Apolipoprotein E Is Localized to the Cytoplasm of Human Cortical Neurons: A Light and Electron Microscopic Study

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Abstract. To clarify the localization of the glial protein apolipoprotein E (apoE) in human cortical neurons, we employed specific immunoelectron microscopy using a monoclonal antibody to human apoE in surgical specimens of temporal lobe. The specimens were rapidly fixed after excision from five patients undergoing surgery for medically intractable seizures, and postmortem material was also taken from one Alzheimer’s disease patient for comparison. Strong apoE immunoreactivity was observed in many astrocytes filling the perinuclear cytoplasm and distal processes completely. Some cortical neurons were also apoE-immunoreactive. ApoE immunoreactivity of neurons was less intense than glial cells and was distributed in a punctate fashion confined to the region of the cell body and proximal dendrites, but not distal processes. These findings suggest that apoE, which is presumably synthesized and stored by astrocytes, may be taken up by cortical neurons in younger adult humans. The presence of apoE in some human neurons may allow apoE to affect neuronal metabolism. Isoform-specific interactions with microtubule-associated proteins, such as tau or MAP2C, could influence the rate of pathology in neurodegenerative diseases such as Alzheimer’s disease.

Key Words: Apolipoprotein E; Astrocytes; Cortical neurons; Human brain; Immunelectron microscopy.

INTRODUCTION

Apolipoprotein E (apoE), a 34-kDa glycoprotein, is the major apolipoprotein in the central nervous system (CNS). ApoE plays an important role in triglyceride-rich lipoprotein metabolism and cholesterol homeostasis (1). ApoE has been postulated to be involved in repair, growth and maintenance of myelin and axonal membranes during development and after injury both in the peripheral and central nervous systems (1–5). Thus, apoE appears to be necessary for the proper functioning and repair of neuronal membranes throughout the normal life of a neuron. The significant role of apoE in membrane maintenance and lipid metabolism suggests the possible involvement of apoE in the pathogenesis of neurodegenerative diseases. In fact, inheritance of specific apoE alleles is a genetic susceptibility factor in late-onset Alzheimer’s disease (AD) (6–11).

Understanding the role of apoE in normal functioning of the CNS and in AD depends critically on the cellular localization of this protein. In the CNS, apoE mRNA is expressed in glial cells, particularly astrocytes (12, 13). Numerous studies have documented immunohistochemical evidence for apoE localization in astrocytes and glial cells of rodent, non-human primates, and patients with AD and age-matched controls (6, 12–22). In lesioned rat hippocampus, apoE expression in astrocytes may be increased during reactive astrogliosis (13, 23, 24). In studies of AD brains, multiple observers have commented on the presence of apoE immunoreactivity in neurons with neurofibrillary tangles (6, 18–22). In our studies we observed the expected intense apoE immunoreactivity of many astrocytes, but also observed definite staining of some neurons in both non-human primate and human brain (6, 20, 22).

Despite the several reports of apoE in cortical neurons with neurofibrillary tangles, the presence of apoE in other neurons is still controversial. In the present study, we have extended our previous studies by determining subcellular distribution of apoE at the ultrastructural level in human temporal lobe obtained from patients undergoing surgery for medically intractable temporal lobe epilepsy and in one patient with AD for comparison. We will describe the presence of apoE in the cell body of human cortical neurons in younger adults with epilepsy as an example of apparent neuronal uptake of a glial protein. If this situation occurs commonly in adult humans, intraneuronal apoE could potentially interact in an isoform-specific fashion with the intraneuronal microtubule-associated protein, tau, and possibly influence the rate of AD pathology (25).

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Fig. 1. Light microscopic appearance of apoE immunoreactivity in paraffin sections of AD brain and in Vibratome sections of temporal lobe surgical specimens. Magnification is the same for A–E and slightly higher in F. A. Rapid autopsy specimen of entorhinal cortex of 77 year old AD patient with apolipoprotein E 4/4 genotype. Immunoreacted section with omission of primary antibody shows lack of staining under control conditions. Although gathered soon after death, tissue was otherwise routinely processed as far as fixation, paraffin embedding, and sectioning. B. ApoE-immunoreacted section from above patient showing immunoreactive plaques and cortical neurons (closed arrow). Other neurons are faintly immunoreactive (open arrow), and others
MATERIALS AND METHODS

Tissue Preparation

Tissue was obtained from the temporal cortex of three male and two female medically intractable seizure patients ranging in age from 21 to 55 years and from one 77 year old male patient with AD. Blocks of temporal lobe were collected during temporal lobe surgery using the resected tissue fragments collected for pathology (less than 5 minutes after removal), or, in the case of the AD patient, 2 hours after death at autopsy. The blocks were trimmed to thickness of 3-5 mm and placed immediately in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Soon thereafter, the blocks from each case were cut into two portions with one portion being left in 4% paraformaldehyde and the other transferred to a solution of 2% paraformaldehyde/0.2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). After immersion fixation for 24 hours at 4°C, both blocks were rinsed in 0.05 M phosphate buffer with normal saline (PBS) and stored in 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) until sectioning for immunocytochemistry.

Pre-embedding Immunocytochemistry

Serial sections were cut on a Vibratome at 35-50 microns and collected free-floating in PBS. Sections were pretreated in freshly prepared 90% formic acid for 3-5 minutes or in 1% methanol-hydrogen peroxide for 5 minutes to enhance immunogenicity and to suppress endogenous peroxidase activity. After a series of three rinses in PBS for 5 minutes each, the sections were incubated for 1 hour at room temperature and then overnight at 4°C in a solution containing a mouse monoclonal antibody against human apoE (3H1, diluted 1:1,000 in 2% normal goat serum) obtained courtesy of Dr. Ross Milne (University of Ottawa, Ottawa, Canada). The 3H1 antibody was discovered using assays of inhibition of heparin-binding sites on apoE and recognizes amino acid residues 243-272 (26). The two other monoclonal antibodies mentioned in this work (ID7 and 6C5) were also tested in pilot runs but did not result in good immunostaining. Staining characteristics of 3H1 in human brain are similar to a rabbit polyclonal antibody raised to human apoE (laboratory of Dr. Karl Weisgraber) used in previous studies on apoE localization in AD brains (20, 22) but are stronger and have less background than a goat polyclonal antibody to rabbit apoE used in our original report (6).

After three washes for 5 minutes each in PBS, tissue sections were then incubated for 30 minutes in biotinylated horse antimouse IgG (diluted 1:50, Vector Laboratories, Burlingame, CA) followed by washes and incubation for 30 minutes in avidin-biotin-peroxidase complex (Vector Laboratories).

Peroxidase activity was visualized by incubating the sections for 7-10 minutes in a solution containing 0.01% 3,3'-diaminobenzidine (DAB) and 0.003% hydrogen peroxide in PBS. In order to rule out nonspecific binding and to ensure specificity, control reactions were carried out on adjacent sections with either omission of primary antiserum or replacement of the relevant secondary antibody by one raised in another species. After the DAB reaction, 35 micron sections were mounted on slides, dehydrated in a series of alcohols, cleared in xylene, and coveredslipped for light microscopy. The 50 micron sections were processed for electron microscopy (see below).

Portions of the tissue blocks from each case were also routinely processed and embedded in paraffin for pathological analysis. Six to eight micron paraffin sections were cut and mounted on coated slides for immunocytochemistry. Sections were deparaffinized, treated with 90% formic acid for 3-5 minutes, washed, and then incubated as for Vibratome sections. Paraffin processing and formic acid treatment results in easier demonstration of apoE immunoreactivity of plaques with some decrease of overall immunoreactivity (22), but there is no appreciable qualitative difference in apoE localization.

Electron Microscopy

The 50 micron immunoreacted sections were postfixed in a solution of 1% osmium tetroxide in 0.1 M phosphate buffer, dehydrated in a graded series of alcohols, and flat-embedded in Epon. After light microscopic observation of the cured sections, small areas containing apoE-immunoreactive neurons and glial cells were trimmed from the section, glued to an Epon post, and sectioned at approximately 80 nm on a Reichert ultramicrotome. These sections were collected on formvar-coated grids with slots and left unstained for observation under a JEOL 1200EXII electron microscope at 80 kV. Sections were examined and areas of interest were photographed at magnifications of 4,400-20,000× for further analysis.

RESULTS

Light Microscopy

By light microscopy, we observed apoE immunoreactivity in sections taken from both the autopsy specimen of temporal lobe of the patient with AD and from the five surgical specimens of temporal lobe (Fig. 1). In both AD and temporal lobe epilepsy material, the pattern of immunoreactivity was specific to anti-apoE antibody and

are unstained. C: Vibratome section of lateral temporal lobe neocortex of young (<30 years old) epilepsy patient showing lack of staining under control conditions. D: ApoE-immunoreacted section from above case showing intense staining of neuropil filled with astrocytic cell bodies and processes, and presence of immunoreactive neurons (arrow). Neurons have unstained nuclei and are only stained in the region of the cell body. E: Another section from above case showing apoE immunoreactivity of astrocytes and their processes terminating on a cortical blood vessel. Many immunoreactive cortical neurons are present but are less obvious since no particular staining of dendrites is observed. F: Higher magnification to show typical staining characteristics of immunoreactive cortical neurons (arrowheads) with unstained nuclei and no staining beyond the region of the nucleus. In the left corner, a stained neuron is observed (arrows) with a more darkly staining satellite glial cell probably apposed to its upper left border (see Fig. 4).
no staining was observed in control sections (Fig. 1A–D). In the AD case, plaques, many glial cells, and some neurons were immunoreactive (Fig. 1B). In the AD case, the pattern of immunoreactivity corresponded to previous observations in our laboratory of apoE localization in human brain specimens obtained at autopsy from 32 patients including normal controls, patients with AD, and patients with Parkinson's disease (22). In the surgical cases where no neuritic plaques or neurofibrillary tangles were observed, only glial cells and neurons were apoE-immunoreactive (Fig. 1D).

We observed apoE immunoreactivity in tissue fixed in 2% paraformaldehyde/0.2% glutaraldehyde as well as in tissue fixed in 4% paraformaldehyde; however, apoE immunoreactivity was more robust in the tissue fixed in only paraformaldehyde. In these tissue blocks, the staining of astrocytic glial cells was particularly dense and complete; intense apoE immunoreactivity was present not only in the thin rim of cytoplasm surrounding the nucleus but also in the processes as they spread throughout the neuropil and as they ended on blood vessels (Fig. 1E). In contrast, the staining of neurons was less intense and confined to the region of cytoplasm just around the nucleus and in proximal processes (Fig. 1F). We occasionally observed apoE-immunoreactive satellite glial cells in close proximity to labeled neurons (arrows, Fig. 1F).

Marked astroglialosis was observed in both the AD brain and the surgical temporal lobe specimens, and intense apoE staining of astrocytes was observed particularly in layer 1 and subcortical white matter. Stained glial cells included cells with the morphology of protoplasmic and fibrillary astrocytes, as well as neuronal satellite glial cells. No apparent immunoreactivity of white matter oligodendrocytes was observed. Some staining of endothelial cells and blood vessel walls was observed and was attributed to heavy envelopment by astrocytic endfoot.

Electron Microscopy

Control sections reacted in parallel with omission of primary antibody were unstained at the light microscopic level (Fig. 1A, C), and we found no immunoprecipitate in their companion thin sections at the ultrastructural level in any cell class (Fig. 2A). In contrast, sections reacted with anti-apoE antibody revealed strongly stained glial cells at the light microscopic level (Fig. 1B, D, E, F) and showed abundant profiles of heavily immunoreactive glial cells with dense horseradish peroxidase-reaction product filling their cell bodies and processes at the electron microscopic level (Fig. 2B). Although immunoreactivity often decorated the external membranes of mitochondria, no specific compartmentalization of the reaction product was observed (Fig. 2B). Most structures were heavily immunolabeled, and we could not specifically ascertain labeling of other pertinent organelles such as Golgi vesicles or cisternae. The same intense immunoreactivity of glial cell somas was also observed in numerous smaller processes in the surrounding neuropil (Fig. 2C). Based on the light microscopic sections and on their intense immunoreactivity, these processes are probably smaller distal processes of immunoreactive glial cells. No associated synaptic densities or vesicles were observed in these processes. The strong immunoreactivity of astrocytes from soma to distal processes is supported by the staining of astrocytic endfeet that ended upon cerebral blood vessels which were heavily immunoreactive for their entire extent (Figs. 1E, 2C).

Much of the neuropil was unstained in apoE-immunoreacted sections. In particular, it was common to see large unstained areas of fine axonal and dendritic processes with occasional profiles of strongly immunoreactive, presumed astrocytic processes. We did not observe any staining of neurons or neuropil in the control sections (Fig. 3A). In apoE-immunoreacted sections examined at the ultrastructural level, it was common to find neurons that contained no immunoreactivity in regions of neuropil that contained immunoreactive processes. However, as suggested by the companion sections prepared for light microscopy, some neurons were clearly apoE-immunoreactive (Fig. 1). In contrast to the glial cells, these neurons contained reaction product that was lighter and in punctate distribution (Fig. 3B, C). As in glial cells, apoE immunoreactivity was not confined to any particular subcellular compartment, but rather was present in clumps in the cytoplasm apparently associated with the membrane of the endoplasmic reticulum or other organelles and cellular structures. We found heavy immunoprecipi-
Fig. 3. Electron microscopic analysis of immunoreacted Vibratome sections from surgical specimen of lateral temporal lobe from another young (<30 years old) epilepsy patient. Fixation 2% paraformaldehyde/0.2% glutaraldehyde. A. Control section showing lack of immunoprecipitate in neuron and neuropil of sections reacted with omission of primary antibody. B. Immunoprecipitate in cytoplasm of cortical neuron in section exposed to 3H1 anti-apoE antibody. Note that nucleus is negative as well as most of adjacent neuropil. C. Area outlined in B is presented at higher magnification (bar indicates 400 nanometers). Immunoprecipitate is located widely in the cytoplasm, although locally dense in some locations. Immunoprecipitate is seen on the
Fig. 4. Electron microscopic analysis of immunoreacted Vibratome section from surgical specimen of lateral temporal lobe from same patient illustrated in Figure 3. The densely immunoreactive cell in right center with relatively condensed nuclear chromatin is assumed to represent a satellite glial cell due to its close apposition to a large, less strongly immunoreactive region (arrowheads indicate border) filling much of the photograph. This region is presumed to represent proximal dendrite or perinuclear soma of a large cortical neuron given the size and shape of this process and the intensity of immunoreaction. On the left side of this lightly immunoreactive cell is a strongly immunoreactive process (arrows) which is also apposed to the presumed neuron. The overall appearance suggests the combined profile of satellite glial cell and neuronal cell body illustrated in Figure 1F.

tate on the outer membranes of structures with the appearance of microbodies as well as on mitochondria (Figs. 2B, 3C). We did not see immunoprecipitate inside definite organelles or on the plasma or nuclear membrane. Unlike the situation for glial cells, we did not find evidence for any immunoreactivity of any identified distal neuronal processes, whether axonal or dendritic, despite inspecting many fields with profiles of distal pre- and post-synaptic neuronal elements closely adjacent to heavily immunoreactive small processes presumed to be glial.

These observations of sections prepared for electron membrane but not inside mitochondria and small, uniformly electron dense organelles that may represent microbodies or peroxisomes. The nuclear membrane and plasma membrane are relatively unstained.
microscopy supported the impression from light microscopic studies that (a) some, but not all, neurons are apoE-immunoreactive, (b) neurons are less immunoreactive than glial cells, and (c) apoE immunoreactivity in neurons appears confined to the cell body and not the distal dendrites or axon. This relationship is demonstrated in Figure 4, where a portion of lightly labeled neuronal cytoplasm (possibly expansion near base of proximal dendrite) is observed with a closely apposed, strongly immunoreactive satellite glial cell. The presence of strongly apoE-immunoreactive processes near the somas of some human cortical neurons is similar to photomicrographs presented by Diedrich and coworkers (12).

**DISCUSSION**

In this study, we demonstrate the immunolocalization of apoE in cortical neurons as well as in glial cells in adults undergoing temporal lobe resection for medically intractable epilepsy. The ultrastructural demonstration of apoE immunoreactivity in human cortical neurons extends and confirms our previous findings in autopsy specimens of human brain of normal aged controls and AD patients (20, 22), and in aged prosimian primates (6, unpublished data). The demonstration of strong apoE immunoreactivity of many astrocytes and glial cells in our material agrees with the results of previous immunohistochemical studies (6, 12, 17-19, 21).

The additional light and electron microscopic evidence for neuronal localization of apoE, however, is in contrast to the commonly held viewpoint that apoE is localized only in astrocytes and glial cells (12-19, 21). Most previous studies on the immunolocalization of apoE have been carried out in rodent brain and have reported the presence of apoE in glial cells and particularly astrocytes. An astrocytic localization for apoE fits with the observation that apoE mRNA is found only in glial cells and not in neurons in the CNS (12, 13). Storage and secretion of apoE by astrocytes would certainly be sufficient to account for the proposed role of apoE in myelin repair and maintenance, and in reverse cholesterol transport during synaptic remodeling. However, unlike "glia-specific" structural glial proteins such as glial fibrillary acidic protein (GFAP), the above role for apoE does not preclude its uptake or presence in neurons, particularly since it is secreted into the extracellular space.

In fact, apoE has been reported in human cortical neurons with neurofibrillary tangles in patients with AD by several different groups (6, 18-22) as well as in the extracellular space in association with amyloid deposits in a variety of diseases (6, 12, 18-22). A receptor for uptake of apoE-containing lipoprotein particles has been demonstrated in human and rodent neurons, LDL receptor-related protein (LRP) (21, 27, 28), and possibly also the LDL receptor (21). The LRP is abundantly located in the soma region of neurons (21, 27) and in proximal processes (28). Given the rich expression of apoE in the nervous system by astrocytes and the presence of relevant receptors on cortical neurons for apoE-lipid particles, the question might well be posed: why is apoE not present in most neurons?

We have demonstrated that (a) apoE is present in some, but not all, cortical neurons, (b) the apparent content of apoE in neurons is less abundant than in glial cells, and (c) apoE in neurons is present in the cytoplasm and is associated with the external membrane surface of some, but not all, intracellular organelles. We used surgical temporal lobe specimens in order to acquire optimally preserved material for apoE immunolocalization, realizing that these specimens do not necessarily represent normal human brain. We also observed some heterogeneity of apoE immunoreactivity in neurons with several cases showing only rare examples. Based on our previous observations in aged normal controls and in prosimian primates, we suggest that apoE in neurons is not a rarity but may be common in adult primate brain (6, 22, unpublished data).

The failure to observe neuronal localization of apoE in other studies could be due to immunoreagents employed, loss of antigen/alteration of antigen during fixation and processing, and species or individual differences in apoE localization. All of these possibilities can be supported.

Firstly, many antisera to apoE that are biochemically active as inhibitors, or are adequate for Western blotting, are poor reagents in immunocytochemistry. In our experience, a polyclonal antibody to human apoE (20, 22) and the present monoclonal antibody to the carboxy terminus (3H11) were the strongest reagents for staining neurons and glia; several other polyclonal antibodies had high backgrounds and two other monoclonal antibodies had no staining of neurons or glia in fixed tissue.

Secondly, fixation of apoE may be a significant factor. We found that light fixation resulted in more intense immunoreactivity, with loss of both neuronal and glial staining intensity with longer fixation times or higher strengths of formaldehyde or glutaraldehyde. Access of immunoreagents to epitopes on chemically fixed apoE expected to be associated with lipids and membranes appears to be limited in non-extracted tissue sections. Alcohol extraction, formic acid treatment, or paraffin embedding results in increased immunoreactivity in any given specimen. Since reactive astroglia are strongly apoE-immunoreactive, the mere demonstration of immunoreactive glia does not ensure that optimal sensitivity of immunocytochemical methods has been achieved for a given tissue preparation.

Finally, most studies reporting absence of apoE in neurons have been performed in rodents. We find that apoE is rarely localized in cortical neurons in rodents, often only in older specimens (work in preparation). In
normal human aged controls, we found localization of apoE in hippocampal neurons in two of six cases and found neuronal apoE localization in essentially all cases of AD and Parkinson's disease (22). These prior results, and the present study, in temporal lobe epilepsy patients suggest that the presence of apoE in neurons may reflect some dynamic change in glial and/or neuronal physiology related to aging, normal tissue repair, or disease. Thus, the presence of apoE in some cortical neurons may be common and may represent an adaptive response.

The neuronal localization of apoE may be important for its role in AD neuronal pathology (25). Further studies are needed to better define the ultrastructural compartmentalization of apoE, any possible specificity of apoE localization related to neuronal class, and the mode of entry of apoE into neurons. Our results suggest a predominantly cytoplasmic localization in human cortical neurons and glial cells. The cytoplasmic localization agrees with the previous report of ultrastructural localization of apoE in rodent glial cells (14), but in our material we could not conclusively demonstrate the presumed localization of apoE in Golgi secretory vesicles and cisternae. In hepatocytes, apoE is localized not only in the cytoplasmic compartment but also in Golgi vesicles and cisternae and peroxisomes (29). Careful attention in future studies will need to be paid to reagent choice, tissue preparation, and species and cell class differences in both in vitro studies and in vivo cell culture studies.

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