Human Pancreatic Secretory Trypsin Inhibitor Stabilizes Intestinal Mucosa against Noxious Agents

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Pancreatic secretory trypsin inhibitor (PSTI) is a serine protease inhibitor, expressed in gut mucosa, whose function is unclear. We, therefore, examined the effects of PSTI on gut stability and repair. Transgenic mice overexpressing human PSTI within the jejunum (FABP^{-1178 to +28} hPSTI construct) showed no change in baseline morphology or morphometry but reduced indomethacin-induced injury in overexpressing hPSTI region by 42% ($P < 0.01$). Systemic recombinant hPSTI did not affect baseline morphology or morphometry but truncated injurious effects in prevention and recovery rat models of dextran-sodium-sulfate-induced colitis. *In vitro* studies showed PSTI stimulated cell migration but not proliferation of human colonic carcinoma HT29 or immortalized mouse colonic YAMC cells. PSTI also induced changes in vectorial ion transport (short-circuit current) when added to basolateral but not apical surfaces of polarized monolayers of Colony-29 cells. Restitution and vectorial ion transport effects of PSTI were dependent on the presence of a functioning epidermal growth factor (EGF) receptor because cells with a disrupted (EGFR^{-/-} immortalized cells) or neutralized (EGFR blocking antibodies or tyrosine kinase inhibitor) receptor prevented these effects. PSTI also reduced the cytokine release of lipopolysaccharide-stimulated dendritic cells. We conclude that administration of PSTI may provide a novel method of stabilizing intestinal mucosa against noxious agents

and stimulating repair after injury. (Am J Pathol 2007, 171:1462–1473; DOI: 10.2353/ajpath.2007.070192)

Pancreatic secretory trypsin inhibitor (PSTI), also known as serine protease inhibitor Kazal type 1 (SPINK1), is a 56-amino acid peptide that protects the pancreas from autodigestion because of premature activation of pancreatic proteases.¹ PSTI expression also occurs in the normal human breast, in the mucus-producing cells of the mucosa of the stomach and large intestine,^{2,3} and is markedly up-regulated in epithelial and other cells in the small intestine at sites of injury, such as in Crohn's disease.^{2,4} The functions of this extrapancreatic PSTI are, however, unclear.

When an injury occurs, one of the earliest repair processes is cell migration (restitution) over the denuded area to re-establish epithelial continuity. We have previously shown that PSTI stimulates cell migration, but not proliferation, of HT29 cells.² As local expression of PSTI increases at sites of injury, PSTI stimulates cell migration *in vitro*, and PSTI shares limited sequence homology with the potent healing factor epidermal growth factor (EGF),⁵ we hypothesized that PSTI expression within the gut has relevance in maintaining mucosal integrity and/or stimulating repair after injury. To examine this idea further, we have now performed a series of related studies examining the effect of PSTI on gut defense, repair, and vectorial ion transport using a combination of *in vitro* and *in vivo* models.

To examine the effect of localized overexpression on gut integrity, as occurs at sites of injury, we produced transgenic mice that chronically overexpress human PSTI within the jejunal mucosa. These mice were then studied under baseline circumstances and in their resistance to the injurious effects of the nonsteroidal anti-inflammatory drug, indomethacin. A further series of *in vivo* models were then used to examine the effect of administering

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exogenous human recombinant PSTI on gut morphometry and morphology under baseline circumstances and on the amount of colitis caused by the well-established damaging agent dextran sodium sulfate (DSS). A series of preliminary *in vitro* studies then examined the effect of PSTI on lipopolysaccharide-induced cytokine release from monocytes as a potential further mechanism of action of PSTI in these models.

In addition to its barrier and absorptive functions, gut homeostasis, which includes electrolyte and fluid balance, requires tight control of vectorial epithelial ion transport. Because several regulatory gut peptides, including growth factors such as EGF,⁶ are known to influence epithelial ion transport, we examined whether PSTI could also modulate this process and compared and contrasted the effects of EGF and PSTI in this system. This was performed by following changes in short-circuit current (SCC) using voltage-clamped, polarized epithelial monolayers of Colony-29 cells. Although PSTI is unlikely to be a direct EGFR ligand,^{7,8} the EGF receptor may still have relevance in mediating some of these effects. In a final series of studies, therefore, we also examined the effect of PSTI administration in a variety of *in vitro* models in which the EGFR pathway could be perturbed.

Materials and Methods

Production of Recombinant Human PSTI

PSTI Expression Vector

The hPSTI cDNA sequence was prepared from PANC-1 cells. RNA from these cells was reverse-transcribed and the coding region for PSTI amplified and cloned into the pQE-30 expression vector (Qiagen, Crawley, UK) with an enterokinase cleavage site engineered to allow removal of the polyhistidine tag. The N-terminal histidine-tagged recombinant protein was prepared by expression in transformed IPTG-induced bacteria followed by isolation using nickel-NTA affinity chromatography and enterokinase treatment.

PSTI Protein Validation and Biological Activity

Gel electrophoresis, colloidal blue staining, and Western blotting of the final product showed a single band of correct size, and further analysis using mass spectroscopy confirmed the theoretical correct molecular weight (data not shown). The biological activity of recombinant peptide was verified using a trypsin inhibitor assay with *N*α-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) as substrate (data not shown) and restitution assays as described in the main text.

Ethical Approval of Procedures Involving Animals and Humans

Genetic modification of animals and all animal procedures were approved by the appropriate local and national authorities. All animals had free access to water,

were kept on standard chow diet *ad libitum*, and were sacrificed by cervical dislocation. For collection of dendritic cells (study series 5) involving collection of blood from human volunteers, appropriate ethical approval was obtained with informed consent.

Study Series 1

Production of Transgenic Mice Using the Rat Intestinal Fatty Acid-Binding Protein (rFABPi) Promoter FABP^{-1178 to +28}-hPSTI Fusion Gene

Nucleotides -1178 to +28 of the rat FABPi promoter, inserted into the EcoRI-SmaI sites of pUC13, was kindly donated by Jeff Gordon, Washington University, St. Louis, MO.⁹ This promoter sequence includes the first 28 bases of the nontranslated region of the FABP. Human PSTI cDNA was obtained by reverse transcription-polymerase chain reaction (RT-PCR) from the human pancreas cell line PANC-1, the hPSTI cDNA was inserted in the BamHI-PstI site of pUC13 directly following the promoter. The final 1573-bp cassette was microinjected into more than 200 recently fertilized oocytes (strain C57 × CBA).

Southern Analyses and RNA Blot Hybridization Studies

Positive offspring were detected using standard Southern methodology.¹⁰ To establish the expression of hPSTI RNA in transgenic-positive animals, total RNA was prepared from ileum and jejunum of several heterozygote-positive mice and negative littermates using standard methods and probed using 364-bp hPSTI cDNA.¹⁰ Mouse GAPDH (Ambion, Warrington, UK) was used as the housekeeping gene to normalize for RNA loading.

Western Blot Studies

Using standard techniques,² Western blots were performed and probed using GERP, a mouse anti-human monoclonal antibody (1/1000),² as primary antibody, a secondary rabbit anti-mouse IgG₁ alkaline phosphatase-conjugated antibody (1/1000; ICN, Irvine, CSA) and visualized using BCIP/NBT alkaline phosphatase substrate.

Immunohistochemistry for hPSTI

Expression of hPSTI peptide was confirmed by immunohistochemical staining as previously described, using the streptavidin-peroxidase method.^{2,3} Two antibodies were used in parallel for these immunostaining studies: T4, a rabbit anti-human polyclonal antibody, was used at a final concentration of 1/400,³ and GERP, a mouse anti-human monoclonal antibody, was used at a final concentration of 1/200.² Both antibodies gave identical results.

Influence of Human PSTI Expression on Baseline Parameters and Sensitivity to Indomethacin-Induced Small Intestinal Damage

This study was performed to examine the effect of hPSTI expression on baseline and post-indomethacin morphometry (microdissected villi), proliferation (bromodeoxyuridine, or BrdU, staining) and cell migration up the crypt and villus (using double labeling with BrdU and [³H]thymidine) using methods previously described.¹⁰ Samples of intestine were collected and assessed as described previously.¹⁰

Study Series 2

Effect of PSTI on Colonic Growth and on DSS-Induced Colitis

For these studies, male Sprague Dawley rats (225 to 275 g, *n* = 6 per group; Harlan Olac Ltd., UK) were housed in standard cages (four animals per cage) and fed standard laboratory chow (Special Diet Services, Essex, UK) and tap water *ad libitum*.

Effects of PSTI Administration on Colonic Growth under Baseline Circumstances

All rats received a 1-ml enema and an intraperitoneal injection daily for 9 days. All solutions were made up in normal saline, and enema solutions contained 2% hydroxy propyl methyl cellulose to delay evacuation. Group 1: placebo enema plus placebo intraperitoneally; group 2: placebo enema plus PSTI (150 mg/kg) intraperitoneally; and group 3: PSTI (150 mg/kg) enema plus placebo intraperitoneally. All rats also received BrdU (50 mg/kg i.p.) 2 hours before sacrifice. After sacrifice, the various segments of gut were collected and weighed and histology sections prepared. The number of BrdU-labeled cells were recorded from 20 individual crypts per site per animal in the small intestine and colon. All tissues were scored by a person unaware of the experimental conditions.

Effects of PSTI Administration on DSS-Induced Colitis

Colitis was induced with 4% (w/v) DSS (molecular mass, 36 to 44 kDa; ICN, Aurora, OH) in the drinking water *ad libitum*. Rats not given DSS or PSTI were used to determine baseline values. Mean DSS and food consumption were noted per cage each day. Rats were weighed daily and visually inspected for signs of distress, diarrhea, and rectal bleeding. The disease activity index (DAI, based on Cooper et al¹¹) was assessed every day after the induction of colitis. The DAI combines the scores of weight loss (calculated to day 1 of DSS administration of the cycle), stool consistency, and bleeding divided by 3. At the end of the study, animals were sacrificed and colonic tissue collected and subsequently analyzed for microscopic damage using the scoring system described by Williams and colleagues.¹² The total histolog-

ical colitis score is derived from the sum of the four subscores of 1) inflammation severity, ii) inflammation extent, iii) crypt damage, and iv) percentage of involvement. Tissue was also analyzed for myeloperoxidase (MPO) activity (used as a marker of neutrophilic infiltration) as described previously.¹³

Study A: Effect of PSTI on Prevention of Colonic Injury Caused by DSS

Rats received PSTI (25 and 300 mg/kg) or placebo by intraperitoneal injection for 9 days (days 1 to 9) with DSS in drinking water also being given to some groups from days 3 to 9. All animals were sacrificed on the morning of day 10.

Study B: Effect of PSTI on Recovery of Colonic Injury Caused by DSS

Colitis was induced in all rats (*n* = 30) for 7 days (days 1 to 7). On day 8, the DSS was withdrawn, and animals began to receive either daily placebo or PSTI (300 mg/kg) intraperitoneal injections. Cohorts of animals were then sacrificed on day 8 (baseline damage) and during the recovery period (days 15 and 22).

Study C: Effect of Route of Administration of PSTI on Prevention of Colonic Injury Caused by DSS

To determine the relative efficacy of PSTI when given via the enema or intraperitoneal routes, PSTI was administered at 50 and 150 mg/kg via daily enema or intraperitoneal injection for 9 days with DSS also being given in the drinking water for the last 7 days. To maintain consistency between groups, all animals received some form of daily enema and injection (see below). All solutions were made up in normal saline, and enema solutions contained 2% hydroxy propyl methyl cellulose to delay evacuation. All animals were sacrificed on the morning of day 10. Group 1: placebo enema plus placebo intraperitoneally; group 2: placebo enema plus placebo intraperitoneally plus DSS; group 3: placebo enema plus PSTI (50 mg/kg) intraperitoneally plus DSS; group 4: placebo enema plus PSTI (150 mg/kg) intraperitoneally plus DSS; group 5: PSTI (50 mg/kg) enema plus placebo intraperitoneally plus DSS; group 6: PSTI (150 mg/kg) enema plus placebo intraperitoneally plus DSS.

Study Series 3

Effects of PSTI Using in Vitro Models of Gut Homeostasis

Effects of PSTI on cell migration—methods. Cell migration assays were performed using our previously published methods.¹⁴ In brief, the various cell lines were grown as a monolayer and a standard wound inflicted across the culture. An inverted microscope (Nikon TS100; Tokyo, Japan) and a Nikon Coolpix 800 digital camera with 125-fold magnification were used to obtain serial pho-

tomicrographs (see Figure 5A). Identical regions were examined at each time point by premarking the base of the plates to facilitate alignment. Twenty measurements per field were performed by placing a transparent grid over the photograph and measuring the distance moved from the original wound line.

Preliminary studies. To confirm the biological activity of recombinant hPSTI, HT29 cells were treated with Dulbecco's modified Eagle's medium (DMEM) (negative control), DMEM plus 10% fetal calf serum (positive control), and DMEM plus hPSTI (at 2 $\mu\text{mol/L}$), which we have shown previously to be the optimum concentration to stimulate restitution.² The specificity of the response to hPSTI was analyzed by culturing additional wells in the presence of recombinant hPSTI (2 $\mu\text{mol/L}$) in combination with a PSTI-neutralizing antibody (GERP, 1:100).²

Influence of the EGF receptor on migratory effects of PSTI—blocking the pathway. To investigate the influence of the EGFR in the restitution response stimulated by recombinant hPSTI, cells were treated with hPSTI or EGF (2 $\mu\text{mol/L}$) alone or in combination with either an anti-EGFR antibody (Cetuximab, 100 mg/ml; Bristol-Myers Squibb Co., Princeton, NJ) or the specific EGFR tyrosine kinase inhibitor tyrphostin (AG1478, 100 nmol/L).

Disrupting the receptor. As a further method of examining the relevance of the EGFR to PSTI-mediated migratory effects, a conditionally immortalized colonic EGFR^{-/-} cell line was derived from colonic cells obtained from an EGFR^{-/-} mouse pup¹⁵ carrying the tsA58 transgene, which has a temperature-sensitive mutation of the simian virus 40 large tumor antigen.¹⁶ As a control for the study of these cells, wild-type colonic cells from mice of the same genetic background were immortalized in a similar manner creating a cell line (YAMC) that does express the EGFR.

The EGFR^{-/-} cells and YAMC cells were grown in RPMI 1640 medium containing 5% fetal calf serum, 1 $\mu\text{g/ml}$ insulin, and 5 U/ml mouse interferon- γ at a permissive temperature of 33°C and 5% CO₂. Restitution assays were performed basically as described above for HT29 cells, but these two cell lines required wells to be coated with 10 $\mu\text{g/ml}$ laminin before seeding otherwise the monolayers detached after wounding. After inflicting the standard damage, cells were treated with RPMI 1640 medium (negative control), RPMI 1640 medium plus 5% fetal calf serum (positive control), recombinant hPSTI (2 $\mu\text{mol/L}$), EGF (2 $\mu\text{mol/L}$), or transforming growth factor (TGF)- β_2 (200 ng/ml). For all studies, results are expressed as the mean and SEM of three separate experiments.

Effect of PSTI on Proliferation

We previously demonstrated that PSTI did not increase proliferation in HT29 cells using BrdU incorporation.² To strengthen this previous report, we performed more detailed analyses in which HT29 or RIE6 cells were incubated with various concentrations of PSTI or EGF and effects on proliferation were analyzed using [³H]thymidine,¹⁷ BrdU incorporation,² or absolute cell number.¹⁷ To assess the rate of cells entering DNA synthesis,

[³H]thymidine (2 $\mu\text{Ci/well}$) or BrdU was included 24 hours after the addition of the test factors and cells were left for a further 24 hours. Absolute cell numbers were determined for all wells. For each condition, the effects of the solutions were measured in six separate wells. Cell viability, determined by the ability to exclude 0.2% trypan blue, was greater than 90% in all cell lines.

Effects of PSTI on Vectorial Epithelial Anion Transport

Colony-29 (an adenocarcinoma cell line that shows polarization) was grown to confluence on collagen-coated Millipore filters (0.45 μm ; Millipore Corporation, Billerica, MA) and placed in Ussing chambers as previously described.⁶ Epithelial monolayers were voltage-clamped (with a W-P I DVC1000), and the resulting SCC ($\mu\text{A cm}^{-2}$) was monitored continuously and allowed to stabilize for 10 minutes. Changes in SSC were measured after the addition of test peptide applied to either basolateral or apical reservoirs. The various compounds tested comprised PSTI added either apically or basolaterally (1 to 10 $\mu\text{mol/L}$) or EGF added basolaterally at a maximal concentration of 10 nmol/L. In each experiment, the muscarinic agonist carbachol (10 $\mu\text{mol/L}$) was the final agonist added basolaterally as an internal control to confirm continued sensitivity of the G_q-protein-coupled epithelial transduction pathway.

As for the restitution studies, to investigate the role of EGFR in the observed responses, some epithelial monolayers were pretreated with the anti-EGFR antibody (cetuximab, 100 mg/ml) or saline vehicle overnight. After pretreatment epithelial monolayers were treated with either hPSTI (3 $\mu\text{mol/L}$) or EGF (10 nmol/L) basolaterally, and changes in SSC were recorded.

Study Series 4

Effects of PSTI on EGF Receptor Phosphorylation

Because we had shown HT29 cells mounted a biological response to PSTI, these cells were used to investigate the effect of PSTI on EGFR phosphorylation using well-established methods.¹⁸ In brief, HT29 cells were grown to confluence and then serum-starved for 16 hours. Cells were treated with either EGF or hPSTI (15 nmol/L) for 1, 3, 10, 20, 40, or 60 minutes at 37°C and washed with ice-cold phosphate-buffered saline (PBS), and clarified cell lysates were prepared.

Lysates were incubated with anti-EGFR antibody (Ab1; Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C for 4 hours. The resulting immune complexes were collected on protein G-Sepharose beads that were added 2 hours after the antibody. Beads were isolated by centrifugation and washed three times in lysis buffer before extraction with sample buffer and fractionation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were electrotransferred to polyvinylidene difluoride membranes, Western-blotted with anti-phosphotyrosine antibody PY20 (61000; BD Biosciences, Lexington, KY), de-

tested with horseradish peroxidase-coupled secondary antibody (Amersham, Little Chalfont, UK), visualized by enhanced chemiluminescence (Amersham), and quantitated by densitometry.

Study Series 5

Effect of PSTI on Dendritic Cell Activation

Monocyte-derived dendritic cells were developed by culture of CD14⁺ selected (Miltenyi Biotech, Guildford, UK) peripheral blood monocytes (from healthy volunteers) for 6 days in the presence of 40 ng/ml GM-CSF and 40 ng/ml interleukin (IL)-4 (First Link Ltd., Birmingham, UK) using X-VIVO 15 serum-free medium (BioWhittaker, Walkersville, MD) supplemented with 10% AB serum. Dendritic cells were plated at 10⁶ cells per well in a 24-well tissue culture plate and treated with a range of PSTI concentrations (0.1 to 10 ng/ml) or equal concentrations of a similarly produced recombinant control peptide (trefoil factor family 1, TFF1).¹⁹ Activation was subsequently induced by the addition of 10 ng/ml gel-purified lipopolysaccharide (Sigma, St. Louis, MO), and cells were then incubated for 24 hours before analyses.

Cytokine Measurement

Twenty-four hours after activation, supernatants were removed and spun to remove cells and debris before being analyzed for cytokine content by capture enzyme-linked immunosorbent assay. Tumor necrosis factor (TNF)- α and IL-12p70 were detected using antibody pairs (Pharmingen, BD Biosciences). Briefly, immunoabsorbent plates (Nunc, Loughborough, UK) were coated with 1 μ g/ml of capture antibody and incubated for 2 hours at 37°C. Plates were washed in PBS/0.1% Tween 20 before incubating with samples and standards (Peprotech, London, UK) overnight. Plates were next incubated with the appropriate biotinylated detecting antibody for 2 hours at 37°C. Finally, results were visualized by incubation for 1 hour with 0.1 μ g/ml avidin-horseradish peroxidase (Biosource Int., Camarillo, CA) followed by addition of substrate solution, TMB (Zymed, South San Francisco, CA). Color development was stopped by the addition of 0.5 mol/L H₂SO₄, and plates were read for optical density at 450 nm (enzyme-linked immunosorbent assay plate reader; Titertek Multiskan; Huntsville, AB).

Statistical Analyses

Data from all experiments are expressed as mean $\pm$ SEM. For the DSS-induced colitis studies, the samples were randomly coded and assessed by two blinded individuals and then analyzed using one-way analysis of variance. For the study on baseline parameters and sensitivity to indomethacin, data were analyzed by two-way analysis of variance using presence of transgene and administration of indomethacin as factors. Where a significant effect ($P < 0.05$) was found, individual t -tests

were performed, based on the group means and residual obtained from the analysis of variance, a method equivalent to repeated measures analyses. Similar two-way analysis of variance analyses were performed for the dendritic cell studies using concentration and treatment as factors. For the voltage-clamp studies, basal and stimulated SSC levels were compared between the treated and control monolayer groups using unpaired two-tailed Student's t -testing ($P < 0.05$ was considered significant). Subsequent analyses were performed as for the indomethacin study.

Results

Study Series 1

Rat Intestinal Fatty Acid-Binding Protein (rFABP) Promoter FABP γ ^{-1178 to +28}-hPSTI Fusion Gene Transgenic Mice

Of the 90 microinjected live-born mice, Southern blot analysis of tailsnip DNA identified two founders that had incorporated the construct (Figure 1A). For all subsequent studies, heterozygotes were used because this allowed negative littermates to act as controls. As expected using this promoter,⁹ Northern (Figure 1B), Western (Figure 1C), and immunostaining (Figure 1D) showed that expression of human PSTI was restricted to the enterocytes of the jejunal villi. Morphometry and morphology of these villi showed no differences between heterozygotes and negative littermates under baseline circumstances (Figure 2, A and B). As expected, control littermates given indomethacin showed marked small intestinal injury with villus shortening and bulbous expansion (Figure 2C) whereas in transgenic animals the degree of injury appeared much less marked in the (PSTI-expressing) jejunal region (Figure 2D). To quantitate these differences, morphometric analyses were performed, showing that within the jejunum the degree of villus shortening in transgenic animals was much less (approximately one third) of that seen in control indomethacin-treated animals (Figure 3A). Importantly, the degree of injury sustained in response to indomethacin treatment was similar in wild-type control and transgenic mice in the (non-PSTI-expressing) ileal region (Figure 3B), showing that this effect was specific to PSTI-expressing areas and was not attributable to some other nonspecific general effect.

These animals were then studied in further detail to examine some of the possible mechanisms of action of the protective effect of regional PSTI overexpression. In the current study and as shown by us previously, using a different transgenic growth factor model,¹⁰ administration of indomethacin to control mice resulted in an increased migration of enterocytes up the villi and an increase in proliferation (Figure 3, C and D). However, although PSTI expression reduced the degree of injury seen within the jejunum, this was not reflected by any differences between transgenic and control animals in terms of amount of increase in migration of enterocytes up the villi (Figure

Figure 1. Generation of rFABPi-hPSTI mice. **A:** The construct cassette includes nucleotides –1178 to +28 of the rFABPi promoter and the first 28 bases of the nontranslated region of the FABP. This was linked to a 364-bp sequence of PSTI cDNA derived by RT-PCR from RNA from the human pancreatic cell line PANC1. **B:** Northern hybridization using a specific 364-bp hPSTI cDNA probe showed expression of hPSTI RNA only within the jejunum (J) of transgenic animals but not in the ileum (I). No hPSTI RNA expression was seen in tissue (J or I) from the wild-type littermates. **C:** Western hybridization probed using GERP, a mouse anti-human monoclonal antibody (1/1000) as primary antibody. Expression of hPSTI peptide can be seen in the jejunum (J) of transgenic animals but not in the ileum (I). As expected, no hPSTI was seen in tissue (J or I) from the wild-type littermates. **D:** Immunohistochemical staining for hPSTI (brown) showed no expression in the villi of the jejunum of wild-type mice [longitudinal (**a**) and transverse (**c**) sections]. In contrast, the jejunal villi of transgenic animals showed strong expression within the enterocytes [longitudinal (**b**) and transverse (**d**) sections]. No hPSTI immunoreactivity was seen in the villi of the ileum of transgenic or control mice (data not shown).

3C) or differences in proliferation (Figure 3D) caused by indomethacin (effect of indomethacin $P < 0.05$ on analysis of variance in both cell position and proliferation studies but no effect or interaction with the presence of transgenic as factor).

Study Series 2

Effect of PSTI on Colonic Growth and on DSS-Induced Colitis

Effects of PSTI administration on colonic growth under baseline circumstances. PSTI administration given via

Figure 2. Effect of indomethacin on small intestinal injury. Under baseline circumstances, jejunal villi of control (**A**) and PSTI-positive animals (**B**) were morphologically identical, showing normal long tapering villi. **C:** Administration of indomethacin to control animals caused a marked shortening of jejunal villus length with some bulbous expansion. **D:** In contrast, the administration of indomethacin to transgenic animals resulted in much less morphological damage to the (PSTI-expressing) villi within the jejunum. The degree of morphological injury seen in response to indomethacin in the villi of the (non-PSTI-expressing) ileal region was, however, similar in both the transgenic and control animals (not shown, but see Figure 3 for morphometry).

enema or intraperitoneal injection did not affect wet weight of the large or small intestine, nor the amount of BrdU-labeled cells/crypt as compared with controls (data not shown).

Effects of PSTI on DSS-induced colitis—study A—effect of PSTI on prevention of colonic injury caused by DSS. As expected, DSS caused a marked increase in edema, areas of surface denudation, and an influx of mixed inflammatory infiltrate. Administration of PSTI caused a dose-dependent reduction in histological colitis scoring (Figure 4A), including a marked reduction in the amount of the mixed inflammatory infiltrate as demonstrated by histology and MPO levels (data not shown). Similar dose response results were seen using the DAI assessment (data not shown).

Study B—effect of PSTI on recovery of colonic injury caused by DSS. Animals that had received DSS alone for 7 days had a histological ulcer score of 27.5 ± 0.8 (Figure 4B). Animals that had received PSTI (300 mg/kg i.p. daily) showed a faster rate of recovery when assessed using histological scoring ($P < 0.01$ for both time points studied). Typical histological appearances at the end of 7 days of DSS (Figure 4Cii) and the effect of PSTI administration on recovery are shown (Figure 4Civ). Similar improvements in the rate of recovery, attributable to PSTI, were seen when damage was assessed using DAI or MPO measurements (data not shown).

Study C—effect of route of administration of PSTI on prevention of colonic injury caused by DSS. For both doses of

Figure 3. Effects of indomethacin on villus height and enterocyte migration in the small intestine. **A:** Administration of indomethacin caused a 40% reduction of villus height in the jejunum of wild-type (WT) animals, whereas transgenic (Trans) animals overexpressing hPSTI within the jejunum had a much-reduced sensitivity. **B:** In contrast to **A**, there was no difference in sensitivity to indomethacin between wild-type and transgenic animals in the (non-PSTI-expressing) ileal region. **C:** Double labeling with BrdU and [³H]thymidine allowed rate of migration of cells up the villi to be determined. Indomethacin treatment caused a similar increase in rate of migration of cells up the jejunal villi in wild-type and transgenic animals. **D:** [³H]thymidine crypt labeling was used to determine changes in proliferation within the jejunum. Indomethacin treatment caused an increase in number of labeled cells, but there were no differences between wild-type and transgenic animals. Data shown as mean $\pm$ SEM. ** $P < 0.01$ comparing the effect of indomethacin within the same genotype.

PSTI tested (50 and 150 mg/kg), administration of PSTI by the enema or intraperitoneal route gave similar results in terms of amount of reduction of injury when assessed by DAI, histology scoring, or MPO assay. For example, histological colitis score at 150 mg/kg PSTI i.p. was 14.3 ± 0.9 versus 14 ± 0.7 when the same dose given by enema. The same animals assessed by MPO levels gave results of 4.18 ± 0.15 versus 3.64 ± 0.32 mU/mg protein. Other data not shown.

Study Series 3

Effects of PSTI Using in Vitro Models of Gut Homeostasis

Effects of PSTI on cell migration. For both peptides, dose-response curves for PSTI or EGF showed maximal promi-

Figure 4. Effects of PSTI on DSS-induced colitis. **A:** PSTI colitis prevention study. DSS was administered in drinking water for 7 days. PSTI was administered once a day via intraperitoneal injection at either 25 or 300 mg/kg, starting 2 days before DSS. Data shown as mean $\pm$ SEM. ** $P < 0.001$, compared with negative control animals receiving DSS but no PSTI. **B:** PSTI colitis recovery study. All animals received DSS in drinking water for 7 days. A cohort was sacrificed on day 8 to assess baseline degree of damage (Δ). Further cohorts of animals who had also received DSS for 7 days were then given a daily intraperitoneal injection of either placebo ($\square$) or PSTI (300 mg/kg, $\circ$) and sacrificed 7 and 14 days later. Data shown as mean $\pm$ SEM. ** $P < 0.001$, compared with baseline control animals; + + $P < 0.001$, comparing animals given PSTI or placebo for the same time period. **C:** PSTI colitis recovery study (histology). **i:** Distal colon (80 to 100%) from a no-DSS-treated animal showing normal architecture. **ii:** DSS caused a marked increase in edema, areas of surface denudation, and an influx of mixed inflammatory infiltrate with loss of cryptal architecture. **iii:** Fourteen days later, animals that had received placebo injection showed signs of recovery although a moderate inflammatory infiltration was still present. **iv:** The degree of recovery and reduction in inflammatory infiltrate was much greater in animals that had received PSTI with a return to nearly normal appearance. Original magnifications, $\times 10$.

gratory effects when added at 2 μ mol/L (dose response data not shown). The addition of an antibody raised against PSTI prevented the promigratory activity of PSTI

Figure 5. Effect of the addition of PSTI on cell migration. **A:** Confluent monolayers of HT29 cells were wounded and had serial photographs taken to follow movement of the leading edge of the wound. **i** and **ii:** Typical appearances of wounded monolayers at time 0. Twenty-four hours later, cells in the well that had PSTI ($2 \mu\text{mol/L}$) added to the medium (**iv**) showed a much greater rate of movement than cells incubated in medium alone (**iii**). **B:** Graph shows distance migrated under various conditions at the 24-hour time point after wounding. The addition of EGF or PSTI (both $2 \mu\text{mol/L}$) caused a two- to threefold increase in movement compared with negative control (DMEM alone). Co-incubation with an EGF receptor-binding antibody ($100 \mu\text{g/ml}$) or a specific EGF receptor tyrosine kinase inhibitor (Tyrphostin AG1478, 1 nmol/L) markedly truncated the promigratory effects of both EGF and PSTI. Data shown as mean $\pm$ SEM. ** $P < 0.01$, compared with relevant peptide alone. **C:** Full time course of wounded monolayers of immortalized mouse colonic cells in which the EGF receptor has been genetically inactivated. These cells do not respond to the addition of EGF (□) or PSTI (●), having a rate of migration virtually identical to cells grown in medium alone (△). These cells were, however, still capable of responding to factors that stimulate migration through other pathways, such as TGF- β_2 (200 ng/ml , ○).

but not EGF and vice versa when an anti-EGF antibody was used (data not shown). In contrast, the addition of the anti-EGFR-binding antibody Cetuximab (Figure 5B) or the EGFR tyrosine kinase inhibitor Tyrphostin (AG1478, Figure 5B) markedly truncated the promigratory activity of both peptides.

Corresponding results were found using the conditionally immortal colonic EGFR^{-/-} cell line. The addition of either PSTI or EGF did not increase the rate of migration of these cells, whereas addition of TGF- β_2 , a positive control acting through a different receptor, was able to stimulate migration (Figure 5C). Similarly treated immortalized YAMC colonic cells, obtained from the same source but where the EGFR was still present, were stimulated to migrate in response to addition of EGF or PSTI (data not shown).

Effect of PSTI on proliferation. Proliferation assays using HT29 or RIE6 cells using [^3H]thymidine, BrdU incorpora-

tion, or absolute cell number showed no proliferative response to the addition of PSTI. Proliferation was, however, increased in both cell lines in response to the addition of EGF. In HT29 cells [^3H]thymidine uptake values in wells treated with PSTI were similar to values seen in wells incubated in DMEM alone (negative control, $3859 \pm 1507 \text{ cpm}$ versus $3863 \pm 1786 \text{ cpm}$, respectively). In contrast, addition of EGF resulted in a significant increase in [^3H] thymidine incorporation ($9111 \pm 2692 \text{ cpm}$). All assays in both cell lines gave similar results (data not shown).

Actions of PSTI on epithelial ion transport. The addition of PSTI to the basolateral, but not the apical, surface of Colony-29 epithelial monolayers resulted in a concentration-dependent increase in SSC (Figure 6A). Although there were obvious similarities, the profile and time course of basolateral-induced changes in SSC in response to PSTI and EGF differed in two important aspects (Figure 6B, profiles and graphs shown in left col-

Figure 6. Effect of addition of PSTI or EGF on SCC in histologically polarized Colony-29 epithelia. Colony-29 cells were grown on membranes as a polarized monolayer and placed in Ussing chambers. Changes in vectorial ion transport were followed by measuring alterations in SCC. Data shown as mean $\pm$ SEM. **A:** The addition of PSTI to the basolateral (●), but not apical (○), surfaces of Colony-29 cells caused a dose-dependent increase in SCC. **B:** Comparison of the responses to basolaterally administered PSTI or EGF showed that, although both peptides caused an increase in SCC, the shape and duration varied between the two peptides. The increase in SCC caused by PSTI had a slower onset but resulted in a more prolonged effect compared with EGF (compare SCC profiles of PSTI and EGF in left column). **C:** The effects of PSTI or EGF on SCC could be abrogated by pretreating monolayers with an EGFR-neutralizing antibody overnight (SCC profiles of right columns). The results of quantitation of these effects at three standard time points (at 5, 10, and 15 minutes after the addition of peptides) are shown underneath each representative trace.

umn). EGF addition induced a rapid onset in electrogenic responses that was relatively short lived, returning to baseline values by ~15 minutes. In contrast, when PSTI was added, although there was usually an initial delay before the increase in SSC occurred, once this did happen, the effect was much more prolonged than that caused by the addition of EGF (lasting for at least 30 minutes). Pretreatment with the anti-EGFR antibody markedly truncated the increases in SSC seen in response to either EGF or PSTI (Figure 6C, profiles and graphs of right column). The addition of PSTI, EGF, or anti-EGFR antibody pretreatment did not influence the increase in SSC in response to addition of the positive control carbachol (data not shown).

Study Series 4

Effects of PSTI on EGF Receptor Phosphorylation

The addition of EGF caused a rapid and intense phosphorylation of the EGFR, but this effect began to diminish within 3 minutes (Figure 7). In contrast, the addition of PSTI resulted in a more gradual, less intense, induction of the EGFR (Figure 7).

Study Series 5

Effect of PSTI on Dendritic Cell Activation

Analyses of the expression of proinflammatory cytokines in the supernatant of lipopolysaccharide-activated

Figure 7. Phosphorylation of the EGFR in response to the addition of PSTI or EGF to HT29 cells. HT29 cultures were serum-starved overnight and then stimulated with either EGF (15 nmol/L) or PSTI (15 nmol/L) for the times indicated. Cells were lysed and fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by Western blotting. **A:** Filters treated with a specific anti-phosphotyrosine EGFR antibody (PY99) showed that both PSTI and EGF caused phosphorylation of the EGFR receptor. The addition of EGF caused a rapid and intense phosphorylation of the EGFR, but this effect began to diminish within 10 minutes. The addition of PSTI resulted in a more gradual induction of phosphorylation of the EGFR compared with EGF (row 1). Equal loading of the filters was confirmed using the pan-specific EGFR antibody (row 2). **B:** Densitometric analyses of filters showing density of the bands. After exposure of cells to EGF (●) or PSTI (○) for various times.

Figure 8. Effect of PSTI on proinflammatory cytokines. Dendritic cells were derived from peripheral blood monocytes of healthy volunteers and treated with a range of either PSTI (○) or recombinant control peptide (□, negative control). Dendritic cells were activated by the addition of lipopolysaccharide and the expression of TNF-α (**A**) and IL-12 (**B**) measured. Unstimulated dendritic cells did not produce measurable amounts of either cytokine (●). Data shown as mean ± SEM. **P < 0.001, compared with control recombinant peptide.

dendritic cells showed that, in the absence of PSTI or control protein, baseline TNF-α concentration was 22.5 + 1.8 ng/ml, and IL-12 was 18.2 + 1.3 pg/ml. PSTI caused a dose-dependent down-regulation of both cytokines whereas the control protein had no effect (Figure 8).

Discussion

Using *in vitro* and *in vivo* studies, we examined the effect of PSTI on various aspects of gut mucosal integrity and repair. Increasing PSTI levels by inducing chronic overexpression or by exogenous administration stabilized the gut against the noxious agents indomethacin and DSS. PSTI stimulated cell migration and vectorial ion transport but did not increase proliferation. PSTI also inhibited cyto-

kine release from lipopolysaccharide-stimulated dendritic cells. At least some of these actions seem dependent on the presence of a functional EGF receptor.

PSTI is a potent serine protease inhibitor initially identified in the pancreas where it helps prevent premature activation of pancreatic proteases.¹ However, its wider distribution, which includes the epithelium of the normal breast, urothelium, and in the stomach and colon^{2,3,4,20} and damaged regions of the small intestine,² suggests that it may play additional roles. These current studies, therefore, examined its potential actions in influencing gut defense and repair.

The amino acid sequence of PSTI across species is highly conserved with mouse and human PSTI sharing ~61% sequence homology. We showed our recombinant PSTI, like the purified form from human pancreas used previously by us,² could stimulate migration of human colonic HT29 cells and then showed it also stimulated migration of mouse colonic YAMC cells demonstrating cross-species activity. Having shown human PSTI can influence mouse intestinal cells, we considered it appropriate to examine the effect of overexpressing human PSTI within a mouse transgenic model.

To reproduce the situation of small bowel regional up-regulation of PSTI, as seen in various clinical scenarios of small intestinal injury, we produced transgenic mice with regional overexpression of PSTI within the jejunum. These animals showed an increased resistance to indomethacin-induced injury in the jejunal area that overexpressed PSTI. Importantly there was no increased resistance in the non-PSTI-expressing ileal region of the same animals, confirming the increased resistance was directly attributable to the presence of the human PSTI. The increased resistance appeared not to be due to influencing the rate of proliferation or migration up the villi, despite the *in vitro* studies showing that PSTI possesses promigratory activity. These results show the need for caution in extrapolating from the *in vitro* to the *in vivo* situation.

Indomethacin causes damage to the gastrointestinal tract by several mechanisms including reduction of mucosal prostaglandin levels, reduction of mucosal blood flow, stimulating neutrophil activation, and possibly also stimulating apoptosis.²¹ Several of these factors may have been influenced by PSTI overexpression, and further work is required to understand this protective effect. This might include the development of (intestinal) site-specific knockout or conditional knockout animals because the production of (global) PSTI^{-/-} knockout mice has recently been shown to result in a lethal phenotype within a few days of birth with virtually complete loss of pancreatic tissue.²²

A second series of *in vivo* studies examined the effect of exogenously administered PSTI on gut function, with particular focus on the colon under baseline circumstances and in its resistance to inflammation. Several models of colitis are available including genetically modified mice with aberrant immune responses (spontaneous models) or by administering noxious compounds rectally or orally (inducible models). We used the DSS rat model of colitis because it is a reproducible model extensively

used by our group and other groups. The exact mechanism by which injury is caused is unclear but is probably dependent on innate immune mechanisms rather than acquired immunity because it also occurs in severe combined immunodeficiency (SCID) mice.²³ In addition, alteration in both Th₁ and Th₂ cytokine profiles have been observed when colitis has been induced using DSS, although the Th₁ response predominates.²⁴

PSTI administration reduced the development of colitis when administered at the same time as the DSS and stimulated repair when given after the DSS had been discontinued. Intraperitoneal and enema preparations were both approximately equally efficacious in reducing the degree of colonic injury and its associated inflammatory infiltrate. PSTI may have mediated these effects via several mechanisms including direct actions on the epithelial cells, immune cells, and on the overlying mucus layer. In support of this mucus preservation role, we previously showed PSTI can stabilize gastric mucus from pancreatic proteases.²⁰ Colonic epithelial contact times when PSTI was administered by the intraperitoneal and enema routes are likely to have been different because the circulating half life of PSTI is ~8 minutes,²⁵ whereas previous studies on stability of PSTI show it to be stable in a neutral environment (as in the enema preparation),³ giving a longer period of exposure although retention times of the enema vary between animals. However, as systemic and luminal PSTI administration were approximately similarly effective in reducing the amount of injury sustained, it is likely that the direct cytoprotective actions of PSTI, such as the promigratory activity and/or immune modulation, are probably at least as important as any mucus preservation effect of PSTI.

The detailed mechanisms of DSS-induced colitis are complex but involve an inflammatory cascade that includes TNF- α , interferon- γ , and IL-6.²⁶ Histological assessment of our *in vivo* studies showed that neutrophil and lymphocyte recruitment to the colon was reduced by administration of PSTI, and this result was supported by parallel reductions in colonic MPO levels. To examine the effect of PSTI on immune function further, we performed *in vitro* studies examining cytokine release from dendritic cells activated by administering bacterial lipopolysaccharide. These results showed dose-dependent reductions in release of the proinflammatory cytokines TNF- α and IL-12 in response to PSTI. Immune effects of PSTI have not been explored in any detail previously, although, given the interaction of PSTI with the EGFR, it is of note that a study examining the effect of administering a murine anti-EGFR antibody (ie, potentially the reverse situation) resulted in immune recruitment and activation.²⁷ Further studies examining immune modulatory functions of PSTI as well as other potential mechanisms of action, such as changes in apoptosis, seem merited.

Patients suffering from ulcerative colitis have a long-term reduction in the mucosal PSTI levels in previously affected areas.⁴ Taken together, our current series of studies suggests that these reduced colonic mucosal PSTI levels may have clinical relevance, leading to increased susceptibility to further episodes of injury. Caution needs to be shown, however, in extrapolating from

the animal to the human situation, particularly because the amounts of PSTI used in the current animal studies are probably pharmacological rather than physiological.

The gut epithelium plays several important roles including acting as a barrier and also being involved in fluid and electrolyte homeostasis. To begin to examine this latter component, an additional series of studies examined the effect of PSTI on vectorial ion transport by following changes in SCC using the well-validated method of a polarized cell monolayer (human colonic Colony-29 cells) voltage clamped in an Ussing chamber. These studies showed that PSTI increased vectorial ion transport when added to basolateral, but not apical, surfaces and that this effect was sustained for a considerable period of time, suggesting changes in its mucosal concentration may have relevance to luminal fluid secretion and mucus fluidity.

The effect of PSTI on vectorial ion transport was markedly abrogated by preincubating with an EGF receptor-neutralizing antibody, suggesting that the EGFR was involved in this process. Similar results were seen when following the promigratory properties of PSTI using the *in vitro* model of wound repair. Perturbation of the EGFR using an EGFR-neutralizing antibody, the EGFR tyrosine kinase inhibitor tyrphostin, or using immortalized cells that had the EGF receptor inactivated (EGFR^{-/-} cells) resulted in the cells failing to migrate in response to PSTI.

Although the EGFR seems involved in many of the PSTI-mediated effects, the relationship between PSTI and the EGFR is probably not that of a direct receptor ligand. PSTI and EGF share a similar three Cys-Cys disulfide bridge structure and have moderate sequence homology (50%). However, most radiolabeled EGF receptor PSTI/EGF displacement studies have reported PSTI is not a direct EGFR ligand.^{7,8} In addition, although PSTI has been reported to stimulate proliferation in a few cell lines, such as the pancreatic cell line AR4-2J,⁸ most studies found a divergence of results in the proliferative activity of EGF and PSTI, with PSTI having little or no proliferative activity when added to various cell lines. Our current studies showed PSTI did not stimulate proliferation of HT29 cells, despite inducing phosphorylation of the EGFR. It, therefore, seems more likely that PSTI is inducing cross phosphorylation of the EGFR and/or influencing its downstream pathways. Further work is required to explore this area in more detail.

In the normal nondamaged human gut²⁸ and in polarized monolayers of Colony-29 cells, the EGF receptor is restricted to basolateral rather than apical membranes. Ussing chamber studies showed a response to basolateral but not apical administration of PSTI, supporting a potential role for a basolateral receptor of some sort (such as the EGFR). It is important to note, however, that at sites of injury, such as occurs in colitis, EGF receptors become exposed to the luminal environment and are potentially available for interaction by direct or indirect EGFR ligands.²⁹

In conclusion, we have shown that PSTI can stabilize both the small and the large intestinal mucosa against injury as well as stimulating the early restitutive phase of repair. In addition to providing some insight into its patho-

physiological role, these studies suggest that additional work examining its mechanisms of action and, in due course, potentially performing clinical trials of PSTI-like agonists could be of interest.

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