Glial fibrillary acidic protein in late life major depressive disorder: an immunocytochemical study

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Objectives: Depression is a common psychiatric disorder in late life. Cerebrovascular disease has been postulated as an important aetiological factor in many cases (the "vascular depression" hypothesis). Consistent with this, an inflammatory response, most probably representing ischaemia, has been reported with increases in intercellular adhesion molecule 1 (ICAM-1), in the dorsolateral prefrontal cortex (DLPFC) in postmortem tissue from elderly depressed subjects. As ischaemia is known to cause astrogliosis, this study has further tested the "vascular depression hypothesis" by investigating the distribution of the astrocytic marker glial fibrillary acidic protein (GFAP) in the DLPFC and in the anterior cingulate cortex (ACC).

Methods: Postmortem tissue was obtained from 20 elderly patients with a history of major depressive disorder (MDD) and 20 control subjects. Sections were stained for GFAP using standard immunocytochemistry. Sets of images were obtained from all cortical layers in the DLPFC and ACC with the exception of layer IV in the ACC, and from gyral and deep white matter in both regions. The percentage of the area of each image occupied by GFAP was calculated using true colour image analysis, and mean values obtained for each region examined.

Results: Immunoreactivity for GFAP was low in grey matter (for example, Mean (SEM) 0.76 (0.2)% in DLPFC layer V in depressed subjects), but higher in white matter (for example, 12.02 (2.2)% in DLPFC deep white matter in depressed subjects). Pronounced gliosis was observed within grey matter in a few cases only. GFAP immunoreactivity was significantly higher in layer I of the DLPFC in depressed subjects 15.8 (2.6)% than in controls 9.7 (1.3)% ($t=2.2$; $df=27.5$, $p=0.04$). No difference was detected in any other region.

Conclusions: The data suggest any increase in GFAP in elderly MDD patients is limited to layer I of the DLPFC. These results provide some support for the vascular depression hypothesis and further implicate DLPFC abnormalities in depression.

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Depression is a common disorder with a prevalence in late life of 2%–3% for major depressive disorder (MDD) and 12%–15% for all depressive syndromes.¹ It is associated with considerable morbidity,² and significantly increased mortality,³ but its aetiology remains unclear. Magnetic resonance imaging studies have shown an increase in white matter lesions (WMLs) or signal hyperintensities in MDD, predominantly in the deep white matter,^{4,5} and subcortical grey matter.^{4,6} Such lesions seem most strongly linked to depression when they involve frontal-subcortical circuits that reciprocally link prefrontal areas (the dorsolateral prefrontal cortex (DLPFC) and the anterior cingulate cortex (ACC)) to the basal ganglia.⁷ This is consistent with the findings of positron emission tomography studies in depression, which have demonstrated hypometabolism in both the DLPFC,^{8,9} and the ACC.¹⁰

Quantitative postmortem neuropathological studies have also found abnormalities in neurones and glia in both the DLPFC,^{11,12} and the ACC.¹³ We have recently demonstrated that such WMLs in the deep white matter are attributable to ischaemia in depression.¹⁴ As depression independently predicts hypertension,¹⁵ stroke,¹⁶ and coronary heart disease,¹⁷ ischaemic disease seems a relevant factor in the aetiology of depression.^{18,19} Ischaemia may be expected to trigger a number of changes in brain tissue, among which is an inflammatory response. Evidence for such a response in elderly MDD subjects in brain regions linked to depression would support the vascular depression hypothesis, which proposes that cerebrovascular disease may contribute to the development of some geriatric depressive syndromes.¹⁸ To date only a few studies have examined this question in MDD. We have previ-

ously reported increased immunoreactivity for the inflammatory marker intercellular adhesion molecule 1 (ICAM-1) in the vasculature of the dorsolateral prefrontal cortex (DLPFC) in elderly MDD.²⁰ Astrogliosis forms part of a classic inflammatory response within the CNS, and is seen in a wide range of disorders including cerebral ischaemia,²¹ AIDS dementia and inflammatory demyelinating diseases,²² and in neurodegenerative diseases such as Alzheimer's disease.^{22,23} Evidence for astrogliosis in MDD subjects would provide further support for the presence of an inflammatory response in major depression, and indicate that this response had a parenchymal as well as an endothelial component. Astrogliosis is characterised by hypertrophy and hyperplasia of astrocytes, which markedly upregulate expression of glial fibrillary acidic protein (GFAP), an intermediate filament protein expressed in astrocytic cell bodies and processes.²⁴ It seems that only two studies have quantified GFAP in brain tissue from MDD patients.^{12,25} The first of these, using proteomic technology, found that three isoforms of GFAP were decreased in frontal cortex (Brodmann area 10) in MDD subjects. The second study,¹² using immunocytochemistry, reported that GFAP immunoreactivity in layers III+IV and V of the DLPFC, measured as an areal fraction, was significantly lower than controls in a younger subgroup of depressed

Abbreviations: ICAM-1; intercellular adhesion molecule 1; DLPFC, dorsolateral prefrontal cortex; GFAP, glial fibrillary acidic protein; ACC, anterior cingulate cortex; MDD, major depressive disorder; WML, white matter lesion

subjects (23–45 years), but was not significantly different from control values in an older subgroup (46–86 years). A significant increase in GFAP immunoreactivity with age in MDD subjects was also detected.¹²

The aim of this study was to examine GFAP immunoreactivity in grey and white matter in the DLPFC and in the ACC in postmortem tissue obtained from 20 elderly depressed subjects age 60 years or over at death, and 20 age matched controls. Our hypothesis was that expression of GFAP, as revealed by GFAP immunoreactivity, and reflecting astrogliosis, would be higher in elderly depressed subjects than in control subjects.

METHODS

Subjects

Brain tissue from 40 elderly subjects (20 depressed and 20 controls) was obtained from our Neuropathology Department Brain Tissue Bank. Postmortem permission for research had been given and ethical approval granted for the study. Cases consisted of 20 subjects with major depression and 20 controls. All subjects were 60 years or over at death. Full details of the clinical and neuropathological assessment of subjects and of exclusion criteria have been published elsewhere.²⁶ Briefly, depressed subjects had suffered at least one documented episode of DSM-IV major depression,²⁷ but had never met DSM-IV criteria for dementia or other major psychiatric disorder. Control subjects met the same criteria but had never suffered a DSM-IV depressive episode. All subjects received a full postmortem examination and were excluded if they met neuropathological criteria for Alzheimer's disease or any neurological disorder. The control and depressed groups were comparable in vascular risk factors.²⁶

Tissue

Full face coronal blocks were taken from the right, formalin fixed, hemisphere in the DLPFC (Brodmann areas (BA) 9 and 46), and the ACC (BA 24). Sections of ACC contained tissue obtained either from the region located immediately rostral to the genu of the corpus callosum, or from the region immediately caudal to this—that is, these last sections contained corpus callosum, flanked by upper and lower cingulate cortex. After embedding in paraffin wax 10 µm sections were prepared on 3"×2" slides.

GFAP immunocytochemistry

Tissue sections were stained for GFAP using standard immunocytochemistry. After dewaxing in xylene and rehydration through graded alcohols, sections were microwaved at 450 W for two five minute bursts in 0.01 M citrate buffer (pH 6) to optimise antigen retrieval.²⁸ Endogenous peroxidase activity in the tissue was neutralised in 0.05 M TRIS buffered saline (TBS, pH 7.6) containing 3% H₂O₂ for 15 minutes. After block with an appropriate serum, sections were incubated with a rabbit polyclonal anti-GFAP antibody (Dako, catalogue no Z0334, used at 1:4000) for one hour. Sections were then incubated for 30 minutes with an appropriate secondary antibody, then for 30 minutes with avidin-biotinylated horseradish peroxidase complex (serum, secondary antibody and ABC complex, "Elite" Vectastain ABC kit, PK-6101, Vector Laboratories). GFAP was then visualised by incubating sections in TBS containing 0.025% diaminobenzidine (DAB) and 0.066% H₂O₂ for five minutes. Sections were given three washes in TBS between all incubation steps except between application of serum and application of primary antibody. Finally, all sections were lightly counterstained in haematoxylin and mounted in DPX.

Image capturing

Analysis was performed on one section for each case. Standard cytoarchitectural maps were used to identify the DLPFC and

the ACC,^{29, 30} and the limits of these cortical regions were marked on each slide with a permanent marker pen. Images were captured using a ×10 objective lens on a Zeiss Axioplan 2 light microscope coupled to a 3-chip CCD true colour video camera (JVC KY F55B). Images were taken from deep white matter (10 images per section), cortical grey matter (five images per section), and gyral white matter (five images per section). Gyral white matter was that area of white matter immediately adjacent to, or contained within the gyral fold of the grey matter of the DLPFC and the ACC. Images were obtained from layers I, II, III, V, VI in the ACC, and layers I, II, III+IV, V, VI, in the DLPFC. Cortical layers were identified by standard morphological criteria for neurons within granular cortex (DLPFC) and agranular cortex (ACC). In both brain regions, the image obtained in layer I was taken immediately inside the edge of the section. We and others have observed differential intensity of stain for GFAP in layer I,¹² and therefore it was necessary to obtain the image from a standard position in all sections. The very edge of the section was excluded from the image to exclude artefactual staining. In all other layers, images were located randomly within the layer in question.

Image analysis

The area of staining for GFAP in each image, expressed as a percentage, was calculated using a commercially available software program that permitted true colour analysis (Image Pro Plus, version 4.0; Media Cybernetics). A macro was set by taking the average of RGB values in 50 separate images measured manually, and images were then rated automatically. Analysis was carried out blind to diagnosis.

Statistics

The Kolmogorov-Smirnov test was first applied to data to determine whether the distribution within each dataset was normal or skewed. Subsequently, data were tested for significance using an independent samples *t* test or a Mann-Whitney test as appropriate. The Pearson correlation test was also applied to investigate whether data correlated to age of subjects, postmortem interval, and duration of fixation.

RESULTS

White matter could usually be distinguished from grey matter by the greater intensity of staining for GFAP in the former. Cell profiles immunoreactive for GFAP displayed classic astrocytic morphology, with GFAP+ve cell bodies and processes (fig 1). Although the intensity of staining for GFAP was generally higher in the ACC as compared with the DLPFC, the comments that follow apply equally to both brain regions. Within the cortex, the highest level of staining was observed in layer I, which invariably contained numerous distinct GFAP+ve cell profiles (fig 1A, B). GFAP+ve processes extended from layer I of the cortex as far as layer III. The degree of staining for GFAP in cortical layers other than layer I was in most cases low (fig 1C). In around one quarter of DLPFC cases, virtually no GFAP+ve cell profiles were detectable in cortical layers II–VI. However, a few cases showed more intense GFAP immunoreactivity in grey matter (fig 1D). Some positive staining of processes within layer VI was evident. GFAP+ve profiles appeared more frequently within white matter, both in the white matter contained within the gyral folds (fig 1E), and within the deep white matter (fig 1F). Substantial variability in the areal fraction for GFAP immunoreactivity was noted both between and within individual cases in both groups. While mean values for the areal fraction of GFAP staining were almost always slightly higher in the depressed group, a significant difference was found between the groups of controls and depressed subjects only in layer I of the grey matter of the DLPFC ($t=2.2$; $df=27.5$, $p=0.04$). No

Figure 1 GFAP immunoreactivity in DLPFC and ACC grey and white matter. (A), (B) Layer I of DLPFC cortex (A, control, B, depressed,); (C) DLPFC grey matter, (D) ACC grey matter, (E) ACC gyral white matter, (F) ACC deep white matter. GFAP immunoreactivity is clearly more pronounced in (B) (depressed) than in (A) (control). (C), (E), and (F) show typical levels of GFAP immunoreactivity in grey matter, gyral white matter, and deep white matter respectively, while (D) shows unusually high GFAP immunoreactivity in ACC grey matter. Sections are 10 µm paraffin wax, stained for GFAP as described in Methods, and counterstained with haematoxylin. Scale bar applicable to all images =50 µm.

significant difference was observed in any other region examined (fig 2); DLPFC white matter $p \geq 0.4$; DLPFC grey matter other than layer I, $p \geq 0.1$; ACC white matter, $p \geq 0.7$; ACC grey matter, $p \geq 0.4$. There was no correlation between GFAP immunoreactivity and age, postmortem interval, or length of time in fixative, with the exception of GFAP immunoreactivity in the gyral white matter of the DLPFC, which correlated positively with duration of fixation ($n=40$, $r=-0.48$, $p=0.002$).

DISCUSSION

This study has investigated the distribution of GFAP immunoreactivity in the grey and white matter of the DLPFC and the ACC of elderly MDD patients and age matched controls. Increased GFAP immunoreactivity in depressed subjects was found only in layer I of the DLPFC, and therefore this study has provided only limited support for the vascular depression hypothesis. Nevertheless we consider this finding important, as representing the first evidence for gliosis in elderly MDD patients in excess of that which accompanies normal aging.

We are unable to directly compare our GFAP grey matter data with those reported previously relating to the distribution of ICAM-1 in MDD,²⁰ as these data were not layer specific. However, our observation of increased ICAM-1 in DLPFC

white matter in MDD in our earlier study,²⁰ when taken with the absence of increased GFAP in DLPFC white matter in this study, suggests that ICAM-1 may be upregulated in white matter in elderly MDD patients in the absence of gliosis. Data from this study show both similarities with and differences from other relevant publications. The general pattern of GFAP immunoreactivity we observed, with lower levels of staining in grey matter than in white matter, conforms to a previous description of the distribution of GFAP in normal human brain.³¹ Our finding that GFAP immunoreactivity in cortical layers III+IV and V of the DLPFC was not different from controls agrees with those of another study in depressed subjects aged 46–86 year.¹² However, the absence of a correlation between age and GFAP immunoreactivity in both groups in our study may be considered surprising given that both GFAP mRNA and GFAP protein have been reported to increase with age in brain tissue of normal human subjects,^{32–33} while GFAP immunoreactivity increases with age in MDD patients.¹² This difference may have been attributable to the age range of subjects, which was considerably narrower in our study than in those just mentioned. Finally, we obtained markedly lower values for the areal fraction of GFAP immunoreactivity in DLPFC grey matter than did another study that also

Figure 2 Areal fraction of GFAP immunoreactivity in DLPFC and ACC grey matter and white matter. Brain tissue was obtained at postmortem examination from 20 elderly subjects who had experienced DSM-IV major depression during life and from 20 age matched control subjects. Tissue was fixed in formalin, embedded in paraffin wax, and 10 μ m sections containing either the DLPFC or the ACC prepared. One section from each case was stained for GFAP using standard immunocytochemistry. Images were obtained from DLPFC and ACC grey and white matter as indicated, and the degree of staining for GFAP was calculated as a percentage of total image area using true colour image analysis. Histograms indicate areal fraction of immunoreactivity for GFAP in (A) DLPFC grey matter, (B) DLPFC white matter, (C) ACC grey matter, (D) ACC white matter. Data are presented as mean (SEM). The figure obtained for each case represents the average of measurements obtained from five separate images in each brain region, except in deep white matter, where 10 images were obtained for each case.

quantified GFAP immunoreactivity in MDD, that of Miguel-Hidalgo and coworker.¹² The reason for this difference is unclear. Our data for DLPFC grey matter were not influenced by postmortem delay or duration of fixation. We used a different embedding material (wax compared with celloidin) and a different primary antibody than Miguel-Hidalgo and coworkers and such technical differences may have contributed to the differences in data obtained in the two studies.³¹ Alternatively, these differences may reflect the variability that has been observed in the GFAP content of human cerebral cortex between different people.³² It is clear that the degree of gliosis in grey matter was relatively mild in most of our sample, and absent from some cases. It has been suggested that age related increases in GFAP protein are secondary to degenerative processes such as synaptic loss, and do not occur in aging in the absence of neurodegeneration,²³ so that age related gliosis may not be inevitable.

Our data do not necessarily indicate that the characteristics of astrocytes do not change in elderly MDD patients in regions other than layer I of the DLPFC. It is possible for instance that the DLPFC and the ACC of MDD patients might contain a lowered density of enlarged GFAP +ve astrocytes. In addition as lower than control levels of GFAP immunoreactivity have been observed in young MDD subjects,¹² it is possible that our findings in the depressed group represent a greater increase in GFAP with age than in normal subjects, although given the generally low levels of GFAP immunoreactivity observed in this study the relevance of this is unclear. Our finding of equivalent levels of GFAP immunoreactivity in the control and in the depressed group in regions other than layer I of the DLPFC does not necessarily indicate that no ischaemic episode occurred. Firstly, long term hypoperfusion, resulting in tissue oligoemia, may represent a sufficient insult to impair neural

functioning and to influence mood, without constituting a sufficient stimulus to initiate gliosis, which is correlated with actual neuronal injury, at least in rodents.³⁴ Secondly, it is possible that periodic circulatory insufficiencies may have triggered a temporary gliotic response in some patients that was not however present at the time of death. Finally, the method used in this study, immunocytochemistry, may not have detected all forms of GFAP present in the original tissue samples. The observation that age related increases and topographical variations in GFAP in human brain were detected biochemically by immunoblotting but not by standard immunocytochemistry on sections from the same subjects,³² suggests that methods additional to that of immunocytochemistry may be required to definitively determine whether expression of GFAP is greater than control levels in elderly MDD patients.

The vascular depression hypothesis suggests that the roots of depression in a subset of elderly patients are different from those that cause depression with early onset. It is clearly relevant to inquire whether our findings and those of other published studies suggest that these two classes of depression are characterised by differing neuropathology. While no study has specifically answered this question, existing reports suggest that the nature of glial alterations may vary between elderly and younger MDD subjects. Our observation of equal or increased immunoreactivity for GFAP in the DLPFC and ACC of control and depressed subjects contrasts with reduced GFAP immunoreactivity in the DLPFC of depressed subjects under 46 years old.¹² The observation of this last study that the packing density of GFAP+ve cell bodies was lower than controls in young MDD subjects, but showed a non-significant tendency towards greater than control values in MDD subjects over 46 years,¹² suggests that astrocytic loss may be a feature of

depression with early onset but not of depression in older subjects. This suggestion is supported by reports that glia are lost from various regions of frontal cortex in MDD subjects aged less than 66 years,^{13, 35} although to our knowledge this parameter has not been quantified in subjects older than this. It is clear that the picture of glial alterations in MDD is incomplete, and is complicated both by the use of different cut off ages in different studies, and by a lack of distinction between subjects suffering from depression with early onset and depression with late onset in elderly MDD groups.

In conclusion, our study has provided partial support for the hypothesis of vascular depression. More research is necessary to further clarify neuropathological differences between elderly and younger MDD subjects, and in particular between early onset and late onset depressives.

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