Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL) syndrome is the most common monogenic inherited form of small vessel disease, characterized by frequent migraine attacks with aura, recurrent strokes and progressive white matter degeneration. Early vascular cognitive impairment progresses into frank dementia of subcortical type later in life. Linked to mutations in the NOTCH3 gene, CADASIL vasculopathy is associated with accumulation of granular osmiophilic material and NOTCH3 extracellular domain around small caliber arteries and arterioles, and eventual loss of vascular smooth muscle cells. Cerebral blood flow dysregulation has been hypothesized as a major mechanism, largely based on evidence from hemodynamic studies in CADASIL patients. Although animal models expressing CADASIL mutations reproduced the pathology and cerebrovascular dysfunction, the phenotypic spectrum has been quite heterogeneous, possibly due to the choice of genetic constructs and obvious species differences between mouse and man. Nevertheless, these experimental models provide new opportunities to explore the molecular and physiological mechanisms of CADASIL, and address the fundamental question of whether CADASIL phenotype represents loss of NOTCH3 function or gain of a novel and pathological function. Here, I provide an overview of existing animal models of CADASIL and the pathophysiological insights gained from these models. (Stroke. 2010;41[suppl 1]:S129-S134.)

Key Words: CADASIL ■ leukoaraiosis ■ small vessel disease ■ vascular cognitive dysfunction

Since its genetic definition in 1990s, Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL) syndrome is a prototypical small vessel disease of the brain with Mendelian inheritance, has emerged as an important cause of stroke and pure vascular dementia in young or middle-aged adults. It is estimated that >10% of patients with stroke and white matter disease under age 50 may carry a CADASIL mutation. The clinical syndrome, albeit heterogeneous, is characterized by recurrent lacunar strokes, leukoaraiosis, migraine headaches, mood disturbances and apathy, and dementia. Despite the conspicuous early absence of vascular risk factors such as hypertension, recurrent acute ischemic events start on average within the fifth decade, almost exclusively lacunar infarcts involving subcortical white matter, deep gray matter nuclei and brain stem. Vascular risk factors, when present, can exacerbate disease progression. The onset of progressive leukoaraiosis precedes lacunar strokes. Indeed, white matter disease is present on neuroimaging in presymptomatic stages, and its penetrance is complete by the end of fourth decade. Cognitive impairment and dementia correlate with the extent of cumulative subcortical pathology, in particular the lacunar infarct burden and brain atrophy.

Pathology and Vascular Dysfunction

Besides the lacunar infarcts, diffuse white matter demyelination and axonal loss sparing the subcortical U-fibers, CADASIL brains also display microhemorrhages often in gray matter and laminar cortical neuronal apoptosis, the latter correlating with and possibly secondary to the subcortical lesion load and axonal loss. However, the characteristic histopathologic finding in CADASIL is a nonhypertensive, nonatherosclerotic, nonamyloid vasculopathy involving the small-caliber (<500 μm) pial and penetrating arteries and arterioles. The pathognomonic accumulation of granular osmiophilic material within the tunica media in proximity to the smooth muscle cell membranes is accompanied by degeneration and loss of smooth muscle cells, adventitial fibrosis and mural thickening, and markedly enlarged perivascular spaces. As a result, luminal stenosis develops in long penetrating arteries supplying subcortical white matter.

Physiological studies on CADASIL patients have shown an age-dependent reduction in resting cerebral blood flow (CBF), volume and dilatory reserve, and increased oxygen extraction fraction. In most studies, hypoperfusion was spatially limited to white matter regions that showed leukoaraiosis, and was comparable to that observed in leukoaraiosis of other etiologies. Endothelial morphological and functional changes have also been noted in systemic vessels.

Genetics

CADASIL is caused by mutations in the NOTCH3 gene, member of an evolutionarily conserved transmembrane receptor...
family (Drosophila Notch homologues 1 to 4) regulating context-dependent cell fate determination during metazoan development. In an adult brain, NOTCH3 is expressed almost exclusively by vascular smooth muscle cells (VSMC), preferentially in small caliber arteries, and by pericytes. NOTCH3 receptor is a heterodimer with a large extracellular segment consisting of 34 tandem epidermal growth factor-like repeats noncovalently attached to a transmembrane domain. Ligand binding to the extracellular domain activates proteolytic cleavages that shed the extracellular domain and allow translocation of the intracellular segment to the nucleus for transcriptional regulation. Each epidermal growth factor-like repeat contains 6 conserved cysteine residues. Pathogenic mutations in CADASIL patients identified to date are predominantly missense mutations within the NOTCH3 extracellular domain, and either add or delete cysteine residues resulting in an odd number of cysteines. This is believed to promote abnormal cysteine-cysteine interactions leading to conformational changes, pathological homo/heterodimerization or multimerization. Indeed, in CADASIL, NOTCH3 extracellular domain accumulates in the cytoplasmic membrane of VSMC.

Because of the clinical heterogeneity even among the affected members of a single CADASIL family, it has been difficult to characterize genotype-phenotype associations. It is possible that the presence of common vascular risk factors or polymorphisms in other genes that modulate NOTCH3 signaling modify the disease progression. As a potential mechanism for clinical heterogeneity, different CADASIL mutations affect NOTCH3 receptor function and processing in different ways. Some mutations have been shown to interfere with proper receptor handling, at least in vitro (ie, maturation, trafficking, and clearance). Most CADASIL mutations preserve NOTCH3 ligand binding and downstream signal transduction, except those within the ligand binding domain (epidermal growth factor-like repeats 10–11). The latter was associated with earlier onset white matter disease and strokes in some families although, paradoxically, progression of disability and dementia appeared to be slower. Earlier death or stroke and disability have been linked to other missense mutations as well, and a small in-frame deletion was associated with a phenotype dominated by migraine with severe aura and a paucity of strokes. Therefore, evidence does suggest distinct genotype-phenotype associations.

### Animal Models

Several Notch3 mutant mouse models have been developed over the past decade (Table). Some reproduced CADASIL-like vasculopathy and white matter disease, and provided important

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*Not a CADASIL model; †Heterozygous knockout was indistinguishable from wild type in all aspects; ‡Normal when expressed on wild type Notch3 background; §Normal at 6 months, but older mice were not tested. PAC, P1-derived artificial chromosome; GOM, granular osmiophilic material; VSMC, vascular smooth muscle cell; CBF, cerebral blood flow; BP, blood pressure; +, present; −, absent; ↓, diminished; ↑, increased; ⇣, unchanged. Blank indicates not studied.
insights into the role of Notch3 in normal vascular structure and function and in CADASIL pathogenesis.

**Notch3 Knockout Mice**

Although not a genetic model for CADASIL, Notch3 knockout mouse models have nevertheless provided important clues on the role of Notch3 in cerebrovascular homeostasis. Notch3 null mouse models reported to date were viable and fertile, and did not show gross anatomic developmental abnormalities, including the circle of Willis and larger conductance arteries. However, ultrastructural studies revealed postnatal VSMC maturation and differentiation defect in small systemic and cerebral resistance arteries of homozygous knockout mice. Affected arteries displayed enlarged diameters and thinner media resembling veins. Although these ultrastructural abnormalities were not noted in another knockout model, transcription of genes associated with muscle development and contraction, as well as the canonical Notch downstream targets, were nevertheless downregulated in isolated enriched cerebral VSMC. Functionally, knockout arteries displayed impaired ability to constrict on increased luminal pressure (ie, myogenic response) both in vivo and in vitro, although pharmacological contractions did not differ from the wild type, arguing against a nonspecific reduction in contractility.

Despite these structural and functional defects, Notch3 knockout mice did not develop any parenchymal pathology resembling CADASIL, but showed increased sensitivity when challenged by induced focal cerebral ischemia. After transient proximal occlusion of the middle cerebral artery using a microvascular clip (arrows) through a temporal craniotomy. Blue pixels indicate regions where CBF was below 30% of baseline 60 minutes after middle cerebral artery occlusion. Imaging field (5x7 mm) was positioned over the right hemisphere as shown in the inset. Along with enlarged infarct volumes in the knockout, these data link Notch3 signaling to a novel stroke phenotype.

**CADASIL Mutant Mice**

To date 4 mutant mouse models expressing common CADASIL mutations (R90C, R169C, C428S, R142C) have been developed and studied in detail. These models differed in their transgenic strategy and expression levels, endogenous Notch3 expression, and the predicted effects of mutations on Notch3 function (Table). Transgenic models variably showed age-dependent ultrastructural abnormalities in cerebral and systemic resistance arteries characteristic for CADASIL, including GOM and NOTCH3 ectodomain deposits; evidence for VSMC degeneration was observed in systemic but not cerebral arteries. At least in the tail arteries, more subtle changes in arterial media including increased actin polymerization and disruption of VSMC anchorage to adjacent cells and matrix appeared to precede the deposits by several months. No vascular pathology was present in transgenic mice overexpressing the wild type NOTCH3. The rate of NOTCH3 deposition in VSMC membranes appeared to be proportional to the expression level of the mutant transgene in different mouse lines. Most notably, in the R169C mutant with highest levels of expression, characteristic NOTCH3 ectodomain aggregates were present as early as 1 to 2 months of age and GOM deposits around 5 months; both progressively increased with age. Interestingly, smooth muscle cells otherwise appeared normal in the R169C mutant even at 20 months, setting a contrast with the R90C and C428S transgenic mice. Instead, capillary density was progressively reduced in white but not gray matter starting as early as 5 months, and decreasing to <50% of wild type Notch3 transgenic mice at 20 months.

As predicted from the vascular morphological changes, mutant mice showed abnormal vasomotor responses that were in general opposite to the knockout phenotype, with a few exceptions. For example, R90C transgenic systemic arteries constricted more on increased luminal pressure, both in vivo and in vitro, and showed impaired ability to dilate in response to a number of physiological stimuli, including hypercapnia, hypotension, and functional cortical activation. Interestingly, in the R169C mutant passive dilatory reserve was reduced in the absence of other structural abnormalities such as arterial wall...
thickness, which may explain the paradoxical attenuation of myogenic tone in this mutant strain. In both R90C and R169C mutants, the onset of vascular dysfunction appeared to precede conspicuous vascular pathology, and in the R90C mutant did not worsen significantly with age, suggesting that vascular dysfunction is not dependent on GOM and NOTCH3 deposition or degeneration of smooth muscle cells. Importantly, resting CBF was measured in the R169C mutant and found to be reduced, albeit mildly in both white and gray matter, starting around 12 months and reaching 10% to 20% reduction at 18 months of age.

Despite the prominent vascular pathology and dysfunction, cerebral parenchymal lesions resembling CADASIL were disappointingly absent in the R90C and C428S mutants. Conspicuous leukoaraiosis did develop in the R169C mutant with the highest level of transgene expression, although lacunar infarcts were still absent.23 Senescent R169C mutants (18 months) showed white matter vacuolization, demyelination, and astrocytosis involving corpus callosum, internal capsule, and other major white matter bundles.

In contrast to the transgenic overexpressors, R142C knockin mice did not develop any anatomic, histopathologic, or ultrastructural abnormalities, although vasomotor function has not been studied.47 This mutation has previously been shown to affect intracellular trafficking but retain Notch3 signaling function in vitro.33 Yet the R142C knockin model showed normal Notch3 receptor expression, cleavage, intracellular trafficking, downstream signaling, and ectodomain clearance. The reasons for the lack of CADASIL phenotype in this knockin model are unclear.

Pathophysiological Insight From Animal Models

Available data support chronic cerebrovascular dysfunction and subcortical ischemia punctuated by acute lacunar infarcts as the main disease mechanism in CADASIL. Leukoaraiosis, by virtue of being the earliest sign of CADASIL (ie, neuroimaging), is the only CADASIL-like parenchymal pathology reproduced in mutant mouse models, and appeared to be, at least by temporal association, secondary to vascular dysfunction and reduced capillary density. However, such a temporal association (ie, cerebral hypoperfusion preceding leukoaraiosis) has not been unequivocally demonstrated in CADASIL patients. In small clinical series, acetazolamide has been reported to partially restore tissue perfusion,49 but whether such drug interventions can impact the progression of subcortical pathology and cognitive dysfunction is not known. Therefore, however plausible, chronic hypoperfusion as the primary cause of leukoaraiosis in CADASIL is far from established. Although Notch3 is exclusively expressed in VSMC and pericytes in adult brain, it is conceivable that CADASIL mutations interfere with normal cellular communication in the neurovascular unit (eg, between smooth muscle cells and astrocyte end feet), thereby disrupting the homeostasis. NOTCH3 signaling is critical for endothelium-pericyte interactions to promote and maintain microvascular networks.51 Disruption of which may result in microcirculatory rarefaction. The mechanism of lacunar strokes in CADASIL is also not clear. Enhanced myogenic contractility as well as mural fibrosis and thickening can conceivably lead to progressive stenosis and occlusions by raising the critical closing pressure of small penetrating arteries. However, significant stenosis does not occur in deep gray matter nuclei where lacunar infarcts also commonly occur in CADASIL patients,52 and the degree of luminal stenosis in white matter does not always correlate with the presence of lacunar infarcts.53 Ultrastructural and functional abnormalities of endothelial cells have been reported in CADASIL mutant mice and patients, and may play a role in the pathogenesis of lacunar infarcts.19–21,46,54

Animal models of CADASIL also provide insight into the nature of CADASIL mutations. Although Notch signaling is known to be dosage sensitive, evidence strongly argues against a simple hypomorphic (ie, loss of function) phenotype in CADASIL. First, Notch3 knockout mice do not develop the characteristic tissue changes observed in CADASIL, and functionally display vasomotor abnormalities that tend to be opposite to those observed in CADASIL mutant mice. Second, majority of CADASIL mutations do not interfere with the membrane trafficking of the receptor or downstream transcriptional signaling. Third, transgenic expression of either functionally active (ie, R90C and R169C) or inactive (ie, C428S) mutant NOTCH3 all reproduce the ultrastructural features of CADASIL vasculopathy. Fourth, transgenic expression of the R90C mutant NOTCH3 on a Notch3 knockout background also reproduces GOMs and NOTCH3 aggregates, despite restored downstream signal transduction by the mutant receptor.55 Taken together with the autosomal dominant nature of the syndrome, it is unlikely that CADASIL represents a simple loss of function phenotype, although this may be a modulator of the clinical phenotype. Data also do not support a simple hypermorphic phenotype, because CADASIL mutations do not increase downstream NOTCH3 signal transduction.55 Interestingly, however, an activating NOTCH3 mutation (L1515P in the extracellular heterodimerization domain that does not alter the number of cysteines) which increases NOTCH3 signaling by several-fold independent of ligand-binding (ie, hypermorphic) also causes small vessel disease with early onset lacunar strokes and leukoaraiosis but without GOM or NOTCH3 deposition, hereby distinct from the CADASIL syndrome.56 The accumulation of GOMs and mutant NOTCH3 would suggest a neomorphic phenotype (ie, gain of a novel, pathological function); however, it is not known whether GOM or NOTCH3 aggregates are causally related to vascular dysfunction, leukoaraiosis, and lacunar infarcts, or whether they are innocent biomarkers. Animal models also addressed the issue of dominant negative effect (ie, mutant NOTCH3 antagonizing wild type NOTCH3) with mixed results. Transgenic expression of C428S (loss of function mutation) mutant NOTCH3 on a heterozygous knockout background (ie, one functional copy of endogenous Notch3, which, alone, is not associated with any structural abnormalities55) inhibited downstream signaling by the normal Notch3 copy when compared to nontransgenic heterozygous knockout;57 however, similar experiments using the R90C mutant did not reveal a dominant negative effect.55 It should be noted that the level of transgene expression was clearly higher in the C428S mutant compared to the R90C. Overall, the data from mutant mouse models...
suggest that one or all of these mechanisms may contribute to or modulate the phenotype, possibly explaining some of the clinical heterogeneity in CADASIL.

Commentary

The caveats of extrapolating data from transgenic mice to CADASIL patients notwithstanding (eg, shorter life span, smaller brain size, overexpression of mutant gene), mutant mouse models have unequivocally shown us that Notch3 is critical for maintaining normal vascular structure and function in adult brain, possibly by acting as a luminal pressure sensor modulating the myogenic response and arterial phenotype of smooth muscle cells. As summarized above, there has been a fair amount of congruence between clinical and experimental observations. Nevertheless, reproduction of leukoaraiosis and lacunar infarcts in mutant mouse models of CADASIL has been problematic. At best, leukoaraiosis appeared only in 1 mutant model and very late in its normal life span (18 months), and required significant overexpression of the mutant gene. Moreover, lacunar infarcts, which appear to be a major determinant of disability clinically, have never been reproduced. Yet, leukoaraiosis and lacunar infarcts are the most relevant end points one would like to target preclinically for novel therapeutic approaches, because the relevance of GOMs, NOTCH3 aggregates, and smooth muscle degeneration for vascular dysfunction, and the relevance of vascular dysfunction for leukoaraiosis and strokes are not yet established. Often in mutant models with subtle but progressive and cumulative abnormalities, phenotypes that otherwise would take a long time to develop can be unveiled by challenging the system. In CADASIL, cerebral vasculature can be experimentally challenged by arterial stenosis or occlusion. This has been tested in the Notch3 knockout mice using an established middle cerebral artery occlusion model, and revealed a novel stroke phenotype in a mutant mouse model which otherwise did not develop parenchymal pathology. Of course, Notch3 knockout is not a CADASIL model, and similar studies in mice expressing specific CADASIL mutations will undoubtedly add to our understanding of CADASIL and help develop preclinical screening tools for novel therapies. In this regard, established experimental models of stroke and cerebral hypoperfusion are valuable to interrogate the cerebral vasculature and parenchyma in CADASIL mutants, despite the fact that none truly represents lacunar pathophysiology, for which there is, unfortunately, no established experimental model.

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References


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