**FULL PAPER** *Internal Medicine*

# High Mobility Group Box 1 (HMGB1) Protein is Present in the Cerebrospinal Fluid of Dogs with Encephalitis

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(Received 7 October 2010/Accepted 11 March 2011/Published online in J-STAGE 25 March 2011)

ABSTRACT. Many cases of encephalitic disease with unknown etiologies have been reported in specific breeds of small dogs. High mobility group box 1 (HMGB1) in neuronal cells was recently found to be a novel cytokine-like mediator that is a marker of neuronal necrosis and inflammation. The aim of this study was to determine whether HMGB1 levels are elevated in the cerebrospinal fluid (CSF) of dogs suspected of having encephalitis. CSF was obtained from 31 dogs that were diagnosed with an encephalitic disease by clinical examinations and magnetic resonance image (MRI) scanning. The CSF samples were analyzed via western blotting (WB) and an enzyme-linked immunosorbent assay (ELISA) with a polyclonal antibody against HMGB1. The mean HMGB1 concentration was significantly higher in the encephalitic dogs than that in the healthy controls. The concentrations of HMGB1 were correlated with the cell counts and total protein concentrations, which are known CSF indicators of the neuronal inflammation associated with encephalitis. These results suggest that HMGB1 protein in CSF confirms the presence of necrosis and inflammation in most cases of canine encephalitis and that HMGB1 will be a new indicator of encephalitis.

KEY WORDS: biomarker, canine, CSF, encephalitis, HMGB1.

*J. Vet. Med. Sci.* 73(7): 917–922, 2011

There are a number of encephalitic diseases that present with neurological symptoms in canines. Due to improvements in computed tomography (CT) and magnetic resonance imaging (MRI) techniques in animal healthcare, diseases of the nervous system that form gross lesions, such as brain tumors and hydrocephalus, can be diagnosed with considerable accuracy. At present, degenerative and inflammatory diseases are diagnosed clinically from imaging data, the results of neurological examinations, blood biochemical tests and cerebrospinal fluid (CSF) analysis. However, accurate diagnosis still requires histological analysis of brain tissue. Granulomatous meningoencephalitis (GME) is a delayed-type, T-cell dependent allergic response in the brain tissue [22]. Additionally, necrotizing meningoencephalitis (NME) is an autoimmune disease that affects brain tissue [13, 25, 27]. Thus, a highly correlated diagnostic marker is needed to correctly diagnose the various forms and progression of encephalitic disease in the clinic.

High mobility group box 1 (HMGB1) is a 27- to 29-kDa protein that is a known transcriptional regulator and a major component of the non histone nuclear protein group [2, 14]. HMGB1 is similar to the amphoterin molecule isolated from the brain as a neurite outgrowth factor that can bind to heparin [18]. HMGB1 was recently found to be a late inflammatory mediator in septic shock in humans and rats [30]. An increase in the concentration of HMGB1 in the blood is associated with many human diseases, including rheumatoid arthritis [24], acute lung injury (ALI) [28], cancer [32], disseminated intravascular coagulation (DIC) [6] and surgery [21]. Recently, it was also shown that HMGB1 is a damage-associated molecular pattern molecule (DAMP). This protein is passively released as an endogenous danger signal from necrotic and inflammatory cells [19] and induces an inflammatory response [12]. Conversely, it has been postulated that the ability of apoptotic cells to retain HMGB1 in the nucleus is a strategy to prevent development of the inflammatory response [5]. The extracellular accumulation of HMGB1 in the brain, as a result of its release from dying neurons during cerebral ischemia, elicits local inflammatory events in mice [8, 16]. Moreover, HMGB1 also promotes neuroinflammation in the post ischemic rat brain [8] and in the neurodegenerative processes associated with Alzheimer's disease [23]. There have only been two reports that the concentration of HMGB1 in the blood increases in canines with systemic inflammatory response syndrome (SIRS) [36] and lymphoma [15]. It has been reported that the HMGB1 receptor is the receptor for advanced glycation end products (RAGE) [7]. HMGB1 functions as an inflammatory cytokine, and the signal from HMGB1/RAGE binding activates NF-κB [1, 20]. HMGB1 also plays a role in inflammation after cerebral ischemia [8] in rats. In particular, HMGB1 can be passively released into the extracellular space during necrosis, whereas it is retained in the nucleus of cells undergoing apoptosis. Immune cells also actively release HMGB1 upon stimulation [5, 19]. Furthermore, extracellular HMGB1 engages membrane receptors on different cells, signaling proliferation, differentiation, cytopro-

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tection and immune activation [3, 4, 8, 17].

In this study, to confirm whether HMGB1 is associated with suspected encephalitis in dogs, we determined the concentration of HMGB1 in CSF samples obtained from canines that were diagnosed with encephalitic disease by MRI and other general evaluations, especially cell count and protein concentration in the CSF.

## MATERIALS AND METHODS

*CSF preparation*: CSF samples were obtained from 31 dogs (median age of 4 y, 11 m; range of 0 y, 8 m to 13 y, 9 m; male/female=11/20) that had been diagnosed with encephalitis by MRI and neurological examinations at the Veterinary Hospital of Rakuno Gakuen University during the period of 2005 to 2007. For controls, CSF samples were obtained from 10 clinically healthy beagles (median age of 7 y, 1 m; range of 1 y, 9 m to 9 y, 10 m; male/female=4/6) that had been bred at Rakuno Gakuen University. The control dogs had never showed neurological symptoms and had normal examination results. The CSF samples were collected from the cisterna magna under general anesthesia. The extracted CSF samples were examined by routine biochemical tests (cell count, protein concentration, specific gravity, glucose concentration, anti-canine distemper virus (CDV) antibody test, fibrin concentration, tryptophan concentration, globulin response, chloride concentration) and were stored at –30ºC until analysis. All of the animals were treated according to the Laboratory Animal Control Guidelines of Rakuno Gakuen University, which conform to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (U.S.A.).

*MRI*: The dogs were anesthetized, and images were obtained in ventral recumbency using a 0.2-Tesla MR scanner (SIGNA Profile OpenSpirit, GE Healthcare Japan, Tokyo, Japan) with a surface array coil for human extremities. T2-weighted, T1-weighted and T2-fluid-attenuated inversion recovery (FLAIR) images were obtained in the transverse and coronal planes with slice thicknesses of 3.5 to 5.0 mm. Enhanced T1-weighted images (WI) were obtained after intravenous administration of meglumine gadopentetate (Magnevist®, Bayer, Osaka, Japan, 0.1 mmol/kg body weight).

*Clinical diagnosis*: All dogs were diagnosed with encephalitis by clearly positive MRI images, and encephalitis was confirmed by serum and CSF analysis. Seventeen dogs had been diagnosed with suspected NME, 2 dogs had been diagnosed with suspected GME, 1 dog had been diagnosed with suspected CDV infection and 9 dogs had been diagnosed with indistinguishable encephalitis by MRI, CSF analysis, CDV analysis and neurological examinations. Two dogs were diagnosed with suppurative encephalitis histologically.

*Western blotting (WB) analysis of canine CSF proteins*: To confirm the presence of HMGB1, which has a molecular weight at 27–29 kDa, in CSF, the CSF proteins from two healthy dogs and eight encephalitic dogs were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to the Laemmli method [9] with slight modifications. The separated proteins were electroblotted from the polyacrylamide gel onto a PVDF membrane (ATTO, Tokyo, Japan) following the method of Towbin *et al*. [26]. The immunoblotting analysis was performed with a rabbit anti-peptide (KPDAAKKGVVKAEK) polyclonal antibody that was highly specific for HMGB1 and demonstrated no cross-reactivity with human HMGB2 (Shino-Test, Tokyo, Japan) as the primary antibody (2.5 $\mu$g/m*l*; the same antibody as used for the ELISA) [31, 33] and a goat horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Bio-Rad, Hercules, CA, U.S.A.) as the secondary antibody (diluted at 1:15,000) as previously described [35]. The immunoreactive bands were detected with ECL™ western blotting detection reagents (GE Healthcare, Buckinghamshire, UK).

*HMGB1 enzyme-linked immunosorbent assay (ELISA)*: The HMGB1 concentrations in the CSF samples of dogs were measured by an ELISA according to the manufacturer's instructions (HMGB1 ELISA Kit II, Shino-Test, Tokyo, Japan). Because the amino acid sequence (accession no. AAN11296) of dog HMGB1 is identical to that of human HMGB1 (accession no. AAH30981: 100% homology) and cross-reactivity was suggested by WB, the antibody can recognize dog HMGB1.

*Statistical analysis*: The data were analyzed by the Mann-Whitney *U* test or the Pearson correlation coefficient test using the StatMate III software (ATMS, Tokyo, Japan). The data are presented as the mean ± the standard error (SE). *P* values < 0.05 (two-tailed) were considered statistically significant.

## RESULTS

*CSF analysis*: CSF samples were obtained from 31 dogs that had suspected encephalitis. The cell counts in the CSF samples ranged from 2 cells/$\mu$*l* to 2,736 cells/$\mu$*l* (median 15 cells/$\mu$*l*), and the protein concentrations ranged from 8 mg/d*l* to 120 mg/d*l* (median 20 mg/d*l*). All healthy dogs had normal values (cell counts < 5 cells/$\mu$*l* and protein concentrations < 25 mg/d*l* [29]).

*MRI images*: The MRI analyses suggested that dogs presenting with neurological symptoms had inflammation of the brain (Fig. 1). In the T2-WI of a representative brain of an encephalitic dog, there was diffuse hyperintensity in both the parietal and temporal lobes, indicating that primarily the gray matter area was damaged (Fig. 1d). The lesions were T1 hypointense, and irregular hyperintensity in the T2 FLAIR image was observed. In the T2 FLAIR image of an encephalitic dog (Fig. 1f), there was a hypointense area in the right temporal lobe, indicating a cavitation area. Because no areas of strong contrast enhancement or peripheral rim enhancement were noted in the encephalitic dog, this patient was suspected to have NME.

*WB analysis*: The CSF samples from 2 healthy and 8 encephalitic dogs were analyzed by WB to examine for the presence of HMGB1. HMGB1 bands of approximately 27–

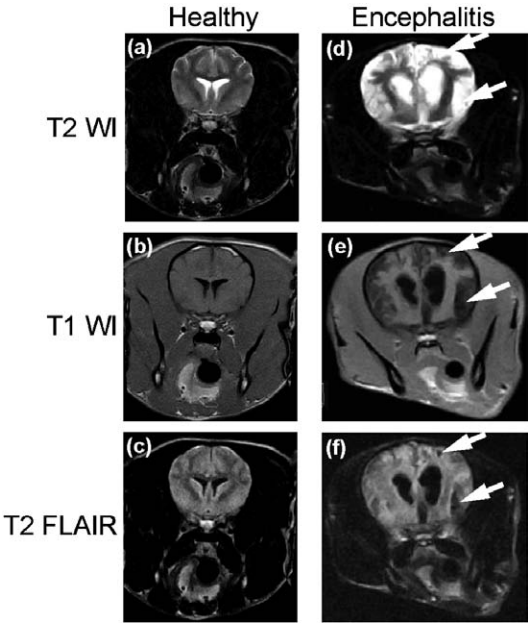


Fig. 1. Transverse MR images of brains from healthy (a, b, c) and encephalitic (d, e, f) canines. T2- (a, d) and T1-WI (b, e) and T2 FLAIR images (c, f) are shown. The gray matter area was the most damaged in the encephalitic dog (d, e and f). T1-hypointense and irregular hyperintensity lesions were observed in T1-WI and T2 FLAIR images in dogs with encephalitis (arrows in d, e, f). The dog shown was clinically diagnosed with NME because there was no area of strong contrast enhancement, but there were cavitary lesions an addition to peripheral rim enhancement.

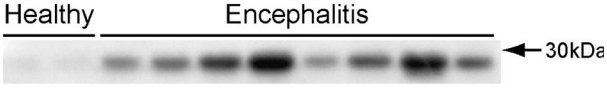


Fig. 2. WB analysis of HMGB1 in CSF samples from 2 healthy and 8 encephalitic dogs. The HMGB1 protein was detected as a single band in CSF samples from 2 healthy and 8 encephalitic dogs.

29 kDa in size were detected in the CSF of encephalitic dogs, and the intensity of the HMGB1 bands was higher in encephalitic dogs compared with the healthy controls, which had only faint bands (Fig. 2).

*ELISA*: The HMGB1 concentrations in the CSF samples were measured by ELISA. The mean HMGB1 concentrations in the CSF samples from the healthy and encephalitis dogs were 0.41 ± 0.13 *n*g/m*l* (n=10) and 2.89 ± 0.91 *n*g/m*l* (n=31), respectively; the mean HMGB1 concentrations were significantly higher in the encephalitic dogs than in the healthy controls ($P<0.001$, using the Mann-Whitney *U* test; Fig. 3).

*Correlations*: The correlations between the HMGB1 concentration and the cell count (a) and the total protein concentration (b), which are regarded as inflammatory signals, in the CSF of the encephalitic dogs are shown in Fig. 4. There were positive correlations between the concentration of HMGB1 and the cell count ($r=0.942$, $P<0.001$) and between the HMGB1 concentration and the total protein concentration ($r=0.742$, $P<0.001$; Fig. 4a and b, respectively).

## DISCUSSION

This is the first report of the detection of HMGB1 in the CSF of dogs having encephalitis. The CSF HMGB1 concentration of the dogs with encephalitis was significantly higher that of the healthy dogs (29/31: 94 %).

It is well known that because CSF proteins reflect the state of the central nervous system well, accurate analysis of CSF can provide significant information about the neurological health of a patient. In this study, HMGB1 was detected in CSF samples from encephalitic dogs that were diagnosed by MRI and additional clinical evaluations. A high cell count and increased protein concentration in the CSF are indicators of canine neurological diseases, such as NME [10]. However, we supposed that degradation of the brain tissue and neuronal cells had not occurred yet in some cases having a low cell count and protein concentration in the CSF. We found that the CSF HMGB1 concentration was correlated with these established disease markers, suggesting that the HMGB1 concentration in the CSF is related to presence of the disease.

Liu *et al*. [11] reported that an anti-HMGB1 monoclonal antibody ameliorated brain infarction induced by two hours of occlusion of the middle cerebral artery in rats and that this effect was associated with a marked reduction in neurological deficits. Currently, treatment with HMGB1 inhibitors has been shown to reduce inflammation in many preclinical animal studies. Effective therapeutic strategies for a wide range of preclinical disease models through HMGB1 inhibition have been discussed [34]. After further investigation of CSF HMGB1 in dogs having encephalitis, HMGB1 will be a significant factor for inspection and diagnosis of this disease.

ACKNOWLEDGMENTS. We thank Dr. Maruyama of Kagoshima University and Dr. Suda of Keio University for their advice. This work was supported by a Grant-in-Aid to the High Technological Research Center (Rakuno Gakuen University) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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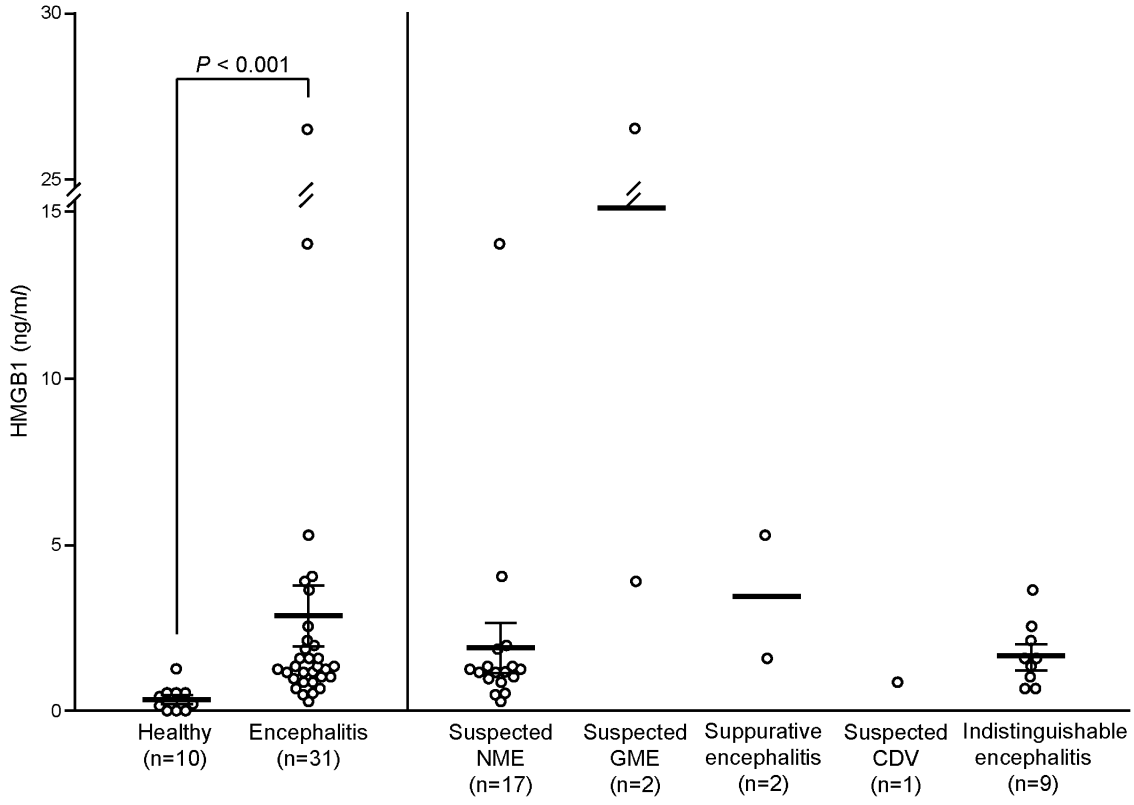


Fig. 3. HMGB1 ELISA analysis of CSF samples from 10 healthy and 31 encephalitic dogs. The HMGB1 concentrations obtained from the ELISA analyses were 0.41 ± 0.13 *n*g/m*l* for the 10 healthy dogs and 2.89 ± 0.91 *n*g/m*l* for the 31 encephalitic dogs. Almost all of the CSF samples from the encephalitic dogs (29 of 31 animals) had an elevated HMGB1 level compared with the calculated mean ± SE of the healthy samples ($P<0.001$). The data were analyzed by the Mann-Whitney *U* test. The data are presented as the mean ± SE.

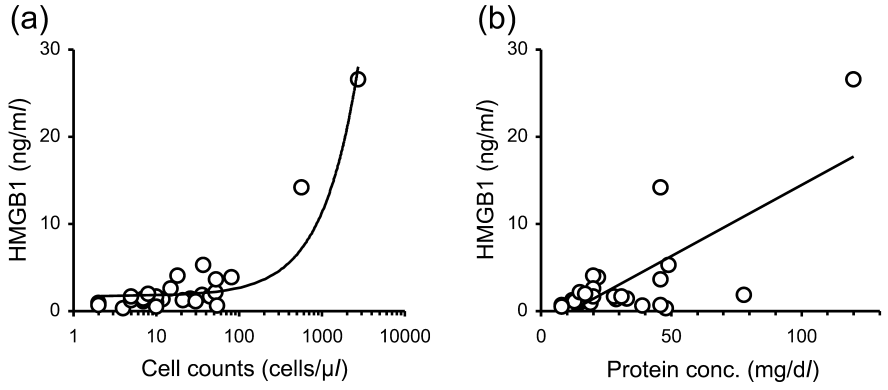


Fig. 4. The relationship between two indicators of encephalitis and HMGB1 expression in the CSF from dogs with encephalitic diseases. (a) The correlation between the logarithm of the cell counts and HMGB1 concentrations in CSF samples from encephalitic dogs. (b) The correlation between the total protein concentration and the HMGB1 concentration in CSF samples from encephalitic dogs. The data were analyzed by the Pearson correlation coefficient test. There were positive correlations between the HMGB1 concentration and the logarithm of the cell count (a) ($r$=0.942, $P$<0.001) and between the HMGB1 concentration and total protein concentration (b) ($r$=0.742, $P$<0.001).

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