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Quantification of molecular interactions between ApoE, amyloid-beta (Aβ) and laminin: Relevance to accumulation of Aβ in Alzheimer's disease☆

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ABSTRACT

Accumulation of amyloid-β (Aβ) in plaques in the brain and in artery walls as cerebral amyloid angiopathy indicates a failure of elimination of Aβ from the brain with age and Alzheimer's disease. A major pathway for elimination of Aβ and other soluble metabolites from the brain is along basement membranes within the walls of cerebral arteries that represent the lymphatic drainage pathways for the brain. The motive force for the elimination of Aβ along this perivascular pathway appears to be the contrary (reflection) wave that follows the arterial pulse wave. Following injection into brain parenchyma, Aβ rapidly drains out of the brain along basement membranes in the walls of cerebral arteries; such drainage is impaired in apolipoprotein E ε4 (ApoE4) mice. For drainage of Aβ to occur in a direction contrary to the pulse wave, some form of attachment to basement membrane would be required to prevent reflux of Aβ back into the brain during the passage of the subsequent pulse wave. In this study, we show first that apolipoprotein E co-localizes with Aβ in basement membrane drainage pathways in the walls of arteries. Secondly, we show by Atomic Force Microscopy that attachment of ApoE4/Aβ complexes to basement membrane laminin is significantly weaker than ApoE3/Aβ complexes. These results suggest that perivascular elimination of ApoE4/Aβ complexes would be less efficient than with other isoforms of apolipoprotein E, thus endowing a higher risk for Alzheimer's disease. Therapeutic correction for ApoE4/Aβ/laminin interactions may increase the efficiency of elimination of Aβ in the prevention of Alzheimer's disease.

1. Introduction

A key feature of Alzheimer's disease pathology is the extracellular accumulation of soluble amyloid-β (Aβ) and of insoluble Aβ as plaques in brain parenchyma and in the walls of cerebral arteries as cerebral amyloid angiopathy (CAA) [1,2]. These features indicate that there is a failure of elimination of Aβ from the brain with increasing age and in Alzheimer's disease [3]. Mechanisms for elimination of Aβ from the brain include enzymatic degradation by neprilysin within brain tissue and artery walls; absorption of Aβ into the blood mediated by low density lipoprotein receptor-1 and elimination by lymphatic drainage along basement membranes in the walls of cerebral capillaries and arteries [4]. Accumulation of insoluble fibrillar Aβ in the walls of capillaries and arteries in CAA reflects failure of elimination of Aβ along lymphatic drainage pathways with age and Alzheimer's disease [5].

When soluble tracers, including Aβ, are injected into the brain parenchyma, they are rapidly eliminated along basement membranes of capillaries towards cervical lymph nodes [6,7]. The pattern of deposition of Aβ in the walls of capillaries and arteries in human CAA exactly mirrors the lymphatic drainage pathways defined in experimental tracer studies [5]. Tracers and Aβ appear to leave the walls of the carotid artery in the neck at the level of cervical lymph nodes as they drain from the brain to regional lymph nodes in the neck [6,8]. Perivascular drainage of Aβ from the brain is impaired with age as shown experimentally and by the presence of CAA in aging humans [9]. Aβ secreted by amyloid precursor protein (APP)-transgenic mice harbouring the Swedish double mutation driven by a neuron specific promoter is observed in the perivascular drainage pathways as CAA co-localized with apolipoprotein E (ApoE) [1]. Furthermore, perivascular drainage of Aβ is impaired in mice expressing human apolipoprotein E ε4 (ApoE4) suggesting that the risk factor for Alzheimer's disease in patients possessing the ε4 allele of ApoE may be related to a failure of elimination of Aβ from brain [10].

Studies on the motive force for perivascular drainage of Aβ from the brain suggest that solutes are driven along basement membranes in the walls of arteries by the contrary (reflection) wave that follows the pulse wave [11]. In order for this mechanism to function effectively, some form of attachment of transported material to basement membrane proteins would be required in order to prevent reflux of material during

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passage of the pulse wave itself. If no attachment activity were present, Aβ and other solutes would oscillate within the basement membrane rather than be driven rapidly out of the brain as has been observed experimentally. One of the major candidates for performing such attachment activity for Aβ is ApoE.

ApoE is the predominant lipoprotein in the brain and regulates transport of cholesterol from astrocytes to neurons [12–14]. Three ApoE alleles (ε2, ε3 and ε4) encode the production of corresponding protein isoforms (E2, E3 and E4). Binding of Aβ to ApoE has been proposed as a mechanism by which Aβ is transported across the blood–brain barrier [4] and levels of ApoE are lower in ApoE4-positive individuals than in ApoE3 carriers [15]. Recent work has demonstrated minimal direct physical interaction between ApoE and soluble Aβ within the cerebrospinal fluid [16]. Thus, the role of ApoE in mediating the clearance of Aβ from the brain remains unresolved.

Since Aβ40 is the predominant type of Aβ found in CAA [17], in the present study we tested the hypothesis that interactions of Aβ40 with protein components of cerebral vascular basement membranes, such as laminin, are stronger in the presence of ApoE3 than in the presence of ApoE4. If this hypothesis is substantiated, it would suggest that perivascular drainage of Aβ in individuals possessing ApoE4 would be less efficient due to defective attachment of Aβ/ApoE4 complexes to basement membranes during perivascular lymphatic drainage. This would ultimately lead to failure of elimination of Aβ from the brain and its deposition in artery walls as CAA.

In order to test the hypothesis, we first identified the location of ApoE in relation to fibrillary Aβ within basement membranes in the walls of arteries in AD. Secondly, we performed single-molecule force spectroscopy with an atomic force microscope (AFM) in order to determine the force of attachment between Aβ/ApoE4 complexes and the basement membrane protein, laminin. We then compared the attachment forces of Aβ/ApoE4 complexes with those of Aβ/ApoE3.

2. Materials and methods

2.1. Materials for AFM tip functionalization

Analytical grade materials used for AFM tip functionalization were obtained from Sigma Aldrich, UK. The following chemicals were used: ethanol, chloroform, ethanolamine hydrochloride (ethanolamine-HCl), dimethylsulfoxide (DMSO), triethylamine (TEA), sodium hydroxide (NaOH), and sodium cyanoborohydride ($NaCNBH_3$). Aldehyde–PEG–NHS linker was purchased from the Institute of Biophysics, University of Linz, Austria. Human recombinant laminin-511 was purchased from (BioLamina, Sweden), while human Aβ40 (referred to as Aβ for the rest of the manuscript), human ApoE3 and human ApoE4 were from Cambridge Bioscience (Cambridge, UK).

2.2. Immunofluorescence of human tissue

Paraffin sections from 5 cases diagnosed with Alzheimer's disease from the South West Dementia Brain Bank, Frenchay Hospital, Bristol were utilised for immunostaining. Sections of middle frontal gyrus were immunostained with antibodies specific for Aβ42 (clone 21F12, 1:4000), pan-apolipoprotein E (pan-apoE, clone 5F6, 1:2000) provided by Elan Pharmaceuticals Inc. (USA). We could not access an antibody specific for Aβ40 that worked on human tissue, so we used Aβ42, as we know that Aβ42 becomes entrapped in the cerebrovascular amyloid deposits [18,19]. Smooth muscle actin (SMA: clone 1A4, Dako, UK, 1:100) was used to identify smooth muscle cells in the blood vessel walls.

Immunostaining was performed using the appropriate antigen retrieval methods for each primary antibody. For Aβ42, pan-apoE and ApoE E4 sections were pre-treated with neat formic acid. Triple immunostaining was detected using AF594 (red, Aβ42) or AF633 (blue, pan-apoE) fluorochromes conjugated with biotinylated secondary antibodies (Life Technologies, UK) and SMA-FITC (Abcam, UK, 1:200, green), respectively. A Leica SP5 confocal scanning microscope was used for imaging.

2.3. Atomic force microscope measurements

2.3.1. Functionalization of AFM measuring tips

The AFM silicon nitride tips (MSNL-10, Bruker, UK) were functionalized with the desired protein following three modification steps: (1) amino functionalization, (2) modification with aldehyde–PEG–NHS linker, and (3) ligand coupling. AFM cantilevers were washed in chloroform three times and dried under a stream of nitrogen before tips were subjected to modification. Amino functionalization was done by esterification with ethanolamine at room temperature [20]. AFM tips were then placed in a closed container with the ethanolamine-HCl solution, left overnight, washed three times in DMSO and ethanol and dried under a stream of nitrogen. Subsequent functionalization steps were performed following a custom tip modification protocol provided by Agilent Technologies, Inc. [21]. The PEG linker was immobilized on aminated AFM probes by the NHS ester terminus (step 2). 3.3 mg of aldehyde–PEG–NHS linker was dissolved in 1 ml chloroform, and transferred into a small glass reaction chamber. 10 μl of triethylamine was added before amino-functionalized AFM tips were immersed into the solution. The chamber was covered to prevent chloroform evaporation. After 1.5 h, tips were removed from the solution, washed three times in chloroform, and dried under the stream of nitrogen. The use of PEG spacer, as an intermittent link for biomolecule attachment to the cantilever, provides important advantages in molecular recognition force spectroscopy [22–26]. The linker is chemically and physically inert, allowing rapid and free reorientation of biomolecules. The spacing between molecule and the tip reduces the likelihood of molecules being crushed during the probe-surface contact. Non-linear elastic properties of PEG make it easy to discriminate between the non-specific and specific interaction events.

Proteins (Laminin, ApoE3, or ApoE4) were immobilized on AFM probes using the amine–amine reactive linker aldehyde–PEG–NHS. A sheet of parafilm was pressed into a glass petri dish. AFM cantilever chips were set onto the film in a circular "wagon wheel" pattern so that the tips were pointed upward and inward. 10–30 μl of the protein solution was applied onto the cantilevers. The proteins were allowed to react for 1 h to couple via intrinsic amino groups to the aldehyde-function of the PEG linker on the tip. After 1 h, 5 μl of 1 M ethanolamine was added to the protein solution drop to inactivate unreacted aldehyde groups.

2.3.2. Substrate preparation for AFM experiments

Substrates for AFM experiments were prepared as follows: 20 μl of protein solution, i.e. Aβ, ApoE3, ApoE4, or complexes of ApoE3 + Aβ (1:1 M ratio), and ApoE4 + Aβ (1:1 M ratio). Complexes were left to react at 4 °C for 1 h. Solutions were added onto a freshly cleaved mica (Agar Scientific, UK) substrate which was already inserted into a liquid cell. 100 μl of dH_2O were added to a cell. Proteins were left to adsorb to the substrate for 30 min. The mica substrate was washed with water to ensure that only adsorbed proteins remained on the substrate.

Aβ has a tendency to form large aggregates with time [27]. To maintain the solubility state of Aβ, fresh protein was deposited on the mica every 4 h, and a new functionalized AFM tip was used in the experiments. Images of Aβ were captured immediately after deposition and before the possible Aβ rearrangement, i.e. after 4 h. No Aβ agglomeration in fibrillar form was observed on the mica during any stage of the experiment.

2.3.3. Single molecule force spectroscopy experiments

Sample imaging and molecular force spectroscopy experiments were performed in water using Agilent 5500 Scanning Probe Microscopy, MAC III, Agilent Technologies, US. Images were acquired using the

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