Elevated Levels of Anti-CD9 Antibodies in the Cerebrospinal Fluid of Patients with Subacute Sclerosing Panencephalitis

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Subacute sclerosing panencephalitis (SSPE) is a slowly progressive and highly lethal disease of the central nervous system that occurs in children and young adults. Although the primary cause of SSPE is believed to be persistent infection of neuron and glial cells by a measles virus, the precise mechanism of the progression of this disease has not yet been elucidated. CD9, a member of the tetraspanin family, is expressed in myelin and other nervous tissues. This study detected significant amounts of anti-CD9 antibodies in the cerebrospinal fluid (CSF) of all patients with SSPE included in the study. Anti-CD9 antibodies were also detected in the CSF of some patients with other neurologic disorders, but those patients had lower levels of anti-CD9 antibodies than did the patients with SSPE. The level of anti-CD9 antibodies was elevated and reached a peak that coincided with the appearance of brain atrophy. These findings shed light on a new aspect of the causes and progression of SSPE.

Subacute sclerosing panencephalitis (SSPE) is a slowly progressive and highly lethal disease of the central nervous system (CNS) that occurs in children and young adults. Although the primary cause of SSPE is believed to be the persistent infection of neuron and glial cells by a defective measles virus [1], the precise mechanism of the progression of this disease has not yet been elucidated. CD9, a member of the tetraspanin superfamily [2]. This protein is expressed in a variety of hematopoietic and nonhematopoietic cells. CD9 is implicated in cell growth, cell adhesion, cell motility, membrane fusion, viral infection, and tumor cell metastasis [2–4]. Nervous systems, including sympathetic neurons and dorsal root ganglion cells, express CD9 [5]. Schwann cells and glial cells of the CNS also express CD9 [6]. Myelin in the CNS and the peripheral nervous system contains abundant CD9 [7, 8]. Although the function of CD9 in myelin has not yet been elucidated, the abundant expression of CD9 suggests that the glycoprotein has a unique function at the surface of myelin.

Autoimmune responses to myelin proteins are considered to be possible causes of the onset and progression of some demyelinating diseases [9]. This led us to examine whether CD9 also could be implicated in demyelinating diseases and other neurologic disorders. In the this study, we examined the cerebrospinal fluid (CSF) levels of anti-CD9 antibodies in patients with neurologic disorders.

Patients, Materials, and Methods

Patients and CSF samples. SSPE was diagnosed on the basis of clinical features and confirmed by the presence of characteristic electroencephalographic findings and measles antibody levels in the CSF. Clinical staging was based on the methods of Jabbour et al. [10]. Diagnoses of other diseases were based on relevant criteria. CSF samples were obtained by lumbar puncture from patients with neurologic disorders, including 12 patients with SSPE (9 male and 3 female). We also determined CSF levels of anti-CD9 antibodies in 5 patients with orthopedic diseases and 3 patients with gynecologic diseases (control subjects).

Glutathione-S-transferase (GST)–CD9 proteins. The recombinant GST fusion proteins containing the second extracellular loop of CD9, which corresponds to aa 113–194 of either monkey CD9 (mkCD9) or human CD9 (hCD9), were produced in Escherichia coli with pGEX-3X plasmids (Pharmacia) inserted in the corresponding cDNA sequences of CD9 at EcoRl/BamHI sites. These forms of GST fusion proteins, referred to as “GST-mkCD9” and “GST-hCD9,” cover most of the extracellular domain of CD9 and are recognized by several mouse monoclonal antibodies (Mabs).
Directed to hCD9 by means of immunoprecipitation and Western blotting. The GST fusion protein containing human heparin-binding epidermal growth factor–like growth factor (GST-HB), which corresponds to aa 106–149, was also produced in E. coli [11]. Fusion proteins were purified, using 2 purification step cycles, with Glutathione Sepharose 4B (Pharmacia), as instructed in the manufacturer’s protocols.

**RIA.** We used a solid-phase RIA to determine the amount of anti-CD9 antibodies in CSF. In all, 30 μg of GST-CD9 (GST-mkCD9 or GST-hCD9) or GST protein was immobilized on a Reacti-Bind glutathione-coated strip well plate (Pierce). The plates were incubated with blocking solution (0.15 M NaCl, 50 mM Tris, 1 mg/mL bovine serum albumin [BSA], and 1% skim milk, pH 8.0) at 37°C for 3 h and at 4°C overnight. CSF from patients was diluted 3 times with blocking solution, and 50 μL of the diluted CSF was added to wells of the GST-CD9– and GST-coated plates. The plates were incubated for 2 h at 37°C and then for 3 h at 4°C. After the wells were washed 3 times with washing solution (50 mM Tris and 0.15 M NaCl, pH 8.0), the plates were incubated with 50 μL of 125I-labeled anti–human IgG (heavy and light chains; 10 μg/mL) in blocking solution at 37°C for 2 h. After another 3 washes with washing solution, the radioactivity remaining on each well was counted by gamma counter. For binding experiments, we used both the GST-CD9–coated plate and the GST-coated plate for each CSF sample. Specific binding was determined by subtracting the nonspecific binding obtained from the GST-coated plate from the total binding obtained from the GST-CD9–coated plate. Data are expressed as the amount of 125I-labeled anti–human IgG that was calculated from the values of the specific binding and the specific activity of the 125I-labeled antibody.

**Western blotting.** GST fusion proteins and GST were subjected to SDS-PAGE and electrotransferred to an Immobilon membrane (Millipore), as described elsewhere [7]. CSF samples were diluted 3 times with Tris-buffered saline–BSA (20 mM Tris-HCl, 100 mM NaCl, and 3% BSA, pH 7.5), and membranes were incubated at 4°C overnight with the diluted GST-CD9 samples. After incubation with 10 ng/mL horseradish peroxidase (HRP)–conjugated anti–human IgG, immunoreacted bands were visualized with an ECL Western blotting kit (Amersham International). Mouse anti-hCD9 MAb (clone KazR-1) and HRP-conjugated anti–mouse IgG were also used as controls.

**Results**

**Elevated anti-CD9 antibodies in CSF samples from patients with SSPE.** CSF samples from patients with neurologic disorders were examined for anti-CD9 antibodies. No anti-CD9 antibodies were observed in CSF samples from the control subjects (n = 8). However, CSF from patients with SSPE contained significantly (P < .005) more anti-CD9 antibodies than were found in CSF from patients with other neurologic disorders and from control subjects (figure 1A). Anti-CD9 antibodies in CSF were elevated in all patients with SSPE who were tested, although the amounts varied by patient and by disease stage (see below). CSF samples from some patients with Japanese B encephalitis (n = 5) and from 1 patient with acute disseminated encephalomyelitis (n = 3) also showed low levels of anti-CD9 antibodies, but the levels were lower than in patients with SSPE (figure 1A). Anti-CD9 antibodies were not detected in the CSF of patients with multiple sclerosis (MS; n = 14), Guillain-Barré syndrome (n = 3), leukodystrophy (n = 2), human T cell leukemia virus (HTLV) type 1–associated myelopathy (n = 3), Machado-Joseph disease (n = 3), brain tumors (n = 3), and collagen disease (n = 18). MS is a typical demyelinating disease, although no anti-CD9 antibodies were detected in any sample from the 14 patients with MS who were tested in this study (8 patients in the acute exacerbation phase and 6 with chronic remitting conditions).

The CSF levels of anti-CD9 antibodies shown in figure 1A were determined with mkCD9. Because mkCD9 differs in 2 amino acid residues from hCD9, we also did assays with GST-CD9 (A) or GST–human CD9 (B). Data are shown as amounts of 125I-labeled anti–human IgG, as described in Patients, Materials, and Methods. Bars indicate mean ± SE.

**Figure 1.** Presence of anti-CD9 antibodies in the cerebrospinal fluid (CSF) of patients with subacute sclerosing panencephalitis (SSPE) or other neurologic disorders. A, CSF levels of anti-CD9 antibodies in patients with SSPE (n = 12), Japanese B encephalitis (JE; n = 5), and acute disseminated encephalomyelitis (ADEM; n = 3). B, CSF levels of anti-CD9 antibodies in patients with SSPE (n = 5), cytomegalovirus encephalitis (CMV; n = 3), human immunodeficiency virus encephalopathy (HIV; n = 4), progressive multifocal leukoencephalopathy (PML; n = 4), and aseptic meningitis (ASM; n = 3). Quantities of anti-CD9 antibodies were determined by solid-phase RIA, using glutathione-S-transferase (GST)–monkey CD9 (A) or GST–human CD9 (B). Data are shown as amounts of 125I-labeled anti–human IgG, as described in Patients, Materials, and Methods. Bars indicate mean ± SE.
hCD9, to confirm that anti-CD9 antibodies observed in CSF samples from patients with subacute sclerosing panencephalitis (SSPE) react with hCD9. Figure 1B shows that levels of anti-CD9 antibodies were elevated in the CSF of all patients with SSPE tested (n = 5). Although we could not examine CSF samples from the other patients with SSPE included in this study (because the available samples were limited), it is evident that the CSF of patients with SSPE contains anti-CD9 autoantibodies. Using a GST-hCD9–coated plate, we also measured the CSF levels of anti-CD9 antibodies among patients with other neurologic disorders caused by infectious diseases. Low levels of anti-CD9 antibodies were observed in CSF samples from only a few patients, but the levels were much lower than in patients with SSPE.

Levels of whole IgG in the CSF of patients with SSPE (n = 5) and patients with other neurologic disorders caused by infectious diseases (n = 11) were 59–276 and 61–504 μg/mL, respectively. The level of anti-CD9 antibodies did not correlate with the level of whole IgG in patients with SSPE.

The presence of anti-CD9 antibodies in the CSF of patients with SSPE was confirmed by Western blot analysis. Although similar amounts of GST and GST-mkCD9 proteins were blotted on membrane for Western blotting, CSF from patients with SSPE preferentially reacted with GST-mkCD9 (figure 2A). Similar results were obtained when GST-hCD9 was used (figure 2B). CSF from patients with MS and from patients infected with human immunodeficiency virus reacted with neither GST-CD9 nor GST (figure 2A and 2B). Moreover, CSF from 1 patient with SSPE did not react with GST-HB (figure 2B), which further supports the suggestion that this patient’s antibodies are specific to CD9. To examine whether the anti-CD9 antibodies detected would react with native CD9 protein, hCD9 (isolated from hCD9-expressing L cells) was also subjected to SDS-PAGE and Western blotting. However, the CSF of patients with SSPE did not react with CD9 isolated from L cells (figure 2C).

Relationships between levels of anti-CD9 antibodies and other clinical data from patients with SSPE. Of the 12 patients with SSPE, 9 were male and 3 were female (age range, 3–22 years). Eight patients were in the early stages of the disease, and 4 were in stages III or IV, by the criteria of Jabbour et al. [10]. Figure 3 illustrates the clinical course in 2 representative cases. Interferon (IFN)–α was administered during the time period indicated in figure 3. In one patient, > 100 pg of anti-
CD9 antibodies was observed in CSF during stage I–II of the disease (figure 3A). Levels of antibodies peaked quickly and then decreased. Computed tomographic (CT) and magnetic resonance imaging (MRI) data obtained during stage I–II (figure 3A, images 1 and 2) appeared to be normal. CT scans obtained ~1 year after the MRI data (figure 3A, image 3) confirmed that moderate brain atrophy had occurred. In the other patient, the highest anti-CD9 antibody levels were observed during stage I, and levels then fluctuated from 150 to 450 pg (figure 3B). CT findings suggested that mild brain atrophy occurred even during stage I (figure 3B, image 1). It is noteworthy that the level of anti-CD9 antibodies in CSF was elevated even before administration of IFN-α, which negates the possibility that IFN treatment was the cause of the elevation.

Discussion

We examined CSF samples from patients with several neurologic disorders for the presence of anti-CD9 antibodies. No significant levels of these antibodies were detected in CSF samples from control subjects or from the majority of patients with other neurologic disorders who were tested, whereas CSF samples from all patients with SSPE contained anti-CD9 antibodies. Western blot analysis confirmed the existence and specificity of anti-CD9 antibodies. Although anti-CD9 autoantibodies have been found in patients with hematologic disorders, including thrombocytopenic purpura [12], we believe that the present study is the first to describe the presence of autoantibodies against CD9 in CSF.

The failure to detect cellular CD9 in CSF samples from patients with SSPE could have several causes. First, the amounts of anti-CD9 antibodies in the CSF and cellular CD9 subjected to Western blotting may have been too low to detect. Second, the anti-CD9 autoantibodies contained in the CSF of patients with SSPE may have been generated by fragmented CD9 or a denatured form of CD9 and therefore might have reacted more efficiently to a partly denatured form of CD9 than to the intact form. Third, CD9 expressed in L cells may be different in its antigenic structure.

A previous MRI study [13] has indicated that brain atrophy progresses during stages II and III of SSPE. In the patient for
whom results are shown in figure 3A, the presence of high levels of anti-CD9 antibodies in CSF during stage I–II was confirmed. CT and MRI data indicated that no or mild atrophy was present at this stage. In the patient for whom results are shown in figure 3B, elevated anti-CD9 antibody levels and mild brain atrophy were observed during stage I. This stage is considered to be the onset of the disease. Although our data do not prove that elevation of anti-CD9 antibodies occurred before brain atrophy, our results suggest that the elevation of CSF anti-CD9 antibodies coincided with brain atrophy, rather than resulting from secondary effects of brain-tissue destruction.

Some reports suggest that CD9 and other tetraspanin proteins play a role in viral infections. CD9 is involved in the susceptibility to or in the virus-induced cell-cell fusion of the canine distemper virus [3, 14]. HTLV-1–induced syncytium formation was inhibited by the antibody against CD82 [15]. These results suggest that tetraspanin has a function in the infection and/or fusion of some enveloped viruses. CD9 is essential for sperm-egg fusion in mouse fertilization, which may suggest that CD9 is involved in membrane fusion [4]. CD46, one of the measles virus receptors, might form complexes with CD9 [16]. Thus, we speculate that CD9 may contribute to both infection and propagation of the measles virus. Further studies are needed to elucidate the link between CD9 and measles virus infection.

In the present study, anti-CD9 antibodies were detected in CSF samples from some patients with neurologic disorders caused by infectious diseases and from 1 patient with acute disseminated encephalomyelitis, although the levels in these patients were lower than the levels found in CSF samples from patients with SSPE. CD9 is also expressed in gray matter, although the amount is much lower than it is in white matter. Thus, neuronal degeneration due to inflammation may cause the elevation of CSF levels of anti-CD9 antibodies. The slow progression of SSPE may result in the high levels of anti-CD9 antibodies that we found.

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References

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