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Phospholipase A_2 gene expression and activity in histologically normal ileal mucosa and in Crohn's ileitis

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Abstract

Increased activity of phospholipase A_2 (PLA_2) in the ileal mucosa may contribute to the inflammation in Crohn's disease. The results of this study showed that (a) three months after ileocolonic resection for Crohn's disease the neoterminal ileal mucosa showed endoscopically new inflammation and had higher PLA_2 activity than at the time of the operation (n=8); no such findings were seen in controls (n=7), (b) histologically normal ileal mucosa (n=3) contained mRNA for three isoforms of PLA_2 (PLA_2-I, PLA_2-II, and $cPLA_2$), but the amounts of PLA_2-II mRNA clearly exceeded the amounts of mRNA for PLA_2-I and $cPLA_2$, (c) ileal mucosa from Crohn's patients (n=2) contained higher values of PLA_2-II mRNA than ileal mucosa from two controls, (d) ileal mucosa from Crohn's patients (n=4) showed increased PLA_2-II mRNA three months after ileocolonic resection. In conclusion, these results show that the predominating PLA_2 mRNA in the human ileal mucosa is type II PLA_2, and that increased synthesis of PLA_2-II might be responsible for the increased PLA_2 activity found in the ileal mucosa accompanying recurrent ileal inflammation in Crohn's disease.

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Keywords: distal ileum, distal small intestine, inflammation, messenger ribonucleic acid, polymerase chain reaction.

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Phospholipase A_2 (PLA_2) is a key enzyme in the formation of arachidonic acid metabolites, platelet activating factor, and lysophosphatidylcholine,[1] all of which may take part in inflammatory reactions in the gastrointestinal tract.[2] Accordingly, the activity of PLA_2 was found to be increased in the ileal mucosa of patients with Crohn's disease, and the increased PLA_2 activity was associated with early symptomatic recurrent ileal inflammation after surgery.[3] Furthermore, although there was endoscopically detectable inflammation specific for Crohn's disease already three months after ileocolonic resection,[4] an increase in mucosal PLA_2 activity was found to precede the endoscopically detectable inflammation.[5] These findings suggest that activation of intestinal PLA_2 may play a part in the pathogenesis of Crohn's disease, although the underlying mechanisms remain to be clarified.

Until now, three genetically different forms of PLA_2 have been identified: two low molecular weight PLA_2, group I PLA_2 (PLA_2-I) and group II PLA_2 (PLA_2-II), and one high molecular weight, cytosolic PLA_2 ($cPLA_2$).[1] PLA_2-I is found in large amounts in the pancreas and is thought to serve mainly as a digestive enzyme. The presence of PLA_2-I, however, in other organs than the pancreas, such as the lung,[6] suggests that PLA_2-I may have additional, yet unidentified, physiological functions. PLA_2-II has been found both associated with cells and tissues and secreted extracellularly. For example, PLA_2-II has been found in secretory granules of the small intestinal Paneth cell,[7 8] increased amounts of PLA_2-II have been shown in rheumatoid synovial fluid,[9] and in psoriatic tissue.[10] High values of PLA_2 activity in serum from patients with Crohn's disease or ulcerative colitis, have been attributed to PLA_2-II, and correlated with disease activity.[11] The high molecular weight $cPLA_2$ has been identified in various inflammatory cells like human platelets, polymorphonuclear leucocytes, and monocytes[12–14] and is thought to play an important part in receptor mediated signal transduction, arachidonic acid liberation, and eicosanoid production.[15] It is not known in detail which type(s) of PLA_2 are present in the human ileal mucosa, or which type(s) could be responsible for the raised ileal PLA_2 activity found in patients with Crohn's disease.

The aim of this study was threefold: (a) to determine the activity of PLA_2 in the ileal mucosa in relation to the development of recurrent inflammation after ileocolonic resection for Crohn's disease, (b) to clarify which type(s) of PLA_2 are present in ileal mucosa, and (c) to examine which type(s) of PLA_2 could be responsible for the raised PLA_2 activity seen in the ileal mucosa of patients with Crohn's disease. We investigated the endoscopic appearance and mucosal PLA_2 activity of the ileal resection margin, at the time of ileocolonic resection and at follow up three months later. In addition, the mRNA expression of different PLA_2 enzymes (PLA_2-I, PLA_2-II and $cPLA_2$) was studied in a small number of patients with Crohn's disease and patients without inflammatory bowel disease.

Methods

Subjects

Mucosal samples for measurements of PLA_2 activity were obtained from the ileum of 15

patients operated on with ileocolonic resection. Eight of these (five men and three women, 17–55 years, mean 35) had Crohn's disease and seven (five women and two men, 43–77 years, mean 66) had colonic cancer. Intraoperatively, the appearance of the ileum was evaluated endoscopically and mucosal biopsy specimens were taken for PLA_2 analysis. The patients were followed up three months after the operation, with colonoileoscopic examination.[5] At that time the severity of recurrent inflammation was assessed by an endoscopic score and new mucosal biopsy specimens were collected for PLA_2 analysis. The biopsy samples were frozen in liquid nitrogen and kept at −70°C until analysis.

To study expression of PLA_2 on the mRNA level, RNA was isolated from the ileal mucosal resection margin of four of the patients described above (17–55 years, mean 38, Figs 4–6). From these four patients, RNA was also isolated from the same area, obtained at endoscopic follow up after three months. In addition, RNA was isolated from the distal ileum of two other patients with Crohn's disease (30 and 40 years, one macroscopically normal and one macroscopically inflamed) and two patients with colonic neoplasm (74 and 81 years, both macroscopically normal, Fig 3). RNA was also isolated from the proximal colon of a patient with colonic neoplasm (79 years, macroscopically normal, Fig 1).

After surgical removal, the bowel specimen was kept on ice, both during transport to the laboratory and while the mucosa was scraped off the muscularis layer. RNA preparations were started within 30 minutes after surgical removal. Biopsy samples were frozen in liquid nitrogen and kept at −70° until analysis.

Ethics

The study was approved by the ethics committee of human experimentation, Linköping.

Primers and probes

For detection of PLA_2 mRNA, primers and probes (synthesised by Scandinavian Gene Synthesis, Köping, Sweden) were chosen from complementary deoxyribonucleic acids. PLA_2-I was purified and sequenced from human lung.[6], PLA_2-II from human platelets and synovial fluid,[9 16] and $cPLA_2$ from the human monoblast U937 cell line.[17] Sequences (Table I) are specific as ascertained by computer assisted search of updated versions of Gene Bank.

TABLE I *Oligonucleotide primer and probe sequences*

	5′-3′	*Amplified product (bp)*
Primers for PCR		
5′ PLA_2-I	GTGACCCCTTCTTGGAATAC	233
3′ PLA_2-I	GGCCTCACACTCTTTGTTTT	
5′ PLA_2-II	AAGCCGCACTCAGTTATGG	238
3′ PLA_2-II	GCAGCAGCCTTATCACACT	
5′ $CPLA_2$	ATGCCCAGACCTACGATTTA	737
3′ $CPLA_2$	AGGGGTTTTCTTCATACTTC	
Probes for PCR product		
5′ PLA_2-I	TGGCAGCACTTGTCCAGTTCATCCA	
5′ PLA_2-II	ATGAGTGACACAGCAGCGATCCGTT	
5′ $cPLA_2$	TTGACATATACCAGGTGGAGCCAGA	
Probe for northern blot		
5′ PLA_2-II	ACACAGCAGCGATCCGTTGCATCCTTGGGG	

RNA preparation

Total RNA was prepared according to Chomczynski and Sacchi[18] as previously described in detail.[19]

PLA_2 enzyme activity

PLA_2 activity was analysed after disintegration of biopsy specimens with a Dounce homogeniser, using 150 mM NaCl as homogenising medium. The PLA_2 activity was analysed with labelled *Escherichia coli* membranes as substrate as previously described.[3] PLA_2 activity was expressed as units (U), representing percentage ^{14}C-oleic acid liberation of total radioactivity per μg protein.

Northern blots

Separation of different sized RNA fragments was performed in a denaturing system as previously described[19] and the fragments transferred to positively charged nylon membranes (Boehringer Mannheim). The original method was modified by labelling the specific oligonucleotide probe, corresponding to base pairs 271–300 in the human PLA_2-II complementary deoxyribonucleic acid, with DIG (digoxigenin) oligonucleotide tailing system according to the manufacturer's instructions (Boehringer Mannheim, Mannheim, Germany). The membrane with bound RNA was prehybridised (60°C) for one hour in a solution containing 0·39 M sodium chloride, 0·03 M sodium citrate, 1% blocking reagent, 0·1% *N*-lauroylsarcosine, and 0·02% laurylsulphate. The PLA_2 hybridisation reaction was then carried out in a solution as previously described and with the addition of 10 nM labelled probe (60°C) for three hours. The membrane was washed and the hybridised product was detected with DIG Luminescent system according to the manufacturer's instructions (Boehringer Mannheim). The resulting blots were subjected to autoradiography on Cronex 4 *x* ray film with intensifying screens (Du Pont de Nemours, Bad Homburg, Germany) at room temperature for 5–30 minutes before development.

Polymerase chain reaction (PCR) assisted mRNA amplification

First strand cDNA synthesis RNA was denatured at 70°C for five minutes and then chilled on ice. One μg total RNA was transcribed to cDNA in a final volume of 20 μl master mix solution containing 5 mM $MgCl_2$, 1x PCR buffer II (50 mM KCl and 10 mM TRIS HCl, pH 8·3), 1 mM of each nucleotide (dGTP, dATP, dTTP, and dCTP), 1 U/μl RNase inhibitor, 2·5 U/μl reverse transcriptase, and 2·5 μM random hexamers. The reaction mixture was incubated at 20°C for 10 minutes, 42°C for 15 minutes, 99°C for five minutes, and 5°C for five minutes (GeneAmp RNA PCR Kit, Perkin-Elmer, Roche

TABLE II *PLA_2 enzyme activity in ileal mucosa from resection margin at time for ileocolonic resection of patients operated on for Crohn's disease or colonic cancer, and from the same area (preanastomotic) at follow up three months later*

	PLA_2 activity (U)		
Patients	*At operation*	*At 3 months*	*p Values*
Crohn's disease (n=8)	7·1 (5·5–11)	10·3 (9·6–11·1)	<0·05
Controls (n=7)	8·1 (5·8–11)	8·2 (7–10·2)	>0·05
p Values	>0·05	<0·01	

PLA_2 activity is expressed as units (U), representing percentage liberated ^{14}C-oleic acid (after background correction) from ^{14}C-oleic acid-labelled *E coli* membranes. Medians and ranges.

Molecular Systems, New Jersey, USA). cDNA from each sample was synthesised in one tube and then divided into separate tubes for the PCR.

PCR

Five µl first strand cDNA was added to a final volume of 25 µl PCR mixture containing 2 mM $MgCl_2$, 1×PCR buffer (10 mM TRIS HCl, 50 mM KCl), and 0·2 µM of each primer, and 0·5 U AmpliTaq DNA Polymerase. The reaction mixture was amplified with a Perkin-Elmer Thermal cycler 9600 for 25–55 cycles. Hot start of the PCR reaction was performed by heating the reaction mixture to 70°C for four minutes in the Thermal cycler before addition of the AmpliTaq DNA Polymerase. The reaction was immediately subjected to a single denaturation step (94°) for 105 seconds, and thereafter to the repeated three step temperature profile of 94°C for 10 seconds (denaturation), 54·5°C (PLA_2I and PLA_2-II) or 51·5°C ($cPLA_2$) for 10 seconds (annealing), and 72° for 10 seconds (PLA_2-I and PLA_2-II) or 15 seconds ($cPLA_2$) (primer extension). A single 10 minutes elongation period finished the PCR reaction.

Detection of PCR products

PCR products were separated on 1·6% agarose gel (SeaKem ME, FMC BioProducts, Rockland, ME, USA), and stained with ethidium bromide. All PCR products showed a single band of expected size when compared with known molecular weight markers (VI and IX, Boehringer Mannheim, Mannheim, Germany).

PCR products were also transferred to cationised nylon membranes (Boehringer Mannheim) in a solution containing 0·39 M sodium chloride and 0·03 M sodium citrate by a microfiltration unit according to the manufacturer's instruction (BioRad). PCR products were bound to the membrane by cross linking with ultraviolet light at 1200 W (Stratalinker, Stratagene) and kept dry until hybridisation. The membrane with PCR products was hybridised under the same conditions as described for northern blots, but with 10 nM DIG labelled oligonucleotide probe, complementary to part of one strand of the PCR product, and the temperatures during hybridisation reaction were adjusted to fit probes for PLA_2-I and PLA_2-II (53°), or $cPLA_2$ (51°) PCR products. The signal intensity was measured with a computerised image system (Bio Image Products, Ann Arbor, MI, USA).

In an attempt to get optimal conditions for comparison between samples, different numbers of amplification cycles were tested for each group of PLA_2. The same batch of reverse transcribed total RNA was divided for use in the analysis of PLA_2-I, PLA_2-II, and $cPLA_2$. Also, the number of cycles used in the amplification of the PCR product, was chosen to give optimal resolution.

Statistical analysis

Comparative statistical analysis was made with Wilcoxon rank sum test (between the groups), and with Wilcoxon signed rank test (within the groups), with $p<0·05$ considered significant.

Results

Ileal PLA_2 activity and endoscopic appearance in Crohn's disease

Three months after ileocolonic resection for Crohn's disease, the PLA_2 activity was significantly increased in the preanastomotic ileal mucosa compared with the mucosa obtained from the same area at the time of resection (Table II). No such difference was found in controls (patients resected for colonic cancer) (Table II). Moreover, three months after ileocolic resection, the PLA_2 activity in ileal mucosa from Crohn's disease patients was significantly higher than in the controls. No macroscopical signs of inflammation were seen at the resection margin at the time of operation in Crohn's disease patients or controls (score=0), while after three months postoperatively, all Crohn's disease patients showed recurrent ileal inflammation (scores between 1 and 4, median 2). No endoscopic signs of inflammation were seen in the control group (score=0).

Ileal PLA_2 mRNA expression

With northern blot analysis, PLA_2-II mRNA was easily detected in histologically normal mucosal samples from the distal ileum and proximal colon. As Fig 1 shows, the expression of PLA_2-II mRNA in the distal ileum clearly exceeded that in the proximal colon. The

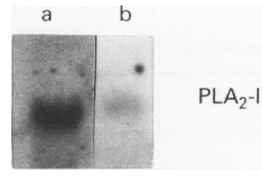

Figure 1: Northern blot analysis of group II PLA_2 mRNA from histologically normal intestinal mucosa. The mucosal samples (a, distal ileum; b, proximal colon) were obtained from two patients operated on for colonic cancer. Each sample contained 30 µg total RNA and was hybridised with a digoxigenin tailed 30 mer oligonucleotide probe corresponding to base pairs 300–329 in the low molecular weight (0·8 kDa) human group II PLA_2 complementary DNA. The Figure shows the results after 30 minutes of autoradiographic exposure time.

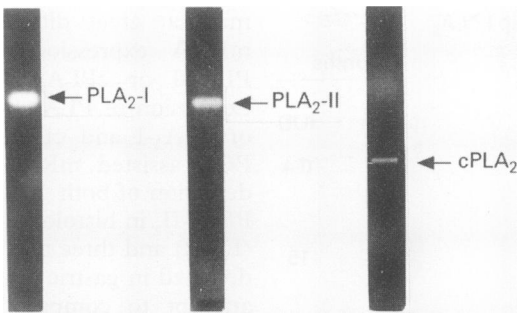

Figure 2: The Figure shows presence of group I PLA_2, group II PLA_2, and $cPLA_2$ in histologically normal ileal mucosa after PCR assisted mRNA amplification. Mucosa were obtained from three patients operated on for colonic cancer. One μg total RNA was transcribed to cDNA, and aliquots from the cDNA preparation were used in three separate PCR reactions with primers specific for human group I PLA_2, group II PLA_2, and $cPLA_2$, respectively (Table I). Each PCR reaction was amplified 30–40 cycles. The resulting PCR products were separated on agarose gel and visualised with ultraviolet light after ethidium bromide staining. The Figure shows data from one of the patients, which are representative for the other two.

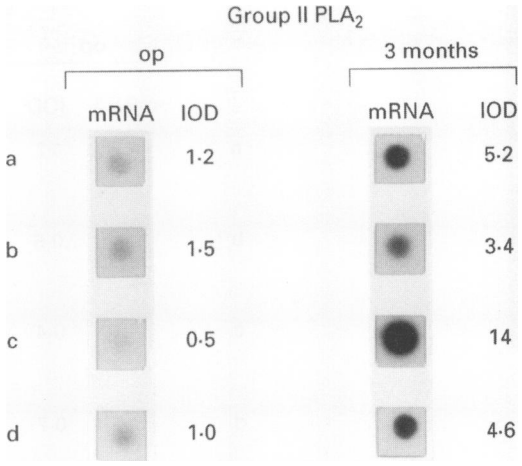

Figure 4: Group II PLA_2 mRNA expression in ileal mucosa from four patients operated on for Crohn's disease (a–d). RNA was isolated from endoscopically normal ileal resection margin at the time of ileocolonic resection (op), and from the same area at endoscopic follow up after three months. The Figure shows spots and integrated optical density units (IOD), of group II PLA_2 mRNA, obtained after dot blot analysis of group II PLA_2 PCR products amplified 25 or 30 cycles. One μg total RNA was transcribed to cDNA with random hexamer primers, and aliquots from the same cDNA preparation were used in the PCR amplification of group II PLA_2 as well as group I PLA_2 and $cPLA_2$. The PCR product was hybridised with a digoxigenin tailed 20 mer oligonucleotide probe corresponding to part of the human group II PLA_2 gene and spanning an area on both sides of an intron. The Figure shows the results obtained after five minutes of autoradiographic exposure time.

expression of PLA_2-II mRNA was thus clearly evident. Using the same conditions for northern blot analysis, however, mRNA for PLA_2-I or $cPLA_2$ could not be detected, either with 30 μg total RNA from each sample, or with up to 10 μg of purified mRNA.

By contrast, PCR assisted mRNA amplification permitted the detection of PLA_2-I and $cPLA_2$ together with PLA_2-II mRNA in histologically normal ileal mucosa (Fig 2). mRNAs for PLA_2-I, PLA_2-II, and $cPLA_2$ were also found in histologically normal gastric and colonic mucosa (data not shown).

Northern blot analysis of samples from the distal ileum showed higher PLA_2-II mRNA expression in two Crohn's disease patients than in two patients with non-inflammatory bowel disease (Fig 3). Both the macroscopically inflamed and the macroscopically normal mucosa of the Crohn's disease patients, showed increased PLA_2-II mRNA expression when compared with patients with non-inflammatory bowel disease (Fig 3).

The ileal mRNA expression of PLA_2-I, PLA_2-II, and $cPLA_2$ was studied in four of the patients with Crohn's disease, previously investigated for ileal PLA_2 activity at ileocolonic resection and at follow up three months later (Table II). The results showed that after three months, PLA_2-II mRNA expression was increased in all four cases (25 or 30 cycles PCR amplification) (Fig 4). Furthermore, although mRNA for PLA_2-I could be detected in all samples after 50 cycles PCR amplification, no clear pattern appeared as to differences between the time of resection and after three months (Fig 5). As Fig 6 shows, it was possible to detect mRNA for $cPLA_2$ in two of the investigated samples at the time of resection and in one sample after three months (55 cycles PCR amplification). No clear pattern was seen as to differences in $cPLA_2$ mRNA between samples at the time of resection and after three months (Fig 6).

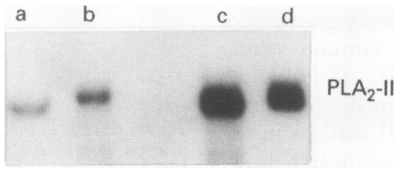

Figure 3: Northern blot analysis of group II PLA_2 mRNA in ileal mucosa obtained from four different patients. Two of these (a and b) were operated on for colonic cancer, and the other two (c and d) for Crohn's disease. The ileal mucosa was histologically normal in a, b, and d, but inflamed in c. Each sample contained 30 μg total RNA and was hybridised with a digoxigenin tailed oligonucleotide probe as characterised in Table I. The Figure shows the results obtained after 15 minutes of autoradiographic exposure time.

Discussion

The rate of postoperative recurrence is high in Crohn's disease, and recurrent ileal inflammation has been found in the neoterminal ileum three months after ileocolonic resection.[4] We previously found that PLA_2 activity was increased in the ileal preanastomotic area, suggesting a role for PLA_2 in the development of the new inflammatory lesions.[5] The results of our investigation confirm and extend these previous findings. Thus, the PLA_2 activity was found to be normal in endoscopically normal ileal mucosa at the time of operation and a raised PLA_2 activity was found accompanying ileal recurrent inflammation after three months (Table II). Thus, the PLA_2 activity does not seem to be generally increased in the ileal mucosa of patients with Crohn's disease. It may be inferred, therefore, that increased PLA_2 activity accompanies an ongoing inflammatory process, which starts in the neoterminal ileum, close to the new junction. It is possible that the close proximity to the colon,

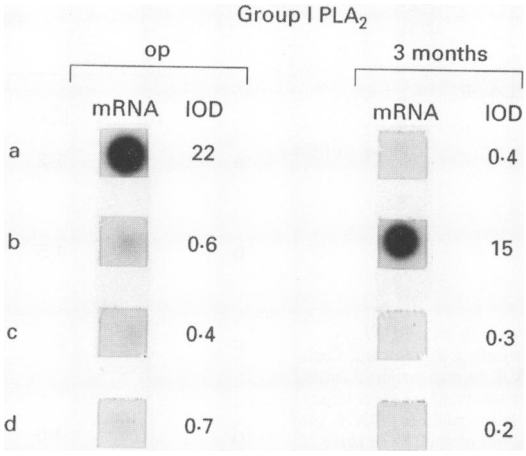

Figure 5: Group I PLA_2 mRNA expression in ileal mucosa from four patients with Crohn's disease (a–d). RNA was isolated from endoscopically normal ileal resection margin at the time of ileocolonic resection (op), and from the same area at endoscopic follow up after three months. The Figure shows spots and integrated optical density units (IOD), of group I PLA_2 mRNA, obtained after dot blot analysis of group I PLA_2 PCR products amplified 50 cycles. The group I PLA_2 PCR products were hybridised with a digoxigenin tailed 20 mer oligonucleotide probe corresponding to part of the PCR product and spanning an area on both sides of an intron of the human group I PLA_2 gene. The Figure shows the result obtained after 20 minutes autoradiographic exposure time.

with the possibility of reflux of colonic substances, might be harmful to ileal mucosa.[5] We have previously shown that colonic microbial substances, like endotoxin from *E coli*,[19] or phospholipase C from *Clostridium perfringens*,[20] may increase PLA_2 activity and synthesis, and PLA_2 dependent arachidonic acid release, respectively.

Previous investigations have not shown the type(s) of PLA_2 that could be responsible for the raised activity found in patients with Crohn's disease. In this study, we analysed mRNA for three different types of PLA_2 in ileal mucosa. Using northern blot analysis we could easily detect PLA_2-II mRNA expression in samples from histologically normal ileal or colonic mucosa (Fig 1). The signals from transcripts of PLA_2-II were much more intense in a sample from the distal ileum than in a sample from the proximal colon. This finding is in accordance with those of Kiyohara *et al*,[8] who reported stronger immunoreactive PLA_2-II signals in ileal than colonic mucosa, and by Minami *et al*,[21] who showed more intense PLA_2-II mRNA expression in ileal than caecal mucosa. Moreover, immunohistochemical studies by Nevalainen *et al*,[7] and Kiyohara *et al*[8] have shown that the secretory granules of human intestinal Paneth cells contain strong immunoreactivity of PLA_2-II. The small intestinal Paneth cells might thus be the source of the large amounts of PLA_2-II mRNA in the ileum. Other cells cannot be excluded, however, particularly not in a mucosa subjected to inflammatory reaction.

With northern blot technique, we could not detect PLA_2-I or $cPLA_2$ in ileal mucosa, not even after purification of the samples to pure mRNA fractions. Because of these striking differences in detection possibilities, it can be deduced that there are, in the human ileal mucosa, great differences in the values of mRNA expression between PLA_2-II and PLA_2-I or $cPLA_2$. Obviously, the mRNA expression of PLA_2-II greatly supersedes that of PLA_2-I and $cPLA_2$. On the other hand, PCR assisted mRNA amplification enabled detection of both group I and $cPLA_2$, besides PLA_2-II, in histologically normal ileal mucosa (Fig 2) and three types of PLA_2 could also be detected in gastric and colonic mucosa. In an attempt to compare PLA_2-I, PLA_2-II, and $cPLA_2$ in biopsy specimens of normal and inflamed mucosa, we analysed mRNA expression for all three types of PLA_2 after PCR assisted mRNA amplification (Figs 4–6). The results suggest that PLA_2-II might contribute to the increased PLA_2 activity seen as soon as three months after ileocolonic resection. No clear changes in mRNA expression for $cPLA_2$ were seen at the time when recurrent inflammation was observed. Nevertheless, a contribution from $cPLA_2$ to the increased PLA_2 activity cannot be excluded. $cPLA_2$ is thought to exert its enzyme activity after phosphorylation,[15] which may be achieved without de novo synthesis of the enzyme.

When PLA_2-II mRNA was determined in ileal mucosa from two Crohn's disease patients and compared with histologically normal ileal mucosa from controls, there were clear differences in PLA_2-II mRNA expression. Both the macroscopically inflamed and the macroscopically normal sample from Crohn's disease patients showed stronger intensity of PLA_2-II transcripts than the controls, showing that PLA_2-II mRNA synthesis may be increased regardless of whether the ileal mucosa is macroscopically inflamed or not. It is possible that the increased group II PLA_2 mRNA synthesis precedes the development of a macroscopically visible inflammation.

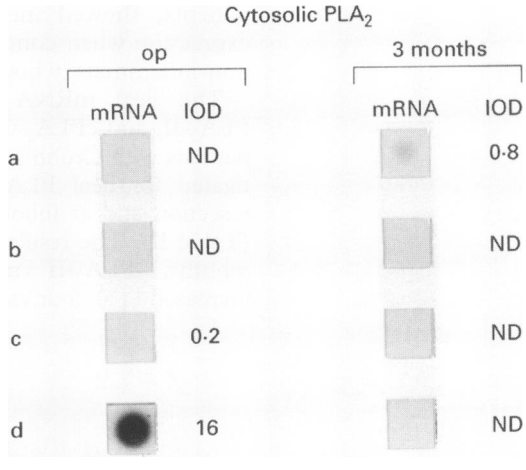

Figure 6: $cPLA_2$ mRNA expression in ileal mucosa from four patients with Crohn's disease (a–d). RNA was isolated from endoscopically normal ileal resection margin at the time of ileocolonic resection (op), and from the same localisation at endoscopic follow up after three months. The Figure shows spots and integrated optical density units (IOD), of $cPLA_2$ mRNA, obtained after dot blot analysis of $cPLA_2$ PCR products amplified 55 cycles. The $cPLA_2$ PCR products were hybridised with a digoxigenin tailed 20 mer oligonucleotide probe corresponding to part of the amplified area of the human $cPLA_2$ gene. The Figure shows the result after 20 minutes of autoradiographic exposure time.

In summary, we have found that the distal ileum is rich in PLA_2-II mRNA and that the expression of this mRNA and the corresponding enzyme activity accompanies recurrent new ileal inflammation as soon as three months after ileocolonic resection for Crohn's disease. As far as we know, this is the first study in which endoscopic findings and PLA_2 enzyme activity are related to group I, group II, and $cPLA_2$ mRNA expression within the same patient at the time of ileocolonic resection and at the same locality at follow up on a later occasion. This study does not clarify, however, which particular cell type(s) is responsible for the increased PLA_2 activity and mRNA expression. This problem might be considered by using in situ hybridisation and immunohistochemical studies of normal and diseased tissue.

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