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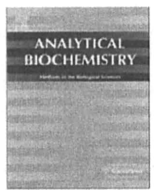

Structural characterization of multibranched oligosaccharides from seal milk by a combination of off-line high-performance liquid chromatography–matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry and sequential exoglycosidase digestion

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ABSTRACT

A complex mixture of diverse oligosaccharides related to the carbohydrates in glycoconjugates involved in various biological events is found in animal milk/colostrum and has been challenging targets for separation and structural studies. In the current study, we isolated oligosaccharides having high molecular masses (MW ~ 3800) from the milk samples of bearded and hooded seals and analyzed their structures by off-line normal-phase–high-performance liquid chromatography–matrix-assisted laser desorption/ionization–time-of-flight (NP-HPLC–MALDI–TOF) mass spectrometry (MS) by combination with sequential exoglycosidase digestion. Initially, a mixture of oligosaccharides from the seal milk was reductively aminated with 2-aminobenzoic acid and analyzed by a combination of HPLC and MALDI–TOF MS. From MS data, these oligosaccharides contained different numbers of lactosamine units attached to the nonreducing lactose (Galβ1–4Glc) and fucose residue. The isolated oligosaccharides were sequentially digested with exoglycosidases and characterized by MALDI–TOF MS. The data revealed that oligosaccharides from both seal species were composed from lacto-*N*-neohexaose (LNnH, Galβ1–4GlcNAcβ1–6[Galβ1–4GlcNAcβ1–3]Galβ1–4Glc) as the common core structure, and most of them contained Fucα1–2 residues at the nonreducing ends. Furthermore, the oligosaccharides from both samples contained multibranched oligosaccharides having two Galβ1–4GlcNAc (*N*-acetyllactosamine, LacNAc) residues on the Galβ1–4GlcNAcβ1–3 branch or both branches of LNnH. Elongation of the chains was observed at 3-OH positions of Gal residues, but most of the internal Gal residues were also substituted with an *N*-acetyllactosamine at the 6-OH position.

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Specific sequences of monosaccharides occur as important structural elements of oligo- and polysaccharides of glycoproteins and glycolipids, and they comprise recognition motifs for ligand–receptor or cell–cell interactions [1–4]. Oligosaccharides are cooperatively synthesized by actions of various glycosyltransferases and are usually present as a complex mixture of diverse oligosaccharides. In particular, the isomeric/branching structure is the major feature, and their structural determination is essential for understanding the biosynthesis and biological significance.

Mammalian milk/colostrum is a rich source of carbohydrates of diverse structures [5–8]. Although the most dominant carbohydrate in mammalian milk is generally lactose, a small amount of characteristic oligosaccharides are also present [9–13]. The milk oligosaccharides usually have a common lactose (Galβ1–4Glc) core that is extended at the 6- and/or 3-OH positions of the Gal as linear/branched mode [14]. Furthermore, the linear/branched chains are frequently fucosylated and/or sialylated and in a few cases are sulfated.

Due to the similarities and complex structures of milk oligosaccharides, structural determination of them has been a big and challenging work. Urashima and coworkers isolated various oligosaccharides from many mammalian species' milk/colostrum and characterized their structural features by a combination of

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some preparative chromatographic techniques and ^{1}H-nuclear magnetic resonance (NMR)[1] spectrometry [14–21]. They reported that milk oligosaccharides contain blood group-related antigens and that their relative abundances are characteristic among animal species. For example, bear milk contains oligosaccharides having an α-Gal epitope (Galα1–3Galβ1–4GlcNAc-R), A blood antigen (GalNAcα1–3[Fucα1–2]Gal-R), B blood antigen (Galα1–3[Fucα1–2]Gal-R), and Lewis^X antigen (Galβ1–4[Fucα1–3]GlcNAc-R) [19]. Urashima and coworkers also reported that milk samples from bearded and hooded seals contain a large amount of neutral oligosaccharides, including lactose (Galβ1–4Glc), 2′-fucosyllactose (Fucα1–2Galβ1–4Glc), and lacto-*N*-fucopentaose (Fucα1–2Galβ1–4GlcNAcβ1–3Galβ1–4Glc) as major components [17,18]. In addition, both milk samples also contain branched oligosaccharides having lacto-*N*-neohexaose (LNnH, Galβ1–4GlcNAcβ1–6[Galβ1–4GlcNAcβ1–3]Galβ1–4Glc) as a core, and most of them have one or two nonreducing α1–2 linked Fuc. Although both milk samples contain sialylated oligosaccharides with high molecular masses, structural studies were not done because of the limited performance of the NMR method. It has also been revealed that milk samples in monotremes such as platypus and echidna contain Lewis^X and Lewis^Y antigens (Fucα1–2Galβ1–4[Fucβ1–3]GlcNAc-R) [10,12,22]. In view of these species-specific structural features and distribution of diverse oligosaccharides in milk/colostrum of different animals, detailed structural studies are not only useful for understanding the underlying evolutionary significance but also promising for using these unique features for biomedical applications.

The dominant carbohydrate in mammalian milk is generally the disaccharide lactose, whereas the milk samples of phocid species, including hooded and bearded seals, contain a variety of oligosaccharides other than lactose [17,18]. The oligosaccharides in bearded and hooded seal milk contain lactose, lacto-*N*-neotetraose (LNnT, Galβ1–4GlcNAcβ1–3Galβ1–4Glc), and LNnH as core units. Furthermore, it is noteworthy that milk oligosaccharides from both species contain type II chain (Galβ1–4GlcNAc-R) but not type I chain (Galβ1–3GlcNAc-R). The presence of α1–3 linked GlcNAc and type II chain suggests that seal mammary glands contain poly-*N*-acetyllactosamines, which are synthesized by β(1–4)galactosyltransferase as well as β(1–3)*N*-acetylglucosaminyltransferase. A search for higher oligosaccharides having poly-*N*-acetyllactosamine structure is an interesting target for understanding the regulation of biosynthesis because they are further modified to form functional oligosaccharides (e.g., sialyl Le^x) and/or branched structures.

NMR spectroscopy is the most important technique that provides sequence information including linkage and α/β-anomeric configurations. However, due to the complexity of extremely overlapping signals of the monosaccharide residues in similar environments, especially in the case of oligosaccharides having poly-Galβ1–4GlcNAc (*N*-acetyllactosamine), it is often difficult to assign the branching pattern by only the NMR technique. In contrast, mass spectrometry (MS) has been an indispensable technique for structural analysis of oligosaccharides and useful for the analysis of higher oligosaccharides with high sensitivity. Finke and coworkers reported a method for the analysis of higher oligosaccharides (MW ~ 3000) by a combination of chromatographic separation and matrix-assisted laser desorption/ionization–time-of-flight (MALDI–TOF) MS [23,24]. Recently, tandem mass spectrometry (MS/MS) with collision-induced dissociation (CID) has been applied to structural analysis of various oligosaccharides [25,26]. Chai and coworkers reported a method for the analysis of a complex mixture of isomeric neutral oligosaccharides in human urine and milk samples by nano-liquid chromatography–electrospray ionization–ion trap (LC–ESI–IT) mass spectrometer and identified three novel isomeric fucosylated lacto-*N*-hexaoses (LNHs) based on the studies using CID–MS/MS experiments [27,28]. However, the MS method often cannot differentiate isomeric branched or linear oligosaccharides such as LNnH and *para*-lacto-*N*-hexaose (pLNH, Galβ1–4GlcNAcβ1–3Galβ1–4GlcNAcβ1–3Galβ1–4Glc). Thus, it is still difficult to characterize anomeric configurations, branching configurations, and epimeric forms. This information is often obtained by the analysis of the digestion products with specific exoglycosidases. The molecular mass obtained from MS analysis after digestion with well-defined exoglycosidases reveals the sequence of oligosaccharides and information on the branching pattern.

In this study, we isolated higher oligosaccharides (MW ~ 3800) from bearded and hooded seal milk samples and analyzed their structural characteristics by normal-phase (NP)–HPLC after desialylation with neuraminidase and also by MALDI–TOF MS. Furthermore, we confirmed the branching pattern of the oligosaccharides by a combination of sequential exoglycosidase digestions and MALDI–TOF MS and MALDI–quadrupole ion trap (QIT)–TOF MS.

Materials and methods

Materials

Milk samples from bearded seal (BS) and hooded seal (HS) were collected from a lactating female in Svalbard, Norway, and from animals on the drifting pack ice in the southern part of the Gulf of St. Lawrence, Canada, respectively. Both milk samples were stored at −20 °C until use. α1–2 Fucosidase derived from *Corynebacterium* sp. and α1–3,4 fucosidase from *Streptomyces* sp. 142 were purchased from Takara Biochemicals (Kusatsu, Japan). α2,3,6,8 Neuraminidase from *Arthrobacter ureafaciens* was kindly donated by Yasuhiro Ohta (Marukin Bio, Kyoto, Japan). β-Galactosidase and β-*N*-acetylhexosaminidase (both from jack beans) were obtained from Seikagaku Kogyo (Tokyo, Japan). All other reagents were analytical or HPLC grade.

Fractions containing acidic oligosaccharides from BS and HS milk samples

Samples of BS and HS milk (40 and 20 ml, respectively) were obtained after delipidation and protein precipitation according to the reported method [17,18]. Briefly, the milk samples were diluted with 4 volumes of distilled water and shaken vigorously with 4 volumes of chloroform/methanol (2:1, v/v). The chloroform layer and denatured protein were discarded. The methanol was removed from the upper layer by a rotary evaporator, and the resulting carbohydrate-containing solution was freeze-dried. Carbohydrate-containing fractions were fractionated on a Biogel P-2 column (2.5 × 100 cm) previously equilibrated with water. An aliquot (0.5 ml) of each fraction was analyzed for hexose by phenol–sulfuric acid assay and for sialic acids by resorcinol assay [29]. Fractions eluted earlier were pooled and lyophilized to dryness (see Fig. 1 in Ref. [17] and Fig. 1 in Ref. [18]). The neutral oligosaccharides of both milk samples and a part of acidic oligosaccharides of bearded seal milk were already characterized in Urashima and coworkers' previous studies [17,18]. The fraction (1.7 mg), which was eluted

[1] *Abbreviations used:* NMR, nuclear magnetic resonance; LNnH, lacto-*N*-neohexaose; LNnT, lacto-*N*-neotetraose; MS, mass spectrometry; MALDI–TOF, matrix-assisted laser desorption/ionization–time-of-flight; MS/MS, tandem mass spectrometry; CID, collision-induced dissociation; LC–ESI–IT, liquid chromatography–electrospray ionization–ion trap; LNH, lacto-*N*-hexaose; pLNH, *para*-lacto-*N*-hexaose; NP, normal-phase; QIT, quadrupole ion trap; BS, bearded seal; HS, hooded seal; 2AA, 2-aminobenzoic acid; DHB, 2,5-dihydroxybenzoic acid; mw, molecular mass; LNnTD, lacto-*N*-neotetradecaose; LNnD, lacto-*N*-neodecaose; LNnDD, lacto-*N*-neododecaose; LNnOD, lacto-*N*-neooctadecaose; GnT, β-*N*-acetylglucosaminyltransferase; iGnT, β3-*N*-acetylglucosaminyltransferase; IGnT, β(1–6)*N*-acetylglucosaminyltransferase.

at void volumes, from the HS milk sample was used in the following preparations and characterization of each oligosaccharide.

The fraction from the BS milk sample was further separated by ion exchange chromatography, as indicated in the previous study [18]. The lyophilized material was dissolved in 50 mM Tris–HCl buffer (pH 8.7, 2.0 ml) and subjected to anion exchange chromatography on a DEAE Sephadex A-50 (1.5 × 35 cm). The unadsorbed oligosaccharide fractions were used for structural study of the oligosaccharides in the previous study [18]. The adsorbed oligosaccharides were eluted by linear gradient elution with changing NaCl concentrations from 0 to 0.25 M in the same buffer. Two fractions (BS1 and BS2) obtained by linear gradient elution were pooled and lyophilized to dryness. The lyophilized material was dissolved in water and passed through a Biogel P-2 column (2.5 × 100 cm). The fractions eluted at the void volume were pooled and lyophilized to dryness to yield a mixture of acidic oligosaccharides (2.0 and 2.3 mg of BS1 and BS2, respectively).

Fluorescent labeling of oligosaccharides with 2AA

Fluorescent labeling of oligosaccharides was performed according to the method reported previously [30,31]. Briefly, a solution (250 μl) of 2-aminobenzoic acid (2AA) and $NaBH_3CN$, prepared by dissolution of both reagents (30 mg each) in methanol (1 ml) containing 4% CH_3COONa and 2% boric acid, was added to a mixture of oligosaccharides (100 μg). The mixture was kept at 80 °C for 60 min. After cooling, water (250 μl) was added, and the mixture was applied to a small column (1 × 50 cm) of Sephadex LH-20 previously equilibrated with 50% aqueous methanol. The earlier eluted fluorescent fractions that contained labeled oligosaccharides were collected and evaporated to dryness. The residue was dissolved in water (1 ml), and the solution was stored at −20 °C until analysis.

Preparation of asialo-oligosaccharides

A mixture of 2AA-labeled acidic oligosaccharides (~10 μg) was dissolved in 20 mM acetate buffer (pH 5.0, 50 μl), and neuraminidase (10 mU, 10 μl) was added to the mixture. After incubation at 37 °C for 24 h, the reaction mixture was kept in the boiling water bath for 5 min. After centrifugation of the mixture at 10,000*g* for 10 min, a portion of the supernatant was used for the analysis.

α-Fucosidase digestion

A mixture of 2AA-labeled asialo-oligosaccharides (~2 μg), as described above, was dissolved in 20 mM phosphate buffer (pH 7.5, 50 μl) for α1-2 fucosidase digestion or in 20 mM phosphate buffer (pH 6.0, 50 μl) for α1-3,4 fucosidase. α1-2 Fucosidase (40 μU, 2 μl) or α1-3,4 fucosidase (10 μU, 10 μl) was added to the mixture. After incubation at 37 °C for 24 h, the reaction mixture was kept in the boiling water bath for 5 min, and centrifuged at 10,000*g* for 10 min. The supernatant was diluted with water to adjust the volume of 200 μl. A portion of each solution (5 μl) was used for NP–HPLC analysis.

Sequential exoglycosidase digestion of oligosaccharides

Each oligosaccharide isolated by NP–HPLC was dissolved in 20 mM citrate buffer (pH 3.5, 8 μl), and β-galactosidase (1 mU, 2 μl) was added to the mixture. After incubation at 37 °C for 12 h, the reaction mixture was kept in the boiling water bath for 5 min. After centrifugation of the mixture, the supernatant was diluted with water (10 μl). A portion of the solution (2 μl) was analyzed by MALDI–TOF MS. Another portion (5 μl) was mixed with 30 mM citrate buffer (pH 5.0, 5 μl) containing β-*N*-acetylhexosaminidase (5 mU), and the reaction mixture was kept at 37 °C for 12 h. The supernatant was diluted with water (10 μl), and then a portion of the solution (2 μl) was also analyzed by MALDI–TOF MS.

Separation of the 2AA-labeled oligosaccharides

HPLC was performed with a Shimadzu apparatus equipped with two LC-6ADvp pumps and an FP-920 fluorescence detector (Waters). Separation was done with an Amide 80 column (TOSOH, 4.6 mm i.d. × 250 mm) using a linear gradient formed by 2% acetic acid in acetonitrile (solvent A) and 5% acetic acid in water containing 3% triethylamine (solvent B). The column was initially equilibrated and eluted with 70% solvent A for 2 min. After 2 min, solvent B was increased to 95% over 80 min and kept for further 20 min [32]. Fluorescence detection was performed at 410 nm by irradiating at 325 nm light.

MALDI–TOF MS

MALDI–TOF mass spectra were acquired with a Voyager DE-PRO mass spectrometer (PE Biosystems, Framingham, MA, USA). A nitrogen laser was used to irradiate samples, and an average shot of 50 times was taken. The instrument was operated in a linear mode at an accelerating voltage of 20 kV. An aqueous sample solution (2 μl) was mixed with a matrix solution (2 μl) of 1% 2,5-dihydroxybenzoic acid (DHB) in methanol/water (1:1). The mixture was applied to a polished stainless-steel target and then dried in atmosphere for a few hours.

MALDI–QIT–TOF MS

MALDI–QIT–TOF mass spectra were acquired on an AXIMA-QIT–TOF mass spectrometer (Shimadzu, Kyoto, Japan). A nitrogen laser was used to irradiate samples, and an average shot of 50 times was taken. Argon was used for CID. The instrument was operated in positive and reflectron mode. An aqueous sample solution (2 μl) was mixed with a matrix solution (2 μl) of 1% DHB in ethanol/water (1:1), and the mixture was applied to a polished stainless-steel target and dried in atmosphere for a few hours.

Results

Acidic oligosaccharides having high molecular masses in BS and HS milk samples

The method for the preparation of the oligosaccharide samples used in the current study was reported previously [17,18]. Two fractions (BS1 and BS2, 2.0 and 2.3 mg, respectively) from the BS milk sample (40 ml) and a fraction containing acidic oligosaccharides (HS, 1.7 mg) were used in the current study. Because the oligosaccharides from the HS and BS milk samples contained type II chain (Galβ1–4GlcNAc-R) but not type I chain (Galβ1–3GlcNAc-R), we add "neo" to all core oligosaccharide structures.

Oligosaccharides obtained from BS1 and BS2 were fluorescently labeled with 2AA and analyzed by MALDI–TOF MS. As shown in Fig. 1A, a large number of ion signals were observed at the range from *m*/*z* 1484.8 to *m*/*z* 3530.4. In BS1, two major molecular ions were observed at *m*/*z* 2362.3 and 2653.3, which were due to monofucosyl LNnH with one and two NeuAc residues (NAc1H4N2F1–2AA and NAc2H4N2F1–2AA). Ions at *m*/*z* 2151.0, 3027.9, and 3318.6 are 80 mass units larger than the *m*/*z* values of monofucosyl LNnH (theoretical molecular mass [mw] 2071.2), difucosyl lacto-*N*-neotetradecaose (LNnTD, theoretical mw 2946.7), and monosialyl difucosyl LNnTD (theoretical mw 3238.6), respectively. These data indicate that these oligosaccharides are substituted with one SO_3H group. In BS2, we observed two major ions at *m*/*z* 2337.2 and

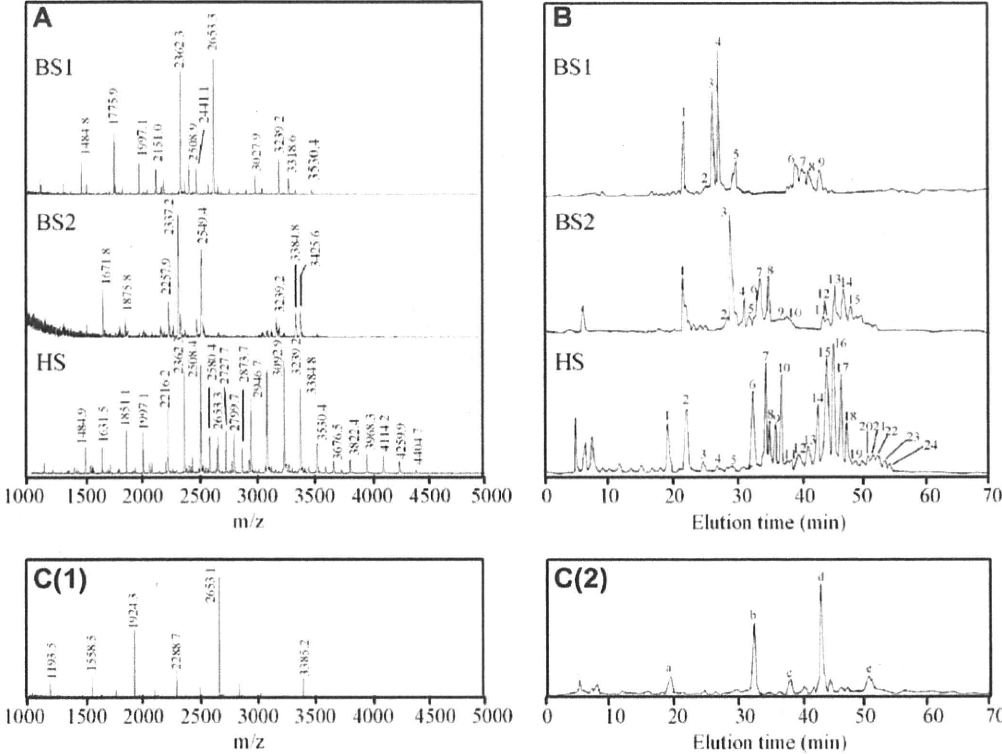

Fig. 1. MALDI–TOF MS and NP–HPLC analysis of higher oligosaccharides from bearded and hooded seal milk. (A) MALDI–TOF MS analysis of sialo-oligosaccharides from bearded and hooded seal milk. (B) NP–HPLC analysis of asialo-oligosaccharides from bearded and hooded seal milk. (C) MALDI–TOF MS and NP–HPLC analysis of defucosylated asialo-oligosaccharides from hooded seal milk. BS1, DEAE-adsorbed fraction 1 in BS milk oligosaccharides; BS2, DEAE-adsorbed fraction 2 in BS milk oligosaccharides; HS, higher oligosaccharide fraction in HS milk oligosaccharides; digestion product of HS with α1–2 fucosidase. The monosaccharide compositions of asialo-oligosaccharides are summarized in Table 1.

2549.4, which were due to difucosyl decaose with one SO_3H group (H5N5F2–SO_3H–2AA) and monosialo–difucosyl neodecaose with one SO_3H group (NAc1H5N5F2–SO_3H–2AA), respectively. The molecular ion observed at *m/z* 3384.8 was due to monosialo LNnTD with three Fuc residues (NAc1H8N6F3–2AA).

HS showed characteristic ladder ions between *m/z* 1400 and *m/z* 4500. These ladder ions were classified into five groups based on the number of lactosamine (Galβ1–4GlcNAc) units. The ions observed at *m/z* 1484.9 and 1631.5 have the composition of NAc1H4N2–2AA and NAc1H4N2F1–2AA, respectively, and are due to mono- and difucosyl LNnH with one NeuAc residue. Molecular ions at *m/z* 1851.1 and 1997.1 are due to the oligosaccharides having compositions of NAc1H5N3–2AA and NAc1H5N3F1–2AA, respectively. A series of the ions at *m/z* 2216.2, 2362.3, 2508.4, and 2653.3 were observed abundantly in HS and are due to the oligosaccharides having monosialo lacto-*N*-neodecaose (LNnD) core (NAc1H6N4–2AA) to which 0, 1, 2, and 3 Fuc residues are attached. Five signals from *m/z* 2946.4 to *m/z* 3530.4 were consistent with oligosaccharides of NAc1H8N6–2AA having 0 to 4 Fuc residues. In addition, we found characteristic glycans having extremely large molecular masses, as observed for the series of ions observed from *m/z* 3676.5 to *m/z* 4404.7. These oligosaccharides are considered to have the core of LNnTD to which 0 to 5 Fuc residues are attached.

To determine linkages of Fuc residues in HS oligosaccharides, we carried out specific fucosidase digestion. A mixture of HS asialo-oligosaccharides was digested with α1–3/4 fucosidase and α1–2 fucosidase, respectively, and the products were analyzed with MALDI–TOF MS. We found that α1–3/4 fucosidase did not act on these oligosaccharides, whereas digestion with α1–2 fucosidase (*Corynebacterium* sp.) caused disappearance of most ions, and six ions were observed at *m/z* 1193.5, 1558.5, 1924.3, 2288.7, 2653.1, and 3385.2 (Fig. 1C(1)). These ions are consistent with the theoretical *m/z* values of H4N2–2AA, H5N3–2AA, H6N4–2AA, H7N5–2AA, H8N6–2AA, and H10N8–2AA, respectively.

The oligosaccharides obtained from milk samples were also analyzed by HPLC using a TSK-Gel Amide-80 column after removing sialic acids with neuraminidase to improve resolution (Fig. 1B) [32]. We collected the major peaks and observed the molecular ions by MALDI–TOF MS. The results are summarized in Table 1.

BS1-1 obtained from the BS1 fraction was assigned as LNnH having two LacNAc units and core Galβ1–4Glc unit from its molecular ion (*m/z* 1193.5) (Fig. 1B, top panel). Molecular ions (*m/z* 2071.8) of BS1-3 and BS1-4 are consistent with the theoretical *m/z* values of H6N4F1–2AA, suggesting the presence of isomers of monofucosyl LNnD. The peak observed at 30 min (BS1-5) gave two molecular ions (*m/z* 2802.6 and 2949.0). The molecular ion at *m/z* 2802.6 is consistent with the theoretical *m/z* value of H8N6F1, suggesting the structure of monofucosyl LNnTD. Likewise, the molecular ion at *m/z* 2949.0 was assigned as difucosyl LNnTD. Minor peaks (BS1-6 to BS1-9) were due to the oligosaccharides having a core structure of LNnD or LNnTD to which a sulfate group is attached (for confirmation of the structure, see the following section).

In the BS2 fraction, BS2-1 and BS2-3 are composed of H3N3F1–2AA and H5N5F2–2AA, respectively. Digestion of BS2-1 and BS2-3 with α1–2 fucosidase caused the loss of one and two fucose residues, respectively. The defucosylated oligosaccharides gave molecular ions corresponding to the theoretical *m/z* values of H3N3–2AA and H5N5–2AA. From the monosaccharide compositions, these oligosaccharides are considered to be hexa- and decasaccharide, having LacNAc units at the reducing end (for confirmation of the structure, see the following section). Oligosaccharides observed be-

Table 1
List of asialo-oligosaccharides observed in bearded and hooded seal milk.

Peak number	Observed mass	Calculated mass	Composition
(a) BS1			
–1	1193.5	1194.1	H4N2–2AA
–2	2110.8	2111.9	H5N5F1–2AA
–3	2071.8	2071.2	H6N4F1–2AA
–4	2071.8	2071.2	H6N4F1–2AA
–5	2802.6	2801.7	H8N6F1–2AA
	2949.0	2947.9	H8N6F2–2AA
–6	2151.0	2151.2	H6N4F1–SO_3H–2AA
	3027.0	3027.9	H8N6F2–SO_3H–2AA
–7	2150.8	2151.2	H6N4F1–SO_3H–2AA
	3026.9	3027.9	H8N6F2–SO_3H–2AA
–8	2150.5	2151.2	H8N6F2–SO_3H–2AA
	3026.6	3027.9	H6N4F1–SO_3H–2AA
–9	2151.2	2151.2	H6N4F1–SO_3H–2AA
(b) BS2			
–1	1381.6	1381.3	H3N3F1–2AA
–2	2111.6	2111.9	H5N5F1–2AA
–3	2259.5	2258.2	H5N5F2–2AA
–4	2217.7	2217.7	H6N4F2–2AA
–5	2217.2	2217.7	H6N4F2–2AA
	2363.5	2363.7	H6N4F3–2AA
–6	2988.7	2988.0	H7N7F2–2AA
	3134.6	3134.1	H7N7F3–2AA
–7	3134.0	3134.1	H7N7F3–2AA
–8	2948.7	2947.9	H8N6F2–2AA
–9	3094.4	3093.5	H8N6F3–2AA
–10	3240.5	3239.2	H8N6F4–2AA
–11	2338.1	2338.2	H5N5F2–SO_3H–2AA
–12	2338.2	2338.2	H5N5F2–SO_3H–2AA
–13	2337.8	2338.2	H5N5F2–SO_3H–2AA
–14	2337.5	2338.2	H5N5F2–SO_3H–2AA
–15	3215.5	3215.1	H7N7F3–SO_3H–2AA
(c) HS			
–1	1193.2	1194.1	H4N2–2AA
–2	1339.3	1340.4	H4N2F1–2AA
–3	1558.6	1559.0	H5N3–2AA
–4	1704.9	1705.4	H5N3F1–2AA
–5	1761.9	1762.8	H5N4–2AA
–6	1923.9	1924.4	H6N4–2AA
–7	2068.7	2070.0	H6N4F1–2AA
–8	2069.5	2070.0	H6N4F1–2AA
–9	2215.2	2216.3	H6N4F2–2AA
–10	2215.5	2216.3	H6N4F2–2AA
–11	2288.8	2289.4	H7N5–2AA
	2363.5	2362.3	H6N4F3–2AA
–12	2435.6	2435.5	H7N5F1–2AA
	2492.7	2492.4	H7N6–2AA
–13	2580.9	2581.5	H7N5F2–2AA
	2637.0	2638.5	H7NF1–2AA
–14	2652.7	2654.0	H8N6–2AA
–15	2798.6	2799.7	H8N6F1–2AA
–16	2944.5	2945.7	H8N6F2–2AA
–17	3089.9	3091.7	H8N6F3–2AA
–18	3236.4	3237.7	H8N6F4–2AA
–19	3236.7	3237.7	H8N6F4–2AA
–20	3382.8	3384.1	H10N8–2AA
–21	3528.8	3530.0	H10N8F1–2AA
–22	3674.6	3676.3	H10N8F2–2AA
–23	3819.8	3822.0	H10N8F3–2AA
–24	3820.0	3822.0	H10N8F3–2AA
(d) Defucosyl HS			
a	1193.5	1194.1	H4N2–2AA (lacto-*N*-neohexaose)
b	1924.2	1924.4	H6N4–2AA (lacto-*N*-neodecaose)
c	2288.4	2289.4	H7N5–2AA (lacto-*N*-neododecaose)
d	2653.1	2654.0	H8N6–2AA (lacto-*N*-neotetradecaose)
e	3382.5	3384.1	H10HN8-2AA (lacto-*N*-neooctadecaose)

tween 30 and 38 min (BS2-4 to BS2-10) are considered to have multi-Fuc residues. BS2-4 and BS2-5 have the core structure of the LNnH unit and contain Fucα1–2 residues at the nonreducing ends because these fucose residues were specifically released by digestion with α1–2 fucosidase (data not shown). BS2-6 and BS2-7 showed molecular ions at *m*/*z* 2988.7 and 3134.6, respectively, which correspond to the compositions of H7N7F2–2AA and H7N7F3–2AA. Peaks BS2-8 to BS2-10 were LNnTD containing multiple Fucα1–2 residues. As a group of characteristic oligosaccharides in BS2, oligosaccharides having 80 mass units larger than BS2-3 were observed between 42 and 49 min. Oligosaccharides (BS2-11 to BS2-14) showed molecular ions at *m*/*z* 2338.2, indicating the composition of H5N5F2–SO_3H–2AA. These oligosaccharides are considered to be isomers having both Fuc and sulfate groups at different positions.

We found 24 oligosaccharide peaks in total in the HS milk sample. These oligosaccharides had LNnH, LNnD, lacto-*N*-neododecaose (LNnDD), LNnTD, and lacto-*N*-neooctadecaose (LNnOD) as core structures (Table 1, part c). HS-1 and HS-2 observed at 20.0 and 21.5 min, respectively, gave molecular ions at *m*/*z* 1193.2 and 1339.3, which correspond to H4N2–2AA and H4N2F1–2AA, respectively. HS-6, -7, -8, -9, and -10 showed molecular ions at *m*/*z* 1923.9, 2068.7, 2069.5, 2215.2, and 2215.5, respectively. The molecular ion of HS-6 is consistent with the theoretical mw of H6N4–2AA, suggesting the structure of LNnD. HS-7/8 (*m*/*z* 2068.7/2069.5) and HS-9/10 (*m*/*z* 2215.2/2215.5) showed larger molecular ions than those of HS-6 by one Fuc (146 mass units) and two Fuc (292 mass units), respectively. From these results, we concluded that these oligosaccharides had the core structure of LNnD to which different numbers of Fuc residues were attached (for confirmation of the structures, see below). The most abundant group of peaks (HS-14 to HS-19) commonly contains LNnTD (HS-14 at *m*/*z* 2652.7) as the core structure. HS-15, -16, -17, and -18/19 showed *m*/*z* values larger than LNnTD by one (146 mass units) to four (584 mass units) Fuc residues. These results indicate that HS-14 to HS-19 have LNnTD unit to which different numbers of Fuc residues are attached. The peaks (HS-20 to HS-24) having high molecular weights (*m*/*z* 3382.8 to 3820.0) were also observed between 50 and 54 min. These ladder peaks contained LNnOD (theoretical mw 3384.1) as the core structure to which one to four fucose residues are attached.

Urashima and coworkers reported that GlcNAc residues of LNnT and LNnH units in BS and HS oligosaccharides are not fucosylated. In contrast, most GlcNAc residues in bear milk oligosaccharides are fucosylated at OH-3 [17,18]. After digestion of asialo-oligosaccharides derived from HS with α1–3,4 fucosidase from *Streptomyces* sp. 142 or α1–2 fucosidase from *Corynebacterium* sp., the digestion products were analyzed by NP–HPLC. α1–3,4 Fucosidase did not act on the HS oligosaccharides, indicating that the oligosaccharides were not substituted at OH-3/4 on GlcNAc residues with fucose residues (data not shown). In contrast, most peaks disappeared on digestion with α1–2 fucosidase, and five peaks were observed at 19 min (peak a), 32 min (peak b), 38 min (peak c), 43 min (peak d), and 51 min (peak e) (Fig. 1C and Table 1, part d). These data indicated that all core oligosaccharides observed in Fig. 1C(2) were composed of one reducing terminal lactose and 2 to 8 LacNAc units. Peaks a and b showed molecular ions at *m*/*z* 1193.5 and 1924.2, which correspond to the molecular masses of LNnH and LNnD, respectively. Peak c showed a molecular ion at *m*/*z* 2288.4 of LNnDD. Peak d was the most abundant one in HS and showed the molecular ion of LNnTD at *m*/*z* 2653.1. In a similar manner, we confirmed that peak e was due to LNnOD.

Characterization of the branching pattern of BS oligosaccharides

The structures of dominant oligosaccharides in BS1 (BS1-1, -3, and -4 in Fig. 1B) were easily assigned. Digestion of BS1-1 with β-galactosidase from jack beans caused loss of two galactose res-

idues ($\Delta m/z$ 324). Further digestion with β-*N*-acetylhexosaminidase gave a molecular ion at *m/z* 461 corresponding to lactose with 2AA (data not shown). From the data, we concluded that BS1-1 was substituted with two Gal-GlcNAc residues at Gal OH-6 and Gal OH-3 of lactose. The BS1-3 and BS1-4 were digested with α1–2 fucosidase to afford an ion at *m/z* 1923.5 corresponding to LNnD. Digestion of the defucosylated oligosaccharide with β-galactosidase caused the loss of three galactose residues ($\Delta m/z$ 486), and the product showed a molecular ion at *m/z* 1435. From these results, we concluded that the core oligosaccharide of BS1-3 and BS1-4 is substituted with two LacNAc units on either branch of LNnH (data not shown). Oligosaccharides observed between 38 and 42 min gave molecular ions H6N4F1–SO_3H–2AA and H8N6F1–SO_3H–2AA (*m/z* 2151.2 and 3026.6, respectively). Among these oligosaccharides, we obtained BS1-9 as nearly pure state (Fig. 2). Digestion of the BS1-9 with α1–2 fucosidase caused the loss of one fucose residue and gave a molecular ion corresponding to LNnD (*m/z* 2005.1) with a sulfate group. Serial digestions of the defucosylated oligosaccharide with β-galactosidase and β-*N*-acetylhexosaminidase caused the loss of two LacNAc units and gave a molecular ion corresponding to the composition of H4N2–SO_3H–2AA (*m/z* 1275.6). These results indicated that the defucosylated oligosaccharide has two nonsubstituted Gal residues at the nonreducing ends. Further digestion of the oligosaccharide with β-galactosidase gave a molecular ion, H3N2–SO_3H–2AA (*m/z* 1113.3). Urashima and coworkers reported that some oligosaccharides in BS milk were sulfated at the nonreducing terminal Gal OH-3 [18]. From this report and our observations, we concluded that the oligosaccharides from BS1-6 to BS1-9 were due to LNnD and LNnTD substituted with one sulfate group at the OH-3 position of the nonreducing terminal Gal residue.

Structures of dominant oligosaccharides in BS2 (BS2-1 and BS2-3) were confirmed in a similar manner. Digestion of BS2-1 with α1–2 fucosidase caused the loss of one fucose residue (Fig. 3A). Further digestion with β-galactosidase gave a molecular ion (*m/z* 911.4) corresponding to H3N3–2AA. Finally, digestion with β-*N*-acetylhexosaminidase gave a molecular ion at *m/z* 505.1 corresponding H1N1–2AA. Accordingly, we concluded that the core disaccharide at the reducing end in BS2-1 was Gal-GlcNAc and that BS2-1 was a hexasaccharide substituted with two LacNAc units at OH-6 and OH-3 of Gal residue of the terminal Gal-GlcNAc. Oligosaccharide BS2-3 was also digested with α1–2 fucosidase to give a glycan showing the molecular ion at *m/z* 1966.5 corresponding to H5N5–2AA (Fig. 3B). Digestion of the core oligosaccharide with β-galactosidase caused the loss of three galactose residues ($\Delta m/z$ 486). Further digestion with β-*N*-acetylhexosaminidase gave a molecular ion (*m/z* 869.9) corresponding to H2N2–2AA. Thus, we concluded that one of the branched units on BS2-1 was further substituted with two LacNAc residues. The characteristic oligosaccharides from BS2-11 to BS2-14 showed molecular ions at *m/z* 2238.1, which are consistent with the composition of H5N5F2–SO_3H–2AA. These oligosaccharides were digested with α1–2 fucosidase to produce a signal at *m/z* 2046.3 corresponding to H5N5–SO_3H–2AA. Further digestion of the defucosylated oligosaccharide with β-galactosidase caused the loss of two Gal residues. These observations indicated that the oligosaccharides from BS2-11 to BS2-14 have two LacNAc branches substituted with α1–2 Fuc residue (data not shown).

Characterization of the branching pattern of HS oligosaccharides

Digestion of the core oligosaccharide (A, peak a in Fig. 1C(2)) with β-galactosidase caused the loss of two galactose residues

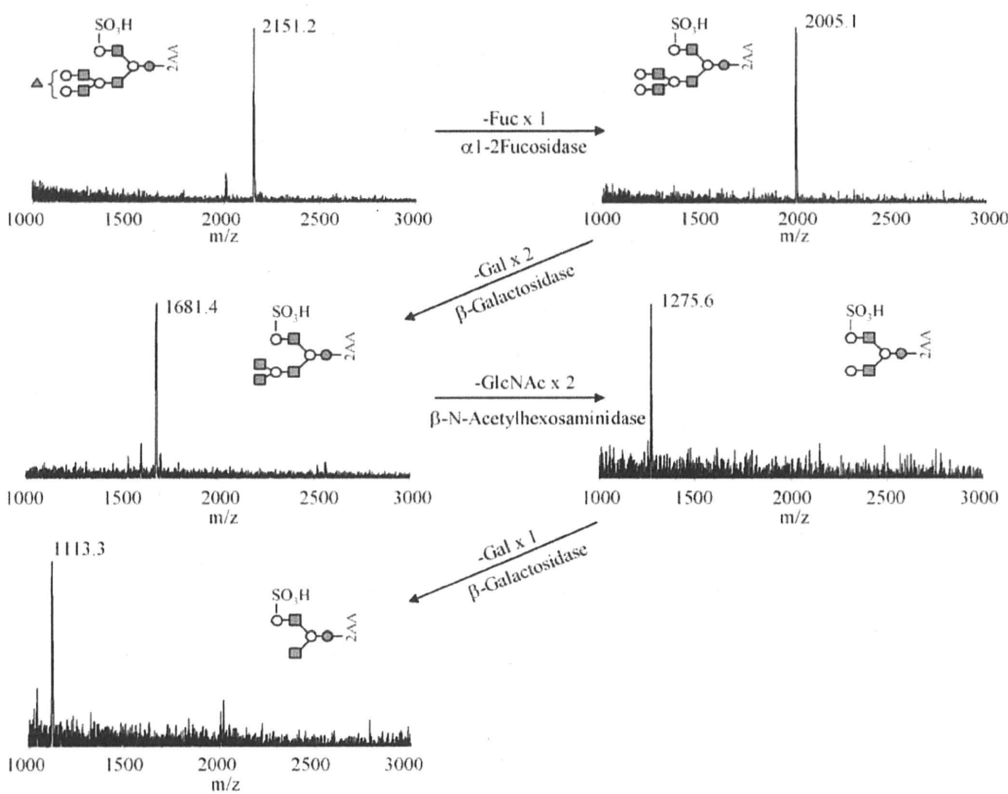

Fig. 2. Stepwise exoglycosidase digestion of characteristic oligosaccharide BS1-9 observed in Fig. 1B. Conditions for the enzymatic reaction with exoglycosidases are shown in Materials and methods. Symbols: open circles; Gal; filled circles, Glc; filled squares, GlcNAc; filled triangles, Fuc. Linkage positions are assigned tentatively.

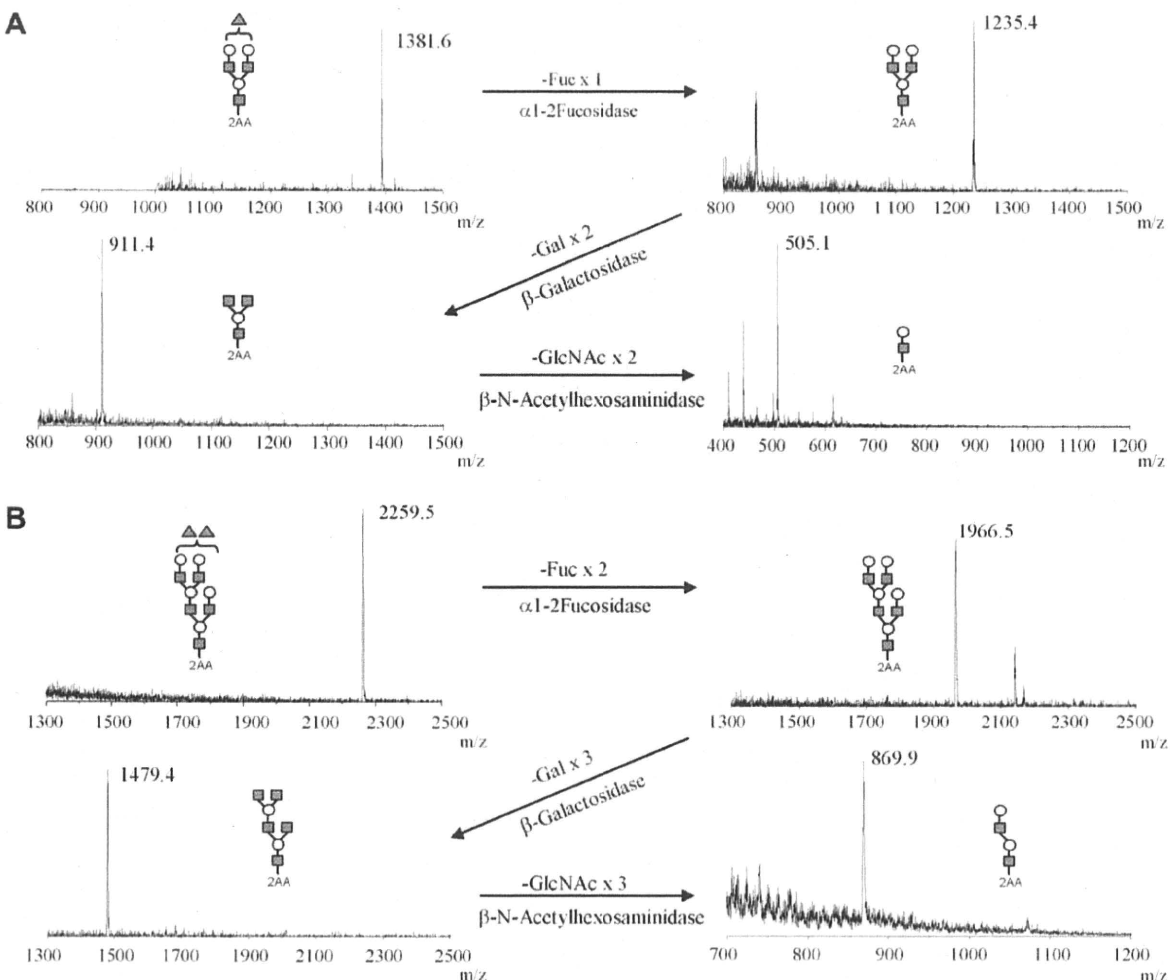

Fig. 3. Stepwise exoglycosidase digestion of characteristic oligosaccharides BS2-1 (A) and BS2-3 (B) observed in Fig. 1B. Symbols: open circles, Gal; filled circles, Glc; filled squares, GlcNAc; filled triangles, Fuc. Linkage positions are assigned tentatively.

($\Delta m/z$ 324) and gave a product ion at m/z 868.8 (Fig. 4A). The oligosaccharide at m/z 868.8 was further digested with β-*N*-acetylhexosaminidase, and a new ion corresponding to Gal-Glc–2AA was observed at m/z 462.4. From these observations, we concluded that the oligosaccharide (peak a) has the structure of Galβ1–4GlcNAcβ1–6[Galβ1–4GlcNAcβ1–3]Galβ1–4Glc (LNnH). Digestion of the core oligosaccharide (B, peak b in Fig. 1C(2)) with β-galactosidase caused the loss of three galactose residues ($\Delta m/z$ 486), and the product showed a molecular ion at m/z 1437.5 (Fig. 4B). The oligosaccharide at m/z 1437.5 was further digested with β-*N*-acetylhexosaminidase to release three GlcNAc residues. These observations indicated that the core oligosaccharide (peak b) has a triantennary structure. The produced oligosaccharide corresponding to Gal-GlcNAc-Gal-Glc–2AA (m/z 826.7) was again digested with β-galactosidase to produce a peak at m/z 665.1 (GlcNAc-Gal-Glc–2AA). The structure was confirmed by comparison of the retention time with that of trisaccharide (GlcNAcβ1–3Galβ1–4Glc–2AA) prepared by digestion of lacto-*N*-tetraose (Galβ1–3GlcNAcβ1–3Galβ1–4Glc) with β-galactosidase using HPLC on an ODS column and capillary electrophoresis (data not shown). These observations indicated that the core oligosaccharide (peak b) has two LacNAc units on the α1–3 LacNAc branch of LNnH. Digestion of the core oligosaccharide (C, peak c in Fig. 1C(2)) with β-galactosidase caused the loss of three Gal residues ($\Delta m/z$ 486), and the product showed a molecular ion at m/z 1801.9. The oligosaccharide at m/z 1801.9 was further digested with β-*N*-acetylhexosaminidase to produce a molecular ion at m/z 1193.8. The oligosaccharide has the structure of H6N4–2AA. These results indicated that peak c has a triantennary structure. The oligosaccharide (m/z 1193.8) was again digested with β-galactosidase to produce a peak at m/z 869.1 by the loss of two galactose residues. The course of digestion by a combination of exoglycosidases revealed that peak c was a dodecasaccharide having three LacNAc residues at nonreducing ends, and we concluded that the oligosaccharide of peak c has two LacNAc units and one LacNAc unit on both branches of LNnH. Digestion of the core oligosaccharide (D, peak d in Fig. 1C(2)) with β-galactosidase caused the loss of four galactose residues ($\Delta m/z$ 648), and the product showed a molecular ion at m/z 2004.5. The product was further digested with β-*N*-acetylhexosaminidase to produce an ion at m/z 1193.8 corresponding to LNnH. These observations indicated that peak d has a tetraantennary structure. The produced oligosaccharide corresponding to LNnH was again digested with β-galactosidase to produce a molecular ion at m/z 869.1. From these results, we concluded that peak d was a tetradecasaccharide having four LacNAc residues at the nonreducing ends and that both branches of LNnH were substituted with two LacNAc units. The oligosaccharide (E, peak e in Fig. 1C(2)) having the largest molecular mass (m/z 3382.5) present in HS milk caused

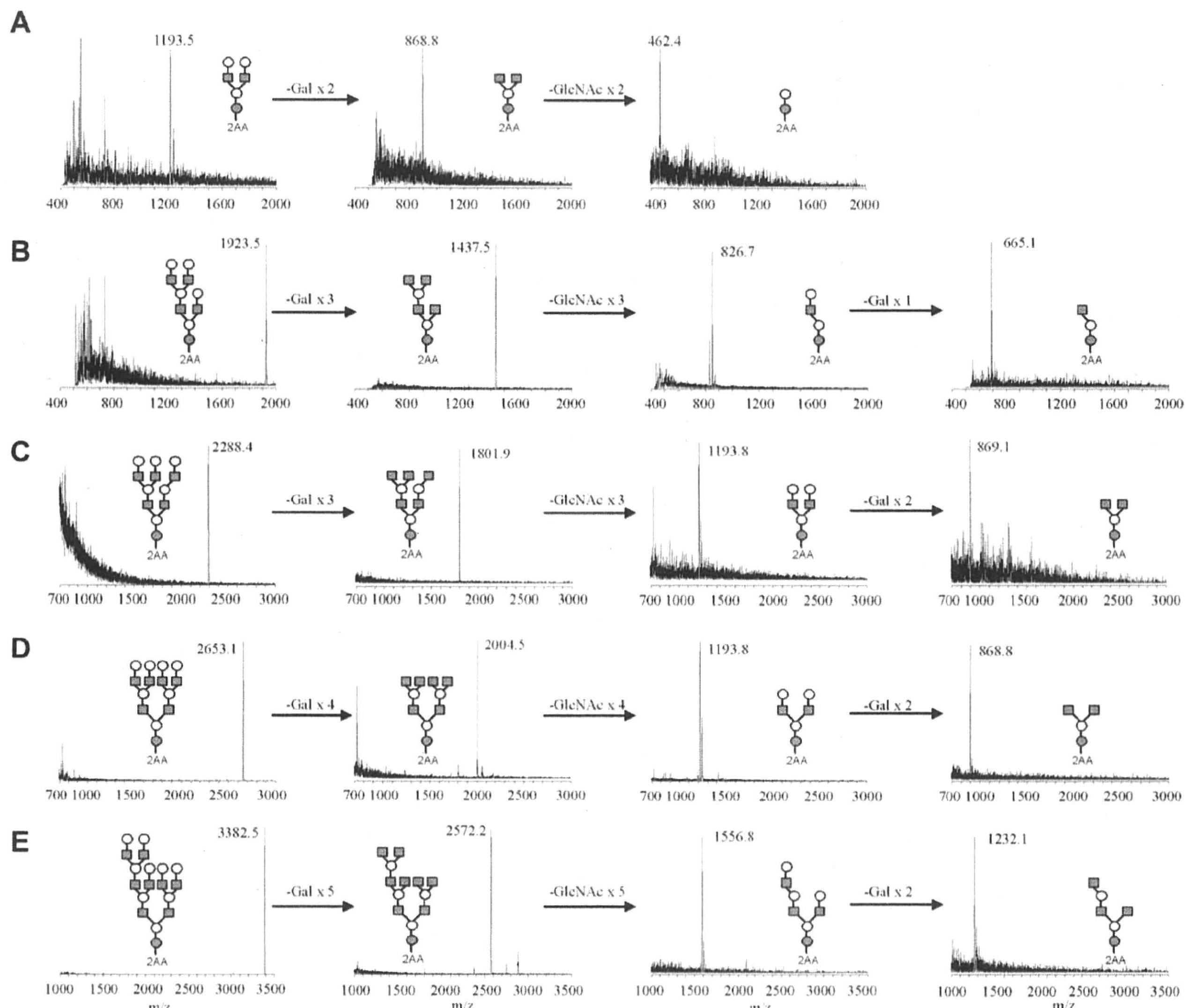

Fig. 4. Stepwise exoglycosidase digestion of core oligosaccharides derived from HS observed in Fig. 1B: (A) lacto-*N*-neohexaose (LNnH); (B) lacto-*N*-neodecaose (LNnD); (C) lacto-*N*-neododecaose (LNnDD); (D) lacto-*N*-neotetradecaose (LNnTD); (E) lacto-*N*-neooctadecaose (LNnOD). Symbols: open circles, Gal; filled circles, Glc; filled squares, GlcNAc.

the loss of five galactose residues ($\Delta m/z$ 810) by digestion with β-galactosidase, and the product showed a molecular ion at m/z 2572.2. The product was further digested with β-*N*-acetylhexosaminidase to produce a molecular ion at m/z 1556.8. The oligosaccharide was again digested with β-galactosidase to produce a peak at m/z 1232.1. From these results, we concluded that the oligosaccharide derived from peak e was an octadecasaccharide, as shown in Fig. 4E.

Structural determination of fucosylated decasaccharides by MALDI–QIT–TOF MS

The core oligosaccharides in HS milk are substituted with a different number of fucose residues, as shown by characteristic ladder patterns (Fig. 1B). We purified monofucosylated LNnD (MFLNnD, HS-7, and HS-8 in Fig. 1B) and difucosylated LNnD (DFLNnD, HS-9, and HS-10 in Fig. 1B) and analyzed them using MALDI–QIT–TOF MS. Fig. 5 shows the MS/MS spectra using $[M + Na]^+$ observed at m/z 2093.1 for the purified MFLNnD (Fig. 5A and B). The Y ion at m/z 1947.5/1947.2 corresponding to the loss of 146 mass units (dHex-18 mass) from $[M + Na]^+$ indicates the presence of a Fuc residue. The Y ions at m/z 1728.0/1728.1 and 1581.9/1582.0 are due to H5N3F1–2AA and H5N3–2AA, respectively. These fragment ions were commonly observed in HS-7 and HS-8. We also found the set of B ion series, $[H2N2]^+$ at m/z 753.5/754.5, $[H3N3]^+$ at m/z 1118.7/1118.8, $[H3N3F1]^+$ at m/z 1264.8/1264.9, and $[H4N3F1]^+$ at m/z 1791. Characteristic ions observed at m/z 1264.8 (Fig. 5A) and m/z 1118.8 (Fig. 5B) suggested the difference in the linkage positions of Fuc residues at the nonreducing Gal residues. The B ion at m/z 1264.8 ($B_{5\alpha}$) indicates that a Fuc residue is linked to the most outer LacNAc residue. The B ion at m/z 1118.8 corresponding to three LacNAc units suggests that one Fuc is attached to the 6-branch side of the reducing terminal lactose. Urashima and coworkers reported that small oligosaccharides in HS milk contained type II LacNAc (Galβ1–4GlcNAc-R) but not type I LacNAc (Galβ1–3GlcNAc-R) [14]. Thus, the oligosaccharides HS-7 and HS-8 are assigned to those as indicated in Fig. 5.

Fig. 6 shows the MS/MS spectra of the ions at m/z 2239.8 for the $[M + Na]^+$ of DFLNnD (HS-9 and HS-10 in Fig. 1B). The Y ions at m/z 2093.3 corresponding to a loss of 146 (dHex-18 mass) from $[M + Na]^+$ indicate the presence of Fuc residue. In a similar manner, in the case of MS/MS of MFLNnD (Fig. 5), the Y ions observed at m/z

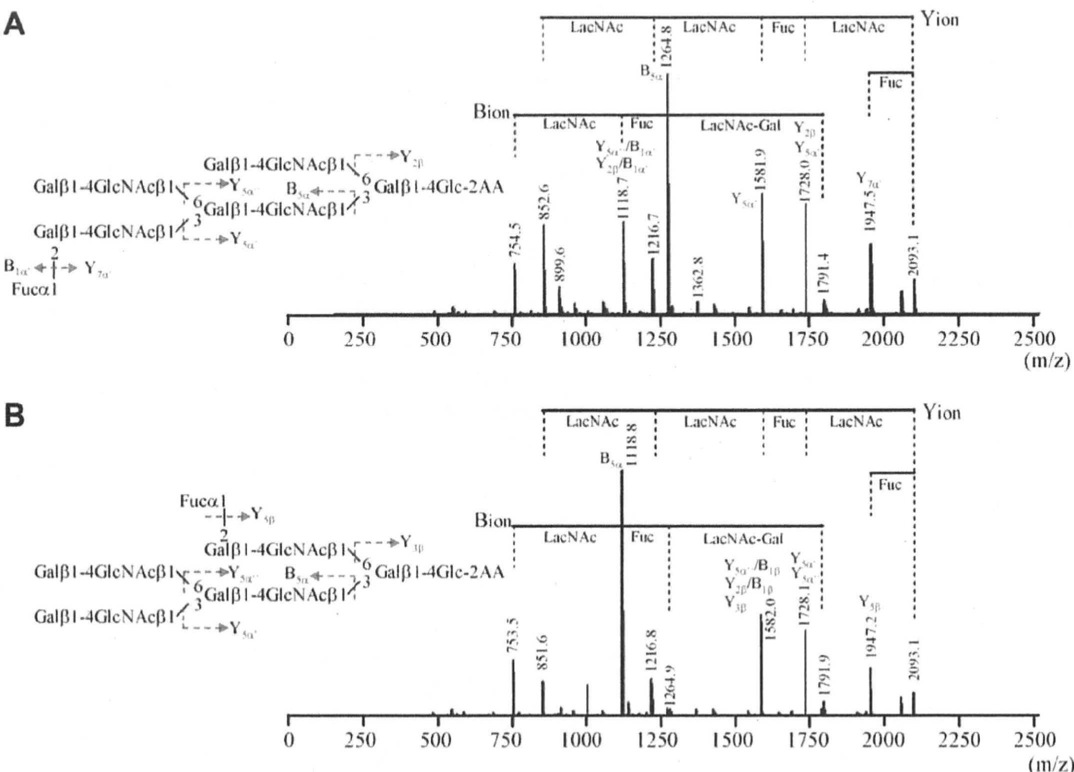

Fig. 5. MS/MS spectra of monofucosyl LNnD HS-7 (A) and HS-8 (B). The dotted lines on the spectra indicate type of cleavage (Y or B ion) according to Domon and Costello's nomenclature [48].

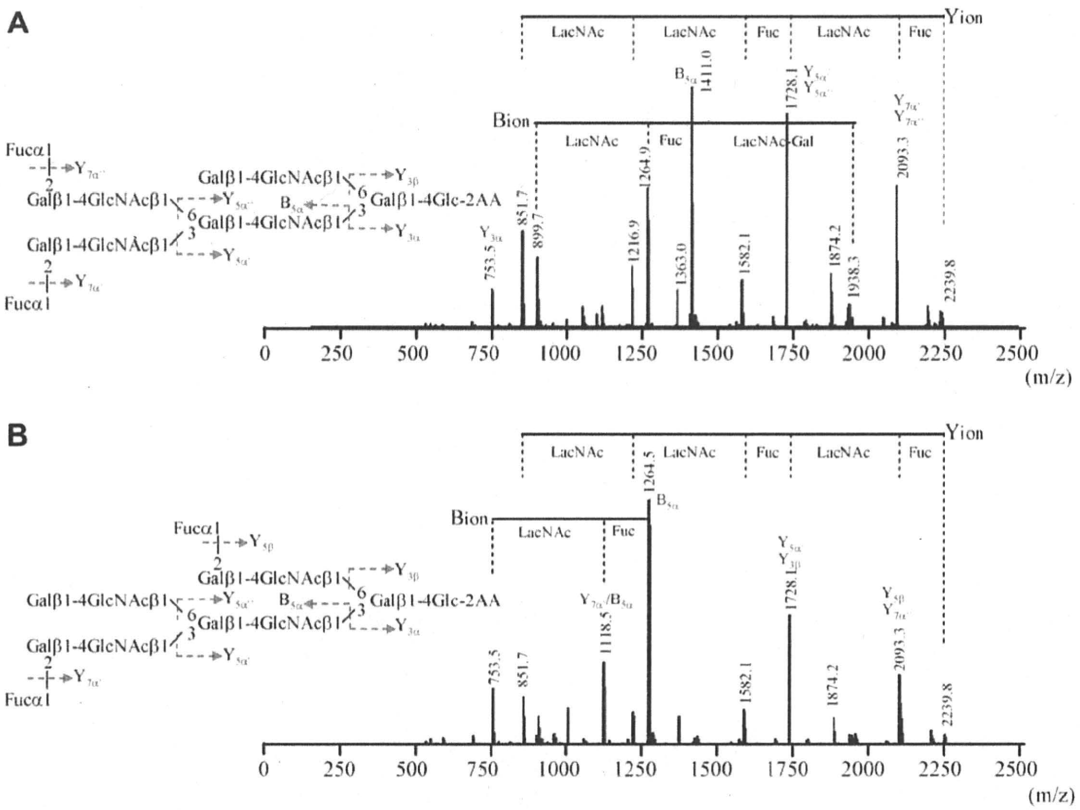

Fig. 6. MS/MS spectra of difucosyl LNnD HS-9 (A) and HS-10 (B). The dotted lines on the spectra indicate type of cleavage (Y or B ion) according to Domon and Costello's nomenclature [48].

1728.1 and 1582.1 correspond to [H5N3F1–2AA] and [H5N3–2AA], respectively. The Y ion at *m/z* 851.7 corresponds to the composition of $[H3N1–2AA]^+$. These fragment ions of the Y ion series were commonly observed in HS-9 and HS-10. We also observed the set of B ion series, $[H2N2]^+$ at *m/z* 753.5, $[H2N2F1]^+$ at *m/z* 1118.5, $[H3N3F1]^+$ at *m/z* 1264.5, and $[H3N3F2]^+$ at *m/z* 1411.0. A characteristic ion at *m/z* 1411.0 observed in HS-9 (Fig. 6A), which corresponds to three LacNAc units having two Fuc residues, indicates that two Fuc residues are attached to the nonreducing Gal residues of both LacNAc branches linked to Galβ1–4GlcNAcβ1–3Galβ1–4Glc, as shown in Fig. 6A. Thus, the structure of HS-9 is assigned as shown in Fig. 6A. In contrast, a characteristic ion at *m/z* 1264.5 ($B_{5\alpha}$) observed in HS-10 (Fig. 6B) indicates the attachment of only one Fuc to the most outer LacNAc residue. Therefore, the oligosaccharide (HS-10) is assigned to the structure as shown in Fig. 6B.

Among LNnD substituted with α1–2 Fuc residues, HS-7 and HS-10 were abundantly present (Fig. 1B) and both oligosaccharides have an α1–2 Fuc residue on either LacNAc on the α1–3 branch of the LNnH core. These observations suggest that the modification of multibranched core oligosaccharides with α1–2 Fuc residues proceeds preferably at LacNAc residues of the elongated branches.

Discussion

We studied structural features of oligosaccharides from the milk samples of bearded and hooded seals by NP–HPLC and MALDI–TOF MS. The combination of sequential digestion of the oligosaccharides with exoglycosidases and MALDI–TOF MS was a useful technique for elucidation of the branching patterns and modification of oligosaccharides with fucose and/or sulfate group(s). Table 2 shows a list of asialo-oligosaccharides found in bearded and hooded seal milk. The oligosaccharides are categorized into nine core structures (A–I) based on the monosaccharide compositions. LNnD and LNnTD (C and E in Table 2) were observed as common core structures in both milk samples, but the two species showed quite different features. The most characteristic fea-

Table 2
Structural features of the oligosaccharides derived from bearded and hooded seal milk.

ID	Core structure	Number of Fuc residues					ID	Core structure	Number of Fuc residues				
		0	1	2	3	4			0	1	2	3	4
A	2AA	HS	HS	–	–	–	F	2AA	HS	HS	HS	HS	HS
B	2AA	HS	HS	–	–	–	G	2AA	–	BS	–	–	–
C	2AA	HS	HS BS	HS BS	HS BS	–	H	2AA	–	BS	BS	–	–
Cs	2AA +SO_3H*	–	BS	–	–	–	Hs	2AA +SO_3H*	–	–	BS	–	–
D	2AA	HS	HS	HS	–	–	I	2AA	–	–	BS	BS	–
E	2AA	HS	HS BS	HS BS	HS BS	HS BS	Is	2AA +SO_3H*	–	–	–	BS	–
Es	2AA +SO_3H*	–	–	BS	–	–							

*Core structures with sulfate group at 3-OH position of nonreducing terminal Gal.

ture of oligosaccharides in both milk samples is that multi-branched oligosaccharides were present and linear oligosaccharides were not detected in the current study.

BS milk contained characteristic oligosaccharides having monosaccharide compositions of H3N3F1 (G in Table 2), H5N5F1 (H), and H7N7F3 (I), and these oligosaccharides were confirmed as Galβ1–4GlcNAcβ1–3[Galβ1–4GlcNAcβ1–6]Galβ1–4GlcNAc and Galβ1–4GlcNAcβ1–3[Galβ1–4GlcNAcβ1–6]GlcNAcβ1–3[Galβ1–4GlcNAcβ1–6]Galβ1–4GlcNAc. Free oligosaccharides having LacNAc at the reducing end have been reported in bovine and caprine colostrum [33-35]. In mammary glands, lactose is synthesized by lactose synthase, a complex of β4 galactosyltransferase I (βGalT-I) and α-lactalbumin [36]. βGalT-I is also involved in the synthesis of Galβ1–4GlcNAc in the case of the absence of α-lactalbumin. However, α-lactalbumin in lactating mammary glands changes the preferred acceptor of βGalT-I from GlcNAc to Glc [37]. Interestingly, bovine colostrum contains oligosaccharides such as NeuAcα2–6Galβ1–4GlcNAc, Galβ1–4(Fucα1–3)GlcNAc, and Galβ1–3(Fucα1–4)GlcNAc, but their concentrations decrease dramatically to the trace level 7 days after parturition [38,39]. The presence of large oligosaccharides such as H, HS, I, and Is in BS milk strongly suggests that biosynthesis starts from LacNAc as the core structure.

HS milk contained varieties of oligosaccharides having multi-branched core structures (i.e., cores E and F in Table 2). In addition, most oligosaccharides were substituted with different numbers of α1–2 Fuc residues at the nonreducing terminal Gal residues. All oligosaccharides in HS milk have LNnH (Galβ1–4GlcNAcβ1–3[Galβ1–4GlcNAcβ1–6]Galβ1–4GlcNAc) as a common core. They are preferentially elongated at the Galβ1–4GlcNAcβ1–3 branch of the LNnH core unit. For example, the core structure having monosaccharide compositions of H6N4 (C in Table 2) has two LacNAc residues on the Galβ1–4GlcNAcβ1–3 branch of LNnH (see Fig. 4B), in contrast to the branching of lacto-*N*-decaose in human milk [40]. Among the β-*N*-acetylglucosaminyltransferase (GnT) family, β3-*N*-acetylglucosaminyltransferase (iGnT), which is a key enzyme for the elongation of LacNAc sequence, prefers type II chain (Galβ1–4Glc/GlcNAc) [41–43]. Urashima and coworkers analyzed small oligosaccharides in HS milk by ^{1}H NMR spectroscopy and revealed that major oligosaccharides contained only type II chains [14]. The addition of Galβ1–4 residue to terminal GlcNAc provides the preferable acceptor for iGnT enzyme. In contrast, HS milk contains multiantennary oligosaccharides (E in Table 2), which have two LacNAc residues on both branches of LNnH, indicating that the Gal residue on the β1–6 branch of LNnH was substituted followed by modification of the β1–3 branch of LNnH. iGnT was considered to be less efficient to the longer LacNAc repeats as the acceptors [44]. Furthermore, the efficiency of iGnT may be decreased by the presence of the Galβ1–4GlcNAcβ1–6 branch to the Gal residue of Galβ1–4GlcNAcβ1–3Galβ1–4GlcNAc. In general, the sequence of Galβ1–4GlcNAcβ1–3Galβ1–4Glc/GlcNAc provides the preferred acceptor for β(1–6)*N*-acetylglucosaminyltransferase (IGnT), which is thought to be a key enzyme for the branching of oligosaccharides [45–47]. It is likely that human milk oligosaccharides preferentially is elongated at the Galβ1–4GlcNAcβ1–6 branch of LNH, and the Galβ1–3GlcNAcβ1–3 branch of LNH does not receive further modification with LacNAc [6]. The presence of multi-branched oligosaccharides in seal milk suggests that enzyme activities of IGnT and β4GalTs in seal mammary glands are higher than those in other eutherian mammals.

In this article, we have focused on characterization of the branching pattern of oligosaccharides of high molecular masses in seal milk samples by means of the combination of MALDI-TOF MS and sequential exoglycosidase digestion. Branching is one of the major structural features of carbohydrates, and a relatively simple set of monosaccharides can form various branching configurations. Techniques based on MS/MS were used for the structural characterization of oligosaccharides. Special emphasis was made so that the combined use of MALDI–TOF MS and sequential exoglycosidase digestion gave unambiguous structural details of multi-branched oligosaccharides, including linkage positions and anomeric configurations.

Acknowledgment

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2. ヒト幹細胞からの肝細胞分化誘導とその創薬非臨床試験への応用

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今日の医薬品開発では，ヒトでの肝毒性および薬物体内動態・薬物相互作用を予測するための非臨床試験において，ヒト肝細胞・肝組織を用いた毒性試験および薬物動態・薬理試験を行うことが非常に重要だと認識されている．しかし，ヒト肝細胞・肝組織の入手の難しさ，個体差およびロット差の問題など，大きな問題点が依然として存在する．本稿では，これらの問題点について解説するとともに，その克服を目指し，ヒト多能性幹細胞から肝細胞を効率的に誘導して肝毒性・代謝研究に応用する試みについて述べる．

はじめに

生体の薬物に対する反応において，ヒトと動物との間にはきわめて大きな種差がある．その主な原因として，標的組織における反応性の相違と薬物の体内動態の相違があげられる．特に，薬物動態関連因子は実験動物とヒトとの間には著しく大きな種差があることが知られている．これらの種差のため，開発中の医薬品候補化合物がヒトでの臨床段階において思わぬ毒性を示したり，期待される薬物動態を示さなかったりするなどして，開発中止になることがある．そこで最近ではヒト細胞・組織を用いた非臨床試験が広く行われるようになった．医薬品開発の初期においてヒト細胞・組織を用いた非臨床試験を行うことは，開発にかかわる意思決定を早めることを可能とし，医薬品開発の効率化と経済性に資するとともに，結果として無駄な動物実験を省くことにも役立つ．

[キーワード&略語]
薬物性肝障害，特異体質，薬物代謝，個人差，幹細胞

ES細胞：embryonic stem cell（胚性幹細胞）
FGF：fibroblast growth factor（線維芽細胞増殖因子）
HGF：hepatocyte growth factor（肝細胞増殖因子）
HNF-3β：hepatocyte nuclear factor-3β（肝細胞核因子3β）
iPS細胞：induced pluripotent stem cell（人工多能性幹細胞）

肝臓はヒト体内では最大の腺組織であり，代謝において中心的な役割を担い，健康な生命の維持に大きく寄与している．このため，薬物誘発性の肝機能障害（薬物性肝障害）は薬物に由来する副作用のなかでも医薬品開発の過程における開発中止，警告表示あるいは販売中止に至る主要な薬物関連有害事象の1つとなっている．したがって，医薬品開発においては，薬物およびその代謝産物のヒト肝臓に対する毒性を可能な限り早期に予測することが大切となる．また，薬物のヒト

In vitro generation of hepatocytes from human stem cells and its application to non-clinical drug development
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表1　薬物性肝障害の分類

	中毒性肝障害	特異体質性肝障害	
		アレルギー性	代謝性
用量依存性	あり	なし	なし
動物での再現性	あり	なし	なし
原因	薬物・代謝産物自体の毒性	反応性代謝産物の抗原性（T細胞依存性肝細胞障害）	薬物代謝関連酵素の個人差（遺伝的特徴）

肝臓での代謝過程を理解することは，「毒性学」の観点からだけでなく，ヒトにおける薬物の体内動態の評価および複数の薬剤を投与する場合の薬物間相互作用の評価という「薬物動態学」または「臨床薬理学」の観点からも重要である．薬物の肝毒性および代謝過程を非臨床試験のなかで評価する際，ヒト肝細胞・肝組織を用いることで種差の問題の多くを克服することが可能となる．しかし，ヒト肝細胞・肝組織には，入手の難しさ，個体差およびロット差など，大きな問題点が存在している．

近年，幹細胞を分化誘導してさまざまな細胞種を効率的に作製する方法の開発が急速に進んでいる．ヒト幹細胞から肝細胞を効率的に誘導し，肝毒性・代謝研究へ応用できれば，これまでのヒト肝細胞・肝組織を用いた非臨床試験における問題点の多くを解決することができると期待されている．

1 薬物性肝障害・薬物応答性

多くの薬物は肝臓で代謝されるため肝障害（肝毒性）を示す可能性がある．薬物性肝障害の肝臓における症状としては，脂肪肝，肝細胞死，毛細胆管性胆汁うっ滞，胆管障害，類洞壁障害，リン脂質症，線維化，硬化，腫瘍などが認められるが，いずれが発生するかは薬物の投与量と頻度，投与期間，および細胞の薬物に対する応答性（薬物動態，薬効・副作用の出方）などによってさまざまである．発症機序をもとにした場合，薬物性肝障害には大きく分けて「中毒性肝障害」と「特異体質性肝障害」とが存在する（**表1**）[1]．

1）中毒性肝障害と特異体質性肝障害

中毒性肝障害の場合は薬物自体またはその代謝産物が肝毒性をもっており，用量依存性であり，動物実験で再現しやすい．したがって，既存の技術でも毒性発現を予測もしくは予防することが論理的には可能である．

これに対し，特異体質性肝障害は薬物に対する特異体質が原因で起きるもので，一般的に用量依存性がなく，動物実験では認められない性格をもち，前もって予測することが困難である．医薬品開発のうえで問題となる薬物性肝障害の多くはこの特異体質性肝障害に属している．特異体質性肝障害は現在ではさらに「アレルギー性特異体質」によるものと「代謝性特異体質」によるものとに分類されている．アレルギー性特異体質性肝障害は薬物の反応性中間代謝産物がハプテンとなり担体タンパク質と結合して抗原性を獲得し，T細胞依存性肝細胞障害により惹起される肝障害である．一方，代謝性特異体質性肝障害は薬物代謝関連酵素の特殊な個人差（遺伝的特徴）に起因する[2]．

2）薬物によって異なる肝障害

中毒性であれ，特異体質性であれ，肝機能障害を惹起しやすいのは化学的に反応性の高い代謝物を生成する薬物であることが知られている．ただし，反応性代謝物を生成しても，ある薬物では惹起される肝障害が中毒性であり，別の薬物では特異体質性となる．薬物によって異なるタイプの肝毒性を引き起こす仕組みは明らかではないが，反応性代謝物が結合する相手のタンパク質種の差による可能性があげられている[1]．いずれにせよ，ヒトにおける反応性代謝物の生成を予測し，その個人差（特に非常に低い比率で存在する遺伝的特徴）と特異体質性肝障害との相関性を評価することは薬物性肝障害の予測において重要である．

3）薬物応答性の個人差

また，肝毒性試験のみならず，薬物動態・薬理試験として肝臓での代謝プロフィールを取得し，薬物の体内動態や複数の薬剤を同時に服用する際にみられる薬物相互作用など，いわゆる「薬物応答性」の個人差を臨床試験開始前に予測することも医薬品開発では重要

表2　非臨床試験においてヒト肝細胞・肝組織を用いる長所・短所

	長所	短所
総体として	・医薬品開発における有効性・安全性の予測 ◆ヒト*in vivo*における代謝プロフィールおよび代謝能レベル ◆薬物輸送に関する置換 ◆薬物相互作用の予測	・入手が困難 ・大きい個体差 ・技術要求 ・バイオハザード対応が必要
ヒト肝細胞・肝組織	・代謝プロフィール・代謝能評価可能（第Ⅰ相，第Ⅱ相） ・薬物誘導能検討可能 ・代謝レベルの薬物間相互作用検討可能 ・*in vivo*-*in vitro*相関が高い	・入手が困難 ・ロット差が大きい ・高い技術レベルが必要 ・ドナー情報が限定
ヒト肝ミクロソーム	・さまざまな代謝能 ・P450発現が高い ・簡便である ・入手しやすい ・P450レベルでの相互作用検討可能	・多くの第Ⅱ相代謝酵素の欠如により，総体的代謝能評価ができない ・個体差が大きい ・ドナー情報が限定
ヒトES細胞・iPS細胞由来肝細胞（予想）	・代謝プロフィール・代謝能評価可能（第Ⅰ相，第Ⅱ相） ・薬物誘導能検討可能 ・代謝レベルの薬物間相互作用検討可能 ・特定ドナー由来の規格化された品質の細胞を大量に入手可能 ・多くのロットを用意することが可能なので個体差情報と母集団情報の双方を取得可能（iPS細胞）	・培養，分化誘導，肝細胞の分離・回収に高い技術が必要 ・匿名化されておりドナー情報が得られない（ES細胞）

（文献3より）

である．薬物応答性の個人差には，薬物代謝・動態関連分子や薬物標的分子の遺伝的多型，ならびにそれらの発現に影響する環境的要因がかかわっている．したがって，薬物応答性の個人差を予測するためには，これら遺伝的・環境的要因とヒト肝臓または肝細胞・肝組織での代謝プロフィールとの関連を明らかにする必要がある．

2 ヒト肝細胞・肝組織を用いた非臨床試験

安全で有効性の高い新薬を効率的に開発するには，ヒトでの薬効や副作用ならびに薬物相互作用を非臨床試験で高精度・高感度に予測し，早期に臨床試験に入ることが求められる．そのため，医薬品開発の初期段階においては，主に肝毒性・薬物応答性の種差の問題の多くを克服するという意味で，ヒト肝細胞・肝組織を用いた試験の重要性が広く認識されている．肝毒性・薬物応答性予測においてヒト肝細胞・肝組織を利用することの長所・短所を**表2**にあげる．ヒト肝組織は，医薬品候補物質のヒト*in vivo*における代謝プロフィールとそのレベルの予測，肝薬物輸送能，および薬物相互作用予測のための優れた実験系である．また，肝ミクロソーム分画はCYP（シトクロムP450）をはじめとする主要な薬物代謝酵素を含み，取り扱いが簡単であること，凍結試料として入手しやすいなどの利点がある．ただし短所として，ミクロソームには，多くの第Ⅱ相薬物代謝※1酵素群が含まれないことから，薬物全体の代謝プロフィールに関するデータを得ることができない．一方，遊離ヒト肝細胞ならびに初代培養ヒト肝細胞にはすべての薬物代謝関連の酵素が含まれることから，薬物の代謝経路および代謝パターンを明らかにできる．米国食品医薬品局（FDA）が2006年に提示した薬物相互作用に関するドラフトガイダンス[4]のなかでは，新薬の申請書類に初代培養ヒト肝細胞を用いた薬物代謝誘導試験などを資料として添付することが推奨されている．

しかしながら，これら従来のヒト肝細胞・肝組織標本を用いた試験系では，標本のドナーの個人差などに起因するロット差が大きいことが問題とされている．

※1　第Ⅱ相薬物代謝

薬物代謝反応は第Ⅰ相と第Ⅱ相に大別される．官能基形成や開裂反応（酸化・還元・加水分解）である第Ⅰ相反応に対し，第Ⅱ相反応は内因性物質（グルクロン酸・硫酸・グリシンなど）との抱合体化である．第Ⅱ相反応を触媒する酵素は主に細胞質にある．

ロット間のばらつきが大きくてもロット数が十分多ければ試験系として問題はないが，ヒト肝細胞・肝組織標本のもう1つの大きな問題として，わが国における法的制約もあり，その入手が困難なことがある．入手先を海外に大きく依存していることから，人種差の評価が難しく，倫理的に問題を孕むことも問題とされている．特異体質性肝障害の発症率は一般に5,000人に1人未満とされており[5]，例えば多数の特異体質性肝障害発症者の肝細胞を得ることなどは著しく困難である．

特異体質性肝障害のような低頻度で観察される肝毒性や薬物応答性の個人差を十分に予測することが可能な非臨床試験を行うためには，① 用いられるヒト肝細胞・肝組織のロット（ドナー）の数をできるだけ多く，かつ各ロットについては② 規格化された品質（細胞の形質・調製過程）のものを③ 安定的に大量に得ることが求められる．しかし，従来の試験系で用いられるようなヒト肝細胞・肝組織標本ではこの3要件を満たすことは容易ではない．また，可能ならば日本人からの標本を用いることが望まれる．これらの問題を解決する手段として，近年，幹細胞から誘導された肝細胞の利用に期待が集まっている．

3 幹細胞からの肝細胞分化誘導

ヒト肝細胞は，たとえ初代培養して増殖させたとしても，徐々に生理機能が減弱し，現在の技術では最長1カ月程度で多くの機能を失ってしまう．また株化された肝細胞由来細胞はごく限られた肝機能しか示さない．胎児の肝臓に存在する肝芽細胞または成体肝臓中に存在するオーバル細胞と呼ばれる細胞は肝幹細胞として肝細胞への分化能をもつが，組織中の細胞数が少ないため，その単離が難しく，非臨床試験に用いることができるほどの体外増殖も困難である．骨髄細胞や臍帯血，羊膜などからも肝細胞様の細胞が誘導されることが報告されているが[6]，肝幹細胞と同様，原材料としての組織・血液の供給量に限りがある．

そこで近年，多能性幹細胞であるヒト胚性幹細胞（ES細胞）やヒト人工多能性幹細胞（iPS細胞）などからヒト肝細胞を誘導しようと試みられている．分化能および自己複製能が有限である体性幹細胞と比較した場合，これらの多能性幹細胞は，ひとたび肝細胞への効率的分化誘導方法が確立すれば，その多能性および無限の自己複製能ゆえに安定した原材料供給が可能となる点で有利である．現時点では肝臓中の肝細胞に比肩するだけの形質をもつ細胞を非臨床試験に用いられるほど大量に人為的に誘導する方法は確立されていないが，ヒトES細胞からの肝細胞様細胞の誘導の効率および生理機能は年々着実に進歩し続けている．ヒトES細胞から肝細胞を誘導する場合，大きく分けて，未分化細胞を内胚葉系に分化させるステップと，内胚葉系細胞を肝細胞へ分化・成熟させるステップとが必要となる．したがって，内胚葉系細胞をいかに効率的に獲得するかが第1の課題となる．

これまでの研究により分化誘導開始時にアクチビンAなどを添加することが内胚葉系細胞への誘導に効果的であることが知られている[6][7]．内胚葉系細胞から肝前駆細胞の誘導にはFGFなどが有効であるとされている．これは，胚発生時に肝前駆細胞を含んだ肝臓前駆組織（肝芽）の出現を誘導する因子の1つがFGFであることと合致する．肝前駆細胞の増殖および成熟にはHGFやオンコスタチンMおよびデキサメタゾンなどが用いられることが多い．

現在，これらの因子の量やタイミングだけでなく新たな液性因子の探索や，転写因子HNF3-βの過剰発現による分化の方向付け，細胞外マトリックス，細胞の足場材料，三次元培養などをさまざまに組合わせた形での培養条件の最適化が精力的に模索されている[8][9]．また，酸素分圧や酸化ストレス状態などの細胞近傍の環境因子，およびES細胞のエピジェネティックな調節などについても最適化の余地があると考えられる．

'07年に初めて報告されたヒトiPS細胞からは，すでに神経細胞，骨細胞，心筋細胞，脂肪細胞，膵臓細胞，血管細胞，造血細胞，内皮細胞など，さまざまな細胞種への分化誘導法が報告されている．ヒトiPS細胞から肝細胞を直接誘導した例はなかなか出なかったが，ごく最近，比較的効率的な肝細胞様細胞の誘導法が報告された[10]．今後の研究の進展が期待される．

4 幹細胞由来肝細胞の非臨床試験への応用

ヒトES細胞およびヒトiPS細胞は高い増殖能・多能性・自己複製能を兼ね備えている．これらを再生医

療・細胞治療に応用する場合はがん化の懸念など，安全性上の高いハードルが存在するが，非臨床試験への応用ではその心配がない．そこで，従来のヒト肝細胞・肝組織を用いた非臨床試験の問題点を克服することを目指し，ヒト多能性幹細胞を効率的に肝細胞に誘導する技術の開発を通じて，信頼性が高くハイスループットの化合物スクリーニング系を樹立する試みが現在続けられている．ヒトES細胞およびヒトiPS細胞から肝細胞への分化については現状の技術では効率または形質の面で十分なものとは言えない．しかし，将来的に優れた分化培養方法が確立されれば，一定の品質のヒト肝細胞を安定的かつ大量に供給することが可能となると考えられる．

ただし，受精胚を原材料とするヒトES細胞は倫理的理由などにより多くのドナーからの樹立が困難と予想され，また制度上ドナーを匿名化する必要があるため，表現型と遺伝型との相関の解析が難しいという問題が存在する．一方，ヒトiPS細胞は原材料としての体細胞を多くのドナーから入手しやすく，またドナーの情報も確保しやすいと考えられる．したがって，多数のドナー由来のヒトiPS細胞由来の肝細胞を用いて薬物の代謝プロフィールおよび遺伝子プロフィールなどを取得し，ドナー情報と照合することが可能になれば，肝毒性・薬物応答性の個人差の詳細なメカニズムが明らかにされることが期待できる．また，もし十分な数のドナーに由来するiPS細胞由来肝細胞を得ることが可能ならば，それらを利用してスクリーニング系を構築し，低頻度の特異体質性肝障害および薬物応答性の個人差を予測することが技術的に可能となることも期待できる．

おわりに

ヒト肝細胞・肝組織を用いた医薬品候補化合物の非臨床試験は，今日の創薬において大変重要なものと認識されているが，原材料の入手をはじめ多くの問題が残されている．ヒトES細胞およびヒトiPS細胞からの肝細胞誘導方法を確立すれば，その多くを解決することができると考えられる．特にヒトiPS細胞は異なるドナー由来の多くのロットをバンク化することが可能であり，特異体質性肝障害および薬物応答性の個人差の予測に威力を発揮することが期待される．ただし，そのためには肝細胞誘導方法の確立のみでは十分ではなく，例えば細胞チップ※2のようなハイスループットのアッセイシステムの構築や，*in vivo*における肝毒性・薬剤応答性と*in vitro*の細胞応答性とをつなぐデータベースおよびインフォマティクスなど，周辺技術の開発も非常に重要になる．

※2　細胞チップ

多くの小さな穴（ウェル）をもち，細胞の反応や変化を観察するために細胞をウェルあたり1つずつ均一に入れることのできるチップ．現在，数十万個〜百万個のウェルをもったシリコンや樹脂製のチップが製作されている．

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<つぶやき>
「『規制科学』という分野に携わっています．一見，威圧感がありますが，実は先端医療の安全性・品質について『何を』『どのように』明らかにすべきかを示すという点で実用化までの電車のレールを敷くような仕事です．」

ヒト細胞・組織加工医薬品などの安全性確保

Safety of human cellular and tissue-based products

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◎再生医療や細胞治療のツールとして使用することを目的に，細胞または組織を加工した製品を“細胞・組織加工医薬品/医療機器”(細胞組織製品)という．わが国では先ごろ，初の細胞組織製品として重症熱傷治療用培養皮膚製品が薬事法上の承認を受け，また世界に先がけてわが国で開発されたヒト人工多能性幹細胞(iPS 細胞)も再生医療・細胞治療への応用に熱い期待が集まっており，国内外で細胞組織製品の熾烈な開発競争が繰り広げられている．しかし，細胞組織製品の本格的な実用化・産業化に至るためには，その安全性評価方法の理解・確立が必須である．本稿ではヒト細胞組織製品の安全性の評価・確保について，最近の厚生労働省の関連指針を軸に概説する．

細胞・組織加工医薬品，細胞・組織加工医療機器，確認申請，品質管理，ヒト幹細胞臨床研究指針

再生医療(患者の組織の再生・修繕または置換を目的とする治療)および細胞治療(加工した細胞を患者の生理的機能の修復・修正または調節を目的として使用する治療)に使用するために，細胞あるいは組織を加工した製品を“細胞・組織加工医薬品”または“細胞・組織加工医療機器”という．本稿では，これらをまとめて“細胞組織製品”とよぶ．わが国では平成 19 年(2007)に重症熱傷治療用培養皮膚製品が，国内初の細胞組織製品として薬事法上の承認を受けた．近い将来にはさらに多くの細胞組織製品が実用化されると見込まれている．

しかし，本格的な細胞組織製品の実用化・産業化を達成するためには，検討すべき課題はまだ多い．なかでも重要な課題は，細胞組織製品の安全性をどう確保したらよいのか，ということである．本稿ではヒト細胞組織製品の安全性の評価・確保のポイントについて，厚生労働省の関連指針を軸に解説する．

ヒト細胞組織製品の品質・安全性ガイドライン

わが国でのヒト細胞組織製品の開発の多くは医師の裁量による臨床研究(あくまで研究が主目的)として行われてきたが，商品化をめざした活動としてのヒト細胞組織製品の開発も近年盛んに行われている．逆に，ヒト細胞組織製品の品質・安全性ガイドラインに関しては商品としてのヒト細胞組織製品に対するものが先行しており，大学などの研究機関での臨床研究を対象にした『ヒト幹細胞を用いる臨床研究に関する指針』(以下“ヒト幹細胞臨床研究指針”，厚生労働省，平成 18 年)においても，臨床研究で用いられるヒト細胞組織製品の品質・安全性に関しては商品としてのヒト細胞組織製品に対する品質・安全性ガイドラインを準用する形となっている．

商品としてのヒト細胞組織製品の製造・販売には薬事法に基づく承認が必要となる．使用目的による分類からすれば，“医薬品”あるいは“医療機器”のいずれかに分類されることになるが，どちらに分類されるかによって安全性確保の基準に自動的に差が出るわけではない．いずれにせよ細胞

組織製品は取扱い方法の分類からすれば“特定生物由来製品”とみなされる可能性が高いと想定され，そうなれば保健衛生上の危害の発生または拡大を防止するための措置を講じる必要がある．とくに細胞組織製品は臨床使用経験が少ないために知見の蓄積も乏しく，リスク予測が難しいため，わが国では治験でヒトに使用する前に製品の安全性と品質の“確認”を厚生労働大臣に求めなければならない(医薬発 906 号通知，平成 11 年)．この手続きは“確認申請”とよばれている．つまり開発者は新規細胞組織製品について治験実施に適うだけの安全性と品質をあらかじめ示さなければならない．

現在わが国には，細胞組織製品の品質・安全性確保についての主幹となるガイドラインとして，①『細胞組織利用医薬品等の取扱い及び使用に関する基本的考え方』(以下，“基本的考え方”；医薬発 1314 号通知別添 1，平成 12 年)，②『ヒト(自己)由来細胞・組織加工医薬品等の品質及び安全性の確保に関する指針』(以下“ヒト自己指針”，薬食発第 0208003 号，平成 20 年)，および③『ヒト(同種)由来細胞・組織加工医薬品等の品質及び安全性の確保に関する指針』(以下“ヒト同種指針”，薬食発第 0912007 号，平成 20 年)が出されている．②および③は“医薬発 1314 号通知別添 2”(平成 12 年)の改訂版として位置づけされている．なお，これらの指針は輸血用血液製剤，移植医療としての骨髄移植，臍帯血移植，ヒト皮膚や骨などを直接利用する医療行為を対象としていない点で欧米の指針とは異なるので，注意を要する．

上記“基本的考え方”は，細胞・組織を取り扱う際の基本的要件を示すとともに，細胞組織製品の品質・安全性，ならびに細胞・組織の取扱いに関する科学的および倫理的妥当性を確保することを目的とし，細胞組織製品の承認後のみならず，治験時においても適用される．“基本的考え方”のなかで細胞組織製品の安全性に関してもっとも強調されているのは，細菌，真菌，ウイルスなどの汚染の危険性への対策である．

“ヒト自己指針”および“ヒト同種指針”はそれぞれ，ヒト(自己)由来およびヒト(同種)由来の細胞組織製品の品質および安全性の確保のための基本的な技術要件についてまとめたもので，製造販売承認申請時のみならず，治験開始前の確認申請で求められる資料について記されている．ヒト(自己)由来製品とヒト(同種)由来製品との間の根本的な差異は，自己由来の細胞・組織を用いる場合にはその細胞・組織を介する感染症伝播のリスクおよび免疫学的な問題が理論上ないことである．しかし，自己由来であっても製造工程におけるクロスコンタミネーションの問題や製造従事者，医療従事者などの安全上の問題は，同種由来の場合と同様に存在する．また，培養工程においてウイルスの増殖するリスクを考慮することが必要な場合もある．さらに自己由来の場合，個別製品の製造となるので，それらの品質のばらつきを最小限にとどめる工夫が必要な反面，製品レベルでの各種試験の実施に試験検体の量的制約がある．それらに留意した合理的な品質確保の方策(製造工程のより厳密な恒常性維持・管理など)を採用する必要がある．なお，自己由来であっても遺伝子改変細胞の場合には相応の留意が必要である．

“ヒト自己指針”および“ヒト同種指針”の安全性確保策

“ヒト自己指針”および“ヒト同種指針”のコンセプトとして細胞組織製品の品質・安全性確保の方針は，原材料となる細胞・組織の適格性をはじめ，原材料の品質管理・培養方法を含めた製造方法の恒常性の確保，工程評価を含めた妥当性の検証，中間製品の品質管理，最終製品の規格設定および品質管理によって担保することになっている．しかし，細胞組織製品の原料となる細胞・組織は複雑な構造と，“生きている”という動的な特性をもつため，従来の医薬品などに適用されてきた品質管理の必要事項がかならずしも適用できるとは限らない．したがって，細胞組織製品の安全性に関しては特別な配慮が必要となってくる．

1. 感染因子

原材料から最終製品までの製造工程を通じ，安全性に関してもっとも強調されているのは，感染因子の汚染の危険性への対策である．細胞組織製品では，従来の医薬品のような高度な精製やウイルスの不活化・除去を製造時に行うことは非常に

難しい．このため，原材料および製造工程での感染因子の混入をいかに防ぐかが課題となる．

ヒト同種由来製品の場合はドナーからの感染リスクの評価が最重要であり，各種感染症に対する試験や既往歴に関する問診・調査を入念に行う必要がある．ウイルス感染の初期には検査で検出不可能なウィンドウ期があることから，適切な時期に再検査することが推奨される．また，製造工程でのウイルス増殖リスクがある場合にも製造工程中の適切な段階で再検査が必要となる場合がある．ヒト自己由来製品の場合，製造時のクロスコンタミネーションや製造従事者らへの伝播の可能性について，とくにB型肝炎ウイルス，C型肝炎ウイルス，ヒト免疫不全ウイルスおよび成人T細胞白血病ウイルス感染には留意する．

2. 最終製品の品質管理

最終製品の品質管理では必要で適切な規格および試験方法を設定し，その根拠を明らかにする必要がある．“ヒト自己指針”および“ヒト同種指針”では一般的な品質管理項目および試験として，①細胞数・生存率，②確認試験，③細胞の純度試験，④細胞由来の目的外生理活性物質に関する試験，⑤製造工程由来不純物試験，⑥無菌試験およびマイコプラズマ否定試験，⑦エンドトキシン試験，⑧ウイルス試験，⑨効能試験，⑩力価試験，⑪力学的適合性試験が例示されている．また，最終製品および重要な中間製品については細胞の生存率・力価などに基づく安定性試験を実施し，妥当な貯法や有効期限を設定することが求められる．

3. 非臨床安全性試験

製品の特性および適用法から評価が必要と考えられる安全性関連事項について，技術的に可能であれば，科学的合理性のある範囲で，適切な動物を用いた試験または *in vitro* での試験を実施する．なお，非細胞・組織成分および製造工程由来の不純物などについては可能なかぎり，動物を用いた試験ではなく理化学的分析法による評価が求められている．ヒト由来の試験用検体は貴重であり，また，ヒト由来の製品を実験動物などで試験してかならずしも意義ある結果が得られるとは限らない．逆に，動物由来の製品モデルを作成し，適切な実験動物に適用する試験系により試験を行うことで，より有用な知見が得られると考えられるような場合には，むしろ，そのような試験系を用いることに科学的合理性がある場合がある．場合によっては細胞を用いる試験系も考慮し，このようなアプローチにより試験を行った際には，その試験系の妥当性について明らかにする必要がある．

ヒト細胞組織製品の安全性において感染症伝播とともに懸念されることとして，製品の造腫瘍性があげられるが，最終製品ごとの造腫瘍性試験を一律に課すのは合理的ではない．たとえば，自己由来細胞でも文献上の知見や類似品の使用経験などから造腫瘍性が考えにくいものについては，規格の培養期間を超えて培養した細胞について目的外の形質転換を起こしていないことを明らかにすることでよい場合もあると考えられる．一方，多分化能を有する幹細胞は体細胞と比較して腫瘍化の可能性が高いとも思われるので，それに配慮した試験を計画する必要があると考えられる．

4. 効力または性能を裏づける試験

現在の科学で技術的に可能かつ科学的合理性のある範囲で，実験動物または細胞などを用い，適切に設計された試験により，細胞・組織加工医薬品などの機能発現，作用持続性および医薬品・医療機器として期待される効果を検討することが求められる．ただし確認申請段階では，当該製品の効力または性能による治療が他の治療法と比較したときはるかに優れて期待できることが国内外の文献または知見などにより合理的に明らかにされれば，かならずしも詳細な実験的検討は必要とされない．

5. 体内動態

製品を構成する細胞・組織および導入遺伝子の発現産物について，技術的に可能かつ科学的合理性がある範囲で，実験動物での吸収および分布などの体内動態に関する試験などにより，患者などに適用された製品中の細胞・組織の生存期間，効果持続期間を推測し，目的とする効果が十分得られることを明らかにする．当該細胞・組織が特定の部位(組織など)に到達して作用する場合には，その局在性を明らかにする．

6. 臨床試験

確認申請の段階における安全性については，臨