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Multifunctional liposomes delay phenotype progression and prevent memory impairment in a presymptomatic stage mouse model of Alzheimer disease

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ABSTRACT

The failure of clinical trials largely focused on mild to moderate stages of Alzheimer disease has suggested to the scientific community that the effectiveness of Amyloid-β (Aβ)-centered treatments should be evaluated starting as early as possible, well before irreversible brain damage has occurred. Accordingly, also the preclinical development of new therapies should be carried out taking into account this suggestion. In the present investigation we evaluated the efficacy of a treatment with liposomes multifunctionalized for crossing the blood-brain barrier and targeting Aβ, carried out on young APP/PS1 Tg mice, taken as a model of pre-symptomatic disease stage.

Liposomes were administered once a week to Tg mice for 7 months, starting at the age of 5 months and up to the age of 12 when they display AD-like cognitive and brain biochemical/anatomical features. The treatment prevented the onset of the long-term memory impairment and slowed down the deposition of brain Aβ; at anatomical level, prevented both ventricle enlargement and entorhinal cortex thickness reduction, otherwise occurring in untreated mice. Strikingly, these effects were maintained 3 months after treatment discontinuation. An increase of Aβ levels in the liver was detected at the end of the treatment, then followed also by reduction of brain Amyloid Precursor Protein and increase of Aβ-degrading enzymes. These results suggest that the treatment promotes brain Aβ clearance by a peripheral 'sink' effect and ultimately affects Aβ turnover in the brain.

Worth of note, the treatment was apparently not toxic for all the organs analyzed, in particular for brain, as suggested by the lower brain TNF-α and MDA levels, and by higher level of SOD activity in treated mice. Together, these findings promote a very early treatment with multi-functional liposomes as a well-tolerated nanomedicine-based approach, potentially suitable for a disease-modifying therapy of AD, able to delay or prevent relevant features of the disease.

1. Introduction

Alzheimer disease (AD) is the most common form of dementia, accounting for 60–80% of cases [1]. Although the cause and progression of AD are still not well understood, the central role of Amyloid-β (Aβ) peptide in AD pathogenesis is widely accepted, even if a variety of additional factors, either dependent or independent from Aβ, appears to contribute [2,3]. In fact, Aβ is thought to directly damage the brain, disrupting the synaptic functionality, which strongly correlates with the cognitive deficits characteristic of the pathology. Given its pivotal role, many Aβ-centered strategies have been attempted and are still in progress; however, several clinical trials focused on mild to moderate AD have been discontinued, suggesting that at that stage Aβ accumulation has already exerted substantial synaptic and neuronal loss, preventing a clinical recovery. As a matter of fact, accumulation and deposition of brain Aβ is a very early event in AD, and probably begins ~10–20 years prior to the onset of clinically detectable symptoms [4].

These evidences suggest that the effectiveness of Aβ-centered treatments should be evaluated starting as early as possible [5], well before irreversible brain damage has occurred and clinical trials are ongoing in this direction. Accordingly, also the preclinical development of new therapies should be carried out taking into account this

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suggestion.

Thus, in the present investigation we evaluated the efficacy of a treatment with Aβ-targeting liposomes carried out on young APP/PS1 Tg mice, taken as a model of presymptomatic stage of the disease, to prevent or slow down the onset of typical AD-like hallmarks, namely brain Aβ accumulation, cerebral anatomical abnormalities and memory impairment.

Within this frame, we utilized liposomes designed for AD treatment (mApoE-PA-LIP), dually functionalized with a synthetic peptide (mApoE) containing the receptor-binding domain of apolipoprotein-E for blood-brain barrier targeting and crossing, and with phosphatidic acid (PA) for Aβ binding [6,7]. These liposomes have been previously utilized for acute treatment of aged symptomatic Tg mice, taken as a severe AD model [8], while in the present investigation their efficacy for therapy of AD at presymptomatic stage has been tested, adapting the animal model, the frequency and duration of the treatment to this specific purpose.

2. Materials and methods

2.1. Liposomes preparation and characterization

Preparation and characterization of mApoE–PA–LIP was carried out as repeatedly reported [7–10]. Briefly, liposomes were prepared by extrusion procedure using polycarbonate filters (100 nm pore size diameter), and were composed of sphingomyelin (Avanti Polar Lipids) and cholesterol (Sigma) (1:1 M ratio), containing 5 mol% of phosphatidic acid (PA; Avanti Polar Lipids) for Aβ binding, and 2.5 mol% of 1,2-stearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(-poly(ethyleneglycol)-2000)] (mal-PEG-PE; Avanti Polar Lipids) for further surface functionalization with mApoE peptide for BBB targeting. mApoE peptide, carrying the amino acid sequence CWG-LRKLRKRLLR and containing residues 141–150 of the receptor-binding protein of human ApoE, was reacted to form a covalent thioether bond with mal-PEG-PE, resulting in formation of mApoE-PA-LIP [9,10].

Liposomes were freshly prepared and characterized for each round of administration, and size and ζ-potential were comparable to those described previously [7–10].

2.2. Animals and experimental design

Twenty APP/PS1 5-month-old Tg male mice (Jackson Laboratory, USA), mean weight of 28–30 g, and 20 non-Tg (WT) age-matched littermates were used. Mice were all drug and behavioral test naïve and no environmental enrichment was used because it notably improves AD pathology in mouse models of AD [11,12]. All procedures involving animals and their care were conducted according to European Union (EEC Council Directive 86/609, OJ L 358,1; December 12, 1987) and Italian (D.L. n.116, G.U., Suppl. 40, February 18, 1992) laws and policies, and in accordance with the United States Department of Agriculture Animal Welfare Act and the National Institutes of Health (Bethesda, MA, USA) policy on Humane Care and Use of Laboratory Animals. They were reviewed and approved by the Mario Negri Institute Animal Care and Use Committee that includes *ad hoc* members for ethical issues (1/04-D).

All animals (Tg or WT) were intraperitoneally injected with mApoE–PA–LIP (100 μl, 73.5 mg of total lipids/kg) or with vehicle (100 μl PBS) once a week for 7 months. Therefore, two experimental groups were treated with mApoE–PA–LIP (Tg and WT mice, n = 10 for each) and two control groups were treated with PBS (Tg and WT, n = 10 for each). The weight of the animals was recorded before each treatment. To minimize the effect of subjective bias, the treatment was performed in blind. Mice were treated always at the same time of the day (9:00–10:00 A.M.) in a specific room inside the animal facility, following a randomized order based on the draw of the animal identification code. At the end of treatment, five animals per group were sacrificed to assess treatment effects. The rest of the animals was kept for other three months without any kind of treatment and then sacrificed to analyze the duration of the effects after treatment discontinuation.

2.3. Novel object recognition test (NORT)

NORT is a memory test that relies on spontaneous animal behavior without the need for stressful elements such as food or water deprivation or foot-shock [13]. In the NORT, mice are introduced into an arena containing two identical objects that they can explore freely. 24 h later, they are reintroduced into the arena, with two objects one of which had already been presented (familiar) and the other new and completely different (novel). The day before the beginning of the treatment, after 4 months from its start and at the end of treatment, mice were tested in an open-square grey arena (40 × 40 cm), 30 cm high, with the floor divided into Twenty-five squares by black lines, placed in a specific room dedicated to behavioral analysis and separate from the operator's room. The following objects were used: a black plastic cylinder (4 × 5 cm), a glass vial with a white cap (3 × 6 cm), and a metal cube (3 × 5 cm). The task started with a habituation trial during which the animals were placed in the empty arena for 5 min and their movements recorded as the number of line-crossings, which provide an indication of both the WT and Tg mouse motor activity. The next day, mice were again placed in the same arena containing two identical objects (familiarization phase). Exploration was recorded in a 10-min trial by an investigator blinded to genotype and treatment. Sniffing, touching, and stretching the head toward the object at a distance of not > 2 cm were scored as object investigation. 24 h later (test phase), mice were again placed in the arena containing two objects: one of the objects presented during the familiarization phase (familiar object), and a new, different one (novel object), and the time spent exploring the two objects was recorded for 10 min. Mice were tested following a predefined scheme (five mice for each treatment group and the remaining mice by following the same scheme) so to precisely maintain the 24 h re-test for each mouse. Results were expressed as the percentage of time spent investigating objects in the 10 min or as a discrimination index (DI), *i.e.* (seconds spent on novel − seconds spent on familiar)/(total time spent on objects). Animals with no memory impairment spent longer investigating the novel object, giving a higher DI.

2.4. MRI analysis

Animals were anesthetized with isoflurane in a mixture of O_2 (30%) and N_2O (70%). Body temperature was maintained at ~37 °C by a warm water circulated heating cradle. Imaging was performed on a 7 T small bore animal Scanner (Bruker Biospec, Ettlingen, Germany). Two actively decoupled radio frequency coils were used: a volume coil of 7.2 cm diameter used as the transmitter and a surface coil as the receiver. A 3D RARE T2-weighted sequence was performed to assess anatomical changes. The morphological images were obtained with a voxel size of 117 × 147 × 147 μm (matrix = 256 × 102 × 102 and Field of View = 3 × 1.5 × 1.5 cm); TR = 2500 ms, effective TE = 50 ms and a RARE factor of 16, for 1 average.

The volume measurements of structural MRI images were obtained using Java-based custom made software. ROIs were manually chosen by a trained expert following the Paxinos' atlas [14]. Total intracranial volume, whole brain, cortex, hippocampus, striatum and the ventricular system were measured. Data from each animal were obtained by the integration of averaged ROI area for slice thickness.

To measure thickness of the entorhinal cortex, nine coronal slices were selected at the level between Bregma − 2.75 mm and Bregma − 3.80 mm based on mouse brain atlases [14] and the thickness was measured below the rhinal fissure [15]. We visually inspected all the coronal acquisitions to choose a reference image. We then registered all