Ectopic Expression of Musashi-1 in Alzheimer Disease and Pick Disease
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Abstract
Abnormal accumulation of proteins in filamentous cytoplasmic inclusions is a hallmark of several neurodegenerative disorders, including Alzheimer disease (AD) and Pick disease (PD). Musashi-1 (Msi-1), an RNA-binding protein associated with neural progenitor cells, has been shown by others to increase the accumulation of tau isomers in intracellular inclusions in frontotemporal dementia and parkinsonism linked to chromosome 17. We investigated the expression of Msi-1 in the hippocampus of AD, PD, and aged normal control subjects using immunohistochemistry. Comparison of immediately adjacent serial sections stained using the modified Bielschowsky method and immunostained for Msi-1 showed that Msi-1 was present in 83 ± 6% of neurofibrillary tangle bearing neurons in AD and 94 ± 14% of Pick bodies in PD specimens. Aged control hippocampus demonstrated virtually no Msi-1 immunostaining. The presence of Msi-1 in a significant percentage of neurons containing cytoplasmic inclusions in 2 different neurodegenerative diseases suggests that it may play a role in the pathogenesis of these lesions.

Key Words: Alzheimer disease, Neurofibrillary tangles (NFT), Pick bodies, tau.

INTRODUCTION
Musashi-1 (Msi-1) is a neural, RNA-binding protein with 2 conserved RNA recognition motifs with a preference for G/A(U) nAGU sequences (1). Musashi-1 is highly expressed in neural progenitor cells (NPC) (2–6) and is widely used as a positive identifier of NPC. In Drosophila, musashi is required for 2 successive asymmetric divisions of sensory organ precursor cells (7). In mammals, Msi-1 expression is enriched in NPC in embryonic central nervous system (3, 6, 8) and is generally downregulated during neural differentiation. Msi-1 is not present in newly generated postmitotic neurons (6), although Msi-1 transcripts have been detected in the early postnatal brain and in adults in proliferating neuronal or glial precursor cells of the subventricular zone (SVZ) of the hippocampus and in cells of an astrocyte lineage (6).

The selective expression of Msi-1 in the cytoplasm of NPC (3, 6) suggests that it may help to maintain cells in an undifferentiated state during posttranslational gene regulation, perhaps by translationally repressing synthesis of Numb, a Notch antagonist (9–11), thus regulating neuronal differentiation (12). In addition, ectopic expression of Msi-1 in N2a cells and in experimental mouse models led to increased production and accumulation of tau transcripts containing exon-10 (13) that is characteristic of intracellular inclusions observed in the brains of subjects with frontotemporal dementia and parkinsonism linked to chromosome 17 (14–17).

Based on these observations, we carried out immunohistochemical studies to determine if Msi-1 protein is observed in lesion-bearing neurons in the brains of patients with Alzheimer disease (AD) and Pick disease (PD) compared with aged normal control subjects.

MATERIALS AND METHODS
Case Selection and Neuropathologic Examination
Serial sections of paraffin-embedded hippocampus were obtained from 5 AD, 4 PD, and 5 aged normal control subjects through the neuropathology core of the University of Kentucky Alzheimer’s Disease Research Center (UK-ADRC). All subjects had annual mental status testing and physical and neuropsychologic examinations. All AD subjects demonstrated progressive intellectual decline and met NINCDS-ADRDA Workgroup (18) criteria for the clinical diagnosis of probable AD. Histopathologic examination of multiple sections of neocortex, hippocampus, entorhinal cortex, amygdala, basal ganglia, nucleus basalis of Meynert, midbrain, pons, medulla, and cerebellum using hematoxylin and eosin and the modified Bielschowsky stains along with 10D-5 (for Aβ) and α-synuclein immunohistochemistry were carried out on all subjects. All patients with AD met accepted criteria for the histopathologic diagnosis of AD (19, 20), typically demonstrated Braak scores of VI, and met high likelihood NIA-Reagan Institute criteria for the histopathologic diagnosis of AD. Control subjects were derived from the longitudinal control population followed by the UK-ADRC. Control subjects demonstrated Braak scores of I or II and met NIA/Reagan low likelihood criteria for the histopathologic diagnosis of AD. All patients with PD showed...
typical clinical features of frontotemporal dementia (personality, emotional, and behavioral alterations, decline in executive function, and subsequent language changes). Frontal and temporal lobe atrophy was observed at autopsy, and microscopic examination showed neuron loss, abundant Pick bodies, and, in most cases, rare balloon neurons. Subject demographic data are shown in the Table.

**Immunohistochemical Staining and Quantitative Comparisons**

Serial sections (10 μm) of paraffin-embedded hippocampus/parahippocampal gyrus (HPG) from 5 AD, 4 PD, and 5 aged normal control subjects were cut using a Shandon Finesse microtome, placed on Plus-slides, and rehydrated through xylene, descending alcohols, and water. Section 1 of the series was stained using the modified Bielschowsky method. The adjacent section was used for Msi-1 immunohistochemistry. The sections were rehydrated to water and incubated for 10 minutes in a 0.75-mg/mL trypsin solution prepared in 150 mM Tris HCl (pH 7.6) containing 3.3 mM CaCl2 at 37°C. After 3 rinses in phosphate-buffered saline, the sections were immunostained using the Dako Envision System (Dako, Carpinteria, CA) per manufacturer’s instructions. Briefly, endogenous peroxidase activity was blocked by incubation for 5 minutes in 3% hydrogen peroxide/methanol. After 3 washes in water and Tris-buffered saline containing 0.1% Tween-20 (TTBS), the sections were incubated for 30 minutes in a 1:100 dilution of rabbit polyclonal raised against amino acids 5 through 21 of human Msi-1 (Chemicon International, Temecula, CA) in TBS containing 1% fetal bovine serum. After 3 to 5 washes in TBS containing 0.1% Tween-20 (TTBS), the sections were incubated in peroxidase-labeled polymer conjugated to goat antirabbit IgG in Tris-HCl total) of SDS loading buffer containing 15% v/v glycerol, mercaptoethanol, and 0.1% bromophenol blue and boiled 5 minutes. The samples were cooled to room temperature and passed through progressively smaller pipette tips, and plated as free-floating single-cell suspensions. The cells were pelleted by centrifugation at 850 x g for 10 minutes and homogenized in 300 μL distilled/deionized water using a micro-Dounce homogenizer. Homogenates of HPG from representative AD and control subjects were prepared by homogenizing ~100 mg tissue in 5 mL phosphate-buffered saline using a chilled Dounce homogenizer. For Western blot analysis, aliquots of protein (20 μg) were mixed with an equal volume (40 μL total) of SDS loading buffer containing 15% v/v glycerol, 0.125 M Tris HCl (pH 6.8), 5 mM Na2EDTA, 2% SDS, 1% 2-mercaptoethanol, and 0.1% bromophenol blue and boiled 5 minutes. The samples were cooled to room temperature and separated on a 4% to 20% gradient SDS-PAGE gel (Bio-Rad, Hercules, CA) and transferred to nitrocellulose. The gels were blocked overnight in 5% dry milk/TTBS and were probed with Msi-1 at a dilution of 1:500. To further verify specificity of the antibody, serial sections of hippocampus from a representative AD subject were immunostained using the antibody as described previously and also using antibody that had been preincubated with immunizing peptide (APQPGLASPDSHPDCK) for 24 hours.

Counts of neurofibrillary tangles (NFTs) and Pick bodies were performed by taking serial photographs of immediately adjacent Bielschowsky-stained sections and Msi-1 immunostained sections of all of the CA1 and subiculum of each case using a 20× objective on a Nikon Eclipse E600 microscope. These micrographs were printed on 8” × 10” paper and each margin defined so as not to allow any overlap. Each NFT and Pick body was circled and counted with the naked eye.

**TABLE. Subject Demographics**

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Sex</th>
<th>PMI (hours)</th>
<th>Braak Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>AD</td>
<td>82.8 ± 4.2</td>
<td>3 M/2 F</td>
<td>3.1 ± 0.3</td>
</tr>
<tr>
<td>PD</td>
<td>66.3 ± 0.8*</td>
<td>3 M/1 F</td>
<td>9.5 ± 3.0*</td>
</tr>
<tr>
<td>Control</td>
<td>81.0 ± 1.5</td>
<td>3 M/2 F</td>
<td>2.9 ± 0.3</td>
</tr>
</tbody>
</table>

* p < 0.05, 2-tailed t-test.
AD, Alzheimer disease; PD, Pick disease; PMI, postmortem interval.

**Statistical Analysis**

Age, postmortem interval (PMI), and Braak scores were compared using a 2-tailed t-test and ABSTAT software (AndersonBell, Arvada, CO).

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RESULTS

Subject Demographics

Statistical comparison of age, PMI, and Braak scores (Table) showed no significant differences in age between AD and control subjects. Pick disease subjects showed a statistically significant (p < 0.05) lower age (66.3 ± 0.8 years) compared with control subjects (82.8 ± 4.2 years). Pick disease subjects showed a significantly higher PMI (9.5 ± 3.0 hours) compared with control subjects (2.9 ± 0.3 hours). There were no other significant differences in PMI. Braak scores were significantly higher (6 ± 0) in AD subjects compared with control subjects. There were no differences in Braak scores for PD subjects compared with control subjects.

Verification of Msi-1 Antibody Specificity

Western blot analysis of 20-μg aliquots of NPC from AD brain and crude homogenates of AD and control HPG probed for Msi-1 showed a predominate band at ~39 kDa consistent with the molecular weight of Msi-1 in NPC. Analysis of crude homogenate from AD and control HPG showed the presence of a band at ~39 kDa and a second band above 85 kDa that may represent aggregated protein. Figure 1 demonstrates that Msi-1 expression is most pronounced in NPC, although there is minimal staining present in crude homogenate of AD brain. To verify specificity of the antibody, sections of a representative AD subject were immunostained using antibody alone and antibody that had been preincubated with immunizing peptide. The immunizing peptide completely blocked Msi-1 immunostaining confirming specificity of the antibody (Fig. 2).

Alzheimer Disease

Sections immunostained with Msi-1 antibody revealed staining of NFT-bearing neurons throughout CA1, subiculum (Fig. 3 and inset), and the parahippocampal gyrus. Immunostaining was present in typical flame-shaped, globular, and hemispeckled NFT. Rare ghost tangles were Msi-1-positive but most were not stained. The staining suggested a filamentous nature of some NFT-bearing neurons, whereas in others, a microgranular appearance was found. In some partially stained neurons, there was a granular character of the staining in the cytoplasm. The nuclei of NFT-bearing neurons were not immunostained. Glial cells were not Msi-1-positive. Although most senile plaques (SP) were not immunopositive, a few neurites of rare neuritic SP in the subiculum were Msi-1-positive. Neuropil threads did not label with the Msi-1 antibody. Counts of NFT-bearing neurons on immediately adjacent sections stained with Msi-1 and the modified Bielschowsky stain showed that Msi-1 immunostained 83 ± 6% of NFT-bearing neurons stained with the Bielschowsky method.

Pick Disease

In Pick disease, immunostaining with Msi-1 showed that abundant Pick bodies were positive in the granule neurons of the dentate gyrus (Fig. 4), CA1, subiculum (Fig. 5), parahippocampal gyrus, and inferior temporal gyrus. The overall regional pattern and staining of individual Pick bodies were similar in adjacent Msi-1 and modified Bielschowsky-stained sections. Most Pick bodies were seen as discreet, round, densely staining cytoplasmic inclusions with sharp borders filling much of the neuron cytoplasm. They were especially abundant in the granule neurons of the dentate gyrus. In the parahippocampal and inferior temporal gyri, they were most prominent in lamina II. The nuclei of neurons containing Pick bodies showed no immunostaining. Counts of Pick bodies in the CA1 and subiculum of adjacent sections stained with Msi-1 and the Bielschowsky stain showed that 94 ± 14% of Pick bodies stained with the modified Bielschowsky stain were immunopositive for Msi-1.
DISCUSSION

Based on the observation that Msi-1 regulates tau RNA maturation in experimental animals and leads to increased accumulation of tau transcripts similar to those observed in filamentous cytoplasmic inclusions in familial frontotemporal dementia and parkinsonism linked to chromosome 17 (14), a tauopathy, we carried out immunohistochemical studies to determine if Msi-1 is increased in neurons containing filamentous cytoplasmic inclusions in other tauopathies (AD and PD). Our results demonstrate that the Msi-1 antibody specifically labels Msi-1 localized in 83 ± 6% of NFT-bearing neurons in 5 AD subjects and 94 ± 14% of Pick bodies in 4 PD subjects. Immunostaining of control brain showed only minimal labeling of neurons.

Because Msi-1 is generally considered a marker of NPC, the observation of this protein in neurons containing cytoplasmic inclusions in AD and PD was initially surprising. Msi-1 consists of 362 amino acids and contains 2 conserved RNA recognition motifs in the N-terminal half of the protein and a nuclear export signal in the C-terminal half of the protein (3, 6). Msi-1 is highly evolutionarily conserved and was originally described in Drosophila where it is required for 2 successive asymmetric cell divisions of sensory organ precursor cells (3). In mammals, Msi-1 expression is enriched in the cytoplasm of proliferating multipotent NPC in embryonic CNS (3, 6, 8) and currently serves as a characteristic identifier of NPC. In mice, Msi-1 expression gradually downregulates during neural differentiation (24) with low levels of expression in adulthood (6). In a detailed study, Sakakibara et al showed Msi-1 protein in proliferating neural or glial precursor cells in the SVZ of the hippocampus and in cells of an apparent astrocyte lineage in postnatal and adult central nervous system (6). Msi-1 expression was observed in early (P3) postnatal brain in tightly packed round cells of the SVZ composed of PCNA-positive proliferating cells and in the posterior region of the SVZ that contains glial precursor cells (25). In adult rodent hippocampus, no cells of the CA1/CA2 or CA3 were immunopositive for Msi-1, although weak staining was observed in the dentate gyrus, hilus, and stratum radiatum (8), presumably arising from NPC.

FIGURE 3. Section of subiculum immunostained with Msi-1 antibody showing abundant neurofibrillary tangles (200×). Inset shows higher magnification of neurofibrillary tangles immunostained with Msi-1 antibody.

FIGURE 4. (A) Section from the dentate gyrus of a patient with Pick disease immunostained with Msi-1 antibody. Note multiple Pick bodies in cytoplasm of dentate neurons. (B) The adjacent area of the same case stained with the Bielschowsky stain. Note the multiple Pick bodies.
Studies of Imai et al show that Msi-1 overexpressed in NIH 3T3 cells is localized to ribosomes in the cytoplasm of NPC and that Msi-1 comigrates with polysomes and ribosome units during sucrose gradient centrifugation (24). The selective expression of Msi-1 in the cytoplasm of NPC suggests that it does not play a role in re-mRNA splicing but rather translationally regulates expression of downstream genes (24) and also suggests that it may help maintain cells in an undifferentiated state during posttranslational gene regulation (26), perhaps through interaction with Numb, a negative regulator of Notch-1.

Numb RNA has a 3’ Msi-1-binding site (24) and Numb protein expression is translationally repressed by Msi-1. Because Numb serves as a Notch-1 antagonist (10, 11, 15, 27), it is hypothesized that Msi-1 may serve as a positive regulator of Notch-1 signaling. Studies of Imai et al (24) show that overexpression of Msi-1 in NIH 3T3 cells activates Notch-1 signaling through a pathway dependent on action of the PRP/JK transcription factors of the CSL family that forms complexes with the intracellular domain of Notch-1 in the nucleus (28). Notch signaling induces self-renewal of NPC (29, 30), although hyperactivation of Notch may lead to apoptosis (26). A recent study by Ishikura et al showed that Notch-1 activation plays a key role in the dendritic atrophy in prion disease (31). Additionally, elevated levels of Notch-1 and dendritic atrophy have been observed in AD (32–34).

In light of its role in maintaining the proliferation of NPC, it is tempting to consider the presence of Msi-1 in degenerating neurons in AD and PD subjects to be evidence of reentry into the cell cycle. Several previous studies demonstrate the presence of a variety of cell division markers in susceptible neurons in AD brain (35–41), including CDK4, CDK5, cyclins D, E, and B, Ki-67, and CDK1s p16 and p21. Early work of Nagy et al (39) suggested that because AD neurons express either cyclin E or B but not A, the cell cycle is activated but not completed. It was hypothesized that under these conditions, some neurons might enter the cell cycle but are not able to pass the G1/S transition and simply exit the cell cycle, whereas others might progress to G2 without DNA replication and undergo apoptosis in a BAX-dependent manner or develop NFT as a result of G2 phase-related CDK activity (42). Additionally, amyloid beta peptide is reported to promote activation of mitotic cycles in differentiated cultured cortical neurons (43) that is driven by cell cycle proteins normally observed in proliferating cells, including cyclin D1, phosphotetanoblastoma, a substituted of cyclin D, cyclin-dependent kinases 4 to 6, cyclin E, and cyclin A (42). Further support for reentry of cell cycles includes the activation of select signal transduction pathways and transcriptional activation that results in cytoskeletal alterations, including tau phosphorylation, increased mitochondrial activity, and DNA replication (9, 44).

Another potential role of Msi-1 in neuron degeneration in AD and PD is in the regulation of tau RNA maturation. Recent studies of Cuadrado et al (13) show that ectopic expression of Msi-1 in N2a neuroblastoma cells and in mice led to accumulation of tau transcripts containing exon 10 and an increased 4R/3R ratio of tau. It is of note that tau transcripts containing exon 10 are present in intraneuronal inclusions in the brain of subjects with familial frontotemporal dementia with parkinsonism linked to chromosome 17 (14). Additionally, human tau mRNA contains a GUGAGU site at the beginning of exon 10 that is homologous to the consensus-binding site of Msi1 (G/A(U)nAGU). This suggests that Msi-1 might have an effect on exon 10 splicing.

Overall our data show that most inclusion-bearing neurons identified by the modified Bielschowsky method in AD and PD also demonstrate ectopic expression of Msi-1. Based on its potential modulation of both Notch-1 (through inhibition of Numb) and tau maturation, it appears Msi-1 may play a role in the neuron degeneration in AD and PD.

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