

## Plasma High-Mobility Group Box 1 (HMGB1) in Dogs with Various Diseases: Comparison with C-Reactive Protein

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**ABSTRACT.** High-mobility group box 1 (HMGB1), a nonhistone chromosomal protein, has recently been suggested as a late mediator of the inflammatory cascade. Blood HMGB1 levels are increased in a number of human diseases, and HMGB1 has been suggested to be a useful marker for disease severity and prognosis. The objective of this study was to assess the clinical usefulness of HMGB1 in dogs. Plasma HMGB1 levels, as well as C-reactive protein (CRP), a typical canine inflammatory marker, were measured in dogs with various diseases, especially systemic inflammatory response syndrome (SIRS), and dogs that had undergone surgery. HMGB1 gradually increased and attained a maximum level 72 hr after surgery, whereas CRP increased rapidly, peaking at 24 hr. Although both HMGB1 and CRP levels were significantly increased in dogs with various diseases compared with the control dogs, no correlation was found between the HMGB1 and CRP values. HMGB1 levels in the SIRS group were significantly elevated compared with those in the non-SIRS group. However, the increase in HMGB1 levels above the reference range was not indicative of SIRS. Instead, the presence of increased HMGB1 and CRP levels above the reference ranges significantly affects the poor outcome of SIRS. The present study indicates that HMGB1 is a novel canine inflammatory marker and is distinct from CRP. However, the additional clinical value of HMGB1 measurement remains unclear, and further studies are warranted.

**KEY WORDS:** canine, CRP, HMGB1, inflammation, SIRS.

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High-mobility group box 1 (HMGB1), a 30-kDa nonhistone chromosomal protein, is a highly conserved protein among various species, with 100% amino acid homology between dog and humans, also mouse and rat, and 99% homology between rodents and humans [8, 10, 15, 18, 27]. HMGB1 is known to have diverse cellular functions, including determination of nucleoside structure and stability, regulation of gene transcription, and activation of steroid hormone receptors [6, 26].

Beyond these intracellular roles, the role of extracellular HMGB1 as a late mediator of the inflammatory cascade has recently been shown. Under inflammatory or injurious conditions, HMGB1 is secreted by particular cells, such as monocytes and macrophages, that have been stimulated with endotoxin or proinflammatory cytokines (*e.g.*, tumor necrosis factor [TNF] and interleukin-1 [IL-1]) [7, 17, 20]. HMGB1 release starts 8–16 hr after stimulation by proinflammatory cytokines and remains elevated for at least 36 hr. Extracellular HMGB1 acts as a proinflammatory cytokine [25, 26]. HMGB1 interacts with macrophages and monocytes through the receptor for advanced glycation end products (RAGE); activates the nuclear factor (NF- $\kappa$ B) signaling pathway and mitogen-activated protein kinase (MARK); and induces prolonged inflammation, organ disorder, tumor growth and metastasis, septicemia, and death

[7, 17, 20, 24]. Thus, HMGB1 is indicated as a late mediator of inflammation.

In human medicine, overexpression of HMGB1 has been detected in a number of human diseases and pathological states, such as sepsis [12, 13, 26], tumors (*e.g.*, gastrointestinal stromal tumors [5], pancreatic cancer [23], hepatocellular carcinoma [4], malignant lymphoma [14]), disseminated intravascular coagulation (DIC) [11], acute pancreatitis [30], and surgery [21]. Increased levels of extracellular HMGB1 have been associated with disease severity and response to treatment [5, 6, 11, 12, 14, 21–23, 30]. HMGB1 is suggested to be a potentially suitable prognostic marker for these diseases.

In the field of veterinary medicine, C-reactive protein (CRP), known as one of the major acute phase proteins, has recently been used as an inflammatory marker. Blood CRP concentration is increased in various inflammatory diseases [9, 30, 16], and its increased level is correlated with the severity and course of the diseases [9, 29]. Although few reports are available on blood HMGB1 levels in diseased dogs [13, 32], Yu *et al.* recently reported increased plasma HMGB1 levels in dogs with systemic inflammatory response syndrome (SIRS) [32]. However, they did not examine the correlation between HMGB1 and other inflammatory markers or its clinical utility as a prognostic factor in dogs with SIRS.

The purpose of this study was to assess the clinical usefulness of HMGB1 as an inflammatory marker for various canine diseases and as a prognostic marker in dogs with SIRS. We investigated HMGB1 levels in dogs after sur-

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ger, with various diseases, and with SIRS, and compared them with blood CRP levels.

## MATERIALS AND METHODS

**Animals and samples:** Dogs referred to the Veterinary Medical Center of the University of Tokyo (VMC-UT) between April 2008 and November 2008 were prospectively recruited for this study. Dogs with multiple diseases or unconfirmed diagnoses and dogs that had already been treated were excluded. Clinically ill dogs were categorized as having SIRS if at least 2 of the following SIRS criteria were found: heart rate < 120 bpm, respiratory rate >40 bpm or PaCO<sub>2</sub><30 mm Hg, rectal temperature >40°C (104°F) or <38°C (100.4°F), and a leukogram with >18,000 leukocytes/ $\mu$ l or <5,000 leukocytes/ $\mu$ l [1].

The study group included 307 dogs (127 female and 180 male). The median age was 8 years (range, 1–18). The diseased dogs were categorized into 10 parts; the dogs with tumors (n=77), hematological disease (n=53), hepatobiliary and pancreatic disease (n=45), gastrointestinal disease (n=41), endocrine disease (n=20), neuromuscular disease (n=19), skin disease (n=15), respiratory disease (n=14), cardiovascular disease (n=11), and with joint disease (n=8).

Thirty-six clinically healthy Beagle dogs (15 female and 21 male) owned by VMC-UT as blood donors were used as controls. The median age was 4 years (range; 1–7). Among these dogs, 5 clinically healthy dogs (median age, 2; range, 1–3) underwent ovariohysterectomy and blood collection before and 1, 2, 6, 12, 24, 36, 48, 72, 96, and 120 hr after surgery. All surgeries were performed by the same person. The study was approved by the University of Tokyo of Veterinary Medicine Institutional Animal Care and Use Committee. All experiments were conducted according to the animal experimentation guideline of the University of Tokyo.

All dogs were subjected to physical examination and standard hematological and biochemical tests. Additional diagnostic procedures (e.g., radiography, ultrasonography, endoscopy, exploratory laparotomy, cytology, and histopathology) for individual dogs were performed depending on the medical condition. Blood was collected by venipuncture, and aliquots were placed in heparin-containing tubes. Samples were centrifuged and plasma was collected and stored at –20°C until analysis.

**HMGB1 and CRP measurement:** Because the amino acid sequence of canine HMGB1 completely matches that of human HMGB1 [15], it was measured with a commercially available human ELISA kit (HMGB1 ELISA Kit II; Shino-Test Corporation, Tokyo, Japan) according to the manufacturer's instructions [28]. The kit has been validated for use in dogs in previous study [13]. All samples were measured in duplicate, and the results were averaged.

Plasma CRP concentration was measured by a canine CRP measurement kit (Laser CRP-2; Arrows Co., Ltd., Osaka, Japan) following the manufacturer's instructions (reference range, <1.00 mg/dl, maximum measurement

limit=20 mg/dl).

**Statistical analysis:** Differences between groups were assessed for statistical significance by using Mann-Whitney's *U* test. Correlation between 2 variables was tested by Pearson's correlation analysis. Samples with CRP values higher than the limit (20 mg/dl) were excluded from the correlation analysis. Relationships of categorical variables were assessed by Fisher's exact test. A *P* value less than 0.05 denoted a statistically significant difference.

## RESULTS

**Plasma HMGB1 level in the control group:** The plasma concentration of HMGB1 in the control group ranged from 0 to 6.62 ng/ml (mean=1.98 ng/ml, SD=1.74 ng/ml). According to this result, the reference range for canine blood HMGB1 concentrations was estimated to be less than 5.46 ng/ml (mean  $\pm$  2 SD).

**Time-course change in HMGB1 and CRP levels after surgery:** Figure 1 shows the time-course change in HMGB1 and CRP levels before and after ovariohysterectomy. After surgery, the mean HMGB1 levels increased to above the reference range at 2 hr, 12 hr, and from 36 hr to 96 hr. The maximal value was attained at 72 hr (mean, 38.74 ng/ml; range, 25.81–50.44 ng/ml); it then returned to the reference range within 5 days. In contrast, the mean CRP levels started to increase at 12 hr after surgery. The maximal value was attained at 24 hr (mean, 6.4 mg/dl; range, 4.6–7.9 mg/dl), and then returned to normal within 5 days.

**Plasma HMGB1 and CRP levels in the dogs with underlying diseases:** The HMGB1 levels increased significantly (*P*<0.001) in the diseased dogs (median, 1.79 ng/ml; range, 0–174.70 ng/ml) compared with the control group. The CRP concentration also increased significantly (*P*<0.001) in the diseased dogs (median, 4.9 mg/dl; range, 0 to >20 mg/dl) compared with the control group (median, 0 mg/dl; range, 0–0.4 mg/dl). No correlation was found between HMGB1 and CRP (*r*=0.064; Fig. 2).

The HMGB1 levels exceeded the reference range in 31 of 77 dogs (40%) with tumors (median, 2.63 ng/ml; range, 0–174.70 ng/ml), 19 of 53 dogs (36%) with hematological disease (2.36 ng/ml; 0–133.33 ng/ml), 18 of 45 dogs (40%) with hepatobiliary and pancreatic disease (1.79 ng/ml; 0–154.00 ng/ml), 9 of 41 dogs (22%) with gastrointestinal disease (1.47 ng/ml; 0–26.43 ng/ml), and 4 of 14 dogs (29%) with respiratory disease (1.81 ng/ml; 0–22.70 ng/ml). However, the HMGB1 levels in none of the dogs exceeded the reference range for endocrine disease (range, 0–2.17 ng/ml), neuromuscular disease (0–5.46 ng/ml), skin disease (0–2.74 ng/ml), cardiovascular disease (0–3.27 ng/ml), or joint disease (0–2.14 ng/ml).

The CRP levels exceeded the reference range in 60 of 77 dogs (78%) with tumors (median, 4.3 mg/dl; range, 0 to >20 mg/dl), 40 of 53 dogs (75%) with hematological disease (3.2 mg/dl; 0 to 20 mg/dl), 36 of 45 dogs (80%) with hepatobiliary and pancreatic disease (9.3 mg/dl; 0 to >20 mg/dl), 16 of 41 dogs (39%) with gastrointestinal disease (1.8 mg/dl;

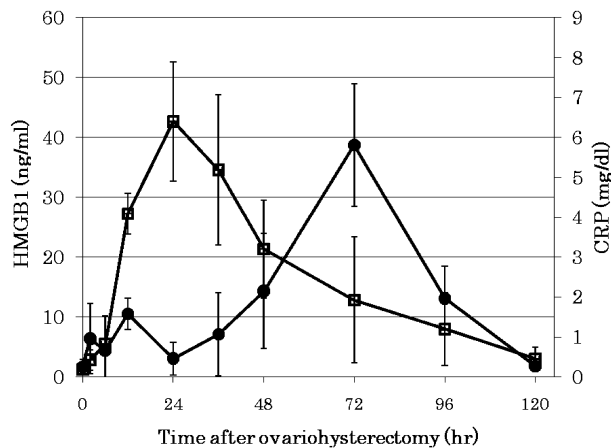


Fig. 1. Time-course changes in plasma HMGB1 (open square) and CRP (closed circle) levels in dogs ( $n=5$ ) before and after ovariohysterectomy. The data are expressed as mean  $\pm$  SD.

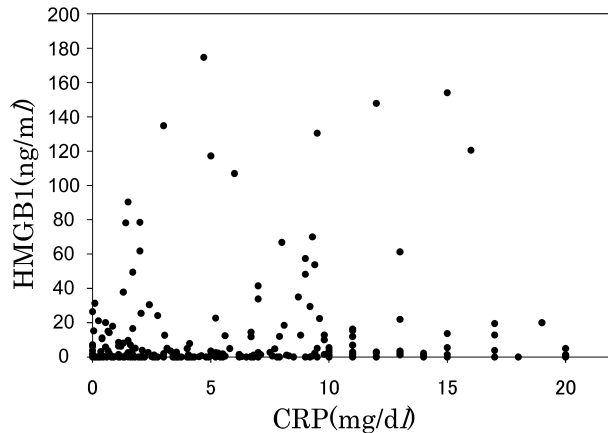


Fig. 2. Correlation of blood HMGB1 and CRP in 302 dogs with various diseases. Pearson's product-moment correlation coefficient ( $r$ ) = 0.064.

0–14.6 mg/dl), 9 of 14 dogs (64%) with respiratory disease (1.81 mg/dl; 0–13.2 mg/dl), 2 of 15 dogs (13%) with skin disease (0.3 mg/dl; 0–2.3 mg/dl), 1 of 11 dogs (9%) with cardiovascular disease (0.1 mg/dl; 0–4.2 mg/dl), and 8 of 8 dogs (100%) with joint disease (12 mg/dl; 2.3 to >20 mg/dl). The CRP levels in the dogs with endocrine (range, 0–0.8 mg/dl) or neuromuscular (0–0.7 mg/dl) disease did not exceed the reference range.

**Levels of HMGB1 and CRP in dogs with SIRS and their association with outcome:** Among the dogs with underlying disease ( $n=307$ ), 133 were diagnosed with SIRS. The HMGB1 levels were significantly higher ( $P<0.001$ ) in dogs with SIRS (median, 2.79 ng/ml; range, 0–174.70 ng/ml) than in those without SIRS (0.25 ng/ml; 0–78.49 ng/ml) (Fig. 3A). The HMGB1 levels of 31 of 133 (23%) dogs with SIRS and 43 of 174 (24%) dogs without SIRS exceeded the reference range. There was no significant difference

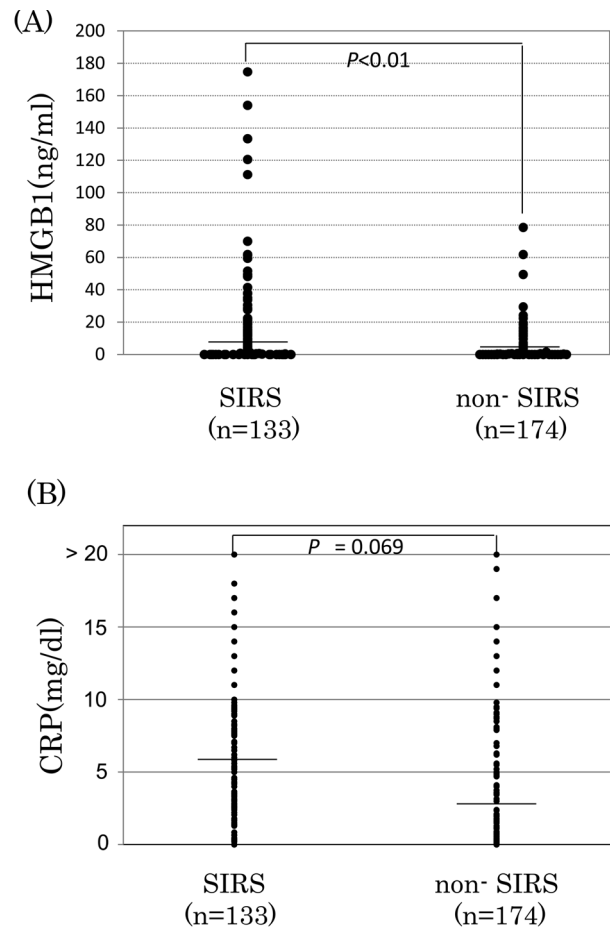


Fig. 3. Plasma concentrations of HMGB1 and CRP in dogs with and without SIRS (non-SIRS). The plasma HMGB1 level was significantly ( $P<0.01$ ) increased in dogs with SIRS compared with that in dogs without SIRS (A). Plasma CRP levels were not significantly different between SIRS and non-SIRS dogs (B). Bars show the medians.

( $P=0.069$ ) in CRP levels between dogs with (median, 5.4 mg/dl; range, 0 to >20 mg/dl) and without SIRS (3.2 mg/dl; 0 to >20 mg/dl) (Fig. 3B). The CRP level of 91 of 133 (69%) dogs with SIRS and of 81 of 174 (47%) dogs without SIRS exceeded the reference range.

The survival rate at 14 days after initial diagnosis in dogs with SIRS was 76% (101/133). The HMGB1 levels were significantly elevated ( $P<0.01$ ) in nonsurvivors (median, 7.1 ng/ml; range, 0–174.70 ng/ml) compared with survivors (2.40 ng/ml; 0–133.3 ng/ml). The survival rates in dogs with SIRS in which the HMGB1 levels were above or within the reference range were 55% (17/31) and 84% (84/102), respectively. HMGB1 levels above the reference range significantly ( $P<0.01$ ) affected the poor outcome in the SIRS group (Table 1).

There was no significant difference ( $P=0.081$ ) in CRP levels between nonsurvivors (median, 5.6 mg/dl; range, 0 to

Table 1. Association between plasma HMGB1 and CRP levels at diagnosis and outcomes at 14 days after diagnosis in dogs with SIRS

Plasma level at diagnosis	Non-survivor (n=32)	Survivor (n=101)	<i>P</i> value*
HMGB1			
Above reference range	14	17	< 0.01
Within reference range	18	84	
CRP			
Above reference range	27	63	< 0.05
Within reference range	5	37	

\* *P* value from Fisher's exact test.

>20 mg/dl) and survivors (3.5 mg/dl; 0 to >20 mg/dl). The survival rates in the SIRS group, in which CRP levels were above or within the reference range, were 70% (64/91) and 30% (37/42), respectively. CRP level above the reference range significantly ( $P<0.05$ ) affected the poor outcome in dogs with SIRS (Table 1).

## DISCUSSION

Because plasma HMGB1 concentrations were significantly elevated in dogs with surgical trauma and various inflammatory diseases, including tumors and hematological disorders, extracellular HMGB1, as well as CRP, could be a new canine inflammatory marker. However, no correlation was found between HMGB1 and CRP levels in the diseased dogs. The concentration of HMGB1 does not correlate with those of CRP even in the diseases that tend to show high concentration of HMGB1 (tumors, hematological disease and hepatobiliary and pancreatic disease) (data not shown). There are several possible explanations for the discrepancy between HMGB1 and CRP.

One is the difference in their release sites and timing. CRP is synthesized mainly by hepatocytes upon activation by proinflammatory cytokines, whereas HMGB1 is released actively by innate immune cells, including macrophages and monocytes, and passively by necrotic cells [7, 17, 19, 24–26]. Furthermore, the kinetic changes in HMGB1 and CRP levels after surgery were different. CRP is first detectable 4 hr after stimulation, and the time of the maximum peak is 24 hr after stimulation according to the present results and those of a previous report [3]. In contrast, HMGB1 gradually increased and reached its maximum level 72 hr after surgical stimulation in the present study. According to previous reports in humans, most HMGB1 released during the first 12 hr after stimulation is derived from a preformed protein pool, and the HMGB1 released after 12 hr is newly synthesized in immune cells [25, 26]. Furthermore, extracellular HMGB1 binds to RAGE and results in activation of the cells through MARK and NF- $\kappa$ B followed by prolonged inflammation [24]. The slightly elevated HMGB1 levels from 2 to 12 hr observed in this study may have been a result of HMGB1 released from damaged cells or from a protein pool of immune cells by surgical stimula-

tion, which might have been gradually synthesized in response to proinflammatory cytokines.

The other possible reason for the discrepancy is that CRP has a higher sensitivity against inflammation than does HMGB1 because there were many diseased dogs with remarkable CRP elevation but without increased HMGB1 concentrations. In this regard, further discussion about kinetic changes in proinflammatory cytokines and HMGB1 and/or CRP in each disease is needed.

In the present study, HMGB1 levels in dogs with SIRS were significantly higher than those in dogs without SIRS, whereas there was no statistical difference in CRP levels. These findings suggest that HMGB1 plays an important role in the pathogenesis of SIRS in dogs as well as humans [21]. Proinflammatory cytokines such as IL-1 and TNF are known to be deeply involved in SIRS as early mediators of systemic inflammation, and are also associated with HMGB1 release from macrophages and monocytes [24, 26]. Although proinflammatory cytokines are also involved in CRP production in the liver [3], there might be differences in susceptibilities between HMGB1 and CRP with regard to the profile of proinflammatory or anti-inflammatory cytokines in SIRS.

The finding that HMGB1 is significantly increased in dogs with SIRS suggests that it might be useful in the diagnosis of canine SIRS. Diagnosis of SIRS is made based on the WBC count and physical examination findings, including body temperature, heart rate, and respiratory rate [1]. Because these parameters are sometimes influenced by excitation of dogs, the HMGB1 level can be useful as a supportive marker for SIRS. In the present study, however, there was no significant difference in the rate of dogs with HMGB1 levels above the reference range between the SIRS and non-SIRS groups, indicating that the clinical application of HMGB1 as a diagnostic marker for SIRS is limited.

In this study, there was significant difference in the HMGB1 levels between nonsurvivors and survivors with SIRS; these results are in agreement with those of previous studies [26]. In human medicine, HMGB1 is described as a prognostic marker for sepsis, acute pancreatitis, DIC, and surgical outcome [11, 21, 26, 30]. Extracellular HMGB1 induced by proinflammatory cytokines also reportedly induces secretion of plasminogen activator inhibitor-1 from

vascular endothelial cells, which may be related to DIC and organ failure [11]. A similar mechanism might be involved in the poor outcome of SIRS dogs with higher HMGB1 levels. Although an increase in HMGB1 levels above the reference range significantly affects the poor outcome of dogs with SIRS, the CRP status also could predict the outcome of dogs with SIRS in our study. The additional clinical value of HMGB1, apart from CRP measurement, as a prognostic marker for SIRS is unclear. Further studies, including assessment of the prognostic value of HMGB1 in specific diseases or serial measurements of HMGB1 in SIRS patients, should be performed.

In conclusion, the plasma HMGB1 level was increased in dogs with various diseases, SIRS, and those that had undergone surgery, indicating its potential use in dogs as a novel inflammatory marker that demonstrates different kinetic changes associated with CRP. Although HMGB1 significantly increased in dogs with SIRS, its clinical usefulness as a diagnostic and prognostic marker for SIRS is unclear; further investigations with a larger numbers of cases are warranted.

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