

Increased group II phospholipase A₂ in colonic mucosa of patients with Crohn's disease and ulcerative colitis

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Abstract

The immunochemical protein content of group II phospholipase A₂ (PLA₂) and PLA₂ enzymatic activity were measured for colonic mucosal biopsy samples obtained from patients with either Crohn's disease of the colon or ulcerative colitis, and control patients without inflammatory bowel disease. Immuno-reactive group II PLA₂ (IR-PLA₂ II) content and PLA₂ activity in actively inflamed colonic mucosa of Crohn's disease patients were significantly higher than those in inactively inflamed mucosa of Crohn's disease patients and the colonic mucosa of controls. IR-PLA₂ II content and PLA₂ activity in severely inflamed mucosa of ulcerative colitis patients were significantly higher than those in the colonic mucosa of the controls. Mucosal PLA₂ enzymatic activity was closely correlated with mucosal IR-PLA₂ II content in patients with Crohn's disease and ulcerative colitis. These results suggest that an increase in PLA₂ enzymatic activity in inflamed colonic mucosa of Crohn's disease and ulcerative colitis was mainly attributed to increased protein content of group II PLA₂, and that an increase in mucosal group II PLA₂ may be involved in the pathogenesis of intestinal inflammation of Crohn's disease and ulcerative colitis.

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Phospholipase A₂ (PLA₂) hydrolyses the fatty acyl ester bond at the sn-2 position of glycerophospholipids and produces free fatty acids and lysophospholipids. Cellular calcium dependent PLA₂s have been thought to participate in both the regulation of phospholipid metabolism in biomembranes and eicosanoid biosynthesis.¹ It has been shown that cellular calcium dependent PLA₂s of mammalian origin can be classified into at least three groups according to their distinct characteristics in primary structure²⁻⁶: the pancreatic type (group I), the viperid and crotalid type (group II), and the cytosolic high molecular weight type. Group I PLA₂ is considered to be one of the digestive enzymes, and it is abundant in pancreatic juice. Human group II PLA₂ was purified from spleen,⁷ platelets, and synovial fluid.⁵ Recently, we purified group II PLA₂ from human ileal mucosa and provided

evidence for the presence of immunoreactivity and messenger RNA (mRNA) of group II PLA₂ in human ileal and colonic mucosa.^{8,9}

Recent studies suggest that calcium dependent PLA₂s, group II enzyme in particular, participate in inflammatory response either through a direct action or through an indirect action by metabolites of phospholipids.¹⁰⁻¹³ It has been reported that immunoreactive group II PLA₂ (IR-PLA₂ II) values and PLA₂ enzymatic activity were increased in serum samples and synovial fluid of patients with rheumatoid arthritis,¹⁴⁻¹⁶ and in serum samples of patients with septic shock^{17,18} and acute pancreatitis.¹⁹

Inflammatory bowel disease - that is, Crohn's disease and ulcerative colitis - is a chronic intestinal inflammatory disease of unknown aetiology. Arachidonate derived chemical mediators may participate in the pathogenesis of inflammatory bowel disease. Raised intestinal contents of leukotriene B₄ and prostaglandins have been reported in inflammatory bowel disease patients.²⁰⁻²² In this context, increased PLA₂ activity has been reported in ileal and colonic mucosa of patients with Crohn's disease.^{23,24} We have recently reported an increase in PLA₂ activity and IR-PLA₂ II values in serum samples of patients with Crohn's disease and ulcerative colitis.^{8,25} The isozymic form, however, of PLA₂ in the inflamed mucosa of patients with inflammatory bowel disease remains to be established.

In this study, to examine the association of group II PLA₂ with intestinal inflammation of inflammatory bowel disease patients, we have measured PLA₂ activity and protein content of group II PLA₂ in colonic mucosal biopsy samples obtained from Crohn's disease and ulcerative colitis patients.

Methods

PATIENTS

In 21 patients with Crohn's disease of the colon (12 men and 9 women) aged 15-35 years (mean (SD) age of 23.5 (1.1)), colonoscopy was performed on a total of 27 occasions. One of 21 Crohn's disease patients had received resection of sigmoid colon. In patients with Crohn's disease, the colonic mucosa was evaluated with regard to erythema, erosions or ulcers on colonoscopic examination. The presence of any of these signs was considered to show active

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inflammation. All Crohn's disease patients were receiving special diets including elementary and low residual diet. Colonic mucosal biopsy for assay of PLA₂ activity and IR-PLA₂ II content was performed in 13 Crohn's disease patients during endoscopically diagnosed active phase, of whom seven were receiving treatment with sulphasalazine (0.5–3.0 g/day), and six were receiving no drugs. A similar biopsy was performed in 14 Crohn's disease patients during endoscopically diagnosed inactive phase, of whom eight were receiving treatment with sulphasalazine (0.5–3.0 g/day), and six were receiving no drugs. In active and inactive Crohn's disease patients biopsy samples were obtained from macroscopically involved mucosa.

In 21 patients with ulcerative colitis (11 men and 10 women) aged 20–58 years (mean (SD) age of 34.3 (2.6)), colonoscopy was performed on a total of 26 occasions. None of the patients had prior surgical treatment. In patients with ulcerative colitis, all colonoscopic findings were graded from 1 to 4 according to the severity of inflammation using the criteria of Matts.²⁶ When the score was 1 or 2, the inflammation was considered to be mild, and when the score was 3 or 4, the inflammation was considered to be severe. Colonic mucosal biopsy samples for assay of PLA₂ activity and IR-PLA₂ II content were obtained from endoscopically diagnosed mildly inflamed mucosa in 13 ulcerative colitis patients, of whom four were receiving no drugs, three were receiving treatment with sulphasalazine (1.5–3.0 g/day), and six were receiving treatment with prednisolone (5–20 mg/day) in addition to sulphasalazine (1.5–4.0 g/day). Similar samples were obtained from severely inflamed mucosa in 13 ulcerative colitis patients, of whom four were receiving no drugs, seven were receiving treatment with sulphasalazine (1.5–4.0 g/day), and two were receiving treatment with prednisolone (5–30 mg/day) in addition to sulphasalazine (1.5–4.0 g/day).

The control group comprised 15 patients (nine men and six women) aged 23–64 years (mean (SD) age of 41.7 (3.4)) undergoing colonoscopy for the follow up after endoscopic polypectomy, investigation of gastrointestinal symptoms, or in addition to radiographic investigation for irregular bowel habit. In five of 15 control patients one colonic polyp was found on colonoscopic examination, and in the other patients no abnormality was found. Nineteen biopsy samples of histologically normal mucosa were obtained from 15 control patients from ascending or transverse or descending colon: we previously reported that PLA₂ activity and IR-PLA₂ II were uniformly distributed in human colonic mucosa.⁹

The biopsy samples obtained from controls, and patients with Crohn's disease and ulcerative colitis were washed twice by cold saline and stored at –35°C until use. Informed consent was obtained in each case of biopsy in controls and patients with Crohn's disease and ulcerative colitis.

ASSAY FOR PLA₂ ACTIVITY

The biopsy samples were homogenised in 30 volumes of 10 mM TRIS-HCl (pH 7.4), and PLA₂ activity was determined as reported^{27, 28} using 0.8 mM 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-glycerol (POPG) as a substrate in the presence of 5 mM cholate. Fatty acids released by PLA₂ action were labelled with 9-anthryl-diazomethane, and the derivatised fatty acids were separated by reverse phase high performance liquid chromatography, and oleic acid was quantitated using marganic acid as an internal standard. Calcium dependent PLA₂ activity was estimated as the difference between the activity assayed in the presence of 5 mM CaCl₂ and that in the presence of 10 mM EDTA.

The protein concentrations of the homogenates of colonic biopsy samples were determined with bicinchoninic acid protein assay reagent (Pierce).

ASSAY FOR IMMUNOREACTIVE GROUP II PLA₂ SOLUBILISED FROM COLONIC BIOPSY SPECIMENS BY 1 M KBr TREATMENT

We previously found that human group II PLA₂ was enriched in the particulate fractions of tissue homogenates, and that it was readily solubilised by treatment with a high concentration of KBr.^{9, 27} For measuring the content of group II PLA₂ in the biopsy samples, an aliquot of the homogenates of the colonic biopsy samples was mixed with an equal volume of 10 mM TRIS-HCl (pH 7.4) containing 2 M KBr. After the mixture was kept on ice for 60 minutes, it was centrifuged at 40 000×g for 40 minutes at 4°C. PLA₂ activity in the supernatant and pellet fractions was measured to estimate its recovery in the former fraction. The supernatant was used for assaying IR-PLA₂ II content. The IR-PLA₂ II content was determined by a sensitive radioimmunoassay system specific for human group II PLA₂, using a monoclonal antibody against human splenic group II PLA₂.²⁹ The antibody used in the radioimmunoassay was not cross reactive with human pancreatic PLA₂. The sensitivity of the radioimmunoassay was 0.78 ng/ml, and the interassay coefficient variance was about 5%. To examine the effect of 1 M KBr on measuring IR-PLA₂ II concentrations, the standard human group II PLA₂ was dissolved with 10 mM TRIS-HCl (pH 7.4) containing 1 M KBr, and the concentrations of IR-PLA₂ II in 10 mM TRIS-HCl (pH 7.4) containing 1 M KBr were compared with the standard curve used in the radioimmunoassay for this study. The presence of 1 M KBr did not affect IR-PLA₂ II concentrations in the assay used.

STATISTICAL ANALYSIS

Results are presented as mean (SEM). Data were analysed by unpaired Wilcoxon's rank test. Regression analysis was used to determine the relations of PLA₂ activity to IR-PLA₂ II content. It was considered significant when $p < 0.05$.

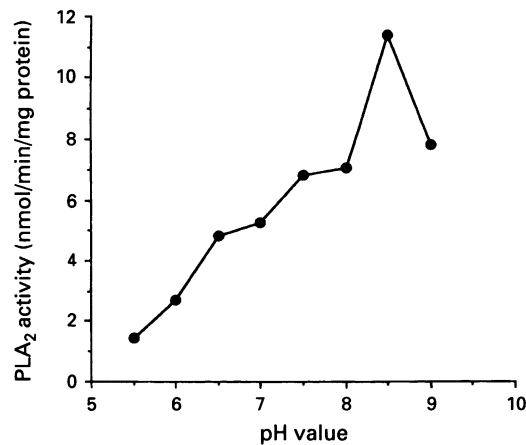


Figure 1: The pH dependence of PLA₂ activity in colonic mucosa of a control patient towards mixed micelles of POPG and cholate. The pH of assay mixtures was adjusted using 0.1 M 2-(morpholino) ethanesulphonic acid (pH 5.5–6.5) and 0.1 M TRIS-HCl buffer (pH 7–9.5).

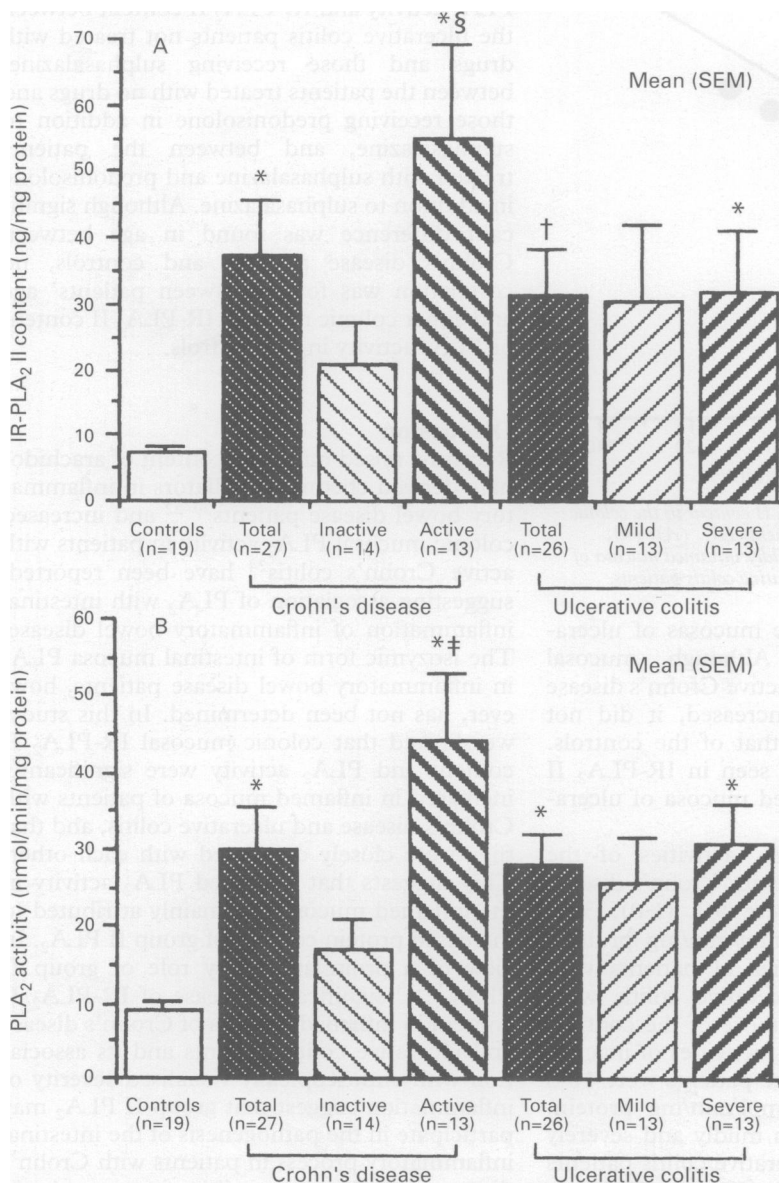


Figure 2: IR-PLA₂ II contents (A) and PLA₂ activities (B) in the colonic mucosa of controls, and patients with Crohn's disease and ulcerative colitis. * $p < 0.01$ compared with controls. † $p < 0.05$ compared with controls. ‡ $p < 0.01$ compared with inactive Crohn's disease. § $p < 0.05$ compared with inactive Crohn's disease.

Results

pH DEPENDENCE OF PLA₂ ACTIVITY IN COLONIC MUCOSA HOMOGENATES

It is well established that group II PLA₂ is alkaline active with optimal pH ranging from 8 to 9.5.⁴ As Almer *et al*³⁰ reported the optimal pH of 6.0 for PLA₂ activity in human colonic mucosa using a labelled *Escherichia coli* assay, we examined the pH dependence of activity towards mixed micelles of POPG and cholate in a homogenate of colonic mucosal biopsy sample obtained from a control patient (Fig 1). The pH was adjusted using 0.1 M TRIS-HCl buffer (pH 7–9.5) or 2-(*N*-morpholino) ethanesulphonic acid (pH 5.5–6.5). The maximal activity was found at pH 8.5, and thus this pH was used in this study.

SOLUBILISATION OF PLA₂ ACTIVITY WITH KBr

We examined whether the PLA₂ activity in inflamed colonic mucosa of patients with Crohn's disease and ulcerative colitis could be extracted by 1 M KBr treatment to the same extent as that in normal colonic mucosa.⁹ The recoveries of PLA₂ activity in the supernatant of the homogenates of the colonic biopsy samples obtained from controls, Crohn's disease patients, and ulcerative colitis patients after the KBr treatment were 94.4% (6.9), 90.4 (4.6), and 91.5 (4.7)%, respectively. Because the PLA₂ was fairly efficiently solubilised into the supernatant fraction of each biopsy sample, an IR-PLA₂ II content was defined as ng IR-PLA₂ II in the supernatant fractions/mg of homogenate proteins.

COLONIC MUCOSAL IR-PLA₂ II CONTENT AND PLA₂ ACTIVITY IN INFLAMMATORY BOWEL DISEASE

Figure 2 summarises the IR-PLA₂ II content and PLA₂ activity in the colonic biopsy samples obtained from the controls, and patients with Crohn's disease and ulcerative colitis. Mucosal IR-PLA₂ II contents of the controls, and all patients with Crohn's disease and ulcerative colitis were 7.2 (0.9), 37.4 (8.1), and 31.1 (7.2) ng/mg protein, respectively. The content of patients with Crohn's disease and ulcerative colitis were significantly higher than that of the controls ($p < 0.01$ and $p < 0.05$, respectively). Colonic mucosal IR-PLA₂ II contents of inactive and active Crohn's disease patients were 20.7 (6.5) and 54.8 (14.1) ng/mg protein, respectively, and those in mildly inflamed and severely inflamed mucosae of ulcerative colitis patients were 30.2 (11.6) and 32.0 (9.1) ng/mg protein, respectively. The IR-PLA₂ II content of active Crohn's disease patients was significantly higher than that of inactive Crohn's disease patients ($p < 0.05$) and controls ($p < 0.01$). The content in the severely inflamed colonic mucosa of ulcerative colitis patients was significantly higher than that in the colonic mucosa of controls ($p < 0.01$). No significant difference in IR-PLA₂ II content was found, however, between the mildly inflamed and

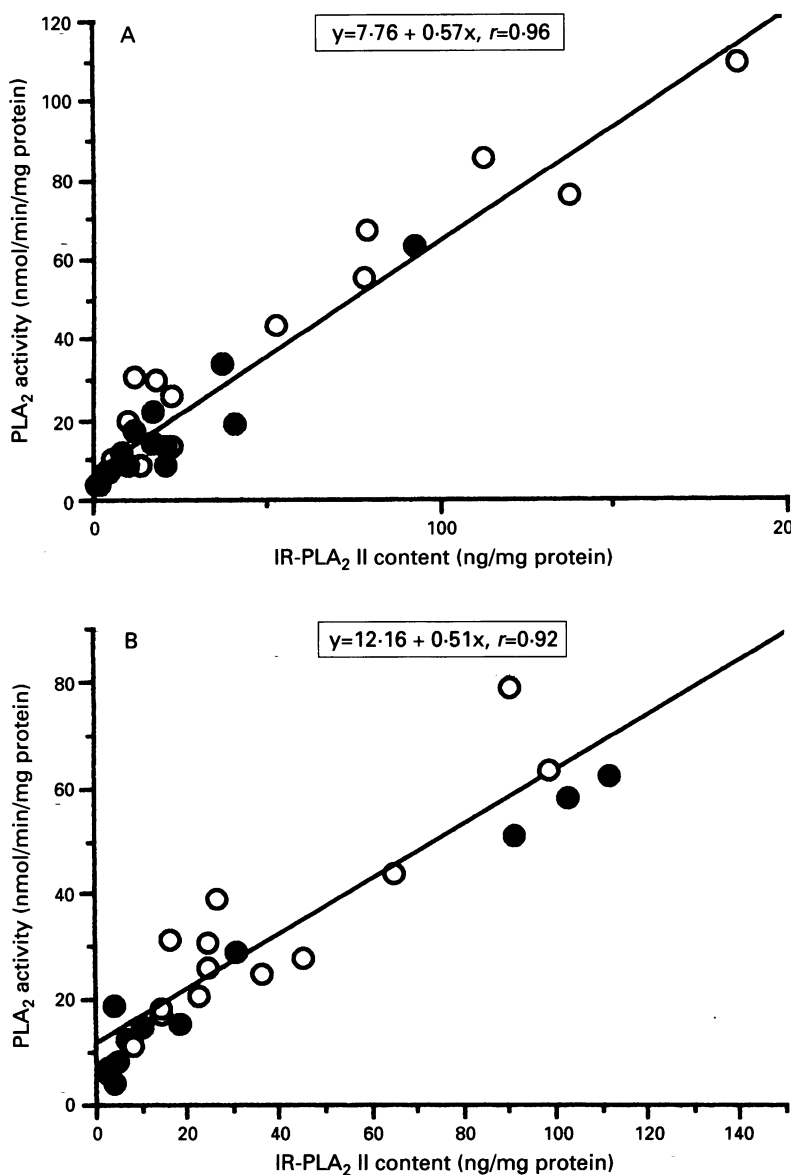


Figure 3: Correlation of PLA₂ enzymatic activity with IR-PLA₂ II content in the colonic mucosa of Crohn's disease patients (A) and ulcerative colitis patients (B). (A): (●), inactive Crohn's colitis, (○), active Crohn's colitis. (B): (●), mildly inflamed mucosa of ulcerative colitis patients, (○), severely inflamed mucosa of ulcerative colitis patients.

severely inflamed colonic mucosas of ulcerative colitis patients. Although mucosal IR-PLA₂ II content of inactive Crohn's disease patients tended to be increased, it did not significantly differ from that of the controls. The same tendency was seen in IR-PLA₂ II content in mildly inflamed mucosa of ulcerative colitis patients.

Colonic mucosal PLA₂ activities of the controls, and all patients with Crohn's disease and ulcerative colitis were 9.1 (1.0), 30.0 (5.4), and 28.0 (4.0) nmol/min/mg protein, respectively. The activities of patients with Crohn's disease and ulcerative colitis were significantly higher than those of the controls ($p < 0.01$). Mucosal PLA₂ activities of inactive and active Crohn's disease patients were 16.8 (4.2) and 44.2 (8.8) nmol/min/mg protein, respectively, and those in mildly and severely inflamed mucosas of ulcerative colitis patients were 25.5 (6.1) and 30.9 (5.1) nmol/min/mg protein, respectively. Mucosal PLA₂ activity of active Crohn's disease patients was significantly higher than that of inactive Crohn's

disease patients ($p < 0.01$) and controls ($p < 0.01$). The activity in severe ulcerative colitis patients was significantly higher than that in the controls ($p < 0.01$). There was no significant difference in PLA₂ activity, however, between mildly inflamed and severely inflamed colonic mucosas of ulcerative colitis patients. Although mucosal PLA₂ activities of inactive Crohn's disease or mild ulcerative colitis patients tended to be increased, there was no significant difference between those of the patients and controls.

On regression analysis, PLA₂ activity was closely correlated with IR-PLA₂ II content in the colonic biopsy samples obtained from the patients with Crohn's disease ($r = 0.96$, $p < 0.01$) or ulcerative colitis ($r = 0.92$, $p < 0.01$) (Fig 3).

There was no significant difference in colonic mucosal PLA₂ activity and IR-PLA₂ II content between the Crohn's disease patients not receiving drugs and those receiving treatment with sulphasalazine. There was also no significant difference found in colonic mucosal PLA₂ activity and IR-PLA₂ II content between the ulcerative colitis patients not treated with drugs and those receiving sulphasalazine, between the patients treated with no drugs and those receiving prednisolone in addition to sulphasalazine, and between the patients treated with sulphasalazine and prednisolone in addition to sulphasalazine. Although significant difference was found in age between Crohn's disease patients and controls, no correlation was found between patients' age and either colonic mucosal IR-PLA₂ II content or PLA₂ activity in the controls.

Discussion

Recently, raised intestinal content of arachidonate derived chemical mediators in inflammatory bowel disease patients²⁰⁻²² and increased colonic mucosal PLA₂ activity in patients with active Crohn's colitis²⁴ have been reported, suggesting association of PLA₂ with intestinal inflammation of inflammatory bowel disease. The isozymic form of intestinal mucosa PLA₂ in inflammatory bowel disease patients, however, has not been determined. In this study, we showed that colonic mucosal IR-PLA₂ II content and PLA₂ activity were significantly increased in inflamed mucosa of patients with Crohn's disease and ulcerative colitis, and that they were closely correlated with each other. This suggests that increased PLA₂ activity in the inflamed mucosa was mainly attributed to increased protein content of group II PLA₂. In view of a proinflammatory role of group II PLA₂,¹¹⁻¹³ significant increase of IR-PLA₂ II content in inflamed mucosa of Crohn's disease and ulcerative colitis patients and its association with endoscopically visualised severity of inflammation suggest that group II PLA₂ may participate in the pathogenesis of the intestinal inflammatory process in patients with Crohn's disease and ulcerative colitis, for example, in the development of inflammation by generation of various proinflammatory mediators such as lysophospholipids, prostaglandins, and

leukotrienes. It has not been shown that group II PLA₂ directly participates in the mobilisation of arachidonic acid; it has been reported that both cytosolic PLA₂ and group II PLA₂ may participate in prostaglandin I₂ synthesis in human umbilical vein endothelial cells.³¹ The functional difference in inflammatory response between cytosolic PLA₂ and group II PLA₂ should be further investigated.

It is possible that the increase in IR-PLA₂ II content seen in inflamed colonic mucosa of Crohn's disease and ulcerative colitis patients is a consequence of infiltration of inflammatory cells. This study did not show the type of cells responsible for these increments. A preliminary study in our laboratory showed that PLA₂ activity of polymorphonuclear and mononuclear leucocytes isolated from peripheral blood cells of patients with Crohn's disease and ulcerative colitis was much less than that of colonic mucosa (data not shown), and it was reported that group II PLA₂ immunoreactivity was not detectable in inflammatory cells infiltrating in synovial tissue of inflamed joints in rheumatoid arthritis.³² The precise localisation of group II PLA₂ in the inflamed mucosa remains to be determined.

Almer *et al*³⁰ reported that no significant difference was found in colonic mucosal PLA₂ activity between ulcerative colitis patients and control patients without inflammatory bowel disease in contrast with the results of this study. In their study, colonic mucosal PLA₂ activity was estimated at an acidic pH value of 6.0 and in the presence of 2 mM CaCl₂. Under these conditions alkaline active calcium dependent and calcium independent PLA₂s, and lysosomal PLA₂s with acidic pH optimum may contribute to the measurable activity. On the other hand, we selected a pH of 8.5 for assaying PLA₂ activity where group II PLA₂ is optimally active and used POPG/cholate mixed micelles, the best substrate for group II PLA₂.^{9,28} Calcium dependent PLA₂ activity was estimated by subtracting the value of activity in the presence of EDTA from that in the presence of Ca²⁺ ion. Therefore, the discrepancy on mucosal PLA₂ activity in ulcerative colitis patients could be explained by the difference in assay conditions used.

In previous studies,^{8,25} we reported that serum PLA₂ activity and IR-PLA₂ II concentrations were increased in active Crohn's disease patients and reflected the colonoscopic severity of inflammation in ulcerative colitis patients. The origin of serum PLA₂ in these patients is still unknown. The results of this study suggest that an increased concentration of group II PLA₂ in serum samples of patients with Crohn's disease and ulcerative colitis may be in part explained by leakage of group II PLA₂ from the inflamed mucosa.

The mechanism for an increase in group II PLA₂ in the inflamed colonic mucosa of patients with Crohn's disease and ulcerative colitis is unknown. Recently, it has been reported that various cytokines, such as interleukins 1 and 6, and tumour necrosis factor alpha caused an increase in group II PLA₂ mRNA and PLA₂ activity.^{12,13,33} High

concentrations of interleukin 1 and interleukin 6 in mucosal biopsy specimens and an increased production of tumour necrosis factor alpha by mononuclear cells isolated from intestinal biopsy specimens have been reported in patients with active inflammatory bowel disease.³⁴⁻³⁶ These findings raise a possibility that these cytokines stimulate intestinal mucosal group II PLA₂ synthesis and secretion, which may play a part in intestinal inflammatory process in inflammatory bowel disease. There is no direct evidence, however, that these cytokines elicit the expression of group II PLA₂ in vivo. The role and regulation of group II PLA₂ in intestinal inflammation of inflammatory bowel disease remain to be clarified.

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