Influence of Apolipoprotein E Genotype on Neuronal Damage and ApoE Immunoreactivity in Human Hippocampus Following Global Ischemia

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Abstract. Apolipoprotein E (apoE) influences the response to and outcome from brain injury possibly through alterations in neuronal repair mechanisms. This study aimed to determine alterations in neuronal and glial apoE after brain injury in patients and sought to determine whether possession of an apoE-ε4 allele influences the degree of apoE immunoreactivity or the degree of neuronal damage following brain injury. ApoE immunoreactivity and neuronal damage were semiquantitatively assessed in the temporal lobe of a group of controls (n = 44) and in a group of patients who had an episode of global ischemia and subsequently died (n = 58, survival ranged from 1 hour to 3 months). There was a significant degree of neuronal damage in all hippocampal sectors and in the neocortex of the global ischemia group compared with controls (p < 0.0001). Glial apoE immunoreactivity was significantly increased in hippocampal sectors (CA1, CA2, CA3/CA4, dentate fascia) in the global ischemia group compared with controls (p < 0.01). Neuronal apoE immunoreactivity was significantly increased in all hippocampal sectors (CA1, CA2, CA3/CA4, dentate fascia) and in the neocortex of the global ischemia group compared with controls (p < 0.0001). There was a significant association between the degree of neuronal apoE immunoreactivity and the degree of neuronal damage in the global ischemia cases (r^2 = 0.691, p < 0.001) and there was not an association in the control group. Possession of an apoE-ε4 allele did not influence the degree of neuronal or glial apoE immunoreactivity or the degree of neuronal damage in the global ischemia cases or the controls. The data indicate apoE is markedly increased in neurons and glia following brain injury. In this study, apoE genotype did not appear to influence neuronal damage, glial apoE or intraneuronal apoE following injury.

Key Words: Apolipoprotein E; Cardiorespiratory arrest; Genotype; Human; Immunoreactivity; Ischemia.

INTRODUCTION

Apolipoprotein E (apoE) exists in humans as three isoforms E2, E3 and E4 encoded by the alleles ε2, ε3, and ε4. ApoE-ε4 allele is recognized as the major genetic risk factor for late-onset familial and sporadic Alzheimer disease (1). There is now evidence that apoE also underlies a genetically determined susceptibility to the effects of different forms of brain damage such as head injury (2), intracerebral hemorrhage (3), and chronic traumatic brain injury in boxers (4). In general, possession of an apoE-ε4 allele is associated with worsened response or outcome following the initial brain injury.

The precise mechanisms by which different isoforms of apoE can influence the response to and outcome from brain injury is unknown and has been limited to some extent by the lack of information regarding the role of apoE in the CNS. In the periphery, apoE regulates lipid transport and metabolism and is dramatically upregulated following peripheral nerve injury (5). A similar role for apoE in the CNS has been described whereby apoE distributes cholesterol and phospholipids to neurons following injury (6, 7). In normal rodent brain apoE is primarily localized to astrocytes and their processes (8). We (9, 10, 11) and others (12, 13) have demonstrated that, following injury, apoE is increased initially in astrocytes and subsequently is localized predominantly to degenerating neurons. In contrast to normal rodent brain, neuronal apoE has been demonstrated in normal human brain (14, 15). Intraneuronal apoE has also been found in the brains of patients with Alzheimer disease (16) and in a preliminary study of patients who died following status epilepticus, hypoglycemia, and global ischemia/hypoxia (17).

Intraneuronal apoE may be a mechanism following injury by which apoE influences neuronal repair, regeneration, and survival. In vitro studies have indicated that apoE can potentially interact with and regulate cytoskeletal proteins such as tau and microtubule-associated proteins and this interaction is isoform dependent (18, 19). Thus, the ability of apoE to regulate neuronal function may be isoform-specific and this could have important clinical and pathological implications.

The aims of this study were firstly to determine whether apoE is increased in neurons and glia following brain injury in humans. For the purpose of this study we selected individuals who had died following a period of global ischemia. This type of brain injury results in a relatively stereotyped pathology in which there is selective neuronal damage in hippocampal regions and neocortex. Secondly we sought to determine if alterations in neuronal and glial apoE are apoE genotype dependent and, thirdly, whether apoE genotype is associated with worsened neuronal damage (as a measure of histopathological outcome) following global ischemia.
MATERIALS AND METHODS

Postmortem Human Brain Tissue

Archival paraffin embedded blocks of medial temporal lobe were selected from 58 patients who died after having survived an episode of global ischemia due to cardiorespiratory arrest (37 males, 21 females; age range 17 to 85 years; mean age 51 ± 2 years). The survival of the patients after the initial episode of global ischemia ranged from 1 hour [h] to 3 months, mean survival, 5.7 ± 1 day. Forty-four control patients (29 males, 15 females; age range from 18 to 82 years, mean age 50 ± 3 years) without clinical or pathological evidence of neurological or psychiatric impairment or significant cardiovascular disease were used in this study.

ApoE Genotype

ApoE genotyping was performed from the paraffin-embedded tissue as previously described using the polymerase chain reaction/restriction enzyme analysis technique (20).

ApoE Immunoreactivity and Ischemic Neuronal Damage

Paraffin-embedded sections (7–8 μm) were dewaxed in an oven at 60°C for 1 h and then in histoclear for 10 minutes (min) and then absolute alcohol for 10 min and 5 min. Sections were stained with hematoxylin and eosin (H&E) to allow identification of the extent of ischemic neuronal damage. Adjacent sections were processed for apoE immunohistochemistry as follows. Sections were put in citric acid buffer (pH 6) and microwaved for 10 min and left in buffer for 30 min. This pretreatment step was known from previous studies of apoE immunohistochemistry to produce optimum apoE immunostaining in formalin fixed human brain tissue. Endogenous peroxidase was eliminated by incubating with 0.5% H2O2 in methanol for 30 min followed by washes in running water (20 min) and PBS (5 min). Nonspecific sites were blocked with 10% normal horse serum and 3% bovine serum albumin in PBS for 1 h and then incubated overnight with primary antibody (apoE 1:7,500, goat polyclonal, Chemicon) in PBS containing 15% normal horse serum and 10% bovine serum albumin. After PBS washing (2 × 10 min) sections were incubated with a biotinylated anti-goat IgG and processed with a Vectastain ABC kit. Colour was developed using a DAB Kit (Vector Labs.) Sections were dehydrated and coverslipped. In the present study, controls for the specificity of the immunostaining included omission of the primary antibody.

Semiquantification of ApoE Immunoreactivity and Ischemic Damage

Semiquantitative assessment of apoE immunoreactivity and ischemic neuronal damage in specific regions within the hippocampus and cortex was performed at ×100 magnification using a graticule in the eyepiece. The cases were coded and the investigator was blind to their identity. Neuronal apoE immunoreactivity was classified as follows: 0 = no neurons stained; 1 = <35% neurons stained; 2 = 35–70% neurons stained; 3 = >70% neurons stained. Glial apoE immunoreactivity was similarly classified. Ischemic neuronal damage was defined in the H&E stained sections as neurons in which the cell bodies were shrunken and nuclei triangular and the cytoplasm intensely stained eosinophilic (21). H&E sections from all control and global ischemia cases were examined for evidence of infarction i.e. damage to glial cells or blood vessels in addition to neuronal damage. Ischemic neuronal damage was classified as follows: 0 = no ischemic neurons; 1 = <35% ischemic neurons; 2 = 35–70% ischemic neurons; 3 = >70% ischemic neurons. An average degree of apoE immunoreactivity (or neuronal damage) for each case was calculated from the sum of the degree of apoE immunoreactivity (or neuronal damage) in CA1 + CA2 + CA3/CA4 + dentate fascia + neocortex divided by 5.

Statistics

Student’s unpaired t-test was used to assess statistical significance of differences in the degree of neuronal or glial apoE immunoreactivity between control and global ischemia groups. Differences in the degree of neuronal or glial apoE immunoreactivity and differences in the degree of neuronal damage were compared in cases with and without an apoE-e4 allele using Student’s unpaired t-test. Linear regression analysis was used to determine whether there was a significant association between the degree of apoE immunoreactivity with age or survival following global ischemia or degree of neuronal damage.

RESULTS

Ischemic Neuronal Damage and ApoE Immunoreactivity

Control and global ischemia groups had similar mean ages and a similar male to female ratio. Following global ischemia, neuronal damage was observed in the hippocampal regions and neocortex and there was minimal neuronal damage in the controls. In all 58 global ischemia cases, there was widespread irreversible damage with a characteristic pattern of selective neuronal vulnerability. In the affected areas, in the H&E stained sections, the neurons were contracted with densely staining nuclei and intensely eosinophilic cytoplasm: encrustations were also seen. In short surviving cases the appearances were those of selective neuronal necrosis. With longer survival, reactive changes were seen in astrocytes and both microglial and macrophage formation became evident. In none of the global ischemia cases was there evidence of infarction (21). Neuronal damage was significantly increased (p < 0.0001) in each of the hippocampal sectors and neocortex compared with controls (Fig. 1). ApoE immunoreactivity was detected in glial cells in both the controls and global ischemia cases. However, semiquantification of the degree of glial apoE immunoreactivity indicated a significant increase in glial apoE in the global ischemia group compared with controls in hippocampal sectors CA1, CA2, CA3/CA4, and dentate fascia (Fig. 2). There was minimal neuronal apoE immunoreactivity detected in the control cases. In only 4 out of the 44 control cases was a moderate (semiquantitative score of 2) amount of neuronal apoE immunoreactivity observed, the majority of control cases had minimal or no neuronal
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Fig. 1. Semiquantitative assessment of the degree of neuronal damage in temporal lobe. Histograms are mean ± SEM values for controls (n = 44) and cases of global ischemia (n = 58). There was a significant increase in the degree of neuronal damage in the global ischemia group compared with controls in each of the regions examined; hippocampal sectors CA1, CA2, CA3/CA4, dentate fascia, and neocortex. ***p < 0.0001 using Student's unpaired t-test.

Fig. 2. Semiquantitative assessment of the degree of glial apoE immunoreactivity in temporal lobe. Histograms are mean ± SEM values for controls (n = 44) and cases of global ischemia (n = 58). There was a significant increase in the degree of apoE glial immunoreactivity in the global ischemia group compared with controls in hippocampal sectors CA1, CA2, CA3/CA4, dentate fascia. *p < 0.01, **p < 0.001 using Student's unpaired t-test.

 apoE immunoreactivity in the hippocampus or neocortex. In contrast, intense neuronal apoE immunoreactivity was observed in the majority of cases following global ischemia. Semiquantification of the degree of neuronal apoE immunoreactivity indicated that there was a statistically significant increase (p < 0.0001) in neuronal apoE immunoreactivity in each of the regions examined (hippocampal sectors CA1, CA2, CA3, CA4, dentate fascia, and neocortex) following global ischemia compared with controls (Fig. 3). It was noted in the global ischemia cases that intraneuronal apoE immunoreactivity was detected in neurons that had the characteristics of ischemic cell change (Fig. 4). Linear regression analysis indicated there was a significant and positive association between the degree of neuronal apoE immunoreactivity and the degree of neuronal damage (Fig. 5) in the global ischemia cases (r² = 0.691, p < 0.001). There was no association between the level of neuronal apoE immunoreactivity and neuronal damage in the controls.

There is some evidence from the literature that neuronal apoE may increase with age (22). There was no association found between the degree of neuronal apoE immunoreactivity and age in the controls in this study (Fig. 6). In addition, there was no association between the degree of neuronal apoE immunoreactivity and survival time of the patients following global ischemia (Fig. 7).

The amount of amyloid β immunoreactivity was semiquantified in each of the cases. In the control group, 8 of the 44 cases had amyloid deposits and in the global ischemia group, 20 of the 58 cases had amyloid deposits. There was no statistical difference between the amount of amyloid deposits between the groups (p < 0.05). In addition, there was no correlation between the amount of amyloid deposits and extent of neuronal damage in the global ischemia group (data not shown).

Influence of ApoE-ε4 allele on ApoE Immunoreactivity and Ischemic Damage

We tested the hypothesis that possession of an apoE-ε4 allele may alter the degree of glial and neuronal apoE immunoreactivity and/or the degree of neuronal damage following global ischemia. The cases were separated into those that contained at least one apoE-ε4 allele and those without an apoE-ε4 allele. In the control group 29 cases were without an apoE-ε4 allele and 13 cases had one ε4
Fig. 4. Illustrative examples of apoE immunostaining (A, C) and ischemic neuronal damage (B, D) in a 50-year-old control (apoE genotype 3/3) (A, B) and a 53-year-old patient (apoE genotype 3/3) who had died 36 h after an episode of global ischemia (C, D). Note the absence of neuronal apoE immunoreactivity and neuronal damage in the control case. In contrast there is intense apoE immunoreactivity localized to neurons in the global ischemia case which exhibit the histological characteristics of ischemic cell change.

allele and in the global ischemia group 40 cases were without an apoE ε4 allele and 18 cases possessed an apoE ε4 allele. The frequency of the apoE-ε2 allele was too low to determine whether possession of this allele had an influence on the parameters measured (2 controls and 3 global ischemia cases had one ε2 allele and one control and one global ischemia case had 2 ε2 alleles). The average degree of glial and neuronal apoE immunoreactivity and average degree of neuronal damage was calculated based on the levels assessed in hippocampal sectors CA1, CA2, CA3/CA4, dentate fascia, and neocortex. There was no significant difference in the degree of neuronal apoE immunoreactivity between global ischemia cases with and without an apoE-ε4 allele (Fig. 8). Similarly in the control group there was no difference in the degree of apoE immunoreactivity in cases with and without apoE-ε4 allele. In each individual region examined (hippocampal sectors CA1, CA2, CA3/CA4, dentate fascia, and neocortex) the degree of apoE immunoreactivity was not influenced by possession of an apoE-ε4 allele. Similarly there was no significant difference in the degree of glial apoE immunoreactivity between cases with and without an apoE-ε4 allele in the control group or following global ischemia (Fig. 9). In addition, possession of an apoE-ε4 allele did not influence the degree of neuronal damage in the global ischemia group or the control group (Fig. 10).

DISCUSSION

This study demonstrated that following global ischemia in humans, apoE is increased in glia and is markedly increased in neurons, predominantly those that show the
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Fig. 5. Correlation of the degree of neuronal apoE immunoreactivity and the degree of neuronal damage in controls and in cases of global ischemia using linear regression analysis. Each point represents one case. There was a significant and positive association between the degree of neuronal apoE immunoreactivity and degree of neuronal damage in the global ischemia group ($r^2 = 0.691, p < 0.001$). There was no association found in the control group.

Fig. 6. Correlation of the degree of neuronal apoE immunoreactivity and age (years) in the control group using linear regression analysis. Each point represents a control case. There was no correlation determined ($r^2 = 0.126, p > 0.05$).

Fig. 7. Correlation of the degree of neuronal apoE immunoreactivity and survival of the patients following an episode of global ischemia using linear regression analysis. Each point represents a case of global ischemia. There was no correlation determined ($r^2 = 0.06, p > 0.05$).

Fig. 8. The average degree of neuronal apoE immunoreactivity in temporal lobe in cases with and without an apoE-e4 allele. Histograms are mean ± SEM values for control and cases of global ischemia. There was no significant difference in the degree of neuronal apoE immunoreactivity in cases with and without an apoE-e4 allele in the global ischemia group or in the control group. $p > 0.05$ using Student's unpaired t-test.

morphological features of ischemic cell change, in agreement with previous observations made in experimental rodent models of injury. We speculated that, in humans, there may be isoform-specific differences in the degree of neuronal apoE immunoreactivity following injury but there was no evidence from this study that apoE genotype influenced either the extent of glial apoE, neuronal apoE or neuronal damage.

ApoE immunostaining was increased in glial cells in patients who had suffered an episode of global ischemia compared with controls. This is consistent with experimental data that indicate apoE is predominantly produced by astrocytes following injury. We previously reported a marked increase in apoE in astrocytes at 24 h following global ischemia in a rat model but apoE immunostaining was reduced to baseline levels at 72 h post-ischemia (9,
Fig. 9. The average degree of glial apoE immunoreactivity in temporal lobe in cases with and without an apoE-e4 allele. Histograms are mean ± SEM values for control and cases of global ischemia. There was no significant difference in the degree of neuronal apoE immunoreactivity in cases with and without an apoE-e4 allele in the global ischemia group or in the control group. p > 0.05 using Student’s unpaired t-test.

Fig. 10. The average degree of neuronal damage in temporal lobe in cases with and without an apoE-e4 allele. Histograms are mean ± SEM values for control and cases of global ischemia. There was no significant difference in the degree of neuronal damage in cases with and without an apoE-e4 allele in the global ischemia group or in the control group. p > 0.05 using Student’s unpaired t-test.

In the present study, the majority of the global ischemia cases had survived longer than a day following the initial injury thus it is possible the data is an underestimation of the degree of astrocytic apoE.

Intraneuronal apoE immunostaining was markedly elevated in all the regions examined in the temporal lobe of patients following global ischemia. Previously, we had demonstrated a dramatic increase in apoE in neurons in selectively vulnerable brain regions such as the CA1 hippocampal sector and caudate nucleus following a period of global ischemia in a rat model (9, 10). In these animal studies, apoE was found in degenerating neurons and was not localized to neurons in regions that do not undergo neuronal degeneration. Other studies have reported an increase in neuronal apoE following brain injury in a rat model of complete cerebral ischemia (13) and in a gerbil model of transient forebrain ischemia (12). Similarly, apoE was localized in human brain following ischemia predominantly to ischemic neurons. In response to injury, apoE is most likely internalized from the extracellular space. In vitro studies have shown apoE is taken up into neurons via neurites. Furthermore, in normal brain there is, to date, no evidence, that apoE is synthesized in neurons but is instead synthesized predominantly in glia and then secreted (23). However, it cannot be discounted that the methods of detection may not be sufficiently sensitive to detect low baseline levels of apoE expression in normal neurons. An additional source of apoE may be that derived from plasma due to leakage of apoE across the blood brain barrier. Experimental animal models have indicated that an increase in permeability of the blood brain barrier occurs within hours following global cerebral ischemia (24). There is minimal information regarding the integrity of the blood brain barrier in humans following global ischemia. However, in the present study we did not find any histological evidence to indicate blood brain barrier breakdown in any of the cases studied. We also failed to observe an increase in perivascular apoE suggesting apoE was not derived from plasma in the present study. The functional significance of apoE accumulation in neurons is unknown. It is speculated that after injury apoE is increased as a protective response and provides cholesterol and lipids for neuronal repair similar to the role described for apoE in the peripheral response to injury.

ApoE has also been localized immunohistochemically to neuronal populations of 'normal' individuals unlike normal rodent brain in which neuronal apoE has not been detected (14, 15, 16).

In keeping with these findings, we also observed neuronal apoE immunostaining in control brain in this study. It has been suggested that intraneuronal apoE may occur as a consequence of aging, apoE being involved in remodelling of synapses and membranes (22). However, in the 4 out of 44 control cases that exhibited moderate levels of apoE immunoreactivity, 2 of these individuals were aged 20 and 24 years. A broad range of control cases was studied (age range from 18 to 82 years) allowing a possible correlation between age and neuronal apoE to be determined but no significant association was found between the degree of neuronal apoE immunoreactivity and age. However, it is possible some individuals were subjected to some form of agonal stress prior to death for which we could not control.

In several human CNS diseases there is a clear association between apoE polymorphism and development of the disease. Inheritance of the apoE-e4 allele is a well-established risk factor for the subsequent development of late-onset Alzheimer disease (1). ApoE polymorphism has also been shown to influence the clinical neurological
outcome following acute head injury (2); in post-traumatic coma (25), spontaneous intracerebral hemorrhage (3) and influence cognition following cardiopulmonary bypass (26). The mechanisms by which apoE genotype can influence the expression of different CNS diseases are unclear. ApoE receptors, including LDLR-LRP and VLDL, have been identified on neuronal cell bodies and dendrites (27, 28) and may be a mechanism by which apoE is taken up into neurons following injury. In vitro studies have provided evidence that apoE isoforms are differentially taken up into cells such that apoE-E2 uptake by the LRP receptor is 40% as effective as apoE-E3 or apoE-E4 uptake into neurons (29). ApoE in the cytoplasm of neurons can then potentially interact with and regulate cytoskeletal proteins such as tau and microtubule-associated proteins in an isoform-dependent manner (18, 19) and may determine the ability of apoE to modulate neuronal function. In vitro at least, apoE has been shown to influence neurite outgrowth in an isoform-specific manner with apoE-E3 promoting neurite outgrowth and apoE-E4 retarding outgrowth (30). On the basis of the in vitro data, we speculated that there may be isoform-specific differences in the degree of neuronal apoE immunoreactivity following brain injury and this may determine genotype-differences in outcome. In the present study we were able only to study differences in neuronal apoE and neuronal damage in cases with and without an apoE e4 allele due to the infrequency of the apoE e2 allele. A clear effect of apoE genotype on apoE alterations or neuronal damage was not determined in the present study. There are limitations to the study which include the inability to control for the severity and duration of ischemic episode and the length of survival following ischemia; variables which may preclude detection of genotype differences. It is possible in this study that the initial ischemic insult induced maximal uptake of apoE into neurons. In addition, it is possible that the initial response to injury is nonspecific and it is the fate of apoE in neurons which is differentially determined. In the present study we did not find an association between the degree of neuronal apoE immunoreactivity and survival following global ischemia. However, the majority of cases had survival times less than 10 days and this may not be long enough to determine the fate of neuronal apoE.

In conclusion, we find that apoE is markedly increased in glia and neurons following global ischemia and is predominantly localized to ischemic neurons. Possession of an apoE e4 allele did not influence either the degree of glial or neuronal apoE or neuronal damage following global ischemia. However, the limitations of the study in terms of variation in severity of insult and survival time may have precluded detectable genotype differences. The recent development of transgenic mice which express human apoE alleles e2, e3, and e4 will allow isoform-specific differences to be determined (31, 32).

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