Role of COL4A1 in Small-Vessel Disease and Hemorrhagic Stroke

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SUMMARY

Small-vessel diseases of the brain underlie 20 to 30 percent of ischemic strokes and a larger proportion of intracerebral hemorrhages. In this report, we show that a mutation in the mouse Col4a1 gene, encoding procollagen type IV α1, predisposes both newborn and adult mice to intracerebral hemorrhage. Surgical delivery of mutant mice alleviated birth-associated trauma and hemorrhage. We identified a COL4A1 mutation in a human family with small-vessel disease. We concluded that mutation of COL4A1 may cause a spectrum of cerebrovascular phenotypes and that persons with COL4A1 mutations may be predisposed to hemorrhage, especially after environmental stress.

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STROKE IS A LEADING CAUSE OF DEATH AND SERIOUS LONG-TERM DISABILITY in developed nations. Survivors often have a severely diminished quality of life, require long-term care, and are at high risk for recurrence. Intracerebral hemorrhage accounts for 10 to 15 percent of strokes and is a particularly severe form of stroke, with disproportionately high rates of death and long-term disability. Hypertension and amyloid angiopathy are important risk factors associated with intracerebral hemorrhage; other risk factors include vascular malformations, coagulation abnormalities, and the use of sympathomimetic drugs. However, intracerebral hemorrhage often occurs in the absence of a history of known risk factors, indicating that other, unidentified, predisposing factors are present in many patients. Small-penetrating-vessel diseases underlie up to 30 percent of ischemic strokes and a larger proportion of intracerebral hemorrhages. Despite extensive investigation, the causes of small-vessel disease or intracerebral hemorrhage are usually unknown, particularly in younger patients.

We recently identified mutations in a gene encoding type IV collagen α1 (COL4A1), a basement-membrane protein, in mice and in human families with porencephaly. Because all the mutant mice were genetically identical, porencephaly in this model may be caused by an interaction between the mutation and the environment. We proposed that pressure on the head during birth induces hemorrhage and that the degree of pressure insult could determine whether porencephaly ensues. We further hypothesized that mutated Col4a1 or COL4A1 could predispose adult mice and people who do not have porencephaly to vascular disease.

In the current investigation, we tested our hypothesis that environmental stress and Col4a1 mutation conspire to induce hemorrhage. We also investigated the broad-
er medical importance of COL4A1 in the pathogenesis of hemorrhagic stroke in adults.

METHODS

ANIMALS
Experiments were conducted in compliance with the guidelines of the institutional animal care and use committee guidelines. Unless otherwise stated, all mice used in experiments were backcrossed a minimum of eight generations to C57BL/6J mice and were matched according to age and strain. To produce CASTB6F1 mice, mutant mice were backcrossed six generations to inbred C57BL/6J mice and the offspring were then crossed to inbred CAST/Eij mice.

SURGICAL DELIVERY
Sperm from four mutant mice was used for in vitro fertilization of oocytes from C57BL/6J female mice, and embryos were then transferred into FVB/NJ female mice. Thirteen female mice gave birth normally, yielding a cohort of control mice. Pups were delivered by hysterectomy in the case of 16 female mice. Each litter was housed with a single foster mother.

ADULT SURVIVAL
The age at death was defined in two-month intervals, with the survival percentage calculated by dividing the number of mice that died at a particular age by the total number of mice in the aging experiment. Because mice were removed for various experiments, the total numbers of mice in successively older age groups decreased. The respective total numbers of control and mutant mice retained in each age group were as follows: 2 to 3.9 months, 1026 and 320; 4 to 5.9 months, 640 and 280; 6 to 7.9 months, 131 and 162; 8 to 9.9 months, 105 and 110; 12 to 13.9 months, 92 and 90; 14 to 15.9 months, 65 and 57; 16 to 17.9 months, 47 and 41; 18 to 19.9 months, 35 and 34; and 20 to 27.9 months, 19 and 18.

HISTOLOGY
For histologic examination of the lungs, the intact trunk was placed in Bouin’s fixative at 4°C overnight. Five-micron sections of paraffin-embedded tissues were stained with hematoxylin and eosin. For histologic examination of brain tissue, deeply anesthetized mice (four to five months old) were transcardially perfused with phosphate-buffered saline, followed by either 4 percent paraformaldehyde or a combination of 0.8 percent paraformaldehyde and 1.2 percent glutaraldehyde in 0.1 M phosphate buffer. Two-millimeter coronal brain slices were examined for gross evidence of intracerebral hemorrhage before being embedded in paraffin and sectioned. Electron microscopy was performed as described previously.

CLINICAL MEASUREMENTS
Fluorescein angiography and blood-pressure measurement were performed as described previously. Urine was analyzed with use of a Synchron CX
clinical analyzer (Beckman Coulter), according to the manufacturer’s protocol.

ANALYSIS OF MUTATIONS IN PATIENTS
A detailed clinical report describing a French family has been published previously. Permission for the study was granted by the institutional review board after written informed consent had been obtained from the patients. Sequence analysis was performed as described previously. Ninety-eight ethnically and geographically matched persons served as controls and were also evaluated for the presence of mutations by direct sequence analysis.
RESULTS

INTERACTION OF TRAUMA AND MUTATION AS A CAUSE OF CEREBRAL HEMORRHAGE

All heterozygous (Col4a1+/Δex40) mutant pups have cerebral hemorrhage, and approximately 50 percent die on the day of birth.3 Thus, the Col4a1 mutation may predispose mice to vascular fragility and act in concert with birth trauma to cause cerebral hemorrhage. To test this possibility, we compared the presence or absence and degree of hemorrhage in pups born naturally with those observed in pups delivered surgically. All naturally born mutant pups had obvious hemorrhages, which were considered severe in most cases (14 of 20) (Fig. 1A). In contrast, surgical delivery of mutant pups had a profound effect (Fig. 1B): none of 26 surgically delivered mutant pups had severe hemorrhage (P<0.001 by the chi-square test). This finding indicates that Col4a1 mutations can weaken vessels and confer substantially increased susceptibility to stress-induced hemorrhage.

RESPIRATORY DEFFECTS IN MUTANT MICE

Although surgical delivery prevented cerebral hemorrhage, it did not prevent perinatal death. Unexpectedly, the viability of surgically delivered mutant pups was less than that of mutant pups born naturally (see the Supplementary Appendix, available with the full text of this article at www.nejm.org). Newborn mutant pups were often cyanotic and in respiratory distress. As compared with wild-type pups, which had expanded lungs and open alveoli, the mutant pups had compact lungs, with few or no terminal air spaces visible (see Fig. S1 of the Supplementary Appendix). This finding suggests that respiratory defects contribute to perinatal mortality.

CEREBRAL HEMORRHAGE DUE TO COL4A1 MUTATION IN ADULT MICE

In addition to perinatal effects, adult mutant mice may have weakened vasculature that predisposes them to later-onset cerebral hemorrhage. To evaluate this possibility, we allowed the mice to age and found that the rate of death was higher among the mutant mice than among their control littermates (Fig. 1C). Some adult mutant mice had sudden overt neurologic deficits, including seizures and hemiparesis. On examination, these mice, but not control mice, often had subarachnoid hemorrhage (Fig. 1E). We therefore concluded that Col4a1 mutation predisposes adult mice to hemorrhagic stroke.

The frequency of hemorrhagic episodes in these mice may be greater than the frequency of clinical manifestations of stroke. To identify clinically silent intracerebral hemorrhage, we examined the brains of mice with no obvious neurologic abnormalities. All the mutant mice, but none of the wild-type mice, had intracerebral hemorrhage (Fig. 1F through 1K). At three months of age (at which time intracerebral hemorrhage was observed in the mutant mice), mutant and wild-type mice had virtually identical blood pressures and heart rates (Fig. S2 of the Supplementary Appendix). Our data indicate that mutant vessels are weak enough to rupture, even in the absence of hypertension.

PREDOMINANCE OF RECURRENT ADULT HEMORRHAGE IN THE BASAL GANGLIA

The presence of hemorrhages at different stages of repair clearly indicates that mutant mice are predisposed to spontaneous, multifocal cerebral hemorrhage as adults. In some cases, mice had multiple fresh hemorrhages (Fig. 1H), some of which were probably induced by artificially elevated intravascular pressure during perfusion before the tissue was harvested. The spontaneous cerebral hemorrhages tended to occur in specific locations. Hemosiderin was almost always observed in the basal ganglia in adult mice (Fig. 1G, 1H, 1J, and 1K). Of the 23 lesions with hemosiderin or fibrosis that were identified, 22 were in the basal ganglia and only 1 was in the cortex.

VASCULAR DEFECTS AT SITES OTHER THAN THE BRAIN

In addition to having multifocal recurrent hemorrhage, mutant mice had other phenotypes that occur in some families with small-vessel disease.7-9 These phenotypes include retinal vascular tortuosity, defects in the glomerular basement membrane, and microalbuminuria (Fig. 2, and the Supplementary Appendix). Vascular tortuosity was evident solely in mice with the C57BL/6J genetic background (Fig. 2, and the Supplementary Appendix).

SMALL-VESSEL DISEASE DUE TO COL4A1 MUTATIONS IN MICE AND HUMANS

The phenotypic similarities between Col4a1-mutant mice and a French family with small-vessel dis-
ease (Fig. 3A) prompted us to assess the family for COL4A1 mutations. All six affected members of this family had retinal arteriolar tortuosity (Fig. 3B), including one member with retinal hemorrhage. In addition, two of the genotypically affected persons had infantile hemiparesis, and three had migraine with aura. Neuroimaging showed diffuse leukoencephalopathy associated with dilated perivascular spaces in all the affected family members (Fig. 3C). Microbleeds in several ar-

Figure 2. Abnormalities of the Retinal and Renal Vasculature.
Fluorescein angiography in control mice (Panel A) and Col4a1-mutant mice (Panel B) with the C57BL/6J genetic background revealed abnormal patterning of the retinal vasculature in mutant mice alone. Mutant retinal vessels were highly tortuous, with more frequent branching than control vessels. As shown in Panel C, retinal vascular tortuosity was affected by the genetic background; the retinal vasculature of mutant CASTB6F1 mice had an essentially normal appearance. Transmission electron microscopy of the glomerular basement membrane (between arrows) from control mice (Panel D) and mutant mice (Panel E) at three months of age revealed focal disruptions (arrowhead) of the glomerular basement membrane in mutant mice alone (images are representative of three experiments). The scale bar in Panel E represents 0.25 μm. Renal function was analyzed by measurement of urinary albumin levels (Panel F). Although none of 12 mutant mice up to 1.2 months of age had any detectable urinary albumin, 36 of 37 mutant mice between 1.3 and 2.4 months of age and 8 of 8 mutant mice that were 24 months of age had detectable urinary albumin. Albumin was detected in 1 of 23 control mice, 2 of 28 control mice, and 3 of 7 control mice, respectively, at those ages. To determine the severity of the albuminuria and whether the severity increased over time, the urinary albumin was quantitated and standardized to the level of urinary creatinine in young mice (nine control and six mutant) and old mice (seven control and eight mutant) (Panel G). The level of albuminuria was marginally elevated in mutant mice at two years of age, but this difference was not significant (P = 0.07 by the t-test). The T bars represent standard errors.
Eas were observed on magnetic resonance imaging (MRI) of the brain (Fig. 3D), suggesting involvement of the cerebral vasculature in this family. Two members of the family had a fatal intracerebral hemorrhage. One died after cerebral trauma, and a second had a fatal intracerebral hemorrhage while receiving anticoagulant therapy.

Sequence analysis revealed a G1769A transition in exon 25, leading to a change from glycine to glutamic acid at position 562 (G562E) in affected family members; this change was not observed in the chromosomes of unaffected family members or in 196 population-matched control chromosomes. The asterisk indicates heterozygosity for the mutant nucleotide.

Figure 3. COL4A1 Mutation in a Family with Retinal Arteriolar Tortuosity, White-Matter Abnormalities, and Cerebral Hemorrhage.

Panel A shows the pedigree of a French family with small-vessel disease. All affected members of the family (solid symbols) had retinal vascular tortuosity and leukoencephalopathy. The genotype (normal [G/G] or mutant [G/A]) is indicated for each family member. Patients II-2 and II-3 died of cerebral hemorrhage after anticoagulant therapy at 40 years of age and head trauma at 33 years of age, respectively. The square symbols represent males, the circles females, and the symbols with a slash a deceased person. Panel B, a red-free photograph of the right eye of Patient III-2 (at 20 years of age), shows marked tortuosity of the medium and small arterioles, particularly in the macula. No changes were apparent in the veins or capillaries, and no microaneurysms were observed. Cerebral MRI in Patient II-1 (at 40 years of age) revealed diffuse periventricular white-matter abnormalities on fluid-attenuated inversion recovery (Panel C, bright signal) and silent microbleeding in the deep cerebellum on gradient echo-weighted imaging (Panel D, arrow). No MRI or retinal abnormalities were detected in the absence of a mutant genotype. As shown in Panel E, genomic-sequence analysis of COL4A1 revealed a G1769A transition in exon 25, leading to a change from glycine to glutamic acid at position 562 (G562E) in affected family members; this change was not observed in the chromosomes of unaffected family members or in 196 population-matched control chromosomes. The asterisk indicates heterozygosity for the mutant nucleotide.
tion in exon 25 of COL4A1 that segregated with the disease and that was not observed in 196 chromosomes from unaffected French persons (Fig. 3E). The mutation changed the glycine residue at position 562 to glutamic acid (G562E) within the triple-helix domain of the protein. Glycine residues are highly conserved within the triple-helix domain of collagen type IV α1, and mutations in codons encoding glycine are pathogenic in multiple species.\textsuperscript{3,4,10}

**DISCUSSION**

We have shown that a semidominant mutation in the mouse Col4a1 gene confers a genetic predisposition to environmental-stress–induced hemorrhage and adult-onset stroke. Furthermore, this genetic predisposition extends beyond hemorrhagic stroke to include retinal and renal vascular defects and white-matter abnormalities. In both humans and mice, there was considerable phenotypic variability; intracerebral hemorrhages ranged from being clinically silent to fatal. Our data suggest that this form of hemorrhagic stroke reflects a strong gene–environment interaction.

Compromised cerebral vasculature in Col4a1 mutants leads to hemorrhagic stroke, especially in the context of trauma. Birth trauma is a major factor contributing to perinatal hemorrhage in mice with mutations in Col4a1. By alleviating this trauma, hemorrhage was dramatically reduced. The risk did not end at birth, however: adult mice also had cerebral hemorrhage. Consistent with this observation was the death from hemorrhage after accidental head trauma of a person with a COL4A1 mutation. These data suggest that in persons with COL4A1 mutations, trauma at birth and during adulthood is a stronger risk factor for intracerebral hemorrhage than it is in persons without these mutations. Persons with such mutations are probably sensitized to other risk factors for cerebral hemorrhage, such as hypertension or exercise-induced stress. A second member of the family described in this report had a fatal cerebral hemorrhage while receiving anticoagulant medication, suggesting that anticoagulant use exacerbates the risk associated with COL4A1 mutations.

The phenotype resulting from mutated Col4a1 varies among mice with different genetic backgrounds. Similarly, affected patients in the family described here had retinal vascular tortuosity, whereas previously described families with COL4A1 mutations did not.\textsuperscript{3,4} Some members of families with porencephaly have only hemorrhagic stroke,\textsuperscript{11} and some patients known to have COL4A1 mutations have only white-matter abnormalities on MRI.\textsuperscript{7,11} These data suggest that any of the phenotypes we have observed could be present as isolated features in a family or an individual patient. The specific phenotype in any single patient probably depends on a combination of factors, including the specific mutant allele, other genetic interactions, and environmental influences.

The location of intracerebral hemorrhage (namely, in the region of small penetrating vessels that supply the basal ganglia) in mutant mice is coincident with that in approximately 75 percent of patients with intracerebral hemorrhage. Thus, unlike other forms of hemorrhagic stroke that are caused by mutation of a single gene (cerebral amyloid angiopathy or cerebral cavernous malformations), intracerebral hemorrhage caused by a Col4a1 mutation is reminiscent of intracerebral hemorrhage in small-penetrating-vessel disease. The presence of a collagen type IV α1 mutation compromises the vascular basement membrane and weakens the vessel. Without neuropathological material showing that hemorrhages in our patients arise from these small vessels or that the vessels give rise to the microaneurysms that characterize typical hypertensive hemorrhages, we cannot judge the extent of similarity between the vascular changes due to COL4A1 mutations and those that arise spontaneously or with hypertension. Nonetheless, we hypothesize that the changes in the basement membrane caused by the mutations may explain the predominance of lesions in the basal ganglia. Microvasculature devoid of supporting cells and branching at large angles would be highly susceptible to hemorrhage. Lenticulostriate vessels, small penetrating vessels that supply the basal ganglia and branch off their parent vessels at right angles, creating a point of increased stress, meet the susceptibility criteria. The majority of hemorrhages in humans appear to involve the lenticulostriate vessels. Taken together, the location of hemorrhages in the basal ganglia and the identification of COL4A1 mutations in patients with intracerebral hemorrhage in the absence of other defects provide support for the need for an evaluation of COL4A1 alleles as risk factors for cerebral small-vessel disease and intracerebral hemorrhage in humans.
As multifactorial diseases, small-vessel diseases and intracerebral hemorrhage are expected to have genetic contributions from multiple genes. On the basis of our results with COL4A1, we propose that alleles of other basement membrane genes, such as those encoding the other collagens, the laminins, nidogen, and perlecans, could also genetically predispose persons to small-vessel disease and intracerebral hemorrhage. Our data support the possibility that LAMB2 may underlie hereditary endotheliopathy, retinopathy, nephropathy, and stroke with linkage to chromosome 3p21. Our findings suggest that prevention of birth trauma (cesarean delivery) and adult trauma and avoidance of risk factors for bleeding may decrease the risk of hemorrhagic stroke in patients with mutated COL4A1.

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