Swollen Cortical Neurons in Creutzfeldt-Jakob Disease Contain a Phosphorylated Neurofilament Epitope

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Abstract. The distribution of swollen neurons and the presence of a phosphorylated neurofilament protein (NFP) epitope in these cells were studied in six cases of Creutzfeldt-Jakob disease (CJD). Swollen neurons are widely distributed in the cerebral cortex and are most abundant in the cingulate and parahippocampal gyri. They are more numerous in the panencephalopathic type of CJD than in the subacute spongiform encephalopathic type. A phosphorylated epitope of NFP was detected in the perikarya of swollen neurons by an immunocytochemical method using a series of monoclonal antibodies that distinguish phosphorylated and nonphosphorylated epitopes of NFP. This abnormal distribution of phosphorylated NFP epitopes indicates that the process of NFP phosphorylation is altered in neurons affected by CJD. This investigation, in accordance with previous studies, suggests that the abnormal posttranslational modification of the neurofilament may play an important role in the pathogenesis of several neurodegenerative disorders.

Key Words: Creutzfeldt-Jakob disease; Immunocytochemistry; Neurofilament; Phosphorylation; Swollen neurons.

INTRODUCTION

Human neurofilaments are composed of three polypeptide subunits with molecular weights of approximately 68, 160 and 200 kilodaltons (kDa) (1). The subunits are synthesized in the neuronal perikarya, assembled into a filamentous form, and then transported centrifugally along the axons by slow axoplasmic flow. During this process, a phosphorylation occurs at multiple sites of the 160 and 200 kDa subunit molecules (2). It has been shown that the phosphorylation of neurofilaments results in their conformational and antigenic changes (3). The role of phosphorylation in neurofilament function, however, is yet undetermined (4). In addition, the phosphorylation level of neurofilaments in a variety of neuronal diseases has not been fully established.

Swollen cortical neurons with eccentrically placed nuclei and chromatolytic glassy cytoplasm are observed in several neurodegenerative diseases (5). The cytoplasm of swollen neurons contain abundant neurofilaments, immunoreactive to neurofilament protein (NFP) antibodies and phosphorylated epitopes of NFP (5–7). Similar swollen neurons have previously been described in the cerebral cortex of patients suffering from Creutzfeldt-Jakob disease (CJD) (6, 8, 9). The precise distribution of these cells and their immunocytochemical nature, however, remains uncertain.

In this study, we describe the distribution of swollen neurons in the brains of patients with CJD and the localization of a phosphorylated NFP epitope in these...
cells by an immunocytochemical method using a series of monoclonal antibodies that distinguish phosphorylated and nonphosphorylated epitopes of NFP.

MATERIALS AND METHODS

Materials

The brains from six patients with Creutzfeldt-Jakob disease (CJD) and from three patients with non-neurological disorders (as age-matched controls) were studied. The tissues were obtained from autopsies performed two to 12 h postmortem, fixed for one to eight weeks in 10% formalin buffered with phosphate and then embedded in paraffin. Sections, three micrometers (μm) thick, were stained with hematoxylin and eosin (H&E), the Klüver-Barrera luxol-fast-blue method, and the Bodian silver protargol method, or processed for immunocytochemical examination. Antisera against the 68 kDa, 160 kDa and 200 kDa subunits of neurofilaments were obtained from rabbits that had been immunized with human neurofilament triplet protein (10). Three monoclonal antibodies (1D, 4C and 7B) were obtained from the culture media of mouse hybridomas, and characterized by immunocytochemical and immunotopological studies (11). The reactivity of the 1D antibody with the 200 kDa and 160 kDa subunits was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immune blotting analysis. This reaction, however, was completely inhibited by a preincubulation of NFP containing nitro-cellulose strips with alkaline phosphatase. In contrast, epitopes recognized with the 4C and 7B antibodies were phosphatase-resistant and localized in the 160 kDa and 68 kDa subunits. The anti-rabbit IgG swine serum and soluble peroxidase-antiperoxidase (PAP) complex were purchased from DAKO (Copenhagen, Denmark), and the biotinylated goat anti-rabbit immunoglobulin serum and peroxidase-conjugated streptavidin from BioGenex Lab (Dublin, CA, USA). The bacterial alkaline phosphatase (type III) was obtained from Sigma (St. Louis, MO, USA).

Methods

The PAP method was used for immunostaining with polyclonal antiserum (10), and the biotin-streptavidin (B-SA) method for that with monoclonal antibodies (12). The anti-neurofilament sera were diluted with 10% normal swine serum in phosphate-buffered saline as follows: anti-68 kDa, 1:150, anti-160 kDa, 1:500, anti-200 kDa, 1:300. Three hybridoma media, containing monoclonal antibodies, were used at dilutions ranging between 1:1 to 1:10. Control sections were prepared by using either normal rabbit serum or tissue culture medium from the parental mouse myeloma cells (P3-X63-Ag8-U1). Some sections were incubated with alkaline phosphatase (20 IU/ml in 0.1 M Tris-HCl buffer, pH 8.0, containing 5 mM phenylmethylsulfonyl fluoride) for 24 h at room temperature just before the incubation with monoclonal antibodies. Control sections were similarly incubated with alkaline phosphatase solution prepared using 0.1 M sodium phosphate buffer, pH 8.0.

RESULTS

Six cases were diagnosed as having CJD on the basis of clinical and pathological features briefly summarized in Table 1. They were classified into two types, subacute spongiform encephalopathic (SSE) and panencephalopathic (PE) types, based on the clinical and pathological criteria (13, 14). Patients with the PE type showed rapid progressive mental deterioration resulting in akinetic mutism within a few months, and thereafter their neurological symptoms were relatively static. In patients with SSE type, however, the neurological signs and symptoms developed insidiously, and constantly progressed during the course. In SSE type brains, a slight to moderate neuronal loss, slight astrocytosis and severe spongy degeneration were found in the cortical and subcortical gray matter. There were minimal changes in the white matter. In contrast, there was severe gray and white matter degeneration of the cerebrum in PE type brains. The cerebral cortex showed severe neuronal loss, marked prolif-

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<td>OC</td>
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<td>FR, IN, TE, PA</td>
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F: female; M: male; (+): present; –: absent; PSD: periodic synchronous discharge; EEG: electroencephalogram; ++++: severe; ++: moderate; +: slight; PE: panencephalopathic type; SSE: subacute spongiform encephalopathy; IN: insular cortex; CI: cingulate gyrus; PH: parahippocampal gyrus; FR: frontal cortex; TE: temporal cortex; PA: parietal cortex; OC: occipital cortex.
Fig. 1. Case 4. Distribution of swollen neurons in the brain from a patient with CJD. One dot represents ten swollen neurons. a: A coronal section of the rostral left frontal lobe. b: A coronal section through the left lateral geniculate body. c: A coronal section of the right occipital and posterior parietal lobes.

Expansion of hypertrophic astrocytes, mobilization of fat granule cells and a slight spongy state. There were loss of myelinated fibers, astrocytic proliferation and abundant fat droplets in the macrophages widely distributed in the white matter. Brains of this type also showed degeneration in the brainstem, cerebellum and spinal cord.

Each brain contained a variable number of swollen neurons. They were most abundant in the deeper cortical layers of the parahippocampal, insular and cingulate gyri (Fig. 1). They were also noted in smaller numbers in the frontal, temporal and parietal cortices. The occipital lobe, however, contained fewer swollen neurons. The brains with the PE type of CJD had more swollen neurons than those with the SSE type of CJD.

The swollen neurons had rounded cell bodies, and were two or three times larger than the adjacent pyramidal neurons. They had eccentrically placed nuclei and abundant, slightly eosinophilic cytoplasm with a ground glass appearance (Fig. 2a). In some cells, the cytoplasm contained ill-defined, eosinophilic inclusions (Fig. 2b). Nissl bodies, if present, were located at the periphery of the cytoplasm. With Bodian’s method a distinct argyrophilia of their cytoplasm was present.

Polyclonal antibodies against the 68 kDa and 160 kDa neurofilament subunits immunostained the swollen neurons (Fig. 3). Most of the swollen neurons showed a homogeneous cytoplasmic staining. However, in some neurons the periphery of the cytoplasm was more intensely immunostained than the center. Antibody to the 200 kDa neurofilament subunit weakly stained some swollen neurons.

The monoclonal antibody 1D, specific to a phosphorylated epitope of NFP, positively immunostained the swollen neurons in all six cases (Fig. 4a, b). The distended cytoplasm of most of the swollen neurons were intensely and homogeneously immunostained with 1D antibody. However, neurons with low levels of, or no detectable immunoreactivity, were occasionally observed. This antibody also labeled axons running through the cortex, but not the perikaryal cytoplasm of non-swollen neurons. The cytoplasmic swelling and selective, intense 1D-immunoreactivity of
Fig. 2. Swollen neurons in the cerebral cortex. a: A cortical neuron with an eccentrically placed nucleus and swollen chromatolytic cytoplasm. b: A swollen neuron containing an ill-defined, eosinophilic inclusion in the cytoplasm. H&E. ×660.

The swollen neurons made it possible to easily identify them in the cerebral cortex under the low-power field of the microscope (Fig. 4a). Some swollen neurons were slightly stained with 7B antibody, but 4C antibody did not stain any of the swollen neurons (Fig. 4c, d). The cytoplasm and dendrites of cortical neurons of age-matched control brains were weakly stained with 4C and 7B antibodies. However, these neurons only rarely showed weak immunoreactivity with 1D antibody. All antibodies intensely immunostained the axons of the white matter.

Fig. 3. Immunocytochemical distribution of neurofilament subunits in swollen neurons. a: Perikaryal cytoplasm of swollen neurons were positive for the 68 kDa NFP and b: 160 kDa NFP. c: Anti-200 kDa serum weakly stained the cytoplasm of swollen cells. Immunoperoxidase with hematoxylin. ×560.
Fig. 4. a: Swollen neurons were localized in the deeper cortical layers of the parahippocampal gyrus and positively immunostained with 1D antibody. Immunoperoxidase with hematoxylin. ×52. b: Swollen cells were positive for a phosphorylated 1D epitope but c: negative for a nonphosphorylated 4C epitope. d: A monoclonal antibody against a nonphosphorylated 7B epitope slightly stained some swollen neurons. Immunoperoxidase with hematoxylin. ×780.

Incubation of the tissue sections with alkaline phosphatase (20 IU/ml in Tris-HCl buffer) abolished the staining of the swollen neurons by 1D antibody (Fig. 5a). Pretreatment of the sections with an alkaline phosphatase solution prepared using phosphate buffer, however, did not change the 1D-immunostaining (Fig. 5b).

DISCUSSION

Swollen neurons have often been described in previous reports on Creutzfeldt-Jakob disease (CJD), and were considered to be one of the histological hallmarks of this disease (8). They were also found in kuru, another type of human spongiform encephalopathy, and even in experimentally induced CJD or kuru (15). Swollen neurons are found in the cerebral cortex, especially in the deeper layers and in the
Fig. 5. a: Immunoreactivity of swollen neurons to 1D monoclonal antibody was inhibited by a pretreatment of the tissue sections with alkaline phosphatase in Tris-HCl buffer. b: The 1D-immunoreactivity was retained after the treatment with alkaline phosphatase prepared in sodium phosphate buffer. Immunoperoxidase with hematoxylin. ×930.

... basal ganglia; however, their detailed distribution has not yet been described. This study showed that swollen neurons were widely distributed in the cerebral cortex, and that they were most abundant in the cingulate and parahippocampal gyri. This distribution, however, could not be directly related to any clinical features of the patients. In the PE type of CJD, in which extensive cortical and medullary degeneration was consistently observed, the swollen cells were more abundant than in the SSE type of CJD. This suggests that the severity of neurodegenerative processes, especially those of the white matter, is correlated with the number of swollen neurons in the deep cortical layers.

Neuronal swellings also occur in brains with a variety of neurological disorders such as in Pick’s and Alzheimer’s diseases (5), as well as in some unknown neurodegenerative conditions (6, 16). Swollen neurons associated with Pick’s disease are widely distributed in all regions of the cerebral cortex and in some deep gray matter (17). In contrast, those of Alzheimer’s disease are confined to the amygdala, claustrum and insular cortex (5, 6). Meanwhile, swollen neurons are considered to be a definitive change in such malnutritional encephalopathies as pellagra, in which central chromatolysis is found in Betz cells, brainstem nuclei and spinal motor neurons (18). Although some nutritional deficiencies have been suggested to be involved in the pathogenesis of cortical neuronal swellings in CJD (8), a distinct topographical difference between the swollen neurons in CJD and pellagra does not support this opinion.

Recent immunohistochemical studies have shown that abnormal neurons in neurodegenerative disorders contain neurofilament protein (NFP) in their perikarya and in cytoplasmic inclusions. Whether abnormally accumulated neurofilaments are phosphorylated or remain nonphosphorylated is a question of importance. Changes in the phosphorylation state have been assumed to be a mechanism for regulating the conversion between the moving non-polymeric form and the stationary polymeric form of neurofilaments (19–21). In normal neurons the phosphorylated epitopes of NFP are present exclusively in axons; however, nonphosphorylated epitopes are widely distributed in perikarya, dendrites and axons (4, 11, 22). An abnormal distribution of phosphorylated NFP occur in experimental animals and in several human diseases. Phosphorylated epitopes of NFP are found in brainstem neurons.
of aluminum-intoxicated rabbits (23), and in the neurons of dorsal root ganglia after axonal injuries (24). In human neurological diseases, phosphorylated NFP epitopes are present in neurofibrillary tangles (25, 26), in Pick bodies (25), and Lewy bodies (27) and in lower motor neurons and axonal spheroids of amyotrophic lateral sclerosis (28, 29). Dickson et al (5) have observed the immunocytochemical localization of phosphorylated epitopes within swollen neurons in a variety of neurodegenerative diseases. This study has shown that the swollen neurons of CJD contain a phosphorylated epitope of neurofilament protein. These data indicate that an abnormal distribution of phosphorylated NFP is a common pathologic feature in various neurodegenerative disorders and, therefore, this abnormality suggests an underlying common mechanism for the functional impairment of affected neurons.

The phosphorylation of neurofilament occurs predominantly in the tail domain of the 160 kDa and 200 kDa subunits (30). These carboxy-terminal domains have been shown to form the side arm projections that connect neurofilaments with each other and neurofilaments and microtubules to produce a cross-linked matrix of the cytoskeleton (3, 30, 31). Lewis and Nixon (20) showed that the phosphorylation of NFP in optic axons advanced in association with the transition of neurofilament from moving to stationary forms. Based on these data, they suggested a hypothesis that the progressive phosphorylation of the tail domain facilitates the radial projection of side arms to favor interactions with axonal elements (20). If this is the case, the abnormal perikaryal accumulation of neurofilaments and the cytoplasmic swelling of neurons may be interpreted as indicating that the perikaryal neurofilaments were consistently phosphorylated in swollen CJD neurons. This abnormal phosphorylation may expedite the conversion of neurofilament from the moving form to cross-linked stationary networks in the perikarya. The stationary form of neurofilaments have been shown to stay in axons longer than the moving form (19). Therefore, the perikaryal phosphorylated neurofilaments may be retained there for a longer period of time and thus increase the cytoplasmic volume of the neurons affected by CJD. This finding suggests that the abnormal posttranslational modification of the neurofilaments may play an important role in the perikaryal and axonal swellings in several neurodegenerative disorders.

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