Autoantibodies against Glial Fibrillary Acidic Protein (GFAP) in Cerebrospinal Fluids from Pug Dogs with Necrotizing Meningoencephalitis

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ABSTRACT. Cerebrospinal fluids (CSFs) from 9 Pug dogs with necrotizing meningoencephalitis (NME: Pug dog encephalitis) were examined to identify the antigens for anti-astrocyte autoantibodies. Each CSF exhibited a positive reaction to the cytoplasm of cultured canine astrocytes by an indirect fluorescent antibody test. In an immunoblotting analysis on normal canine brain proteins, eight of 9 CSFs showed a common band of 52 kDa, corresponding to glial fibrillary acidic protein (GFAP), and all of 9 CSFs reacted with purified bovine GFAP. From these results, GFAP is one of the common autoantigens in Pug dogs with NME. On the other hand, the reactivity of CSFs to chymotrypsin-digested bovine GFAP fragments were variable among dogs, indicating that the antibodies in the CSFs recognized different epitopes on GFAP.

KEY WORDS: autoantibody, canine, CSF, GFAP, NME.

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Canine necrotizing meningoencephalitis (NME: Pug dog encephalitis) is an idiopathic inflammatory disease in the brain, affecting specific small-sized breeds such as Pugs [4–6, 18, 22]. Histopathology of NME is characterized by lymphocytic inflammation and subsequent necrosis in the cerebral cortex [20]. While the pathogenesis of NME is unclear, Uchida *et al.* [22] and our previous report [12] demonstrated that dogs with NME possessed anti-astrocyte autoantibodies in their cerebrospinal fluids (CSFs), suggesting that NME is an autoimmune disease. In addition, Suzuki *et al.* [20] reported the immunoglobulin and complement deposition on astrocytes in an NME case, supporting the pathological roles of anti-astrocyte autoantibodies. In the present study, we identified a common autoantigen for the anti-astrocyte autoantibodies in CSFs from Pug dogs with NME.

MATERIALS AND METHODS

CSFs: Between January 2001 to April 2004, 9 Pug dogs (11-month to 7-year of age, 5 males and 4 females) were referred to University of Tokyo Veterinary Medical Center, and were diagnosed as NME by magnetic resonance imaging (MRI) and postmortem pathological examinations. CSFs were collected from the cisterna magna under general anesthesia from 7 cases and during necropsy from 2 cases. A CSF sample from a clinically healthy Pug dog was used as a control.

Indirect fluorescent antibody test (IFA) on cultured astrocytes: Primary cultures of astrocytes were prepared from the forebrain of full-term fetuses of a clinically healthy Beagle

dog as described by McCarthy *et al.* [14]. The purity of obtained astrocytes was confirmed by an immunofluorescence assay with an anti-human GFAP monoclonal antibody (YLEM, Roma, Italy). Over 99% of cultured cells were GFAP-positive astrocytes. Astrocytes were maintained in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich, St. Louis, MO, U.S.A.) with 20% fetal calf serum (Invitrogen, Carlsbad, CA, U.S.A.) under 5% CO₂/95% air at 37°C.

The reaction of CSFs on astrocytes was examined by an IFA test. That is, astrocytes were cultured on 22-mm-diameter glass coverslips for 48 hr, rinsed with HBSS, fixed with cold acetone for 5 min and dried. After 3 washes with phosphate-buffered saline (PBS), the cells were incubated with diluted CSF (1:10) at 37°C for 60 min. After 3 washes, fluorescein isothiocyanate (FITC)-conjugated goat anti-canine IgG (Bethyl, Montgomery, TX, U.S.A.) was applied at 1:400 at 37°C for 60 min. Then, the cells were washed, dried, mounted with Vectashield (Vector Labs, Burlingame, CA, U.S.A.), and analyzed by a fluorescent microscope (Optiphot-2, Nikon, Tokyo, Japan) equipped with an image analyzing system (ORCA-1394, Hamamatsu Photonics, Hamamatsu, Japan).

Immunoblot on canine brain proteins: A piece of the cerebral cortex from a healthy female Beagle dog was kindly gifted from Professor Tsujimoto, Department of Veterinary Internal Medicine, The University of Tokyo. An approximate 1 g of the cerebral cortex was washed 3 times in ice-cold PBS and homogenized in 10 ml of sucrose buffer (250 mM sucrose, 1 mM EDTA, 1.6 mM phenylmethanesulfonyl fluoride, 10 µg/ml pepstatin A, 10 µg/ml leupeptin, 10 µg/ml aprotinin, pH 7.4) on ice. The protein concentration of the homogenate was determined with a commercial kit (Protein Assay kit, Bio-Rad Laboratories, Hercules, CA,

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U.S.A.), using bovine serum albumin as a standard.

For electrophoresis, approximately 10 μ g of brain proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gels, and then stained with Coomassie Brilliant Blue or transferred onto nitrocellulose membranes (Hybond ECL, Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.). The membranes were blocked by 5% skim milk (Becton Dickinson, Sparks, MD, U.S.A.) in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 hr, and incubated with diluted CSFs (1:200) for 1 hr. After washes, autoantibody was reacted with horseradish peroxidase (HRP)-conjugated goat anti-canine IgG (1:4,000, Bethyl) for 1 hr and visualized by a chemiluminescence kit (ECL and Hyperfilm ECL, Amersham).

Mass spectrometry of proteins: Protein bands on stained gels, which corresponded to positive bands on the immunoblot membranes, were recovered, in-gel digested with trypsin, and identified with matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) tandem mass spectrometry (MS/MS: Model 4700 Proteomics Analyzer, Applied Biosystems, U.S.A.) as previously described [15]. The MS/MS spectra were analyzed by using the Mascot software program (Matrix Science, London, UK).

Immunoblot on intact/digested bovine GFAP: One μ g/lane of intact bovine GFAP (Progen Biotechnik, Heidelberg, Germany) was separated by SDS-PAGE on 10% gels and was transferred onto nitrocellulose membranes. Digestion of bovine GFAP by α -chymotrypsin (Sigma-Aldrich: GFAP to enzyme weight ratio was 5:1) was performed in 10 mM Tris (pH 8.8) on ice for 30 min. The digests were separated by SDS-PAGE on 13.5% gels, and were transferred onto nitrocellulose membranes. These membranes were submitted to immunostaining with CSFs as described above.

RESULTS

Distribution of autoantigen(s) in cultured astrocytes: Figure 1 shows the results of IFA tests on cultured canine astrocytes. All CSFs from NME cases clearly reacted with astrocytes, showing the positive signal in the cytoplasm within the cell body and processes. The reaction pattern of CSFs was similar to that of an anti-human GFAP monoclonal antibody (mAb), whereas the CSF from the healthy control (C) did not react with astrocytes.

Immunoblot on canine brain proteins and mass spectrometry of proteins: Results of immunoblotting on canine brain proteins are shown in Fig. 2. In case 1–7 and 9, a common band of 52 kDa was detected (arrow), which corresponded to GFAP by the amino acid sequence analysis with TOF-MASS (Table 1). In some cases, additional bands were observed at different molecular weights (arrow heads).

Immunoblot on intact/digested bovine GFAP: Figure 3 shows the results of immunoblotting on intact bovine GFAP. All CSFs from NME cases (1–9), as well as anti-human GFAP monoclonal antibody (mAb), clearly reacted to bovine GFAP at approximately 50 kDa, whereas neither

Fig. 1. Indirect fluorescent antibody test (IFA) on cultured canine astrocytes. All CSFs from NME cases (1–9) clearly reacted to astrocytes, showing positive signals in the cytoplasm within the cell body and processes. The reaction patterns were similar to that of anti-human GFAP monoclonal antibody (mAb), whereas no reaction was observed in CSF from a healthy control (C).

the control CSF (C) nor second antibody alone (N: goat anti-canine IgG) reacted to bovine GFAP.

CSFs from 4 cases (case 4–7) were examined on digested GFAP to evaluate the epitopes on GFAP (Fig. 4). In all of 4 cases, a common band was observed at 25 kDa. On the other hand, CSF of case 6 strongly reacted to the digested GFAP fragment at approximately 16 kDa (arrow head). The band patterns were variable among tested dogs.

DISCUSSION

The present study was designed to identify the autoantigen for anti-astrocyte autoantibodies, which were frequently detected in the CSF from NME cases [12]. All of the present 9 cases also possessed anti-astrocyte autoantibodies, which were detected by an IFA test on frozen sections of the normal canine cerebrum (data not shown). The IFA test on cultured fetal canine astrocyte was done to evaluate the distribution of autoantigen(s) in astrocytes. All CSFs strongly reacted to the cytoplasm of astrocytes, while minimal reaction was detected on the plasma membrane. To identify the cytoplasmic autoantigen, an immunoblot on normal canine brain proteins was performed, and GFAP was one of the major candidates of autoantigens. The reactivity of CSFs with GFAP was well confirmed by the immunoblotting on purified bovine GFAP. On the other hand, the reactivity of

Fig. 2. Immunoblot on canine brain proteins with CSFs from NME cases (1–9), the control CSF (C) and the second antibody alone (N). In case 1–7 and 9, a common band at approximately 52 kDa was detected (arrow), which corresponded to GFAP. In some cases, additional bands were observed at different molecular weights (arrow heads).

Table 1. Tryptic peptides of a common band reacted with CSFs and identified as GFAP by mass spectrometry

No.	Mass		Position (residue)		Matched peptides
	Observed	Calculated	Start	End	
1	1098.63	1097.62	96	105	ALAAELNQLR
2	1177.63	1176.61	112	121	LADVYQAE LR
3	1265.65	1264.64	259	270	SKFADLTDA AAR
4	1050.56	1049.51	261	270	FADLTDA AAR
5	1060.53	1059.48	280	287	HEANDYRR
6	1208.58	1207.58	320	330	EAASYQEALAR
7	1499.8	1498.78	345	356	HLQEYQDLLNVK

Fig. 3. Immunoblot on intact bovine GFAP. All CSFs from NME cases (1–9), as well as anti-human GFAP monoclonal antibody (mAb), clearly reacted to bovine GFAP at 50 kDa, while neither the control CSF (C) nor the second antibody alone (N) reacted to bovine GFAP.

CSFs to bovine GFAP digested by α -chymotrypsin were variable among dogs, suggesting the antibodies in those CSFs recognized different epitopes on GFAP. In humans, only one patient with systemic lupus erythematosus (SLE) has been reported to be positive for an anti-GFAP autoantibody in the CSF [19], although the anti-GFAP autoantibodies are often detected in human sera. In the present study, we detected anti-GFAP autoantibodies in the CSFs from all NME cases, indicating a unique feature of canine NME.

GFAP is the major protein of intermediate filaments in mature astrocytes [7], and is widely used as a marker of

astrocytes [9]. Studies on GFAP reported that GFAP has significant roles in maintaining the morphology of astrocytes [8], forming the integrity of blood-brain barrier [11], and modulating the synaptic efficacy [13]. Pekny and Pekna [17] reported that GFAP has essential roles in reactive gliosis during central nervous system (CNS) pathologies, and in nerve regeneration after brain trauma. In addition, human GFAP gene mutations have been reported in fatal [2] and infant [1] CNS diseases.

The present study does not provide information whether the anti-GFAP autoantibody is a cause, consequence or

Fig. 4. Immunoblot on digested bovine GFAP. CSFs from 4 cases (case 4–7) were examined. Although a common band was observed at 25 kDa, the band patterns were variable. Case 6 strongly reacted to the fragment of 16 kDa (arrow head). CBB; digested GFAP stained by Coomassie Brilliant Blue, M; molecular weight markers.

epiphenomenon of canine NME. Production of anti-GFAP autoantibody may be caused by GFAP leakage from damaged astrocytes, since GFAP is the principal cytoplasmic protein in mature astrocytes. However, autoantibodies against cytoplasmic autoantigens were also detected in autoimmune hepatitis [23] and type I diabetes [21] in human patients, and were considered to play essential roles in the pathogenesis of diseases. In canine NME, IgG and complement deposition was detected on astrocytes, suggesting a possibility of autoantibodies to disrupt the astrocyte functions [20]. Astrocyte dysfunction was reported to induce disturbances in the CNS, since astrocytes modulate the extracellular concentrations of excitatory amino acids such as glutamate [10, 16]. Excessive accumulation of glutamate in the extracellular space induces neuronal cell death by the excitatory neurotoxicity [3].

In conclusion, GFAP is one of common autoantigens in canine NME, and the anti-GFAP autoantibodies in the CSF may be closely related to the pathogenesis and/or pathophysiological states of NME, although further studies are necessary to clarify the underlying pathological mechanisms.

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