Cationization increases brain distribution of an amyloid-beta protofibril selective F(ab’)2 fragment

Stina Syvänen a, Desirée Edén b, Dag Sehlin a, *

a Department of Public Health and Caring Sciences/Geriatrics, Uppsala University, Rudbeck Laboratory, SE-751 85 Uppsala, Sweden
b Department of Medical Sciences, Clinical Chemistry, Uppsala University Hospital, SE-751 85 Uppsala, Sweden

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 administered 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
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 to be changed the buffer of F(ab')2-h158 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 (125I) 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 different pH (4.7 or 5.5) to achieve different levels of cationization. In short, the cationization was performed as follows: Zebar™ 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 mQH2O 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 (125I) was performed using Chloramine-T [22]. The method is based on electrophilic attack of the phenolic ring of tyrosine residues by in situ oxidized 125I. Briefly, 250 pmole of pF(ab')2-h158, 125I 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 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 isoflorane at 2, 4, 6-8 or 24 h after a single intraperitoneal (i.p.) injection of 49 ± 3.3 MBq [125I]pF(ab')2-h158 or [125I]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-
blood (K_p) concentration ratio was calculated as following:

\[
K_p = \frac{\text{Measured radioactivity per gram brain tissue}}{\text{Measured radioactivity per gram blood}}
\]  

(1)

In addition, concentrations in brain and blood were quantified as % of injected dose (% ID/g) and was calculated as following:

\[
\%ID_g = \frac{\text{Measured radioactivity per gram brain tissue (or blood)}}{\text{Injected radioactivity}}
\]  

(2)

For SPECT imaging, 17-18 month old tg-ArcSwe mice were i.v. injected with either [125I]pF(ab')-h158 or [125I]F(ab')-h158. Starting 1 h post injection, mice were SPECT scanned for 60 min using a Triumph trimodality System (TriFoil Imaging, Inc., Northridge, CA, USA), followed by a 3 min CT examination. Mice were kept anesthetized with 1.5–2.0% isoflurane in 50% medical oxygen and 50% air during the SPECT and CT scans and then perfused as above.

2.7. Statistical analyses

Results reported are always mean ± standard deviation. Data were statistically analyzed with unpaired Student’s t-test. Significance levels were labelled *p < 0.05 and **p < 0.01.

3. Results

The humanized Aβ protofibril specific antibody h158 was enzymatically cleaved below the hinge region, yielding a pure preparation of F(ab')2-h158 (Fig. 1A), with retained Aβ binding properties, as compared to h158 (Fig. 1B). F(ab')2-h158 was then cationized at two different pH, 4.7 or 5.5 and analyzed with IEX spin columns and ELISA to determine the level of cationization. A reaction pH of 4.7 and 5.5 resulted in elution from the IEX column at pH 11.4 and pH 11.1, respectively, compared to the unmodified fragment eluting at pH 9.6. Cationization of F(ab')2-h158 was reproducible and did not alter the affinity to Aβ protofibrils (Fig. 1C), but resulted, as expected, in increased unspecific binding to BSA (Fig. 1D).

The fragment modified at pH 4.7, hereafter referred to as pF(ab')2-h158, was chosen for the subsequent preclinical experiments on the basis of being the most cationized fragment. It was anticipated that the level of unspecific binding was acceptable and even beneficial for interaction with the endothelial cells of the BBB. Radiolabeling with 125I did not alter the binding capacity of pF(ab')2-h158 to protofibrils and only slightly increased the BSA binding compared to unlabeled pF(ab')2-h158. Labeling yield, calculated based on the added radioactivity and the radioactivity in the purified radioligand solution, was 45 ± 3% and 77 ± 9% for pF(ab')2-h158 and F(ab')2-h158, respectively.
Pharmacokinetics in blood, studied after i.p. injection of \([125I]\) F(ab')\(_2\)-h158 and \([125I]\)pF(ab')\(_2\)-h158 in 4 month old wt mice, is shown in Fig. 2A. The elimination half-life in blood was similar for both proteins; 3.5 ± 0.1 h for \([125I]\)F(ab')\(_2\)-h158 and 3.3 ± 0.3 h for \([125I]\)pF(ab')\(_2\)-h158 (p > 0.05 n.s.). However, \([125I]\)pF(ab')\(_2\)-h158 had a lower C\(_{\text{max}}\) that occurred somewhat later than C\(_{\text{max}}\) for \([125I]\) F(ab')\(_2\)-h158, indicating a somewhat lower bioavailability of the cationized antibody fragment (Fig. 2A).

Both radiolabeled proteins displayed an increasing brain-to-blood concentration ratio up to 4 h followed by a subsequent decrease. The brain-to-blood concentration ratio (K\(_p\)) of the cationized fragment, \([125I]\)pF(ab')\(_2\)-h158, was at least doubled at all time points studied (except 1 h post injection) compared to unmodified \([125I]\)F(ab')\(_2\)-h158 (Fig. 2B, Table 1). The brain concentrations, measured as %ID/g (Equation (2)) was increased 60% at 2 h (Table 1).

SPECT imaging was performed in 17–18 month old tg-ArcSwe mice 2 h after injection of \([125I]\)pF(ab')\(_2\)-h158 or \([125I]\)F(ab')\(_2\)-h158 (Fig. 3). A SPECT image from the mouse injected with the cationized antibody fragment showed accumulation of radioactivity in central parts of the brain, i.e. near the third ventricle (Fig. 3A) while the mouse injected with the unmodified fragment did not display any radioactive signal in the brain (Fig. 3B). The cationized \([125I]\)pF(ab')\(_2\)-h158 was also diffusely distributed in regions where A\(_\beta\) pathology is generally deposited (Fig. 3C).

### Table 1

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>([125I])F(ab')(_2)-h158</th>
<th>([125I])pF(ab')(_2)-h158</th>
<th>Difference (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.015 ± 0.007</td>
<td>0.013 ± 0.005</td>
<td>0.8</td>
</tr>
<tr>
<td>2</td>
<td>0.007 ± 0.006</td>
<td>0.019 ± 0.003</td>
<td>2.8*</td>
</tr>
<tr>
<td>4</td>
<td>0.012 ± 0.002</td>
<td>0.022 ± 0.003</td>
<td>1.8*</td>
</tr>
<tr>
<td>6</td>
<td>0.005 ± 0.001</td>
<td>0.015 ± 0.001</td>
<td>3.0</td>
</tr>
</tbody>
</table>

% of injected dose per gram brain (%ID/g)

<table>
<thead>
<tr>
<th>% of injected dose per gram brain (%ID/g)</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>0.04 ± 0.01</td>
<td>0.03 ± 0.02</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>2.0</td>
<td>0.04 ± 0.01</td>
<td>0.06 ± 0.01</td>
<td>1.6*</td>
<td></td>
</tr>
<tr>
<td>4.0</td>
<td>0.06 ± 0.02</td>
<td>0.09 ± 0.01</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>6–8</td>
<td>0.02 ± 0.005</td>
<td>0.02</td>
<td>1.0</td>
<td></td>
</tr>
</tbody>
</table>

All values are means ± SD. *p < 0.05, **p < 0.01. In all groups, n ≥ 3, except the 6–8h \([125I]\)pF(ab')\(_2\)-h158 group, where n = 1.

The lower brain concentrations after cationization was probably due to larger distribution also to other organs than the brain, an observation reported also in previous studies [15,16]. This was confirmed by the unchanged half-life in blood, i.e. elimination itself was not affected by cationization. The increased K\(_p\) of the cationized fragment (Fig. 2B), which was mainly a result of decreased blood concentrations, is especially important for imaging where a large amount of radioactivity in the blood pool of the brain (around 5%) would otherwise give rise to a confounding signal.

The brain distribution of \([125I]\)F(ab')\(_2\)-h158 and \([125I]\)F(ab')\(_2\)-h158 was then visualized \(in vivo\) with the imaging technique SPECT. Whereas \([125I]\)F(ab')\(_2\)-h158 was clearly distributed to the center of the brain, likely representing the third ventricle, \([125I]\)F(ab')\(_2\)-h158 did not generate any SPECT signal (Fig. 3A-B). This observation is in line with our previous finding that mAb158 accumulates in the choroid plexus [21], a phenomenon that could be enhanced by cationization. The cationized fragment also displayed a diffuse uptake in frontal cortical brain regions (Fig. 3C), known to contain large amounts of A\(_\beta\) plaques in this mouse model [26]. As soluble A\(_\beta\) oligomers and protofibrils have been reported to be generated around plaques [27], the SPECT signals seen here is likely to represent the presence of such A\(_\beta\) species, further supported by our previous findings with PET imaging using the same antibody fragment engineered for receptor mediated transcytosis [20]. In vivo quantification of soluble A\(_\beta\) aggregates could potentially be used as a diagnostic tool to predict disease stage in AD patients, likely including the group of patients displaying diffuse plaque pathology that today are diagnosed falsely as amyloid-negative.
with the available amyloid PET radioligands.

Both cationization and radiolabeling methods were stable and reproducible. Unspecific binding increased somewhat after cationization, but as a moderate increase of the unspecific binding was the goal with the cationization in the first place, this was not considered problematic. Labeling yield was somewhat higher for unmodified F(\(\text{ab'}\))\(_2\)-h158, possibly due to the introduction of polyamines on the protein surface, but importantly, radiolabeling did not alter the affinity to \(\text{A}\beta\) protofibrils which could potentially happen if \(^{125}\text{I}\) is attached to tyrosine residues in the antigen binding region. Radionuclide \(^{125}\text{I}\) is suitable for SPECT imaging which is a less sophisticated, non-quantitative imaging modality with lower sensitivity in comparison to PET, that is used for imaging of \(\text{A}\beta\) plaques in the human brain. To use pF(\(\text{ab'}\))\(_2\)-h158 for PET imaging, the same labeling method can be used with PET radionuclide iodine-124 \((^{124}\text{I})\). Alternatively, since pF(\(\text{ab'}\))\(_2\)-h158 has a very short biological half-life it may be advantageous to develop labeling with fluorine-18 \((^{18}\text{F})\), which is regarded as the most suitable radionuclide due to its short half-life (110 min) and its high degree of positron emission.

In addition to having developed a new potential radioligand targeting soluble \(\text{A}\beta\) protofibrils, we believe that the development of methods to increase antibody and large protein delivery to the brain is important not only for this specific project, but also as a general technique that can be applied to increase brain concentrations of therapeutic or diagnostic antibodies and biologics in a wide range of neurological disorders. While approaches such as receptor mediated transcytosis are powerful techniques to achieve this goal [28], the method described here is uncomplicated, reproducible and well suited for translation. Cationization could thus serve as an alternative or complement to more elaborate ways to increase brain distribution of large molecules.

Acknowledgements

We would like to thank Veronika Asplund and Ram Kumar Selvaraju for assistance with SPECT scanning. We are also grateful to BioArctic AB for providing a F(\(\text{ab'}\))\(_2\) fragment of the humanized variant of the \(\text{A}\beta\) protofibril selective mouse monoclonal antibody mAb158. This work was supported by grants from the Swedish Research Council (#2012-1593). Funders were not involved in planning and conducting the research or writing the manuscript.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2017.09.065.

Author contributions

S.S. and D.S. designed the research; S.S., D.E. and D.S. performed the research; S.S., D.E. and D.S. analyzed the data; S.S. and D.S. wrote the paper.

Conflict of interest

The authors report no conflicting financial interests.

References
