Increased Levels of ε and γ Isoforms of 14-3-3 Proteins in Cerebrospinal Fluid in Patients with Creutzfeldt-Jakob Disease

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We established four hybridoma cell lines producing monoclonal antibodies (MABs) against 14-3-3 proteins. Immunoblot analysis revealed that ε and γ isoforms were specifically increased in premortem cerebrospinal fluid samples from patients with sporadic Creutzfeldt-Jakob disease. Furthermore, dot immunoblot analysis showed that MABs were more specific for native antigen than polyclonal antibodies were.

The transmissible spongiform encephalopathies (TSE) include Creutzfeldt-Jakob disease (CJD), kuru, Gerstmann-Sträussler-Scheinker syndrome, and fatal familial insomnia in humans (2), scrapie in sheep and goats, and bovine spongiform encephalopathy (BSE) in cattle (9), as well as other scrapie-like diseases among wild and domestic animals, such as transmissible mink encephalopathy and chronic wasting disease of mule deer. Epidemic BSE in the United Kingdom, which presumably resulted from the feeding of cattle with scrapie- or BSE-contaminated bovine meal (1), has been molecularly linked to a novel, variant form of CJD, termed new variant CJD (10). This event has called into question the safety of the human food supply and has generated enormous interest in the development of rapid, sensitive, and specific assays for the premortem diagnosis of TSE in humans and domesticated animals.

In 1986, Harrington and colleagues detected two proteins in cerebrospinal fluid (CSF) from CJD patients, termed p130/131, by two-dimensional gel electrophoresis (3). With the subsequent demonstration that these proteins are members of the 14-3-3 family (4), tests for the detection of 14-3-3 proteins in CSF from humans and animals with TSE have been developed (4, 8, 11, 12). However, since at least eight isoforms of 14-3-3 proteins exist in humans, we sought to improve future diagnostic tests by developing monoclonal antibodies (MABs) which would detect an isoform-specific increase of 14-3-3 proteins in CSF from CJD patients.

Since a polyclonal antibody (Santa Cruz Biotechnology) against β-isofoms peptides was used in initial experiments (4), we amplified human cDNA (Clontech) of the 14-3-3 β isofoms to prepare fusion proteins between glutathione S-transferase (GST) or thioredoxin and human 14-3-3 protein (5). Amplified products were cloned into plasmids, pGEX 2T (Pharmacia) for the GST–14-3-3 fusion protein and pTrxFus (Invitrogen) for the thioredoxin–14-3-3 fusion protein, expressed in Escherichia coli, and affinity-purified in accordance with the manufacturer’s instructions. Five 6-week-old female BALB/c mice were immunized subcutaneously on day 0 with 20 μg of purified GST–14-3-3 fusion protein in 0.2 ml of complete Freund’s adjuvant. On days 7, 14, and 21, all mice were reinjected subcutaneously with 20 μg of purified GST–14-3-3 fusion protein in 0.2 ml of incomplete Freund’s adjuvant. The two mice with the highest antibody titers by immunoblot analysis with thioredoxin–14-3-3 fusion protein (β isofoms) were injected intravenously with 10 μg of purified GST–14-3-3 fusion protein on day 35. Three days later, spleen cells from these mice were fused with the SP2/0 myeloma cell line. After selection of hybridomas in hypoxanthine-aminopterin-thymidine medium, antibody-producing cells were screened by immunoblot analysis with GST- or thioredoxin–14-3-3 fusion proteins. The immunoblot procedure employed for screening was similar to that used for testing CSF samples and is described later. Specifically, media from 30 pools, each containing 10 clones, were selected, and the 4 positive pools were further subcloned to identify the 4 hybridoma clones producing MABs against 14-3-3 protein. All MABs showed the immunoglobulin G1 (IgG1) subtype.

The four MABs and two polyclonal antibodies (Santa Cruz Biotechnology) were examined by immunoblot analysis for reactivity to 14-3-3 proteins in CSF from patients with sporadic CJD. CSF samples were submitted to the National Institutes of Health. CJD was assigned to one of three diagnostic categories on the basis of clinical information provided by the referring physicians: pathologically confirmed, clinically definite (rapidly progressive dementia, myoclonus, and characteristic electroencephalographic findings), or clinically probable (progressive dementia and myoclonus, ataxia, or characteristic electroencephalographic findings) (4). All CSF samples from CJD patients used in this study were confirmed by pathological examination. CSF from patients with dementia who were later pathologically confirmed not to have CJD served as the non-CJD patient control. The pathological diagnoses were based on routine neuropathological analysis. CSF (10 μl) was mixed with 10 μl of 2X sample loading buffer (1× 50 mM Tris-HCl [pH 6.8], 100 mM dithiothreitol, 2% sodium dodecyl sulfate [SDS], 0.1% bromophenol blue, 10% glycerol), heated for 10 min at 100°C, separated by SDS–15% polyacrylamide gel electrophoresis (SDS–15% PAGE), and then transferred to Immobilon polyvinylidene difluoride (PVDF) membranes (Millipore Corp.). Membranes were incubated with MABs (1 μg/ml)
or polyclonal antibodies (1 μg/ml) in phosphate-buffered saline containing 0.2% Tween 20. After washing, bound antibodies were detected by goat anti-mouse IgG (1:5,000) or goat anti-rabbit IgG (1:5,000) conjugated with horseradish peroxidase (Amersham Pharmacia) followed by chemiluminescence (ECL; Amersham Pharmacia). MAb 9 reacted to two proteins in CSF of CJD patients (Fig. 1). The larger band was about 32 kDa, and the smaller band was 28 kDa. From cDNA data (5, 6), only the ε isoform was expected to be 32 kDa and the other isoforms were 28 kDa. We therefore suspected that the larger band represented the ε isoform. The 32-kDa band was detected only in CSF from CJD patients, whereas the 28-kDa band was detected by other MAbs (MAbs 7 and 13) and was not specific for CSF from CJD patients. Only MAb 9 stained neuronal cells and axons in formalin-fixed cerebral tissue. To verify that the 32-kDa band was the ε isoform of 14-3-3, we used polyclonal antibodies (Santa Cruz Biotechnology) to isoform-specific peptides. Rabbit anti-ε antibody detected the 32-kDa band (Fig. 1, lanes 2 and 3) in the same CSF samples. Therefore, we concluded that MAb 9 detected the ε isoform and other isoforms with weaker affinity and that the ε isoform was specifically increased in CSF from CJD patients. Rabbit anti-γ isoform antibody revealed that this isoform also increased specifically in CSF from CJD patients. In summary, the ε and γ isoforms were increased specifically in CSF from CJD patients. These immunoblot results suggest the potential utility of MAb 9 and γ-isoform-specific polyclonal antibody in the premortem diagnosis of CJD. Previously, in screening numerous CSF samples sent to the Laboratory of Central Nervous System Studies, National Institute of Neurological Disorders and Stroke, by using a rabbit polyclonal antibody to the β isoform, a similar increase of 14-3-3 proteins in CSF was observed (4). We suspected that this isoform-nonspecific polyclonal antibody could detect the total increase of 14-3-3 isoform proteins. MAbs 3 (data not shown), 7, and 13 showed weak affinity to denatured 14-3-3 protein isoforms, and these were not specific for CSF from CJD patients.

We then tried to classify the reactivity of these antibodies to native 14-3-3 proteins by using dot hybridization. Since the full-length cDNA encoding the γ isoform has not been cloned, we prepared recombinant 14-3-3 proteins, including ε, ζ, η, τ, and β isoforms. Recombinant human 14-3-3 τ-, ζ-, η-, β-, and ε-isoform fusion proteins with a hexahistidine tag on the amino terminus were produced from human 14-3-3 cDNAs by PCR. cDNA products were subcloned into pET-21a vector (Novagen), transformed into E. coli BL21(DE3), and purified by TALON metal-affinity resin chromatography (Clontech Laboratory) according to the manufacturer’s instructions. Homogeneity was confirmed by SDS-PAGE followed by Coomassie brilliant blue staining. Each 14-3-3 protein (100 ng) was dotted onto Immobilon PVDF membranes, dried for 30 min, blocked with 2% skim milk (Yukijirushi), and then reacted for 2 h with MAbs or polyclonal antibodies which had been diluted to 1 μg/ml. Bound antibodies were detected by goat anti-mouse or anti-rabbit IgG (1:5,000) conjugated with horseradish peroxidase followed by chemiluminescence. Detection without primary antibodies or dot blot of other nonspecific histidine-tagged proteins did not show any signals (data not shown). ε-, ζ-, and τ-specific polyclonal antibodies reacted with ε, ζ, and τ antigens with highest affinity (Fig. 2A). However, they also reacted to other isoforms with a weaker affinity. A polyclonal antibody preimmunized against β-isoform peptides showed broad isoform affinity and reacted relatively equally (Fig. 2A). MAb 3 reacted with the ε isoform, MAb 7 reacted with the τ isoform, and MAb 13 reacted with the ζ isoform, whereas MAb 9 revealed affinity for two isoforms, namely, the τ and ζ isoforms (Fig. 2B). In comparison with the broad reactivities of polyclonal antibodies to native antigen, MAbs reacted more specifically and the polyclonal antibodies lost their specificity to native 14-3-3 proteins.

Although mice were immunized with the β isoform, none of the newly generated MAbs reacted to the native β isoform. By contrast, polyclonal antibodies prepared against β-isoform.
specific peptides showed broad affinity to several isoforms. Since 14-3-3 proteins are cytosolic chaperone-like proteins (7), we suspect that dynamic protein folding of 14-3-3 fusion proteins used for immunization produced other isoform-specific epitopes. These and other antibodies need further testing on a larger number of CSF from patients or animals with suspected or pathologically proven TSE to define their utility as diagnostic reagents. In addition, 14-3-3 isoform-specific analysis using MABs or polyclonal antibodies may provide further insight into the kinetics of 14-3-3 proteins in prion diseases.

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