IMMUNOCHEMICAL AND MORPHOMETRIC FEATURES OF ASTROCYTE REACTIVITY vs. PLAQUE LOCATION IN ALZHEIMER’S DISEASE

MARIA C. VANZANI1, RUBEN F. IACONO2, ROBERTO L. CACCURI1, MARIA I. BERRIA1

1Departamento de Microbiología, Facultad de Medicina; 2Cátedra de Inmunología, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires

Abstract The quantitative relationship between glial fibrillary acidic protein (GFAP) hyper-reactivity and β-amyloid protein (βAP) deposition was investigated by double immunoperoxidase labeling of hippocampal and entorhinal cortex sections from five Alzheimer’s disease (AD) cases and five age-matched controls. βAP plaques, which were absent in controls, were found in all AD samples, without significant differences in number or perimeter according to their location among the regions studied. In contrast, the mean number of GFAP (+) cells was significantly greater in the hippocampus than in the entorhinal cortex from AD cases (49 vs. 39). Although at lower values (30 vs. 20), predominance of astrocyte hyperplasia in hippocampus as compared with entorhinal cortex was also found in control samples. Concomitant astrocyte hypertrophy, as defined by surface density (Sv) values of GFAP-immunoreactive material exceeding those of control means, affected a similar proportion of cells in the hippocampus (73%) and the entorhinal cortex (74%) from AD cases. Since an increased number of GFAP (+) cells in the hippocampus was not accompanied by an increased number and/or perimeter of neighbouring plaques, such differential hyper-reactivity in samples from AD patients, as well as in those with normal aging, seems to depend partially on the regional location of the involved astrocyte.

Key words: hippocampus, entorhinal cortex, GFAP, βAP, quantitative analysis

Resumen Características inmunoquímicas y morfométricas de reactividad astrocitaria vs. localización de placas en enfermedad de Alzheimer. La relación cuantitativa entre la hiperreactividad de la proteína gliofibrilar ácida (GFAP) y los depósitos de proteína β-amiloide (βAP) fue investigada mediante doble marcación por inmunoperoxidasa en cortes histológicos de hipocampo y corteza entorrinal correspondientes a cinco casos de enfermedad de Alzheimer (AD) y cinco controles de edades similares. Las placas βAP, ausentes en controles, se encontraron en cambio en todas las muestras AD, donde no se observaron diferencias significativas en número o perímetro según su localización en las regiones estudiadas. En cambio, el número de células GFAP (+) fue significativamente mayor en hipocampo que en corteza entorrinal (49 vs. 39). Aunque a menores valores (30 vs. 20), el predominio de hiperplasia astrocitaria en hipocampo con respecto a corteza entorrinal, también se observó en controles. En AD, una concomitante hipertrofia astrocitaria, definida por valores de densidad de superficie (Sv) del material inmunorreactivo para GFAP, afectó a un número similar de células en hipocampo (73%) y corteza entorrinal (74%). Dado que el aumentado número de células GFAP(+) no se acompañó de mayor número y/o perímetro de placas vecinas, la hiperreactividad regional exhibida tanto por AD como por envejecimiento normal, parecería depender de la localización del subtipo astrocitario involucrado.

Palabras clave: hipocampo, corteza entorrinal, GFAP, βAP, análisis cuantitativo

Astrocyte activation, known as the earliest CNS response to injury and initially described by enhanced number and size of such cells, is currently evidenced by the overimmunoreactivity of glial fibrillary acidic protein (GFAP), the main 8-9 nm intermediate filament of reactive astrocyte1. In this connection, a significant increase in GFAP expression has been observed in many neurodegenerative disorders, including Alzheimer’s disease (AD), the most common dementia in the elderly. Though the abundance of diffuse and dense-core amyloid plaques in specific brain areas is a neuropathological hallmark of AD, the concomitant presence of astrocyte activation is also a salient feature. Since the original description of astrocyte association with senile plaques2 has been repeatedly confirmed3-5, the pathogenic relevance of astrocyte response, either as a primary or early secondary reaction to amyloid deposition, has deserved growing attention6-8.
of astrocytes, is spatially related to the number and the surface density (Sv) of amyloid plaques, we compared the distribution of activated astrocytes with that of βAP (+) deposits in brain tissues. In these astrocytes, signs of mitotic activity were searched through the detection of a specific marker such as proliferating cell nuclear antigen (PCNA).

Taking into account that entorhinal cortex and hippocampus are representative brain areas for the histopathological diagnosis of AD, they were chosen for the present study. Since GFAP immunoreactivity is known to increase throughout adult life span, postmortem brain samples from age-matched subjects were used as controls.

Material and Methods

**Samples.** Postmortem hippocampus and entorhinal cortex from five patients (mean 69 years, range 60-75) with a clinical diagnosis of primary degenerative dementia (DSM IV-CERAD) as well as from controls represented by five age-matched deceased individuals lacking neurological disease (mean 67.2 years, range 60-77) were obtained from the Division of Pathology, Francisco Santojanni Foundation Hospital, Buenos Aires, Argentina. For initial screening, 5-µm thick sections from paraffin-embedded tissues were stained with hematoxylin-eosin.

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**Single immunohistochemistry.** Serially obtained histological sections were microwaved before overnight incubation at 4 °C with a 1/1600 dilution of a rabbit serum against bovine GFAP (Dako A/S, Denmark), or a 1/100 dilution of a mono-clonal antibody against beta-amylloid Clone 6F/3D (Dako), or a 1/800 dilution of a monoclonal anti-PCNA antibody (BioGenex, USA), followed by incubation with a 1/200 dilution of biotinylated goat antibodies against rabbit immunoglobulins (Dako) or biotinylated rabbit antibodies to mouse immunoglobulins (Dako), depending on the species of the first antibody. Peroxidase-conjugated streptavidin (Dako) at 1/200 dilution was finally added. Second and third antibody incubations were performed at room temperature during 30 min, while reaction development was achieved by 10-min exposure to 0.03% dianaminobenzidine tetrahydrochloride (DAB, Fluka AG, Switzerland) plus 0.02% hydrogen peroxide.

**Double immunohistochemistry.** The peroxidase activity of GFAP-labeled sections was blocked by treatment with methanol-hydrogen peroxide 5% for 30 min, and samples were subsequently incubated with anti-beta amyloid or anti-PCNA antibodies, followed by biotinylated rabbit anti-mouse antiserum in both cases. Samples were incubated with peroxidase-conjugated streptavidin and the reaction was developed with the AEC Chromogen Kit (Immunotech, France).

**Quantitative evaluation of immunolabeled sections.** Each double immunolabeled section was observed at 400x magnification through a Zeiss microscope. The number of GFAP (+) cells and SAP (+) plaques was recorded in 8 areas of hippocampus (CA1 to CA4) and entorhinal cortex, which were determined by sequential displacement of a test square grid delimiting 0.01 mm² in the section. Only process-bearing astrocytes with their nuclei in the plane of the section were considered. For counting, βAP (+) plaques were recorded separately as diffuse (i.e., lacking-morphologically identified substructures) or dense core (i.e., exhibiting a compact central mass surrounded by an outer sphere).

Resorting to a computer-assisted approach already described, morphometric analysis of both GFAP (+) cells and βAP (+) plaques was carried out by means of a stereological grid, following the point-counting method as applied in studies of rat brain tissue. To this end, 8 microscopic fields in each histological section, in which the number of GFAP (+) cells and βAP (+) plaques had been recorded, were chosen for measurement of surface density of their respective immunoreactivity.

Counting and morphometric analysis were carried out by at least two independent observers.

All data were analyzed using Student’s t-test for comparison of the means, taking p<0.05 as significance level.

**Results**

Consistent labeling clearly distinguishable from the immunonegative background was achieved for βAP as well as for GFAP, thus allowing accurate quantification of the number and perimeter of plaques and astrocytes, respectively.

βAP diffuse and dense-core plaques were found in hippocampus (Fig. 1-a) and entorhinal cortex (Fig. 1-b) from all five AD cases, while such deposits were completely absent in equivalent samples from aged-matched controls. Plaques were mostly observed in CA1 to CA2 areas in the hippocampus, while their distribution was...
more homogeneous in entorhinal cortex. As shown in Table 1, βAP deposits did not differ significantly (p<0.05) in number or size according to their brain location among the regions studied. Nevertheless, a significant predominance of diffuse over dense-core plaques was recorded in both hippocampus and entorhinal cortex.

Differences in the number of GFAP (+) cells were found when the two areas selected for study were compared (Table 1). In histological sections corresponding to the five AD cases, GFAP-immunoreactive astrocytes were more numerous in hippocampus than in entorhinal cortex (49 ± 2 vs. 39 ± 2). Such regional increase was also observed in brains from age-matched controls, but at values significantly lower than in AD (30 ± 2 vs. 20 ± 1).

Evident cell hypertrophy suggested by cursory inspection of AD samples as compared with those of controls (Fig. 2) was later confirmed by astrocyte morphometry, since significantly increased SvGFAP values were found in comparison with those of controls. On the basis of a cell size estimated by the number of intersections of the superimposed stereological grid with immunolabeled body perimeter and emerging processes (SvGFAP), 73% of labeled astrocytes in AD hippocampus and 74% in AD entorhinal cortex exceeded the mean values +SD in controls (Fig 3). A further peculiarity of GFAP-immunoreactive profile in AD was a greater number, width and branching of astrocyte processes as compared to those of controls.

PCNA labeling, as evidenced by reddish nuclei, was not found in GFAP (+) astrocytes, either in AD or in controls.

**Discussion**

As expected, astrocyte hyperplasia was significantly higher in hippocampal and entorhinal samples from AD patients than in those from controls. Since we did not find signs of mitotic activity in GFAP (+) cells, whether in AD or control samples, increased GFAP (+) cell counts appear to result from activation of former quiescent astrocytes rather than from proliferation. Nevertheless, attempts to disclose such sign of mitotic activity had been proved positive in experimental studies related to astrocyte aging.

<table>
<thead>
<tr>
<th>Region</th>
<th>Alzheimer disease (n=5)</th>
<th>Control (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hippocampus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number</td>
<td>βAP Plaque</td>
<td>GFAP Astrocyte</td>
</tr>
<tr>
<td>Diffuse/Dense core</td>
<td>0.0260 ± 0.0148</td>
<td>0.0376 ± 0.0064</td>
</tr>
<tr>
<td>Diffuse</td>
<td>18 ± 1</td>
<td>49 ± 2</td>
</tr>
<tr>
<td>Dense core</td>
<td>0.0195 ± 0.0069</td>
<td>0.0376 ± 0.0064</td>
</tr>
<tr>
<td><strong>Entorhinal cortex</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number</td>
<td>βAP Plaque</td>
<td>GFAP Astrocyte</td>
</tr>
<tr>
<td>Diffuse/Dense core</td>
<td>0.0378 ± 0.0229</td>
<td>0.0370 ± 0.0058</td>
</tr>
<tr>
<td>Diffuse</td>
<td>36 ± 1</td>
<td>39 ± 2</td>
</tr>
<tr>
<td>Dense core</td>
<td>0.0270 ± 0.01663</td>
<td>0.0370 ± 0.0058</td>
</tr>
</tbody>
</table>

PCNA labeling, as evidenced by reddish nuclei, was not found in GFAP (+) astrocytes, either in AD or in controls.
both in vivo and in vitro, but in which harvest and fixation of samples had been experimentally controlled. It is known that mitosis is a relatively short event that may progress to completion even after bodily death, as long as individual cells survive.

In spite of similar distribution of βAP (+) plaques in the two evaluated brain areas, GFAP (+) astrocyte counts were higher in the hippocampus than in the entorhinal cortex of AD patients. Although at lower values than in AD, controls also exhibited greater GFAP-immunoreactive cells in the hippocampus. It may be assumed that in both AD and normal aging such differential GFAP reactivity is partly attributable to the regional location of astrocytes in brain. On the basis of GFAP quantitation by immunoblotting in diverse brain areas in the course of aging, it has been demonstrated that astrogliosis starts in hippocampus, since it has been never observed in the entorhinal cortex alone and, once manifested in this brain area, it is systematically accompanied by a higher reaction in the hippocampus.

According to SvGFAP values, astrocyte hypertrophy was significantly greater in AD, involving almost identical percentages of GFAP (+) cells in hippocampal and entorhinal samples. Therefore, the recorded differences in the number of reactive astrocytes in hippocampus vs. entorhinal cortex were not found for SvGFAP values, which were similarly increased in both brain areas. Such lack of correlation between astrocyte density and cell size has been found in aging rats exhibiting pronounced hyperplasia in the hippocampus and accentuated hypertrophy in the frontal cortex. In turn, aging rhesus monkeys show an increase in GFAP (+) cell size but not in cell density in all subcortical white matter areas of the frontal, temporal, and parietal cortices. Furthermore, in frontal cortices and subcortical white matter of individuals displaying a variety of other diseases, ranging from AIDS to infarction, the extent of gliosis is reflected by an increase in cell size but not in the density or intensity of the GFAP staining of astrocytes.
Although at cursory inspection βAPP deposits appeared as more pronounced in hippocampus, no significant differences in plaque number and size were found according to their localization, either in hippocampus or in entorhinal cortex. On the basis of the proposed sequence of βAPP deposition in AD, entorhinal cortex and CA1 hippocampal sectors were involved subsequently to neocortex. Such temporospatial progression suggests that hippocampus and entorhinal cortex exhibit similar susceptibility to become involved, once they have received afferent input from previously affected neocortex. In this connection, topographical studies including entorhinal region, perirhinal cortex and hippocampal formation, indicate that neuritic plaques gradually develop in the projection areas of tangle-bearing neurons. As Muramori et al suggested, attribution of AD changes to dementia should be neglected when confined to the entorhinal cortex, but appreciated when they spread to the hippocampal subiculum and/or cornus ammonis.

Since our characterization of plaques was carried out exclusively by βAPP immunolabeling, thus overlooking detection of other plaque components identifiable by specific markers of glial cells and dystrophic neurites, the predominance of diffuse over dense-core plaques herein observed can hardly be attributed to a prevailing earlier stage of plaque maturation alone, as the presence of late burnt-out plaques cannot be ruled out. Taking into account that amyloid plaque arrangement varies markedly among non-demented elderly individuals and is even absent in numerous cases, the lack of βAPP reactivity in our five controls (mean age 67.2 years) is not unexpected.

To sum up, quantitation of astrocyte activation performed herein has allowed a more accurate characterization of astrocyte changes, including number and size, that take place in hippocampus and entorhinal cortex which, together with neocortex, are the first brain areas to evidence AD histopathological alterations. However, no quantitative correlation could be shown between astrocyte activation and βAPP deposition in either hippocampus or entorhinal cortex. A better knowledge of the role played by glial cells, whether microglial or astroglial, may contribute to the development of therapeutic strategies designed to modulate the inflammatory processes recognized as contributory factors in the progression of CNS neurodegeneration. Likewise, the unraveling of mechanisms responsible for the abundance of reactive astrocytes and activated microglia, may provide a deeper insight into the pathophysiology of AD. To this end, all histocytomorphometric approaches enabling more objective characterization of activated glia, as well as its regional location, may help to validate the relevance of such cell response in the course of AD, as well as in normal aging.

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References


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Tout cède à la continuité d’un sentiment énergique. Chaque rêve finit par trouver sa forme; il y a des ondes pour toutes les soifs, de l’amour pour tous les coeurs.

Todo cede ante la continuidad de un sentimiento enérgico. Todos los sueños acaban por tomar forma; para toda sed se encuentra agua, y para todos los corazones amor.

Gustave Flaubert (1821-1880)