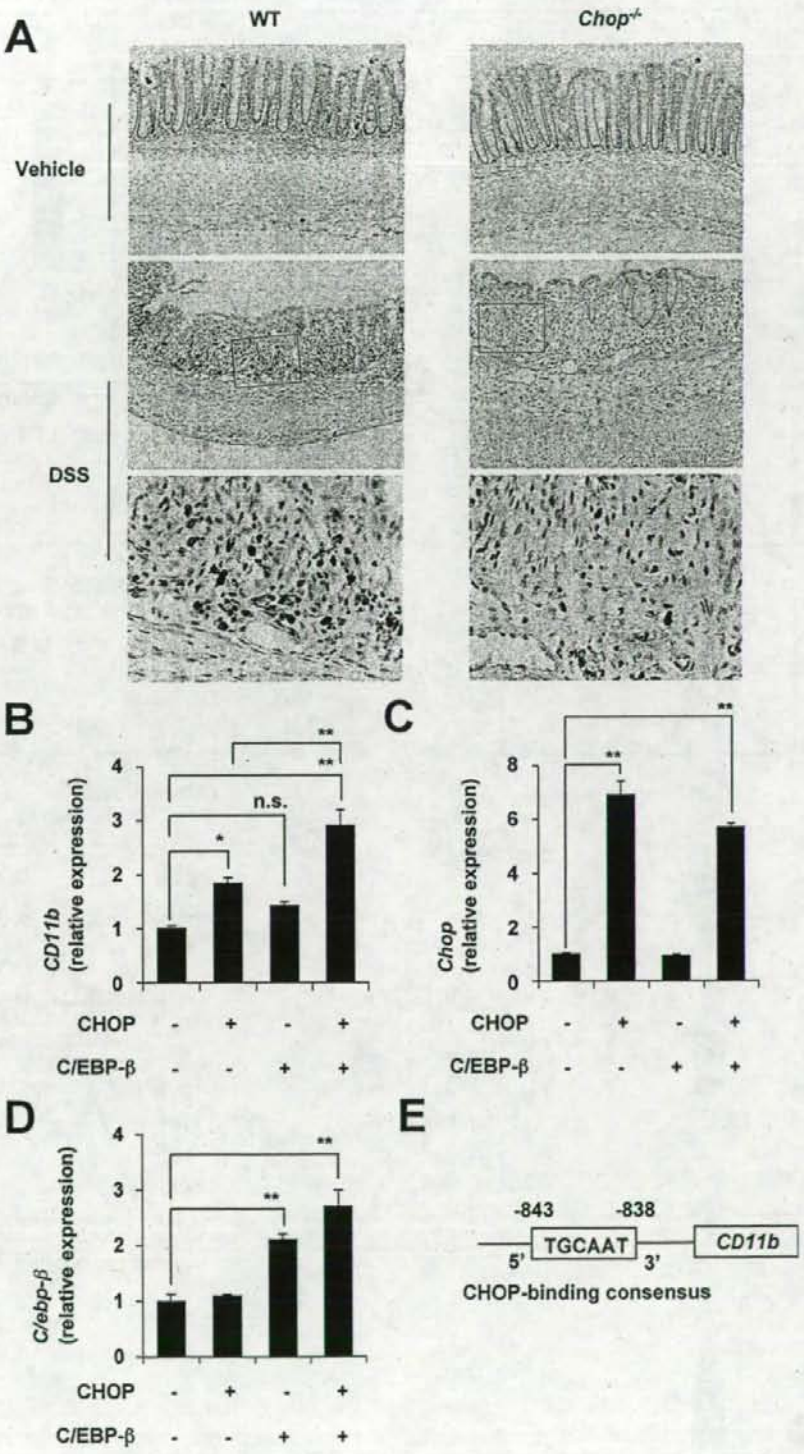
Namba *et al.* Fig.7



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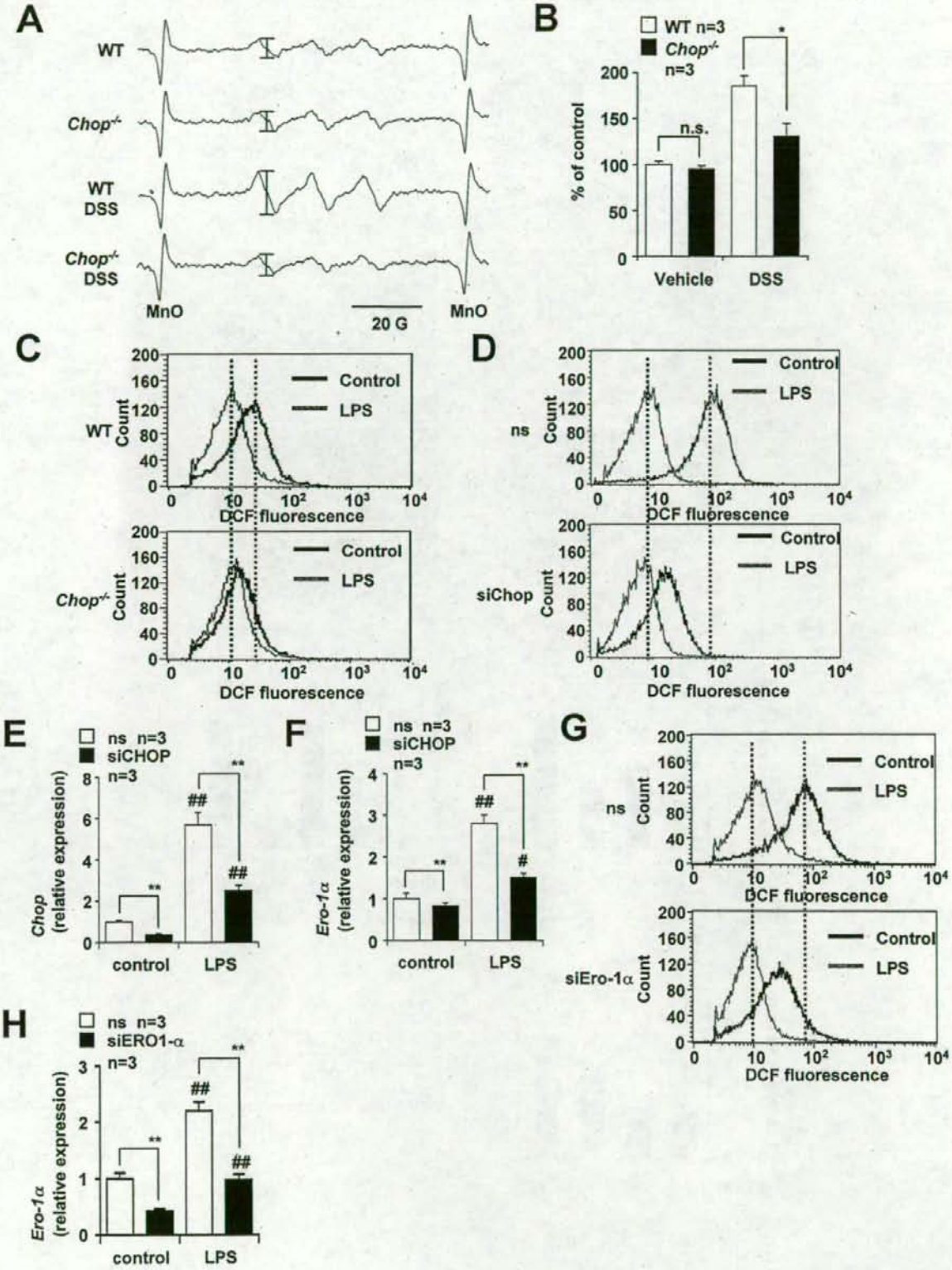
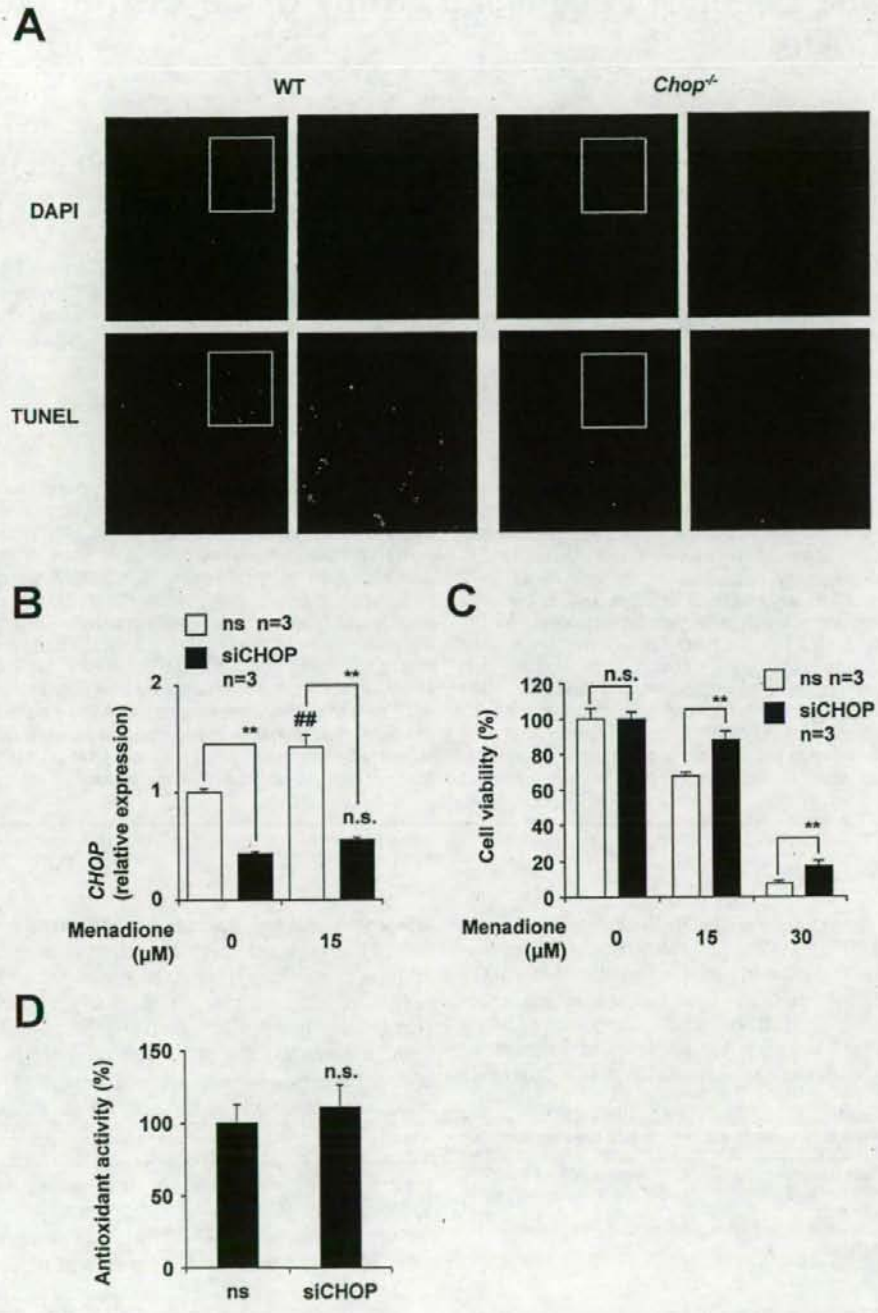


Namba *et al.* Fig.9



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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*-dianisidine, 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-1β*, 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

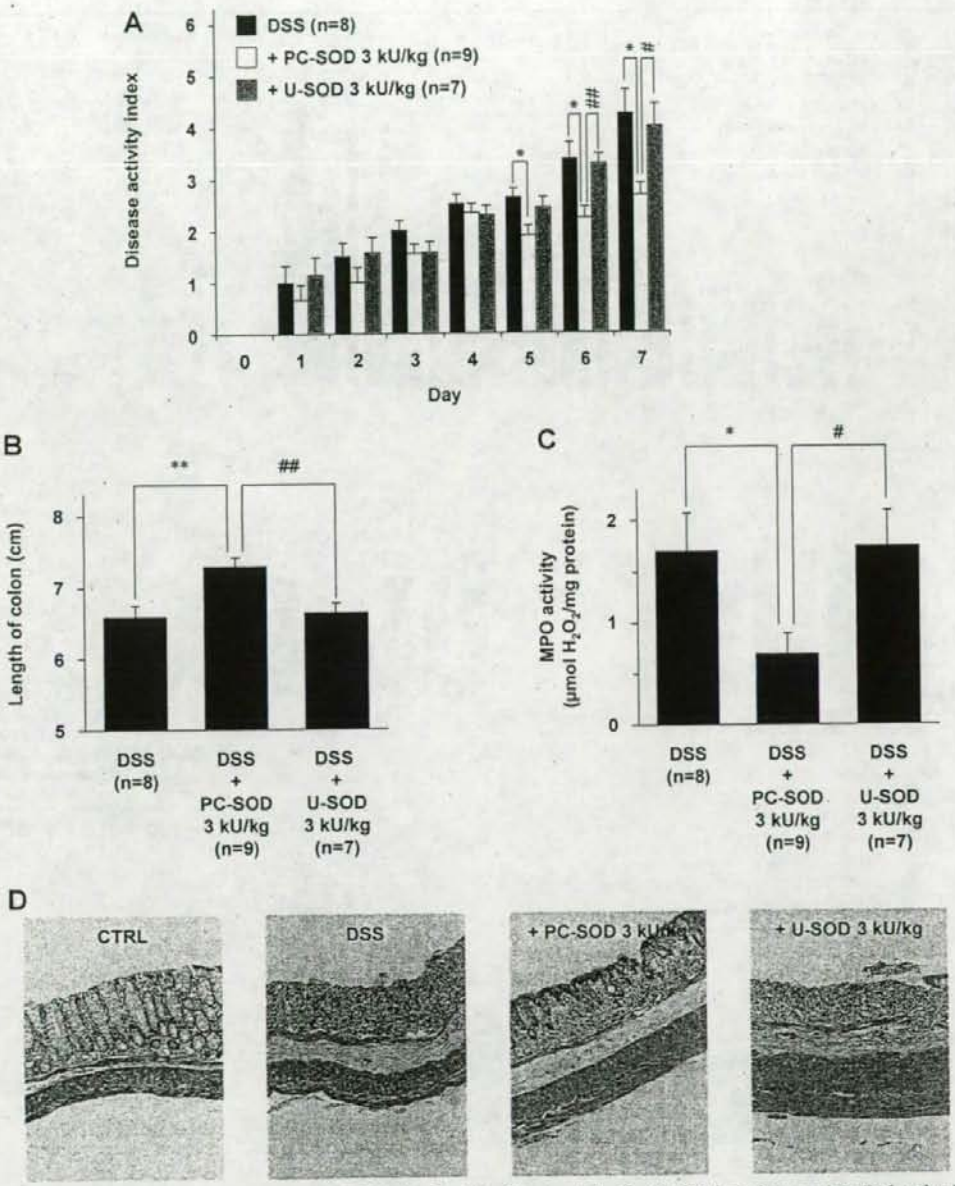

Fig. 1. Effect of PC-SOD and U-SOD on development of DSS-induced colitis. Mice treated with 4% DSS for 7 days, as described under *Materials and Methods*, were intravenously administered PC-SOD or U-SOD once daily. DAI was measured daily (A). The length of the colon (B) and colonic MPO activity (C) were 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

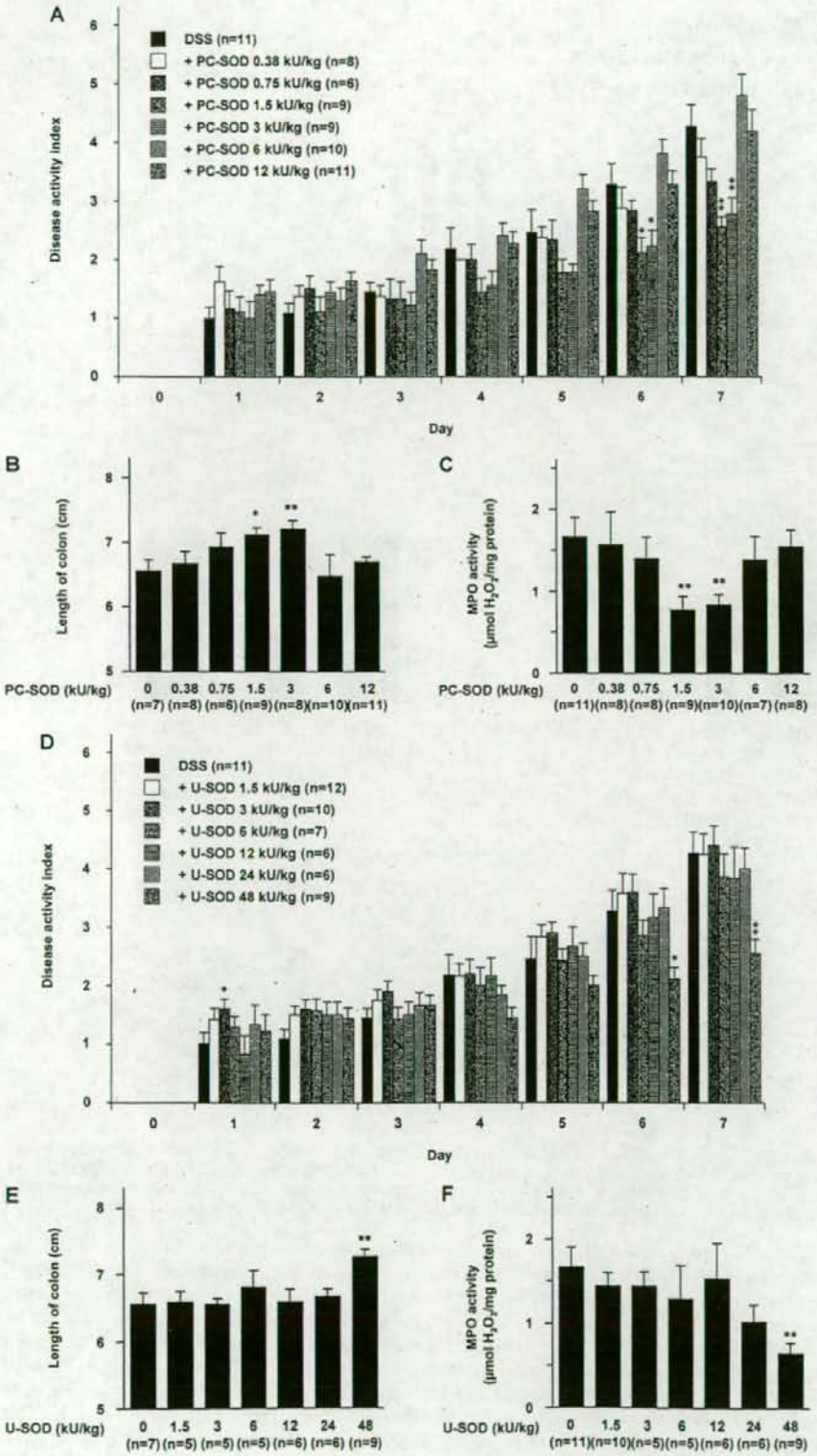

Fig. 2. Dose-response profile of effect of PC-SOD and U-SOD on development of DSS-induced colitis. Mice were treated with DSS and PC-SOD (A–C) or U-SOD (D–F), and colitis was assessed as described in the legend of Fig. 1. Values are mean ± S.E.M. *, $P < 0.05$; **, $P < 0.01$ (versus without PC-SOD or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

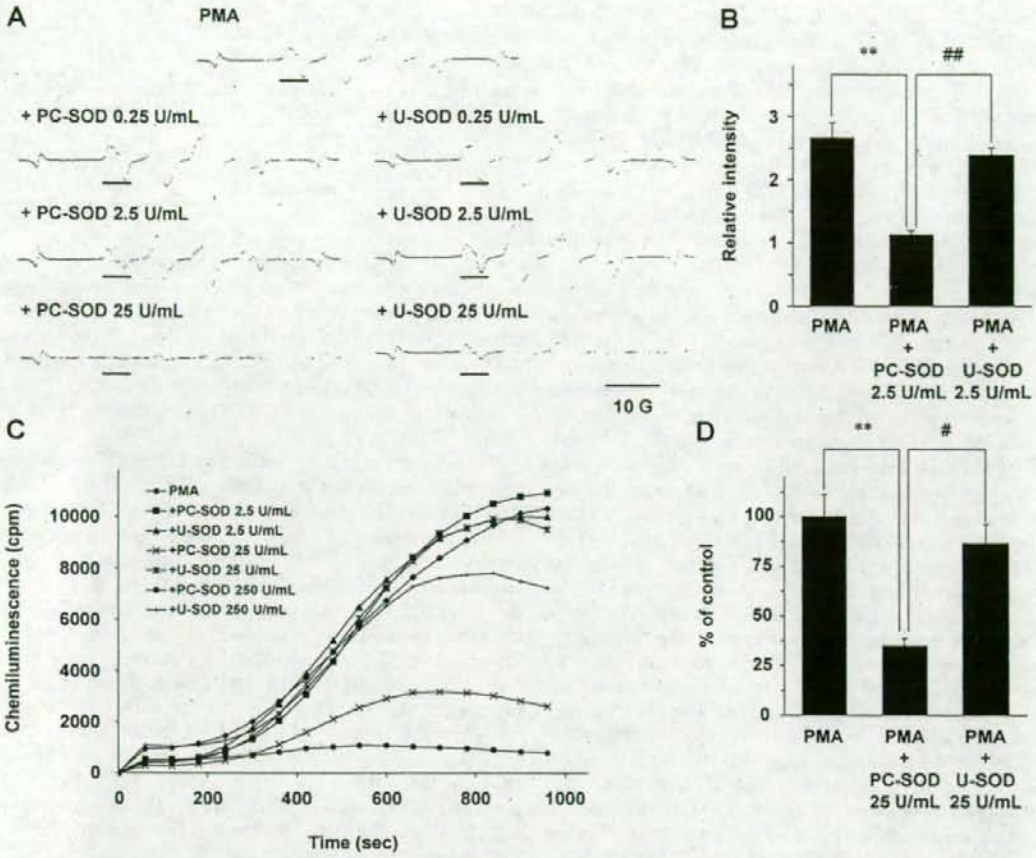

Fig. 3. Effect of PC-SOD or U-SOD on the amount of superoxide anion in vitro. Human neutrophils were preincubated with PC-SOD or U-SOD for 1 h and washed with medium. Neutrophils were then activated with PMA, and the amount of superoxide anion was measured by ESR (A and B) or CL analysis (C and D). The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

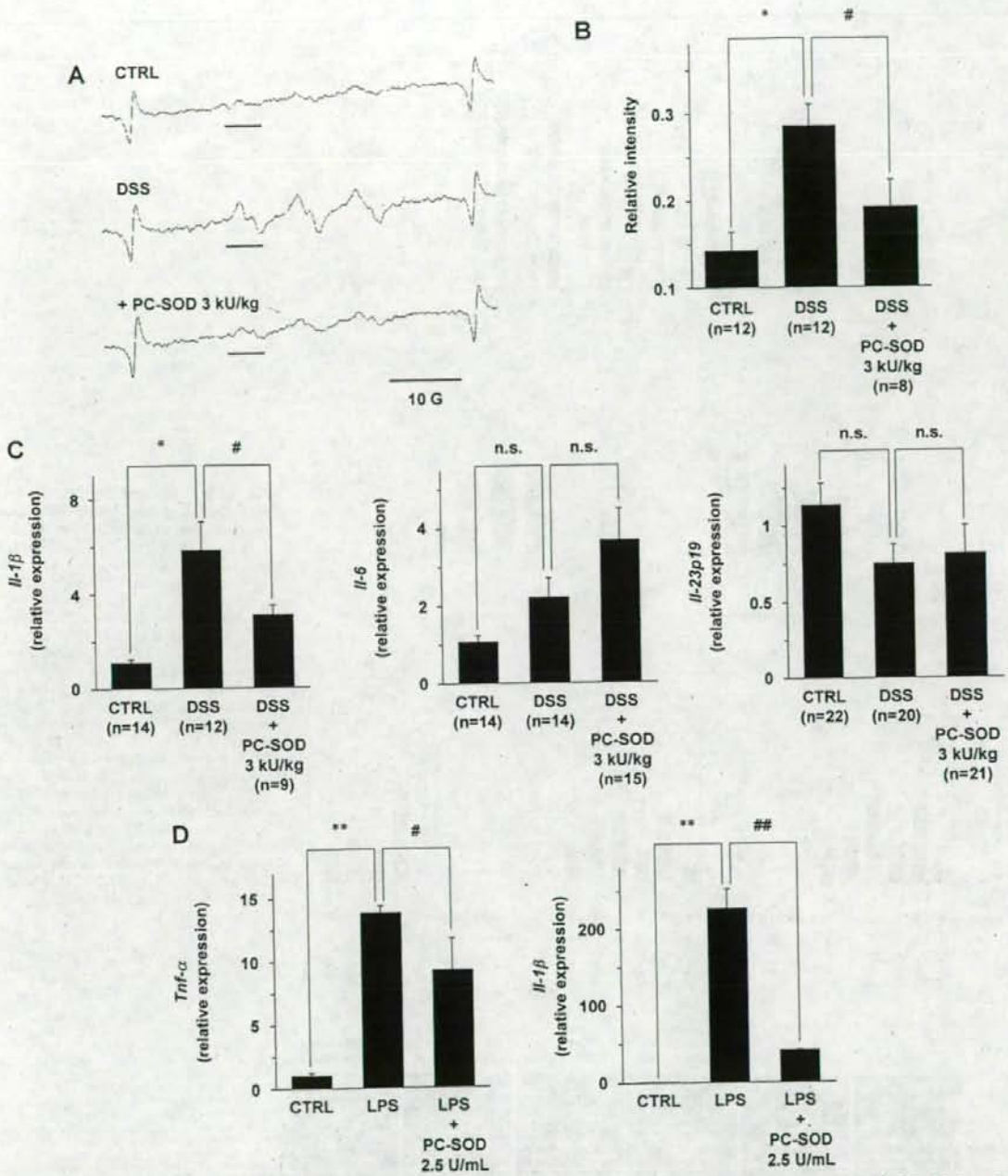

Fig. 4. Effect of PC-SOD on the level of ROS and expression of cytokines. DSS and PC-SOD were administered to mice, as described in the legend of Fig. 1 (A–C). After 7 days, POBN was administered and the colons were dissected and subjected to radical adduct ESR spectrum analysis (A). The intensity of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

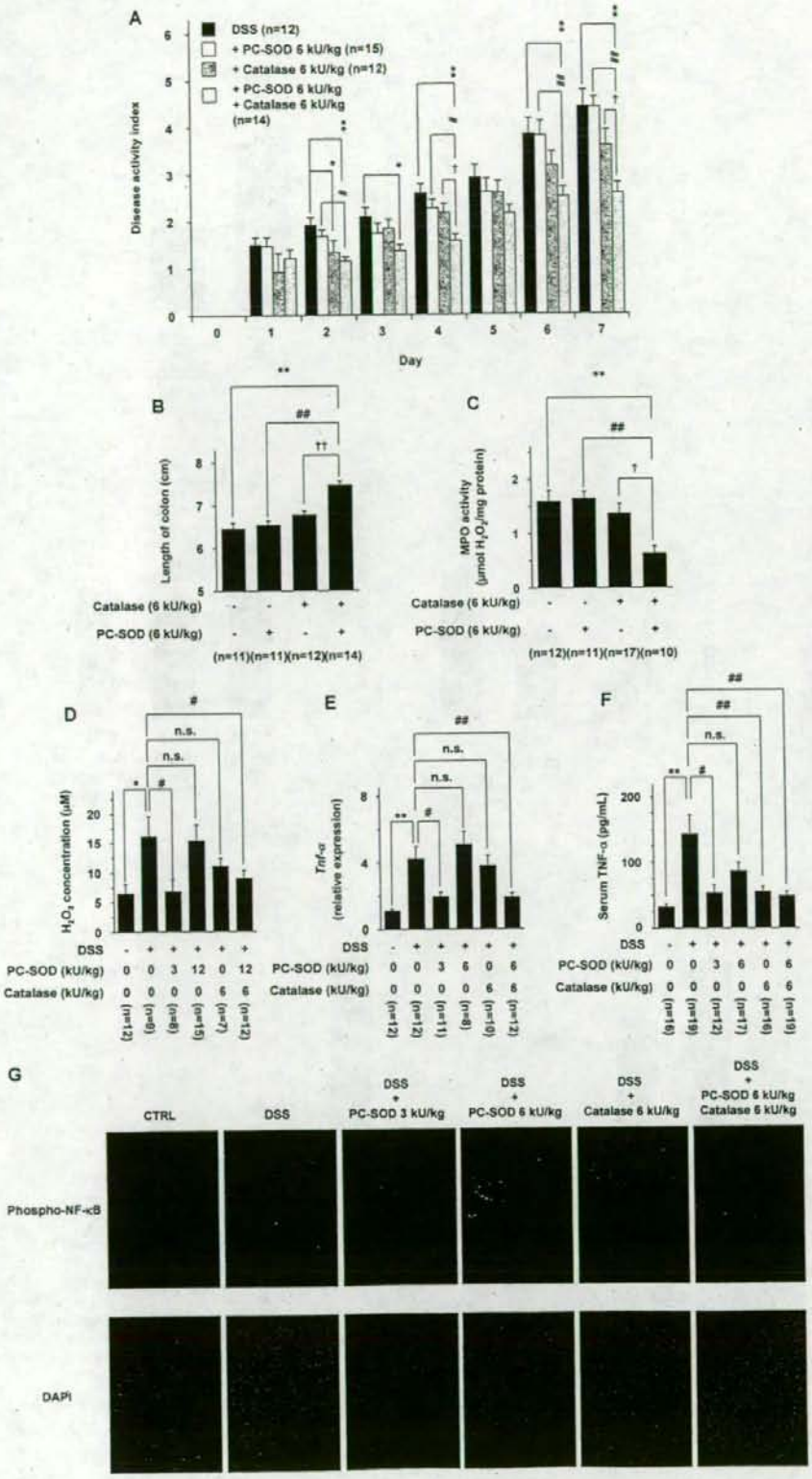

Fig. 5. The effect of simultaneous administration of catalase on the ameliorative effect of PC-SOD on DSS-induced colitis, colonic level of hydrogen peroxide, TNF-α, and NF-κB. Mice were treated with DSS and PC-SOD (A–G), and colitis was assessed (A–C), as described in the legend of Fig. 1. Catalase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

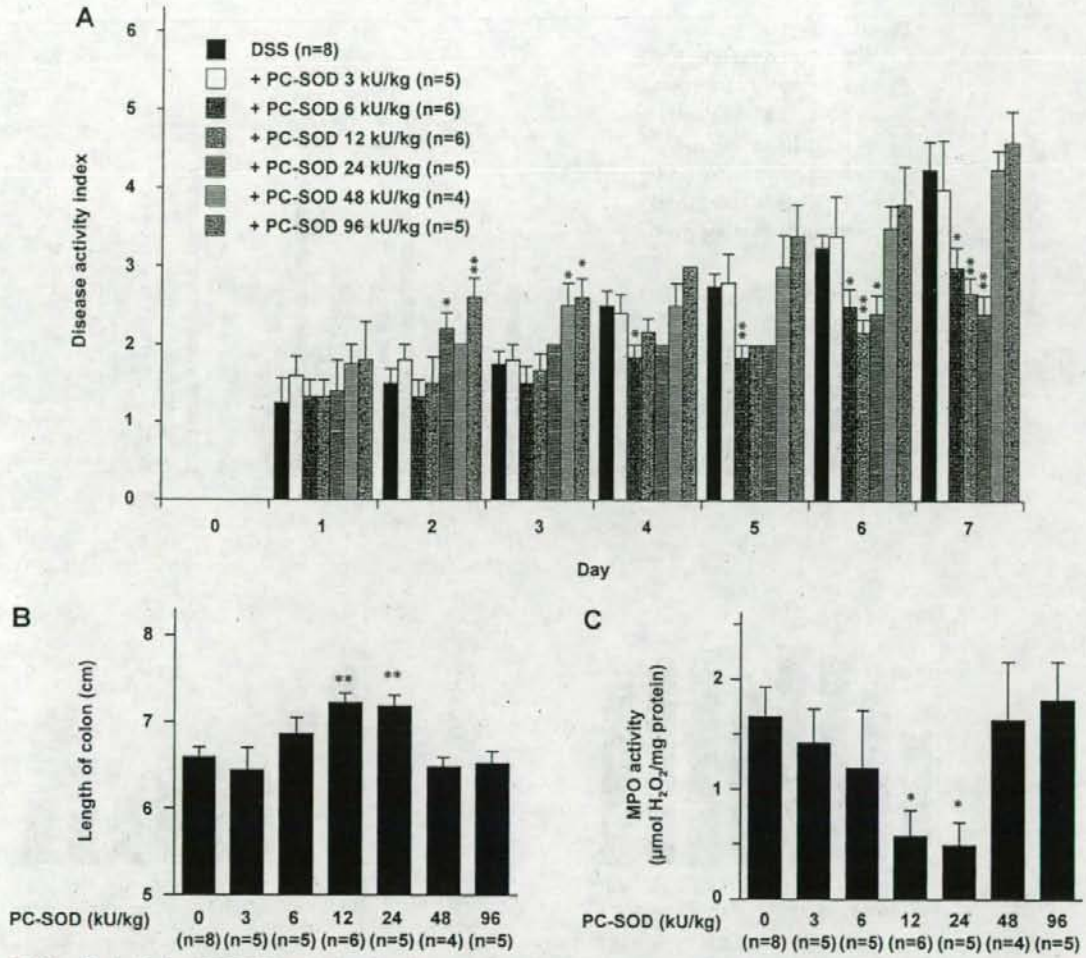

Fig. 6. Effect of a single dose of PC-SOD on the development of DSS-induced colitis. PC-SOD was intravenously administered to mice once at the start of DSS treatment (day 0). Colitis was subsequently assessed, as described in the legend of Fig. 1. Values are mean ± S.E.M. *, $P < 0.05$; **, $P < 0.01$.

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

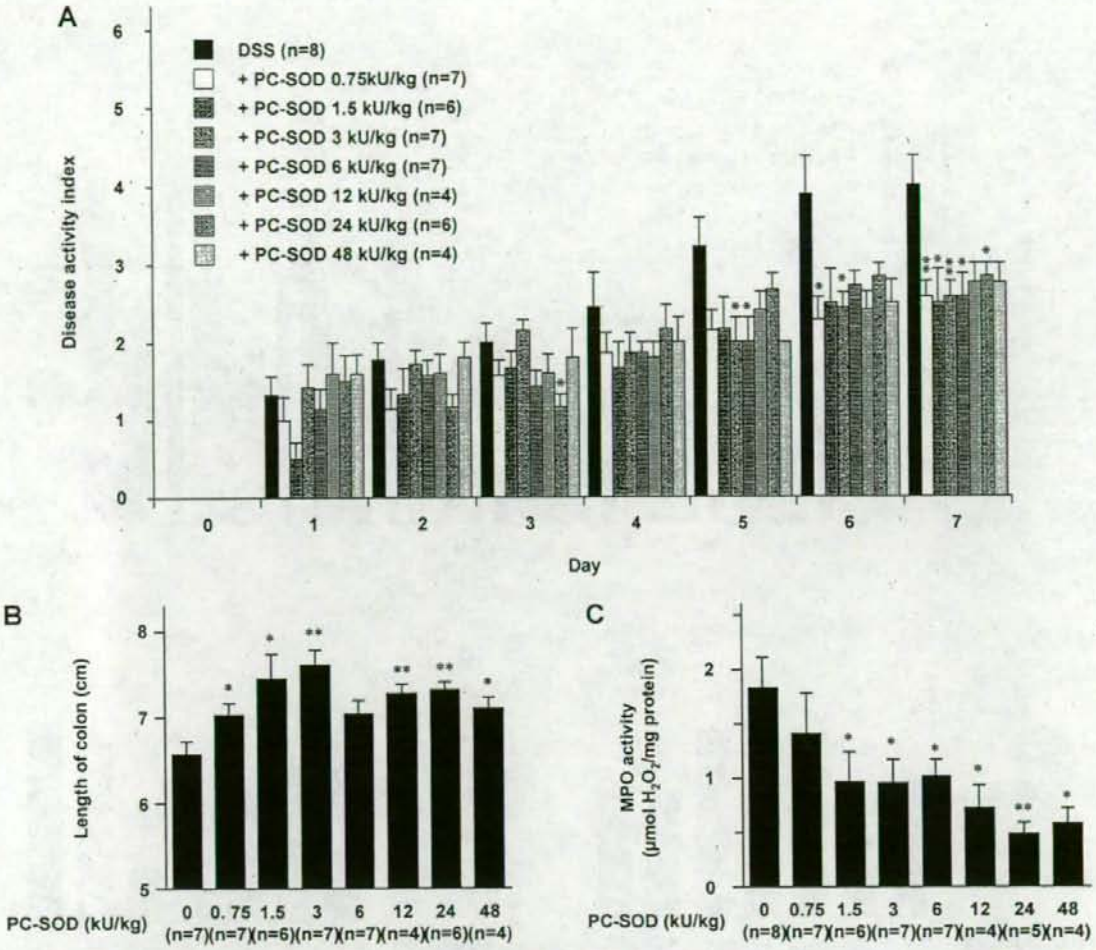

Fig. 7. Effect of oral administration of PC-SOD on development of DSS-induced colitis. PC-SOD was orally administered to mice once daily. Colitis was assessed, as described in the legend of Fig. 1. Values are mean ± S.E.M. *, $P < 0.05$; **, $P < 0.01$.

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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Analysis of Origin Recognition Complex in *Saccharomyces cerevisiae* by Use of Degron Mutants

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Origin recognition complex (ORC), a six-protein complex (Orc1p-Orc6p), may deeply involve in initiation of chromosomal DNA replication. However, since most temperature-sensitive *orc* mutants of *Saccharomyces cerevisiae* show the accumulation of cells with nearly 2C DNA content, the exact stage at which ORC acts is not fully understood. In this study, we constructed a heat-inducible degron mutant for each ORC subunit. As well as each targeted subunit, other subunits of ORC were also rapidly degraded under non-permissive conditions. In the *orc5* degron mutant, incubation under the non-permissive conditions caused accumulation of cells with nearly 2C DNA content, and phosphorylation of Rad53p. When Orc5p (ORC) is depleted, this inhibits G1/S transition and formation of a pre-replicative complex (pre-RC). For pre-RC to form, and G1/S transition to proceed, Orc5p (ORC) must be present in late G1, rather than early G1, or G2/M. Block and release experiments revealed that Orc5p (ORC) is not necessary for S and G2/M phase progression. We therefore propose that ORC is necessary for the G1/S transition and pre-RC formation, and accumulation of cells with nearly 2C DNA content seen in various *orc* mutants is due to inefficient pre-RC formation, and/or induction of checkpoint systems.

Key words: DNA replication, heat inducible degron mutant, ORC, Orc5p, pre-replicative complex.

Abbreviations: α-factor, α mating factor; DHFR, dihydrofolate reductase; FACS, fluorescence-activated cell sorter; HA, haemagglutinin; HU, hydroxyurea; MCM, mini-chromosome maintenance complex of proteins; ORC, origin recognition complex; pre-RC, pre-replication complex.

The initiation of chromosomal DNA replication is tightly regulated to replicate the genome just once per cell cycle. To reveal the underlying molecular mechanism for this regulation, it is important to understand the initiator of chromosomal DNA replication. In *Escherichia coli*, DnaA is the initiator of chromosomal DNA replication: temperature-sensitive *dnaA* mutants show defects in initiation of DNA replication, and an *in vitro* chromosomal DNA replication system, reconstituted from purified enzyme, is dependent on DnaA (*1*). In eukaryotes, origin recognition complex (ORC) is the most likely initiator. ORC was originally identified as a six-protein complex that specifically binds to *Saccharomyces cerevisiae* (*S. cerevisiae*) origins of chromosomal DNA replication (*2*) (in this manuscript, 'ORC' refers to *S. cerevisiae* ORC). ORC homologues have been found in various eukaryotic species, including humans (*3*). Although there is only weak homology in amino acid sequence between ORC and DnaA, these two factors share a number of functions; (i) both bind to each origin of chromosomal DNA replication (*1*, *2*); (ii) both bind to ATP and ADP, they have intrinsic ATPase activity, and adenine nucleotides bound to them regulate their activities (*4–10*) (iii) both proteins interact with replicative DNA helicase and recruit it to each origin of DNA replication (*1*, *10*, *11*). These observations strongly suggest that ORC is the initiator of chromosomal DNA replication. However, an origin-dependent *in vitro* chromosomal DNA replication system has not yet been developed for eukaryotes, so the dependency of replication on ORC has not been formally proved.

Most temperature-sensitive S. cerevisiae orc mutants show the accumulation of cells with nearly 2C DNA content at non-permissive temperatures (*12–16*), suggesting that ORC may be involved in the G2/M progression rather than the G1/S transition. This observation argues against the idea that ORC initiates DNA replication. On the other hand, the contribution of ORC to the initiation has been suggested by using temperature-sensitive orc mutants and block and release experiments, microscopic observation and 2D gel experiments (*13*, *16–19*). Thus, exact role of ORC in vivo is not yet fully understood. There are significant problems in interpreting results with such temperature-sensitive mutants; some mutant proteins show gain-of-function, and the mutant protein may maintain some functions even at non-permissive temperatures.

To address this issue, we used genetic systems that cause rapid and conditional elimination of the target protein. In yeast, we generally use a genetic shut-off

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system, for example, locating the target gene under the *GAL* promoter and changing the carbon source of culture medium from galactose to glucose to suppress transcription. However, in this system, the target protein is depleted according to its natural half-life. Therefore, stable proteins such as ORC are depleted slowly, and thus other approaches seem necessary (*15*).

Creating a heat-inducible degron mutant is a very useful way to achieve rapid conditional degradation of a target protein. The N-terminal of the target protein is fused with the N-terminal fragment of mutated (temperature-sensitive) mouse dihydrofolate reductase (DHFR) gene, the so-called 'heat-inducible degron'. When cells are incubated at the non-permissive temperature (37°C), the fused protein is easily ubiquitinated, resulting in its rapid degradation by ubiquitin-proteasome system (*20*). Diffley and co-workers (*21–23*) improved this method by *GAL* promoter-regulated simultaneous overproduction of Ubr1p, the E3 ubiquitin ligase, which stimulates this degradation. This improvement has proved useful for genetic analysis of DNA replication-related proteins, such as Cdc45p, and the mini-chromosome maintenance complex of proteins (MCM). Kanemaki *et al.* (*24*) clearly showed that Mcm4p can be rapidly depleted in a heat-inducible degron mutant with the overproduction of Ubr1p, but not in cells with a genetic shut-off system. In this study, we constructed a heat-inducible degron mutant for each ORC subunit. Under non-permissive conditions (incubation at 37°C in the presence of galactose), each targeted subunit, and also other subunits of ORC, were rapidly degraded. All *orc* degron mutants showed a temperature-dependent galactose-stimulated growth defect. Block and release experiments using the *orc5* mutant showed that Orc5p (ORC) is necessary at late G1, rather than early G1 and G2/M phases, for the G1/S transition and pre-replicative complex (pre-RC) formation, suggesting that ORC functions as the initiator of chromosomal DNA in budding yeast.

MATERIALS AND METHODS

Strains, Plasmids and Medium—S. cerevisiae strains are listed in Table 1 (*25*). Cells were cultured in YP medium (1% yeast extract and 2% Bacto-peptone) containing 2% galactose, raffinose or glucose. Plasmids, pPW66R and pKL54, were gifts from Dr. Diffley. Plasmids, pRS404, pFA6a-3HA-*TRP1* and pFA6a-13Myc-*TRP1*, were from our laboratory stock.

The degron mutant for each *ORC* gene was constructed as described previously (*21, 23*). The plasmid pKL54 (*GAL-UBR1*) was digested with *Pme*I and transformed into W303-1A. The construct of the resultant strain (YYM101) was confirmed by colony PCR. DNA encoding the N-terminal region of each ORC subunit was then amplified by PCR and the amplified DNA was ligated into the *Hin*dIII-*Xho*I region of pPW66R. The resultant plasmid was digested with a single-cutting restriction enzyme and transformed into YYM101. The construction of resultant strains (YYM102–107) was confirmed by colony PCR.

Modification of the *ORC2* gene with 13Myc, or of the *ORC6* gene with 3HA (haemagglutinin, HA) was performed as described previously (*26*). PCR was performed using pFA6a-3HA-*TRP1* or pFA6a-13Myc-*TRP1* plasmid as template, with primers to the C-terminal region of the *ORC6* or *ORC2* genes, respectively. The amplified DNA was transformed into W303-1A, YYM104 or YYM106. The construction of resultant strains (YYM108–113) was confirmed by colony PCR.

Table 1. **Yeast strains**

Strain	Genotype	Reference
W303-1A	*Mata ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	Thomas and Rothstein (*25*)
YYM101	W303-1A *ubr1*Δ*::GAL-Myc-UBR1::HIS3*	This study
YYM102	YYM101 *orc1-td*	This study
YYM103	YYM101 *orc2-td*	This study
YYM104	YYM101 *orc3-td*	This study
YYM105	YYM101 *orc4-td*	This study
YYM106	YYM101 *orc5-td*	This study
YYM107	YYM101 *orc6-td*	This study
YYM108	W303-1A *ORC2-13Myc::TRP1*	This study
YYM109	W303-1A *ORC6-3HA::TRP1*	This study
YYM110	YYM104 *ORC2-13Myc::TRP1*	This study
YYM111	YYM104 *ORC6-3HA::TRP1*	This study
YYM112	YYM106 *ORC2-13Myc::TRP1*	This study
YYM113	YYM106 *ORC6-3HA::TRP1*	This study
YYM114	W303-1A *bar1*Δ*::TRP1*	This study
YYM115	YYM106 *bar1*Δ*::TRP1*	This study

Disruption of the *BAR1* gene in W303-1A and YY106 was performed as described previously (*27*). PCR of the *BAR1* gene was performed using pRS404 (a plasmid with *TRP1*) as template, and primers to the 5′ upstream region of the N-terminal or 3′ downstream region of the C-terminal. The amplified DNA was transformed into W303-1A or YYM106. The construct of resultant strains (YYM114–115) was confirmed by colony PCR.

Fluorescence-Activated Cell Sorter (FACS) Analysis—Samples were prepared as previously described (*15*) with some modifications. Cells were pelleted by centrifugation, washed with sterilized water and fixed in 70% ethanol for 12 h. Cells were again pelleted, re-suspended in 50 mM sodium citrate, sonicated for 1 min, treated with 0.25 mg/ml RNase A (SIGMA) for 1 h at 50°C and then with 1 mg/ml Proteinase K (Wako) for 1 h at 50°C. DNA was stained with 50 μg/ml of propidium iodide (SIGMA) and 20,000 cells from each sample were scanned with a FACSCalibur (Becton Dickinson).

Chromatin-Binding Analysis—Yeast spheroplasts were lysed with Triton X-100 and samples were processed into soluble (supernatant) and chromatin (insoluble precipitate) fractions by centrifugation (*15*). Equivalent amounts (total protein) of chromatin fractions were electrophoresed on 7.5% polyacrylamide gels containing SDS, transferred to PVDF membrane–and probed with monoclonal antibodies against Orc3p (SB3), Orc5p (SB5), Mcm2 (Mcm2–28), HA (12CA5) or Myc (9E10) (*28–31*).

Block and Release Experiments for Cell Cycle Progression—Yeast cells were cultured at 24°C to early log phase and cell cycle progression was blocked at the G1, S or G2/M phase by incubation with 0.05–5 μg/ml with α mating factor (α-factor) (SIGMA), 0.1 M

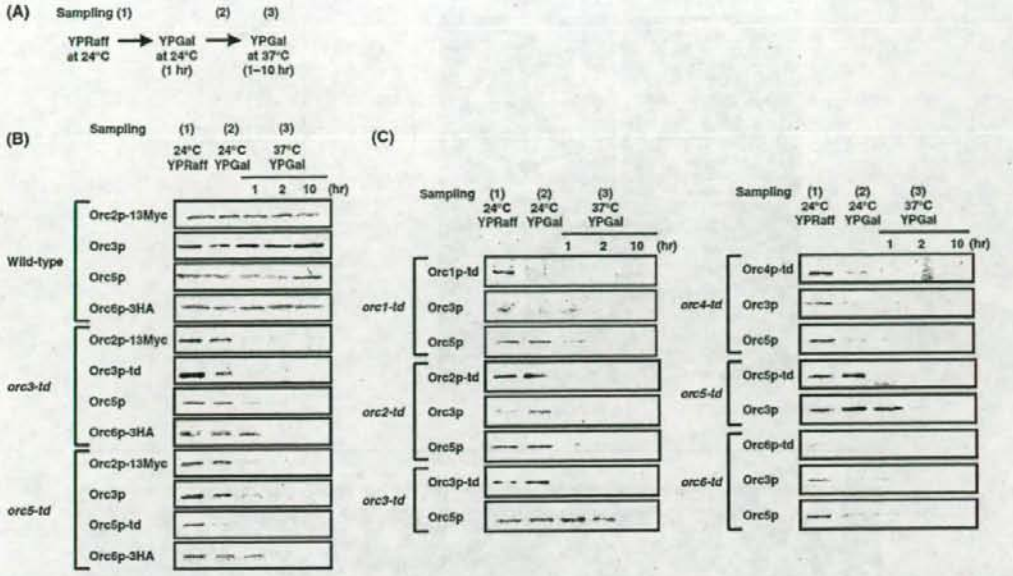

Fig. 1. **Degradation of ORC subunits in *orc* degron mutants.** (A) Experimental outline and timing of sampling. (B) W303-1A, YYM108, YYM109 (wild-type); YYM104, YYM110, YYM111 (*orc3-td*); and YYM106, YYM112, YYM113 (*orc5-td*) cells were cultured in YP medium with 2% raffinose (YPRaff) to logarithmic phase at 24°C, and then cultured in YP medium with 2% galactose (YPGal) for 1 h at 24°C. Cells were further incubated at 37°C in the same medium, and a small portion of culture was taken when indicated. Chromatin fractions were prepared and analysed by immunoblotting using monoclonal antibodies specific for Orc3p, Orc5p, Myc (for Orc2p-13Myc) and HA (for Orc6p-3HA and each td-subunit). (C) YYM102-107 (*orc1-td*, *orc2-td*, *orc3-td*, *orc4-td* *orc5-td* and *orc6-td*) cells were cultured and analysed as above.

hydroxyurea (HU) (SIGMA) or 3 μg/ml nocodazole (SIGMA), respectively. Cells were released from the block by washing and re-suspending in fresh medium without the blocking agents.

RESULTS

Construction of a Heat-inducible Degron Mutant for Each ORC Subunit—To achieve temperature- and galactose-dependent rapid degradation of the targeted subunit of ORC, we inserted the 'heat-inducible degron' sequence at the N-terminal of each subunit of ORC, and transformed a plasmid containing the *UBR1* gene under the *GAL* promoter. Pre-incubation of *orc* degron mutants at 24°C for 1 h in YP medium with galactose (Fig. 1A) stimulated the subsequent degradation of the targeted subunit at 37°C (data not shown), so we routinely performed this pre-incubation. As shown in Fig. 1B, in the *orc3* degron mutant (*orc3-td*), Orc3p disappeared within 1 h of temperature shift (from 24°C to 37°C). Furthermore, following Orc3p-degradation, other subunits (myc-tagged Orc2p, Orc5p, HA-tagged Orc6p) also disappeared (Fig. 1B). Degradation of Orc1p and Orc4p could not be tested, as antibodies against Orc1p and Orc4p did not work under our experimental conditions, and we could not insert any tag into either the *ORC1* gene or the *ORC4* gene. Similar results were obtained with the *orc5* degron mutant (*orc5-td*); rapid degradation of Orc5p and subsequent degradation of other subunits (myc-tagged Orc2p, Orc3p, HA-tagged Orc6p; Fig. 1B). We confirmed that in wild-type cells the amount of each subunit of ORC is constant under the experimental conditions used (Fig. 1B).

For other heat-inducible *orc* degron mutants (*orc1-td*, *orc2-td*, *orc4-td* and *orc6-td*), there was rapid degradation of each targeted subunit and subsequent degradation of other subunits (Orc3p and Orc5p; Fig. 1C). Based on these results, we concluded that we had constructed heat-inducible degron mutant for every ORC subunit. From the results in Fig. 1, it can be suggested that ORC becomes unstable when any one of its subunits is degraded.

Growth Phenotypes of the Heat-Inducible orc Mutants—The effect of incubation temperatures and galactose on growth of each *orc* degron mutant strain was tested on YP agar plates (Fig. 2A). Compared to wild-type cells, the growth of all mutants was slow at 37°C, especially with galactose. At 24°C, growth of mutants was indistinguishable from that of wild-type cells (Fig. 2A). Since the transformation of *UBR1*-expression plasmid (*GAL-UBR1*) into the wild-type strain did not affect growth (Fig. 2A), the inhibition seen in mutants must be due to degron-dependent degradation of the targeted subunit and other subunits.

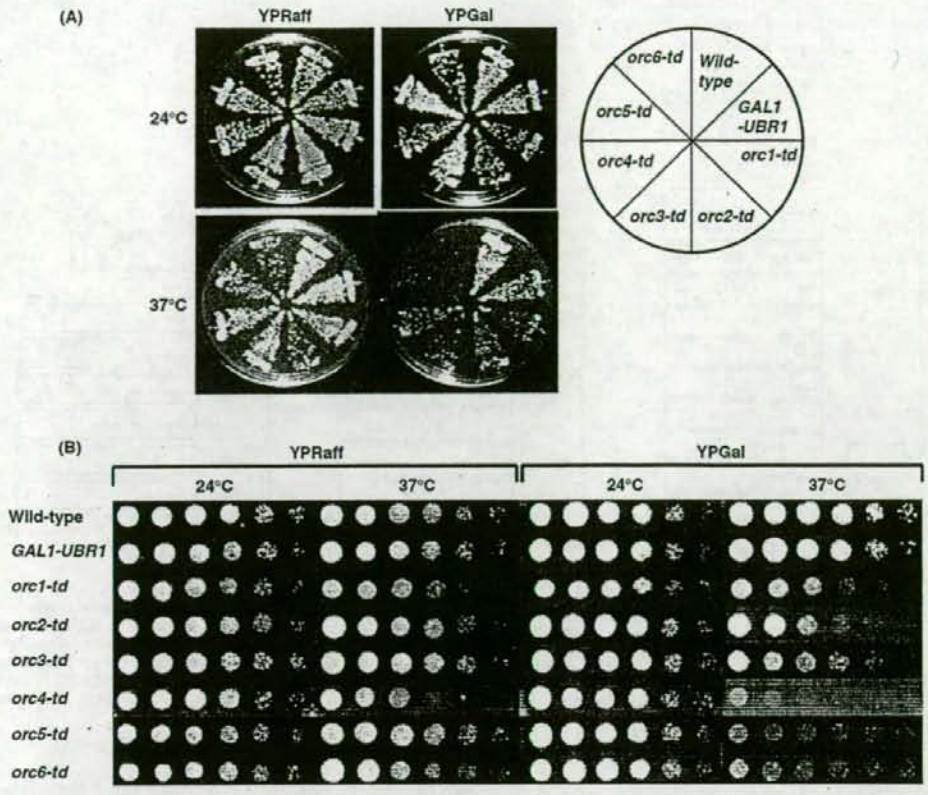

Fig. 2. **Growth phenotypes of ORC degron mutants.** W303-1A (wild-type), YYM101 (*GAL-UBR1*) and YYM102-107 (*orc1-td, orc2-td, orc3-td, orc4-td orc5-td* and *orc6-td*) cells were streaked on YP agar plates with 2% raffinose (YPRaff) or galactose (YPGal) and incubated at 24°C or 37°C for 2 days (A). Cell suspensions of each strain ($O.D._{600}=1$, 0.25, 0.13, 0.068, 0.034, 0.017; from left to right) were dropped onto YPRaff or YPGal and incubated at 24°C or 37°C for 2 days (B).

For quantitative monitoring of the growth of *orc* degron mutants, we performed a dilution assay. Every mutant showed a temperature-dependent and galactose-stimulated growth defect (Fig. 2B), which is consistent with the results in Fig. 2A. The extents of growth defects differed among the various *orc* degron mutants; *orc4*, *orc5* and *orc6* mutants showed relatively clear phenotype. We used some not all *orc* degron mutants in the following experiments.

Cell Cycle Progression in the Heat-Inducible orc Mutants—The results in Fig. 2 indicate that under the non-permissive conditions the mutants have defects in cell cycle progression. To determine which phase of the cell cycle is blocked, *orc3* and *orc5* mutants and wild-type cells were asynchronously grown at 24°C in YP medium with raffinose, then with galactose, the incubation temperature was shifted to 37°C, and their DNA contents were determined by FACS analysis (Fig. 3A). Compared to the wild-type cells, the proportion of cells with nearly 2C DNA content increased over time in *orc3* and *orc5* mutants (Fig. 3B), suggesting that most cells were blocked in late S phase or G2/M phase. This accumulation was observed earlier in the *orc5* mutant than in the *orc3* mutant, which is consistent with the greater growth defect in *orc5* mutant (Fig. 2B). The transformation of *UBR1*-expression plasmid into wild-type cells did not affect the cell cycle progression (Fig. 3B), suggesting that it is the degron-dependent degradation of ORC, which is responsible for this defect of cell cycle progression.

Eukaryotic cells have checkpoint systems, which detect DNA damage and defects in DNA replication and stop cell cycle progression (*32*, *33*). Phosphorylation of Rad53p plays an important role in the checkpoint systems, and in some temperature-sensitive *orc* mutants Rad53p was phosphorylated at non-permissive temperatures (*32–34*). Thus, checkpoint systems may become induced in *orc* degron mutants under non-permissive conditions. We monitored Rad53p phosphorylation in the *orc5* mutant by immunoblotting under the same conditions as the FACS analysis. As shown in Fig. 3C, after incubation in