

Increased colonic apelin production in rodents with experimental colitis and in humans with IBD

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Abstract

Apelin and its receptor, the APJ receptor, are expressed in the gastrointestinal tract. The aims of this study were to examine the effects of sodium dextran sulfate (DSS)-induced experimental colitis in rats and mice and inflammatory bowel disease (IBD) in humans on intestinal apelin production, and the influence of exogenous apelin on colonic epithelial cell proliferation in mice. In rodents with experimental colitis, colonic apelin mRNA levels were elevated during the inflammatory reaction as well as during the tissue repair phase that ensues after DSS withdrawal. Fluctuations in colonic apelin expression were paralleled by similar changes in apelin immunostaining. Apelin immunostaining was increased in the surface epithelium, in epithelial cells along the length of the tubular gland and in the stem cell region at the gland base. In ulcerative colitis (UC) and Crohn's disease patients, apelin immunostaining revealed a pattern of increased intestinal apelin content similar to that observed in rodents with experimental colitis. Administration of synthetic apelin to mice during the recovery phase of DSS-induced colitis stimulated colonic epithelial cell proliferation significantly. Our observations that colonic apelin production is increased during and after DSS exposure indicate that apelin plays multiple roles during the different stages of colitis. Additionally, the stimulatory action of exogenous apelin on colonic epithelial proliferation suggests that the increased apelin production during intestinal recovery stage may contribute to the repair of the intestinal epithelium in experimental rodent models of colitis and in IBD patients.

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1. Introduction

Apelin, a peptide characterized originally in extracts of the bovine stomach, is the endogenous ligand for the APJ receptor [1–6]. Apelin and APJ are found in the brain, kidney, heart, lung, adipose tissue, gastrointestinal tract and mammary gland [3,7–12]. Apelin has a broad range of activities including effects on heart contractility and blood pressure, appetite and drinking behavior, the hypothalamic–pituitary–adrenal axis, gastric acid secretion, and on insulin and cholecystokinin (CCK) secretion [12–19].

In humans, inflammatory bowel disease (IBD, ulcerative colitis and Crohn's disease) is a chronic disease of the gastrointestinal tract characterized by intestinal inflammation that is triggered by poorly healing mucosal ulcerations [20–22]. The extent to which intestinal inflammation influences apelin expression is not known. The aim of these studies, therefore, was to investigate whether colonic apelin production is altered in rodents with experimental colitis and in humans with IBD. Additionally, the effect of apelin administration on colonic epithelial proliferation was tested in mice.

In this study, we show that intestinal apelin production is increased in rodents with experimental colitis and in humans with IBD. Additionally, we demonstrate that apelin administration to mice increases colonic proliferation. These findings imply that the increased apelin expression during the recovery stage of DSS-

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induced intestinal inflammation contributes to the repair of the intestinal epithelium in experimental rodent models of colitis and in IBD patients.

2. Materials and methods

2.1. Animals

All animal experiments were conducted in accordance with mandated standards of humane care and were approved by the Institutional Animal Care and Use Committee. Adult male C57/BL6 mice and Sprague–Dawley rats were maintained in air-conditioned and light-regulated rooms (lights on, 0600–1800 h) and given access to food and water ad libitum.

2.2. Induction of experimental colitis in rodents

Colitis was induced in mice and rats by giving 5% dextran sodium sulfate (DSS) in drinking water for 7 and 5 days, respectively [23,24]. At various times after initiation of DSS treatment, colonic tissue was harvested for preparation of histological sections and total cellular RNA.

2.3. Human IBD tissues

Intestinal histological sections were prepared from specimens obtained from patients undergoing bowel resection. The study was approved by the institutional review board at Cleveland Clinic and informed consent was obtained from each patient. Tissues were fixed in buffered formalin and paraffin embedded. Paraffin-embedded tissue sections (5 μ m) were immunostained for apelin as described.

2.4. Effect of exogenous apelin on colonic epithelial proliferation during the recovery stage of DSS-induced colitis in mice

Either vehicle (0.05% bovine serum albumin in saline) or synthetic pyro-apelin-13 (50 μ g/3 \times /day, SC) was given to adult male mice for 3 days pre-treated with DSS (5%). Apelin was given during the recovery phase, when tap water replaced DSS drinking water. Sixteen to eighteen hours after the last apelin administration, mice were sacrificed 90 min after administration of BrdU (250 mg/kg, IP). Colons were removed, fixed in formalin and processed for BrdU immunostaining.

2.5. Northern blotting and real time RT-PCR analyses of apelin expression

Colonic apelin expression was measured by either Northern blotting or real time RT-PCR analyses as described previously [12,25]. In brief, full thickness colon tissue samples were removed quickly after animals were sacrificed and immediately homogenized in 4 M guanidinium isothiocyanate containing 25 mM sodium citrate, pH 7.0, 0.5% sodium lauroylsarcosine, and 0.1 M β -mercaptoethanol. Homogenates were frozen at -80 °C until purification of total cellular RNA by ultracentrifugation over a cesium chloride cushion (2 ml,

5.7 M). Poly[A]⁺ RNA was prepared as described previously [12,26] and separated on a 1% agarose gel (~ 10 μ g/lane) in a 20 mM 3-[*N*-morpholino] propanesulfonic acid running buffer system, transferred to a nylon membrane and subjected to Northern hybridization. ³²P labeled riboprobes prepared from Strip-EZ RNA kits (Ambion Inc.) were used for Northern hybridizations. The rat apelin riboprobe was supplied by Lee et al. [14]. Expression levels of apelin and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes were quantitated by densitometry. Real time RT-PCR assays were done using an Applied Biosystems (Foster City, CA) 7000 sequence detection system. Applied Biosystems Assays-By-Design containing a 20 \times assay mix of primers and TaqMan MGB probes (6-FAM dye-labeled probe) were used for the target genes: mouse apelin (accession no. NM013912); and, a pre-developed rat 18S rRNA (VIC dye-labeled probe). TaqMan assay reagent (P/N 4319413E) was used for the internal control. Primers were designed to span exon–exon junctions so as not to detect genomic DNA. Probe sequences were searched against the Celera database [12].

2.6. Apelin immunohistochemistry (IHC)

Pieces of terminal colon harvested from either control rodents or rodents with experimental colitis were fixed in buffered formalin and processed for apelin immunostaining as described previously [12,27]. Buffered formalin-fixed, paraffin-embedded tissue sections (5 μ m) were deparaffinized and rehydrated by passage through xylene and graded ethanol solutions. After deparaffinization, slides were treated with 1% H₂O₂ in PBS for 15 min, followed by microwave antigen retrieval at 100 °C for 10 min in DAKO Target Retrieval Solution (DAKO Corporation, Carpinteria, CA) in an H2800 Microwave Processor (Energy Beam Sciences, Inc; Agawam, MA). Following sequential 15 min incubations with 0.1% avidin and 0.01% biotin (Vector Laboratories, Inc; Burlingame, CA) to block endogenous avidin and biotin, slides were incubated in 0.05% casein (Sigma, St. Louis, MO)/0.05% Tween-20/PBS for 30 min to block nonspecific protein binding. For rodent tissue sections, a primary rabbit apelin antiserum generated against apelin-19 (apelin-17 with TYR-CYS at N-terminal to facilitate radiolabeling and conjugation to a larger protein) and apelin-36 coupled to keyhole limpet hemocyanin (KLH) was applied to sections at a 1:1200–2000 dilution for 60 min. For human tissue sections, a primary rabbit antiserum generated against apelin-36 (Phoenix Pharmaceuticals, Belmont, CA) was applied to sections at a $\sim 1:400$ dilution for 60 min. Rabbit serum Ready-to-Use (InnoGenex, San Ramon, CA) was applied as a negative control. Biotinylated F(ab')₂ fragment of swine anti-rabbit immunoglobulins (DAKO Corp.) served as the secondary antibody and was detected by Streptavidin–HRP, and colorized by DAB (DAKO Corp.). Slides were counter-stained with Mayer's Modified Hematoxylin (Poly Scientific, Bay Shore, NY) before mounting, viewed under a Nikon Eclipse E600 microscope and images captured with a Nikon DXM1200 digital camera and ACT-1 (v.2.00) program. All antibody incubations and detection procedures were performed on a DAKO Autostainer or

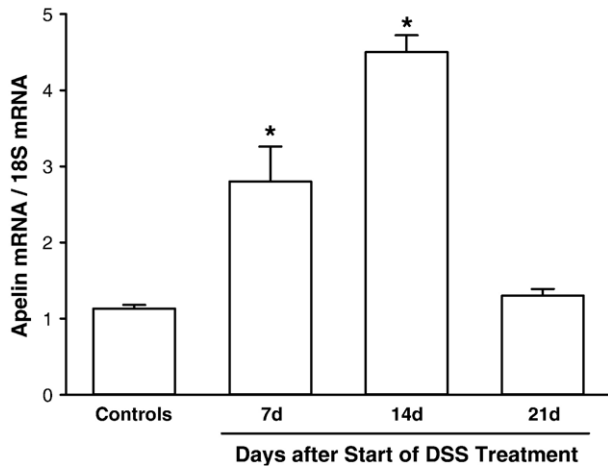


Fig. 1. Real time RT-PCR analysis of apelin expression in mice with DSS-induced colitis. DSS (5%) was given in drinking water ad lib for 7 days. DSS-containing drinking water was replaced with tap water at the end of day 7. Colonic apelin and ribosomal 18S mRNA levels were measured by real time RT-PCR. Apelin expression was normalized to a stomach RNA specimen that contained nominal levels of apelin mRNA and then to colon ribosomal 18S mRNA levels. * $P < 0.05$ vs controls. $N = 4$ mice/group.

manually. Histological sections of colons harvested from mice and rats with DSS-induced colitis were also stained with hematoxylin and eosin (H & E).

2.7. BrdU IHC

To visualize proliferating colonic epithelial cells in control and apelin-treated mice, BrdU was given 90 min before sacrifice. Formalin-fixed, paraffin-embedded sections were deparaffinized, treated with 1% H_2O_2 for 15 min and then subjected to antigen retrieval at 100 °C for 10 min in DAKO Target Retrieval Solution in a H2800 Microwave Processor. Slides were treated with 0.1% of avidin and 0.01% of biotin sequentially, then 0.03% casein in 0.05% Tween/PBS before application of a biotinylated mouse anti-BrdU antibody (Molecular Probes, Eugene, OR). Mouse Ig Ready-to-Use (InnoGenex, San Ramon, CA) served as a negative control. Antibody incubations, detection with universal DAKO LSAB2 HRP system and colorization with DAB were done with a DAKO Autostainer. Slides were counter-stained with Mayer's Modified Hematoxylin (Poly Scientific, Bay Shore, NY) before mounting. The number of BrdU-stained cells per field was examined in vehicle and apelin-treated groups.

2.8. Myeloperoxidase (MPO) assay

Tissues (20–50 mg) were homogenized in potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide, followed by three cycles of sonication and freeze–thawing. Tissue homogenates were centrifuged at 1200 g for 5 min and supernatants were assayed for MPO activity. Substrate for the MPO assay was made by adding 1 μ l of hydrogen peroxide and 6.7 mg of *o*-dianisidine dihydrochloride (Sigma) to 40 ml of potassium phosphate buffer. Substrate was

dispensed in 200 μ l aliquots into a 96-well plate. Five microliters of test supernatants were added to the wells containing the substrate and the plate was incubated at room temperature for 15 min and then read at 490 nm.

2.9. Statistical analyses

Results are expressed as the mean \pm SEM. Data were analyzed by *t*-test or one-way ANOVA and subsequently with Newman–Keuls test when appropriate. $P < 0.05$ was considered significant.

3. Results

3.1. Experimental colitis in rodents increases colonic apelin expression and content

Experimental colitis was induced in mice and rats by giving 5% DSS in drinking water ad lib for 7 and 5 days, respectively. DSS-containing drinking water was then replaced with tap drinking water. Mice and rats were sacrificed either immediately following the initial DSS exposure, or 14 and 21 days after the start of DSS treatment. In mice, signs of DSS-induced colitis included decreased body weights (controls: 24 ± 0.5 g vs DSS mice: 19 ± 0.4 g), anal bleeding, a shortened colon (control: 8.7 ± 0.4 cm vs DSS mice: 6.4 ± 0.2 cm) and increased myeloperoxidase (MPO) levels (control mice: 7.9 ± 1.4 vs DSS mice: 26.0 ± 6.2 μ g/g tissue). Examination of hematoxylin and eosin (H & E) stained colon sections verified the presence of colitis in mice (Fig. 3, panel B) and rats (sections not shown).

In mice with DSS-induced colitis, colonic apelin mRNA levels were increased two to three-fold 7 and 14 days after induction of colitis (Fig. 1). Colonic apelin mRNA levels then decreased to control apelin mRNA levels 21 days after induction of colitis (14 days after cessation of DSS treatment).

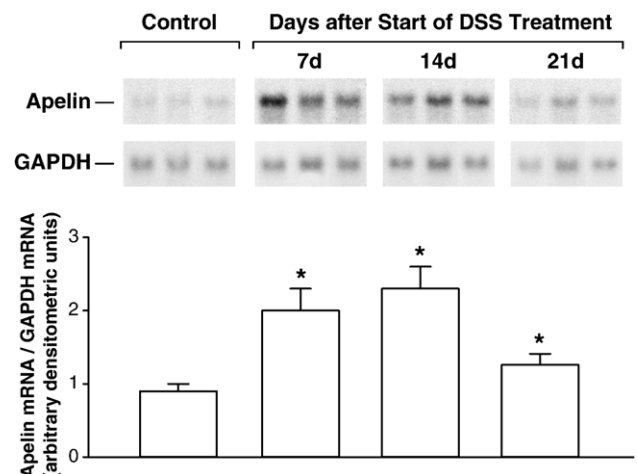


Fig. 2. Northern analysis of colonic apelin expression in rats with DSS-induced colitis. DSS was given in drinking water ad lib for 5 days and replaced with tap water at the end of day 5. Colonic apelin mRNA levels were measured using poly(A)⁺ RNA extracts of colon tissue harvested at different times after induction of colitis. Apelin expression was normalized to colon GAPDH mRNA levels. The mean \pm SEM of three rats is shown. * $P < 0.05$ vs controls.

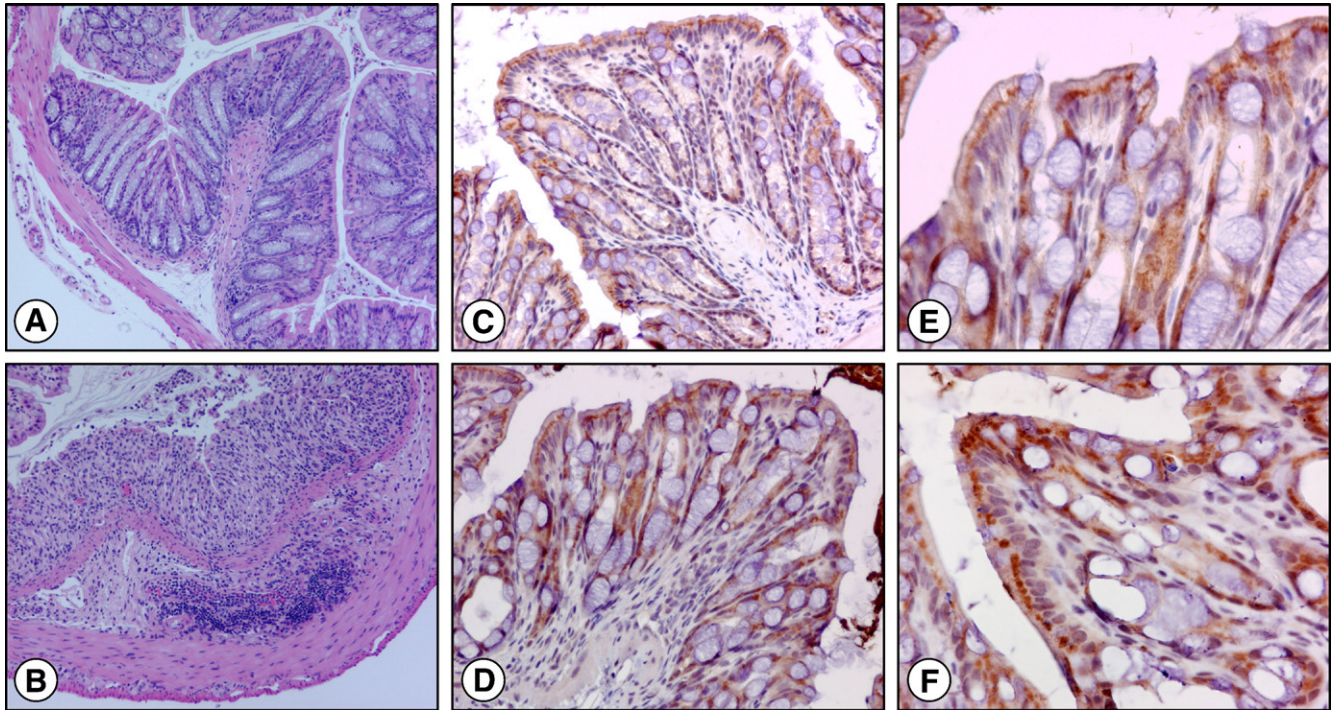


Fig. 3. Increased colonic apelin peptide production during DSS-induced colitis in mice. (A) Control mouse colon, H & E stained. (B) Mouse colon with DSS-induced colitis. Notice presence of lesion with loss of crypts, retainment of surface epithelium and inflammatory infiltrate. H & E stained. (C) Control mouse colon immunostained for apelin. Notice light apelin immunostaining primarily in surface epithelium (brown color). (D, E and F) Apelin immunostaining in mouse colon with colitis, 1 week after start of DSS treatment. Notice the more intense apelin immunostaining in surface epithelium and in epithelial cells along the length of the tubular gland into the stem cell region of the crypts.

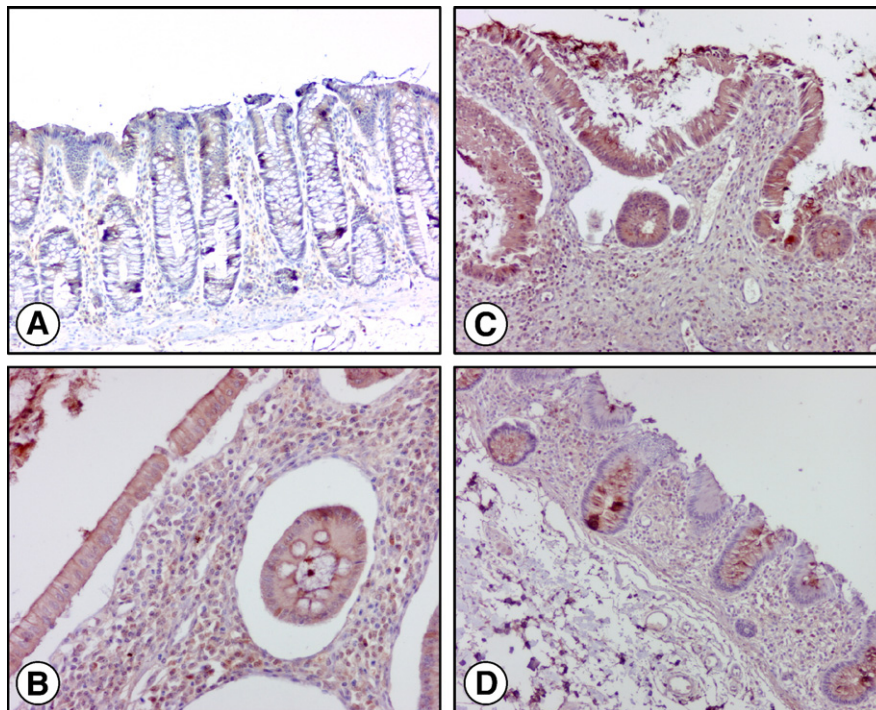


Fig. 4. Increased colonic apelin peptide production in UC and Crohn's disease patients. (A) Control human colonic mucosa. Epithelium shows a low apelin immunostaining (brown-red color). (B) Colonic mucosa of an ulcerative colitis (UC) patient showing strong apelin immunostaining in the surface epithelium. Positive apelin immunostaining is also seen in some immune cells. (C) Colonic mucosa of a Crohn's disease patient showing strong apelin immunostaining in the surface epithelium. (D) Colonic mucosa of a Crohn's disease patient showing strong apelin immunostaining in epithelial cells along the length of the tubular gland into the stem cell region of the crypts.

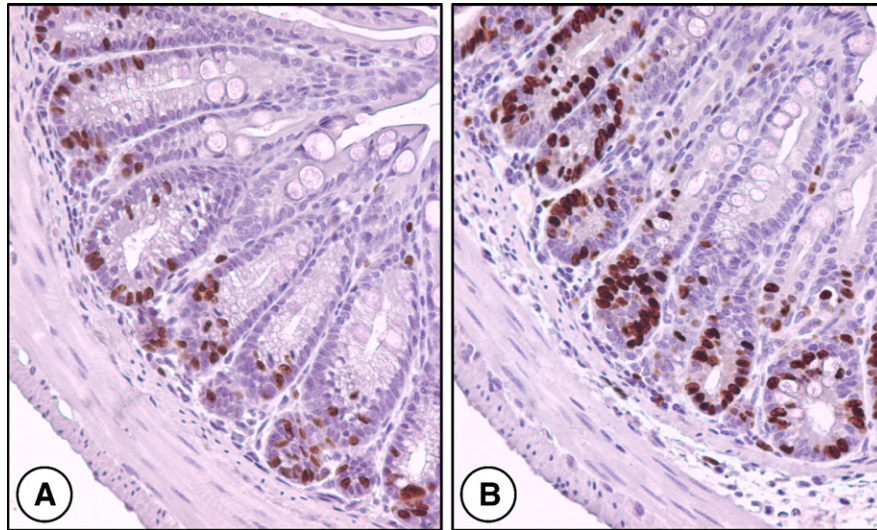


Fig. 5. Increased BrdU (yellow-brown staining) incorporation into colonic epithelium of (B) apelin-treated mouse when compared to (A) control mouse. BrdU was given IP to control and apelin-treated mice 90 min before sacrifice and colons were harvested and processed for measurement of BrdU incorporation as described in Methods and materials.

In rats, colonic apelin mRNA levels were increased significantly ($P < 0.05$ vs control rats) 7, 14 and 21 days after induction of colitis (Fig. 2). Colonic apelin mRNA levels increased approximately one-fold in rats with colitis. Colonic apelin mRNA levels then decreased 21 days after start of DSS treatment but remained significantly elevated when compared to colonic apelin mRNA levels of control rats.

In control mice, apelin immunostaining is present primarily in the surface epithelium of the colon (Fig. 3, panel C). In mice with colitis, sacrificed 7 days after the start of DSS treatment, colonic apelin immunostaining is widespread and intense. Apelin immunostaining is evident in the surface epithelium and in epithelial cells along the length of the tubular gland into the stem cell region of the crypts (Fig. 3, panels D–F). Colonic apelin immunostaining is cytoplasmic and granular in nature. Two weeks after the start of DSS treatment (one week after cessation of DSS) the intensity of apelin immunostaining is decreased when compared to mice sacrificed one week after start of DSS treatment. Three weeks after the start of DSS treatment the widespread pattern of colonic apelin immunostaining is not present and the intensity of apelin immunostaining in the surface epithelium is similar to control mice (data not shown).

The apelin IHC findings in rats with DSS-induced colitis are similar to those made in mice with DSS-induced colitis. In rats with colitis, sacrificed 7 days after the start of DSS treatment, colonic apelin immunostaining is widespread and intense. Apelin immunostaining in the surface epithelium and in epithelial cells into the stem cell region of the crypts is more intense when compared to control rats (data not shown). In rats 14 days after initiation of DSS treatment the intensity of colonic apelin immunostaining is reduced. Twenty-one days after initiation of DSS treatment the widespread pattern of apelin immunostaining is absent and the intensity of apelin immunostaining in the surface epithelium is similar to control rats (data not shown).

3.2. Intestinal apelin immunostaining in patients with IBD

Histological sections of intestinal tissue specimens harvested from ten UC, ten Crohn's disease and five controls were immunostained for apelin. Apelin immunostaining was clearly increased in intestinal tissue sections prepared from IBD patients when compared to apelin immunostaining in control tissue sections (Fig. 4, panels B–D). As observed in rodents with experimental colitis, apelin immunostaining was increased in the surface epithelium and in epithelial cells along the length of the tubular gland into the stem cell region of the crypts of patients with UC and Crohn's disease.

3.3. Influence of exogenous apelin on colonic cell proliferation in mice

Apelin or vehicle was given for 3 days to mice pre-treated with DSS (5%). Apelin was given during the recovery phase, when tap water replaced DSS drinking water. Apelin treatment resulted in a higher incorporation of BrdU into the colonic epithelium when compared to vehicle-treated mice indicating that apelin stimulates colonic epithelial proliferation during the

Table 1
Apelin stimulates colonic epithelial proliferation during recovery stage of DSS-induced colitis

Group	Number of BrdU positive cells/field
Control	17+1
Apelin treated	25+2*

Apelin (50 μ g, 3 \times /day, SC) or vehicle was given for 3 days to mice pre-treated with DSS (5%). Apelin was given during the recovery phase, when tap water replaced DSS drinking water. The influence of apelin on colonic epithelial proliferation was assessed by giving BrdU (290 mg/kg) 90 min before sacrifice and harvest of colons. Colonic epithelial cells that incorporated BrdU were identified as described in the Materials and methods. * $P < 0.05$, $N = 5$ mice/group; one field/mouse was examined.

recovery stage of experimental induced colitis. The number of colonic epithelial cells immunostained for BrdU was significantly higher in apelin-treated mice when compared to control mice (Fig. 5, Table 1).

4. Discussion

The primary findings of the present study are that intestinal apelin production is up-regulated in rodents with experimental colitis and in humans with IBD, and that exogenous apelin stimulates colonic epithelial cell proliferation in mice previously exposed to DSS. This is the first report to demonstrate that intestinal inflammation influences colonic apelin production and that apelin affects intestinal epithelial proliferation. In another model of inflammation our laboratory has found that intestinal apelin expression is increased in LPS treated rodents (Greeley et al., unpublished data).

Although an underlying molecular mechanism for the increased intestinal apelin production was not explored in the present study, our laboratory (Wang et al., unpublished data) has identified putative binding sites in the 5'-upstream regions of the rat and human apelin genes for AP-1 and NF κ B, two transcription factors sensitive to inflammation [28,29]. AP-1 and NF κ B have been shown to activate numerous genes during inflammation including proinflammatory cytokines, ICAM-1 and matrix metalloproteinase-1 (MMP-1) [28,30,31].

Interestingly, colonic apelin expression and peptide levels are increased during the inflammatory reaction in response to DSS drinking water as well as during the tissue repair phase that ensues after DSS withdrawal suggesting that apelin plays multiple roles during the different stages of colitis in the DSS model. Alterations in colonic apelin expression during and after DSS exposure are paralleled by corresponding changes in colonic apelin immunostaining, that reflect apelin peptide contents, with one exception. In rats with DSS-induced colitis, colonic apelin mRNA levels remain elevated three weeks after initiation of DSS treatment whereas colonic apelin immunostaining has returned to control levels. The finding that apelin stimulates colonic epithelial proliferation together with the increased apelin expression during the recovery stage of experimental colitis implies that endogenous apelin contributes to the repair of the intestinal epithelium in rodents with experimental colitis and in humans with IBD. We have also found in other studies that apelin increases colonic epithelial proliferation in normal mice by 20–30% (GH Greeley, unreported data). Our observation that apelin elevates colonic epithelial proliferation concurs with earlier reports showing that apelin stimulates endothelial cell proliferation [32–34] and exerts a chemokine effect on cell migration [35].

In humans, ulcerative colitis is a chronic inflammatory disease that usually shows a course of intermittent exacerbation and remission [20–22]. In the present study an increased apelin immunostaining was evident in the surface epithelium and stem cell region in histological specimens harvested from active UC and Crohn's disease patients. These findings agree with and extend the apelin immunostaining pattern observed in rodents having experimental colitis. Although the cell types which ex-

press the apelin receptor and the APJ receptor in the intestine are not known, localization of apelin in the stem cell region and its influence on epithelial proliferation imply that APJ is expressed in the stem cell region. Alternatively, the increased surface epithelial production of apelin may act luminally and in a paracrine manner to stimulate epithelial proliferation. In contrast to the stimulatory effects of colitis on colonic apelin mRNA levels, preliminary experiments show that expression levels of the APJ receptor are unchanged in rodents with experimentally-induced colitis (GH Greeley, unreported data).

The present study indicates that apelin exerts a stimulatory action on colonic epithelial proliferation since BrdU incorporation into the proliferating epithelium was increased in mice given exogenous apelin. This finding implies that the increased apelin production during the recovery stage of DSS-induced colitis promotes epithelial proliferation. Although crypt regeneration is an integral component of mucosal healing after acute intestinal inflammation and mucosal damage [36,37], earlier reports have described a reduced BrdU incorporation in regions of intestinal ulceration in rats [38]. Furthermore, earlier studies show that colonic expression of trefoil factor is activated during experimental colitis in rodents and is thought to be part of the endogenous mucosal restitution response [39,40].

The difficult nature of making interpretations on changes in levels of regulatory peptides should be pointed out due to the multiple activities of these substances and the unstable nature of inflamed tissue [41]. It is worth pointing out that some regulatory peptides augment tissue damage as well as exert a protective action [42,43].

In summary, we have shown an up-regulation of colonic apelin production in rodents with experimental colitis and in humans with IBD. The increased apelin expression during intestinal inflammation suggests that apelin contributes to the repair of the intestinal epithelium in rodents with experimentally-induced colitis and in humans with IBD.

Acknowledgments

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