Serum Glial Fibrillary Acidic Protein as a Diagnostic Biomarker in Dogs with Progressive Myelomalacia

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ABSTRACT. In humans, increased levels of GFAP in the CSF and blood have been reported with various neural diseases. However, there has been no study describing the usefulness of GFAP in the blood for disease of the spinal cord in dogs. The aim of this study was to describe the utility of GFAP in serum for a diagnosis of progressive myelomalacia. Fifty-six dogs with acute thoracolumbar IVDD diagnosed by computed tomography with myelography or MRI were included. Serum specimens were collected at initial presentation from all cases and at follow-up examinations from some cases. Serum samples were assayed for GFAP concentrations using a commercially available GFAP ELISA Kit. Progressive myelomalacia was the final diagnosis in 8/51 cases (15.6%). Eight dogs had clinical signs suggestive of progressive myelomalacia, of which 6 were positive and 2 were negative by GFAP. Seven dogs had a detectable level of serum GFAP, of which 6 had the onset of progressive myelomalacia. The sensitivity and specificity of the GFAP to progressive myelomalacia were 75% and 97.7%, respectively. The results suggest the utility of GFAP in serum in the diagnosis of progressive myelomalacia.

KEY WORDS: canine, diagnosis, glia, neurology.


Progressive myelomalacia is thought to result in ischemic and hemorrhagic necrosis of the spinal cord, which can occur following acute spinal cord injury, and represents extensive damage of the intramedullary spinal vasculature [5, 8, 14]. Although the exact pathophysiological mechanism of myelomalacia is poorly understood, it seems to be the result of the concussive effects of trauma followed by ischemia, which results in release of vasoactive substances, oxygen-free radicals and cellular enzymes [14]. Progressive myelomalacia frequently results from thoracolumbar intervertebral disc disease (IVDD) in dogs [4, 6, 12, 15]. The prognosis of thoracolumbar IVDD is good, if the treatment is initiated early in the course of the disease [4, 6]. However, when the onset of progressive myelomalacia is suspected, it is necessary to suspend surgical treatment. Though the possibility of using the cutaneous trunci muscle reflex for diagnosing progressive myelomalacia was suggested in a previous report [10], clinical signs and abnormal findings in a neurological examination for progressive myelomalacia take several days to appear after the development of paraplegia [12, 14]. There is currently no effective way to detect progressive myelomalacia at the early stage. Recently, diagnostic imaging, such as myelography, and MRI have been used to attempt diagnosis of progressive myelomalacia, but the accuracy has not been sufficient [11, 14]. Nevertheless, differentiation of progressive myelomalacia from IVDD is of clinical importance for the choice of appropriate treatment.

Glia fibrillary acidic protein (GFAP) is the major protein constituent of glial filaments in differentiated astrocytes of the central nervous system [1]. Astrocytes are an important cellular component for maintenance of the blood brain barrier [2, 7] and for modulation of the efficacy of synapses [9]. GFAP is tightly packed into intermediate filament polymers and is generally insoluble [13]. However, when astrocytes are damaged, GFAP is released into the cerebrospinal fluid (CSF) as soluble fragments [6, 8]. In humans, increased levels of GFAP in the CSF and blood have been reported with various neural diseases, for example, systemic lupus erythematosus, multiple sclerosis, Alzheimer’s disease, subarachnoid hemorrhage and traumatic brain injury [3, 16, 17]. We postulated that GFAP may be released to peripheral blood in progressive myelomalacia following neural damage. However, we were unable to find any studies that show the usefulness of GFAP for disease of the spinal cord in dogs. This study describes the utility of GFAP in the blood for an early diagnosis of progressive myelomalacia.
MATERIALS AND METHODS

The databases of the Animal Hospital of Iwate University and Maizuru Animal Medical Center were reviewed for cases of canine IVDD from April to November in 2011. Of a total of 97 cases with a diagnosis of IVDD with diagnostic imaging, computed tomography and myelography or MRI, 51 were selected for this study on the basis of the following case definition: Herniation was located in lumbar vertebra and hindlimb paralysis was presented. After diagnosis, operations were performed exclusive of six cases of progressive myelomalacia. Decompression of the extruded disk material was confirmed in all operated cases. Follow-up evaluation was performed until three months after operation. The owners of the dogs that had not made a successful recovery by discharge were contacted again to determine whether the dogs had recovered in an actual examination.

Neurological examination: All dogs had a neurological examination at presentation, and a grade was recorded in the medical records: grade 1 – spinal pain, grade 2 – ambulatory paraparesis and/or ataxia and/or proprioceptive deficits, grade 3 – nonambulatory paraparesis, grade 4 – paraplegia with intact deep pain perception and grade 5 – paraplegia with no deep pain perception.

Collection of serum: Serum specimens were collected at all cases at the first medical examination, and unscheduled sampling was performed in some cases. The specimens were centrifuged at 12,000 × g for 2 min at 4°C to remove the cells, and the supernatant was stored at −80°C until biomarker assays were performed. All samples were prepared within 30 min.

GFAP assay for the serum: Serum samples were assayed for GFAP using a commercially available GFAP ELISA system (BioVendor Laboratory Medicine, Candler, NC, U.S.A). The samples were assayed according to the protocol provided by the kit manufacturer. Briefly, 100 μl of the prepared standard and 100 μl of each sample were incubated at 25°C for 2 hr. The wells were then aspirated, washed, and incubated with 100 μl of biotin-labeled antibody at 25°C for 1 hr. Next, the wells were aspirated, washed, and incubated with 100 μl of the streptavidin-HRP conjugate solution at 25°C 1 hr. Then, the wells were aspirated, washed and incubated with 100 μl of the substrate solution at room temperature for 10–15 min. This reaction was stopped by the addition of 100 μl of stop solution, and the optical density was measured at 450 nm using a spectrophotometer (Tosoh Corporation, Tokyo, Japan). The concentrations of GFAP were determined according to a standard curve and reported in ng/ml.

Statistical analysis: Sensitivity, specificity, positive predictive value and negative predictive value for the final diagnosis of progressive myelomalacia were calculated for the serum GFAP test. Logistic regression was used to calculate relative risk and 95 percent confidence intervals (95% CI) summarizing the association between the onset of progressive myelomalacia and serum GFAP. A P-value of 0.05 or less was considered statistically significant.

RESULTS

A total of 51 dogs fulfilled the inclusion criteria. Eighty percent (41/51) were dachshunds, and 84% (43/51) were chondrodystrophoid. The dogs were 4–14 years of age (mean 6.5 years).

Progressive myelomalacia was the final diagnosis in 8/51 (15.6%) cases with characteristic symptom as follows: flaccid paraplegia, total areflexia of the pelvic limbs, tail and anus, loss of deep pain perception caudal to the site of spinal cord injury, flaccid abdominal musculature, depressed mental state and respiratory difficulty due to intercostal and diaphragmatic paralysis. No marked findings suggesting progressive myelomalacia were found using diagnostic images. The clinical signs in the remaining cases improved in clinical signs regardless of whether the surgical treatment was performed (Table 1).

An intraoperative aspect suggestive of progressive myelomalacia was found in one case (case 42) in which serum GFAP was not detected during the first visit. The serum GFAP level of this case was never evaluated again. Serum GFAP was detected in case 37 two days after developing paraplegia. Four days later, the dog was euthanatized, because of the development of progressive myelomalacia. Similarly, 6 of the 8 dogs with onset progressive myelomalacia were a positive for serum GFAP at 2 days after developing paraplegia.

The symptoms with regard to progressive myelomalacia gradually progressed toward the head in all cases (Table 2). In this research, cranial movement of the border of the CTM reflex, lower motor neuron signs in the forelimb, respiratory disturbance and Horner syndrome were occurred successively in most of the dogs. This information is in agreement with a past report. In particular, cranial movement of the CTM reflex was strongly associated with progressive myelomalacia in a previous report [10]. Ultimately, all dogs that developed progressive signs consistent with progressive myelomalacia died (6 by nature death and 2 by euthanasia). The period

| Table 1. GFAP values of dogs in each grade |
| Number | Not detected | Detected | GFAP value (ng/ml) |
| Grade III | 10 | 10 | 0 | – |
| Grade IV | 22 | 22 | 0 | – |
| Grade V | PM - | 11 | 10 | 1 | 1.6 (1.6) |
| + | 8 | 2 | 6 | 3.04 (0.75–6.7) |

Data for number of cases, detection of GFAP and blood concentration of GFAP are presented.
The biomarker for progressive myelomalacia in dog

from paraplegia onset to death was 6.8 (3–12) days.

Eight dogs showed the onset of progressive myelomalacia, of which 6 were positive and 2 were negative for GFAP. In 7 dogs, serum GFAP was detected, of which 6 showed the onset of progressive myelomalacia. The sensitivity and specificity of the GFAP for progressive myelomalacia were 75% and 97.7%, respectively. A significant association between progressive myelomalacia and serum GFAP was shown (RR=18.857 (4.709–75.514)) (Table 3).

Of the 43 dogs that did not develop progressive myelomalacia, postoperative recovery in 3 dogs was not evaluated after discharge. Follow-up evaluation was performed on the remaining 40 dogs for 3 months postoperatively. All 32 dogs grouped into grades III and IV showed an improvement in motor function until three months after operation.

Eight of the 11 dogs that were classified as grade V were subjected to postoperative examination; 4 dogs recovered motor function, 1 dog recovered nociception and 3 dogs showed no improvement until 3 months after operation.

DISCUSSION

To the best of our knowledge, this is the first study on a biomarker of progressive myelomalacia in the blood. IVDD is commonly encountered in small animal practice. As a consequence of advances in diagnosis and treatment for it, the outcome can be stabilized. In fact, all the dogs other than those with progressive myelomalacia in this study

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**Table 2. Process of the symptoms of progressive myelomalacia**

<table>
<thead>
<tr>
<th>Case</th>
<th>Serum GFAP</th>
<th>Specific symptom of MP and its onset day</th>
</tr>
</thead>
<tbody>
<tr>
<td>37</td>
<td>Negative</td>
<td>at day 2</td>
</tr>
<tr>
<td>38</td>
<td>Positive</td>
<td>at day 2</td>
</tr>
<tr>
<td>40</td>
<td>Positive</td>
<td>at day 2</td>
</tr>
<tr>
<td>42</td>
<td>Negative</td>
<td>at day 2</td>
</tr>
<tr>
<td>45</td>
<td>Positive</td>
<td>at day 2</td>
</tr>
<tr>
<td>47</td>
<td>Positive</td>
<td>at day 2</td>
</tr>
<tr>
<td>48</td>
<td>Negative</td>
<td>at day 1</td>
</tr>
<tr>
<td>49</td>
<td>Negative</td>
<td>at day 2</td>
</tr>
<tr>
<td>50</td>
<td>Negative</td>
<td>at day 2</td>
</tr>
</tbody>
</table>

The day of developing paraplegia is defined as day 0.

**Table 3. RR, sensitivity and specificity of GFAP for progressive myelomalacia**

<table>
<thead>
<tr>
<th>GFAP</th>
<th>Progressive Myelomalacia</th>
<th>Onset</th>
<th>No onset</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detected</td>
<td>6</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Not detected</td>
<td>2</td>
<td>42</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Relative risk</td>
<td>18.857</td>
<td>4.709–75.514</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sensitivity</td>
<td>0.75</td>
<td>0.483–0.850</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Specificity</td>
<td>0.977</td>
<td>0.927–0.995</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
showed improved symptoms with treatment. However, the outcome in progressive myelomalacia is poor regardless of therapy, because its lesion is thought to be irreversible and progressive. Therefore, an index indicating whether myelomalacia is progressive is needed for adequate prediction of treatment outcome. The cranial movement of the cutaneous trunci reflex indicated that progressive myelomalacia develops about 5–7 days after the appearance of paraplegia [12]. In fact, GFAP was detected after less than 5 day in serum from the dogs with progressive myelomalacia in this study and was detected in serum earlier than the onset of highly characteristic clinical presentation of progressive myelomalacia. These results support the usefulness of GFAP in serum for an early diagnosis of progressive myelomalacia.

The sensitivity and specificity of GFAP for differentiation of progressive myelomalacia in this study are thought to be sufficient. However, GFAP was negative in 2 dogs with progressive myelomalacia. In the event of injuring the spinal cord, a time lag might be needed before GFAP appears in the serum from the affected area of the spinal cord. In 2 negative cases, the level of GFAP in the serum was measured at only the first visit; this might have been why it was not detected in serum. Collection of blood samples at the appropriate time after appearance of paraplegia may be important in measuring the level of GFAP in serum for prediction of progressive myelomalacia. Therefore, it might be better to test for GFAP repeatedly, if it is not detected in the first examination. In fact, a dog that was GFAP negative at the first examination was found to have GFAP in the serum in the second test. Although the time course of GFAP appearing in the blood was unclear in the present study, it was suggested that at least 24 hr needs to have passed after advancing to paralysis for GFAP to be detected in the serum. This is because GFAP appeared in blood 24 hr after paralysis onset in all cases in the present study. If the 2 cases in which GFAP was not detected in serum had been tested over time and not just in the first examination, GFAP might have been detected. As a result, the sensitivity of GFAP for progressive myelomalacia may be higher than that in this study.

GFAP was detected in 1 case of thoracolumbar IVDD without progressive myelomalacia in this study. No marked findings were found in diagnostic images of this dog. In the end, this case remained nonambulatory paraparetic at 3 months after the operation. On the other hand, in the dogs classified as grades III and IV, GFAP was not found in the serum and their postoperative outcomes were good. These results suggested a correlation between detecting GFAP and the severity of spinal cord lesion. The concentrations of GFAP in the serum of the dog with progressive myelomalacia were significantly higher than those of the dogs with thoracolumbar IVDD without progressive myelomalacia and those of the healthy dogs. This suggests the possibility that astrocytes were more severely damaged in myelomalacia than thoracolumbar IVDD. As the impact of injury may be an important factor in the progression of myelomalacia, the low value indicated in IVDD reflected that the lesion was mild. The other possibility is that there are two types of pathological status in myelomalacia. The first type is progressive myelomalacia. Progressive myelomalacia is thought to be ischemic or hemorrhagic necrosis of the spinal cord that can occur following acute spinal cord injury and represents extensive damage of the intramedullary spinal vasculature. Dogs that develop progressive myelomalacia die quickly. The second type is a focal lesion. This type of lesion was confined to a minimal area and did not expand, so recovery might be expected [4, 12]. It is difficult to distinguish between thoracolumbar IVDD and the focal lesion type of myelomalacia. This type of lesion is the reason why one dog that did not develop progressive myelomalacia showed GFAP in the blood in this research. The pathway of GFAP in CSF leakage into systemic blood was not investigated in this study. However, two possibilities were speculated about this pathway. The first possibility is destruction of the blood-brain barrier. GFAP is the principal intermediate filament in mature astrocytes of the central nervous system [1]. Astrocytes are involved in the integrity of the blood brain barrier [2, 7], so when the BBB breaks down as a result of astrocytes damaged by progressive myelomalacia, GFAP is able to be detected in the blood. The second possibility is that GFAP appears in the blood in a concentration-dependent manner. In dogs with progressive myelomalacia, the concentrations of GFAP in CSF may be very high, resulting in leakage into the blood. This hypothesis might be evaluated by examination of the GFAP in CSF compared with in the blood. Unfortunately, GFAP in CSF was not measured in this study.

A definite diagnosis of progressive myelomalacia should be based on pathological examination; however, sampling a biopsy specimen from the spinal cord of a live patient is impossible. Consequently, in the clinical setting, progressive myelomalacia is diagnosed based on the characteristic clinical presentation as follows: flaccid paraplegia, total areflexia of the pelvic limbs, tail and anus, loss of deep pain perception caudal to the site of spinal cord injury, flaccid abdominal musculature, depressed mental state and respiratory difficulty due to intercostal and diaphragmatic paralysis. This was how it was diagnosed in the current study.

In the current study, GFAP was measured by using a commercial ELISA kit for human GFAP. This kit was used in a previous study for measuring the GFAP of dogs. To validate the availability of the ELISA kit for dogs, the cross-reactivities of anti-human GFAP antibodies in the kit with canine GFAP were confirmed by an indirect immunofluorescence assay using cultured canine astrocytes [16].

In this study, the efficacy of GFAP in the blood as a predictive factor for progressive myelomalacia was demonstrated by the strong correlation. This outcome will contribute to deciding whether surgical repair is applied for paralysis.

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REFERENCES