INFLAMMATORY BOWEL DISEASE

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

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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 characterise 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 nuclear factor kB (NFkB) activation, proinflammatory cytokine production, and endothelial adhesion molecule expression were also analysed 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 histological severity of inflammatory lesions. PAP localised to colonic Paneth cells. Incubation of mucosa from active Crohn’s disease with PAP dose dependently reduced proinflammatory cytokines secretion. PAP prevented TNF-α induced NFkB activation in monocytic, epithelial, and endothelial cells and reduced proinflammatory cytokine mRNA levels and adhesion molecule expression.

Conclusions: PAP is synthesised by Paneth cells and is overexpressed in colonic tissue of active IBD. PAP inhibits NFkB 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.

METHODS
The study was approved by the ethics committee of the Hospital Clinic de Barcelona, and all patients gave their written informed consent before enrolment.

Analysis of serum PAP in IBD
PAP levels in serum were analysed 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, and in UC with the Lichtiger modified Truelove and Witts clinical activity index. Biological markers of IBD activity (CRP and ESR) were also determined.

Colonic disease extension was determined by colonoscopy performed in all cases within six months before inclusion in the study. In CD patients, small intestinal involvement was assessed by a follow through performed within the same time period. Location of CD was categorised into three subgroups: ileal, colonic, and ileocolonic. Location of UC was categorised

Abbreviations: CD, Crohn’s disease; IBD, inflammatory bowel disease; 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; NFkB, nuclear factor kB; RT-PCR, reverse transcription-polymerase chain reaction
Intestinal PAP synthesis in IBD

Intestinal PAP synthesis was analysed 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 receiving 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 categorised as: inactive: no lesions; mild: 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 (RM). Assessment of histological severity of inflammation in biopsy samples was performed using previously described grading scores for UC9 or CD.13

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

Localisation 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 antihuman PAP antibody was generated by one of the investigators (JLI), as previously described.14 Localisation of PAP in Paneth cells was confirmed by immunostaining for lysozyme (1/400; EnVision System, Dako, Copenhagen, Denmark).

Effects of PAP on intestinal proinflammatory cytokine production

Sets of four 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 37°C with 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.16 Results are expressed as pg of cytokine per mg of tissue.

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

Assessment of NFκB activation by immunofluorescence in HT29 colonic cells

Human HT29 epithelial colonic cell line was cultured at 37°C with 5% CO2. Cells were preincubated for one hour with various doses of PAP (50, 500, or 1000 ng/ml) and challenged with 10 ng/ml of TNF-α (Sigma, St Louis, Missouri, USA) for 30 minutes at 37°C. Thereafter, cells were incubated with a rabbit polyclonal anti-NFκB p65 (C-20) antibody (Santa Cruz Biotechnology, Santa Cruz, California, USA) for one hour, followed by incubation with a goat antirabbit FITC antibody (Santa Cruz Biotechnology) for one hour in the dark. After washing, slides were mounted for fluorescence microscopy.

Quantification of PAP effects on NFκB 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 with 5% CO2. Cells were preincubated for one hour with various doses of PAP (50, 100, 500, or 1000 ng/ml) and activated or not with 10 ng/ml TNF-α for 30 minutes at 37°C. Then, nuclear extracts were obtained using a nuclear extract kit from Active Motif (Rixensart, Belgium) and activation of NFκB was measured with a commercial ELISA kit (TransAM; Active Motif). Results are expressed as fold induction of NFκB after TNF-α stimulation.

Assessment of PAP effects on stimulated TNF-α expression in HT29 cells

HT29 cells maintained at 37°C with 5% CO2 were preincubated for one hour with various doses of PAP (50, 100, 500, or 1000 ng/ml) and stimulated or not with 10 ng/ml TNF-α for 30 minutes at 37°C. Then, total RNA was obtained to evaluate changes in TNF-α mRNA expression using quantitative real time reverse transcription-polymerase chain reaction (RT-PCR) analysis on a LightCycler detection system (Roche Applied Science, Basel, Switzerland). Expression levels of TNF gene (encoding the TATA binding protein) were used as an internal control. First strand cDNA was synthesised 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, Cologne, Germany), following the manufacturer’s instructions, using the dual fluorescent Taqman probes (Qiagen Operon) 5′-FAM-TAG CCT ATG TTT TAG CAA ACC CTC AAG CT-TAMRA-3′ (position 435) and 5′-FAM-TCC CAA GCC GTT TG CTC GGT A-TAMRA-3′ (position 811), respectively. The following primers were used: TBP forward (position 791) 5′-GCC CGA AAC GCC GAA TAT A-3′ and TBP reverse 5′-CGT GCC TCT CCT CTT A TC A CTG A-3′ (position 855); TNF-α forward 5′-TCT TCT CGA ACC CCG AGT GA-3′ (position 407) and TNF-α reverse 5′-CCT CGT ATG GCA CCA CCA G-3′ (position 557). Quantitative real time RT-PCR was performed in a total volume of 20 μl containing 1× amplification buffer and 5 μl cDNA template. Samples were heated for 15 minutes at 95°C and amplified for 45 cycles (denaturation at 95°C for 10 seconds, annealing and elongation at 60°C for 60 seconds with a transition rate of 20°C/s). All samples were analysed 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 with 5% CO₂ and various concentrations of PAP (25, 50, 100, 250, or 1000 ng/ml) in the presence or absence of 10 ng/ml TNF-α. After incubation, cells were washed, and incubated for 45 minutes 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 antimouse IgG antibody (Caltag, Burlingame, California, USA) for 30 minutes and a developing solution of OPD (Sigma). Absorbance was read at a wavelength of 450 nm. Results are expressed as per cent of OD₄₅₀ nm reduction versus OD₄₅₀ nm of TNF-α activated cells.

Figure 1  | Serum pancreatitis associated protein (PAP) levels in inflammatory bowel disease (IBD) patients with disease of diverse severity. Inactive Crohn’s disease (CD, n = 63) or ulcerative colitis (UC, n = 34) patients had higher PAP levels than healthy controls (n = 29) or inflammatory controls (infectious diarrhoea, n = 14). In IBD patients, PAP levels increased in parallel with disease severity, categorised as: mild (CD n = 23, UC n = 14), moderate (CD n = 18, UC n = 9), or severe (CD n = 4, UC n = 6). *p<0.05 versus healthy controls and inflammatory controls; †p<0.05 versus inactive disease; ‡p<0.05 versus mild disease; §p<0.05 versus moderate disease; ††p<0.05 versus severe UC. Correlation of serum PAP levels with Crohn’s disease activity index (CDAI) in CD patients (B) and with the Lichtiger modified Truelove and Witts clinical activity index in UC patients (C). Correlation of serum PAP levels with serum C reactive protein (CRP) levels in CD (D) and UC (E) patients. Correlation of serum PAP levels with erythrocyte sedimentation rate (ESR) in CD (F) and UC (G) patients. (H) Serum PAP levels in CD patients according to location of intestinal lesions: ileal (n = 26), ileocolonic (n = 24), or colonic (n = 20). (I) 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.
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**RESULTS**

**Study 1: analysis of serum PAP in IBD**

The 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 association with severity of disease, as measured by the corresponding clinical activity indexes (fig 1A). Serum PAP levels had a positive and significant correlation with clinical activity indexes in both CD (r = 0.70, p<0.0001) (fig 1B) and UC (r = 0.44, p<0.001) (fig 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. In 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 (fig 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 were observed when patients with ileal, ileocolonic, or colonic lesions of similar clinical severity were compared (fig 1H). In UC, serum PAP levels were also not related to disease extension; similar levels were found in distal and extensive colitis of similar clinical severity (fig 1I). When patients with CD and UC were stratified according to disease severity, PAP levels were similar in both diseases in mild and moderate cases but were significantly higher in severe CD compared with severe UC.

**Study 2: characterisation 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) compared with 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 (fig 2A). Levels of secreted PAP also correlated with histological severity of intestinal inflammation in both CD (r = 0.870; p = 0.0004) (fig 2B) and UC (r = 0.616; p = 0.0001) (fig 2C). When levels of secreted PAP of healthy and inflamed colonic mucosa from the same patient were compared (n = 10), 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 inflamed tissue (1.94 (0.21) ng/mg tissue; p<0.001) (fig 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...
Pancreatitis associated protein (PAP) in the colon of Crohn’s disease (CD) and ulcerative colitis (UC) patients is synthesised in Paneth cells. Immunohistochemistry of consecutive colon paraffin sections are shown. Representative images from a UC patient have been chosen. Haematoxylin-eosin staining demonstrated the presence of metaplastic Paneth cells in the descending colon (A, 200×; B, 600×). Immunostaining for lysozyme precisely identified Paneth cells at the base of crypts (C, 200×; D, 600×). Immunostaining for PAP is seen in metaplastic Paneth cells (E, 200×; F, 600×). No staining was observed in sections incubated without primary antibody (data not shown). All sections were counterstained with haematoxylin.

Study 3: effects of PAP on intestinal inflammation

Proinflammatory cytokine secretion in CD and UC biopsies

Active CD is characterised by increased production of several proinflammatory cytokines such as TNF-α, IL-6, IL-8, IFN-γ, IL-12, and IL-18. Secretion of all of these proinflammatory cytokines by the inflamed mucosa from patients with CD (n = 6) was significantly reduced on incubation with 50 ng/ml PAP to a variable extend (19–55%), with the most marked reductions observed with IFN-γ, TNF-α, and IL-6 (fig 4A). Higher concentrations of PAP (500 or 2000 ng/ml) did not induce further inhibition of cytokine secretion, whereas incubation with 25 ng/ml had a significantly weaker inhibiting effect, as shown in fig 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 were low (data not shown). To rule out possible interference of PAP with the cytokine ELISA assays, PAP was added just before the 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.
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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 proinflammatory 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 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 increase the proportion of responses (data not shown). We did not analyse IL-18 or IL-12 secretion in UC biopsies because levels of these cytokines were not increased during inflammation, a finding in keeping with previous observations.21 22

To determine whether exogenous PAP can penetrate into cultured colonic tissue, biopsies incubated in the presence or absence of this protein were immunostained for PAP. As shown in fig 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 the main target for PAP and that these cells are able to internalise it.

NFκB activation in different cell types

Expression of cytokines that are downregulated on incubation of inflamed CD tissue with PAP is in part regulated by NFκB.23–28 We hypothesised that inhibition of NFκB 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 NFκB activation in the colonic epithelial cell line HT29 challenged with TNF-α. In non-stimulated cells, NFκB p65 staining was detected only in the cytoplasm (fig 6A). Stimulation of these cells with 10 ng/ml TNF-α induced translocation of NFκB p65 to the nucleus (fig 6B). Incubation of stimulated cells with 50, 500, or 1000 ng/ml PAP inhibited translocation of NFκB p65 in most cells (fig 6C, D, E), as staining remained predominantly cytoplasmic. 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 NFκB (fig 6F, G, H).

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

Effects of PAP on stimulated TNF-α transcription
To test the possible link between the effects of PAP on NFκB activation and cytokine production in cell lines, we monitored expression of TNF-α mRNA in stimulated HT29 cells after incubation with PAP by real time RT-PCR. As shown in fig 8, 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 proinflammatory cytokine production through transcription regulation.

Endothelial adhesion molecule expression
Endothelial cells are activated in active IBD 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 NFκB activation. 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 (fig 9).

DISCUSSION
In this study, we have shown that serum PAP levels were increased in patients with IBD relative to healthy controls, and this increase seemed to be specific for chronic intestinal inflammation as 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. In the current study, we explored whether disease type, location, or severity influenced PAP production. We provided evidence that in both CD and UC, 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 to type or location of disease. This observation is at odds with a recent study involving CD patients which found increased PAP levels only in those with ileal disease. 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 to the severity of intestinal inflammation was confirmed in the ex vivo studies measuring PAP production in 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 localised in Paneth cells in ileal and also in...
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and stimulated with 10 ng/ml tumour necrosis factor with increasing PAP concentrations (0, 50, 100, 500, or 1000 ng/ml) umbilical vein endothelial cells (C). Cells were preincubated for one hour k

Effects of pancreatitis associated protein (PAP) on nuclear PAP dose; and all results are expressed as mean (SEM).

NFκB activity (fold induction)NFκB activation in THP-1 cells (A), HT29 cells (B), and human colonic tissue. This observation is in keeping with a recent study and may explain the marginal but significant increase in serum PAP in inactive IBD as hyperplasia and metaplasia of Paneth cells is maintained during 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 demonstrated that secretion of proinflammatory cytokines by colonic tissue of patients with active CD was inhibited by addition of PAP, and that downregulation of cytokine production occurred through a mechanism involving inhibition of NFκB activation. PAP inhibited TNF-α induced NFκB activation in three cell types that participate in the initiation and perpetuation of intestinal inflammation—that is, monocytes, epithelial cells, and endothelial cells.

Increased expression of adhesion molecules is another important factor involved in the pathogenesis of IBD. We asked whether PAP could directly affect these determinants of leucocyte recruitment by monitoring expression of E-selectin, ICAM-1, and VCAM-1, as these molecules are upregulated through a NFκB dependent mechanism. 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 leucocyte 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 knockout animal models.

In the current study, we observed that supplementation with exogenous PAP did 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

Pancreatitis associated protein (PAP) on nuclear factor κB (NFκB) activation in THP-1 cells (A), HT29 cells (B), and human umbilical vein endothelial cells (C). Cells were preincubated for one hour with increasing PAP concentrations (0, 50, 100, 500, or 1000 ng/ml) and stimulated with 10 ng/ml TNF-α for 30 minutes. Total RNA was extracted and TNF-α mRNA expression was quantified by quantitative real time reverse transcription-polymerase chain reaction. TNF-α expression in stimulated cells was 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 (SEM) of four different experiments. *p<0.05 versus previous PAP dose; †p<0.05 versus control (TNF-α (–) PAP 0 ng/ml).

Figure 8 Tumour necrosis factor α (TNF-α) mRNA expression normalised to endogenous control TBP gene. HT29 cells were preincubated for one hour with increasing pancreatitis associated protein (PAP) concentrations (0, 50, 100, 500, or 1000 ng/ml) and stimulated with 10 ng/ml TNF-α for 30 minutes. Total RNA was extracted and TNF-α mRNA expression was quantified by quantitative real time reverse transcription-polymerase chain reaction. TNF-α mRNA expression in stimulated cells was 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 (SEM) of four different experiments. *p<0.05 versus previous PAP dose; †p<0.05 versus control (TNF-α (–) PAP 0 ng/ml).

Figure 7 Effects of pancreatitis associated protein (PAP) on nuclear factor κB (NFκB) activation in THP-1 cells (A), HT29 cells (B), and human umbilical vein endothelial cells (C). Cells were preincubated for one hour with increasing PAP concentrations (0, 50, 100, 500, or 1000 ng/ml) and stimulated with 10 ng/ml tumour necrosis factor α (TNF-α) for 30 minutes. Nuclear extracts were obtained, and activated NFκB was measured using an oligonucleotide based specific ELISA (TransAM). NFκB activation in TNF-α stimulated cells was significantly reduced in a dose dependent manner by incubation with PAP. Results are expressed as NFκB-fold induction. Experiments were performed in quadruplicate and all results are expressed as mean (SEM). *p<0.05 versus previous PAP dose; †p<0.05 versus TNF-α (–) PAP 0 ng/ml.
TNF-α, and IL-18, and a clinical response to immunoblockade of some of these cytokines such as TNF-α or IL-12, 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 ciclosporin 38-40 or methotrexate, 39 40 has been previously documented in clinical controlled trials. Thus it is conceivable that if 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 NFKB. Although the finding of increased levels of a potential anti-inflammatory factor paralleling disease severity may be puzzling, it may reflect 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 IFN-γ. 41 42 In that regard, increased PAP levels in inactive disease may also have a role in maintaining a balance between anti- and proinflammatory 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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**REFERENCES**

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