厚生労働科学研究費補助金

創薬基盤推進研究事業

トランスクリプトソーム解析による医薬品の副作用機構の解明と、その副作用感受性診断、及び創薬への応用

平成 21 年度　総括研究報告書

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トランスクリプトソーム解析による医薬品の副作用機構の解明と、その副作用感受性診断、及び創薬への応用

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研究要旨

今年度我々は、これまで我々が確立した薬剤性間質性肺炎に関する動物モデルの改善に取り組み、簡便、かつ再現性のよい動物モデルの構築に成功した。このモデルにおいてレフルノミドによる間質性肺炎の発症機構を検討し、レフルノミドが上皮細胞の上皮間葉転換を起こすことがその原因であること、及びその上皮間葉転換は、ウリジンの枯渇、Notch シグナルの活性化、転写因子 SIP1 の活性化を介して起こることを発見した。

A. 研究目的

医薬品の副作用、特に副作用感受性に関する個人差が臨床現場で大きな問題になっている。問題は、その副作用の発症機構が充分に理解されていないため、新薬候補品の副作用、及び患者の副作用感受性を予測出来ない点である。そこで本研究で我々はトランスクリプトソーム解析を用いて医薬品の副作用発症機構を解明し、新薬候補品の副作用、及び副作用感受性に関する個人差を予測する方法を確立する。この研究は以下に述べるように、新薬の開発にも繋がる。以下に我々がこれまで行ってきた、非ステロイド系抗炎症薬（NSAID）に関する研究の成果を述べる。

アスピリンを代表とする NSAID は優れた抗炎症薬として世界中でよく使用されているが、その胃潰瘍副作用（NSAID 潰瘍）が臨床現場で大きな問題になっている（米国では年間 16500 人が NSAID 潰瘍で亡くなっている)。我々は NSAID が誘導する遺伝子をストレス遺伝子チップ（自ら開発した、ストレス遺伝子に特化した DNA チップ）で解析し、NSAID が胃粘膜細胞死を誘導すること、及びこの細胞死が NSAID 潰瘍の原因であることを見出した。実際、市販されている NSAID の細胞傷害性と胃潰瘍副作用の間には有意な相関性が見られた（副作用予測システムの確立)。この結果は、細胞傷害性の少ない NSAID は胃潰瘍副作用の少ない NSAID になることを示している。実際我々はそのような NSAID の合成に成功し、それらが充分な抗炎症作用を示すにも関わらず、ほとんど胃潰瘍を起こさないことを見いだした（現在前臨床試験中)。またこの細胞傷害に影響を及ぼす複数の遺伝子を同定し、その遺伝子多型により細胞の NSAID 感受性が変化することを見出したので、本研究で我々はこの成果を基に、患者の NSAID 潰瘍感受性を予測する方法を確立する。

また最近我々は、臨床現場で間質性肺炎副作用が問題になっている抗癌剤や抗リウマチ薬に関しても、ストレス遺伝子チップによるトランスクリプトソーム解析を行った。その結果、これらの医薬品が抗炎症作用を持つタンパク質の発現を強く抑えることを見出した。またこれまで成功していなかった薬剤性間質性肺炎の実験動物モデルの確立に成功し、これらの抗炎症タンパク質の減少が薬剤性間質性肺炎の原因になっていることを示唆した。以上の成果を受けて本研究で我々は、薬剤性間質性肺炎、及び他の医薬品副作用に関して、その発症機構を解明し、新薬候補品の副作用、及び患者の副作用感受性を予測する方法を確立すると共に、副作用の少ない新薬の開発に向けた研究も行う。

B. 研究方法

抗癌剤（ゲフィチニブ（イレッサ）な

ど)、抗リウマチ薬（レフルノミド、エタネルセプトなど)、漢方薬(小柴胡湯など)による間質性肺炎副作用（薬剤性間質性肺炎）が臨床現場で大きな問題になっているが、その発症メカニズムはほとんど分かっていない。また欧米では、我が国ほど薬剤性間質性肺炎は問題になっていない。そこで薬剤性間質性肺炎発症機構を解明し、新薬候補品の副作用、及び患者の副作用感受性を予測する方法を確立すると共に、副作用の少ない新薬を開発することは大変重要である。これまでに我々は、これらの医薬品がSOD、HO-1、Nrf2、HSPなど抗炎症作用を持つタンパク質の発現を強く抑えることを見出している。

薬剤性間質性肺炎研究が遅れていたのは、その動物モデルが確立されていなかったためである。我々は、TNF-α（薬剤性間質性肺炎において重要な役割を果たしている)、及び低用量ブレオマイシン（高用量ブレオマイシン単独で、間質性肺炎症状が現れる）をあらかじめ投与したマウスに、レフルノミドやエタネルセプトを投与すると、間質性肺炎症状が現れることを見出し、薬剤性間質性肺炎モデルを確立したと考えている。このモデルにおいて、PC-SOD（SODを修飾し安定性を高めた製剤で、現在、間質性肺炎治療薬としての臨床試験中)、及びHO-1の誘導剤により、この間質性肺炎様症状が改善することを見出した。以上の結果は、これらの医薬品が抗炎症タンパク質を低下させることにより、間質性肺炎を引き起こしている可能性を示している。

そこで我々は、他の薬剤性間質性肺炎を起こす薬剤、及びその他の薬剤をこのモデルで検討し、このモデルが新薬候補品の間質性肺炎副作用を予測するシステムとして使用出来るかを検討する。また、SOD、Nrf2、HSPのノックアウトマウスや過剰発現マウスにおける薬剤性間質性肺炎を調べることにより、これらの因子が薬剤性間質性肺炎に関与していることを証明する。

C. 研究結果

昨年度我々は、以前に確立した薬剤性間質性肺炎に関する動物モデルにおいて、薬剤性間質性肺炎を起こす種々の薬剤が間質性肺炎様症状（組織傷害、炎症性細胞の浸潤、組織の繊維化、肺機能の低下）を起こすことを見出した。またPC-SODやピルフェニドンなど間質性肺炎に対して有効性を示した医薬品により、この症状が改善することを見出した。この結果は、我々の確立したこの動物モデルが新薬候補品の間質性肺炎副作用を予測するシステム、及び間質性肺炎治療薬の評価システムとして有用であることを示唆している。

一方我々は昨年度、中用量ブレオマイシンをあらかじめ投与したマウスにレフルノミドを投与すると、TNF-αを投与し

なくても間質性肺炎様症状が誘導されることを見出した（より簡便な動物モデルの確立)。またこの時、上皮細胞の上皮間葉転換（EMT、最近間質性肺炎に深く関与していることが報告）が起こることを見出したので、今年度はさらに解析を進めた。まずレフルノミド依存の EMT を試験管内で再現し、これがレフルノミドのピリミジン合成阻害作用に依存していること、及びこの EMT が Notch シグナル系を介する新しい機構で誘導されることを見出した。さらに我々はレフルノミドを化学的に修飾し、EMT 誘導作用、及び間質性肺炎誘導作用を弱めることに成功した。以上の結果は、レフルノミドは上皮細胞の EMT を起こすことにより、薬剤性間質性肺炎を誘導することを示唆している。

さらに我々はウリジンを経気道投与することにより、レフルノミド投与依存の肺での EMT 誘導、及び肺の繊維化をほぼ完全に抑制できることを見出した。この結果は、レフルノミド依存の薬剤肺線維症の治療法の確立に繋がると考えている。

このように本研究により、これまでほとんど分かっていなかった薬剤性間質性肺炎誘導機構がかなり明らかになった。特に今年度の我々の結果から、薬剤性間質性肺炎に上皮細胞の EMT が関与することが初めて示唆された。この結果は、薬剤性間質性肺炎の予防（新薬候補品の間質性肺炎副作用の予測)、及びその治療薬の開発に大きく貢献すると思われる。

D.考察

結果の欄に記載した

E.結論

このように平成 20 年度の我々の研究により、これまでほとんど分かっていなかった薬剤性間質性肺炎誘導機構がかなり明らかになり、また我々の確立した動物モデルが有用であることが示唆された。

F.健康危険情報

該当なし

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H.知的財産権の出願・登録状況

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研究成果に刊行に関する一覧表

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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.

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

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

Materials and Methods

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Results

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

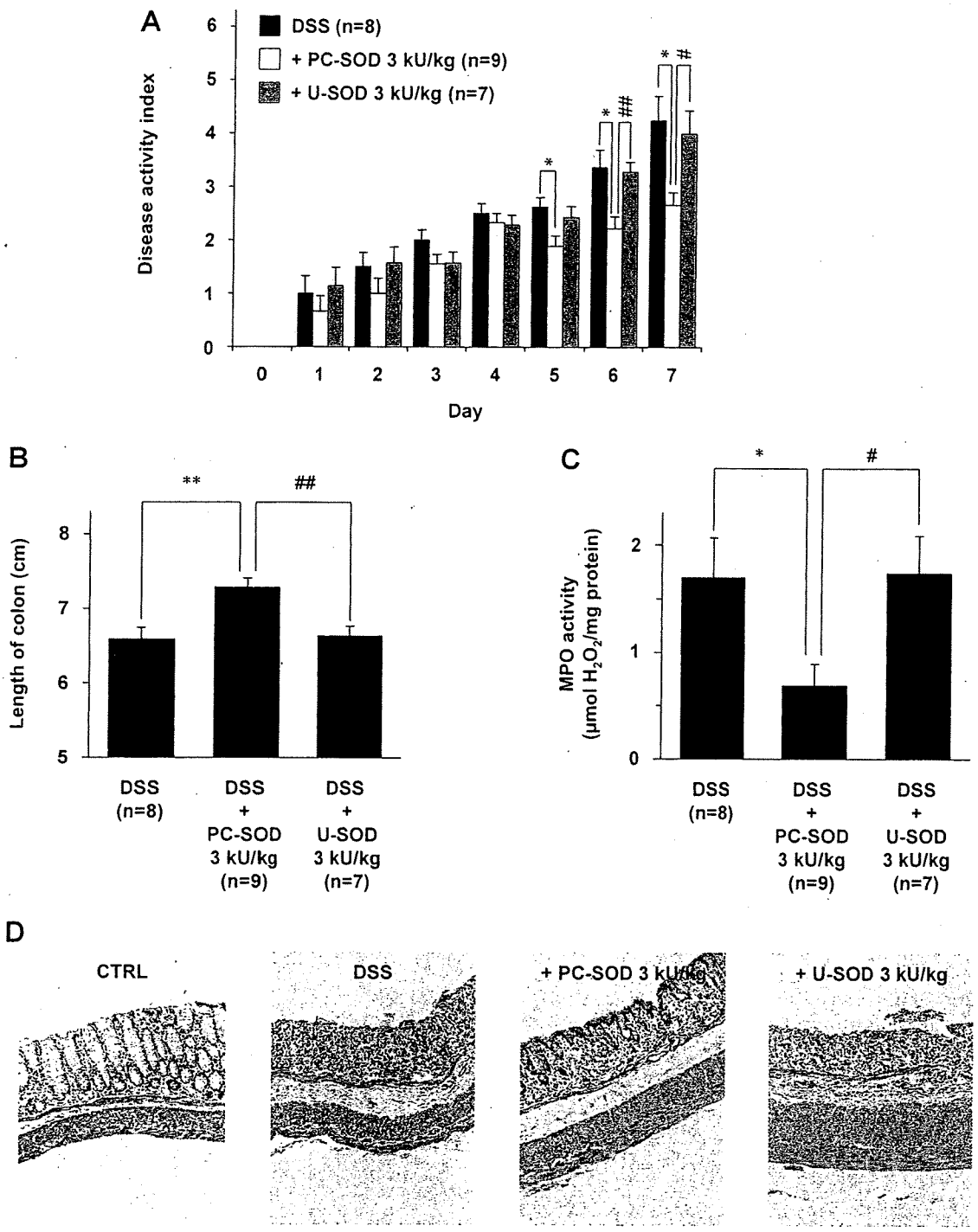

Fig. 1. Effect of PC-SOD and U-SOD on development of DSS-induced colitis. Mice treated with 4% DSS for 7 days, as described under *Materials and Methods*, were intravenously administered PC-SOD or U-SOD once daily. DAI was measured daily (A). The length of the colon (B) and colonic MPO activity (C) were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

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

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

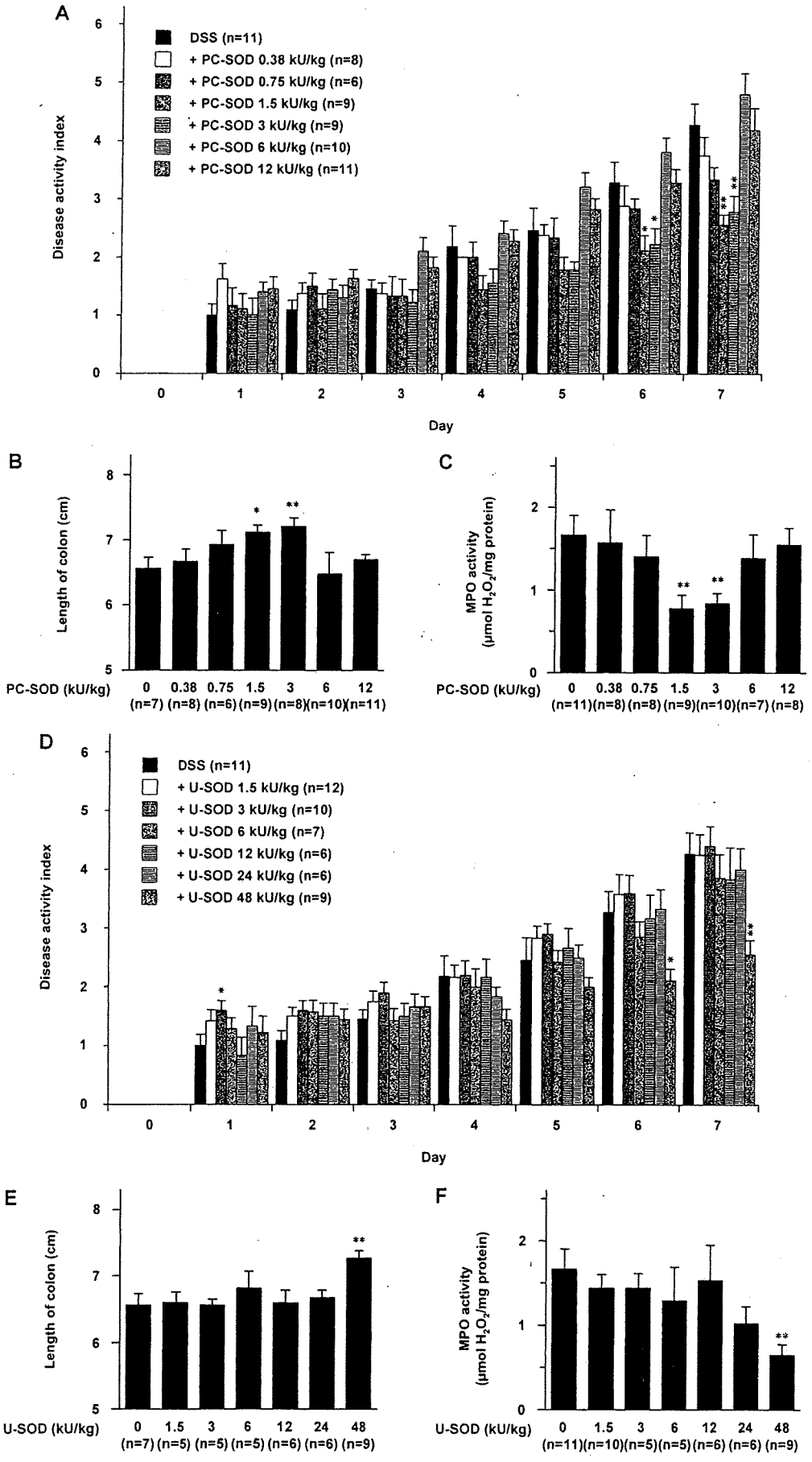

Fig. 2. Dose-response profile of effect of PC-SOD and U-SOD on development of DSS-induced colitis. Mice were treated with DSS and PC-SOD (A–C) or U-SOD (D–F), and colitis was assessed as described in the legend of Fig. 1. Values are mean ± S.E.M. *, $P < 0.05$; **, $P < 0.01$ (versus without PC-SOD or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

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

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

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

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

TABLE 1

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

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

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

TABLE 2

Serum and colonic levels of PC-SOD

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

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

* $P < 0.05$.

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

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

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