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Short Communication

Distribution and Expression of Pancreatic Secretory Trypsin Inhibitor and Its Possible Role in Epithelial Restitution

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Pancreatic secretory trypsin inhibitor (PSTI) is a potent serine protease inhibitor that prevents excessive digestion of the gastrointestinal mucus but may also directly affect epithelial function. We therefore examined the distribution of PSTI in the human adult and fetus using immunohistochemistry and* in situ *hybridization and examined its effects on cell proliferation and migration* in vitro. *PSTI peptide and mRNA were found in the exocrine pancreas, mucus-producing cells of the normal gastrointestinal tract, acinar component of the normal breast, and surface epithelial cells at the edge of benign gastric ulcers. Peptide, but not message, was identified in the renal proximal tubule, probably reflecting reabsorption of filtered peptide. Purified human PSTI did not affect proliferation of the human colonic cell line HT-29 but caused a threefold increase in the rate of migration in an* in vitro *wounding model of restitution. This effect was inhibited by co-administering a PSTI-neutralizing antibody, a transforming growth factor-β-neutralizing antibody, or an epidermal growth factor receptor-blocking antibody. As PSTI is widely distributed in several human organ systems and stimulates cell migration* in vitro, *we conclude that PSTI is likely to have additional roles to that of preserving the gastrointestinal mucous layer from excessive digestion. (Am J Pathol 1996, 148:715–722)

Pancreatic secretory trypsin inhibitor (PSTI) is a 56-amino-acid peptide that potently inhibits the proteolytic activity of trypsin and other serine proteases. It was originally isolated from the pancreas,[1] where its sole function was thought to be to prevent premature activation of pancreatic proteases. However, it has subsequently been identified in the mucus-producing cells throughout the gastrointestinal tract and is the only protease inhibitor known to be secreted into the intestinal lumen.[2] PSTI has also been shown to stimulate proliferation of human fibroblasts,[3] human endothelial cells,[3] and the rat pancreatic cancer cell line AR4–2J.[4] These findings suggest that PSTI may be an important regulatory peptide, involved in controlling cellular function, in addition to acting as a protease inhibitor to prevent excessive digestion of gastrointestinal mucus. To gain additional insight into its function *in vivo*, we examined the distribution of PSTI peptide and mRNA in the normal human fetus and adult. In addition, as we have recently shown that the mucosal concentrations of PSTI are reduced in the stomachs of patients with benign gastric ulcers,[5] we performed detailed analyses of the distribution of gastric PSTI expression at sites distant from the ulcer and at the ulcer edge. These studies showed that, although the expression of PSTI

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was generally reduced in the surface cells of the gastric epithelium, there was strong expression of PSTI in the cells involved in migration and regeneration at the ulcer edge. We therefore went on to examine the effect of PSTI on cell migration and proliferation using an *in vitro* model of cell wounding.

Materials and Methods

Tissue Samples

A series of formalin-fixed, paraffin-embedded fetal and adult tissues were used for both the immunohistochemistry and the *in situ* hybridization studies. Frozen tissues for use in Northern and Western analyses were obtained from the Histopathology Unit (Imperial Cancer Research Fund) tissue bank and satisfied statutory and local ethical considerations.

Purification of PSTI Peptide Used for the Production of the Monoclonal Antibody and for the in Vitro Studies

PSTI was purified from pancreatic juice obtained from postoperative pancreatic drains using our previously published protocol.[6] Purity of samples was confirmed by high pressure liquid chromatography followed by radioimmunoassay using antibody T4.[6]

Immunohistochemistry

Sections of tissue were analyzed for PSTI-like immunoreactive cells using a streptavidin/biotin-peroxidase (Dako, Glostrup, Denmark) technique as previously described.[7] Two antibodies were used in parallel, a rabbit anti-human polyclonal antibody, T4, used at a final concentration of 1/1000, as previously described,[6] and a mouse anti-human PSTI monoclonal antibody, GERP, which had been raised against the full-length purified human PSTI, used at a final concentration of 1/200. Neither antibody cross-reacted with epidermal growth factor (EGF) or transforming growth factor (TGF)-α. The specificity of the PSTI staining was checked in parallel sections by using antibody that had been preincubated with excess purified PSTI.

In Situ Hybridization

The distribution of mRNAs encoding PSTI was established by *in situ* hybridization using a ^{35}S-labeled antisense riboprobe. This probe consisted of a 159-bp sequence corresponding to nucleotides 124 to 283 of the coding region of PSTI,[8] which was produced by reverse transcription polymerase chain reaction using RNA isolated from a pancreatic tumor cell line, PANC-I. Five-micron sections of formalin-fixed material were mounted on 3-aminopropyl-triethoxysilane-treated glass slides and treated as described by Senior.[9] Slides were exposed for 6 days at 4°C before development of silver grains and were counterstained with toluidine blue to visualize histology.

Northern Blot Analyses

Total RNA was prepared using standard methods,[10] and 20-μg samples of total RNA were electrophoresed through a 1% (v/v) formaldehyde/agarose gel before transfer to nylon filters (Hybond-N, Amersham International, Amersham, UK). Northern analyses were performed using a ^{32}P-labeled probe (produced using a nick-translation kit, Gibco BRL, Paisley, UK) consisting of an identical DNA sequence to that used for the *in situ* hybridization studies. Filters were sequentially washed in 2X, 1X, and 0.5X standard saline citrate containing 0.1% sodium dodecyl sulfate at 55°C for 30 minutes.

Western Blot Analyses

Tissues were dispersed by sonication in 1X Laemmli suspension buffer and heat denatured, and 10-μg protein samples were run on pre-cast 18% (w/v) Tris-glycine polyacrylamide gels (Novex, San Diego, CA). After electrophoresis, proteins were transferred electrophoretically onto nitrocellulose filters and fixed using 0.2% (w/v) gluteraldehyde. Blots were incubated in 5% goat serum to reduce nonspecific binding and then incubated successively in the primary PSTI antibody (T4 or GERP) for 2 hours at room temperature, followed by an alkaline phosphatase-conjugated goat anti-mouse or anti-rabbit immunoglobulin (Sigma Chemical Co., St. Louis, MO) for 1 hour. The enzymatic reaction was visualized using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate dissolved in phosphate-buffered saline (PBS).

Effect of PSTI on Cell Migration and Proliferation

To examine the effect of PSTI on epithelial restitution, we used our previously published modification[11] of the *in vitro* wounding assay described by Sato and Rifkin.[12] Confluent layers of the human colorectal cell line HT-29 were grown in Petri dishes in Dulbecco's minimal essential medium containing 10% fetal calf serum at 37°C

in a humidified atmosphere of 5% CO_2 in air. Wounds were made by scraping a disposable pipette tip across the dishes. Cells were washed immediately with serum-free medium to remove released soluble factors and subsequently cultured for an additional 24 hours in fresh serum-free medium in the presence or absence of purified PSTI (0, 1, 5, 10, or 25 μg/ml) to obtain a dose-response curve. Cells were also incubated with 10 μg/ml PSTI in combination with mitomycin C (0.5 μg/ml; an inhibitor of cell proliferation), an EGF receptor (EGF-R)-blocking antibody (100 μg/ml; kindly donated by W. Gullick[13]), or a TGF-β-neutralizing antibody (100 μg/ml; British-Biotechnology, Avingdon, UK). In addition, to examine whether migration was dependent on the antiprotease activity of PSTI, cells were cultured in the presence of soya bean trypsin inhibitor (0, 10, 50, or 100 μg/ml, Sigma Ltd., Poole, UK), which has previously been shown to have a similar inhibitor profile to PSTI.

We also examined the proliferative effect of PSTI on HT-29 cells using 5-bromo-2′-deoxyuridine (BrdU) labeling as a measure of proliferation.[14] Briefly, nonconfluent HT-29 cells were grown in Dulbecco's minimal essential medium containing 10% fetal calf serum for 24 hours, washed three times in serum-free medium, and incubated in serum-free medium containing various concentrations of PSTI for 24 and 48 hours, and 1 hour before the final time point, BrdU (1 nmol/L) was added to all wells. At 1 hour later, cells were fixed in ice-cold 5% methanol/5% acetic acid (v/v), washed three times in ice-cold PBS, air dried, and stained using a commercially available BrdU detection kit (Boehringer Mannheim, Mannheim, Germany). The percentage of BrdU-immunoreactive cells were then assessed microscopically. All studies were performed in quadruplicate.

Statistical Analyses

Studies of the effect of PSTI on cell migration were analyzed by analysis of variance using treatment and dose as factors. When a significant effect was seen ($P < 0.05$), individual comparisons were performed based on the residuals and degrees of freedom obtained from the analysis of variance.

Results

Distribution of PSTI Expression in Normal Tissue

The distribution of PSTI-like immunoreactivity was identical when using the monoclonal antibody GERP or the polyclonal antibody T4. PSTI-like immunoreactivity was always blocked by preincubating the antibodies in purified PSTI but not by adding excess EGF or TGF-α.

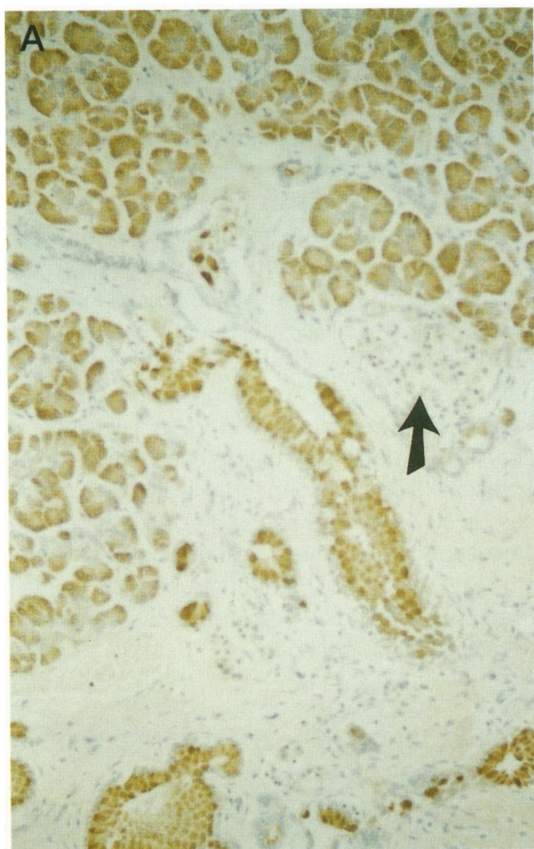
PSTI mRNA and peptide were strongly expressed in an identical distribution in the acinar cells of human fetal and adult pancreas, the mucus-producing cells of the stomach, small intestine, and colon, and in the acinar component of the normal breast (see Figures 1 and 2). In the fetal and adult kidney, PSTI-like immunoreactivity was seen in the collecting tubules (Figure 1D) and the transitional epithelium of the pelvis, although staining was much weaker in adult tissue. In contrast, PSTI mRNA was seen in the transitional epithelium of the renal pelvis but was not present in the collecting tubules. Western and Northern analyses confirmed the presence of peptide and message in all of these tissues (Figure 2, A and B), the identification of PSTI mRNA positivity in the renal sample reflecting its presence in the transitional epithelium of the renal pelvis.

Distribution of PSTI in the Stomach of Patients with Gastric Ulcers

The intensity of staining for PSTI-like immunoreactivity or mRNA was generally reduced in areas distant from the ulcer and was mainly confined to the gland bases (Figure 3A; compare with Figure 2C). These changes were particularly severe in biopsies showing intestinal metaplasia. In contrast, examination of the ulcer edge showed strong staining for both peptide and message in the regenerative monolayer of epithelial cells that cover the ulcer base during the healing process (Figure 3B).

Effect of PSTI on Cell Migration and Proliferation in Vitro

The addition of purified PSTI to wounded monolayers of HT-29 cells stimulated cell migration in a dose-dependent manner (Figure 3C). Concentrations of PSTI greater than 10 μg/ml did not cause any additional increase in the rate of cell migration (data not shown). In wounded monolayers to which 10 μg/ml PSTI had been added, the co-presence of a PSTI-neutralizing antibody (T4, data not shown) or an EGF-R-blocking antibody reduced cell migration to control levels, whereas the addition of a TGF-β-neutralizing antibody removed approximately 40% of the stimulatory effect of PSTI (Figure 3D). The presence of an inhibitor of cell proliferation, mitomycin C, did not affect cell migration in response to PSTI (data not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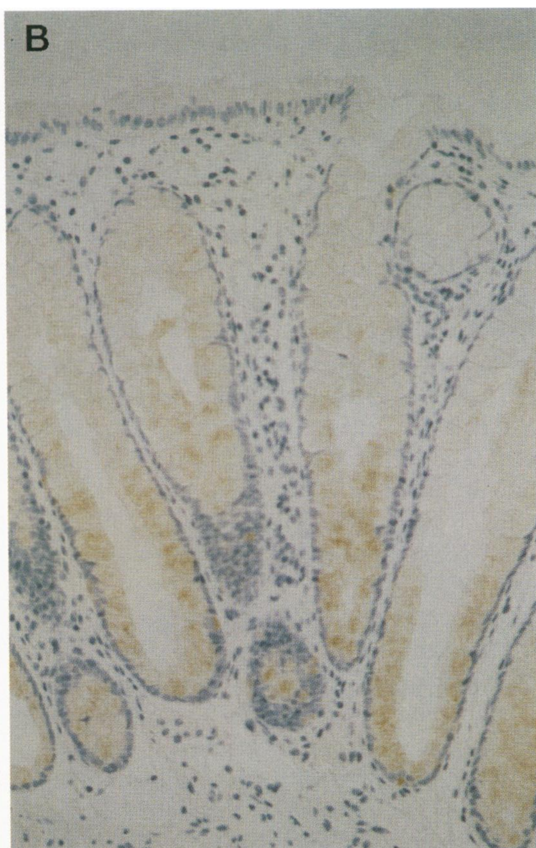
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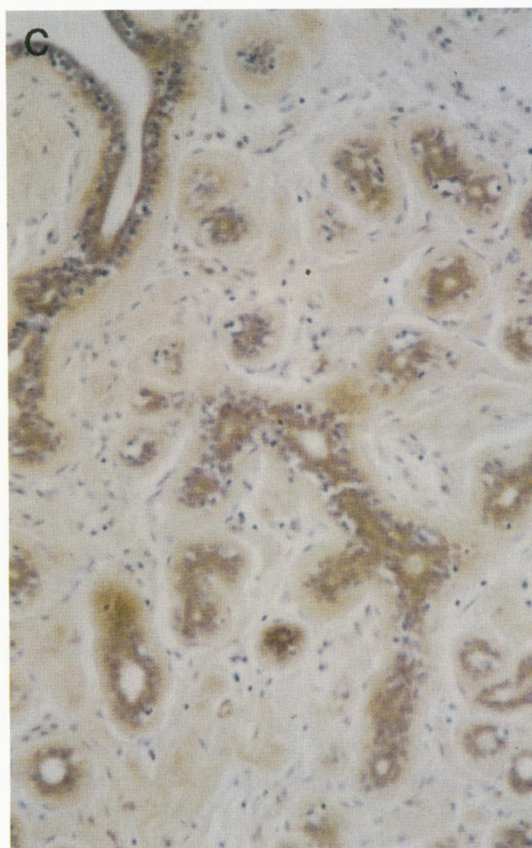
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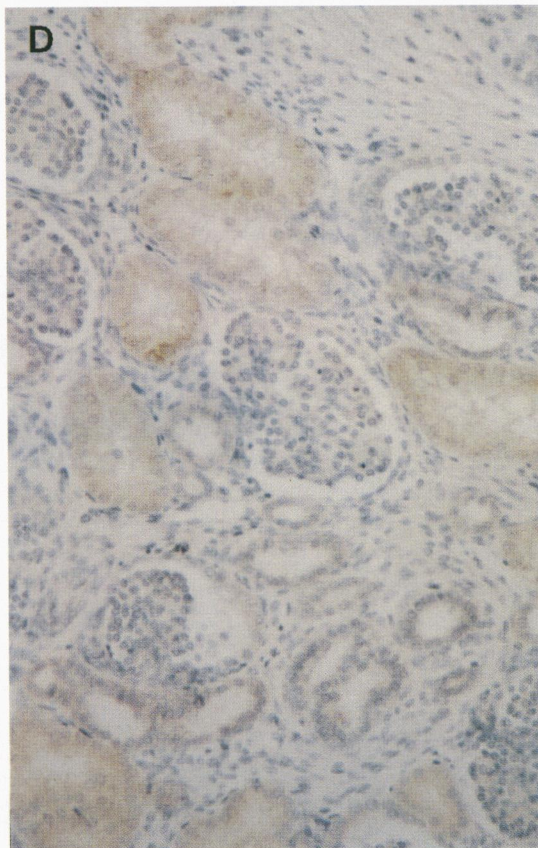
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shown). The addition of soya bean trypsin inhibitor, which has a similar serine protease inhibitor profile to PSTI but is structurally distinct, had no effect on cell migration (data not shown). PSTI had no effect on proliferation at any of the doses tested (1 to 5000 ng/ml, data not shown).

Discussion

PSTI is a potent serine protease inhibitor that is secreted into the gut lumen by the pancreas and by the mucus-secreting cells of the gastrointestinal tract. We have shown that PSTI is also produced in the normal adult breast and is strongly expressed in the regenerating epithelium at the edge of benign gastric ulcers. In addition, using an *in vitro* model of cell wounding, we have shown that PSTI stimulates cell migration of the human colonic cell line HT-29. These results suggest that PSTI may play additional roles to that of preventing excessive digestion of gastrointestinal mucus and, in particular, may be important in stimulating the healing of breaches in gastrointestinal mucosal integrity.

PSTI was originally identified in the pancreas and, because of its protease inhibitor activity, it was suggested that its primary role is to prevent premature activation of pancreatic proteases. The identification of PSTI in mucus-producing cells of the gastrointestinal tract led to the suggestion that it may also be important in preventing excessive digestion of gastrointestinal mucus. This idea was also supported by our finding that patients with gastritis and/or gastric ulcers, who are known to have an abnormal structurally weaker mucous layer, have decreased gastric tissue PSTI levels.[5] Additional roles for PSTI, acting directly on cellular function, were also suggested by the realization that PSTI has weak structural homology with EGF[15] and that PSTI stimulates proliferation of human fibroblasts[3] and endothelial cells[3] and the rat pancreatic cancer cell line AR4–2J.[4]

We are aware of only two previous reports examining the distribution of PSTI in multiple organ systems. In these studies, Fukayama used immunohistochemical localization[16] whereas Shibata used radioimmunoassay of tissue homogenates.[17] Our results are broadly in keeping with their findings, although we found that the normal human breast expresses PSTI whereas Fukayama reported that the breast was negative (Shibata did not measure PSTI in breast tissue). The reasons for this difference between our results and those of Fukayama are unclear, although this group only used a single rabbit anti-human polyclonal antibody for their studies whereas we obtained consistently positive results using a rabbit anti-human polyclonal and a mouse anti-human monoclonal antibody and *in situ* hybridization. Our finding raises the interesting possibility that PSTI is secreted into breast milk where its antiprotease and/or potential mitogenic properties may have important functions in neonatal gut development. The value of using *in situ* hybridization in combination with immunohistochemistry is supported by our finding that the proximal tubule contains PSTI peptide but not message. This is probably because PSTI is filtered in the kidney and taken up by the proximal tubules where it is digested.[18] We have previously shown that the stomachs of patients who have gastric ulcers have significantly reduced PSTI levels at sites distant to the ulcer and that this reflects the almost invariable presence of gastritis.[5] The present study confirms our previous findings, but we have extended our previous results to show strong expression of PSTI peptide and message in the epithelial cells involved in the regenerative process at the ulcer edge and migrating over the ulcer base. The mechanism by which this probable local up-regulation of PSTI occurs in these cells is unclear but may reflect local changes in prostaglandin concentrations as we have previously shown gastric output of PSTI can be stimulated by administration of prostaglandin E_2.[19]

The function of up-regulation of PSTI in these cells is unclear, but it is known that, when a breach in mucosal integrity of the stomach, small intestine, or colon occurs, surviving cells from the edge of the wound migrate over the denuded area to re-establish epithelial continuity. This is followed by a later increase in proliferation and architectural remodeling.[20] Because we found that cells involved in this process strongly express PSTI, we used a well validated model of cell wounding to examine its potential pro-migratory and/or proliferative activity. We chose HT-29 cells as they are derived from the human colon and are known to respond to other pro-migratory peptides such as EGF and the trefoil peptides[11,21]; however, caution must always be shown in extrapolating results obtained using carcinoma cell lines to the normal (or damaged) stomach. Our stud-

Figure 1. *Distribution of PSTI in normal human tissue visualized by immunohistochemistry. PSTI-like immunoreactivity (shown as brown staining) was present in the exocrine component of the pancreas* (**A**) *but was absent in the islets of Langerhans* (**arrow**). *PSTI-like immunoreactivity was also present in the mucus-producing cells of the colon* (**B**) *and the acinar component of the breast* (**C**). *PSTI immunoreactivity was also seen in the convoluted tubules and collecting ducts of the renal cortex* (**D**).

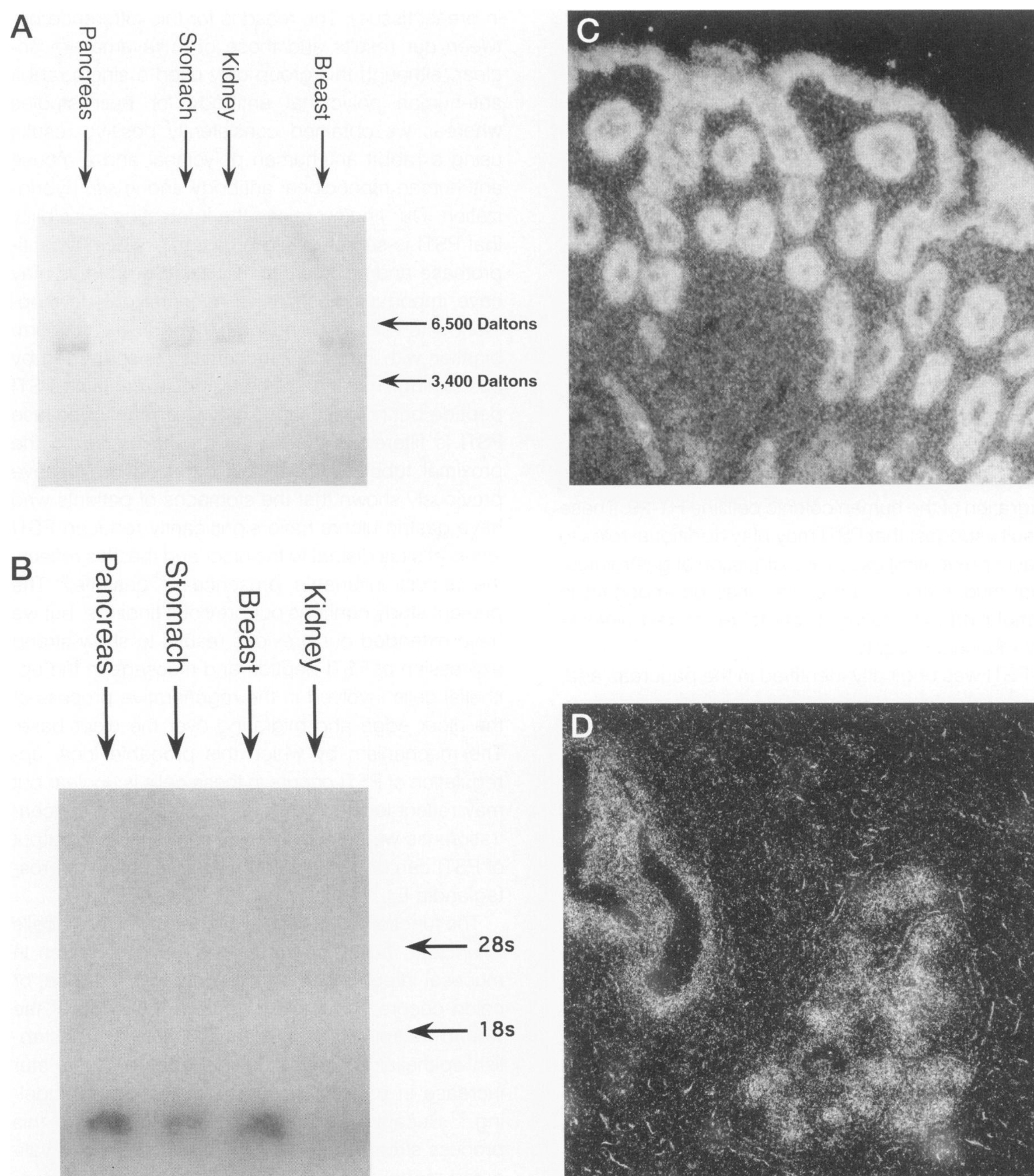

Figure 2. *Distribution of PSTI in normal human tissue visualized by Western blot, Northern blot, and* in situ *hybridization.* A: *Western blots using protein extracted from adult tissue (10 μg of total protein/lane) gave a single band of the anticipated size (approximately 6 kd) in the pancreas* (lane 1), *stomach* (lane 3), *kidney* (lane 4), *and breast* (lane 6). B: *Northern blots (20 μg of total RNA/lane) isolated from the same tissues gave similar results, although the band obtained from renal tissue (which included transitional epithelium from the renal pelvis) was fainter than that seen in pancreas, stomach, and breast.* C *and* D: *Similar results were seen using* in situ *hybridization techniques with strong staining for PSTI mRNA seen in the mucus-producing cells of the stomach* (C) *and acinar component of the breast* (D).

ies showed a marked pro-migratory effect of PSTI that was probably independent of its trypsin inhibitor activity as soya bean trypsin inhibitor, which has a similar protease inhibitory profile, did not have a pro-migratory effect. The pro-migratory activity of PSTI was partially inhibited by addition of a TGF-β-neutralizing antibody. A similar TGF-β-dependent effect has been shown in the pro-migratory actions of EGF, TGF-α, and interleukin-1β using the rat small intestinal cell line IEC-6.[22] This is probably due to the ligand causing the cells to release TGF-β, which in turn stimulates the cells to migrate in an autocrine

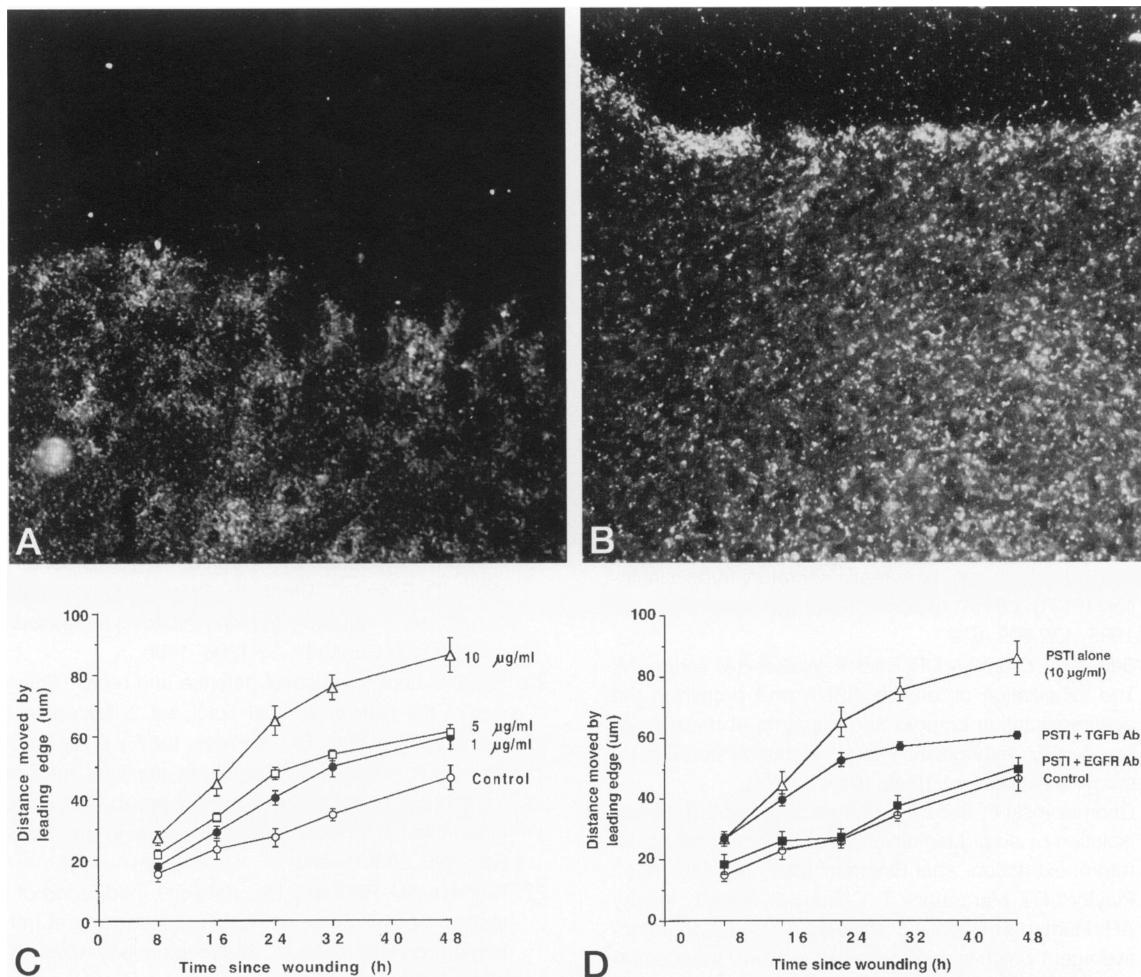

Figure 3. *Distribution of PSTI at the edge of benign gastric ulcers and effect of purified PSTI on cell migration.* **A** *and* **B**: *Patients with benign gastric ulcers had markedly reduced PSTI immunoreactivity and mRNA* (**A**) *in the surface epithelium at areas distant from the ulcer (compare with Figure 2C). However, there was strong PSTI expression in cells at the ulcer edge, particularly in the regenerative monolayer of epithelial cells covering the ulcer base* (**B**). **C**: *Effect of PSTI on the rate of migration of the human colonic cell line HT29. Addition of PSTI to the incubation medium (final concentrations, 1 to 10 µg PSTI/ml) caused a dose-dependent increase in the rate of cell migration (for all concentrations of PSTI,* $P < 0.01$ versus *control after 8 hours). Values are expressed as mean ± SEM (n = 6).* **D**: *Effect of TGF-β-neutralizing and EGF-R-blocking antibodies on the pro-migratory effects of PSTI. Addition of a TGF-β-neutralizing antibody caused a 40% reduction in the rate of migration induced by 10 µg/ml PSTI, whereas the addition an EGF-R-blocking antibody reduced the rate of migration to levels seen in cells to which no PSTI peptide had been added (control). Values are expressed as mean ± SEM (n = 6).*

fashion.[22] Our finding that migration induced by PSTI could be inhibited by adding an EGF-R-blocking antibody suggests that PSTI mediated this effect through binding to the EGF-R. This idea is supported by a previous report that rat PSTI competed with mouse EGF for binding to mouse Swiss 3T3 cells,[23] although Niinobu et al[24] reported that the binding of human ^{125}I-labeled PSTI to the same cells could be displaced by cold PSTI but not EGF, suggesting a separate receptor.

Taken together, our findings that PSTI is strongly expressed in cells involved in the regenerative process at the ulcer edge and that PSTI stimulates cell migration in a human intestinal cell line suggests a previously unrecognized role in maintaining mucosal integrity and in stimulating repair. Additional studies therefore seem appropriate.

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