Human Milk Bioactivity: Lessons from the Evolution of Lactation

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Lactation has evolved over the past 200 million years since the appearance of the aplacental, egg laying monotremes. Subsequently, there has been an extensive adaptation to reproduction, particularly in lactational strategies, when the Theria (subclass of mammals) split into the Metatheria (Mesomammalia) and Eutheria (Placentalia) lineages over 140 million years ago (1). The role of milk in providing nutrition to the suckled young in mammals is well established. However, over the past few decades a focus on the functionality of milk has confirmed that it also delivers a range of bioactives that are either present in milk in the mammary gland or are released when the milk is processed in the oral cavity and the gut of the neonate. These bioactives may include proteins, peptides, complex oligosaccharides and miRNA and have the potential to stimulate development, regulate gut flora and provide protection for the young from infection (2-8). An interesting and often neglected group of bioactives is now being identified; they have a function locally within the mammary gland to remodel the mammary tissue (stimulate growth or signal cell death) and can provide protection from infection and inflammation when the mammary gland is susceptible to these challenges. This review will focus predominantly on the relationship between milk proteins and proteases, resulting in the release of bioactive peptides.

Milk Bioactives – Impact on Development of the Young

To better identify bioactives, scientists have increasingly used comparative genomics and bioinformatics to explore the lactational strategies of the Australian monotremes and marsupials. The lactation cycle in the only two extant monotremes, the echidna (Tachyglossus and Zaglossus genera) and platypus (Ornithorhynchus anatinus), and an Australian marsupial, the tammar wallaby (Macropus eugenii) have been studied extensively (9,10). The echidna lays shelled eggs (11) and the hatchlings are altricial (undeveloped) and not immune competent (12). Therefore, milk is not only important for growth and development but particularly for protection of the young from disease while they are in a non-sterile environment (13,14). Reproduction in the tammar is characterised by a short gestation period (26.5 days), birth of immature young and a long lactation period (approximately 300 days), during which the concentration of all the major milk constituents, and many minor milk bioactives progressively change (15) (Fig. 1A). There is increasing evidence that these changes in milk composition regulate growth of the tammar pouch young (10,16), particularly during the first 100 days postpartum, when the development of the neonate is similar to a late stage eutherian fetus. Therefore the signalling factors involved in the development of the eutherian fetus are most likely delivered to marsupials in the milk of the mother (17) (Fig. 1B). Fostering experiments demonstrated that transferring the early phase pouch young to a late phase lactating tammar can accelerate the growth and physical development of pouch young (18,19), and also accelerate maturation of specific organs such as the stomach (20). More recent studies using in vitro models have shown that milk collected from marsupials during early lactation (day 20–100), but not late lactation (day 100–300), stimulated proliferation and differentiation of whole lung cultures from mouse embryos (21). Therefore the temporal delivery of these bioactives is most likely crucial for the development of the suckled young.

The regulation of the lactation cycle in the tammar wallaby has fascinated and challenged scientists for many decades and the interesting interplay between the endocrine, autocrine and paracrine mechanisms that are implicated in this process are now beginning to be better understood. If these animals are genuine biomedical research models that offer new insights into reproduction in eutherians, it must be assumed that ‘evolution has not reinvented the wheel’ and that the basic mechanisms regulating cellular responses have been conserved over the past 140 million years.

This assumption appears to be reasonably sound. Research over the past 50 years has shown that insulin, glucocorticoids and prolactin are required for lactation in eutherians and a number of in vitro studies have shown these hormones are a basic requirement for successful lactation in the tammar and echidna (22-26). It is now accepted that the mammary extracellular matrix (ECM) has...
an integral role and is essential for the development of the mammary gland during gestation, the overt differentiation of mammary epithelial cells for galactopoesis and remodelling of the mammary gland during involution (27-29). More recent studies have shown that the ECM in the tammar has additional roles in regulating the cellular program for the lactation cycle in the tammar, particularly in controlling the progressive changes in milk composition (30,31). Perhaps one of the most exciting and compelling studies on the regulation of lactation was reported recently by Jane Visvader’s laboratory (29). The experiments using a mouse model showed that binuclear mammary epithelial cells appeared in the mammary gland at lactogenesis and disappeared at involution. The presence of these cells in the mammary gland was essential for successful lactation. However it was fascinating to note that these binuclear cells were also present in lactating tissue from the human and wallaby, indicating that this basic regulatory process was conserved during the evolution of lactation.

It is ironic that marsupials may be considered a primitive mammal. However, the reality is that the mammary gland in these species is very sophisticated in terms of its capacity for temporal delivery of bioactives for multiple targets. The eutherian mammary gland is less sophisticated as many of its previous functions have evolved to be delivered by multiple tissues, particularly a well-developed placenta and the amniotic fluid. It is clear that the marsupial provides a unique opportunity to more easily identify bioactives that potentially play a role in the early development of the fetus. However, it may suggest that an increased focus on human colostrum would be appropriate to get a better understanding of its potential role in regulating early development of the baby, particularly at a time when the gut is amenable to increased transfer of milk components.

We have known for some time that significantly premature and low birthweight babies have acute challenges for survival, largely due to limited development of their lungs and gut, and these babies may show an increased frequency of mature onset disease (32). Studies using the tammar wallaby may lead to a new range of

Fig. 1. The lactation cycle of the tammar wallaby.

A. The lactation cycle in the tammar has been divided into four phases, characterised by changes in milk composition and the sucking pattern of the pouch young.

B. Development of the tammar pouch young from day 6 to day 220 postpartum (upper panels) compared to development of the human embryo at day 23 and preterm baby at 24 weeks of age (lower panels).
human fortifiers that include bioactives with the potential to specifically target the growth and development of tissues in the human neonate to improve outcomes for premature and low birthweight babies (33,34).

Therefore, developmental clocks are set in the neonate and any potential disruption to this process may subsequently impact on mature onset disease. The tammar provides a new model to better understand this process of developmental programming. It is not known if this developmental program is set during the short gestation or whether the milk provided in early lactation includes signals to the altricial neonate that have a role in this process. The option of cross fostering neonates to mothers at advanced stages of lactation to exclude the delivery of putative milk bioactives to the suckled young (33-35) may shed new light on the initiation of developmental programming.

Milk Bioactives That Impact on Mammary Function

The early work of Li et al. (36) showed that ligating the teats on one side of a lactating mouse led to these mammary glands progressing only to involution, despite all mammary glands being exposed to the same hormonal milieu. This was clear evidence that milk bioactivity could impact locally on mammary gland physiology. More recent work using the tammar has shown that the mammary gland has a sophisticated capacity to use milk when under challenge from infection and that milk can stimulate growth, and program cell death of the tissue when appropriate (36,37). Mammary genes coding for milk proteins can be multifunctional and demonstrate a temporal domain-specific bioactivity resulting from alternate splicing (38). For example, cathelicidin usually exists in an inactive proform and is cleaved by specific proteases to provide the two domains of cathelicidin that may have a variety of functions (39). The tammar cathelicidin 1 gene (MaeuCath1) is differentially expressed in the mammary gland throughout the lactation cycle as two splice variants (MaeuCath1a and 1b) (38). The level of MaeuCath1a transcripts are upregulated only during early lactation (2A) and late involution whereas MaeuCath1b transcripts are expressed throughout lactation and steadily increase from late lactation until early involution. The protein corresponding to MaeuCath1a significantly inhibits a range of bacteria (38). The expression of this transcript in the first 48 hours postpartum to day 80 postpartum is consistent with a need to provide protection from pathogens (40). MaeuCath1a expression at day 10 of involution suggests an additional antibacterial role at a time when the mammary gland is more susceptible to pathogen-mediated mastitis (41). The continued expression of the MaeuCath1b transcript when the neonate has developed adaptive immunity suggests additional roles for the maintenance and proliferation of mammary epithelia during increasing milk production (23,42). Indeed, several studies have suggested a role for cathelicidins in epithelial cell proliferation during wound healing, maintenance and re-establishment of the intestinal barrier integrity and proliferation of lung epithelial cells (43-45). More recent in vitro studies have shown wallaby mammary epithelial cells exhibiting increased proliferation following inclusion of MaeuCath1b in the media, confirming this hypothesis (38).

Another interesting tammar milk protein, WAP four-disulphide domain protein-2 (WFDC2) is part of a large family of whey acidic protein (WAP) four disulfide core proteins (46-49). Tammar WFDC2 is comprised of two four-disulfide core domains annotated as domain III on the amino terminal end and domain II at the carboxyl terminal end (50). Expression of the WFDC2 gene is elevated only in pregnancy, early lactation and during involution (51). Studies by Watt et al. (52) showed domain II of the protein had antibacterial activity against a range of pathogenic bacteria but no antibacterial activity against Enterococcus faecalis. The elevated expression of WFDC2 during pregnancy and involution correlates with the timing of increased risk of infection in the mammary gland (40,52,53), largely resulting from Staphylococcus aureus, Streptococcus spp. and Escherichia coli in the mammary tissue (54-56). However, expression of this gene during the first 100 days postpartum suggests an additional role in protecting the pouch young when it is not immune competent (52). Therefore, the antibacterial effect of WFDC2 appears to be directed to pathogenic bacteria and not commensal bacteria (40,53,57). The down regulation of WFDC2 around 100 days postpartum, when the young detaches from the teat, correlates with the development of an immune response in the young.

Human Milk Bioactivity – Acute Response to Challenge

The examples of the temporal regulation of either intact proteins or domain-specific expression of proteins to provide the kind of milk bioactivity required by the tammar mammary gland and the suckled young is dependent on sophisticated programming of the mammary gland. However, as discussed previously, there is a reduced need for this level of progressive change in the delivery of specific bioactives in human milk, although it is evident that human milk composition can change during lactation and in response to specific challenges to the breast (58,59).

A recent study by Sharp et al. (2016) using microarrays to analyse the expression of genes in cells present in breast milk at days 24, 48 and 101 of lactation and days 7 and 14 of involution identified a total of 206 protease genes (60). A large component of these genes were predicted to code for proteins with a signal peptide. Predicted classes of secreted proteases included threonine, aspartate, cysteine, serine and metallo proteases. Some of these genes were expressed throughout lactation whereas other genes were expressed only at specific time points during lactation. Bioactive milk peptides resulting from proteolytic digestion had functions that included immunomodulatory (61,62), antimicrobial (63-65), and antithrombotic (66,67) activity, and these peptides could act as opioid agonists (68-71), ACE inhibitors (72) and proliferative factors (73). In recent studies, skim breast milk was incubated at 37 degrees over a period of 5 days to allow digestion of milk protein by endogenous proteases (Watt, Nicholas and Sharp, unpublished data). Peptides were predominantly derived from cassein proteins, and antibacterial assays
using mastitis causing *S. aureus* showed that incubating this hydrolysate significantly increased antimicrobial activity. Similarly, these peptides showed anti-inflammatory activity. Importantly, the peptides did not show any capacity to program apoptotic activity in human mammary epithelial cells. Therefore these peptides may play a specific role in the breast to reduce infection and inflammation if there is an interruption to breastfeeding.

**Acute Responses to Mastitic Challenge**

Determination of gene expression in normal lactating women demonstrated the utility of RNA obtained directly from human milk cells to detect mammary epithelial cell (MEC)-specific gene expression. Therefore milk cell RNA collected during mastitis was also analysed by human Affymetrix arrays and revealed regulation of a unique set of genes specific to this disease state, whilst maintaining regulation of milk synthesis genes (60). Genes specific to infection and related to immune function, antimicrobial response, and anti-inflammatory response were upregulated compared to healthy milk (Fig. 2). More recent studies (Watt, Nicholas and Sharp, unpublished data) have used human MECs cultured on a range of ECMs which allows the formation of three-dimensional acini (mammospheres) that respond to insulin, cortisol and prolactin to express milk protein genes. These mammospheres were challenged with lipoteichoic acid (LTA) and showed similar transcriptional responses to milk-derived cells from women with mastitis (Fig. 3). These *in vitro* acini therefore represent a robust and relevant human mammary model to better understand the acute molecular responses to mastitic challenge, and may therefore provide new opportunities for improved treatment of breast infection.

This review has focused on the relationship between milk proteins and peptide bioactivity. Although it is becoming evident that breastmilk bioactivity for the development of the young has been diminishing with a greater contribution from the placenta, there is still much to explore with regard to the complexity of human milk. The established role of complex carbohydrate on gut flora and the general health of the young and the emerging role of miRNA as legitimate signalling molecules for both the breast and the young (74,75) emphasise the complexity of the multiple roles of milk, in addition to its role in nutrition. However, it is increasingly evident that the interrogation of the evolutionary history of lactation has great promise for the application of comparative approaches to obtain a better understanding the role of milk in acute and chronic wellbeing of the baby.

**References**

Fig. 3. Mastitis-specific gene expression mimicked by in vitro mammospheres.
Expression profiles of genes which were shown to be upregulated in mastitis were also induced when mammospheres were incubated with LTA for 4 and 24 hours.

A. Haematoxylin and eosin stain of paraffin embedded milk cells collected from mature milk. Arrows indicate presence of intact cells showing stained nuclei and cytoplasm. Binucleated cells can be observed.

B–D. Gene expression profile of milk cells collected from the different stages of lactation (24, 48 and 101 days) and mastitis (day 23).

E. Bright-field microscopy of human mammospheres grown for 7 days.

F–H. Gene expression profiles of 7 day mammospheres treated with and without LTA for 4 and 24 hours.
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