Immunoreactive Epidermal Growth Factor Receptors in Neuritic Plaques from Patients with Alzheimer’s Disease

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Abstract. Alzheimer’s disease (AD) is characterized neuropathologically by the presence of neuritic plaques (NP) in cerebral cortex and hippocampus, as well as intraneuronal neurofibrillary tangles and granulovacuolar degeneration. The etiology of plaque formation has remained obscure, but morphologically NP are known to contain amyloid cores surrounded by astrocytes and degenerating neurons. Although growth factors are important in growth, differentiation and regrowth in response to injury, studies relating growth factors to AD have been lacking. Epidermal growth factor (EGF) plays an important role outside the central nervous system (CNS) through interaction with its specific receptor, EGF-R. Using an antibody to EGF-R (three-step immunoperoxidase staining) in conjunction with fluorescence staining, we found that the majority of NP from patients with pathologically confirmed AD as well as those few NP in the normal aging brain showed intense EGF-R immunoreactivity. Specific staining was seen at the periphery of plaques but not in the central amyloid core. Tissue sections from AD cases were also reacted with antibodies to both glial fibrillary acidic protein (GFAP) and paired helical filaments (PHF) in an attempt to identify which component of the NP was reactive for EGF-R. The antibody to PHF densely stained the periphery of NP but not the central core in a majority of NP. The antibody to GFAP stained a few reactive astrocytes that bordered plaques in only a small proportion of all plaques present. We conclude that the neuron and its processes although not exclusively may be the site of EGF-R immunoreactivity. An EGF/EGF-R system within the CNS may play an important part in scar formation in response to neuronal injury and death or it may function as a trophic factor important in axonal or dendritic sprouting. It is also possible that EGF could serve as a neurotransmitter/neuromodulator in the CNS.

Key Words: Alzheimer’s disease; Epidermal growth factor; Glial fibrillary acidic protein (GFAP); Growth factors; Neuritic plaques; Paired helical filaments (PHF).

INTRODUCTION

Alzheimer’s disease (AD) is a dementing disorder characterized clinically by progressive deterioration of intellectual function and memory loss. Typical neuropathologic changes include the presence of neuritic (senile) plaques (NP) in gray matter, intraneuronal neurofibrillary tangles and granulovacuolar degeneration within nerve cells (1). The presence and concentration of neuritic plaques has been correlated with the severity of dementia (2). Terry and Wiśniewski (3) recognized and characterized at least three different types of NP. These authors theorized that the NP forms
because of the degenerated neurons' attraction for reactive astrocytes, which in turn deposit amyloid, a characteristic part of the NP (4).

Some of the earliest biochemical studies on AD focused on the changing metabolism of traditional neurotransmitters, including acetylcholine, catecholamines and serotonin (5). Recently, neuropeptides have come under scrutiny in relation to abnormal brain function in AD (6).

Numerous growth factors are known to play important roles in cellular development, growth and regrowth in response to injury. These effects have been shown to be both stimulatory and inhibitory and are in response to interaction with other growth factors, rather than a specific response to any one factor. One peptide growth hormone that plays an important role at sites of injury, epidermal growth factor (EGF), has been well characterized outside the CNS (7–9), and its effects are mediated by binding to a specific receptor, EGF-R. In recent reports, EGF has been immunocytochemically localized to the CNS in developing and mature rat brain (10). We have recently shown that EGF-R can be immunocytochemically localized in neurons from normal human brain tissue taken at autopsy (11). This study compares the distribution of EGF-R in normal human brain tissue to that in brain tissue from patients with AD.

MATERIALS AND METHODS

Samples

Postmortem brain tissue was collected from thirteen patients who had died with a clinical diagnosis of AD. This population ranged in age from 57 to 95 years (mean = 75). Postmortem brain tissue from a group of aged individuals who died without a history of neurologic or psychiatric disorder was used as control tissue. This population (n = 9) ranged in age from 60 to 87 years (mean = 73). Brain tissue, taken at autopsy after postmortem delays of 4 to 18 hours, was fixed in 10% neutral buffered formalin for a minimum of two weeks. Tissue blocks were taken from the superior frontal gyrus, hippocampus, and cerebellum. All tissue was processed for paraffin-embedding at temperatures not exceeding 55°C, and serially sectioned at 8 µm. Sections were mounted on gel-coated slides and heated to 50°C for 30 minutes (min) to ensure adhesion to the slide.

Techniques for Diagnosis

Sections from hippocampus and cerebral cortex from all patients were stained with Feigin silver impregnation and Thioflavin S fluorescent stains. Feigin sections were examined using low power (×20) light microscopy, and a diagnosis of AD was established if 18 ± 2 NP were identified in any field. Thioflavin S stain was used to confirm these results, with NP being identified by their characteristic fluorescence when viewed with ultraviolet light.

EGF Receptor Antibody

The anti-EGF-R serum was produced by immunizing rabbits with an EGF-R preparation purified from A-431 human epithelioid carcinoma cells (12). Antiserum #451 has been previously characterized and is known to immunoprecipitate native, occupied, some precursor and degradation forms of the EGF-R (12–14). Absorbed antiserum, to serve as a control, was produced by adding A-431 membranes to the EGF-R antiserum until the serum no longer blocked EGF binding to human fibroblasts.

Antibody to Glial Fibrillary Acidic Protein (GFAP)

The antibody to GFAP (Biomedical Technologies, Inc., Stoughton, MA) was produced by immunizing rabbits with homogenous GFAP isolated from bovine spinal cords. This antibody was monospecific to GFAP using transblots of adult rat spinal cord cytoskeletal protein. Optimal dilution was 1:200.
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Antibody to Paired Helical Filaments (PHF)

The antibody to PHF (ICN ImmunoBiologics, Lisle, IL) was produced by immunizing rabbits with a highly purified preparation of paired helical filaments isolated from human patients with AD. A 1:200 dilution was optimal.

Immunocytochemical Staining

Previously described in situ immunocytochemical staining procedures for the identification of EGF-R were used (11, 15–17) with the following modifications. Sections were deparaffinized through two changes of xylene, hydrated, and preincubated in normal 3% porcine serum for 20 min to saturate nonspecific tissue binding sites of serum components. Preimmune serum was rinsed from the slides and specimens were then incubated in either anti-EGF receptor serum (#451), anti-bovine GFAP serum, rabbit anti-human PHF, normal rabbit serum (#180) or absorbed #451, all of which were diluted 1:200 in phosphate buffered saline (PBS) containing 3% goat serum and 1 mg/ml glycine. All primary antibody incubations were performed overnight at 4°C in a humidified chamber, except for the anti-GFAP which worked optimally when incubated for one hour at room temperature. Following incubation with the primary antibody, tissue was rinsed for ten min in PBS and subjected to further incubation using a three-step immunoperoxidase procedure (DAKO PAP kit, Santa Barbara, CA). Immunoreactive EGF-R, GFAP and PHF were visualized using 3-3-diaminobenzidine (DAB). The DAB was prepared using 0.05% DAB in 0.05 M Tris buffer at pH 7.6 with 0.01% H₂O₂ added just before the reaction initiation. DAB sections were counterstained with a filtered 1% aqueous solution of Thioflavin S for 20–40 min and then rapidly differentiated in three changes of 80% ethanol, dehydrated and coverslipped using fluoromount. Both DAB and Thioflavin S labeled NP could be identified in the same tissue section when this double staining procedure was used. Adjacent serial sections were stained using the Thioflavin S procedure alone.

RESULTS

Neuritic Plaque Staining with Feigin and Thioflavin S Methods

Feigin and Thioflavin S stains confirmed the presence of NP in all 13 cases with the clinical diagnosis of AD. Neuritic plaques were especially prominent in hippocampus, subiculum and cerebral cortex, with numerous areas exhibiting 18 ± 2 plaques per low power field (×20). Those patients who had been clinically free of neurologic disease showed rare NP in hippocampus and cortex.

Distribution of EGF-R Immunoreactivity

In the present study we observed prominent EGF-R immunoreactivity localized in the periphery of the majority of NP from the AD cases (Figs. 1A, 2A, 3C). This localization of immunoreactive EGF-R could be easily visualized with DAB and showed a similar pattern of distribution with dark immunoreactivity in clusters surrounding an unstained amyloid core in NP. The immunoreactivity was not, however, limited to typical NP but rather was seen in a full spectrum of types of NP, from those with degenerating neurites, but no amyloid core, to those that consisted primarily of amorphous material. The amyloid-positive areas in blood vessels, as seen with the Thioflavin Stain, did not show EGF-R immunoreactivity. In previous experiments using the antibody to EGF-R (11), we found that the distribution of the receptor for EGF in the human nervous system was widespread, though discrete, and was usually localized to large neurons in many varied regions such as the anterior horn, dentate nucleus, cerebral cortex, cerebellum and hippocampus. The pattern of staining was consistent in each of these areas, with moderate to intense cytoplasmic staining extending into dendritic processes and often showing
Fig. 1. Hippocampal region from an AD brain. Intense DAB reaction product indicating EGF immunoreactivity is deposited within immature NP. ×225.

punctate perinuclear deposits (11). The capillary endothelium also exhibited EGF-R immunoreactivity as did the smooth muscle in the media of larger vessels. In this study, oligodendrocytes and resting astrocytes showed no staining when reacted with antiserum #451; however, the activated astrocytes surrounding an area of cerebral infarction were strongly positive for EGF-R crossreactive material (data not shown). Immunoreactivity was seen in neurons and glia in hippocampus, cerebellum, and frontal cortex in AD and in the aged control brains in a pattern similar to that previously described for normal human brain (11).

Serial control sections from AD cases treated identically except that #180 (normal rabbit serum) or preabsorbed antiserum was used instead of antiserum #451 showed no EGF-R immunoreactivity (Fig. 2C); however, numerous NP could be visualized

Fig. 2. Neuritic plaque in the frontal cortex of an AD patient photographed under a variety of conditions. This mature NP shows EGF-R immunoreactivity centered around an unreactive central amyloid core. ×550. A. Neuritic plaque with dense DAB reaction product in its periphery but an unreactive, unstained central core. B. The identical microscopic field also stained with Thioflavin S and photographed using ultraviolet light. The central amyloid core is brilliantly fluorescent with Thioflavin S. Additional fluorescence can be detected in the periphery of the NP. C. Adjacent serial section photographed under bright field conditions showing the same microscopic field seen in A and B. This section was reacted with control antiserum (#180) and then counterstained with Thioflavin S. Note the absence of any DAB
reaction product. D. Fluorescence image of the same field seen in C, indicating that this is an amyloid-containing neuritic plaque. Note the more extensive staining when compared to B, indicating that the DAB reaction product overlaps and obscures areas showing fluorescence in the periphery.
using the Thioflavin S stain (Fig. 2D). The normal aged brains showed rare NP in cortex and hippocampus using both immunocytochemical and fluorescent staining techniques; there were no NP in cerebellum. The same pattern and intensity of immunoreactive EGF-R was seen in the few NP that were present (data not shown).

One case with severe degenerative changes and numerous NP was examined for NP in other brain regions known to be associated with pathways for memory such as the dorsomedial and anterior nuclei of the thalamus (18). Both of these areas contained NP with moderate immunoreactivity with EGF-R antiserum.

**GFAP Immunoreactivity**

The pattern of immunoreactivity seen in NP of AD brains as well as in normal aged controls was the same when both were treated with antiserum to GFAP. Only a few NP showed positive staining when compared to the adjacent Thioflavin S-stained control. Positively-stained NP showed a dense staining in both the cytoplasm and processes of the astrocytes that bordered NP with and without amyloid cores (Fig. 3B). There was no overlap between fluorescent-stained areas and those showing GFAP immunoreactivity; in fact, the astrocytic component of the positively-stained NP seemed to circle the plaque instead of being an intrinsic component.

Many of the AD brains also showed a relative astrocytosis not associated with NP within the cortex and hippocampus. These astrocytes stained in the same manner as those found in the periphery of the NP. No positive staining was seen in any section treated with the preimmune rabbit serum.

**Immunoreactivity to Paired Helical Filaments**

Unlike the tissue sections treated with an antibody to GFAP, those which were reacted with antiserum to PHF showed dark immunoreactivity in clusters in a majority of NP in both hippocampus and frontal cortex from AD patients. The quality and pattern of staining was similar but not identical to that seen with EGF-R antibody; in fact, most plaques that were immunoreactive for EGF-R were also immunoreactive for PHF and vice versa. The PHF antibody produced a reaction product only in the peripheral zone when a central amyloid core was present but stained most elements of the NP without a core or so-called (Fig. 3A). In addition, neurofibrillary tangles in both hippocampus and cortex were densely stained.

**Interaction of Immunocytochemical and Fluorescent Staining**

Staining with Thioflavin S confirmed the fact that those areas containing immunoreactive EGF-R, PHF and GFAP were localized in the periphery of amyloid-
containing NP (Fig. 2B, D) and throughout immature plaques. There was considerable overlap between fluorescent staining and EGF-R and PHF immunoreaction product in the periphery of plaques; in fact, the DAB reaction product interfered with the visualization of fluorescence in the sections that were double labeled. The adjacent control sections counterstained with Thioflavin S confirmed this masking effect (Fig. 2D).

Qualitative Observations

The intensity of the immunocytochemical staining for EGF-R varied among the NP and ranged from moderate to severe. This variation existed both within a case and from case to case, as well as within a particular section from a single case, possibly indicating the existence of subsets of NP. The NP with moderate immunoreactivity with antiserum to EGF-R were also the same plaques with less intense fluorescence with Thioflavin S. Not all NP with fluorescent staining contained EGF-R immunoreactivity, but the majority did. All NP visualized with antiserum to EGF-R contained at least a focus of fluorescence, when the identical plaque was seen in an adjacent section stained only with Thioflavin S, so that the NP was not obscured by the DAB reaction product. The plaques with immunoreactive EGF-R did not appear to be confined to a particular layer of neocortex. There was a variation from case to case, with several cases showing prominent plaques in layers 4–6, others with most plaques in layers 1–3, and still others with equal distribution throughout all layers. Similarly, plaques varied in size from brain to brain and also between and within regions in a particular case.

DISCUSSION

In this study the majority of NP found in postmortem brain tissue from individuals with AD exhibited well localized immunoreactive EGF-R in sections incubated with EGF-R antiserum. We also demonstrated EGF-R localization in the rare NP found in non-demented aged human brains. Their identical localization and appearance suggests a similar pathogenesis for these NP which may be accelerated in the disease state. Furthermore, this pattern of immunoreactivity in NP is similar, though not identical, to that seen when AD tissue is treated with an antiserum to PHF, suggesting that one location for the EGF-R may be in the neuronal population. The astrocytic component of the plaques, identified with an antibody to GFAP, were found in only a minority of NP, but these cells cannot be ruled out as another site of the EGF receptor in this study. Our observations suggest a CNS function for EGF/EGF-R. This system may be activated in response to injury, serve as a trophic factor (19) or neurotransmitter/neuromodulator, or merely accumulate in NP because of their neuronal origin.

Epidermal growth factor (EGF) has well-defined mitogenic effects and induces differentiation and proliferation in a variety of tissues (15–17, 20–25). In addition EGF may have non-mitogenic roles (26) and be involved in ion transport. Like other gut/brain peptides, EGF shows hormonal activity in the gastrointestinal tract by its ability to inhibit hydrochloric acid secretion (27). In cells derived from nervous tissues such as the PC-G2 (28) and PC-12 rat pheochromocytoma cell lines, EGF has also been reported to induce a number of effects. In these cells, considered to be models of sympathetic neurons, EGF has been shown to effect phosphorylation reactions (29), induce both tyrosine hydroxylase (30) and ornithine decarboxylase (31), increase cellular adhesion (32) and alter morphology (33). The detection in
cerebrospinal fluid (CSF) of EGF (34), which does not cross from blood into brain or CSF (35), indicates that EGF may be elaborated in the CNS as part of an intrinsic EGF system in brain tissue (19). Furthermore, EGF has been immunocytochemically localized in both developing and mature rat brain to globus pallidus, striatum, and substantia nigra (10).

Recent studies have shown that EGF-R can be discretely localized and identified immunohistochemically in pyramidal cells of the cerebral cortex and hippocampus, Purkinje cells of the cerebellum, anterior horn cells of the spinal cord, dorsal root ganglion neurons, neurons of the dentate nucleus and in ependymal cells and choroid plexus (11). The EGF-R was not seen in resting astrocytes or oligodendrocytes; however, intense immunocytochemical staining with antisera to EGF-R was seen in the cytoplasm of reactive astrocytes that proliferate around an area of cerebral infarction (unpublished observations). This observation has led us to wonder if the paucity of cytoplasm in the resting astrocyte is the reason for lack of visualization of the EGF receptor?

Several possibilities exist for the role of EGF/EGF-R in aging as seen in this study. Neuritic plaques may not be an attempt at scar formation in the CNS but just the opposite, i.e. an attempt at neuritic regeneration. Geddes et al (36) have shown that the hippocampus is capable of a plastic response in AD, with expansion of a specific receptor population in response to injury, so that growth may occur along with degeneration in AD. Similarly, Probst et al (37) looked at NP in the hippocampus of patients with AD using a Golgi-Cox method; they found frequent abnormal dendritic and axonal segments in these areas, a finding in agreement with other authors. Their data showed occasional axonal and dendritic sprouting. This finding and the report of trophic stimulation of cultured neurons by Morrison et al (19) may indicate some trophic or even reparative activity within the NP.

Since our data show a similar pattern of immunoreactivity in NP with antiserum to both EGF-R and PHF, we believe that at least a portion of the EGF-R are located in the neuronal population, consistent with our previous findings in the normal human brain (11). The EGF/EGF-R system may function as a neurotransmitter/ neuromodulator system within the CNS, as do over 50 other gut/brain peptides. Others have demonstrated substance P levels (9), somatostatin, enkephalin-like immunoreactivity and corticotropin-releasing-factor-like immunoreactivity in terminal dendrites within neuritic plaques from AD brains (38–41). However, the EGF-R pattern of immunoreactivity within NP differs morphologically from that seen using antibody to somatostatin. The predominantly cytoplasmic distribution of EGF-R has been reported in previous studies from our laboratory (11, 15–17) and from Damjanov et al (42). In addition, ultrastructural data indicate that EGF-R are widely distributed in the cytoplasm (43). Thus, numerous reports indicate that in an in vivo equilibrium the internalized EGF-R would not be seen only on the membrane.

A possibility that can neither be confirmed or ignored in this study is that the astrocytic component of NP may either contain EGF-R, produce the ligand for the receptor, or induce the receptor on the degenerating neuronal component. Astrocytes in culture have been shown to proliferate (44) and differentiate (45) in response to EGF as well as possess the ability to bind 125I-EGF (46). When the CNS sustains an injury, astrocytes increase in number, size and appearance in response to unknown morphogens and mitogens. Evidence now exists that the brain is capable of producing endogenous mitogenic factors that cause changes in the glial population and may thus produce its own scar (47). Specifically, the neuronal death which occurs in response to an as yet unknown insult in AD may activate the intrinsic EGF/
EGF-R system in the brain. This activation may then stimulate astrocytes, particularly in the regions of the degenerated neuritic debris, to participate in various stages of development of the NP. This hypothesis could explain the observation of different stages of plaques, as well as the differences we observed in the intensity of the EGF-R immunohistochemical reaction product among NP.

In conclusion, we find EGF-R immunoreactivity within the cortical and hippocampal NP in patients with AD and in the rare plaques in normal aged patients' brains. This report, coupled with reports that EGF is present in CSF (34), strongly suggests that another well-characterized peptide (EGF) is biologically active in brain tissue.

ACKNOWLEDGMENTS

We thank Jesse Britton, Mary McKissack, Mike Pritchard and Tracie Reynolds for excellent technical assistance as well as Marissa Garcia and Tom Ebers for typing the manuscript.

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(Received 10 February 1987/Accepted 30 October 1987)
MS 87-07