Differential Relationships of Reactive Astrocytes and Microglia to Fibrillar Amyloid Deposits in Alzheimer Disease

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Abstract

Although it is clear that astrocytes and microglia cluster around dense-core amyloid plaques in Alzheimer disease (AD), whether they are primarily attracted to amyloid deposits or are just reacting to plaque-associated neuritic damage remains elusive. We postulate that astrocytes and microglia may differentially respond to fibrillar amyloid. Therefore, we quantified the size distribution of dense-core thioflavin-S (ThioS)-positive plaques in the temporal neocortex of 40 AD patients and the microglial and astrocyte responses in their vicinity (<50 μm) and performed correlations between both measures. As expected, both astrocytes and microglia were clearly spatially associated with ThioS-positive plaques (p = 0.0001; ≤50 μm vs >50 μm from their edge), but their relationship to ThioS-positive plaque size differed: larger ThioS-positive plaques were associated with more surrounding activated microglia (p = 0.0026), but this effect was not observed with reactive astrocytes. Microglial response to dense-core plaques seems to be proportional to their size, which we postulate reflects a chemotactic effect of amyloid β. By contrast, plaque-associated astrocytic response does not correlate with plaque size and seems to parallel the behavior of plaque-associated neuritic damage.

Key Words: Alzheimer disease, Amyloid plaques, Apolipoprotein E, Astrocytes, Microglia.

INTRODUCTION

Dense-core amyloid plaques are one of the pathologic hallmarks present in the brain of patients with Alzheimer disease (AD). They are defined by the presence of a compact core of fibrillar amyloid that can be stained with dyes specific for protein aggregates rich in β-pleated sheet structure, such as thioflavin-S (ThioS) and Congo red. The microenvironment within and around these dense-core plaques is characterized by the presence of both neuritic dystrophies and astrocytic and microglial responses (1–12).

Plaque-associated neuritic changes include distorted dendrites and axons, as well as bulbous varicosities or swellings that are generally thought to form as a result of the toxic effect of plaques on the surrounding neuropil (1–4). We have previously reported that, besides these obvious neuritic dystrophies, a more subtle expression of neuritic change consisting of an increased curvature of the trajectory of otherwise normal-looking neurites occurs in the plaque microenvironment (i.e. within 50 μm) (13–15). Importantly, this increased curvature ratio of neurites surrounding dense-core plaques is independent of plaque size, indicating that 1) it is not explained by a “mass effect” of plaques on surrounding neurites, and 2) there is no proportionality between the extent of amyloid fibrils deposited and this local neurotoxic feature of dense-core plaques (15). Similarly, synaptic loss radiates out from the surface of dense-core plaques and is another feature of this 50-μm halo of neuronal damage that exists around them (16, 17).

The roles of plaque-associated glial responses in AD remain controversial (5–11, 18–20). Reactive astrocytes and activated microglial cells may be primarily attracted to plaques by amyloid β (Aβ) species and/or proinflammatory cytokines; however, an alternative and equally plausible explanation is that these glial cells cluster around dense-core plaques because they react to the plaque-induced neuritic damage, perhaps exerting neuroprotective effects (19).

We have previously shown that plaque-associated neuritic curvature is largely independent of plaque size (15); herein, we investigated the relationship between reactive glia and plaque size. We reasoned that if plaque-associated glial responses were chemotactically driven by Aβ deposition, then their magnitude would correlate with plaque size; whereas, if glial cells were responding primarily to plaque-associated neuritic changes, their numbers would not be influenced by plaque size. We performed stereology-based quantitative measures of reactive astrocytes and activated microglia and correlated them with the size distributions of dense-core (ThioS-positive) amyloid plaques from the temporal neocortex of a large group of AD patients. Our results suggest that astrocytes and microglia differentially respond to fibrillary Aβ deposits. The proportionality between plaque-associated microglial response and plaque size favors the idea of a direct chemotactic effect of Aβ on microglia, whereas the independence of plaque-associated astrocytic response from...
plaque size resembles the behavior of plaque-associated neuritic changes.

**MATERIALS AND METHODS**

**Subjects**

Forty AD patients were selected from the Massachusetts Alzheimer Disease Research Center Brain Bank. Demographic and clinical characteristics of this sample have been reported before (21, 22) (Table 1). All the study subjects or their next of kin gave informed consent for the brain donation, and the Massachusetts General Hospital Institutional Review Board approved the study protocol. All the subjects fulfilled the NINCDS-ADRDA criteria for probable AD (23) and the NIA-Reagan criteria for high likelihood of AD (24). Because cerebrovascular disease is a major cause of focal gliosis, cases with cerebrovascular disease considered severe enough to contribute to a dementia syndrome were excluded. Cases with Lewy body pathology were also excluded.

**Brain Specimens and Immunohistochemical Studies**

Eight-micrometer-thick, formalin-fixed, paraffin-embedded sections from the temporal association isocortex (BA38) were deparaffinized for immunohistochemistry by standard methods. This region was selected because it is a multimodal association area that frequently has moderate numbers of dense-core plaques in AD sufficient to allow the sampling of 100 of these plaques per section (25, 26). Primary and fluorescently labeled secondary antibodies are listed in Table 2. Sections were microwaved for 20 minutes at 95°C in boiling citrate buffer (0.01 mol/L, pH 6.0, 0.05% Tween-20) for antigen retrieval. Gliarial fibrillary acidic protein (GFAP) was used as a marker for reactive astrocytes (27). Ionized calcium-binding adaptor molecule 1 (Iba1) and CD68 were used as markers for activated microglia. Ionized calcium-binding adaptor molecule 1 is a cytosolic protein upregulated in activated microglia (28), whereas CD68 is a widely used marker for cells with a phagocytic phenotype, including macrophages and microglia, and is mainly located in the lysosomal membrane (29). After immunostaining, the sections were counterstained with ThioS (Sigma-Aldrich, St. Louis, MO) 0.05% in 50% ethanol for 8 minutes and coverslipped with Vectashield mounting media with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA).

**Dense-Core Plaque Size Distribution**

The size distribution of dense-core plaques in the 40 AD cases was obtained by manually outlining the perimeter of the ThioS-positive plaques photographed for the analysis of glial responses close to plaques with the appropriate tool of the public domain software ImageJ (http://rsbweb.nih.gov/ij/). The fields photographed in each section (142,606 μm²) were randomly selected under the 20 × objective of a BX51 Olympus epifluorescence microscope equipped with a DP70 CCD camera and a motorized stage controlled by the CAST stereology software (Olympus, Tokyo, Japan). To reach the end point of 100 plaques while ensuring an adequate representation of all 6 cortical layers and a sufficient representation of the entire cortical ribbon of the specimens, the meander sampling was set at 1%. Cross-sectional areas of plaques from anti-GFAP, -CD68, and –Iba1–immunostained sections of the same case were pooled because the low meander sampling used made the double measurement of the same plaque in nearly adjacent sections very unlikely (n = 300).

**Stereology-Based Quantitation of Reactive Glial Cells Close to Dense-Core Plaques**

Sections from the 40 AD patients were fluorescently stained for reactive astrocytes (GFAP) or activated microglia (both CD68 and Iba1) and dense-core plaques (ThioS positive). The reactive glial cells surrounding dense-core plaques were quantified using 2 different approaches with the CAST-Olympus system.

First, in a glia-centered approach, 100 GFAP-positive astrocytes or Iba1-positive microglial cells per section were randomly selected under the 20 × or the 40 × objective, respectively, and their distance with respect to the closest dense-core plaque was measured with the appropriate tool of the CAST software, as previously described (15, 21, 30) (Fig. 1).
Briefly, the entire cortical ribbon of the specimen was outlined as the region of interest. To reach the end point of 100 cells while ensuring that all 6 cortical layers in the entire cortical ribbon were evenly covered, the meander sampling was tailored to each specimen and typically set between 1% and 4%. The size of the optical disector was set at 10% \( (3,565.2 \mu m^2 \text{ for Iba1-positive microglia and } 14,260.2 \mu m^2 \text{ for GFAP-positive astrocytes}) \). Only glial cells with a visible nucleus in the DAPI staining were counted and only if their cell soma fell within the counting frame or touching one of the 2 green sides of the counting frame (but none of the red sides) were considered. (C, D) Next, the presence of a ThioS-positive dense-core plaque was evaluated in the blue-green channel (C), and, if present, the distance between the glial cells and the closest plaque was measured with the appropriate tool of the CAST stereology software (D). Glial cells were considered close to plaques if their soma was located within 50 \( \mu m \) from the nearest plaque edge and far from them if located beyond this boundary.

FIGURE 1. Glia-centered stereology-based study. (A) Diagram represents a typical disector map resulting from the random sampling of a paraffin section of temporal cortex using the parameters described in the Materials and Methods section. (B) Only glial cells (in this example, GFAP-positive astrocytes, numbered 1, 2, 3) with a DAPI-visible nucleus and whose soma fell within the counting frame and/or touched one of the 2 green sides of the counting frame (but none of the red sides) were considered. (C, D) Next, the presence of a ThioS-positive dense-core plaque was evaluated in the blue-green channel (C), and, if present, the distance between the glial cells and the closest plaque was measured with the appropriate tool of the CAST stereology software (D). Glial cells were considered close to plaques if their soma was located within 50 \( \mu m \) from the nearest plaque edge and far from them if located beyond this boundary.
tool of ImageJ (Fig. 2). Glial cells located close to 2 or more dense-core plaques according to this criterion were “split” among those plaques (i.e. 0.5 astrocytes or microglial cells for those close to 2 plaques, 0.33 for those close to 3 plaques, 0.25 for those close to 4 plaques, etc.). This conservative criterion was implemented to avoid possible bias of the results caused by double counting of glial cells close to dense-core plaques in fields with a high density of plaques. These counts were then normalized to the size of the corresponding plaques (including the 50-μm boundary) to avoid differential sampling of glial cells within the neuropil because of differential plaque sizes. Thus, these results are expressed as the number of glial cells/mm² of area, including the plaque and a 50-μm perimeter around the plaque. Again, only glial cells with a visible nucleus in the DAPI staining were considered.

APOE Genotyping

APOE genotype was determined in all the study subjects by restriction fragment length polymorphism analysis as previously described (31).

Statistics

Normality of data sets was assessed with the D’Agostino-Pearson omnibus test. Plaque size distributions are not Gaussian because they typically exhibit a positive skew (22, 32, 33). To correlate the stereology-based quantitative measures of glial responses with the plaque size distributions, we fit mixed-effects regression models using all the plaques measures from all AD subjects and allowing for correlation within subjects. We tested whether the slopes of the regression lines were different from zero at a 2-sided significance level of p < 0.05.
We also compared the distributions of glial cells per dense-core plaque (number of cells within 50 μm of the nearest plaque edge) of AD APOEε4 carriers and noncarriers using the clustered Wilcoxon rank-sum test at a 1-sided significance level of p < 0.05 (34). Last, to compare the density of glial cells in the proximity versus far away from dense-core plaques in each APOE group, we performed a Wilcoxon sign-rank t-test for paired values. To compare these densities between both APOE groups, we used the Wilcoxon unpaired t-test. Statistical analyses were run in SAS (version 9.2; SAS Institute, Cary, NC) and GraphPad Prism for Mac (version 4.0; GraphPad Software Inc., La Jolla, CA). Graphs were performed with GraphPad Prism for Mac (version 4.0).

RESULTS

Relationship Between Reactive Glia and Dense-Core Plaque Size

To address the relationship between reactive glia and plaque size in the microenvironment around plaques, we...
quantified the density of reactive astrocytes and activated microglial cells close to dense-core plaques (≤50 μm) in sections doubly stained for GFAP or Iba1 and ThioS and then correlated these densities with the size measures of dense-core plaques. First, we observed a significantly increased density of GFAP-positive astrocytes and Iba1-positive microglia in the vicinity of dense-core plaques, as compared with further away (>50 μm) from them (p = 0.0001 for both, Fig. 3), thereby confirming the expected clustering of reactive glia around dense-core plaques. The size of dense-core plaques correlated positively with the density of surrounding Iba1-positive activated microglia (p = 0.0026), although not with the density of GFAP-positive reactive astrocytes (p = 0.1564) (Table 3). These results suggest that larger dense-core plaques are associated with more activated microglial cells in their vicinity than smaller dense-core plaques.

To evaluate the relationship between glia and the size of fibrillar amyloid deposits further, we repeated the quantitative analyses in a plaque-centered fashion. We counted the number of GFAP-positive astrocytes and Iba1-positive microglial cells in the vicinity of randomly selected dense-core plaques (≤50 μm from their edge) and correlated these counts with the size of those individual dense-core plaques. We confirmed the above significant positive correlation between the size of dense-core plaques and the number of surrounding Iba1-positive microglial cells (p < 0.0001). Similar analyses for CD68-positive (phagocytic) microglia yielded the same results (p < 0.0001). By contrast, again, GFAP-positive astrocytes did not correlate with plaque size (p = 0.6124) (Table 3; Fig. 4).

The APOE4 Genotype Does Not Impact Glial Responses to Plaques

Previous postmortem quantitative studies have established that the APOE4 allele is associated with a higher cortical amyloid plaque burden (33, 35–38). More recent studies have shown that apoE is a cofactor for the clearance of Aβ by astrocytes and microglia (39, 40) and that the efficiency of this clearance might be isoform dependent, with apoE4 being less effective than apoE3 (41, 42). In addition, APOE4 knock-in mice exhibit enhanced astrocytic and microglial activation on treatment with lipopolysaccharide as compared with APOE3 knock-in mice (43), and apoE has been involved in the migration of microglia toward common chemotactic stimuli, such as the complement factor C5a and ATP, also in an isoform-differential fashion (apoE3 > apoE4) (44).

We therefore investigated whether astrocytic and microglial responses to dense-core plaques are similar in APOE4 carriers versus noncarriers. Both glia and plaque-centered analyses revealed a quantitatively similar association of reactive astrocytes and activated microglial cells to dense-core plaques in APOE4 carriers and noncarriers (Fig. 5). Moreover, controlling for the presence of the APOE4 allele or for the number of APOE4 alleles had no significant influence on the relationship between plaque size and associated astrocytic or microglial responses (Table 3).

DISCUSSION

The nature of the interaction between amyloid plaques and reactive glia has been debated since the first descriptions of clusters of glial cells within and around senile plaques (5–7). We approached this question by studying the relationship between plaque-associated (within 50 μm from the nearest plaque edge) reactive glia and plaque size. Although postmortem studies cannot establish causal relationships, the results of our present neuropathologic quantitative study indicate that these 2 glial cell types may have differential responses to ThioS-positive fibrillar dense-core deposits.

Microglial Response to Amyloid Plaques

Our observation that microglial clustering in the vicinity of dense-core plaques is proportional to their size can be interpreted as evidence favoring a model in which a chemo- tactic signal engages microglia. Although whether such a signal is Aβ or another plaque-associated component remains

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**TABLE 3. Summary of the Results of This Study**

<table>
<thead>
<tr>
<th>Glia-Centered Analyses</th>
<th>Model 1</th>
<th>Model 2</th>
<th>Model 3</th>
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<tbody>
<tr>
<td></td>
<td>Estimate</td>
<td>Estimate</td>
<td>Estimate</td>
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<tr>
<td></td>
<td>(SD)</td>
<td>p</td>
<td>(SD)</td>
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<tr>
<td>Dense-core plaque size vs GFAP-positive astrocytes close</td>
<td>0.8721</td>
<td>0.1564</td>
<td>0.8723</td>
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<td></td>
<td>(0.6152)</td>
<td></td>
<td>(0.6237)</td>
</tr>
<tr>
<td>Dense-core plaque size vs Iba1-positive microglia close</td>
<td>1.8182</td>
<td>0.0026</td>
<td>1.8581</td>
</tr>
<tr>
<td></td>
<td>(0.6030)</td>
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<td>(0.6166)</td>
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Plaque-centered analyses

<table>
<thead>
<tr>
<th></th>
<th>Model 1</th>
<th>Model 2</th>
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<tr>
<td></td>
<td>Estimate</td>
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<tr>
<td></td>
<td>(SD)</td>
<td>p</td>
<td>(SD)</td>
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<tr>
<td>Dense-core plaque size vs GFAP-positive astrocytes close</td>
<td>0.0727</td>
<td>0.1642</td>
<td>0.1637</td>
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<tr>
<td></td>
<td>(0.1435)</td>
<td></td>
<td>(0.1314)</td>
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<tr>
<td>Dense-core plaque size vs Iba1-positive microglia close</td>
<td>0.8396</td>
<td>&lt;0.0001</td>
<td>0.6950</td>
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<tr>
<td></td>
<td>(0.1170)</td>
<td></td>
<td>(0.1019)</td>
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<tr>
<td>Dense-core plaque size vs CD68-positive microglia close</td>
<td>0.7537</td>
<td>&lt;0.0001</td>
<td>0.7485</td>
</tr>
<tr>
<td></td>
<td>(0.0970)</td>
<td></td>
<td>(0.0905)</td>
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</table>

Model 1 indicates the results of the bivariate regression analyses depicted in the first column. Model 2 is similar to Model 1 but controlling for the presence of at least one APOE4 allele (i.e., APOE4 carriers vs noncarriers). Model 3 is similar to Model 1 but controlling for the number of APOE4 alleles (i.e., none vs 1 vs 2 alleles). Values represent the estimates (SD) of the slopes and the p values of the corresponding regression models.

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J Neuropathol Exp Neurol • Volume 72, Number 6, June 2013

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unclear (44–48), substantial in vitro data suggest that the Aβ peptide is a strong candidate (49–52). It should be noted that although compact plaques are defined by the presence of a dense core of insoluble Aβ fibrils, they also exhibit a peripheral halo of more soluble and toxic oligomeric species of Aβ (16, 17, 53, 54); hence, our results do not distinguish the effect of fibrillar Aβ from that of soluble oligomeric Aβ.

Our observation is also consistent with a relatively ineffective role of microglia at clearing amyloid fibrils (55–59) or at least argues against a major role of microglia in plaque removal in the AD brain (60, 61). On the other hand, microglial responses to plaques may contribute to stabilizing plaque size, which remains relatively stable throughout the clinical course of the disease (22, 33), perhaps by effectively phagocytosing and degrading the smaller and more soluble Aβ species in the halo around dense-core plaques (59).

Astrocytic Response to Amyloid Plaques

In sharp contrast to the microglial response, the magnitude of astrocytic reaction did not correlate with plaque size. This observation resembles our previous finding of the absence of correlation between plaque size and neurite curvature ratio—an expression of plaque-induced neuritic damage subtler than plaque-embedded neuritic dystrophies (15)—and suggests that plaque-associated reactive astrocytes are preferentially reacting to plaque-induced neuritic damage rather than to Aβ itself. Because periplaque Aβ oligomers can directly induce the morphologic neurodegenerative triad of spine loss, dendritic simplification, and neuritic dystrophies (62), it is difficult to dissect the primary trigger of astrocytic reaction. However, our interpretation is supported by a recent study in APP/PS1 mice with a GFAP and vimentin null background. Deletion of both glial filament proteins prevented the contact of astrocytes with plaques and resulted in a marked increase in the number of dystrophic neurites per plaque, suggesting that plaque-associated reactive astrocytes actually protect neurons surrounding the plaques (19). Further studies in experimental systems will be necessary to evaluate the primary cause of plaque-associated astrocytic responses.

Role of APOE Genotype in Glial Responses to Amyloid Plaques

Importantly, carrying the APOEε4 allele had no significant impact on the previous results; this argues against a differential effect of the apoE isoforms on glial cell activation and migration toward plaques and extends our previous observation of a similar magnitude of glial responses at postmortem
examination between APOE4 carriers and noncarriers (21, 63). However, because the apolipoprotein E is mainly secreted by astrocytes and, to a lesser extent, microglia, the APOE genotype may still influence other aspects or features of the astrocytic and microglial phenotypic change that occurs in AD by as yet unknown autocrine or paracrine mechanisms (64).

In summary, we observed that activated microglia and reactive astrocytes differentially interact with ThioS-positive fibrillar amyloid deposits. Microglial responses to dense-core plaques seem to be proportional to plaque size, whereas plaque-associated astrocytic responses do not correlate with plaque size. Amyloid β itself may be the primary chemotactic signal for activated microglia, whereas astrocytes might primarily respond to plaque-associated neuritic damage.

ACKNOWLEDGMENT

The authors thank the patients and caregivers involved in the research at Massachusetts General Hospital.

REFERENCES


