Nanoparticles for therapy of Alzheimer Disease

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ABSTRACT

NAD project (large cooperative; 19 partners, funded by the E.C., FP72007-2013, G.A. n° 212043) is aiming to the therapy and diagnosis, even combined, of Alzheimer Disease, by creating different multiple-functionalized nanoparticles, able to bind, detect and remove β-amyloid peptide (Aβ) from the brain and from the blood. The results obtained so far show the ability of liposomes functionalized to bind Abeta, to cross the blood-brain barrier either in vitro and in vivo and to reduce the Aβ burden from the brain of transgenic animal models of the disease, thus opening new vistas for the possible therapy of Alzheimer Disease in humans.

Keywords: liposomes, Alzheimer, Abeta

1 INTRODUCTION

Alzheimer Disease (AD) causes continuous deterioration of higher nervous functions, due to progressive and irreversible neurodegeneration of the limbic and association cortices, leading to the total loss of autonomy and eventually to death. Although substantial progress has been made in the scientific understanding of AD, there remains an urgent need to identify effective therapies and early detection strategies, in order to avert a financially overwhelming public health problem. Several evidences suggest that the overproduction and subsequent accumulation of β-amyloid (Aβ), a proteolytic fragment composed of 40 to 42 a.a. residues, derived from the membrane-associated amyloid precursor protein (APP), plays a central role in AD. This peptide is released from cells in a soluble form that progressively aggregates, forming oligomers, fibrils and ending with the deposition of extracellular plaques, detectable post-mortem in AD brains. Plaques are one of the morphological hallmarks of the disease, along with intracellular fibrillary tangles constituted of hyper-phosphorylated protein tau. Finally, brain and blood Aβ are in equilibrium through the blood brain barrier (BBB), and sequestration of Aβ in the blood may shift this equilibrium, drawing out the excess from the brain (“sink” effect).

2 MATERIALS AND METHODS

2.1 Preparation of liposomes

Liposomes were prepared by an extrusion method in 10 mM phosphate buffered saline buffer (PBS), pH 7.4 through a 100-nm pores polycarbonate filters and were composed of sphingomyelin (Sm) and cholesterol (Chol) at 1:1 molar ratio. Sm and Ch were mixed with 5% molar phosphatidic acid (PA). Liposomes contained also 2.5 molar% of mal-PEG-PE for subsequent functionalization. Briefly, lipids were mixed in chloroform/methanol (2:1, v/v) and dried under a gentle stream of nitrogen followed by a vacuum pump for 3 h to remove traces of organic solvent. The resulting lipid film was rehydrated in phosphate-buffered saline (PBS), vortexed and then extruded 10 times at 55 °C through a stack of two polycarbonate filters (100-nm pore size diameter) under 20 bar nitrogen pressure with an extruder. Liposomes were separated from possible unincorporated material by size-exclusion chromatography using PD-10 column and PBS as the eluent. Lipid recovery after PD-10 column was assessed by assaying the individual components or by measuring the radioactivity associated. Phospholipid recovery after extrusion was determined by phosphorous assay using the method of Stewart.

Finally, for some experiments liposomes were also containing containing a small amount of fluorescent sphingomyelin (BODIPY-Sm, 0.5 molar%) in the case of confocal microscopy experiments. Two different radioactive lipid tracers (3H-Sm and 14C-PA) were also present for quantification of cellular uptake, permeability measurements across the blood brain barrier and binding experiments.

Lipid recovery after extrusion was evaluated by assaying the individual components: size, polydispersity index and zeta potential were obtained using a ZetaPlus particle sizer and zeta-potential analyzer (Brookhaven Instruments, Holtsville, New York) at 25°C in PBS by dynamic light scattering with a 652-nm laser beam. size and polydispersity were obtained from the intensity autocorrelation function of the light scattered at a fixed angle of 90 degrees. The correlation function was analyzed by means of a two-cumulant expansion. Each measurement was performed under an electrical field of 29.7 V/cm.22 Standard deviations were calculated from at least three measurements. Stability was measured in buffer byfollowing size and polydispersity index by dynamic light scattering for 3 days.

2.2 Functionalization of liposomes with ApoE peptides or tat-peptide
The sequence corresponding to a.a. residues 141–150 of human ApoE peptide and its tandem dimer repeat (141–150) were synthesized on an automated synthesizer. The peptides were bearing at the C-terminal a tryptophan residue for fluorescence monitoring and ended with cysteine residue for covalent coupling with mal-PEG-PE in the liposome bilayer.

The sequence corresponding to residues 48-57 of human TAT protein was synthesized on an automated Applied Biosystems synthesizer model 433A (Applied Biosystems, Foster City, California) at 0.1 mM scale with NOVASYN-TGA resin, using Fmoc-protected L-amino acids. The peptides were bearing at the C-terminal a tryptophan residue for monitoring the peptide by fluorescence and ended with cysteine residue for covalent coupling with mal-PEG-DSPE. Amino acids were activated by reaction with O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium tetrafluoroborate and N,N-diisopropylethylamine. A capping step with acetic anhydride after the last coupling cycle of each amino acid was included. Peptides were cleaved from the resin with trifluoroacetic acid/water/3,6-dioxa-1,8-octanediethiol (90:5:5 vol/vol/vol, 180 min at 25°C), precipitated, and washed with diethyl ether. Crude peptide was then purified by reverse-phase high-performance liquid chromatography on a semipreparative C18 column (Symmetry 300; Waters Corporation, Milford, Massachusetts), and peaks collected were characterized by matrix assisted laser desorption/ionization mass spectrometry (MALDI-TOF). The sequence was GRKKRRQRRPPQGWC (2065.47 g/mol) and purity was higher than 95%.

mApoE or dApoE or tat-peptide was added to liposomes in PBS to give a final peptide:mal-PEG-PE molar ratio of 1:2:1, and incubated overnight at room temperature to form a thioether bond with mal-PEG-PE. Liposomes decorated with the peptide (ApoE-liposomes) were separated from unbound peptide using PD-10 column. The yield of coupling of the peptide to liposomes was assessed by measuring the tryptophan fluorescence intensity (λex = 280 nm) of the incubation mixture and of ApoE-liposomes recovered from the PD-10 column. Spectra were recorded between 300 and 450 nm using a Cary Eclipse spectrofluorometer (Varian). The amount of peptide bound to liposomes was calculated from the fluorescence intensity of a known amount of the peptide dissolved in PBS, taken as the standard.

2.3 Functionalization of PA-LIP with RI7217 or OX26 by covalent coupling

To covalently bind RI7217 or OX26 antibody to maleimide-containing LIP, free thiol groups were generated by reacting the antibody with Traut’s reagent in 0.15 M Na-borate buffer with 0.1 mM EDTA, pH 8.5. After incubation for 90 min at 25°C under N2, RI7217 solution was concentrated and the buffer exchanged with PBS (pH 7.4) using an Amicon filter device. Ellman’s reagent was used to determine the number of sulphydryl groups on the antibody, using acetyl-cysteine for the calibration curve. Using a antibody -iminothiolane (Traut’s reagent) molar ratio of 1:10, an average of 3 primary amines per antibody were thiolated. Thiolated RI7217 or OX26 was then incubated with LIP (4 mM) containing PE-PEG-mal overnight at 25°C at a molar ratio Antibody/ phospholipids of 1:1000. To remove the unbound antibody, the LIP suspension was passed through a Sepharose 4B-CL column (25x1 cm). The amount of RI7217 or OX26 bound to LIP and unbound was quantified by Bradford assay. Background absorbance values of buffer and control LIP without antibody were subtracted from sample absorbance values. Phospholipids recovery after column was determined as above described.

2.4 Permeability across an in vitro model of BBB

For transport experiments across a cell monolayer, Human brain endothelial cells (hCMEC/D3) were obtained from Institut National de la Santé et de la Recherche Médicale (INSERM, Paris, France), hCMEC/D3 cells cultured between passage 25 and 35 were used. For culturing, the cells were seeded at a concentration of 27000 cells/cm² and grown in tissue culture flasks coated with 0.1mg/ml rat tail collagen type 1. Under these culture conditions, hCMEC/D3 cells express P-gp polarised on the apical membrane.

A cell monolayer was usually formed 3 days after seeding, as judged by three criteria: (1) the cells formed a confluent monolayer without visible spaces between cells under a light microscope; (2) the height of the culture medium in the upper chamber had to be at least 2 mm higher than that in the lower chamber for at least 24 hours; and (3) a constant transendothelial electrical resistance (TEER) value, measured using an EVOM Endothelial chamber (World Precision Instruments, Sarasota, Florida) was obtained (typically between days 6 and 8). Wells were used when TEER value was higher than 100 Ω·cm².

All the permeability experiments were performed in serum-free medium at 37°C. In parallel experiments, the efflux of the hydrophilic marker [14C]sucrose (200 µM) added in the upper chamber was measured to evaluate the paracellular permeability. At the end of the experiments, TEER and [14C]sucrose PEs were again determined so as to confirm that sample application had resulted in no adverse effects on tight junction function. We also added to the mixture a very small amount of radiolabelled lipids, [3H]-sphingomyelin and [14C]-phosphatidic acid, as tracers of liposomes. The cellular transport of liposomes across hCMEC/D3 cell monolayer was determined by measuring the radioactivity in the bottom compartment of transwell system between 10-120 min of incubation at 37°C with liposomes, and calculated as described [4].
2.5 In vivo administration of ApoE-PA-liposomes to APP/PS1 Tg mice

Double transgenic mice containing the mutant versions of APP-PS1 human genes (strain - B6.Cg-TG(APPswe,Psen1dE9)85Dbo/J) were utilized as a mouse model of Alzheimer disease that develop "senile plaques" after 7-8 m ("amyloidosis")

Transgenic male mice (8 month-old) were treated intraperitoneally (i.p.) for 24 days, 3 times a week, with 100 µl (2.5mmol) of 40mM liposomes or with phosphate buffered saline as control. Mice were sacrificed under CO₂ and brain and blood were taken. Ab40 and Ab42 were measured in plasma and in brain homogenate by ELISA assay after extraction with formic acid. One hemibrain was fixed for 24 h in 4% paraformaldehyde in phosphate buffered saline (pH 7.4) and cryoprotected in graded concentrations of sucrose (15-30%) in PBS. The fixed tissue was then analyzed for Ab content by immunohistochemical analysis using mAb6E10.

3 RESULTS

NAD partners have realized liposomes specifically functionalized with PA to bind Aβ with high affinity: KD 60 nM as assessed by SPR. These NP proved their potential to be used for the therapy of AD by preventing, in vitro, neurotoxicity of Aβ peptide, and Aβ-induced Tau phosphorylation [1-3].

Liposomes were characterized by DLS. Sm/Chol liposomes containing 5% PA displayed a mean diameter of 114 nm, a PDI of 0.318 and a Z potential of −28.38±0.059 mV.

Further functionalizations for crossing the BBB have been performed and tested on a BBB in vitro transwell model utilizing brain capillary endothelial cells, human hCMEC/D3. The following ligands have been tested: i) Antibody anti-Transferrin-receptor; ii) ApoE-derived peptides; iii) Tat-1 peptide. The permeability was assessed by measuring the recovery of radioactivity in the lower chamber of the traswell apparatus. As shown, the best performing functionalization was attained using the monomeric ApoE peptide (fig. 1). After coupling with ApoE peptide, the size of liposomes slightly increased to 136 nm with PDI 0.198 and Z potential of −27.74±0.018.

The high negative zeta potential and the low PDI of ApoE-liposomes indicate that the liposomes are stable after functionalization with ApoE peptide, in according with previous data [5,6]. The size of the ApoE-liposomes remained unchanged for at least 48 h (data not shown). Moreover, since the ratio between 14C-PA and 3H-Sm in the upper chamber (where liposomes are added) and in the lower chamber (where the material crossing the BBB model is recovered) are comparable, it is likely that liposomes cross intactly the barrier model.

Double functionalization (both for binding Abeta and for crossing the BBB) enhances NP cell uptake and transport across the BBB model without affecting the affinity for Abeta (data not shown).

Fig. 1 Permeability of liposomes across HCMEC/D3 monolayer.
Permeability of Liposomes containing PA and different BBB ligands and radiolabeled with 14C-PA and 3H-Sm was measured by recovery of radioactivity across a transwell cellular model. Lipid dose = 200 nmols of total lipids, 2h incubation.

Experiments in vivo carried out on animal models of Alzheimer disease (single and double TG mice) show that after treatment with single or double-functionalized NP, the levels of β-amyloid circulating in blood and β-amyloid oligomers in brain have a tendency to decrease (data not shown).

Finally, parallel experiments suggest that treatment of TG mice with double-functionalized NP can decrease the content of Abeta in brain (Fig. 2).

Fig. 2 - Histochemical staining of brain sections of AD TG mice (9 months of age) treated with phosphate buffered saline (left panel) or with liposomes functionalized with PA and ApoE peptide (right panel) administered i.p. for 24 days, 3 times a week.

4 DISCUSSION

Liposomes composed of Sm/Ch, containing PA and functionalized with ApoE peptide display a strong affinity
in vitro against Abeta peptide in fibrillar and oligomeric form. The same liposomes are displayed the ability to cross a BBB in vitro model. When administered i.p. to TG mice models of Alzheimer disease, liposomes are able to reduce Abeta peptide burden in the brain. Taken all together, these results open new possibilities for the treatment of Alzheimer Disease.

REFERENCES