Cortical Neuronal Apoptosis in CADASIL

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Background and Purpose—Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is caused by mutations of the NOTCH3 gene and is a model of pure vascular dementia. Cortical atrophy has been reported to be associated with cognitive decline in the disease, although the underlying mechanism is unknown. We postulated that apoptosis may be involved in this process.

Methods—We report the clinical history, magnetic resonance imaging findings, and pathologic examinations of 4 patients (2 of whom were demented) who died from complications of the disease. Apoptosis was evaluated in brain tissue using antibodies against activated caspase3 and in situ end labeling assays for DNA fragmentation.

Results—Widespread neuronal apoptosis in the cerebral cortex (predominantly in layers 3 and 5) was observed in all patients. This was not seen in 3 non-CADASIL controls. Semiquantitative analysis suggested that apoptosis was more extensive in the presence of larger load of subcortical ischemic lesions and smaller brain volumes.

Conclusions—Neuronal apoptosis may be involved in cortical atrophy in CADASIL and appears related to the burden of subcortical ischemic lesions. These findings may have important implications in other small vessel diseases and may provide a potential target for future therapeutic interventions. (Stroke. 2006;37:2690-2695.)

Key Words: apoptosis  ■ CADASIL  ■ cortex  ■ cortical atrophy  ■ lacunar infarction  ■ white matter damage

Evidence suggests that apoptotic pathways play an important role in delayed brain injury after ischemic infarction. Although necrotic cell death occurs at the core of an infarction where oxygen supply is most reduced, peri-infarct areas apoptotic cell death largely predominates. Apoptosis may also be involved in delayed neuronal degeneration in the central nervous system after distal axonal injury. Because these mechanisms may contribute to brain damage after ischemic injury, further elucidation of this pathway has the potential to offer new therapeutic avenues in treatment of stroke-related diseases, including vascular dementia.

Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is a hereditary small vessel disease caused by mutations in the NOTCH3 gene and is considered a model of pure vascular dementia.

Pathologic studies demonstrate electron-dense osmiophilic granular material within the media of small arteries and capillaries in close association with degenerating smooth muscle cells. These microstructural changes are presumably responsible for vessel wall dysfunction with decreased basal perfusion and hemodynamic reserve leading to extensive subcortical ischemic lesions. Loss of cortical neurons in CADASIL has been demonstrated in a single case.

On MRI, there is evidence of widespread white matter hyperintensities (WMH) on T2-weighted images, and old cerebral microhemorrhages on gradient-echo images. Recently, it has been shown that cortical atrophy may progress over the course of the disease and is associated with cognitive decline in CADASIL.

We postulated that apoptotic cell death may be involved in the pathway of cerebral atrophy in this disease. Herein, we report the clinical, MRI and pathologic findings in 4 patients with CADASIL who died after complications of the disease.

Subjects and Methods

Four patients with CADASIL were seen and followed at Hospital Lariboisière until death. The diagnosis was confirmed by identification of a typical mutation in the NOTCH3 gene (Table). Evaluation of neurologic disability and cognitive function was performed within 1 year of death in all patients. Gross and microscopic postmortem neuropathologic examination was performed. Three subjects without neurologic disease at death (1 motor vehicle accident death, 2 gunshot related deaths) served as controls (time between injury and death within hours). The mean age of the controls was 46.3 years old versus 64 years old in the subjects.

MRI and Image Processing and Analysis

MRI imaging was performed in all patients in the year before death. Quantitative imaging was obtained in 2 patients (patients 1 and 3) who underwent MRI with identical parameters on a 1.5-T scanner. The total volume of WMH on FLAIR images was normalized to the intracranial cavity in each patient (nWMH), as well as the total...
volume of lacunes on T1-weighted images (nLV) and the total brain volume (nTBV). The number of microhemorrhages was determined on gradient-echo sequences.

Pathologic Examination

In patient 1, autopsy was performed within 24 hours after death and limited to the brain. A complete postmortem examination was performed 12 hours after death in patients 2 and 4, and within 36 hours after death in patient 3. Brains of control patients were similarly assessed within 24 hours of death.

Gross examination of the brain was performed after 1 month 10% buffered formalin fixation. Large slices involving the cerebral hemispheres and the brainstem/cerebellum at the level of the dentate nuclei were embedded in paraffin and 15-μm-thick sections were stained by hematoxylin and eosin and cresyl violet combined with Luxol fast blue (Klüver and Barrera stain). Smaller blocks were taken from the cerebral cortex with underlying white matter, deep gray nuclei, hypothalamus, midbrain, cerebellum, brainstem. Five-μm-thick sections were stained with hematoxylin and eosin, Masson’s trichrome, and Bodian silver impregnation combined with Luxol fast blue.

On selected 5-μm-thick sections of samples from the frontal lobe at the level of the rostrum of corpus callosum (F1), occipital lobe at the level of the calcarine sulcus, hippocampus, basal ganglia, thalamus and cerebellum, immunohistochemistry was performed using an avidin-biotin complex, peroxidase-based method with the following antibodies (Ab): a monoclonal Ab raised against the precursor of the protein β-amyloid (anti-Alzheimer Precursor protein A4; Boehringer Mannheim GmbH; 1/200) to identify axonal damage,16 a polyclonal Ab raised against activated caspase3 (affinity-purified rabbit anti-human/mouse caspase3 active; R&D Systems Europe, Lille, France; 1/1000) to identify apoptotic cells, and a monoclonal Ab raised against N3ECD (kindly provided by Dr A. Joutel, INSERM U270, Faculté de Médecine Lariboisière, Paris) to demonstrate Notch3 deposition.17 For all techniques, controls included the omission of the primary antibody and simultaneous staining of known positive or normal material. Endothelial cells, included the omission of the primary antibody and simultaneous staining of known positive or normal material. Endothelial cells, served as internal positive control.18

The presence of apoptotic cells was also examined using in situ end labeling (ISEL) to identify internucleosomal DNA fragmentation. This was performed using the ApopTag kit (QBiogene, MP Biomech) according to the manufacturer’s recommendations and modified using an alkaline phosphatase technique to avoid false positivity related to lipofuscin as previously described.19

Semiquantitative evaluation was performed by 2 independent neuropathologists (F.G., M.B.) blinded to the clinical data. The intensity of Notch3 and β-APP expression was scored as follows: 0 = absent, 1 = mild, 2 = marked, and 3 = intense. The severity of neuronal apoptosis was evaluated on ISEL and scored as follows: 0 = no apoptotic cells; 1 = occasional isolated apoptotic neurons; 2 = occasional groups of apoptotic neurons; and 3 = frequent apoptotic neurons.

Results

The clinical data, MRI findings, and pathologic examinations for the 4 patients and 3 controls are summarized in the Table. Severe neuronal apoptosis was found in the cerebral cortex, predominantly in layers 3 and 5, as demonstrated by ISEL and expression of activated caspase3 in patient 1 (Figure 1B and 1C, arrowheads) and patient 4 (Table). There were extensive lesions of the underlying white matter with severe axonal damage in subcortical fibers as identified by β-APP expression (Figure 1D, stained in brown). By contrast, moderate neuronal apoptosis was found in layers 3 and 5 of the cerebral cortex of patient 2 (Table) and patient 3 (Figure 1F and 1G, arrowheads). There was some evidence of ischemic lesions in the underlying white matter and axonal damage in subcortical fibers (Figure 1H and Table). In all patients, apoptotic neurons were not localized within or around the rare cortical microinfarcts and the intensity of apoptosis was not related to that of Notch3 deposition. We were unable to quantify the total number of cortical microinfarcts because of the microscopic nature of these lesions and because we examined only limited number of samples. However, the preservation of neurons in close proximity to these lesions was striking (Figure 2). There was also evidence of astrocytic and oligodendrocytic apoptosis in the white matter. These apoptotic glial cells were commonly located at a distance from the focal subcortical

![Figure 1](http://stroke.ahajournals.org//image-handling/10.1161/01.str.111.16.2691.f1a.png)
ischemic lesions, mainly at the cortico-subcortical junction in spared or mildly edematous white matter (Figure 3). Minimal neuronal apoptosis was seen in the hippocampus (Figure 4A and 4B) where endothelial cell apoptosis served as a positive control (Figure 4A, arrowhead). Caspase3 immunopositive neurons were always positively stained by ISEL on successive sections but ISEL-positive neurons were much more numerous. Caspase3 immunopositive neurons usually were normal in appearance, consistent with the fact that caspase3 activation is an early phenomenon preceding DNA strand breakage and terminal nuclear and cytoplasmic changes.20 A proportion of ISEL-positive neurons demonstrated the characteristic morphology of apoptotic cells with shrunken cytoplasm and pyknotic nuclei consistent with evidence showing that although ISEL positivity precedes terminal morphological changes and persists in the later stages of the apoptotic process,21 there was no evidence of either caspase3 immunostaining or ISEL-positive neurons in the cortex of the 3 control subjects (Table).

Figure 2. Example of cortical microinfarction in CADASIL. Normal, nonapoptotic neurons (arrowheads) are present close to an ischemic focus (delineated by dotted lines) that can be identified by the presence of microglial cells positively stained by ISEL (apoptosis normally occurs in macrophage/microglial cells after phagocytosis).

### Discussion

To our knowledge, this is the first report to describe widespread neuronal apoptosis in the cerebral cortex in CADASIL, a model of pure vascular dementia. Neuronal apoptosis occurs in layers 3 and 5 of the cerebral cortex and its degree seems to be related to the extent of associated subcortical white matter damage but not to areas of cortical ischemia. Neuronal apoptosis was not found within or close to the occasional cortical microinfarcts present in the patients. It was not observed in the hippocampus despite of the presence of some ischemic neurons. Additionally, the degree of cortical apoptosis appears to be related to cognitive impairment and to cerebral atrophy.

Based on semiquantitative neuropathologic comparisons in all of our patients, we found that the number of apoptotic neurons in layers 3 and 5 was associated with the extent of white matter lesions and the intensity of axonal damage in the subcortical white matter. These data are also supported by volumetric MRI analysis in 2 of the patients demonstrating that in the individual with more extensive cortical apoptosis (patient 1), nWMH and nLV were considerably greater than in the individual where apoptosis was less severe (patient 3). Additionally, in all patients

### Table: Semiquantitative Evaluation of Neuronal Apoptosis in 4 CADASIL Patients and 3 Controls

<table>
<thead>
<tr>
<th>Cases</th>
<th>NOTCH3 Mutation</th>
<th>Age/Sex</th>
<th>Dementia</th>
<th>MMSE</th>
<th>mRS</th>
<th>Quantitative MRI</th>
<th>Frontal Lobe</th>
<th>Neuronal Apoptosis</th>
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β-APP IHC indicates β-amyloid precursor protein immunohistochemistry; Caspase IHC, caspase3 immunohistochemistry; MMSE, Mini-Mental Status Examination; nt, nucleotide position; mRS, modified Rankin Scale.

Notch3 and β-APP expression: 0=absent, 1=mild, 2=marked, and 3=intense. Apoptosis: 0=no apoptotic cells, 1=occasional isolated apoptotic neuron, 2=occasional groups of apoptotic neurons, and 3=frequent apoptotic neurons.
apoptosis was rare or absent in the hippocampus and the cerebellar cortex, where the amount of associated subcortical lesions was considerably less. Layer 3 of the cerebral cortex contains pyramidal neurons that have been shown to have extensive connections with cortical layer 5,22,23 where large pyramidal neurons have prominent subcortical projections.24,25 Experimentally, selected cortical pyramidal cell neurons have been shown to undergo apoptosis after axonal damage in an animal model.26 Our results suggest that apoptosis in cortical neurons in CADASIL may be secondary to axonal damage in the underlying white matter through deafferentation or retrograde neuronal degeneration.

Cortical apoptosis in layer 3 has been previously implicated in the neurodegenerative process in Alzheimer disease.27 In addition, layers 3 and 5 of the cerebral cortex have been shown to contain a large number of pyramidal neurons with high levels of acetylcholinesterase activity.28 A decrease of this activity has been reported in patients with Alzheimer dementia.29 A significant loss of this activity has also been reported in a demented CADASIL patient with relative sparing of hippocampal areas11 in line with the regional pattern of apoptosis observed in our study. This is consistent with the preservation of memory encoding processes even in advanced stages of CADASIL and after the occurrence of dementia.7,30

In patients 1 and 3 who underwent quantitative MRI analysis, the severity of apoptosis appeared related to normalized brain volume. Although the MRI data are limited to only 2 cases, one may hypothesize that the apoptotic pathway contributes to cerebral atrophy in CADASIL. Recent results showed that degree of atrophy has a significant clinical impact in the disease.14 Our finding of many apoptotic neurons (by ISEL but also by activated caspase3 immunostaining) in our post-mortem cases deserves further comment. Evidence suggests that once the apoptotic program is initiated in a neuron, the neuron is rapidly cleared.31 Thus, the large number of apoptotic neurons that we observed, particularly on ISEL staining, is an unexpected finding. However, initiation of apoptosis is a 2-step process.18 The first step (known as priming) designates which cells will undergo programmed cell death. In the second step (known as triggering), primed cells undergo irreversible fragmentation of DNA which then leads to cell death.18 It has been suggested that agonic events and death may themselves be triggering stimuli, revealing many primed neurons that would have otherwise died more progressively.32 However, as these triggering stimuli only initiate DNA fragmentation in primed cells,18,33 reliable evaluation of apoptosis can still be made. In other words, the agonic events themselves are unlikely to cause the apoptosis observed in

![Figure 3](http://stroke.ahajournals.org/)

**Figure 3.** Glial cell apoptosis in affected white matter areas illustrated in patient 1. A, Gross section of occipital lobe (Klüver & Barrera stain) showing multiple areas of white matter damage. *Area of microscopic section in adjacent panels. B, ISEL in the occipital lobe demonstrating oligodendrocytic (arrowheads) apoptosis. C, Caspase3 immunostaining in the occipital lobe demonstrating astrocytic (arrowheads) apoptosis.
the present study. The lack of cortical apoptosis observed in our 3 controls further supports this hypothesis.

Our finding of cortical apoptosis in these 4 cases suggests that programmed cell death may be a common feature in CADASIL and associated with cortical atrophy. However, the small number of cases analyzed in this study limits our ability to draw definitive conclusions. We cannot exclude the fact that apoptosis and cortical atrophy in CADASIL are in fact independent processes. Additionally, other unmeasured factors may contribute to, and partially explain the differences in brain volume seen in our patients.

More generally, it has been recently reported that cognitive impairment associated with subcortical ischemic vascular disease may primarily result from loss of cortical gray matter.\textsuperscript{34} Whether cortical apoptosis represents a general feature in vascular dementia requires further investigation. Our findings, if confirmed in larger studies, may also suggest that anti-apoptotic drugs\textsuperscript{35} are of potential therapeutic interest in CADASIL and possibly in other types of subcortical ischemic dementias.

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**Disclosures**

None.

**References**


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