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 bone meal (1), has been molecularly linked to a novel, variant form of CJD, termed new variant CJD (10). Since a polyclonal antibody (Santa Cruz Biotechnology) against β-isofrom peptides was used in initial experiments (4), we amplified human cDNA (Clontech) of the 14-3-3 β isoform 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 isoform) 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 SP2O 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 2× 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). MAbs 9 reacted to two proteins in CSF of CJD patients (Fig. 1). The larger band was about 32 kDa. We therefore suspected that the larger band represented the isoform of 14-3-3, 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). MAbs 3 reacted with the ε isoform, MAbs 7 reacted with the τ isoform, and MAbs 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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REFERENCES