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Cationization increases brain distribution of an amyloid-beta protofibril selective F(ab')$_2$ fragment

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ABSTRACT

Antibodies and fragments thereof are, because of high selectivity for their targets, considered as potential therapeutics and biomarkers for several neurological disorders. However, due to their large molecular size, antibodies/fragments do not easily penetrate into the brain. The aim of the present study was to improve the brain distribution via adsorptive-mediated transcytosis of an amyloid-beta (Aβ) protofibril selective F(ab')$_2$ fragment (F(ab')$_2$-h158). F(ab')$_2$-h158 was cationized to different extents and the specific and unspecific binding was studied *in vitro*. Next, cationized F(ab')$_2$-h158 was labelled with iodine-125 and its brain distribution and pharmacokinetics was studied in mice. Cationization did not alter the *in vitro* affinity to Aβ protofibrils, but increased the unspecific binding somewhat. *Ex vivo* experiments revealed a doubling of brain concentrations compared with unmodified F(ab')$_2$-h158 and *in vivo* imaging with single photon emission computed tomography (SPECT) showed that the cationized F(ab')$_2$-h158, but not the unmodified F(ab')$_2$-h158 could be visualized in the brain. To conclude, cationization is a means to increase brain concentrations of therapeutic antibodies or fragments and may facilitate the use of antibodies/fragments as imaging biomarkers in the brain.

1. Introduction

Central to Alzheimer's disease is amyloid-beta (Aβ), a 40-42 amino acid long hydrophobic and self-aggregating peptide. Aβ monomers gradually aggregate into soluble oligomeric assemblies and eventually into insoluble fibrils, the main constituents of amyloid plaques which are a hallmark of AD. It is generally accepted today that aggregated soluble forms of Aβ (oligomers/protofibrils) rather than insoluble fibrils, are the toxic form of Aβ, causing the synaptic failure that eventually leads to dementia [1–7]. Moreover, soluble forms of Aβ correlate better than fibrils with disease severity [1,8,9]. Thus, efforts are today devoted towards therapeutic targeting and diagnosis of soluble Aβ aggregates [10]. Immunotherapy utilizing therapeutic proteins such as antibodies and fragments thereof is considered as a potential treatment strategy in several neurological diseases including AD [11]. Antibodies and fragments (Fab, F(ab')$_2$), scFv etc.) are large molecules, and hence, do not readily cross the blood-brain barrier (BBB). For example, it is generally anticipated that less than 0.1% of the injected antibody dose reaches the brain [12]. The low concentrations that reach the brain is one of the main hurdles for therapeutic use of protein drugs for neurological disorders. Large doses thus have to be administrated to achieve therapeutic concentrations in the brain. In addition to the cost (protein drugs are in general much more expensive than small molecular drugs), systemic side effects may hinder the use of the protein drug at doses needed for achieving a therapeutic effect on a brain target. Several approaches have been investigated to increase large protein delivery to the brain [13]; osmotic opening of the BBB, different types of carrier particles (liposomes, nanoparticles), receptor-mediated transcytosis and modifications of surface charges, e.g. cationization. Some of the methods can be used in combination; liposomes that are carrying a net positive charge have been conjugated to proteins aimed at enhancing BBB or passage via receptor-mediated transcytosis [14].

Various glycoproteins attached to the surface of the endothelial cells of the BBB contribute to the negatively charged surface membrane of the BBB. Increased unspecific interaction between proteins aimed for targets inside the brain and the BBB can thus be

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achieved by increasing the positive charges on the protein through cationization. This leads to an increased probability of the protein to be taken up by and transported across the endothelial cells of the BBB. This process is called adsorptive-mediated transcytosis and has been described as a promising strategy for *in vivo* delivery of macromolecules across the BBB [15,16]. In the present study a F(ab')$_2$ fragment (F(ab')$_2$-h158) was generated by cleavage of a humanized variant of the Aβ protofibril selective mouse monoclonal antibody mAb158 [17]. F(ab')$_2$-h158 was then cationized with the polyamine putrescine with the objective of increasing its brain distribution while retaining its affinity to Aβ protofibrils. The putrescine cationized F(ab')$_2$-h158 (pF(ab')$_2$-h158) was labelled with iodine-125 (^{125}I) and brain distribution was studied *ex vivo* and *in vivo* using single photon emission computed tomography (SPECT).

2. Methods

2.1. Generation of a F(ab')$_2$-fragment

A humanized variant of the mouse monoclonal antibody mAb158, selectively binding to Aβ protofibrils [17,18] was enzymatically cleaved with the enzyme IdeS, specifically cleaving just below the hinge region [19], according to a previously published method, to generate F(ab')$_2$-h158 [20].

2.2. ELISA binding experiments

ELISA was used to evaluate the specific binding of the antibody/fragments to Aβ monomers and protofibrils (here defined as soluble Aβ aggregates larger than 100 kDa, eluting in the void volume on a Size Exclusion Superdex 75 column) and to evaluate any increased nonspecific binding to proteins in general. A 96-well ELISA plate was coated with 10 nM Aβ monomers or protofibrils, prepared as previously described [21], or PBS for nonspecific binding analysis, and blocked for 1 h with BSA. The antibody/fragment to test (h158, unmodified F(ab')$_2$-h158 or pF(ab')$_2$h158, modified at different pH), was added in dilution series and incubated for 2 h on a shaker. Secondary antibody; HRP-conjugated goat-anti human IgG, F(ab')$_2$ fragment specific (Jackson ImmunoResearch Inc.) diluted 1:2000 was added to each well and the plates were incubated 1 h on a shaker. Signals were developed with K blue aqueous TMB substrate (Neogen Corp., Lexington, KY, USA) and read with a spectrophotometer at 450 nm. All antibody dilutions were made in ELISA incubation buffer (0.1% BSA and 0.05% Tween-20 in PBS with 0.15% Kathon.

2.3. Cationization

F(ab')$_2$-h158 was cationized using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) as a crosslinker between F(ab')$_2$-h158 and the polyamine putrescine as previously described [16]. The reaction was carried out at two different pH (4.7 or 5.5) to achieve different levels of cationization. In short, the cationization was performed as following: Zeba™ micro spin desalting columns (Thermo Scientific, Rockford, IL, USA) were used to change the buffer of F(ab')$_2$-h158 to 0.1 M 2-(*N*-morpholino) ethanesulfonic acid (MES) with a pH of 4.7 or 5.5. The samples were mixed with equal amount of 1 M putrescine dihydrochloride in corresponding 0.1 M MES buffer. EDC-hydrochloride was diluted in mQ-H_2O to a concentration of 30 mg/ml and added to each sample to a final concentration of 2 mg/ml. The samples were incubated on a shaker for 120 min at 1000 rpm before dialysis with Slide-A-Lyzer 3 × 30 min in 500 ml of PBS. Following dialysis, the concentration of F(ab')$_2$-h158, after cationization termed pF(ab')$_2$-h158, was measured with a spectrophotometer.

To evaluate the level of cationization in the samples, Vivapure ion exchange (IEX) mini H spin columns (Sartorius Stedim Biotech GmbH, Goettingen, Germany) were used. Cationized pF(ab')$_2$-h158 was allowed to bind to the column matrix and was then eluted with buffer of increasing pH, ranging from pH 8 to pH 14. The higher level of cationization, the higher pH was required for the elution of pF(ab')$_2$-h158, which was accomplished around the isoelectric point of the protein. Each sample was analyzed with ELISA to investigate at what pH the cationized pF(ab')$_2$h158 fragments were eluted.

2.4. Radiolabeling

Direct radioiodination of pF(ab')$_2$h158 or unmodified F(ab')$_2$h158 with iodine-125 (^{125}I) was performed using Chloramine-T [22]. The method is based on electrophilic attack of the phenolic ring of tyrosine residues by *in situ* oxidized ^{125}I. Briefly, 250 pmoles of pF(ab')$_2$-h158, ^{125}I stock solution (Perkin-Elmer Inc., Waltham, MA, USA) and 5 μg Chloramine-T (Sigma, St. Louis, MO, USA) were mixed in PBS to a final volume of 110 μl. The reaction was allowed to proceed for 90 s and subsequently quenched by addition of 10 μg of sodium metabisulfite (Sigma) and dilution to 500 μl in PBS. The radiolabeled proteins were immediately purified from free iodine and low-molecular weight components with a disposable NAP-5 size exclusion column (GE Healthcare AB, Uppsala, Sweden) according to the manufacturer's instructions (cut-off 5 kDa) and eluted in 1 ml of PBS. Labeling was always performed on the same day as the experiment.

2.5. Animals

All animals experiments described in this paper were approved by the Uppsala County Animal Ethics board (#C216/11 and #C110/11) following the rules and regulations of the Swedish Animal Welfare Agency and the EU Directive 2010/63/EU for animal experiments. The transgenic model used was the tg-ArcSwe model harboring the *Arctic (AβPP E693G)* and *Swedish (AβPP KM670/671NL)* mutations. Tg-ArcSwe mice show elevated levels of large soluble Aβ aggregates already at a very young age and abundant and rapidly developing plaque pathology starting at around 6 months of age [23–25]. The animals were housed with free access to food and water in rooms with controlled temperature and humidity in an animal facility at Uppsala University.

2.6. Ex vivo and SPECT experiments

Tg-ArcSwe (>16 months; n = 29) and wild-type (wt) mice (4 months; n = 6) were anesthetized with isoflurane at 2, 4, 6-8 or 24 h after a single intraperitoneal (i.p.) injection of 4.9 ± 1.3 MBq [^{125}I]pF(ab')$_2$-h158 or [^{125}I]F(ab')$_2$-h158 with a specific activity of 95 ± 26 MBq/nmole. A blood sample was obtained from the heart followed by intracardiac perfusion with 50 ml physiological saline during 2 min. Following perfusion, brains were isolated and radioactivity was measured with a γ-counter (1480 Wizard™, Wallac Oy, Turku, Finland).

In addition to the terminal blood samples obtained in all animals, blood samples (8 μl) were obtained from the tail vein for a subset of animals also at 0.5, 1, 2, 3, 4, 6, 8, 12 and 24 h after injection.

Two measures of brain distribution was used; The brain-to-