Anti-inflammatory effects of pancreatitis associated protein in inflammatory bowel disease

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Abbreviations: CD, Crohn’s disease; IBD, inflammatory bowel diseases; UC, ulcerative colitis; PAP, pancreatitis associated protein; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; HUVEC, human umbilical vein endothelial cells; IL, interleukin; IFN-γ, interferon γ; TNF-α, tumour necrosis factor α; NF-κB, nuclear factor κB.

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ABSTRACT:

Background and aims: Increased pancreatitis associated protein (PAP) mRNA has been reported in active inflammatory bowel disease (IBD). The aims of the current study were to characterize PAP production in IBD and the effects of PAP on inflammation.

Patients and methods: Serum PAP levels were determined in healthy controls (n=29), inflammatory controls (n=14) and IBD patients (n=171). Ex vivo PAP secretion in intestinal tissue was measured in 56 IBD patients and 13 healthy controls. Cellular origin of PAP was determined by immunohistochemistry. The effects of exogenous PAP on NF-κB activation, pro-inflammatory cytokine production and endothelial adhesion molecule expression were also analyzed ex-vivo.

Results: Patients with active IBD had increased serum PAP levels compared with controls, and these levels correlated with clinical and endoscopic disease severity. Ex vivo intestinal PAP synthesis was increased in active IBD and correlated with endoscopic and histologic severity of inflammatory lesions. PAP localized to colonic Paneth cells. Incubation of mucosa from active Crohn’s disease (CD) with PAP dose-dependently reduced pro-inflammatory cytokines secretion. PAP prevented TNF-α–induced NF-κB-activation in monocytic, epithelial, and endothelial cells and reduced pro-inflammatory cytokine mRNA levels and adhesion molecule expression.

Conclusions: PAP is synthesized by Paneth cells and is overexpressed in colonic tissue of active IBD. PAP inhibits NF-κB activation and downregulates cytokine production and adhesion molecule expression in inflamed tissue. It may represent an anti-inflammatory mechanism and new therapeutic strategy in IBD.
INTRODUCTION
Pancreatitis associated protein I (PAP I) is a member of the type III subclass of the REG gene family that was first identified in rat pancreatic juice after experimental pancreatitis.[1] PAP I is also expressed in mice and humans, the aminoacid sequences of these proteins showing a high degree of conservation.[2] Human PAP is constitutively expressed in the pancreas and small intestine.[3] Increased PAP mRNA has been documented in colonic mucosa from patients with active inflammatory bowel disease (IBD) [4][5][6] as well as in experimental models of colitis [6][7].
Although several functions have been proposed for PAP, the physiological relevance of PAP upregulation in inflammatory diseases remains unknown. Recent observations suggest that PAP may have a protective effect against inflammatory damage in pancreatic [8][9] and extrapancreatic [10] inflammatory conditions.
In the current study we characterized PAP synthesis in IBD, examining its relationship with the type of disease, ulcerative colitis (UC) or Crohn’s disease (CD), and its severity, based on clinical, endoscopic and histological parameters. Given the protective action of PAP against inflammatory damage previously observed, we assessed whether PAP supplementation modulates signaling pathways in active IBD in ex vivo studies, particularly whether it alters pro-inflammatory cytokine synthesis in colonic inflamed mucosa from patients with CD and UC. After observing that this turned out to be the case, and in order to gain a further mechanistic insight, we assessed whether PAP modulates activation of the nuclear factor κB (NF-κB) in three different cell lines as models of the cell types that orchestrate the chronic inflammation present in IBD, namely monocytes, epithelial and endothelial cells. Finally, we analyzed if PAP could inhibit adhesion molecule expression in stimulated endothelial cells.

METHODS
The study was approved by the ethical committee of the Hospital Clínic de Barcelona, and all patients gave their written informed consent before enrollment.
Analysis of serum PAP in IBD
PAP levels in serum were analyzed in active or inactive IBD patients (n=171), in healthy control subjects (n=29), and in a group of patients with documented acute infectious gastroenteritis (positive stool culture for Gram negative bacteria) as intestinal inflammatory controls (n=14). Patients or controls with previously known intestinal or inflammatory diseases other than IBD, acute or chronic pancreatitis, previous intestinal resection, or chronic renal failure were excluded. In CD, clinical severity of the disease was estimated using the CD activity index (CDAI) [11], and in UC with the Lichtiger-modified Truelove and Witts clinical activity index.[12] Biologic markers of IBD activity, namely C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) were also determined.
Colonic disease extension was determined by colonoscopy performed in all cases within 6 months before inclusion into the study. In CD patients small intestinal involvement was assessed by a follow-through performed within the same time period. Location of CD was categorized in three subgroups: ileal, colonic or ileocolonic. Location of UC was categorized in two subgroups: distal, when lesions were confined to the rectosigmoid region, and extensive.
Serum PAP was measured using a commercially available ELISA kit (Dynabio S.A., Marseille, France) and results are expressed as ng of PAP per ml of serum.
**Intestinal PAP synthesis in IBD**

Intestinal PAP synthesis was analyzed using *ex vivo* culture of intestinal biopsy samples in 56 patients with IBD (41 with UC and 15 with CD) and 13 healthy controls. Colonic biopsy specimens were obtained from inflamed and/or non-inflamed mucosa of patients with active or inactive IBD. Patients under steroid treatment were excluded. Mucosal biopsies were taken from control patients free of intestinal inflammatory disease who underwent colonoscopy for cancer screening, in whom colonoscopy excluded the presence of lesions. Endoscopic severity of UC was assessed following a previously described scoring system.[13] Evaluation of endoscopic severity in CD was referred to the area where biopsies were taken and categorized as: inactive: no lesions; mild: circumscribed aphthous lesions; moderate: superficial ulcers < 1 cm in length; severe: deep ulcerations or ulcers >1 cm in length. Biopsy specimens were examined blindly by a single gastrointestinal pathologist (R.M.). Assessment of histological severity of inflammation in biopsy samples was performed using previously described grading scores for UC [14] or CD.[15]

For measurement of PAP secretion by colonic tissue, colonic biopsy samples weighing approximately 15-30 mg were cultured at 37ºC and 5%CO₂ for 24h. At the end of the culture period, supernatants were recovered and stored at –80ºC for later analysis. Levels of PAP protein secreted were analyzed measuring PAP concentration in the supernatant by ELISA (Dynabio S.A.). Results are expressed as ng PAP per mg of tissue.

**Localization of PAP in intestinal biopsies**

Cellular origin of PAP was determined using immunohistochemistry. Colon or ileum paraffin sections from biopsy samples of healthy subjects, patients with UC and patients with CD (n=3 per group) were stained with haematoxylin-eosin, or immunostained with a rabbit polyclonal antibody against human PAP (1/50). This anti-human PAP antibody was generated by one of the investigators (JLI) as previously described.[16] Localization of PAP in Paneth cells was confirmed by immunostaining for lysozyme (1/400; EnVision system, DAKO, Copenhagen, Denmark).

**Effects of PAP on intestinal pro-inflammatory cytokine production**

Sets of 4 endoscopic biopsies from controls and patients with active or inactive CD or UC were seeded on a well with medium alone or supplemented with various concentrations of PAP (25, 50, 500, or 2000 ng/ml). Biopsies were cultured at 37ºC and 5% CO₂ for 24 hours. Then supernatants were recovered. Concentrations of interleukin-6 (IL-6), tumour necrosis factor α (TNF-α), interferon γ (IFN-γ), interleukin-12p70 (IL-12p70), interleukin-18 (IL-18) and interleukin-8 (IL-8) in tissue culture supernatants were measured using ELISA kits supplied by Diaclone (Besançon, France). PAP protein was obtained from human pancreatic juice collected by endoscopic retrograde pancreatography and purified using immunoaffinity chromatography as previously described [10][16]. Results are expressed as pg of cytokine per mg of tissue.

In order to determine whether PAP can penetrate into the cultured tissue, colonic biopsy samples from CD patients (n=3) incubated for 24h with medium alone or supplemented with 50ng/ml PAP were fixed with 4% formaldehyde and immunohistochemistry against PAP as described previously was performed.

**Assessment of NF-κB activation by immunofluorescence in HT29 colonic cells**

Human HT29 epithelial colonic cell line was cultured at 37ºC and 5% CO₂. Cells were pre-incubated for 1 hour with various doses of PAP (50, 500, or 1000 ng/ml) and
challenged with 10 ng/ml of TNF-α (Sigma, Saint Louis, MO) for 30 min at 37°C. Thereafter, cells were incubated with a rabbit polyclonal anti-NF-κB p65 (C-20) antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour, followed by incubation with a goat anti-rabbit FITC antibody (Santa Cruz Biotechnology) for 1 hour in the dark. After washing, the slides were mounted for fluorescence microscopy.

Quantification of PAP effects on NF-κB activation in different cell types
Human promonocytic THP-1 cells as model of immune cells, human HT29 epithelial colonic cells, and human umbilical vein endothelial cells (HUVECs) were used. All cell lines were maintained at 37°C and 5% CO₂. Cells were pre-incubated for 1 hour with various doses of PAP (50, 100, 500, or 1000 ng/ml) and activated or not with 10 ng/ml of TNF-α (Sigma) for 30 min at 37°C. Then nuclear extracts were obtained using a nuclear extract kit from Active Motif (Rixensart, Belgium) and activation of NF-κB was measured with a commercial ELISA kit (TransAM, Active Motif). Results are expressed as fold induction of NF-κB after TNF-α stimulation.

Assessment of PAP effects on stimulated TNF-α expression in HT29 cells
HT29 cells maintained at 37°C and 5% CO₂ were pre-incubated for 1 hour with various doses of PAP (50, 100, 500, or 1000 ng/ml) and stimulated or not with 10 ng/ml of TNF-α for 30 min at 37°C. Then, total RNA was obtained to evaluate changes in TNF-α mRNA expression using quantitative real-time RT-PCR analysis on a LightCycler detection system (Roche Applied Science). Expression levels of TBP gene (encoding the TATA binding protein) were used as an internal control. First strand cDNA was synthesized from 2 µg of total RNA using random hexamers and expanded by reverse transcriptase according to the manufacturer’s instructions (Roche Applied Science), subsequently diluted 1:10 with water, and stored at -20°C until use. TNF-α and TBP PCR products were detected using the QuantiTect Probe PCR kit (QIAGEN Operon) following the manufacturer’s instructions using the dual-fluorescent Taqman probes (QIAGEN Operon) 5'-FAM-TAGCCCATATGTTAGCAACCCTCAAGCT-TAMRA-3' (position 435) and 5'-FAM-TCCAAGCGGTTTGCTGCGGTA-TAMRA-3' (position 811), respectively. The following primers were used: TBP forward (position 791) 5'-GCCGAAACGCGGAATATA-3'; TBP reverse 5'-CGTGGCTCTTATCCTATGTA-3' (position 855); TNF-α forward 5'-TCTTCTGAACCCCGAGTGA-3' (position 407) and TNF-α reverse 5'-CCTCTGATGGCACCACCAG-3' (position 557). The quantitative real-time RT-PCR was done in a total volume of 20 µl containing 1x amplification buffer and 5µl cDNA template. Samples were heated for 15 min at 95°C and amplified for 45 cycles (denaturation at 95°C for 10 sec, annealing and elongation at 60°C for 60 sec with a transition rate of 20°C/sec). All samples were analyzed in duplicate. Data evaluation was performed using the LightCycler data analysis software (version 3.5).

Effects of PAP on adhesion molecule expression in endothelial cells
HUVECs were incubated for 20 hours at 37°C and 5% CO₂ with various concentrations of PAP (25, 50, 100, 250, or 1000 ng/ml) in the presence or absence of 10 ng/ml TNF-α (Sigma), then washed and incubated for 45 min with an anti-E-selectin antibody (68-5H11), an anti-ICAM-1 antibody (HA58) or an anti-VCAM-1 antibody (51-10C9). Antibodies were purchased from BD Pharmingen (Heidelberg, Germany). After several washes, cells were incubated with a horseradish peroxidase conjugated secondary anti-mouse IgG antibody (CALTAG, Burlingame, CA) for 30 min and a developing solution
of OPD (Sigma). Absorbance was read at a wavelength of 450nm. Results are expressed as % of O.D.450nm reduction vs. O.D.450nm of TNF-α activated cells.

Statistical analysis
Data were analyzed using ANOVA with Bonferroni post-hoc test or the nonparametric Kruskal-Wallis test with Dunns post-hoc test for multiple group comparisons, when appropriate. Repeated measures for the same patient were analyzed by using Student’s paired t test. Correlation Z test was used to establish correlations between two quantitative variables. Values are expressed as mean ± SEM. Statistical significance was set at p< 0.05.

RESULTS
Study 1: Analysis of serum PAP in IBD.
Presence of chronic intestinal inflammation was associated with increased production of PAP, as estimated from serum levels of the protein. In comparison with healthy controls (n=29), increased PAP levels in serum were already detected in patients with clinically inactive CD (n=63) or UC (n=34), and were further increased in those with active IBD (CD n=45; UC n=29), in correlation with the severity of the disease as measured by the corresponding clinical activity indexes (Figure 1A). Serum PAP levels had a positive and significant correlation with clinical activity indexes in both CD (r=0.70, p<0.0001) (Figure 1B), and UC (r=0.44, p<0.001) (Figure 1C). PAP levels also correlated positively and significantly with serum CRP levels (CD r=0.28, p<0.02; UC r=0.45, p<0.001) and with ESR (CD r=0.27, p<0.01; UC r=0.45, p<0.001), although these correlations were weak. By contrast, serum levels of PAP in a group of patients with acute intestinal inflammation due to intestinal infection (n=14) were identical to those of healthy control subjects (Figure 1A). CRP values of this group of inflammatory controls (4.26 ± 1.78) were not different from those of IBD patients with active disease (3.89± 0.66).

In CD, serum PAP levels were not influenced by location of inflammatory lesions; similar values being observed when patients with ileal, ileocolonic or colonic lesions of similar clinical severity were compared (Figure 1D). In UC, serum PAP levels were also not related to disease extension; similar levels being found in distal and extensive colitis of similar clinical severity (Figure1E). When patients with CD and UC were stratified according to disease severity, PAP levels turned out to be similar in both diseases in mild and moderate cases, but were significantly higher in severe CD as compared to severe UC.

Study 2: Characterization of intestinal PAP production in IBD.
Ex vivo analysis of PAP secretion in colonic tissue from IBD patients
PAP secretion was significantly increased in the mucosa of patients with active CD (n=10) and UC (n=36) as compared to mucosa from patients with inactive disease (CD n=5; UC n=5) or control patients (n=13). In patients with IBD ex-vivo PAP secretion paralleled the severity of colonic inflammation assessed by endoscopy (Figure 2A). Levels of secreted PAP also correlated with histological severity of intestinal inflammation in both CD (r=0.870; p=0.0004) (Figure 2B) and UC (r=0.616; p=0.0001) (Figure 2C). When the levels of secreted PAP of healthy and inflamed colonic mucosa from the same patient were compared (n=10), the low basal levels of PAP (0.01±0.01 ng/mg tissue) secreted by the healthy tissue contrasted with the significantly higher levels observed in the inflamed tissue (1.94±0.21 ng/mg tissue; p<0.01) (Figure 2D),
indicating that increased PAP secretion is restricted to areas involved by the chronic inflammatory process.

**Paneth cells express PAP in active IBD**

Immunostaining of human colonic mucosa from healthy controls did not reveal any positive signal for PAP. In contrast, positive staining for PAP protein in CD and UC colonic mucosa was clearly detected in epithelial cells located at the bottom of crypts (Figure 3E,F). Hematoxylin and eosin staining of colonic serial sections indicated that PAP-expressing cells exhibited a characteristic morphology of Paneth cells, including the presence of acidophilic granules. Lysozyme staining confirmed the localization of PAP to Paneth cells in UC and in CD as well (Figure 3 C,D).

**Study 3: Effects of PAP on intestinal inflammation.**

**Pro-inflammatory cytokine secretion in CD and UC biopsies**

Active CD is characterized by an increased production of several pro-inflammatory cytokines such as TNF-α, IL-6, IL-8 [17][18][19], IFN-γ [20], IL-12 [21] and IL-18.[22] Secretion of all these pro-inflammatory cytokines by the inflamed mucosa from patients with CD (n=6) was significantly reduced upon incubation with 50 ng/ml PAP to a variable extend (19%-55%), with the most marked reductions observed in IFN-γ, TNF-α and IL-6 (Figure 4A). Higher concentrations of PAP (500 or 2000ng/ml) did not induce further inhibition of cytokine secretion, whereas incubation with 25 ng/ml had a significantly weaker inhibiting effect, as shown in figure 4B. PAP had no significant effects on cytokine secretion by colonic mucosa from patients with inactive CD (n=4) or from healthy controls (n=4) in which baseline levels of cytokine secretion are low (data not shown). In order to rule out a possible interference of PAP with the cytokine ELISA assays, PAP was added just before cytokine assays to the culture medium of tissue samples incubated without PAP. Addition of PAP to the assay medium did not alter cytokine levels measured by ELISA.

In contrast with the uniform response observed in CD, incubation of inflamed colonic mucosa from patients with UC in the presence of PAP showed variable results. Globally, incubation of inflamed colonic mucosa with PAP 50 ng/ml did not significantly modify secretion of the pro-inflammatory cytokines relative to samples incubated in the absence of PAP: TNF-α (-17.3±23.4; n=8), IL-6 (+3.3±22.8; n=8), INF-γ (-43.6±19.7; n=4) or IL-8 (-22.7±19.3; n=4). Nevertheless, 50% of the patients studied responded with a decrease in the production of these cytokines after incubation with PAP. Higher concentrations of PAP (500 or 2000 ng/ml) did not induce further inhibition or increased the proportion of responses (data not shown). We did not analyze IL-18 and IL-12 secretion in UC biopsies because levels of these cytokines in tissue culture supernatants were not increased during inflammation, a finding in keeping with previous observations.[21][22]

In order to determine whether exogenous PAP can penetrate into the cultured colonic tissue, biopsies incubated in the presence or absence of this protein were immunostained for PAP. As shown in figure 5, immunostaining was detected in the cytoplasm of epithelial cells of the samples incubated with this protein, whereas in biopsies incubated with medium alone the signal was only present in Paneth cells as mentioned above. This result indicates that epithelial cells are a main target for PAP and that these cells are able to internalize it.

**NF-κB activation in different cell types**
Expression of cytokines that were downregulated upon incubation of inflamed CD tissue with PAP is in part regulated by NF-κB.\textsuperscript{[23]}\textsuperscript{[24]}\textsuperscript{[25]}\textsuperscript{[26]}\textsuperscript{[27]}\textsuperscript{[28]} We hypothesized that inhibition of NF-κB activation might be one of the mechanisms mediating the anti-inflammatory effect of PAP.

Initially immunofluorescence studies were used to monitor the effects of PAP on NF-κB activation in the colonic epithelial cell line HT29 challenged with TNF-α. In non-stimulated cells, NF-κB p65 staining was detected only in the cytoplasm (Figure 6A). Stimulation of these cells with 10 ng/ml TNF-α induced translocation of NF-κB p65 to the nucleus (Figure 6B). Incubation of stimulated cells with 50, 500, or 1000 ng/ml PAP inhibited translocation of NF-κB p65 in most cells (Figure 6C,D,E), as staining remained predominantly cytoplasmatic. Inhibition was stronger with 500 ng/ml PAP than with 50 ng/ml, but did not further increase when a concentration of 1000 ng/ml was used. Incubation of non-stimulated cells with PAP did not result in activation of NF-κB (Figure 6F,G,H).

To confirm the inhibitory ability of PAP on TNF-α-induced NF-κB activation, and explore its differential effects in different cell types, we used a quantitative NF-κB activation assay (TransAM). Monocytes, epithelial cells and endothelial cells have been implicated in the pathogenesis of IBD \textsuperscript{[29]}, and these cell types show an activation of NF-κB in the inflamed intestinal mucosa.\textsuperscript{[30]}\textsuperscript{[31]} To investigate which cell types may be affected by PAP, NF-κB activation was measured in stimulated human THP-1 monocytic cells, HT29 epithelial cells and HUVECs. As shown in figure 7, PAP inhibited TNF-α-induced NF-κB activation in all three cell types studied, in a dose dependent manner, with THP-1 cells showing the highest degree of inhibition. Addition of 100 to 500 ng/ml of PAP to the culture medium in non stimulated cells did not alter NF-κB activation in any case.

**Effects of PAP on stimulated TNF-α transcription**

To test the possible link between the effects of PAP on NF-κB activation and cytokine production in cell lines, we monitored the expression of TNF-α mRNA in stimulated HT29 cells after incubation with PAP by real-time RT-PCR. As shown in figure 8, the addition of PAP to the culture medium reduced TNF-α induced transcript levels in a dose-dependent manner, confirming the inhibitory action of PAP on pro-inflammatory cytokine production through transcription regulation.

**Endothelial adhesion molecule expression**

Endothelial cells are activated in active IBD \textsuperscript{[32]}\textsuperscript{[33]} and this activation is accompanied by an increase in adhesion molecule expression. Endothelial expression of E-selectin, ICAM-1 and VCAM-1 is strongly induced by TNF-α and is dependent on NF-κB-activation.\textsuperscript{[34]} We explored whether PAP could also affect TNF-α-induced adhesion molecule expression in HUVECs. Indeed, PAP significantly inhibited TNF-α-induced adhesion molecule upregulation in a dose dependent manner. E-selectin upregulation was most sensitive to the effects of PAP, whereas ICAM-1 and VCAM-1 upregulation were only affected by the highest doses of PAP (Figure 9).

**DISCUSSION**

In this study we show that serum PAP levels are increased in patients with IBD relative to healthy controls, and this increase seems to be specific for chronic intestinal inflammation, since serum PAP was not increased in patients with intestinal inflammation in the context of infectious diarrhoea. The concept that intestinal PAP
production is elevated in active IBD had already been put forward in studies based on measurement of PAP mRNA in intestinal samples.[4][5][6] In the current study we explored whether disease type, location or severity influence PAP production. We provide evidence that in both CD and CU serum PAP levels parallel disease severity. Nevertheless, patients with inactive disease had still higher serum PAP levels than those of control healthy subjects. The increase in PAP production in active disease bore no relationship with type or location of disease. This observation is at odds with a recent study including only patients with CD and observing increased PAP levels only in patients with ileal disease.[35] Our results including a significant number of CD patients with disease limited to the colon and, even more convincingly, the observation of increased PAP levels in patients with UC, clearly indicates that production of this protein is also increased in the presence of inflammation limited to the colon. The notion that PAP production is increased in relation with the severity of intestinal inflammation is confirmed in the ex vivo studies measuring PAP production by inflamed intestinal tissue, in which highly significant correlations were found between histological and endoscopic severity of intestinal inflammation and PAP liberation to the culture medium.

Increased PAP production in both ileal and colonic disease is probably related to hyperplasia and metaplasia of Paneth cells. In the immunohistochemistry study performed in tissue samples obtained from patients with active IBD, we observed that PAP protein localized in Paneth cells in ileal and also colonic tissue. This observation is in keeping with a recent study[6] and may explain the marginal but significant increase of serum PAP in inactive IBD, since hyperplasia and metaplasia of Paneth cells is maintained in periods of quiescent disease.

The role that increased PAP production may have on the course of intestinal inflammation is currently unknown. Here we provide evidence that exogenous PAP supplementation can penetrate into intestinal tissue, specifically into epithelial cells, and oppose the inflammatory process that takes place in IBD. We demonstrate that secretion of pro-inflammatory cytokines by colonic tissue of patients with active CD was inhibited by addition of PAP, and that downregulation of cytokine production occurs through a mechanism involving inhibition of NF-κB activation. PAP inhibited TNF-α-induced NF-κB activation in three cell types that participate in the initiation and perpetuation of intestinal inflammation such as monocytes, epithelial and endothelial cells.[29]

Increased expression of adhesion molecules is another important factor involved in the pathogenesis of IBD.[36] We asked whether PAP could directly affect these determinants of leukocyte recruitment by monitoring expression of E-selectin, ICAM-1 and VCAM-1, since these molecules are upregulated through a NF-κB-dependent mechanism.[34] TNF-α-induced expression of these adhesion molecules was significantly reduced when cells were challenged in the presence of PAP. Hence, PAP could also counteract the inflammatory response by inhibiting leukocyte recruitment into the intestine. Unfortunately, we could not directly assess the anti-inflammatory role of endogenous PAP due to the lack of blocking antibodies and absence of knock-out animal models.

In the current study we observed that supplementation with exogenous PAP does not have the same anti-inflammatory effect in the inflamed mucosa of CD and UC. We observed a significant and consistent reduction of cytokine production in all samples of inflamed tissue from CD patients, whereas only 50% of patients with UC responded with a reduction in cytokine production, and this decrease did not reach statistical significance when we examined the UC group as a whole. Such difference could be
attributable to the fact that immune cell activation patterns present in CD and UC are different. Activation of immune response in CD is of Th1-type, with increased production of TNF-α, IL-12, IFN-γ, and IL-18, and a clinical response to immunoblockade of some of these cytokines such as TNF-α or IL-12 [37], whereas the pattern of immune cell activation in UC is more complex. Discrepancies between the response of UC and CD to various forms of treatment, such as cyclosporine [12][38] or methotrexate [39][40] has been previously documented in clinical controlled trials. Thus, it is conceivable that if the blockade of cytokines involved in the Th1-type response is a key element in reducing activation of the immune system by PAP in CD, this may not have the same effect in UC.

In conclusion, this study demonstrates that PAP synthesis is increased in IBD, to an extent that parallels the severity of intestinal inflammation. This increment is probably related to hyperplasia/metaplasia of Paneth cells, which is in keeping with the notion that these cells exert mostly a protective function in intestinal homeostasis. PAP secreted by these cells may dampen the inflammatory damage by affecting diverse components of the inflammatory response, including cytokine production, adhesion molecule expression and activation of NF-kB. Although the finding of increased levels of a potential anti-inflammatory factor paralleling disease severity may be puzzling, it may reflect the activation of regulatory functions to limit tissue damage, as has been described for other molecules that downregulate various elements of the inflammatory cascade such as IL-10 and interferon-γ [41][42]. In that regard, increased PAP levels in inactive disease may also have a role in maintaining a balance between anti- and pro-inflammatory factors in an inflammation prone intestine. If PAP was eventually shown to be effective in the treatment of human CD, the use of a small human recombinant protein may have numerous advantages over antibody-based therapies in terms of tolerance and immunogenicity.
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FIGURE LEGENDS

Figure 1. (A) Serum PAP levels in IBD patients with disease of diverse severity. Inactive CD (n=63) or UC (n=34) patients have higher PAP levels than healthy controls (n=29) or inflammatory controls (infectious diarrhea, n=14). In IBD patients PAP levels increased in parallel with disease severity categorized as: mild (CD n=23, UC n=14), moderate (CD n= 18, UC n=9), and severe (CD n= 4, UC n= 6). * p<0.05 vs. healthy control and inflammatory control; # p<0.05 vs. inactive; $ p<0.05 vs. Mild; & p<0.05 vs. Moderate; a p<0.05 vs. UC Severe. Correlation of serum PAP levels with the clinical activity index CDAI in CD patients (B), and with the Lichtiger-modified Truelove and Witts clinical activity index in UC patients (C). (D) Serum PAP levels in CD patients according to location of intestinal lesions: ileal (n=26), ileocolonic (n=24) or colonic (n=20). (E) Serum PAP levels in UC patients according to disease extension: distal (n=21) or extensive (n=42). Results are expressed as ng PAP/ml serum.

Figure 2. Ex vivo PAP production in intestinal tissue of patients with IBD and controls (ng PAP/mg tissue). (A) PAP secretion paralleled the endoscopic severity of lesions in CD (n=15) and UC (n=41) #, p<0.05 vs. inactive; *, p<0.05 vs. mild. Correlation of PAP secretion levels with the histological index in CD (B) and UC (C). (D) Samples of healthy and inflamed intestinal mucosa from 10 IBD patients were assayed in parallel. Inflamed mucosa secreted significantly more PAP than healthy tissue (paired t test, p<0.01).

Figure 3. PAP protein in the colon of CD and UC patients is synthesized in Paneth cells. Immunohistochemistry of consecutive colon paraffin sections are shown in the photographs. Representative images from a UC patient have been chosen. Haematoxylin-eosin staining demonstrates presence of metaplastic Paneth cells in the descending colon (A: 200x, B: 600x). Immunostaining for lysozyme precisely identifies Paneth cells at the base of the crypts (C: 200x, D: 600x). Immunostaining for PAP is seen in metaplastic Paneth cells, (E:200x, F: 600x). No staining was observed in sections incubated without primary antibody (data not shown). All sections were counterstained with hematoxylin.

Figure 4. Reduction of cytokine secretion from mucosa samples incubated with PAP. (A) Colonic biopsies from inflamed mucosa of patients with CD (n=6) were cultured for 24 hours in medium alone or with 50 ng/ml PAP and concentrations of pro-inflammatory cytokines in culture supernatants were measured by ELISA. Secretion of the following cytokines was monitored: TNF-α; IL-6; IFN-γ; IL-18; IL-12; IL-8. (B) Dose-response relationship between PAP (25, 50, 500, 2000 ng/ml) supplemented to the medium and TNF-α secretion in the same biopsies as above. Results are expressed as % changes in cytokine secretion in tissue samples incubated with PAP relative to samples incubated with medium alone. *, p<0.05 for each cytokine in the paired t test.

Figure 5. Presence of PAP in epithelial cells of colonic biopsies incubated with this protein. Immunohistochemistry for PAP in colonic sections from a patient with CD incubated for 24h with medium alone (A) or with 50 ng/ml PAP (B). (A) In the absence of PAP in the medium immunostaining for PAP is only found in Paneth cells at the base of the crypts; no signal is found in other epithelial cells (400x). (B) In tissue samples incubated in the presence of exogenous PAP the protein is diffusely identified in the
cytoplasm of all epithelial cells (600x). All sections were counterstained with hematoxylin.

**Figure 6.** Effect of PAP on nuclear translocation of NF-κB in colonic epithelial cells. The intracellular location of NF-κB p65 was determined in HT29 cells by immunofluorescence using an anti-NF-κB p65 antibody with FITC labelling. In non-stimulated cells incubated with medium alone staining is cytoplasmatic (A). (B) Cells stimulated with 10 ng/ml TNF-α show a diffuse immunostaining indicating that NF-κB has translocated from the cytoplasm to the nucleus. (C) Stimulated cells in the presence of 50 ng/ml PAP maintain a predominantly cytoplasmic NF-κB immunostaining, indicating an inhibition of NF-κB translocation. Incubation of stimulated cells with 500 ng/ml (D) or 1000 ng/ml (E) PAP resulted in nearly complete restriction of immunostaining to the cytoplasm, indicating a stronger inhibition of NF-κB translocation. In cells incubated with PAP 50 ng/ml (F), 500 ng/ml (G) and 1000 ng/ml (H) in the absence of TNF, immunostaining of NF-κB is restricted to the cytoplasm. All preparations were blindly examined in a fluorescence microscope with 1000x.

**Figure 7.** Effects of PAP on NF-κB activation in THP-1 cells (A); HT29 cells (B) and HUVECs (C). Cells were pre-incubated for 1 hour with increasing PAP concentrations (0, 50, 100, 500 or 1000 ng/ml) and stimulated with 10 ng/ml TNF-α for 30 min. Nuclear extracts were obtained, and activated NF-κB was measured using an oligonucleotide-based specific ELISA (TransAM). NF-κB activation in TNF-α stimulated cells is significantly reduced in a dose-dependent manner by incubation with PAP. Results are expressed as NF-κB fold induction. Experiments were performed in quadruplicate and all results are expressed as mean ± S.E. # p<0.05 vs. TNF-α (−) PAP 0 ng/ml; * p<0.05 vs. previous PAP dose.

**Figure 8.** TNF-α mRNA expression normalized to the endogenous control TBP gene. HT29 cells were pre-incubated for 1 hour with increasing PAP concentrations (0, 50, 100, 500 or 1000 ng/ml) and stimulated with 10 ng/ml TNF-α for 30 min. Total RNA was extracted and TNF-α mRNA expression was quantified by quantitative real-time RT-PCR. TNF-α expression in stimulated cells is significantly reduced in a dose-dependent manner by incubation with PAP. Results are shown as percentage relative to TNF-α expression in control cells (100%) given as mean ± S.E. of four different experiments. # p<0.05 vs. control (TNF-α (−) PAP 0 ng/ml); * p<0.05 vs. previous PAP dose.

**Figure 9.** Effect of PAP on expression of E-selectin (A), ICAM-1 (B) and VCAM-1 (C) in endothelial cells. HUVECs were stimulated with 10 ng/ml TNF-α and incubated with increasing PAP concentrations (0, 25, 50, 100, 250 or 1000 ng/ml). TNF-α stimulation induced an increase in E-selectin, ICAM-1 and VCAM-1 expression and this expression was significantly reduced by co-incubation with PAP in all cases. Results are expressed as percentage of adhesion molecule expression relative to TNF-α−stimulated cells. Experiments were performed in quadruplicate and all results are expressed as mean values ± S.E.
REFERENCES


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