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Role of group IIA phospholipase A_2 in rat colitis induced by dextran sulfate sodium

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Abstract

Although group IIA phospholipase A_2 has been suggested to be implicated in inflammatory bowel disease, its pathophysiological role in colitis remains unclear. We investigated whether group IIA phospholipase A_2 had pro-inflammatory roles in dextran sulfate sodium-induced colitis in the rat. Secretory phospholipase A_2 activity was markedly increased in the distal colon with two peaks. Strong immunostaining for group IIA phospholipase A_2 was found in subepithelial tissue and lamina propria at early stage and in deeper tissues of the erosion area at later stage. Treatment with a specific group IIA phospholipase A_2 inhibitor, S-3013/LY333013 (methyl[[3-(aminooxoacetyl)-2-ethyl-1-(phenylmethyl)]-1*H*-indol-4yl]oxy) acetate), reduced erosion area, shortening of colon and colonic inflammation, and strongly inhibited the increase in secretory phospholipase A_2 activity and moderately reduced myeloperoxidase activity in the colon in vivo, while eicosanoid levels were not affected. Marked group IIA phospholipase A_2 expression in the erosion area and the improvement of colitis by the group IIA phospholipase A_2 inhibitor strongly suggest that this enzyme plays pro-inflammatory roles in this colitis model.

Keywords: Group IIA phospholipase A_2; Colitis; Dextran sulfate sodium; S-3013/LY333013

1. Introduction

Phospholipase A_2 comprises a diverse family of lipolytic enzymes that hydrolyze the *sn*-2 fatty acid ester bond of glycerophospholipids to produce free fatty acid and lysophospholipids (Dennis, 1994). Over the past two decades, a number of phospholipase A_2s have been identified and characterized. From their structural and biochemical features, these phospholipase A_2s are classified into several families, including secretory phospholipase A_2, arachidonoyl-specific cytosolic phospholipase A_2 and Ca^{2+}-independent phospholipase A_2 (Six et al., 2000; Balsinde et al., 1999). Among these, the non-pancreatic group IIA phospholipase A_2 has been postulated to play important roles in the pathophysiology of various diseases, such as acute pancreatitis, septic shock, acute lung injury, rheumatoid arthritis and inflammatory bowel disease (Vadas et al., 1993; Minami et al., 1992; Nevalainen et al., 2000).

Although the etiology of inflammatory bowel disease is unknown, many factors and mediators such as environmental and genetic factors, inflammatory cytokines and lipid mediators including prostaglandins and leukotrienes have been postulated to be involved in the pathogenesis of inflammatory bowel disease (Fiocchi, 1998). Of particular interest, increased levels of serum group IIA phospholipase A_2 are correlated well with the severity of inflammatory bowel disease (Minami et al., 1992), and increases in both the mRNA and group IIA phospholipase A_2 protein were found in the affected colonic mucosa of patients with inflammatory bowel disease (Haapamäki et al., 1997; Minami et al., 1994; Ribardo et al., 2001). Since group IIA phospholipase A_2 is involved in the production of eicosanoid metabolites such as prostaglandins, leukotrienes and platelet activating factor (Dennis, 1994; Six et al., 2000), it may amplify the inflammatory reaction in the intestinal mucosa, suggesting a pro-

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inflammatory role of this enzyme, as we already reported in a model of acute lung injury (Furue et al., 1999; Koike et al., 2000). On the other hand, group IIA phospholipase A_2 has been postulated to have an antibacterial role against gram-positive and gram-negative organisms (Weinrauch et al., 1996; Madsen et al., 1996), implying a protective effect against bacterial infection. Thus, it is still unknown whether group IIA phospholipase A_2 has detrimental or protective effects in inflammatory bowel disease.

To address this issue, we employed dextran sulfate sodium-induced colitis in rats as a clinically relevant colitis model (Okayasu et al., 1990; Elson et al., 1995; Gaudio et al., 1999), and examined changes in phospholipase A_2 activity and immunohistochemistry through the course of colitis. We further studied the efficacy of S-3013/LY333013 ((methyl[[3-(aminooxoacetyl)-2-ethyl-1-(phenylmethyl)]-1*H*-indol-4yl]oxy) acetate; Draheim et al., 1996), an orally available specific inhibitor of group IIA phospholipase A_2, to elucidate whether this enzyme has a pro-inflammatory role in this colitis model.

2. Material and methods

2.1. Drugs

The specific group IIA phospholipase A_2 inhibitor S-3013/LY333013 (Lot. 323SB7-A) was synthesized by Lilly Research Laboratories (Indianapolis, IN). S-3013/LY333013 is the methyl ester of the free acid S-5920/LY315920 and is rapidly converted to S-5920/LY315920, such that only S-5920/LY315920 was detected in the plasma (Draheim et al., 1996). S-5920/LY315920 inhibits human group IIA phospholipase A_2 activity stoichiometrically through optimized tight-binding interactions at the enzyme catalytic site (Draheim et al., 1996; Snyder et al., 1999). Its IC_{50} values (nM) for human group IIA phospholipase A_2 and -IB were 9 and 228 nM, respectively (Draheim et al., 1996). It also inhibited rat group IIA phospholipase A_2 and -IB with IC_{50} values (nM) of 2 and 27 (Koike et al., 2000), respectively. Furthermore, S-5920/LY315920 was inactive against cyclooxygenases and cytosolic phospholipase A_2 (Snyder et al., 1999). Both S-3013/LY333013 and S-5920/LY315920 were co-developed by Shionogi Company and Eli Lilly (Indianapolis, IN). S-3013/LY333013 was suspended in 0.6% Arabic gum and administered orally in a volume of 5 ml/kg twice a day on weekdays or 10 ml/kg once a day on weekends.

2.2. Induction of colitis

All animal experiments were approved by the Animal Care and Use Committee of Shionogi Research Laboratories, Osaka, Japan. Colitis was induced in rats according to the method of Kimura et al. (1995). Seven-week-old male Sprague–Dawley rats (Charles River Japan, Tokyo) were used in the experiments. Animals received 3% dextran sulfate sodium (mean mol. wt. 5000, Wako, Osaka) in their drinking water for 11 or 12 days. After day 11 or 12, rats were fed 1% dextran sulfate sodium until the end of the experiments. Normal control rats were given tap water.

In drug efficacy experiments, we selected animals for use on day 12 by the following criteria: (1) loss of weight was no more than 20 g; (2) concentration of hemoglobin was higher than 10 mg/dl; (3) moderate bloody stool observed on both days 11 and 12, because the severity of colitis was very variable with about 20% of animals having severe colitis, 20% no or slight, and remaining 60% showing moderate colitis that was suitable for drug efficacy study. After selection, animals were housed individually, given 1% dextran sulfate sodium in their drinking water and administered drug or vehicle (p.o.) for further 14 days. Utilizing this protocol, efficacy of clinically effective drugs such as prednisolone, sulfasalazine and 5-aminosalicylate was already documented (Kimura et al., 1998).

2.3. Assessment of colitis

The symptom of bloody stool was graded using a bloody stool score: Grade 0, stool without blood; Grade 1, stool with blood; Grade 2, constant bloody stool; Grade 3, severe bloody stool. On day 26, animals were anesthetized with 50 mg/kg of pentobarbital (Nembutal® Injection, Dainippon Pharmaceuticals, Osaka, Japan) and killed by exsanguination. The large intestine (from the colon to the rectum) was excised, opened longitudinally and fixed in 10% of formalin for 1 week. The area of erosion was stained with Alcian blue and determined by image analysis software (Optimas®, Optimas, Bothell, WA) (Kimura et al., 1995).

2.4. Histology

Following the measurement of erosion area, histological examination was performed with hematoxylin and eosin staining after preparing 3-μm paraffin sections of two longitudinal tissue sections of the rectum (3 cm in length from the anus) in each rat. Under the light microscope with a micrometer, the length of erosion, inflammatory granulation beneath the erosion and non-erosive granulation with gland loss were measured and summed up for two longitudinal sections, respectively.

2.5. Immunohistochemistry

Tissue samples were embedded in Tissue-Tek® O.C.T. Compound (Sakura Finetek USA, Torrance, CA) and frozen on dry ice, and stored at −80 °C. Immunohistochemical analysis was performed using the standard avidin–biotin–peroxidase procedure (Vectastain ABC-Elite Kit; Vector Laboratories, Burlingame, CA) and 3,3′ -diaminobenzidine hydrochloride as the chromogen. Briefly, serial frozen sections (6 μm) were fixed in Zamboni fixative for 30 min and incubated overnight for 4 °C with optimal concen-

trations (1 μg/ml) of primary antibodies after treatment with 0.3% H_2O_2/methanol. The primary antibodies used were rabbit anti-rat group IIA phospholipase A_2 immunoglobuline G (IgG) for group IIA phospholipase A_2 and rabbit anti-rat pancreatic group IB phospholipase A_2 IgG for group IB phospholipase A_2 (Nakano and Arita, 1990; Nomura et al., 1994). The rabbit anti-rat group IIA phospholipase A_2 antibody was raised against rat platelet group IIA phospholipase A_2 and does not cross-react with rat group IB phospholipase A_2, group X phospholipase A_2 (rat group V phospholipase A_2 not yet obtained), human group IB phospholipase A_2, group IIA phospholipase A_2, group V phospholipase A_2 or group X phospholipase A_2, nor does it cross-react with human cytosolic phospholipase A_2. For negative control, normal rabbit serum (× 100) was used instead of primary antibody. Counterstaining of nuclei was performed with methyl green solution.

2.6. Tissue sampling for biochemical analyses

On given days, the lower and upper parts of the large intestine (ca. 3 cm length; Fig. 1) were excised and washed gently with ice-cold saline to remove blood and feces. Each part was divided longitudinally into three portions and immediately frozen on dry ice (for secretory phospholipase A_2, cytosolic phospholipase A_2 and myeloperoxidase activity assay) or in liquid nitrogen (for eicosanoid and lysophosphatidylcholine assay). Samples were stored at − 80 °C until assayed.

2.7. Phospholipase A_2 activity assay

After thawing on ice, to the intestinal tissue was added 3 μl/mg tissue of 7.7 mM ethylene–diaminetetraacetic acid (EDTA) containing 1.5 μg/ml prostaglandin E_1 and homogenized with a Polytron type homogenizer (HG-30, Hitachi, Tokyo, Japan) for 30–40 s. The activity of secretory phospholipase A_2 in the tissue homogenate was measured using phosphatidlyglycerol, as preferred substrate for secretory phospholipase A_2, by the method of Tojo et al. (1993). Samples (1.2–127 μg protein) were incubated for 30 min at 40 °C with mixed micelles (final volume 100 μl) consisting of 1 mM 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (Avanti Polar Lipids, Alabaster, AL) and 2 mM sodium deoxycholate in 0.1 M Tris–HCl buffer (pH 8.0) containing 150 mM NaCl and 10 mM $CaCl_2$ and 1 mg/ml bovine serum albumin. The reaction was stopped by addition of 400 μl of Dole's reagent. Hydrolyzed oleic acid was extracted into the upper heptane layer and the extracted fatty acid was derivatized with 9-anthyldiazomethane and analyzed by high performance liquid chromatography (HPLC). The secretory phospholipase A_2 activity was calculated by subtraction of non-enzymatic hydrolysis of the substrate (less than 1% of the total phospholipid added).

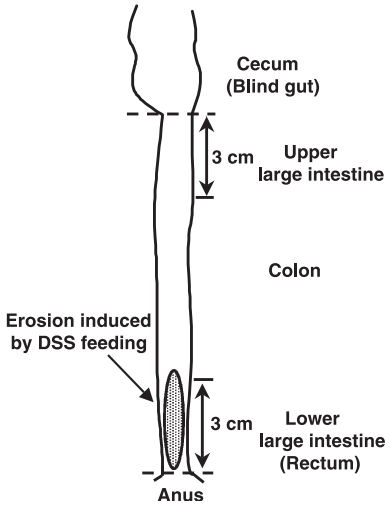

Fig. 1. Illustration of the excised intestinal tract. Intestinal tract 3 cm from the bottom of the cecum was used as the upper large intestine. Three centimeters of intestinal tract from the anus was used as the lower large intestine where most of the mucosal erosion was induced by dextran sulfate sodium (DSS) feeding.

In the next in vitro experiments, secretory phospholipase A_2 activity in homogenates was measured with the in vitro addition of S-5920/LY315920, EDTA or anti-rat group IIA phospholipase A_2 antibody as described previously (Koike et al., 2000).

The activity of cytosolic phospholipase A_2 was determined by the method of Kramer et al. (Kramer et al., 1993). Aliquots of 20 μl homogenate were incubated for 15 min at 37 °C in a mixed micelle suspension/solution (final volume 200 μl) consisting of 1-palmitoyl-2-[^{14}C]arachidonyl-phosphatidylcholine (2.5 μM, 667 Bq/nmol, NEN, Boston, MA and Avanti Polar Lipids) and 1.25 μM of 1,2-dioleoylglycerol (Avanti Polar Lipids) in 100 mM glycine–NaOH buffer (pH 9.5) containing 150 mM NaCl, 5 mM $CaCl_2$ and 2 mM dithiothreitol. The reaction was stopped by addition of 1.25 ml of Dole's regent. Free [^{14}C]arachidonic acid was extracted in *n*-heptane and the radioactivity was counted in a liquid-scintillation counter. Specific cytosolic phospholipase A_2 activity was calculated by subtraction of non-enzymatic hydrolysis of the substrate (less than 0.5% of the total radioactivity added). Also, anti-human cytosolic phospholipase $A_2\alpha$ antibody (no. 690/2T, kindly gifted from Dr. Roehm, Eli Lilly, USA), which cross-reacts with rat cytosolic phospholipase A_2 was used to confirm the cytosolic phospholipase A_2 activity in the homogenate.

Protein was measured using BCA protein assay reagent (Pierce, Rockford, IL).

2.8. Myeloperoxidase activity assay

Myeloperoxidase activity was measured according to Krawisz et al. (1984). Myeloperoxidase was extracted from homogenized tissue in the buffer with 0.5% hexadecyltrimethylammonium bromide (HTAB) in 50 mM potassium phosphate buffer, pH 6.0. Suspensions were centrifuged (30,000 × *g*, 20 min, 4 °C). Myeloperoxidase was assayed

spectrophotometrically: 0.1 ml of the sample to be measured was mixed with 2.9 ml of 50 mM phosphate buffer, pH 6.0, containing 0.167 mg/ml *o*-dianisidine dihydrochloride and 0.015% hydrogen peroxide. The change in absorbance at 460 nm was measured.

2.9. Eicosanoid and lysophosphatidylcholine assay

Eicosanoids in the intestinal tissue were measured according to Hori et al. (1996). Tissue samples snap-frozen in liquid nitrogen were pulverized and stored at −80 °C until assayed. To the sample was added 3 ml of 80% ethanol and homogenized for 30 s. The homogenate was centrifuged at 250 × *g* for 20 min at 4 °C and the eicosanoids in the supernatant were adsorbed to C18 and finally extracted with ethyl acetate. The following eicosanoids were measured: thromboxane B_2 (a metabolite of thromboxane A_2) and prostaglandin E_2 were quantified with respective [^{125}I]radioimmunoassay (RIA) kits (New England Nuclear (NEN), Boston, MA) and leukotriene B_4 was quantified with a [^{3}H]RIA kit (NEN).

Lysophosphatidylcholine was quantified as follows. Lipids were extracted from the tissue homogenate according to the method of Bligh and Dyer (1959). To tissue samples quick frozen in liquid nitrogen was added 19 ml/g tissue of chloroform and homogenized for 30 s. The lower phase of the homogenate was used as the phospholipid extract. The phospholipids were separated by thin layer chromatography on precoated activated silica-gel type 60 G thin-layer plates. The lipid area of chromatograms was visually detected by exposure to I_2 vapor. The spot corresponding to lysophosphatidylcholine was scraped off for phosphorous determination.

2.10. Statistical analysis

All values are indicated as means ± S.E.M. Welch's test, Dunnett's test and Steel's test were used to analyze the differences between groups, with $P < 0.05$ taken as significant.

3. Results

3.1. Changes in secretory phospholipase A_2, cytosolic phospholipase A_2 and myeloperoxidase activity

To test whether secretory phospholipase A_2 or cytosolic phospholipase A_2 was involved in the rat dextran sulfate sodium-induced colitis, the time courses of changes in the activities of secretory phospholipase A_2 and cytosolic phospholipase A_2 in the homogenized tissue of the upper and lower large intestine of dextran sulfate sodium-treated rats were examined. The secretory phospholipase A_2 activity was markedly increased only in the lower large intestine of dextran sulfate sodium-treated rats with two peaks on days 11 and 22–25 (Fig. 2A), while a slight increase was

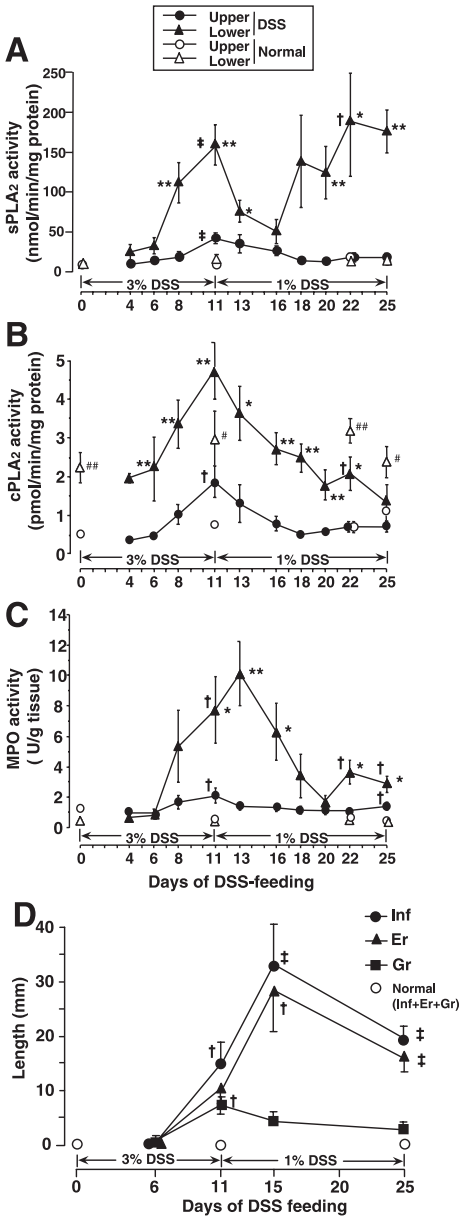

Fig. 2. Changes in (A) secretory phospholipase A_2, (B) cytosolic phospholipase A_2 and (C) myeloperoxidase activities in DSS-induced colitis in rats. The activities in the upper large intestine (solid circles) or lower large intestine (solid triangles) of dextran sulfate sodium-treated rats and those in the upper large intestine (open circles) or lower large intestine (open triangles) of normal control rats are shown. $^*P<0.05$, $^{**}P<0.01$ versus upper large intestine of dextran sulfate sodium-treated rats (Welch's test). $^{\#}P<0.05$, $^{\#\#}P<0.01$ versus upper large intestine of normal control rats (Welch's test). $^{\dagger}P<0.05$, $^{\ddagger}P<0.01$ versus normal upper or lower large intestine. (D) Histological changes. The length of erosion (Er, solid triangles) or length of inflammatory granulation beneath the erosion (Inf, solid circles) or the length of non-erosive granulation with gland loss (Gr, solid squares) in the lower large intestine of dextran sulfate sodium-treated rats, and Inf + Er + Gr in the lower large intestine of normal rats (open circles) are shown. $^{\dagger}P<0.05$, $^{\ddagger}P<0.01$ versus normal at day 0 (Welch's test). Each point represents the mean ± S.E.M. of 5–10 animals for dextran sulfate sodium group and 4–5 for normal group.

observed in the upper large intestine around day 11. In contrast plasma secretory phospholipase A_2 activity was only slightly increased on day 25 by dextran sulfate sodium feeding (normal rats 6.0 ± 0.3 nmol/min/ml, $n = 4$; dextran sulfate sodium-treated rats 8.1 ± 1.0 nmol/min/ml, $n = 10$, $P < 0.05$).

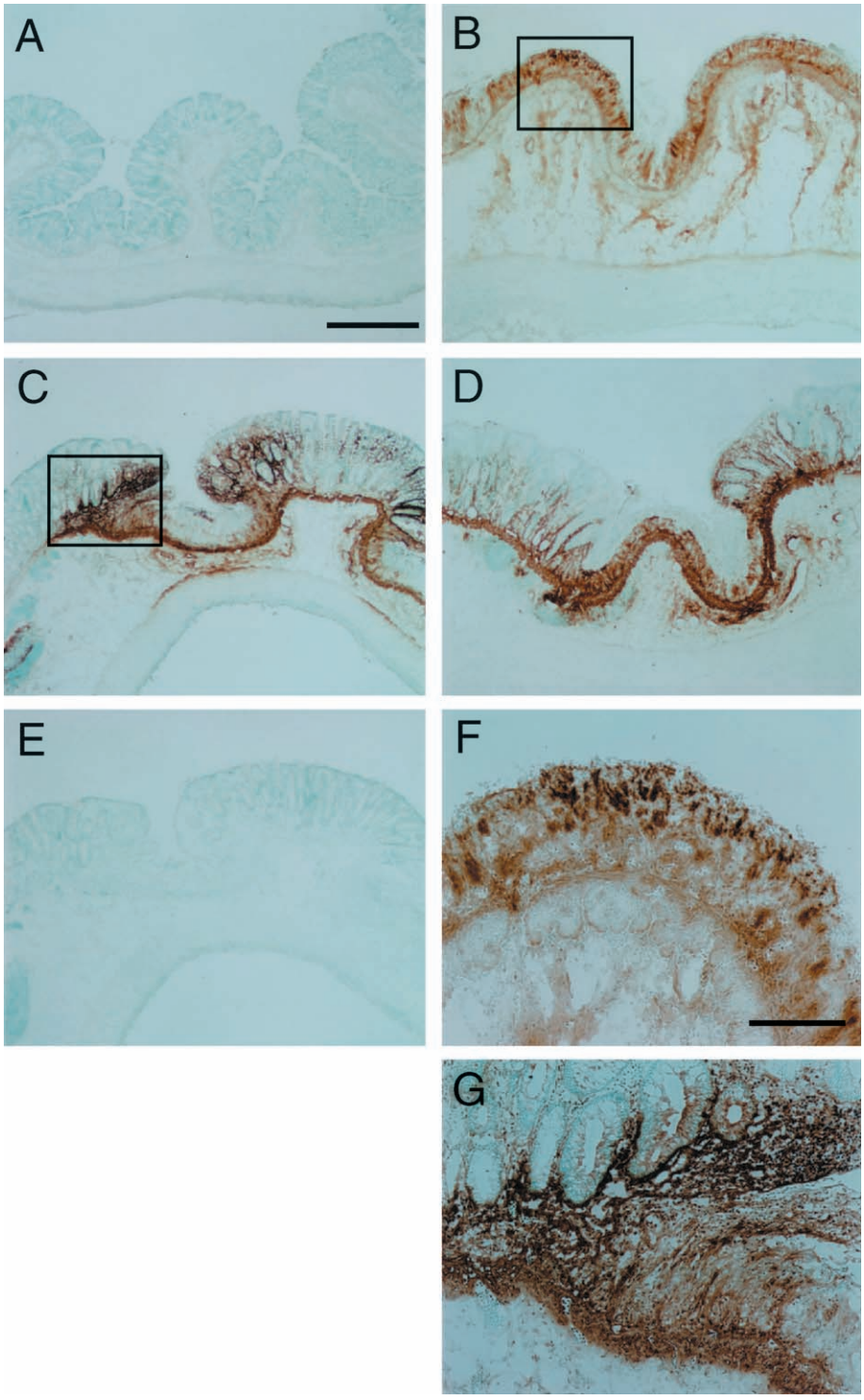

Fig. 3. Immunohistochemical localization of group IIA phospholipase A_2 and group phospholipase A_2-IB in dextran sulfate sodium-induced colitis in rats. Immunohistochemistry of group IIA phospholipase A_2 in the lower large intestine collected from (A) normal tap water-fed rat on day 22, (B) dextran sulfate sodium-fed rat on day 11, (C) dextran sulfate sodium-fed rat on day 22, (D) dextran sulfate sodium-fed rat on day 25. (E) Immunohistochemistry of group IB phospholipase A_2 in the section adjacent to (C). (F, G) Enlarged figures of (B) and (C), respectively. Bar = 1 mm in (A)–(C), and 250 μm in (F) and (G). The figure shown is representative from three animals each.

The cytosolic phospholipase A_2 activity was slightly increased with a peak on day 11 in both the upper and lower large intestine (Fig. 2B) of dextran sulfate sodium-treated rats. The specificity of the peak cytosolic phospholipase A_2 activity was confirmed by adding anti-cytosolic phospholipase A_2 specific antibody into the homogenate, which abrogated more than 90% of the phospholipase A_2 activity (% control; 8.4 ± 1.3, $n = 4$). Interestingly, cytosolic phospholipase A_2 activity in the lower large intestine was significantly higher than that in the upper large intestine in both normal and dextran sulfate sodium-treated rats. Myeloperoxidase activity, measured as an indicator of the degree of inflammation, was markedly raised only in the lower intestine of dextran sulfate sodium-treated rats with peaks on days 13 and 25 of dextran sulfate sodium feeding (Fig. 2C).

These observations clearly indicate that secretory phospholipase A_2 but not cytosolic phospholipase A_2 was markedly increased concomitant with the increase in myeloperoxidase activity in the lower large intestine where most of the large erosive lesions were formed by dextran sulfate sodium feeding.

Another set of experiment shows the time-course of histological changes in the tissue of the large intestine of dextran sulfate sodium-treated or non-treated rats (Fig. 2D). The length of erosion increased from days 6 to 15, and slightly decreased at day 25, showing that the size of erosion reached a peak with a lag of 3 days after the first peak of secretory phospholipase A_2 activity. The ratio of erosion to inflammatory granulation beneath the erosion remained constant (day 11, 0.79 ± 0.07, $n = 10$; day 15, 0.76 ± 0.09, $n = 6$; day 25, 0.78 ± 0.05, $n = 10$). Most of the cells (more than 95%) that infiltrated into inflamed region were neutrophils from days 6 to 25, followed by very few number of eosinophils and mononuclear cells (data not shown).

3.2. Immunohistochemistry of group IIA and IB phospholipase A_2

To determine the type of secretory phospholipase A_2 responsible for the increase in secretory phospholipase A_2 activity by dextran sulfate sodium feeding, immunohistochemical analyses of group IIA phospholipase A_2 and group IB phospholipase A_2 in the lower large intestine were performed. Neither group IIA phospholipase A_2 nor group IB phospholipase A_2 immunostaining was noted in the normal tissue (Fig. 3A). On day 11 of dextran sulfate sodium-feeding, strong immunostaining for group IIA phospholipase A_2 was noted in the order of: epithelial cells> lamina propria>muscularis mucosae = blood vessels in submucosa>submucosa (Fig. 3B,F). Histologically, destruction of epithelial cells and marked edema of the submucosal area were prominent. On days 22–25, strong group IIA phospholipase A_2 immunostaining was shifted to the deeper tissues in the order of: lamina propria = muscularis mucosae>blood vessels in submucosa = submucosa>epithelial cells (Fig. 3C,D,G). A portion of the epithelial cells seemed to have regenerated and submucosal edema had subsided. The strong immunostaining was confined to the tissues beneath the erosion. Strong immunoreactive cell types were vascular smooth muscle cells, infiltrating leukocytes (neutrophils, lymphocytes and macrophages) and epithelial cells (Fig. 3G). In contrast, almost no immunoreactivity of pancreatic type group IB phospholipase A_2 was detected in the specimen adjacent to the Fig. 3C on day 22 (Fig. 3E). In the upper large intestine, only slight immunostaining for group IIA phospholipase A_2 was observed coinciding with tiny erosions on day 22, while no group IB phospholipase A_2 immunostaining was noted in normal or dextran sulfate sodium-treated rats (data not shown).

These findings strongly suggested that group IIA phospholipase A_2 is responsible for the biphasic increase of secretory phospholipase A_2 activity induced by dextran sulfate sodium feeding.

3.3. Effect of S-3013/LY333013 on erosion and colon length

To determine whether the increased group IIA phospholipase A_2 plays a pro-inflammatory role in the rat dextran sulfate sodium colitis, we next examined the therapeutic effect of a specific group IIA phospholipase A_2 inhibitor, S-3013/LY333013, with focusing on the second peak of secre-

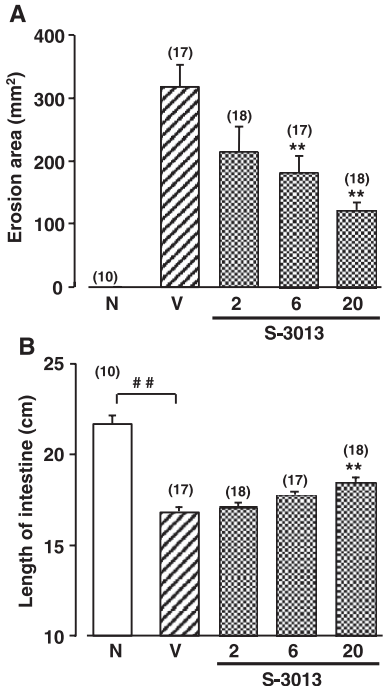

Fig. 4. Therapeutic effect of S-3013/LY333013 on DSS-induced colitis in rats. (A) Erosion area and (B) length of the large intestine are shown. N: normal control rats, V: vehicle-treated dextran sulfate sodium-fed rats. Drug treatment was commenced with the start of 1% dextran sulfate sodium feeding (day 12) and continued for 14 days. S-3013/LY333013 was administered (p.o.) twice a day (mg/kg doses, as given per day). Each column represents the mean ± S.E.M. Numbers in parentheses indicate numbers of animals used. $^{\#\#}P < 0.01$ versus vehicle (Welch's test). $^{*}P < 0.05$, $^{**}P < 0.01$ versus vehicle (Dunnett's test).

tory phospholipase A_2 activity in this model (days 22–25). Drug treatment was commenced from the start of 1% dextran sulfate sodium feeding (day 12) and continued for 14 days. S-3013 at dose of 1, 3 or 10 mg/kg twice daily (p.o.) decreased the erosion area (mm^2) in the large intestine in a dose-dependent manner (by 32.8%, 43.5% ($P<0.01$) and 62.1% ($P<0.01$), respectively; Fig. 4A). S-3013/LY333013 also prevented the shortening of the large intestine by dextran sulfate sodium-feeding with % inhibition of 4.2%, 18.8% and 33.3% ($P<0.01$), respectively (Fig. 4B). Furthermore, the inhibitor significantly decreased the grade of bloody stool: mean bloody stool grade during treatment period for vehicle group, 2, 6 and 20 mg/kg/day groups ($n=17–18$) were 1.94 ± 0.07, 1.79 ± 0.08, 1.67 ± 0.04 ($P<0.01$) and 1.67 ± 0.08 ($P=0.051$), respectively (Steel's test).

Histological examination also revealed that S-3013/LY333013 decreased the length of erosion and inflammatory granulation beneath the erosion, depending on the dose administered, in comparison with the vehicle control (Figs. 5 and 6). Particularly, active erosion was significantly inhibited in 6 or 20 mg/kg/day group (Fig. 6; $P<0.05$ and 0.01, respectively), showing a good correlation with erosion

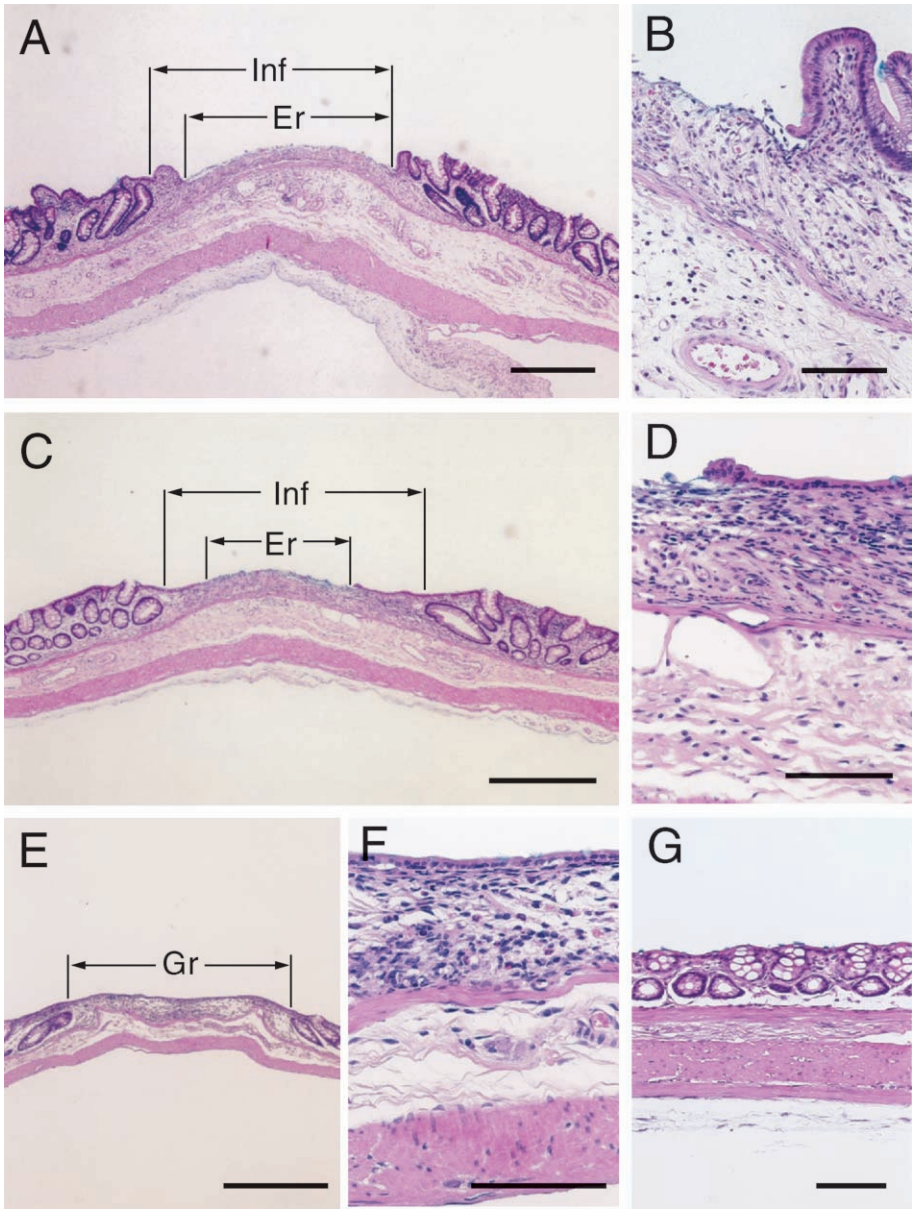

Fig. 5. Histological assessment of dextran sulfate sodium-induced colitis in rats. Specimens were collected on day 26 after dextran sulfate sodium feeding and stained with H&E. (A) Vehicle-treated dextran sulfate sodium-fed rat. Er, the length of erosion; Inf, the length of inflammatory granulation beneath the erosion. (B) Enlarged figure of (A). Active erosion is noticeable. (C) S-3013/LY333013-treated dextran sulfate sodium-fed rat (20 mg/kg/day from days 12 to 26). (D) Enlarged figure of (C). Lining epithelia are relatively well preserved around the erosion. (E) Vehicle-treated dextran sulfate sodium-fed rats. Gr, the length of non-erosive granulation with gland loss. (F) Enlarged figure of (E). Granulation without erosion is noted. (G) Normal control rat. Bar = 500 μm in (A), (C) and (E), and 100 μm in (B), (D), (F) and (G).

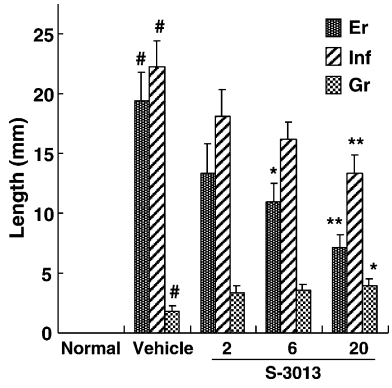

Fig. 6. Effects of S-3013/LY333013 on histological changes of colonic tissue in dextran sulfate sodium-induced colitis in rats. Er, the length of erosion; Inf, the length of inflammatory granulation beneath the erosion; Gr, the length of non-erosive granulation with gland loss. Each column represents the mean ± S.E.M. $N = 17–18$ except for the normal group ($n = 10$). $^{\#}P < 0.05$ versus normal (Welch's test). $^{*}P < 0.05$, $^{**}P < 0.01$ versus vehicle (Dunnett's test).

area. A significant decrease of inflammatory granulation beneath the area of erosion was also observed in the 20 mg/kg/day group ($P < 0.01$), in comparison with the vehicle control. Conversely, non-erosive granulation with gland loss was significantly increased in the 20 mg/kg/day group ($P < 0.05$). Also, the ratio of the erosion to the inflammatory granulation was dose-dependently decreased by S-3013/LY333013 treatment (vehicle 0.82 ± 0.04, $n = 17$; 20 mg/kg/day 0.53 ± 0.04, $n = 18$, $P < 0.01$).

These results clearly indicated that group IIA phospholipase A_2 plays a definite pro-inflammatory role in the later stage of rat dextran sulfate sodium colitis.

3.4. Effect of S-3013/LY333013 on secretory phospholipase A_2 and myeloperoxidase activity

Next, we examined the effect of S-3013/LY333013 on secretory phospholipase A_2 and myeloperoxidase activity in the tissue of the large intestine of dextran sulfate sodium-induced colitis rats. On day 26, secretory phospholipase A_2 activity in the lower large intestine in the dextran sulfate sodium-treated vehicle group (96.5 ± 9.1 nmol/min/mg protein) was 12-fold ($P < 0.01$) higher than that in the normal controls (7.9 ± 1.3 nmol/min/mg protein) (Fig. 7A). The elevation of secretory phospholipase A_2 activity by dextran sulfate sodium feeding was markedly reduced in vivo by 10 mg/kg of S-3013 (p.o.) twice a day (7.0 ± 1.1 nmol/min/mg protein, $P < 0.01$ vs. vehicle; Fig. 7A). Also, in vitro, the secretory phospholipase A_2 activity in homogenized tissue from the vehicle group was markedly inhibited by addition of 1, 10 or 100 nmol/l of S-5920/LY315920, the active metabolite of S-3013/LY333013 (% control; 49.5%, 10.8% and 5.0%, respectively; Fig. 7B). The increase in secretory phospholipase A_2 activity in the large intestine was also inhibited by 5 mM EDTA or 1% anti-rat group IIA phospholipase A_2 antibody in vitro (% control; 4.5% or 19.2%, respectively; Fig. 7B). Myeloperoxidase activity was markedly increased in the large intestine in the vehicle group (4.1 ± 0.8 U/g tissue, $P < 0.01$) compared to control group (0.3 ± 0.1 U/g tissue). The main source of the activity should be neutrophils because more than 95% of the infiltrated cells were neutrophils at day 25 as described in the Section 3.1. This myeloperoxidase activity was significantly reduced by S-3013 at 10 mg/kg twice daily (2.2 ± 0.28 U/g tissue, $P < 0.05$ vs. vehicle group).

These results confirmed that the dominant secretory phospholipase A_2 isozyme increased in the inflamed large intestine was group IIA phospholipase A_2, and that S-3013/LY333013 (S-5920/LY315920) strongly inhibited the increase in secretory phospholipase A_2 activity in vitro and in vivo accompanying the significant decrease of myeloperoxidase activity in vivo.

3.5. Effects of S-3013/LY333013 on eicosanoid and lysophosphatidylcholine levels

Next, we examined whether S-3013/LY333013 affected the levels of eicosanoids and lysophosphatidylcholine,

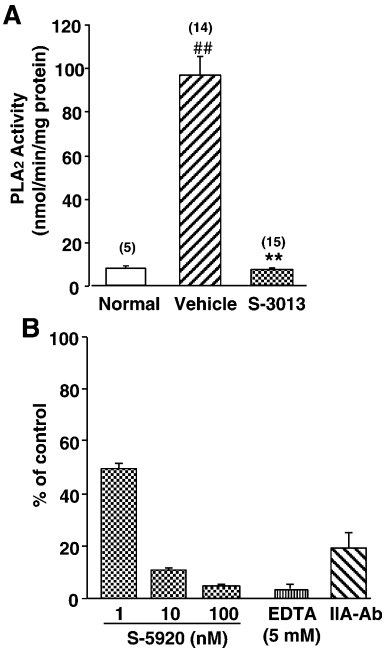

Fig. 7. Effect of S-3013/LY333013 on colonic secretory phospholipase A_2 activity of dextran sulfate sodium-induced colitis in rats. (A) In vivo effect of S-3013/LY333013 on secretory phospholipase A_2 activity in the lower large intestine of colitis rats. Normal: normal control rats. Vehicle: vehicle-treated dextran sulfate sodium-fed rats. S-3013/LY333013 (10 mg/kg, p.o.) was administered twice a day from the start of 1% dextran sulfate sodium feeding (day 12) and continued for 14 days. The colonic tissues were collected 2 h after the last dosing of the drug. (B) In vitro effects of S-5920/LY315920 (active metabolite of S-3013/LY333013), 5 mM EDTA and 1% anti-rat group IIA phospholipase A_2 rabbit antibody (IIA-Ab) on increased colonic secretory phospholipase A_2 activity. The homogenized tissues from the vehicle group were incubated with these agents ($n = 5$). Each column represents the mean ± S.E.M. Numbers in parentheses indicate numbers of animals used. $^{**}P < 0.01$ versus vehicle (Welch's test).

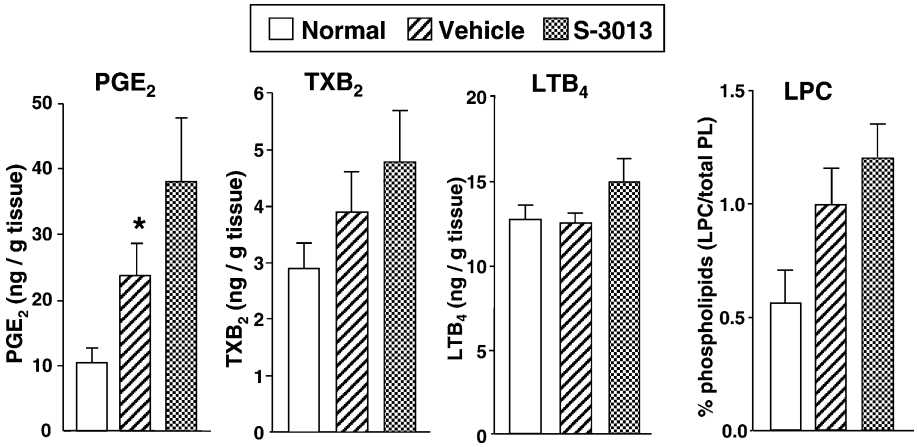

Fig. 8. Effect of S-3013/LY333013 on colonic prostaglandin E_2, leukotriene B_4, thromboxane B_2 and lysophosphatidylcholine levels in dextran sulfate sodium-induced colitis in rats. The lower large intestine was collected on day 26 after dextran sulfate sodium feeding from normal control rats (N), vehicle-treated dextran sulfate sodium-fed rats (V) and from S-3013/LY333013-treated dextran sulfate sodium-fed rats (S). S-3013/LY333013 (10 mg/kg, p.o.) was administered twice a day from the start of 1% dextran sulfate sodium feeding (day 12) and continued for 14 days. The colonic tissues were collected 2 h after the last dosing of the drug. Each column represents the mean ± S.E.M. ($n = 9–10$). *$P < 0.05$ versus normal (Welch's test).

which are thought to be increased by up-regulation of phospholipase A_2 activity, in the tissue of the lower large intestine of colitis rats. Prostaglandin E_2 level in the vehicle-treated dextran sulfate sodium-fed group was two-fold higher than that in the normal controls (Fig. 8). Unexpectedly, prostaglandin E_2 level tended to be increased by S-3013/LY-333013 (10 mg/kg twice a day) compared to the vehicle group. Neither thromboxane B_2 nor leukotriene B_4 was affected by dextran sulfate sodium feeding or S-3013/LY333013 treatment. Although lysophosphatidylcholine level was elevated by dextran sulfate sodium, S-3013/LY-333013 did not affect the increase in lysophosphatidylcholine.

4. Discussion

Dextran sulfate sodium is a sulfated polymer and induces colitis in rodents (Elson et al., 1995; Okayasu et al., 1990), and rat dextran sulfate sodium colitis resembles human ulcerative colitis both histologically and topologically (Gaudio et al., 1999). Dextran sulfate sodium is thought to cause colitis by interfering with intestinal epithelial cell function and stimulating colonic inflammation through the up-regulation of inflammatory cytokines and mediators (Elson et al., 1995). In this study, we demonstrated clearly that group IIA phospholipase A_2 plays pro-inflammatory roles in this well-established model of colitis, as evidenced by the marked increase in both secretory phospholipase A_2 activity and group IIA phospholipase A_2 immunostaining in the affected colonic tissue, and further by the protective effect of a strong specific group IIA phospholipase A_2 inhibitor against colonic erosion and inflammation.

The biphasic increase in secretory phospholipase A_2 activity in the colonic tissue paralleled the group IIA phospholipase A_2 immunostaining that appeared exclusively beneath the erosion area. Furthermore, very strong group IIA phospholipase A_2 immunoreactivity was observed in broader tissues such as epithelial cells, lamina propria, muscularis mucosae, blood vessels in submucosa and submucosa. Of interest, strong immunostaining was noted near the luminal area such as epithelial cells in the early stage of colitis (day 11), while the deeper tissues such as the muscularis mucosae were strongly stained in the later stage (days 22–25). The cause for the shift of immunostaining is not clear, but this phenomenon strongly suggested a critical role of group IIA phospholipase A_2 during the course of rat dextran sulfate sodium colitis, regardless of whether it plays a pro-inflammatory or protective role, and its pro-inflammatory role was confirmed by the apparent efficacy of S-3013/LY333013 in the present model. The length of erosion at day 15 was longer than that at day 11 when the secretory phospholipase A_2 activity showed the first peak. This indicates that erosive lesion continued to be formed after the secretory phospholipase A_2 activity began to decrease by reducing dextran sulfate sodium from 3% to 1% from day 11. The erosion length at day 25 was shorter than that at day 15, and the ratio of the erosion length to the inflamed tissue length remained constant. Interestingly, S-3013/LY333013 treatment dose-dependently decreased this ratio (Fig. 6), suggesting acceleration of the repair process as well as inhibition of erosive change. These findings imply the coexistence of the repair process and persistent injury and that secretory phospholipase A_2 in the second peak could damage the tissue and keep the lesions competing with this repair process.

Recently, several novel secretory phospholipase A_2 isozymes such as group V and X phospholipase A_2 were identified (Six et al., 2000). The inhibitory activities of S-5920/LY315920, active metabolite of S-3013/LY333013, against human group V and X phospholipase A_2 were 15- to 177-fold and 6- to 13-fold, respectively, weaker than

those for group IIA phospholipase A_2 in vitro (T. Ono, unpublished data), though the relative inhibitory activity varies depending on the substrates used because each secretory phospholipase A_2 has its order of preference for substrate phospholipids. Its inhibitory activity against rat group X phospholipase A_2 was also about 50-fold weaker than that for rat group IIA phospholipase A_2 when phosphatidylglycerol was used as a substrate (T. Ono, unpublished data; rat group V phospholipase A_2 not yet obtained). Most of the secretory phospholipase A_2 activity in the late phase of the colitis (days 22–25) can be ascribed to rat group IIA phospholipase A_2, because its specific antibody abrogated 80% of the secretory phospholipase A_2 activity in the erosive colitis tissue (Fig. 7B). The remaining small part of secretory phospholipase A_2 activity in the lesion seems to be due to other isotypes of phospholipase A_2 such as group IB, V and X, since the activity was almost completely diminished by S-5920/LY315920 that is capable of inhibiting group IB, V and X phospholipase A_2 as well as group IIA phospholipase A_2 at higher concentrations. Furthermore participation of calcium-independent phospholipase A_2 is thought to be very small because the increased phospholipase A_2 activity was completely diminished by the addition of EDTA in vitro (Fig. 7B). Further study is needed to define the increase of secretory phospholipase A_2 activity in the early phase of this colitis model.

In human inflammatory bowel disease patients, group IIA phospholipase A_2 protein was shown to be strictly localized in Paneth's cells and/or in the epithelial cells of actively inflamed sites, while inflammatory cells including macrophages, lymphocytes and granulocytes were negative (Haapamäki et al., 1997; Minami et al., 1997). In contrast, broader tissue areas and inflammatory cells were positively stained in the present rat colitis model. The reason for these differences is not clear, but it might mirror the differences in the magnitude of involvement of group IIA phospholipase A_2 between human inflammatory bowel disease and the rat colitis model. Alternatively, it might reflect the differences in sections used: we used frozen sections of inflamed tissue, while paraffin sections were used for human inflammatory bowel disease samples, suggesting that the processing of paraffin sections could partially denature and/or wash out the antigen during fixation or removal of paraffin. Indeed, localization of group IIA phospholipase A_2 mRNA on the frozen sections from ulcerative colitis patients seemed to be broader than that of group IIA phospholipase A_2 protein on the paraffin sections (Haapamäki et al., 1997).

In the present study, the severity of colitis was morphologically assessed by measurement of the length of the large intestine and erosion areas in the lower large intestine, using direct staining of the erosive colonic mucosa with Alcian blue. The Alcian blue-positive erosion areas were correlated well with the histological longitudinal length of erosion with inflammatory granulation, indicating the accurate estimation of erosion.

S-3013/LY333013 clearly improved erosion and inflammation in dextran sulfate sodium-induced colitis. Prednisolone (2 mg/kg p.o. daily) and sulfasalazine (200 mg/kg p.o. daily) also showed comparable efficacy as 20 mg/kg daily (p.o.) of S-3013/LY333013 in this model (data not shown). Interestingly, histological findings indicated that S-3013/LY333013 preferentially reduced active erosion in comparison to the underlying inflammatory granulation or non-erosive granulation with gland loss, suggesting that group IIA phospholipase A_2 might be involved in the healing process of the injured epithelial cells, a process that also involves many growth factors and lipid mediators. Although group IIA phospholipase A_2 has been postulated to have an antibacterial role against gram-positive and gram-negative organism (Weinrauch et al., 1996; Madsen et al., 1996), uncontrolled and excessive increase of this enzyme could overwhelm its protective effect against infection and result in chronic inflammation of the intestinal mucosa (Haapamäki et al., 1998). Indeed, a significant decrease by S-3013/LY333013 of erosion area would lead to not only the decrease of bleeding but also less chance for the entry of luminal intestinal bacteria from the lesioned mucosa into the systemic circulation. These observations further support the pro-inflammatory role of group IIA phospholipase A_2 in the present colitis model.

Dextran sulfate sodium feeding significantly increased prostaglandin E_2 level in the lower large intestine on day 26 concomitant with a tendency of increase in thromboxane B_2 and lysophosphatidylcholine levels, while leukotriene B_4 level was not changed. Unexpectedly, S-3013/LY333013 did not inhibit these increases but rather enhanced the increase of all eicosanoids and lysophosphatidylcholine despite the almost complete inhibition of secretory phospholipase A_2 activity in the inflamed intestinal tissue. These findings suggested that: (1) cytosolic phospholipase A_2 rather than secretory phospholipase A_2 could mainly be involved in the production of eicosanoids and lysophosphatidylcholine—indeed, cytosolic phospholipase A_2 is closely linked to cyclooxygenases by providing arachidonic acid around the nuclear membranes (Murakami et al., 1999); (2) the significant decrease of erosion area by S-3013/LY333013 could result in the relative increase of non-erosive granulation with gland loss that is capable of producing larger amounts of prostaglandin E_2 than the lesioned tissues. Interestingly, prostaglandin E_2 was recently proposed to be a protective mediator based on the findings that dimethyl prostaglandin E_2 prevented decrease epithelial cell proliferation associated with dextran sulfate sodium (Tessner et al., 1998) and that the colonic lesion was less severe in the colonic mucosa with higher levels of endogenous prostaglandin E_2 in the early phase (day 14) of dextran sulfate sodium-induced colitis in rats (Hirata et al., 2001). Recently, it was reported that prostaglandin E_2 protects intestinal tract via EP4 receptor in dextran sulfate sodium-induce colitis in mice (Kabashima et al., 2002). These observations are consistent with the present findings

that S-3013/LY333013 protected dextran sulfate sodium-induced colonic injury but did not reduce eicosanoid levels.

Thus, although the precise mechanism(s) by which S-3013/LY333013 exerted its efficacy is not clear at preset, its failure to affect eicosanoids and lysophosphatidylcholine production despite the marked inhibition of secretory phospholipase A_2 activity in the lesioned tissues leads us to speculate that group IIA phospholipase A_2 may directly destroy the phospholipid bilayer of cell membranes in intestinal tissues as documented for its bactericidal effects, and that the group IIA phospholipase A_2 inhibitor could interfere with this process by directly inhibiting the enzymatic action of group IIA phospholipase A_2 on cell membranes. In the present study secretory phospholipase A_2 activity was increased in the inflamed tissue concomitant with lesion formation, and furthermore the group IIA phospholipase A_2 inhibitor suppressed about 50–60% of erosion and myeloperoxydase activity (neutrophil infiltration) with nearly complete inhibition of secretory phospholipase A_2 activity, suggesting that about 50% of the inflammation in this colitis might be mediated by group IIA phospholipase A_2.

Although the involvement of arachidonic acid metabolites seems to be partial in this colitis model, we cannot rule out the possibility that other eicosanoids (hydroxy-6,8,11,14-heptadecatetraenoic acid, leukotriene C_4 and D_4) participate in the pathogenesis of this colitis model in concert with group IIA phospholipase A_2. Especially, such eicosanoids might cause inflammation at the earlier stage of the colitis, because marked edema was seen in the tissue of large intestine at the early stage (day 11) as shown in Fig. 3B.

In conclusion, group IIA phospholipase A_2 plays definite pro-inflammatory roles in dextran sulfate sodium-induced colitis in rats. Determination of the clinical relevance of the present findings must await clinical trials of group IIA phospholipase A_2 inhibitors for human inflammatory bowel disease.

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