Research Reports

Cholinergic Neuronal Deficits in CADASIL

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Background and Purpose—Previous evidence from MRI and acetylcholinesterase histochemistry suggests cholinergic fibers are affected in cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL).

Methods—As a measure of cholinergic function, we assessed choline acetyltransferase (ChAT) activities in the frontal and temporal neocortices and the immunocytochemical distribution of ChAT and p75 neurotrophin receptor (P75NTR) by in vitro imaging in the nucleus basalis of Meynert of CADASIL subjects.

Results—ChAT activities were significantly reduced by 60% to 70% in frontal and temporal cortices of CADASIL cases, as were ChAT and P75NTR immunoreactivities in the nucleus basalis.

Conclusions—Our findings suggest cholinergic neuronal impairment in CADASIL and implicate cholinomimetic therapy for subcortical vascular dementias. (Stroke. 2007;38:188-191.)

Key Words: CADASIL ■ cholinergic neurones ■ cognitive impairment ■ small vessel disease ■ vascular dementia.

Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is the most common form of hereditary small vessel disease which leads to cognitive decline and dementia.1,2 MRI has enabled quantification of the burden of leukoaraiosis and lacunar infarction in the subcortical structures. Hyperintensities in the deep white matter (WM), internal and external capsules and the anterior temporal pole are characteristic of CADASIL. CADASIL is linked to mutations within the epidermal growth factor–like repeat region of the Notch3 gene located on chromosome 19p13.3 To date, >80 mutations have been described, all involving either a gain or loss of cysteine residue(s). The pathology is characterized by profound demyelination and axonal damage as well as an arteriopathy involving distinctive degeneration of the arterial smooth muscle cells in the brain and peripheral organs. Electron microscopy enables visualization of granular osmiophilic material deposits, which are diagnostic for CADASIL.2

Little is known about the neurochemical pathology of CADASIL. A single case report indicated that substantial cholinergic denervation may occur in CADASIL.4 It was suggested that the subcortical lesions alone are sufficient to cause cholinergic loss without necessarily affecting the nucleus basalis of Meynert (nbM). In the absence of any Alzheimer disease (AD)–type lesions in CADASIL, this study disclosed therapeutic implications for sporadic cerebrovascular disease characterized by small vessel disease pathology.5 Previous studies in Binswanger disease (hypertensive encephalopathy) and spontaneously hypertensive rats demonstrated decreased WM volume and loss of cholinergic neuronal markers6 with significantly decreased acetylcholine concentrations in the cerebrospinal fluid, hippocampus and cortex.7

To test the hypothesis that CADASIL cases exhibit profound cholinergic dysfunction, we measured cortical choline acetyltransferase (ChAT) enzyme activity and assessed the immunocytochemical distribution of ChAT and of p75 neurotrophin receptor (P75NTR) in postmortem brain tissue from genetically confirmed CADASIL cases and age-matched and elderly controls.

Materials and Methods

Samples of brain tissue from CADASIL and controls were obtained from various sources including the Newcastle Brain Tissue Resource Centre, the Institute of Psychiatry, London (courtesy of Dr Safar Al Sarraj), Southern General Hospital, Glasgow (Prof David Graham), University of Helsinki (Drs Marc Baumann, Raimo Sulkava, and Tuomo Polvikoski) and Frenchay Hospital, Bristol (Dr Tim Moss). Available case notes indicated that the CADASIL subjects met the minimum criteria for cognitive impairment per our poststroke study.8 None of the controls had clear neurological or pathological evidence for cerebrovascular or neurodegenerative disease.

Frozen samples from frontal (Brodmann 9 and 10) and temporal (Brodmann 20 to 21) cortices were collected from a total of 9 CADASIL cases with mean (±SEM) age of 58±3 years (range 52 to 74 years; 7 males [m], 2 females [f]), 14 age-matched with mean age 57±3 years (53 to 74 years; 7 m, 7 f) and 9 older controls with mean age 87±3 years (79 to 102 years; 4 m, 5 f). Both regions were not available from 1 CADASIL and 5 of the control brains. Fixed paraffin sections of basal ganglia cut serially at the level of the anterior commissure taking in the same coronal segment of the nbM.

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were used for immunocytochemistry and comprised 4 CADASIL cases (52 to 74 years; 3 m, 1 f) and 4 age-matched controls (43 to 74 years; 4 m). There were no apparent correlations with the measures and length of fixation or postmortem interval from death to freezing/fixation of tissue between the groups. *Notch3* gene mutations in the CADASIL patients were confirmed by direct sequencing of DNA and were all determined to be in exon 4 except one as follows: R133C (n=3), R141C, (3) R169C (4) and R558C (1). Disease duration from the first cerebrovascular event in the CADASIL cases ranged from 15 to 24 years.

ChAT activity was measured as described previously modified from the original method of Fonnum.9 For the immunocytochemical studies, 10-μm sections containing the whole caudate-putamen were stained with antibodies against ChAT, P75NTR and amyloid precursor protein (APP) immunoreactivities using either pressure cooking in 0.1 mol/L EDTA for 1 minute (ChAT) or microwaving in 0.01 mol/L citrate buffer for 10 minutes (P75NTR, APP). Primary antibody concentrations for ChAT were 1:200 (Chemicon), for P75NTR were 1:400 (clone 7F10, Novacastra) and APP were 1:500 (clone 22c11, Chemicon). The immunoreactive product was visualized by nickel enhanced 3,3-diaminobenzidine or by fluorescein (APP) and assessed quantitatively using Image Pro Plus software version 4.5.129 (Media Cybernetics). For each case at least 4 square boxes were randomly overlaid onto images of the nbM taken at x20 magnification. The percentage area covered by immunoreactivity was measured and the mean value taken. All assessments including biochemical analyses were carried out blind to cases or controls.

**Statistical Analysis**

Standard PC-based statistical software was used. Differences between the groups were compared using the Mann–Whitney U test (2-group comparisons) and 1-way analysis of variance (3-group comparisons).

**Results**

CADASIL cases showed typical vascular lesions,10 without AD pathology. Mean ChAT activities were significantly decreased by 74% in the frontal (P<0.001) and 78% in the temporal (P<0.05) cortex of CADASIL cases compared with age-matched controls. ChAT activities were also reduced in the CADASIL group when compared with elderly controls although significance was apparent only in the frontal cortex (Figure 1). The observed differences between the elderly and young controls is likely attributable to age-related loss.7 These results suggest decreased specific ChAT enzyme activities in 2 different neocortical regions.

To verify the biochemical data, we assessed CADASIL cases for the immunocytochemical distribution of ChAT and P75NTR, well characterized markers of cholinergic neurones. With respect to the nbM, both markers demonstrated clear loss of immunoreactivity in all 4 CADASIL cases (Figure 2) showing slightly smaller-sized neurones with a vacuolated appearance and reduced arbour compared with controls. Quantitative assessments revealed that both ChAT (P<0.05) and P75NTR (P<0.01) immunoreactivities in the cases were significantly reduced in the nbM compared with those of age-matched controls (Figure 2). Interestingly, the large ChAT positive intrinsic neurones in the putamen that were strongly immunoreactive in the soma with long protruding arbours were also affected in CADASIL cases. The interrupted ChAT positive fibers were distributed among the complex mosaic of varicosities forming patches of light and dark (striosomal) staining. In CADASIL cases positive neurones often appeared less frequent, smaller and shrivelled, with reduced density of nodes or projections (not shown).

We further examined P75NTR immunoreactivity in lateral cholinergic pathway by imaging tracts coursing from the nbM around the putamen and lower parts of the external capsule. Although these showed differential immunostaining in CADASIL cases, qualitative assessment revealed distinct differences between CADASIL and controls (Figure 3B through 3D). Controls showed P75NTR positive fibers robustly stained with continuous fiber tracts in discreet bundles. However, CADASIL cases showed punctate deposits indicating interrupted P75NTR positive immunoreactivity particularly along the external capsule. These findings collectively suggest that cholinergic neurones and fibers emanating from the nbM are disrupted in CADASIL. Further evidence for disruption of cholinergic fibers in CADASIL cases was obtained by comparing immunostaining for APP and ChAT (Figure 3). Sections stained for APP (which is regularly transported along axons) from 4 cases revealed widespread accumulation of immunoreactivity along fibers in subcortical WM. However, several damaged fibers in the lateral pathway, indicated by colocalization of APP and ChAT immunoreactive products, suggested high disruption of cholinergic axons within these (Figure 3E through 3G). We did not observe any relationship between the *Notch3* mutation site (genotype) and changes in ChAT activities or morphological measures.

**Discussion**

Our observations show anomalies in cholinergic neuronal markers in CADASIL consistent with the single case report.4 We also found cholinergic axon projections, labeled by P75NTR, along WM tracts to the frontal cortex to be affected. The characteristic small vessel disease and leukoencephalop-
athy apparent in CADASIL likely targets the cholinergic tracts following along the lateral ventricles and around the frontal horn first.7,11 This is consistent with the previous analysis on Binswanger disease cases suggesting damage to cholinergic fibers of the external capsule in the absence of coexisting AD pathology12 and that small vessel dementia is associated with strategic damage to the external capsule resulting in executive dysfunction.13 Indeed, MRI studies suggest that the external capsule (highly traversed by the lenticulostriate arteries) and frontal lobe WM are often affected in CADASIL patients.14

We also showed reduced immunoreactivity of ChAT and the P75NTR in the nbM. It is plausible that as the disease progresses in each CADASIL subject, the variable degree of WM tract destruction and cholinergic fiber impairment causes differential retrograde (Wallerian type) degeneration tracking all the way back to the nbM.

Recently, it was reported that there is no significant change in ChAT activity in Brodmann 36 (an area with severe AD-type pathology) of “pure” vascular dementia cases.9 They argue that any cholinergic deficits to be observed in vascular dementia may be attributable to concurrent AD-type pathology. CADASIL subjects seldom show concurrent Alzheimer lesions. None of the cases we analyzed here exhibited AD pathology.

In summary, our findings show cholinergic neuronal deficits by both enzyme activity and immunocytochemistry in CADASIL cases. As for AD, these observations implicate cholinomimetic therapy in CADASIL and more broadly in subcortical vascular dementia.5,15

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Figure 2. The extent of loss of cholinergic markers in the nbM in CADASIL postmortem tissue compared with age-matched controls, as demonstrated by reduced immunoreactivity of 2 cholinergic markers; ChAT in panels A through D and P75NTR in panels E through H. Panels A, B, E and F illustrate immunoreactivity in the nbM of 2 age-matched control cases and panels C, D, G and H in 2 CADASIL brains. Dot-plots (horizontal line shows median value) indicate that although only 3 to 4 cases could be analyzed at the same coronal levels in nbM, there is a significant difference between CADASIL cases and age-matched controls (**P<0.01, *P<0.05; Mann–Whitney U test). Scale bars=100 μm.

Figure 3. Disruption of subcortical cholinergic fibers in CADASIL postmortem tissue evaluated by P75NTR, ChAT and APP immunoreactivities. Coronal sections containing the putamen and capsules (box in A) were immunostained to demonstrate extent of tract disruption in the lateral pathway of CADASIL cases. This is apparent by comparing P75NTR immunoreactivity in an age-matched control (B) and 2 CADASIL brains (C and D). P75NTR is known to be located axonally because it binds cortical nerve growth factor for retrograde transport to the nbM. Similarly, colocalization (panel G, yellow-green) of APP (panel E, green) and ChAT (panel F, red) immunoreactivity by confocal microscopy in a basal ganglia section from a CADASIL subject suggests disruption of axonal transport in fibers along the external capsule. Panel A shows several lacunes in a T2-weighted MR scan at the coronal plane from a 65-year-old CADASIL patient. Scale bars=100 μm.
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Disclosures

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