Progression of Cerebral Amyloid Angiopathy in Transgenic Mouse Models of Alzheimer Disease

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Abstract
Cerebral amyloid angiopathy (CAA), the deposition of β-amyloid (Aβ) in cerebral vessels, has been implicated as a common cause of hemorrhagic stroke and other forms of vascular disease. CAA is also a frequent concomitant of Alzheimer disease (AD). While the long-term consequences of CAA are well recognized from clinical and pathologic studies, numerous questions remain unanswered regarding the progression of the disease. Examination of CAA in traditional histologic sections does not easily allow for characterization of CAA, particularly in leptomeningeal vessels. In order to approach this topic, we used low magnification imaging of intact, postmortem brains from transgenic mouse models of AD-like pathology to define the spatial and temporal characteristics of CAA in leptomeningeal vessels. Imaging of brains from 10- to 26-month-old animals demonstrated a stereotypical pattern to the development of CAA, with vessels over the dorsal surface of the brain showing an anterior-to-posterior and large-to-small vessel gradient of involvement. High magnification imaging revealed that CAA deposition began with a banding pattern determined by the organization of the vascular smooth muscle cells. Further analysis of the pattern of amyloid deposits showed shrinkage and disappearance of the gaps between clusters of amyloid bands, gradually reaching a confluent pattern. These data led to a classification system to describe the severity of CAA deposition and demonstrate the potential of using intact brains to generate maps defining the progression and kinetics of CAA. This approach should lead to more informed analysis of the consequences of evolving therapeutic options for AD on this related form of vascular pathology.

Key Words: Alzheimer, Amyloid-beta, Angiopathy, Mouse, Multiphoton, Transgenic, Vascular.

INTRODUCTION
Cerebral amyloid angiopathy (CAA) is the aggregation of beta-amyloid peptide (Aβ) in cerebral arteries, eventually resulting in smooth muscle cell death (1–3). CAA is often found in the presence of lobar hemorrhages and cerebral infarcts (4–7). Aβ, which is cleaved from the amyloid precursor protein (APP) and ranges from 39 to 43 amino acids long, is also a key component of the senile plaques characteristic of Alzheimer disease (AD). Although CAA can occur independently, it is more often associated with AD; for example, Yamada found CAA in 35% of postmortem nonAD brains compared with a frequency of CAA in postmortem AD brains of 87% (8). Despite the growing number of people affected with AD and CAA, there is no method for visualizing CAA in humans in vivo and therefore little opportunity for understanding when or how CAA pathology develops. An appreciation for the progression of CAA and CAA-related vascular injury is important as potential therapeutics for AD and CAA are considered.

In the past decade, transgenic mouse models of brain Aβ deposition have played an important role in the understanding of AD. Through the overexpression of forms of APP carrying AD- or CAA-linked mutations, sometimes in combination with mutations in PS1, these animals generate high levels of Aβ peptides. These animals develop Aβ deposits in the form of plaques and represent a model of one component of the neuropathology of AD (9, 10). In addition, many of these models develop extensive CAA (11–14). As with the human disease, these murine models of CAA are age dependent and the deposition of Aβ in vessel walls has deleterious effects including impaired reactivity (15–17), microhemorrhages (18, 19), and loss of smooth muscle cells (12, 14, 19).

Previous studies that have examined CAA in murine models have focused on examination of histologic sections, an approach that can obscure the relationship between different sites of Aβ deposition along the length of an individual vessel and the overall pattern of involvement throughout the brain. In order to define the spatial and temporal parameters of CAA development and progression, we examined intact whole...
brains from transgenic mice to ask when and where CAA initially deposits and the path it follows once it has deposited. An accurate spatial and temporal framework for CAA will provide useful information about the factors that govern Aβ deposition and shed light on how therapeutic treatment might be integrated into the pathways of Aβ production and deposition to halt the negative effects of CAA on vascular function.

**MATERIALS AND METHODS**

**Tissue Samples**

Postmortem brains from four different transgenic mouse models that develop Aβ deposits were studied. These included Tg2576 mice on the B6SJL/J strain overexpressing the Swedish APP double mutation driven by the hamster prion protein promoter (PrP) (10); Tg2576 mice crossed with FvB/N mice overexpressing the Presenilin-1 M146L mutation driven by the platelet-derived growth factor B-chain promoter (PDGF-B) (20); PDAPP transgenic mice on the C57BL/6 background overexpressing the APP V717F mutation under the control of the PDGF-B promoter (9); and TgCRND8 mice on the 129S6/SvEvTac background overexpressing both the Swedish double mutation and the APP V717F mutation under the control of the PrP promoter (15). Animals had been previously euthanized either by CO2 asphyxiation or lethal injection of the anesthetic Avertin (Tribromoethanol, 250 mg/kg intraperitoneally). The brains, some of which had been perfused, were fixed in paraformaldehyde for at least 24 hours and stored in 4°C until use.

**Staining**

Brains were rinsed in 1× TBS for 10 minutes and then immersed in thioflavin S (0.001% in TBS, Sigma Aldrich, St. Louis, MO) for 20 minutes at 4°C. Brains were washed in 1× TBS for 10 minutes to remove unbound thioflavin S. Vascular smooth muscle cells were stained with phallacidin, an isolated phallotoxin that binds to F-actin, as described (21) with minor modifications. Briefly, whole brains previously fixed in paraformaldehyde were washed with 50 mM glycine in PBS for 15 minutes. Brains were treated for 20 minutes with Triton-X 0.1%, followed by 30 minutes preincubation in 2% bovine serum albumin (BSA) in 50 mM glycine-PBS, to block nonspecific binding. The tissue was incubated for 45 minutes with 4U Bodipy TR-X phallacidin (Molecular Probes, Eugene, OR) in 2% BSA-50 mM glycine-PBS. Brains were then washed with 50 mM glycine-PBS and stained for amyloid with thioflavin S as described above.

**Imaging Method**

Brains were imaged with a Bio-Rad 1024 MP microscope (Bio-Rad, Hercules, CA), as previously described (12, 22). Two-photon fluorescence was generated at an excitation wavelength of 785 nm by a Ti:Sapphire laser (Mai Tai, Spectra Physics, Mountain View, CA). Emitted light was selectively collected by external detectors (Hamamatsu Photonics, Bridgewater, NJ) in the range of 380 to 480 nm, corresponding to the emission wavelength of thioflavin S, and 560 to 650 nm for Bodipy TR-X phallacidin. Z-series were taken using an Olympus 2X objective (XL Fluor, NA = 0.14) to a depth of 4 mm with 100 μm z-steps in a 6158 μm × 6158 μm field. Four z-series were required to image each dorsal and each ventral surface. In addition, high magnification z-series of individual vessel segments of these same brains were taken with an Olympus UMPPlanFI 20× objective (NA = 0.95) to a depth of 200 μm with 5 μm z-steps in a 615.8 μm × 615.8 μm field. The z-series were projected according to maximum intensity algorithms using the Scion Image and ImageJ programs. For low magnification images requiring multiple z-series, photomerges were performed using Adobe Photoshop Elements 2.0 to obtain composite whole brain images.

**RESULTS**

We coupled a relatively low magnification, high numerical aperture objective (Olympus 2×, NA = 0.14) with multi-photon microscopy to image surface fluorescence from intact brains that were fixed and removed from APP transgenic mice. This approach provided a macroscopic view of entire brains. Higher magnification images with submicron resolution of individual vessels were also obtained from the same specimen. By treating the brains with the histochemical fluorophore thioflavin S, plaques and CAA were labeled with high contrast while preserving the leptomeningeal vessels (which are commonly damaged or lost when processing rodent brains for traditional histologic sections). This ability to assay the surface vasculature across multiple brains from transgenic animals of different ages and severity of disease allowed for an understanding of the spatial progression of the disease in a manner not available in studies using other approaches.

**Stereotyped Pattern of CAA in Different Lines of Transgenic Mice**

Figure 1 compares 4 different mouse models of AD, each of which overexpresses human APP and develops both parenchymal Aβ deposits and CAA. The whole brain images indicate the pattern and degree of affected vessels in these mouse models; Aβ deposits as plaques are also visible at this magnification. The Tg2576 (24 months; Fig. 1A) and Tg2576 × PS1 (22 months; Fig. 1B) animals exhibited similar maximal CAA burdens at this extreme age. CAA was more lightly deposited in similar aged PDAPP mouse brains (22 months; Fig. 1C), TgCRND8 mice (11 months; Fig. 1D) developed comparable extents of CAA as Tg2576 and Tg2576 × PS1 mice but at much earlier ages; however, breeding difficulties with this line precluded additional investigations. The Tg2576 mouse model of Aβ deposition was selected for subsequent studies because of its ready availability, robust disease, and the extensive literature regarding other aspects of this model.

**Progression of CAA in Tg2576 Mice**

We used whole brain multiphoton imaging to examine brains of Tg2576 mice at different ages, studying brains from animals that were 10 months (n = 4), 16 months (n = 4), and >22 months (n = 4) old. Examination of the pattern of CAA involvement in visible leptomeningeal vessels over the dorsal and ventral surfaces of the brain revealed a consistent set of patterns (Fig. 2). Along the dorsal surface of the brain a
“limited” stage of involvement could be defined as CAA involving the portions of anterior and middle cerebral arteries as they extended into the field of view from the interhemispheric fissure and around the lateral aspect of the brain, respectively. From the ventral aspect this stage was characterized by involvement of large caliber portions of arteries distinct from the circle of Willis. At the other extreme of severity a “widespread” stage of CAA was typified by the presence of CAA extending to involve nearly all vessels evident from the dorsal surface, and reaching towards — but not involving — the circle of Willis on the ventral surface. An “intermediate” stage was characterized most consistently by the involvement of smaller caliber vessels across the brain surface in the anterior half of the dorsal surface, with comparably less involvement of similar caliber vessels posteriorly. Blood vessels in the posterior fossa were inconsistently involved with CAA, although there was greatest involvement in older mice. Because these patterns were defined by the existence of uninvolved areas of the vasculature, the integrity of these leptomeningeal vessels that lacked CAA was confirmed through staining with phallacidin (Fig. 3).

The consistency of the pattern of limited, intermediate, and widespread CAA was confirmed by examination of dorsal and ventral brain composite images by 2 examiners blinded to the age of the animal. There was complete interobserver agreement as to stage of CAA, and the 3 identified stages were well correlated with animal age (Fig. 4), indicating that leptomeningeal CAA is a consistent disease process across animals.

**Pattern of Deposition of CAA in Tg2576 Mice**

Affected vessel segments were examined at higher magnification to characterize the pattern of CAA in more detail. Deposits of thioflavin S-positive material could be found as either short segments that did not completely span the

**FIGURE 1.** The pattern of expression of amyloid deposits in different APP transgenic mouse models. Four different APP mouse models exhibit different patterns of expression of CAA. These are representative images of intact mouse brains acquired with multiphoton microscopy after staining the brains with thioflavin S. Tg2576 (A) and P51xTg2576 (B) develop extensive CAA by 22 months of age. At 24 months of age, PDAPP mice (C) have detectable CAA, but it is not very widespread. TgCRND8 mice (D) have severe vascular deposition of Aβ at 11 months. Scale bar = 2 mm.

**FIGURE 2.** Chronic progression of CAA in Tg2576 mice. (A–C) Representative images of the dorsal surface of intact mouse brains at 10, 16, and 23 months of age, respectively. Angiopathy first accumulates in the anterior region of the brain in large arteries (A) progressing to involvement of all arteries (B, C). Posterior fossa remain relatively spared. On the ventral surface of the same brains, the circle of Willis remains free of CAA throughout advanced stages of disease (D–F). Vessels close to the circle of Willis develop CAA later than ventral surface vasculature located more distally. Scale bar = 2 mm.
circumference of the involved vessel or, particularly in older animals, as bands of completely circumferential amyloid (Fig. 5). Although direct progression from incomplete to complete bands could not be observed in this static study of brains harvested at different ages, the shift towards more complete circumferential wraps of CAA in older animals suggested that this reflected progression of the disease. Double-labeling with thioflavin S and Bodipy-conjugated phallacidin (Fig. 3) indicated that the bands of CAA appeared between vascular smooth muscle cells. This relationship was maintained across the age spectrum examined, although as mice reached older ages there was some loss of vascular smooth muscle cells.

**Banding Pattern and Gaps**

CAA appeared in vessel segments as clusters of closely spaced bands interrupted by uninvolved segments (gaps). To examine what influences the spacing of amyloid deposits and gaps in affected leptomeningeal vessels, we performed a quantitative analysis of the fluorescence intensity profile along single vessel segments (Fig. 6). We began this analysis by focusing on the most heavily involved vessel segments in older mice. The distribution of mean spacing between bands of CAA was narrow, with a mean of 9.3 ± 3.3 µm. The average width of a smooth muscle cell in the same region of the mouse leptomeningeal vasculature, as measured by the width of phallacidin staining, was 7.7 ± 0.5 µm (n = 42 cells). These results confirmed our impression that deposition of CAA occurs between smooth muscle cells.

In younger animals with limited involvement, clusters of bands of CAA were separated by larger gaps that were distinguishable qualitatively (Fig. 6A vs. 6C) and quantitatively (Fig. 6B vs. 6D). We examined the progression of CAA via “filling in” of these unaffected gaps. As the severity of CAA deposition increased, we observed both a decrease in the size of these gaps, suggesting that progression occurs via propagation of existing deposits, as well as a decrease in the number of these unaffected areas, indicating new initiation of deposits. These results suggest that progression of CAA probably occurs via both initiation of new deposits and propagation from existing ones.

**DISCUSSION**

We have approached the study of CAA deposition by looking at vascular amyloid in whole brains rather than the single vessel profiles seen in histologic sections. The advantage of this approach is the ability to understand progression over long, contiguous vascular segments (up to several mm in length). The maps of the superficial vessels show that Aβ accumulation in cerebral arteries begins as early as 9 months in Tg2576 mice and occurs simultaneously on both the dorsal and ventral sides. PS1xTg2576 and TgCRND8 mice also showed all superficial vessels affected by the end stage of
FIGURE 5. Progression of CAA in matched vessels. High magnification images of proximal and distal regions of the anterior cerebral artery were obtained from mouse brains at different ages. At 10 months, angiopathy has already accumulated in cerebral arteries (A). However, smaller vessels distal from the midline and lateral side of the brain have little accumulation (D). As the disease progresses (16 months), arteries in all regions continue to fill in with angiopathy (B, E). By the most advanced stages of disease (23 months), the majority of each vessel is affected (C, F).

FIGURE 6. Qualitative and quantitative measurements of CAA band spacing. 20× images of thioflavin S labeled CAA represent vessels with intermediate (A) and widespread (C) involvement. Note in panel (A) that bands of CAA occurred in clusters separated by gaps. This band spacing in the clusters, or widespread involvement (C) approached the width of smooth muscle cells. Gaps were much larger than the spacing of individual bands and become more rare with widespread involvement. Panels (B) and (D) show the corresponding histogram of band spacing obtained with quantitative analysis of intensity “peaks” that occur across a longitudinal profile of a vessel. The histograms demonstrate that closest band spacing occurs at ~9 μm (approaching the width of a smooth muscle cell), but that broader spacing of CAA (gaps) extends out to more than 60 μm in cases with intermediate involvement. Scale bar = (A, C) 50 μm.
disease, whereas aged PDAPP mice developed significant, but not such extensive CAA. These results suggest that different AD-related mutations (with or without accompanying mutant PS1) can produce similar patterns of vascular amyloid deposition. Although this form of analysis of amyloid deposition in the cerebrovasculature does not allow us to unequivocally define the nature of the blood vessels affected, previous work on CAA in humans (2, 3, 5) and mouse models (12, 14) has determined that this form of pathology is primarily associated with arteries and arterioles; the coincident labeling of affected vessels by staining with phallacidin confirms that the vessels under study in the leptomeninges of these animals were on the arterial side of the vasculature.

A question addressed in this study was whether particular superficial vessels were predisposed to amyloid deposition. Based on the whole brain images, we conclude that CAA follows a predictable pattern of deposition depending on both vessel location and size. Larger caliber vessels in the anterior region of the brain were targeted early in the course of the disease, while smaller vessels were affected much later after most regions of the brain had already become involved. Based on this consistent pattern of angiopathy accumulation, we were able to classify CAA into three stages of vessel involvement: limited, intermediate, and widespread. This deposition of Aβ in leptomeningeal vessels is roughly synchronous with the deposition of Aβ (in the form of plaques) in the brains of these mice, as assessed through comparison with studies performed using both morphologic and biochemical approaches (10, 23, 24).

Previous studies have classified CAA severity in individual vessels, such as the scheme of Vonsattel (3) defining CAA severity as “mild,” “moderate,” and “severe” based on degradation of smooth muscle cells and other vasculopathic changes in the most severely affected vessels. We propose the scheme used in the current report as a complementary method for systematically classifying extent of CAA burden across superficial vessels in the whole brain in mouse models. There do not appear to have been comparable studies of the progression of CAA in humans; such investigations would be most appropriate in the context of genetically determined forms of the diseases, such as those associated with the E693N mutation or trisomy 21.

We noted that vessels corresponding to the circle of Willis did not develop CAA even at advanced stages of disease. Furthermore, arteries next to the circle of Willis developed CAA later than all other ventral vasculature; the portion of vessel closest to the circle of Willis was the last part of the vessel to develop CAA regardless of its size. This finding was consistent across all 4 mouse lines tested, again supporting vessel composition and location, in addition to the specific biochemical characteristics of Aβ generation, as a key determinant of amyloid deposition. While the arteries at the base of the brain are typically of larger caliber than many of the leptomeningeal vessels, this is not always true, and it is possible that vessel caliber is not the only determining factor for the relative under-involvement of these vessels. These vessels sit completely within the subarachnoid space and are furthest from direct contact with brain parenchyma; this difference may play into the relative resistance of these vessels towards CAA.

We found an interband distance of approximately 9 μm as the closest spacing of amyloid deposits in fully involved vessel segments. Intercalated between these bands of amyloid is F-actin staining. A typical vascular smooth muscle cell from other vascular beds in the mouse is approximately 2 μm wide and wraps around the vessel wall several times (25)—a finding that we have confirmed for leptomeningeal blood vessels through scanning electron microscopy in the Tg2576 mice (data not shown). The distance between bands of amyloid thus appears to represent 4 to 5 wraps of a vascular smooth muscle cell around the circumference of the vessel and suggest that amyloid deposits preferentially between smooth muscle cells rather than between wraps of a single smooth muscle cell. This was confirmed by our measurement of the width of smooth muscle cell units in these same vessels through the use of phallacidin staining. Our attempts to directly visualize the Aβ deposits in relationship to the smooth muscle cells through the use of immunogold labeling combined with scanning electron microscopy were not successful, although this remains a potential avenue for further exploration.

A second question explored here was how cerebrovascular amyloid progresses. Two plausible models for progression are propagation of existing deposits or continual random seeding throughout the vasculature. A previous study in human histologic sections found evidence of increased Aβ40 per affected cortical vessel (rather than increasing numbers of affected vessels) with advancing disease severity (26), supporting a model of propagation of existing deposits. The current analysis found CAA deposits in clusters, also supporting the idea of propagation of existing deposits. Smaller islands of vascular amyloid can also be identified, however, suggesting some random deposition as well. Either model of progression (propagation or seeding) would predict that the gaps between clusters of CAA would first shrink and then disappear, as we observed. In order to assess the magnitude of the 2 pathways, direct serial observations of CAA progression will need to be performed.

CAA is well recognized as a risk factor for lobar hemorrhage in humans (2, 3) and there have been reports of CAA-related hemorrhages in mouse models of Aβ deposition, including the Tg2576 model (19, 27–30). Despite these other observations, we did not observe evidence of hemorrhage among the brains examined in this study. Several possible reasons for this apparent discrepancy include the ages of the animals studied (most of the animals discussed in this work are younger than those which developed spontaneous hemorrhages), the absence of additional manipulations that may induce hemorrhage and the focus in this work on leptomeningeal vessels. It remains possible that small hemorrhages were present within these brains, although there was no external evidence of this.

In conclusion, this report describes the progression of CAA in mouse models of Aβ deposition at both a macroscopic and microscopic scale. The results lead to a new classification method to compare the severity of CAA and demonstrate that progression of vascular deposits is predictable in mouse models. This insight will allow evaluation of anti-Aβ therapeutics on prevention or removal of CAA in an informative manner. It should be pointed out that the findings of this study are limited only to surface vasculature and may not generalize
to vessels affected by CAA in the deeper cortical layers. Likewise, this investigation, similar to studies with postmortem human tissue, allowed analysis of pathology at a single time point only. An important future step, made possible by in vivo techniques for amyloid labeling and detection (13, 22, 31), will be to follow the progression of CAA in individual vessels in living animals over time.

REFERENCES