Early Human Development 91 (2015) 7–11

Contents lists available at ScienceDirect

Early Human Development

journal homepage: www.elsevier.com/locate/earlhumdev

Effect of gestation length on the levels of five innate defence proteins in human milk

Marita Broadhurst [a], Keryn Beddis [a], Janet Black [b], Harold Henderson [a], Arun Nair [b], Thomas Wheeler [a,*]

[a] AgResearch, Ruakura Research Centre, Hamilton, New Zealand
[b] Neonatal Unit, Waikato Hospital, Private Bag 3200, Hamilton, New Zealand

ARTICLE INFO

Article history:
Received 20 September 2013
Received in revised form 3 November 2014
Accepted 6 November 2014

Keywords:
Lactoferrin
Immunoglobulin A
Immunoglobulin G
Complement C3
Secretory component protein

ABSTRACT

Background: Human milk contains a range of host defence proteins that appear to contribute to health and wellbeing, but their variability in abundance among individuals has not been very well characterised. Milk from mothers of premature infants has altered composition, but the effect of gestation length on the host-defence properties of milk is not known. A study was therefore undertaken to determine the variability and effect of gestation length on the abundance of five host-defence proteins in milk; lactoferrin, secretory IgA, IgG, secretory component, and complement C3.

Methods: Milk was obtained from 30 mothers at their second and fifth week of lactation. These were from three groups of ten mothers having had very premature (V; 28–32 weeks gestation), premature (P; 33–36 weeks) or full term deliveries (T; 37–41 weeks). The concentration of each of the five proteins was measured in each milk sample by either ELISA or quantitative western blotting.

Results: The concentration of IgG, and complement C3 ranged 22- and 17-fold respectively between mothers, while lactoferrin, secretory IgA, and secretory component ranged 7-, 9-, and 4-fold, respectively. The V group had significantly lower concentrations of four of the five proteins, the exception being IgG. Levels of these four proteins also decreased between weeks 2 and 5 of lactation in the P and T groups. Significant correlation was found between the concentrations of the host defence proteins within individual mothers, indicating some degree of co-ordinate regulation.

Conclusions: Mothers vary widely in the levels of host defence proteins in milk. Very short gestation length results in decreased abundance of host-defence proteins in milk. This may have functional implications for very premature infants.

1. Introduction

Milk plays a critical role in providing nutrition to the newborn mammal as well as protecting the neonate against infection. This latter functionality of milk is delivered in part through a range of antimicrobial, pathogen-recognition and other host defence proteins which form part of the complement of minor proteins in milk [1–4]. The abundance of some of these proteins varies considerably between individuals. Some milk proteins are up-regulated as part of the inflammatory response that occurs during infection of the mammary gland [5–7]. Some host defence proteins are also known to be altered in abundance with the stage of lactation [8–11]. This variability in the protein composition of milk, at least in part, may reflect the changing need of the offspring during its growth and development.

Premature babies have specific nutritional requirements [12] and infection is of particular concern. The composition of the milk from preterm mothers is known to be altered compared with full-term mothers [13,14]. However it is not clear whether this is a response to addressing the premature infant's needs, whether it is due to immaturity of mammary development, or for other reasons. Several studies have addressed whether a shorter than normal gestation length affects the host defence proteins in milk. Immunoglobulin A has been reported to be higher in milk from short gestation mothers compared with normal length gestation [15–18]. In contrast, other studies have reported lower IgA levels in milk from mothers of very pre-term babies [19,20]. These studies revealed a high degree of variability in the concentration of IgA between the individual donors. The levels of another host defence protein, lactoferrin, has also been reported to be either lower [18,19] or higher [21] in milk from mothers of premature babies. Thus there is a lack of clarity as to what extent the concentration of host defence proteins vary among individuals, and if the composition of these host defence proteins is altered in pre-term mothers' milk compared with full term mothers.

In a study of 30 mothers (10 in each of three gestation length groups) we wished to determine the extent of within-mother and between-mother variability in the abundance of a range of host defence

* Corresponding author at: AgResearch, Ruakura Research Centre, Private Bag 3123, Hamilton, 3240 New Zealand.
E-mail address: tom.wheeler@agresearch.co.nz (T. Wheeler).

http://dx.doi.org/10.1016/j.earlhumdev.2014.11.006
0378-3782/

proteins in milk during established lactation, and the extent to which the levels of these proteins were altered by gestation length. The levels of five host defence proteins were measured; lactoferrin, secretory IgA, IgG, secretory component, and complement C3. Large variations were found for each of the proteins, with some differences between the gestation-length groups. These data will provide a basis for developing approaches to provide better natural defence against infection for premature infants.

2. Materials and methods

2.1. Collection of milk samples

Between 3 and 5 ml of breast milk was collected either manually or using a breast pump from a total of 30 mothers on two separate occasions; two weeks and five weeks after giving birth. Ten of the mothers had delivered between 28 and 32 weeks of gestation (very premature, V), another ten had delivered between 33 and 36 weeks of gestation (premature, P), while the remaining ten had delivered between 37 and 41 weeks of gestation (normal term, T). The mothers were recruited to the study at the Waikato Hospital Neonatal Unit, Hamilton, New Zealand over a seven month period based on willingness to participate, the absence of any health issue in the mother–infant dyad at time of each collection, and no signs of mastitis since giving birth. A formal informed consent was obtained from each mother. The recruitment procedure was approved by the Northern Y Regional Ethics Committee, based in Hamilton, New Zealand. All the mothers underwent a spontaneous parturition, with 12 of them having a caesarean section delivery. All were non-smokers, and there were no significant differences among the groups in age of the mother, parity or the sex of the babies. However, the P group was more diverse in age of the mother compared to the other groups, and there were 3, 2 and 1 multiple births in the V, P and T groups, respectively.

The milk samples were collected at home and chilled to approximately 6 °C within 10 min of collection and transported on ice to the laboratory within 24 h. The protease inhibitor phenylmethanesulfonyl fluoride (PMSF) was added to a concentration of 1 mM. The milk was centrifuged at 800 *g* for 10 min at 4 °C. The fat layer was removed and aliquots of the skim milk were transferred into fresh tubes and frozen at −20 °C. These were used for all the subsequent analysis. The integrity of the milk proteins was confirmed by electrophoretic analysis.

2.2. Analysis of milk samples

Total protein concentration of each skimmed milk sample was determined by Bradford assays using commercially supplied reagents (Bio-Rad, Hercules, CA). The concentrations of lactoferrin, secretory IgA, and IgG in skimmed milk were measured by ELISA using commercially supplied kits (Bethyl Laboratories, Montgomery, TX). The concentration of the complement factor C3 was also measured by ELISA using a commercially supplied kit (Genway Biotech, San Diego, CA). Each sample was diluted 1/3000 and 1/5000 for lactoferrin, secretory IgA, and IgG assays, and 1/80,000 and 1/160,000 for the C3 assay. Each dilution was measured in duplicate alongside a set of standards and the results averaged. No matrix effects were observed for the sample analyses at the dilutions used.

The concentration of secretory component in the milk samples was measured using quantitative western blotting using a commercially available polyclonal anti-human secretory component antibody raised in sheep (Sigma, St Louis, MO). Five serial dilutions of purified secretory IgA (Sigma) were used as a standard and analysed on the same gel as the milk samples to be quantified. The quantity of secretory component was calculated based on its known stoichiometry in secretory IgA. Samples and standards were resolved by SDS polyacrylamide gel electrophoresis, transferred to nitrocellulose, blocked with 4% (w/v) nonfat milk powder and probed with anti-secretory component antibody at 53 ng/ml. After washing, the blot was probed with anti-sheep IgG conjugated to horseradish peroxidase (Sigma). After three washes the blot was incubated for 2 min in a freshly prepared mixture of 1.25 mM luminol (Sigma), 67 μM p-coumaric acid (Sigma) and 0.01% (w/v) hydrogen peroxide in 0.1 M Tris.HCl, pH 8.8. The luminol and p-coumaric acid were added from stocks of 500 mM luminol and 168 mM p-coumaric acid in DMSO. The hydrogen peroxide was added from a 30% (v/v) stock solution (Scharlau, Sentiment, Spain). The chemiluminescent signal was captured using a CCD camera-based detector (Chemidoc, Bio-Rad) and processed to produce quantities of secretory component in the samples and standards using the Quantity One software package (Bio-Rad). Between two and eight replicate analyses of each sample were performed, at a range of dilutions.

2.3. Statistical analysis

The data were subjected to ANOVA using GenStat. Statistical analysis of the total protein concentration, and the logarithm of individual protein concentrations were analysed with a linear mixed model analysis in GenStat with gestation group, week of lactation and their interaction as fixed effects, and individual mother and individual mother-within-week as random effects. Within-assay variability was assessed for individual protein concentrations, with coefficients of variation attained between 10 and 15%. Pearson's correlation analysis was performed using the GenStat software package.

3. Results

The abundance of total protein, as well as that of five host-defence proteins (lactoferrin, secretory IgA, IgG, complement C3 and secretory component) was determined in milk samples collected from each of 30 mothers, falling into one of three groups; 10 having had a normal term gestation (T) of 37 to 40 weeks, 10 having had a premature delivery (P) between 33 and 37 weeks, and 10 having had a very premature delivery (V) of between 26 and 33 weeks. For all but one of the pre-term mothers, samples were obtained at both week 2 and week 5 of lactation. The concentration of total protein obtained from one of the term mother's week 5 sample was very much higher than the others (Fig. 1). This sample also had very high levels of the individual proteins — between 2 fold and 40 fold higher than the average for the remaining samples. This outlier was most likely due to an inflammatory response as a result of a subclinical infection of the mammary gland at the time of collection. The values obtained from this sample were therefore removed from subsequent statistical analyses and figures. A large and significant degree of variation among the mothers was observed for each of the proteins.

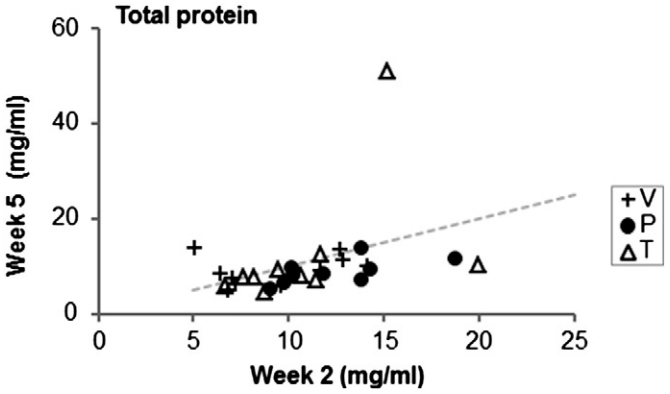

Fig. 1. Total protein concentration of milk from mothers at weeks 2 and 5 of lactation. The protein concentrations (mg/ml) obtained from milk samples from each mother at week 5 was plotted against the protein concentration of the milk sample obtained at week 2 from the same mother, for each of the 10 mothers in each of the groups (very premature (V), premature (P), and full term (T)). The dashed line represents equal concentration for weeks 2 and 5.