Pharmacogenomic Effects of Apolipoprotein E on Intracerebral Hemorrhage

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Background and Purpose—The purpose of the study was to evaluate the effect of APOE genotype and the feasibility of administering an apolipoprotein E-mimetic therapeutic to modify outcomes in a murine model of intracerebral hemorrhage.

Methods—Intracerebral hemorrhage was induced via stereotactic injection of 0.1 U Clostridial collagenase into the left basal ganglia of wild-type and apolipoprotein-E targeted-replacement mice, consisting of either homozygous 3/3 or 4/4 genotypes. Animals were randomized to receive either vehicle or apolipoprotein E-mimetic peptide. Outcomes included functional neurological tests (21-point neuroseverity score and Rotorod latency) over the initial 7 days after injury, radiographic and histological hemorrhage size at 3 and 7 days, brain water content for cerebral edema at 24 hours, and quantitative polymerase chain reaction for inflammatory markers at 6, 24, and 48 hours.

Results—Apolipoprotein-E targeted-replacement mice consisting of homozygous 3/3 demonstrated superior neuroseverity scores and Rotorod latencies over the first 3 days after intracerebral hemorrhage, decreased cerebral edema at 24 hours, and reduced upregulation of IL-6 and endothelial nitric oxide synthase when compared to their apolipoprotein-E targeted-replacement mice consisting of homozygous 4/4 counterparts. After intravenous administration of 1 mg/kg apolipoprotein E-mimetic peptide, both wild-type and apolipoprotein-E targeted-replacement mice consisting of homozygous 4/4 exhibited improved functional outcomes over 7 days after intracerebral hemorrhage, less edema at 24 hours, and reduced upregulation of IL-6 and endothelial nitric oxide synthase when compared to mice that did not receive the peptide.

Conclusions—Our data indicate that APOE genotype influences neurological outcome after intracerebral hemorrhage in a murine model. In particular APOE4 is associated with poor functional outcome and increased cerebral edema. Additionally, this outcome can be modified by the addition of an apolipoprotein E mimetic-peptide, COG1410. 

Key Words: apolipoprotein E □ gene therapy □ inflammation □ intracerebral hemorrhage □ mouse

A polipoprotein E (apoE) is a 34-kDa protein known for its role in cholesterol metabolism; however, there is emerging evidence suggesting a singularly important role for apoE in the injured central nervous system (CNS). There are 3 common human isoforms of apoE, designated apoE2, apoE3, and apoE4 (APOE=gene; apoE=protein) that differ by single amino acid substitutions at residues 112 and 158. Presence of the APOE4 allele is associated with poor prognosis in a variety of acute and chronic neurological diseases, including intracerebral hemorrhage (ICH). Although a full understanding of the mechanism by which apoE affects the CNS response to injury remains elusive, it is clear that presence of the APOE4 allele is an independent risk factor for ICH, and there is increasing evidence supporting its role in downregulating endogenous inflammatory responses in an isoform-specific fashion, which is consistent with its known immunomodulatory properties. Recent observations demonstrating the effects of apoE in modifying secretion of inflammatory mediators and generation of cerebral edema have been shown after closed head injury and systemic inflammation responses. Furthermore, ICH is associated with glial activation and release of inflammatory mediators, which contribute to breakdown of the blood–brain barrier, enhancement of secondary neuronal injury, and development of cerebral edema. Therefore, the isoform-specific effect of apoE on neuroinflammation and acute injury responses may be particularly relevant in modifying outcomes after ICH, and clinical studies have implicated the presence of APOE4 with poor outcome in this setting.

Given the importance of apoE in modifying acute brain injury responses after ICH, one possible therapeutic strategy

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632
The targeted embryonic stem cells were injected into C57BL6/J mice for 8 generations. This resulted in targeted replacement mice created by replacing only the coding regions of mouse APOE with human APOE allele-specific genomic construct into or APOE3TR, and APOE4TR mice were used in these experiments to avoid potential interaction of vascular deterioration because of advanced age. Before injury, mice were prerandomized to treatment or vehicle groups. The trachea was intubated after anesthesia induction with 4.6% isoflurane and the lungs were mechanically ventilated with 1.6% isoflurane in 30% O2/70% N2. Rectal temperature was maintained at 37±0.5°C by underbody warming system. The animal’s head was secured in a stereotactic frame, local anesthetic was injected, and the scalp was incised. After exposure of the skull, a burr hole was created 2 mm left lateral to bregma, and a 0.5-µL syringe needle (Hamilton) was advanced to a depth of 3 mm from cortex. Type IV-S Clostridial collagenase (Sigma) was injected over 5 minutes (0.1 U in 0.4 µL normal saline [NS]). The incision was then closed, and animals were allowed to recover spontaneous ventilation with subsequent extubation.

**Synthesis and Administration of Peptide**

Peptides were synthesized by NeoMPS to a purity of 95%. COG1410 is acetyl-AS-Aib-LRKLAib-KRLL-amide, which is derived from apoE residues 138 to 149 with Aib (amino isobutyric acid) substitutions at positions 140 and 145. For all experiments, peptides were dissolved in sterile saline immediately before use and injected via the tail vein with 100 µL of 250 µg/mL COG1410 peptides or 100 µL of sterile saline vehicle as control.

**Testing of Neurological Deficits**

An automated Rotorod (Ugo Basile) was used to assess vestibulomotor function. On the day before hemorrhage induction, mice underwent 2 consecutive conditioning trials at a set rotational speed (16 revolutions/min) for 60 sec followed by 3 additional trials with an accelerating rotational speed. The average time to fall from the rotating cylinder in the latter 3 trials was recorded as baseline latency. After injury, mice underwent consecutive daily testing with 3 trials of accelerating rotational speed (interrital interval of 15 min). Average latency to fall from the rod was recorded. Mice unable to grasp the rotating rod were given a latency of 0 sec.

Neuroseverity scoring was assessed after injury by using a neurobehavioral examination (scoring scale, 7–21) as previously described (Table).

Motor score (4–12) was derived from spontaneous activity, symmetry of limb movements, climbing, balance, and coordination. Sensory score (3–9) was derived from body proprioception, vibrissae, visual, and tactile responses. Sensory tests examined function from both cerebral hemispheres.

**Histology**

Mice were anesthetized and euthanized on day 3 or 7 after injury. Brains were removed and frozen at −20°C. Coronal sections of 20-µm thickness were taken at 320-µm intervals over the rostral-caudal extent of the lesion. Sections were stained with hematoxylin and eosin, and lesion volume was measured by digitally sampling stained sections with an image analyzer. Lesion volumes (mm³) were computed as running sums of lesion area multiplied by the known interval (eg, 320 µm) between sections over the extent of the lesion expressed as an orthogonal projection.

**MRI**

MRI was performed using a 7-T Bruker MRI (Bruker Biospin). Animals were anesthetized via inhalation of 1.5% isoflurane in room air and cardiopulmonary parameters were monitored continuously. Core temperature was maintained at 37±0.5°C via a circulating water bath. Each MRI session lasted 20 minutes, which was equivalent across animal groups. Images were acquired at a 256×256 matrix and a 4-cm² field of view. A relaxation enhancement (RARE) sequence was performed for both T1-weighted (echo time/relaxation time [TE/TR]=7.5/5300) and T2-weighted (TE/TR 12/4200) imaging. Eighteen slices at 1-mm thickness were performed for entire brain coverage.
Assessment of Cerebral Edema

To coincide with the beginning of the period of maximal inflammatory effect,21 mice were anesthetized, euthanized at 24 hours after injury, and perfused with 30 mL of phosphate-buffered saline via transcardiac puncture. Brains were sectioned midsagittally with removal of cerebellum and brain stem, and each hemisphere was weighed immediately (“wet” weight). Hemispheres were allowed to dehydrate over 24 hours at 100°C and then reweighed (“dry” weight). To compare across genotypes, cerebral edema was expressed as water content calculated as a percentage of wet weight (wet weight/dry weight)/(wet weight) × 100.22

Quantification of mRNA for Tumor Necrosis Factor-α, IL-6, and Endothelial Nitric Oxide Synthase

Mice were anesthetized, euthanized, and perfused with 30 mL of phosphate-buffered saline via transcardiac puncture at baseline, 6, 24, or 48 hours after injury. Brains were sectioned midsagittally, flash frozen at −20°C, and stored at −80°C. RNA was extracted from pulverized frozen hemispheres using the PerfectPure RNA Tissue Kit (5 PRIME) according to manufacturer’s instructions. RNA was quantified using a Bio-Rad SmartSpec 3000 Spectrophotometer (Bio-Rad) and was reverse-transcribed to cDNA using the High-Capacity cDNA Archive Kit (Applied Biosystems) with MultiScribe reverse-transcriptase and random primers. The levels of mRNA expression were determined by quantitative real-time polymerase chain reaction performed on a ABI 7300 Sequence Detection Software system (Applied Biosystems), using 100 ng cDNA per reaction, adding Taqman Universal PCR master mix and TaqMan Assays-on-Demand Gene Expression primer/probe sets (all from Applied Biosystems) for the murine cytokines. Relative mRNA quantification was calculated using the 2−∆∆CT method.23 The threshold cycle number of the target gene for each sample is normalized using a housekeeping gene (endogenous 18S) and calibrated to the control samples (uninjured animals). Final results were expressed as fold changes over uninjured animals.

Statistical Analysis

Rotorod performance and neuroseverity scoring were compared with repeated measures analysis of variance with time as the repeated variable. Hemorrhage size, interhemispheric water weight difference, and quantitative real-time polymerase chain reaction fold changes were compared using Student t test. Statistical significance was assumed with P<0.05, and values expressed as mean±SEM.

Results

Effects of Endogenous APOE Polymorphism

To evaluate the endogenous effects of APOE polymorphism after ICH, collagenase-induced hemorrhage was performed in the left basal ganglia of APOETR mice. Hematoxylin and eosin staining revealed the characteristic development of a well-circumscribed hematoma and the development of cerebral edema (Figure 1). Seventy-two hours after injury, hematoma volumes were comparable when measured in APOE4TR and APOE3TR animals (n=8/group, 23.6±2.03 mm³ in APOE4TR vs 24.4±2.03 mm³ in APOE3TR). Additional information about the endogenous APOE polymorphism is provided in the Table. Neurosensitivity Scoring.
ally, MRI was performed on a separate cohort of animals (n=5/group) given 0.075 U of collagenase to assess correlation with volumetric measurement by hematoxylin and eosin staining. Hemorrhage volume was assessed by MRI at 2, 24, and 72 hours and by hematoxylin and eosin histology at 72 hours in the same set of animals. MRI revealed a stable hematoma volume in all animals (10.2±2.65 at 2 hours vs 12.8±4.01 at 24 hours vs 11.3±3.16 mm³ at 72 hours) without evidence of expansion but with transition from homogenous signal present with blood to a more heterogeneous lesion with the formation of iron breakdown products (Figure 2). Furthermore, assessment of volume by MRI at each of the time points did not demonstrate any differences between the APOE4TR groups (2 hours: 10.8±5.24 in APOE3TR vs 9.4±2.68 in APOE4TR; 24 hours: 12.7±7.11 in APOE3TR vs 12.9±5.46 in APOE4TR; 72 hours: 11.0±5.39 in APOE3TR vs 11.5±4.55 mm³ in APOE4TR).

Finally, hemorrhage volumes measured by MRI at 72 hours correlated well with histology at 72 hours after injury in the same set of animals (72 hours: 11.3±3.16 in MRI vs 13.4±3.19 mm³ in hematoxylin and eosin; r²=0.98). Although there was no difference in hemorrhage volume as a function of apoE isoform, APOE4TR animals did have a significant increase in brain water content in the injured hemisphere at 24 hours as compared to their APOE3TR counterparts (80.0±4.0 in APOE4TR vs 75.1±1.0% in APOE3TR; P<0.01).

To assess whether apoE might directly influence early inflammatory events in the CNS in an isoform-specific fashion, quantitative real-time polymerase chain reaction was performed for the inflammatory cytokines IL-6, endothelial nitric oxide synthase (eNOS), and tumor necrosis factor (TNF)-α at 6, 24, and 48 hours (n=5/group/time point) after ICH induction. RNA levels for all proteins were increased at 6 hours in the injured hemisphere compared to the uninjured hemisphere (TNF-α: 15.05±2.64 in injured, 0.86±0.26 in uninjured; Figure 3A; eNOS: 1.11±0.2 in injured, 0.64±0.22 in uninjured; IL-6: 22.88±0.87 in injured, 1.22±0.45 in uninjured; P<0.01) with return to baseline within 48 hours after injury. Although IL-6 and eNOS were upregulated in all animals, these cytokines were significantly elevated in APOE4TR animals as compared to APOE3TR at 6 hours (IL-6: 22.81±0.87 in APOE4TR vs 13.93±1.13 in APOE3TR; eNOS: 1.11±0.19 in APOE4TR vs 0.57±0.06 in APOE3TR; P<0.01; Figure 3B,3D); additionally, TNF-α RNA upregulation was not significant (P<0.09; Figure 3C). Collectively, these results suggest that although APOE genotype does not appear to have a primary effect on hematoma formation, presence of the APOE4 allele is associated with a greater degree of inflammation, leading to increased cerebral edema.

Finally, to determine whether increases in inflammation and cerebral edema in the APOE4TR animals were associated with worsened functional deficit, behavioral testing with serial Rotorod and neuroseverity assessment were performed daily for 3 days after injury on a separate cohort of APOE3TR (n=9) and APOE4TR (n=11) animals. APOE4TR animals had a greater degree of functional deficit as compared to the APOE3TR mice, as quantified by shorter Rotorod latencies (P<0.01; Figure 4A) and poorer neuroseverity scores (P<0.01; Figure 4B) over the course of the 3 days tested; furthermore, these functional outcome measures appeared to mirror each other in terms of recovery. It should be noted that sham-operated animals of both genotypes (APOE3TR and APOE4TR) did not exhibit significant functional neurological deficit by either Rotorod latency or neuroseverity scoring over the course of any of the days tested. Together these data suggest the clinical relevance of this
model of ICH given that endogenous APOE genotype influences functional neurological outcomes and that the presence of an APOE4 allele results in increased cerebral edema with poorer recovery.

Effects of Exogenous ApoE-Mimetic Peptide
To demonstrate whether the beneficial effects of endogenous apoE3 could be harnessed as a therapeutic strategy, vehicle or COG1410 (1 mg/kg) was injected intravenously in C57-BL/6 mice at 30 minutes and 4 hours after ICH induction (n=8/group). Although there was no difference in hemorrhage volume assessed at 7 days (24.3±1.17 in vehicle-treated vs 23.5±3.02 mm³ in COG1410-treated), animals treated with doses of apoE-mimetic peptide demonstrated a durable improvement in functional outcomes, extended to 7 days to assess the possibility of a “ceiling effect” for COG1410 by Rotorod latencies (Figure 5A; P<0.01) and neuroseverity scores (P<0.01) that persisted over the course of testing. Additionally, sham-operated animals did not demonstrate any functional deficits after injury. This was associated with a reduction in the brain water content in the injured hemisphere attributable to cerebral edema at 24 hours after injury (n=5/group; 78.7±0.9 in vehicle-treated vs 75.5±0.5% in COG1410-treated; P<0.05). Finally, to evaluate the role of inflammation in the development of cerebral edema and its influence on functional outcomes, we performed quantitative real-time polymerase chain reaction in a separate cohort of animals (n=5/group) at 6 hours after ICH induction for the inflammatory cytokines eNOS, IL-6, and TNF-α. Hemorrhage was again associated with an upregulation of all cytokines at 6 hours in the injured hemisphere of WT mice (TNF-α: 13.86±1.86 injured vs 4.03±0.81 uninjured; IL-6: 22.77±3.24 injured vs 3.87±1.02 uninjured; eNOS: 1.21±0.08 injured vs 0.99±0.07 uninjured; P<0.01). How-
ever, when given the apoE-mimetic peptide, both IL-6 and eNOS were significantly reduced (IL-6: 22.77±3.24 in vehicle-treated vs 9.52±2.95 in COG1410-treated; eNOS: 1.52±0.05 in vehicle-treated vs 1.21±0.08 in COG1410-treated; P<0.01). These data suggest that the administration of an apoE-mimetic peptide represents a potentially viable therapeutic strategy that may improve outcome independent of the endogenous apoE isoform.

Discussion

In the current study, we extend clinical observations implicating a role for APOE polymorphism in modifying functional outcomes after supratentorial ICH using a transgenic murine model of collagenase-induced basal ganglia hemorrhage. Consistent with the clinical literature, we found that presence of the human APOE4 gene and its apoE4 products were associated with poor functional outcome, independent of any direct effect on hematoma volume. Moreover, we demonstrate that treatment with an apoE-mimetic therapeutic peptide, whose sequence does not encompass any of holo protein’s polymorphic residues, improved durable functional outcome in WT, APOE3TR, and APOE4TR animals.

Although originally described in the context of cholesterol metabolism, clinical observations suggest that APOE polymorphism plays a uniquely important role in modifying the CNS response to acute and chronic injury.1,24 In particular, presence of the APOE4 allele has been associated with poor neurological outcome after ICH2 and traumatic brain injury.25 Although the mechanism by which apoE affects the CNS response to injury remains poorly defined, one unifying hypothesis is that apoE modifies neuroinflammatory responses in an isoform-specific fashion by modulating microglial activation. This function of apoE in the generation of inflammation in the brain has been well-described in a number of in vitro and preclinical models26 and has recently been translated into the clinical setting, in which APOE4 is associated with more robust inflammatory responses after ex vivo stimulation of human monocyte-derived macrophages,27 cardiopulmonary bypass,28 and in the critically ill population.29

In the current study, we demonstrate that the expression of APOE4 is associated with enhanced neuroinflammatory responses and impaired functional outcomes independent of hematoma volume after ICH. CNS inflammation, characterized by glial activation, release of inflammatory mediators, and breakdown of the blood–brain barrier may be particularly relevant in the development of cerebral edema and secondary neuronal injury after ICH. One possible mechanism by which apoE may confer a neuroprotective effect is via downregulation of microglial activation, an effect that has been demonstrated in vitro30 and in vivo.5 This effect is evidenced by a decrease in inflammatory mediators such as IL-6 and eNOS after ICH in our model. Therefore, it appears that our murine model of ICH is clinically relevant as the isoform-specific
effects of apoE that we observed in the model are mirrored in the human condition. Our results are consistent with the hypothesis that apoE exerts an isoform-specific effect on neuroinflammatory responses independent of any direct effect on hematoma volume, because presence of an APOE4 allele was associated with increased cerebral edema in the company of upregulated markers of inflammation, with similar findings being published after ICH in humans. It is, therefore, logical to assume that our clinically relevant murine model of ICH should be used to evaluate potential targeted pharmacogenomic interactions.

In addition to providing mechanistic insight into the role of APOE polymorphism in acute brain injury, a more complete understanding of the function of apoE in the injured CNS may also raise the possibility of developing novel therapeutic strategies. Although apoE does not cross the blood–brain barrier, many of its adaptive properties appear to be mediated by signaling cascades initiated by specific interaction with cell surface receptors.31 Based on these receptor interactions, a series of peptides derived from the receptor-binding domain of apoE were created and demonstrated to exert many of the same adaptive functional effects as the intact holoprotein. These studies have been extended to preclinical models of traumatic brain injury, where a peptide derived from the receptor-binding region, apoE(133–149), was well-tolerated, crossed the blood–brain barrier, and was associated with improved functional and histological outcomes.6 The peptide used in the current study, COG1410, represents a second-generation apoE-based therapeutic in which the helicity and antiinflammatory potency of the peptide was enhanced by the introduction of 2 non-naturally occurring Aib residues.1 In the current study, systemic administration of COG1410 reduced functional disability and histological injury, a finding that is consistent with its palliative effects in other models of acute brain injury.32

Thus, the current experimental paradigm offers the opportunity to study pharmacogenomic interactions between an apoE-based therapeutic and background humanized APOE genotype. In fact, we provide evidence that intravenous administration of apoE-mimetic peptide (COG1410) reduces inflammation and improves functional outcome regardless of the humanized APOE genetic background. This effect appears most robust in WT and APOE4TR mice, and when examined closely, administration of apoE-mimetic peptide results in neurological outcomes that are similar to their APOE3TR counterparts. These functional improvements are coupled with decreases in markers of microglial activation, inflammation, and cerebral edema. Together these data provide evidence that small mimetic peptides may represent a rational targeted therapeutic strategy.

Several limitations to this study should be addressed. Although collagenase-induced ICH is a commonly used model, there are theoretical concerns that bacterial collagenase might induce an inflammatory response independent of that elicited by parenchymal blood. Although in this model it is impossible to tease out exact contributions to inflammation from exogenous (collagenase) vs endogenous (blood) substances, the antiinflammatory properties of endogenous apoE and exogenous apoE-mimetic peptide are consistent throughout, and demonstration of the isoform-specificity of apoE is consistent with published data in the human condition, decreasing the likelihood that outcomes present in this preclinical model are related to inflammation secondary to bacterial collagenase alone. Second, although presence of the APOE4 allele was associated with significantly increased levels of IL-6 and eNOS RNA at 6 hours after hemorrhage, an increase TNF-α was not wholly demonstrated. However, TNF-α elevation is an ultra-early event after ICH, and levels may have peaked before 6 hours.7 Finally, the reason for the divergence in treatment effect of the mimetic peptide between the different genotypes is unclear. Although the apoE-mimetic peptide reduced inflammation and outcome in both APOE3TR and APOE4TR animals, it had a greater effect in the presence of APOE4. This might suggest that apoE4 is associated with a loss of adaptive antiinflammatory function of endogenous apoE, and that this may be overcome with exogenous administration of an apoE-mimetic peptide. This pharmacogenomic interaction suggests that this therapeutic strategy might be of particular benefit to patients with an APOE4 allele who are at highest risk for poorer functional outcomes.

**Summary**
In summary, we provide data demonstrating that the presence of APOE4 is associated with enhanced neuroinflammatory responses and cerebral edema after ICH in our murine model. This is consistent with isoform-specific differences that have been demonstrated in a variety of both preclinical and clinical acute brain injury paradigms. The protective effect of the apoE3 isoform can be simulated by the administration of COG1410, an apoE-mimetic peptide, in our model of ICH. This therapeutic approach improves outcomes in both APOE3TR and APOE4TR animals, suggesting that it may be a viable, rational strategy for targeted pharmacogenomic therapy humans after ICH.

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