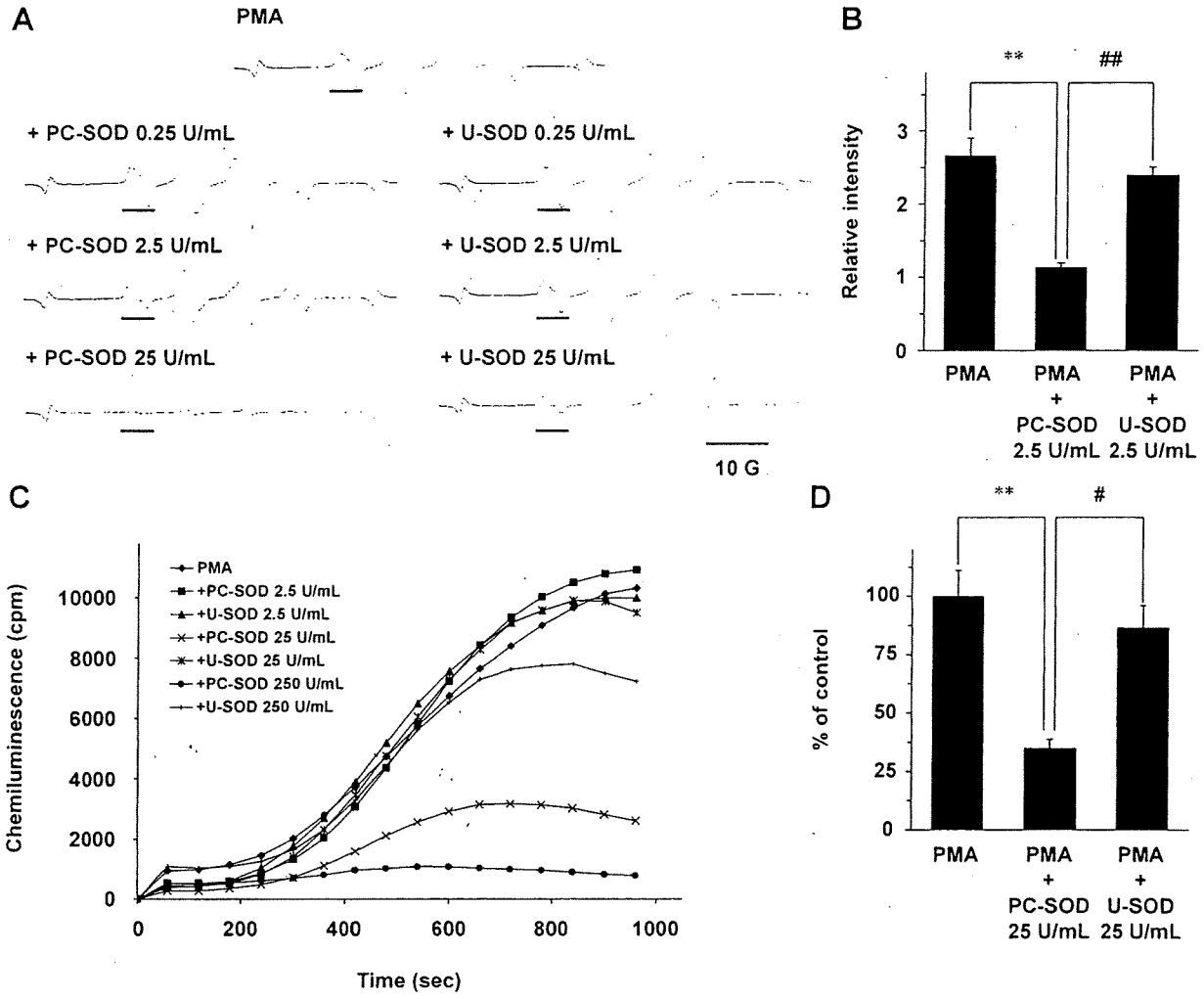


Fig. 3. Effect of PC-SOD or U-SOD on the amount of superoxide anion in vitro. Human neutrophils were preincubated with PC-SOD or U-SOD for 1 h and washed with medium. Neutrophils were then activated with PMA, and the amount of superoxide anion was measured by ESR (A and B) or CL analysis (C and D). The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

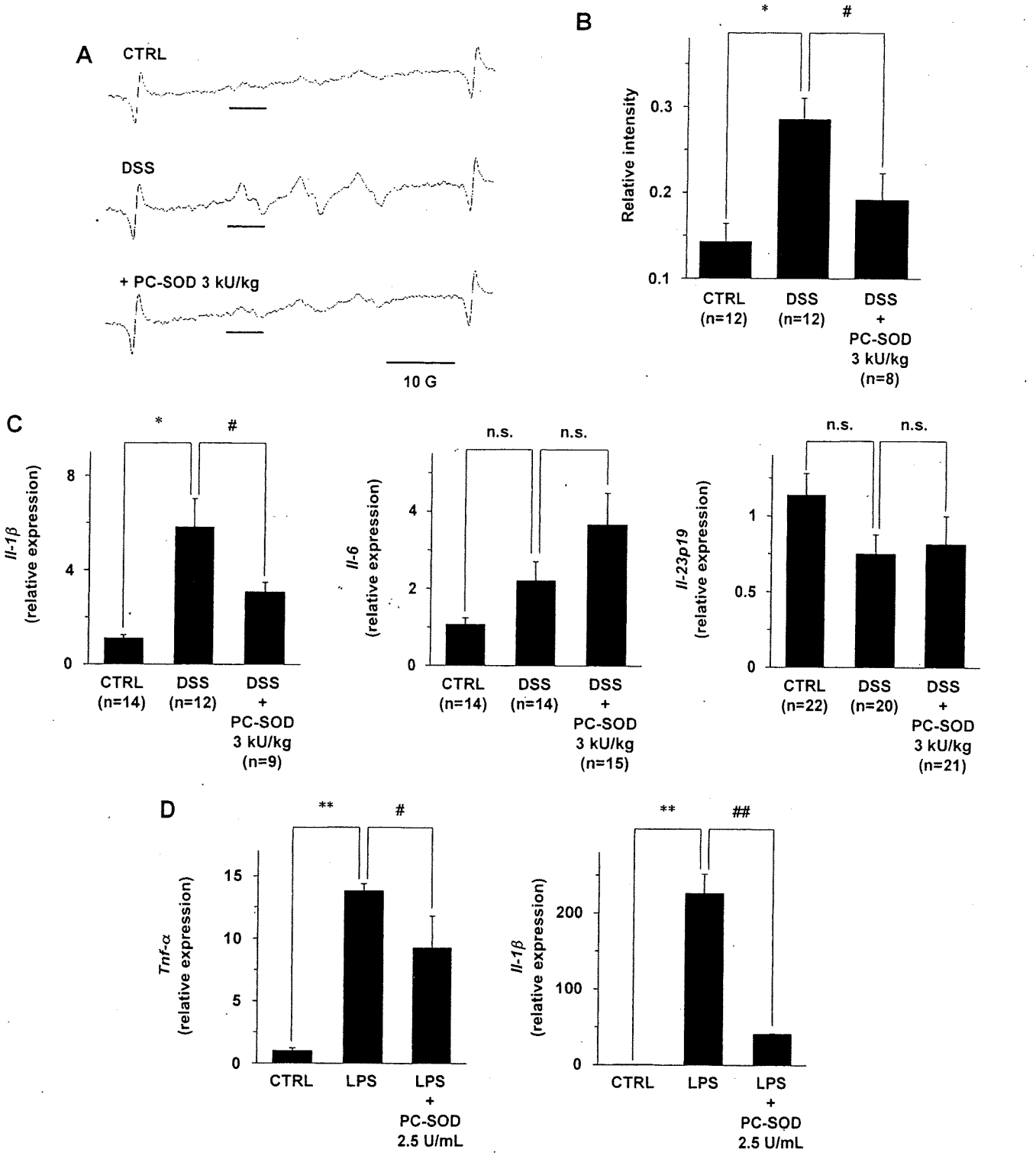


Fig. 4. Effect of PC-SOD on the level of ROS and expression of cytokines. DSS and PC-SOD were administered to mice, as described in the legend of Fig. 1 (A–C). After 7 days, POBN was administered and the colons were dissected and subjected to radical adduct ESR spectrum analysis (A). The intensity of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

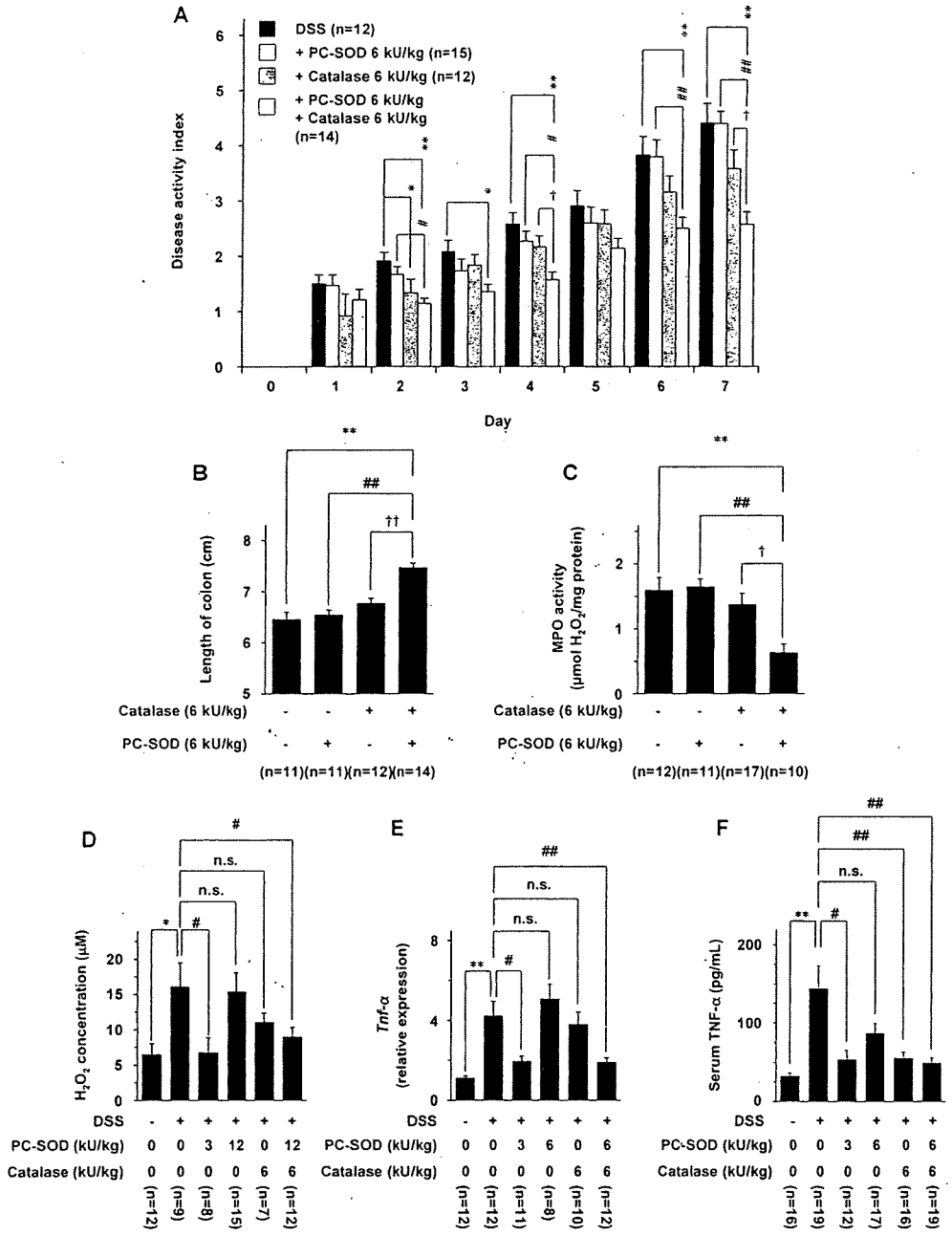


Fig. 5. The effect of simultaneous administration of catalase on the ameliorative effect of PC-SOD on DSS-induced colitis, colonic level of hydrogen peroxide, TNF-α, and NF-κB. Mice were treated with DSS and PC-SOD (A–G), and colitis was assessed (A–C), as described in the legend of Fig. 1. Catalase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

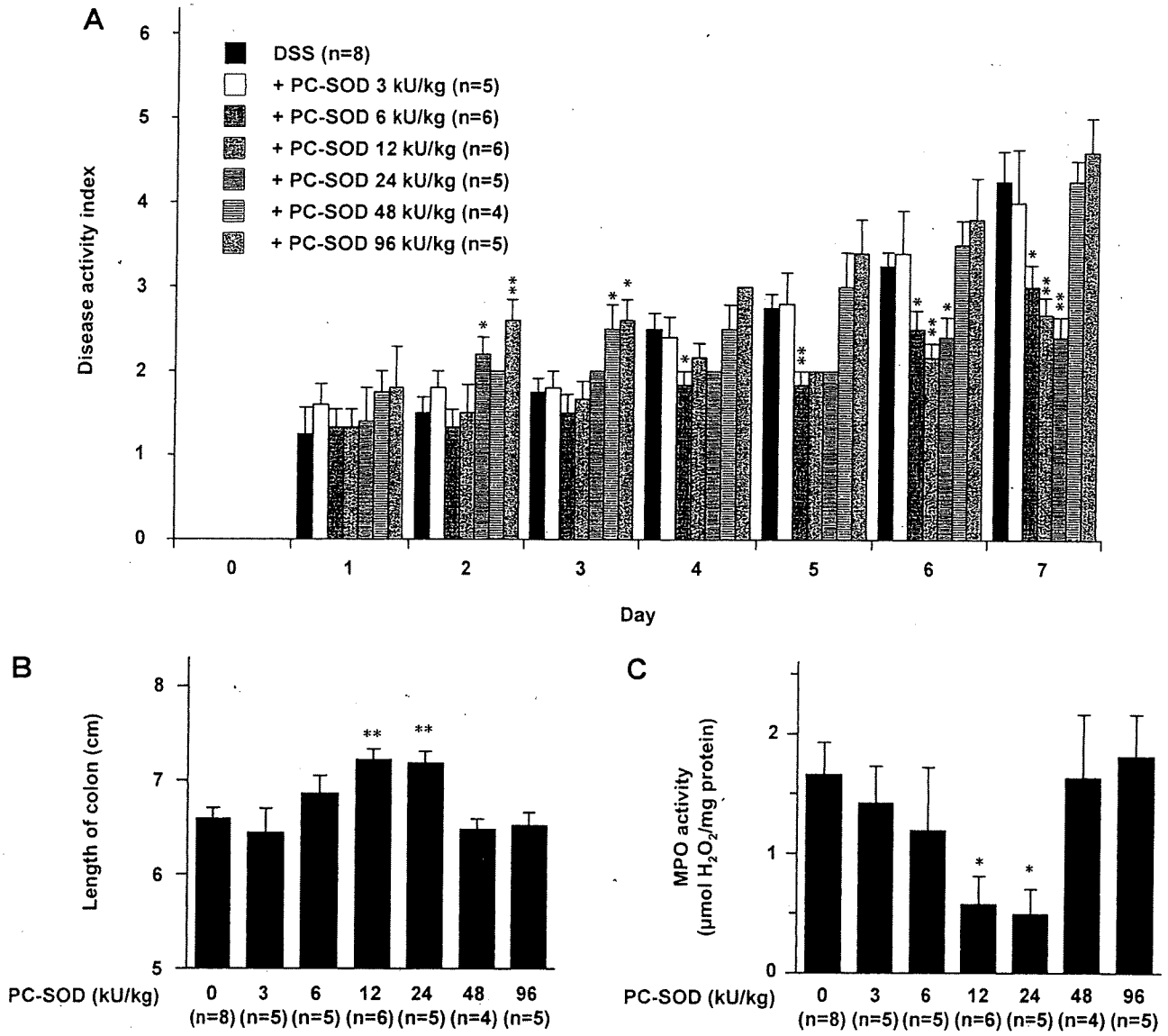


Fig. 6. Effect of a single dose of PC-SOD on the development of DSS-induced colitis. PC-SOD was intravenously administered to mice once at the start of DSS treatment (day 0). Colitis was subsequently assessed, as described in the legend of Fig. 1. Values are mean ± S.E.M. *, $P < 0.05$; **, $P < 0.01$.

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

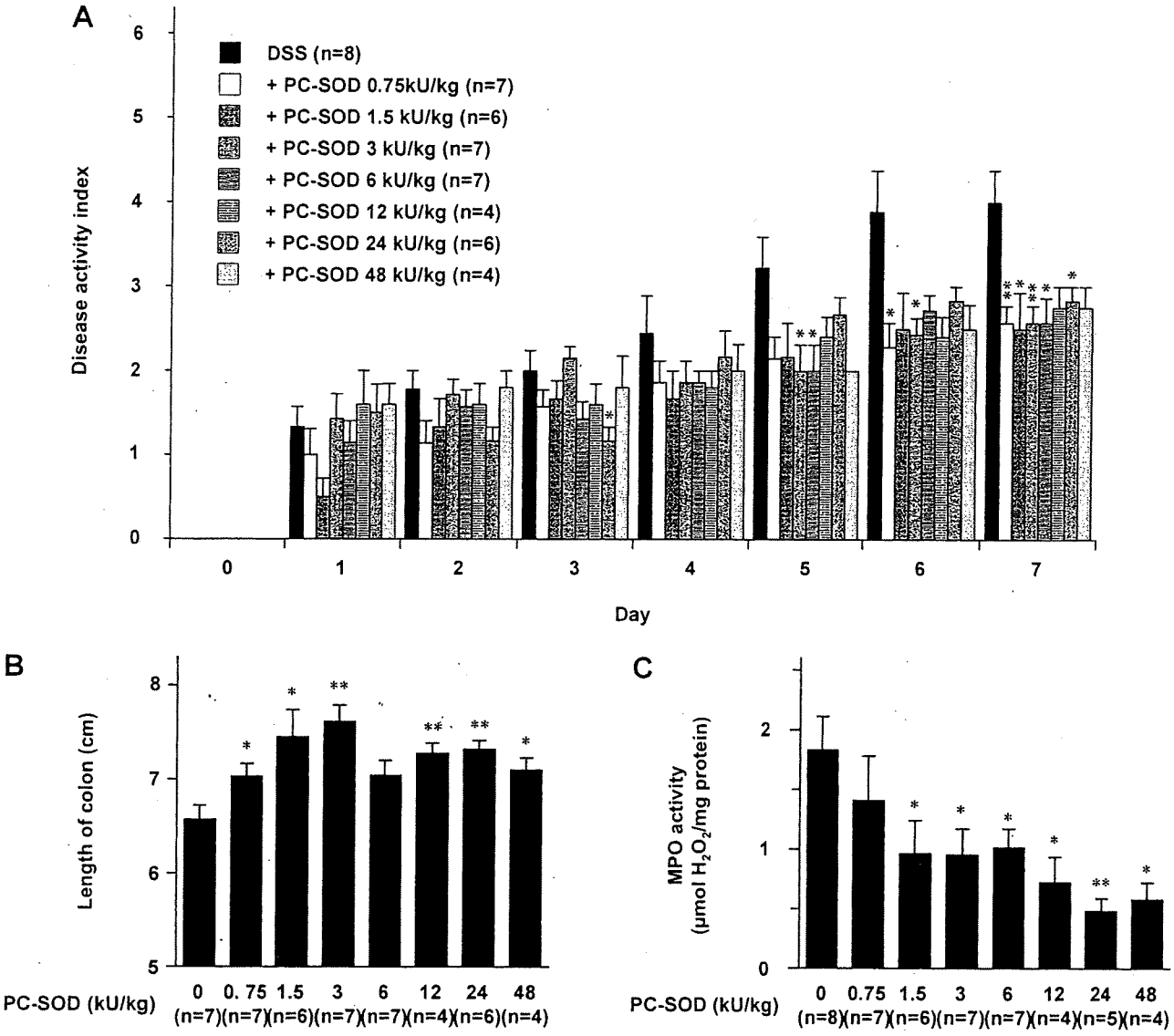


Fig. 7. Effect of oral administration of PC-SOD on development of DSS-induced colitis. PC-SOD was orally administered to mice once daily. Colitis was assessed, as described in the legend of Fig. 1. Values are mean ± S.E.M. *, $P < 0.05$; **, $P < 0.01$.

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.

References

Baumgart DC and Sandborn WJ (2007) Inflammatory bowel disease: clinical aspects and established and evolving therapies. *Lancet* **369**:1641–1657.

Bongartz T, Sutton AJ, Sweeting MJ, Buchan I, Matteson EL, and Montori V (2006) Anti-TNF antibody therapy in rheumatoid arthritis and the risk of serious infections and malignancies: systematic review and meta-analysis of rare harmful effects in randomized controlled trials. *JAMA* **295**:2275–2285.

Broeyer FJ, van Aken BE, Suzuki J, Kemme MJ, Schoemaker HC, Cohen AF, Mizushima Y, and Burggraaf J (2008) The pharmacokinetics and effects of a long-acting preparation of superoxide dismutase (PC-SOD) in man. *Br J Clin Pharmacol* **65**:22–29.

Chikawa T, Ikata T, Katoh S, Hamada Y, Kogure K, and Fukuzawa K (2001) Preventive effects of lecithinized superoxide dismutase and methylprednisolone on spinal cord injury in rats: transcriptional regulation of inflammatory and neurotrophic genes. *J Neurotrauma* **18**:93–103.

Cuzzocrea S (2003) Emerging biotherapies for inflammatory bowel disease. *Expert Opin Emerg Drugs* **8**:339–347.

den Hartog GJ, Haenen GR, Boven E, van der Vijgh WJ, and Bast A (2004) Lecithinized copper,zinc-superoxide dismutase as a protector against doxorubicin-induced cardiotoxicity in mice. *Toxicol Appl Pharmacol* **194**:180–188.

Greenwald RA (1990) Superoxide dismutase and catalase as therapeutic agents for human diseases: a critical review. *Free Radic Biol Med* **8**:201–209.

Herfarth H, Brand K, Rath HC, Rogler G, Schölmerich J, and Falk W (2000) Nuclear factor-kappa B activity and intestinal inflammation in dextran sulphate sodium (DSS)-induced colitis in mice is suppressed by gliotoxin. *Clin Exp Immunol* **120**: 59–65.

Hori Y, Hoshino J, Yamazaki C, Sekiguchi T, Miyauchi S, Mizuno S, and Horie K (1997) Effect of lecithinized-superoxide dismutase on the rat colitis model induced by dextran sulfate sodium. *Jpn J Pharmacol* **74**:99–103.

Igarashi R, Hoshino J, Ochiai A, Morizawa Y, and Mizushima Y (1994) Lecithinized superoxide dismutase enhances its pharmacologic potency by increasing its cell membrane affinity. *J Pharmacol Exp Ther* **271**:1672–1677.

Igarashi R, Hoshino J, Takenaga M, Kawai S, Morizawa Y, Yasuda A, Otani M, and Mizushima Y (1992) Lecithinization of superoxide dismutase potentiates its protective effect against Forssman antiserum-induced elevation in guinea pig airway resistance. *J Pharmacol Exp Ther* **262**:1214–1219.

Institute of Laboratory Animal Resources (1996) *Guide for the Care and Use of Laboratory Animals*, 7th ed. Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, Washington, DC.

Karakawa T, Sato K, Muramoto Y, Mitani Y, Kitamado M, Iwanaga T, Nabeshima T, Maruyama K, Nakagawa K, Ishida K, et al. (2008) Applicability of new spin trap agent, 2-diphenylphosphinoyl-2-methyl-3,4-dihydro-2*H*-pyrrole *N*-oxide, in biological system. *Biochem Biophys Res Commun* **370**:93–97.

Keane J, Gershon S, Wise RP, Mirabile-Levens E, Kasznica J, Schwieterman WD,

Siegel JN, and Braun MM (2001) Tuberculosis associated with infliximab, a tumor necrosis factor alpha-neutralizing agent. *N Engl J Med* **345**:1098–1104.
Keshavarzian A, Morgan G, Sedghi S, Gordon JH, and Doria M (1990) Role of reactive oxygen metabolites in experimental colitis. *Gut* **31**:786–790.
Kruidenier L, Kuiper I, van Duijn W, Marklund SL, van Hogezand RA, Lamers CB, and Verspaget HW (2003a) Differential mucosal expression of three superoxide dismutase isoforms in inflammatory bowel disease. *J Pathol* **201**:7–16.
Kruidenier L, Kuiper I, Van Duijn W, Mieremet-Ooms MA, van Hogezand RA, Lamers CB, and Verspaget HW (2003b) Imbalanced secondary mucosal antioxidant response in inflammatory bowel disease. *J Pathol* **201**:17–27.
Kruidenier L, van Meeteren ME, Kuiper I, Jaarsma D, Lamers CB, Zijlstra FJ, and Verspaget HW (2003c) Attenuated mild colonic inflammation and improved survival from severe DSS-colitis of transgenic Cu/Zn-SOD mice. *Free Radic Biol Med* **34**:753–765.
Kruidenier L and Verspaget HW (2002) Review article: oxidative stress as a pathogenic factor in inflammatory bowel disease–radicals or ridiculous? *Aliment Pharmacol Ther* **16**:1997–2015.
Mahgoub AA, El-Medany AA, Hager HH, Mustafa AA, and El-Sabah DM (2003) Evaluating the prophylactic potential of zafirlukast against the toxic effects of acetic acid on the rat colon. *Toxicol Lett* **145**:79–87.
Mao GD, Thomas PD, Lopaschuk GD, and Poznansky MJ (1993) Superoxide dismutase (SOD)-catalase conjugates: role of hydrogen peroxide and the Fenton reaction in SOD toxicity. *J Biol Chem* **268**:416–420.
Marikovsky M, Ziv V, Nevo N, Harris-Cerruti C, and Mahler O (2003) Cu/Zn superoxide dismutase plays important role in immune response. *J Immunol* **170**: 2993–3001.
Mima S, Tsutsumi S, Ushijima H, Takeda M, Fukuda I, Yokomizo K, Suzuki K, Sano K, Nakanishi T, Tomisato W, et al. (2005) Induction of claudin-4 by nonsteroidal anti-inflammatory drugs and its contribution to their chemopreventive effect. *Cancer Res* **65**:1868–1876.
Muranaka H, Suga M, Sato K, Nakagawa K, Akaike T, Okamoto T, Maeda H, and Ando M (1997) Superoxide scavenging activity of erythromycin-iron complex. *Biochem Biophys Res Commun* **232**:183–187.
Nakajima H, Hangaishi M, Ishizaka N, Taguchi J, Igarashi R, Mizushima Y, Nagai R, and Ohno M (2001) Lecithinized copper, zinc-superoxide dismutase ameliorates ischemia-induced myocardial damage. *Life Sci* **69**:935–944.
Podolsky DK (2002) Inflammatory bowel disease. *N Engl J Med* **347**:417–429.
Salimuddin, Nagasaki A, Gotoh T, Isobe H, and Mori M (1999) Regulation of the genes for arginase isoforms and related enzymes in mouse macrophages by lipopolysaccharide. *Am J Physiol* **277**:E110–E117.
Sato K, Akaike T, Kohno M, Ando M, and Maeda H (1992) Hydroxyl radical production by H_2O_2 plus Cu,Zn-superoxide dismutase reflects the activity of free copper released from the oxidatively damaged enzyme. *J Biol Chem* **267**:25371–25377.
Sato K, Kadiiska MB, Ghio AJ, Corbett J, Fann YC, Holland SM, Thurman RG, and Mason RP (2002) In vivo lipid-derived free radical formation by NADPH oxidase in acute lung injury induced by lipopolysaccharide: a model for ARDS. *FASEB J* **16**:1713–1720.
Schmidt KN, Amstad P, Cerutti P, and Baeuerle PA (1995) The roles of hydrogen peroxide and superoxide as messengers in the activation of transcription factor NF-kappa B. *Chem Biol* **2**:13–22.
Schreiber S, Nikolaus S, and Hampe J (1998) Activation of nuclear factor kappa B inflammatory bowel disease. *Gut* **42**:477–484.
Seguí J, Gironella M, Sans M, Granell S, Gil F, Gimeno M, Coronel P, Piqué JM, and Panés J (2004) Superoxide dismutase ameliorates TNBS-induced colitis by reducing oxidative stress, adhesion molecule expression, and leukocyte recruitment into the inflamed intestine. *J Leukoc Biol* **76**:537–544.
Simmonds NJ, Allen RE, Stevens TR, Van Someren RN, Blake DR, and Rampton DS (1992) Chemiluminescence assay of mucosal reactive oxygen metabolites in inflammatory bowel disease. *Gastroenterology* **103**:186–196.
Suzuki J, Broeyer F, Cohen A, Takebe M, Burggraaf J, and Mizushima Y (2008a) Pharmacokinetics of PC-SOD, a lecithinized recombinant superoxide dismutase, after single- and multiple-dose administration to healthy Japanese and Caucasian volunteers. *J Clin Pharmacol* **48**:184–192.
Suzuki Y, Matsumoto T, Okamoto S, and Hibi T (2008b) A lecithinized superoxide dismutase (PC-SOD) improves ulcerative colitis. *Colorectal Dis* **10**:931–934.
Tamagawa K, Taooka Y, Maeda A, Hiyama K, Ishioka S, and Yamakido M (2000) Inhibitory effects of a lecithinized superoxide dismutase on bleomycin-induced pulmonary fibrosis in mice. *Am J Respir Crit Care Med* **161**:1279–1284.
Tanaka K, Namba T, Arai Y, Fujimoto M, Adachi H, Sobue G, Takeuchi K, Nakai A, and Mizushima T (2007) Genetic evidence for a protective role for heat shock factor 1 and heat shock protein 70 against Colitis. *J Biol Chem* **282**:23240–23252.
Tsao C, Greene P, Odlind B, and Brater DC (1991) Pharmacokinetics of recombinant human superoxide dismutase in healthy volunteers. *Clin Pharmacol Ther* **50**:713–720.
Tsubokawa T, Jadhav V, Solaroglu I, Shiokawa Y, Konishi Y, and Zhang JH (2007) Lecithinized superoxide dismutase improves outcomes and attenuates focal cerebral ischemic injury via antiapoptotic mechanisms in rats. *Stroke* **38**:1057–1062.
Yasui K and Baba A (2006) Therapeutic potential of superoxide dismutase (SOD) for resolution of inflammation. *Inflamm Res* **55**:359–363.

Address correspondence to: Dr. Tohru Mizushima, Graduate School of Medical and Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-honmachi, Kumamoto 862-0973, Japan. E-mail: mizu@gpo.kumamoto-u.ac.jp

European Journal of Pharmacology 603 (2009) 120–132

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Immunopharmacology and Inflammation

Inhibition of both COX-1 and COX-2 and resulting decrease in the level of prostaglandins E_2 is responsible for non-steroidal anti-inflammatory drug (NSAID)-dependent exacerbation of colitis ☆

Ken-Ichiro Tanaka, Shintaro Suemasu, Tomoaki Ishihara, Yuichi Tasaka, Yasuhiro Arai, Tohru Mizushima *

Graduate School of Medical and Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-honmachi, Kumamoto 862-0973, Japan

ARTICLE INFO

Article history:
Received 10 July 2008
Received in revised form 7 November 2008
Accepted 24 November 2008
Available online 6 December 2008

Keywords:
NSAIDs
Cyclooxygenase-1
Cyclooxygenase-2
Prostaglandins E_2
Colitis

ABSTRACT

A number of clinical studies have shown that non-steroidal anti-inflammatory drugs (NSAIDs) exacerbate inflammatory bowel disease; however the molecular mechanism whereby this occurs remains unclear. NSAIDs inhibit cyclooxygenase (COX), which has subtypes COX-1 and COX-2. In this study, we have examined the effect of various types of NSAIDs on the development of dextran sulfate sodium (DSS)-induced colitis, an animal model of inflammatory bowel disease. The DSS-induced colitis was worsened by administration of non-selective NSAIDs but not by COX-1 or COX-2 selective inhibitors. However, administration of a combination of both COX-1- and COX-2-selective inhibitors exacerbated the colitis. The intestinal level of PGE_2 dramatically decreased in response to administration of COX-1- and COX-2-selective inhibitors, and exogenously administered PGE_2 suppressed the exacerbation of colitis by NSAIDs. The expression of mucin proteins, which protect the intestinal mucosa, was suppressed by non-selective NSAIDs and this expression was restored by PGE_2, both *in vivo* and *in vitro*. Intestinal mucosal cell growth was inhibited by non-selective NSAIDs and this cell growth was restored by PGE_2, both *in vivo* and *in vitro*. This study provides evidence that inhibition of both COX-1 and COX-2 and the resulting dramatic decrease in the intestinal level of PGE_2 is responsible for NSAID-dependent exacerbation of DSS-induced colitis. Furthermore, expression of mucin proteins and intestinal mucosal cell growth seems to be involved in this exacerbation and its suppression by PGE_2.

1. Introduction

Inflammatory bowel disease has become substantial health problems. Recent studies suggest that inflammatory bowel disease involves chronic inflammatory disorders in the intestine due to the infiltration of activated leukocytes into intestinal tissues, the subsequent intestinal mucosal damage induced by reactive oxygen species that are released from the activated leukocytes, and, as a result of this mucosal damage, invasion of intestinal pathogenic bacteria across the intestinal mucosa (Podolsky, 2002). To develop a clinical protocol for the treatment of inflammatory bowel disease and to avoid accidental exacerbation of inflammatory bowel disease by clinically used drugs, it is important to know what type of drugs ameliorate or exacerbate the development of inflammatory bowel disease and to understand the underlying molecular mechanism. For this purpose, experimental animal colitis models, in particular the dextran sulfate sodium (DSS)-induced colitis models, are useful (Jurjus et al., 2004).

Non-steroidal anti-inflammatory drugs (NSAIDs) are a useful family of therapeutics and the anti-inflammatory actions of NSAIDs are mediated through their inhibitory effects on cyclooxygenase (COX) activity. COX is an enzyme essential for the synthesis of prostaglandins, which have a strong capacity to induce inflammation. Therefore, it was tentatively proposed that NSAIDs would be effective for the treatment of inflammatory bowel disease; however, clinical and animal studies have shown that NSAIDs exacerbate the development of inflammatory bowel disease and inflammatory bowel disease-related experimental colitis (Evans et al., 1997; Felder et al., 2000; Kabashima et al., 2002; Yamada et al., 1993). This seems to be due to the protective effects of prostaglandins on the intestinal mucosa through various mechanisms such as stimulation of mucin production, stimulation of mucosal cell growth, inhibition of mucosal apoptosis and inhibition of the production of pro-inflammatory cytokines (Kabashima et al., 2002). Supporting this notion, it was reported that inflammatory bowel disease-related experimental colitis can be attenuated by pre-treatment with exogenous prostaglandins (Tessner et al., 1998). However, it is not clear that NSAIDs exacerbate inflammatory bowel disease through decreasing the intestinal level of prostaglandins, because recent studies on gastric mucosa have shown that NSAIDs directly, in other words in a manner

☆ This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Health, Labour, and Welfare of Japan, as well as Japan Science and Technology Agency and Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

* Corresponding author. Tel./fax: +81 96 371 4323.
E-mail address: mizu@gpo.kumamoto-u.ac.jp (T. Mizushima).

0014-2999/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2008.11.058

independent of COX-inhibition, damage gastric mucosal cells, which contributes to the formation of NSAID-induced gastric lesions (Tomisato et al., 2004). Furthermore, some studies have shown contradictory results which indicate that NSAIDs are safe for the treatment of inflammatory bowel disease and inflammatory bowel disease-related experimental colitis (Bonner et al., 2000; Takeuchi et al., 2006).

COX has two subtypes, COX-1 and COX-2. COX-1 is constitutively expressed in various tissues and play an important role in physiological homeostasis, whereas COX-2 is induced under inflammatory conditions, including in the intestine of inflammatory bowel disease patients and animal models of inflammatory bowel disease (Fukata et al., 2006; Singer et al., 1998; Tessner et al., 1998). Recently, COX-2-selective inhibitors have been developed as NSAIDs that are safer for gastric mucosa. Clinical and animal studies suggest that COX-2-selective inhibitors are also safe and, in some cases, beneficial for the treatment of inflammatory bowel disease patients and inflammatory bowel disease-related experimental colitis (El Miedany et al., 2006; El-Medany et al., 2005; Mahadevan et al., 2002; Martin et al., 2005); however, the mechanism by which they act is unclear. Furthermore, some studies have shown conflicting results: COX-2-selective inhibitors exacerbated the development of inflammatory bowel disease and colitis in animal models of inflammatory bowel disease (Bonner, 2001; Okayama et al., 2007; Reuter et al., 1996). On the other hand, the involvement of COX-1 in inflammatory bowel disease remains unclear. Since it is not uncommon for NSAIDs to be administered to inflammatory bowel disease patients accidentally or intensively (Evans et al., 1997), it is important to know what types of NSAIDs (for example, COX-1- and COX-2-selective inhibitors) are safe for inflammatory bowel disease patients. In this study, we have examined the effects of various NSAIDs on the development of DSS-induced colitis and found that the colitis was exacerbated by treatment with both COX-1- and COX-2-selective inhibitors. We also suggest that this exacerbation is due to a decrease in the intestinal level of PGE_2. Furthermore, we suggest that the protective effect of PGE_2 against DSS-induced colitis is mediated by various mechanisms, such as by induction of the expression of mucin proteins, stimulation of epithelial cell proliferation and suppression of reactive oxygen species-induced cell death in the intestine.

Table 1
Effect of various NSAIDs on colon shortening associated with DSS-induced colitis

Treatment	Length of colon (cm)
Vehicle	8.6±0.06
DSS	6.9±0.25
+SC-560 (2.5 mg/kg)	6.5±0.19
+SC-560 (5 mg/kg)	6.4±0.28
+SC-560 (10 mg/kg)	6.9±0.35
+Celecoxib (5 mg/kg)	6.9±0.25
+Celecoxib (10 mg/kg)	6.8±0.24
+Celecoxib (20 mg/kg)	7.2±0.28
+SC-560 (5 mg/kg) and Celecoxib (10 mg/kg)	5.8±0.24[a]
+Indomethacin (1 mg/kg)	5.8±0.06[a]

ICR mice were treated with or without 3% DSS for 7 days.
The indicated dose of each NSAID was administered daily. After 7 days, the colon lengths were determined as described in the Materials and methods. Values are mean±S.E.M. (n=4–15).
[a] $P<0.05$, vs. Control.

2. Materials and methods

2.1. Chemicals and animals

Celecoxib was from LKT Laboratories (St. Paul, MN). Paraformaldehyde, peroxidase standard, fetal bovine serum (FBS), o-dianisidine, 5-bromo-2′-deoxyuridine (BrdU), 3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), menadione, hexadecyl trimethyl ammonium bromide (HTAB) and 2′-(4-ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5′-bi-1H-benzimidazole trihydrochloride (Hoechst 33342) were obtained from Sigma (St. Louis, MO). The antibody to BrdU was from Santa Cruz Biotechnology Inc (Santa Cruz, CA). Indomethacin and DSS (M.W. 5000, 15–20% sulfur content) were from WAKO Pure Chemicals (Tokyo, Japan). Lipopolysaccharide (LPS) was

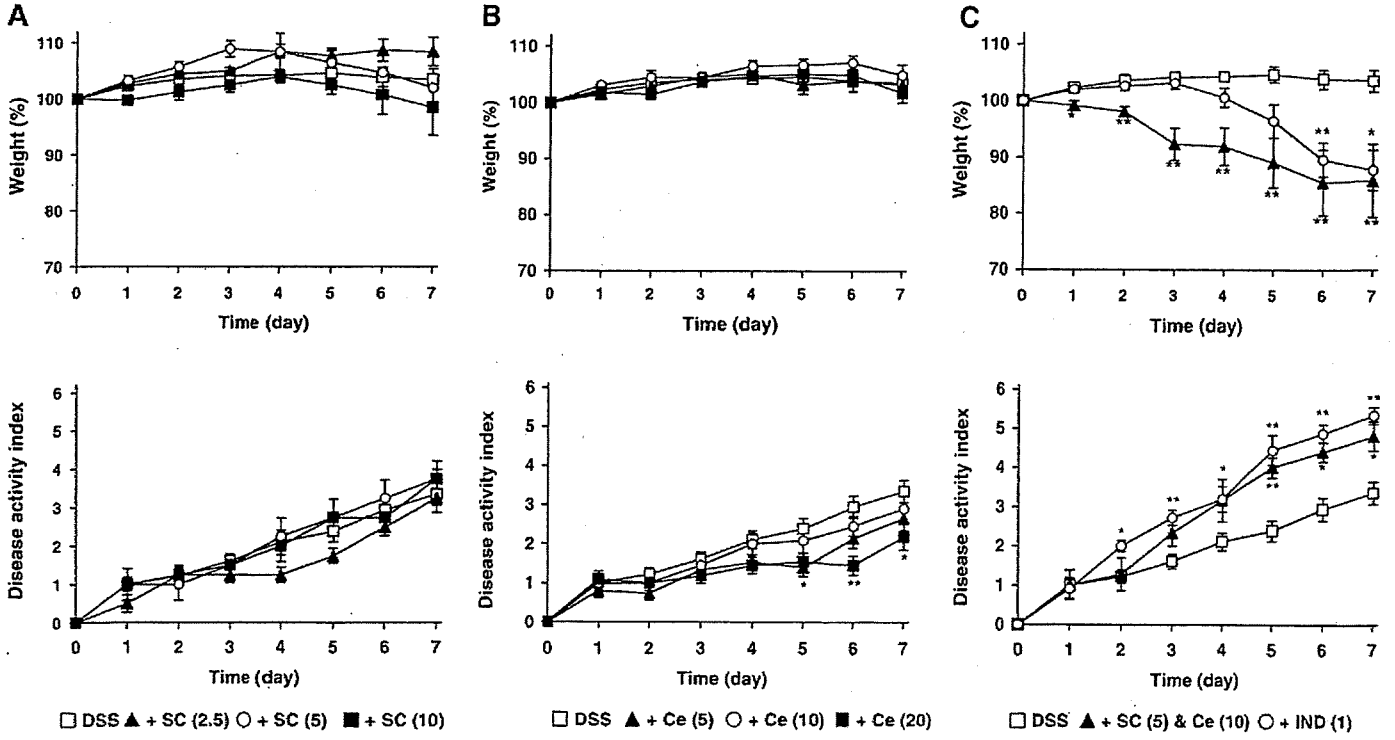


Fig. 1. Effect of various NSAIDs on DSS-induced colitis. DSS-induced colitis was developed as described in the legend of Table 1. The indicated dose (mg/kg) of SC-560 (SC) (A, C), celecoxib (Ce) (B, C) or indomethacin (IND) (C) was administered daily. Body weight and disease activity index were measured daily. Values are mean±S.E.M. (n=4–18). **$P<0.01$; *$P<0.05$.

from List Biological Laboratories, Inc (Campbell, CA). Optimal cutting temperature (O.C.T.) compound was from Sakura Finetek Japan (Tokyo, Japan). Mayer's hematoxylin and malinol were from MUTO Pure Chemicals (Tokyo, Japan). Terminal deoxynucleotidyl transferase (TdTase) was obtained from TOYOBO (Osaka, Japan). Alcian blue was from Nacalei Tesque (Kyoto, Japan). Nuclear fast red was from Merck KGaA (Darmstadt, Germany). The Envision kit was from Dako Co (Carpinteria, CA). Biotin 14-ATP and Alexa Fluor 488 conjugated with streptavidin were purchased from Invitrogen (Carlsbad, CA). VECTASHIELD was from Vector Laboratories (Burlingame, CA). 4′, 6-diamidino-2-phenylindole-, dihydrochloride (DAPI) was from Dojindo Laboratories (Kumamoto, Japan). The RNeasy kit was obtained from Qiagen (Valencia, CA), first-strand cDNA synthesis kit was from GE Healthcare (Little Chalfont, UK) and iQ SYBR Green Supermix was from Bio-Rad (Hercules, CA). 5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-(trifluoromethyl)-1H-pyrazole (SC-560), the ELISA kit for PGE_2 and 16, 16-dimethyl PGE_2 (dm-PGE_2) were from Cayman Chemicals (An Arbor, MI). IEC6 (rat intestinal epithelial cell) and RAW264 (mouse leukemic monocyte) cells were from RIKEN BioResource Center (Tsukuba, Japan) and bEnd.3 (mouse

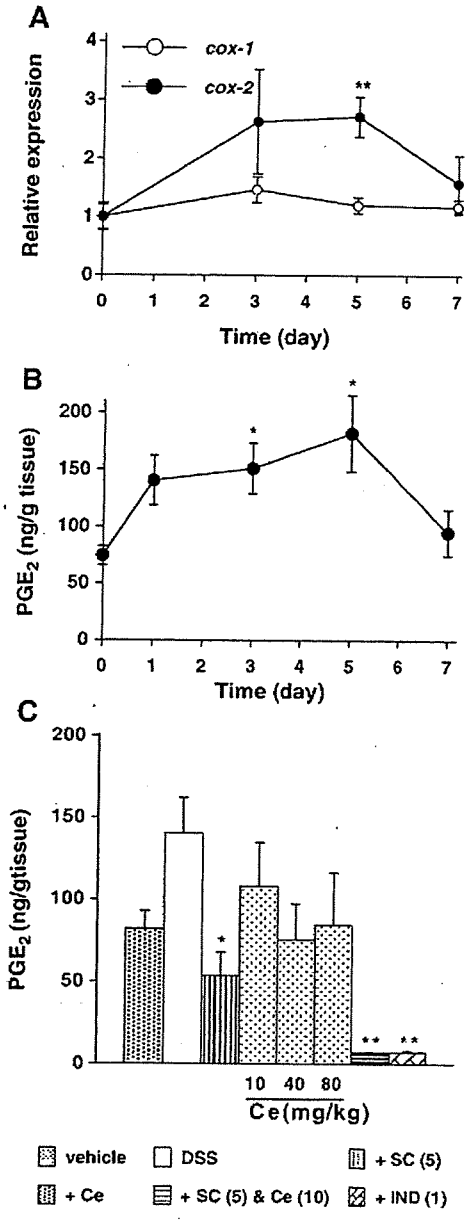


Fig. 2. The intestinal level of PGE_2 in DSS-treated mice. DSS-induced colitis was developed (A–C) and NSAIDs were administered (C) as described in the legend of Fig. 1. Colonic tissues were removed periodically and total RNA was extracted. Samples were subjected to real-time RT-PCR, using a specific primer set for *cox-1* or *cox-2*. Values were normalized to the *GAPDH* gene, expressed relative to the control sample (i.e. mice without DSS-treatment) (A). After the indicated number of days (B) or 3 days (C), colonic tissues were removed and PGE_2 levels were determined as described in the Materials and methods (B, C). Values are mean ± S.E.M. (n=4–18). **P<0.01; *P<0.05.

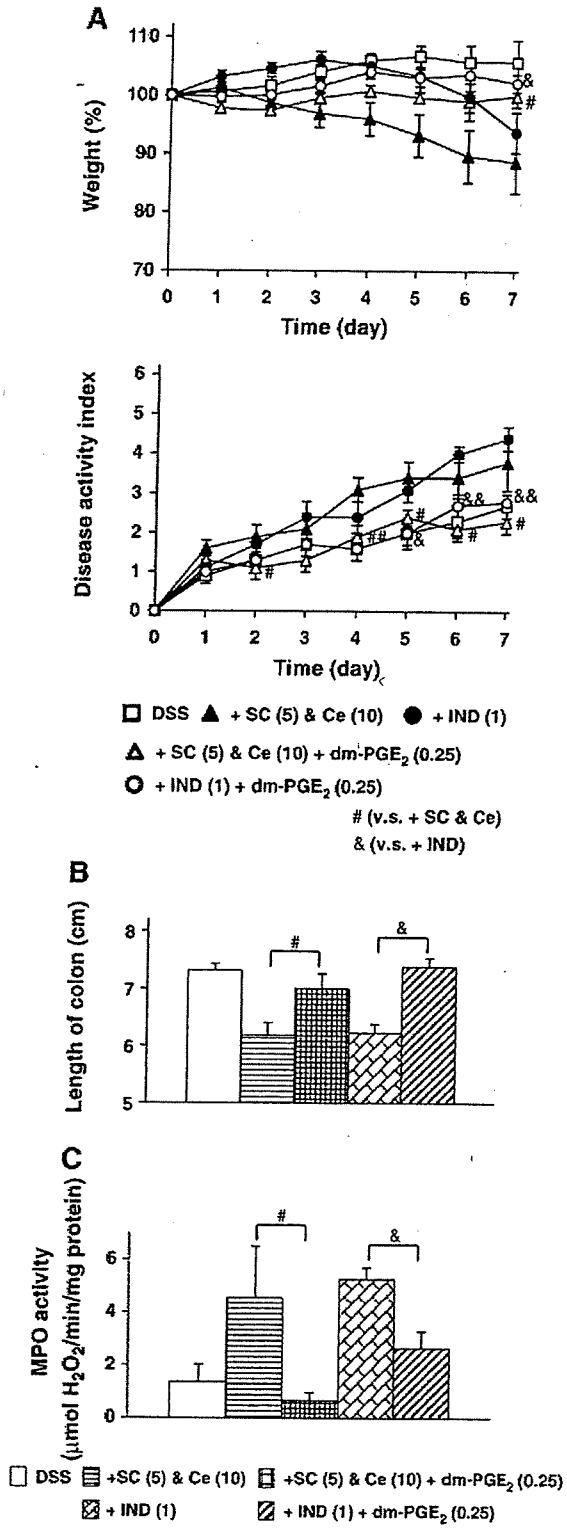


Fig. 3. Effect of dm-PGE_2 on exacerbation of DSS-induced colitis by NSAIDs. DSS-induced colitis was developed and NSAIDs were administered as described in the legend of Fig. 1. dm-PGE_2 (0.25 mg/kg) was administered twice per day. Body weight and (A) and colon length (B) were measured as described in the legend of Fig. 1 and Table 1, respectively. MPO activity was measured as described in the Materials and methods (C). Values are mean ± S.E.M. (n=4–11). ## (or &&)P<0.01; # (or &)P<0.05.

brain endothelioma) cells were from the American Type Culture Collection (Rockville, MD). We used IEC6 cells because it is normal cell line and RAW264 and bEnd.3 cells because they are standard cell lines and used in many previous paper. ICR mice were from Kyudo Co. (Kumamoto, Japan). 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 Institute of Health, and were approved by the Animal Care Committee of Kumamoto University.

2.2. Development of DSS-induced colitis and measurement of colon length and disease activity index

Colitis was induced in mice by the addition of 3% DSS (w/v, final concentration) to their drinking water as described previously (Tanaka et al., 2007). The animals were allowed free access to the DSS-containing water for 7 days. Over that time, NSAIDs were orally administered daily and dm-PGE_2 was intraperitoneally administered twice per day.

After 7 days, animals were placed under deep ether anaesthesia and sacrificed, the colons were dissected and their length measured from the ileocecal junction to the anal verge.

The disease activity index was determined macroscopically by an observer unaware of the treatment they had received, according to previously reported criteria (Tanaka et al., 2007). Briefly, the disease activity index 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: normal color stool; 1: brown color stool; 2: reddish color stool; 3: bloody stool).

For histopathological observation, measurement of PGE_2, myeloperoxidase (MPO), various mRNAs, mucin as well as apoptosis and proliferation study, we used rectum and distal colon.

For labelling with BrdU to examine epithelial cell proliferation, 1 h before the mice were sacrificed, BrdU (100 mg/kg) was injected intraperitoneally as described previously (Kabashima et al., 2002).

The intestinal level of PGE_2 was determined by ELISA according to the manufacturer's protocol, as previously described (Futaki et al., 1993).

2.3. Myeloperoxidase (MPO) activity

MPO activity in the colonic tissues was measured as previously described (Krawisz et al., 1984; Tanaka et al., 2007). After 7 days of DSS treatment, animals were placed under deep ether anaesthesia and sacrificed. Colons were dissected, rinsed with cold saline and cut into small pieces. Samples were homogenized, freeze-thawed and centrifuged. The protein concentrations of the supernatants were determined using the Bradford method (Bradford, 1976). MPO activity was determined in 10 mM phosphate buffer with 0.5 mM o-dianidisine, 0.00005% (w/v) hydrogen peroxide and 20 μg protein. MPO activity was obtained from the slope of the reaction curve and its specific activity was expressed as the number of hydrogen peroxide molecules converted per min per mg protein.

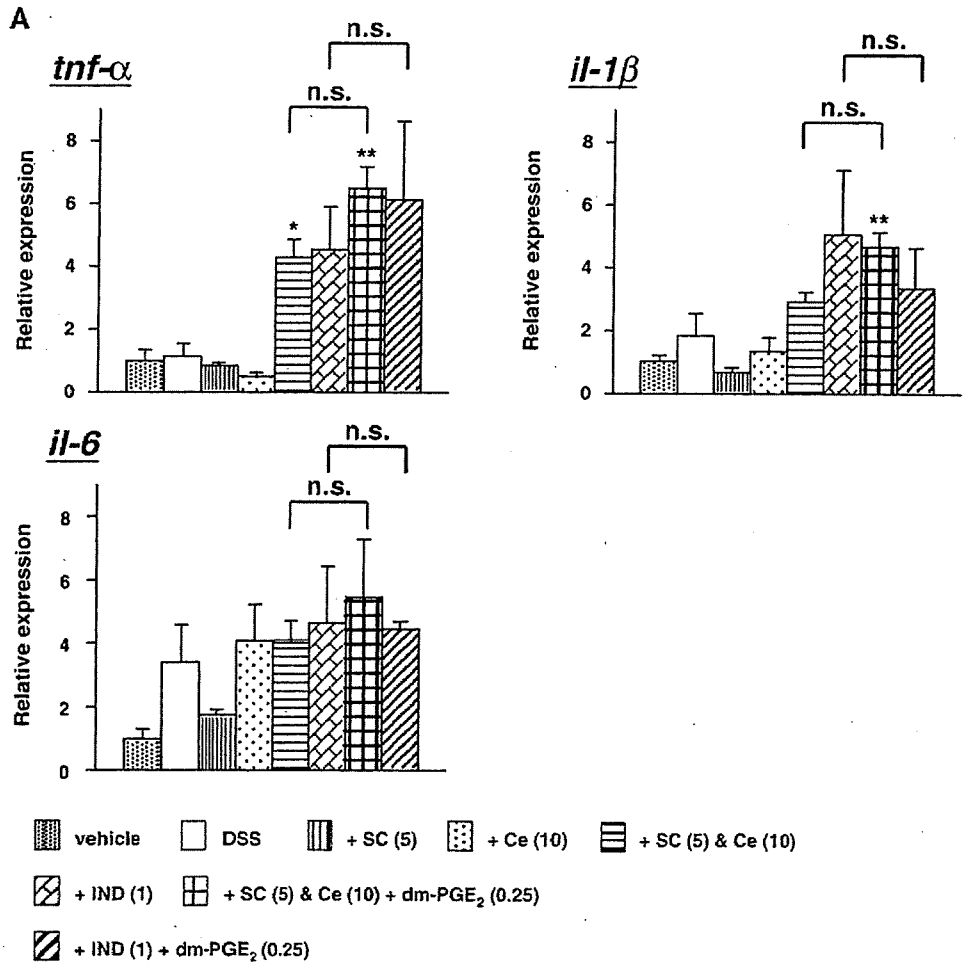


Fig. 4. Effect of NSAIDs and dm-PGE_2 on the mRNA expression of various proteins in the intestinal tissues of DSS-treated mice. DSS-induced colitis was developed and NSAIDs and dm-PGE_2 were administered as described in the legend of Fig. 3. The relative mRNA expression of each gene in the intestinal tissues was monitored and expressed as described in the legend of Fig. 2A. Values are mean ± S.E.M. (n=3–7). **(or ## or &&)P<0.01; *(or # or &)P<0.05; n.s., not significant.

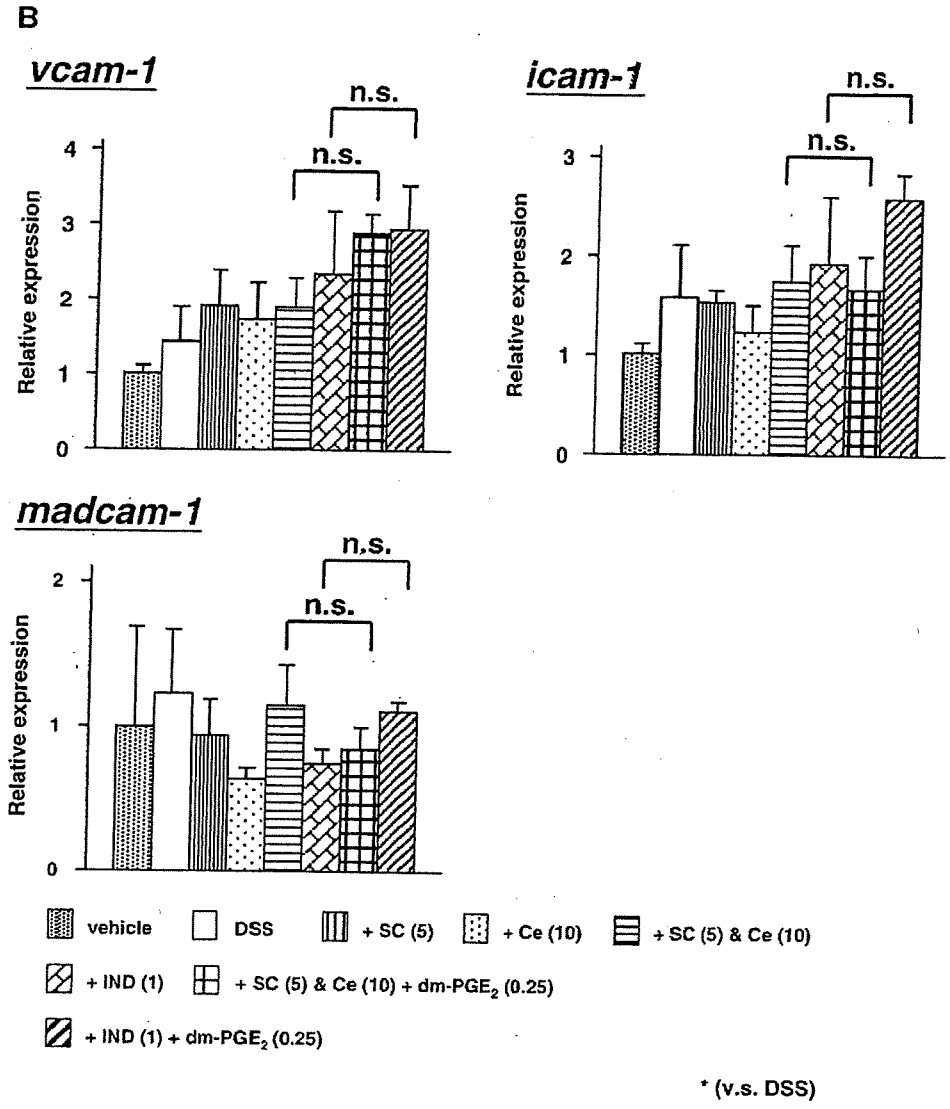


Fig. 4 (*continued*).

2.4. Real-time RT-PCR analysis

Total RNA was extracted from intestinal tissues using an RNeasy kit according to the manufacturer's protocol. Samples (2.5 μg RNA) were reverse-transcribed using a first-strand cDNA synthesis kit according to the manufacturer's instructions. Synthesized cDNA was used in real-time RT-PCR (Chromo 4 instrument (Bio-Rad)) experiments using iQ SYBR GREEN Supermix and analyzed with Opticon Monitor Software according to the manufacturer's instructions. The real-time PCR cycle conditions were 2 min at 50 °C, followed by 10 min at 90 °C and finally 45 cycles of 95 °C for 30 s and 63 °C for 60 s. 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 website (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The primers used were (name: forward primer, reverse primer): for mouse; *tumor necrosis factor (tnf)-α*: 5′-cgtcagccgatttgctatct-3′, 5′-cggactccgcaaagtctaag-3′; *interleukin (il)-1β*: 5′-gatcccaagcaatacccaaa-3′, 5′-ggggaactctgcagactcaa-3′; *il-6*: 5′-ctggagtcacagaaggagtgg-3′, 5′-ggtttgccgagtagatctcaa-3′; *vascular cell adhesion molecule (vcam)-1*: 5′-ctcctgcacttgtggaaatg-3′, 5′-tgtacgagccatccacagac-3′; *intercellular adhesion molecule (icam)-1*: 5′-tcgtgatggcagcctcttat-3′, 5′-gggcttgtcccttgagtttt-3′; *mucosal addressin cell adhesion molecule (madcam)-1*: 5′-gcaggctgggagctactct-3′, 5′-tccctcttgtggtaggttgc-3′; *cox-1*: 5′-cggtgacatcgatgctttag-3′, 5′-ggagcccccatctctatcat-3′; *cox-2*: 5′-tgctatctttggggagacca-3′, 5′-gctcggcttccagtattgag-3′; *muc2*: 5′-gctgacgagtggttggtgaatg-3′, 5′-gatgaggtggcagacaggagac-3′; *muc3*: 5′-cgtggtcaactgcgagaatgg-3′, 5′-cggctctatctctacgctctcc-3′; for rat; actin: 5′-gatcattgctcctcctgagc-3′, 5′-actcctgcttgctgatccac-3′; *muc2*: 5′-gaggacaggcccatctatga-3′, 5′-cagatcctccaggtgggtag-3′; *muc3*: 5′-atgcacaaaggcaagagtcc-3′, 5′-ctcaaagccaatgttgtgga-3′.

2.5. Histological and immunohistochemical analysis

Colonic tissue samples were fixed in 4% buffered paraformaldehyde, embedded in O.C.T. compound and cryosectioned.

For immunohistochemical analysis, sections were treated in a microwave oven with 0.01 M citric acid buffer (pH 6.0) for antigen activation and incubated with 0.3% hydrogen peroxide-containing methanol for removal of endogenous peroxidase. Sections were blocked with 2.5% goat serum for 10 min, incubated for 12 h with antibody against BrdU (1:100 dilution) in the presence of 2.5% BSA and then incubated for 1 h with peroxidase-labelled polymer conjugated to goat anti-mouse immunoglobulins (Envision kit). Then, 3, 3′-diaminobenzidine (DAB) was applied to the sections and the sections were finally incubated with Mayer's hematoxylin. Samples were mounted with malinol and inspected using a fluorescence microscope (Olympus BX51).

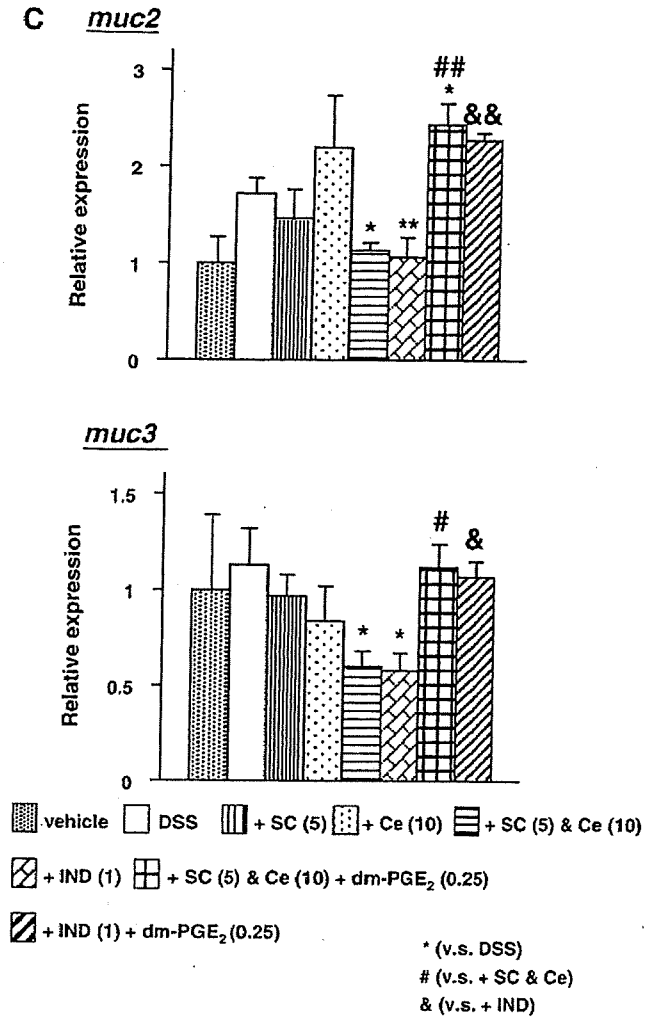


Fig. 4 (*continued*).

For staining of mucus with Alcian blue, sections were placed in 3% acetic acid for 3 min, then in 1% Alcian blue and 3% acetic acid (pH 2.5) for 10 min. Sections were then incubated in 0.1% nuclear fast red for 1 min as a counter stain and mounted with malinol.

2.6. TdT-mediated dUTP-biotin end labelling (TUNEL) assay

Colonic tissue samples were fixed in 4% buffered paraformaldehyde, embedded in O.C.T. compound and cryosectioned. Sections were incubated first with proteinase K (20 μg/ml) for 15 min at 37 °C, then with TdTase and biotin 14-ATP for 1 h at 37 °C and finally with Alexa Fluor 488 conjugated with streptavidin and DAPI (5 μg/ml) for 2 h. Samples were mounted with VECTASHIELD and inspected using a fluorescence microscope (Olympus BX51).

2.7. Cell culture and staining with Hoechst 33342

Cells were cultured in RPMI1640 medium supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere of 95% air with 5% CO_2 at 37 °C. Unless otherwise noted, cells (0.8×10^4 cells per well in 24-well plates, 4×10^4 cells per well in 6-well plates, 6×10^5 cells in 100-mm plates) were cultured for 24 h prior to use in experiments. Apoptotic chromatin condensation was monitored as described previously (Tsutsumi et al., 2002). Cells were washed with PBS, stained with 10 μg/ml Hoechst 33342 and observed under a fluorescence microscope.

2.8. Statistical analysis

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

3. Results

3.1. Effect of various NSAIDs on DSS-induced colitis

The severity of DSS-induced colitis can be monitored by various indices, such as body weight, disease activity index and length of colon. We examined the effect of SC-560, a COX-1-selective inhibitor, on the time-course of development of colitis induced by 3% DSS administration by monitoring body weight and disease activity index. Administration of 3% DSS caused a mild increase in the (Fig. 1A), which is consistent with our previous results (Tanaka et al., 2007). Administration of SC-560 did not affect the DSS-induced increase in the disease activity index and alteration in body weight even at the highest tested dose (10 mg/kg) (Fig. 1A). Celecoxib, a COX-2-selective inhibitor did not worsen the DSS-induced colitis (Fig. 1B). It was reported that either SC-560 or celecoxib worsened the DSS-induced colitis (Okayama et al., 2007) and the discrepancy may be due to the difference in animal species and dose of DSS. Furthermore, disease activity index data showed weak amelioration of DSS-induced colitis by celecoxib (Fig. 1B). We also examined the effect of administration of a combination of SC-560 and celecoxib on development of DSS-induced colitis. As shown in Fig. 1C, administration of this combination to DSS-treated mice not only reduced body weight but also stimulated an increase in the disease activity index. We confirmed that in mice that had not been treated with DSS, administration of the SC-560/celecoxib combination did not affect body weight and the data not shown. Based on previous reports (Kato et al., 2001), the concentration of SC-560 or celecoxib employed in the experiments described in Fig. 1 C should have been sufficient to inhibit COX-1 or COX-2, respectively. Furthermore, administration of a non-selective NSAID (indomethacin) alone decreased the body weight and stimulated an increase in the disease activity index of DSS-treated mice to a similar extent as treatment with SC-560/celecoxib (Fig. 1C). The exacerbation of DSS-induced colitis by administration of SC-560/celecoxib or indomethacin was confirmed by monitoring another index of colitis, DSS-induced colon shortening, which is used as a morphometric measure for the degree of inflammation (Table 1). All of these results suggest that inhibition of both COX-1 and COX-2 exacerbates DSS-induced colitis.

In order to test this idea, we measured the intestinal level of PGE_2. At first we confirmed, by real-time PCR, that *cox-1* and *cox-2* mRNAs were expressed in the intestinal tissues (Fig. 2A). DSS-administration up-regulated the expression of *cox-2* mRNA but not of *cox-1* mRNA, as described previously (Fukata et al., 2006; Singer et al., 1998), however, the time-course profile was not consistent with data reported previously (Okayama et al., 2007). This may be due to the difference in animal species and dose of DSS. The results displayed in Fig. 2B show how the intestinal level of PGE_2 is altered with the development of DSS-induced colitis. The intestinal level of PGE_2 increased gradually for 5 days after initiation of DSS-treatment and then returned to the original level (Fig. 2B). This transient profile was not observed in previous report and again this may be due to the difference in animal species and dose of DSS. Fig. 2C shows the effect of administration of various NSAIDs on the intestinal level of PGE_2 in DSS-treated mice. Administration of SC-560 weakly decreased the intestinal level of PGE_2; however, celecoxib did not affect the level significantly (Fig. 2C). Furthermore, administration of SC-560/celecoxib dramatically decreased the intestinal level of PGE_2 to a similar extent as was observed in response to indomethacin administration (Fig. 2C). Combining the results summarised in Table 1, Figs. 1 and 2, it seems

that the observed large decrease in the intestinal level of PGE_2 is required for the exacerbation of DSS-induced colitis by NSAIDs.

3.2. Effect of PGE_2 on the exacerbation of DSS-induced colitis by NSAIDs

To confirm the role of PGE_2 in exacerbation of DSS-induced colitis by NSAIDs, we have examined the effect of exogenously administered PGE_2 in this disease model. As shown in Fig. 3A, administration of dm-PGE_2 (a stable analogue of PGE_2) returned the body weight and disease activity index of SC-560/celecoxib- or indomethacin-administered DSS-treated mice to a similar level to that observed in control mice (without NSAID-administration). Similar results were obtained with another index of colitis, colon length (Fig. 3B). Colonic MPO activity, an indicator of leukocyte infiltration, was also increased by administration of SC-560/celecoxib or indomethacin and this increase was suppressed by simultaneous administration of dm-PGE_2 (Fig. 3C). The results shown in Fig. 3 show that administration of dm-PGE_2 suppresses the exacerbation of DSS-induced colitis by NSAIDs and suggest that the decrease in the intestinal level of PGE_2 is responsible for the exacerbation of DSS-induced colitis by NSAIDs.

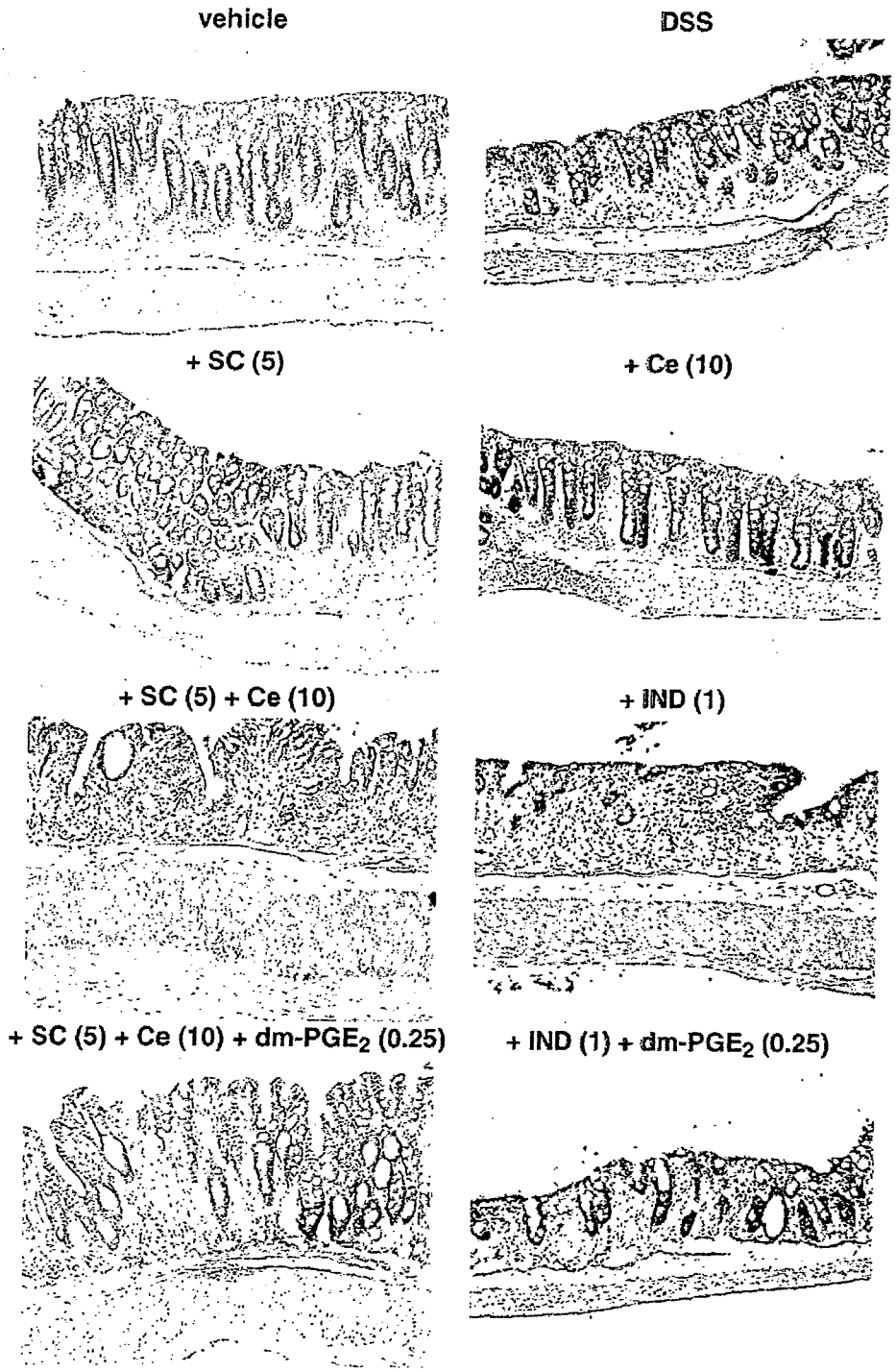


Fig. 5. Effect of NSAIDs and dm-PGE_2 on the amount of mucus in the intestinal mucosa of DSS-treated mice. DSS-induced colitis was developed and NSAIDs and dm-PGE_2 were administered as described in the legend of Fig. 3. Sections of colonic tissues were prepared and subjected to staining with Alcian blue as described in the Materials and methods. Magnification of all photomicrographs is ×100.

3.3. Involvement of cytokines, cell adhesion molecules and mucin proteins in exacerbation of DSS-induced colitis by NSAIDs

Pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-6 and cell adhesion molecules, such as ICAM-1, VCAM-1 and MAdCAM-1, play an important role in the activation and infiltration of leukocytes that is associated with inflammatory bowel disease. In order to understand the mechanism governing the exacerbation of DSS-induced colitis by NSAIDs and its suppression by PGE_2, we compared the mRNA expression of various inflammatory bowel disease-related proteins, such as cytokines, cell adhesion molecules and mucin proteins in the intestinal tissues of DSS-treated mice. As shown in Fig. 4A, *tnf-α* mRNA expression was induced by administration of SC-560/celecoxib; however, this induction was not suppressed by simultaneous administration of dm-PGE_2. Neither SC-560/celecoxib nor dm-PGE_2 affected *il-1β* or *il-6* mRNA expression (Fig. 4A). Likewise, *vcam-1*, *icam-1* and *madcam-1* mRNA expression was not affected by SC-560/celecoxib or dm-PGE_2 (Fig. 4B). We previously reported that DSS-treatment up-regulated the mRNA expression of these cytokines and cell adhesion molecules (Tanaka et al., 2007). The ineffectiveness of DSS on expressions of these factors may be due to that we measured 3 days (instead of 7 days in our previous paper) after the initiation of DSS treatment. On the other hand, *muc2* and *muc3* mRNA expression was inhibited by administration of SC-560/celecoxib or indomethacin, and simultaneous administration of dm-PGE_2 counteracted this effect (Fig. 4C). Staining of tissue with Alcian blue demonstrated that the administration of indomethacin or SC-560/celecoxib decreased the amount of mucus (blue-staining spots) in the intestinal mucosa of DSS-treated mice. Simultaneous administration of dm-PGE_2 counteracted this effect, with mucin levels in these mice equivalent to that of controls (Fig. 5). The results displayed in Figs. 4 and 5 suggest that expression of mucin proteins rather than expression of cytokines and cell adhesion molecules is involved in the observed exacerbation of DSS-induced colitis by NSAIDs and its suppression by PGE_2.

We also examined the effect of NSAIDs and PGE_2 on the mRNA expression of cytokines, cell adhesion molecules and mucin proteins *in vitro*. In RAW264 cells, indomethacin did not affect *tnf-α*, *il-1β* and *il-6* mRNA expression (Table 2A). Similarly, in bEnd.3 cells, indomethacin did not affect *vcam-1*, *icam-1* and *madcam-1* mRNA expression (Table 2B). We confirmed that treatment of these cells with LPS up-regulated the mRNA expression of these genes (up-regulation of *tnf-α* mRNA expression was observed 3 h (data not shown) but not 24 h (Table 2A) after the addition of LPS), which is consistent with previous results (Gupta et al., 1995). On the other hand, in IEC6 cells, *muc2* and *muc3* mRNA expression was inhibited by indomethacin (Table 2C). Treatment of cells with dm-PGE_2 stimulated mRNA expression of these genes (Table 2C), suggesting that indomethacin directly inhibits mRNA expression of mucin proteins through decreasing the level of PGE_2. This effect may be responsible for the decrease in the expression of mucin proteins observed after the administration of indomethacin or SC-560/celecoxib in DSS-treated mice (Figs. 4 and 5).

3.4. Involvement of intestinal mucosal apoptosis and cell growth in the exacerbation of DSS-induced colitis by NSAIDs

Inhibition of cell growth and induction of cell death at the intestinal mucosa also play an important role in the pathogenesis of inflammatory bowel disease, because the intestinal mucosa functions as a barrier against pathogenic bacterial invasion. Inhibition of cell growth and induction of apoptosis at the intestinal mucosa was reported in both inflammatory bowel disease patients and animal models of inflammatory bowel disease (Kabashima et al., 2002; Souza et al., 2005). The cell death associated with inflammatory bowel disease seems to be induced mainly by reactive oxygen species. Mucin proteins (such as MUC2 and MUC3) also function as a barrier to bacterial invasion and mucosal damage by reactive oxygen species (Van der Sluis et al., 2006). Therefore we examined, using the TUNEL assay, the effect of indomethacin administration on the level of apoptosis observed in the intestinal mucosa of DSS-treated mice. More TUNEL-positive cells (apoptotic cells) were observed in the intestinal mucosa of the indomethacin- or SC-560/celecoxib-administered mice than the control mice (Fig. 6A). Furthermore, simultaneous administration of dm-PGE_2 counteracted this effect, with the extent of apoptosis in those animals similar to that of controls (Fig. 6A).

We also examined, using a BrdU-staining method, the effect of indomethacin administration on intestinal mucosal cell proliferation in DSS-treated mice. Less BrdU-positive cells (growing cells, cells stained brown) were observed at the intestinal mucosa of indomethacin- or SC-560/celecoxib-administered mice than the control mice (Fig. 6B, C). Furthermore, simultaneous administration of dm-PGE_2 with the indomethacin resulted in similar numbers of BrdU-positive cells to the control (Fig. 6B, C). The results shown in Fig. 6 suggest that alterations to the levels of cell proliferation and apoptosis in intestinal mucosa are involved in the exacerbation of DSS-induced colitis by NSAIDs and its suppression by PGE_2.

We also examined the effect of indomethacin and PGE_2 on apoptosis and cell growth *in vitro*. As shown in Fig. 7A, the relative number of IEC6 cells with apoptotic chromatin condensation was not increased by treatment with indomethacin, showing that indomethacin did not induce apoptosis under these experimental conditions.

We also used menadione, a superoxide anion (a representative reactive oxygen species) releasing drug, to examine the effect of PGE_2 on reactive oxygen species-induced cell death. As shown in Fig. 7B, cell death induced by treatment with menadione for 24 h was partially suppressed by simultaneous treatment of cells with 0.5 μM dm-PGE_2. We concluded that the cell death described in Fig. 7B was mediated by apoptosis, based on monitoring apoptotic chromatin condensation (data not shown). These results show that PGE_2 protects intestinal cells from reactive oxygen species-induced apoptosis.

Indomethacin significantly inhibited the growth of IEC6 cells and this growth inhibition was suppressed by simultaneous treatment of cells with dm-PGE_2 (Fig. 7C). This suggests that indomethacin directly

Table 2
Effect of indomethacin and dm-PGE_2 on mRNA expression of various proteins *in vitro*

A

Treatment	Relative expression		
	tnf-α	*il-1β*	il-6
Control	1.00±0.09	1.00±0.08	1.00±0.09
Indomethacin (50 μM)	0.94±0.06	1.28±0.05	1.12±0.06
Indomethacin (100 μM)	0.90±0.05	1.26±0.07	0.91±0.09
LPS	1.11±0.08	3.00±0.25[b]	110±6.40[b]

B

Treatment	Relative expression		
	vcam-1	*icam-1*	*madcam-1*
Control	1.00±0.02	1.00±0.05	1.00±0.09
Indomethacin (50 μM)	0.98±0.07	1.15±0.06	1.12±0.06
Indomethacin (100 μM)	0.91±0.06	1.02±0.07	0.91±0.09
LPS	11.1±0.37[b]	4.94±0.37[b]	44.6±3.54[b]

C

Treatment	Relative expression	
	muc-2	*muc-3*
Control	1.00±0.09	1.00±0.07
Indomethacin (100 μM)	0.49±0.12[b]	0.24±0.01[a]
Indomethacin (100 μM)+ dm-PGE_2 (0.5 μM)	2.00±0.13[b,c]	5.05±0.13[a,c]

RAW264 (A) or bEnd.3 (B) cells were incubated with the indicated concentrations of indomethacin (or 5 μg/ml LPS) for 24 h or 18 h, respectively (A, B). IEC6 cells were pre-incubated with or without 0.5 μM dm-PGE_2 for 1 h and further incubated with the indicated concentrations of indomethacin for 24 h in the presence of the same concentration of dm-PGE_2 as was used in the pre-incubation step (C). Relative mRNA expression of each gene was monitored and expressed as described in the legend of Fig. 2A. Values shown are mean±S.E.M. (n=3).
[a]P<0.05, vs. Control; [b]P<0.01, vs. Control; [c]P<0.01, vs. Indomethacin (100 μM).

inhibited the growth of the intestinal cells through decreasing the level of PGE_2 and this effect may be responsible for the inhibition of epithelial cell proliferation in the intestine of DSS-treated mice after the administration of indomethacin *in vivo* (Fig. 6B and C).

4. Discussion

In both clinical and animal studies, it is debatable as to whether NSAIDs are of benefit, have no effect or aggravate inflammatory bowel disease and inflammatory bowel disease-related experimental colitis (Bonner, 2001; Bonner et al., 2000; El Miedany et al., 2006; Evans et al., 1997; Felder et al., 2000; Mahadevan et al., 2002; Takeuchi et al., 2006; Yamada et al., 1993). To utilize NSAIDs for the clinical treatment of inflammatory bowel disease but avoid exacerbation of inflammatory bowel disease by NSAIDs, it is important to know what types of NSAIDs ameliorate or exacerbate the development of inflammatory bowel disease and to understand the underlying molecular mechanisms. In this study, focusing on COX-1/COX-2 specificity, we have examined the effect of various NSAIDs on DSS-induced colitis. Administration of either a COX-1-selective inhibitor (SC-560) or of a COX-2-selective inhibitor (celecoxib) did not affect DSS-induced colitis, however, the colitis was clearly exacerbated by the administration of these drugs in combination or administration of the non-selective NSAID indomethacin. For the combination experiment (the results of which are shown in Fig. 1 and Table 1),

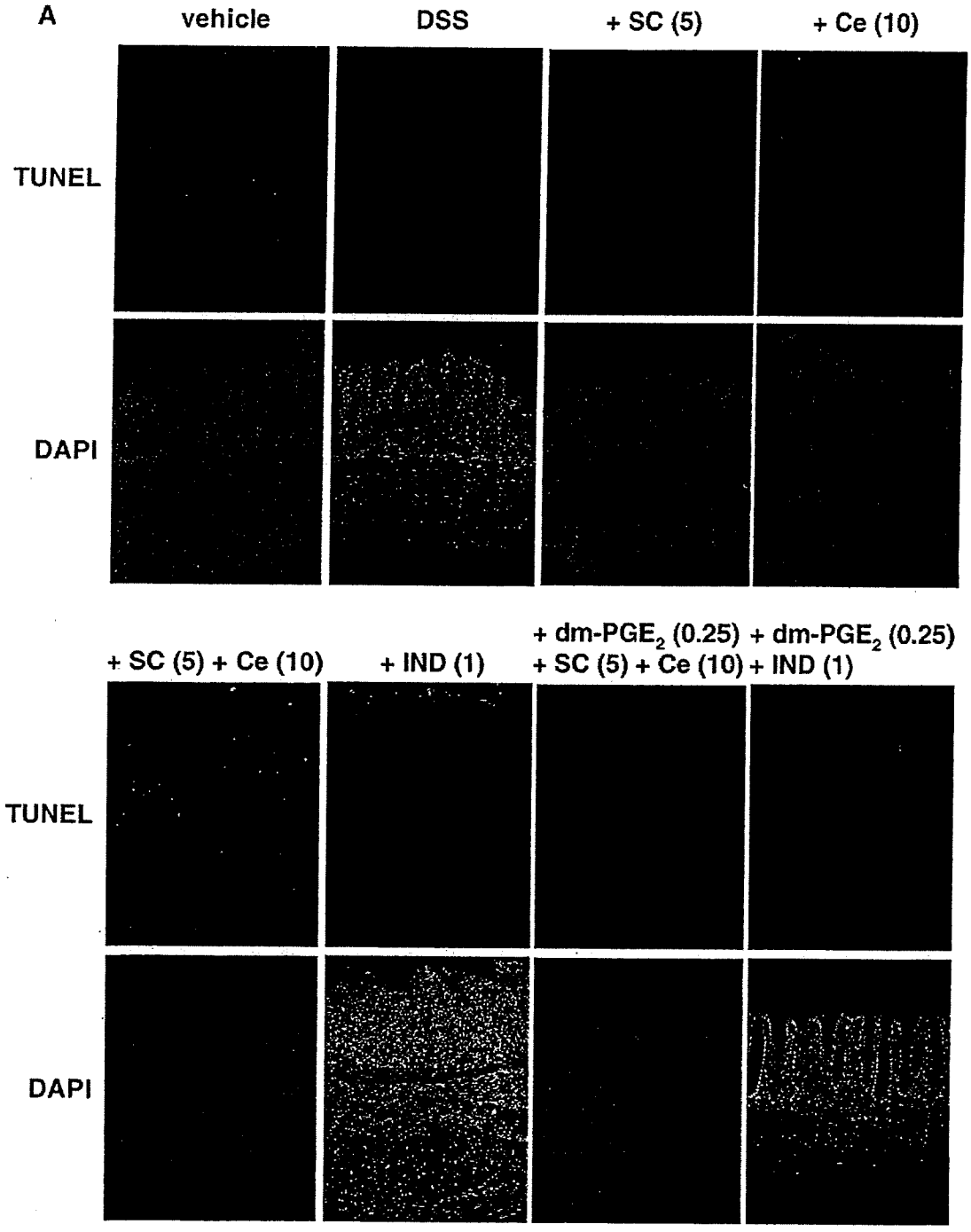
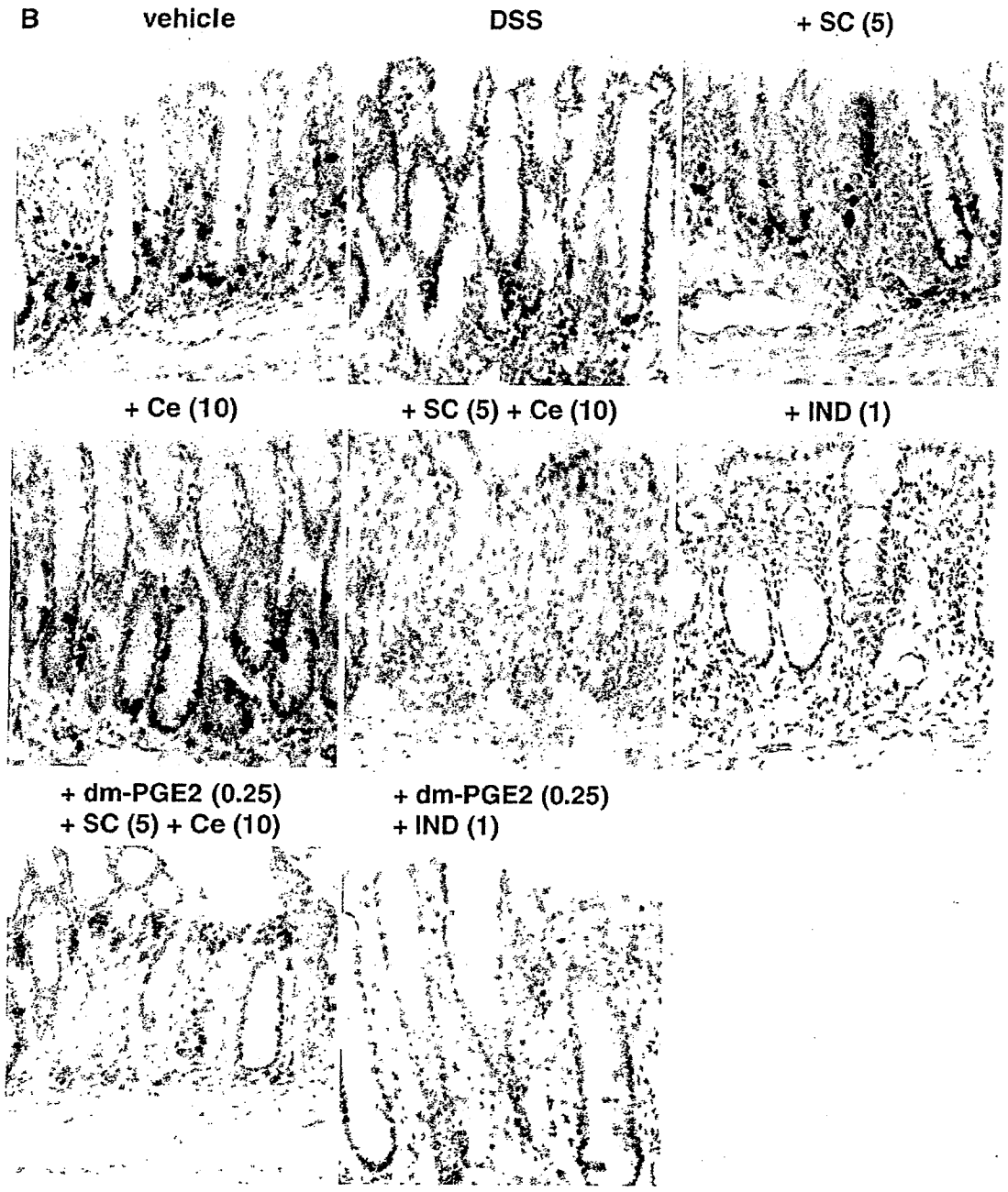


Fig. 6. Effect of NSAIDs and dm-PGE_2 on the level of apoptosis and cell proliferation in the intestinal mucosa of DSS-treated mice. DSS-induced colitis was developed and NSAIDs and dm-PGE_2 were administered as described in the legend of Fig. 3. Sections of intestinal tissues were prepared and subjected to the TUNEL assay and DAPI staining (A) or to immunohistochemical analysis with an antibody against BrdU (B). Cells (more than 400 cells) were counted for staining with BrdU in four independent sections. Values shown are mean±S.E.M. **(or ## or &&)$P<0.01$ (C). Magnification of all photomicrographs is ×100 (A) or ×200 (B).



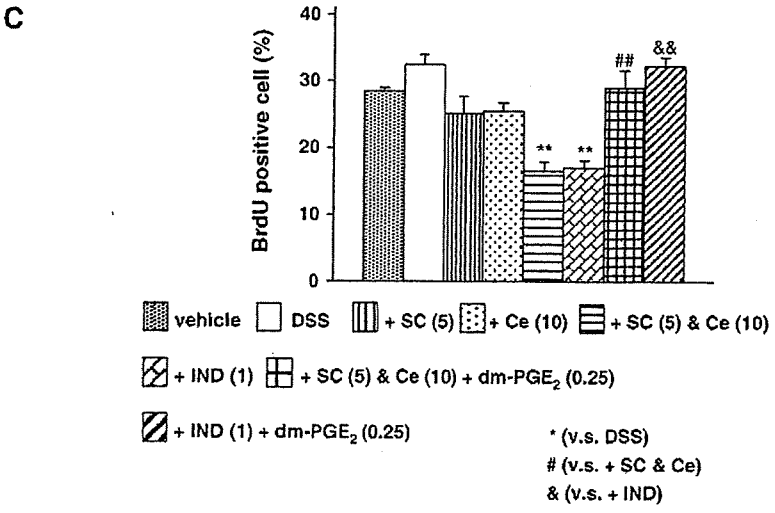


Fig. 6 (*continued*).

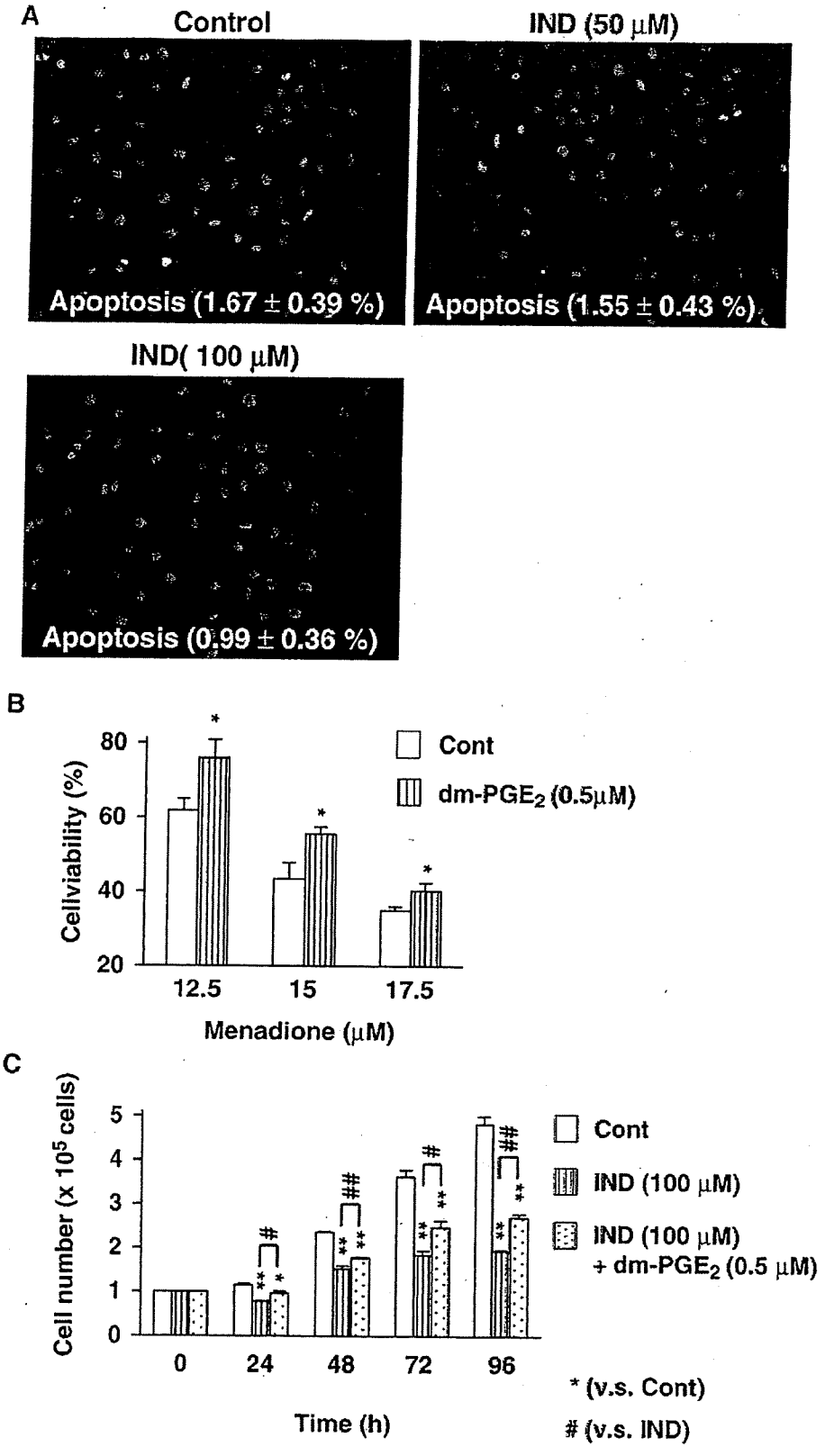


Fig. 7. Effect of indomethacin and dm-PGE_2 on apoptosis and cell growth *in vitro*. IEC6 cells were incubated with the indicated concentrations of indomethacin for 24 h, stained with Hoechst 33342 and the relative number of Hoechst 33342-positive cells was determined. Magnification of all photomicrographs is ×100 (A). Cells were pre-incubated with the indicated concentration of dm-PGE_2 for 1 h and further incubated with the indicated concentrations of menadione (B) or 100 μM indomethacin (C) for the indicated periods in the presence of the same concentrations of dm-PGE_2 as in the pre-incubation step. Cell viability was determined by MTT assay (B). Total viable cell numbers were counted (C). Values shown are mean±S.E.M. ($n=3$). **(or ##)$P<0.01$; *(or #)$P<0.05$.

we used half of the highest dose of SC-560 or celecoxib that was used in the other experiments (single NSAID administration). It may therefore be concluded that the effects of SC-560 and celecoxib are synergistic. These results suggest that non-selective NSAIDs, rather than COX-1- or COX-2-selective inhibitors, exacerbate the development of DSS-induced colitis and inflammatory bowel disease. This idea is supported by the majority of

previous clinical and animal studies, although some results are inconsistent with this idea (Bonner, 2001; El Miedany et al., 2006; El-Medany et al., 2005; Evans et al., 1997; Felder et al., 2000; Mahadevan et al., 2002; Martin et al., 2005; Okayama et al., 2007; Reuter et al., 1996; Yamada et al., 1993). The importance of COX-1 and COX-2 in protecting against inflammatory bowel disease and inflammatory bowel disease-related experimental colitis was also suggested by other studies (Okayama et al., 2007). For example, both COX-1- and COX-2 deficient mice exhibited a phenotype of sensitivity to DSS-induced colitis (Morteau et al., 2000). Furthermore, rapid development of colitis in IL-10-deficient mice (without DSS) was observed in response to administration of both COX-1- and COX-2-selective inhibitors (Berg et al., 2002).

As for the primary mechanism for the exacerbation of DSS-induced colitis by NSAIDs (SC-560/celecoxib or indomethacin), we conclude that the intestinal level of PGE_2 plays a major role, based on the following observations. In DSS-treated mice, administration of a combination of SC-560 and celecoxib or of indomethacin dramatically decreased the intestinal level of PGE_2, however, administration of SC-560 or celecoxib alone did not greatly decrease the level of PGE_2. Simultaneous administration of dm-PGE_2 suppressed the exacerbation of DSS-induced colitis by NSAIDs. These results also suggest that PGE_2 synthesis at intestinal tissues involves both COX-1 and COX-2. Other evidences are consistent with PGE_2 playing an important role in the protection of intestinal tissues against DSS-induced colitis. For example, administration of PGE_2 suppressed the development of DSS-induced colitis, and PGE_2 has been reported to be involved in the regeneration of epithelial crypts after DSS-induced damage (Cohn et al., 1997). It is well known that COX-2 is up-regulated and that the level of PGE_2 is increased in the intestinal tissues of inflammatory bowel disease patients relative to unaffected individuals (Singer et al., 1998). The results of the current study suggest that these responses ameliorate the development of inflammatory bowel disease.

Pro-inflammatory cytokines and cell adhesion molecules positively contribute to the progression of inflammatory bowel disease and colitis in animal models of inflammatory bowel disease. Pro-inflammatory cytokines activate, and thereby stimulate the release of reactive oxygen species from, leukocytes and cell adhesion molecules are essential for recruitment of blood circulating leukocytes into inflamed intestinal tissues (Danese et al., 2005; Kinoshita et al., 2006). However, in this study, we have shown that administration of PGE_2 does not significantly affect the mRNA levels of these proteins. This suggests that the expression of pro-inflammatory cytokines and cell adhesion molecules is not involved in the suppression of NSAID-dependent exacerbation of DSS-induced colitis by PGE_2. However, we assume that this is due to our experimental conditions and that pro-inflammatory cytokines and cell adhesion molecules are involved in the protective effects of PGE_2 against inflammatory bowel disease and inflammatory bowel disease-related experimental colitis, because a number of previous studies have clearly shown that PGE_2 inhibits the production of pro-inflammatory cytokines (particularly TNF-α and cell adhesion molecules (Kabashima et al., 2002; Kunkel et al., 1988).

Invasion of pathogenic bacteria across the intestinal mucosa is responsible for the development of inflammatory bowel disease. Therefore, the decrease in the intestinal mucosal cell number due to stimulation of apoptosis and inhibition of cell proliferation stimulates the development of inflammatory bowel disease (Kabashima et al., 2002; Tessner et al., 1998). In this study, we have shown that in DSS-treated mice the level of apoptosis and epithelial cell growth at the intestinal mucosa is stimulated and inhibited, respectively, by administration of NSAIDs (SC-560/celecoxib or indomethacin) and that this alteration is suppressed by simultaneous administration of PGE_2. This suggests that the alterations in the levels of intestinal mucosal cell growth and apoptosis are involved in the exacerbation of DSS-induced colitis by NSAIDs and its suppression by PGE_2. In accordance with this idea, it has been reported that PGE_2 stimulates intestinal epithelial growth (Tessner et al., 1998). We have also shown that indomethacin inhibited the growth of cultured intestinal cells and that addition of exogenous dm-PGE_2 recovered this cell growth, suggesting that NSAIDs affect cell growth partially through decreasing the level of PGE_2. We could not detect apoptosis induced by indomethacin *in vitro*. Based on our previous results with gastric mucosal cells (Tomisato et al., 2001), this is probably due to the low concentrations of indomethacin used. Higher concentrations of indomethacin may induce apoptosis in the intestinal cells; however, such high concentrations may not be achieved *in vivo*. Alternatively, we consider that reactive oxygen species released from activated leukocytes are responsible for the increased apoptosis in the presence of NSAIDs (SC-560/celecoxib or indomethacin) *in vivo* and for its suppression by PGE_2, because we have shown that PGE_2 protects intestinal cells from menadione-induced apoptosis. We have also demonstrated that NSAIDs (SC-560/celecoxib or indomethacin) inhibit the mRNA expression of mucin proteins and that PGE_2 suppresses this inhibition both in *vivo* and *in vitro*. These results suggest that NSAIDs directly affect the expression of mucin proteins through decreasing the level of PGE_2 and that this is involved in the exacerbation of DSS-induced colitis by NSAIDs and its suppression by PGE_2. The protective role of mucin proteins against DSS-induced colitis was genetically confirmed recently, using *muc2*-deficient mice (Van der Sluis et al., 2006).

In accordance with previous results (Cuzzocrea et al., 2001), we have shown that administration of celecoxib ameliorates the progression of DSS-induced colitis, suggesting that celecoxib is beneficial for the treatment of inflammatory bowel disease. In fact, some previous clinical studies support this idea (El Miedany et al., 2006; El-Medany et al., 2005). Our results also show that celecoxib, under conditions where it was observed to have a protective effect against DSS-induced colitis, did not significantly affect the intestinal level of PGE_2. Furthermore, we recently found that some other COX-2 selective inhibitors do not ameliorate DSS-induced colitis (Tanaka K. et al. unpublished results). These results suggest that a mechanism other than COX inhibition is also involved in conferring the protective effect of celecoxib against DSS-induced colitis. It was reported that celecoxib has various COX-independent effects on cells, such as induction of the endoplasmic reticulum (ER) stress response, inactivation of phosphatidylinositol 3-kinase (PI3K)/3-phosphoinositide dependent kinase-1 (PDK1)/Akt and inactivation of mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) (Ding et al., 2005; Tsutsumi et al., 2004; Zhang et al., 2004). Identification of a COX-independent mechanism that confers the protective effect of celecoxib against DSS-induced colitis will be important for application of this drug in the clinical treatment of inflammatory bowel disease.

References

Berg, D.J., Zhang, J., Weinstock, J.V., Ismail, H.F., Earle, K.A., Alila, H., Pamukcu, R., Moore, S., Lynch, R.G., 2002. Rapid development of colitis in NSAID-treated IL-10-deficient mice. Gastroenterology 123, 1527–1542.

Bonner, G.F., 2001. Exacerbation of inflammatory bowel disease associated with use of celecoxib. Am. J. Gastroenterol 96, 1306–1308.

Bonner, G.F., Walczak, M., Kitchen, L., Bayona, M., 2000. Tolerance of nonsteroidal antiinflammatory drugs in patients with inflammatory bowel disease. Am. J. Gastroenterol 95, 1946–1948.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem 72, 248–254.

Cohn, S.M., Schloemann, S., Tessner, T., Seibert, K., Stenson, W.F., 1997. Crypt stem cell survival in the mouse intestinal epithelium is regulated by prostaglandins synthesized through cyclooxygenase-1. J. Clin. Invest 99, 1367–1379.

Cuzzocrea, S., Mazzon, E., Serraino, I., Dugo, L., Centorrino, T., Ciccolo, A., Sautebin, L., Caputi, A.P., 2001. Celecoxib, a selective cyclo-oxygenase-2 inhibitor reduces the severity of experimental colitis induced by dinitrobenzene sulfonic acid in rats. Eur. J. Pharmacol 431, 91–102.

Danese, S., Semeraro, S., Marini, M., Roberto, I., Armuzzi, A., Papa, A., Gasbarrini, A., 2005. Adhesion molecules in inflammatory bowel disease: therapeutic implications for gut inflammation. Dig. Liver. Dis 37, 811–818.

Ding, H., Han, C., Zhu, J., Chen, C.S., D'Ambrosio, S.M., 2005. Celecoxib derivatives induce apoptosis via the disruption of mitochondrial membrane potential and activation of caspase 9. Int J. Cancer 113, 803–810.

El Miedany, Y., Youssef, S., Ahmed, I., El Gaafary, M., 2006. The gastrointestinal safety and effect on disease activity of etoricoxib, a selective cox-2 inhibitor in inflammatory bowel diseases. Am. J. Gastroenterol 101, 311–317.

El-Medany, A., Mahgoub, A., Mustafa, A., Arafa, M., Morsi, M., 2005. The effects of selective cyclooxygenase-2 inhibitors, celecoxib and rofecoxib, on experimental colitis induced by acetic acid in rats. Eur. J. Pharmacol 507, 291–299.

Evans, J.M., McMahon, A.D., Murray, F.E., McDevitt, D.G., MacDonald, T.M., 1997. Non-steroidal anti-inflammatory drugs are associated with emergency admission to hospital for colitis due to inflammatory bowel disease. Gut 40, 619–622.

Felder, J.B., Korelitz, B.I., Rajapakse, R., Schwarz, S., Horatagis, A.P., Gleim, G., 2000. Effects of nonsteroidal antiinflammatory drugs on inflammatory bowel disease: a case-control study. Am. J. Gastroenterol 95, 1949–1954.

Fukata, M., Chen, A., Klepper, A., Krishnareddy, S., Vamadevan, A.S., Thomas, L.S., Xu, R., Inoue, H., Arditi, M., Dannenberg, A.J., Abreu, M.T., 2006. Cox-2 is regulated by Toll-like receptor-4 (TLR4) signaling: role in proliferation and apoptosis in the intestine. Gastroenterology 131, 862–877.

Futaki, N., Arai, I., Hamasaka, Y., Takahashi, S., Higuchi, S., Otomo, S., 1993. Selective inhibition of NS-398 on prostanoid production in inflamed tissue in rat carrageenan-air-pouch inflammation. J. Pharm. Pharmacol 45, 753–755.

Gupta, D., Jin, Y.P., Dziarski, R., 1995. Peptidoglycan induces transcription and secretion of TNF-alpha and activation of lyn, extracellular signal-regulated kinase, and rsk signal transduction proteins in mouse macrophages. J. Immunol 155, 2620–2630.

Jurjus, A.R., Khoury, N.N., Reimund, J.M., 2004. Animal models of inflammatory bowel disease. J. Pharmacol. Toxicol. Methods 50, 81–92.

Kabashima, K., Saji, T., Murata, T., Nagamachi, M., Matsuoka, T., Segi, E., Tsuboi, K., Sugimoto, Y., Kobayashi, T., Miyachi, Y., Ichikawa, A., Narumiya, S., 2002. The prostaglandin receptor EP4 suppresses colitis, mucosal damage and CD4 cell activation in the gut. J. Clin. Invest 109, 883–893.

Kato, M., Nishida, S., Kitasato, H., Sakata, N., Kawai, S., 2001. Cyclooxygenase-1 and cyclooxygenase-2 selectivity of non-steroidal anti-inflammatory drugs: investigation using human peripheral monocytes. J. Pharm. Pharmacol 53, 1679–1685.

Kinoshita, K., Hori, M., Fujisawa, M., Sato, K., Ohama, T., Momotani, E., Ozaki, H., 2006. Role of TNF-alpha in muscularis inflammation and motility disorder in a TNBS-induced colitis model: clues from TNF-alpha-deficient mice. Neurogastroenterol. Motil 18, 578–588.

Krawisz, J.E., Sharon, P., Stenson, W.F., 1984. Quantitative assay for acute intestinal inflammation based on myeloperoxidase activity. Assessment of inflammation in rat and hamster models. Gastroenterology 87, 1344–1350.

Kunkel, S.L., Spengler, M., May, M.A., Spengler, R., Larrick, J., Remick, D., 1988. Prostaglandin E2 regulates macrophage-derived tumor necrosis factor gene expression. J. Biol. Chem 263, 5380–5384.

Mahadevan, U., Loftus Jr., E.V., Tremaine, W.J., Sandborn, W.J., 2002. Safety of selective cyclooxygenase-2 inhibitors in inflammatory bowel disease. Am. J. Gastroenterol 97, 910–914.

Martin, A.R., Villegas, I., Alarcon de la Lastra, C., 2005. The COX-2 inhibitor, rofecoxib, ameliorates dextran sulphate sodium induced colitis in mice. Inflamm. Res 54, 145–151.

Morteau, O., Morham, S.G., Sellon, R., Dieleman, L.A., Langenbach, R., Smithies, O., Sartor, R.B., 2000. Impaired mucosal defense to acute colonic injury in mice lacking cyclooxygenase-1 or cyclooxygenase-2. J. Clin. Invest 105, 469–478.

Okayama, M., Hayashi, S., Aoi, Y., Nishio, H., Kato, S., Takeuchi, K., 2007. Aggravation by selective COX-1 and COX-2 inhibitors of dextran sulfate sodium (DSS)-induced colon lesions in rats. Dig. Dis. Sci 52, 2095–2103.

Podolsky, D.K., 2002. Inflammatory bowel disease. N. Engl. J. Med 347, 417–429.

Reuter, B.K., Asfaha, S., Buret, A., Sharkey, K.A., Wallace, J.L., 1996. Exacerbation of inflammation-associated colonic injury in rat through inhibition of cyclooxygenase-2. J. Clin. Invest 98, 2076–2085.

Singer, II, Kawka, D.W., Schloemann, S., Tessner, T., Riehl, T., Stenson, W.F., 1998. Cyclooxygenase 2 is induced in colonic epithelial cells in inflammatory bowel disease. Gastroenterology 115, 297–306.

Souza, H.S., Tortori, C.J., Castelo-Branco, M.T., Carvalho, A.T., Margallo, V.S., Delgado, C.F., Dines, I., Elia, C.C., 2005. Apoptosis in the intestinal mucosa of patients with inflammatory bowel disease: evidence of altered expression of FasL and perforin cytotoxic pathways. Int J. Colorectal. Dis 20, 277–286.

Takeuchi, K., Smale, S., Premchand, P., Maiden, L., Sherwood, R., Thjodleifsson, B., Bjornsson, E., Bjarnason, I., 2006. Prevalence and mechanism of nonsteroidal anti-inflammatory drug-induced clinical relapse in patients with inflammatory bowel disease. Clin. Gastroenterol. Hepatol 4, 196–202.

Tanaka, K., Namba, T., Arai, Y., Fujimoto, M., Adachi, H., Sobue, G., Takeuchi, K., Nakai, A., Mizushima, T., 2007. Genetic evidence for a protective role for heat shock factor 1 and heat shock protein 70 against colitis. J. Biol. Chem 282, 23240–23252.

Tessner, T.G., Cohn, S.M., Schloemann, S., Stenson, W.F., 1998. Prostaglandins prevent decreased epithelial cell proliferation associated with dextran sodium sulfate injury in mice. Gastroenterology 115, 874–882.

Tomisato, W., Tsutsumi, S., Hoshino, T., Hwang, H.J., Mio, M., Tsuchiya, T., Mizushima, T., 2004. Role of direct cytotoxic effects of NSAIDs in the induction of gastric lesions. Biochem. Pharmacol 67, 575–585.

Tomisato, W., Tsutsumi, S., Rokutan, K., Tsuchiya, T., Mizushima, T., 2001. NSAIDs induce both necrosis and apoptosis in guinea pig gastric mucosal cells in primary culture. Am. J. Physiol. Gastrointest. Liver. Physiol 281, G1092–1100.

Tsutsumi, S., Gotoh, T., Tomisato, W., Mima, S., Hoshino, T., Hwang, H.J., Takenaka, H., Tsuchiya, T., Mori, M., Mizushima, T., 2004. Endoplasmic reticulum stress response is involved in nonsteroidal anti-inflammatory drug-induced apoptosis. Cell. Death. Differ 11, 1009–1016.

Tsutsumi, S., Tomisato, W., Takano, T., Rokutan, K., Tsuchiya, T., Mizushima, T., 2002. Gastric irritant-induced apoptosis in guinea pig gastric mucosal cells in primary culture. Biochim. Biophys. Acta 1589, 168–180.

Van der Sluis, M., De Koning, B.A., De Bruijn, A.C., Velcich, A., Meijerink, J.P., Van Goudoever, J.B., Buller, H.A., Dekker, J., Van Seuningen, I., Renes, I.B., Einerhand, A.W., 2006. Muc2-deficient mice spontaneously develop colitis, indicating that MUC2 is critical for colonic protection. Gastroenterology 131, 117–129.

Yamada, T., Deitch, E., Specian, R.D., Perry, M.A., Sartor, R.B., Grisham, M.B., 1993. Mechanisms of acute and chronic intestinal inflammation induced by indomethacin. Inflammation 17, 641–662.

Zhang, Z., Lai, G.H., Sirica, A.E., 2004. Celecoxib-induced apoptosis in rat cholangiocarcinoma cells mediated by Akt inactivation and Bax translocation. Hepatology 39, 1028–1037.