Molecular Determinants of Milk Lactoferrin as a Bioactive Compound in Early Neurodevelopment and Cognition

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Lactoferrin is a sialic acid-rich, iron-binding milk glycoprotein, known to have multifunctional health benefits, including its ability to modulate immune function and facilitate iron absorption, as well as its antibacterial and anti-inflammatory actions. Human milk contains significantly higher lactoferrin levels than bovine milk at the same stages of lactation. The purpose of this review is to discuss the current state of knowledge of lactoferrin as a conditional nutrient for neurodevelopment, neuroprotection, and cognitive function during the period of rapid brain growth. *(J Pediatr 2016;173S:S29-36)*.

Nutrients can significantly affect multiple neural developmental processes by regulating neurotransmitter pathways, synaptic transmission, signal-transduction pathways, and synaptic plasticity and, thus, have a long-term influence on cognitive events well into adulthood. Infants are born with all of their neurons formed, with a very limited degree of neurogenesis in the postnatal period. However, the growth of synaptic connections between these neurons and the acquisition, development, and formation of myelin sheath around nerve fibers are in large part established and elaborated in the early postnatal life.[1] The first 2 years of a child's life are critical, as the brain reaches 80% of its adult weight.[1,2] The rapidly developing neonatal and postnatal brain is most vulnerable to nutrient insufficiency, yet, also possesses a high degree of plasticity. Many crucial molecules are known to impact neural development, including iron, zinc, omega-3 fatty acids, choline, and sialylated glycoconjugates, suggesting the beneficial structure-function relationship in the developing brain.[1,3,4] Human milk contains all the nutrients an infant needs in the first 6 months of life. Lactoferrin, with its iron-binding property, is one of most abundant sialylated glycoprotein in human milk. Iron is an essential nutrient, which has structural and functional role in improving cognitive and motor development. Sialic acid (Sia) is a key monosaccharide for synthesis of brain gangliosides and sialylated glycoproteins, including polysialic acid (polySia) on the neural cell adhesion molecules (NCAMs). The brain gangliosides and polySia NCAMs are of critical importance in brain and cognitive development. Therefore, we hypothesized that dietary lactoferrin may have a beneficial impact on neural development, cognition, and memory and support the developing brain to function to its full potential. We tested this hypothesis, using our established piglet animal model[5] to elucidate the molecular basis of how lactoferrin improves neurodevelopment, neuroprotection, and cognitive function. This article sheds some light on the recent work, to strengthen the role of lactoferrin in neurodevelopment and cognition, and to review ongoing work.

Chemical Features of Lactoferrin

Lactoferrin is about 80-kDa glycoprotein composed of 703 amino acid residues and is a member of the transferrin family.[6,7] As a member of the transferrin family of iron-binding proteins, lactoferrin shares more than 60% homology at the amino acid level with transferrin,[8] and a 77% homology exists between human and bovine species,[9] as well as similar predicted 3-dimensional structures. An important functional property of lactoferrin is its high affinity for binding iron.[6] Based on the extent of lactoferrin saturation with iron, lactoferrin can be apo lactoferrin (iron free), monoferric lactoferrin (1 iron atom bound), or holo lactoferrin (2 iron atoms bound).[10,11] The iron saturation of lactoferrin in human milk (human lactoferrin [hLF]) and cow's milk (bovine lactoferrin [bLF]) is around 11% and 13%, respectively,[12] which represent about 0.15%-3.0% of the total iron in milk. The functional groups exposed at the protein surface are different among the apo and the holo lactoferrin forms and, as a consequence, the iron status of lactoferrin most likely affects its physiological functions. Lönnerdal et al[12] reported that the apo forms of band hLF were able to stimulate the proliferation and differentiation of human intestinal epithelial cells, and cell incubation with the apo lactoferrin increased the expression of transforming growth factor-β1, whereas

BDNF	Brain-derived neurotrophic factor
bLF	Bovine lactoferrin
CA	Cornu ammonis
DEX	Dexamethasone
HI	Hypoxia-ischemia
hLF	Human lactoferrin
IUGR	Intrauterine growth restriction
NCAM	Neural cell adhesion molecule
polySia	Polysialic acid
Sia	Sialic acid

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the holo lactoferrin did not have any effect on transforming growth factor-β1, but increased the production of the pro-inflammatory cytokine interleukin-18.[12] Thus, iron status can modulate lactoferrin's effects on intestinal epithelium growth and maturation, and immune response.

hLF is the most abundant glycoprotein. It contains 1-4 Sia residues at the terminal position of N-linked glycan chains for each lactoferrin molecule.[7,8,13] hLF contains 50% more Sia (N-acetylneuraminic acid, Neu5Ac) than recombinant hLF.[14] The characterization of hLF glycosylation using nuclear magnetic resonance spectroscopy method showed that hLF carries biantennary glycans that are mono- or bi-sialylated, as well as mono- or bi-fucosylated glycans.[15] Yu et al[14] demonstrated that N-glycans of hLF are comprised entirely of highly branched, highly sialylated and highly fucosylated complex-type structures, and many contain Lewis(x) epitopes.[14] bLF is also glycosylated but is characterized by having α1-3-linked galactose residues at the terminal nonreducing position.[16] Expression profiles of the glycan groups of hLF from the milk of 5 donors during the first 10 weeks of lactation, showed that hLF is more diverse and undergoes remarkable dynamic changes in glycosylation (eg, there was a decrease in the extent of hLF glycosylation in all mothers from birth to the first 2 weeks of lactation, after which fucosylation increased).[17] The decrease in glycosylation of lactoferrin may be related to the protective role of glycosylation for the polypeptide backbone that protects the protein from protease digestion.[17] This period accompanies extensive changes in the gut flora population.[18] The glycosylation of lactoferrin is tightly regulated by gene expression.[17] Recent reports using lectin microarrays suggested that more diverse complex-type oligosaccharide structures were present on bLF during early lactation, with an abundance of oligomannose-type glycans in later lactation.[19] N-linked glycosylation in hLH is about 5%, 85%, and 9% of the molecules occurring at one (Asn479), 2 (Asn138 and 479), or 3 sites (Asn138, 479, and 624), respectively.[20,21] In bLF, however, Asn233, 368, 476, and 545 are always used,[22] and it makes up about 30% of the glycosylation molecules occurring at Asn281 in the N-lobe in bovine colostrum and about 15% in mature milk.[21,23,24] Comparison of the tryptic proteolysis of hLF and bLF showed that hLF is about 100-fold more resistant than bLF.[21] These differences in the glycol profiles of lactoferrin in milk between species or lactation stages suggest that these may have different functionality in vivo.

Glycans attached to proteins play key roles in mediating cell signaling and cell-cell recognition events.[25] However, the significance of glycosylation for lactoferrin is not completely understood, although protection against proteases, such as the pancreatic enzyme, trypsin, has been suggested.[21] In 2010, Ando et al[26] demonstrated that the carbohydrate chains of hLF are responsible for Toll-like receptor 4 activation. This is an intriguing feature and may disentangle the complicated mechanisms behind the immunomodulating effect of lactoferrin. An in vivo study proved that hLF glycans play a key role in protecting the intestinal mucosa from different pathogens and are, thus, involved in gut-microbiota interaction in the neonatal period.[17] It is well known that Sia play numerous roles in many aspects of immunity. Sialylated oligosaccharides in human milk prevent the binding of rotavirus[27,28] and cholera toxin[29] associated with infant diarrhea, as well as *Escherichia coli* strains associated with neonatal meningitis and sepsis.[30] The role of Sia in infant nutrition and neurodevelopment and cognition has been extensively reviewed in our previous articles.[1,2]

Milk Lactoferrin and Metabolism

Lactoferrin is secreted mainly by the mammary glands but also in various exocrine mammalian secretions.[31] The concentration of lactoferrin in milk varies as function of animal species and duration of lactation. In human milk, lactoferrin concentration varies between 1-7 g/L, peaks in colostrum, and then decreases to become stable in mature milk at 1 g/L.[32] Mature preterm milk, however, contains higher concentration of lactoferrin than full-term milk.[33] The decline in lactoferrin concentration is slower in preterm mother's milk than in full-term mother's milk.[32,34] Lactoferrin also is found in cow's milk, but the levels in cow's milk are significantly lower than those in human milk, ranging between 0.03 and 0.1 g/L of mature bovine milk.

Both hLF and bLF partially resist proteolysis in the digestive tract of neonates and bind to enterocytes through specific receptors. Lactoferrin receptors have been identified in the gastrointestinal tract on leukocytes and macrophages, platelets, and on bacteria. hLF also can be absorbed by the immature intestine, as demonstrated by the fact that intact lactoferrin from maternal origin was detected in the urine of preterm infants fed breast milk.[35,36] However, the ability of lactoferrin to survive digestion most likely depends on host-related factors, including gastric pH, emptying rate, and activity of the gastrointestinal proteases. The magnitude of lactoferrin digestion is different in preterm, term neonates, and older infants, as the gastric pH decreases as the infant matures and maximum hydrolysis of hLF in vitro with preterm gastric fluid was measured at pH3.2.[37] The lactoferrin excretion in stool samples of breastfed infants is higher than that of formula-fed infants.[38] Significant amounts of lactoferrin excreted by the infants decreased during the postnatal age in a trend similar to its decline in the milk.[39] However, lactoferrin intake does not correlate with its excretion,[40] suggesting that other factors might influence levels in digestion. The bLF is transferred from the intestine into peripheral blood in a form with intact molecular weight and is localized within 10-20 minutes after oral administration in the liver, kidneys, gall bladder, spleen, and brain in mice.[41]

The glycosylation or iron status of lactoferrin also can modulate the digestive fate of the molecule (eg, hLF is about 100-fold more resistant to tryptic hydrolysis than bLF) based on the differences in conformation, with major cleavage sites being less accessible to trypsin.[21] Furthermore, specific glycosylation sites of lactoferrin have a protective effect against digestion.[21] In adults, orally-administered bLF survives passage through the stomach. Also, holo lactoferrin, assessed