Pancreatic secretory trypsin inhibitor is a major motogenic and protective factor in human breast milk.

Tania Marchbank¹, Gillian Weaver², Marit Nilsen-Hamilton³, Raymond J Playford¹.

1. Centre for Gastroenterology, Institute of Cell and Molecular Science, Barts & The London School of Medicine, Queen Mary University of London, London, UK, 2. Milk Bank Manager, Queen Charlotte’s Hospital, London W12 0HS, UK, 3. Department of Biochemistry, Biophysics and Molecular Biology, Iowa State University, Ames, Iowa, IA50011, USA.

Running head: PSTI, colostrum and milk

Correspondence Prof. RJ Playford,

Professor of Medicine,

Barts & The London School of Medicine and Dentistry,

Queen Mary University of London,

Turner Street,

London E1 2AD, UK

Telephone +44(0)20 7882 2260

Fax +44 (0)20 7377 7607

Email r.playford@qmul.ac.uk

Abbreviations: DMEM; Dulbecco’s modified Eagle medium, NSAID; non-steroidal anti-inflammatory drugs, PSTI; pancreatic secretory trypsin inhibitor, EGF; epidermal growth factor, TGFα; transforming growth factor alpha, NGAL; neutrophil gelatinase associated lipocalin.
ABSTRACT

Background: Colostrum is the first milk produced after birth and is rich in immunoglobulins and bioactive molecules.

Objective: We examined if human colostrum and milk contained pancreatic secretory trypsin inhibitor, a peptide of potential relevance for mucosal defence and using in vitro and in vivo models, determined if its presence influenced gut integrity and repair.

Designs: Human milk was collected from individuals over various times from parturition and PSTI concentrations determined using immunoassay. Human milk samples were analysed for proliferation & pro-migratory activity (wounded monolayers) and anti-apoptotic activity (caspase-3 activity) using intestinal HT29 cells +/- neutralizing antibodies to PSTI and EGF. Rats were restrained and given indomethacin to induce gastric injury. Effect of gavage with human breast milk +/- neutralizing antibodies on amount of injury were compared with animals receiving a commercial formula feed.

Results. PSTI is secreted into human milk with colostrum containing a much higher concentration of PSTI than human milk obtained later. Human milk stimulated migration and proliferation about three-fold and reduced indomethacin-induced apoptosis by about 70-80%. Sixty-five percent of the migratory effect of human milk could be removed by immunoneutralisation of PSTI. PSTI worked synergistically with EGF in mediating these effects. Gastric damage in rats was reduced by about 75% in the presence of human milk and was more efficacious than the formula feed (p<0·001). Protective effects of the milk were reduced by about 60% by PSTI immunoneutralisation.

Conclusions: PSTI is secreted into human milk at concentrations which have probable pathophysiological relevance.
Key words: Repair, injury, nutrition
INTRODUCTION

Colostrum is the first milk produced after birth and is rich in immunoglobulins, antimicrobial peptides and other bioactive molecules including growth factors. In combination with the milk that is subsequently produced, it is important for the nutrition, growth and development of the newborn infant, and contributes to the immunological defence of the neonate. There are continuous changes in the composition of the mammary secretions throughout the suckling period, although colostrum is generally regarded as being that produced for the first few days (usually up to about day 5-7) after birth.

Much of the major focus on the value of breast feeding or administering human or bovine colostrum has been around its value in transferring passive immunity. During the first few days after birth, many species (including humans) probably have enhanced gut permeability for macromolecules, leading to some of the maternal IgG antibodies present in the milk crossing the gut wall in an intact form to reach the systemic circulation.

In addition to its antibody and antimicrobial molecules, colostrum and, to a lesser extent, milk are rich in a variety of molecules which can influence cell growth, differentiation and function. These molecules include non peptide factors (eg glutamine, polyamines and nucleotides), hormones (eg prolactin) and more ‘classical’ growth factors such as epidermal growth factor (EGF), for general review see ref 19.

Pancreatic secretory trypsin inhibitor (PSTI), also known as serine protease inhibitor Kazal type 1 (SPINK1), is a 56 amino acid peptide (7) that protects the pancreas from
auto-digestion due to premature activation of pancreatic proteases, mutations of which are associated with familial pancreatitis (3). PSTI probably has additional functions as shown by our findings that PSTI is expressed in the mucus producing cells of the stomach and large intestine and secreted into gastric juice where it protects the mucus layer from excessive digestion (5). We have also shown that PSTI is expressed in the glandular system of the human breast where its function is unclear (10).

In the current studies, we examined whether PSTI was secreted into human breast milk and whether the concentration of PSTI changed over time. We also examined the concentrations of the potent growth factor EGF, known to be present in human milk, and transforming growth factor alpha (TGF\(\alpha\)) which binds to the same receptor as EGF. We also took the opportunity to measure levels of the lipocalin neutrophil gelatinase associated lipocalin (NGAL) as the mouse homologue (24p3) has recently been shown to be present in murine milk where it may influence immune function or gut integrity (20, 25) and we have also shown both 24p3 and NGAL are capable of stimulating restitution of human intestinal cells (20). Finally, we performed a series of in vitro and in vivo experiments examining the potential protective and healing effects of PSTI within human colostrum and milk.

**MATERIALS AND METHODS**

All chemicals were purchased from Sigma (Dorset).
Ethics

All animal and human studies were approved by appropriate regulatory authorities.

Preparation of human milk.

Human milk was obtained from the Milk Bank at Queen Charlotte’s Hospital. This was milk donated but surplus to requirement and consent for its use in the study was obtained from the donors. For the time course studies, milk from 7 individuals was collected at various time points following delivery. Milk was stored at -20°C, defrosted before use and defatted by centrifugation at 13,000g for 10 minutes at 4°C.

Study series 1. Quantitation of bioactive peptide concentrations in human milk.

PSTI concentrations were determined by radioimmunoassay as previously described (5). EGF and TGFα concentrations were determined using commercial ELISA kits (R&D Systems Ltd, Abingdon, UK). NGAL concentrations were determined using well validated ELISA and Western blotting techniques (25).

Study series 2. Effect of human milk on restitution and proliferation in vitro.

Background to methods.

One of the earliest repair responses following injury is migration of surviving cells over any denuded area to re-establish epithelial integrity. Cell migration assays were performed using our previously published methods (21), using human colonic carcinoma cell line HT29. All results are expressed as mean +/- SEM of three separate experiments.
Study protocols

1. Preliminary concentration finding study

Wells were incubated with 5%, 25%, 50% or 75% (vol/vol) human milk in serum free medium. DMEM + 10% foetal calf serum (FCS) was used as a positive control and DMEM alone was used as a negative control. The effect of these various concentration on restitution were then determined. 5% colostrum increased restitution by approximately 15% and 25% colostrum increased it by 55%. Higher doses showed no further increase in the rate of migration and at 75%, the cells showed signs of toxicity (increased detachment of cells). As 25% human milk gave the optimum restitution effect, this was, therefore, used in all subsequent experiments (Figure 1A).

2. Pro-migratory effects of human milk collected at various days post-partum.

Wells were incubated with samples from day 5, day 10 or day 65 following birth, all at a concentration of 25% (vol/vol).

3. Influence of specific bioactive peptides within the human milk on pro-migratory effects.

A series of incubation conditions were set up using various anti-peptide or receptor neutralizing antibodies or an EGFR tyrosine kinase inhibitor. Wells were incubated in 25% human milk collected at day 5 post partum in the absence and presence of either mouse monoclonal IgG1 anti-hEGF antibody (MAB636, R&D systems, 1:50), mouse monoclonal IgG1 anti-hPSTI antibody (GERP, 1:100 (10)), mouse monoclonal IgG1 anti-
lipocalin-2/NGAL antibody (MAB1757, R&D systems, 1:50), anti-EGFR antibody (Cetuximab, Bristol-Myers Squibb Company, Princeton, 100 mg/ml) or EGFR tyrosine kinase inhibitor, Tyrphostin (AG1478, 100nM). Changes in the distance migrated by wounded monolayers in the presence of these various factors were then determined.

4. Pro-migratory effects of recombinant EGF and PSTI when given alone and in combination.

To investigate any synergistic effect between PSTI and EGF, HT29 wounded monolayers were incubated in either DMEM containing 10nM or 20nM PSTI, 10nM or 20nM EGF, or both 10nM EGF and 10nM PSTI.

**Cell proliferation as a model of wound repair**

Cell proliferation assays utilised Alamar blue (Invitrogen, Paisley, UK, (13)) as per manufacturers instructions measuring changes in absorbance at 570 nm.

1. **Effect of human milk on proliferation**

Briefly, cells were seeded at 2000 cells/well, grown in DMEM and 10% FCS in 96 well plates overnight. The following day, cells were washed with DMEM alone and various human milk concentrations (5-75% (vol/vol), n=6) were tested under serum starved conditions for 24 hours and then quantitated using Alamar blue. Foetal calf serum was used as a positive control.

2. **Influence of specific bioactive peptides on proliferative effect of milk**
As for the migratory studies, a series of anti-peptide or receptor blocking antibodies or a tyrosine kinase inhibitor (all at same concentrations as described for migration assays) were added to wells in the presence of 10% (vol/vol) human milk.

**Study series 3. Effect of human milk on apoptosis *in vitro.***

Increased apoptosis is likely to be relevant in the gastrointestinal damaging side effects of NSAID use (17). We, therefore, used a well validated *in vitro* model which measures changes in caspase-3 activity as a marker of apoptosis in cells that had been treated with indomethacin. Previous studies have shown that apoptosis is increased in intestinal cells after 4 hours incubation with indomethacin (8) and this timing of collection was, therefore, used.

Protocol: HT29 cells were seeded at $5 \times 10^5$ cells/flask in T25cm flasks in DMEM containing 10% FCS, and were grown for 24 h. Cells were then treated for 4 hours in medium containing FCS, PSTI (10 nM-2μM), EGF (10 nM-2μM) or 25% human milk collected at day 5 *post partum* in the absence and presence of the various antibodies and EGFR tyrosine kinase inhibitor at the same concentrations as for study series 2 (above) and in the presence or absence of indomethacin (800μM).

Cells were washed in ice cold PBS, lysed in lysis buffer (50mM Hepes, 5 mM DTT, 0.1 mM EDTA, 0.1% CHAPS, pH 7.4) for 5 minutes on ice. Lysates were cleared by centrifugation at 10,000g for 10 minutes at 4°C. Protein concentrations were determined using a standard BCA method (Pierce, Rockford, IL). Caspase-3 activity was measured
using the caspase-3 cellular activity assay kit (235419, Merck Chemicals limited, Nottingham, UK) following the manufacturers instructions using recombinant caspase-3 (30U) as a positive control. 100 μg of protein was used in triplicate wells for each treatment, additional wells containing cell lysates had the specific caspase-3 inhibitor (Ac-DEVD-CHO) added to show that any activity detected was caspase-3 specific. Absorbance at 405 nm was determined at 10 minute intervals over a 2 hour period.

**Study series 4: Effect of the human milk on gastric damage in vivo.**

The ability of human milk to reduce gastric damage was assessed using a well-validated model (21). In addition, the importance of specific factors within the milk that elicited beneficial effects were determined using specific peptide-neutralizing or receptor blocking antibodies.

Briefly, following an overnight fast Sprague Dawley rats (225–275 g) were randomised to receive (n=8), by gastric gavage, one of the following factors; 2ml of saline (negative control), human milk (day 5 sample) or human milk containing blocking antibodies (concentrations as for migration assays) or formula milk (SMA gold, SMA nutrition, Berkshire, UK). All gavage solutions also contained 2% hydroxypropyl-methylcellulose to delay gastric emptying. Thirty minutes after gavage, all rats received indomethacin (20 mg/kg subcutaneously) and were placed in Bollman-type restraint cages. Three hours later, animals were killed, their stomachs removed and the intragastric pH determined using a micro pH electrode. This pH assessment was performed to determine if pH
remained below 4, as pH rises above this level influence the degree of injury that occurs using this model (18). Stomachs were then processed as previously described to determine the degree of macroscopic (mm2/stomach) and microscopic injury (scale 0-4) (21). In addition, sections were stained for the presence of active caspase-3 following the method of Marshman and co-workers (12) using 1:200 rabbit polyclonal anti–active caspase-3 antibody (AF835, R&D Systems, Abingdon, UK).

**Statistical analyses**

Data from all experiments are expressed as mean ± SEM. One way ANOVA were used as appropriate. Where a significant effect was seen (p<0.05), individual comparisons were performed using t-tests based on the group means, residual and degrees of freedom obtained from the ANOVA, a method equivalent to repeated measures analyses.

**RESULTS**

**Study series 1. Growth factor concentrations in human milk.**

PSTI levels in human milk were the highest in the earliest samples collected (at about 150 ng/ml) falling to about half this level by day 8, eventually reaching a plateau at about 20 ng/ml by day 20 (Figure 2A). EGF concentrations were roughly similar to those of PSTI (highest concentrations about 190 ng/ml on day 3) and showed a similar fall over time to stabilise at about 35 ng/ml by day 35 (Figure 2B). TGFα levels in human milk were only about 1/1000 that of peak EGF or PSTI concentrations at about 70 pg/ml (data not shown). No NGAL was detected in any of the samples.
Study series 2. Effect of human milk on restitution and proliferation *in vitro*.

Pro-migratory effects of human milk collected at various days *post-partum*.

Human milk from day 5 after birth increased cell migration by about three-fold compared to DMEM alone (negative control, \( p < 0.01 \)). Compared to this day 5 sample, the increase in migration was reduced by about 23% and 60% when human milk from day 10 & day 65 was used (\( p = 0.052 \) & \( p < 0.05 \), Figure 1B).

Origin of pro-migratory effects within the milk.

The addition of either monoclonal hEGF or hPSTI neutralizing IgG1 antibody to the migration assays both resulted in a significant reduction in the amount of additional restitution stimulated by the human milk above baseline values by about 65% (\( p < 0.01 \), Figure 1C).

The presence of the monoclonal hNGAL neutralizing antibody did not significantly affect migration. In contrast, the presence of the anti-EGFR antibody or the specific EGFR tyrosine kinase inhibitor reduced the rate of migration to baseline levels (Figure 1C).

Pro-migratory effects of recombinant EGF and PSTI when given alone and in combination.

When given alone, incubation of damaged monolayers with either 10 nM EGF or 10 nM PSTI did not significantly affect the rate of migration compared to baseline (Figure 3).
However, when given together, 10 nM EGF & PSTI showed a synergistic effect, resulting in a significantly greater amount of migration compared with using 20 nM of either peptide alone (p < 0·01 vs. either peptide alone at 20 nM).

Cell proliferation

Effect of human milk on proliferation

A linear relationship was seen between the number of cells added and changes in absorbance readings. All concentrations of milk above 5% significantly increased proliferation (as assessed by changes in absorbance values), the optimum concentration was found to be 10% milk. Concentrations above this showed no additional effect (Figure 4A).

Influence of specific bioactive peptides on proliferative effect of human milk

The presence of day 5, 10% milk solution in the incubation medium caused a doubling of absorbance values (Figure 4B). Addition of monoclonal hPSTI or hNGAL neutralizing antibodies had no significant effect on the increase of proliferation (absorbance) caused by the milk. The addition of monoclonal hEGF neutralizing antibody reduced the additional increase in proliferation (absorbance) effect of human milk above baseline values by about 50% (p < 0·01). The increase in proliferation (absorbance) caused by the milk could be virtually completely abrogated by the presence of either the anti-EGFR antibody or the specific EGFR tyrosine kinase inhibitor (Figure 4B).

A low level of caspase-3 activity was seen in HT29 cells following incubation in medium alone and results were not significantly different in cells that had also received human milk +/- neutralizing antibodies, PSTI or EGF without indomethacin. In contrast, cells incubated with indomethacin alone had about a three-fold rise in Caspase-3 levels (Fig 5A). This pro-apoptotic effect of indomethacin effect was markedly truncated by the co-presence of PSTI, EGF or human milk (Fig 5A & B, p<0.001). The protective effect of human milk against indomethacin-induced apoptosis was truncated in the presence of PSTI or EGF neutralizing antibodies given alone or in combination where additive/synergistic responses were seen (p<0.01, Fig 5B). Similarly, the protective effect of milk was virtually completely removed if Tyrphostin was also present. Changes in absorbance (used as a marker of caspase-3 activity) were shown to be specific as these effects were not seen when the capase-3 inhibitor were also added to the cells (data not shown).

**Study series 4: Effect of human milk on gastric damage in vivo.**

Animals that received a negative control gavage had a macroscopic gastric damage score of 60 ± 2 mm²/stomach. Co-administration of human milk caused a 75% reduction in amount of injury (Figure 6A, also see Figure 6B). Compared to the presence of human milk, gavage with commercially produced milk formula was much less efficient in reducing injury, resulting in only a 30% decrease in induced damage (macroscopic score of 42 ± 2 mm²/stomach in formula milk gavage vs. 15 ± 3 mm²/stomach in rats that received human milk, p < 0.01).
The co-presence of the PSTI or EGF neutralizing antibody in the milk gavage resulted in a truncation of the protective effects (reducing protection by 60 and 55% respectively, p < 0·001, Figure 6A). However, the NGAL neutralizing antibody did not diminish the protective effect of human milk.

Histological assessment using the microscopic scoring system gave similar results to those obtained using macroscopic assessment of injury (data not shown). In keeping with the results from the *in vitro* studies, caspase-3 staining was markedly increased in animals given indomethacin alone, but the amount of staining was much lower in animals that had also received human milk (Fig 6B). Gastric pH assessment showed all animals had pH's in the range of 1-3.

**DISCUSSION**

We have shown PSTI is secreted into human milk at concentrations similar to those of EGF. Milk produced during first few days after birth (colostrum) contains much higher concentrations of PSTI compared to milk obtained later *post partum*. The PSTI within human milk was of sufficient concentration to stimulate cell migration of an intestinal cell line *in vitro* and to reduce indomethacin-induced apoptosis. PSTI acted synergistically with EGF in mediating these effects. Using an *in vivo* rat model of gastric damage, we showed human milk was more effective in reducing damage than a standard formula milk feed and that at least a proportion of the protective effect of human milk in this model was due its PSTI content.
Although PSTI was initially found in the pancreas, its much wider distribution suggests that it may play additional roles. Its potential relevance for mucosal defence and repair is strengthened by our findings that PSTI administration reduced injury in an animal model of colitis and also had immune modulatory actions when administered to human dendritic cells (11).

For the *in vitro* studies, HT29 cells were used to examine restitution, proliferation and apoptotic effects as these are of human large intestinal origin and have been used by other groups for similar types of studies (25). For the *in vivo* study, the effect of the human milk on gastric injury were performed using rats as we have previously validated this model for other nutritional-based growth factor products (for example ref 4). The use of caspase-3 analyses to determine apoptosis was based on well validated methods (12) and the anti-apoptotic effects of the milk seen in the *in vitro* study was reproduced using the *in vivo* system, showing that this result was not restricted to one species. Caution always has to be shown, however, in extrapolating results obtained from *in vitro* cancer cell lines and animal models to the human situation.

Concentrations of PSTI in the milk during the first few days after birth were about 160 ng/ml, similar to those reported for EGF (23). Both EGF and PSTI showed roughly parallel falls to about 20–40 ng/ml over the next 35 days. In contrast, TGFα concentrations in the milk were about 1,000 fold lower and NGAL levels were
unrecordably low. As marked differences in growth factor constituents of milk occur between species (eg 16), the source of colostrum or milk given to an infant will markedly affect its biologically active constituents.

The perinatal period is a time of rapid change including increase in pancreatic and gastric enzyme and acid secretions, associated with onset of feeding and continuing digestive maturation (24). These alterations associated with onset of feeding probably contribute to the risk of breakdown of integrity and may partially explain why the levels of the protective factors EGF and PSTI are particularly high during this period.

When an acute mucosal injury occurs, surviving cells from the edge of the wound rapidly migrate over the denuded area in a process called restitution. Our in vitro studies showed pro-motogenic activity of EGF and PSTI and, for the first time, that synergistic responses were seen if PSTI & EGF are co-administered. The signalling mechanism behind this finding is unclear although the EGF receptor and tyrosine kinase signalling are probably involved as demonstrated by our previous findings that EGFR-blocking antibodies or tyrosine kinase inhibitors can prevent the pro-migratory effects of PSTI or EGF (11). However, the relationship between PSTI and the EGFR is probably not that of a direct receptor ligand as demonstrated by the negative result of most radiolabelled displacement studies (6, 14) and the divergence of results in the pro-proliferative activity of EGF and PSTI against various cell lines (6, 10, 14). It, therefore, seems likely that PSTI is inducing cross phosphorylation of the EGFR and/or influencing its downstream pathways as well as possibly acting via a distinct pathway.
In the normal non-damaged adult human gut, EGF receptors are restricted to basolateral rather than apical membranes (22) and any ingested EGF or PSTI within milk is unlikely to influence gut function. However, in the neonatal normal gut and in the adult damaged gut, increased gut permeability probably allows exposure of EGF receptors to interaction by direct or indirect EGFR ligand (22).

Various models of gut injury are available involving adult or neonatal animals. Neonatal animal models are usually reserved for extreme scenarios, such as induction of necrotising enterocolitis. As we wished to study a less extreme model, reflecting a more standard situation and the fact that indomethacin exposure in the perinatal period increases the risk of gastric mucosal injury (15), we chose the rat indomethacin-stress induced model. Human colostrum markedly reduced gastric injury in this model and was much more efficacious than commercial formula milk, suggesting that feeding neonates with human colostrum, which contains multiple bioactive molecules, may offer advantages over formula feeds in establishing and maintaining gut integrity.

Indomethacin causes damage to the gut by several mechanisms including reduction of mucosal prostaglandin levels and mucosal blood flow, stimulating neutrophil activation, and stimulating apoptosis (9). Although our in vitro studies demonstrated pro-migratory effects of human milk, this mechanism was probably not relevant in our short term in vivo model as histological examination showed no evidence of epithelial migration over the damaged area. In this particular model, it is more likely that the human milk reduced
the degree of initial damage through other mechanisms. Although several compounds within the milk were probably involved in this protective effect, possibly acting via more than one pathway, our studies employing immunoneutralisation suggested that both EGF and PSTI were key peptides in mediating protection. In addition to showing the pro-migratory effects of milk and PSTI, we showed they reduced NSAID-induced apoptosis. Indomethacin influences apoptosis via several pathways including enhancing degradation of *survivin* via the ubiquitin proteasome machinery (as shown in cultured gastric epithelial cells and in human and rat gastric mucosa, 2) and by dissociating BAX from Bcl-X(L), thereby promoting BAX mitochondrial translocation and multimerization (as shown using colon cancer cell lines, 1). Further work is required to examine if PSTI and milk influenced these or other pathways in mediating their anti-apoptotic effects and also examining other mechanisms such as effects on proliferation *in vivo*.

We conclude that PSTI is a major bioactive peptide present in human milk that can influence restitution, apoptosis, gut mucosal protection and repair. In addition, our studies suggest that feeding neonates with human colostrum, that contains multiple bioactive molecules, may also offer advantages over formula feeds in establishing and maintaining gut integrity.

**Acknowledgements**

We are grateful for the contribution of Lee Bendickson.

**Funding**
This work was partially funded by the Wexham Park Gastrointestinal trust grant number 2004/6772.
REFERENCES


FIGURE LEGENDS

Figure 1. Alteration of pro-migratory effect of human milk at various dates following birth and influence of specific factors within the milk.

Human colonic cancer cell line HT29 was grown as a monolayer and a standard wound inflicted at time zero. Serial photographs were then taken over the next 24 h.

A. Quantitative assessment using various concentrations of human milk, from a mother day 5 following birth, in the medium showed that optimal migration was achieved at 25% (vol/vol). Higher doses of milk showed no additional benefit (data not shown)

B. Wounded monolayers were incubated with human milk (25% vol/vol) obtained at various times following birth. Distance migrated at the 24 h time point following injury to the monolayers are shown. ** signifies $p < 0.01$ vs. negative control, $$ signifies $p < 0.01$ vs. distance migrated in presence of milk from mothers 5 days following birth.

C. Samples of human milk obtained from mothers 5 days after giving birth were incubated as above at 25% (vol/vol) in the presence and absence of various neutralizing antibodies or tyrosine kinase inhibitors. Effect on distance migrated 24 h following wounding the monolayer are shown. ** signifies $p < 0.01$ vs. negative control, $$ signifies $p < 0.01$ vs. distance migrated in presence of milk alone.
Figure 2. Bioactive peptide concentrations in human milk

Human milk samples obtained at various times following birth were analysed using specific immunoassays. Concentrations of PSTI (A) and EGF (B) within the milk are shown.

Figure 3. Pro-migratory effects of recombinant EGF and PSTI when given alone and in combination.

Distance migrated by wounded monolayers of HT29 cells at the 24 h time points are shown. Influence of addition of various doses of PSTI and EGF when given alone or in combination are shown. ** signifies $p < 0.01$ vs. negative control, $$ signifies $p < 0.01$ vs. PSTI or EGF given separately.

Figure 4. Effect of human milk on proliferation.

A. HT29 cells were incubated for 29 h in presence of various concentrations of human milk obtained from mothers 5 days following birth. Foetal calf serum was used as a positive control. Changes in proliferation were assessed by adding Alamar blue and measuring changes in absorbance at 570 nm. ** signifies $p < 0.01$ vs. negative control.

B. The importance of specific factors within the milk in inducing pro-proliferative effects were examined by adding various neutralizing antibodies or tyrosine kinase inhibitors to wells which contained 10% (vol/vol) human milk. ** signifies $p < 0.01$ vs. negative control. $$ signifies $p < 0.01$ vs. human milk alone.
Figure 5. Effect of PSTI, EGF and human milk on apoptosis.

A. HT29 cells were incubated with indomethacin (to induce apoptosis) in the presence and absence of various concentrations of PSTI or EGF. Apoptosis was assessed using caspase-3 activity. Baseline values of cells in which indomethacin was not added are also shown.

B. Effect of human milk +/- neutralizing antibodies to PSTI and EGF on indomethacin-induced apoptosis are shown. In addition, the effect of the tyrosine kinase inhibitor Tyrphostin in the presence of milk on indomethacin-induced apoptosis is shown. Data presented as mean +/- SEM. ** signifies p < 0.01 vs. cells incubated with indomethacin alone.

Figure 6. Effect of the human milk on gastric damage in vivo.

Rats received indomethacin (20mg/kg) and were restrained for three hours. Animals also received a 2 ml gavage containing saline (negative control), human milk or formula milk. Additional animals received human milk with neutralizing antibodies to PSTI, EGF or NGAL. The degree of macroscopic and microscopic injury were subsequently assessed.

A. Macroscopic quantitative assessment of gastric damage. Animals that received indomethacin and a saline ‘negative control’ gavage had extensive damage. Gavage with human milk reduced the degree of injury. Co-presence of neutralizing antibodies to human EGF or PSTI truncated the protective effect of human milk whereas co-presence of neutralizing antibody to NGAL did not.
Gavage with formula milk was far less efficacious than human milk. Microscopic quantitative assessment showed similar results (data not shown). ** signifies p < 0.01 vs. negative control. $$$ signifies p < 0.01 vs. injury in animals that had received human milk without neutralizing antibody.

B. Representative histology of stomachs stained for active caspase-3, collected under same conditions as Figure 6A. 1. Normal (rats did not receive indomethacin or restraint) had virtually no caspase-3 immunoreactivity, 2. Animals that underwent indomethacin and restraint with saline gavage had much more severe injury and this was associated with marked caspase-3 immunoreactivity, 3. Gavage with human milk markedly reduced the degree of microscopic injury and caspase-3 immunoreactivity. 4. Gavage with formula milk was far less effective in reducing injury and had greater caspase-3 immunoreactivity than animals given human milk (compare 3 & 4). Magnification X20 in all photos.