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

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Marchbank T, Weaver G, Nilsen-Hamilton M, Playford RJ. Pancreatic secretory trypsin inhibitor is a major motogenic and protective factor in human breast milk. Am J Physiol Gastrointest Liver Physiol 296: G697–G703, 2009. First published January 15, 2009; doi:10.1152/ajpgi.90565.2008.—Colostrum is the first milk produced after birth and is rich in immunoglobulins and bioactive molecules. We examined whether human colostrum and milk contained pancreatic secretory trypsin inhibitor (PSTI), a peptide of potential relevance for mucosal defense and, using in vitro and in vivo models, determined whether its presence influenced gut integrity and repair. Human milk was collected from individuals over various times from parturition and PSTI concentrations determined with the use of immunoassay. Human milk samples were analyzed for proliferation and migratory activity (wounded monolayers) and antiapoptotic activity (caspase-3 activity) with the use of intestinal HT29 cells with or without neutralizing antibodies to PSTI and epidermal growth factor (EGF). Rats were restrained and given indomethacin to induce gastric injury. Effect of gavage with human breast milk with or without neutralizing antibodies to PSTI and epidermal growth factor (EGF); for general review see Ref 19. Pancreatic secretory trypsin inhibitor (PSTI), also known as serine protease inhibitor Kazal type 1, is a 56-amino acid peptide (7) that protects the pancreas from autodigestion attributable 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 present study, 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-α (TGF-α), which binds to the same receptor as EGF. We also took the opportunity to measure levels of the lipocalin neutrophil gelatinase-associated lipocalin (NGAL) because the mouse homolog (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 that 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, UK).

Ethics

All animal and human studies were approved by appropriate regulatory authorities at the Home Office, Queen Mary’s School of Medicine and Dentistry and Queen Charlotte’s Hospital.

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Preparation of Human Milk

Human milk was obtained from the Milk Bank at Queen Charlotte’s Hospital. This was donated milk but surplus to requirement, and consent for its use in the study was obtained from the donors. For the time course studies, milk from seven individuals was collected at various time points following delivery. Milk was stored at −20°C, defrosted before use, and defatted by centrifugation at 13,000 g for 10 min 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 TGF-α concentrations were determined with commercial ELISA kits (R & D Systems, Abingdon, UK). NGAL concentrations were determined with 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 reestablish epithelial integrity. Cell migration assays were performed using our previously published methods (21) with the use of human colonic carcinoma cell line HT29. All results are expressed as mean ± SE of three separate experiments.

Study Protocols

Preliminary concentration-finding study. Wells were incubated with 5, 25, 50, or 75% (vol/vol) human milk in serum-free medium. DMEM + 10% FCS was used as a positive control, and DMEM alone was used as a negative control. The effects of these various concentrations on restitution were then determined. Five percent colostrum increased restitution by 35%, 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). Because 25% human milk gave the optimum restitution effect, this was, therefore, used in all subsequent experiments (Fig. 1A).

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

Influence of specific bioactive peptides within the human milk on promigratory effects. A series of incubation conditions were set up using various antipeptide or receptor-neutralizing antibodies or an EGF receptor (EGFR) tyrosine kinase inhibitor. Wells were incubated in 25% human milk collected at day 5 postpartum 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, Ref. 10), mouse monoclonal IgG1 anti-EGFR antibody (Cetuximab, 100 mg/ml; Bristol-Myers Squibb, Princeton, NJ), or EGFR tyrosine kinase inhibitor, tyrphostin (AG1478, 100 nM). Changes in the distance migrated by wounded monolayers in the presence of these various factors were then determined.

Promigratory 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 10 or 20 nM PSTI, 10 or 20 nM EGF, or both 10 nM EGF and 10 nM PSTI.

Cell Proliferation as a Model of Wound Repair

Cell proliferation assays utilized Alamar blue (Invitrogen, Paisley, UK; Ref. 13) per manufacturer’s instructions measuring changes in absorbance at 570 nm.

Fig. 1. Alteration of promigratory effect of human milk at various dates following birth and influence of specific factors within the milk. Human colon cancer cell line HT29 was grown as a monolayer and a standard wound inflicted at time 0. 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. **P < 0.01 vs. negative control (−ve); $SP < 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. Effects on distance migrated 24 h following wounding the monolayer are shown. **P < 0.01 vs. negative control; $SP < 0.01 vs. distance migrated in presence of milk alone. PSTI, pancreatic secretory trypsin inhibitor; Ab, antibody; EGF, epidermal growth factor; Ngal, neutrophil gelatinase-associated lipocalin; Tyroph, tyrphostin; EGFR, EGF receptor.
Effect of human milk on proliferation. Briefly, cells were seeded at 2,000 cells/well and 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 h and then quantitated using Alamar blue. FCS was used as a positive control.

Influence of specific bioactive peptides on proliferative effect of milk. As for the migratory studies, a series of antipeptide 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 nonsteroidal anti-inflammatory drug use (17). We therefore used a well-validated in vitro model that 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-h incubation with indomethacin (8), and this timing of collection was therefore used.

For this protocol, HT29 cells were seeded at 5 × 10⁴ cells/flask in T 25-cm² flasks in DMEM containing 10% FCS and were grown for 24 h. Cells were then treated for 4 h in medium containing FCS, PSTI (10 nM-2 μM), EGF (10 nM-2 μM), or 25% human milk collected at day 5 postpartum 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 (800 μM).

Cells were washed in ice-cold PBS and lysed in lysis buffer (50 mM HEPES, 5 mM DTT, 0.1 mM EDTA, 0.1% CHAPS, pH 7.4) for 5 min on ice. Lysates were cleared by centrifugation at 10,000 g for 10 min 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, Nottingham, UK) following the manufacturer’s instructions using recombinant caspase-3 (30 U) as a positive control. A sample (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 min intervals over a 2-h 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 randomized to receive (n = 8), by gastric gavage, one of the following factors: 2 ml 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 euthanized, their stomachs removed, and the intragastric pH determined using a micro-pH electrode. This pH assessment was performed to determine whether pH remained below 4 because 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 coworkers (12) using 1:200 rabbit polyclonal antiactive caspase-3 antibody (AF835, R & D Systems).

Statistical Analyses

Data from all experiments are expressed as means ± SE. One-way ANOVA was used as appropriate. Where a significant effect was seen (P < 0.05), individual comparisons were performed using t-tests on the basis of 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 (Fig. 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 stabilize at about 35 ng/ml by day 35 (Fig. 2B). TGF-α levels in human milk were only about 1/1,000 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

Promigratory effects of human milk collected at various days postpartum. Human milk from day 5 after birth increased cell migration by about threefold compared with DMEM alone (negative control, \( P < 0.01 \)). Compared with this day-5 sample, the increase in migration was reduced by about 23% and 60% when human milk from days 10 and 65 was used (\( P < 0.01 \), Fig. 1B).

Origin of promigratory 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% (\( P < 0.01 \), Fig. 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 (Fig. 1C).

Promigratory 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 with baseline (Fig. 3). However, when given together, 10 nM EGF and 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), and the optimum concentration was found to be 10% milk. Concentrations above this showed no additional effect (Fig. 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 (Fig. 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 (Fig. 4B).

Study Series 3. Effect of Human Milk on Apoptosis In Vitro

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.
milk with or without neutralizing antibodies, PSTI, or EGF without indomethacin. In contrast, cells incubated with indomethacin alone had about a threefold rise in caspase-3 levels (Fig. 5A). This proapoptotic effect of indomethacin was markedly truncated by the copresence of PSTI, EGF, or human milk (Fig. 5, A and 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 because these effects were not seen when the caspase-3 inhibitor was 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. Coadministration of human milk caused a 75% reduction in amount of injury (Fig. 6, A and B). Compared with 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 copresence 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, Fig. 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 pHs in the range of 1–3.

DISCUSSION

We have shown that PSTI is secreted into human milk at concentrations similar to those of EGF. Milk produced during the first few days after birth (colostrum) contains much higher concentrations of PSTI compared with milk obtained later postpartum. 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 attributable to 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 defense 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 because 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 was performed using rats because we have previously validated this model for other nutritional-based growth factor products (for example, see Ref. 4). The use of caspase-3 analyses to determine apoptosis was based on well-validated methods (12), and the antiapoptotic effects of the milk seen in the in vitro study were 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 and applying them 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, TGF-α
concentrations in the milk were about 1,000-fold lower, and NGAL levels were unrecordably low. Because marked differences in growth factor constituents of milk occur between species (e.g., 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 promotogenic activity of EGF and PSTI and, for the first time, that synergistic responses were seen if PSTI and EGF are coadministered. The signaling mechanism behind this finding is unclear although the EGF receptor and tyrosine kinase signaling are probably involved as demonstrated by our previous findings that EGFR-blocking antibodies or tyrosine kinase inhibitors can prevent the promigratory 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 radiolabeled displacement studies (6, 14) and the divergence of results in the proproliferative activity of EGF and PSTI against various cell lines (6, 10, 14). It, therefore, seems likely that PSTI is inducing crossphosphorylation of the EGFR and/or influencing its downstream pathways as well as possibly acting via a distinct pathway.

In the normal nondamaged 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 necrotizing enterocolitis. Because 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 promigratory effects of human milk, this mechanism...
was probably not relevant in our short-term in vivo model because 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 immunoneutralization suggested that both EGF and PSTI were key peptides in mediating protection.

In addition to showing the promigratory effects of milk and PSTI, we showed they reduced nonsteroidal anti-inflammatory drug-induced apoptosis. Indomethacin influences apoptosis via several pathways including enhancing degradation of survivin via the ubiquitin proteasome machinery (as shown using colon cancer cell lines, 1). Further work is required to examine whether PSTI and milk influenced these or other pathways in mediating their antiapoptotic 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, which contains multiple bioactive molecules, may also offer advantages over that feeding neonates with human colostrum, which contains in human milk that can influence restitution, apoptosis, gut mucosa, 2) and by dissociating basal cell lymphomnal-2-associated protein (BAX) from basal cell lymphomnal extra large, thereby promoting BAX mitochondrial translocation and multimerization (as shown using colon cancer cell lines, 1).

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GRANTS

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