Increased Expression of Neuronal Apolipoprotein E in Human Brain With Cerebral Infarction

Kazuko Aoki, MD, PhD; Toshiki Uchihara, MD, PhD; Nobuo Sanjo, MD, PhD; Ayako Nakamura; Kenji Ikeda, MD, PhD; Kuniaki Tsuchiya, MD, PhD; Yoshihiro Wakayama, MD, PhD

**Background and Purpose**—Cellular origin of apolipoprotein E (ApoE) in the human brain and its roles in physiological and pathological conditions remain to be clarified.

**Methods**—Immunolocalization of ApoE was investigated in a series of autopsied human brains with or without infarction. ApoE expression was also estimated on immunoblot on protein extracts from autopsied brains and a cultured neuroblastoma cell line of human origin (GOTO) subjected to an oxidative stress induced by exposure to hydrogen peroxide (0.2 mmol/L).

**Results**—In addition to astrocytes and microglia, neurons and degenerated axons in and around the ischemic foci contained ApoE-like immunoreactivity, which was more intense in recent ischemic foci. Immunoblot demonstrated an increase in expression of ApoE in brain extracts from ischemic lesion, and this increase was also pronounced in the cultured neuroblastoma cell line after the stress.

**Conclusions**—Accumulation of ApoE in neurons in and around ischemic foci of the human brain is related to an increase in ApoE synthesis in neurons, as seen in cultured neuronal cells after oxidative stress. Intrinsic regenerative activity of neuron in reaction to external insults may be related to this increase in ApoE of neuronal origin. *(Stroke. 2003;34:875-880.)*

**Key Words:** apolipoproteins • cerebral infarction • neurons • oxidative stress

A polipoprotein E (ApoE) is synthesized in various organs, including liver, spleen, kidney, and brain, and is supposed to play multiple roles. There is accumulating evidence that ApoE in the central nervous system is involved not only in stable physiological conditions but also in more dynamic situations such as development, remodeling, degeneration, and regeneration.1–6 Moreover, the cellular source of ApoE in the central nervous system, initially considered to be restricted to astrocytes,7,8 is currently considered to be more variable according to conditions.9–14

In this study we attempted to examine the manner in which ApoE is expressed in human brain with ischemic foci to clarify how an insult to the central nervous system modifies the expression of ApoE, which has been reported to play certain roles in repair of the central nervous system.1,15–17 A parallel in vitro study on a cultured cell line corroborated these in vivo findings.18,19

**Methods**

Autopsied brains from 9 patients with cerebral infarction, 6 patients with Alzheimer disease (AD), and 8 patients without neurological disorders were enrolled in this study. Clinical data of the patients are summarized in the Table. The time from the onset of the ischemic attack to death was between 8 days and 10 months, determined from the clinical records.

**Immunohistochemistry**

Formalin-fixed, paraffin-embedded blocks, including both the area of ischemic necrosis and the surrounding nonnecrotic area, were obtained at 10-μm thickness. Sections were deparaffinized and treated with a microwave oven in citrate buffer 3 times for 5 minutes, treated with 1% hydrogen peroxide for 30 minutes, then incubated for 3 days at 4°C with either ApoEC (1:2000; anti-human ApoE polyclonal antibody raised against a synthetic peptide EKVQAVGTSAPVPSDNH equivalent to the C-terminal amino acid sequence 299 to 317 of human apolipoprotein E; IBL) or ApoEAB94720 (1:2000; anti-human recombinant ApoE; Chemicon) diluted with PBS containing 0.03% Triton-X 100 and the corresponding blocking serum. The sections were then incubated for 2 hours with a biotinylated secondary antibody (1:1000; Vector), followed by avidin-biotin-peroxidase complex (1:1000; ABC Elite, Vector). The peroxidase labeling was visualized with diaminobenzidine as chromogen, which yielded a brown reaction product after approximately 40 minutes, and then the stained sections were counterstained with hematoxylin.
Cellular localization of ApoE epitope was examined on double-labeled sections with the following combinations of probes: anti-carbimidin (1:3000; anti-spot 35, rabbit polyclonal antibody; generous gift from Dr Yamakuni, Tohoku University) as a marker for neurons/ApoEAB947; anti–glial fibrillary acidic protein (1:1000; mouse monoclonal antibody; DAKO) as a marker for astrocytes/ApoE; or biotinylated Ricinus communis agglutinin (1:1000; RCA-120; Seikagakukouyou) as a marker for microglia/ApoE. After one of the probes (antibody or lectin) was visualized with diamobenzidine and nickel ammonium sulfate as purple, the sections were subjected to second-cycle immunostaining with the other probe. They were then treated similarly except that diamobenzidine was used without nickel ammonium sulfate to yield brown reaction products.

To localize ApoE-like immunoreactivity in relation to axons, double immunolabeling with ApoEC and anti-neurofilament (SMI31; Sternberger Monoclonal) was performed. Deperaffinized sections were incubated with SMI31 (1:1000), and the epitope was then immunofluorolabeled by FITC-conjugated anti-mouse IgG (1:200; Cappel). The FITC signal was observed under the fluorescence microscope combined with a laser confocal system (TCS-SP; Leica), and the images were captured and recorded on magnetic optical disks. The same section was subjected to second-cycle immunostaining with ApoEC (1:2000), visualized with the ABC method. The already photographed axons were identified with the use of various structures such as lipofuscin granules or blood vessels as landmarks. The relationship between ApoE-like immunoreactivity and neurofilaments was assessed on the same field.

**Cell Culture, Cell Stimulation, and Protein Extraction**

The human neuroblastoma cell line GOTO was cultured in RPMI 1640 medium supplemented with 20% fetal bovine serum. At 3 days in culture, the incubation medium was replaced with RPMI 1640 without fetal bovine serum. Hydrogen peroxide was added directly to the medium to produce a final concentration of 0.2 mmol/L and incubated for appropriate times. At fixed intervals (4, 24, and 48 hours) after the challenge with hydrogen peroxide, the incubation medium was rapidly aspirated, and the cells were washed twice with ice-cold PBS and fixed by 10% trichloroacetic acid for 30 minutes at 4°C. After the dishes were scraped with a rubber policeman, the lysate was centrifuged at 15 000g for 5 minutes, and the supernatant was discarded. Then each pellet of GOTO cell, at different intervals after the exposure to hydrogen peroxide, was resuspended by sonication in a sample buffer containing 9 mol/L urea, 2% Triton X, and 5% 2-mercaptoethanol. Then one fifth volume of 10% lithium dodecyl sulfate solution and approximately 2 μL of 1 mol/L Tris solution were added to the sample buffer, and the samples were sonicated again. (1) Each human brain homogenate from the autopsied brains (2 AD brains, 2 brains with infarction, and 4 control brains) was also fixed by 10% trichloroacetic acid, and extracted protein was treated in the same way as the GOTO cell.

**Western Blot Analyses**

Lysates containing equal amounts of protein (GOTO, 10 μg; autopsy brains, 10 μg) were subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Proteins were then transferred to a polyvinylidene difluoride membrane. The blots were blocked with 10% (wt/vol) skim milk and 0.1% Tween 20 in Tris-buffered saline (TBS) at room temperature for 1 hour and washed in 1% (wt/vol) bovine serum albumin/TBS at room temperature for 10 minutes. Then the blots were probed with ApoEC (1:4000) or ApoEAB947 (1:4000) in 1% bovine serum albumin/TBS solution at 4°C for 3 days. After 3 washes with 1% (wt/vol) skim milk and 0.1% Tween 20 in TBS at room temperature for 30 minutes, the blots were incubated with horseradish peroxidase–coupled goat anti-rabbit IgG secondary antibody (Pierce) diluted to 1:2000 with 1% skim milk/TBS at room temperature for 2 hours. Then the blots were washed 3 times with 0.1% Tween 20/TBS and visualized with the use of an enhanced chemiluminescence system (Amersham). The same blots were reprobed with anti–β-actin (1:40 000; Sigma) and visualized with horseradish peroxidase–coupled anti-mouse IgG secondary antibody (Kirkegaard and Perry Laboratory Inc). ApoE-immunoreactive bands were digitally captured, and their relative intensities were quantified with NIH Image (version 1.62). Because the number of the samples was not very large, quantified data were analyzed not only with ANOVA and the Fisher protected least significant difference test but also with the nonparametric rank method of Kruskal-Wallis.

**Results**

**Immunolocalization of ApoE in Human Brains**

Neurons exhibited an intense ApoE-like immunoreactivity at the periphery of ischemic focus (Figure 1A). This ApoE-like immunoreactivity was completely abolished throughout the adjacent section when ApoEC was coincubated with the antigen peptide (Figure 1B, the same area as Figure 1A), which confirmed the specificity of ApoE-like immunoreactivity with ApoEC. ApoEAB947 gave essentially the same...
results as ApoEC. This ApoE-like immunoreactivity in neurons was more pronounced in recent foci than in older foci. None of these neurons were atrophic or ischemic.

Double-labeled sections of ischemic foci demonstrated that ApoE-like immunoreactivity was present variably in neurons, some ballooned neurons (Figure 2A), astrocytes (Figure 2B), and microglia (Figure 2C). Double-labeling immunohistochemistry with anti-neurofilament (SMI-31; Figure 3A) and ApoEC (Figure 3B) antibodies clarified colocalization of these 2 epitopes, which confirmed that accumulation of ApoE was also extended into axons. ApoE-positive glial cells were, however, rarely observed where ApoE accumulated in neurons or axons and vice versa: neurons and axons immunopositive for ApoE were rarely in close contact with glial cells containing ApoE-like immunoreactivity (Figures 1 to 3).

Differences in immunolocalization of ApoE and in its immunoreactivity according to pathological conditions are shown in Figure 4. In control brains, ApoE-like immunoreactivity was present variably in neurons, some ballooned neurons (Figure 4A) or in subpial astroglia. In AD brains, ApoE-like immunoreactivity was accumulated in senile plaques and neurofibrillary tangles (Figure 4B), while other neuronal labeling was not comparable to that seen at the periphery of an ischemic focus (Figure 4C). Comparison of the ischemic area (Figure 4D, left) and its adjacent less affected area (Figure 4D, right) demonstrated that ApoE-like immunoreactivity in neurons was apparently more intense in areas involved in the ischemic process. Even in brains with ischemic focus, ApoE-like immunoreactivity in neurons was barely detectable in intact areas closely adjacent to the ischemic focus (Figure 4D).

**Western Blot Analyses**

Probing human brain extracts with ApoEC on Western blot demonstrated a major band at approximately 36 kDa (Figure 5A), as was detected similarly when probed with ApoEAB947. Quantitative analysis of the relative intensity of ApoE-immunoreactive bands (Figure 5B) demonstrated that expression of ApoE protein was significantly increased ($P = 0.0498$, nonparametric method of Kruskal-Wallis) in AD brains (214%, mean relative to mean of controls, indicated as 1; $P = 0.0320$, ANOVA) and further increased in brains with infarction (247%, mean relative to mean of controls; $P = 0.0132$, ANOVA).

Probing extracts from human neuroblastoma cell line (GOTO) with ApoEC visualized bands similar to those seen in human brains (Figure 6A). Four independent sessions were followed up to 48 hours after exposure to hydrogen peroxide, and the density of ApoE-immunoreactive bands was quantified relative to the baseline (0 hours) of each session, indicated as 1 (Figure 6B). Quantitative analysis of the relative intensity of ApoE-immunoreactive bands demonstrated that expression of ApoE protein was dependent on intervals ($P = 0.0088$, nonparametric method of Kruskal-Wallis).
Wallis) after exposure to hydrogen peroxide. It increased at 4 hours (133%, mean relative to mean before exposure; \( P=0.0213, \) ANOVA). At 24 hours, this increase was more significant (168%; \( P=0.0002, \) ANOVA), which was progressive up to 48 hours (187%; \( P=0.0002, \) ANOVA). A consistent amount of \( \beta \)-actin immunoreactivity is seen at the bottom of Figure 5A and Figure 6A, suggesting that the amount of loaded protein was consistent.

**Discussion**

The major cellular source of ApoE in the brain has been considered to be astrocytes.7,8 On the other hand, immunolocalization of ApoE to other cellular components, such as neurons in the hippocampus or frontal cortex, microglia, oligodendrocytes, and blood vessels, has also been reported.9–14 It still remains to be clarified how ApoE is produced and handled in the brain. Moreover, little is known regarding how the metabolism of ApoE is altered in diseased brains. In the present study immunohistochemical investigation on human autopsied brains with ischemic lesions demonstrated that astrocytes and microglia contained ApoE-like immunoreactivity, as reported previously.11,13 In addition to these glial components, neocortical neurons around the ischemic foci also contained abundant ApoE-like immunoreactivity. At least 2 possibilities have been proposed regarding the cellular source of ApoE in neurons. Because ApoE could be internalized through several receptors on plasma membrane,19 it is speculated that ApoE in neurons is derived from astrocytes and is internalized through these carrier molecules.1,23 Another possibility is that ApoE is produced in neurons, as demonstrated previously by in situ hybridization.24 Although these 2 pathways are not mutually exclusive, the present study demonstrated that neurons were one of the major sites of ApoE synthesis in ischemic foci, in addition to glial cells. The absence of ApoE-positive glial cells around ApoE-positive neurons in ischemic foci suggests that ApoE is synthesized in these neurons and is not derived exogenously. Moreover, accumulation of ApoE was not only restricted to neuronal soma but also extended into degenerated axons (Figure 3B), again not in contact with ApoE-positive glial cells, which suggests a neuronal origin of ApoE. Although it has been reported that some neurons without disease possibly exhibit ApoE-like immunoreactivity in human brain,12,25 it was at most faint in the present study. Because the same antibody visualized a significant amount of ApoE on Western blot (Figure 5A), it is likely that sampling, routine fixation in formalin, and processing in paraffin, as used in the present study, might have attenuated immunohistochemical labeling of ApoE in normal neurons. Fixation with another fixative (such as paraformaldehyde) and use of free-floating sections

![Figure 4. ApoE-like immunoreactivity (probed with ApoEC) in various pathological conditions (counterstained with hematoxylin).](image)

A, Control. Some neurons exhibit ApoE-like immunoreactivity, although it is at most very faint (bar=50 μm). B, AD. ApoE-like immunoreactivity is accumulated in senile plaques, while neuronal labeling is absent outside neurofibrillary tangles (bar=50 μm). C, At the periphery of an ischemic focus. ApoE-like immunoreactivity is intense in neurons (bar=50 μm). D, Low-power view of C. ApoE-immunoreactive neurons are abundant in the affected area (left), while they are absent in less affected area (right; bar=100 μm).

![Figure 5. Western blot of extracts from human brains probed with ApoEC (A) and its densitometric quantification (B).](image)

A, ApoEC-immunoreactive bands at approximately 36 kDa, derived from brains with infarction (Infarct) and from those with AD, are more intense than those from normal brains (Normal). Arrowhead: 36 kDa. \( \beta \)-Actin-immunoreactive bands are shown as an internal control (bottom). B, Relative intensity of ApoEC-immunoreactive bands (mean±SD) was quantified and expressed with mean of normal brains (\( n=4 \)) as a reference, indicated as 1. ApoE-immunoreactive bands are more intense in AD brains (\( n=2 \)) and in brains with infarction (\( n=2 \)) relative to normal brains. \( ^* P<0.05; \) broken line indicates not significant by ANOVA.
might have visualized ApoE immunoreactivity even in normal neurons, while brain tissue with ischemic focus is usually too fragile to be subjected to such a free-floating method. More importantly, ApoE-like immunoreactivity in neurons was more evident around ischemic foci. Immunoblots from brain extracts demonstrated that full-length form (34 to 37 kDa) of ApoE was present regardless of the disease conditions. Quantification of relative intensity of ApoE-immunoreactive bands demonstrated that the amount of ApoE is increased to 114% (>2-fold) in AD and more significantly increased to 146% in ischemic foci (Figure 5B). Because glial cells in ischemic foci also contain ApoE-like immunoreactivity, it is difficult to decide what kind of cell (neuronal or glial) is responsible for this increase seen on Western blot. Although synthesis of ApoE in other neuroblastoma cell lines has been reported, accumulation of ApoE protein in neuronal cells after exposure to hydrogen peroxide may have some pathological significance. While synthesis of ApoE could be regulated through interaction between glia and neurons, possibly mediated, for example, by nuclear factor-κB, the present study on a neuroblastoma cell line demonstrated that neuronal accumulation of ApoE protein could occur independently of glial cells. Therefore, the increase in ApoE, as we observed in ischemic foci of human brains, is explained, at least in part, by upregulation of ApoE synthesis in neurons. Intense ApoE-like immunoreactivity in these neurons in ischemic focus also corroborated this interpretation.

Several epidemiological studies demonstrated that APOE genotype e4 was possibly linked to some neurological disorders (eg, Lewy body disease, amyotrophic lateral sclerosis, AIDS) other than AD. Other studies demonstrated that recovery from cerebrovascular diseases was also influenced by APOE genotype. Recovery from cerebrovascular diseases was also influenced by APOE genotype.33,35 These data suggest that ApoE in the brain plays certain essential roles during destruction and recovery of the nervous system, as well as in pathological conditions. It still remains to be proven how ApoE plays certain roles in ischemic foci. The present study suggested an upregulation of ApoE in neurons after cellular stresses such as ischemia or oxidative stress. If this upregulation is linked to recovery of neurons after cellular stresses in general, regulation of ApoE expression will provide a clue to constructing a therapeutic strategy aimed at improving recovery from a wide variety of neurological diseases. It is therefore essential to know how the expression of ApoE is regulated in physiological and pathological conditions.

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References


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