Potential role of protease-activated receptor-2-stimulated activation of cytosolic phospholipase A2 in intestinal myofibroblast proliferation: Implications for stricture formation in Crohn's disease

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Abstract
Background and aims: Myofibroblast hyperplasia contributes to muscularis mucosae thickening and stricture formation in Crohn's disease (CD). Protease-activated receptor-2 (PAR-2) and cytosolic phospholipase A2 (cPLA2) are known regulators of cell growth, but their significance in intestinal myofibroblast proliferation remain to be elucidated. The principle aims of the present study were to investigate if PAR-2 is expressed in the expanded muscularis mucosa in ileal CD specimens, if inflammatory cytokines may stimulate PAR-2 expression in intestinal myofibroblasts, and if PAR-2 and cPLA2 may regulate intestinal myofibroblast growth.

Methods: Immunohistochemistry was used for detection of PAR-2 in ileal CD specimens. Studies on PAR-2 expression, PLA2 activation and cell growth were performed in a human intestinal myofibroblast cell line, CCD-18Co. PAR-2 expression was investigated by RT-PCR and immunocytochemistry. PLA2 activity was analyzed by quantification of released 14C-arachidonic acid (14C-AA). Cell growth was examined by 3H-thymidine incorporation and cell counting.

Abbreviations: AA, arachidonic acid; AACOCF3, arachidonyl trifluoromethyl ketone; BEL, bromoenol lactone; CD, Crohn's disease; MAFP, methyl arachidonyl fluorophosphonate; PAR-2, protease-activated receptor-2; PLA2, phospholipase A2; cPLA2, cytosolic phospholipase A2; iPLA2, Ca2+-independent phospholipase A2; PMA, phorbol myristate acetate; TNF-α, tumor necrosis factor-α.

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1. Introduction

Formation of strictures, leading to intestinal obstruction, is a common clinical problem in Crohn's disease (CD). Overgrowth and thickening of the muscularis mucosae is frequently seen in CD, and contributes to the stricture formation. The expansion of muscularis mucosae is associated with an increased number of myofibroblasts, possibly as a result of transdifferentiation and proliferation of resident smooth muscle cells in this layer.

Protease-activated receptor-2 (PAR-2) is a G-protein coupled receptor that is activated upon proteolytic cleavage by specific serine proteases, such as trypsin and mast cell tryptase. PAR-2 is expressed by various different cell types in the intestine of mammals, including intestinal myofibroblasts. PAR-2 activation has been shown to increase normal cell proliferation, and to augment growth of tumor cells. However, the significance of PAR-2 in regulating growth of intestinal myofibroblasts has never been investigated.

CD is associated with a diminished intestinal epithelial barrier to macromolecules, and it seems likely, thus, that increased amounts of luminal trypsin may cross the bowel wall in CD. Moreover, activation of intestinal mast cells is a feature of CD, and activated mast cells are known to release large amount of the PAR-2 activator tryptase. Taken together, there are reasons to believe that CD is associated with an increased content of PAR-2 activating proteases in the bowel wall. In addition, CD is associated with increased concentrations of tumor necrosis factor-α (TNF-α) in the intestinal mucosa. It is widely accepted that this cytokine is a major mediator in the pathophysiology of CD, and anti-TNF-α therapy is highly effective in the clinical management of CD. TNF-α has been shown to up-regulate PAR-2 expression in experimental studies. One may speculate, thus, that such a possible PAR-2 up-regulation in CD might render cells of the bowel mucosa more responsive to PAR-2-mediated activation by proteases.

Activation of phospholipase A₂ (PLA₂) and production of arachidonic acid (AA) metabolites have been implicated in the pathophysiology of various inflammatory diseases, including CD, and also in the regulation of cell growth. It has been reported that stimulation of PAR-2 may activate intracellular, high-molecular weight, PLA₂, and PAR-2-stimulated proliferation has been associated with an increased production of AA metabolites in some studies. Indeed, there are several lines of evidence that PAR-2 may regulate normal intestinal processes, such as motility and secretion, via the production of AA metabolites. It is still unknown, however, if PLA₂ activation affects intestinal myofibroblast proliferation, and if PAR-2 stimulation activates PLA₂ in this cell type.

Increased levels of several AA metabolites in the CD intestine positively correlate with disease activity, and it is generally accepted that AA metabolites are involved in the pathophysiology of CD. Most mammalian cells express two main types of intracellular, high-molecular weight, PLA₂, commonly named cytosolic PLA₂ (cPLA₂) and Ca²⁺-independent PLA₂ (iPLA₂). However, cPLA₂ is the only PLA₂ type that shows selectivity toward AA, and it is generally believed, therefore, that cPLA₂ plays a key role in the biosynthesis of AA metabolites. No studies concerning PLA₂ activity in intestinal myofibroblasts have been reported, and the relative contribution of different PLA₂ types in the release of AA in this cell type remains to be investigated.

The principal aim of the present study was to obtain further information about cellular mechanisms involved in the myofibroblast hyperplasia of the muscularis mucosae in CD strictures. Based on the obtained results, we hypothesize that TNF-α-induced PAR-2 up-regulation and PAR-2-mediated cPLA₂ activation might be of importance in intestinal myofibroblast proliferation.

2. Material and methods

2.1. Intestinal tissue samples

Full-thickness ileal samples with microscopically established muscularis mucosae overgrowth were obtained from four patients (three men and one woman, 36–56 years, mean 47) operated on for Crohn's ileitis at the university hospital of Linköping. All specimens were taken from macroscopically normal mucosa, but specimens from one of the patients showed signs of microscopic inflammation (neutrophil infiltration). At the time of operation, one of the patients was on medication with azathioprine, and one (the one with microscopically inflamed mucosa) was on medication with mesalazine. Full-thickness specimens of normal ileum were obtained from five patients operated on for colonic cancer (four women and one man, 70–92 years, mean 81), and used as comparative controls. Specimens were snap frozen, cryosectioned in 4 μm slices, and analyzed for PAR-2 by immunohistochemistry. The study was approved by the ethics committee of research on human subjects (the regional ethical review board in Linköping), and carried out in accordance with the Declaration of Helsinki.

2.2. Cells

The human cell line CCD-18Co (ATCC, no. CRL-1459) was obtained from LGC Promochem (Middlesex, UK). CCD-18Co cells exhibit many of the known characteristics of intestinal...
myofibroblasts, and were cultured as previously described. Cell viability was routinely determined by the trypan blue exclusion assay, and was not affected by the different treatments used. However, to maintain the viability of the cells, the concentrations of some agents had to be adjusted depending on the experimental layout (i.e. in the studies on proliferation, the concentration of trypsin had to be lowered because of the relatively long incubation time, and the concentration of AACOCF₃ had to be lowered because of an increased toxicity of this substance during serum-free conditions). If not otherwise indicated, pre-confluent cells were used for the studies.

2.3. Stimulation of PAR-2 expression

Cells were seeded in culture dishes (20 cm²) or on chamber slides (0.8 cm²; Nalge Nunc International, Rochester, NY), and cultured for up to 72 h with TNF-α (25 ng/ml). Cells in dishes were analyzed for PAR-2 by RT-PCR, whereas cells on chamber slides were supplied to immunocytochemical analysis.

2.4. RT-PCR

Hexamer-primed cDNA was generated (OmniScript reverse transcript RT Kit, Qiagen, Solna, Sweden) from 500 ng isolated total RNA (Ultracspe II RNA isolation system, Nordic BioSite, Täby, Sweden) and amplified using PuReTaq RTG PCR beads (Amersham Biosciences, GE Healthcare, Freiburg, Germany) and primers (Invitrogen Life Technologies Ltd, Paisley, UK) specific for human PAR-2 (forward, 5′-CTG CTG TCT TCA GTG GA; reverse, 5′-GAC ACT TCG GCA AAG GAG AG; product size 531 bp). Two amplification programs for PAR-2 were used, either 1 cycle 94 °C, 5 min; followed by 45 cycles of 94 °C, 30 s; 50 °C, 1 min; and a final cycle of 72 °C, 15 min, or (for semi-quantitative evaluation after TNF-α stimulation) 1 cycle 94 °C, 2 min; followed by 45 cycles of 94 °C, 30 s; 58 °C, 1 min; and a final cycle of 72 °C, 10 min. The amplification program for 18SrRNA was: 1 cycle 94 °C, 2 min; followed by 45 cycles of 94 °C, 30 s; 58 °C, 30 s; 72 °C, 1.5 min; and a final cycle of 72 °C, 8 min. The PCR-products were identified by size after gel electrophoresis. The identity of the PAR-2 product was also verified by sequencing (MWG Biotech AG, Martinsried, Germany). Caco-2 cells were used as positive control for PAR-2, and untranscribed total RNA was used as negative control.

2.5. Immunohistochemistry and immunocytochemistry

Cryosections (fixed in ice-cold acetone) and cells on chamber slides (fixed in 4% paraformaldehyde) were incubated with mouse anti-human PAR-2 monoclonal antibody (FITC-conjugated SAM-11, diluted 1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4 °C for 16 h. Before incubation, cryosections were blocked with 50% normal rabbit serum (DakoCytomation, Glostrup, Denmark) for 1 h at room temperature. An isotype-matched, irrelevant, mouse IgG₂a antibody (DakoCytomation) was used as negative control. After incubation with the irrelevant antibody (not FITC-conjugated) a secondary, biotin-conjugated, rabbit anti-mouse antibody (DakoCytomation) and FITC-conjugated streptavidin (DakoCytomation) were added. For visualization of cells on chamber slides, actin filaments were stained with rhodamine–phalloidin (Molecular Probes, Eugene, OR, USA). Slides were examined using NikonEclipse E600 Confocal Microscope (Nikon, Japan) and Nikon Confocal Microscope EZ-C1 Software version 2.0.

2.6. Release of AA

Cells seeded in 20 cm² culture dishes were labeled for 24 h with 0.1 μCi 14C-labeled arachidonic acid (14C-AA) (Amersham Biosciences, GE Healthcare, Freiburg, Germany) as described, before treated with appropriate stimulators and inhibitors for various times and concentrations. If cells were pre-treated with TNF-α, this cytokine was added to the growing cells for 48 h, before new medium with TNF-α and 14C-AA was added for further 24 h. All treatments of 14C-labeled cells with stimulators and inhibitors were done in the absence of serum. The amount of 14C-AA released to the culture medium was analyzed by liquid scintillation counting. Trypsin (type IX-S from porcine pancreas) and the peptide SLIGRL-NH₂ (Peptides International, Louisville, KY, USA) were used as PAR-2 activators. Methyl arachidonyl fluorophosphonate (MAFP) was used as combined inhibitor of cPLA₂ and iPLA₂, whereas bromoeno lactone (BEL) was used as a specific inhibitor of iPLA₂. Phorbol myristate acetate (PMA) and the calcium ionophore A23187 were used to specifically stimulate cPLA₂-mediated AA release. The specificity for AA was verified by a comparable stimulation of 14C-oleic acid (New England Nuclear, Perkin Elmer, Wellesley, MA, USA) labeled cells. If not otherwise indicated, all stimulators and inhibitors were obtained from Sigma (St. Louis, MO, USA).

2.7. Cell proliferation

Cells seeded in 9 cm² culture dishes were incubated with the combined cPLA₂ and iPLA₂ inhibitor AACOCF₃ (10 μM) or the specific iPLA₂ inhibitor BEL (10 μM) for 3 days, before pulse-labeled for 4 h with 0.4 μCi ³H-thymidine (Amersham Biosciences, GE Healthcare, Freiburg, Germany). The amount of incorporated radioactivity was analyzed as described. Unlabeled cells in parallel dishes were counted in a haemocytometer.

Cells seeded in 20 cm² culture dishes were serum-starved for 2 days, before the PAR-2 activator trypsin (type IX-S from porcine pancreas) (1 nM), with or without 5 μM AACOCF₃, was added. After 5 days with trypsin, and or AACOCF₃, cells were counted in a haemocytometer.

Trypsin and PLA₂ inhibitors were obtained from Sigma. AACOCF₃ was used instead of MAFP in the studies on proliferation to avoid possible oxidative inactivation of MAFP during the relatively long incubation times used in these experiments.

2.8. Statistical analysis

Values are given as mean ± SEM. GraphPad Prism 4.0 software (GraphPad Software, San Diego, CA, USA) was used for statistical evaluations. Depending on the experimental
3. Results

3.1. PAR-2 immunolabelling of the ileal mucosa

Mucosal PAR-2 immunolabelling was, as expected from previous studies, found in the epithelium and scattered through the lamina propria both in CD patients and controls (Fig. 1a and b). The PAR-2 immunolabelling was, however, very strongly expressed in the expanded muscularis mucosae of the CD specimens (Fig. 1b).

TNF-α, known to be present at increased levels in the CD mucosa, has been reported to up-regulate PAR-2 in some cell types. Our next step was, therefore, to investigate if the PAR-2 expression of intestinal myofibroblast may be affected by this inflammatory cytokine.

3.2. TNF-α stimulates PAR-2 mRNA expression in intestinal myofibroblasts

The myofibroblast cell line CCD-18Co was found to express a basal level of PAR-2 mRNA (Fig. 2a). This expression was evident in both pre-confluent, proliferating cells, and in confluent cells that had reached mitotic silence (Fig. 2a).

To simplify the detection of any increased PAR-2 mRNA expression after TNF-α stimulation, the PCR-reaction was adjusted so that the basal expression was just below the detection limit. Stimulation with TNF-α increased the PAR-2 mRNA expression, and this increase was time-dependent, being more obvious after 48 h than after 24 h of stimulation (Fig. 2b). Moreover, the effect of TNF-α on the PAR-2 mRNA expression seemed to be more pronounced in pre-confluent cells, than in confluent cells (Fig. 2b).

Since only the use of pre-confluent cells is scientifically justified when studying cellular events related to proliferation, only pre-confluent CCD-18Co cells were used in the subsequent studies.

3.3. TNF-α stimulates PAR-2 protein expression in intestinal myofibroblasts

PAR-2 was detected upon immunocytochemical analysis of untreated control cells, and the expression was increased in cells stimulated with TNF-α (Fig. 3).

Because of our finding that PAR-2 was up-regulated by TNF-α, together with the reported ability of PAR-2 activators to stimulate intracellular PLA2 activity in other cell types, we next investigated if up-regulation of PAR-2 by TNF-α affected AA mobilization in intestinal myofibroblasts.

3.4. TNF-α potentiates PAR-2-stimulated AA release in intestinal myofibroblasts

The PAR-2 activator trypsin caused a dose-dependent increase in the basal release of AA (Fig. 4). Pre-incubation with 10–100 ng/ml TNF-α for 72 h was without effect on the basal release of AA, but significantly potentiated the trypsin-stimulated AA release (Fig. 5a). This effect by TNF-α was independent of dose within the studied range (Fig. 5a). On the other hand, the trypsin-stimulated AA release after pre-incubation with 100 ng/ml TNF-α was dependent on the trypsin dose (Fig. 5b).

Like trypsin, the specific PAR-2 activating peptide, SLIGRL-NH2, significantly increased the AA release from TNF-α-treated cells (Fig. 5c), although it was unable to release AA per se at the concentration used in this study (Fig. 5c).

The release of AA after trypsin-stimulation of TNF-α-treated cells was reduced by pre-treatment with MAFP, a combined inhibitor of cPLA2 and iPLA2 (Fig. 6a), but not by the specific iPLA2 inhibitor BEL (Fig. 6b).

Since TNF-α per se is a known stimulator of cPLA2 expression and activity in many cells, we next investigated

Figure 1  Immunolabelling (green) of PAR-2 in ileal specimens from (a) a control patient with colonic cancer, and (b) microscopically uninflamed ileum of a patient with Crohn’s ileitis. E = epithelium, MM = muscularis mucosae, SM = submucosa. Note the prominent PAR-2 expression of the expanded muscularis mucosae of the Crohn patient (b). Isotype-matched irrelevant negative control antibody showed no staining (c). Confocal images at a magnification of ×200. Scale bar = 30 μm. Image a and b represent typical results obtained in five control patients and four Crohn patients, respectively. Note, however, that the increased epithelial PAR-2 expression suggested in panel b was not shown in all CD samples investigated.
if the synergism between TNF-α and trypsin could merely be explained by TNF-α-mediated stimulation of cPLA2.

3.5. TNF-α alone does not affect cPLA2-mediated release of AA in intestinal myofibroblasts

The protein kinase C stimulator PMA and the calcium ionophore A23187 were found to synergistically increase the release of AA, but not the release of the control fatty acid, 14C-oleic acid, (Fig. 7a). Moreover, this synergistic effect was reduced by MAFP (Fig. 7b). These results suggest that the AA release after combined stimulation with PMA and A23187 was due to the activation of an AA-specific, intracellular, high-molecular weight PLA2, that was dependent on both phosphorylation and an increased intracellular calcium level for its activity, i.e. cPLA2.43 Pre-incubation of the cells with TNF-α, according to the same protocol as used in the experiments on trypsin-stimulated AA release, did not affect this cPLA2-mediated AA release (Fig. 7c).

Our finding that PAR-2 stimulation activates cPLA2 in intestinal myofibroblasts, prompted us to investigate if PAR-
2 stimulation and cPLA<sub>2</sub> was of importance in the regulation of intestinal myofibroblast growth.

### 3.6. cPLA<sub>2</sub> activity and PAR-2 activation affects intestinal myofibroblast growth

Incubation with the combined cPLA<sub>2</sub> and iPLA<sub>2</sub> inhibitor, AACOCF<sub>3</sub>, but not the specific iPLA<sub>2</sub> inhibitor, BEL, reduced the incorporation of <sup>3</sup>H-thymidin per cell (Fig. 8a), i.e. the rate of DNA synthesis.

Stimulation of serum-starved cells for 5 days with 1 nM trypsin caused a 25% increase in cell number, compared to unstimulated control cells (Fig. 8a). This growth-promoting effect of trypsin was abolished in the presence of AACOCF<sub>3</sub> (Fig. 8b).

### 4. Discussion

Morphological and biochemical characterization of cells in the thickened muscularis mucosae have indicated that myofibroblasts, rather than conventional smooth muscle cells, are responsible for the overgrowth of this layer in CD, and thereby important in the formation of intestinal strictures. In the present study, strong expression of PAR-2 was demonstrated in the thickened muscularis mucosae from all four CD patients. We therefore hypothesized that the expansion of muscularis mucosae in CD is associated with proliferation of PAR-2 expressing myofibroblasts.

The present study is the first to demonstrate that TNF-α may up-regulate PAR-2 expression and potentiate PAR-2-stimulated AA mobilization in intestinal myofibroblasts, thereby suggesting a novel mechanism of myofibroblast proliferation in intestinal inflammation. Increased amounts of TNF-α are found in the CD mucosa, even in endoscopic normal tissue. In addition, the concentrations of PAR-2 activating proteases, such as trypsin and tryptase, are likely to be increased, because of a diminished epithelial barrier and increased mast cell activation. It seems possible, thus, that TNF-α may up-regulate PAR-2 on intestinal myofibroblasts, and that this, together with increased levels of proteases in the bowel wall, may result in increased PAR-2-mediated myofibroblast activation in CD. The stimulatory effect of TNF-α on PAR-2 expression in intestinal myofibroblasts agrees with previous studies, showing up-regulation of PAR-2 in response to inflammatory cytokines and in various inflammatory conditions. It is worth noticing, however, that up-regulation of PAR-2 by TNF-α is not the only possible connection between TNF-α and PAR-2 in intestinal myofibroblasts. Indeed, intestinal myofibroblasts express TNF-α and PAR-2 activation has been shown to stimulate the release of TNF-α from other cell types. It is quite possible, thus, that stimulation of PAR-2 may release TNF-α also from intestinal myofibroblasts, although this remains to be investigated.

TNF-α and PAR-2 stimulators, including trypsin and a specific PAR-2 stimulator, SLIGRL-NH<sub>2</sub>, were found to synergistically increase the AA release from intestinal myofibroblasts. The trypsin effect was reduced by MAFP, a

![Figure 5](https://academic.oup.com/ecco-jcc/article-abstract/3/1/15/2392955/1)
combined inhibitor of cPLA2 and iPLA2, but not by the specific iPLA2 inhibitor BEL. It seems likely, thus, that pre-stimulation with TNF-α up-regulates PAR-2 and that subsequent stimulation with trypsin results in amplification of cPLA2-mediated AA release. The synergism between TNF-α and trypsin was dose-dependent with regard to trypsin, but not with regard to TNF-α. It is possible that this was due to a maximal up-regulation of PAR-2 expression already at the lowest TNF-α concentration used, and that the AA release was only dependent on the degree of receptor stimulation.

Stimulation with SLIGRL-NH₂ without previous TNF-α stimulation did not cause any release of AA. It is known that SLIGRL-NH₂ has much lower potency compared to protease-mediated activation. Obviously, the concentration of SLIGRL-NH₂ used in our study was not high enough to activate the cells, unless the amount of PAR-2 receptors was up-regulated by previous stimulation by TNF-α.

Figure 6  Effect of PLA₂ inhibitors on trypsin-stimulated release of radiolabeled arachidonic acid (1⁴C-AA) from TNF-α pre-treated human intestinal myofibroblasts (CCD-18Co). Cells were pre-treated with TNF-α (25 ng/ml) for 72 h, and then incubated with the PLA₂ inhibitor for 30 min, before being stimulated with trypsin (100 nM) for 10 min. Control cells were pre-treated with TNF-α, but otherwise treated with culture medium only. (a) Effect of the combined cPLA₂ and iPLA₂ inhibitor MAFP (10 μM). Data from five independent experiments. **p<0.001 versus control cells. (b) Effect of the specific iPLA₂ inhibitor BEL (10 μM). Data from four independent experiments. ***p<0.001 versus control cells.

Figure 7  Stimulation of cPLA₂ activity in human intestinal myofibroblasts (CCD-18Co). Cells were incubated with PMA (200 nM) for 1 h, and then with A23187 (4 μM) for further 10 min. Control cells were incubated with culture medium only. (a) Effect on the release of radiolabeled arachidonic and oleic acid. Data from eight (arachidonic acid) and four (oleic acid) independent experiments. **p<0.001 versus control cells. (b) Effect of pre-treatment with the combined cPLA₂ and iPLA₂ inhibitor MAFP (10 μM) for 30 min. Data from four independent experiments. ***p<0.001 versus control cells. (c) Effect of pre-treatment with TNF-α (25 ng/ml) for 72 h. Data from four independent experiments. **p<0.001 versus control cells.
Thus, that the synergism between TNF-α-release induced by PMA and A23187. It seems more likely, PLA2s stimulation may activate intracellular high-molecular weight to up-regulation of PAR-2.

In conclusion, this study demonstrates (i) that PAR-2 is strongly expressed in the expanded muscularis mucosae in CD, (ii) that TNF-α may up-regulate the PAR-2 receptor and increase PAR-2-mediated AA mobilization by cPLA2 in intestinal myofibroblasts, and (iii) that activation of PAR-2 may stimulate proliferation of intestinal myofibroblasts by a cPLA2 dependent mechanism. Based on these results, we hypothesize that TNF-α-induced PAR-2 up-regulation and PAR-2-mediated cPLA2 activation might contribute to the intestinal myofibroblast proliferation associated with stricture formation in CD. However, further experimental work will be needed to investigate the effect of TNF-α on PAR-2 stimulated myofibroblast proliferation, and to elucidate which specific PLA2-derived lipid mediators mediate the proliferative effect upon PAR-2 stimulation. Moreover, to better mimic the in vivo situation, future studies should include studies on primary myofibroblasts isolated from patients, as well as studies on combinations of cytokines and growth factors found in the CD mucosa. One growth factor of particular interest might be TGFβ3, since this factor is of prime importance in CD fibrogenesis, has been shown to increase the expression of PAR-2 in intestinal myofibroblasts of extra-intestinal origin, and also might be an inducer of PAR-2 cleaving proteases in the intestine.

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Statement of authorship. UC participated in the design of the study, carried out most of the experimental studies on
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