TGF- β 2, a Protective Intestinal Cytokine, Is Abundant in Maternal Human Milk and Human-Derived Fortifiers but Not in Donor Human Milk

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Abstract

Objective: This study compared cytokines (in particular transforming growth factor [TGF]- β 2) and lactoferrin in maternal human milk (MHM), human-derived milk fortifier (HDMF), and donor human milk (DHM).

Materials and Methods: MHM was randomly collected from breastfeeding mothers who had no infectious illness at the time of milk expression. HDMF and DHM were products derived from human milk processed by Holder pasteurization. MHM samples were collected at different times (early/late) and gestations (preterm/term). Lactoferrin was analyzed by western blotting, and cytokines were quantified using commercial enzyme-linked immunosorbent assays. Significance was determined using analysis of variance.

Results: In the 164 samples analyzed, TGF- β 2 concentrations in HDMF and preterm MHM (at all collection times) were fivefold higher than in DHM ($p < 0.05$). Early preterm MHM had levels of interleukin (IL)-10 and IL-18, 11-fold higher than DHM ($p < 0.05$). IL-6 in DHM was 0.3% of the content found in MHM. IL-18 was fourfold higher in early MHM versus late MHM regardless of gestational age ($p < 0.05$). Lactoferrin concentration in DHM was 6% of that found in MHM.

Conclusions: Pasteurization decreases concentrations of most cytokines and lactoferrin in DHM. TGF- β 2, a protective intestinal cytokine, has comparable concentrations in HDMF to MHM despite pasteurization.

Introduction

THE USE OF MATERNAL HUMAN MILK (MHM) has been shown to decrease the incidence of necrotizing enterocolitis (NEC) compared with formula.¹ The protection against NEC has been postulated to be due to heightened immunity and defense against infection.¹ Some of the key components of human milk, such as lactoferrin and cytokines, may play a role in intestinal disease.^{2,3} Lactoferrin is one of the major whey proteins in human milk, has antimicrobial properties, sequesters iron, and has been shown to reduce the incidence of late-onset sepsis in neonates weighing less than 1,000 g.² In addition, a systematic review of lactoferrin in combination with the probiotic *Lactobacillus rhamnosus* GG has shown a reduction in NEC.⁴ A beneficial effect of lactoferrin on iron acquisition in the gut is well documented. That process involves a receptor-mediated absorption of iron-bound lactoferrin through intestinal epithelial cells.⁵ The role of lactoferrin in transfer of iron from maternal milk is of utmost importance

because it alters the gut microbiome and, in turn, may lead to intestinal disease.⁶

Transforming growth factor (TGF)- β 2 has recently been found to play a key role in intestinal injury. Decreased TGF- β 2 expression and bioactivity in animal and human intestinal tissues have been associated with NEC.⁷ TGF- β is presumed to promote gut barrier function, immune tolerance, and mucosal repair in the neonatal gastrointestinal tract.⁸ The neonatal immune system undergoes extensive postnatal development, and the acquisition of intestinal microbiota is a major determinant of early immune development and may play a key role for the development of intestinal disease in the preterm infant.⁶ Specific roles of commensal microbiota and breastmilk have been documented in the induction of Toll-like receptor expression in human adult and fetal epithelial cells⁶; these effects may be modulated by various cytokines present in breastmilk and altered by pasteurization and/or time of lactation.

The use of an exclusive human-derived nutrition (to include human-derived milk fortifiers [HDMFs]) has reduced

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the incidence of NEC in preterm infants compared with use of combined bovine human milk fortifiers/formula.⁹ Donor human milk (DHM) has become increasingly used when MHM is unavailable; the use of DHM has been shown to decrease the use of formula in preterm infants and not affect the use of MHM.¹⁰ In a retrospective single-center study, the use of DHM decreased the incidence of surgical NEC.¹⁰ Contrary to this result, in a randomized trial, DHM used as a supplement to MHM was not superior to preterm formula for the reduction of NEC in preterm infants, but none of the groups had a strict human-derived diet when it has been shown to be beneficial.^{9,11} DHM undergoes pasteurization, which decreases bacterial and viral counts, including human immunodeficiency virus and cytomegalovirus, but levels of many of the beneficial immunologic factors decrease as well.¹² The amounts of these factors in DHM, HDMF, and MHM and the effect of pasteurization remain relatively understudied. Furthermore, changes in the concentrations of immune factors depending on the time of lactation may have an impact on their content in breastmilk and, in turn, may decrease the protective effect for NEC.

The purpose of this study was to compare the concentrations of lactoferrin, TGF- β 2, and other cytokines in MHM, DHM, and HDMF and to evaluate the effects of pasteurization and time of collection.

Materials and Methods

Subjects

Mothers delivering at University Hospital, San Antonio, TX, who planned on breastfeeding were randomly approached between January 26, 2009 and January 31, 2011 and asked to participate in this study. Inclusion criteria were all mothers who were planning to breastfeed. Mothers excluded from the study were those who chose not to breastfeed, had impaired decision-making capacity, had clinical evidence of mastitis, were taking medications that were contraindicated for breastfeeding (chemotherapeutics, radiation therapy, methotrexate, etc.), or who had human immunodeficiency virus, hepatitis B virus, or hepatitis C virus infection. This study was approved by the University of Texas Health Science Center at San Antonio Institutional Review Board.

Collection and processing of breastmilk

After informed consent was obtained, mothers provided a small sample (2–5 mL) of expressed breastmilk. The de-identified expressed breastmilk samples were collected and labeled as either early (<48 hours after delivery) or late (>48 hours after delivery) and either term ($\geq$ 37 weeks of gestation) or preterm (<37 weeks of gestation). The DHM samples were collected from the University Hospital Neonatal Intensive Care Unit supply in a random manner. The DHM samples were purchased from the Mother's Milk Bank at Austin (Austin, TX). The HDMF samples were donated by Prolacta Bioscience (Monrovia, CA). After the samples were collected and labeled, 0.5–1-mL aliquots were transferred into Eppendorf tubes and then centrifuged (Microcentrifuge; Beckman, Fullerton, CA) at 4°C for 10 minutes, the fat layer was removed, and the supernatant-rich whey was collected and placed into a new Eppendorf tube. The process was repeated a second time. Samples were then stored at –80°C in a Harris freezer until testing. Freeze–thaw cycles were minimized.

Pasteurization

Whole milk samples were pasteurized by either the Holder method or flash pasteurization. After aliquoting into Eppendorf tubes as described above, whole milk samples undergoing Holder pasteurization were heated to 63°C for 30 minutes and then cooled. Whole milk samples designated to undergo flash pasteurization were heated to 74°C for 30 seconds and then allowed to cool. After pasteurization, all whole milk samples were centrifuged as described above and treated similarly to unpasteurized samples.

Western blot analysis

Protein concentrations in centrifuged milk samples were determined using the Bio-Rad (Hercules, CA) DC protein assay with bovine serum albumin used as the standard. Ten micrograms of total protein was separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Bio-Rad). The separated proteins were transferred electrophoretically to polyvinylidene membranes (Millipore, Bedford, MA) using a semidry transfer blot system and blocked in Tris-buffered saline with 5% nonfat dry milk for 2 hours. The membranes were incubated overnight at 4°C with the lactoferrin primary antibody (Aldrich, St. Louis, MO) in Tris-buffered saline containing Tween-20 and 5% nonfat dried milk powder. The blots were then incubated with secondary antibody (Bio-Rad) conjugated to horseradish peroxidase appropriately diluted in the same buffer for 1 hour. Peroxidase-labeled proteins were detected with an enhanced chemiluminescence assay kit. Electrophoresis reagents were purchased from Bio-Rad, and enhanced chemiluminescence reagents, western blot reagents, and film were from Amersham Pharmacia Biotech (Little Chalfont, United Kingdom).

Enzyme immunoassays

The aqueous phase of human milk was quantified for lactoferrin, interleukin (IL)-6, IL-10, IL-18, and TGF- β 2 by enzyme immunoassays as described by the manufacturer. To quantify IL-6 in MHM, the specimens were diluted 50-fold but not in the DHM samples. To quantify lactoferrin concentrations in MHM, specimens were diluted 10,000-fold, and DHM samples were diluted 1,000-fold. TGF- β 2 was activated into an immunoreactive form using polypropylene tubes using 1 N HCl and then neutralized with 1.2 N NaOH/0.5 M HEPES as described in the manufacturer's directions. Enzyme-linked immunosorbent assay kits for lactoferrin, IL-6, IL-10, and TGF- β 2 were purchased from R&D Systems, Inc. (Minneapolis, MN). IL-18 enzyme-linked immunosorbent assay kits were purchased from MBL International (Woburn, MA).

Statistical analysis

Statistical calculations were performed with SPSS for Windows version 16.5 (SPSS, Inc., Chicago, IL). Differences between groups were determined using one-way analysis of variance, followed by Bonferroni's or Tukey's test. A value of $p < 0.05$ was considered to be statistically significant.

Results

In total, 357 mothers were approached; of these, 185 declined to participate or did not meet inclusion criteria. Of the

FIG. 1. (A) Representative western blot of human lactoferrin protein in a unpasteurized maternal human milk (MHM) sample versus one pasteurized by the Holder method. (B) Representative western blots for lactoferrin in preterm and term early (<48 hours) MHM, preterm and term late (>48 hours) MHM, donor human milk (DHM), and formula. The graph shows lactoferrin concentrations by enzyme-linked immunosorbent assay in the corresponding samples. Graphical data are mean $\pm$ SE values. * $p < 0.001$ compared with DHM and formula.

172 mothers who were recruited, 65 were unable to donate because of insufficient milk production. Therefore, in total, 107 mothers donated 107 samples. In total, 36 DHM samples were collected from the neonatal intensive care unit supply, and 21 HDMF samples were donated from Prolacta

Bioscience from at least eight different batch numbers. No demographic characteristics are presented as all samples were de-identified.

Recombinant lactoferrin protein was significantly decreased by pasteurization as shown in Figure 1A. DHM had

FIG. 2. Comparison of transforming growth factor (TGF)- β 2 concentrations in maternal human milk (MHM), donor human milk (DHM), and human-derived milk fortifier (HDMF). Data are mean $\pm$ SE values. * $p < 0.05$ compared with DHM, ** $p < 0.05$ for HDMF compared with term late MHM.

50% less lactoferrin protein expression by western blot analysis than all of the MHM samples irrespective of gestational age or time of collection ($p < 0.05$). Similarly, the lactoferrin concentrations of DHM was 6–7% of the amount found in any of the MHM samples regardless of gestation and time of collection when measured by enzyme-linked immunosorbent assay analysis ($p < 0.01$), and no lactoferrin was found in formula samples (Fig. 1B).

TGF- β 2 concentrations were significantly higher in preterm early MHM, preterm late MHM, and HDMF samples compared with DHM ($7,415 \pm 1,489$ pg/mL, $9,216 \pm 1,344$ pg/mL, and $11,079 \pm 665$ pg/mL, respectively, versus $1,538 \pm 345$ pg/mL; $p < 0.05$). In addition, TGF- β 2 levels in HDMF were also significantly higher than term late MHM samples ($p < 0.05$) (Fig. 2). In total, 10 HDMF and MHM samples with the highest levels of TGF- β 2 were then pasteurized by Holder and flash pasteurization. Levels of TGF- β 2 were reduced by 14% after Holder pasteurization and 38% after flash pasteurization.

FIG. 3. (A) Interleukin (IL)-6 and (B) IL-10 concentrations in maternal human milk (MHM), donor human milk (DHM), and human-derived milk fortifier (HDMF). Data are mean $\pm$ SE values. * $p < 0.05$ compared with DHM and HDMF.

FIG. 4. (A) Comparison of interleukin (IL)-18 concentrations in preterm early and late maternal human milk (MHM), term early and late donor human milk (DHM), and human-derived milk fortifier (HDMF). * $p < 0.05$ compared with DHM and HDMF. (B) Comparison of IL-18 levels in all early MHM samples, all late MHM samples, and DHM samples. * $p < 0.05$ compared with late MHM and DHM.

For IL-6, preterm early MHM, term early MHM, and term late MHM had significantly higher levels compared with both DHM and HDMF (135 ± 46 pg/mL, 160 ± 25 pg/mL, and 131 ± 41 pg/mL, respectively, vs. 0.45 ± 0.08 pg/mL and 0 pg/mL, respectively; $p < 0.05$) (Fig. 3A). Only preterm early MHM had significantly higher levels of IL-10 versus DHM (28.1 ± 9.9 pg/mL vs. 2.4 ± 0.5 pg/mL, $p < 0.05$). No other significant differences were found among MHM stages or compared with HDMF with regard to IL-10 (Fig. 3B).

IL-18 levels of preterm early MHM were significantly higher than those of DHM and HDMF (500 ± 249 pg/mL vs. 42 ± 32 pg/mL and 2 ± 1.3 pg/mL, respectively; $p = 0.01$) with a trend toward higher levels in term early MHM versus DHM and HDMF (Fig. 4A). When all early MHM samples were combined and compared with late MHM and DHM samples, early MHM samples had significantly higher IL-18 levels compared with late MHM and DHM (487 ± 158 pg/mL vs.

104±37 pg/mL and 42±32 pg/mL, respectively; $p < 0.01$) (Fig. 4B).

Discussion

MHM has large amounts of lactoferrin and key cytokines related to intestinal health/injury compared with pasteurized human milk products. We have shown that lactoferrin concentrations are significantly decreased in DHM and that pasteurization of DHM is a factor in the decrease of lactoferrin concentration. Studies have shown that pasteurization decreases or inactivates bioactive components of breastmilk, which may impact the ability of human milk to protect against NEC.^{13,14} Other factors, such as exposure to freeze-thaw cycles, may play a role in the decreased lactoferrin concentrations. However, these studies have not controlled for freeze-thaw cycling or pasteurization alone and the association with decreased bioactivity and anti-infective properties of milk.¹² In our study, freeze-thaw cycles are not a factor because we minimized to one freeze-thaw cycle at most, and therefore the results are solely due to the effects of pasteurization. Proteolysis of lactoferrin occurs under acidic conditions (as it occurs in the stomach), and peptides with enhanced antimicrobial activity are released¹⁵; we speculate that proteolysis of both lactoferrin and peptides occurs during pasteurization, and therefore their activity is blunted after heat exposure.

Lactoferrin modulates cytokine and/or chemokine production by the gut-associated lymphoid tissue, which then enters the systemic circulation and influences circulating leukocytes.⁵ Lactoferrin creates an environment for the growth of beneficial bacteria in the gut, reducing colonization with pathogenic bacteria.^{2,4} In vitro studies have shown that lactoferrin acts synergistically with anti-staphylococcal and anti-*Candida* agents.¹⁶ The facts that intestinal receptors for lactoferrin have been demonstrated and that lactoferrin has the ability to modulate intestinal cell differentiation and proliferation¹⁷ make lactoferrin a promising agent in the prevention or treatment of NEC. Bovine lactoferrin supplementation was studied in a randomized trial and found to significantly decrease late-onset sepsis episodes and approached significance for decreased occurrence of NEC of stage 2 or greater and death compared with controls.² In this setting, it only reduced the incidence of NEC when bovine lactoferrin was used in conjunction with *L. rhamnosus* GG. This effect may have been due to an interaction of the *Lactobacillus* with bovine lactoferrin to boost the defenses of an immature intestine or the cumulative effect of iron trapping for pathogenic bacteria along with enhancement of benign microflora.² Currently, bovine lactoferrin has not been approved for use in the United States. With the recent findings of a reduction of NEC with a total human-derived nutrition⁹ and the lower concentrations of lactoferrin found in DHM and HDMF in this study, recombinant human lactoferrin supplementation of DHM may be warranted.

We also found that TGF- β 2 was consistently abundant in HDMF. The importance of this finding is enhanced by recent data from our group where premature baboon intestine expressed less TGF- β 2 than term intestine, in particular in those baboons that developed NEC spontaneously.¹⁸ In human and murine models, a protective effect of TGF- β 2 is seen because

of decreased intestinal cell apoptosis.⁷ In addition, TGF- β 2 suppresses macrophage cytokine expression and mucosal inflammatory responses in the developing human intestine by specifically attenuating IL-1 β -induced inflammatory responses.^{7,8,19} Furthermore, data from other inflammatory intestinal diseases have shown a clear role of TGF- β 2. For example, in children with Crohn's disease, a polymeric diet rich in TGF- β 2 for 8 weeks induced remission, promoted mucosal healing, and decreased levels of biochemical markers of inflammation and pro-inflammatory cytokines like tumor necrosis factor- α , IL-8, interferon- γ , and IL-1 β .^{19,20} Furthermore, a randomized controlled trial of 32 children with active Crohn's disease reported remission as well as improved disease, endoscopic, and histologic scores in the nutritional therapy group (diet rich in TGF- β 2) and concluded that a diet rich in TGF- β 2 was as efficacious as corticosteroids.²¹ In adult studies, one retrospective report showed 50% clinical remission rates, but the efficacy in adults remains to be fully elucidated.²²

The high content of TGF- β 2 in HDMF may explain the decreased incidence and severity of NEC (less surgical NEC) seen in preterm infants allocated to receive an exclusive human-derived nutrition.⁹ In preclinical models, it has been shown that enterally administered TGF- β 2 can protect against intestinal injury similar to NEC, an inflammatory bowel necrosis of premature infants.^{7,19} It was surprising that DHM had minimal amounts of TGF- β 2, whereas HDMF did not. The striking differences between HDMF and DHM may be related to the processing of HDMF; it comes from pooled samples of the breastmilk cream, which are highly concentrated, and as a result TGF- β 2 might be concentrated as well. The bioactivity in comparison with MHM was not determined in this study. Thus, the anti-inflammatory properties of TGF- β 2, especially in the setting of an inflammatory disease such as NEC, need to be further investigated with HDMF and/or with recombinant TGF- β 2 as a potential supplement to MHM or DHM.

IL-18, a pro-inflammatory cytokine that is produced by macrophages, keratinocytes, and intestinal epithelial cells, was found in minimal quantities in DHM and HDMF. In the rat model of NEC, IL-18 is up-regulated in ileal tissue, and higher IL-18 levels correlated with more tissue damage.²³ IL-18 was also found to be higher in human milk from mothers with preterm delivery or pregnancy complications.²⁴ It is surprising that we found that all early MHM samples had higher levels of IL-18, but because of the wide variations in concentrations of this particular cytokine, the implications of these findings are unknown. Larger studies are needed to elucidate if a critical "dose" is related to intestinal injury.

IL-6 was present in MHM at all time points, whereas DHM and HDMF had minimal levels of this cytokine. IL-6 is a pro-inflammatory and anti-inflammatory cytokine and has an uncertain role in NEC. It has been found to be elevated in level in ascitic fluid and plasma in infants with NEC.^{25,26} There are limited data with regard to the intestinal effects of IL-6 supplementation and/or concentrations found in MHM. Therefore, it is unknown if the concentrations of IL-6 in enteral feeds will predispose infants for NEC. This study provides additional data of the content of IL-6 in MHM at different time points and gestations, and because it was found consistently, it will provide the foundation for further prospective studies.

On the other hand, IL-10, which promotes immunoglobulin A production,²⁷ was found in high concentrations only in the early preterm milk group. The increased levels of IL-10 may be protective early in the course of prematurity. Previous studies have found that the IL-10 level is reduced after Holder pasteurization, which is consistent with the reduced levels in DHM and HDMF found in this study.¹⁴ It is interesting that IL-10 has been found to be absent in up to 86% of maternal milk in those infants who developed NEC.^{27,28} IL-10 knockout mice develop an enterocolitis similar to that of NEC seen in preterm infants.²⁹ These findings enhance the importance of early feeds, in particular those of colostrum of preterm milk, which has the highest concentrations of IL-10.

The choice of infant feeding may hold important health consequences in preterm infants. In particular, alterations in the gut microbiome might exist depending on the type of enteral feeding regimen. Studies have shown significant differences in the development of the gut microbiome in formula-fed infants compared with breastfed infants,³⁰ and those with exclusive human milk diets may have decreased risk of intestinal disease even when breastmilk has been pasteurized.

In conclusion, early milk/colostrum is rich in lactoferrin and potentially protective cytokines, whereas DHM is not. HDMF has large quantities of TGF- β 2, which in turn may have a protective effect for NEC in preterm infants. Providing early MHM to preterm infants along with an exclusive human nutrition with human-derived supplements may offer additional intestinal protection.

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