Progressive Neuronal DNA Damage Associated with Neurofibrillary Tangle Formation in Alzheimer Disease

JIN G. SHENG, MD, ROBERT E. MIRAK, MD, PhD, AND W. SUE T. GRIFFIN, PhD

Abstract. DNA damage, as demonstrated by in situ Tdt-mediated dUTP-X-nick end labeling (TUNEL), is widespread in the cerebral cortex in end-stage Alzheimer disease, but has not been previously correlated with stages of neurofibrillary tangle formation. To assess possible relationships between neurofibrillary tangle formation and DNA damage, we used tau immunohistochemistry and TUNEL in tangle-rich fields of tissue sections of subiculum and parahippocampal cortex tissue from 12 Alzheimer and 6 control patients. Structures were classified and quantified as tau+/TUNEL-, tau/-TUNEL+, tau/-TUNEL-, or tau+/TUNEL+. Tau+ structures were subclassified into 4 stages (0–3) based on neurofibrillary tangle morphology. The total number of TUNEL+ neurons was significantly less in control than in Alzheimer patients (35 ± 7.2 vs 90 ± 9.3/mm², mean ± SEM; p < 0.05). The number of tau+/TUNEL+ neurons (40 ± 1/mm²) was less than that of tau+/TUNEL− neurons (68 ± 7/mm²) or tau−/TUNEL+ neurons in the same fields (50 ± 4/mm², p < 0.0001). Tau+/TUNEL+ structures were fewer in number (21 ± 1/mm²), with a third of these representing acellular “ghost tangles” (stage 3). Tau− neurons were more likely than tau+ neurons to be TUNEL− (64 ± 6% vs 44 ± 2%; mean ± SEM; p < 0.01), although most TUNEL− neurons were tau−, even in these selected, tangle-rich fields. TUNEL+ positivity was not uniformly distributed among tangle stages. TUNEL positivity was less common among early (stage 0) tangles than in tau− neurons (21 ± 6% vs 44 ± 2%; p < 0.001), but this rose to 53% among intermediate (stage 1) tangles, and to 87% among late (stage 2) tangles. We suggest that early stages of neurofibrillary tangle formation occur in a subpopulation of relatively healthy (TUNEL−) neurons, and that tangle progression is accompanied by increasing neuronal morbidity.

Key Words: Alzheimer disease; DNA damage; Neurofibrillary tangles; Tau; TUNEL.

INTRODUCTION

Alzheimer disease is the most common cause of dementia in older adults. The degree of cognitive impairment in Alzheimer disease correlates with loss of neuronal synaptic contacts (1), and with the incidence (2) of a characteristic histopathological feature of Alzheimer disease—the neurofibrillary tangle (3, 4). These intraneuronal structures have been closely associated with neuronal dysfunction (5), degeneration, and loss in Alzheimer disease, although the extent of neuronal injury and loss in Alzheimer disease greatly exceeds the incidence of neurofibrillary tangles (6).

The recent development of the Tdt-mediated dUTP-X-nick end labeling (TUNEL) technique has made possible the identification of early stages of cell injury and degeneration in standard paraffin tissue sections (7). This technique catalyzes polymerization of nucleotides to free 3′-OH DNA ends in a template-independent manner to label DNA strand breaks. Application of this technique to Alzheimer disease has shown extensive TUNEL labeling of cerebral cortical neurons (6, 8–12), but little relationship to neurofibrillary tangle formation (6, 12). Initially interpreted as an indicator of apoptotic cell death (8, 10), TUNEL positivity in human postmortem Alzheimer material is now thought to reflect either overt or incipient DNA damage (DNA “vulnerability”) (11, 12). In this study, we used a dual label technique (TUNEL combined with immunohistochemical labeling of tau-immunoreactive neurofibrillary tangles) to define the relationship between formation of neurofibrillary tangles and neuronal DNA damage in Alzheimer disease.

MATERIALS AND METHODS

Patients and Tissues

Twelve clinically demented patients (2 female, 10 male; 79 ± 2 years of age, postmortem interval 8 ± 2 hours, mean ± SEM) with postmortem neuropathological confirmation of Alzheimer disease according to CERAD criteria (13) and 6 neurologically normal controls (2 female, 4 male, 73 ± 4 years, 7 ± 2 hours), were used in this study. The ages and postmortem intervals of the 2 groups were not statistically different. Right half brains were immersion-fixed in 20% phosphate-buffered formalin (8% formaldehyde) for 10–14 days. The brains were then coronally sectioned and tissue blocks obtained of the hippocampus and adjacent parahippocampal cortex at the level of the lateral geniculate nucleus. These were embedded in paraffin and sectioned at a thickness of 10 μm.

Immunohistochemical Labeling

Immunohistochemical labeling was performed as previously described (14) except that deparaffinized sections were treated
with 99% formic acid for 5 min in preparation for TUNEL. There were no discernible differences in neurofibrillary tangle immunoreaction arising from inclusion of the formic acid treatment step. Rehydrated sections were permeabilized in 0.5% Triton X-100 for 10 minutes followed by 0.2N HCl for 20 minutes. Endogenous peroxidase was blocked with 3% H2O2 in 97% methanol for 30 minutes. The primary antibody was a monoclonal mouse anti-tau (tau2, a gift from Dr. L. I. Binder; Northwestern University, Chicago, Ill), which is commercially available from Sigma (St. Louis, Mo). This was diluted 1:200 with 2% normal goat serum in tris-buffered saline and was incubated on the sections overnight at room temperature. Mouse peroxidase-anti-peroxidase (DAKO, Carpinteria, Calif) labeled with fast red was used to visualize tau immunoreactive (tau+) structures.

TUNEL

TUNEL was performed on tau-immunoreacted tissue sections using an In Situ Cell Death Detection Kit-POD (Boehringer-Mannheim Biochemica, Indianapolis, Ind). Labeling was performed according to the protocol provided in the kit. Briefly, tissue sections were incubated in 3% H2O2 in 97% methanol for 30 minutes (min) and then incubated in proteinase K (10 μg/ml in phosphate-buffered saline [PBS]) at room temperature for 15 min followed by 3 washes in PBS. Terminal deoxynucleotidyl transferase mixed with nucleotide (total volume 50 μl) was applied directly to the tissue sections, which were then covered with a coverslip. The slides were incubated in a humidified chamber at 60 min at 37°C, and rinsed 3 times with PBS. Converter-POD (50 μl) was applied to the section, which was then coverslipped and incubated in the humidified chamber for another 30 min at 37°C. Sections were then lightly counterstained with hematoxylin. TUNEL+ neurons were visualized after incubation with metal-enhanced diaminobenzidine substrate (Boehringer Mannheim).

Classification of Neurofibrillary Tangle Stages

Neurofibrillary tangles were classified into 4 stages, based on patterns of tau2 immunoreactivity and neuronal morphology, as previously described (15) (Fig. 1). Briefly, neurons showing fine granular tau immunoreactivity were classified as stage 0; those showing fibrillar tau2 immunoreactivity with normal neuronal shape and nuclei were classified as stage 1; those showing dense cytoplasmic tau2 immunoreactivity with peripheral displacement of the nucleus were classified as stage 2; and acellular, fibrillar tau2+ structures ("ghost tangles") were classified as stage 3.

Quantification of TUNEL+ Neurons and Tau+ Neurons

TUNEL+ cells included neurons, astrocytes, and microglia, identified using nuclear and cytoplasmic morphology. Neurons were classified as tau+/ TUNEL-, tau+/ TUNEL+, tau+/ TUNEL-, and tau+/ TUNEL+. The numbers of each of these different neuron cell types were counted in tangle-rich fields of subiculum/parahippocampal cortex, using an eyepiece grid, in ten 40× microscopic fields (0.126 mm2/field) in double-labeled tissue sections. Counted tau+ neurons were further classified according to the stage of neurofibrillary tangle formation as described above. Accuracy of total neuronal counts was verified on adjacent sections stained with hematoxylin.

Statistical Analysis

ANOVA followed by Fisher’s test was used to assess the significance of differences between numbers of TUNEL+ and TUNEL- neuronal classes, between neuronal populations in Alzheimer and control tissue, and between TUNEL+ neurons associated with different tangle stages (defined above) representing consecutive steps in tangle evolution, e.g. the number of TUNEL+ neurons associated with stage 0 tangles was compared with the number associated with stage 1 tangles, and these latter were, in turn, compared with the number associated with stage 2 tangles.

RESULTS

Tissue sections from Alzheimer patients showed TUNEL positivity in neurons (Fig. 1), in astrocytes, and in microglia. In the tangle-rich fields selected, tau+ neurons represented approximately one-third of all neurons, and TUNEL+ neurons represented approximately half of all neurons (Table 1). The total number of TUNEL+ neurons was significantly less in control than in Alzheimer patients (35 ± 7.2 vs 90 ± 9.3; p < 0.05). The number of tau+/ TUNEL+ neurons was less than that of tau- / TUNEL- neurons or tau-/ TUNEL+ neurons in the same fields (p < 0.0001). Tau+/ TUNEL- structures were fewer in number, and a third of these represented acellular "ghost tangles" (stage 3). Tau+ neurons were more likely than tau- neurons to be TUNEL+ (64 ± 6% vs 44 ± 2%; p < 0.01).

Neuronal TUNEL positivity did not vary with postmortem interval in our study, and the numbers of TUNEL+ neurons in Alzheimer patients was higher than that of control patients at all postmortem intervals (Fig. 2). The greatest number of TUNEL+ neurons was found in the Alzheimer patient with the longest postmortem interval (21 hours), but the second highest count of TUNEL+ neurons occurred in the Alzheimer patient with the shortest postmortem interval (1.5 hours). Among controls, the greatest number of TUNEL+ neurons occurred in the patient with the second-shortest postmortem interval (4 hours).

Tau+ neurons were subclassified according to stage of neurofibrillary tangle formation (Fig. 1). Classic stage 2 tangles represented 70% of tau+/ TUNEL+ neurons, stage 1 tangles represented 26% of tau+/ TUNEL+ neurons, and stage 0 tangles represented only 3% of tau+/ TUNEL+ neurons. Across tangle stages 0–2, there was a progressive increase in incidence of TUNEL positivity (Fig. 3): 21% of stage 0 tangles were TUNEL+, 53% of stage 1 tangles were TUNEL+, and 87% of stage 2 tangles were TUNEL+. A few acellular stage 3 tangles (5%) showed TUNEL positivity, probably representing necrotic cellular remnants. These latter structures constituted only 1% of all tau+/ TUNEL+ structures.

Tau- / TUNEL+ neurons were significantly more numerous than those of either tau+/ TUNEL+ neurons or
Fig. 1. Photomicrographs of tau2 (red)/TUNEL (brown) double labeled neurons showing progressive stages of neurofibrillary tangle formation in Alzheimer disease. (A) A stage 0 (S0) neurofibrillary tangle in a TUNEL- neuron. (B) A stage 1 (S1) neurofibrillary tangle in a TUNEL+ neuron. (C) A stage 2 (S2) neurofibrillary tangle in a TUNEL+ neuron. (D) A stage 3 (S3) extracellular neurofibrillary tangle ("ghost tangle") with associated TUNEL+ glial cells. (E) Tau- TUNEL+ neurons (arrows). (F) A tau- TUNEL+ neuron (open arrow) adjacent to a stage 2 tau- TUNEL+ neuron (closed arrow). Bars = 15 μm.
TABLE 1

<table>
<thead>
<tr>
<th>Neuronal type</th>
<th>Alzheimer</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>tau*/TUNEL+</td>
<td>$40 \pm 1$ (23%)*</td>
<td>$3 \pm 1$ (1.5%)</td>
</tr>
<tr>
<td>tau*/TUNEL-</td>
<td>$21 \pm 1$ (12%)</td>
<td>$1 \pm 1$ (0.5%)</td>
</tr>
<tr>
<td>tau/TUNEL+</td>
<td>$50 \pm 4$ (28%)</td>
<td>$32 \pm 8$ (16%)</td>
</tr>
<tr>
<td>tau/TUNEL-</td>
<td>$68 \pm 7$ (37%)</td>
<td>$165 \pm 7$ (82%)</td>
</tr>
</tbody>
</table>

* Data presented as mean ± SEM (% of all neurons).

Fig. 2. The numbers of TUNEL+ neurons was independent of postmortem interval for Alzheimer cases ($r = 0.4, p = 0.3$), for controls ($r = 0.2, p = 0.7$), and for the group as a whole ($r = 0.3, p = 0.2$).

tau*/ TUNEL- neurons, even in the selected, tangle-rich areas examined here ($p < 0.0001$). Furthermore, the number of tau*/ TUNEL+ neurons were significantly greater than the number of tau*/ TUNEL- neurons ($p < 0.001$).

DISCUSSION

Our results show a progressive increase in the incidence of TUNEL positivity with stage of neurofibrillary tangle formation, from 21% of stage 0 tangle-bearing neurons to 53% and 87%, respectively, of stage 1 and stage 2 tangle-bearing neurons. This is the first demonstration of a progressive association between stage of neurofibrillary tangle formation and a molecular in situ index of neuronal cell injury.

The TUNEL technique identifies free 3'-OH DNA ends in a template-independent manner to label DNA strand breaks (7). Such breaks identify injured cells, and are characteristic of the later stages of apoptotic cell death, but are also found in cells undergoing non-apoptotic necrosis (16). TUNEL+ neurons are prominent in postmortem brain tissue sections from Alzheimer patients, and this has been interpreted as evidence of DNA damage in vivo that is rendered manifest through addition of agonal and perhaps immediate postmortem insults (11, 12). TUNEL thus appears to identify a population of injured neurons, vulnerable to degeneration and cell death, in Alzheimer disease. The effect of postmortem interval on TUNEL

Fig. 3. Incidence of TUNEL positivity in total, and in the tangle-bearing subset of neurons in tangle-rich fields of subicular and parahippocampic gyrus of control and Alzheimer patients. (A) Incidence of tangle-bearing (tau+) and of TUNEL+ neurons in control and Alzheimer patients. Starred values are significantly greater than corresponding control values ($^{*}p < 0.05$, $^{**}p < 0.001$). (B) Numbers of TUNEL+ and of TUNEL- neurons bearing different stages of neurofibrillary tangles in Alzheimer disease. TUNEL+ stage 2 tangles were significantly more common than TUNEL+ tangles at any other stage ($^{*}p < 0.0001$). (C) Percentage of neurons showing TUNEL positivity at each stage in Alzheimer disease. The incidences of TUNEL positivity in stages 1 and 2 were significantly greater than those of the corresponding preceding tangle stage ($^{**}p < 0.001$ in each case).
positivity has been previously examined. Some investigators have reported increased numbers of TUNEL\(^*\) neurons after postmortem intervals of 8.5–10 hours (11, 17), while others have found no increases in numbers of TUNEL\(^*\) neurons with postmortem intervals as long as 48–72 hours (6, 18). We find no obvious correlation between numbers of TUNEL\(^*\) neurons and postmortem interval, for either Alzheimer patients or controls, over a postmortem interval range of 1.5–15 hours (see Fig. 2). Only one patient in our study had a longer postmortem interval (21 hours), and this latter patient, who had Alzheimer disease, had the highest number of TUNEL\(^*\) neurons. This might suggest an effect of long (greater than 15 hours) postmortem interval on TUNEL positivity, but this is not supported by our finding of the second highest number of TUNEL\(^*\) neurons in the patient with the shortest postmortem interval in our study, 1.5 hours. Similarly, among controls, the highest number of TUNEL\(^*\) neurons occurs in the patient with the second-shortest postmortem interval, 4 hours. Our finding that 44% of non–tangle-bearing neurons show TUNEL positivity in selected tangle-rich fields of a highly involved region of end-stage Alzheimer brain is consistent with previous reports of numerous TUNEL\(^*\) neurons in the brains of Alzheimer patients (8–12), as well as biochemical demonstration of increased DNA breaks in the brains of Alzheimer patients (17). In this study, we compared TUNEL positivity in neurons at different stages of neurofibrillary tangle formation, all of which were present within the same fields of the same tissue sections. As such, any and all factors affecting those tissue sections, including age, state, postmortem interval, fixation techniques, embedding, sectioning, etc., will be identical for each of the different tangle stages analyzed in a given section.

Among neurons showing the earliest manifestations of neurofibrillary tangle formation (stage 0), the incidence of TUNEL positivity was actually lower than that seen among neurons devoid of tau immunoreactivity (21% vs 44%). This is despite the fact that, overall, tau\(^*\) neurons showed a greater incidence of TUNEL positivity than did tau\(^−\) neurons (64% vs 44%). This result suggests that neurofibrillary tangle formation commences among a subpopulation of relatively healthy neurons, and that neurofibrillary tangle formation precedes, rather than follows, neuronal injury and death detectable with the TUNEL technique. Conversely, the high incidence of tau\(^−\) TUNEL\(^*\) neurons (56% of all TUNEL\(^*\) neurons and 44% of all tau\(^−\) neurons), even in fields selected for their rich content of tau\(^*\) neurofibrillary tangles, suggests that DNA damage and neuronal injury occurs via mechanisms independent of neurofibrillary tangle formation for most neurons in Alzheimer disease, in accordance with previous findings (6, 12). The existence of both tangle-dependent and tangle-independent pathways for neuronal injury and development of TUNEL positivity is further supported by our finding that age-matched controls have a number of TUNEL\(^*\) neurons that do not exhibit tau immunoreactivity.

In a previous study of neurofibrillary tangle stages in Alzheimer disease, we showed an increasing association of tangle-bearing neurons, representing successive tangle stages, with activated microglia and activated astrocytes, overexpressing the neurotrophic cytokines interleukin-1 and S100\(β\) (15). Our present finding of an increasing incidence of TUNEL positivity with neurofibrillary tangle stage correlates closely with these previous results. Interleukin-1 is neurotoxic at high levels (19), suggesting that microglial interleukin-1 overexpression by tangle-associated microglia may contribute to cell injury (20). Similarly, overexpression of S100\(β\) by tangle-associated astrocytes (15) may contribute to neuronal cell injury via S100\(β\)-induced increases in intraneuronal free calcium concentrations (21). Such increases could promote calcium-mediated intracellular processes, including the abnormal phosphorylation of tau protein (22) that accompanies neurofibrillary tangle formation. These observations collectively suggest that a complex interaction between neuronal cytoskeletal degeneration, microglial and astrocytic activation with cytokine overexpression, and neuronal DNA damage occurs in that subset of neurons that have the neurofibrillary tangles characteristic of Alzheimer disease.

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