Selective Nuclear Shrinkage of Oligodendrocytes Lacking Glial Cytoplasmic Inclusions in Multiple System Atrophy: A 3-Dimensional Volumetric Study

Naoto Uyama, MD, Toshiki Uchihara, MD, PhD, Yoko Mochizuki, MD, PhD, Ayako Nakamura, Ryosuke Takahashi, MD, PhD, and Toshio Mizutani, MD, PhD

Abstract
Gliai cytoplasmic inclusions (GCIs) are a pathologic hallmark of multiple system atrophy (MSA), but their pathogenetic roles need to be clarified. To determine possible roles of GCIs in individual cells, serial optical sections obtained by confocal microscopy were reconstructed to yield 3-dimensional (3D) images of the nuclei to quantify nuclear volume. Oligodendroglial nuclear volumes were determined in the pons of 6 MSA and 7 control patients. The nuclear volumes were significantly smaller in the MSA group as a whole (135.81 ± 60.83 µm³, mean ± SD; n = 404) than in the control group (188.05 ± 55.71 µm³; n = 308; p < 0.001). This difference was due to a significantly smaller nuclear volume of oligodendrocytes without GCIs (GCI− group, 91.26 ± 23.77 µm³; n = 210) compared with the control group (p < 0.001) and compared with the oligodendrocytes with GCIs (GCI+ group, 184.03 ± 51.18 µm³; n = 194; p < 0.001); the difference between the latter GCI+ and control groups was not significant (p > 0.05). This selective decrease in nuclear volume restricted to the GCI+ group cannot be explained if nuclear shrinkage was accelerated in the presence of GCIs. Conversely, GCI formation might be linked, either directly or indirectly, to a mechanism that counteracts rather than accelerates nuclear shrinkage. This novel 3-dimensional strategy provides pivotal data that link GCI formation and degeneration in MSA.

Key Words: Gliai cytoplasmic inclusions, Multiple system atrophy, Nuclear volume, Three-dimensional reconstruction.

INTRODUCTION
Multiple system atrophy (MSA) (1) is characterized by the distribution of degenerative lesions and the presence of glial cytoplasmic inclusions (GCIs) (2, 3). Because GCIs are a pathologic hallmark of MSA, they are believed to be tightly linked to the pathogenetic mechanism of MSA; numerous studies have attempted to clarify possible relationships between the presence of GCIs and the extent and severity of degeneration (4–7). Reported data are, however, highly variable, and none of the previous neuropathologic studies have established a pathogenetic role for GCIs. Although it has been reported that nuclei of GCI-bearing oligodendrocytes (GCI+) usually appear larger than those of oligodendrocytes that do not harbor GCIs (GCI−) (8, 9), it is unclear whether the presence of GCIs is linked to accelerated neurodegeneration. Because the degenerative cascade of MSA leads both to GCI formation and cellular degeneration, the morphologic parameters (e.g. nuclear volume) of an individual oligodendrocyte might be influenced by the formation of GCIs. In this study, we focus on the nuclear volume of oligodendrocytes with or without GCIs. Instead of using conventional indirect methods of volume estimation (10–12), we measured the actual nuclear volume of each oligodendrocyte reconstructed into a 3-dimensional (3D) image from serial optical sections (~200 sections at an interval of 0.1 µm) spanning the entire oligodendrocyte. This straightforward method has been used mainly in the field of diagnostic radiology and provides estimates of the real volume regardless of the shape of the targets (13–15) and is readily applicable to serial data obtained with confocal microscopy (16). This is the first successful attempt to measure directly the actual nuclear volume of oligodendrocytes. Because a decrease in nuclear volume relative to the control was significant only in GCI− oligodendrocytes but not in GCI+ oligodendrocytes, it is possible to conclude that nuclear shrinkage is accelerated in the absence of GCIs.

MATERIALS AND METHODS
Six patients with the clinical and neuropathologic diagnosis of MSA (3 men, 3 women; age range at death, 54–74 years) autopsied at the Department of Pathology, Tokyo Metropolitan Neurological Hospital, Tokyo, Japan, and 7 control patients (6 men, 1 woman, age range at death, 47–84 years) with no...
disease involving the central nervous system were studied. The durations of MSA ranged from 4 to 7 years (Table 1). The diagnosis of MSA was confirmed postmortem on the basis of neuronal loss and the presence of GCIs in relevant regions, including the putamen, cerebellar white matter, and motor cortex (4). Oligodendrocytes in pontine transverse fibers were chosen for analysis because the size and appearance of oligodendrogial nuclei were relatively homogeneous, which made it easier to quantify and interpret their morphologic changes. Moreover, the high frequency of GCIs in the pons of MSA brains is another advantage in estimating their possible effects.

Formalin-fixed blocks were obtained from the midpons of MSA and control brains, washed in 0.1 mol/L phosphate buffer 3 times, then kept in 15% sucrose/0.1 mol/L phosphate buffer solution until they sank. Free-floating sections 30 μm thick were prepared on a freezing microtome and kept at 4°C for 5 minutes at room temperature. The sections were then immersed in 0.01 mol/L PBS containing 0.3% Triton X-100 twice the diameter of nuclei) along transverse fibers and with linear alignment in the stretch of 5 to 10 contiguous oligodendrocytes along the long axis of nerve fibers. Larger nuclei of astrocytes or small angular nuclei of microglia (18) (both not in linear alignment) were easily distinguishable. Only cells with round nuclei serially lined in proximity (within twice the diameter of nuclei) along transverse fibers and with comparatively uniform size in the stretches were identified as

### TABLE 1. Demographic Features of Patients With MSA and Controls

<table>
<thead>
<tr>
<th>Case</th>
<th>Age of Onset/Death, Years</th>
<th>Sex*</th>
<th>Cause of Death</th>
<th>Brain Weight, g</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSA 1</td>
<td>47/54</td>
<td>F</td>
<td>Sudden death</td>
<td>1,310</td>
</tr>
<tr>
<td>MSA 2</td>
<td>51/58</td>
<td>M</td>
<td>Pneumonia</td>
<td>1,318</td>
</tr>
<tr>
<td>MSA 3</td>
<td>52/59</td>
<td>F</td>
<td>Pulmonary insufficiency</td>
<td>1,230</td>
</tr>
<tr>
<td>MSA 4</td>
<td>57/61</td>
<td>F</td>
<td>Pulmonary insufficiency</td>
<td>1,296</td>
</tr>
<tr>
<td>MSA 5</td>
<td>63/69</td>
<td>M</td>
<td>Cardiac insufficiency</td>
<td>1,225</td>
</tr>
<tr>
<td>MSA 6</td>
<td>70/74</td>
<td>M</td>
<td>Pulmonary insufficiency</td>
<td>1,285</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>56.7 ± 8.5/62.5 ± 7.5†</td>
<td></td>
<td></td>
<td>1,277.3 ± 40.3‡</td>
</tr>
</tbody>
</table>

*No significant difference between control and MSA groups (by Fisher exact test).
†p < 0.05 compared with control group (by Student t-test).
‡p > 0.05 compared with control group (by Student t-test).
CIDP, chronic inflammatory demyelinating polyradiculoneuropathy; F, female; M, male; MSA, multiple system atrophy.

### TABLE 2. Nuclear Volume of Oligodendroglia in MSA and Control Cases

<table>
<thead>
<tr>
<th>Oligodendrocyte</th>
<th>Control Cases, n = 7</th>
<th>Total Oligodendrocytes in MSA Cases, n = 6</th>
<th>Oligodendrocytes in MSA Cases With GCI</th>
<th>Oligodendrocytes in MSA Cases Without GCI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume (mean ± SD), μm³</td>
<td>188.05 ± 55.71</td>
<td>135.81 ± 60.83*</td>
<td>184.03 ± 51.18‡</td>
<td>91.26 ± 23.77‡‡</td>
</tr>
</tbody>
</table>

*No significant difference compared with control group (by 1-way ANOVA).
†No significant difference compared with control group (by 1-way ANOVA)
‡p < 0.001 compared with control group (by 1-way ANOVA).
‡‡p < 0.001 compared with GCI+ group (by 1-way ANOVA).
GCI, glial cytoplasmic inclusion; MSA, multiple system atrophy.

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oligodendrocytes. Serial optical sections at an interval of 0.1 μm were obtained to encompass the entire structure of each oligodendrocyte. Usually, 200 serial optical sections were sufficient to encompass the entire structure of an oligodendrocyte, including GCIs when they were present. Each 3D image data set consisted of a series of 2-dimensional (2D) images (Fig. 1 and Video, Supplemental Digital Content 1, http://links.lww.com/NEN/A60). Each image frame had a 63.5-μm field of view with a 512 × 512-pixel array, yielding 0.124 × 0.124-μm/pixel dimensions. The data were reconstructed for 3D observation and quantification by using software (TRI/3D SRFII-64 Release 4, Ratoc, Tokyo, Japan) running on the Windows platform on a 64-bit basis. To estimate nuclear volume with this software, the 2D contour of each nucleus, along the nuclear membrane labeled with 4,6-diamidino-2-phenylindole, dihydrochloride, was traced manually on some of the 2D images every 4 to 5 images. The relative position of the manually defined contour and the fluorescence intensity of 4,6-diamidino-2-phenylindole, dihydrochloride were used to calculate automatically the nuclear contour in intervening optical sections; these were later confirmed by inspection. This yielded the binary representation of the nuclear contour of an oligodendrocyte on a 3D basis consisting of noncubic voxels (0.124 × 0.124 × 0.1 μm). Subsequently, after discarding spurious voxels from the binary oligodendroglial nucleus representation, these data sets were reconstructed for 3D observation and quantification (Video, Supplemental Digital Content 2, http://links.lww.com/NEN/A61), and the total number of voxels, which substitute the nucleus, was obtained on the basis of a known voxel size of 0.0015376 μm³, yielding the actual volume size (13–16).

Statistical analyses were performed using Dr. SPSSII for Windows software (SPSS Japan, Tokyo, Japan). Size distributions according to nuclear volume were estimated by the Kolmogorov-Smirnov method to determine whether they were compatible with the normal distribution in each patient or group (control, MSA total [GCI+ and GCI−], GCI+, GCI−). Because the size distribution of the MSA total group did not exhibit normality, its comparison with that of the control group was estimated using the Mann-Whitney U test with Bonferroni correction. Otherwise, the 1-way analysis of variance (ANOVA) was used to compare nuclear volume among the different groups when nuclear sizes exhibited a normal distribution.

RESULTS

There were no significant differences among groups with respect to age or brain weight (by Student t-test) or sex (by Fisher exact test; Table 1). The nuclear volume size distributions are shown in Table 2 and Figures 2 and 3. Because the size distributions showed normal distributions (as determined by the Kolmogorov-Smirnov test when each patient or each group was analyzed), our sampling and volumetric analysis procedure yielded data representative of a homogenous cell group not only in each patient but also in each group. The quantile-quantile plots of data of the GCI+, GCI−, and control groups show almost linear distributions.
along the lines representing the normal distribution (Fig. 4). Although the control group exhibited a normal size distribution with a single peak, the data from the MSA total group (consisting of GCI+ and GCI− groups) were not in a normal distribution. The nuclear volumes were significantly smaller in the MSA total group (135.81 ± 60.83 μm³; mean ± SD; n = 404) compared with the control group (188.05 ± 55.71 μm³; n = 308; p < 0.001 by the Mann-Whitney U test with Bonferroni correction). This difference was due to the significantly smaller nuclear volume of GCI− than of the control, whereas the nuclear volume of GCI+ was equivalent to that of the control. The technical novelty of this study includes 3D reconstruction of target structures (nuclei), followed by direct quantification of the entire structures.

Routine evaluation of histologic sections is usually performed on 2D basis on thin-sample specimens in which cell loss is perceived as a decrease in local density and atrophy as the volume loss of the cell body. One-dimensional indices such as diameter and perimeter are ready candidates for representing structure sizes, but sophisticated 2D indices such as cross-sectional area of the structures allow more precise determinations of their size. Using 2D quantification of neuronal size in the substantia nigra, Ma et al (19) demonstrated a 3.2% decrease per decade, whereas Cruz-Sánchez et al (20) reported progressive swelling with aging by simple comparisons of images. Similar discrepancies

![FIGURE 2. Histograms of size distribution of oligodendroglial nuclei. The nuclear volumes in cubic microns are expressed on the abscissa. The ordinate represents the relative frequency of calculated nuclear size. The lower inverted histograms represent the controls (unfilled bars with black lines) and the multiple system atrophy (MSA) group as a whole (MSA total group, bars filled with gray); the latter shows neither unimodal (single peak) nor bimodal (double peak) curves. The upper histograms indicate the MSA group with glial cytoplasmic inclusions (GCIs) (GCI+ group, bars filled with gray) or without GCIs (GCI− group, unfilled bars with black lines).](http://jnen.oxfordjournals.org/)

![FIGURE 3. Comparison of size distribution of oligodendroglial nuclei from each group. Box plots show the nuclear volumes for each group (control; glial cytoplasmic inclusion [GCI]+, oligodendrocytes with GCIs from multiple system atrophy [MSA] cases; GCI−, oligodendrocytes without GCIs from MSA cases, MSA total, all oligodendrocytes from MSA cases). The boxes enclose the middle half of the values bounded by upper and lower quartiles. The bold lines represent the medians. The lower whisker indicates the smallest nonoutlier observation; the upper whisker indicates the nonoutlier observation. *, p < 0.001 when compared with control group (by the Mann-Whitney U test; Bonferroni correction); †, p < 0.001 when compared with control group (by 1-way analysis of variance [ANOVA]); ‡, NS, no significant difference when compared with control group (by 1-way ANOVA); §, p < 0.001 when compared with GCI+ group (by 1-way ANOVA).](http://jnen.oxfordjournals.org/)
among the studies have also been observed in Parkinson disease (21, 22), suggesting that 2D representation of 3D reality into a single plane is not sufficient.

Various stereologic methods have been developed by synthesizing 2D data sets to estimate volumes (10–12). These methods essentially include 2 steps: theoretically unbiased sampling of 2D raw data set and virtual estimation of the volume. Although still indirect, these innovative strategies made it possible to estimate the volume on a 3D basis. Even with these 3D quantification methods, however, Rudow et al (23) reported an age-associated increase of neuronal size on multiple system atrophy (MSA), open squares; control, gray crosses. Q-Q, quantile-quantile.

Changes in Relation to Aging and Degeneration

Table 3. Three-Dimensional Volume Estimates and Their Changes in Relation to Aging and Degeneration

<table>
<thead>
<tr>
<th>Aging</th>
<th>Overall Change, %</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AD ACG, PCG, CA1, PVC nuclei, neuron</td>
<td>Hypertrophy in CA1/ACG of asymptomatic AD</td>
<td>26</td>
</tr>
<tr>
<td>PD SN neuron*</td>
<td>Reduction</td>
<td>23</td>
</tr>
<tr>
<td>ALS MC nuclei, neuron†</td>
<td>No difference</td>
<td>27</td>
</tr>
<tr>
<td>TLE TC neuron*</td>
<td>+28 in GM,+55 in WM</td>
<td>28</td>
</tr>
<tr>
<td>Alcoholics Purkinje cell/nuclei, neuron†</td>
<td>−24 in cell body, −16 in nuclei</td>
<td>29</td>
</tr>
</tbody>
</table>

*The nuclear method and its modifications.
†The rotator method and its modifications.

ACG, anterior cingulate gyrus; AD, Alzheimer disease; ALS, amyotrophic lateral sclerosis; CA1, Cornu ammonis 1; GM, gray matter; m+, melanin-positive; MC, motor cortex; NC, neocortex; PCG, posterior cingulate gyrus; PD, Parkinson disease; PVC, primary visual cortex; SN, substantia nigra; TC, temporal cortex; TH+, tyrosine hydroxylase–positive neuron; TLE, temporal lobe epilepsy; WM, white matter.
shrinkage in aging and neurodegenerative processes. As expected, therefore, we demonstrated that the nuclear volumes of oligodendroglia in MSA cases as a whole are significantly reduced. Table 3 shows the summary of indirect 3D estimation of the volume of the nucleoli/nuclei/cell and their relation to aging and diseases; neurodegenerative processes are not necessarily related to volume reduction of neurons (26–28). Because these data were obtained without distinguishing the presence or absence of pathologic inclusions, however, how the disease-related changes identified are linked to pathologic inclusions is unclear. Indeed, several 2D morphometric studies have focused on the size of individual cells to identify cellular changes and their relationships to pathologic inclusions (Table 4). For example, transected nuclear areas of pontine neurons harboring nuclear inclusions are uniformly larger than those not harboring nuclear inclusions (32–34). Two-dimensional observations of a thin histologic plane may, however, not identify inclusions within the cell that are not present in the histologic plane. Our 3D reconstruction analysis spans the entire target structure, including pathologic inclusions, and therefore definitively determines whether or not pathologic inclusions are present. Although the relationships between disease-related changes and inclusion-related changes remain to be clarified, it is now possible to quantify 3D changes of each cell without bias after definitively determining the presence or absence of pathologic inclusions.

It is not surprising that the pathologic inclusions in MSA are related to a larger cell/nuclear size or a lesser extent of degeneration. There are similar examples in cell culture models (36, 37), transgenic animals (38, 39), and a variety of human conditions (30–35). It is not clear whether oligodendrocytes with and without GCI undergo completely different degenerative processes or whether they represent different aspects of a shared process. Because the distribution of the nuclear volume of MSA oligodendrocytes as a whole was neither unimodal (single peak) nor bimodal (double peaks; Fig. 2), it is likely that the GCI⁺ and GCI⁻ groups undergo some common mechanism of the degeneration. The selective decrease in nuclear volume restricted to the GCI⁺ group cannot be explained if the nuclear shrinkage is accelerated in the presence of GCIs. Preserved nuclear volume of GCI⁺ oligodendrocytes does not necessarily imply that they are free from the degenerative process.

Some studies have attempted to simulate the oligodendroglial pathology of MSA. For example, overexpression of human α-synuclein leads to the cytotoxicity in cultured oligodendrocytic progenitor cells (40), and transgenic mice that overexpress human wild-type α-synuclein show progressive locomotor dysfunction; histlogic analyses showed the accumulation of detergent-insoluble α-synuclein similar to GCIs (41, 42) and system-oriented degeneration but with some differences from MSA (43, 44). Numerous studies of MSA patient brains have attempted to find possible correlations between GCIs and degeneration. In MSA, GCIs show a “system-oriented distribution in the supraspinal motor systems (i.e. primary motor and higher motor areas of the cerebral cortex, pyramidal, extrapyramidal, and corticocerebellar systems) in the supraspinal autonomic systems and in their targets” (4), and changes in neurons follow a similar distribution pattern (2, 4, 6). Nevertheless, the density of GCIs and the severity of neuronal loss and their possible correlations claimed by previous studies are highly conflicting (2, 4, 6, 7, 45). Apoptosis-related proteins, including Bax (46) and activated caspase 3 (47), are reportedly present mainly in oligodendrocytes, often showing α-synuclein–positive inclusions (47). Interestingly, the cytoplasmic expression of Bcl-2, a putative anti-apoptotic protein, has been reported in oligodendrocytes with α-synuclein coexpression in approximately 25% of GCI⁺ cells (46).

Although the major question of whether GCIs are cytotoxic or cytoprotective remains to be answered, apoptosis with the morphologic feature of nuclear shrinkage (48) is considered to account for at least some of the oligodendroglial death in MSA (46, 47). In Parkinson disease and dementia with Lewy bodies (other degenerative processes with α-synuclein deposits [49, 50]), it is hypothesized that the aggregation of α-synuclein prevents or delays neuronal degeneration possibly by sequestering toxic protein species (51, 52). It remains to be determined whether a similar scenario may also explain the role of aggregation of α-synuclein as GCIs in MSA. The relative preservation of nuclear volume with GCI and accelerated shrinkage in the absence of GCIs in human brain are compatible with this possibility.

It is now possible to analyze the pathologic expression of specific molecules in relation to nuclear size. Molecular mechanisms bridging cellular/nuclear atrophy and GCI formation or other pathologic deposits are now candidate subjects for further study in a quantitative fashion rather than a “black and white” dualism such as neuronal death versus survival. The novel strategy for human brain studies herein provides a pivotal viewpoint regarding the pathologic significance of GCIs and will be applicable to degenerative processes other than MSA.

**TABLE 4. Two-Dimensional Area Quantification Studies and Their Dependence on Index Lesions**

<table>
<thead>
<tr>
<th>Target + Index Lesion/Area</th>
<th>Change of Area Dependent on the Index Lesion, %</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PD</td>
<td>SN neuron ± LBs/nucleus, neuron</td>
<td>No difference</td>
</tr>
<tr>
<td>AD</td>
<td>Hippocampal neuron ± NFTs/nucleus, neuron</td>
<td>No difference</td>
</tr>
<tr>
<td>MJD</td>
<td>Pontine neuron ± NIs/nucleus</td>
<td>−21% with NAs, −41% without NAs</td>
</tr>
<tr>
<td>NIID</td>
<td>Pontine neuron ± NIs/nucleus</td>
<td>+22% with NIs, −14% without NIs</td>
</tr>
<tr>
<td>SCA1</td>
<td>Pontine neuron ± NIs/nucleus</td>
<td>No difference with NIs, −12% without NIs</td>
</tr>
<tr>
<td>DRPLA</td>
<td>Cerebellar granule cell ± NIs/nucleus</td>
<td>Larger with NIs</td>
</tr>
</tbody>
</table>

[Note: The table entries are placeholders for actual data, as the provided text is incomplete.]

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