioWhittaker Europe (Verviers, Belgium). Type IV collagen, heparin, trypsin-EDTA, endothelial cell trypsin, HRP, sodium deoxycholic acid, TMB liquid substrate, SATA, TCEP, HEPES, L-cystein and cholesterol were obtained from Sigma (Zwijndrecht, the Netherlands) and fibronectin from Boehringer Mannheim (Almere, the Netherlands). Bovine holo-Tf, BSA and hydroxylamine-HCl were obtained from ICN Pharmaceuticals (Zoetermeer, the Netherlands) and sodium chloride from Merck (Amsterdam, the Netherlands). EPC-35 was purchased from Lipoid GmbH (Ludwigshafen, Germany), PEG_{2000}-DSPE from Avanti Polar Lipids Inc. (Alabaster, AL, USA) and PEG_{2000}-maleimide-DSPE from Shear Water Corporation (Huntsville, AL, USA). Vivaspin columns are obtained from Vivascience AG (Hannover, Germany) and polycarbonate filters for extrusion from Nuclepore (Pleasanton, USA). The Biorad protein assay was obtained from Bio-Rad Laboratories (Veenendaal, the Netherlands).

2.2. Cell culture

Primary brain capillary endothelial cells (BCEC) were cultured from isolated bovine brain capillaries as described before (Gaillard et al., 2001). Briefly, brain capillaries were seeded in type IV collagen and fibronectin-coated plastic culture flasks and cultured in a 1:1 mixture of DMEM (containing 2 mM L-glutamin, 100 U/ml penicillin, 100 μg/ml streptomycin, non-essential amino acids and 10% fetal calf serum) and astrocyte-conditioned medium (ACM), supplemented with 125 μg/ml heparin at 37 °C, 10% CO_2 for 4–5 days. ACM was obtained as described before (Gaillard et al., 2001). ACM is added to the cell culture medium to induce BBB properties of the BCEC, while the TfR is not influenced by addition of ACM (Visser et al., 2004a). At 70% confluence the BCEC were passaged with trypsin-EDTA and seeded into a type IV collagen coated 96 wells plate at a density of 15,000 cells/well. Subsequently, BCEC were cultured for 4–5 days at 37 °C, 10% CO_2.

Brain capillaries as well as BCEC were cultured without the addition of iron or iron scavengers. Under these conditions we have found a good TfR expression and internalisation (Visser et al., 2004a).

2.3. Preparation of liposomes containing HRP, using the post-insertion technique

Bovine holo-Tf (containing two iron atoms) was modified with a thiol group using SATA (1:8 molar ratio) as described before (Visser et al., 2004c). Micelles of PEG_{2000}-DSPE and PEG_{2000}-maleimide-DSPE (in a molar ratio of 1:1) were prepared by hydration of the lipid film in 0.25 ml HEPES buffered saline (HBS), pH 6.5. Directly after hydration micelles were incubated with SATA modified Tf (1 or 3 mg Tf per 9 μmol phospholipid) in the presence of TCEP (0.01 mM) for 2 h at room temperature under constant shaking. To block all excess maleimide groups, micelles were incubated with L-cystein for 30 min, subsequently 1 mM NEM was added to block free thiol groups. Free maleimide groups need to be blocked to prevent non-specific reactions with other components during the preparation or the use of liposomes. To obtain control liposomes, PEG micelles were incubated with L-cystein for 2 h, followed by a subsequent addition of NEM.

Liposomes were prepared of egg phosphatidyl choline (EPC-35) and cholesterol in a 2:1 molar ratio, by resuspending a lipid film in HBS with a final HRP concentration of 300 μg/μmol phospholipid (PL). Liposomes (approximately 10 mM PL) were extruded stepwise, through 200, 100, 80 and 50 nm polycarbonate filters (four times per filter size)