cells. GSIs have almost the same effect on progenitor cells of the normal and regenerating crypt, which becomes critically toxic once the epithelia have been damaged. Thus caution is needed with the use of GSIs when intestinal tissue damage is present.

Although studies have revealed various extrinsic factors promoting the regeneration of the intestinal epithelia (2, 3), the intracellular mechanism mediating the regenerative process has not been fully elucidated (15). Our data show that Notch activation maintains the larger number of IECs in the immature state, thereby promoting the proliferation and supporting the rapid recovery of IECs needed to restore proper epithelial structure. Thus we identified Notch signaling as one of the main intracellular pathways mediating the organized regenerative response of the intestinal epithelia. Although we know that several ligands and receptors of the Notch pathway are expressed in the intestine (24, 26), we do not know the precise mechanism by which these ligands activate Notch receptors, in particular IECs. A recent study by Riccio et al. (22) clearly showed that both Notch1 and Notch2 function redundantly in the intestinal epithelia and that they directly regulate the cell cycle progression of crypt progenitor cells. Thus an analysis of the Notch ligand expression is needed to understand the mechanism by which these Notch receptors could be activated during epithelial regeneration and the mechanism by which such activation could be downregulated at the later stage of regeneration.

One of our surprising findings was the upregulation of PLA2G2A in Notch-activated IECs, suggesting that Notch might also modulate immune functions of IECs. PLA2G2A is usually expressed in Paneth cells, and it is known to have an antimicrobial effect (4). The loss of the continuity of the epithelial layer allows various and abundant microorganisms to invade the submucosal area, thereby promoting inflammation and further destruction of the mucosa. Thus the local secretion of PLA2G2A at the damaged mucosal area may be quite beneficial for limiting bacterial invasion and providing a proper environment for regeneration. However, previous reports have shown that PLA2G2A is also expressed by neutrophils and macrophages accumulating at the inflamed mucosa of colitis (29, 39). Consistent with this, we observed an infiltration of PLA2G2A-positive cells in the lamina propria of inflamed mucosa in UC (Fig. 9, *C–F*). An RT-PCR analysis of DSS-colitis showed a significant upregulation of PLA2G2A expression in the inflamed colonic mucosa (Supplemental Fig. S3). However, such an upregulation was not inhibited with LY411,575 treatment, suggesting that the expression of PLA2G2A by neutrophils or macrophages might be less dependent on Notch activation. In those cells, intracellular pathways such as NF-κB might function to promote expression of PLA2G2A in the inflamed colonic mucosa (37). Also, our histological analysis suggested that the upregulation of PLA2G2A secretion was not a general but a partial response in Notch-activated IECs, indicating that an additional condition is required for ectopic expression of PLA2G2A.

Our microarray analysis also revealed a number of genes other than PLA2G2A that are regulated by NICD1 in IECs. Although the results did not show an upregulation of other genes specific to Paneth cells such as lysozyme or α-defensins, our quantitative RT-PCR confirmed that genes such as clusterin or spermidine/spermine N1-acetyltransferase were also upregulated upon Notch1 activation in LS174T cells (data not shown). Trefoil factor-1 may also promote Notch-mediated tissue regeneration because it is known to be a key factor in restitution (11). The group of genes shown in the present analysis was quite distinct from the previous microarray analysis comparing GSI-treated and untreated intestinal tissues (14, 27). Because we used an in vitro IEC-based assay, the group of genes identified can be recognized as candidates of the IEC-specific target genes of Notch. However, because only a limited number of genes were analyzed (up to 10,000 annotated genes) in the present study, a further analysis including a larger group of genes may elucidate additional genes that are regulated downstream of Notch.

In conclusion, Notch signaling acts as an indispensable intracellular signaling pathway in IECs, especially during tissue regeneration. It regulates not only the differentiation, but also the proliferation of IECs, and it also regulates the immune function of IECs. We have shown for the first time that such functions of Notch are also present in the human intestine, both under normal conditions and when tissue damage has occurred. The present study provides a novel molecular basis for the advanced understanding of the regeneration process in the human intestinal epithelia, which may be utilized to establish alternative therapies for refractory ulcers caused by various intestinal diseases.

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ORIGINAL ARTICLE

Lack of macrophage migration inhibitory factor suppresses innate immune response in murine dextran sulfate sodium-induced colitis

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Abstract

Objective. Macrophage migration inhibitory factor (MIF) plays an important role in the development of inflammatory diseases. Recent studies have indicated an association of MIF with gastrointestinal inflammation including colitis, but the mechanism by which MIF exacerbates gut inflammation has not been fully clarified. In this study, in order further to clarify the role of MIF in intestinal inflammation, we investigated the association of MIF with innate immunity in experimental colitis using MIF-deficient mice. ***Material and methods.*** Colitis was induced by treating mice with 3% dextran sulfate sodium (DSS) solution for 7 days. The expressions of chemokines in the colon were determined by reverse transcriptase-polymerase chain reaction (RT-PCR). Myeloperoxidase activity in the colon was measured and immunohistochemistry for F4/80 was analyzed. ***Results.*** DSS treatment increased the level of myeloperoxidase activity and infiltration of F4/80-stained cells in the colon, and up-regulated the mRNA expressions in macrophage inflammatory protein (MIP)-1α, MIP-2, macrophage chemotaxic protein (MCP)-1, and interferon inducible protein (IP)-10 in wild-type mice. In contrast, this increase and up-regulation were not observed in the colon of MIF-deficient mice treated with DSS. ***Conclusion.*** Our findings indicate that a lack of MIF suppresses the innate immune response in DSS-induced colitis.

Key Words: *Chemokine, colitis, innate immunity, macrophage migration inhibitory factor*

Introduction

Macrophage migration inhibitory factor (MIF) was originally discovered as a molecule associated with the mechanism of delayed-type hypersensitivity [1,2] and is known to be a pluripotent cytokine involved in a broad spectrum of physiological and pathological events [3]. As a new biological aspect, it has been reported that MIF genetic polymorphism is associated with the development of autoimmune diseases such as juvenile-onset systemic idiopathic arthritis [4,5]. On the one hand, MIF is ubiquitously expressed in a variety of cells and exerts an array of biological functions relevant not only to immune responsiveness [6–9] but also to tumorigenesis [10]. On the other hand, MIF secretion is associated with a multitude of diseases in humans, and it is suggested that MIF is the key mediator for the expression of inflammatory cytokines, particularly TNF-α [6].

Inflammatory bowel diseases (IBDs) such as ulcerative colitis and Crohn's disease are characterized by chronically relapsing inflammation of the bowel of unknown etiology [11]. Although the mechanisms of the inflammation and immune responses in IBD have not yet been fully elucidated, it has been suggested that various inflammatory mediators, such as TNF-α, are involved in their pathogenesis and exacerbation [12,13]. Recently, an

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association between MIF and intestinal inflammation in animals and humans has been suggested in several studies [14–17].

Some experimental colitis models mimic IBD to analyze the role of molecules. Among experimental colitis models, the dextran sulfate sodium (DSS)-induced colitis model is useful because of its reliability and simplicity [18,19]. We have previously demonstrated that an anti-MIF antibody is effective in preventing DSS-induced colitis in mice through the suppression of pro-inflammatory cytokines such as TNF-α, but we have not fully clarified the mechanism by which MIF regulates the severity of colitis [15]. In particular, we have not investigated the effect of MIF on innate immunity in colitis. In this study, in order further to clarify the role of MIF in the inflammatory states in colitis, we investigated the association of MIF with innate immune response in the colons of mice during DSS-induced colitis using MIF-deficient mice.

Materials and methods

Mice

Eight to 10-week-old male MIF-deficient mice (bred onto a BALB/c background) were established as previously described, and maintained under specific pathogen-free conditions [20]. Age-matched specific pathogen-free BALB/c male mice were purchased from Japan Charles River Co. (Shizuoka, Japan). All animal procedures were conducted according to the guidelines of the Hokkaido University Institutional Animal Care and Use Committee under an approved protocol.

Induction of DSS colitis and assessment of clinical features

Mice were given 3.0% DSS (M.W. 40 kDa; ICN Biomedicals, Costa Mesa, Calif., USA) in distilled water *ad libitum*. Clinical features including weight loss, bloody stool, and diarrhea were scored by disease activity index (DAI) as described previously (Table I) [21]. For histological and molecular analyses, the mice were killed with thiopental i.p. on day 7 post-DSS and then the colon tissues were removed. The severity of colitis was also evaluated by colon length and histology, while survival rates were assessed in mice treated with high-dose DSS (10%) in distilled water.

Histology

The colon tissue was opened longitudinally and fractionalized. Half of the tissue was fixed with 10% neutral buffered formalin, and embedded in paraffin. The other half section of tissue was used for molecular analyses. After deparaffinizing of thin tissue sections (4 μm) on glass slides, the samples were stained with hematoxylin and eosin (H&E). The sections were microscopically evaluated using a histological scoring system described previously in a blind fashion [15]. Briefly, the tissue damage was categorized into six grades: Grade 0, normal mucosa; grade 1, infiltration of inflammatory cells; grade 2, shortening of the crypt by less than half; grade 3, shortening of the crypt by more than half; grade 4, crypt loss; and grade 5, destruction of epithelial cells (ulceration and erosion). The extent of the lesions in the total colon was classified into six grades; grade 0, 0%; grade 1, 1–20%; grade 2, 21–40%; grade 3, 41–60%; grade 4, 61–80%; grade 5, 81–100%.

Table I. Scoring system for the disease activity index (DAI).

Score	Weight loss (%)	Stool consistency	Occult/gross rectal bleeding
0	<1	Normal	Negative
1	1–5		
2	5–10	Loose stool	Hemo-occult positive
3	10–20		
4	>20	Diarrhea	Gross bleeding

These clinical criteria were used to evaluate the severity of colitis in mice.
Scores were tailed for each category and then divided by 3 to obtain the DAI.

Measurement of tissue MPO activity

Tissue myeloperoxidase (MPO) activity in the colon was measured using a standard enzymatic procedure as described previously [17].

Reverse transcriptase-polymerase chain reaction for chemokines

Total RNA was extracted from colon tissues of MIF-deficient and WT mice using an Isogen RNA extraction kit (Isogen; Nippongene, Tokyo, Japan) according to the manufacturer's protocol. In brief, the colon tissues removed from the mice were frozen in liquid nitrogen. The samples were homogenized in Isogen using a polytron homogenizer. Total RNA (2 μg/ml) was incubated at 65°C for 10 min for denaturation. Denatured RNA (2 μg/ ml), 5 × RT buffer (1 × RT buffer: 50 mM Tris, pH8.3, 50 mM KCl, 8.0 mM $MgCl_2$, and 10 mM dithiothreitol), 2.5 mM deoxyoligonucleoside triphosphate (dNTP), 100 pM oligo-dT, and 0.5 ml monkey murine leukemia virus reverse transcriptase (Invitrogen, Carisbad, Calif., USA), and 0.4 μl RNase

inhibitor were incubated at room temperature for 10 min. After this process, 20 μl of the mixture (1 μg RNA) was incubated at 42°C for 1 h. Two microliter of the double-stranded product was then mixed with 10 × Taq/RT buffer (1 × Taq/RT buffer: 10 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatine, and 2.0 mM dithiothreitol), 500 μM dNTP mix, 25 mM $MgCl_2$, 500 mM each of sense and antisense oligonucleotide, and 0.25 ml Taq polymerase (Promega, Madison, Wis., USA). The polymerase chain reaction (PCR) primers for amplification of mouse macrophage inflammatory protein (MIP)-1α, MIP-2, macrophage chemotaxic protein (MCP)-1, inducible protein (IP)-10, and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) were designed (Table II). After the PCR heating step at 94°C for 5 min, subsequent amplification was carried out under the following conditions: 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min for 30 cycles, followed by final extension at 72°C for 7 min (Perkin Elmer DNA thermal cycler). The products were analyzed using 2% gel electrophoresis. GAPDH mRNA expression was used as a loading control.

Immunohistochemistry

Immunohistochemistry for F4/80 was analyzed using a Vectastain ABC kit (Vector Laboratories, Burlingame, Calif., USA) according to the manufacturer's protocol. The paraffin-embedded colon tissues were cut into 4-μm-thick sections. The sections were incubated with 3% H_2O_2 for 10 min at 4°C and then treated with 10% normal goat serum for 30 min at room temperature followed by overnight incubation with the anti-F4/80 antibody (diluted 100:1, Biosource, Camarillo, Calif., USA) at 4°C. F4/80-positive staining was visualized with diaminobenzidine as a chromogen. After F4/80 staining, the number of positively stained cells was counted in the colonic mucosa per mm^2 using a microscope. Three areas of mucosa in each mouse were evaluated in 5 mice from each group.

Statistical analysis

Data are presented as mean values ± standard deviation (SD). The results were statistically analyzed using the Mann-Whitney U-test and the log-rank test (StatView, SAS Institute, Cary, N.C., USA). A p-value of less than 0.05 was considered to be statistically significant.

Results

Clinical and histological features

We assessed the clinical signs and pathologic features of the colons of MIF-deficient mice given 3.0% DSS. The DAI at 7 day in DSS-induced colitis was markedly increased in all of the DSS-treated wild-type (WT) mice (3.3 ± 0.6). In contrast, DAI was not increased in all MIF-deficient mice even when they were treated with DSS (0.1 ± 0.3, $p < 0.01$ versus WT mice given DSS) (Figure 1). In addition, all WT mice were killed by 10% DSS treatment within 18 days, but all MIF-deficient mice survived for more than 18 days (Figure 2).

On histological examination, severe colitis with marked infiltration of mononuclear cells, crypt loss, and destruction of epithelial cells was observed in WT mice 7 days after 3% DSS treatment (tissue damage, 4.5 ± 0.3, extent of lesion, 3.3 ± 0.2). In contrast, no histological features of colitis were found in the colon of any of the MIF-deficient mice treated with 3% DSS for 7 days (tissue damage, 0.1 ± 0.1, extent of lesion, 0.1 ± 0.1, $p < 0.01$ versus WT mice given DSS).

Table II. The primers used in the RT-PCR.

Primers (5' 3') (Accession no.)			Product (bp)
MIP-1α	Sense	GCCCTTGCTGTTCTTCTCTGT	260
(NM011337)	Antisense	GGCATTCAGTTCCAGGTCAGT	
MIP-2	Sense	ACCCTGCCAAGGGTTGACTTG	287
(NM009140)	Antisense	GGCACATCAGGTACGATCCAG	
MCP-1	Sense	CTCACCTGCTGCTACTCATTC	320
(NM011333)	Antisense	GCTTGAGGTGGTTGTGGAAAA	
IP-10	Sense	CCTATCCTGCCCACGTGTTG	433
(BC030067)	Antisense	CGCACCTCCACATAGCTTACA	
GAPDH	Sense	TGAAGGTCGGTGTCAACGGATTTGGC	983
	Antisense	CATGTAGGCCATGAGGTCGACCAC	

Abbreviations: RT-PCR = reverse transcriptase-polymerase chain reaction; MIP = macrophage inflammatory protein; MCP = macrophage chemotaxic protein; IP-10 = interferon inducible protein-10; GAPDH = glyceraldehydes-3-phosphate dehydrogenase.

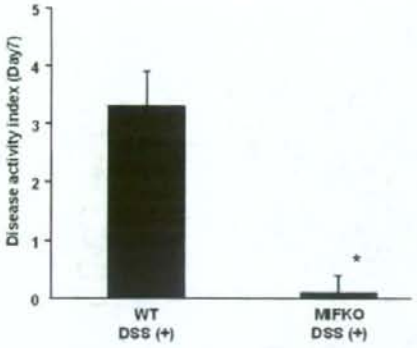

Figure 1. Scores of disease activity index (DAI) in dextran sulfate sodium (DSS)-induced colitis in mice ($n=10$, each group). The DAI scores in macrophage migration inhibitory factor (MIF)-deficient mice treated with 3% DSS solution for 7 days were significantly lower than those in wild-type (WT) mice given DSS. *$P<0.01$ as compared with DSS-treated WT mice. MIFKO = MIF-deficient mice.

Myeloperoxidase activity in the colon tissue

MPO is an enzyme produced mainly by polymorphonuclear leukocytes and is associated with granulocyte contents of tissues. There was no difference in the level of MPO activity in MIF-deficient mice an that in WT mice before DSS treatment (1.5 ± 0.2 and 1.7 ± 0.5 U/g protein, respectively) (Figure 3), whereas the level of MPO activity was significantly lower in MIF-deficient mice than that in WT mice when the mice were treated with 3.0% DSS for 7 days (1.9 ± 0.4 and 7.2 ± 1.0 U/g protein, $p<0.01$, respectively) (Figure 3). There was no significant difference between non-treated MIF-deficient and DSS-treated MIF-deficient mice (1.5 ± 0.2 and 1.9 ± 0.4 U/g protein, respectively) (Figure 3).

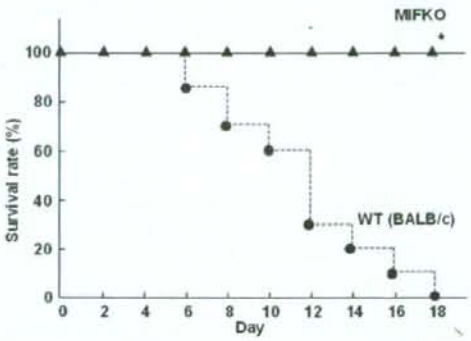

Figure 2. Survival rate of mice in dextran sulfate sodium (DSS)-induced colitis. High-dose DSS (10%) was provided *ad libitum*, and the survival rate was assessed every 24 h. Up to 18 days post-DSS stimulation, there were no deaths among migration inhibitory factor (MIF)-deficient mice ($n=10$), whereas all the wild-type (WT) mice ($n=10$) died of severe diarrhea and rectal bleeding. The two groups were compared statistically using the log-rank test; *$p<0.001$ as compared with DSS-treated WT mice. MIFKO = MIF-deficient mice.

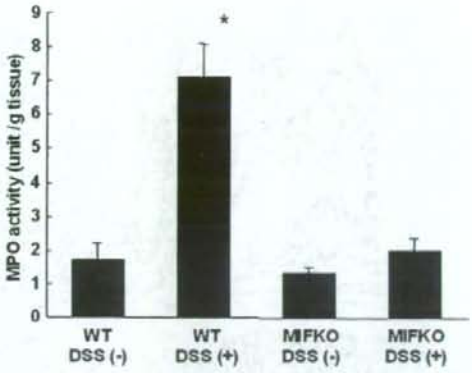

Figure 3. Myeloperoxidase (MPO) activity in the colon of mice exposed to 3% dextran sulfate sodium (DSS) for 7 days (each group; $n=6$). Wild-type (WT) mice treated with DSS significantly exhibited higher levels of MPO activity compared with non-treated WT mice, non-treated migration inhibitory factor (MIF)-deficient mice, and DSS-treated MIF-deficient mice. There was no significant difference in MPO activity among non-treated WT mice, non-treated MIF-deficient mice, and DSS-treated MIF-deficient mice; *$p<0.01$ as compared with non-treated WT mice. MIFKO = MIF-deficient mice.

F4/80 macrophages infiltrating in the colonic mucosa

F4/80-positive cells, which represent macrophages, were analyzed immunohistochemically in the colon tissues of mice treated with or without 3.0% DSS. Cells with F4/80-positive staining were found in the colons of WT mice (23 ± 6 cells/mm^2) (Figure 4A and 5). The number of these positive cells markedly increased in the lamina propria of the colons in WT mice 7 days after 3.0% DSS treatment (68 ± 7 cells/mm^2, $p<0.01$ versus non-treated WT mice) (Figure 4B and 5). In the colons of non-treated MIF-deficient mice, the number of F4/80-positive cells in the colon was similar to that in non-treated WT mice (21 ± 8 cells/mm^2) (Figure 4C and 5). However, the number of F4/80-positive cells was not increased in the colons of MIF-deficient mice even when the mice were treated with DSS for 7 days (23 ± 9 cells/mm^2, $p<0.01$ versus DSS-treated WT mice) (Figure 4D and 5). There was no significant difference in the number of F4/80-positive cells between non-treated MIF-deficient mice and DSS-treated MIF-deficient mice.

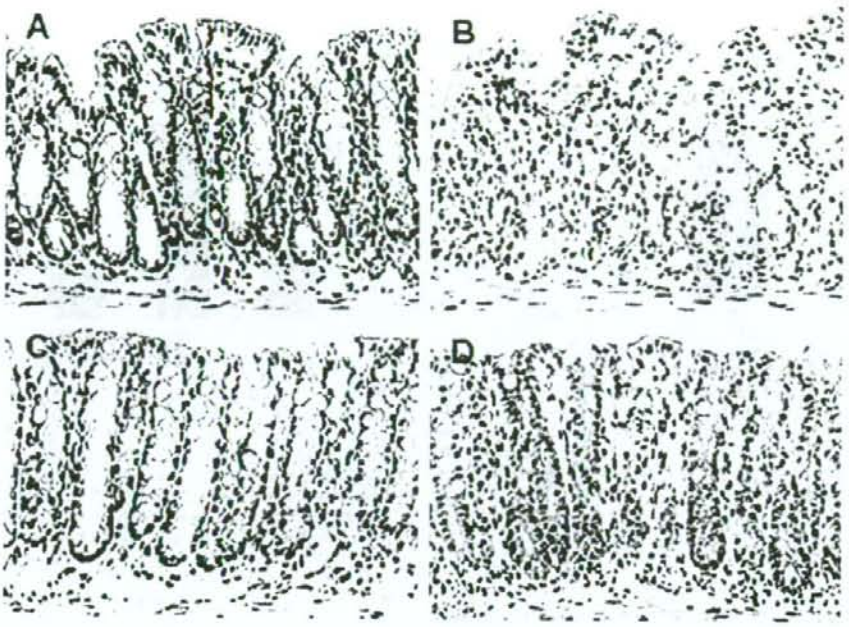

Figure 4. Immunohistochemical analysis for F4/80 in the colon. F4/80-positive staining was seen mainly in the mononuclear cells infiltrating the colonic mucosa. A. Non-treated wild-type (WT) mice. B. WT mice treated with 3% dextran sulfate sodium (DSS) for 7 days. C. Migration inhibitory factor (MIF)-deficient mice. D. MIF-deficient mice treated with 3% DSS for 7 days. Representative photomicrographs are shown. Similar appearances were observed in the colons of the other mice. Original magnification ×200.

Levels of MIP-1α, MIP-2, MCP-1, and IP-10 mRNA in the colon tissue

Reverse transcriptase-PCR (RT-PCR) for the expressions of MIP-1α, MIP-2, MCP-1, and IP-10 mRNA in the colon of mice was analyzed. Under normal conditions, there was little difference in the expressions of MIP-1α, MIP-2, MCP-1, and IP-10 mRNA in the colons of MIF-deficient and WT mice (Figure 6). However, the expression of these mRNAs was remarkably up-regulated in the colon of WT mice on 7 day after 3.0% DSS treatment. In contrast, all these mRNAs were not detected in the colon from MIF-deficient mice even when the mice were treated with DSS for 7 days (Figure 6).

Discussion

MIF is known to be a multifunctional protein involved in inflammatory disorders, including septic shock [8,22], inflammatory lung disease [23], autoimmune disease [24], and response to stimulus [25,26]. Recently, de Jong et al. have shown the pathological role of MIF in T-cell-mediated colitis, following the revelation of its pathogenic role in colitis [14]. We also have demonstrated that MIF plays an important role in the development of DSS-induced colitis in mice [15,17]. Furthermore, we have previously found no feature of DSS-induced colitis in MIF-deficient mice [27]. Consistent with our previous findings, in this study, we found that mortality and clinical and histological findings induced by DSS treatment were completely suppressed in MIF-deficient mice.

At the acute inflammatory site, innate immune cells such as neutrophils and macrophages play an important role in the development of inflammation. It is suggested that MIF affects neutrophil accumulation and activation in inflammatory disease. Makita et al. have reported that MIF modulates neutrophil accumulation in a model of acute pneumonitis in rat [28]. Administration of lipopolysaccharide (LPS) up-regulated MIF expression and increased neutrophil accumulation in the alveoli. Moreover, neutralization of MIF by anti-MIF antibody reduced neutrophil accumulation in the alveoli. For assessment of neutrophil accumulation, MPO activity is useful for assessment of neutrophil accumulation in tissues because MPO activity is reflected in granulocyte content but not in the content of

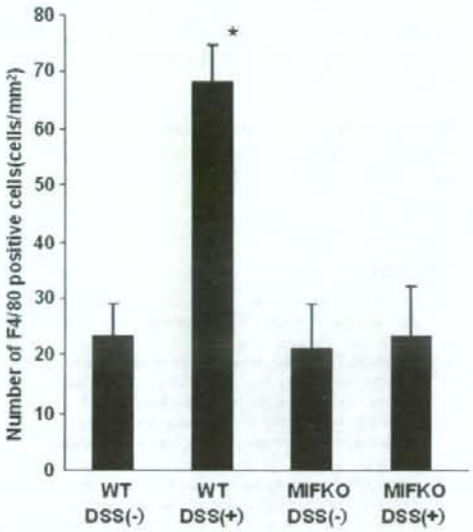

Figure 5. Number of infiltrating F4/80-positive cells. Immunohistochemistry for F4/80 was performed in the colons of mice. The number of cells with F4/80-positive staining was counted in three areas of the colonic mucosa and evaluated in 5 mice in each group. Average numbers of cell counts were calculated. After dextran sulfate sodium (DSS) treatment, the number of F4/80 positive cells was increased in wild-type (WT) mice, but was essentially unchanged in migration inhibitory factor (MIF)-deficient mice; $^{*}p < 0.01$ as compared with non-treated WT mice. MIFKO = MIF-deficient mice.

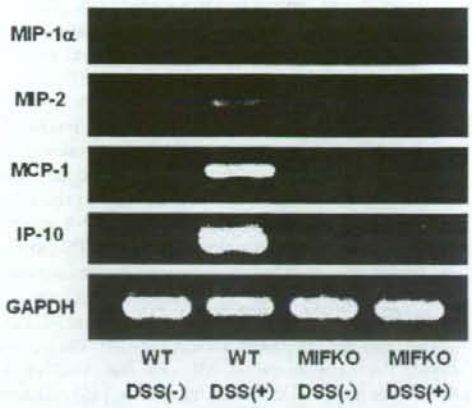

Figure 6. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of the expression of chemokines in the colon of mice. Expressions of macrophage inflammatory protein (MIP)-1α, MIP-2, monocyte chemoattractive protein (MCP)-1, and interferon inducible protein (IP)-10 were remarkably enhanced in the colons of wild-type (WT) mice exposed to 7 days of treatment with dextran sulfate sodium (DSS). In contrast, DSS dose did not enhance the expression of these chemokines in migration inhibitory factor (MIF)-deficient mice. Similar results were obtained from three complete experiments. GAPDH = glyceraldehydes-3-phosphate dehydrogenase; MIFKO = MIF-deficient mice.

other immunocytes in the tissues [29]. We have previously demonstrated that the level of MPO activity was increased in the colon of MIF-transgenic mice with DSS-induced colitis [17]. In this study, we found that the level of MPO activity was significantly lower in the colons of MIF-deficient mice compared with that in WT mice when the mice were treated with DSS for 7 days. Our current data support the evidence obtained from previous studies that MIF modulates the accumulation of neutrophils into the tissues, including the colon.

MIF affects infiltration of macrophages into the inflammatory site [30]. In this study, to assess the effect of MIF on infiltration of macrophages, we carried out an immunohistochemical analysis of colon tissues using F4/80 monoclonal antibody, which is widely used as a marker molecule for mouse macrophages. In fact, Kobayashi et al. have demonstrated that marked infiltration of F4/80-positive stained cells reflecting macrophages was observed in the liver of mice with acute hepatitis induced by bacille Calmette-Guerin and lipopolysaccharide [30]. Moreover, neutralization by anti-MIF antibody suppressed infiltration of F4/80-positive stained cells into the liver of mice with acute hepatitis. de Jong et al. also proposed that MIF contributed to innate immune response including activation of macrophages in a T-cell-mediated colitis model [14]. In this study, we found that MIF-deficient mice showed no increase in infiltrating F4/80-positive stained cells into colonic mucosa. Our results are consistent with those of previous studies and clearly indicate that MIF affects accumulation of macrophages into the colon of mice with DSS-induced colitis.

Regarding involvement of innate immune cells in DSS-induced colitis, Dieleman et al. have demonstrated that a lack of B- and T cells does not influence the induction of colitis by DSS using SCID mice [31]. They suggest the innate immune cells are involved in the development of DSS-induced colitis. Our findings also support their evidence and suggest that MIF partly affects the accumulation of innate immune cells such as neutrophils and macrophages in the colon of mice with DSS-induced colitis.

In the accumulation and activation of innate immune cells at the inflammatory site, chemokines are key molecules. Numerous studies have reported the up-regulation of chemokines in the colon of patients with IBD and in experimental colitis [32–40]. The expressions of IP-10 (CXCL10), IL-8, and MCP-1 are up-regulated in the colonic

epithelium and lamina propria under both physiological and pathological conditions in ulcerative colitis [32,34–37]. On the one hand, in animal models, blockade of IP-10 protects mice from DSS-induced colitis and spontaneous colitis [38,39], while on the other hand, MIP-2 is the chemoattractant for neutrophils and monocytes. Overexpression of MIP-2 enhances the severity of DSS-induced colitis and increases the level of MPO activity in the colon of mice [40]. It is suggested that MIP-2 plays a crucial role in the development of DSS-induced colitis via enhancement of neutrophil accumulation. MCP-1 plays a role as a chemoattractant and activates the function of monocytes, natural killer (NK) cells, and memory T cells [41–44]. On the one hand, the expressions of MIP-1α and MCP-1 are also increased in DSS-induced colitis [45], whereas in recent *in vivo* studies it is shown that MIF modulates the expression of MIP-1α, MIP-2, and MCP-1 [28,46–48]. Thus, we focused on the expression levels of chemokines in the colon of MIF-deficient mice. In this study, we demonstrated that the expressions of MIP-1α, MIP-2, MCP-1, and IP-10 mRNA were up-regulated in the colon of WT mice given DSS. In contrast, the mRNA expression levels of these chemokines were not up-regulated in MIF-deficient mice treated with DSS. These findings suggest that MIF affects the induction of MIP-2, MIP-1α, MCP-1, and IP-10 in the colon of mice during colitis. Suppression of up-regulation of these chemokines may prevent the accumulation of granulocytes and macrophages into the colonic mucosa in MIF-deficient mice during colitis. However, we did not investigate the localization of the expressions of these chemokines and whether the severity of colitis is changed by administration of MIF or these chemokines. Further investigation is needed to confirm the mechanism behind the effect of MIF in modulating innate immunity during DSS-induced colitis.

In conclusion, we discovered that disruption of the MIF gene prevented the up-regulation of chemokines and the accumulation of innate immune cells in the colon of mice treated with DSS. Our findings suggest that MIF plays an important role in the regulation of innate immune response, and that modulation of MIF could be a potential target for treatment of colitis, including IBD.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Therapeutic Effect of Lecithinized Superoxide Dismutase against Colitis

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ABSTRACT

Ulcerative colitis (UC) involves intestinal mucosal damage induced by reactive oxygen species (ROS), in particular, superoxide anion. Superoxide dismutase (SOD) catalyzes dismutation of superoxide anion to hydrogen peroxide, which is subsequently detoxified by catalase. Lecithinized SOD (PC-SOD) is a new modified form of SOD that has overcome previous clinical limitations of SOD. In this study, we examined the action of PC-SOD using an animal model of UC, dextran sulfate sodium (DSS)-induced colitis. DSS-induced colitis was ameliorated by daily intravenous administration of PC-SOD. Unmodified SOD produced a similar effect but only at more than 30 times the concentration of PC-SOD. In vivo electron spin resonance analysis confirmed that the increase in the colonic level of ROS associated with development of colitis was suppressed by PC-SOD administration. The dose-response profile of PC-SOD was bell-shaped, but simultaneous administration of catalase restored the ameliorative effect at high doses of PC-SOD. Accumulation of hydrogen peroxide was observed with the administration of high doses of PC-SOD, an effect that was suppressed by the simultaneous administration of catalase. We also found that either a weekly intravenous administration or daily oral administration of PC-SOD conferred protection. These results suggest that PC-SOD achieves its ameliorative effect against colitis through decreasing the colonic level of ROS and that its ineffectiveness at higher doses is because of the accumulation of hydrogen peroxide. Furthermore, we consider that intermittent or oral administration of PC-SOD can be applied clinically to improve the quality of life of UC patients.

Inflammatory bowel disease (IBD), Crohn's disease, and ulcerative colitis (UC) have become substantial health problems (Cuzzocrea, 2003). Recent studies suggest that IBD is chronic inflammatory disorder occurs in the intestine because of "a vicious cycle"; infiltration of leukocytes into intestinal tissues causes mucosal damage induced by reactive oxygen species (ROS) that are released from the activated leukocytes, and this damage further stimulates the infiltration of leukocytes through induction of proinflammatory cytokines, in particular, tumor necrosis factor (TNF)-α (Podolsky, 2002). Among the various ROS, superoxide anion is particularly important because it has a potent ability to damage cells and leads to the formation of other ROS, such as hydroxy radicals (Kruidenier and Verspaget, 2002). A positive correlation between the severity of IBD and the intestinal level of ROS has been reported (Simmonds et al., 1992). Thus, antioxidant molecules (radical scavengers) have attracted considerable attention as therapeutic candidates for the treatment of IBD.

Superoxide dismutase (SOD) is one such antioxidant protein. SOD catalyzes the dismutation of superoxide anion to

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ABBREVIATIONS: IBD, inflammatory bowel disease; UC, ulcerative colitis; ROS, reactive oxygen species; TNF, tumor necrosis factor; SOD, superoxide dismutase; PC-SOD, lecithinized superoxide dismutase; DSS, dextran sulfate sodium; DAI, disease activity index; U-SOD, unmodified superoxide dismutase; QOL, quality of life; ESR, electron spin resonance; PMA, phorbol 12-myristate 13-acetate; LPS, lipopolysaccharide; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; RT, reverse transcriptase; PCR, polymerase chain reaction; POBN, α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone; DTPA, diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaactic acid; DAPI, 4,6-diamidino-2-phenylindole; NF, nuclear factor; MPO, myeloperoxidase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL, interleukin; CL, chemiluminescence; ELISA, enzyme-linked immunosorbent assay.

hydrogen peroxide, which is subsequently detoxified to oxygen and water by catalase or glutathione peroxidase (Kruidenier and Verspaget, 2002). Among three isoforms of human SOD, Cu/Zn-SOD mainly contributes to the SOD activity in IBD patients (Kruidenier et al., 2003a). Decreased expression of SOD, especially Cu/Zn-SOD, has been observed in IBD patients (Kruidenier et al., 2003a,b). Furthermore, administration of Cu/Zn-SOD suppresses the development of IBD-related colitis in the experimental animal models (Keshavarzian et al., 1990; Segui et al., 2004). These findings raised the prospect that SOD could be of therapeutic benefit in the treatment of IBD. However, subsequent clinical trials of Cu/Zn-SOD have proven unsuccessful, mostly because of its low affinity to the cell membrane, where superoxide anion is produced, and its low stability in plasma, with a half-life of only a few minutes (Greenwald, 1990; Tsao et al., 1991; Igarashi et al., 1992, 1994). Therefore, various drug delivery systems have been applied to SOD to overcome these limitations (Keshavarzian et al., 1990; Igarashi et al., 1992, 1994; Yasui and Baba, 2006).

Among these applications, lecithinized SOD (PC-SOD) is potentially beneficial for clinical treatment of IBD, especially UC. PC-SOD is lecithinized human Cu/Zn-SOD, in which four phosphatidylcholine-derivative molecules are covalently bound to each SOD dimer (Igarashi et al., 1992). In vitro experiments using cultured cells have shown that this modification drastically improves the cell membrane affinity of SOD without decreasing its SOD activity (Igarashi et al., 1992, 1994), whereas in vivo experiments using rats have demonstrated that it also greatly improves plasma stability (Igarashi et al., 1992). In phase I clinical studies, intravenously administered PC-SOD (40–160 mg) had a terminal half-life of more than 24 h, with good safety and tolerability (Broeyer et al., 2008; Suzuki et al., 2008a). Furthermore, intravenously administered PC-SOD ameliorated dextran sulfate sodium (DSS)-induced colitis in rats, an IBD-related colitis animal model (Hori et al., 1997), suggesting that PC-SOD is effective for the treatment of IBD patients. In fact, recent published results of phase II clinical study have shown that intravenously administered PC-SOD (40 or 80 mg) significantly improved the disease activity index (DAI) scores of UC patients (Suzuki et al., 2008b). However, the comparison of PC-SOD with unmodified SOD (U-SOD) based on pharmacological activity against colitis has not been undertaken, and a decrease in the ROS level with PC-SOD administration has not been demonstrated in vivo. In addition to U-SOD, a bell-shaped dose-response profile of PC-SOD has been reported for various pharmacological activities, including anticolitis activity (Mao et al., 1993; Hori et al., 1997; Tamagawa et al., 2000; Tsubokawa et al., 2007). However, its underlying mechanism has remained unknown. Furthermore, when considering the quality of life (QOL) of patients, the present clinical protocol of PC-SOD administration (intravenous infusion once daily for 4 weeks) is expected to be improved. In this study, we compared PC-SOD and U-SOD for their pharmacological activity against DSS-induced colitis and found that PC-SOD has more than 30 times higher activity. In vivo electron spin resonance (ESR) analysis showed that administration of PC-SOD suppressed the increase in the ROS level induced by DSS treatment. We also provide evidence that the ineffectiveness of higher doses of PC-SOD is because of accumulation of hydrogen peroxide at the intestine. Furthermore, based on results obtained here, we propose that intermittent administration or oral administration of PC-SOD is a clinically viable option to improve the QOL of UC patients.

Materials and Methods

Chemicals and Animals. Paraformaldehyde, *O*-dianisidine, phorbol 12-myristate 13-acetate (PMA), fetal bovine serum, and catalase from bovine liver (1340 U/mg) were obtained from Sigma-Aldrich (St. Louis, MO). RPMI 1640 was from Nissui (Tokyo, Japan). Enzymatic digest of animal tissue (Proteose peptone) was from BD Biosciences (San Jose, CA). LPS was from List Biological Laboratories Inc. (Campbell, CA). Alexa Fluor 488 goat anti-rabbit immunoglobulin G was purchased from Invitrogen (Carlsbad, CA). Mounting medium for immunohistochemical analysis (Vectashield) was from Vector Laboratories (Burlingame, CA). 5,5-Dimethyl-1-pyrroline-*N*-oxide (DMPO) was purchased from Labotec (Midrand, South Africa), lymphocyte isolation sterile solution (Ficoll-Paque PluS) from GE Healthcare (Chalfont St. Giles, UK), DSS (mol. wt., 5000; 15–20% sulfur content) and luminol from Wako Pure Chemicals (Tokyo, Japan), and Mayer's hematoxylin, 1% eosin alcohol solution, and mounting medium for histological examination (Malinol) from MUTO Pure Chemicals (Tokyo, Japan). The RNeasy kit was obtained from QIAGEN (Valencia, CA), the PrimeScript 1st strand cDNA Synthesis Kit was purchased from Takara (Kyoto, Japan), and mix for real-time RT-PCR (iQ SYBR Green Supermix) was from Bio-Rad Laboratories (Hercules, CA). α-(4-Pyridyl-1-oxide)-*N*-*tert*-butylnitrone (POBN) was from Alexis Laboratories (San Diego, CA). U-SOD (5190 U/mg) and PC-SOD (3000 U/mg) were from our laboratory stock (Igarashi et al., 1992). SODs were dissolved in 5% xylitol and administered intravenously (tail vein) or orally. Diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaactic acid (DTPA) and 4,6-diamidino-2-phenylindole (DAPI) were from DOJINDO Laboratories (Kumamoto, Japan). An antibody against phospho-nuclear factor (NF) κB p65 (Ser536) was from Cell Signaling Technology Inc. (Danvers, MA). Wild-type mice (8 weeks old, ICR, male) were used throughout. The experiments and procedures described here were carried out in accordance with the *Guide for the Care and Use of Laboratory Animals* as adopted and promulgated by the National Institutes of Health (Institute of Laboratory Animal Resources, 1996) and were approved by the Animal Care Committee of Kumamoto University.

Development of DSS-Induced Colitis and Measurement of Colon Length and DAI. DSS-induced colitis was induced in mice by the addition of 4% DSS (w/v, final concentration) to their drinking water as described previously (Tanaka et al., 2007). The first administration of PC-SOD was done just before the start of DSS administration. The animals were allowed free access to the DSS-containing water for 7 days. For measurement of myeloperoxidase (MPO) activity, expression of mRNAs, and the ROS level, we used rectum and distal colon tissue. After 7 days, animals were placed under deep ether anesthesia and sacrificed, the colons were dissected, and their length was measured from the ileocecal junction to the anal verge.

The DAI was determined macroscopically by an observer unaware of the treatment the mice had received, according to previously reported criteria (Tanaka et al., 2007). In brief, the DAI was calculated as the sum of the diarrheal stool score (0, normal stool; 1, mildly soft stool; 2, very soft stool; 3, watery stool) and the bloody stool score (0, normally colored stool; 1, brown stool; 2, reddish stool; 3, bloody stool).

MPO Activity. MPO activity in the colonic tissues was measured as previously described (Tanaka et al., 2007). After DSS treatment, colons were dissected, rinsed with cold saline, and cut into small pieces. Samples were homogenized, and protein concentrations of the samples were determined using the Bradford method. MPO activity was determined in 10 mM phosphate buffer with 0.5 mM *O*-dianidisine, 0.00005% (w/v) hydrogen peroxide, and 20 μg of protein. MPO activity was obtained from the slope of the reaction curve, and

specific activity was expressed as the number of hydrogen peroxide molecules converted per minute per milligram of protein.

Real-Time RT-PCR Analysis. Real-time RT-PCR was performed as previously described (Mima et al., 2005), with some modifications. Total RNA was extracted from intestinal tissues or mouse peritoneal macrophages using an RNeasy kit according to the manufacturer's protocol. Samples (2.5 μg of RNA) were reverse-transcribed using a first-strand cDNA synthesis kit. Synthesized cDNA was used in real-time RT-PCR (Chromo 4 instrument; Bio-Rad Laboratories) experiments using mix for real time RT-PCR and analyzed with Opticon Monitor Software. Specificity was confirmed by electrophoretic analysis of the reaction products and by inclusion of template- or reverse transcriptase-free controls. To normalize the amount of total RNA present in each reaction, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was used as an internal standard.

Primers were designed using the Primer3 Web site (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The primers used were as follows: *Tnf*-α, 5'-cgtcagccgatttgctatct-3' (forward) and 5'-cggactccgcaaagtctaag-3' (reverse); *Gapdh*, 5'-aactttggcattgtggaagg-3' (forward) and 5'-acacattgggggtaggaaca-3' (reverse); *Il-1*β, 5'-gatcccaagcaatacccaaa-3' (forward) and 5'-ggggaactctgcagactcaa-3' (reverse); *Il-6*, 5'-ctggagtcacagaaggagtgg-3' (forward) and 5'-ggtttgccgagtagatctcaa-3' (reverse); and *Il-23p19*, 5'-gccccgtatccagtgtgaag-3' (forward) and 5'-cggatcctttgcaagcagaa-3' (reverse).

Histological and Immunohistochemical Analysis. Colonic tissue samples were fixed in 4% buffered paraformaldehyde, then embedded in paraffin before being cut into 4-μm sections. For histological examination, sections were stained first with Mayer's hematoxylin and then with 1% eosin alcohol solution. Samples were mounted with mounting medium and inspected with the aid of an Olympus BX51 microscope (Olympus, Tokyo, Japan).

For immunohistochemical analysis, sections were blocked with 3% bovine serum albumin for 30 min, incubated for 12 h with antibody against phospho-NF-κB (1:100 dilution) in the presence of 2.5% bovine serum albumin, and finally incubated for 1 h with Alexa Fluor 488 goat anti-mouse immunoglobulin G in the presence of DAPI (5 μg/ml). Samples were mounted with mounting medium and inspected using fluorescence microscopy (Olympus BX51).

Measurement of ROS in Neutrophils in Vitro. Human neutrophils were prepared as described previously (Karakawa et al., 2008). In brief, polymorphonuclear leukocytes and mononuclear cells were separated using a gradient of lymphocyte isolation sterile solution. Red blood cells remaining in the polymorphonuclear leukocyte fractions were lysed with 0.2% NaCl.

The chemiluminescence (CL) response induced by the superoxide anion released from neutrophils was measured as described (Muranaka et al., 1997). Prepared neutrophils were mixed with 25 ng/ml PMA in RPMI 1640 medium containing 10 μM luminol and 500 μM DTPA. The CL response was continuously recorded for 10 min at room temperature using a luminometer (Advantec Co., Tokyo, Japan).

The level of superoxide anion was also assayed by ESR spin trapping with DMPO as previously described (Karakawa et al., 2008). Prepared neutrophils were incubated with 10 ng/ml PMA in RPMI 1640 medium containing 500 μM DTPA and 25 mM DMPO for 5 min at room temperature. ESR spectra were recorded at room temperature on a JES-TE200 ESR spectrometer (JEOL, Tokyo, Japan) under the following conditions: modulation frequency, 100 kHz; microwave frequency, 9.43 GHz; microwave power, 40 mW; scanning field, 335.2 ± 5 mT; sweep time, 2 min; field modulation width, 0.25 mT; receiver gain, 100; and time count, 0.3 s. After recording the ESR spectra, the signal intensities of the DMPO-OOH adducts were normalized against that of a manganese oxide marker.

Determination of ROS Level and the Amount of Hydrogen Peroxide in Vivo. In vivo ESR analysis was performed as described previously (Sato et al., 1992, 2002), with some modifications. After DSS administration for 7 days, animals were placed under deep anesthesia with chloral hydrate (250 mg/kg) and injected with POBN intraperitoneally (4 mmol/kg). After 1 h, mice were sacrificed, the colons were dissected, and the lipid phase from the samples was extracted as described elsewhere (Sato et al., 1992, 2002). After evaporating the sample, ESR spectra were immediately recorded at room temperature in a JES-TE200 spectrometer under the following conditions: modulation frequency, 100 kHz; microwave frequency, 9.43 GHz; microwave power, 40 mW; scanning field, 335.2 ± 5 mT; sweep time, 2 min; field modulation width, 0.25 mT; receiver gain, 630; and time count, 0.3 s. Every buffer and solutions of the reaction mixture used for ESR measurement were treated with chelex 100 resin (Bio-Rad Laboratories) before use to remove metals.

For determination of hydrogen peroxide levels, colons were dissected, cut into small pieces, suspended in phosphate-buffered saline, and incubated for 30 min at room temperature with rotation. After centrifugation, the supernatants were applied to the NWLSS NWK-HYP01 assay kit (Northwest Life Science Specialties, LLC, Vancouver, WA).

Determination of the Amount of PC-SOD and TNF-α in Vivo. Determination of the amount of PC-SOD was carried out as described previously (Igarashi et al., 1992). After administration of PC-SOD, the blood was collected, and serum samples were obtained by centrifugation. On the other hand, colons were dissected, cut into small pieces, homogenized, and centrifuged to obtain the supernatants. Samples were analyzed using a human Cu/Zn-SOD enzyme-linked immunosorbent assay (ELISA) kit (Bender MedSystems Inc., Burlingame, CA). We used PC-SOD (or U-SOD) for drawing the standard curve of ELISA and determined the amount of PC-SOD (or U-SOD). The amount of TNF-α in serum was determined similarly by use of its ELISA kit from Pierce Chemical (Rockford, IL).

Preparation of Mouse Peritoneal Macrophages. Mouse peritoneal macrophages were prepared as described previously (Salimuddin et al., 1999). Mice were given 2 ml of 10% enzymatic digest of animal tissue by intraperitoneal injection, and peritoneal cells were harvested 3 days later. The cells were seeded in 60-mm culture dishes at 4×10^6 cells/dish in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum. After incubation for 4 h, nonadherent cells were removed, and the adherent cells were cultured for use in the experiments. Virtually all of the adherent cells were macrophages, as previously described (Salimuddin et al., 1999).

Statistical Analysis. All values are expressed as the mean ± S.E.M. Two-way analysis of variance followed by the Tukey test or the Student's *t* test for unpaired results were used to evaluate differences between more than three groups or between two groups, respectively. Differences were considered to be significant for values of $P < 0.05$.

Results

A Comparison of the Effect of PC-SOD and U-SOD on DSS-Induced Colitis. The severity of DSS-induced colitis can be monitored by various indices, such as DAI, length of colon, MPO activity, and histological analysis. We compared PC-SOD and U-SOD for their effect on the development of colitis induced by 4% DSS administration. The clinical study was performed with 40 and 80 mg PC-SOD (Suzuki et al., 2008b), which corresponds to 2 and 4 kU/kg; therefore, we chose the dose of 3 kU/kg for the following experiments. PC-SOD and U-SOD were intravenously administered once daily. There was no significant difference in the volume of water consumed by each group of mice (data not shown). Administration of 4% DSS increased the DAI, and this increase was significantly suppressed by the administration of PC-SOD (3 kU/kg) but not U-SOD (3 kU/kg) (Fig. 1A). DSS-induced colon shortening, used as a morphometric measure for the degree of inflammation, was significantly ameliorated

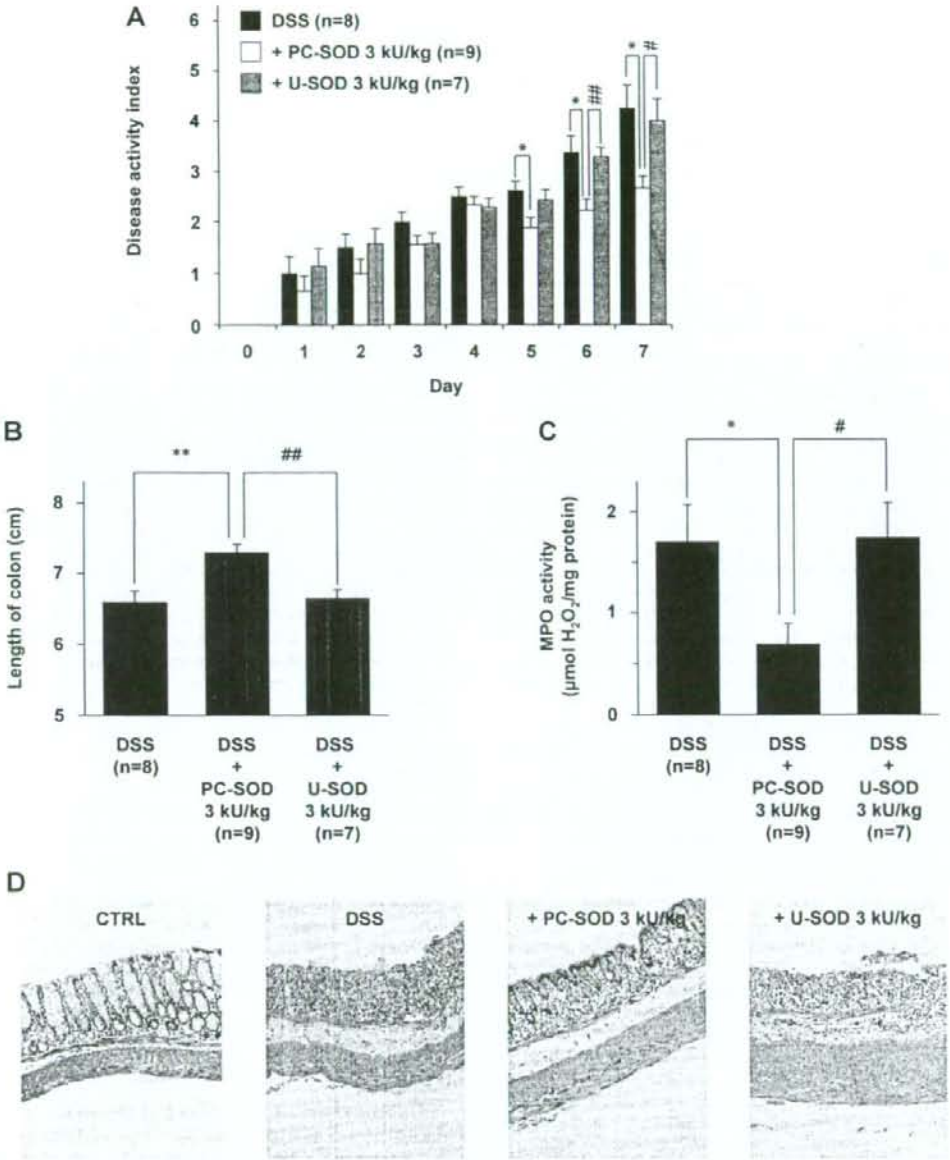

Fig. 1. Effect of PC-SOD and U-SOD on development of DSS-induced colitis. Mice treated with 4% DSS for 7 days, as described under *Materials and Methods*, were intravenously administered PC-SOD or U-SOD once daily. DAI was measured daily (A). The length of the colon (B) and colonic MPO activity (C) were determined at the end of the experimental period. Sections of colonic tissue were also prepared and subjected to histological examination by hematoxylin and eosin staining (D). CTRL, control without DSS treatment. Values are mean ± S.E.M. * or #, $P < 0.05$; ** or ##, $P < 0.01$.

in the PC-SOD-treated animals (Fig. 1B), as was colonic MPO activity, an indicator of leukocyte infiltration (Fig. 1C). Figure 1D shows the results of histological analyses of colonic tissues. Crypt loss and infiltration of leukocytes were observed in DSS-treated mice, and these phenotypes were improved by administration of PC-SOD and, to a lesser extent, U-SOD (Fig. 1D). Taken together, these findings demonstrate that PC-SOD is more effective than U-SOD for the amelioration of DSS-induced colitis.

To compare the specific activity of PC-SOD and U-SOD, we determined their dose-response profiles. As shown in Fig. 2A, PC-SOD produced the maximum beneficial effect at 1.5 to 3 kU/kg, whereas higher doses (6–12 kU/kg) had no significant effect on DAI. A similar bell-shaped profile has also been reported in a rat model of DSS-induced colitis (Hori et al., 1997). In the case of colon shortening and colonic MPO activation, the maximal effect was again observed in response to 1.5 to 3 kU/kg PC-SOD (Fig. 2, B and C). In contrast, U-SOD at the much higher concentration of 48 kU/kg only ameliorated DSS-induced colitis to a similar extent to that obtained

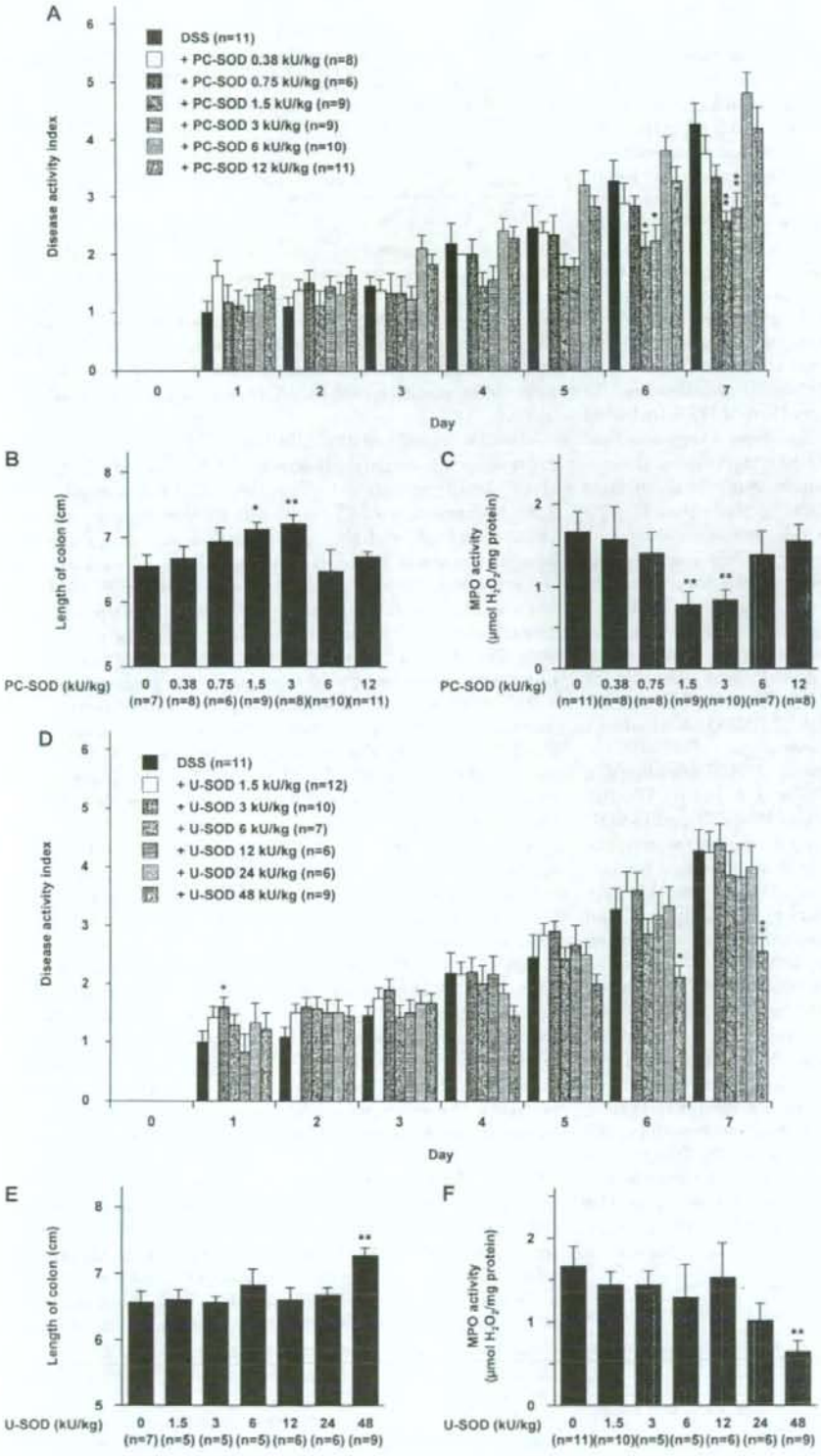

Fig. 2. Dose-response profile of effect of PC-SOD and U-SOD on development of DSS-induced colitis. Mice were treated with DSS and PC-SOD (A–C) or U-SOD (D–F), and colitis was assessed as described in the legend of Fig. 1. Values are mean ± S.E.M. *, $P < 0.05$; **, $P < 0.01$ (versus without PC-SOD or U-SOD).

with 1.5 kU/kg PC-SOD (Fig. 2, D–F), indicating that the specific activity of PC-SOD is more than 30 times that of U-SOD. We next used an ELISA assay to compare the level of PC-SOD and U-SOD in serum and colonic tissues after their intravenous administration. As shown in Table 1, after daily intravenous administration for 7 days and 6 h after the final injection, PC-SOD was detected in serum and colonic tissues at a concentration of 3.3 μg/ml and 0.23 ng/mg, respectively. However, U-SOD was not detected in either preparation, suggesting that the enhanced activity of PC-SOD is partly because of its greater stability in vivo.

We also examined the effect of DSS-treatment on the level of PC-SOD after intravenous administration. As shown in Table 2, DSS treatment significantly increased the level of PC-SOD in colonic tissues but not so clearly in serum, suggesting that PC-SOD can be enriched in damaged or inflamed tissues, which is an advantage for its clinical application for UC.

Involvement of ROS in Amelioration of DSS-Induced Colitis by PC-SOD. Previous studies have suggested that PC-SOD is more potent than U-SOD for decreasing the superoxide anion released from activated neutrophils in vitro by showing that PC-SOD showed higher activity than U-SOD for the suppression of endothelial cell damage induced by activated neutrophils and that more PC-SOD than U-SOD remains on the surface of neutrophils after washing (Igarashi et al., 1994). In this study, we investigated this directly by measuring the superoxide anion by ESR. As shown in Fig. 3A, a radical spin adduct of the ESR spectrum corresponding to superoxide anion (DMPO-OOH adduct) was observed for PMA-activated human neutrophils. Preincubation of neutrophils with PC-SOD lowered the peak of DMPO-OOH adduct in a dose-dependent manner, showing that PC-SOD decreases the level of superoxide anion. U-SOD produced a similar, but less pronounced, effect (Fig. 3, A and B). We also quantitatively measured the activity of PC-SOD and U-SOD based on CL analysis. An increase in CL, in other words, the amount of superoxide anion released from activated neutrophils, decreased in the presence of PC-SOD and U-SOD. Again, PC-SOD was more potent than U-SOD (Fig. 3, C and D), perhaps because of its higher cell membrane affinity, as previously reported (Igarashi et al., 1994).

Although it is generally believed that administration of PC-SOD decreases the level of ROS in vivo, no direct evidence in support of this idea has been reported. In this study, we examined the effect of PC-SOD administration on the intestinal level of ROS measuring the lipid-derived free radical spin adduct with ESR spectroscopy and spin trap POBN, which reacts with ROS to form a radical spin adduct. We have reported recently that this method, in vivo free radical production and ex vivo detection, is effective for monitoring ROS level in the intestine (T. Namba, unpublished data). The hyperfine $\alpha^N = 14.92 \pm 0.06$ G and $\alpha_\beta^H = 2.44 \pm 0.05$ G, which are similar to previous data (Namba et al., unpublished data). Therefore, our detected radical was determined as a lipid-derived free radical. As shown in Fig. 4, A and B, the intestinal level of ROS (the height of the ESR peak shown by the bar) was increased by DSS administration, an effect that was suppressed by the administration of PC-SOD. This is the first direct evidence that PC-SOD decreases the level of ROS in vivo.

TABLE 1

Serum and colonic levels of PC-SOD and U-SOD

Mice were intravenously administered the indicated dose of PC-SOD or U-SOD once daily for 7 days. They were also treated with DSS, as described in the legend of Fig. 1. Blood and colonic tissues were taken 6 h after the final administration. The levels of PC-SOD or U-SOD in the samples were determined by ELISA. Values are mean ± S.E.M.

	DSS + PC-SOD (3 kU/kg)	DSS + U-SOD (3 kU/kg)
Serum (μg/ml)	3.3 ± 0.42	<0.063
Tissue (ng/mg)	0.23 ± 0.038	<0.013

TABLE 2

Serum and colonic levels of PC-SOD

Mice were intravenously administered the indicated dose of PC-SOD once daily for 7 days. They were also treated with or without DSS, as described in the legend of Fig. 1. Blood and colonic tissues were taken 6 h after the final administration. The levels of PC-SOD in the samples were determined by ELISA. Values are mean ± S.E.M.

	PC-SOD (3 kU/kg)	DSS + PC-SOD (3 kU/kg)
Serum (μg/ml)	3.5 ± 0.21	4.0 ± 0.61
Tissue (ng/mg)	0.066 ± 0.029	0.24 ± 0.043*

* $P < 0.05$.

We also examined the effect of PC-SOD on the mRNA expression of various cytokines (TNF-α, IL-1β, IL-6, and IL-23) in the intestine by real-time RT-PCR analysis. The mRNA expression of *Tnf*-α and *Il-1*β was up-regulated by DSS treatment, and this up-regulation was suppressed by administration of PC-SOD (3 kU/kg) (Figs. 4C and 5E). On the other hand, administration of either DSS or PC-SOD did not affect the mRNA expression of *Il-6* and *Il-23p19* (Fig. 4C). The effect of PC-SOD on mRNA expression of *Tnf*-α and *Il-1*β was also examined in vitro. Treatment of peritoneal macrophages prepared from wild-type mice with LPS induced the mRNA expression of *Tnf*-α and *Il-1*β, and this induction was partially suppressed by simultaneous treatment of cells with PC-SOD (Fig. 4D). These results suggest that PC-SOD suppresses the expression of TNF-α and IL-1β by lowering the intestinal level of ROS, resulting in the amelioration of DSS-induced colitis.

As described in the introduction, a bell-shaped dose-response profile has been observed for various pharmacological activities of PC-SOD (Hori et al., 1997; Tamagawa et al., 2000; Tsubokawa et al., 2007). One possible explanation for the ineffectiveness of high doses of PC-SOD is the accumulation of hydrogen peroxide because of the relatively higher activity of SOD compared with catalase (Mao et al., 1993); however, this idea has not been proved. In this study, we tested this idea by examining the effect of simultaneous administration of catalase; if the ineffectiveness of higher doses of PC-SOD is because of the accumulation of hydrogen peroxide, the effect would be restored by simultaneous administration of catalase that detoxifies hydrogen peroxide into oxygen and water. Administration of a high dose of PC-SOD (6 kU/kg) improved the DAI score in DSS-treated mice in the presence of simultaneous intravenous administration of catalase but not in its absence (Fig. 5A). Administration of catalase alone tended to improve the DAI, but this effect was not statistically significant (Fig. 5A). Similar results were observed for DSS-induced colon shortening and colonic MPO activation (Fig. 5, B and C). These findings suggest that the ineffectiveness of high doses of PC-SOD on DSS-induced colitis is caused by accumulation of hydrogen peroxide.

We further tested this idea by direct measurement of the colonic level of hydrogen peroxide. As shown in Fig. 5D, DSS treatment increased the colonic level of hydrogen peroxide,

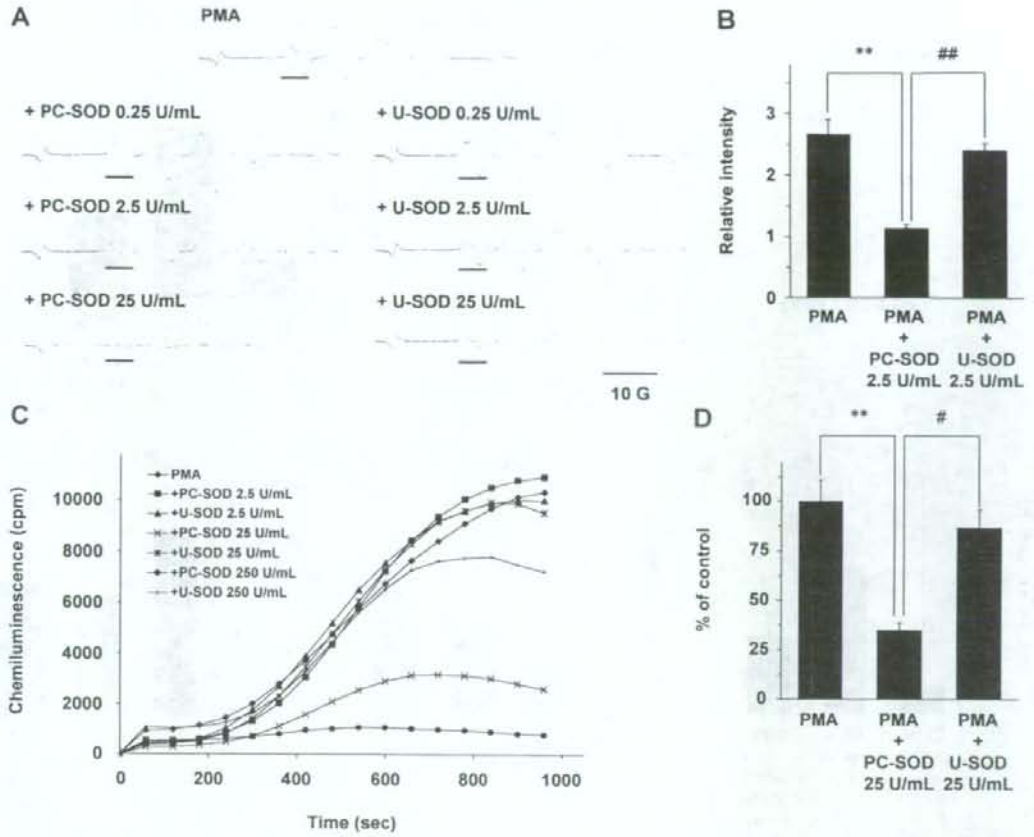

Fig. 3. Effect of PC-SOD or U-SOD on the amount of superoxide anion in vitro. Human neutrophils were preincubated with PC-SOD or U-SOD for 1 h and washed with medium. Neutrophils were then activated with PMA, and the amount of superoxide anion was measured by ESR (A and B) or CL analysis (C and D). The intensity of the ESR signal of the superoxide anion adduct (DMPO-OOH adduct shown by the bar in A) was determined (B). The area under the graph (C) was also determined and is presented as a relative measure in D. Values are given as the mean ± S.E.M. ($n = 3$). #, $P < 0.05$; ** or ##, $P < 0.01$.

and administration of a low dose (3 kU/kg) of PC-SOD suppressed this effect. However, in the case of a high dose of PC-SOD (12 kU/kg), a significant effect was only observed in the presence of simultaneous administration of catalase (Fig. 5D), which alone did not significantly suppress the increase (Fig. 5D). Similar, but less pronounced, results were obtained with 6 kU/kg PC-SOD as compared with a 12 kU/kg dose (data not shown).

We also examined the effect of catalase on the mRNA expression of *Tnf*-α in the presence of a high dose of PC-SOD. The up-regulated of the mRNA expression of *Tnf*-α by DSS treatment was not suppressed by administration of a high (6 kU/kg) dose of PC-SOD (Fig. 5E). However, simultaneous administration of catalase with the high dose of PC-SOD significantly suppressed the DSS-induced mRNA expression of *Tnf*-α (Fig. 5E). We also determined the serum level of TNF-α and found that the level was increased by DSS treatment, and this increase was suppressed by administration of a low (3 kU/kg) dose of PC-SOD and a high (6 kU/kg) dose of PC-SOD with catalase (Fig. 5F). Being different from the case of mRNA expression in the intestine (Fig. 5E), the serum level of TNF-α was decreased by administration of catalase alone. These results suggest that TNF-α plays an important role in the ameliorative effect of PC-SOD against DSS-induced colitis.

Immunohistochemical analysis with antibody against the phosphorylated form of NF-κB p65 at Ser536 (active form of NF-κB p65) demonstrated that DSS administration increases the level of active NF-κB in the colonic mucosa (Fig. 5G), suggesting that the inflammatory response occurs in epithelial cells and infiltrated leukocytes. Again, this increase in the level of active NF-κB expression was suppressed by administration of a low (3 kU/kg) dose of PC-SOD and a high (6 kU/kg) dose of PC-SOD with catalase.

Effect of Modified Methods of PC-SOD Administration. To obtain some useful clues for refining the clinical guidelines for administration of PC-SOD, we tested the outcome of other protocols and routes of administration in the treatment of DSS-induced colitis. As illustrated in Fig. 6A, we first intravenously administered PC-SOD once at the start of DSS treatment (day 0), then monitored the DAI for 7 days. Although the dose found to be effective with daily administration (3 kU/kg) did not improve the DAI score, a higher dose (6–24 kU/kg) produced a significant improve-

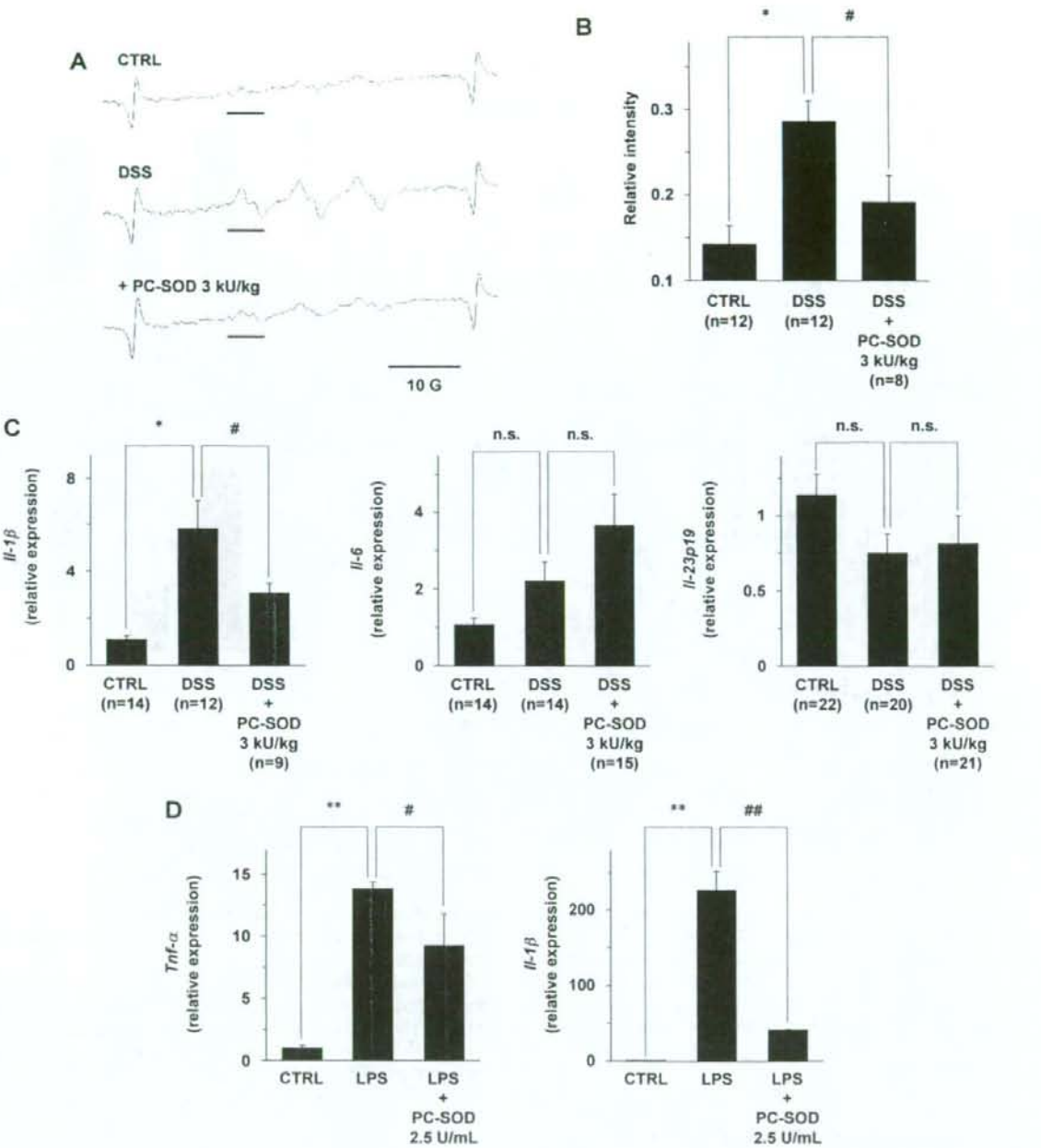

Fig. 4. Effect of PC-SOD on the level of ROS and expression of cytokines. DSS and PC-SOD were administered to mice, as described in the legend of Fig. 1 (A–C). After 7 days, POBN was administered and the colons were dissected and subjected to radical adduct ESR spectrum analysis (A). The intensity of the ESR signal (shown by the bar in A) was determined (B). Samples of colonic mRNA were subjected to real-time RT-PCR, using a specific primer set for *Il-1β*, *Il-6*, and *Il-23p19*. Values were normalized to *Gapdh*, expressed relative to the control sample (i.e., without DSS treatment) (C). Mouse peritoneal macrophages were preincubated with or without indicated concentration of PC-SOD for 1 h and further treated with LPS (1 μg/ml) in the presence of same concentration of PC-SOD as in the preincubation step for 3 h. The mRNA fractions were prepared and analyzed by real-time RT-PCR as described above (D). Values are given as the mean ± S.E.M. ($n = 3$) (B and C) or S.D. (D). * or #, $P < 0.05$; ** or ##, $P < 0.01$; n.s., not significant; CTRL, control.

ment (Fig. 6A). However, higher doses of 48 to 96 kU/kg worsened the DAI during the early stage of colitis development (Fig. 6A). The effectiveness of a one-shot administration of PC-SOD (12 – 24 kU/kg) was also shown by measuring colon shortening and colonic MPO activation (Fig. 6, B and C). The findings suggest that intermittent (for example, once weekly) administration of PC-SOD, a regime that greatly improves the QOL of UC patients, is a viable clinical protocol.

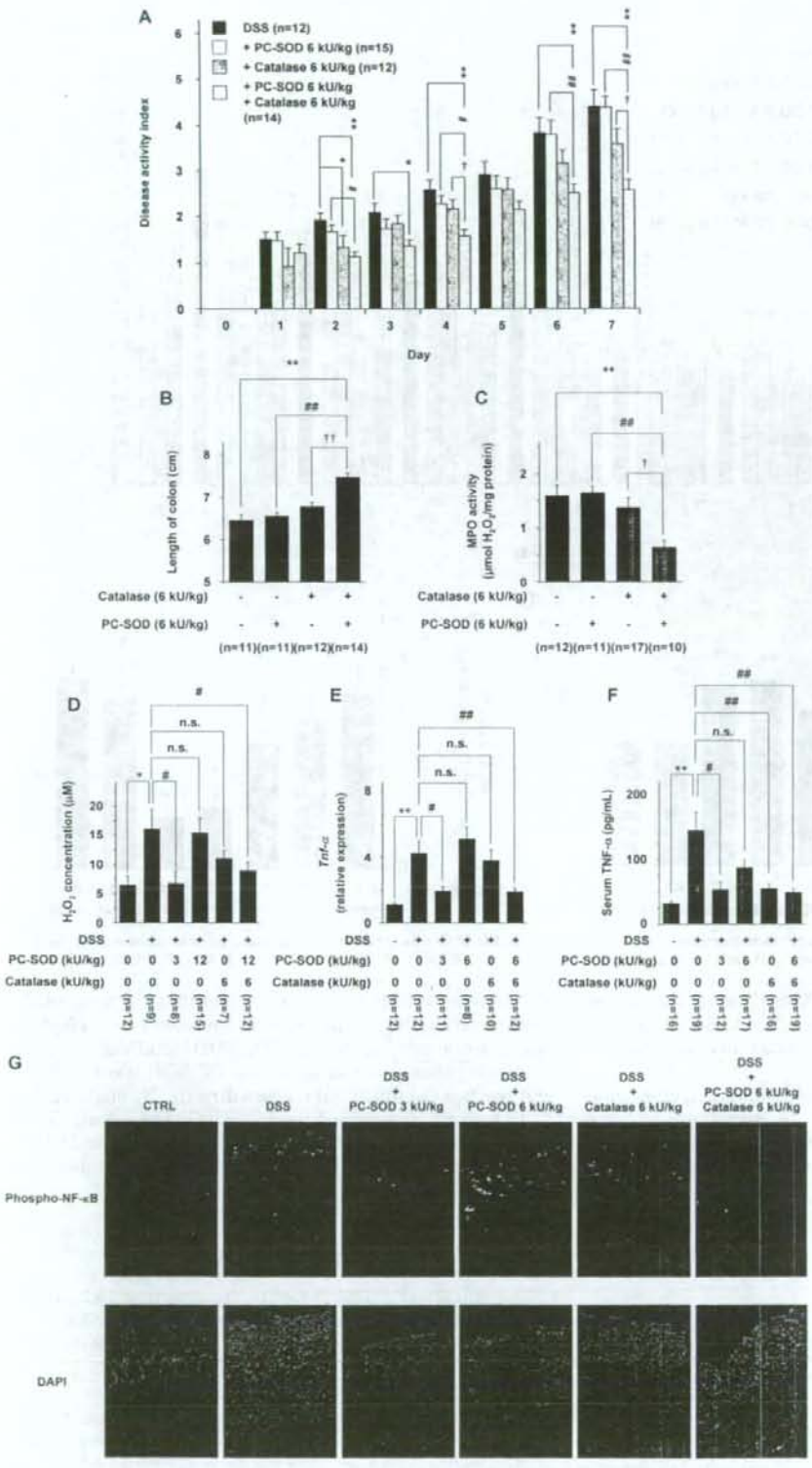

Fig. 5. The effect of simultaneous administration of catalase on the ameliorative effect of PC-SOD on DSS-induced colitis, colonic level of hydrogen peroxide, TNF-α, and NF-κB. Mice were treated with DSS and PC-SOD (A–G), and colitis was assessed (A–C), as described in the legend of Fig. 1. Catalase was intravenously administered once daily (A–G). Colonic tissues were removed and the amount of hydrogen peroxide was determined (D). Samples of colonic mRNA were subjected to real-time RT-PCR as described in the legend of Fig. 4 (E). The serum levels of TNF-α were determined by ELISA (F). Sections of intestinal tissues were prepared and subjected to immunohistochemical analysis with an antibody against phospho-NF-κB p65 and DAPI staining as described under *Materials and Methods* (G). Values are mean ± S.E.M. *, #, or †, $P < 0.05$; **, ##, or ††, $P < 0.01$; n.s., not significant.