Modifying the protease/anti-protease pattern protects from colitis in mice: benefits from elafin over-expression

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Abstract (225 words)

**Background & Aims:** Increased proteolytic activity has been shown to be released from tissues of Inflammatory Bowel Disease (IBD) patients, but no studies have clearly addressed the role of the protease/anti-protease balance in the pathogenesis of colitis. We hypothesized that by changing the pattern of expression of protease inhibitors in the course of colitis, protective effects against the development of inflammation could be achieved. **Methods:** Using both a genetic approach with transgenic mice expressing human elafin, and mice genetically disrupted for the neutrophilic enzymes neutrophil elastase (NE) and proteinase-3 (PR-3), and a pharmacological approach by treating mice with replication-deficient adenovirus vector over-expressing human elafin, we investigated the effects of over-expressing human elafin, a low molecular weight molecule that potently and naturally inhibits NE and PR-3, during the course of colitis. **Results:** we showed that elafin expression/delivery re-equilibrated the proteolytic balance in mouse inflamed colon. In two IBD models (the trinitrobenzene sulfonic acid and the dextran sodium sulphate-induced colitis), transgenic mice expressing elafin and mice genetically disrupted for NE and PR-3 were protected from the development of colitis. Similarly, adenoviral delivery of elafin significantly inhibited inflammatory parameters. Elafin was able to modulate a variety of inflammatory mediators *in vitro* and *in vivo* and to strengthen intestinal epithelial barrier functions. **Conclusions:** these results
highlight elafin as a promising therapeutic agent for chronic intestinal inflammation such as IBD.

**Keywords:** Inflammatory Bowel Disease, Colitis, Proteases, Protease Inhibitors, Elafin, Inflammation.

**Background and Aims**

In the setting of chronic inflammatory diseases such as Inflammatory Bowel Diseases (IBD), uncontrolled inflammatory response, ultimately leading to proteolytic tissue destruction constitutes the basis of the pathology. Although the aetiology of such diseases is not established yet, some mediators seem to play a prominent role in the pathogenesis of chronic inflammatory disorders. Recent studies have highlighted the role of proteases and their receptors in the pathogenesis of colitis. In addition, an increased arginine-specific cleavage proteolytic activity has been shown to be released by tissues from IBD patients. Surprisingly, the regulation of the protease/anti-protease balance in the gut has scarcely been addressed and the therapeutic potential of anti-protease supplementation seldom investigated. Proteolytic enzymes are largely released at sites of inflammation, by resident cells, infiltrated inflammatory cells, or by microorganisms. Concomitantly, endogenous protease inhibitors are synthesized and released within organs, maintaining a physiological proteolytic balance in tissues. Interestingly, in tissues from IBD patients, the expression of elafin and secretory leukocyte protease inhibitor, (two inhibitors of serine proteases expressed at mucosal surfaces and in the skin in inflammatory conditions in humans) was shown to be attenuated upon inflammation, thereby suggesting a disruption of the protease/anti-protease balance in
chronic inflammatory states of the gut. We therefore hypothesized that by changing the pattern of expression of protease inhibitors in the course of colitis, protective effects against the development of inflammation could be achieved. To that aim, we chose to investigate, during the course of colitis, the effects of over-expressing human elafin, one of the two inhibitors down-regulated in IBD. Human elafin is an efficient inhibitor of murine inflammatory proteases such as murine neutrophil elastase (NE) and proteinase-3 (PR-3)\(^9\), validating our present approach (henceforth in the manuscript, the term ‘elafin’ will always refer to the human ortholog). We used two different and independent strategies to over-express elafin into the mouse colon: a genetic strategy using mice transgenic for the expression of elafin and a pharmacological approach administrating intracolonically elafin-expressing adenoviral (Ad) vector, henceforth called Ad-elafin\(^{14}\), in two different models of IBD. Further, in IBD models, we investigated inflammatory parameters in mice deficient for the expression of proteases (NE and PR-3)\(^{15}\).

These approaches allowed us to evaluate the general therapeutic effects of modifying the protease/anti-protease expression pattern in IBD, and in particular, to evaluate the therapeutic potential of elafin expression in the gut.
Methods

Animals

C57/Bl6 male mice (6-10 weeks) were either purchased from Janvier (Le Genest Saint Isle, France), or bred at the Institut Pasteur Animal Care facility for MCMV-elafin transgenics (MCMV-EL), Endothelin-elafin transgenics (Endo-EL) and littermates. MCMV-EL (elafin expressed under the mouse cytomegalovirus promoter) homozygotes mice were originally created on a mixed C57/Bl6 X CBA background\textsuperscript{16} and Endo-EL mice (elafin expressed under the endothelin promoter) were originally obtained as heterozygous animals on a C57BL6 X SJL background\textsuperscript{17}. Both strains were fully backcrossed (9 generations) on a pure C57BL6 background. Mice genetically disrupted for NE and PR-3 were bred at the faculty of medicine in Reims\textsuperscript{15}. All animals were maintained under 12h-light/dark cycles having free access to food and water, except for being fasted the day before the induction of colitis. All procedures were approved by institutional animal care committees and veterinary services.

Induction of colitis

Colonic inflammation in mice was induced by intracolonic administration of trinitrobenzene sulphonic acid (TNBS) (2 mg per mouse dissolved in 50\% ethanol), or by addition to the drinking water of dextran sodium sulfate (DSS) (3 or 5\%), as previously described\textsuperscript{1,2} (water consumption was equal in all groups for the DSS protocols). Body weight and survival rate were measured daily after the induction of colitis. Seven days after TNBS or DSS treatments, mice were sacrificed, and macroscopic score was assessed on whole colonic tissues, while samples were harvested for histology,
immunohistochemistry, myeloperoxidase (MPO) activity, cytokine expression and protease activity (trypsin-like and elastase/PR-3).

Adenovirus constructs and in vivo treatments

Ad-elafin, Ad-dl70-3 (referred hereafter as ‘Ad-null’, as it has no promoter, nor transgene) and Ad-IκBα are all replication-deficient adenovirus vectors which have been described before\textsuperscript{14} and were amplified using well established protocols. Ad-NF-KB-luciferase was obtained from the Iowa Gene Transfer Vector Core (University of Iowa) at a titer of $3.10^{10}$ pfu/ml and was diluted in PBS to obtain final working doses.  

In vivo : Three days (for Ad-null and Ad-elafin) or 5 days (for Ad-NF-κB-luciferase) after drinking either water or DSS, each C57BL/6 male mice was anaesthetized and received intracolonically 100µl of PBS alone (vehicle) or either $5.10^{8}$ pfu Ad-null or Ad5-elafin, or $2.5.10^{9}$ pfu Ad-NF-κB-luciferase. The same treatment (water or DSS) was applied until day 7, when mice were sacrificed and tissues were collected for inflammatory parameter measurements, as described below.

Assessment of inflammation

Macroscopic damage scores were evaluated as previously described\textsuperscript{18-20}. For microscopic damage, we chose to score (ranging from 0 to 10) the three more salient parameters, i.e epithelial cell injury, mucosal gland destruction and inflammatory cells infiltration in mucosa/submucosa\textsuperscript{18-20}. MPO activity was measured as an index of granulocyte infiltration as previously described in colonic tissues harvested at the time of sacrifice\textsuperscript{18-20}.  


Serine protease activity in colonic tissues and luminal washes

As previously described, upon sacrifice, the entire colon was excised and 1ml PBS was instilled and washed twice through the lumen. A piece of colonic tissue was then kept and proteolytic activity (trypsin-like and elastase activity) were measured both in luminal washes and tissues. Trypsin-like and elastase/PR-3-like activities were measured using tosyl-Gly-Pro-Arg-p-nitroanilide (150µM, Sigma) and MeO-succinyl-Ala-Ala-Pro-Val-p-nitroanilide (100µM, Sigma, Saint Quentin Fallavier, France) respectively as substrates. Samples (20 µl for trypsin activity or 10 µl for elastase/PR-3 activity) were resuspended in their respective buffer: 100mM Tris/HCl, 1mM CaCl₂, pH=8 for trypsin activity and 50 mM Tris-HCl, 500 mM NaCl, 0.1% Triton X100 for elastase/PR-3 activity. The change in absorbance at 405 nm was determined over 30-minutes at 37°C with a microplate reader NOVOstar™ (BMG Labtech, France). Activity was compared to known standard dilution of trypsin from porcine pancreas (Sigma) or human NE (Sigma). Protein concentration was determined using a BCA kit® (Pierce, Thermo Scientific, Courtaboeuf, France).

Cytokine expression

Frozen colonic samples of transgenic and adenovirus treated mice were homogenized 30 seconds with a Polytron™ at 4°C in 500µl of cell lysis buffer (20mM Tris-Hcl, pH7.5, 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1mM beta-glycerophosphate, 1 mM Na3VO4, 1 µg/ml leupeptin; Cell Signalling, Sigma) supplemented with anti-proteases (Roche Diagnostics, Meylan,
France) cocktail. After centrifugation (10000Xg, 10 min, 4°C), supernatants were filtered on QIAshredder columns (Qiagen, France). Fifty microliters of this homogenate was used for simultaneous dosage of KC (keratinocyte chemoattractant), MCP-1/CCL2 (Monocyte chemoattractant proteins), RANTES/CCL5 (Regulated upon Activation, Normal T-cell Expressed, and Secreted), TNF-α, IFN-γ, Interleukins (IL) IL-2, IL-4, IL-5, IL-6, IL-10, IL-13 and IL-17A using cytometric bead array (CBA) on fluorescent cell sorter FACSCalibur according to the manufacturer’s instructions (BD Biosciences, Le Pont de Claix, France). Raw values were normalized to tissue weight (average from 30 to 50 mg) and cytokine concentrations were extrapolated from standard curves with the help of FCAP Array® software. Only values above the limit of cytokine detection were considered.

Measurement of NF-κB activity in vivo and bioluminescence

Five days after starting water or DSS treatments, mice received Ad-NF-κB-luciferase intracolonically. Two days later, mice received, an intra-peritoneal injection of D-Luciferin potassium salt (Synchem OHG, ref. BC219) (150mg/kg), 10-minutes before imaging. Mice were then kept anaesthetised under isoflurane inhalation in an IVIS CCD (charge-coupled device) camera coupled to the LivingImage software package (Xenogen, Biosciences, Cranbury, NJ). Bioluminescence signals were imaged during 20 minutes and a digital false-color photon emission image of the mouse was generated. Photons were counted within a constant defined area corresponding to the surface of the abdomen encompassing the whole colonic region. Photon emission was recorded as photons/second/cm2/steradian (p/s/cm2/sr) emitted. Images were analyzed and
quantified with Living Image® 3.0 (Xenogen) software.

**Histology, Immunohistochemistry, and in situ hybridization**

For histology, tissue samples from colons were fixed in 4% neutral buffered paraformaldehyde and embedded in paraffin. Five-micrometer sections were cut and stained with hematoxylin and eosin (H&E) for light microscopic examination. Microscopic damage scores were assessed on histological samples of colons by trained pathologists (M. Huerre, X. Zing) blinded from treatments. Cellular infiltration, epithelial cell ulceration and mucosal gland destruction were given scores ranging from 0 to 10, incremental scores denoting increased inflammation. See supplemental data for immunohistochemistry and *in situ* hybridization protocols.

**Cell culture**

Caco-2 cells were purchased from American Type Culture Collection (ATCC, Molsheim, France, HTB-37) and grown in Dulbecco’s modified Eagle’s medium Glutamax™ (DMEM, Invitrogen Cergy-Pontoise, France). The media was supplemented with 10% fetal bovine serum (FBS, Invitrogen) and 100U/100µg penicillin/streptomycin (Invitrogen). Cultures were kept at 37°C in a 5% CO₂ environment and culture medium was changed every 2 days. Confluent cells were sub-cultured after Ca²⁺/Mg²⁺ free PBS washes and partial digestion with trypsin (10 minutes, 37°C).

HT29 cells were cultured to sub-confluence in 6-well plates and were left either uninfected or were infected during 24hr with recombinant Ad vectors (Ad-null, Ad-elafin, Ad-κBα, all at moi=5) in RPMI medium containing 0.5% of fetal calf serum.
Cells were then left unstimulated or were stimulated with bacterial LPS (final concentration 1µg/ml) or TNF-α (final concentrations 0.1 or 1 ng/ml). 24hr post stimulation, media were harvested and IL-8 protein secretion was measured by ELISA (R&D), following manufacturer’s instructions.

*Paracellular permeability studies*

Caco-2 were grown as monolayer in 12-wells polycarbonate Transwell® plates (2.10^5 cells/well, 1.12cm^2 surface area of semi-permeable filter membrane) until confluence and permeability was measured as previously described^22-24^. Briefly, trans-epithelial resistance (TER) was measured every 3 days using an electrovoltage-meter (World Precision Instruments, France). After 21 days of culture (reaching TER>500 Ω.cm^2), cells were exposed to apical and basolateral cytokines (TNFα, 10 ng/ml), PBS or Ad-elafin (all at moi =5) for 48 h. Apical and basolateral chamber contained 0.5 mL and 1.5 mL of medium, respectively. Paracellular permeability was assessed by apical-to-basal transport of a Dextran probe (MW3000) conjugated to fluorescein isothiocyanate (FITC). After treatments, dextran-FITC (100µM) was added into the apical chamber. After 2 h of incubation (37°C in 5% CO2), 300µl were collected from the basal chamber (triplicates) for fluorometric measurement on a microplate reader NOVOstar™ with an excitation wavelength of 496 nm, emission at 524 nm, and cutoff at 515 nm. Paracellular permeability to Dextran-FITC was expressed as percentage of paracellular flux measured in untreated wells.

*Statistics*
Comparisons among groups were made using a 2-tailed Student’s t test with Bonferroni correction. Data are expressed as mean ± SEM, and a P value less than 0.05 was considered significant.
Results

Proteolytic balance is modified by constitutive elafin expression during colitis

We used here two elafin transgenic mouse lines, MCMV-EL and Endo-EL (which express elafin under the mouse cytomegalovirus and endothelin promoters, respectively, in a variety of tissues, including the gut (\textsuperscript{16, 17}, and not shown)) to modify the protease/anti-protease expression pattern and consequently the proteolytic burden in mouse colon. Elafin expression in transgenic mice but not in wild-type littermates (WT) was detected by quantitative RT-PCR (Endo-EL: Ct 32±1; MCMV-EL: Ct / 30±2, and not detectable in WT) and was shown to be, largely expressed in the epithelium, when analyzed by FISH (see Supplementary Fig 1). We then investigated active protease levels in colonic tissues of elafin transgenic versus wild-type mice (WT). Elastase/PR-3 activity was detected in colonic tissues of mice drinking water and was not different in these basal non-inflamed conditions in wild-type or elafin-transgenic mice (both MCMV-EL and Endo-EL) (Fig. 1A). However, colitis induced either by DSS or TNBS provoked a significant increase in the activity of elastase/PR-3 in colons from WT mice (Fig. 1 A,B). This increase was significantly inhibited in elafin-transgenic mice (both MCMV-EL and Endo-EL) (Fig. 1 A,B), which showed levels similar to non-inflamed tissues. Similarly, trypsin-like activity was equivalent in all non-inflamed colonic tissues from WT, MCMV-EL, Endo-EL (Fig. 1 C, D). Seven days after the induction of colitis (by DSS or TNBS), trypsin-like activity was significantly increased in WT mice, but this increase was not observed in MCMV-EL, Endo-EL elafin-transgenic mice (Fig. 1 C, D). Similar patterns of elastase/PR-3 activity and trypsin-like activity were observed when
activity was measured in luminal lavages collected in PBS (data not shown). Taken together, these results showed that the expression of elafin did not modify basal elastase/PR-3 and trypsin-like activities, but was able to completely inhibit their increase upon the induction of colitis. Therefore, constitutive elafin transgenic expression modifies the protease/anti-protease balance in the colon during colitis, towards an inhibition of proteolytic activity.

*Elafin expression or NE-PR-3 deficiency protects from the development of colitis*

Colitis induced by DSS or intracolonic administration of TNBS provoked, as expected, several signs of inflammation, including weight loss (Supplementary Fig 2), macroscopic damage (DSS Fig. 2A, TNBS Fig 2B), and increased granulocyte infiltration (MPO activity in DSS Fig. 2C, TNBS Fig 2D). Compared to WT mice, transgenic mice constitutively expressing elafin (MCMV-EL and Endo-EL) showed a significant reduction in the observed inflammatory parameters. Macroscopic damage score was significantly reduced in both MCMV-EL and Endo-EL for both types of inflammatory insults (DSS Fig. 2A and TNBS Fig. 2B). MPO activity was significantly reduced both in MCMV-EL and Endo-EL mice when colitis was induced by either DSS (Fig. 2C), or TNBS (Fig. 2D). Microscopically, DSS colitis caused mostly mucosal injuries with epithelial cell destruction (arrows), mucus cell depletion and inflammatory cell infiltration mostly in the mucosa (arrow heads) (Fig. 3A). TNBS colitis caused transmural inflammation with muscle thickening (double arrows), goblet cell depletion, sometimes, crypt abscesses and transmural inflammatory cell infiltration (arrow heads) (Fig. 3A). In the DSS model, both MCMV-EL (Fig. 3B) and Endo-EL (Fig. 3C) showed less inflammation at the microscopic level than WT, with a most striking effect on
epithelial cell injury, mucosal gland destruction and inflammatory cell infiltration (Supplementary data Table 1). In the TNBS model, the inflammatory parameters mostly reduced in both elafin transgenic strains were the inflammatory cell infiltration, and to a lesser extent, mucosal gland destruction in Endo-EL mice (Table 1 Suppl.). Interestingly, we observed a strong expression of elafin in colonic biopsies from control subjects, mostly located in the epithelium. This expression was very weak in tissues from patients with Crohn’s disease or ulcerative colitis (Supplementary Fig 3).

The pro-inflammatory role of NE and PR-3 was demonstrated in mice genetically disrupted for both enzymes. Heterozygote mice partly deficient for the expression of NE and PR-3 expressed significantly reduced amounts of colonic elastase/PR-3 activity (supplementary Fig 4) and were significantly protected against DSS-colitis, as assessed by macroscopic score and MPO levels (Supplementary Fig 4).

_Cytokine-Chemokine expression and release are modified by elafin expression_

In order to further investigate the effects of elafin expression on the modulation of innate immune responses, we measured the mRNA and protein levels of a variety of cytokines and chemokine from WT and elafin transgenic mouse colonic tissues in basal (non-inflamed) or colitis (DSS) conditions. mRNA levels of the chemokines KC, MIP1α, MIP2, and RANTES were significantly reduced both in MCMV-EL and Endo-EL mice compared to WT mice after the induction of DSS colitis (Supplementary Table 2). At the protein level, among the cytokines analyzed, we observed that the levels of IL-6 and those of the neutrophil chemo-attractant proteins KC and IL-17A were significantly reduced in elafin-expressing mice (MCMV-EL) after colitis, when compared to WT mice.
The protein levels of RANTES, TNFα, INFγ, IL-2, MCP1 but also IL-4, IL-10, IL-5 or IL-13 (not shown) were unchanged between elafin transgenic mice and WT mice after colitis induction. Interestingly, RANTES mRNA was down-regulated in elafin transgenics (see Supplementary data, Table 2), suggesting post-transcriptional regulation.

In vivo NFκB activation is modulated by Elafin

Because the pro-inflammatory cytokines and chemokines expression analyzed are under the partial control of the nuclear factor NF-κB, we next tested whether NF-κB activity was modulated in vivo by elafin expression. Using a replication-deficient Ad-NF-κB-luciferase reporter system (Ad-NF-κB instilled intracolonically, Fig 4A) and bioluminescence in vivo recording, we showed for both murine elafin-expressing strains (MCMV-EL and Endo-EL), that elafin expression resulted in drastic down-regulation of NF-κB activity in the gut (Fig 4B, C). For those experiments, inflammatory parameters (macroscopic score, MPO, etc.) were also recorded and confirmed here again, that elafin transgenic mice (MCMV-EL and Endo-EL) had reduced inflammation (Fig 4D shows macroscopic damage scores). This also demonstrated that the use of the Ad vector had no confounding effects in our experimental system.

A role for Elafin in epithelial functions: barrier and defense

Intestinal epithelial cells constitute the very first line of defense in the course of colitis. First through its barrier function epithelium blocks the passage of luminal content into the tissues, and second, upon activation, by secreting chemokines such as the IL-8, it alerts the innate immune system, inducing thereby the recruitment of inflammatory cells.
Because elafin is strongly expressed at human epithelial surfaces, including that of the gut, we investigated the effects of elafin overexpression on those two major functions of the gut epithelium: barrier, and IL-8 secretion. Intestinal epithelial cells monolayers exposed to TNFα allowed the para-cellular passage of dextran. Incubation of cells with the adenoviral vector expressing elafin (Ad-elafin), but not with the null adenovector (Ad-null), induced a significant inhibition of TNF-α induced increased permeability (Fig. 5). For all conditions, changes in permeability could not be attributed to cell death as cell survival was the same in all groups (not shown). Intestinal epithelial cells stimulated either by TNFα or LPS released significant increased levels of IL-8 (Table 1). Treatment of those cells by Ad-elafin significantly reduced this release of IL-8, while Ad-null had no effect (Table 1). Remarkably, the extent of inhibition obtained by treatment of cells with Ad-elafin was similar to that conferred by Ad-Iκα, an Ad vector coding for Iκα, the canonical NF-κB inhibitor. Interestingly, in intestinal epithelial cells, Ad-elafin did not decrease, but instead significantly increased NF-κB activity (Table 1).

Colonic Adenovirus-elafin expression in WT mice recapitulates the elafin protection observed in transgenic mice

We also wanted to evaluate the therapeutic potential of transient delivery of elafin in the inflamed colon, and to investigate whether a transient re-equilibration of the balance proteases/anti-proteases, could modify the course of colitis. Intra-colonic administration of Ad-elafin to wild-type mice (natural ‘KO’ for the elafin gene, see above) after the induction of DSS colitis significantly reduced both elastase (Fig. 6A) and trypsin-like activity (Fig. 6B), while Ad-null had no effect. This decreased proteolytic activity in
tissues from inflamed mice treated with Ad-elafin was associated with a decreased macroscopic damage score (Fig. 6C), characteristic of protection against inflammation. At the microscopic level, mice treated with Ad-elafin showed significantly reduced mucosal injury and a preserved epithelium (Fig. 6D arrows), compared to mice that were treated with Ad-null or mice treated with vehicle. These protective features were also associated with an impressive reduction in chemokines and pro-inflammatory cytokines in colonic tissues (Fig. 6E). TNF-α, IFN-γ, IL-6, MCP-1 KC and IL-17 levels were all significantly increased in DSS-drinking mice, compared to water-drinking mice, and all those cytokines and chemokines were significantly reduced in tissues from mice treated with Ad-elafin, compared to mice treated with PBS or with Ad-null (Fig. 6E). Although RANTES levels were increased by the induction of colitis, this was not significantly reduced by Ad-null or Ad-elafin treatments. Colonic expression of IL-2, IL-4, IL-10, IL-5, and IL-13 were not different in Ad-elafin, Ad-null or PBS-treated mice after the induction of colitis by DSS (Fig. 6E and not shown). DSS-induced colitis disrupted the diffuse immunostaining of the zonula occludens tight junction protein ZO-1 provoking a rearrangement of this protein in, clusters, in a pattern characteristic of intestinal epithelial barrier disruption. Ad-elafin, but not Ad-null treatment administered intra-colonically to DSS-treated mice largely maintained the diffuse immunostaining of ZO-1 in mouse colon (Supplementary Fig 5). Interestingly, Ad-elafin treatment conferred no extra protection in mice genetically disrupted for NE and PR-3 expression (Supplementary Fig 6).

Discussion
In the present study, we showed in different murine models of colitis that the proteolytic balance of tissues is disrupted towards an increased proteolytic activity, potentially providing mechanistic clues about what was previously observed in tissues from IBD patients. Further, we established that restoring this proteolytic balance by the expression of the protease inhibitor elafin, in 3 independent models of over-expression (2 transgenic and 1 adenovirus-mediated expression) is associated with a strong protective effect against the development of colitis. These results not only provide definitive insights into the importance of the proteolytic balance in gut inflammation, but also point to a specific protease inhibitor: elafin, as a possible protective molecule in chronic inflammatory disorders of the gut.

Elafin is a specific inhibitor of NE and PR-3, two enzymes that are released by neutrophils. In our study, whether elafin was expressed constitutively in transgenic mice, or transiently in Ad-elafin-treated mice, colitis-induced elastase/PR-3 and trypsin-like proteolytic activities in colonic tissues and luminal washes (not shown) were significantly inhibited (Fig. 1 and 6). The fact that mice deficient for NE and PR-3 are protected against colitis, and that elafin delivery by adenoviral vector (Ad-elafin) treatment did not confer further protection in those mice strongly suggest that elafin protection could be largely due to its inhibitory activity on elastase and PR-3. Because no inhibitory effect has ever been reported for elafin against trypsin-like enzymes, the decreased trypsin-like activity observed in elafin-expressing tissues could be due to indirect effects. Elafin, by inhibiting NE/PR-3 activity, could reduce a potential stimulatory effect of these enzymes on the production of trypsinogen/trypsin by colonic tissues. Elafin reduced not only
elastase/PR-3 and trypsin-like activities, but also the expression of several inflammatory genes that are, at least in part, under the control of NF-κB, therefore explaining the significant reduction in levels of pro-inflammatory cytokines and chemokines detected in our models. It is noteworthy, in keeping with the type 1 nature of the cytokine profiles triggered by TNBS and DSS at the observed time-points, that elafin modulatory effects were limited to pro-inflammatory cytokines (IL-6, KC, IL-17, TNF-α, MCP-1) as well as to IFN-γ, the prototypic type 1 cytokine, when elafin was administered via the adenoviral vector. We show here in vivo that elafin-expressing transgenic mice have strongly reduced levels of activated NF-κB after the induction of colitis compared to wild-type mice. Importantly, this is the first direct evidence for elafin of such activity on transcription factors in vivo, and more specifically, at sites of inflammation in the colon.

Many cell types could be theoretically targets of the anti-inflammatory properties of elafin in the gut, including monocytes, macrophages, and endothelial cells. Activation of all those cell types has been shown to be modulated by elafin expression, through inhibition of transcription factors, and could participate in synergy to the general anti-inflammatory properties of elafin. However, because elafin is primarily an epithelial cell product, and because of the importance of intestinal epithelial cells in maintaining tissue homeostasis during colitis, we investigated whether this cell type could also be a target of elafin-induced protective properties. Our results show that in human intestinal epithelial cell lines, Ad-elafin was able to significantly decrease LPS and TNFα-induced IL-8 release. Interestingly, this effect was not associated with a reduced, but instead with a significant increased epithelial NF-κB expression. This is in keeping with the notion that NF-κB activation plays an important protective role specifically in
epithelial cells, maintaining intestinal homeostasis\textsuperscript{28}. Therefore, these results suggest that depending on the cell types, elafin could either favour or inhibit NF-κB activation, with the overall goal to exert protection against inflammation. In addition, we also demonstrated here direct effects of elafin on epithelial cell permeability functions: Ad-elafin was able \textit{in vitro} to significantly inhibit TNFα-induced increased intestinal monolayer\textsuperscript{29} permeability (Fig. 5) and, \textit{in vivo}, to restore tight junction organization, after DSS-induced colitis (Fig 5, Supplementary).

As indicated above, the protective effect of elafin could be mediated, at least in part, by tightening mucosal barrier. Indeed, intestinal mucosa permeability is considered a critical element of innate defense, and loss of the barrier function can lead to over-activation of immune cells\textsuperscript{30,31}. Indeed, upon epithelial barrier leakage, over-zealous luminal content-mediated activation of innate immune system has been implicated in the pathogenesis of IBD\textsuperscript{32}. Taken together, our results provide the first evidence that re-equilibration of the proteolytic balance in the inflamed colon is associated with an impressive reduction in inflammatory parameters. Delivery and/or expression of the endogenous protease inhibitor elafin seem to be most effective to achieve a balanced proteolytic activity in the gut and to foster anti-inflammatory signals. Particularly noteworthy and impressive are results stemming from the Ad-elafin protocol which was designed as a ‘therapeutic’ approach, since the Ad-elafin vector was delivered locally 3 days after the onset of inflammation. These results highlight elafin as a strong potential therapeutic agent in the context of chronic intestinal inflammation such as IBD.

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Figure legends

Fig 1 Modulation of colonic protease activity by elafin after DSS- or TNBS-induced colitis.

Male C57/Bl6 WT, MCMV-EL and Endo-EL mice drank either water (n= 9, 6, 5 respectively) or DSS (n= 11, 8, 8, respectively), or received intracolonically TNBS (n = 7, 5, 4 respectively) and were sacrificed 7-days later. Activity of elastase/PR-3 (A, B) and trypsin activity (C, D) were assessed in colonic extracts.

Significant differences compared to WT-inflamed is * for p<0.05 and *** for p<0.005.

Fig 2 Modulation of colonic macroscopic damage and MPO activity in elafin transgenic mice after DSS- or TNBS-induced colitis

Male C57/Bl6 WT, MCMV-EL, Endo-EL mice (after H2O, DSS, or TNBS treatment) were assessed for colonic damage and MPO activity at day 7.

Significant differences compared to WT-inflamed group is * for p<0.05, ** for p<0.01, and *** for p<0.005.

Fig 3 Modulation of tissue architecture and pro-inflammatory cytokines in elafin transgenic mice after DSS- or TNBS-induced colitis

Histology of colons (A-C) and cytokine protein content (D) from C57/Bl6 WT, MCMV-EL, Endo-EL mice, 7-days after H2O, DSS, or TNBS treatment. Scale bar is 20µm.

Significant differences compared to WT corresponding treatment is * for p<0.05.
**Fig 4**  Modulation of in vivo NF-κB activity in elafin transgenic mice following DSS-mediated colitis

Study design (A), luciferase bioluminescence measurements (B: pictures, C: quantification) and macroscopic damage score (D) in male C57/Bl6 WT, MCMV-EL and Endo-EL mice that drank H₂O or DSS and received intracolonically 2.5.10⁹ pfu Ad-NF-κB-luciferase, 5-days after starting water or DSS treatments. Numbers represent mean +/- SD. * represents statistical significance (p<0.05) compared to ‘WT water’, ** represents statistical significance (p<0.05) compared to ‘WT DSS’, *** represents statistical difference (p<0.005) compared to ‘WT Adeno-NFκB + DSS’ treated mice.

**Fig 5** Inhibition of TNF-α-stimulated permeability by Ad-elafin in intestinal epithelial cells

Permeability (apical-to basal transport of a Dextran probe conjugated to FITC) was measured in Caco-2 cells grown as monolayer and exposed to apical and basolateral cytokines (TNFα, 10 ng/ml), and Ad-null or Ad-elafin (all at moi =5) for 48 h. Data (triplicate) are expressed as a percentage of paracellular flux compared to vehicle-treated cells and are representative of n=12 for the TNF group and n=6 for the TNF + Ad-EL group. Significant difference compared to TNF group was noted * for p<0.05.

**Fig 6** Colonic Adenovirus-elafin expression in WT mice recapitulates the elafin protection observed in transgenic mice
Three days after the induction of colitis by DSS (water was given to controls, n=5), male C57/Bl6 WT mice were injected intracolonically with either PBS (n=8) or 2.10^8 pfu of either Ad-null (n=6) or Ad-elafin (n=7). Elastase/PR-3 (A), trypsin activity (B), colonic inflammation (C), histology (D), and cytokine content, (E) were assessed in tissues of those mice 7-days after the induction of colitis. For A-B, significant difference compared to PBS is * for p<0.05 and ** for p<0.01. Ψ represents significant difference compared to Ad-null group for p<0.05. In C, significant difference compared to PBS is *** for p<0.005. ΨΨΨ represents significant difference compared to Ad-null group for p<0.005. Scale bar is 20 µm (D). In E, significant differences compared to DSS alone is * for p<0.05. Ψ represents significant difference compared to DSS +Ad-null-treated group, for p<0.05.

Table 1: Modulation of IL-8 secretion and NF-κB activity in colonic epithelial cells by Adenovirus-delivered elafin or IκB in response to stimulation by bacterial LPS or TNF-α

HT29 cells were treated or not (NI: non-infected) for 24hr with recombinant Ad vectors (Ad-null, Ad-elafin, Ad-IκBα, Ad-NF-κB-luciferase, all at moi =5), and were then stimulated or not with bacterial LPS (final concentration 1µg/ml) or TNF-α (final concentrations 0.1 or 1 ng/ml). Twenty four hours later, IL-8 protein secretion was measured by ELISA in cell supernatants. Values represent mean IL-8 concentrations from 3 experiments, and SD are in brackets. NF-κB activity was measured in lysed cells, data were presented as firefly luciferase activities arbitrary units and expressed as an average of three independent experiments, SD are in brackets. * denotes statistical significance (p<0.05), compared to corresponding stimulation in Ad-null respective
conditions. # denotes statistical significance (p<0.01), compared to the corresponding non-infected (NI) cells.

Reference List


