Quantitative Neuropathological Changes in Presymptomatic Huntington's Disease

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Morphometric studies of the tail of the caudate nucleus, the site where the pathology is first seen, were performed on 16 brain specimens collected from individuals at risk for inheriting Huntington's disease (HD). Medical records and information obtained from immediate family members indicated that all had died without symptoms of HD. Six individuals had 37 or more CAG repeats and were designated HD gene carriers, whereas 10 were determined to be non-carriers. Cell counts of the tail of the caudate nucleus revealed an increased density of oligodendrocytes among the presymptomatic HD gene carriers (mean cells/field: carriers = 40.0, noncarrier = 21.3; age, sex, repeated measure adjusted $F[126] = 11.7$, $p = 0.0008$). No statistically significant differences were found between HD carriers and noncarriers in the density of neurons (carriers = 16.9, noncarriers = 15.5), astrocytes (carriers = 27.8, noncarriers = 21.3) or microglial cells (carriers = 7.9, noncarriers = 5.6). Ubiquitin immunostaining performed in 3 gene carriers revealed intranuclear inclusions in all 3 cases, including 1, with 37 repeats, who died 3 decades before the expected age for onset of the clinical syndrome. Normal densities of other cell types and careful macroscopic examination suggest that the increase in oligodendroglial density is not a consequence of atrophy and may instead reflect a developmental effect of the HD gene.

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Huntington's disease (HD) is a neurodegenerative disorder caused by the abnormal expansion of CAG repeats in the IT15 gene on chromosome 4p16.3.[1] The striatum is the primary structure affected, with a generalized loss of medium-sized neurons progressing along caudal to rostral, dorsal to ventral, and medial to lateral axes.[2] In very mildly affected cases, neuropathologic examinations have shown scattered islands of reactive astrocytosis and neuronal cell loss within the striosomal compartments of the striatum,[3] and quantitative studies have revealed increased oligodendroglial and decreased neuronal cell densities.[4] Although astroglia proliferate in response to neuronal cell death, oligodendroglia are not considered responsive to neurodegeneration. Therefore, increased oligodendroglia might reflect alterations in the morphogenesis of the brain in HD gene carriers.[4]

In this study we sought to test this hypothesis by quantitating cell types in brain specimens of persons dying before the onset of HD. No neuronal or glial changes have been detected by conventional microscopic examination in presymptomatic HD gene carriers.[5] However, no quantitative analysis has been reported previously, probably because of the paucity of these brain samples. The tail of the caudate nucleus was examined because this is the region where the pathology is first reliably detected.[2]

Materials and Methods

Subjects

Sixteen brain specimens were collected by the Brain Tissue Resource Center (McLean Hospital, Belmont, MA) from individuals at risk for inheriting HD (Table 1). Medical records were reviewed and additional information was obtained directly from immediate family members to determine the health status and cause of death. None were reported to have signs of choreic involuntary movements or cognitive deterioration.

Neuropathological Study

Tissue blocks were dissected according to a standardized protocol.[6] Adjacent 7 μm sections were stained with cresyl violet (CV) (Nissl method) and by Luxol fast-blue and hematoxylin and eosin (LH&E). The tail of the caudate nucleus was examined at the level of the lateral geniculate body (block 5 of Vonsattel and coworkers[6]). The borders of the

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Table 1. Clinical and Genetic Features in the 16 Individuals at Risk for Huntington's Disease (HD)

Case No.	Sex	Age at Death (yr)	CAG Repeat Size	Affected Parent	Expected Onset (yr)
HD carriers					
1	M	30	17 /41	Father	58
2	M	40	16 /40	Mother	63
3	F	44	19 /37	Mother	79
4	F	57	17 /42	Mother	54
5	M	60	22 /40	Mother	63
6	M	30	17 /50	Mother	29
Non-carriers					
1	M	0.8	17 /17	Father	—
2	M	29	12 /20	Father	—
3	F	36	17 /23	Father	—
4	M	43	21 /27	Mother	—
5	M	58	20 /22	Father	—
6	M	74	18 /17	Father	—
7	M	79	20 /21	Father	—
8	F	39	17 /23	Mother	—
9	F	86	15 /17	Mother	—
10	F	27	Normal	?	—

M = male; F = female.

tail were outlined by superimposing the CV-stained slide over the LH&E slide.

Cell counts were made on the CV-stained slides as previously reported.[2,4] A Zeiss standard microscope with a magnification of ×400 (×40 objective and ×10 eyepiece) with camera lucida attachment was utilized. Each microscopic field was defined by an eyepiece grid covering a surface of 0.585 mm^2. Whenever possible, 10 microscope fields were recorded for each case. For 4 of the cases, the size of the tail of the caudate precluded an assessment of ten fields. In these instances as many fields as were available were examined (8 fields in 2 cases, 7 fields in 1 case, and 5 fields in 1 case). The numbers of neurons, oligodendroglia, astrocytes, microglia, and cells of undetermined type were recorded. All cell type designations were performed blind to carrier state of the case. Morphologic features combined with cell sizes, as previously described,[4] were used for the definition of cell types. Neurons were identified as cells with granular cytoplasm and discrete nucleolus or nucleus with granular chromatin. Astrocytes were characterized by oval or bean-shaped nuclei, about 10 μm in diameter, with pale, finely textured chromatin and neither nucleoli nor visible cytoplasm. They were distinguished from small neurons on the basis that the latter have an optically empty rim or halo between the cell body and neuropil. Oligodendrocytes were identified as rounded, dark nuclei, sized 5 to 7 μm, without visible cytoplasm. Microglia were defined as rod-shaped cells with darkly stained nuclei, having a process at each pole, and were distinguished from endothelial cells by greater chromatin density and absence of association with vessels. Any cell which could not be unequivocally categorized was included as an "unknown" group.

Adjacent paraffin sections of 3 gene carriers and 3 noncarriers were immunostained with antibodies against microglia (anti-human B lymphocyte LN-3, ICN Biomedicals, OH)[7] and astrocytes (antiglial fibrillary acidic protein, Dako, Denmark).[8] Microglia were identified using the marker lycopersicon esculentum (tomato) lectin (Vector, Burlingame, CA).[9] Frozen sections (40 μm thick) from a gene carrier were immunostained with the antibody neuN (Chemicon, Temecula, CA), which targets a neuron-specific nuclear protein and stains the vast majority of neuronal cell types.[10] Sections from these 3 carriers were stained with an antibody against ubiquitin (Dako, Denmark) and huntingtin[11] to search for intranuclear inclusions. A biotin-conjugated secondary antibody was applied followed by streptavidin-peroxidase, and immunoprecipitates were stained with diaminobenzidine/H^2O^2. Negative control tissue was incubated without primary antibody.

Carrier Testing

Carrier testing for HD was determined by polymerase chain reaction quantification of the number of CAG repeats[1] eliminating an adjacent proline (CCG) repeat. Cases with 37 or more repeats were designated HD gene carriers.[12]

Statistical Analyses

Cell densities were calculated as the number of cells per field. Comparisons of cell densities between gene carriers and noncarriers were made by repeated-measures analysis of variance controlling for sex and age at death of the individual, using the Proc Mixed procedure of the SAS program to adjust for the multiple microscope fields per specimen, and for the correlation among multiple observations for a given subject (SAS, 1990, Cary, NC).[13]

Age at onset was estimated by applying the equation [onset = $10^{(3.118 - 0.033[CAG])}$] derived from a previously studied sample.[12] This equation is recognized to provide the best fit of the relationship of the HD repeat to onset age.[12,14,15]

Results

Genetic Testing and Clinical Features

Six of the 16 brain specimens were determined to be HD gene carriers, and 10 were determined to be noncarriers (Table 1). Causes of death in the HD carriers were cardiac arrest, suicide, melanoma, colon surgery, aortic aneurysm, and was unspecified in 1 case. Causes of death in the noncarriers included bowel obstruction, suicide, Crohn's disease, trauma, lymphoma, pneumonia (two cases), myocardial infarction, asthma, and was unspecified in 1 case.

Three of the carriers died at age 23 to 35 years before their expected onset given their HD repeat size, whereas the other 3 were within 3 years of their expected age at onset.

Neuropathological Examination

None of the cases showed gross atrophy of the caudate nucleus or atrophy of the tail on the macroscopic exam. Three brain specimens were reported to have subtle changes indicative of early HD (grade 1) and all 3 of these (#1, #4, and #5) were found to be HD gene carriers. These changes included the following: for #1 mild neuronal depletion and subtle signs of fibrillary astrocytosis, for #4 mild neuronal loss and astrocytosis involving the dorsomedial portion of the head and of the tail of the caudate nucleus and the dorsal tip of the putamen, and for #5 astrocytosis of caudate nucleus and putamen without neuronal depletion. One noncarrier (#6) had occasional fibrillary astrocytes in the paraventricular portion of the head of the caudate. The tail of the caudate nucleus was judged to be smaller than expected, with mild neuronal loss and occasional fibrillary astrocytes, which was interpreted as suggestive of early signs of HD. Noncarrier case #8 was found to have evidence of a degenerative involvement of the cerebellum, but with a normal striatum. Noncarrier #9 was found to have occasional cortical plaques and tangles suggestive of early Alzheimer's disease with normal striatal pathology.

Cell Counts

The HD gene carriers did not have evidence of a smaller area of cross-section of the tail of the caudate nucleus at the level of the lateral geniculate body. The cross-section of the tail of the caudate nucleus in all gene carriers could accommodate ten fields. However, 4 of the 10 noncarriers could not accommodate 10 fields.

The average density of oligodendroglia among the HD gene carriers was approximately double that found in the non–gene carriers (Table 2, Fig 1). In analyses controlling for repeated measures, age, and sex, the HD gene carriers were found to have significantly more oligodendroglia ($F[126] = 11.7$, $p = 0.0008$) than the noncarriers. The density of neurons, astrocytes, microglia, and unidentified cells in the HD gene carriers was not statistically different from that of the non-carriers (Table 2).

No relationship of age was found for the densities of cell types in either the HD carriers or noncarriers, and the increase in oligodendroglial cell density was not related to age or to the proximity to estimated onset.

Immunocytochemistry

The immunostaining with antibodies or markers against microglia and astrocytes (Fig 2A–B) confirmed that there were not quantitative differences in these cell types between HD gene carriers and noncarriers. The cells found to be increased in the tail of the caudate of the gene carriers did not stain with antibodies against microglia, astrocytes or neurons, reinforcing our observation with CV stain that these cells are oligodendrocytes. Commercially available antibodies reported to recognize oligodendrocytes were unsuccessful in staining these cells in either the HD carriers or noncarriers. Immunohistochemistry with antibodies against oligodendroglia has more variable results than antibodies recognizing other cell types, and conditions such as the fixation of the tissue or the paraffin embedding may have altered oligodendrocytes' antigenicity.

Staining with ubiquitin antibodies in 3 of the HD carriers (#3, #4, and #6) revealed intranuclear inclusions in all three (Fig 2C–D). In the 2 cases close to their expected age at onset (#4 and #6) there were several intranuclear inclusions per section of the tail of the caudate. In the case (#3) with 37 CAG repeats and more than 30 years before the expected onset, the inclusions were smaller and fewer (three were seen in two 7-μm-thick sections). Similar but weaker stain was

Table 2. Cell Densities in the Tail of the Caudate Nucleus in Huntington's Disease (HD) Gene Carriers and Noncarriers

	Cells per Field, Mean (SD)				
	Neurons	Oligodendroglia	Astroglia	Microglia	Unknown
HD carriers	16.9 (6.4)	40.0 (11.7)	27.8 (11.2)	7.9 (3.3)	6.9 (3.1)
Noncarriers	15.5 (2.4)	21.3 (11.0)	21.3 (8.3)	5.6 (3.0)	6.9 (2.5)
	$p = 0.54$	$p = 0.0008$	$p = 0.21$	$p = 0.18$	$p = 0.95$

Comparisons are controlled for age, sex, and repeated measurements.

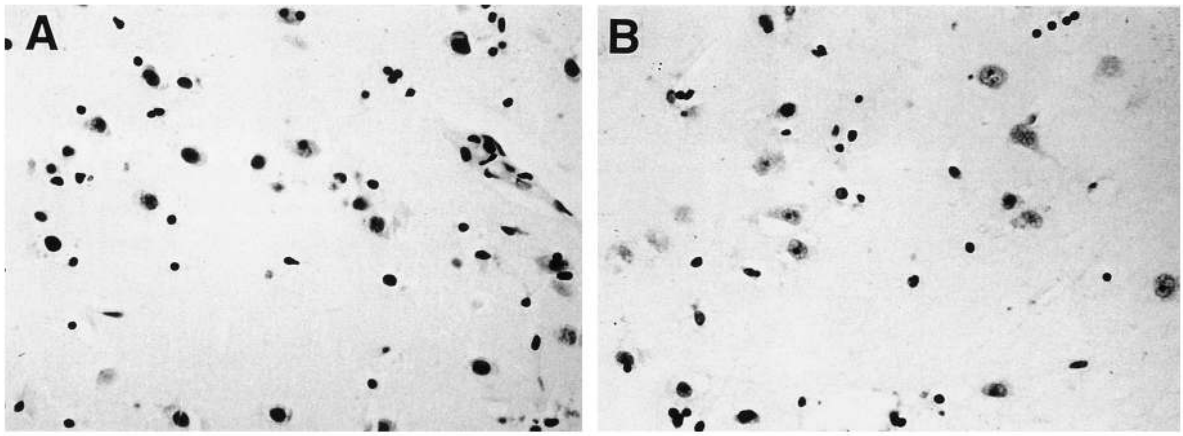

Fig 1. Tail of the caudate nucleus stained with cresyl violet in a Huntington's disease gene carrier (A) and a noncarrier (B). The density of oligodendrocytes is significantly higher in the gene carrier. ×228.

seen with huntingtin immunostaining. No non-nuclear aggregates were noted in these three cases.

Discussion

In a study of 6 presymptomatic HD gene carriers and 10 noncarriers, a significantly increased density of oligodendroglia was found among the carriers. No difference in the densities of neurons, astrocytes, or microglia was found. It appears unlikely that the increased density of oligodendroglia is a consequence of selective sparing of these cells, because the densities of neurons and astrocytes were not different between carriers and noncarriers. Although we cannot rule out that some atrophy in the caudate nucleus has occurred, that oligodendroglia alone are involved makes it unlikely that this observation is a consequence of atrophy among the gene carriers.

The weakness of quantitating densities rather than total number of cells is that the presence of atrophy may "normalize" or mask cell loss.[16] However, the calculation of the total number of cells requires an estimate of the volume of the nucleus, and this cannot be done reliably for the tail of the caudate, because it lacks clear boundaries and because of individual variability in its morphology. Nevertheless, none of the 6 HD carriers showed gross atrophy of the head or tail of the caudate nucleus on careful macroscopic exam. Although the area of cross-section of the tail of the caudate nucleus for 4 of the 10 noncarriers was such that 10 microscope fields could not be accommodated, all of the HD gene carrier specimens could accommodate 10 fields. Thus, although some modest caudate nucleus atrophy may be present among the gene carriers, it is unlikely that it can account for these changes in oligodendroglial density. Finally, previous magnetic resonance imaging studies of presymptomatic HD gene carriers about 15 years prior to disease onset show basal ganglia volumes within 90% of the value for the controls,[17] which suggests that atrophy would be expected to be modest among the 3 carriers in the current study, who were 23 to 35 years before onset of the disease.

The role of the oligodendrocyte is primarily to provide myelin to neuronal axons. The significance of altered oligodendroglial densities is uncertain and may reflect either (1) a gradual modification of the density of this cell type in mid-life or (2) an alteration in the densities of these cells in the formation of the basal ganglia. A doubling in the densities of oligodendroglia among asymptomatic carriers both far from and close to onset suggests that the effects of the HD gene on this cell type occur early in life. The finding that, as the disease progresses, oligodendrocyte density remains almost stable, while that of neurons steadily declines and that of astrocytes increases,[4] suggests that the increase in oligodendroglia is not a response to neurodegeneration and may reflect an alteration in the morphogenesis of the brain in HD gene carriers.

Intranuclear neuronal inclusions are a characteristic pathological feature in disorders caused by CAG expansions.[18] We found that intranuclear ubiquitinated aggregates are present long before the onset of the clinical syndrome and also before neuronal loss. This temporal profile is consistent with findings in transgenic mouse models of HD, which reveal the development of nuclear inclusions accompanied by only subtle neuropathological changes consisting of shrinkage of neurons in the striatum,[19] and that inclusions occur prior to a neurological phenotype.[20] Recent studies suggest that the formation of huntingtin-ubiquitinated inclusions may represent a compensatory protective mechanism. More aggregates are seen in the cortex than in the striatum[21] and within spared NADPH-diaphorase neurons than in the vulnerable calbindin striatal neurons.[22] Furthermore, nuclear aggregations do not

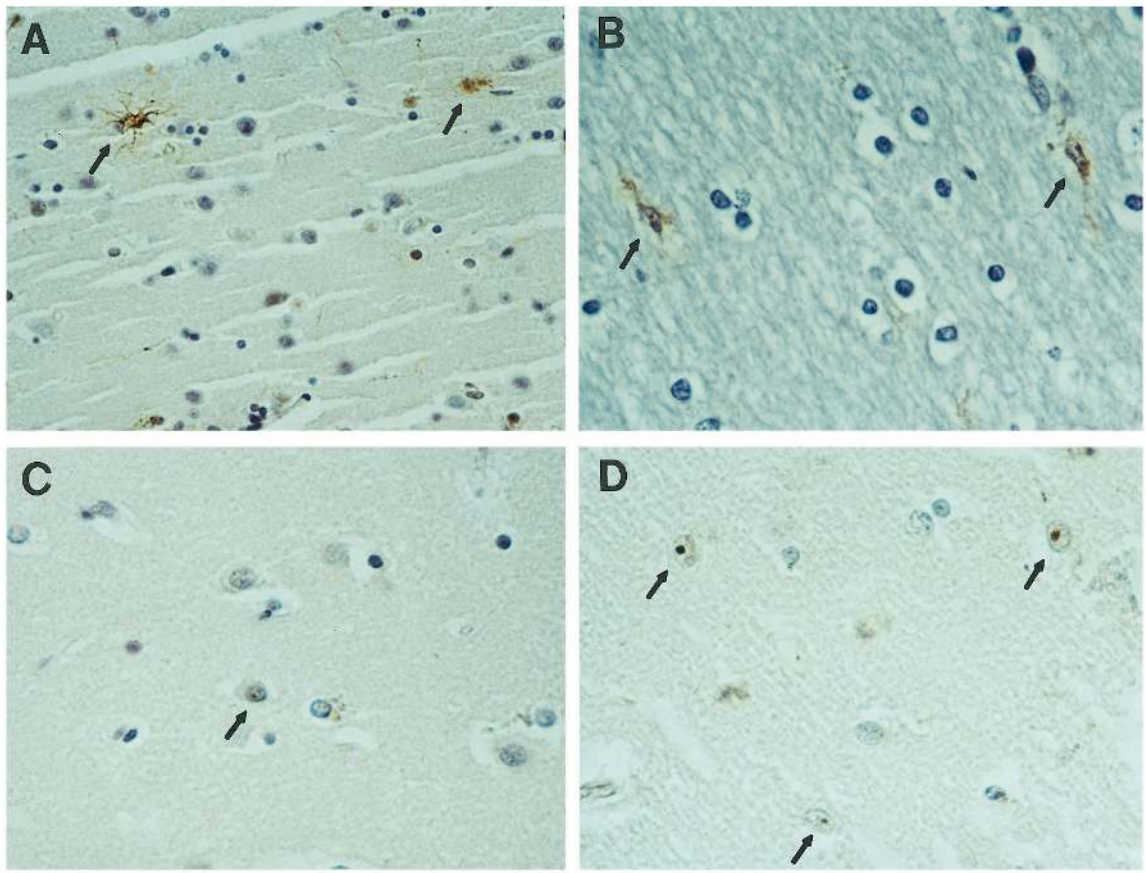

Fig 2. Tail of the caudate in Huntington's disease gene carriers immunostained with antibodies against glial fibrillary acidic protein (GFAP)(A), anti-human B lymphocyte (LN-3)(B) and ubiquitin (C and D). The density of astrocytes (A) and microglia (B) was not different from that of noncarriers. Ubiquitin immunostaining shows a small intranuclear inclusion in a 44-year-old carrier of 37 CAG repeats (case #3) (C) and three clear inclusions in a 30-year-old carrier with 50 CAG repeats (case #6) (D). A, ×225, B–D, ×360.

correlate with huntingtin-induced death[23] and can be disassociated from neuronal death in cell cultures.[24] The presence of nuclear aggregates in the brains of presymptomatic HD carriers, and especially in the case with 37 CAG repeats, dying more than 3 decades before the expected clinical onset, suggests that the brain response to mutant huntingtin starts much earlier than the clinical phenotype.

In summary, the presence of intranuclear inclusions and increased oligodendroglia in presymptomatic carriers long before the expected onset of the clinical phenotype suggests that the pathological process in HD begins early in the life of the gene carrier. As proposed by Penney and coworkers,[25] this process may develop from birth. This study suggests that the impact of the HD gene may follow different temporal sequences in different cell types. Increased oligodendroglia may occur early, even in the morphogenesis of the brain, whereas neuronal death occurs later in the course of the disease. The finding that neuronal density is not altered among presymptomatic carriers suggests that, if mechanisms to protect these cells from the pathologic effects of the gene can be identified, neurological deterioration could be prevented.

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