Serum matrix metalloproteinase 9 (MMP9) as a biochemical marker for wasting marmoset syndrome

Takuro YOSHIMOTO¹⁾, Kimie NIIMI¹⁾* and Eiki TAKAHASHI¹⁾

¹⁾Research Resources Center, RIKEN Brain Science Institute, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan

(Received 26 November 2015/Accepted 27 January 2016/Published online in J-STAGE 12 February 2016)

ABSTRACT. Use of the common marmoset (*Callithrix jacchus*) as a non-human primate experimental animal has increased in recent years. Although wasting marmoset syndrome (WMS) is one of the biggest problems in captive marmoset colonies, the molecular mechanisms, biochemical markers for accurate diagnosis and a reliable treatment remain unknown. In this study, as a first step to finding biochemical marker(s) for the accurate diagnosis of WMS, we conducted blood cell counts, including hematocrit, hemoglobin and platelets, and examined serum chemistry values, including albumin, calcium and levels of serum matrix metalloproteinase 9 (MMP9), using a colony of marmosets with and without weight loss. MMP9 is thought to be an enzyme responsible for the degradation of extracellular matrix components and participates in the pathogenesis of inflammatory conditions, such as human and murine inflammatory bowel disease, which, like WMS, are characterized histologically by inflammatory cell infiltrations in the intestines. The values of hematocrit and hemoglobin and levels of serum albumin and calcium in the WMS group were significantly decreased versus the control group. The platelet values and serum MMP9 concentrations were increased significantly in the WMS group compared with the control group. MMP9 could be a new and useful marker for the diagnosis of WMS in addition to hematocrit, hemoglobin, serum albumin and calcium. Our results also indicate that MMP9 could be a useful molecular candidate for treatment.

KEY WORDS: anemia, body weight, IBD, MMP9, wasting marmoset syndrome

doi: 10.1292/jvms.15-0675; *J. Vet. Med. Sci.* 78(5): 837–843, 2016

The common marmoset (*Callithrix jacchus*) is a member of the New World monkeys that lives in northern and eastern Brazil. Relative to the Old World monkeys, such as macaques, they have several advantages, such as smaller body size, ease of handling, ease of breeding in captivity and absence of severe zoonotic issues. For these reasons, common marmosets have been used as experimental animals in many fields, such as reproductive biology, drug development and infectious disease [1, 3, 19, 21, 35, 36]. In addition, production of transgenic marmosets has become possible in recent years [33]. Since the Brain Mapping by Integrated Neurotechnologies for Disease Studies project (Brain/Minds project) started in Japan [26], the number of studies using common marmosets has increased, particularly in the field of brain sciences [21, 30].

Serious problems, typified by ‘wasting marmoset syndrome’ (WMS), are a concern in the management of common marmosets. The main symptoms of WMS are weight loss, decreased muscle mass and chronic diarrhea, and some studies reported that 28–60% of captive marmosets suffer from and 50–80% of deaths involve WMS [2, 5, 7, 12, 18, 19, 27]. Thus, WMS is one of the biggest problems in operating captive marmoset colonies. WMS is considered an inherent disease in this species, and no effective treatment

has yet been established [19, 27].

Baxter *et al.* reported that lower body weight, under 325 g, identified most marmosets affected by WMS, and progressive body weight loss of 0.05% of the peak body weight per day identified 100% of marmosets affected by WMS [2]. Thus, a presumptive diagnosis of WMS can be made based on this, but the molecular mechanisms involved in the disease remain unclear.

In several reports, it was mentioned that the main disease state in WMS is an inflammatory bowel disease (IBD), anchored by chronic enteritis [2, 19, 25]. In humans, IBDs, as represented by Crohn’s disease and ulcerative colitis, are intractable diseases, and no curative treatment is yet known [9, 37]. Although a specific cause of IBD remains poorly defined, it is thought that interactions between genetic and environmental factors and uncontrollable autoimmunity cause the disease. Matrix metalloproteinase 9 (MMP9) is directly and indirectly involved in tissue remodeling, tumor growth and inflammation by means of controlling inflammatory cytokine activity [6, 8, 14, 20]. Activation of inflammatory cytokines and the involvement of MMP9 in IBD have been reported in humans and mice [11, 31, 34, 37, 38], but these molecular activities in WMS have yet to be characterized.

Thus, this study aimed to determine new target(s) for the diagnosis and therapy of WMS. We compared blood values of WMS animals with those of high-body-weight animals as a control group, using complete blood count (CBC), serum chemistry tests and a serum MMP9 concentration test.

MATERIALS AND METHODS

Animals: The research was approved and overseen by the

*CORRESPONDENCE TO: NIIMI, K., Research Resources Center, RIKEN Brain Science Institute, 2-1 Hirosawa, Wako, Saitama, 351-0198, Japan. e-mail: kimie@brain.riken.jp

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Table 1. Sex, age, body weight, the highest body weight, current body weight/the highest body weight ratio and body weight gain/loss ratio per day in individual animals are represented

	Sex	Age (years)	BW (g)	The highest BW (g)	BW/the highest BW ratio (%)	BW gain or loss ratio per day (%)
WMS 1	Female	5	274.3	437.0	62.8	-0.63
WMS 2	Female	3	220.4	351.1	62.8	-0.10
WMS 3	Female	4	207.8	348.1	59.7	-1.23
WMS 4	Female	4	201.6	290.0	69.5	-0.35
WMS 5	Female	4	220.3	375.2	58.7	-0.44
WMS 6	Female	4	250.0	320.0	78.1	-0.40
WMS 7	Male	4	255.0	360.0	70.8	-0.30
Control 1	Female	4	455.0	464.5	98.0	0.04
Control 2	Male	3	397.1	476.4	83.4	0.04
Control 3	Female	4	387.9	391.5	99.1	0.04
Control 4	Female	2	439.7	470.8	93.4	0.07
Control 5	Female	4	461.2	461.2	100	0.18
Control 6	Female	4	434.3	434.3	100	0.14
Control 7	Male	2	432.8	432.8	100	0.29

animal experiments committee of RIKEN (Wako, Japan) and was conducted in accordance with the Institutional Guidelines for Experiments using Animals.

Common marmosets were reared at the RIKEN Brain Science Institute (Wako, Japan), maintained at 27°C and 50% humidity on a 12/12-hr light/dark cycle. All marmosets in this study were chosen from animals between 2 and 6 years old. Marmosets were allowed *ad libitum* access to water and food pellets (CMS-1M, Clea Japan Inc., Tokyo, Japan) with added vitamin C, D, calcium and acidophilus. Hot water and comb honey were also added to soften the pellets and to improve the animals' preference for the food. Animals were given a piece of Calorie Mate (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) or castella (Castella, Yamazaki Baking Co., Ltd., Tokyo, Japan) as a treat.

WMS animals were determined according to a previous report [2]. Briefly, individuals with body weights less than 325 g with 0.05% body weight loss per day were defined as the WMS group ($n=7$). Individuals with body weight higher than 375 g (a median value of 350–400 g was reported as the average weight of adult marmosets [35]), with 0.036% weight gain per day (average weight gain ratio of normal marmosets reported in [2]), were defined as the control group ($n=7$). The highest body weight was obtained from laboratory records. Body weight, the highest body weight, current body weight/the highest body weight ratio and gain/loss ratio per day are listed in Table 1.

Appearance check: First, all animals underwent an appearance check, including fur and posture (with or without unkempt fur, pale face, undervitalized appearance, alopecia, curved back, pigeon-toed and stiff movement).

Blood collection: Blood samples were drawn from an individual's femoral vein using 26-gauge needles. For the duration of blood collection, animals were under manual retention. Part of the collected blood was used for CBC. The rest of the blood was centrifuged (1,800×g, 20 min and 4°C) after standing for 1 hr at room temperature. The purified serum was stored at -80°C until used for serum chemistry

and serum MMP9 concentration tests.

CBC and serum chemistry tests: A CBC analysis, including white blood cells, red blood cells, hematocrit, hemoglobin, platelets, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC), was performed using Celltac α (MEK-6450, NIHON KOHDEN Co., Ltd., Tokyo, Japan). A serum chemistry panel (albumin and calcium) was performed using a Drychem 4,000 system (FUJIFILM Co., Ltd., Tokyo, Japan).

Serum MMP9 concentration test: We performed serum tests of MMP9 concentrations using commercial ELISA kits (Quantikine ELISA Human MMP-9, SMP900; R&D Systems, Inc., Minneapolis, MN, U.S.A.).

Anatomical study: Marmoset colons were dissected after perfusion with saline followed by 4% paraformaldehyde under deep anesthesia. The dissected colons were fixed with Tissue Fixative (Genostaff, Co., Ltd., Tokyo, Japan), then embedded in paraffin wax and sectioned at 6 μ m for hematoxylin and eosin (HE) staining.

Statistical analysis: Two-tailed Mann-Whitney U-tests were used to compare the WMS and control groups, and Spearman's rank coefficient (two tailed) was used to measure correlations between body weight and each data point (GraphPad Prism, ver. 6 for Windows; GraphPad Software Inc., La Jolla, CA, U.S.A.). Data are presented as means $\pm$ standard error of the mean (SEM). Results were considered significant at 5% or less probability of error.

RESULTS

Appearance check: Pigeon-toe, which was considered to be caused by stiff movement in the hind limb (Fig. 1A and 1B), and alopecia (Fig. 1C), especially on the tail base (Fig. 1D), were observed in all seven animals in the WMS group. Neither abnormality was observed in any of the seven animals in the control group.

CBC: The hematocrit and hemoglobin values differed

Fig. 1. Marmosets in the WMS group show alopecia and pigeon toe. (A) Overall body posture of a marmoset in the WMS group. (B) Magnification of the white square in A. Hind limbs are pigeon-toed. (C) Appearance of a marmoset in the WMS group. Red arrow indicates an area of alopecia. The black square indicates tail-base alopecia. (D) Magnification of the black square in C. Red arrow indicates an area of alopecia, and the black arrow indicates ulceration.

significantly between the animals in the two groups ($P < 0.01$, Fig. 2A, and $P < 0.01$, Fig. 2B, respectively). The mean hematocrit values of the WMS group and the control group were $24.8 \pm 1.92\%$ and $40.7 \pm 1.48\%$, respectively. The mean hemoglobin values of the WMS group and the control group were 8.26 ± 0.808 g/dl and 12.97 ± 0.451 g/dl, respectively. Significant correlations between body weight and hematocrit and hemoglobin values were observed ($P < 0.01$, $r_s = 0.837$, Fig. 2C, and $P < 0.01$, $r_s = 0.851$, Fig. 2D, respectively).

The platelet value was also significantly different between the two groups ($P < 0.01$, Fig. 2E). The mean platelet values of the WMS group and control group were $118.3 \pm 11.61 \times 10^4/\mu\text{l}$ and $45.9 \pm 4.36 \times 10^4/\mu\text{l}$, respectively. Platelet values showed a significant negative correlation with body weight ($P < 0.01$, $r_s = -0.695$, Fig. 2F).

Although there was also a significant difference between the two groups in red blood cell values (mean $335.4 \pm 80.75 \times 10^4/\mu\text{l}$ in the MWS group and $618.9 \pm 66.04 \times 10^4/\mu\text{l}$ in the control group, $P < 0.01$), there was no significant difference ($P > 0.05$) between the groups in MCV (mean 67.8 ± 0.82 fl in the WMS group and 65.8 ± 1.74 fl in the control group),

MCH (mean 22.4 ± 0.87 pg in the WMS group and 21.0 ± 0.34 pg in the control group), MCHC (mean 33.2 ± 1.42 g/dl in the WMS group and 31.9 ± 0.16 g/dl in the control group) or white blood cell values (mean $71.4 \pm 28.23 \times 10^2/\mu\text{l}$ in the MWS group and $57.9 \pm 7.30 \times 10^4/\mu\text{l}$ in the control group).

Serum chemistry test: The levels of serum albumin differed significantly between animals in the two groups ($P < 0.01$, Fig. 3A). The mean albumin levels in the WMS and control groups were 3.49 ± 0.221 g/dl and 5.53 ± 0.300 g/dl, respectively. A significant correlation between body weight and serum albumin level was observed ($P < 0.01$, $r_s = 0.879$, Fig. 3B).

Similarly, the levels of serum calcium differed significantly between animals in the two groups ($P < 0.01$, Fig. 3C). The mean calcium levels in the WMS and control groups were 8.8 ± 0.40 mg/dl and 11.3 ± 0.56 mg/dl, respectively. A significant correlation between body weight and serum calcium level was observed ($P < 0.01$, $r_s = 0.734$, Fig. 3D).

Serum MMP9 concentration: There was a significant difference in serum MMP9 concentration between the two groups ($P < 0.01$, Fig. 4A). The mean MMP9 concentrations

Fig. 2. Hematocrit and hemoglobin values were decreased in marmosets with WMS. (A) Hematocrit values and (B) hemoglobin values of marmosets in the WMS and control groups. Solid horizontal lines indicate mean values for each group. Correlations between (C) body weight and hematocrit value and (D) body weight and hemoglobin value in individual animals. The dotted lines represent best-fit lines of the data points. Platelet values were increased in marmosets with WMS. (E) Platelet values of marmosets in the WMS and control groups. Solid horizontal lines represent mean values for each group. (F) Correlations between body weight and platelet values in individual animals. The dotted lines represent best-fit lines of the data points.

Fig. 3. Serum albumin and serum calcium values were decreased in marmosets with WMS. (A) Serum albumin values and (B) serum calcium values of marmosets in the WMS and control groups. Solid horizontal lines represent mean values for each group. Correlations between (C) body weight and serum albumin value and (D) body weight and serum calcium value in individual animals. The dotted lines represent best-fit lines of the data points.

in the WMS and control groups were 91.7 ± 21.79 ng/ml and 17.4 ± 2.73 ng/ml, respectively. A significant negative corre-

lation between body weight and serum MMP9 concentration was observed ($P < 0.05$, $r_s = -0.660$, Fig. 4B).

Fig. 4. Blood MMP9 values are increased in marmosets with WMS. (A) Blood MMP9 values of marmosets in the WMS and control groups. Solid horizontal lines represent mean values for each group. (B) Correlations between body weight, and blood MMP9 values in individual animals. The dotted lines represent best-fit lines of the data points.

Bowel section of animals in the WMS group: There were no polyps or cancer in the colons in either the WMS or control group (data not shown). Epithelial damage and disruption of crypt architecture were observed in the transverse colon samples (Fig. 5A and 5B) and ascending colon samples (Fig. 5C and 5D). The colonic tissues revealed inflammatory cell infiltration in the lamina propria of the mucosa, atrophy of intestinal villus and decreased numbers of goblet cells (Fig. 5B and 5D). There were no abnormalities in the colons in the control group (data not shown).

DISCUSSION

Common marmosets are often used for long-term research experiments, such as behavioral analysis, in the field of brain science [2]. A long duration is needed for the production of transgenic marmosets, at least 5 months for the duration of pregnancy and a litter size of 2–4 infants, so each animal is very valuable.

WMS can occur in any animal used for ongoing research. Due to this morbidity and mortality, the disease is a large risk factor for wasted research resources. It was reported that some treatments, such as long-term administration of steroids with relatively few side effects, such as budesonide, can cause temporary remission [27]. However, because steroid treatment is relatively ineffective in animals in the terminal stage and is not persistent, steroid treatment cannot be said to be adequate. Thus, early identification of diseased animals, removal from experiments and prompt treatment of these animals are important.

As other reports have mentioned, alopecia [18, 25], pigeon-

toe [18, 25], anemia [18], thrombocytosis [18], hypoalbuminemia [2, 18] and hypocalcemia [2] were also observed in the WMS group in this study. These results support the hypothesis that hematocrit, hemoglobin, platelets, serum albumin and serum calcium can be used for the diagnosis of WMS.

It was suggested that the anemia and hypoalbuminemia seen in the animals reflect poor nutritional condition, caused by an uptake disorder or protein and/or iron absorption defect. However, because no significant difference or even trend was seen in MCV, MCH or MCHC between the WMS and control groups, we suggest that the anemia and hypoalbuminemia observed in this study were due to other factor(s) depending on the individuals. Anemia is a major symptom of IBD in humans [13, 29], and iron deficiency is the largest contributing factor. However, it has been suggested that erythropoietin depression [17], vitamin insufficiency [13] and autoimmune hemolytic disease in IBD patients [24] could also cause anemia. Given that several factors other than iron deficiency could have caused anemia in WMS, similar to the situation in human IBD, further investigation is needed.

It is known that MMPs function to process several inflammatory cytokines, including tumor necrosis factor alpha (TNF- α) [10]. MMP9 is thought to be the main metalloproteinase implicated in the development of IBD [32], and the increase in MMP9 in mouse and rat IBD has been reported [4, 22]. Although there are other biomarkers used for diagnosis of IBD, such as C-reactive proteins (CRP) or erythrocyte sedimentation rate (ESR), it was reported that MMP9 is a more useful biomarker for diagnosis of human IBD than other biochemical markers in terms of that MMP9 can reflect disease state because MMP9 has much higher specificity in distinguishing between active IBD and inactive IBD than other parameters [23]. In our study, the WMS group showed significantly increased serum MMP9 concentrations, suggesting that MMP9 is connected with the pathological condition of WMS. Our results suggest that serum MMP9 might have an important role in the mechanism of WMS and could be useful for the diagnosis of WMS. A MMP9-targeted inhibitor was developed in rats and is hoped to be a specific medicine for IBD [22]. MMP9 might also be a target for the treatment of WMS. To our knowledge, this is the first report of increased MMP9 levels in marmosets with WMS. We previously reported that fecal calprotectin can be a marker for detecting colonic inflammation [25], and it was reported that fecal MMP9 can be used for detecting human IBD as well as fecal calprotectin [15]. So, further investigations of fecal MMP9 as a marker for detecting WMS should be performed. We propose that the WMS marmoset could be an animal model of human IBD with respect to MMP9. Further investigations of other molecules involved in human IBD need to be performed in marmosets to explore this possibility. TNF- α and interleukin-6 (IL-6) are major cytokines in inflammation, and there is a report regarding the involvement of TNF- α and IL-6 in human autoimmune diseases, including IBD [28]. Although TNF- α and IL-6 might be involved in WMS, little has been reported on the behavior of this molecule. Because it is known that MMP9 is regulated

Fig. 5. Representative photomicrographs of sections of the colon in marmosets with WMS. (A) Transverse colon. Scale bar: 100 μ m. (B) Ascending colon. Scale bar: 100 μ m. (C) Magnification of the black square in A. Scale bar: 50 μ m. (D) Magnification of the black square in B. Scale bar: 50 μ m. Red arrows in C and D indicate goblet cells.

by IL-6 [16] and affects TNF- α [10], future experiments aimed at investigating the relationship between MMP9 and TNF- α , or IL-6 in WMS are needed.

ACKNOWLEDGMENTS. We thank Dr. Chitoshi Itakura for providing the opportunity to perform the present study. We also thank the rearing staff of the animal facility for their generous help.

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