NOTE *Laboratory Animal Science*

Screening for Intestinal Microflora Influencing Superoxide Dismutase Activity in Mouse Cecal Mucosa

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ABSTRACT. We have suggested that intestinal microflora reduces the activity of the antioxidant enzyme superoxide dismutase (SOD) in the mouse cecal mucosa. In this study, gnotobiotic mice were used to examine the species of intestinal microflora influencing SOD activity in the cecal mucosa. The total SOD activity in the cecal mucosa of each germ-free (GF), gnotobiotic mouse with *Escherichia coli*, *Lactobacillus* and *Bacteroides* was significantly higher than that in the cecal mucosa of gnotobiotic mice with chloroform-treated feces (CHF), conventionalized (CVz) mice and conventional (CV) mice ($P<0.05$). In addition, CuZnSOD mRNA expression showed similar tendencies. Our results suggest that the antioxidant defense status in the cecal mucosa is influenced by CHF inoculation.

KEY WORDS: cecal mucosa, chloroform-treated feces, germ-free, superoxide dismutase.

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Antioxidant enzymes play an important role in preventing oxidative stress by acting on reactive oxidative species (ROS) generated *in vivo*. While physiological concentrations of ROS *in vivo* have beneficial effects involving cell signaling pathways and the killing of invading pathogens, the unbalanced and elevated levels of ROS may contribute to the development of various diseases, such as cancer, hypertension, diabetes, atherosclerosis, inflammation and premature aging [18]. Superoxide dismutases (SODs) [11] are the most important antioxidant enzymes in the antioxidant defense system against ROS of superoxide ($O_2^{\bullet-}$). At present, three distinct SOD isoforms have been identified in mammals. One SOD isoform has Cu and Zn in its catalytic center (CuZnSOD) and exists in the intracellular cytoplasmic compartment; the second isoform has manganese (Mn) in its catalytic center (MnSOD) and exists in the mitochondria of aerobic cells; and the third isoform is an extracellular SOD [18]. SODs are the first line of defense for dismutation of excess $O_2^{\bullet-}$, which causes tissue disorders, because SODs convert $O_2^{\bullet-}$ to molecular oxygen and H_2O_2 [16]. Of the different SOD enzymes, CuZnSOD is the most abundant and widely distributed enzyme in many tissues [10].

We have reported that upregulation of total SOD and CuZnSOD activities and CuZnSOD mRNA expression occurs in the mucosa of germ-free (GF) mice [3, 4]. In addition, total SOD and CuZnSOD activities in the duodenal, jejunal, ileal, cecal and colonic mucosae of GF mice were significantly higher than those in the mucosa of conventional (CV) mice [4]. Consistent with these results, the total SOD activity in conventionalized (CVz) mice decreased to the level of the total SOD activity observed in the ceca of CV mice [3]. These results suggested that the antioxidant defense system in the five sites of intestinal mucosa is influenced by intestinal microflora, which downregulates SOD activity [3, 4]. Screening for specific intestinal microflora influencing SOD activity in the mouse cecal mucosa is critical for understanding this event. In this study, we examined the intestinal microflora influencing SOD activity and CuZnSOD mRNA expression in the cecal mucosa using gnotobiotic mice.

All experiments were performed using 9-week-old male GF or CV IQI mice, which were bred in our animal facility and originated from CLEA Japan, Inc. (Tokyo, Japan). GF mice were maintained under GF conditions in flexible vinyl isolators. Cages, bedding and water for GF mice were sterilized either in an autoclave or with chlorine dioxide (Exspor; Alcide Co., Redmond, WA, U.S.A.). A commercial diet sterilized with 50-kGy gamma irradiation (CMF; Oriental Yeast Co., Ltd., Tokyo, Japan) and water were provided *ad libitum* to all mice. CV mice were kept in clean racks and were confirmed to be free of serum antibodies against *Clostridium piliforme*, *Mycoplasma pulmonis*, HVJ and MHV. They were also negative for *Pseudomonas aeruginosa*, *Salmonella spp*., *Pasteurella pneumotropica*, *Citrobacter rodentium*, *Corynebacterium kutscheri*, *Mycoplasma spp*., Dermatophytes, *Giardia spp*., *Spironucleus muris* and *Syphacia spp*. GF and CV mice were maintained in the same room under controlled conditions of temperature (23–25°C), relative humidity (40–70%) and light (12 hr, 7:00–19:00 hr). Mice were treated according to the provisions for animal welfare of the Nippon Veterinary and Life Science University, which fol-

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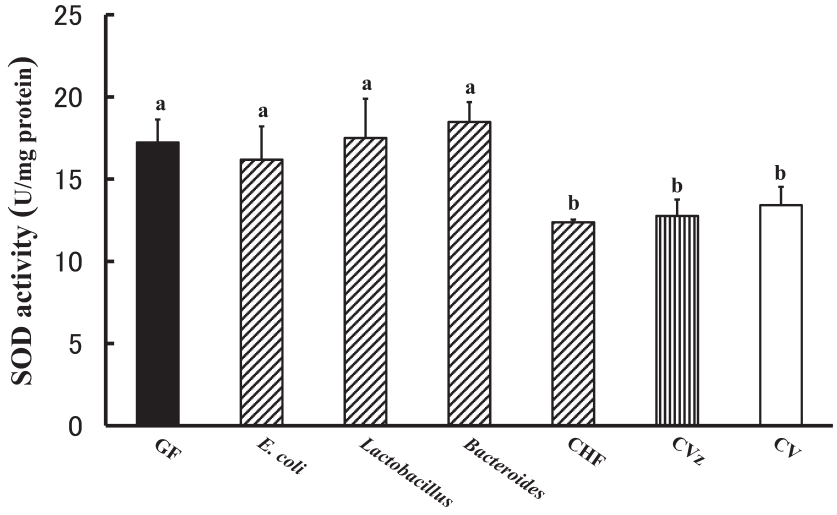

Fig. 1. Comparison of total SOD activities in cecal mucosa of gnotobiotic mice or GF, CVz and CV mice. Each group of animals consisted of 4–8 male animals. Values represent the means ± SD. ■: GF animals; ▨: gnotobiotic animals; ▩: CVz animals; □: CV animals. ab: With significant difference between different marks $P<0.05$.

lows the Guidelines for Animal Experimentation issued by the Japanese Association for Laboratory Animal Science [9].

Gnotobiotic mice were produced by the inoculation of *Escherichia coli* E-17, *Lactobacillus*, *Bacteroides* or chloroform-treated feces (CHF) into GF IQI mice. The fact that GF animals have enlarged ceca is well known [8], and the cecal size of GF mice has been reported to be shrunk to less than that of CV mice by inoculation with a mixture of clostridia obtained from the CHF of CV mice [6, 13]. Clostridium species of CHF are dominant in the mouse intestine, and they play an important role in morphological and physiological normalization of ceca. Because the CHF was viable but non-culturable (VNC) bacteria, murine CHF was prepared from freshly voided feces of CV IQI mice. A 1:100 fecal suspension was prepared with anaerobic trypticase soy broth without dextrose (TSB) (BBL, Sparks, MD, U.S.A.) supplemented with 0.5% of agar, 79 mM of Na_2CO_3 and 33 mM of L-cysteine·HCl·H_2O^{-1}, adjusted to pH 7.2. Chloroform was added to the suspension at a final concentration of 3%. After vigorous shaking and incubation at 37°C for 1 hr, chloroform was evaporated by CO_2 gas bubbling [6, 13]. The culture of *Lactobacillus*(6.0×10^7 CFU/300 μl/mice) [*L. acidophilus* strain 129 (*L. johnsonii*), *L. murinus* strain 91 and, *L. fermentum* strain 106 (*L. reuteri*)] [7], *Bacteroides* (3.6×10^8 CFU/300 μl/mice) [*B. vulgatus* 1 strains and *B. acidifaciens* 6 strains isolated from the cecum of mice] [12] or CHF (300 μl/mice) was orally inoculated into GF mice that had previously been administered *E. coli* E-17 (3.6×10^8 CFU/300 μl/mice). CVz (1:100 fecal/300 μl/mice) mice monoassociated with *E. coli* E-17 (3.6×10^8 CFU/300 μl/mice) for 2 days were inoculated through the fresh feces of CV IQI mice. At 3 weeks after inoculation, these mice were sacrificed by decapitation, and the cecal mucosa was rapidly frozen in liquid nitrogen followed by storage at −80°C until assay. Similar treatments were performed on 12-week-old GF and CV IQI mice for control of gnotobiotic mice. Samples were homogenized by sonication in a sucrose buffer solution (10 mM Tris–HCl, pH 7.4, 0.25 M sucrose and 1 mM EDTA). The total SOD activity was determined in the supernatant obtained after centrifugation at 20,000 g for 20 min at 4°C using the SOD Assay Kit-WST (Dojindo Molecular Technologies Ltd., Kumamoto, Japan). First, the SOD concentration (U/m*l*) that produced 50% inhibition of the WST reaction was determined (IC_{50}) using a standard SOD concentration (MP Biomedical, LLC, Solon, OH, U.S.A.). Following this, the dilution rate of the mouse cecal mucosal extract that established IC_{50} was determined, and the unit concentration (U/m*l*) of the extract was calculated. Each sample was analyzed in duplicates, and the results were expressed as enzyme activity per mg protein. The protein concentration was determined by the Bradford method [2] using bovine serum albumin as the standard.

Total RNA was extracted from frozen cecal mucosa using Trizol reagent (Invitrogen Co., Carlsbad, CA, U.S.A.). The amount of total RNA was measured by spectrophotometry. Total RNA (500 *n*g) was reverse transcribed in a solution of 10 μl of 1 × PrimeScript buffer, 25 pmol of oligo dT primer, 50 pmol of random 6-mer primer and 0.5 μl of PrimeScript RT Enzyme Mix 1 at 37°C for 15 min (Takara Bio Inc., Otsu, Japan). The reaction product was subjected to quantitative real-time polymerase chain reaction (PCR) performed following the instructions for the 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, U.S.A.). After cDNA denaturation at 95°C for 30 sec, PCR was performed according to the following thermal cycling protocol: 95°C for 5 sec and 60°C for 34 sec in 20 μl of buffer containing SYBR Premix Ex Taq (Takara Bio Inc.) and 0.8 μM each of forward CuZnSOD (5′-GGGTTCCACGTCCATCAGT-3′) and re-

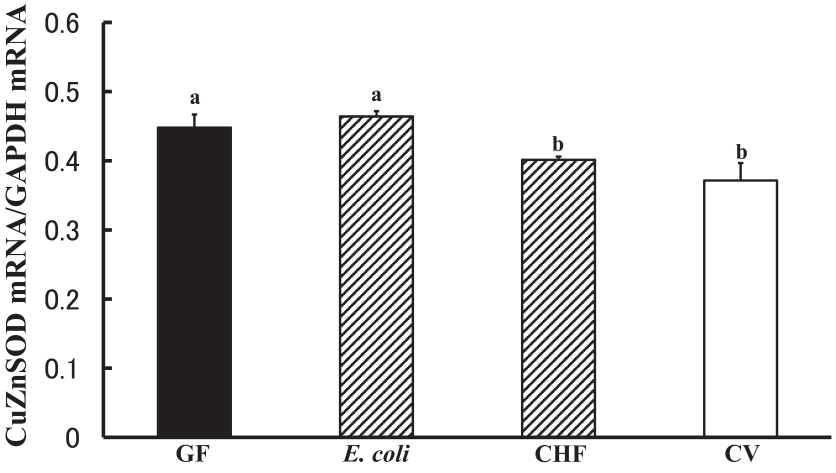

Fig. 2. Comparison of CuZnSOD mRNA expression in cecal mucosa of gnotobiotic mice or GF and CV mice. Each group of animals consisted of 3–5 male animals. Values represent the means ± SD. ■: GF animals; ▨: gnotobiotic animals; □: CV animals. ab: With significant difference between different marks $P<0.01$.

verse CuZnSOD (5′-CACACGATCTTCAATGGACAC-3′) primers [3, 4]. The cDNA sequence was obtained from GenBank (accession number, NM_011434), and forward and reverse primers were designed to span different exons to avoid genomic DNA amplification. Quantitative measurements were performed by establishing a linear amplification curve from serial dilutions of cloned mouse CuZnSOD mRNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA [14]. The amount of CuZnSOD mRNA was normalized to the amount of GAPDH mRNA.

The total bacterial count in the cecal content of 3 weeks after inoculation *E.coli*-, *Lactobacillus*- or *Bacteroides*-inoculated mice was not significantly different compared with those in CVz IQI mice (data not shown). The total SOD activity in the cecal mucosa of GF and *E. coli*-, *Lactobacillus*- or *Bacteroides*-inoculated mice was significantly higher than that in the cecal mucosa of CHF-treated, CVz and CV mice ($P<0.05$, Fig. 1). In addition, CuZnSOD mRNA expression in the cecal mucosa of GF and *E. coli*-treated mice was significantly higher than that in the cecal mucosa of CHF-treated and CV mice ($P<0.01$, Fig. 2). These results clearly demonstrate that the total SOD activity and CuZnSOD mRNA expression in the cecal mucosa of CHF-treated mice were significantly lower than those in the cecal mucosa of GF mice. In the cecal mucosa of CHF mice, the total SOD activity and CuZnSOD mRNA expression decreased to the level of the values observed in CV mice. These findings are in agreement with the results of our previous report on the total SOD activity of the entire cecal body in CVz mice [3]. The observation that GF animals have enlarged ceca is well known [8]; however, the enlarged ceca are reduced in size in GF mice inoculated with a mixture of clostridia obtained from CHF of CV mice [6, 13]. Similar results were obtained in this study (data not shown), although the interaction between SOD activity and cecal size remains unknown. Thus, our data suggest that SOD activity of the mouse cecal mucosa is influenced by CHF of colonization, i.e., CHF downregulates SOD activity of the mouse cecal mucosa. On the other hand, the mechanism by which bacterial colonization, components or metabolite suppresses SOD activity in the cecal mucosa remains unclear.

The phenotypes of intraepithelial lymphocytes in GF mice were changed to those in CV mice after the inoculation of CHF [15]. Clostridia, which are one of the most prominent gram-positive and spore-forming bacteria indigenous to the murine gastrointestinal tract [13], are major components of CHF. Moreover, *Clostridium* clusters IV and XIVa (also known as the *C. leptum* and coccoides groups, respectively) have been implicated in the maintenance of mucosal homeostasis and prevention of inflammatory bowel disease (IBD) [5, 17]. In a recent study, *Clostridium spp*. induced to expand Foxp3+ regulatory T-cells (Treg) [1]. Identifying these metabolites and the molecular mechanisms underlying the CHF–cecal mucosa crosstalk will provide invaluable information toward understanding how the gut microbiota regulates antioxidant enzymes and may suggest potential therapeutic options for treating IBD.

In conclusion, we suggest for the first time that SOD activity in the cecal mucosa is downregulated by CHF inoculation.

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