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Differential recognition of vascular and parenchymal beta amyloid deposition

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Abstract

By phage display, llama-derived heavy chain antibody fragments were selected from non-immune and immune libraries and tested for their affinity and specificity for beta amyloid by phage-ELISA, immunohistochemistry and surface plasmon resonance.

We identified 8 distinct heavy chain antibody fragments specific for beta amyloid. Whilst three of them recognized vascular and parenchymal beta amyloid deposits, the remaining five heavy chain antibody fragments recognized vascular beta amyloid specifically, failing to bind to parenchymal beta amyloid.

These heavy chain antibody fragments, selected from different libraries, demonstrated differential affinity for different epitopes when used for immunohistochemistry. These observations indicate that the llama heavy chain antibody fragments are the first immunologic probes with the ability to differentiate between parenchymal and vascular beta amyloid aggregates. This indicates that vascular and parenchymal beta amyloid deposits are heterogeneous in epitope presence/availability. The properties of these heavy chain antibody fragments make them potential candidates for use in *in vivo* differential diagnosis of Alzheimer disease and cerebral amyloid angiopathy. Continued use and characterization of these reagents will be necessary to fully understand the performance of these immunoreagents.

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5.1 Disclosure statement for authors

All coauthors have seen and agree with the contents of the manuscript and that the manuscript is not under review at any other publication. Two authors (SMM and CTV) hold a patent on two of the HCAB fragments (ni3A and ni8B) described in this study.

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Keywords

Alzheimer's disease; Amyloid beta; Antibody; Histochemistry; Imaging; Immunoreactivity

1. Introduction

Alzheimer disease (AD) is the most common cause of dementia and is characterized by the aggregation of beta amyloid (A β) in the form of plaques and neurofibrillary degeneration, including neurofibrillary tangles, dystrophic neurites and neuropil threads. The amyloid cascade theory proposes that A β plays a central role in the pathogenesis of AD (Hardy and Selkoe, 2002). The deposition of A β as primary neuropathologic feature is shared by AD and cerebral amyloid angiopathy (CAA), although the location of the deposits differ between the two diseases (Hardy and Selkoe, 2002; van Duinen et al., 1987).

CAA describes a group of disorders that is characterized by heterogeneous amyloidogenic protein deposition in cerebral vessel walls. CAA is the most common cause of non-hypertensive intracerebral hemorrhage in the elderly (Revesz et al., 2002). Hereditary cerebral hemorrhage with amyloidosis-Dutch type (HCHWA-D) is a hereditary form of CAA and commonly considered a paradigm for CAA by virtue of being the first familial CAA to be characterized and by far the best described (Bornebroek et al., 1996).

Although AD and CAA frequently coexist in a patient, they generally are distinct clinical and histological entities (Ellis et al., 1996). Growing evidence suggests that patients with severe CAA suffer from cognitive impairment, independent of the presence of AD pathology (Grabowski et al., 2001; Natte et al., 2001).

Currently, neuropathologic examination is required for definitive diagnosis of AD and CAA. Conventional clinical tests are relatively insensitive for the early changes of cerebral amyloidoses, and the changes that are detected are non-specific. With a new generation of neuroprotective agents emerging, there is a need to detect these diseases in early stages.

Molecular imaging techniques specific to amyloid and other structural changes associated with neurodegenerative disease may begin to overcome this predicament, with these techniques relying on agents that are capable of detecting molecular and cellular changes in vivo. It has been demonstrated that agents targeted at A β and detectable by PET can detect cerebral A β accumulations in patients with AD and CAA (Johnson et al., 2007). However, these agents detect both vascular and parenchymal A β , and are thus not well suited to differentiate between AD and CAA.

A relatively new source of immunologic agents has been derived from the Camelid heavy-chain antibody repertoire (HCAb). In addition to conventional immunoglobulins, *Camelidae* express antibodies which are devoid of light chains. Their single N-terminal domain (VHH) is fully capable of antigen binding with affinities comparable with those reported for conventional antibodies (Hamers-Casterman et al., 1993; Zhang et al., 2004). VHHs have several potential advantages as immunologic tool which might allow them to be used for non-invasive, early and differential in vivo A β detection in the brain: because of their small size VHHs rapidly pass the renal filter resulting in a fast blood clearance and rapid tissue penetration; they are reported to be able to cross the blood-brain-barrier (BBB); and they have not shown any immunogenicity in mice (Muruganandam et al., 2002; Stijlemans et al., 2004).

Here we describe the selection of VHHs against A β generated from non-immunized animals and from animals immunized with vascular amyloid deposits from a patient with HCHWA-

D or grey matter from a patient with Down Syndrome and AD pathologic findings. The intent was to obtain high affinity VHHs specific for distinctive AD or CAA A β epitopes. The VHHs selected from the non-immune library solitarily recognize vascular A β depositions, similar to VHHs selected from the vascular CAA library. In contrast, the VHHs generated from the grey matter AD library show predominantly high affinity for A β epitopes that are specific for, or enriched in parenchymal plaques in addition to vascular depositions.

2. Materials and methods

2.1 Selection of antibody fragment by phage display

For the selection of non-immune VHHs an existing llama-derived phagemid library was used, kindly provided by BAC, The Netherlands (Frenken et al., 2000). VHHs were selected in two rounds of micropanning by direct immobilization of A β 1-42, essentially described before (Verheesen et al., 2006). The selection procedure of the second round of selection was identical to the first round with the exception that 10^9 input phages and 1 μ g of antigen was used.

Two Llama-derived immune phagemid libraries were generated. For the vascular CAA library immunization was performed with recombinant beta-amyloid (A β 1-42, r-peptide, Georgia, USA) and with affected blood vessels of a HCHWA-D patient. For the grey matter AD library, brain parenchyma homogenates of a DS patient with extensive plaque formation was used as the immunogen. Phage display selection from the immune libraries were performed as described for the naive library with the exception that for the vascular CAA library selections 0.2–10 μ g of different antigens (A β 1-42, A β 1-16, A β 11-22, A β 25-35 and A β 17-42, r-peptide, Georgia, USA) and 10 μ l phages (4.8×10^8 phages) were used as input. For A β 1-42 one round of selection was sufficient. One round of selection was performed with the grey matter AD library, with 10 and 50 μ l input phages and 0.1–10 μ g antigen (A β 1-42) coated.

2.2 Phage ELISA and diversity screening of selected VHHs

Phage productions and ELISA were performed as described before (Verheesen et al., 2006). In order to assess the diversity of the selected naive VHHs, DNA fingerprints were obtained as described before (van Koningsbruggen et al., 2003). For the immune VHHs the diversity was screened by means of surface Plasmon resonance (SPR) analysis.

2.3 Epitope mapping of A β specific VHHs

Epitope mapping was performed by phage ELISA experiments as described before (Verheesen et al., 2006). The reactivity of the phage-VHHs is tested on synthetic A β fragments 1–40, 1–42, 1–16, 11–22, 22–35, 25–35, 17–42 (rPeptide, Georgia, USA) and control protein. The phage-ELISA data were analyzed by one-way ANOVA followed by a LSD posthoc test. Threshold value for statistical significance was set at $p = 0.05$. SPSS 15.0.1 was used for all analysis (SPSS for Windows, Rel. 15.0.1. 2006, SPSS Inc, Chicago, Illinois, USA).

2.4 Subcloning, Production and purification of VHHs

The VHH genes specific for A β were subcloned into the pUR 5850VSV production vector (van Koningsbruggen et al., 2003), produced in *E. coli* and purified from the periplasmic supernatants as described (Kazemier et al., 1996). This vector contains a C-terminal His6 tag for purification and myc tag for detection. Talon metal affinity resin columns (Clontech, CA, USA) were used for purification according to the supplier's instructions.

2.5 Patient material

Autopsy brain tissue of patients with a genetically confirmed diagnosis of HCHWA-D and patients with AD, CAA, DS or HCHWA-I, and controls were used. The neuropathological evaluation was confirmed by S.G.v.D. and M.L.C.M-S. All HCHWA-D patients gave informed consent to use their tissues for research purposes. Tissue from all other patients and the controls was encoded and used in agreement with the code as defined in the Netherlands (Code Goed Gebruik). For the study of cases from Massachusetts General Hospital, all neuropathologic evaluation was confirmed by M.P.F; tissue was collected and used in the study in accordance with institutional approval for de-identified autopsy tissue.

2.6 Immunohistochemistry

Frozen brain tissue sections (5µm) of DS, HCHWA-D, HCHWA-I, AD, CAA patients and normal control tissue were rinsed in PBS, fixed with ice-cold acetone for 10 min, incubated with peroxidase blocking reagent (Dako Cytomation) for 20 min, washed in PBS and incubated with the anti-Aβ VHHs (0.625–20ng/ul) in 1% BSA/PBS overnight in a wet chamber. Thereafter, sections were rinsed with PBS and incubated with mouse-anti-VSV (1:1000) or mouse-anti-c-myc (1:6000) for 1 hour and subsequently with EnVision⁺® system labelled Polymer-Hrp anti-Mouse (Dako Cytomation) for 30 min. Detection was performed with Liquid DAB⁺ Substrate Chromogen System (Dako Cytomation). Hemotoxylin counterstaining was performed and the sections were dehydrated and mounted in micromount mounting medium (Surgipath, Richmond, IL, USA). 4G8 (1:200) and R1282 (1:1500) were used as a positive control for Aβ staining (Haass et al., 1992). Final preparations were analyzed with an Olympus Provis AX70TF light microscope and images were obtained using the Olympus digital DP12 camera.

2.7 Surface plasmon resonance spectroscopy (SPR)

All SPR binding studies were conducted at 25°C in HBS-EP running buffer using a Biacore 3000 biosensor system (GE Healthcare). Aβ1-42 was amine coupled (EDC/NHS) to a CM-5 sensor chip (research grade, GE) using 10 mM NaAc (pH 4.0) as coupling buffer, and HBS-EP as running buffer. Immobilization levels between 150 and 1000 RU could be obtained, however only surfaces of at least 350 RU gave reproducible results. Therefore, we analyzed VHH binding on surfaces at 350, 450 and 1000RU. As a reference channel an unrelated peptide, of the same size as Aβ1-42, derived from the poly A-binding protein nuclear 1 (containing an alpha-helical domain) was immobilized (Kerwitz et al., 2003). Binding experiments of VHHs were performed, using increasing concentration series from 5 nM to 4 µM at a flow rate of 30 µl/min and injection time of 8,3 min to reach equilibrium binding. The surfaces were regenerated after each cycle with two 60-s pulses of 1 M GnHCl and 10 mM NaOH, followed by a stabilization time of 5 min. Biacore data were analyzed with BiaEvaluation v. 4.1 (GE). Reference surface and blanks were subtracted from all binding curves. The dissociation constant (K_D) for the interaction between VHHs and Aβ1-42 was measured from saturation binding experiments. The value for K_D was obtained using a non-linear least squares analysis (steady state analysis) with $AB_{eq} = AB_{max} / (1 + K_D/[A])$ where AB_{eq} is the average of the response signal at equilibrium in defined intervals for each concentration of VHHs [A] and AB_{max} is the maximum response that can be obtained for VHHs binding depending on the number of binding sites available on the Aβ1-42.

3. Results

3.1 Selection and screening of VHHs

To obtain VHHs from the non-immune library against beta amyloid, two successive rounds of selection were performed by phage display. Synthetic Aβ 1-42 was directly coated onto

Nunc maxisorp plates in both rounds. The progress of selection was monitored by colony count of the enriched polyclonal outputs. In each round an increase in output/input ratio was observed, indicative for enrichment of VHHs directed against A β 1-42 in the polyclonal phage-pool. After the second round of selection 96 monoclonal phage-clones were randomly chosen. Clones were arrayed and tested for A β 1-42 affinity by dot blot analysis (data not shown). Several clones showed specific binding to the antigen. Their diversity was screened by DNA fingerprint analysis showing a large diversity in fingerprint patterns. Subsequently, a subset of clones with different fingerprints was sequenced, resulting in a subset of different VHHs specific for A β 1-42. Two of the VHHs, ni3A and ni8B, were chosen for further characterization.

In order to select high affinity VHHs potentially specific for epitopes unique to CAA A β , phage selection was also performed utilizing the vascular CAA immune library. For A β 1-42 specific VHH, one round of selection was sufficient to obtain high affinity VHHs directed against CAA A β epitopes: the output of the selection plate was significantly higher compared with the background plate after one round of selection. In order to aim at a panel of VHHs directed against diverse A β epitopes, phage selection was performed with different A β fragments. Two successive rounds of selection were necessary judged by the colony count.

Because of the heterogeneous character of A β presentation in CAA and AD we also aimed to select VHHs from a library that was generated by immunizing a llama with a protein homogenate of brain parenchyma with extensive AD pathology. We anticipated that, if there are conformational differences between vascular and parenchymal amyloid deposition, this library would yield VHHs with high affinity for AD A β epitopes that are specific for parenchymal plaques. Judged by colony count, one round of selection was sufficient for the selection of specific VHHs from this library.

After the selection procedure from the vascular and brain parenchyma libraries, >300 monoclonal phage-clones were randomly chosen from each selection and screened by phage ELISA. Many clones showed specific binding to the antigen. Their diversity was screened by means of off rate differences in SPR analysis. As a consequence of the concentration independence of this parameter, production differences between VHH clones are not of influence. A large diversity in SPR curves was detected. Subsequently, a subset of clones with different off rates was sequenced. This yielded several unique high affinity VHHs specific for their respective antigens (va2E, vaE2 and va1G for the vascular library and pa4D, pa11E and pa2H for the parenchymal library). All selected VHHs were subcloned into the pUR5850-VSV production vector to increase the production yield. The characteristics of all VHHs selected for further analysis are summarized in (table 1).

3.2 Epitope mapping of A β specific VHHs

Epitope mapping of the VHHs was performed by phage ELISA experiments. Ni3A and ni8B have a significantly higher affinity preference for A β 1-42 compared to all other antigens. Va2E recognizes 1-42 and 17-42 significantly better than other fragments, although A β 1-40 recognition is clearly detected. VaE2 and va1G are not phage-VHH ELISA reactive. Pa4D, pa11E, pa2H have affinity preference for A β 1-42 and A β 1-40 (figure 1). Thus, VHHs selected from the different libraries show distinct epitope specificity, consistent with the distinct immunogens used prior to the phage-ELISA experiment.

3.3 Determination of affinity constants using SPR biosensor analysis

The binding of the various VHHs over a dynamic range of concentrations from 5 nM to 4 μ M were determined on three different densities of immobilized A β 1-42 (350, 450 and 1000

RUs) to evaluate the affinity constant (KD). Sensorgrams with double referenced binding curves of VHH pa11E are shown for the 450 and 1000 RU surface density (Figure 2A and 2C, respectively) and steady state binding (Req) levels were determined (indicated by an asterisks in figure 2A and 2C). The Req levels were plotted versus concentration (Fig 2B and 2D) and subsequently the KD values were determined using steady state analysis. The average of the KDs for all VHHs is summarized in Table 1 and range between 39 and 1200nM. The KD value of each VHH was found to be consistent and independent of the immobilization level of A β 1-42.

3.4 Immunohistochemistry

The VHHs directed against A β which were derived from the non-immune library (ni3A, ni8B) showed specific staining of vascular A β , with equal affinity for these deposits in cases of AD, HCHWA-D and CAA (figure 3), (table 1).

In order to confirm specificity for A β and to rule out a contribution from the well-established non-specific endothelial cross-reactivity of the VHHs, immunohistochemistry was performed on control tissue lacking evidence of A β deposits. Additionally, staining with a traditional anti-A β antibody (4G8/R1282) was performed on adjacent tissue sections. The absence of staining in control tissue and an identical vascular and/or parenchymal staining pattern obtained with 4G8/R1282 in all patients (figure 4) confirmed A β specificity. Because conformational specificity has been reported for VHHs selected against A β , selected VHHs were also tested on tissue sections of HCHWA-I patients in which the amyloid consists of mutant cystatin (Habicht et al., 2007; Ghiso et al., 1986). Selected VHHs showed absence of staining on HCHWA-I sections and consequently do not detect amyloid epitopes shared by all species of amyloid. Therefore, the selected VHHs are considered to be β -amyloid sequence specific (figures 3,5,6), (table 1).

VHHs selected from the vascular CAA library (vaE2, va1G and va2E) showed vascular A β immuno-reactivity. In contrast with the non-immune VHHs they displayed a higher affinity for vascular A β in HCHWA-D patients when compared with AD (figure 5, table 1).

The VHHs selected from the grey matter AD library (pa2H, pa11E, and pa4D), recognized A β epitopes that are specific for, or enriched in parenchymal plaques. In contrast with the VHHs selected from the vascular CAA library, they showed specificity for parenchymal A β depositions of AD patients. (figure 6, table 1).

As VHHs derived from post-immunization libraries seemed to show a preference for A β deposits which corresponded to the disease state used for immunization, while those from the non-immune library did not show a preference for AD or HCHWA-D A β deposits, all VHHs were tested in a larger panel of sporadic CAA patients. An example of immune reactivity of pa2H and ni8B in a series of 4 sporadic CAA patients is shown in figure 7. Pa2H and ni8B also in these patients recognized CAA, like all other VHH derived from the non-immune and parenchymal libraries. We therefore consider the affinity preference for these VHHs to be disease- and not patient-specific. Furthermore, the overall preference for HCHWA-D of the vascular CAA library derived VHH was further corroborated with the observation that only one sporadic CAA patient showed vascular reactivity for these VHHs (data not shown).

4. Discussion

AD is the most common form of dementia and CAA is a major cause of intracerebral hemorrhage in elderly (Revesz et al., 2002a). Both are progressive neurodegenerative diseases that share A β deposition as etiological event: a misbalance in the homeostasis

between A β production and clearance that promotes accumulation of A β in the brain is supposed to represent the earliest event in the disease process in many patients (Blennow et al., 2006; Seubert et al., 1993). Therefore, A β is a pathologic hallmark of AD, CAA and related neurodegenerative diseases. Although cerebral accumulation and aggregation of A β is the primary neuropathologic feature shared by AD and CAA the two diseases are heterogeneous in A β etiology and clinical presentation (Hardy and Selkoe, 2002; van Duinen et al., 1987). The availability of diagnostic probes for early detection of cerebral A β in living patients which preferably discriminate between vascular and parenchymal aggregates would contribute significantly to the early diagnosis and treatment of this group of disorders.

In this study a panel of immunologic reagents directed against A β were examined with the long term objective of developing tools for the early non-invasive differential in vivo imaging of AD and CAA. VHHs have several potential advantages which might allow them to be used for in vivo imaging of neurological disorders. VHHs have a molecular mass of ~15 kDa and because of their small size VHHs show quick tissue penetration, are rapidly cleared from the blood and can have the propensity to cross the BBB. (Zhang et al., 2004; Harmsen et al., 2005; Muruganandam et al., 2002). Furthermore, in animal models, VHHs have shown little immunogenicity and can be produced in a quick and economical manner in microorganisms (Cortez-Retamozo et al., 2002; Frenken et al., 2000; Arbabi-Ghahroudi et al., 2005; Coppieters et al., 2006).

In this study, we compared the performance of VHHs selected from immune- and non-immune libraries in immunohistochemistry. First VHHs were selected from a non-immune and vascular CAA library. This selection from the vascular CAA library resulted in VHHs that only recognize vascular A β depositions and fail to recognize parenchymal depositions of A β . Because the major component of vascular A β in these diseases is A β 40, high affinity of the VHHs for A β 40 could explain this selective recognition of vascular beta amyloid (Gravina et al., 1995). We therefore tested affinity preference of our selected VHHs for different A β peptides by phage-ELISA. Surprisingly, most VHHs showed highest preference for A β 1-42. From these results we conclude that although in both vessels and parenchyma progressive cerebral accumulation and aggregation of A β is present, A β deposition at these two types of structures seems to differ in A β epitope availability.

In order to obtain VHHs that preferentially recognize A β epitopes that are specific for, or enriched in parenchymal plaques, we immunized a llama with brain parenchyma of a patient with extensive AD pathology. In contrast to the VHHs selected from the vascular library, VHHs selected from this library show high affinity for A β present in parenchymal plaques in addition to vascular affinity. The results from the vascular and parenchymal immune libraries suggest that different epitopes are present or exposed in vascular and parenchymal A β deposits. While vascular A β epitopes recognized by the VHHs are not available in parenchymal A β deposition, A β epitopes available and recognized in the brain parenchyma are also present in vascular A β deposition. This phenomenon may be related to differences in the conformation state of the A β present in the vessels and the parenchyma.

In addition to this discrimination between vascular and parenchymal A β , VHHs selected from the grey matter AD library recognize parenchymal A β selectively in patients with AD pathology but not in HCHWA-D patients. This finding suggests that these VHHs discriminate amyloid plaques from plaques containing (predominantly) non-fibrillar A β .

Overall, the VHHs selected from the different libraries showed distinct patterns of specificity when used for immunohistochemistry, with the highest A β immune reactivity being observed in the disease condition that was used for immunization. This is consistent

with the phage-ELISA data and therefore supporting the presence/availability and recognition by the VHHs of different epitopes present in CAA and AD. Nevertheless, the discriminatory propensity of the VHHs derived from the non-immune and CAA libraries was consistent in sporadic and hereditary CAA.

The formation of highly insoluble fibrils with a β -pleated structure (amyloid) is a common biochemical characteristic shared by proteins with diverse amino acid sequences. This aggregation and fibrillization process is observed in many neurological/neurodegenerative disorders (Westermarck et al., 2002). In AD and CAA, A β is the major constituent of these aggregates, while in HCHWA-I the amyloid consists mainly of cystatin C (L68Q) (Ghisso et al., 1986). Given the absence of immunohistochemical staining of HCHWA-I protein depositions, we conclude our VHHs are specific for A β in its amyloidogenic confirmation. This distinguishes them from some of the recently published antibodies which recognize the amyloid structure independent of its protein composition (Kayed et al., 2007).

Plasma IgGs specific for known toxic A β and amyloidogenic non-A β species are common in healthy individuals and some of these antibody levels decline with age and AD progression (Britschgi et al., 2009). It has been suggested that these A β sequence- or conformation-specific antibodies can prevent or delay A β pathology, possibly through a number of different pathways. Although additional studies are required to establish the therapeutic significance of specific A β antibodies, a preventive measure toward AD may come from further development of conformation-specific A β antibodies such as the VHH described in this study.

In conclusion, we selected sequence specific VHHs against A β . Different immunization strategies resulted in the selection of high affinity VHHs specific for different A β epitopes. To our knowledge, the VHHs presented here are the first immunologic agents that are capable of discriminating vascular and parenchymal A β deposits, though further studies are required to elucidate the basis of this specificity. Additionally, because of the favourable in vivo characteristics of VHHs, these reagents have the potential to serve as tools for in vivo imaging to discriminate between vascular and parenchymal beta amyloid deposition.

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Figure 1.

Epitope mapping of Aβ specific VHHs. Depicted are Phage-VHH Elisa results. Data is represented as the corrected OD (minus negative control) measured at 490nm.

Figure 2. Example of the binding measurements performed with VHH 11E (0nM to 750nM) to 450 RUs (A) and 100RUs (C) immobilized Aβ 1-42. The sensorgrams were double referenced and Req values were determined (indicated by an asterisk) and plotted versus the concentration (B,D) to evaluate the KD using the steady state analysis provided by BiaEval 4.1.

Figure 3. Immunohistochemistry of VHH ni3A derived from non-immune library. In the upper panel a 10x magnification of AD, HCHWA-D, HCHWA-I cryosections is shown. The lower panel is showing a 40x magnification of AD, HCHWA-D and control tissue cryosections. Ni3A shows immunoreactivity with vascular A β in AD and HCHWA-D patient sections. No immunoreactivity was present in HCHWA-I patient sections and control tissue sections.

Figure 4.

Immunohistochemistry with the conventional monoclonal antibody 4G8. The upper panel is showing a 10x magnification and the lower panel a 40x magnification of, from left to right, HCHWA-D with vascular A β immunoreactivity, AD with vascular and parenchymal immunoreactivity and control tissue. Identical vascular A β staining was observed with VHHs ni3A, ni8B, va2E, vaE2 and va1G. Identical parenchymal staining patterns, in addition to vascular A β immune reactivity was observed with VHHs pa2H, pa11E, pa4D.

Figure 5.

Immunohistochemistry of VHH va1G, va2E and vaE2 derived from the vascular amyloid library. **A)** The upper panel shows a 10x magnification of the immunohistochemical staining with va1G on AD, HCHWA-D, HCHWA-I and control tissue cryosections. In the lower panel a 10x magnification of the immunoreactivity with va2E on, from left to right, AD, HCHWA-D, HCHWA-I tissue sections and a 40x magnification of the control tissue is shown. **B)** The upper panel shows a 10x magnification of the immunoreactivity with vaE2 on AD, HCHWA-D, HCHWA-I and control tissue cryosections. In the lower panel a 40x magnification of the immunoreactivity with vaE2 is shown for the same tissue sections. The VHHs in panel A and B show vascular beta amyloid reactivity, with an overall A β affinity preference for HCHWA-D patients, especially va2E, which has no immune reactivity for A β in AD.

Figure 6.

Immunohistochemistry of VHH pa4D, pa11E and pa2H, derived from the grey matter library. **A)** The upper panel shows a 10x magnification of the immunoreactivity with pa4D on AD, HCHWA-D, HCHWA-I and a 10x magnification of control tissue cryosections. In the lower panel a 10x magnification of the staining with pa11E on the same tissue sections from left to right, AD, HCHWA-D, HCHWA-I tissue sections and a 40x magnification of the control tissue is shown. **B)** All the panels show immunoreactivity VHH pa2H, from on AD, HCHWA-D, HCHWA-I and control tissue cryosections. The upper panel is showing a 10x magnification. In the middle panel the 40x magnification is shown. The lower panel shows 10 and 40x magnifications AD and DS cryosections. The VHHs displayed in panel A and B show high affinity for parenchymal A β epitopes, in addition to vascular affinity.

Figure 7. Immunohistochemistry of VHH pa2H and ni8B. VHHs are tested on four different sporadic CAA patients. Two of the patients are Leiden CAA patients and two are Boston CAA patients.

Table 1

Overview of VHHs selected from the non-immune, vascular CAA and parenchymal libraries, with their characteristics.

VHH	Library	Antigen used for selection	Immunohistochemistry						KD Average (nM)	STDV (nM)
			Vascular Aβ reactivity		Parenchymal Aβ reactivity					
			HCHWA-D	AD	HCHWA-D	AD				
			HCHWA-D	AD	HCHWA-D	AD				
ni3A	Non-immune	Aβ 1-42	++	++	–	–	39	12		
ni8B	Non-immune	Aβ 1-42	++	++	–	–	109	36		
va2E	Vascular	Aβ 17-42	++	+	–	–	650	157		
vaE2	vascular	Aβ 1-42	++	+	–	–	1180	443		
va1G	vascular	Aβ 11-22	++	+	–	–	179	73		
pa4D	grey matter	Aβ 1-42	++	+++	–	+++	100	9		
pa11E	grey matter	Aβ 1-42	+++	+++	–	+++	129	45		
pa2H	grey matter	Aβ 1-42	+++	+++	–	+++	160	21		

+ weak intensity, ++ strong intensity, +++ very strong intensity.