Acta Biomaterialia 8 (2012) 2056–2067

Contents lists available at SciVerse ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

Antibody-conjugated PEGylated cerium oxide nanoparticles for specific targeting of Aβ aggregates modulate neuronal survival pathways

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ARTICLE INFO

Article history:
Received 5 October 2011
Received in revised form 11 January 2012
Accepted 31 January 2012
Available online 8 February 2012

Keywords:
Cerium oxide nanoparticle
Targeted therapy
Neurodegeneration
Nanoparticles
Neurotrophins

ABSTRACT

Oxidative stress has been found to be associated with the progression of neurodegenerative diseases such as Alzheimer's, Parkinson's, Lou Gehrig's, etc. In the recent years, cerium oxide nanoparticles (CNPs) have been studied as potent antioxidant agents able to exert neuroprotective effects. This work reports polyethylene glycol (PEG)-coated and antibody-conjugated CNPs for the selective delivering to Aβ aggregates, and the protective effect against oxidative stress/Aβ-mediated neurodegeneration. In this study PEG-coated and anti-Aβ antibody-conjugated antioxidant nanoparticles (Aβ-CNPs-PEG) were developed, and their effects on neuronal survival and brain-derived neurotrophic factor (BDNF) signaling pathway were examined. Aβ-CNPs-PEG specifically targets the Aβ aggregates, and concomitant rescue of neuronal survival better than Aβ-CNPs, by modulating the BDNF signaling pathway. This proof of concept work may allow in the future, once validated in vivo, for the selective delivery of CNPs only to affected brain areas.

1. Introduction

Amyloid-beta 1–42 (Aβ), local inflammation and the consequent production of reactive oxygen species are considered the major etiological and pathological factors in the promotion of neurodegenerative diseases such as Alzheimer's disease (AD) [1–6]. To date, the use of multiple doses of antioxidants to counteract these pathological conditions has met with only limited success [7]. Recently, we have discovered that cerium oxide nanoparticles (CNPs) are redox active and biocompatible materials with both superoxide dismutase [8] and catalase mimetic activity [9]. Among the lanthanide series of elements, cerium is distinctive in that it has two partially filled subshells of electrons, 4*f* and 5*d*, with many excited substates, resulting in a valence structure that undergoes significant alterations depending on the chemical environment [10–13]. Predominate +3 oxidation states on the surface of CNPs are responsible for their unique antioxidant properties [14,15]. We have shown that a single dose of CNPs prevents retinal degeneration induced by peroxides [16]. In an in vitro model, one low dose exerted radical scavenging activity and neuroprotective effects for a long duration and under multiple insults, suggesting the possibility of regenerative activity. Therefore, CNPs have been suggested as a nanopharmacological approach to diseases associated with oxidative stress [17–22]. Previously, using an AD human in vitro model, we have confirmed the antioxidant properties of bare CNPs. We have also demonstrated that CNPs do not act as mere antioxidant agents, but seem to regulate signal transduction pathways involved in neuroprotection [23]. The novelty of the approach described in this work takes advantage of nano- and biotechnological approaches to enhance the specific target of CNPs. We synthesized PEG-coated CNPs and subsequently conjugated anti-amyloid β antibody to the PEG-coated CNPs, obtaining selective delivery to the Aβ plaques and a concomitant increase in neuronal survival. Our results demonstrate that CNPs-Ab may be a potential candidate for anti-neurodegenerative therapy.

2. Materials and methods

2.1. Materials

Triton X-100, dimethylsulfoxide (DMSO), sodium dodecylsulfate (SDS), Tween20, bovine serum albumine (BSA), L-glutamine,

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1742-7061/$ - see front matter Published by Elsevier Ltd. on behalf of Acta Materialia Inc.
doi:10.1016/j.actbio.2012.01.035

4′,6-diamino-2-phenylindole dilactate (DAPI), Nonidet P40, sodium deoxycolate, ethylene diamine tetraacetate (EDTA), phenylmethanesulphonylfluoride (PMSF), superoxide dismustase (SOD) assay kit, sodium fluoride, sodium pyrophosphate, orthovanadate, leupeptin, aprotinin, pepstatin, NaCl, polyvinylidene difluoride (PVDF) sheets, fluorescein-labeled antirabbit and antimouse IgG antibodies, mouse anti-GAP-43, anti-heavy neurofilament (NF-H), anti-P 75 NTR antibodies, Aβ (25–35), Aß (1–42) and anti-β-amyloid (1–42) antibody produced in rabbit were all purchased from Sigma Chemical Co. (St Louis, CO, USA). Trypsin–EDTA solution and streptomycin–penicillin were from Gibco Invitrogen GmbH (Austria); Carboxy-PEG-amine [A(PEG)$_4$] was purchased from Thermo Scientific (Thermo Fisher Scientific Inc., Rockford, IL, USA); mouse anti-p-ERK1/2, rabbit anti-ERK1, rabbit anti-brain-derived neurotrophic factor (BDNF), rabbit anti-TrkB, anti-caspase 3, anti-pro-caspase 9 antibodies, horseradish peroxidase (HRP)-conjugated anti-rabbit and anti-mouse for immunoblotting experiments were from St Cruz Biotechnology (Santa Cruz, CA, USA); mouse anti-β-tubulin III antibody was from Promega (Mannheim, Germany); rabbit anti-phospho Ras/extracellular signal-regulated kinase 5 (pErk5) antibodies were purchased from Upstate, Millipore SpA (Milan, Italy). RPMI-1640 medium and fetal bovine serum (FBS) were from Euroclone Ltd (UK); the apoptosis assay kit was from Roche Diagnostic (Indianapolis, IN, USA); the micro-BCA protein detection kit was from Pierce (Rockford, IL, USA). Vectashield was purchased from Vector Laboratories (Burlingame, CA, USA). All other chemicals were of the highest analytical grade.

2.2. Methods

2.2.1. Synthesis and characterization of CNPs

CNPs 3–5 nm in size were synthesized by a microemulsion method as described elsewhere [36]. After preparation, the particles were washed with acetone and water 6–8 times to remove the surfactant and other impurities. High-resolution transmission electron microscopy (HRTEM), with a FEI Tecnai F30 equipped with an energy dispersive X-ray (EDX) analyzer, was carried out to study the size and morphology of nanoparticles.

2.2.2. Amine functionalization of CNPs

Prepared CNPs were suspended in 0.1 M NaOH solution and stirred for 5 min. 5 ml of distilled epichlorohydrin and 0.5 ml of 2 M NaOH were added and stirred at room temperature (RT). The nanoparticles were then recovered by centrifugation and washed with water several times. Then, the nanoparticles were suspended in water and 30% ammonium hydroxide solution was added to the suspension followed by stirring for several hours. Finally, amine-functionalized nanoparticles were recovered by centrifugation, washed with water (3 or 4 times), and dried [24,25].

XPS was used to confirm the amine functionalization; specta were obtained with a 5400 PHI ESCA spectrometer. The base pressure during XPS analysis was 10^{-10} Torr and Mg K_α X-radiation (1253.6 eV) at a power of 350 W was used.

2.2.3. Preparation of PEG-CNPs

Bifunctional PEG with carboxy- and amino-terminals, having a PEG spacer arm of 18.1 Å was selected for the study. We choose bifunctional PEG, as one end can connect to amine-functionalized nanoparticle and the other end to the antibody. The carboxy-terminal of the bifunctionalized PEG molecule was coupled to the amine-functionalized CNPs using 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) and *N*-hydroxysulfosuccinimide (Sulfo-NHS) coupling chemistry. Briefly, 1 mg ml^{-1} CA(PEG)$_4$ was dissolved in 0.05 M NaCl buffer, pH 6. 2 mM EDC and 5 mM Sulfo-NHS were added to the CA(PEG)$_4$ solution and stirred at RT. Amine-functionalized CNPs were then resuspended in sodium phosphate buffer and added to the reaction mixture and stirred. The molar ratio of amine-functionalized ceria:CA(PEG)$_4$ used for the reaction was 1:4. PEG-CNPs were recovered by centrifugation, washed with water (3 or 4 times), and dried. UV–visible spectroscopy and Fourier transform infrared (FTIR) spectra were collected to confirm presence of the PEG molecule on the nanoparticle surface using PerkinElmer Lamda750S and PerkinElmer Spectrum spectrometers, respectively. The SOD mimetic activity of the PEG-conjugated CNPs was estimated using a SOD Assay kit (Sigma–Aldrich) according to the manufacturer's instructions.

2.2.4. Conjugation of Aβ antibody with PEG-CNP

In the first step, sodium azide and other salts were removed from the anti-Aβ antibody (1–42) produced in rabbit (Sigma–Aldrich) by centrifuging through 10 kD cut-off centricon. This antibody recognizes Aβ (1–42), used in this work, which is the form present in the plaques, in vivo. Antibody (1 mg ml^{-1} concentration) was diluted in NaCl buffer, pH 6. PEG-CNPs were resuspended in sodium phosphate buffer, and added to the antibody and stirred for several hours for proper alignment of the antibody on the surface of the nanoparticles. Then, the EDC/Sulfo-NHS coupling reaction was used to conjugate the antibody to the nanoparticles. 2 mM EDC and 5 mM Sulfo-NHS were added to the reaction mixture and stirred at RT. The molar ratio of PEG-CNPs:Aβ used for the reaction was 1:5. PEG-CNPs-Ab (CNPs-Ab) were recovered by centrifugation, washed with water (3 or 4 times), and resuspended in distilled water. The concentration of CNPs after antibody conjugation was assayed by UV–visible spectroscopy. Bradford assay was performed to confirm the antibody conjugation to the PEG-CNPs.

2.2.5. Cell cultures

Human neuroblastoma-SH-SY5Y cells (ATCC) were cultured at 1×10^4 cells cm^{-2} for 7 DIV in FBS-free RPMI-1640 medium containing N2 supplement in order to obtain neuronal differentiation.

2.2.6. Aβ fibril formation

Aβ (25–35) was used as previously described [23] since it mimics the toxicological and aggregation properties of the full-length peptide, being more toxic to neurons and causing more severe oxidative damage [26]. The Aβ (25–35) stock solution (500 μM) was prepared in FBS-free medium containing N2 supplement (pH 7.4) [27]. Aβ (25–35) stock solution was incubated at 37 °C for 8 days to obtain the amyloid fibrils.

2.2.7. Fluorimetric assay

The Thioflavin T (ThT) fluorescence method was used to follow amyloid polymerization [28–30]. Following 8 days incubation, Aβ fibrils were diluted in 1.5 μM ThT to a final volume of 2 ml of 20 mM Tris–HCl buffer, pH 8.0, and analyzed using a spectrofluorimeter with excitation at 450 nm and emission at 485 nm, as previously described [31].

2.2.8. Treatments

Neurons were incubated with Aβ (25–35) (12.5 μM, f.c.) aggregates for 24 h. For nanoparticle treatment, cells, subjected for 4 h to acute insult with Aβ (25–35) aggregates, were then treated for another 20 h with 200 nM (f.c.) CNPs conjugated to anti-Aβ antibody (CNPs-Ab).

2.2.9. Aβ aggregate detection

In order to asses if the ligation of antibody to the nanoparticles allows specific binding to Aβ aggregates, double-immunofluorescence staining was performed. Briefly, cells were grown on coverslips and then fixed in 4% paraformaldehyde for 10 min at RT. Cells were then incubated with a 0.05% solution of ThT and