

Invited Commentary

Human milk oligosaccharides – the plot thickens

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Human milk is a complex physiological fluid that provides nutrients as well as bioactive factors for the infant⁽¹⁾. A distinctive property of human milk among all other species is its remarkable content and structural diversity of oligosaccharides^(2–4). A broad range of functions has been attributed to human milk oligosaccharides (HMO), including serving as a component of the innate immunity of human milk by preventing attachment of potential pathogens to the intestinal lining^(5,6) and by serving as a prebiotic to promote colonization by a healthy gut microbiota^(2–4). HMO are resistant to digestion⁽⁷⁾, thus little focus had been placed on the potential impact of HMO on the host epithelial cells. However, two recent articles published in the *British Journal of Nutrition* by Kuntz *et al.*^(8,9) provide compelling evidence that HMO modulate intestinal cell proliferation, differentiation and apoptosis via receptor binding and MAP-kinase signalling. These reports provide novel insight into a new contribution of HMO to neonatal intestinal development.

HMO are the third most predominant component in human milk after lactose and lipid, averaging about 20 g/l in colostrum and 5–10 g/l in mature milk^(10,11). HMO differ in molecular weight and structure, constituent sugar or sugar derivatives and pH⁽³⁾. They are composed of both neutral and anionic components. HMO are synthesized in the mammary gland with a lactose core at the reducing end and elongation by *N*-acetylglucosamine units, with greater structural diversity provided by extensive fucosylation and/or sialylation wherein fucose and sialic acid residues are added at the terminal positions^(2–4). HMO vary in size from three to thirty-two sugars⁽⁴⁾ and consist of linear and branched polymers linked together by at least twelve different types of glycosidic bonds⁽³⁾. Current analytical methods have detected approximately 200 distinct molecular species of HMO consisting of mostly neutral and fucosylated oligosaccharides^(4,12).

HMO differ among women and four phenotypic groups, consistent with the Lewis blood group system, have been recognized based on the expression and activity of two fucosyltransferases⁽¹³⁾. The HMO components were recently identified in five donors by microfluidic chips and MS⁽¹⁴⁾. The dominant oligosaccharides components were lacto-*N*-tetraose (LNT), lacto-*N*-neotetraose and lacto-*N*-fucopentaose I/V. A neutral oligosaccharide with neutral mass 709.3 Da (3Hex, 1HexNAc–LNT) was the most prominent oligosaccharide that was present in all human milk samples⁽¹⁴⁾. Consistent with the bifidogenic activity of HMO, this HMO has been previously shown to be preferentially fermented by *Bifidobacterium longum* biovar *infantis*, an isolate from the

infant gut^(15,16). The next most common species with relatively strong abundances consisted of neutral fucosylated oligosaccharides and a fucosylated species with a sialic acid residue.

HMO resist digestion within the stomach and intestine and a significant proportion of HMO passes into the lower gastrointestinal tract⁽⁷⁾, where they are selectively metabolized by beneficial micro-organisms. The bifidogenic potential of HMO is frequently cited as an important reason why the gastrointestinal microbiota of breast-fed infants contains proportionally more bifidobacteria than that of formula-fed infants⁽¹⁷⁾. To gain insight into the mechanisms underlying the bifidogenic properties of human milk, the transcriptome of *B. longum* LMG 13 197 grown in human milk or formula containing galacto-oligosaccharides and long-chain fructo-oligosaccharides was compared with each other and with bacteria grown on glucose-containing media⁽¹⁸⁾. Common genes that were highly up-regulated by both human milk and formula included putative genes for cell surface type 2 glycoprotein-binding fimbriae, which are implicated in attachment and colonization in the intestine⁽¹⁸⁾. Genes involved in carbohydrate metabolism formed the dominant group specifically up-regulated in breast milk and included putative genes for *N*-acetylglucosamine degradation and for metabolism of mucin and HMO via the galactose/lacto-*N*-biose gene cluster⁽¹⁸⁾. Thus, HMO exert bifidogenic properties by serving as a fermentable substrate^(15,16) and, in turn, modulate the bacterium's transcriptome to support its catalytic activity. Indeed, the genomic sequence of bifidobacterium revealed 700 genes that are unique to *B. longum infantis*, relative to other bifidobacterium including a variety of co-regulated glycosidases, suggesting a co-evolution of this strain of bifidobacterium to be uniquely suited for colonization of the human milk-fed infant⁽⁴⁾.

Certain HMO share common epitopes to those present on the infant's intestinal epithelia and known receptors for pathogens and, thus, act as decoys to prevent binding of pathogens to the epithelial cells^(5,6,19). The significant immunological protection afforded by HMO has been partly attributed to the presence of $\alpha(1-2)$ -linked fucosylated oligosaccharides⁽⁶⁾. Fucosylated oligosaccharides are capable of preventing diarrhoeal illness through diverse mechanisms, including inhibition of *Escherichia coli* activity by binding and blocking access to target receptors, prevention of *Campylobacter* adhesion to intestinal cells and competitive inhibition of the binding of Norovirus to the intestinal epithelium⁽⁵⁾.

Last, HMO also can be absorbed into the circulatory system and are excreted in the urine of breast-fed infants⁽²⁰⁾. HMO are transported via the circulation to other sites, such as the urinary tract, where they are thought to block pathogen adhesion^(7,20). HMO have structural similarities to selectin ligands, which mediate important cell–cell interactions in the immune system. Leucocyte infiltration into tissues is associated with many inflammatory conditions. Acidic HMO inhibited rolling and adhesion of leucocytes isolated from human blood to cultured human umbilical vein endothelial cells in a concentration-dependent manner⁽²¹⁾. Furthermore, Bode *et al.*⁽²²⁾ demonstrated that acidic (sialic-containing) HMO, but not neutral HMO, serve as anti-inflammatory components by inhibiting the formation of platelet–neutrophil complexes (PNC) and neutrophil activation. These findings support a role for HMO anti-inflammatory factors which may contribute to the lower incidence and severity of inflammatory diseases in breast-fed infants^(19,21,22).

In a study published earlier this year in the *British Journal of Nutrition*, Kuntz *et al.*⁽⁸⁾ tested the hypothesis that HMO would affect intestinal epithelial cell dynamics. Effects of isolated acidic HMO and neutral HMO fractions or individual oligosaccharides on proliferation, differentiation and apoptosis were assessed in preconfluent transformed human intestinal cells (HT-29 and Caco-2) and non-transformed small-intestinal epithelial crypt cells of fetal origin (HIEC). Growth inhibition was induced by neutral and acidic HMO fractions in all cell lines in a dose-dependent manner. However, transformed cell lines (HT-29 and Caco-2 cells) were more sensitive than HIEC cells. In contrast, HMO induced differentiation (alkaline phosphatase activity) in HT-29 and HIEC cells, but not Caco-2 cells. Among individual oligosaccharides, only sialyllactoses induced differentiation. Induction of apoptosis was also detected in HT-29 and HIEC cells, but for neutral oligosaccharides, not for acidic fractions. Thus, for the first time, acidic and neutral HMO were shown to induce growth inhibition in intestinal cells through at least two different mechanisms, by suppressing cell cycle progression through the induction of differentiation and/or by influencing apoptosis⁽⁸⁾.

To further elucidate the underlying molecular mechanisms of action of HMO on cell cycle dynamics, in a study published in this issue of the *British Journal of Nutrition*, Kuntz *et al.*⁽⁹⁾ exposed HT-29, HIEC and Caco-2 cells to neutral or acidic HMO and investigated cell cycle events via flow cytometry and expression levels of cell cycle regulators by using quantitative real-time RT-PCR. Consistent with their previous study⁽⁹⁾, both acidic and neutral fractions induced a concentration-dependent decrease in proliferation, which was evidenced by G₂/M-arrest and associated changes in cyclin A and B mRNA expression. In addition, they observed that the expression of the cyclin-dependent kinase inhibitors p21^{kip1} and p27^{kip1} p21^{kip1} was p53-independent and necessary for arresting cells in the G₂/M phase, while p27^{kip1} was associated with differentiation effects. Lastly, both neutral and acidic HMO induced phosphorylation of the epidermal growth factor receptor and stimulated the MAP-kinase signalling pathway (ERK1/2 and p38 phosphorylation)⁽⁹⁾.

Further studies are needed to determine the mechanism(s) whereby HMO are able to stimulate EGFR signalling and whether these effects can be blocked by specific inhibitors of

EGFR- and p38-signalling pathways. In addition, the potential physiological relevance of these *in vitro* observations should be established by translation to preclinical animal models to determine whether HMO affect intestinal cell dynamics within the complex milieu of the gastrointestinal tract.

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