Phospholipase A₂ gene expression and activity in histologically normal ileal mucosa and in Crohn's ileitis

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Abstract
Increased activity of phospholipase A₂ (PLA₂) 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 nonterminal ileal mucosa showed endoscopically new inflammation and had higher PLA₂ 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 PLA₂ (PLA₂-I, PLA₂-II, and cPLA₂), but the amounts of PLA₂-II mRNA clearly exceeded the amounts of mRNA for PLA₂-I and cPLA₂, (c) ileal mucosa from Crohn's patients (n=2) contained higher values of PLA₂-II mRNA than ileal mucosa from two controls, (d) ileal mucosa from Crohn's patients (n=4) showed increased PLA₂-II mRNA three months after ileocolonic resection. In conclusion, these results show that the predominating PLA₂ mRNA in the human ileal mucosa is type II PLA₂, and that increased synthesis of PLA₂-II might be responsible for the increased PLA₂ activity found in the ileal mucosa of patients with recurrent ileal inflammation in Crohn's disease.

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

Phospholipase A₂ (PLA₂) 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 PLA₂ was found to be increased in the ileal mucosa of patients with Crohn's disease, and the increased PLA₂ 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 PLA₂ activity was found to precede the endoscopically detectable inflammation.⁵ These findings suggest that activation of intestinal PLA₂ 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 PLA₂ have been identified: two low molecular weight PLA₂, group I PLA₂ (PLA₂-I) and group II PLA₂ (PLA₂-II), and one high molecular weight, cytosolic PLA₂ (cPLA₂).¹ PLA₂-I is found in large amounts in the pancreas and is thought to serve mainly as a digestive enzyme. The presence of PLA₂-I, however, in other organs than the pancreas, such as the lung,⁶ suggests that PLA₂-I may have additional, yet unidentified, physiological functions. PLA₂-II has been found both associated with cells and tissues and secreted extracellularly. For example, PLA₂-II has been found in secretory granules of the small intestinal Paneth cell,⁷,⁸ increased amounts of PLA₂-II have been shown in rheumatoid synovial fluid,⁹ and in psoriatic tissue.¹⁰ High values of PLA₂ activity in serum from patients with Crohn's disease or ulcerative colitis, have been attributed to PLA₂-II, and correlated with disease activity.¹¹ The high molecular weight cPLA₂ has been identified in various inflammatory cells like human platelets, polymorphonuclear leucocytes, and monocytes¹²-¹⁴ 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 PLA₂ are present in the human ileal mucosa, or which type(s) could be responsible for the raised ileal PLA₂ activity found in patients with Crohn's disease.

The aim of this study was threefold: (a) to determine the activity of PLA₂ 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 PLA₂ are present in ileal mucosa, and (c) to examine which type(s) of PLA₂ could be responsible for the raised PLA₂ activity seen in the ileal mucosa of patients with Crohn's disease. We investigated the endoscopic appearance and mucosal PLA₂ 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 PLA₂ enzymes (PLA₂-I, PLA₂-II and cPLA₂) 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 PLA₂ 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 PLA2 analysis. The patients were followed up three months after the operation, with colonoscopical examination. At that time the severity of recurrent inflammation was assessed by an endoscopic score and new mucosal biopsy specimens were collected for PLA2 analysis. The biopsy samples were frozen in liquid nitrogen and kept at −70°C until analysis.

To study expression of PLA2 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°C until analysis.

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

Primers and probes
For detection of PLA2 mRNA, primers and probes (synthesised by Scandinavian Gene Synthesis, Köping, Sweden) were chosen from complementary deoxyribonucleic acids. PLA2-I was purified and sequenced from human lung,6 PLA2-II from human platelet and synovial fluid,7,8,10 and cPLA2 from the human monoblast U937 cell line.11 Sequences (Table I) are specific as ascertained by computer assisted search of updated versions of Gene Bank.

RNA preparation
Total RNA was prepared according to Chomczynski and Sacchi18 as previously described in detail.19

PLA2 enzyme activity
PLA2 activity was analysed after disintegration of biopsy specimens with a Dounce homogeniser, using 150 mM NaCl as homogenising medium. The PLA2 activity was analysed with labelled Escherichia coli membranes as substrate as previously described.3 PLA2 activity was expressed as units (U), representing percentage 14C-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 described19 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 PLA2-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-lauroyl-sarcosine, and 0.02% laurylsulphate. The PLA2 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 MgCl2, 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>
<thead>
<tr>
<th>Primer/Probe</th>
<th>Amplified product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5' PLA2-I</td>
<td>GTGACCCCTTCTCTGGAATAC</td>
</tr>
<tr>
<td>3' PLA2-I</td>
<td>GGCCCTCAACATCTTTTTTT</td>
</tr>
<tr>
<td>5' PLA2-II</td>
<td>GAGCGAGGGGATCAGGATG</td>
</tr>
<tr>
<td>3' PLA2-II</td>
<td>AGGGTGGTTTCTTCAAATTC</td>
</tr>
<tr>
<td>5' cPLA2</td>
<td>ATGCGGCAGCTCAGTT</td>
</tr>
<tr>
<td>3' cPLA2</td>
<td>GGGGCGGTTTCTTCAAATTC</td>
</tr>
<tr>
<td>Probe for northern blot</td>
<td>TAGCAGACGCGGCTCAGTCTTCTATCC</td>
</tr>
<tr>
<td>5' PLA2-II</td>
<td>ACGACAGCGGCTCAGTCTTCTATCC</td>
</tr>
</tbody>
</table>

Table I: Oligonucleotide primer and probe sequences
**TABLE 1**  
PLA₂ 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

<table>
<thead>
<tr>
<th>Patients</th>
<th>PLA₂ activity (U)</th>
<th>At operation</th>
<th>At 3 months</th>
<th>p Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crohn’s disease  (n=8)</td>
<td>7.1 (5.5–11)</td>
<td>10.3 (9.6–11.1)</td>
<td>&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>Controls (n=7)</td>
<td>8.1 (5.8–11)</td>
<td>8.2 (7.1–10.2)</td>
<td>&gt;0.05</td>
<td></td>
</tr>
</tbody>
</table>

PLA₂ activity is expressed as units (U), representing percentage liberation of 14C-oleic acid (after background correction) from 14C-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 MgCl₂, 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°C) for 105 seconds, and thereafter to the repeated three step temperature profile of 94°C for 10 seconds (denaturation), 54-55°C (PLA₂-I and PLA₂-II) or 51-5°C (cPLA₂) for 10 seconds (annealing), and 72°C for 10 seconds (PLA₂-I and PLA₂-II) or 15 seconds (cPLA₂) (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 PLA₂-I and PLA₂-II (53°C), or cPLA₂ (51°C) 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 PLA₂. The same batch of reverse transcribed total RNA was divided for use in the analysis of PLA₂-I, PLA₂-II, and cPLA₂. 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 PLA₂ activity and endoscopic appearance in Crohn’s disease**

Three months after ileocolonic resection for Crohn’s disease, the PLA₂ activity was significantly increased in the preanastomotic ileal mucosa compared with the mucosa obtained from the same area at the time of resection (Table I). No such difference was found in controls (patients resected for colonic cancer) (Table II). Moreover, three months after ileocolonic resection, the PLA₂ 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 post-operatively, 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 PLA₂ mRNA expression**

With northern blot analysis, PLA₂-II mRNA was easily detected in histologically normal mucosal samples from the distal ileum and proximal colon. As Fig 1 shows, the expression of PLA₂-II mRNA in the distal ileum clearly exceeded that in the proximal colon. The

![Figure 1: Northern blot analysis of group II PLA₂ 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 PLA₂ complementary DNA. The Figure shows the results after 30 minutes of autoradiographic exposure time.](http://gut.bmj.com/)
expression of PL2-II mRNA was thus clearly evident. Using the same conditions for northern blot analysis, however, mRNA for PL2-II or cPLA2 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 PL2-I and cPLA2 together with PL2-II mRNA in histologically normal ileal mucosa (Fig 2). mRNAs for PL2-I, PL2-II, and cPLA2 were also found in histologically normal gastric and colonic mucosa (data not shown).

Northern blot analysis of samples from the distal ileum showed higher PL2-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 PL2-II mRNA expression when compared with patients with non-inflammatory bowel disease (Fig 3).

The ileal mRNA expression of PL2-I, PL2-II, and cPLA2 was studied in four of the patients with Crohn's disease, previously investigated for ileal PL2 activity at ileocolonic resection and at follow up three months later (Table II). The results showed that after three months, PL2-II mRNA expression was increased in all four cases (25 or 30 cycles PCR amplification) (Fig 4). Furthermore, although mRNA for PL2-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 cPLA2 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 cPLA2 mRNA between samples at the time of resection and after three months (Fig 6).

**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. We previously found that PL2 activity was increased in the ileal preanastomotic area, suggesting a role for PL2 in the development of the new inflammatory lesions. The results of our investigation confirm and extend these previous findings. Thus, the PL2 activity was found to be normal in endoscopically normal ileal mucosa at the time of operation and a raised PL2 activity was found accompanying ileal recurrent inflammation after three months (Table II). Thus, the PL2 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 PL2 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,
with the possibility of reflux of colonic substances, might be harmful to ileal mucosa. We have previously shown that colonic microbial substances, like endotoxin from *E. coli*, or phospholipase C from *Clostridium perfringens*, may increase PLA₂ activity and synthesis, and PLA₂ dependent arachidonic acid release, respectively.

Previous investigations have not shown the type(s) of PLAs 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 PLAs in ileal mucosa. Using northern blot analysis we could easily detect PLA₂-II mRNA expression in samples from histologically normal ileal or colonic mucosa (Fig 1). The signals from transcripts of PLA₂-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., who reported stronger immunoreactive PLA₂-II signals in ileal than colonic mucosa, and by Minami et al. who showed more intense PLA₂-II mRNA expression in ileal than caecal mucosa. Moreover, immunohistochemical studies by Nevalainen et al. and Kiyohara et al. have shown that the secretory granules of human intestinal Paneth cells contain strong immunoreactivity of PLA₂-II. The small intestinal Paneth cells might thus be the source of the large amounts of PLA₂-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 PLA₁-I or cPLA₂ 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 PLA₁-II and PLA₂-I or cPLA₂. Obviously, the mRNA expression of PLA₂-II greatly superseded that of PLA₁-I and cPLA₂. On the other hand, PCR assisted mRNA amplification enabled detection of both group I and cPLA₂, besides PLA₂-II, in histologically normal ileal mucosa (Fig 2) and three types of PLA₂ could also be detected in gastric and colonic mucosa. In an attempt to compare PLA₁-I, PLA₂-II, and cPLA₂ in biopsy specimens of normal and inflamed mucosa, we analysed mRNA expression for all three types of PLA₂ after PCR assisted mRNA amplification (Figs 4–6). The results suggest that PLA₂-II might contribute to the increased PLA₂ activity seen as soon as three months after ileocolonic resection. No clear changes in mRNA expression for cPLA₂ were seen at the time when recurrent inflammation was observed. Nevertheless, a contribution from cPLA₂ to the increased PLA₂ activity cannot be excluded. cPLA₂ is thought to exert its enzyme activity after phosphorylation, which may be achieved without de novo synthesis of the enzyme.

When PLA₂-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 PLA₂-II mRNA expression. Both the macroscopically inflamed and the macroscopically normal sample from Crohn’s disease patients showed stronger intensity of PLA₂-II transcripts than the controls, showing that PLA₂-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 PLA₂ mRNA synthesis precedes the development of a macroscopically visible inflammation.
In summary, we have found that the distal ileum is rich in PLA2-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 PLA2 enzyme activity are related to group I, group II, and cPLA2 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 PLA2 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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