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EXPRESSION OF GLIA MATURATION FACTOR IN NEUROPATHOLOGICAL LESIONS OF ALZHEIMER'S DISEASE

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Abstract

Aims—The pathology of Alzheimers's disease (AD) is characterized by the presence of amyloid plaques (APs), neurofibrillary tangles (NFTs), degenerating neurons, and an abundance of reactive astrocytes and microglia. We aim to examine the association between glia maturation factor (GMF) expression, activated astrocytes/microglia, APs, and NFTs in AD affected brain regions.

Methods—Brain sections were stained with Thioflavin-S to study AD pathology and sequentially immunolabeled with antibodies against GMF, glial fibrillary acidic protein (GFAP, marker for reactive astrocytes), and Ionized calcium binding adaptor molecule 1 (Iba1, marker for activated microglia) followed by visualization with avidin-biotin peroxidase complex.

Results—Our double immunofluorescence labeling with cell-specific markers demonstrated the glial localization of GMF. The immunohistochemical data showed that APs and NFTs are associated with increased expression of GMF in reactive glia of AD brains compared to non-AD controls.

Conclusions—This is the first report that shows GMF, a mediator of CNS inflammation, is expressed in the brain regions affected in AD and that GMF is mainly localized in reactive astrocytes surrounding APs/NFTs. The distribution of GMF-immunoreactive cells in and around Thioflavin-S stained APs and NFTs suggests involvement of GMF in inflammatory responses through reactive glia and a role of GMF in AD pathology.

Keywords

Glia maturation factor (GMF); Alzheimer's disease (AD); Neuropathology; Neuroinflammation; Reactive glia; Amyloid plaques (APs); Neurofibrillary tangles (NFTs); Tau-protein

INTRODUCTION

Alzheimer's disease is a neurodegenerative disorder, neuropathologically characterized by the presence of amyloid plaques (APs) and neurofibrillary tangles (NFTs) in the cerebral cortex of AD brain. The APs contain dense deposits of aggregated amyloid precursor protein. Most often, the APs are associated with inflammation as noted by the presence of reactive astrocytes, activated microglia, and dystrophic neuritic processes in AD. It has been previously described that there is a role for specific forms of amyloid beta peptide in inducing production of proinflammatory cytokines/chemokines by activated microglia and

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reactive astrocytes [1–3]. In our previous study, we demonstrated that the glia maturation factor (GMF) is a prominent mediator of inflammation in the CNS leading to the death of neurons [4]. Due to GMF's ability to activate astrocyte/microglia and induce several inflammatory mediators, we hypothesize that GMF is involved in the pathogenesis of AD.

In this study, we have examined the presence of GMF expression in AD affected brains. We focused on the co-localization of GMF with APs and NFTs in AD affected regions and observed associations between GMF, reactive astrocytes/microglia, and neuropathological lesions. The presence of activated microglia and reactive astrocytes and their association with APs in the AD brain has been observed previously [5, 6]. This is the first study to demonstrate the expression of GMF in reactive glia and its association with APs/NFTs in AD affected brains.

EXPERIMENTAL PROCEDURES

Human AD brain tissue

Postmortem human AD brains (eight brains; four female and four male) with neuropathological criteria for definite AD were obtained from the University of Iowa Deeded body program (Table 1). The tissues were collected within 3–8 h of death. The temporal lobe blocks were dissected and immersion-fixed in 4% paraformaldehyde solution. Blocks were cryoprotected with 30% sucrose until sunk, and then frozen sections were cut at 40 μm on a sliding microtome. All experimental procedures used were in accordance with the Institutes approved guidelines.

Immunohistochemistry

Immunohistochemical (IHC) procedure was performed according to our previously published methods [7, 8] and outlined below. In brief, we used free-floating sections from the temporal cortex of each brain. Sections were processed by using the standard avidin-biotin-peroxidase complex (ABC) technique. Tissue sections were first treated with 0.3% H_2O_2 in phosphate buffered saline (PBS) for 20 min to eliminate endogenous peroxidase activity. After three washes in PBS, sections were placed in blocking buffer (5% normal goat serum (NGS) and 3 % bovine serum albumin (BSA) diluted in PBS containing 0.3% Triton-X 100) for 1 h, followed by incubation overnight at 4°C with one of the following primary antibodies: GMF (G2-09 mouse monoclonal antibody; 1:100), GFAP (rabbit polyclonal, 1:200, ABCAM, USA) to detect reactive astrocytes, Iba-1 (rabbit polyclonal, 1:1000, WAKO Chemicals, USA) to detect activated microglia, or AT8 (mouse monoclonal, 1:2000; Innogenetics, Belgium) to detect hyperphosphorylated tau protein within NFTs and APs. Primary antibodies were diluted with blocking buffer. After overnight incubation, sections were rinsed three times with PBS for 10 min and then incubated for 2 h with corresponding biotinylated secondary goat anti-mouse IgG or goat anti-rabbit IgG antibodies (Vector Laboratories, Burlingame, CA). Following three rinses with PBS, sections were developed using an ABC standard solution (Vector Laboratories, Burlingame CA) diluted 1:2000 in PBS for 1–2 h. The sections were then washed with PBS again. After washes, sections were placed in a peroxidase reaction solution containing diaminobenzidine (DAB substrate kit for peroxidase, Vector Laboratories, Burlingame, CA) to detect the bound antibody-antigen enzyme complexes. For negative control, sections were identically processed without the primary antibody. The sections were rinsed with distilled water, mounted on slides, and dried overnight at room temperature. Slides were then dehydrated in a graded series of ethanol, cleared in xylene, and coverslipped with Permount (Fisher Scientific, USA).

Immunofluoresence and Thioflavin-S histochemistry

Floating tissue sections from temporal cortex of AD brains were pretreated with 0.3% H_2O_2 in PBS to block the endogenous peroxidase activity. Sections were then rinsed with PBS three times, followed by 1 h incubation with blocking buffer (5% normal goat serum and 3 % BSA diluted in PBS-T). The sections were then incubated overnight at 4°C with GFAP antibody (rabbit polyclonal; ABCAM, 1:100) diluted in blocking solution. On the following day, the sections were washed three times with PBS and incubated for 1 h with goat anti-rabbit IgG conjugated to Alexa Fluor 568 (Invitrogen-Molecular Probes, Eugene, OR) diluted 1:200 in blocking buffer. Sections were then rinsed with PBS several times. For immunofluoresence double-immunostaining, the rinsed sections were counterstained with Thioflavin-S fluorescent marker to observe the neuropathological lesions of NFTs and APs. The sections were mounted onto slides, and cover slipped with aqueous mounting medium with anti-fading agents (EM Sciences, Hatfield, PA). The slides were observed under the fluorescence microscope to visualize the GFAP-immunoreactive astrocytes and their association with NFTs and APs.

Triple labeling

For triple labeling, AT8 was fist detected with DAB substrate followed by GFAP and G2-O9 double immunofluorescence staining. Immunohistochemistry was performed as previously described [7, 8]. The sections were double-immunolabeled for simultaneous detection of GMF and GFAP immunoreactive cells. For immunofluorescence double-labeling, free-floating sections were pretreated with 0.3% Triton-X/ 5% NGS/3% BSA (blocking buffer) in PBS. The sections were then incubated at 4°C with both GFAP polyclonal antibody diluted at 1:100 and G2-09 antibody diluted at 1:20 in blocking solution. Next, the sections were washed in PBS and incubated for 2 h at room temperature with goat anti-rabbit IgG conjugated to Alexa Fluor 488 and goat anti-mouse IgG conjugated to Alexa fluor 568 diluted 1:200. After three rinses with PBS, the sections were mounted onto slides, and cover slipped with aqueous mounting medium with anti-fading agents. The primary antibodies were omitted for non-specific staining in the control.

Microscopic Images

Images were obtained using a Nikon microscope equipped with FITC and rhodamine filters. Single or combined (merged) images were adjusted for contrast and brightness using Adobe Photoshop C2S software or Adobe Photoshop 7.0.

RESULTS

GMF expression in specific brain regions in AD affected cases

Thioflavin-S histochemistry revealed that a large number of neurofibrillary tangles (NFTs) and amyloid plaques (APs) were present in the temporal cortical regions of all AD brains examined (Fig.1A). Expression of GMF in the various layers of temporal cortex was observed in AD brain sections (Fig. 1B). Compared to APs, glia maturation factor (GMF) immunoreactivity was more widely distributed across all layers of the temporal cortex. However, in grey matter GMF-immunoreactivity was observed predominantly along the more superficial layers, and in AD temporal cortex, it was found that layers II through IV expressed more GMF-immunoreactive cells. We also observed a high intensity of GMF-immunoreactivity in numerous APs in these layers. Furthermore, a large number of GMF-positive amyloid plaques were stained in the hippocampus and parahippocampal cortex. GMF immunostaining was present both around the periphery and within the APs. Note that the majority of GMF immunolabeled morphologically identifiable glial cells were localized in lower cortical layers near Thioflavin-S stained APs in AD (Fig.1C). The relative GMF

immunostaining intensity and the laminar distribution pattern of NFTs and APs in AD brain sections are depicted in Fig. 1C.

Qualitative analysis of GMF, NFTs and APs

For qualitative analyses we utilized both single and double immunostaining. IHC staining of temporal cortex from the AD cases showed the presence of GMF immunostaining mainly associated with NFTs and APs. We examined the GMF expression and neuropathological changes in AD affected brain regions. We studied the co-localization of tau (AT8) immunoreactive NFTs and APs with GMF and also Thioflavin-S positive NFTs and APs with GMF, and astrocytes.

Relation between GMF expression and AD pathology

To determine whether GMF-immunopositive cells are associated with APs in AD, brain sections were processed by IHC for GMF and Thioflavin-S concomitantly. Many GMF-immunoreactive cells were co-localized with Thioflavin-S stained APs in AD cases. Furthermore, many of the amyloid deposits presented appeared to be closely associated with GMF. Glial reactivity was also observed in the vicinity of APs in AD brain sections (Fig.1 D–F). GMF-immunoreactive cells that were clustered around the APs in deep cortical layers were also found near Thioflavin-S stained NFTs. This suggests a close association between GMF and dying neurons in AD. An increased expression of GMF is related to AD pathology that often shows NFTs and APs in the vicinity. Elevated GMF in APs and surrounding hypertrophic glial cells also suggests a role for GMF in the processing of amyloid protein related to the pathogenesis of AD.

Association of reactive astrocytes with NFTs and APs in AD brains

To visualize APs and reactive astrocytes simultaneously we used immunofluoresence staining with GFAP for reactive astrocytes and Thioflavin-S histochemical staining for NFTs and APs in AD brain sections (Fig. 2A). The major astroglial reaction was observed primarily in the cells surrounding APs in AD. As shown in Fig. 2B, there were a large number of GFAP-positive astrocytes present in the AD brain. The subsequent co-localization of APs and GFAP-immunoreactive astrocytes showed an increased association of reactive astrocytes with APs in AD brain (Fig. 2C). In addition, we also found that most of the astrocytes surrounding APs showed typical reactive astrocytic features such as thick processes and enlarged cell bodies. Fig. 2D and 2E demonstrate the co-localization of a mature (crown) core-containing plaque with GFAP- immunoreactive astrocytes. The presence of astrocytes in the neuropil intervals was also observed (Fig. 2F). Likewise, GFAP-immunoreactive astrocytes were found around NFTs and amyloid deposits (Fig.2 G–I). We also found that the GMF-positive astrocytes (Fig.3 A, D) and GFAP-reactive astrocytes (Fig.3 B, E) surrounded the NFTs (Fig. 3 C, F), which contain hyperphosphorylated tau protein. Tau immunoreactivity was observed both within APs and in neuritic processes (Fig. 4A). Reactive astrocytes were localized at both the periphery and in the centre of Tau-positive APs and displayed altered morphology including enlarged processes that often enveloped the tau-positive APs (Fig. 4B–D). Likewise, GMF-positive astrocytes predominated at both the periphery and center of APs (Fig. 4 B, C). GMF-positive astrocytes were co-localized with tau-positive APs (Fig. 4D). Triple labeling for Tau (Fig. 4 E, I), GFAP-immunoreactive astrocytes (Fig. 4 G, K), and GMF-positive astrocytes (Fig. 4 F, J) was performed. We observed the association between GMF-positive astrocytes with AT8-immunostained APs. The presence of GMF-positive astrocytes was detected primarily at the periphery of Tau-positive APs and adjacent to NFTs (Fig 4 H, L). GMF immunofluoresence staining was present within the amyloid deposits, and GMF-immunoreactive astrocytes, in specific, were associated with the core of the APs. This close

relationship between GMF-positive astrocytes and APs, particularly with the core of APs, suggests the involvement of GMF-positive astrocytes in AD pathology.

DISCUSSION

Neurofibrillary tangles (NFTs) and amyloid plaques (APs) are neuropathological hallmarks and unique features of inflammation in regions of AD cortex that are associated with neurodegeneration and correlated with the clinical expression of dementia [9–11]. This study describes the relationship of GMF with APs and NFTs in the temporal cortex of AD patients. We observed GMF-positive astrocytes to be extensive and in close association with APs and features of AD inflammation. Furthermore, GMF-immunoreactive astrocytes were localized both around the plaque periphery and within the amyloid plaque core. Earlier studies have shown that this glial reaction occurs, to some extent, during normal aging in addition to AD [12, 13]. GMF-positive astrocytes are strongly associated with Tau (AT8) immunostained APs in affected AD cortex. Similarly, Chao et al. [14] showed a close association between IL-1 α-positive microglia and tau immunoreactive APs in affected areas of AD brain. Interestingly, GMF immunoreactivity is present both within and around APs, and GMF expression is seen in both dense core and diffuse APs. Similarly, previous studies have shown that activated microglia and reactive astrocytes are associated with both types of APs [15–19]. Additionally, a recent study showed that tau-positive structures (NFTs, APs, and neuropil threads) were co-localized with severely fragmented microglial cells, which cluster around these structures [20]. The recruitment of reactive glia to the APs and production of proinflammatory cytokines may contribute to degeneration of neurons in AD [14, 21–23].

Glia Maturation Factor (GMF) is a novel inflammatory protein first discovered, purified, sequenced, and sub-cloned from bovine brain in our laboratory [24–26]. GMF is a highly conserved, unique brain protein and has a molecular weight of 17 kDa [25]. The amino acid sequence of GMF is highly conserved among several species, suggesting that it plays basic roles across many species. The GMF gene has been identified in chromosome 14 by our laboratory and by the Human Genome Sequence Consortium [27]. The GMF gene is 7 kb in length, and contains six introns and seven exons. Earlier, in cell-free assays, we have demonstrated that GMF could be phosphorylated by protein kinase A (PKA) and protein kinase C (PKC) [28]; and the phosphorylated form of GMF is an inhibitor of the ERK isoform of MAP kinase [29] while at the same time a stimulator of the p38 isoform [30], which implicates GMF protein in stress-activated inflammatory responses. GMF is an intracellular inflammatory signal transduction regulator known to play a role in neuroinflammation and neurodegeneration. Our research has shown that GMF is an upstream molecular messenger that activates the stress kinase p38 MAPK, leading to activation of nuclear transcription factor kappa-B (NF-kB), followed by increased production of inflammatory mediators such as TNF-α, IL-6 and IL-1β [4]. In C6 as well as in normal astrocytes, GMF overexpression stimulates p38 MAP kinase activity, and activates the redox enzyme CuZn superoxide dismutase (CuZnSOD) and the transcription factors NF-κB [31, 32]. Overexpression of GMF in PC12 pheochromocytoma cells activates p38 MAP kinase, its downstream MAPKAP-kinase 2, and the final effector tyrosine hydroxylase, accompanied by an increased phosphorylation of these proteins [33]. A study by Kaimori et al. [34] demonstrated an important role for GMF in the pathophysiology of a renal disease by enhancing oxidative stress. The authors discovered that, although normally absent in the kidney, GMF is inducible in the proximal renal tubules under the stress of proteinuria. They subsequently demonstrated that overexpression of GMF in kidney cell lines and 3T3 fibroblasts renders the cells susceptible, by an apoptotic mechanism, to oxidative stress such as the application of TNF-α, angiotensin II, BSO and H_2O_2. They further showed that this increased susceptibility is due to accumulation of intracellular H_2O_2

secondary to an increased expression of CuZn-SOD with a concomitant decrease in catalase, glutathione peroxidase and glutathione. The apoptosis was inhibited by the p38 MAP kinase inhibitor SB-203580. Similarly, Baldwin et al. [35] demonstrated that the transient overexpression of GMF in glioblastoma cells activated p38 MAPK signaling and also increased susceptibility of the tumour cells to cytotoxicity of cisplatin, a chemotherapeutic agent. These results corroborate our earlier report that GMF is involved in p38 MAPK stress-response mechanism. In another recent study, Li et al. [36] analyzed GMF protein expression by immunohistochemistry in 246 patients with various degrees of tumour lesions and found significantly enhanced expression of GMF in adenoma tissues than that in normal epithelium. They also demonstrated significantly higher levels of GMF in twenty human malignant lesion by using 2-DE combined with MALDI-TOF/TOF and quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR). They correlated overexpression of GMF with the clinicopathological features in cancer patients. In another study, we infected a mixed culture of primary glial cells with a replication-defective adenovirus carrying GMF cDNA [37]. A comprehensive survey of gene expression by Affymetrix microarray analysis showed a big increase in several major histocompatibility complex (MHC) class II proteins and several classical proinflammatory cytokines. Recently, we studied inflammatory cytokine/chemokine profiles following GMF overexpression in primary glial cells [4]. The results demonstrated that overexpression of GMF is necessary for the induction of granulocyte-macrophage-colony stimulating factor (GM-CSF) in astrocytes and that GM-CSF is one of the key molecules for GMF-dependent production of inflammatory cytokines in microglial cells. The results also demonstrated that the increased release of GMF-dependent microglial proinflammatory cytokines is cytotoxic to neurons [4]. GM-CSF is a haematopoietic cytokine that is not detected under normal conditions but can be induced by activated glial cells [38]. Its gene is under the transcriptional control of NF-κB [39]. It has been reported that GM-CSF enhances the expression of several pro inflammatory cytokine/chemokine, thus playing an important role in the amplification of immune and inflammatory processes. A report by Tarkowski et al. [40] clearly demonstrated that the levels of GM-CSF were significantly increased in patients with AD and with vascular dementia. Moreover, they also found a significant correlation between the enhanced levels of GM-CSF and that of Fas/APO-1 (proapoptotic factors) and Tau protein in dementia. Based on these results they suggested a crucial role for GM-CSF in neuronal cell damage in AD. Recently, we reported [41] that intraventricular infusion of amyloid beta peptide1-42 (Aβ1-42) in wild type mice brains caused activation of astrocytes and microglia, several fold increase in the production of proinflammatory cytokines/chemokines and memory deficit. Whereas, all these effects of Aβ1-42 infusion, including the memory deficit, were suppressed in GMF deficient mice. In this regard it is important to note that chronic production of inflammatory cytokines/chemokines by reactive glia is known to launch the neuroinflammatory cascade in AD. In conclusion, we provided the first time evidence for association of high level GMF with AD affected cortical regions of human brains where GMF was prominently localized in APs and NFTs, possibly to facilitate AD associated neuroinflammation and memory deficit.

In summary, this is the first report that shows expression of GMF, a mediator of CNS inflammation, is abnormally increased in the cerebral cortex of AD brain where GMF is mainly localized in astrocytes and also associated with APs and NFTs. The distribution of GMF-immunoreactive cells in and around the Thioflavin-S stained APs and NFTs suggests involvement of GMF in inflammatory responses through reactive glia in AD brain and a role for GMF in AD pathology.

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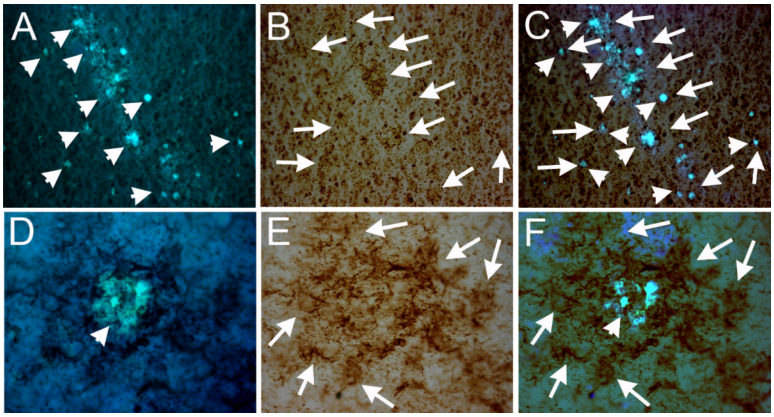

Figure 1.

Double-labeling of temporal cortex in AD brain. (A) Thioflavin-S stained amyloid plaques (green) indicated by arrowheads. (B) GMF immunostaining (brown) shows immunoreactive cells indicated by arrows. GMF immunoreactivity is widely distributed across all cortical layers. (C) Merged image shows GMF immunoreactive cells surrounding the Thioflavin-S stained amyloid plaques. (D) GMF is present in Thioflavin-S stained amyloid plaques in AD temporal cortex. Thioflavin-S staining shows amyloid plaque (green) and same section immunostained with GMF antibody. (E) GMF-positive cells are indicated by white arrows. (F) Merged image shows GMF immunoreactive cells both within and around the plaque. A–C, 20×; D–F, 40×.

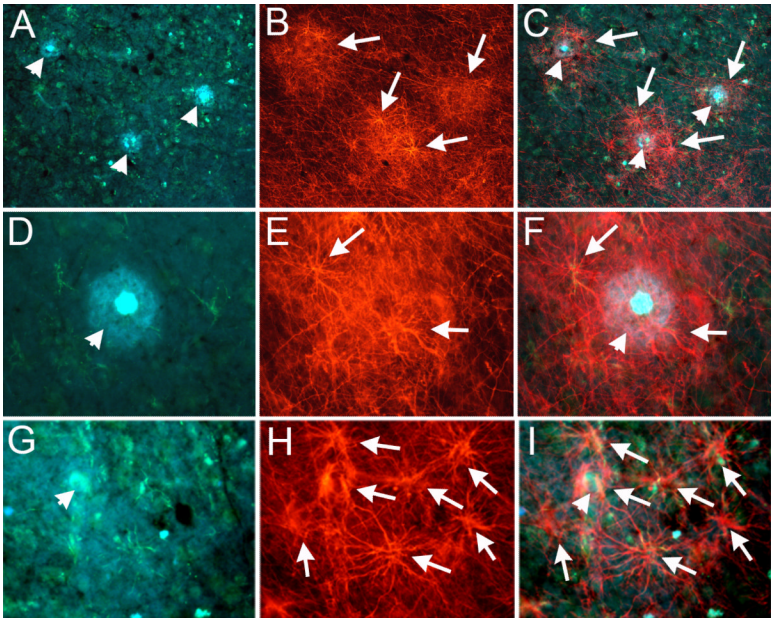

Figure 2.

Double-labeling of AD cortex. (A) Thioflavin-S stained amyloid plaques (green) indicated by arrowheads. (B) Reactive astrocytes (red) stained with immunofluorescent GFAP indicated by arrows. (C) Merged image of A and B shows a large number of GFAP positive astrocytes surrounding the amyloid plaques. (D) High magnification of double labeled AD temporal cortex showing Thioflavin-S stained amyloid plaques and immunofluorescent reactive astrocytes stained with GFAP. Example of a mature amyloid plaque containing a central core is indicated by arrowhead. (E) Reactive astrocytes stained with GFAP shown by arrows. (F) Merged image of D and E shows reactive astrocytes surrounding the amyloid plaque core. (G) Thioflavin-S histochemistry and (H) GFAP immunofluorescence labeling of the temporal cortex of AD brain. Thioflavin-S stained neurofibrillary tangle is indicated by arrowhead. Reactive astrocytes stained with GFAP are indicated with arrow. (I) Merged image of G and H shows the GFAP positive astrocytes around and also in NFTs and amyloid deposits. A–C, 20×; D–I, 40×.

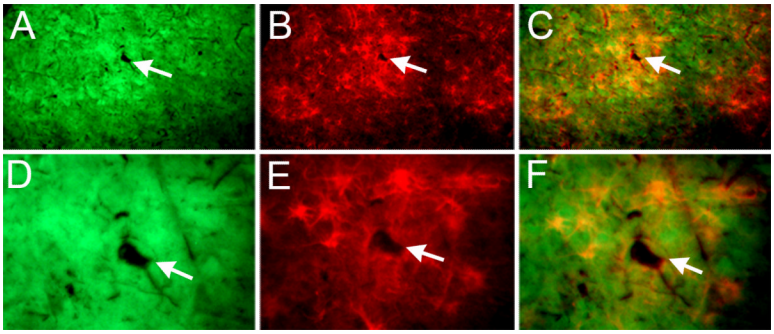

Figure 3.

Triple-immunolabeled image of AD cortex. GMF positive cells (green) surrounding AT8 (Tau) immunostained NFT (black) indicated by arrow (A, D). Immunofluorescence staining of GFAP antibody showing reactive astrocytes (red) also surrounding the Tau stained NFT (B, E). Merged images of both pairs show GMF positive astrocytes (yellow colour representing overlapping signal) around the NFT (C, F). A–C, 20×; D–F, 40×.

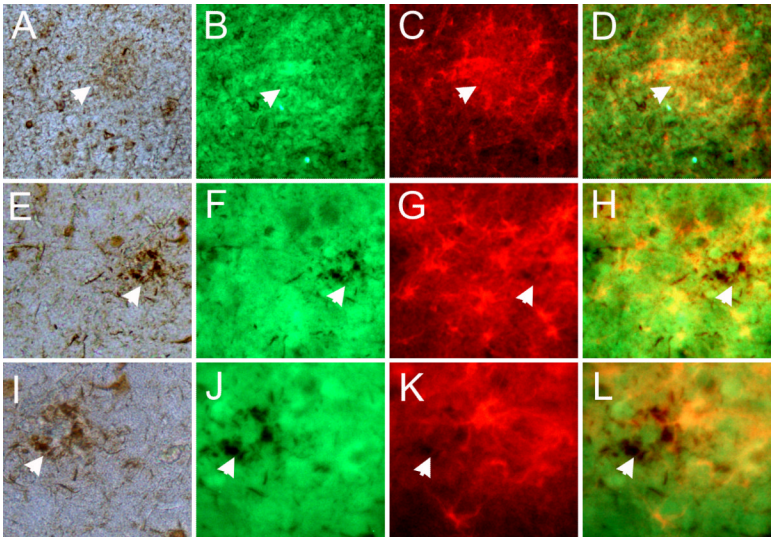

Figure 4.

Triple labeling of AD temporal cortex. (A) AT8 antibody stain of a diffuse amyloid plaque (brown) indicated by arrowhead (arrowhead shown throughout images to maintain point of reference). (B) GMF fluorescent stain (green) of immunoreactive astrocytes. (C) GFAP fluorescent strain (red) of activated astrocyte. (D) Merged image shows co-localization of GMF positive astrocytes (yellow color representing overlapping signal) and the Tau stained amyloid plaque. (E and I) AT8 immunostaining (brown) shows NFTs indicated by arrowhead. (F and J) GMF positive astrocytes are shown with immunofluorescence (green). (G and K) GFAP reactive astrocytes stained with immunofluorescence (red). Merged images of E, F, & G and I, J, & K show GMF positive astrocytes (yellow color representing overlapping signal) surrounding the Tau stained amyloid plaques and NFTs (H, L). A–H, 20×; I–L, 40×.

Table 1

Summary of samples examined in the present study

Case	Brain Weight (Grams)	Age (Years)	Gender	Duration Of AD (Years)
AD-1	974	68	F	11+
AD-2	956	78	F	11
AD-3	NA	88	F	12
AD-4	1050	86	F	10
AD-5	1430	68	M	8
AD-6	1265	74	M	10
AD-7	NA	87	M	6+
AD-8	1630	89	M	15