Phosphorylated Neurofilament Antigens in Neurofibrillary Tangles in Alzheimer’s Disease

LINDA C. CORK, D.V.M., PH.D., NANCY H. STERNBERGER, PH.D., LUDWIG A. STERNBERGER, M.D., MANUEL F. CASANOVA, M.D., ROBERT G. STRUBLE, PH.D., AND DONALD L. PRICE, M.D.

Abstract. Neurofibrillary tangles (NFT) are a hallmark of Alzheimer’s disease (AD), and their presence correlates with the presence of dementia. A major constituent of NFT is the insoluble paired helical filament which shares some antigenic relationships with normal cytoskeletal elements, particularly neurofilaments. If neurofilament proteins (200, 145–160, and 68 kilodaltons [kd]) participate in the formation of NFT, the distribution of these constituents might be expected to be abnormal. To examine this issue, we used immunocytochemical methods to localize phosphorylated and nonphosphorylated epitopes of neurofilament proteins in hippocampal neurons of controls and patients with AD. Normally, the 200-kd neurofilament protein is not phosphorylated in the perikarya of neurons. However, in AD, many pyramidal neurons contained immunoreactive phosphorylated neurofilaments. Patterns of immunoreactivity (linear, flame-shaped, or skein-like within perikarya) greatly resembled the appearance of silver-stained NFT. This pattern of immunoreactivity was not present in hippocampal pyramidal neurons in controls, except in one aged patient in whom adjacent silver-stained sections revealed a few NFT. Patterns of immunoreactivity with antibodies for nonphosphorylated neurofilament proteins were similar in control and AD neurons. Our results indicate that some NFT are associated with abnormal distributions of high molecular weight phosphorylated neurofilament proteins. One domain of the 200-kd protein is believed to be a component of the side arms which link neurofilaments and interact with microtubules. Abnormal interactions of perikaryal neurofilaments could play a role in the genesis of NFT, and this abnormality of the cytoskeleton could contribute to the dysfunction of neurons at risk in AD.

Key Words: Alzheimer’s disease; Cytoskeleton; Neurofibrillary tangles; Neurofilaments.

INTRODUCTION

The neurofilament triplet proteins (200, 145–160, and 68 kilodaltons [kd]), synthesized in the cell bodies of neurons, are slowly transported anterograde as neurofilaments (1, 2). Within different parts of individual neurons (perikarya, dendrites, and axon), there are differences in the relative amounts of these proteins (3) and in their degree of phosphorylation (4). For example, the 200-kd protein may undergo considerable phosphorylation in axons (4). These observations suggest that there may be as yet undefined relationships between neurofilament proteins, their post-
TABLE 1
Neurofilament Epitopes in Fibers and Perikarya

<table>
<thead>
<tr>
<th>Structure</th>
<th>Non-phosphorylated</th>
<th>Phosphorylated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>02-40</td>
<td>06-17</td>
</tr>
<tr>
<td>Fibers in gray matter</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Fibers in white matter</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Pyramidal neurons:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No NFT</td>
<td>++++</td>
<td></td>
</tr>
<tr>
<td>With NFT</td>
<td>*</td>
<td>*</td>
</tr>
</tbody>
</table>

+++ indicates intensity of immunoreactivity.
± indicates variable immunoreactivity.

* No differences could be detected between neurons containing neurofibrillary tangles and those lacking neurofibrillary tangles with antibody 02-40.

Patients with Alzheimer's disease (AD) develop neurofibrillary tangles (NFT) within the perikarya and proximal dendrites of a variety of neuronal populations including cells within the locus coeruleus (6), basal forebrain cholinergic system (7, 8), hippocampus (9, 10), amygdala (11), and neocortex (12, 13). Neurofibrillary tangles (NFT) primarily contain individual 10-nm diameter filaments forming paired helical filaments (PHF) (14), but 15-nm straight filaments and normal-appearing 10-nm neurofilaments may be associated with NFT (15). Paired helical filaments, which are composed of highly insoluble proteins (16), share some antigenic relationships with neurofilament proteins (17–23), microtubule-associated proteins (MAP2) (24) and, possibly, noncytoskeletal brain proteins (25, 26).

The sequence of events leading to the formation of NFT is not known, but an early event may be the maldistribution of neurofilament proteins. Our immunocytochemical studies on phosphorylated and nonphosphorylated epitopes of these proteins indicate that, in AD, the phosphorylated 200-kd neurofilament protein, not normally identified in perikarya of hippocampal pyramidal cells, is located abnormally within cell bodies of these neurons and that this pattern of immunoreactivity resembles NFT as visualized by other methods.

MATERIALS AND METHODS

Antibodies

In these studies, we used four antibodies: 02-40 reacts against nonphosphorylated epitopes of the 200- and 145- to 160-kd neurofilament proteins; 03-44 is directed against a phosphorylated epitope of the 200-kd neurofilament protein; antibodies 06-17 and 07-5 are directed against phosphorylated epitopes shared by the 200- and 145- to 160-kd neurofilament proteins (4, 27, 28).
Case Material, Tissue Preparation, and Immunocytochemistry

Four controls 53–63 years of age and eight patients with AD ranging in age from 52–80 years were included in our case material. The diagnosis of AD was based on a clinical history of dementia, the presence of more than 15 senile plaques per low-power field (×100 magnification) in the hippocampus, and the presence of NFT and senile plaques in neocortex. From each of these brains, sections of hippocampus fixed in 10% neutral-buffered formalin were dissected and processed for paraffin embedding. Some sections were stained with hematoxylin and eosin or silver (Nauomenko-Feigin/periodic acid Schiff). Additional sections (6–12 μm) were deparaffinized to water and stained immunocytochemically with a 1:1,000 dilution of primary antibodies (Table 1). In addition to paraffin-processed material, some formalin-fixed tissue blocks were cryoprotected in 10% sucrose, frozen, sectioned at 40 μm, and stained as described below. Sections were washed and incubated for 30 minutes (min) with the secondary antibody (goat antimouse) diluted 1:20; after washing, sections were incubated for 30 min in mouse ClonoPAP (peroxidase-antiperoxidase complex) (diluted 1:200) (29). These preparations were developed in 0.05% diaminobenzidine tetrahydrochloride/0.01% hydrogen peroxide (eight min). All washes were in 0.05% M Tris buffer, 1.5% saline, and all incubations were carried out at room temperature. Sections were coverslipped with Permount and examined in a Zeiss photomicroscope.

RESULTS

Controls

In conventional stains, structural abnormalities were not seen in the hippocampi of three controls, but the hippocampus of the 63-year-old control showed scattered NFT in pyramidal neurons and occasional senile plaques. Antibody 02-40 demonstrated a nonphosphorylated epitope of neurofilament proteins in the perikarya and proximal dendrites of hippocampal pyramidal neurons, as well as in fibers within the hippocampus (Table 1; Fig. 1A). Fibers in gray matter were more intensely stained than those in white matter. Antibodies against phosphorylated neurofilament epitopes (06-17, 03-44, and 07-5) showed different patterns of staining. With these three antibodies, fibers in white matter were readily visualized, but perikarya generally appeared as negative images. With antibodies 06-17 and 03-44, the immunoreactivity of fibers in white matter was greater than that of fibers in gray matter; antibody 07-5 showed less robust staining of fibers in both gray and white matter.

In the 63-year-old control, antibody 07-5 disclosed a few linear-to-globose fibrillary accumulations of immunoreactivity within perikarya and proximal dendrites, and silver stains revealed NFT in occasional neurons in these same regions.

Alzheimer’s Disease

In patients with AD, plaques were frequent in the hippocampus, and many pyramidal neurons contained NFT (Fig. 2A) and granulovacuolar degeneration. Some NFT were not associated with nuclei and were interpreted as representing accumulations of insoluble PHF which persisted after degeneration of the neuron. As in controls, antibody 02-40 demonstrated a nonphosphorylated epitope in perikarya of pyramidal neurons and proximal dendrites (Fig. 1B); with this antibody, immunoreactivity of NFT did not differ from staining normally seen in perikarya. Staining of some hippocampal pyramidal neurons appeared to be somewhat reduced as compared to staining in neurons of the lateral geniculate body present on the same section. With antibody 07-5, perikarya of many pyramidal neurons of the hippocampus showed abnormal accumulations of phosphorylated neurofilament protein-like immunoreactivity (Fig. 2B); these aggregates appeared as linear fibrils, flame-shaped structures, or skeins of fibrils in a globose arrangement, patterns strik-
Fig. 1. Hippocampal pyramidal neurons with antibody 02-40 against nonphosphorylated neurofilament epitopes. Immunoreactivity is present in perikarya and dendrites of neurons (A) in the 63-year control and (B) in the 77-year-old with AD. Peroxidase–antiperoxidase. ×700.
Fig. 2. A. Neurofibrillary tangles in hippocampal pyramidal neurons in a 74-year-old with AD are sharply delineated in silver stains as fibrillar, flame-like cytoplasmic arrays. Nau­menko-Feigin. × 700. B. Neurofibrillary tangle in hippocampal pyramidal neuron in AD case in Figure 2A showing pattern of robust fibrillary cytoplasmic immunoreactivity with antibody 07-5 against phosphorylated neurofilament epitopes. × 700. C. In a 77-year-old patient with AD, a similar pattern of immunoreactivity within perikarya is also seen using antibody 03-44 against phosphorylated neurofilament epitopes. × 700.
ingly similar to the images of NFT seen in silver stains (Fig. 2A and 2B). A similar pattern of perikaryal immunoreactivity was demonstrated with antibody 03-44 (Fig. 2C), but fewer neurons showed this abnormal pattern. Antibody 06-17 did not stain perikarya or NFT.

**DISCUSSION**

Using immunocytochemical methods, the 68- and 145- to 160-kd neurofilament proteins have been documented previously in perikarya of pyramidal neurons of the hippocampus (3). In our studies, immunoreactivity against nonphosphorylated epitopes of the 200- and 145- to 160-kd neurofilament proteins was readily discernable in perikarya and dendrites, while phosphorylated proteins, particularly the 200-kd protein, were enriched in axons (4). The neurofilament core consists of an alpha helical coiled coil-rod domain containing 68-, 145- to 160-, and 200-kd proteins; the tailpiece domain of the 200-kd protein may link neurofilaments with other organelles (30-34). Phosphorylation of the 200-kd protein may alter interactions between neurofilaments and other organelles (e.g. increased crossbridges) and thereby alter their transport into and along the axon.

Other investigators have shown that some antibodies directed against neurofilament proteins, particularly the 200-kd component, stained NFf in tissues (17-21). Our studies demonstrated a more specific abnormality—phosphorylated 200-kd neurofilament protein accumulated within cell bodies of hippocampal pyramidal neurons. Antibodies directed against epitopes of the phosphorylated 200-kd protein did not stain NFT equally, i.e. the aberrant immunoreactivity was primarily that of epitopes visualized with antibody 07-5 and, to a lesser extent, with antibody 03-44. Striking similarities were observed in patterns obtained with silver stains and immunocytochemical methods (Fig. 2). These studies do not provide information concerning the relationships between phosphorylated neurofilaments and the development of PHF; however, our observations do suggest that the maldistribution of phosphorylated neurofilament proteins in cell bodies is associated with the formation of NFT. As such, these studies are consistent with recent elegant ultrastructural immunocytochemical investigations showing that colloidal gold-labeled antibodies against 200-kd neurofilament proteins decorate isolated PHF (23).

We do not know whether phosphorylated neurofilaments accumulate first within perikarya with the subsequent formation of PHF or whether PHF appear initially and entrap neurofilament proteins which then become phosphorylated. By examining the distribution of various normal and abnormal antigens in hippocampal pyramidal neurons in individuals in early and late stages of disease, it may be possible to identify a vector of evolution. In one hypothetical model, cell dysfunction involving phosphorylation of 200-kd neurofilament proteins would lead to perikaryal accumulation of neurofilaments which, by mechanisms unknown, may become components of PHF. Recent studies suggest that MAP2 or MAP2 fragments may also be present in NFT (24, 35). Both neurofilament proteins and MAP2 are phosphorylated (4, 36-38). Perhaps abnormalities in phosphorylation of these constituents are an early abnormality in this disorder.

Studies of the aluminum poliomyelopathy model provide support for the concept that accumulation of phosphorylated neurofilament proteins is associated with the development of perikaryal neurofibrillary abnormalities (39) (P. Gambetti, personal communication). Rabbits given intrathecal injections of aluminum chloride develop accumulations of neurofilaments in perikarya, dendrites, and proximal axons of motor neurons (40-42) and show a decrease in the rate of transport of neurofilament proteins.
proteins (43, 44). However, patterns of immunoreactivity are somewhat different in the aluminum model than in AD, in that aluminum-induced neurofibrillary accumulations are associated with immunoreactivities demonstrated by antibodies 06-17 and 03-44 but not antibody 07-5. Thus, this experimental model and AD show differences in the patterns of immunoreactivity of various neurofilament epitopes. Nevertheless, our observations suggest that, in both settings, 200-kd neurofilament proteins are abnormally phosphorylated within perikarya and that this change in location of phosphorylated neurofilaments is associated with the development of neurofibrillary abnormalities.

In conclusion, this study indicates that NFT in hippocampal pyramidal cells are associated with abnormal distributions of phosphorylated neurofilament proteins. Moreover, these pyramidal neurons also show abnormalities of other cytoskeletal elements, including unusual tubulin-like immunoreactivity in the granules of granulovacuolar degeneration (45) and actin in Hirano bodies (46). These cytoskeletal changes in hippocampal neurons may contribute to dysfunction and death of these nerve cells (10), a neuronal system known to be important in normal memory processing (47), and, when affected by diseases like AD, may play an important role in the expression of dementia (8).

Note Added In Proof: Recently, two of us (NHS, LAS) reported similar findings in pyramidal neurons of two additional cases of AD and in an older individual with Down’s syndrome (Sternberger et al. Proc Natl Acad Sci USA (in press)).

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