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Human milk oligosaccharides: the novel modulator of intestinal microbiota

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Human milk, which nourishes the early infants, is a source of bioactive components for the infant growth, development and commensal formulation as well. Human milk oligosaccharide is a group of complex and diverse glycans that is apparently not absorbed in human gastrointestinal tract. Although most mammalian milk contains oligosaccharides, oligosaccharides in human milk exhibit unique features in terms of their types, amounts, sizes, and functionalities. In addition to the prevention of infectious bacteria and the development of early immune system, human milk oligosaccharides are able to facilitate the healthy intestinal microbiota. Bifidobacterial intestinal microbiota appears to be established by the unilateral interaction between milk oligosaccharides, human intestinal activity and commensals. Digestibility, membrane transportation and catabolic activity by bacteria and intestinal epithelial cells, all of which are linked to the structural of human milk oligosaccharides, are crucial in determining intestinal microbiota. [BMB Reports 2012; 45(8): 433-441]

INTRODUCTION

Human body is the habitat of a numerous microorganisms (1). These microorganisms continuously interact with the host, resulting in the significant impact on the acute and long term health of human (2, 3). In the human microbiome, the gastrointestinal (GI) microflora has a strong correlation with the individual health condition of their host (4-6). Even the monozygotic twins who have identical genotypes have the different bacterial populations depending on their health condition (7, 8). Recently a common core population of microbiome was observed, however, the health condition of human subject dramatically changes the major bacterial populations (7, 9, 10). Among the human and bacterial interactions, the microflora of the infant GI tract has been focused since the clear correlation (11). It has been witnessed that the infants nourished by mothers' milk are generally healthier than those who diet formula milk, and have a unique microflora of *Bifidobacterium* (12). The human milk does not simply provide nutrients to the early infants, it also contains the active components that aid in developing the immune and cognitive system, preventing the infectious and toxic pathogens and establishing the intestinal microbiota (13-17). Considerable efforts have been made to elucidate the functional component in human milk. Aside from milk proteins and lipids, the oligosaccharides in human milk have recently been the subject of much attention due their unique functionalities. Human milk oligosaccharide (HMO) is a diverse and complex group of oligosaccharides whose concentration is as high as the portion of proteins in human milk (18, 19). The structural complexity within a single oligosaccharide and the diversity of a group of oligosaccharides hamper the quantitative and qualitative study of HMO and their utilization (20). Recent advance in glycoprofiling by mass spectrometry provides the information of HMO in detail enabling the fundamental study of structure based HMO-bacterial interactions (21, 22).

In this review, we introduce the HMO in terms of their uniqueness as a milk oligosaccharides, the diversity and the commonality of their structures, and the interaction with bacteria as a growth substrate, in order to understand the HMO as an essential and functional component in human milk.

HUMAN MILK OLIGOSACCHARIDE

Human milk is rich in HMO. The amount of HMO is vary depending on individuals and the lactation periods, however, it can reach up to 15 g/L which is a similar to, or more than, the amount of proteins in human milk (23, 24). Though the function of HMO is not completely understood, it has been postulated that HMO is responsible for the development of immune system, the prevention of pathogenic infection and, moreover, the modulation of infant GI tract to bifidogenic microbiota (16, 25-30).

The chemical structures of HMO are similar to those of epithelial cell surface glycans such as mucin or glycolipids that bacteria or virus often use for binding (31-33). Due to the structural similarity, thus, HMO competes with the ligand, possibly inhibit-

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ing pathogenic infection (27, 29, 34). The development and stimulation of the infant immune system are affected by HMO as well. It has been reported that HMO has a direct correlation with selectins, integrins, and toll-like receptors (35-37) and affect the interactions between leukocyte-endothelial and leukocyte-platelet cells (35, 38-40).

One of the vital roles of HMO in mother's milk is a prebiotic activity that drives an infant gut microbiota to the bifidogenic population. Since Moro found the presence of bifidobacteria on breast-fed infant feces, human milk is believed to have a specific growth factor to enrich these bacteria (41). With the historical recognition between breast-fed infants and the bifidobacterial microbiota, recent evidences suggest that HMO results in a cognate gastrointestinal population of infant. The growth study reveals that most bacteria commonly found in GI tract cannot grow using HMO as a sole carbon source in minimal medium (22, 42-45). *Escherichia coli, Enterococcus* sp., *Streptococcus thermophiles, Lactococcus lactis, Eubacterium rectile, Clostridium perfringens, Veilonella parvular,* and even a probiotic strain of *Lactobacillus* sp. such as *L. gasseri* did grow on HMO. Among the *Bifidobacterium* sp. only *Bifidobacterium infantis* and *Bifidobacterium bifidum* which are the mainly found in breast-fed infant feces can grow using a HMO as a sole carbon source. *Bifidobacterium breve, Bifidobacterium adolescentis,* and *Bifidobacterium longum,* which are often associated to the adult GI tract, cannot grow on HMO as well (46).

It is noteworthy that HMO is minimally hydrolyzed in infant GI tract and not directly consumed for the nourishment (47-50). Milk oligosaccharides were detected in the urine of breast-fed infant. Further, transportation study using Caco-2 cell reveal less than 1.5% of oligosaccharides were moved from the apical to the basolateral compartment (48, 51). Once ingested with human milk, therefore, HMO flows through the GI tack staying or accumulating at the intestine. Although lactose is the most abundant carbon source in milk, it is absorbed mostly by human. After completely consumption of lactose, therefore, indigested HMO at intestine can serve as a selective agent for the bacterial cell growth. In addition to the environmental impact, the ability of catabolizing HMO appears to be crucial for bacteria to dominate the intestinal microbiota. It suggests that the intestinal microbiota is established, not by a unilateral interaction, but rather by the mutual interactions between HMOs, human and bacteria.

STRUCTURES AND COMPOSITIONS

HMO is a collective terms that refers a group of diverse oligosaccharides present in human mother's milk. As shown in Fig. 1A, galactose (Gal), glucose (Glc), N-acetylglucosamine (GlcNAc), fucose (Fuc) and N-acetylneuraminic acid (sialic acid or NeuAc) are used for the basic building of milk oligosaccharides (18, 19, 31, 33, 52). More than 58 compositional isomers, which have different sugar constituents, were observed by matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS) (20, 53, 54). LC-Chip/TOF-MS analysis revealed more than 200 structural isomers with different glycosidic linkages between base sugar units (30, 55-57). Among them, the structures of 45 neutral free oligosaccharides were elucidated by a tandem mass spectrometry combined with the linkage specific enzyme digestion (Fig. 1B and C: the structural isomers of two abundant HMO).

Although human milk oligosaccharide (HMO) is a free glycan in bodily fluid, their structures are similar to those of O-linked glycans (20, 30, 33, 56). HMO has a lactose residue as a reducing end. GlcNAc is connected linearly to galactose residue via β 1-3 linkages, or branched when attached on both β 1-3 and β 1-6 position. Then, another galactose is added to GlcNAc via β 1-3 or β 1-4 glycosidic bond. These GlcNAc and galactose are elongated in this manner forming oligosaccharide backbone. HMOs are rich in fucosylation and/or sialylation on oligosaccharides. 70% of HMO has fucose residues and 20-50% of HMO has NeuAc depending on the individuals and lactation period. Fucose is attached both GlcNAc or Gal via α 1-3, or 1-4 linkages, or occasionally α 1-2 linkages at terminal position of oligosaccharides. NeuAc is connected via α 2-6 or 2-3 linkages (Fig. 1B and C).

It has been shown that the milks from different mother and lactation periods have the qualitative and quantitative variation of their oligosaccharides profiles (58-60). The observation of HMO profile over the course of 3 month lactation period from

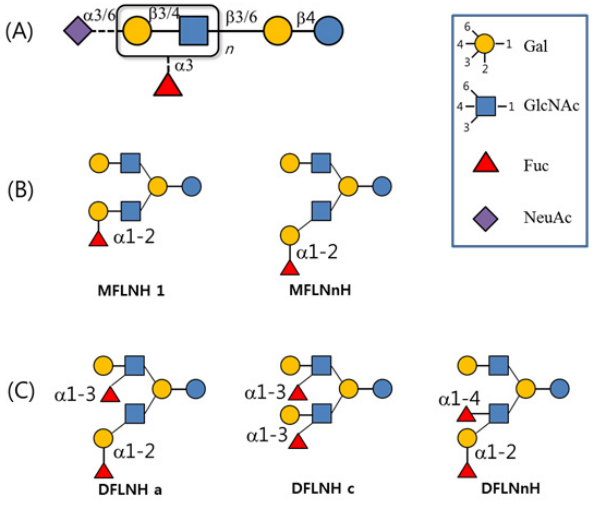

Fig. 1. The basic structure of HMO [Adapted from Wu et al. (2010). Data has been modified]. (A) Monosaccharide backbone of HMO. Sugar annotation is inside the box. Gal, galactose; GlcNAc, N-acetylglucosamine; Fuc, fucose; NeuAc, N-acetylneuraminic acid (sialic aicd). (B) and (C) are the structural isomers of the abundant oligosaccharides in human milk. The glycosidic bonds were confirmed by MS/MS. (B) 4Hex:2HexNAc:1Fuc, MFLNH1 (Monofucosylated lacto-N-hexaose 1) and MFLNnH (Monofucosylated lacto-N-neohexaose), (C) 4Hex:2HexNAc:2Fuc, DFLNH a (Difucosylated lacto-N-hexoase a), DFLNH c (Difucosylated lacto-N-hexoase c), and DFLNnH (Difucosylated lacto-N-neohexaose).

five individual donors revealed the minor changes in the amount of each HMO showing the same trait at different stages of lactation within individual donors (61). Although each donor is internally consistent, the specific profiles of HMOs between donors were apparently different. The level of fucosylation and the sialylation differed up to two fold between donors and the most abundant oligosaccharide was not same across the donors. However, it is worth to note that the major heterogeneity was observed in the minor glycans. All human donors including five Caucasian, Gambian, and pooled samples share the unique features that the great diversity, long length and structures of oligosaccharides that could distinguished them from other mammals (20, 54, 56, 61). In addition, 7-8 glycans among the most abundant 10 glycans were observed in each individual donors regardless of ethnicity or lactation periods showing the commonality of general HMO profiles.

UNIQUE CHARACTERISTICS OF HMO

Mammals produce milk for nurturing their newborns and oligosaccharides are commonly present in mammalian milk. Milk oligosaccharides from various mammals exhibit unique features which might correlate to their phylogenetic characteristics (56, 62-65). For example, the milk oligosaccharides from bovine and porcine are quite different from HMO but similar to each other (62, 63). While the concentration of HMO in milk is up to 15 g/L, both the amount of porcine and bovine milk oligosaccharide (PMO and BMO, respectively) are less than 1 g/L. In terms of their structural diversity, less than 30 isomers were observed by MALDI-FT-ICR and LC-MS/MS analysis of BMO and PMO. Although the basic structures of PMO and BMO are similar to that of HMO comprising Gal, NeuAc, GluNAc, and Fuc, the composition of predominant oligosaccharides are quite different. More than 40% of BMO and PMO are sialyllactose (3`SL; NeuAcα1−3Gal β1−4Glc and 6`SL; NeuAcα1−6Galβ1−4Glc), which is rare in HMO. Majority of OS (~80%) are sialylated and only a trace level of fucosylation was observed in BMO and PMO.

The oligosaccharides in primate milks share more similarity with HMO than the oligosaccharides in bovine and porcine milk (56). The analysis of milk oligosaccharides from apes (chimpanzee, gorilla, siamang), New World monkeys (golden lion tammarin, common marmoset) and Old World monkeys (rhesus) showed that the amounts of OS from these primates were much higher than those of BMO or PMO but less than HMO. Sialyllactose, which is a major glycan in PMO/BMO, was not observed or, if identified, a trace level of OS in primate milks. Instead, human and primate milk shares common OS as major glycans. Lacto-N-tetraose (LNT), lacto-N-*neo*-pentose 1 (LNnP 1), Lacto-N-*neo*hexose1 (LNnH 1), MFLNnH (Monofucosyllacto-N-neohexose), MSMFLNnH (Monosialyl-monofucosyllacto-N-neohexose) were observed in all primates samples and human milk. In addition, 20 different oligosaccharides present in human milk were also found from more than 5 different primate milks. OS in primate milks are moderately fucosylated showing 18-50% of total OS except Siamang and Golden lion tamarin. Sialylation of OS between HMO and primate milk oligosaccharide were similar with 20-30% albeit the Siamang showed relatively high sialylation with 50% of total OS.

Separate study using HPLC coupled with NMR for the structural identification exhibited similar traits (66, 67). The analysis of milk oligosaccharides from great galago, aye-aye, Coquerel's sifaka and mongoose lemeur found the fucosylated and sialylated oligosaccharides. Relative total content of oligosaccharides calculated from the HPLC peak area were similar to other primates in the range between 10-30 % of lactose peak. The oligosaccharides commonly witnessed by Tao *et al*. were observed as well.

Despite these similarities, HMOs still have distinct features that distinguish from the oligosaccharides in other primate milks (56). The total amounts of oligosaccharide in primates are, although they are higher than bovine and porcine, still smaller than those in human milk. Diversity of oligosaccharides in human milk is much higher than those in other primate milks as well. While highly diversified HMO requires more than 50 different oligosaccharides to compose 90% of the amount of total OS, primate milk oligosaccharides requires only 15-30 oligosaccharides to fulfill the 90% of total amount.

Interestingly, chimpanzees, which are a close relative to human, showed similar profiles in their milk oligosaccharides. Considering the major glycans that comprise 90% of the total amount of milk oligosaccharides, the degree of sialylation and fucosylation of chimpanzee are same as human. Further, it was shown that the number of major glycans of chimpanzee and human are similar at 43 and 50, respectively.

Another notable feature of HMOs is their size. The degree of polymerizations (DP) of primate milk oligosaccharides is less than 9 with the length of average DP 5. Meanwhile, the length of HMO has been extended up to DP 13 with an average of DP 9. This elongation of milk oligosaccharide is a unique characteristic of humans. Although the milk oligosaccharides from chimpanzees are as diverse as from human, their average size is similar to those from other primates (56, 67).

The comparison of milk oligosaccharides profiles across evolution did not exactly match the primate phylogeny showing a nonsequential developmental pattern (56, 66, 67). However, it is also clear that the oligosaccharides profiles of primate milk are more similar to each other than those of non-primate suggesting that the variation is correlated to the general population within the species.

BACTERIAL CONSUMPTION

Besides the development of the immune system or the prevention of pathogenic infection, it has been witnessed that HMO has a function that drives the intestinal microbiota toward bifidogenic population (30, 68, 69). The detail has not proven yet,

strong evidence suggests the catabolic capability of HMO plays a key role in modulating the bacterial population (30, 43, 44, 70-72). Two of infant associated Bifidus, *Bifidobacterium infantis* and *Bifidobacterium bifidum,* are able to grow on HMO as a sole carbon source (73, 74).

To consume HMO, microorganism has to through three barriers. First, microorganisms need to catabolize the unit monosaccharides and are able to gain enough ATPs for cell growth from those monosaccharides. Most of intestinal bacteria are able to metabolize galactose via the Leloir pathway or tagatose monophosphate pathway (75-77). Since N-acetyl glucosamine is a building block for the bacterial cell wall, most bacteria have been able to synthesize or catabolize a GlcNAc (78-81). GlcNAc monophosphate is generally converted to glucosamine by deacetylase (82, 83), and then become fructose phosphate by the action of deaminase (78-85). Catabolic pathways of fucose and NueAc are not clearly determined. However, considering the fucose is dehydroxygalactose, catabolism of fucose seems to be complex and thermodynamically unfavorable to produce ATP. NeuAc can be degraded to N-acetylmannosamine and pyruvate (86-88). Two intermediates can be incorporated into glycolysis but requires specific sets of enzymes.

Although HMO consists of four different monosaccharides, the quantities of each monosaccharide are not equal. The amount of each monosaccharide units can be estimated from the MS analysis of HMO (Table 1). When compared to lactose, HMOs is able to provide enough galactose and GlcNAc for cell growth. While the complete hydrolysis of 20 g/L of lactose can produce 117 mM of hexose, the same amount of HMOs can be converted to 66.2 mM of hexose, 31.9 mM of GlcNAc, 13.6 mM of fucose, and 1.6 mM of NeuAc. Without the utilization of fucose and sialic acid, once completely hydrolyzed, 98 mM of hexose and GlcNAc are available to bacteria for their cell growth. Even if bacteria cannot use GlcNAc, 66.2 mM of hexose, mostly galactose, can be provided which is equivalent to the 57% of what is obtained from 20 g/L of lactose.

Table 1. Availability of HMO as a carbon source for bacterial growth. (A) The mole concentration of each oligosaccharide which constitutes 90% HMO. (Data was adapted from Ninonuevo *et al*. (2006) and processed as described below). (B) Mole concentration of monosaccharides in 20 g/L of lactose and HMO after complete hydrolysis

(A)

No.	Intesntiy[a]	MW	Hex	HexNAc	Fuc	NeuAc	% (mol/mol)[b]	Accu. %[c]
1	2,562.66	1,220.4540	4	2	1		20.40%	20.40%
2	2,127.79	709.2640	3	1			16.90%	37.20%
3	1,378.41	1,366.5120	4	2	2		10.90%	48.20%
4	997.23	1,074.3960	4	2			7.90%	56.10%
5	735.45	1,731.6440	5	3	2		5.80%	62.00%
6	603.75	1,585.5860	5	3		1	4.80%	66.80%
7	505.39	1,877.7020	5	3	3		4.00%	70.80%
8	385.71	1,511.5490	4	2	1	1	3.10%	73.80%
9	369.28	2,096.7760	6	4	2		2.90%	76.80%
10	314.30	1,000.3590	3	1	1		2.50%	79.30%
11	296.27	1,950.7180	6	4	1		2.40%	81.60%
12	273.07	1,365.4920	4	2		1	2.20%	83.80%
13	202.62	1,439.5280	5	3			1.60%	85.40%
14	190.50	1,512.5700	4	2	3		1.50%	86.90%
15	181.02	2,242.8340	6	4	3		1.40%	88.30%
16	145.75	1,058.4010	3	2	1		1.20%	89.50%

[a]Absolute peak intensity which linearly correlated to the number of molecules.
[b]Percentile mole concentration. %(mol/mol) of HMO molecule a= $I_a / \sum_{i=1}^{n} I_i$, where n= total number of HMO oligosaccharides, I_a = the peak intensity of HMO molecule a[c]Accumulated percentile mole concentration. Table listed the individual oligosaccharides consist of 90% of total mole of HMO.

(B)

	Total[a]	After hydrolysis[b]			
		Hex	HexNAc	Fuc	NeuAc
Lactose	58.4	116.8			
HMO	16.9	66.2	31.9	13.6	1.6

[a]Total mole concentration of 20 g/L of lactose and HMO.
[b]Mole concentration of 20 g/L of lactose and HMO after complete hydrolysis. For example, the mole concentration of Hex = $\sum_{i=1}^{n} Hex_i \times \%(mol/mol)_i$, where n = total number of HMO oligosaccharides, Hex_i =number of hexose in individual HMO oligosaccharide i, $\%(mol/mol)_i$= the mole % concentration of individual HMO oligosaccharide i.

Given the fact that HMO could provide enough monosaccharaides for bacterial cell growth, their degradation become critical when bacteria grow on HMO. To hydrolyze HMO, four types of glycosyl hydrolase are required. Fucose and sialic acid are disconnected by the action of α-fucosidase and α-sialidase, respectively (42, 88, 89). Then, GlcNAc is detached from the HMO by hexosaminidase as a monosaccharide or by lacto-N-biosidase with galactose (90-93). Galactose residue attached on the GlcNAc is cleaved by β-galactosidase. Since major β-galactosidase and hexosaminidase are exo-type glycosyl hydrolase, the activity of fucosidase and sialidase could be the key step for the utilization of HMO. Indeed, the expression of fucosidase, sialidase, and hexosaminidase are observed from *Bacteroides fragelis, Bacteroides thetaiotaomicron, B. infantis* and, *B. bifidum* when they grow on HMO (72-74, 94). Further, it has been revealed that the genes encode fucosidase and sialidase were not found from *Bifidobacterium* strains that did not grow on HMO such as *B. breve, B. adolesacntis, B. animals,* and *B. dentinum* (69).

Another consideration regarding HMO utilization is the membrane transportation depending on the location of HMO hydrolyzing enzymes. If the hydrolysis of HMO occurs in the extracellular space, existing monosaccharide transport system is could be used. However, the specific set of HMO transport systems should be required, if the HMO is hydrolyzed at cytosol after membrane transportation. For example, *B. infantis* encodes several sets of ABC transport systems with the family 1, solute binding proteins (F1_SBPs), particularly, in HMO cluster (74). HMO cluster is ~40 kbp gene cluster that contains the genes necessary for the HMO catabolism including fucosidase, hexosaminidase, sialidase, β-galactosidase and transport system. F1_SBP is an oligosaccharide binding protein that displays on the cell surface next to the ABC transporter in order to hold the specific oligosaccharide. The F1_SPBs in the HMO cluster are expressed with other catabolic enzymes when grown on HMO suggesting the correlation to the HMO translocation (95). Meanwhile, the SBP mediated transport system was not found on the *B. bifidum* genome suggesting that the hydrolysis of HMO at the extracellular space followed by the uptake of monosaccharide units (73).

CONCLUSION

In terms of quantity and its functional quality, human milk oligosaccharide is one of the major components in mother milk. Infants are depending on the mother milk not only for their gain of weight and height but also for the balanced development and decent health. HMO seems to be a functional component that is responsible for the "quality" of infant growth and development. Without consumption by human digestive system, HMO travels through the GI tract providing various functions that regulate the harmony between host and bacteria community, whether a beneficial or infectious, that evolved together. Not only the infant, the regulation of intestinal microbiota to beneficial population for individual host could improve the adult health as well. Fucose and sialic acid play a functional role in the development of body systems such as immune or mucosa. Further, it was recently reported that they are signature sugars whose changes in human glycome can be a signal of various disease. Despite these critical roles, biological functions and interactions of HMO in complex environment still remain as questions to be answered.

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