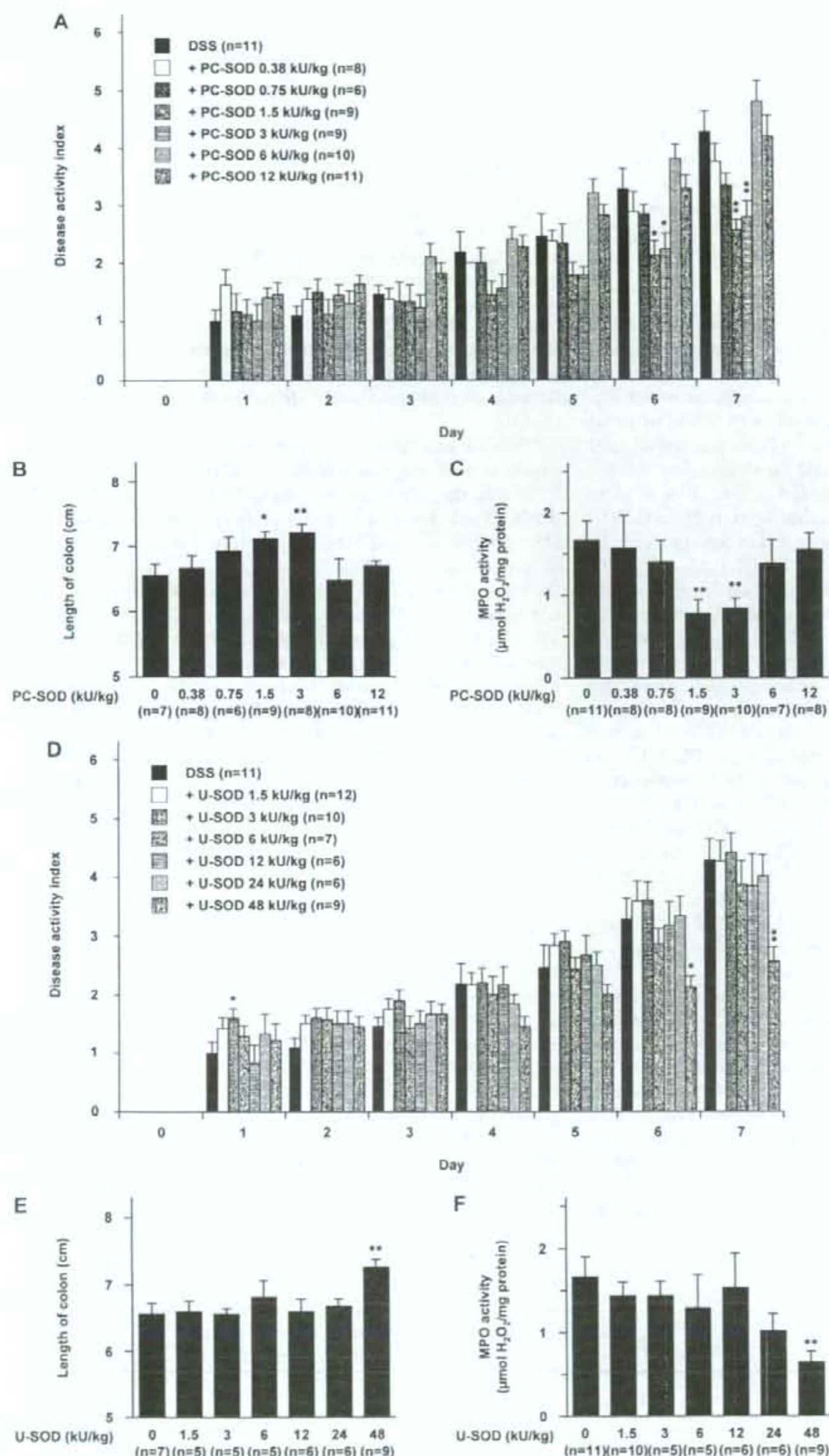

Fig. 2. Dose-response profile of effect of PC-SOD and U-SOD on development of DSS-induced colitis. Mice were treated with DSS and PC-SOD (A–C) or U-SOD (D–F), and colitis was assessed as described in the legend of Fig. 1. Values are mean ± S.E.M. *, $P < 0.05$; **, $P < 0.01$ (versus without PC-SOD or 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 $\alpha^{H}_{\beta} = 2.44 \pm 0.05$ G, which

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*

* $P < 0.05$.

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.

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-1β* 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-1β* 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-1β*, 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,

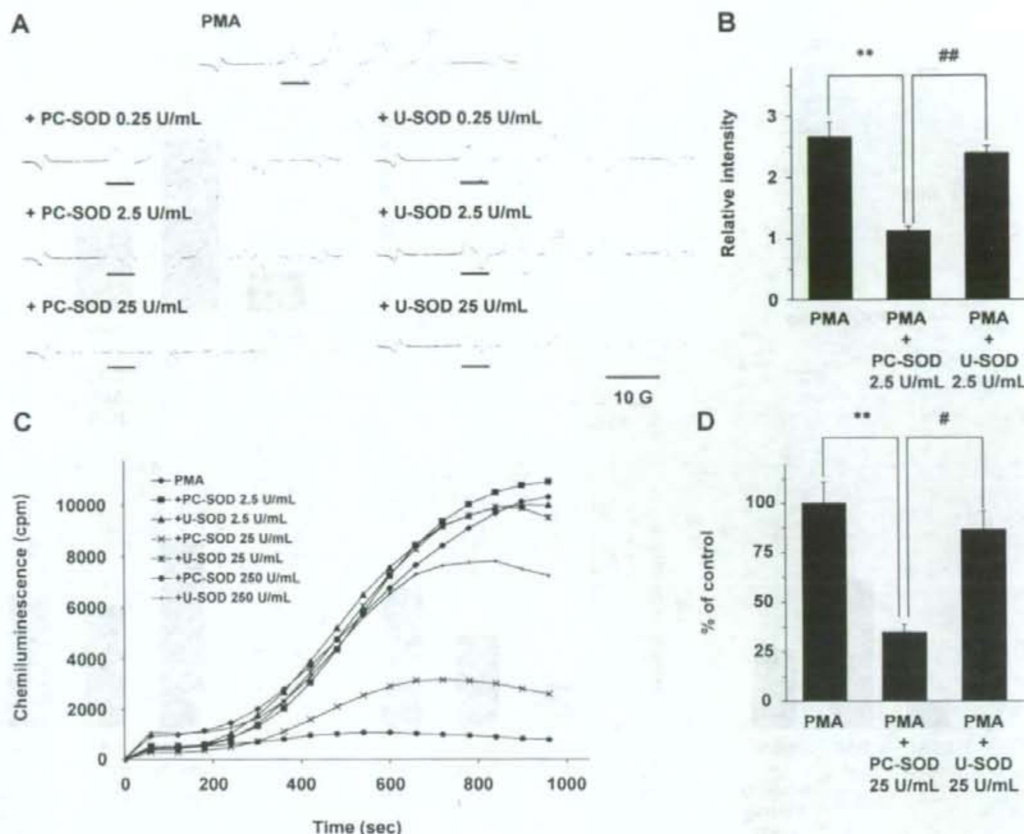

Fig. 3. Effect of PC-SOD or U-SOD on the amount of superoxide anion in vitro. Human neutrophils were preincubated with PC-SOD or U-SOD for 1 h and washed with medium. Neutrophils were then activated with PMA, and the amount of superoxide anion was measured by ESR (A and B) or CL analysis (C and D). The 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-

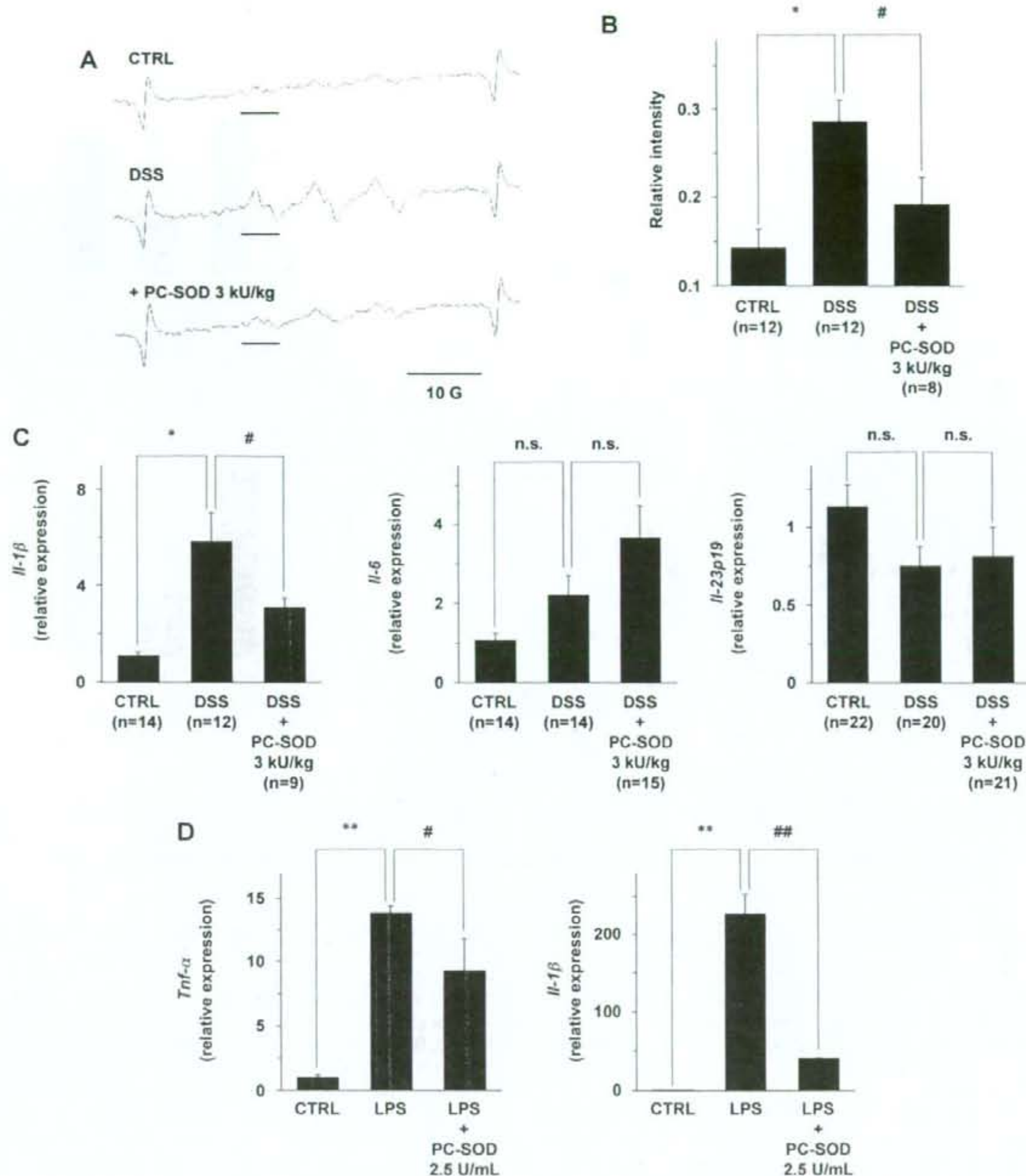

Fig. 4. Effect of PC-SOD on the level of ROS and expression of cytokines. DSS and PC-SOD were administered to mice, as described in the legend of Fig. 1 (A–C). After 7 days, POBN was administered and the colons were dissected and subjected to radical adduct ESR spectrum analysis (A). The intensity of 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.

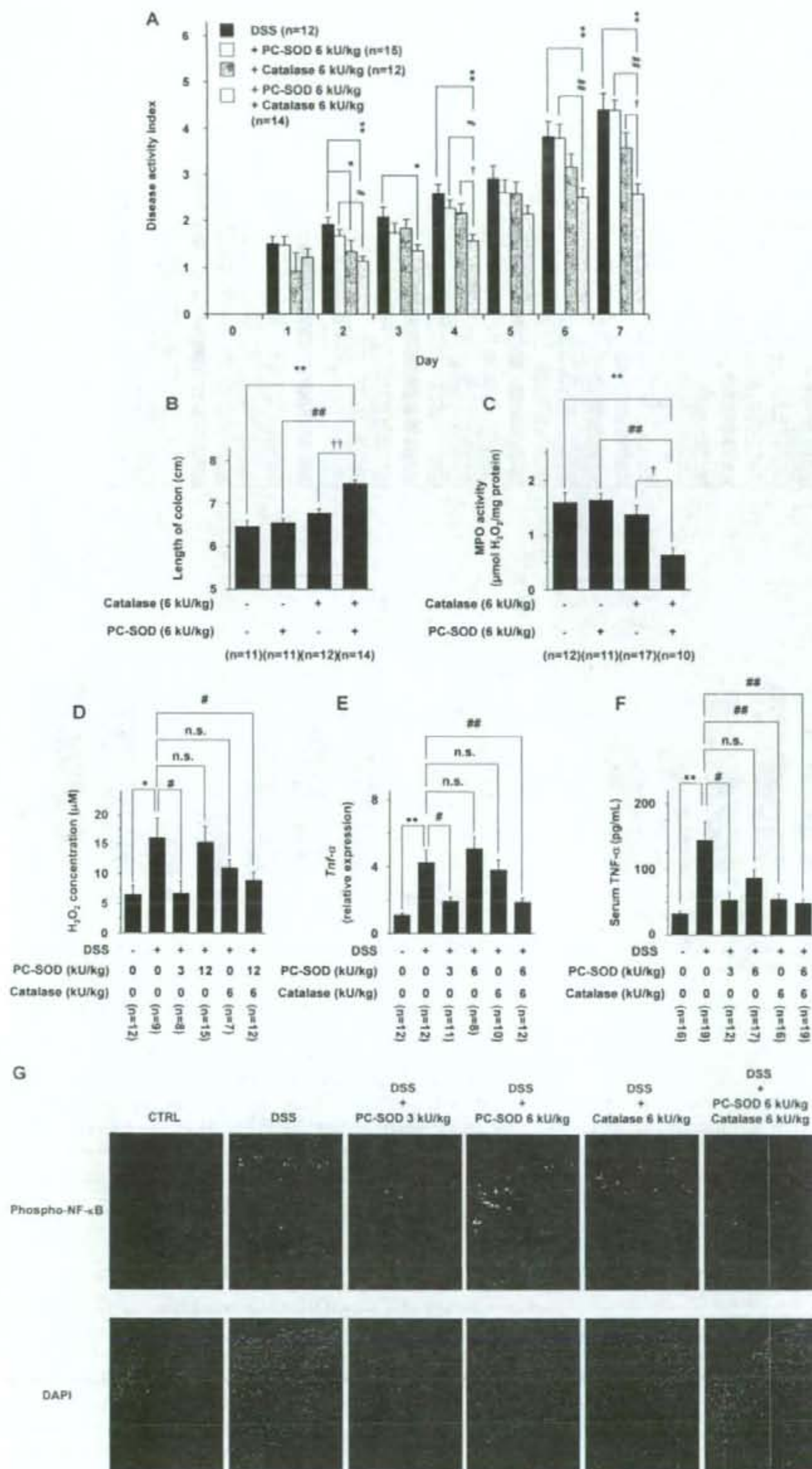

Fig. 5. The effect of simultaneous administration of catalase on the ameliorative effect of PC-SOD on DSS-induced colitis, colonic level of hydrogen peroxide, TNF-α, and NF-κB. Mice were treated with DSS and PC-SOD (A–G), and colitis was assessed (A–C), as described in the legend of Fig. 1. Catalase 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.

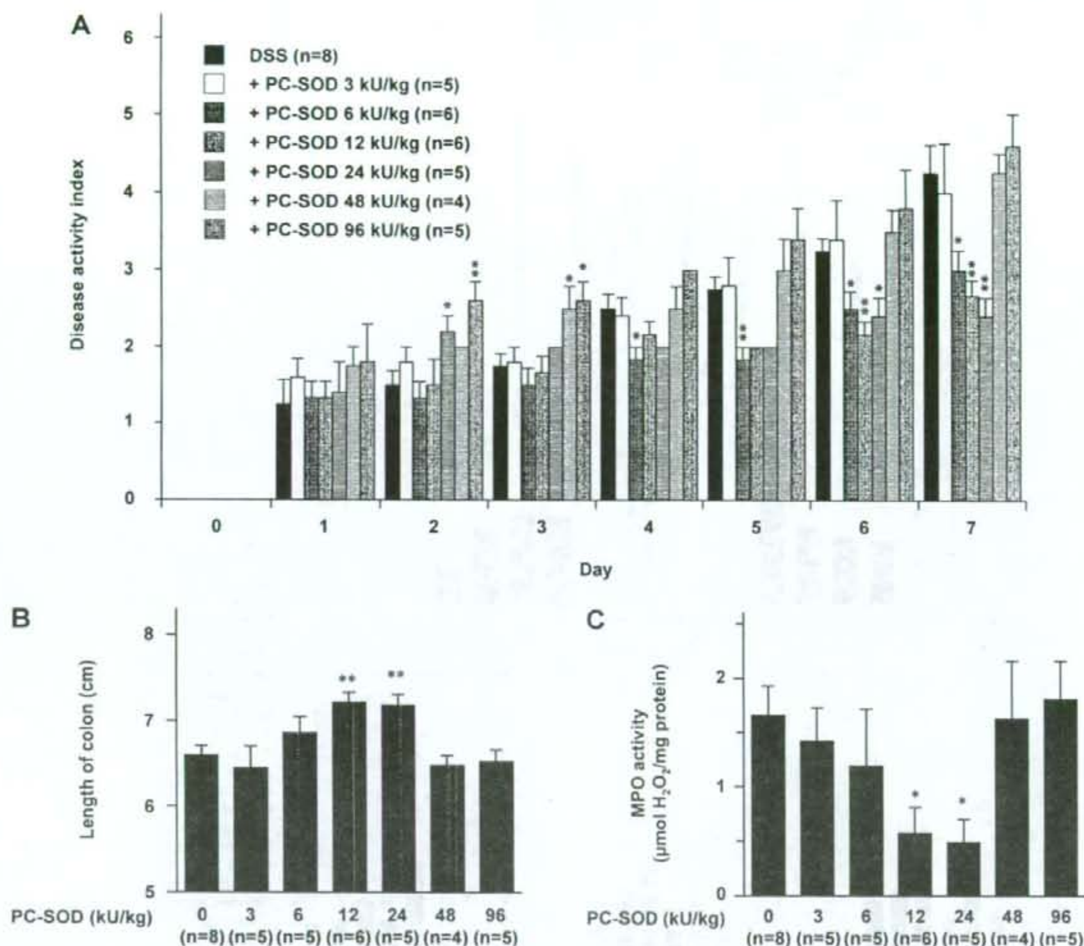

Fig. 6. Effect of a single dose of PC-SOD on the development of DSS-induced colitis. PC-SOD was intravenously administered to mice once at the start of DSS treatment (day 0). Colitis was subsequently assessed, as described in the legend of Fig. 1. Values are mean ± S.E.M. *, $P < 0.05$; **, $P < 0.01$.

When we monitored the level of PC-SOD after this single-dose administration (12 kU/kg), we found that it dropped below detectable limits 72 (in serum) or 24 (in colonic tissues) h after the injection (Table 3).

We also examined the effect of oral administration (once daily) of PC-SOD. As shown in Fig. 7A, significant improvement in the DAI score was observed at most of the doses tested. The ameliorative effect of oral administration of PC-SOD was also observed in terms of colon shortening and colonic MPO activation (Fig. 7, B and C). This suggests that oral administration of PC-SOD, a regime that greatly improves the QOL of UC patients, is a viable clinical protocol and is also clinically viable. We found that the level of PC-SOD in serum did not increase at any time points (6–48 h) after the oral administration of PC-SOD (48 kU/kg) (Table 4), suggesting that orally administered PC-SOD is not absorbed and reaches the intestinal mucosa directly. By employing an ELISA assay, we detected the PC-SOD in the colonic tissues 24 h after its oral administration (48 kU/kg) (Table 4). However, low doses (0.75–1.5 kU/kg) proved undetectable (data not shown).

TABLE 3

Serum and colonic levels of PC-SOD

Mice were intravenously administered 12 kU/kg PC-SOD once on day 0 and treated with DSS for 3 days. Blood and colonic tissue samples were taken periodically. The levels of PC-SOD in the samples were determined by ELISA. Values are mean ± S.E.M.

	15 min	24 h	48 h	72 h
Serum (μg/ml)	170 ± 5.7	2.3 ± 0.67	0.41 ± 0.12	<0.16
Tissue (ng/mg)	1.6 ± 0.69	<0.031	<0.031	<0.031

Discussion

The efficacy of PC-SOD for the treatment of UC patients has already been demonstrated by a phase II clinical study (Suzuki et al., 2008b). However, the mechanism of its action is not fully understood. Given that determining the underlying mechanism is important to advance the further development of this drug, in the present study, we examined the action of PC-SOD in an animal model of UC, DSS-induced colitis. Furthermore, because the current clinical protocol for

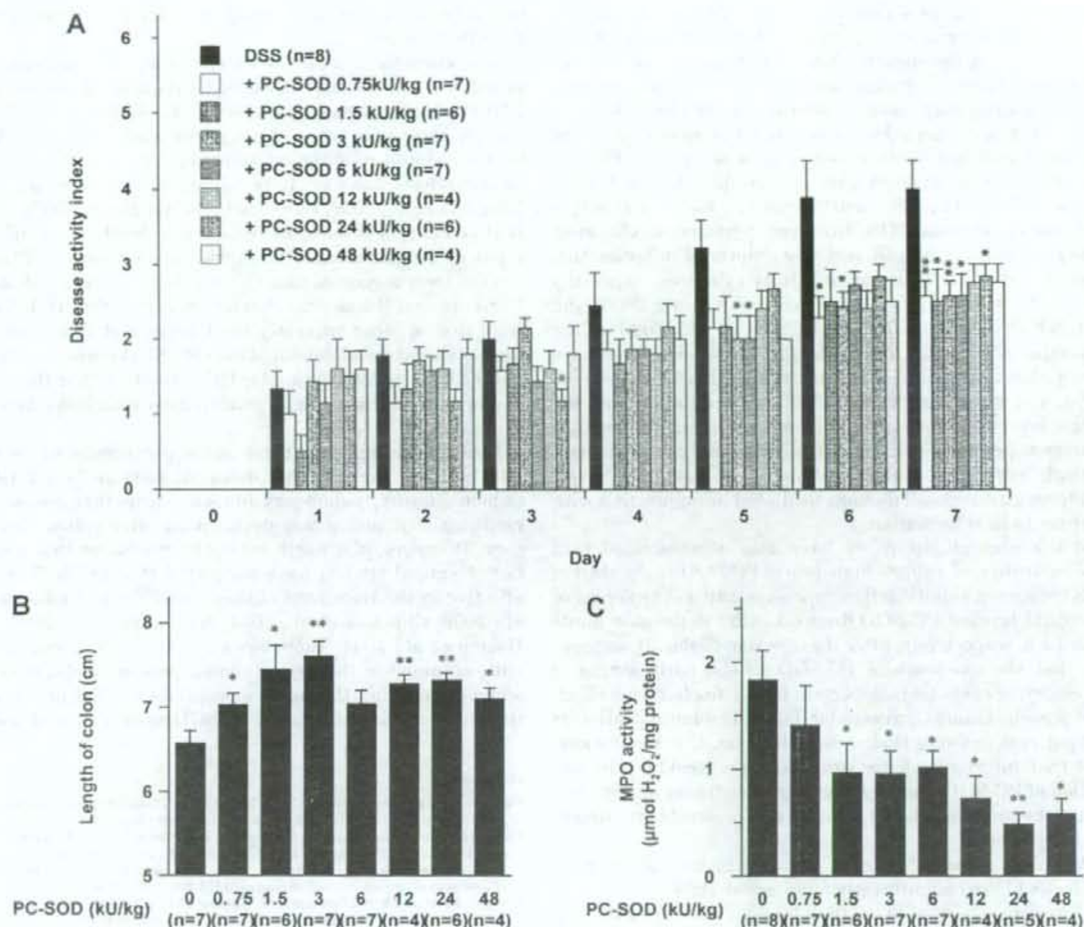

Fig. 7. Effect of oral administration of PC-SOD on development of DSS-induced colitis. PC-SOD was orally administered to mice once daily. Colitis was assessed, as described in the legend of Fig. 1. Values are mean ± S.E.M. *, $P < 0.05$; **, $P < 0.01$.

TABLE 4

Serum and colonic levels of PC-SOD

Mice were orally administered 48 kU/kg PC-SOD once and treated with DSS for 2 days. Blood and colonic tissue samples were taken periodically. The levels of PC-SOD in the samples were determined by ELISA. Values are mean ± S.E.M.

	6 h	12 h	24 h	48 h
Serum (μg/ml)	<0.16	<0.16	<0.16	<0.16
Tissue (ng/mg)	<0.031	<0.031	0.46 ± 0.24	<0.031

the administration of PC-SOD (once daily intravenous infusion for 4 weeks) does not provide patients with good QOL, we also tested other dosing regimes in our animal model.

The superior character of PC-SOD to U-SOD has been shown both in vitro (high cell membrane affinity) and in vivo (high stability in plasma). However, a direct comparison of their pharmacological activity has not been reported. In this study, we have demonstrated that the ameliorative effect of PC-SOD against DSS-induced colitis is more than 30 times higher than that of U-SOD. The higher stability in serum (Table 1) and higher activity for decreasing superoxide anion (Fig. 3) of PC-SOD could contribute to this effect. Analysis of intestinal ROS level in vivo is difficult; thus, the decrease in the level of ROS by PC-SOD has not been directly shown. In this study, we have demonstrated this by use of radical spin adduct ESR spectrum analysis. This analysis should also be useful for detecting the alteration in the intestinal level of ROS associated with various other diseases and for evaluation of drugs for the treatment of such conditions.

The bell-shaped dose-response profile of PC-SOD is of clinical concern because this may reflect side effects of the drug. In this study, we have revealed that the efficacy of higher doses of PC-SOD is restored by simultaneous administration of catalase that converts hydrogen peroxide to water and oxygen. Furthermore, we have directly determined the colonic level of hydrogen peroxide and found that low doses of PC-SOD suppress the DSS-induced increase in the intestinal level of hydrogen peroxide and that simultaneous administration of catalase with high doses of PC-SOD but not the PC-SOD alone produces a similar effect. These results suggest that the ineffectiveness of high doses of PC-SOD on DSS-induced colitis is caused by accumulation of hydrogen peroxide. Although catalase is abundant, recent studies have

suggested that its activity fluctuates during the development of colitis (Kruidenier et al., 2003b,c; Mahgoub et al., 2003). This may affect the clinical efficacy of PC-SOD, and individual examination of catalase activity before the administration of the drug may result in safer and more effective treatment. Hydrogen peroxide is not itself a major cause of ROS-mediated cell damage, but it does react with Fe^{2+} to produce a potent hydroxy radical according to the Fenton reaction (Mao et al., 1993). Furthermore, it has been reported that among various ROS, hydrogen peroxide is the most potent activator of NF-κB, a strong inducer of inflammation through induction of proinflammatory cytokines, especially TNF-α (Schmidt et al., 1995; Marikovsky et al., 2003) and that NF-κB plays an important role in intestinal colitis (Schreiber et al., 1998; Herfarth et al., 2000). Here, we have shown that activation of NF-κB, the mRNA expression of *Tnf-α*, and the serum level of TNF-α are induced when the colonic level of hydrogen peroxide increases, suggesting that hydrogen peroxide damages the intestinal mucosa both through induction of inflammation via activation of NF-κB and through direct cell damage mediated in conjunction with hydroxy radical formation.

In the present study, we have also demonstrated that administration of a single high dose of PC-SOD at the start of DSS treatment significantly suppresses colitis. The serum or intestinal level of PC-SOD dropped below detectable limits 72 or 24 h, respectively, after the injection (Table 3), suggesting that the existence of PC-SOD in the early stages of development of colitis is important for its ameliorative effect. The present clinical protocol for PC-SOD administration to UC patients enforces their hospitalization. Our results suggest that intermittent (for example, once weekly) administration of PC-SOD, thereby allowing ambulatory administration of the drug, may be an effective and preferable treatment for UC patients.

We also found that oral administration of PC-SOD (0.75–48 kU/kg) significantly suppressed DSS-induced colitis. The serum level of PC-SOD did not increase after the oral administration, suggesting that orally administered PC-SOD is not absorbed but reaches the colonic mucosa directly. However, based on ELISA assay, we only detected PC-SOD in colonic tissues after administration of the highest dose tested (48 kU/kg), the tissue level being similar to that obtained after intravenous administration of a 3 kU/kg dose (Table 1). Thus, it seems that a very small amount of PC-SOD (under the limit of detection) is effective when administered orally. The distribution of PC-SOD at the intestine may differ depending on the route of administration, and this may contribute to the higher specific activity of PC-SOD after oral treatment. The fact that a bell-shaped dose-response profile was not observed upon oral administration of PC-SOD (Fig. 7) supports this idea. On the other hand, it is also possible that PC-SOD is modified at the gastric and intestinal mucosa in such a way that it is not recognized by the ELISA assay, although its activity is maintained. If oral administration of PC-SOD is applied clinically, it should greatly improve the QOL of patients treated with the drug. We used human SOD, not mouse protein, for the examination of the effect of oral administration of PC-SOD on DSS-induced colitis in mice. Human SOD may modify the mucosal immunological reaction in mice, and this modification may stimulate the beneficial effect of PC-SOD against colitis in mice. In this case, oral administration of PC-SOD may not give beneficial effects for human.

Glucocorticoids, 5-aminosalicylic acid, and immunosuppressive drugs are currently used for the clinical treatment of IBD (Podolsky, 2002; Baumgart and Sandborn, 2007). Although some new types of drugs, such as infliximab, have been developed recently, a number of clinical problems, such as side effects, have yet to be addressed (Keane et al., 2001; Bongartz et al., 2006; Baumgart and Sandborn, 2007). Thus, IBD remains a disease for which the development of new types of drugs remains therapeutically important. Clinical studies have suggested that PC-SOD is a relatively safe drug. Furthermore, it has a mechanism of action that is different from that of other currently used drugs and those that are under clinical development. Thus, PC-SOD remains a therapeutically beneficial option for UC patients, either through a single administration or in combination with other types of drugs.

ROS play an important role in the progression of not only IBD but also various other diseases, such as focal cerebral ischemic injury, pulmonary fibrosis, chemotherapy-induced cardiotoxicity, and motor dysfunction after spinal cord injury. Therefore, it is worth noting in conclusion that a number of animal studies have suggested that PC-SOD is also effective in the treatment of these conditions (Tamagawa et al., 2000; Chikawa et al., 2001; Nakajima et al., 2001; den Hartog et al., 2004; Tsubokawa et al., 2007). Thus, the results obtained in this paper should provide useful evidence when considering the most appropriate clinical protocol for therapeutic administration of PC-SOD against these diseases.

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Scandinavian Journal of Gastroenterology
2008, 1–9, iFirst article

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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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(Received 10 December 2007; accepted 15 June 2008)

ISSN 0036-5521 print/ISSN 1502-7708 online © 2008 Informa UK Ltd.
DOI: 10.1080/00365520802273017

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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.

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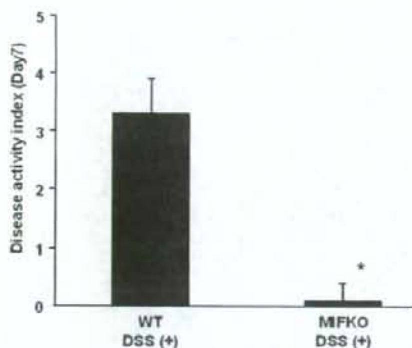

Figure 1. Scores of disease activity index (DAI) in dextran sulfate sodium (DSS)-induced colitis in mice ($n = 10$, each group). The DAI scores in macrophage migration inhibitory factor (MIF)-deficient mice treated with 3% DSS solution for 7 days were significantly lower than those in wild-type (WT)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

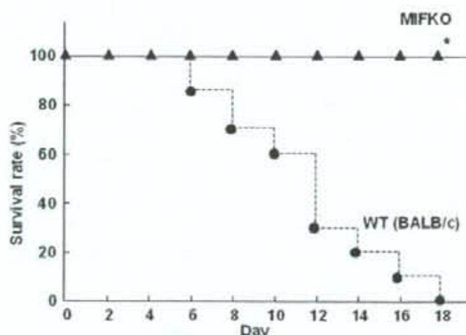

Figure 2. Survival rate of mice in dextran sulfate sodium (DSS)-induced colitis. High-dose DSS (10%) was provided *ad libitum*, and the survival rate was assessed every 24 h. Up to 18 days post-DSS stimulation, there were no deaths among migration inhibitory factor (MIF)-deficient mice ($n = 10$), whereas 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.

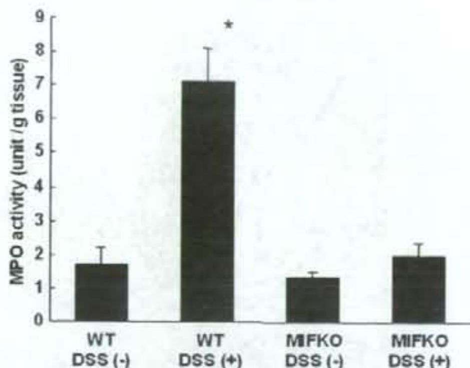

Figure 3. Myeloperoxidase (MPO) activity in the colon of mice exposed to 3% dextran sulfate sodium (DSS) for 7 days (each group; $n = 6$). Wild-type (WT) mice treated with DSS significantly exhibited higher levels of MPO activity compared with non-treated WT mice, non-treated migration inhibitory factor (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.

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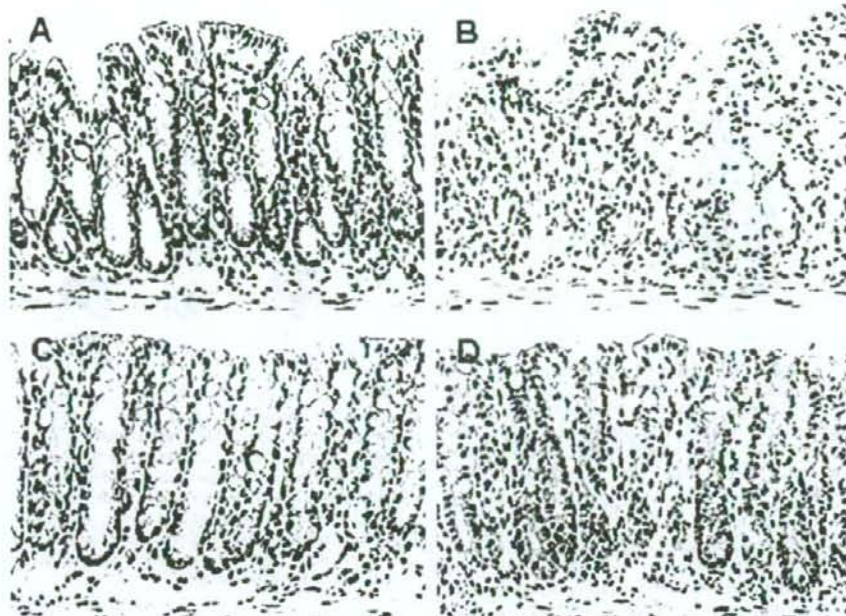

Figure 4. Immunohistochemical analysis for F4/80 in the colon. F4/80-positive staining was seen mainly in the mononuclear cells infiltrating the colonic mucosa. A. Non-treated wild-type (WT) mice. B. WT mice treated with 3% dextran sulfate sodium (DSS) for 7 days. C. Migration inhibitory factor (MIF)-deficient 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.

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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

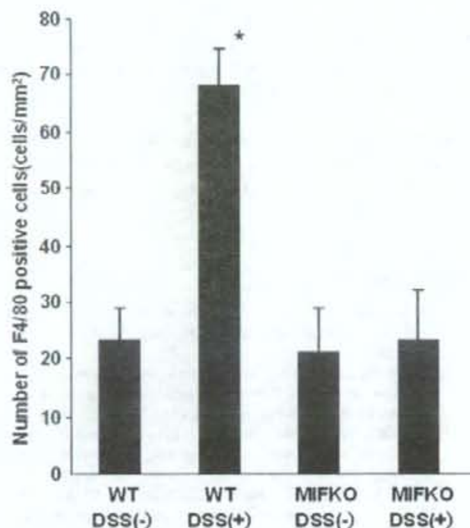

Figure 5. Number of infiltrating F4/80-positive cells. Immunohistochemistry for F4/80 was performed in the colons of mice. The number of cells with F4/80-positive staining was counted in three areas of the colonic mucosa and evaluated in 5 mice in each group. Average numbers of cell counts were calculated. 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.

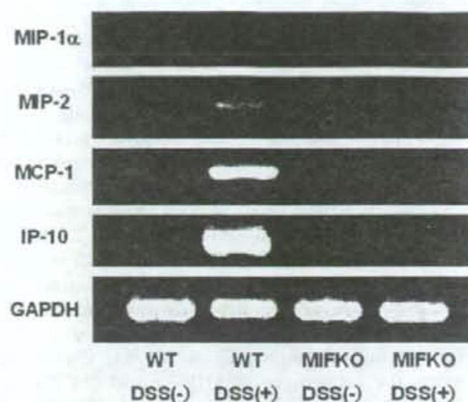

Figure 6. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of the expression of chemokines in the colon of mice. Expressions of macrophage inflammatory protein (MIP)-1α, MIP-2, monocyte chemoattractive protein (MCP)-1, and interferon inducible protein (IP)-10 were remarkably enhanced 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

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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.

Acknowledgements

We thank Rika Nagasima for excellent technical assistance. This research was partly supported by a Grant-in-Aid of Research from the Japanese Ministry of Health, Welfare and Labor, and partly by a Grant-in-Aid from the Ministry of Science and Education (18590665) (T.O.), and by the Program for Promotion of Fundamental Studies in Health Science of the National Institute of Biomedical Inovation (NIBIO) (J.N).

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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International Society for Cellular Therapy ISCT

Cytotherapy (2008) Vol. 10, No. 7, 698–710

informa healthcare

Preparation of functionally preserved CD4$^+$ CD25^{high} regulatory T cells from leukapheresis products from ulcerative colitis patients, applicable to regulatory T-cell transfer therapy

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Background

Ulcerative colitis (UC) is an intractable disease; therefore new therapies need to be developed. CD4$^+$ CD25^{high} regulatory T cells (Treg) significantly ameliorate colitis in animal models. In active UC patients, although Treg are functionally preserved, their proportion in peripheral blood decreases. Thus Treg transfer therapy is expected to be efficacious for UC. During leukapheresis for UC, Treg are depleted, as well as colitogenic effector leukocytes. We therefore designed a leukapheresis/Treg transfer therapy in which Treg are isolated from leukapheresis products and transfused to patients, and studied large-scale germ-free methods of Treg preparation.

Methods

Using the CliniMACS cell selection system, we conducted Treg isolation experiments from leukapheresis products in which B and CD8$^+$ T cells were depleted, followed by positive selection of CD25$^+$ cells. In some experiments, isolated Treg or non-Treg were expanded with interleukin-2 (IL-2) $\pm$ transforming growth factor (TGF)-β1. Expression of a Treg-specific marker, FOXP3, and gut-homing receptors, and suppressor activity of isolated or cultured cells, were analyzed.

Results

CD4$^+$ CD25^{high} T cells were collected and efficiently enriched with a good recovery rate. Isolated cells preferentially expressed FOXP3 and significantly suppressed T-cell proliferation in vitro. *In addition, isolated Treg could be efficiently expanded, and Treg could be induced from non-Treg with TGF-β1* in vitro. *TGF-β1 significantly up-regulated $\alpha E\beta 7$ and $\alpha 4\beta 7$ integrins.*

Discussion

We have established a method of Treg isolation from leukapheresis products that can be used clinically; therefore, Treg transfer therapy is feasible in combination with leukapheresis for UC. Expansion or induction of Treg in vitro *may be another approach to Treg-based immunotherapy.*

Keywords

adoptive transfer, leukapheresis, regulatory T cell, ulcerative colitis.

Introduction

Ulcerative colitis (UC), a form of inflammatory bowel disease (IBD), is a chronic inflammatory disorder that causes persistent colonic inflammation. It mainly affects adolescents and young adults and deeply impairs their quality of life for a long period of time. Its etiology remains unknown; therefore, immunomodulators, including corticosteroids, which suppress immune responses in a non-specific manner, have been the mainstay of treatment for this disease. However, the development of innovative therapy is desperately needed because, despite conventional therapy, the majority of patients experience repeated recurrence of the disease following remission, and a considerable number of patients with severe UC ultimately undergo colectomy.

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DOI: 10.1080/14653240802345812

Through much research, a gradual understanding of the pathophysiologic process of this disease has been gained, and the evidence suggests that an imbalance between colitogenic effector and the regulatory function of the intestinal immune system is responsible for colonic inflammation.

T cells with immunosuppressive capability play an important role in the maintenance of homeostasis by actively regulating immune reactions. One such subset of T cells, naturally occurring $CD4^+$ $CD25^+$ regulatory T cells (Treg), is characterized by their constitutive expression of CD25, cytotoxic T-lymphocyte antigen-4 (CTLA-4), glucocorticoid-induced tumor necrosis factor (TNF) receptor, and the transcription factor forkhead box P3 (FOXP3) [1]. Treg exhibit a hyporesponsiveness to stimuli through T-cell receptors, and possess the ability to suppress $CD4^+$ $CD25^-$ T-cell and $CD8^+$ T-cell proliferation *in vitro*. Treg are essential for the prevention of intestinal inflammation and thus for the maintenance of gut homeostasis. The transfer of naive $CD4^+$ $CD45RB^{high}$ T cells into immunodeficient recipient mice leads to the development of chronic persistent colitis, which resembles human IBD. This phenomenon is the result of the absence of Treg in the transferred cell population, and the co-transfer of Treg into such recipient mice clearly prevents and even cures colonic inflammation [2,3]. The existence of Treg was first demonstrated in mice [4–7] and they have also been identified in human peripheral blood [8,9]. In mice, Treg constitute a typically distinct population of $CD25^+$ cells that comprise 5–10% of thymic, lymph node and splenic $CD4^+$ T cells. In humans, however, up to 40% of peripheral blood $CD4^+$ T cells express CD25 to some extent. It has been shown that only a minority of human $CD4^+$ T cells that express the highest levels of CD25 ($CD25^{high}$) have suppressor activity [8]. The function of Treg seems to be qualitatively preserved in UC patients, as isolated Treg from peripheral blood or intestinal lamina propria in IBD patients reveal suppressor activity *in vitro*, similar to those in healthy subjects [10–12]. However, Treg seem to be quantitatively reduced, as the percentage of Treg is significantly reduced in peripheral blood of active UC patients, in inverse correlation with disease activity [13]. Thus Treg-based cell therapy is expected to be efficacious for the treatment of UC.

Apheresis that depletes large numbers of leukocytes from circulating peripheral blood has been developed for the treatment of UC [14–17]. It is now considered a standard therapy for patients with refractory UC who are steroid-resistant or -dependent in Japan, and its use is spreading to other countries. There are three methods of extracorporeal circulation for leukapheresis: (1) leukapheresis using a centrifugal cell separator; (2) leukapheresis by an adsorptive membrane filter (Cellsorba™); and (3) granulocyte and monocyte/macrophage apheresis by a column filled with cellulose diacetate beads (Adacolumn™). Each method removes different cell populations and thus seems to work through different mechanisms. The former two methods deplete a large number of lymphocytes. As the depletion of lymphocytes by these methods is non-selective, numerous Treg are removed during leukapheresis, together with colitogenic effector lymphocytes. We have considered a possible new therapy in which Treg are isolated from the lymphocytes removed during leukapheresis, and retransfused into UC patients, and named it leukapheresis/Treg transfer therapy. By such therapy, selective depletion of colitogenic effector T cells from UC patients and an increase in the Treg/effector ratio are theoretically possible. To conduct such adoptive transfer therapy with Treg, a large-scale Treg isolation method, which is clinically applicable, needs to be established. We chose the CliniMACS cell selection system from Miltenyi Biotec for this purpose. CliniMACS is a magnetic bead separation method developed for large-scale sterile isolation of cells for clinical application, and has already been applied clinically for the enrichment of $CD34^+$ cells for peripheral blood stem cell transplantation [18,19]. The aim of this study was to establish an appropriate method for *in vitro* Treg-enrichment from apheresis products from UC patients, which is applicable to Treg transfer therapy.

Methods

Patients

Four patients with moderate to severe UC, admitted to Kyushu University Hospital, Fukuoka, Japan, between 2006 and 2007, were enrolled in this study. The protocol of this study was approved by the institutional ethical committee at the Graduate School of Medical Sciences, Kyushu University. Written informed consent was obtained from all patients. Patients underwent centrifugal leukapheresis therapy weekly for 5–11 weeks. Clinical characteristics of the patients were summarized in Table 1. Patient 1 was given two separate courses of leukapheresis because she relapsed after remission induced by the first treatment. An isolation experiment of Treg was conducted