Association of Vascular Amyloid β and Cells of the Mononuclear Phagocyte System in Hereditary Cerebral Hemorrhage with Amyloidosis (Dutch) and Alzheimer Disease

MARIAN L. C. MAAT-SCHIEMAN, SJOERD G. VAN DUINEN, ANNEMIEKE J. M. ROZEMULLER, JOOST HAAN, AND RAYMUND A. C. ROOS

Abstract. Arterial and arteriolar amyloid-β (Aβ) deposition in hereditary cerebral hemorrhage with amyloidosis (Dutch) (HCHWA-D) and Alzheimer disease (AD) cerebral amyloid angiopathy (CAA) were studied as to morphology, extent, and association with mononuclear phagocyte system (MPS) cells using Aβ, a-smooth muscle actin, and monocyte/macrophage marker (HLA-DR, CD68, CD11c, CD45) immunohistochemistry. The HCHWA-D/AD arterial/arteriolar media showed compact Aβ deposits, first appearing at the media/adventitia junction, and concomitant smooth muscle loss. Only HCHWA-D CAA featured (a) severe involvement of larger arteries and (b) arterioles showing a single or double ring of radial Aβ surrounding compact Aβ. Radial Aβ appeared to develop at the media/adventitia junction. Monocyte/macrophage marker-positive fasci/cells co-localized with HCHWA-D arterial Aβ. Focal HLA-DR/CD11c positivity was observed at the media/adventitia junction of AD/HCHWA-D arteries in the absence of local Aβ, but not in controls. Monocyte/macrophage marker positivity co-localized with radial Aβ appeared continuous with perivascular cells and microglia clustering perivascularly. These results suggest that (a) MPS cells are topographically associated with HCHWA-D arterial Aβ and radial arteriolar Aβ, and (b) HLA-DR/CD11c immunoreactivity may appear at the media/adventitia junction prior to Aβ. The latter finding and the assumed formation of radial Aβ at the media/adventitia junction may relate to involvement of the abluminal basement membrane in CAA pathogenesis. The role of MPS cells in this process remains to be established.

Key Words: Alzheimer disease; Amyloid β; Cerebral amyloid angiopathy; Class II major histocompatibility antigen HLA-DR; Hereditary cerebral hemorrhage with amyloidosis (Dutch); Mononuclear phagocyte system.

INTRODUCTION

Cerebral amyloid angiopathy (CAA) due to deposition of amyloid β (Aβ) is the pathological hallmark of hereditary cerebral hemorrhage with amyloidosis (Dutch) (HCHWA-D).

Hereditary cerebral hemorrhage with amyloidosis (Dutch) is an autosomal dominant disease, clinically characterized by recurrent strokes with an onset between 45 and 60 years and by dementia (1–4). The cause of the disease is a codon 693 point mutation of the β protein precursor (BPP) gene on chromosome 21 (5, 6). Cerebral amyloid angiopathy may be a cause of sporadic cerebral hemorrhage and an accompanying feature of Alzheimer disease (AD), Down syndrome, and aging (7).

Vascular smooth muscle cells (SMC) have been implicated as the source of arterial and arteriolar Aβ (8–10). Smooth muscle cells are immunoreactive with antibodies to BPP (8, 9), and they secrete BPP in culture (11). Furthermore, immunohistochemical studies in AD have revealed that arterial and arteriolar Aβ are first deposited in the outer tunica media (9), nonfibrillar Aβ accumulating in and between SMC in the early stages of vascular Aβ formation (10, 12, 13). The production of capillary amyloid fibrils has been attributed to perivascular cells (PVC) (10, 14). Perivascular cells are located outside and within the vascular basement membrane (VBM) and constitutively express class II MHC antigen HLA-DR (15–17). β protein precursor has been localized to PVC by immuno-electronmicroscopy (18). Interactions of Aβ and BPP with extracellular matrix proteins of the VBM may play a role in the process of vascular Aβ formation (19, 20). Notably, Aβ fibrils first appear in the abluminal side of the outer VBM (21).

Many cells other than SMC and PVC, including blood cells, endothelial, neuronal, and glial cells, express BPP. In addition, a soluble form of Aβ (sAβ), primarily sAβ40 and minor amounts of the more amyloidogenic sAβ42, appears to be a normal product of BPP metabolism that is present in biological fluids (22–25). Both sAβ40 and sAβ42 can cross the blood-brain barrier and sAβ40 is extensively sequestered in the cerebral microvasculature (26). Hence, it has been hypothesized that circulating sAβ could be a precursor of cerebrovascular amyloidosis (26). Aβ40 and Aβ42 have been variously identified as the major component of vascular Aβ (27, 28).

The close association of SMC and PVC with amyloid fibrils in the arterial and capillary BM, respectively, has been likened to the morphological association between microglia and amyloid fibrils in AD senile plaques (29). Perivascular cells and microglia putatively belong to the mononuclear phagocyte system (MPS) (15, 16, 30, 31). Notably, strong peripheral staining for monocyte/macrophage markers has been found in cortical angiopathic blood vessels in HCHWA-D, but not in AD (32). The present study addresses the issue of whether this discrep-
MATERIALS AND METHODS

Pieces of leptomeningeal tissue that were isolated from the cerebral cortex and pieces of frontal cortex with the leptomeninges in situ were obtained at autopsy from 10 patients with HCHWA-D, 5 patients with AD (without cerebral hemorrhage), and 7 nondenominated controls without cerebral hemorrhage or CAA (Table 1). Tissue pieces were fixed in 10% buffered formalin (the duration of fixation varied from 24 hours to several months) for paraffin sections or frozen in liquid nitrogen for cryostat-sectioning. Hematoxylin and eosin staining was performed routinely. Paraffin sections of frontal cortex were also stained by the methenamine Bodian method (33).

Immunohistochemistry

The primary antibodies used are listed in Table 2, together with their specificity, sources, selected references and dilution (34–41). For immunohistochemical staining on formalin-fixed tissue, 5-mm-thick paraffin sections were dehydrated in ethanol and preincubated in 0.3% H2O2 to block endogenous peroxidase. Immunohistochemical staining for Aβ on paraffin sections was enhanced by trypsinization (0.5% trypsin in 0.5% CaCl2) and treatment with 85% formic acid for 30 minutes (min). Immunohistochemical staining for HLA-DR and CD68 on 24 h formalin-fixed and paraffin-embedded sections, available from 4 patients with HCHWA-D, was enhanced by trypsinization, either preceded or not preceded by boiling the sections in citrate buffer (pH 6.0) for 25 min. For immunohistochemical staining on frozen tissue, 10-mm-thick cryostat sections were fixed in acetone for 10 min before use. The specificity of each antibody against macrophages was evaluated in lymphoid tissue (spleen, tonsil). The technique that was used for each antibody on lymphoid tissue was used on meningoocortical tissue. All antibodies were appropriately diluted in phosphate-buffered saline (PBS), pH 7.4, containing 1% bovine serum albumin. Secondary antisera and reagents were tested for cross-reactivity and nonspecific staining. Deparaflainized rehydrated sections or acetone-fixed cryostat sections were preincubated with normal goat serum, followed by overnight incubation with the primary antibodies. In the second step, sections were incubated with biotinylated rabbit anti-mouse immunoglobulin (DAKO, Glostrup,

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<th>Antibody (clone)</th>
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<th>Specificity</th>
<th>Source (reference)</th>
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<td>Monocytes/macrophages</td>
<td>DA (41)</td>
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DA = Dakopatts, Denmark; S = Sigma; Cl = Clonab, Biotest, Germany; BD = Becton and Dickinson; P = paraffin; C = cryostat.

Denmark) for 30 min, followed by peroxidase-conjugated streptavidin (DAKO, Glostrup, Denmark) for 30 min. Peroxidase activity was visualized using 3,3'-diaminobenzidine (DAB) (5 mg DAB in 10 ml PBS, pH 7.4, containing 0.02% H2O2), for 3 to 5 min. Negative controls included staining with deletion of one or more steps or staining with nonspecific antibodies of the same immunoglobulin class.

RESULTS

Aβ Immunostaining

The meningeocortical blood vessels in control patients did not show Aβ deposits. Focal to continuous compact Aβ deposition was observed in the outer tunica media at the junction with the adventitia of large meningeal arteries in the patients with HCHWA-D. Extensive Aβ deposition was seen in meningeal veins. Compact Aβ, often containing vacuoles from SMC that had disappeared, largely occupied the media of small meningeal arteries and cortical arteries. Besides, cortical arteries were observed with compact Aβ surrounded by a ring of radially arranged Aβ, peripherally limited by a thin line. Others showed tufts of radial Aβ protruding from this line to form an incomplete second ring. Arterioles with a complete, dense, and broad second ring of radial Aβ were found in the oldest HCHWA-D patients (Fig. 1A, B). Radial Aβ was virtually absent from meningeal blood vessels, even though they were heavily laden with compact Aβ. It appeared after the entrance of the blood vessel into the cerebral cortex (Fig. 1C). In the AD patients, small meningeal arteries and cortical arteries were observed with compact Aβ deposits occupying varying portions of the media, from the outer media to the entire media (Fig. 2A). In one patient several focal Aβ deposits were found at the media/adventitia junction of larger meningeal arteries. Meningeal veins were not affected.

SMA4 Immunostaining

In controls, SMA4 immunoreactivity was localized to SMC of the arterial and arteriolar media and of venous walls. Loss of SMA4 immunostaining was observed at sites of Aβ deposition in HCHWA-D and AD blood vessels. Usually, few lumen-sided SMC or no SMA4-positive SMC were left in small angiopathic meningeal arteries and cortical arteries (see Fig. 3C).

Monocyte/Macrophage Markers

HLA-DR, CD68, CD11c, and weakly CD45 immunoreactive cells were observed apposed to and within the adventitia of large meningeal arteries either in controls or in HCHWA-D and AD patients (see Fig. 4C, F). Similarly immunoreactive cells were apposed to small meningeal arteries and cortical arteries with or without Aβ (Fig. 2B). Endothelial cells showed inconsistent HLA-DR immunoreactivity in controls and patients. HLA-DR and CD11c immunostaining frequently, but not invariably, obscured the rings of radial Aβ of HCHWA-D arterioles in cryostat sections, whereas radial Aβ showed irregularly distributed HLA-DR and slight CD68 immunoreactivity in paraffin sections. This immunoreactivity appeared to be continuous with the HLA-DR and CD68 immunoreactive cells apposed to arterioles with one radial ring of Aβ as well as with HLA-DR- and CD68-positive cells with the morphology of microglial cells clustering in particular around arterioles with a partial or complete second ring (Fig. 3A–E).

Numerous irregularly shaped foci of strong HLA-DR immunoreactivity and a lesser number of CD68-, CD11c-, and weakly CD45-positive foci co-localized with Aβ deposits in the outer media of large HCHWA-D arterioles in cryostat sections (Fig. 4A–F). These foci were difficult to identify as cells because of poor morphological detail. HLA-DR and CD68 immunoreactive deposits were more readily identifiable as cells in paraffin sections (Fig. 5A–C), but they occurred in lesser numbers than in cryostat sections. Short tissue-fixation and pretreatment (trypsinization and boiling) enhanced HLA-DR and CD68 immunostaining in paraffin sections, but the results did not equal those in cryostat sections.

As evidenced in adjacent cryostat sections, irregularly shaped HLA-DR-positive foci were observed along the media/adventitia junction of AD and HCHWA-D meningeal arteries in the absence of local Aβ deposits. Delineate foci of CD11c immunoreactivity were found in the same location (Fig. 6A–C). By contrast, the media/adventitia junction of control arteries did not show any sporadically showed HLA-DR- or LeuM5-positive deposits.

DISCUSSION

Comparison of vascular Aβ deposition in the patients with HCHWA-D and AD of this study revealed that the tunica media of small meningeal arteries and of cortical arterioles shows compact Aβ deposits in both disorders. Severe involvement of large meningeal arteries and veins was observed in HCHWA-D. As in AD CAA (9), the media/adventitia junction appeared to be the site of initial Aβ deposition in HCHWA-D arterioles and arterioles, while vascular SMC disappeared along with Aβ deposition. In HCHWA-D, but not in AD, cortical arterioles were observed showing a single or double ring of radially arranged Aβ adjacent to an inner layer of compact Aβ. Since CAA appeared to be most advanced in the patients with HCHWA-D, the development of radial Aβ may be a manifestation of severe CAA. Alternatively, it may be the consequence of a specific etiology of CAA. In case of HCHWA-D, faster aggregation, accelerated formation of more stable fibrils, and enhanced toxicity to SMC, as observed for HCHWA-D Aβ in vitro, may play a role in...
Fig. 1. (A) HCHWA-D cortical arteriole showing an inner layer of compact Aβ (arrowhead), which is surrounded by a ring of radial Aβ (asterisk). The ring is bordered by a thin line (arrow), from which tufts of radial Aβ (open arrows) protrude to form an incomplete outer ring. (B) Cortical arteriole in an 81-year-old patient with HCHWA-D showing 2 complete rings of radial amyloid surrounding a layer of compact amyloid that shows vacuoles in places where SMC have disappeared. (C) Transition of an angiopathic meningeal artery to a cortical arteriole in HCHWA-D. Radial amyloid (arrows) appears next to vacuolated compact...
this respect (42–47). Furthermore, the mutant Aβ may have binding properties other than nonmutated Aβ (48, 49). Differences between vascular Aβ formation in HCHWA-D and AD have been hypothesized previously in relation to strong βPP immunoreactivity of HCHWA-D CAA as compared to AD CAA (50).

Arterioles were observed (a) with one ring of radial Aβ bordered by a thin line, (b) with tufts of radial Aβ protruding from this line, and (c), only in aged patients, with 2 prominent rings of radial Aβ separated by a thin line. Ultrastructural studies of cortical blood vessels with radial amyloid in a 65-year-old demented patient have

amyloid after the entrance of the vessel into the cerebral cortex (asterisks, pia mater). In the left upper corner a meningeal artery laden with compact amyloid is seen in cross section. (A) Aβ immunostaining, (B) methenamine Bodian method, and (C) hematoxylin and eosin staining of paraffin sections. (A–C), ×170.
Fig. 3. (A) HCHWA-D cortical arteriole showing a layer of compact Aβ surrounded by one radial ring of Aβ. Several nuclei (arrows) of cells in a perivascular position are discernible. (B) HLA-DR immunoreactivity is irregularly distributed within the radial ring and appears continuous with perivascularly localized HLA-DR immunoreactive cells (arrows). (C) SMC (arrowheads) are left only in the innermost media. Nuclei of cells apposed to radial Aβ in a perivascular position are discernible (arrows). (D) Perivascularly localized CD68-positive cells are apposed to an HCHWA-D cortical arteriole with one ring of radial amyloid. Slight CD68 immunoreactivity extends into the radial ring. (E) HCHWA-D cortical arteriole with an inner and an incomplete outer ring of radial Aβ (not shown). Radial amyloid shows strong, irregularly distributed HLA-DR immunoreactivity continuous with HLA-DR-positive cells with the morphology of microglia clustering around the vessel. (A) Aβ, (B) HLA-DR, and (C) SM1/A4 immunostaining of adjacent paraffin sections, (D) CD68 and (E) HLA-DR immunostaining of paraffin sections. (A–E), ×170.
Fig. 4. (A) Large meningeal artery in HCHWA-D showing Aβ immunoreactivity in the outer media and (B) numerous irregularly shaped HLA-DR-positive deposits in the same area, closely following Aβ deposition along the length of the vessel. (C) Higher magnification of HLA-DR-positive deposits co-localizing with Aβ deposits (not shown) in the outer media of an HCHWA-D artery and reaching as far into the media as Aβ deposition. Several elongated HLA-DR-positive cells (arrowheads) are present in or along the adventitia. (D) CD68, (E) CD11c, and (F) weak CD45 immunoreactivity (arrows) co-localizes with amyloid in the outer media of HCHWA-D arteries, an elongated CD45-positive cell (arrowhead) is present in the adventitia, l lumen, m tunica media, a tunica adventitia. (A) Aβ and (B) HLA-DR immunostaining of adjacent cryostat sections, (C) HLA-DR, (D) CD68, (E) CD11c, and (F) CD45 immunostaining of cryostat sections. (A–B), ×42.5; (C–F), ×170.
Fig. 5. (A) Several HLA-DR immunoreactive cells (asterisks) and deposits co-localize with (B) Aβ occupying the outer part of the media of an HCHWA-D meningeal artery. (C) CD68-positive cells (asterisks) also co-localize with Aβ, l lumen, m tunica media, a tunica adventitia. (A) HLA-DR, (B) Aβ, and (C) CD68 immunostaining of paraffin sections. (A–C), ×170.
shown that radially arranged bundles of amyloid fibrils compose the inner and outer ring. The thin line in between is localized at the media/adventitia junction and contains longitudinally running amyloid fibrils and collagenous fibers. The inner ring merges centrally with compact amyloid, i.e. mostly longitudinally arranged amyloid fibrils in between vacuoles of SMC that have disappeared. The outer ring extends within the confines of the adventitia (51). Together, these observations may suggest that the formation of the outer ring takes place at the media/adventitia junction and that the outer ring develops after the inner one and not at the same time. The former notion may relate to the ultrastructural observation that amyloid fibrils develop within the abluminal side of the outer VBM (21). The latter one implies that the outer ring develops at a stage in which all or all but the innermost SMC have disappeared from the vessel wall. Therefore, Aβ from sources other than SMC might contribute to radial Aβ deposition. The possibility that sAβ circulating in CSF or serum may play a role in this regard (26, 52, 53) is supported by the finding that sAβ binds to pre-existing Aβ deposits of CAA in organ cultures of canine leptomeninges (54). However, it is difficult to reconcile with the observation in this and a previous study by others (55) that meningeal blood vessels lack radial Aβ. The formation of radial Aβ in these vessels might be a matter of time, but its absence in meningeal vessels in the oldest HCHWA-D patient of this study, an octogenarian, may argue against this option. Presumably, the development of radial Aβ is determined by local factors in or around the vessel wall.

The HLA-DR+, CD68+, CD11c–, and weakly CD45-positive cells observed along arteries and arterioles with and without Aβ deposition likely represent PVC (17). This study extends the recent finding of monocyte/macrophage marker immunoreactivity in the peripheral part of the congophilic wall of HCHWA-D cortical blood vessels (32) with the observation that this immunoreactivity is localized within radial arteriolar Aβ. It appears to be associated with PVC apposed to and with microglial cells clustering around the vessel wall, both presumably extending their processes between the radially arranged bundles of amyloid fibrils. Aβ has been shown to be chemotactic for MPS cells, including microglia, and to activate macrophages (56, 57). Furthermore, it has been demonstrated that PVC in rat brain act as scavengers in the perivascular space (58) and that rat microglia and human macrophage-like cells are able to degrade Aβ (59). Thus, with regard to the present findings, it may be hypothesized that PVC are engaged in the removal of radial Aβ in the first instance, whereas microglia become involved in this process once the parenchymal BM is breached, the latter event evoking a neuritic and astroglial reaction as well (60–62). Activated macrophages can cause cytotoxicity through the release of reactive oxygen.

Fig. 6. (A) Numerous irregular HLA-DR-positive deposits and (B) delicate CD11c-positive deposits at the junction of media and adventitia of AD arteries without Aβ deposition in adjacent sections (not shown). (C) Higher magnification of CD11c immunoreactivity (arrows) at the junction of media and adventitia of an AD artery without Aβ (not shown). (A) HLA-DR, (B–C) CD11c immunostaining of cryostat sections. (A–B), ×42.5; (C), ×170.
and/or nitrogen intermediates and cytokines such as interleukin-1 (IL-1) (63). Free radical damage has been shown to induce the aggregation of $\alpha$B (64). In addition, IL-1 upregulates $\beta$PP expression and stimulates $\beta$PP processing (65). Furthermore, microglia and macrophages have the ability to process exogenously derived $\beta$PP (66). Thus, PVC and microglia, possibly involved in the removal of $\alpha$B or other components of the vascular wall, may contribute to the degeneration of SMC and foster $\alpha$B deposition.

Monocyte/macrophage immunoreactive foci were found to co-localize with $\alpha$B in the media of HCHWA-D meningeal arteries. They were more numerous in the cryostat than in paraffin sections and only in the latter were they readily identifiable as cells. The slight extent of $\alpha$B deposition in AD meningeal arteries in this study did not allow a conclusion as to the co-localization of MPS cells with AD arterial $\alpha$B. Others have reported the absence of macrophages/leukocytes at sites of $\alpha$B formation in AD arteries (12). Technical factors, in terms of tissue fixation and pretreatment of the sections, may account for the different findings in HCHWA-D. However, a recent study employing cryostat sections showed absence of the inflammation-inducible intercellular adhesion molecule-1 in arterial $\alpha$B deposits in AD (67). The MPS cells co-localizing with HCHWA-D arterial $\alpha$B may have a function comparable to that of PVC and microglia associated with HCHWA-D arteriolar $\alpha$B.

The presence of HLA-DR-positive and CD11c-positive foci at the junction of media and adventitia of AD and HCHWA-D arteries in the absence of local $\alpha$B deposits may imply that the appearance of HLA-DR and CD11c immunoreactivity precedes arterial $\alpha$B deposition detectable by light microscopy. This finding and the recent observation by others (68) of accumulation of apolipoprotein E (apoE) immunoreactivity at the junction between media and adventitia prior to $\alpha$B deposition may relate to pathological changes at the site of the outer BM in the early stages of $\alpha$B formation. In this regard, the ultrastructural observation that the first fibrils appear at the abluminal side of the outer BM (21) may be significant. The local formation of fibrils may be due to disturbances of the mechanism clearing $\alpha$B from the extracellular space. Scavenging of complexes between $\alpha$B and $\alpha$B-sequestering proteins like apoE may be involved in this process (69). A larger number of patients and controls has to be investigated to gain information about the specificity and the significance of the present observation.

In conclusion, this immunohistochemical study demonstrates the presence of radial arteriolar $\alpha$B in HCHWA-D, but not in AD. Further studies are needed to clarify whether the development of radial $\alpha$B in addition to compact $\alpha$B depends on the specific cause of CAA or relates to the more advanced degree of CAA in HCHWA-D as compared to AD. Furthermore, the topographical association of MPS cells with HCHWA-D arterial $\alpha$B and of putative MPS cells in the form of PVC and microglial cells with radial arteriolar $\alpha$B is shown. The function of these cells remains to be established. This study also suggests that radial arteriolar $\alpha$B develops at the media/adventitia junction and that the appearance of monocyte/macrophage immunoreactivity at the media/adventitia junction precedes $\alpha$B deposition. The latter observations may relate to the enigmatic role of the outer VBM in vascular $\alpha$B formation (21).

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