

## Detection of Calprotectin and Apoptotic Activity in the Colon of Marmosets with Chronic Diarrhea

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**ABSTRACT.** The common marmoset (*Callithrix jacchus*) is used as a non-human primate laboratory animal. Marmoset wasting syndrome (MWS) is a disease endemic to captive colonies, and the pathogenesis is unclear. In the present study, marmosets with chronic bloody high-viscosity diarrhea, which is a contributing factor to MWS, were evaluated, and inflammation in the colon was found. Calprotectin is a surrogate marker of intestinal inflammation and induces apoptosis. Marmosets with chronic diarrhea exhibited higher levels of fecal calprotectin. Histochemical analyses showed high expression of calprotectin in the extravascular neutrophils and apoptosis in the chronic colitis lesions. No internal microbiological diseases were identified. Although the cause of chronic colitis was not identified, the marmoset could be a useful model of inflammatory bowel disease.

**KEY WORDS:** apoptosis, calprotectin, colitis, diarrhea, marmoset.

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The common marmoset (*Callithrix jacchus*) is a small New World primate that is native to eastern Brazil and has been used in biomedical research in areas, such as neuroscience, reproductive biology, infectious disease and drug development [6]. Compared to *Macaca* species, the common marmoset has several advantages, such as a small body size, availability, easy breeding in captivity, low husbandry cost, avoidable zoonotic issues, early sexual maturation and a short gestation period [1, 11]. Thus, the number of studies using common marmosets as a non-human primate laboratory animal has increased in recent years [6, 9]. However, marmoset wasting syndrome (MWS), which includes chronic diarrhea, decreased muscle mass and weight loss, is a disease endemic to captive colonies of common marmosets [5, 13]. Although numerous studies have been conducted on the nutritional and infectious factors, the pathogenesis of this life-threatening disease remains unclear. In a previous study, chronic colitis was considered one of the most important contributing factors for the development of MWS [5].

Fecal calprotectin assays have been used to predict intestinal inflammation among patients with chronic diarrhea [3]. Calprotectin, which is a combination of the 8 kDa S100A8 (also called Calgranulin A or myeloid related protein 8, MRP8) and the 14 kDa S100A9 (also called Calgranulin B or myeloid related protein 14, MRP14), is a member of the S100 protein family, a group of calcium-binding molecules with an important role in immunity modulation. Calprotectin is a major protein in neutrophilic granulocytes, which accounts for 60% of the total proteins in the cytosol fraction [8], and

its presence in feces is directly proportional to inflammatory neutrophil migration towards the intestinal tract [3, 15]. Calprotectin induces secretions of various chemokines and promotes apoptosis [3, 15].

Recently, marmosets in our colony showed clinical signs similar to marmosets with MWS, especially chronic diarrhea. As a first step toward understanding the mechanisms of chronic diarrhea, we investigated the presence of inflammation, calprotectin and apoptosis in the colon of marmosets with chronic diarrhea.

The Animal Experiments Committee of RIKEN (Wako, Japan) approved the animal procedures, and the animals were handled in accordance with the Institutional Guidelines for Experiments using Animals. Common marmosets (*Callithrix jacchus*) from a breeding colony at the RIKEN Brain Science Institute (Wako, Japan) were maintained at 27°C and 50% humidity on a 12-hr light–dark cycle. Chronic diarrhea was defined as persistent high-viscosity diarrhea for more than 2 weeks. A control group consisted of marmosets without diarrhea for more than 2 months. The marmosets had no external injury or chronic vomiting and were free of the following microorganisms: Cytomegalovirus, *Shigella* spp., *Salmonella enteritidis*, *Vibrio parahaemolyticus*, *Campylobacter jejuni*, *Campylobacter coli*, *Clostridium difficile*, *Clostridium perfringens*, *Mycobacterium tuberculosis*, *Helicobacter pylori*, *Chlamydia trachomatis*, *Chlamydia pneumonia* and *Entamoeba histolytica*. These microorganisms were measured in the clinical laboratory of the Health Sciences Research Institute (Yokohama, Japan). Diarrheagenic *Escherichia coli* including enteropathogenic, enteroinvasive, enterotoxigenic, enterohemorrhagic and enteroaggregative strains was examined using PowerChek™ diarrheal *E. coli* Detection Kits (Kogene Biotech Co., Ltd., Seoul, Korea). We performed fecal occult blood tests using Quick Chaser (MIZUHO MEDY Co., Ltd., Saga, Japan) to detect subtle blood loss in the intestinal tract. To conduct

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Table 1. Result of biological markers for each marmoset

	Sex	Age (months)	Bloody fecal	Hemoglobin (g/dl)	Hematocrit (%)	RBCs ( $\times 10^6/\mu\text{l}$ )	Calprotectin (ng/ml)	WBCs ( $\times 10^3/\mu\text{l}$ )	Platelets ( $\times 10^5/\mu\text{l}$ )	Albumin (g/dl)
CD 1	Male	72	Positive	5.1	17.3	2.6	315.7	9.1	3.5	2.0
CD 2	Female	46	Positive	4.6	15.5	2.3	304.3	9.8	4.3	2.4
CD 3	Female	73	Positive	5.5	17.4	2.7	226.1	8.9	4.4	2.0
CD 4	Female	66	Positive	4.8	15.5	2.2	380.7	9.5	2.4	2.6
Control 1	Male	58	Negative	13.3	41.1	5.4	30.0	8.1	3.4	2.4
Control 2	Male	40	Negative	13.2	40.1	5.4	33.6	8.0	3.2	2.1
Control 3	Male	42	Negative	12.9	39.9	4.9	12.8	7.5	3.6	2.3
Control 4	Female	51	Negative	14.2	42.2	5.2	24.8	8.2	3.4	1.8
Control 5	Female	66	Negative	12.2	37.1	4.8	12.6	7.6	4.3	2.4
Control 6	Female	43	Negative	14.1	42.2	5.6	16.4	8.0	3.7	2.5

blood biochemical tests, blood was collected through the femoral vein using heparin-treated syringes. Red blood cells (RBCs), hematocrit, white blood cells (WBCs) and platelets were counted using the Celltac  $\alpha$  (MEK-6450, NIHON KOHDEN, Tokyo, Japan). To purify plasma, blood was centrifuged at  $1,800 \times g$  for 15 min within 1 hr of blood collection. Plasma hemoglobin and albumin were measured using the Drychem 4000 system (FUJIFILM, Tokyo, Japan). Fecal calprotectin level was analyzed using PhiCal<sup>®</sup> Calprotectin ELISA Kit (Immundiagnostik AG, Bensheim, Germany). Data were analyzed using Student's *t*-tests (Excel Statistics 2006, SSRI, Tokyo, Japan) and are presented as means  $\pm$  standard error of the mean (SEM). Results were considered significant at 5% or less probability of error. Marmosets were anesthetized with an overdose of sodium pentobarbital and perfused with saline followed by 4% paraformaldehyde. Each marmoset colon was dissected after perfusion, fixed with Tissue Fixative (Gonostaff, Co., Ltd., Tokyo, Japan), then embedded in paraffin and sectioned at 6  $\mu\text{m}$  for hematoxylin and eosin (HE) staining, immunocytochemistry and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay. The protocols for immunocytochemistry [4] and TUNEL assay [12] were conducted as previously reported. To examine calprotectin expression with immunocytochemistry, a primary antibody to calprotectin (ab92331, Abcam plc., Cambridge, MA, U.S.A.) was used at a dilution of 1:750, and the secondary antibody (Polyclonal goat anti-rabbit immunoglobulins/biotinylated, E0432, Dako, Glostrup, Denmark) was used at a dilution of 1:600. TUNEL staining was performed using *in situ* Apoptosis Detection Kit (MK500, TAKARA BIO INC, Tokyo, Japan). The sections were counterstained with Mayer's Hematoxylin (Muto Pure Chemicals Co., Ltd., Tokyo, Japan), dehydrated and then mounted with Malinol (Muto Pure Chemicals Co., Ltd.).

Four animals (one male and three females) aged between 46 and 73 months were included in the chronic diarrhea (CD) group. Six animals (three males and three females) aged between 40 and 66 months were included in the control group. Diarrheagenic *Escherichia coli* was not detected in either group (data not shown). Data regarding biological markers for each animal are listed in Table 1. Although controls did

not produce bloody feces, the CD group had bloody diarrhea in the fecal occult blood test. The biochemical markers were significantly different among groups in hemoglobin (g/dl) (CD and control:  $5.0 \pm 0.20$  and  $13.3 \pm 0.31$ , respectively;  $P < 0.01$ ), hematocrit (%) (CD and control:  $16.4 \pm 0.53$  and  $40.4 \pm 0.78$ , respectively;  $P < 0.01$ ), RBCs ( $\times 10^6/\mu\text{l}$ ) (CD and control:  $2.5 \pm 0.12$  and  $5.2 \pm 0.13$ , respectively;  $P < 0.01$ ), calprotectin (ng/ml) (CD and control:  $306.7 \pm 31.70$  and  $21.7 \pm 3.70$ , respectively;  $P < 0.01$ ) and WBCs ( $\times 10^3/\mu\text{l}$ ) (CD and control:  $9.3 \pm 0.20$  and  $7.9 \pm 0.12$ , respectively;  $P < 0.05$ ). There were not significant differences between groups in platelets ( $\times 10^5/\mu\text{l}$ ) (CD and control:  $3.7 \pm 0.46$  and  $3.6 \pm 0.16$ , respectively;  $P > 0.05$ ) and albumin (g/dl) (CD and control:  $2.3 \pm 0.15$  and  $2.3 \pm 0.11$ , respectively;  $P > 0.05$ ). To investigate the presence of inflammation, expression of calprotectin and activation of apoptosis, we analyzed four diarrhea and six control tissue specimens via histopathological tests including HE staining, immunocytochemistry and TUNEL assay. There were no polyps or cancer in the colon in any of the animals (data not shown). The colon specimens from the CD group revealed epithelial damage and disruption of crypt architecture (Fig. 1). Signs of colonic inflammation, including neutrophilic infiltration in the lamina propria of the mucosa, were evident in the colonic tissues of marmosets in the CD group. In the immunocytochemistry analysis, colon specimens from the CD group had increased numbers of specifically labeled calprotectin-positive cells in the lamina propria with respect to controls (Fig. 2A). TUNEL assay revealed apoptosis induction in all colitis specimens compared to controls (Fig. 2B).

MWS is one of the most important and least understood problems in laboratory-bred marmosets. Because the most common chronic symptom is diarrhea and pathological change is colitis [5], we focused on examining the colon of marmosets with chronic diarrhea using biochemical marker assays and histochemical examinations.

Fecal occult blood tests showed bloody, high-viscosity stools in the CD group, suggesting subtle blood loss in the intestinal tract. The levels of hemoglobin and hematocrit were lower in the CD group than in controls. The number of RBCs was also lower in the CD group. These results indicate anemia in the CD group caused by blood loss in the intestine.

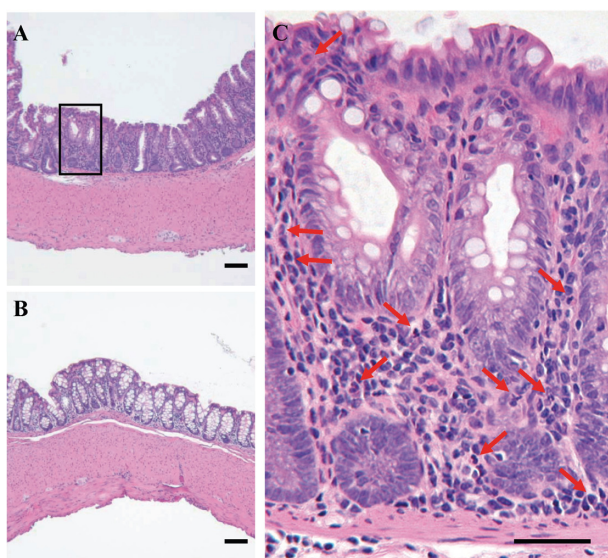


Fig. 1. Representative photomicrographs of sections of the colon in marmosets. HE staining showed the presence of inflammation in the colon of marmosets with chronic diarrhea (CD) group (A) compared with control (B). Scale bar, 100  $\mu\text{m}$ . Magnification of A showed the migration of neutrophils (red arrows) in the lamina propria of the mucosa of CD group (C). Scale bar, 50  $\mu\text{m}$ .

Conversely, platelet number was unaffected. Fecal calprotectin has been used as a non-invasive surrogate marker of intestinal inflammation diseases, such as inflammatory bowel disease [7]. Fecal calprotectin tests showed higher calprotectin concentrations in the CD group than in controls. To confirm the presence of inflammation, other biochemical marker was used. The number of WBCs was higher in the CD group than in controls, indicating that an inflammatory response occurred. No significant changes were observed in albumin parameters between CD and control groups, as previously reported [5]. Albumin concentration is frequently used to define the state of health, especially nutritional status [14]. However, our results indicate that albumin is not a suitable marker for the identification of chronic diarrhea. Reportedly, inflammatory diarrhea is caused by viral, bacterial or parasitic infections as well as by polyps, cancer or immune problems [7]. In the present study, microbiological tests showed negative results in both the CD and control groups. Anatomical findings showed no polyps or cancer in the colon of either group. These results indicate that inflammatory diarrhea is caused by aberrant activation of the colonic immune system. In HE staining, one of the main pathological features was infiltration of neutrophils in the colonic tissues of the CD group. In inflammatory reactions, calprotectin is detectable in elevated amounts that correlate to elevated levels of

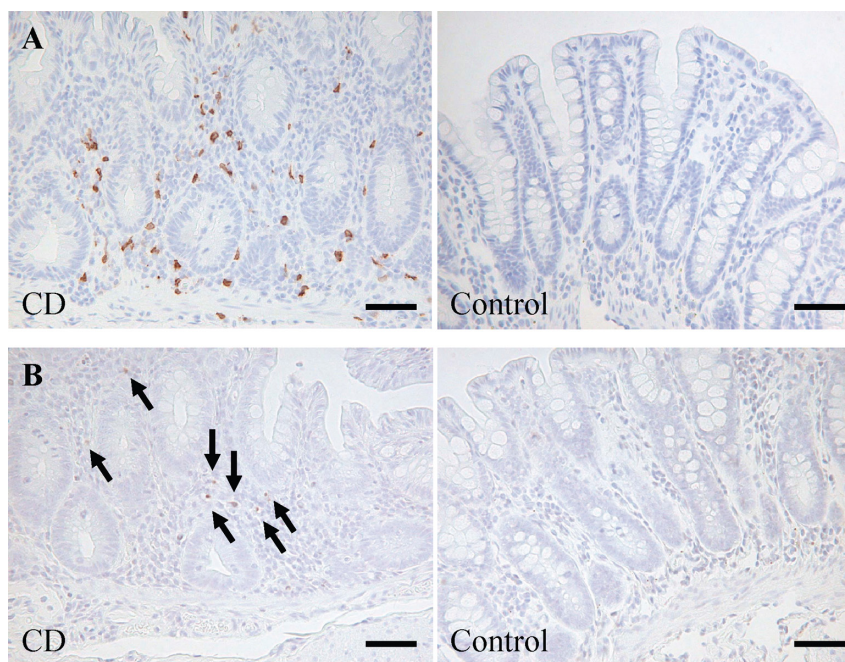


Fig. 2. Representative photomicrographs of calprotectin and apoptosis induction in the mucosa of colon in marmosets. Correlation between calprotectin expression and severity of inflammation was detected in the lamina propria of CD group (A, left panel) compared with controls (A, right panel). Scale bar, 50  $\mu\text{m}$ . TUNEL assay revealed apoptosis induction in the lamina propria of CD group (B, left panel) compared with control (B, right panel). The apoptotic cells were indicated by black arrows. Scale bar, 50  $\mu\text{m}$ .



inflammatory neutrophils [3]. We examined the calprotectin expression pattern in colitis tissues in the CD group using immunocytochemistry. The CD group exhibited calprotectin expression in the extravascular cells. Conversely, there is little extravascular calprotectin-positive cell in the control group. These results indicate that calprotectin detected in the CD group feces was derived from inflammatory neutrophils in the colon.

Calprotectin has the capacity to induce apoptosis in various cells [3]. We performed a TUNEL assay to detect fragmented DNA associated with apoptosis. Although apoptotic cell death has been implicated as a major homeostatic mechanism of intestinal epithelium [2], we found that significantly increased apoptosis was correlated with the severity of colitis. These results suggest that calprotectin functions as a critical mediator of apoptosis and plays a central role in diarrhea.

The colon specimens from the CD group revealed the disruption of crypt architecture. In the present study, we could not clarify whether the loss of stem cells occurs nor not in the crypt of CD group. Although the effect of inflammatory chemokines on stem cells has been unclear, our data suggest that the damage with inflammation and apoptosis might induce abnormal number and differentiation of stem cells. It might be possible that inflammatory chemokines induced by calprotectin affect homeostatic mechanism of the colonic epithelium.

This study is the first report to show that marmosets with chronic diarrhea have a higher calprotectin expression and apoptosis in the inflammatory colon. Because calprotectin induces various inflammatory chemokines [3], which cause decreased muscle mass [10], chronic inflammation may be a primary insult in a majority of marmosets with MWS, and the release of proinflammatory cytokines secondarily causes decreased muscle mass and other syndrome manifestations. We will examine whether other inflammation indicators of marmosets with chronic diarrhea are larger than those of controls in the biochemical tests, since their comparisons between marmosets with chronic diarrhea and controls give more insight in the inflammatory background. Although further studies are needed to characterize colonic inflammation of marmosets with chronic diarrhea, our results suggest that fecal calprotectin can be a useful marker for detecting and monitoring colonic inflammation in marmosets with chronic diarrhea and that marmosets with chronic colitis could be a model of inflammatory bowel diseases.

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