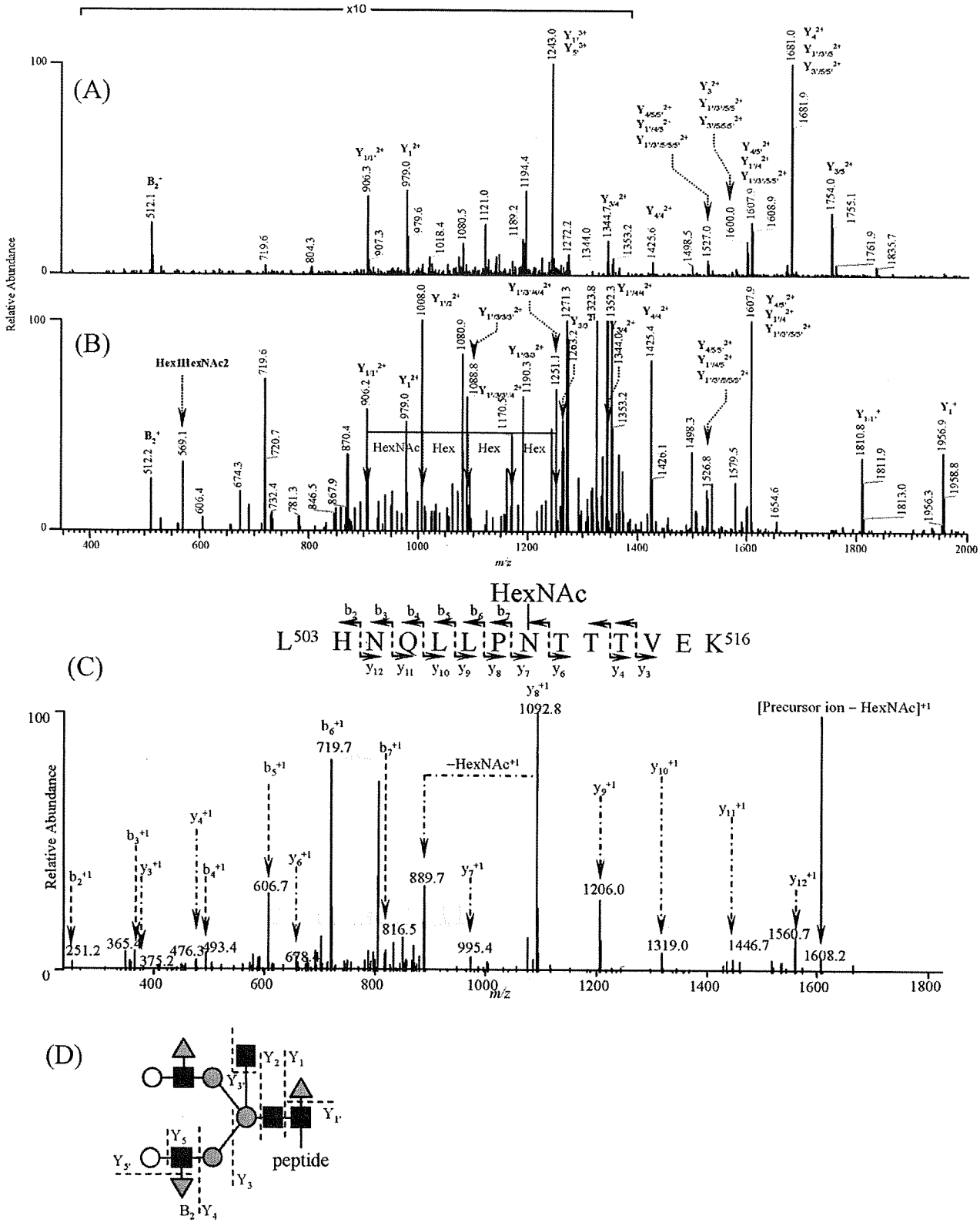


Figure 4. Identification of glycopeptide 8. (A) MS/MS spectrum acquired from the molecular ion $[M + 3H]^{3+}$ (*m/z* 1291.9) of glycopeptide 8 in Figure 2A. (B) MS/MS/MS spectrum acquired from the most intense ion (*m/z* 1681.0) in the MS/MS. (C) MS/MS/MS/MS spectrum acquired from the product ion (*m/z* 906.2) in the MS/MS/MS of glycopeptide 8, and amino acid sequence deduced from the results of database search analysis. (D) Deduced oligosaccharide structure. dHex, deoxyhexose; Hex, hexose; HexNAc, *N*-acetylhexosamine: white circle, galactose; gray circle, mannose; black square, *N*-acetylglucosamine; gray triangle, fucose.

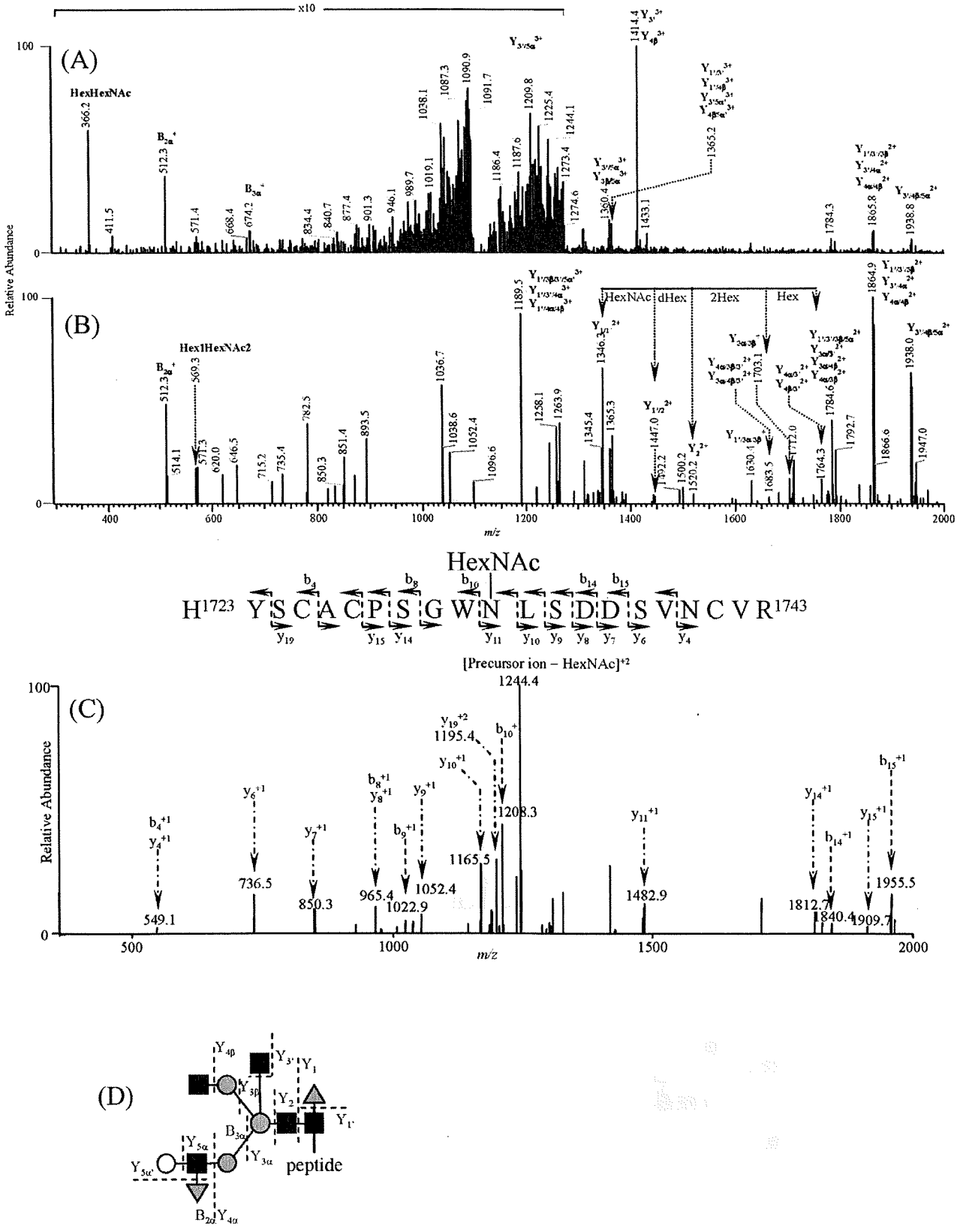


Figure 5. Identification of glycopeptide 15. (A) MS/MS spectrum acquired from the molecular ion $[M + 4H]^{4+}$ (*m/z* 1111.5) of glycopeptide 15 in Figure 2A. (B) MS/MS/MS spectrum acquired from the most intense ion (*m/z* 1414.4) in the MS/MS. (C) MS/MS/MS/MS spectrum acquired from the product ion (*m/z* 1346.3) in the MS/MS/MS of glycopeptide 15, and amino acid sequence deduced from the results of database search analysis. (D) Deduced oligosaccharide structure.

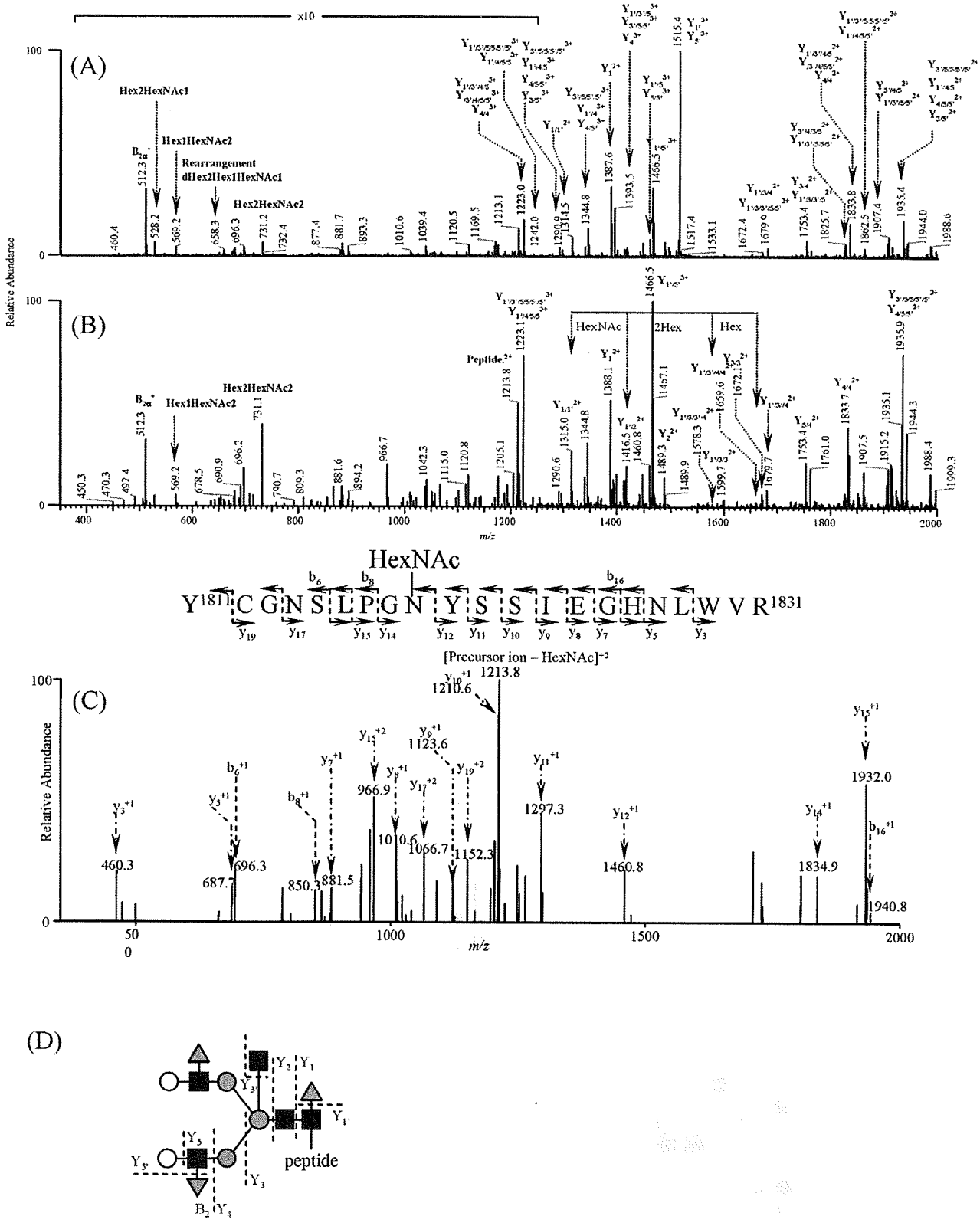
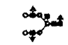
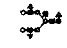
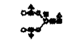
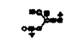
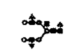
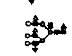
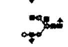
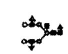
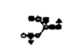
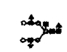
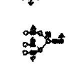
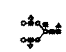
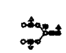
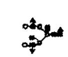


Figure 6. Identification of glycopeptide 17. (A) MS/MS spectrum acquired from the molecular ion $[M + 3H]^{3+}$ (m/z 1564.0) of glycopeptide 17 in Figure 2A. (B) MS/MS/MS spectrum acquired from the most intense ion (m/z 1515.4) in the MS/MS. (C) MS/MS/MS/MS spectrum acquired from the product ion (m/z 1315.0) in the MS/MS/MS of glycopeptide 17, and amino acid sequence deduced from the results of database search analysis. (D) Deduced oligosaccharide structure.

Table 1. Summary of Oligosaccharide Structures and Amino Acid Sequences of Le^x-Glycopeptides Detected by LC−MS^n and Database Search Analysis

	Candidate Le^x-glycopeptide					Peptide						Deglycosylated peptide prepared by PNGaseF treatment					Oligosaccharide	
No.	Observed m/z value of molecular-related ion[a]	Charge state	Observed m/z value of peptide-related ion[b]	Charge state	Additional sugar residue	Amino acid sequence	Xcorr[c]	Protein identified by database search analysis	GI no.[d]	Theoretical mass[e]	Calculated mass[f]	Observed m/z value of molecular-related ion[a]	Charge state	Xcorr[c]	Theoretical mass[e]	Calculated mass	Deduced Structure	Theoretical mass[g]
1	1136.129	[M+2H]$^{2+}$	673.8	2	HexNAc	FGN*ATGHLMGR	1.32	Cubilin precursor	88909288	1141.550	1141.550	572.286	2	2.57	1142.515	1142.545		2281.845
2	1170.147	[M+3H]$^{3+}$	1448.5	1	HexNAc	N*LSYLAAPDIK	1.57	cadherin 16	6680960	1243.583	1243.584	673.291	2	3.43	1244.567	1244.567		2281.845
3	1152.145	[M+3H]$^{3+}$	770.7	2	HexNAc+dHex	RN*WTETEVR	1.33	dipeptidase 1	6681213	1189.584	1189.579	566.290	2	2.87	1190.568	1190.565		2281.845
4	1293.853	[M+3H]$^{3+}$	910.3	2	HexNAc	YYN*QSAGGSHTLQR	1.28	H-2 class I histocompatibility antigen, K-K alpha precursor	122155	1614.718	1614.717	808.865	2	4.19	1615.702	1615.715		2281.845
5	1225.510	[M+3H]$^{3+}$	1613.7	1	HexNAc	TWSAFQN*GTDKR	1.32	low[-]density lipoprotein receptor-related protein 2 (LRP2)	124487372	1409.669	1409.673	471.227	3	1.66	1410.653	1410.659		2281.845
6	1122.804	[M+3H]$^{3+}$	807.7	2	HexNAc	TWSAFQN*GTDKR	1.30	low[-]density lipoprotein receptor-related protein 2 (LRP2)	124487372	1409.669	1409.666	471.227	3	1.66	1410.653	1410.659		1973.735
7	1231.310	[M+3H]$^{3+}$	816.2	2	HexNAc	—[h]	—	—	—	—	—	—	—	—	—	—	—	—
8	1291.243	[M+3H]$^{3+}$	906.2	2	HexNAc	LHNQLLN*TTIVEK	4.12	γ-glutamyltransferase 1 (γ-GTP1)	6679995	1666.868	1666.871	834.937	2	4.12	1667.852	1667.859		2281.845
9	1156.466	[M+5H]$^{5+}$	1070.0	3	HexNAc	HHCELQN*CSSTEFTCINSRPPNKR	—	low[-]density lipoprotein receptor-related protein 2 (LRP2)	124487372	3002.281	3002.269	1001.663	3	4.77	3003.265	3003.239		2793.635
10	1160.210	[M+4H]$^{4+}$	1170.6	2	HexNAc	SGQLDHVWLDVEKN*QSAK	1.53	alanyl (membrane) aminopeptidase	226437487.00	2132.976	2132.976	—	—	—	—	—		2281.845
11	1123.900	[M+5H]$^{5+}$	915.6	3	HexNAc	—	—	—	—	—	—	—	—	—	—	—	—	—
12	1237.506	[M+3H]$^{3+}$	1649.6	1	HexNAc	N*MSSLFYATQLR	1.73	γ-glutamyltransferase 1 (γ-GTP1)	6679995	1445.661	1445.660	724.340	2	3.78	1446.645	1446.665		2281.845
13	1134.803	[M+3H]$^{3+}$	1649.7	1	HexNAc	N*MSSLFYATQLR	1.70	γ-glutamyltransferase 1 (γ-GTP1)	6679995	1445.661	1445.664	724.340	2	3.78	1446.645	1446.665		1973.735
14	1163.044	[M+4H]$^{4+}$	1295.4	2	HexNAc	VVLVN*FTHKPTDHVLHPYR	1.79	low[-]density lipoprotein receptor-related protein 2 (LRP2)	124487372	2384.311	2384.312	796.118	3	4.71	2385.295	2385.312		2281.845
15	1111.453	[M+4H]$^{4+}$	1346.3	2	HexNAc	HYSCACPSGWN*LSDDSVNCVR	2.82	low[-]density lipoprotein receptor-related protein 2 (LRP2)	124487372	2486.660	2486.659	—	—	—	—	—		1973.735
16	1145.460	[M+4H]$^{4+}$	1157.2	2	HexNAc	—	—	—	—	—	—	—	—	—	—	—	—	—
17	1563.318	[M+3H]$^{3+}$	1315.0	2	HexNAc	VCGNSLPGN*YSSILGHNLWVR	2.02	Cubilin precursor	88909288	2473.097	2473.098	—	—	—	—	—		2281.845
18	1481.528	[M+2H+Na]$^{3+}$	925.1	2	HexNAc	VGVTDVFPN*GTQFSR	—	Meprin A subunit beta precursor (endopeptidase-2)	2499913	1622.805	1622.573	812.903	2	3.14	1623.789	1623.791		2793.635
19	1156.990	[M+4H]$^{4+}$	1282.5	2	HexNAc	—	—	—	—	—	—	—	—	—	—	—	—	—
20	1557.387	[M+3H]$^{3+}$	1304.5	2	non-substituted	NQSVVMYSVPQPLGHAIHPSR	1.38	low[-]density lipoprotein receptor-related protein 2 (LRP2)	124487372	2465.249	2465.290	—	—	—	—	—		2281.845
21	1311.210	[M+H+2Na]$^{3+}$	924.9	2	HexNAc	VGVTDVFPN*GTQFSR	—	Meprin A subunit beta precursor (endopeptidase-2)	2499913	1622.805	1622.810	812.903	2	3.14	1623.789	1623.791		2281.845
22	1386.270	[M+2Na]$^{2+}$	948.4	2	HexNAc	VGVTDVFPN*GTQFSR	—	Meprin A subunit beta precursor (endopeptidase-2)	2499913	1622.805	1622.928	812.903	2	3.14	1623.789	1623.791		2484.925

[a] Value obtained by FTICR−MS. [b] Value obtained by IT−MS^n. [c] Value of cross correlation obtained by database search analysis. [d] GenInfo Identifier number. [e] Monoisotopic value. [f] Value calculated by subtraction of the theoretical masses of deduced oligosaccharides from the observed monoisotopic masses of the candidate Le^x-glycopeptide. [g] Monoisotoic value. [h] No data. *, modification with HexNAc; #, modification with HexNAc+dHex. Bold portions of amino acid sequences, consensus sequences of -linked oligosaccharides.

possible modification at Asn with HexNAc (203.1 u) and with dHex + HexNAc (349.1 u), seven glycopeptides were successfully sequenced with a high cross-correlation score (charge +1, Xcorr > 1.5; charge +2, Xcorr > 2.0; charge +3, Xcorr > 2.5; charge +4, Xcorr > 3.0). Figure 4C shows the MS/MS/MS/MS spectrum acquired from glycopeptide 8 (precursor ion: [peptide + HexNAc + 2H]$^{2+}$, m/z 906.2). The database search analysis resulted in Leu^{503}-Lys^{516} in γ-glutamyl transpeptidase 1 (γ-GTP1) (charge +2, Xcorr: 4.12) (Table 1). The linkage of GlcNAc at Asn^{510} in the *N*-glycosylation consensus sequence, Asn-Thr-Thr, was suggested by the good agreement between the experimental b/y-ion pattern and the predicted pattern.

The MS/MS/MS/MS spectrum acquired from [peptide + HexNAc + 2H]$^{2+}$ (m/z 1346.3, glycopeptide 15) is shown in Figure 5C. This peptide was identified as His^{1723}-Arg^{1743} in low-density lipoprotein receptor-related protein 2 (LRP2, megalin) (charge +2, Xcorr: 2.82). The b- and y-ion pattern suggested the linkage of GlcNAc at Asn^{1733} in the *N*-glycosylation consensus sequence, Asn-Lys-Ser.

Figure 6C shows the MS/MS/MS/MS spectrum acquired from another expected Le^x-conjugated glycopeptide (glycopeptide 17; precursor ion: [peptide + HexNAc + 2H]$^{2+}$, m/z 1315.0). Database search analysis revealed that this peptide could be Tyr^{1811}-Arg^{1831} in the cubilin precursor (charge +2, Xcorr: 2.02) (Table 1). It was also suggested that the linkage position of GlcNAc was Asn^{1819} in the *N*-glycosylation consensus sequence of Asn^{1819}-Tyr-Ser^{1821}.

Glycopeptides 2, 10, 12, and 14 were also successfully identified as Asn^{519}-Lys^{529} in cadherin 16 (glycosylation site: Asn^{519}; charge +1, Xcorr: 1.52), Ser^{593}-Lys^{610} in alanyl (membrane) aminopeptidase (glycosylation site: Asn^{606}; charge +2, Xcorr: 1.53), Asn^{343}-Arg^{354} in γ-GTP1 (glycosylation site: Asn^{343}; charge +1, Xcorr: 1.73) and Val^{3444}-Lys^{3463} in LRP2 (glycosylation site: Asn^{3448}; charge +2, Xcorr: 1.79), respectively. Additionally, we deduced that glycopeptides 1, 3–6, 13, and 20 could be Le^x-conjugated glycopeptides from tolerable scores (charges +1 and +2 Xcorr > 1.30) (Table 1). All identified or probable glycopeptides contained consensus sequences of *N*-linked oligosaccharides.

By the present method, three glycoproteins were identified as proteins carrying multiple Le^x-conjugated oligosaccharides—namely, γ-GTP1 (glycosylation site: Asn^{343} and Asn^{510}; glycopeptides 8, 12 and 13), LRP2 (glycosylation site: Asn^{1497}, Asn^{1676}, Asn^{1733} and Asn^{3448}; glycopeptides 5, 6, 14, 15 and 20), and a cubilin precursor (glycosylation site: Asn^{1802}, Asn^{1819}, glycopeptides 1 and 17). Only one glycopeptide was sequenced, but it was deduced that cadherin 16, dipeptidase 1, H-2 class I histocompatibility antigen, and K–K alpha precursor (H2–K(k)), and alanyl (membrane) aminopeptidase could be the Le^x-conjugated glycoproteins.

The sequences of the Le^x-conjugated glycopeptides were confirmed by an additional LC–MS/MS of deglycosylated peptides prepared by PNGaseF-treatment. Because of the deamination of Asn residues by PNGase F treatment, we set the m/z values of [peptide + nH + 0.984 u monoisotopic mass]$^{n+}$ (n = 2–5) as precursor ions on the MS/MS. By this conventional method, 8 peptides that were sequenced by our method were identified as shown in Table 1. Moreover, two peptides which could not be sequenced by our method were also identified as His^{2943}-Lys^{2965} in LRP2 (glycopeptide 9), and Val^{540}-Lys^{554} in the meprin A β subunit precursor (endopeptidase-2, glycopeptides 18, 21 and 22). On the other hand, four glycopeptides that were sequenced by our method were not identified by the conventional method. Using both methods, we failed in the sequencing of four glycopeptides.

Structural Analyses of the Oligosaccharides in the Le^x-Conjugated Glycopeptides. The carbohydrate structures of the Le^x-conjugated glycopeptides were deduced from the fragment patterns and molecular masses obtained by the first run using FTICR-MS. The structural assignment of glycopeptide 8 is shown in Figure 4A and B. The carbohydrate composition was estimated to be 3dHex 5Hex 5HexNAc from the molecular mass of the carbohydrate moiety (calculated molecular mass of the glycan moiety: 2281.850). The fragment ions at m/z 1681.0 and m/z 1425.6 in Figure 4A were assigned to Y_1^{2+} and $Y_{4/4'}^{2+}$, which arose from [M + 2H]$^{2+}$ (m/z 1681.8) by the dissociation of two molecules of the Lewis-motifs. The presence of B_2^+ (m/z 512) in both Figure 4A and B also suggested the binding of two Lewis-motifs. The Y_4^{2+} further yielded [Hex + 2HexNAc]$^+$ (m/z 569.1), $Y_{1'/3/3'}^{3+}$ (m/z 1190.3) and $Y_{3/3'}^{2+}$ (m/z 1263.2) on the MS/MS/MS, which suggested the presence of bisecting GlcNAc. The fucosylation of reducing-end GlcNAc was proven by the detection of $Y_{1/1'}^{2+}$ ([peptide + HexNAc + 2H]$^{2+}$, m/z 906.2) and Y_1^{2+} ([peptide + dHex + HexNAc + 2H]$^{2+}$, m/z 979.0). Consequently, the glycan of glycopeptide 8 was characterized as a bisected and core-fucosylated oligosaccharide carrying two molecules of Le^x-motifs (Figure 4D). The possibility of the deduced structure was confirmed by the good agreement between the experimental mass (2281.850) and the theoretical mass (2281.845) (Table 1).

Figure 5A and B show the assignments of the carbohydrate moiety in the glycopeptide 15. The predominant ion (m/z 1414.4) in the MS/MS spectrum was assigned to [M – HexNAc + 3H]$^{3+}$ (Y_3^{3+} or $Y_{4/}^{3+}$). This Y_3^{3+} ($Y_{4/}^{3+}$) ion yielded the B_2^+ (m/z 512.3) by MS/MS/MS, suggesting the presence of only one molecule of the Lewis-motif. The presence of $Y_{1/1'}^{2+}$ (m/z 1346.3), $Y_{1'/2}^{2+}$ (m/z 1447.0) and Y_2^{2+} (m/z 1520.2) suggested the fucosylation at the reducing end of GlcNAc. The presence of bisecting GlcNAc was deduced from the detection of the ion [Hex + 2HexNAc]$^+$ (m/z 569.3) and $Y_{1'/3\alpha/3\beta}F^{2+}$ (m/z 1630.4). From these fragments, the oligosaccharide structure was characterized as a bisected and core-fucosylated oligosaccharide carrying one molecule of the Le^x-motif (Figure 5D).

The deduced carbohydrate structure of glycopeptide 17 is indicated in Figure 6D. In the MS/MS spectrum, the fragments at m/z 1515.4 and m/z 1393.5 were assigned to [M – dHex + 3H]$^{3+}$ (Y_1^{3+} or Y_3^{3+}) and [M – dHex – Hex – HexNAc + 3H]$^{3+}$ (Y_4^{3+}), respectively (Figure 6A). The detection of B_2^+ (m/z 512.3) in both the MS/MS and MS/MS/MS spectra revealed the binding of two Le^x-motifs (Figure 6A and 6B). The presence of bisecting GlcNAc was suggested by the detection of the ion [Hex + 2HexNAc]$^+$ (m/z 569.2), $Y_{1'/3/3'}^{2+}$ (m/z 1599.7) and $Y_{3/3'}^{2+}$ (m/z 1672.1) in the MS/MS/MS spectrum (Figure 6B). The ions $Y_{1/1'}^{2+}$ ([peptide + HexNAc + 2H]$^{2+}$, m/z 1315.0) and Y_1^{2+} ([peptide + dHex + HexNAc + 2H]$^{2+}$, m/z 1388.1) revealed the fucosylation at the reducing end of GlcNAc. We also found the presence of a distinctive ion of the Lewis y (Le^y) motif, (Fucα1–2)Galβ1–4(Fucα1–3)GlcNAc, at m/z 658.3 in the MS/MS spectrum. To determine whether *N*-linked oligosaccharides contained the Le^y-motif, *N*-linked oligosaccharides were released from mouse kidney proteins and treated with α1–2 fucosidase. Then the glycan profiles of the fucosidase-treated and -untreated oligosaccharides were compared by LC/MS. No change was found in the mass spectrometric glycan profiles between the two samples, but the fragment (m/z 658) was still detected in the MS/MS spectra of the enzyme-treated

glycopeptide 17 (data not shown). These results suggest the absence of α1−2 fucose on the glycopeptides. Consequently, we assigned the glycans of glycopeptide 17 to a bisected and core-fucosylated oligosaccharide carrying two Le^x-motifs (Figure 6D).

The oligosaccharide structures of other Le^x-conjugated glycopeptides were deduced from their B- and Y-type ions as well as the molecular masses obtained by FTICR−MS in the same manner (Table 1 and Figure 7). The most common structure was a bisected and fucosylated complex-type biantennary oligosaccharide carrying two Le^x-motifs (glycopeptides 1−5, 8, 10, 12, 14, 17, 20 and 21). A bisected and core-fucosylated complex-type biantennary oligosaccharide carrying one Le^x-motif was found in glycopeptides 6, 13, and 15. A bisected and core-fucosylated complex-type triantennary oligosaccharide carrying three Le^x-motifs was found in glycopeptides 9 and 18. The oligosaccharide structure of the glycopeptide in glycopeptide 22 was a triantennary carrying two Le^x-motifs. All experimental molecular masses of the deduced glycopeptides were identical to their theoretical masses (Table 1).

Discussion

Several glycan-epitopes, including Lewis antigens, HNK-1, and polysialic acid, have been widely shown to be involved in the physiological functions of glycoproteins and certain diseases. Some oligosaccharide-related antigens are being used as diagnostic markers of tumors in a clinical stage.[42,43] However, only a few proteins are known to carry the glycan-epitopes. To understand the physiological roles of the glycan-epitopes and to develop more effective diagnostic markers, we need methods that allow for the identification of target proteins carrying the glycan motif of interest. Glycan-epitopes are often detected by two-dimensional (2D)-electrophoresis in combination with lectin or immuno-blotting. The stained spots are subjected to in-gel tryptic digestion followed by protein identification by MS/MS and database search analysis. There are still problems in this procedure with the verification of the glycan structure in the identified protein. In addition, the procedure cannot be employed on hydrophobic membrane proteins having a high molecular weight.

In the present study, all proteins in the mouse kidney were digested into peptides, and the fucosylated glycopeptides were enriched by lectin-affinity chromatography. The resulting fucosylated glycopeptides were subjected to two different runs of LC−MS^n. In the first run, the elution positions of Le^x-conjugated glycopeptides in the tryptic peptide map were located based on the presence of Le^x-motif-distinctive ions. We picked out the product ion spectra of expected Le^x-conjugated glycopeptides from the elution positions and carefully assigned the peptide + HexNAc, peptide + (dHex)HexNAc, and peptide fragment. Then the fucosylated glycopeptides were subjected to a second run in which the peptide-related ions were set as precursor ions. We successfully identified γ-GTP1, LRP2, and the cubilin precursor as Le^x-conjugated glycoproteins by sequencing of 2−5 glycopeptides. Although only one glycopeptide was sequenced, cadherin 16, dipeptidase 1, H2−K(k) and alanyl (membrane) aminopeptidase were characterized as Le^x-conjugated glycoproteins based on the good agreement between the experimental and theoretical masses of glycopeptides and their fragment patterns. Some of these were membrane proteins with high molecular masses over 400 kDa, the identification of which might have been difficult by 2D-electrophoresis with Western blotting.

Carbohydrate structures of the identified glycopeptides were deduced from the accurate molecular masses as well as fragment patterns obtained by the first run. We confirmed that all glycopeptides contained a bisected and core-fucosylated oligosaccharide carrying one or two molecules of Le^x-motifs at the *N*-linked oligosaccharide consensus sequence. Our model tissue was a mouse kidney in which we had previously confirmed the presence of Lewis x [Galβ1−4(Fucα1−3)GlcNAc] and/or y [(Fucα1−2)Galβ1−4(Fucα1−3)GlcNAc] motifs as well as the absence of Lewis a [Galβ1−3(Fucα1−4)GlcNAc] or b [(Fucα1−2)Galβ1−3(Fucα1−4)GlcNAc] motifs.[39] In this study, the Le^x-distinctive ions, (2dHex + Hex + HexNAc)$^-$ (*m*/*z* 658), were found in all MS/MS spectra of Lewis-conjugated peptides. However, treatment of α 1−2 fucosidase led to no change in the mass spectrometric glycan profile, suggesting the absence of the Le^x-motif. Recently, several groups have reported the internal migration of fucose residues in the ESI−CID of underived or derived carbohydrates.[44−48] Fucose residues are transferred between branches in liberated *N*-linked oligosaccharides by the ESI−CID.[49] Our finding suggests that the rearrangement of fucose residues also occurs by the ESI−CID of glycopeptides. This phenomenon makes it difficult to deduce the oligosaccharide structure from only the fragmentation pattern. A simultaneous use of lectins and/or antibodies would be crucial for the identification of the desired glycoproteins.

γ-Glutamyl transpeptidase 1 is associated with glutathione salvage, metabolism of endogenous mediators such as leukotrienes and prostaglandins. The attachment of Le^x-conjugated oligosaccharide to mouse γ-GTP 1 has already been demonstrated by Yamashita et al.[51] They determined the carbohydrate structures by the purification of γ-GTP 1 and the sequential exoglycosidase digestion in combination with methylation analysis. The oligosaccharide structures deduced from the MS/MS and MS/MS/MS spectra were in good agreement with those they reported. Furthermore, we revealed the heterogeneity of glycosylation on Asn^{543}.

Dipeptidase 1 is a glycosylphosphatidylinositol-anchored membrane glycoprotein. This protein is highly expressed in the kidney and small intestine and plays an important role in the degradation of cysteinyl-glycine, a glutathione produced by the removal of the glutamyl group from γ-glutamyl cysteinyl-glycine by γ-GTP.[52] The present study is the first report on the oligosaccharide structures of a mouse renal dipeptidase.

Cubilin, which is highly expressed in the renal proximal tubules, is a 460 kDa membrane glycoprotein consisting of 27 CUB (complement components C1r/C1s, Uegf, and bone morphogenic protein-1) domains. Cubilin is an endocytic receptor for intrinsic factor vitamin B12, albumin, apolipoprotein A-I, receptor-associated protein, immune globulin light chain and high-density lipoprotein.[50] These factors bind to cubilin through their CUB domains. The Le^x-conjugated oligosaccharides we found were all located on Asn^{1802} and Asn^{1819} in the CUB12 domain (Figure 8).

Low-density lipoprotein receptor-related protein 2, a high molecular weight membrane protein (520 kDa), is an endocytic receptor for several ligands, vitamin-binding proteins, apolipoproteins, hormones and enzymes. Cubilin and LRP2 are coexpressed in the renal proximal tubules and are associated with tubular protein reabsorption, vitamin metabolism and calcium homeostasis. Low-density lipoprotein receptor-related protein 2 consists of four ligand-binding sites containing cysteine-rich complement-type repeats and epidermal growth

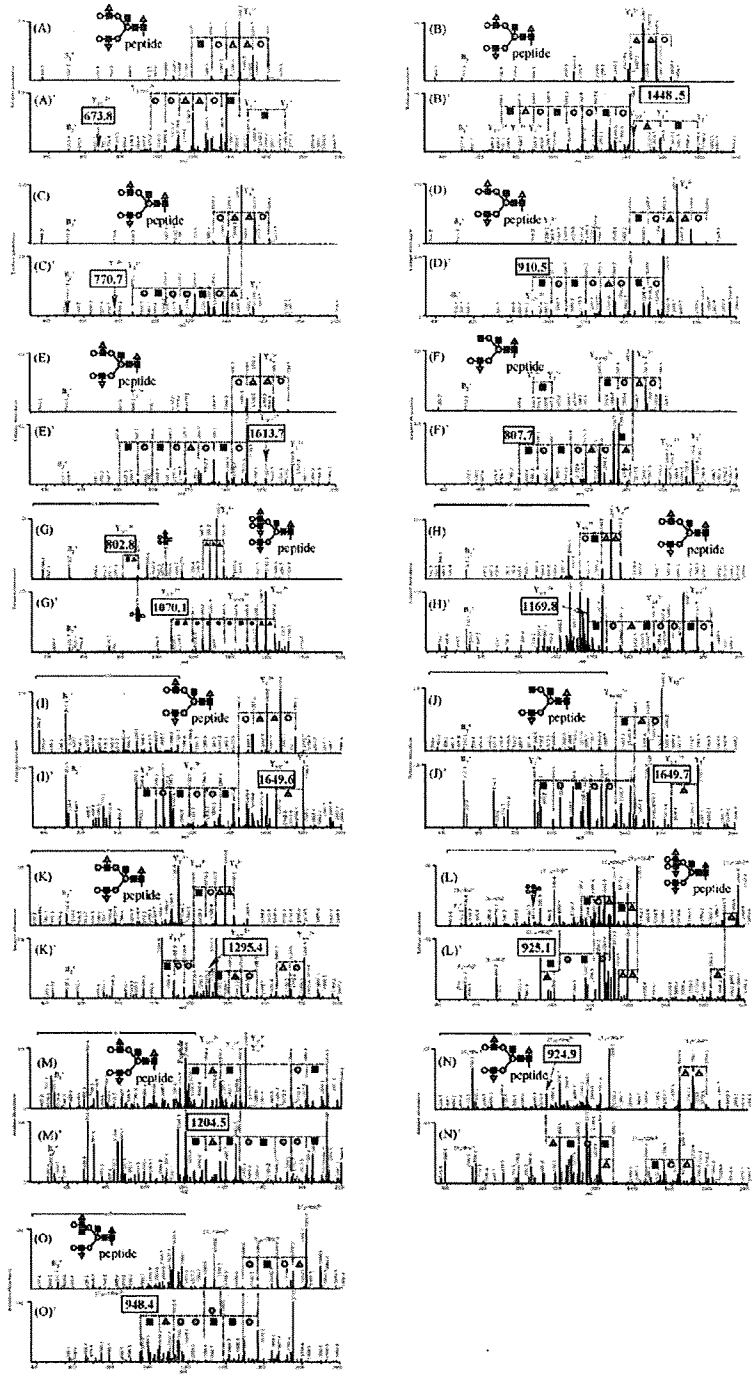


Figure 7. MS/MS and the MS/MS/MS spectra of glycopeptides 1−6, 9, 10, 12−14, 18 and 20−22, and deduced oligosaccharide structures. Boxed values are peptide-related ions. (A) MS/MS spectrum of the molecular ion (*m/z* 1136.4) of glycopeptide 1. (A′) MS/MS/MS spectrum of the predominant ion (*m/z* 1448.2) of (A). (B) MS/MS spectrum of glycopeptide 2 (*m/z* 1170.8). (B′) MS/MS/MS spectrum of the ion (*m/z* 1499.3) in (B). (C) MS/MS spectrum of glycopeptide 3(*m/z* 1152.5). (C′) MS/MS/MS spectrum of the ion (*m/z* 1472.5) in (C). (D) MS/MS spectrum of glycopeptide 4 (*m/z* 1294.2). (D′) MS/MS/MS spectrum of the ion (*m/z* 1685.0) in (D). (E) MS/MS spectrum of glycopeptide 5 (*m/z* 1226.0). (E′) MS/MS/MS spectrum of the ion (*m/z* 1582.6) in (E). (F) MS/MS spectrum of glycopeptide 6(*m/z* 1123.4). (F′) MS/MS/MS spectrum of the ion (*m/z* 1428.4) in (F). (G) MS/MS spectrum of glycopeptide 9 (*m/z* 1156.9). (G′) MS/MS/MS spectrum of the ion (*m/z* 1318.5) in (G). (H) MS/MS spectrum of glycopeptide 10 (*m/z* 1100.8). (H′) MS/MS/MS spectrum of the ion (*m/z* 1297.1) in (H). (I) MS/MS spectrum of glycopeptide 12 (*m/z* 1238.1). (I′) MS/MS/MS spectrum of the ion (*m/z* 1673.6) in (I). (J) MS/MS spectrum of glycopeptide 13 (*m/z* 1135.5). (J′) MS/MS/MS spectrum of the ion (*m/z* 1600.3) in (J). (K) MS/MS spectrum of glycopeptide 14 (*m/z* 1163.5). (K′) MS/MS/MS spectrum of the predominant ion (*m/z* 1380.8) in (K). (L) MS/MS spectrum of glycopeptide 18 (*m/z* 1482.6). (L′) MS/MS/MS spectrum of the predominant ion (*m/z* 1433.9) in (L). (M) MS/MS spectrum of glycopeptide 20 (*m/z* 1558.1). (M′) MS/MS/MS spectrum of the predominant ion (*m/z* 1509.8) in (M). (N) MS/MS spectrum of glycopeptide 21 (*m/z* 1279.2). (N′) MS/MS/MS spectrum of the predominant ion (*m/z* 1263.5) in (N). (O) MS/MS spectrum of glycopeptide 22 (*m/z* 1387.5). (O′) MS/MS/MS spectrum of the predominant ion (*m/z* 1825.5) in (O). White circle, galactose; gray circle, mannose; black square, *N*-acetylglucosamine; gray triangle, fucose.

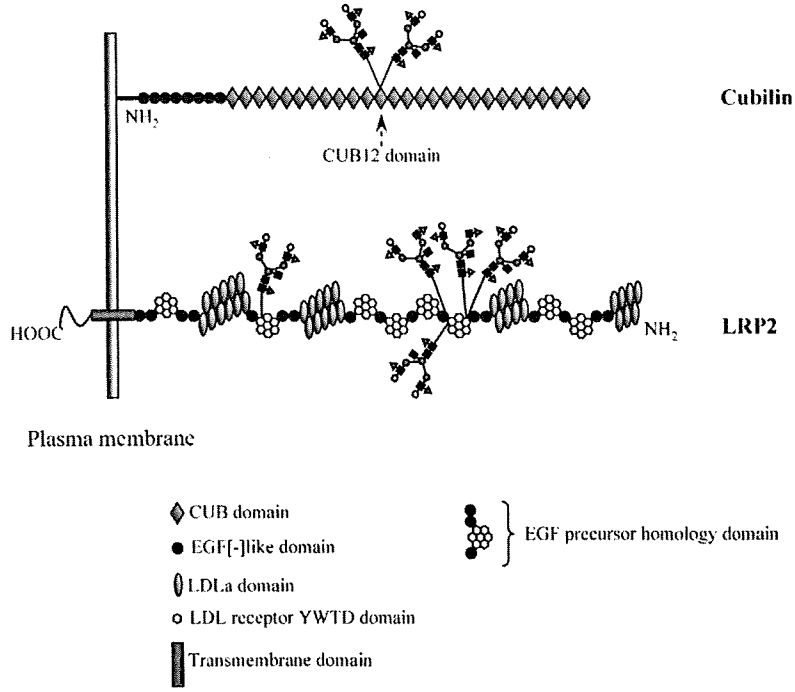


Figure 8. Location of Le^x-conjugated oligosaccharides on cubilin and LRP2. CUB, C1r/C1s, Uegf, and bone morphogenic protein-1; LDLa, Low-density lipoprotein receptor domain class A.

factor (EGF) precursor homology domains, which are associated with pH-dependent ligand dissociation. A previous study demonstrated the linkage of high-mannose-type and bi- and triantennary complex-type oligosaccharides bearing core fucose and bisecting GlcNAc. But there have been no reports on the presence of Le^x-conjugated oligosaccharides in rat kidney LRP2.[53] Interestingly, all of the Le^x-conjugated oligosaccharides we found were located in the EGF precursor homology domains (Asn^{1497}, Asn^{1676}, Asn^{1733} and Asn^{3448}). Furthermore, heterogeneity of glycosylation on Asn^{1497} was observed (Figure 8).

The cadherin 16, H2−K(k) protein and alanyl (membrane) aminopeptidase (aminopeptidase N) were also identified as Le^x-conjugated glycoproteins. Cadherin 16 is a kidney-specific cadherin that is associated with Ca^{2+}-dependent cell−cell adhesion.[54] The H2−K(k) protein, which is a mouse-specific histocompatibility antigen (H-2 antigen), is involved in the presentation of foreign antigens to the immune system. Alanyl (membrane) aminopeptidase (aminopeptidase N) is a transmembrane protein that is expressed predominantly in intestinal mucosa and kidney tissue.[55,56] It was reported that this enzyme is involved in several biological events such as tumorigenesis and immune system.[57] These proteins were unknown to be Le^x-conjugated proteins.

Using the present method, we successfully identified 14 Le^x-conjugated glycopeptides (12 peptides). Some peptides were found to be glycosylated with different Le^x-conjugated oligosaccharides (glycopeptides 5 and 6; glycopeptides 12 and 13). In most cases only a peptide carrying a major oligosaccharide was identified as a Le^x-conjugated glycopeptide. Minor Le^x-conjugated glycopeptides were not subjected to MS/MS/MS in the first run because they were less intense. Such minor glycopeptides might be identified by an additional run in which the glycopeptides identified in the first run are excluded. In addition, our method tended to fail in the identification of glycopeptides having high molecular mass (>4500 Da), glycopeptides detected as triply charged ions, and glycopeptides containing triantennary oligosaccharides. These glycopeptides yielded a smaller number of peptide-related ions, which was insufficient for further CID, and database search analysis resulted in false-positive proteins, for example, a peptide not containing *N*-glycan consensus sequences. Using a conventional approach that included LC−MS/MS of the PNGase F-treated tryptic digest, we found two additional Le^x-expected peptides. One was a high molecular mass peptide (3002 Da) containing a triantennary oligosaccharide, and the other was a peptide detected as sodium adducts in our method. The conventional approach has the advantage of peptide sequencing, but does not allow confirmation of the Lewis-motif in the oligosaccharide. Unlike the classical glycomic approaches that are used for the comprehensive analysis of glycopeptides, our method focused only on Lewis-conjugated glycopeptides. Accordingly, it could be applicable to the identification and screening of glycoproteins carrying target glycan-motifs.

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Current Topics

New Era of Glycoscience: Intrinsic and Extrinsic Functions Performed by Glycans

The Significance of Glycosylation Analysis in Development of Biopharmaceuticals

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Many glycoproteins and glycosaminoglycans are approved for clinical use. Carbohydrate moieties in biopharmaceuticals affect not only their physicochemical properties and thermal stability, but also their reactivity with their receptors and circulating half-life. Modification of glycans is one target of drug design for enhancement of efficacy. Meanwhile, there have been reports of serious adverse events caused by some carbohydrates. It is crucial to maintain the constancy of carbohydrate moieties for the efficient and safe use of glycosylated biopharmaceuticals. On the other hand, for scientific, safety-related, and economic reasons, changes in the manufacturing process are frequently made either during the development or after the approval of new biopharmaceuticals. Furthermore, the development of biosimilar glycoprotein products has been attempted by different manufacturers. Changes in pharmaceutical manufacturing processes possibly cause alteration of glycosylation and raise concerns about alteration of their quality, safety, and efficacy. In this review we provide some current topics of glycosylated biopharmaceuticals from the viewpoints of efficacy, safety, and the manufacturing process and discuss the significance of glycosylation analysis for development of biopharmaceuticals.

Key words glycoprotein; glycosaminoglycan; biopharmaceutical; efficacy; safety; manufacturing process

1. INTRODUCTION

Many biological molecules containing carbohydrates are approved for clinical use in Japan (Table 1). In the beginning, most therapeutic glycoconjugates were naturally occurring glycoproteins and glycosaminoglycans (GAGs) derived from human and healthy animal tissues, such as gonadotropins from human urine, and heparins from the porcine intestine. Recombinant glycoproteins, including erythropoietin and tissue plasminogen activator (tPA), have been developed as copies of native human glycoproteins since the early 1990s in Japan. In many cases their carbohydrate moieties were different from the original human ones. Carbohydrate moieties in glycoproteins affects not only their physicochemical properties and thermal stability but also their reactivity with their receptors and circulating half-life.[1] Modification of carbohydrate moieties is one target of drug design to enhance the efficacy of the original ones. Meanwhile, there have been reports from around the world of serious adverse events caused by carbohydrate-related drugs. For the efficient and safe use of glycoprotein/GAG products, it is necessary to maintain the structures and heterogeneity of carbohydrate moieties in glycosylated biopharmaceuticals.

On the other hand, carbohydrate moieties in biotechnology-derived drugs are variable when the manufacturing process is changed. For scientific, safety-related and economic reasons, it has become common for companies to change the manufacturing process for their approved products. Furthermore, biosimilar glycoprotein products, which are manufactured by different companies, have been developing in many regions.[2,3] One of the main issues for the devel-

Table 1. Glycosylated Biopharmaceuticals in Japan

Origin	Japanese accepted name
Granulocyte-colony stimulating factor	Lenograstim
Granulocyte macrophage colony-stimulating factor	Mirimostim
Interferon	Interferon Alfa (NAMALWA), Interferon Alfa (BALL-1), Interferon Beta, Interferon Beta-1a, Interferon Gamma-n1
Erythropoetin	Epoetin Alfa, Epoetin Beta, Darbepoetin Alfa
Monoclonal antibody	Ibritumomab Tiuxetan, Basiliximab, Infliximab, Rituximab, Cetuximab, Gemtuzumab Ozogamicin, Palivizumab, Tocilizumab, Trastuzumab , Bevacizumab, Adalimumab
Receptor	Etanercept
Follicle stimulating hormone	Follitropin Alfa, Follitoropin Beta
Gonadotropin	Human Menopausal Gonadotropin, Human Chorionic Gonadotropin, Serum Gonadotropin
Factor VII	Eptacog Alfa (Activated)
Factor VIII	Octocog Alfa, Rurioctocog Alfa
Thrombomodulin	Thrombomodulin Alfa
Urokinase	Urokinase
Pro-urokinase	Nasaruplase
Tissue-plasminogen activator	Alteplase, Monteplase, Pamiteplase
Enzymes	Kallidinogenase, Agalsidase Alfa, Agalsidase Beta, Alglucosidase Alfa, Alglucerase, Idursulfase, Imiglucerase, Laronidase, Galsulfase
Heparins	Heparin Sodium, Heparin Calcium, Parnaparin Sodium, Dalteparin Sodium, Enoxaparin Sodium, Reviparin Sodium
Hyaruronate	Sodium Hyaruronate
Chondroitin sulfate	Chondroitin sulfate

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opment of biosimilar products is how to assure the similarity of carbohydrate moieties between the biosimilar products and the reference products.

In this review we provide some current topics of glycosylated biopharmaceuticals in terms of efficacy, safety, and manufacturing process and discuss the significance of glycosylation analysis in the development of biopharmaceuticals.

2. ROLE OF CARBOHYDRATES ON EFFICACY

Glycosylation in some biopharmaceuticals is crucial for their biological activity. Lysosomal storage diseases are characterized by deficiencies of lysosomal enzymes that degrade the glycoconjugates, such as mucopolysaccharides and glycolipids, and consequent cellular damages by their accumulated metabolites.[4] For use in enzyme-replacement therapy against lysosomal storage diseases, several recombinant glycoprotein products have been approved, namely *agalsidase alfa* and *agalsidase beta* for Fabry's disease, *alglucosidase alfa* for Pompe's disease, and *laronidase*, *idursulfase*, and *galsulfase* for mucopolysaccharidosis I, II, and VI, respectively.[4—8] These drugs contain *N*-linked oligosaccharides attached to mannose 6-phosphate (M-6-P), and the secreted enzymes are transported to an acidified prelysosomal compartment through the M-6-P receptor.[9,10] The carbohydrate residue is essential for lysosomal targeting and to exhibit complete enzyme activity in lysosomes. *Imiglucerase* is an analog of human β-glucocerebrosidase, which is used for the treatment of Gausher's disease. This glycoprotein is produced by recombinant DNA technology and exoglycosidase treatment to expose mannose residues in *N*-linked oligosaccharides. The carbohydrate modification facilitates incorporation of this drug into macrophages through mannose-binding receptors.[11] These recombinant lysosomal enzymes have achieved dramatic therapeutic effects against the lysosomal storage diseases.

Several human glycoprotein analogs whose carbohydrates are modified to enhance their efficacy have been developed in recent years. Erythropoietin is a glycoprotein containing three *N*-glycans and one *O*-glycan, and sialylation on its nonreducing ends is closely associated with its circulating half-life.[12] *Darbepoetin alfa* is an erythropoietin analog to which two additional *N*-glycans are attached by replacement of five amino acid residues.[13] The modification of glycosylation prolongs the half-time of this analog compared to its native form. Similar genetic engineering for improvement of half lives has been successfully attempted in antithrombolytic drugs. T-PA consists of finger, epidermal growth factor (EGF), kringle1, kringle2 and catalytic domains, and three *N*-glycans. High-mannose type oligosaccharides at the kringle1 domain and EGF domain are related to the short circulation half-life of t-PA.[14] Extension of half-life in blood has been achieved by eliminating the kringle1 domain from human t-PA and replacing one amino acid residue (*pamiteplase*).[15] These improvements have contributed to reducing the frequency and dose of administration.

Recombinant monoclonal antibodies, which have been successfully used in the treatment of cancers and immune diseases, might be the next target of drug design by glyco-engineering. Several anti-tumor monoclonal antibodies that contain a constant region derived from immunoglobulin (Ig) G have antibody-dependent cellular cytotoxicity (ADCC), and removal of a fucose (Fuc) residue from *N*-linked oligosaccharides at the constant region causes the enhancement of ADCC.[16,17] A non-fucosylated IgG-derived antibody is expected to improve the therapeutic effects of these antitumor pharmaceuticals.

Glycosylation has also been used for the site-selective modification of proteins with polyethylene glycol (PEGylation).[18] In the GlycoPEGylation method, sialic acid covalently substituted with polyethylene glycol (PEG) can be enzymatically transferred to *O*-glycans at serine or threonine positions in proteins produced in *Escherichia coli*. This strategy has overcome the problems of the previous PEGylation, which provided a heterogeneous mixture of PEG positional isomers. There is increasing interest in utilization and modification of glycans in the development of biopharmaceuticals.

3. IMPACT OF CARBOHYDRATES ON SAFETY

Some glycans have caused serious adverse events in clinical stages. Heparin is a highly sulfated GAG composed of a disaccharide unit, β1-4 linked α-D-glucosamine and α-D-iduronic acid or β-D-glucuronic acid (averaging 2.5 sulfate groups per disaccharide). In 2007—2008, a serious adverse event associated with *heparin sodium*, including over eighty deaths, occurred in the United States (US).[19] Over-sulfated chondroitin sulfate (OSCS), which consists of fully sulfated β1-3 linked α-D-*N*-acetylgalactosamine (GalNAc) and a β-D-glucuronic acid unit, was identified as the contaminant in the heparin sodium that had caused hypersensitivity reactions.[20,21] It has been reported that OSCS activated the kinin-kallikrein system and induced generation of C3a and C5a, potent anaphylatoxins derived from complement proteins. The contaminated heparin sodium was distributed to at least twelve countries and raised concern about a shortage of heparin products. For ensuring the safety of heparin products, pharmacopoeias in Japan, the US and EU immediately amended their heparin sodium monograph to confirm the absence of OSCS by ^{1}H-NMR and/or capillary electrophoresis (Fig. 1A). This adverse event has left concerns about the safety of GAGs and motivated the development of a sensitive and selective analytical method for GAGs products all over the globe (Fig. 1B).

An alternative concern for a carbohydrate-related adverse event is immunogenicity of nonhuman glycan motifs. The galactose-α1,3-galactose (Gal(α1-3)Gal) motif is known as one of the major problems in the transplantation of organs from pigs to humans.[22] Mouse myeloma cell lines, which are often used for production of recombinant glycoproteins, also expresses the Gal(α1-3)Gal motif in *N*-linked oligosaccharides. Recently, a high prevalence of hypersensitivity reactions was reported in patients who had been injected with *cetuximab*, which is produced in mouse myeloma cells.[23] This chimeric mouse-human antibody is attached to *N*-linked oligosaccharide containing the Gal(α1-3)Gal motif at the Fab region.[24] It is reported that IgE antibodies against the Gal(α1-3)Gal motif had been present in serum before therapy, and these antibodies caused hypersensitive reactions after cetuximab treatment. Another immunogenic nonhuman glycan is *N*-glycolylneuraminic acid (NeuGc), a sialic acid.[25]

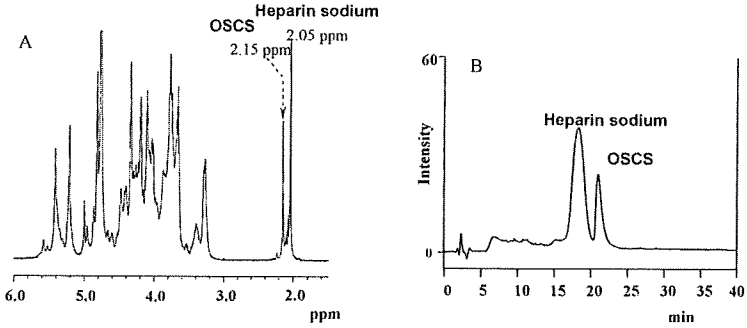

Fig. 1. ^{1}H-NMR Spectra (A) and Anion-Exchange Chromatogram (B) of Heparin Sodium Containing 10% OSCS

^{1}H-NMR spectrum was obtained at 298 K using a 500 MHz JEOL JNM-ECA500 instrument equipped with a 5-mm filed gradient tunable probe with standard JEOL software. Chemical shifts were referenced to the signal of 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt (TSP) as internal standard (0.00 ppm). Anion-exchange chromatogram was obtained using TSKgel DEAE-5PW (21.5 mm I.D.×15 cm, 10 μm) at a flow rate of 1.0 ml/min at 40 °C. The UV detector was set at 230 nm. GAGs were eluted by a linear gradient of 0—100% B buffer (buffer: A, 20 mM Tris–HCl (pH 8.0); B, 20 mM Tris–HCl (pH 8.0) containing 2 M NaCl) within 40 min.

Since Varki *et al.* reported the incorporation of NeuGc into human embryonic stem (ES) cells through the animal serum and the feeder layer, contamination of cell therapy products with NeuGc has become a serious issue in clinics.[26] These reports imply the significance of glycan analysis in recombinant glycoprotein products and risk assessment of nonhuman glycans.

4. ALTERATION OF CARBOHYDRATES BY CHANGES IN EXPRESSION SYSTEM AND MANUFACTURING PROCESS

In addition to recombinant technology using mammalian cells, a transgenic technique has been also focusing on large-scale production of therapeutic glycoproteins.[27,28] Antithrombin III is an anticoagulant factor, and a freeze-dried preparation of human antithrombin III has been approved for treatment of disseminated intravascular coagulation and thrombogenic tendencies. In 2006, the European Medicine Agency (EMEA) authorized the marketing of *antithrombin alfa*, a recombinant human antithrombin III produced from the milk of transgenic goats. Detailed structural analysis revealed that these two glycoproteins were structurally identical except for a difference in glycosylation.[27] An alternative approach for the large-scale and low-cost production of biopharmaceuticals is the use of transgenic plants.[29] Plant cells express nonhuman glycans, such as Fuc (α1-3) *N*-acetylglucosamine (GlcNAc), xylose (Xyl) (β1-2) GlcNAc and Gal (β1-3) GlcNAc at the reducing-end of complex-type *N*-linked oligosaccharides. Currently, plant glyco-engineering, including modification of glycosyltransferases, is developing to overcome limitations in the production of glycoprotein products.[30]

For various scientific, safety-related and economic reasons, changes in manufacturing processes for biopharmaceuticals are often attempted during the development phase and after marketing authorization. Meanwhile recombinant glycoprotein products that have been claimed to be similar to a reference medical product already authorized (biogeneric/biosimilar/follow-on biologics) have been approved by different manufacturers. The changes in the manufacturing process possibly cause the alteration of glycosylation in the glycoprotein products and consequent changes in quality, efficacy and safety. The first biosimilar glycosylated pharmaceutical, *epoetin alfa*, was approved in the EU recently. According to the Japanese guidelines for the biosimilar products, the applicants have to submit some efficacy and safety study data but not all if they can show the data on structural properties and physicochemical/biological similarity between the authorized and biosimilar products. One of the challenging issues in the development of biosimilar glycoprotein products is a comparison of glycosylation between the authorized products and the new entry biosimilar products.

Using some *epoetin* products, we have studied the possibility of several analytical methods for comparison of the glycosylation between closely related biopharmaceuticals. Commercially available *epoetin* products (products A—D) were electrophoresed, and *N*-linked oligosaccharides were released from bands at 30 kDa by an in-gel glycopeptidase F digestion (Fig. 2A). The resulting oligosaccharides were reduced with $NaBH_4$ and subjected to LC/MS. In Fig. 2, *International nonproprietary names* (*INN*) of *epoetins* contained in the products A—C and D are tentatively named as *epoetin* α and β, respectively. Products A and B are marketed in country X, while products C and D are manufactured and distributed in country Y. Figure 2B shows the total ion chromatograms of *N*-linked oligosaccharides released from four *epoetin* products, and the mass spectra acquired from the most intense peaks (peak z_{1-4}) are shown in Fig. 2C. The *m/z* values of the most intense ions (*m/z* 1226.8) and a series of triply charged ions with *m/z* 14 spacing pattern reveal that the most abundant glycan in *epoetin* products are a tetrasialyl fucosylated-tetraantennary oligosaccharide in common but there are tangible differences in acetylation of sialic acids between products A—C (*epoetin* α) and product D (*epoetin* β) (Fig. 2C). Even among *epoetin* α products there were some significant differences, such as the non-fucosylated oligosaccharides that were found in *epoetin* α products in country X (products A and B) but not in country Y (product C). The latest analytical technology allows us to evaluate the similarity of the glycosylation of closely related biopharmaceuticals.[31,32]

5. CONCLUSION

As described above, glycosylation in most biopharmaceuticals affects their efficacy and safety, and the glycosylation is dependent on the manufacturing process and the expres-

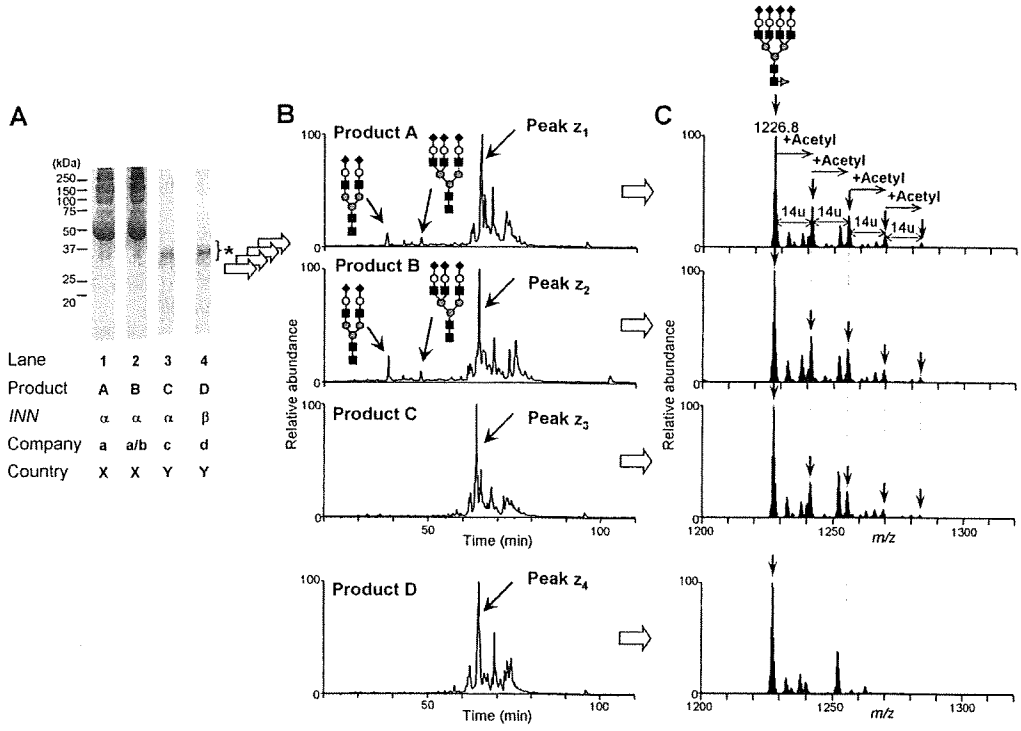

Fig. 2. Glycosylation Analysis of *Epoetin* Products

(A) SDS-PAGE images. Sample: lane 1, product A; lane 2, product B; lane 3, product C; lane 4, product D. (B) *N*-glycan profiles of products A—D acquired by LC/MS in the negative ion mode. (C) Mass spectra of peaks z_{1-4}. Product: A, an *epoetin* α product manufactured/distributed by company a in country X; B, an *epoetin* α product manufactured by company a and distributed by company b in country X; C, an *epoetin* α product manufactured/distributed by company c in country Y; D, an *epoetin* β product manufactured/distributed by company d in country Y. *INN* of products A—C, and D are tentatively named as *epoetin* α and *epoetin* β, respectively. Symbols: ●, Man; ○, Gal; ■, GlcNAc; ▲, Fuc; ◆, NeuAc. * Bands of *epoetins*. LC: instrument, nanoFrontier nLC system (Hitachi High-Technologies Corporation); column, graphitized carbon (0.075×150 mm, ThermoFisher Scientific); flow rate, 200 nl/min; buffer A, 5 mM ammonium acetate with 2% acetonitrile (pH 9.6); buffer B, 5 mM ammonium acetate with 80% acetonitrile (pH 9.6); gradient condition, 5—35% B (110 min). MS: instrument, LTQ-TF (ThermoFisher Scientific); electron voltage, 2.0 kV (negative ion mode).

sion system. Physicochemical and biological characterization of such glycosylation is crucial at various stages, namely the development of new biotherapeutic glycoproteins, the establishment of changes in the manufacturing process, and the development of biosimilar products. Appropriate glycan testing must be adopted if the carbohydrate moiety influences safety and efficacy of the pharmaceutical. Furthermore, an *in vivo* assay could be replaced by the glycan test if the glycan profile is strongly associated with *in vivo* activity. Advances in analytical techniques for carbohydrate moieties are expected to facilitate the development of biopharmaceuticals.

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早期臨床開発段階でのバイオ医薬品の品質・安全性確保

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Quality and safety issues of biotechnological products used in early clinical studies

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Abstract

In the last decade, an increasing number of second-generation (engineered) protein products such as humanized monoclonal antibodies, fusion proteins or chemically modified proteins, have been developed. In the development of such products, selection of the optimal product from several candidates is a critical step. However, the pharmacological effects and safety profiles of these non-natural protein products in humans are difficult to predict. In addition, non-clinical study data on these products are not sufficient, due to species specificity or technical limitations. Therefore, early exploratory clinical studies might be one possible approach for improving the development success rate of engineered protein products.

To ensure the quality and safety of biotechnological products, the following two hurdles must be overcome : 1) establishing a robust manufacturing process and 2) setting specifications based on data from product characterization and non-clinical/clinical studies. In the early development stage, however, the production process might not be fully established or information for setting the specifications might be limited. Here we discuss approaches for ensuring the quality and safety of biotechnological investigational products used for early and exploratory clinical studies. One of the indispensable tests is the viral safety evaluation of the master cell bank and unprocessed bulk. Studies on biological properties and the potency of products using human cell/tissue preparations should be useful for predicting the safety profile of the products. When the manufacturing process of the investigational product has been changed, comparability studies should provide sufficient assurance that no resulting product differences will have an adverse impact on the product characteristics. The discussions herein will hopefully be useful in current efforts to develop new biotechnological products.

Key words

biotechnological products, quality, safety, manufacturing process, IND

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1. 序論

21世紀に入ってバイオ医薬品の開発は急速に広がっており，特に抗体医薬品や改変タンパク質医薬品など，旧来のバイオ医薬品とは異なる製品の開発が進んでいる．第二世代のバイオ医薬品とも言えるこれらの製品では，同時に複数の医薬品候補分子について開発が進められることが多く，適切な医薬品候補分子の選択が開発戦略において重要な位置を占めている．しかし，天然型のタンパク質医薬品と異なり，非天然型のタンパク質医薬品ではヒトにおける薬理作用や免疫反応性などの安全性の予測が容易ではないことに加え，タンパク質医薬品全般に言えることであるが標的分子との相互作用の種特異性が高いことなどにより，動物モデルでの評価では開発候補品の選択に十分な有効性や安全性の情報が得られないことも多い．一方で，医薬品開発において，開発候補品の特性に関する知識や，治療標的の妥当性について，より早くヒトのデータを入手することが有益な場合があることが認められている[1]．このような背景のもと，バイオ医薬品開発の効率化を目指して，臨床試験を早期に実施して開発候補品のヒトでの安全性や有効性を評価していくことが検討され始めており，臨床開発初期に用いるバイオ医薬品について如何に品質や安全性を確保していくかが大きな課題となっている*．

化学薬品に関しては，通常のフェーズⅠ試験の前段階に限定的な投与量で実施される早期探索的臨床試験を含め開発初期における品質・安全性確保の要件について様々な議論が行われ，その一つとしてマイクロドーズ臨床試験の実施に関するガイダンスも公表されている．しかし，バイオ医薬品の品質・安全性確保に求められる要件は，高分子のもつ複雑な物質特性，不安定性，品質の一定性を確保するための原薬製造工程の恒常性の重要性，さらには，ウイルス等の感染因子に対する安全性確保など，化学薬品とは大きく異なっている．そのため，化学薬品の品質・安全性確保の手法をそのままバイオ医薬品に応用することは適切ではない．

バイオ医薬品の品質・安全性確保は，1)頑健な製造方法の構築と工程管理法の確立，2)製品の特性解析・非臨床試験・臨床試験・安定性試験結果などに基づいた規格及び試験方法の設定等により達成される．承認申請製品の品質・有効性・安全性確保を目指して，バイオ医薬品の開発はステップバイステップで進められるものであり，開発ステージの進行とともに，製法や品質特性，安全性に関するデータが蓄積されていくものである．従って，臨床開発初期，すなわち治験開始に当たって求められるデータと承認申請データパッケージとして求められる要件は自ずと異なってくる．バイオ医薬品の臨床開発の初期段階では，製法や各種試験法が十分に確立されていないことも少なくない．しかしその一方で，前述のように抗体医薬品などでは同時に複数の候補品について開発を進めていることも多く，同時平行して開発が進められる複数の開発候補品について，承認時と同様のデータを得るには膨大なリソースと時間が必要である．このようなバイオ医薬品開発の特色を考慮したときに，臨床開発初期の段階で求められる安全性評価や，安全性を担保するための品質評価・製法等について考察することは，バイオ医薬品開発の効率化や成功率向上のためにも有用であると考えられる．

承認申請要件としてのバイオ医薬品の品質・安全性確保についてはこれまで十分に議論が重ねられているが，バイオ医薬品の臨床開発初期における品質・安全性に関する議論は十分とは言えない状況である．欧米では治験薬の品質・安全性確保に関するガイドラインの整備が進められており，我が国においてもバイオ医薬品開発推進のため，

＊治験に用いられる医薬品候補物質は薬事法上の「医薬品」ではないが，本稿では治験に用いられるものを含めてバイオ医薬品と記載する．

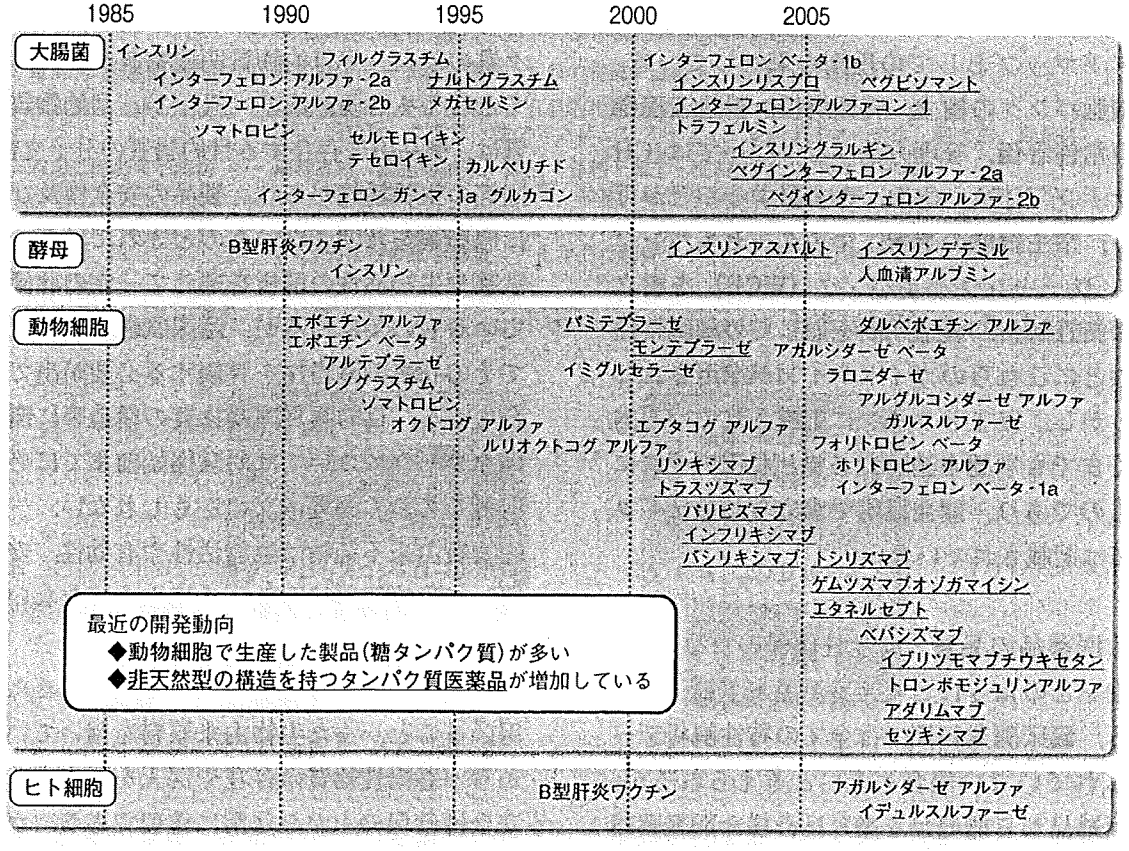
議論を進めることが望まれる．本稿では，臨床開発初期におけるバイオ医薬品の品質・安全性確保に関する議論の端緒として，バイオ医薬品の開発，製法と品質特性，安全性について概説した上で，製法と品質特性の観点から臨床開発初期（主としてフェーズⅠ）におけるバイオ医薬品の品質・安全性評価に関する試案を考察した．また，バイオ医薬品の早期探索的臨床試験についても考察を加えた．

2. バイオ医薬品の開発

1980～90年代のバイオ医薬品開発では，大腸菌やCHO細胞等に目的とするタンパク質の遺伝子を導入してヒト有用タンパク質のコピーを製造する，いわゆる天然型タンパク質製品が主であった．これまでにインスリンや成長ホルモン，エリスロポエチンなど数多くの有用タンパク質が開発され，疾病治療に不可欠な存在となっている．その後21世紀に入って，それまでの天然型製品に加えて，抗体医薬品や改変タンパク質医薬品など非天然型組換えタンパク質製品の開発が急速に進んできている（Fig. 1）．現在，各社のパイプラインに存在するバイオ医薬品の多くも非天然型製品であることから，今後もこのような開発傾向が続くものと推定される．

一般的にバイオ医薬品の製法開発では，遺伝子組換えにより作製した目的タンパク質発現細胞の様々なクローンから，最も生産性が高く，かつ製造期間を通じて安定した製造を行える生産細胞を選択することが行われる．従来の多くのバイオ医薬品は，有用な生物活性を持つ生体由来タンパク質を見出し，天然のタンパク質と同一の一次構造をもつ製品を大量に製造することを目指してきており，目的とするタンパク質に関してはそれほど大きな選択の幅があったわけではなかった．このような場合，より適切な翻訳後修飾やタンパク質の安定性などを考慮することはあっても，わずか

Fig. 1 Approval year of biotechnological products in Japan, and cell substrates used for their production



な例外を除いて一次構造の異なる改変タンパク質製品を開発することはまれであった.

一方，現在最も活発に開発が進められている抗体医薬品や改変タンパク質医薬品では，一次構造の異なる一連の候補群から最適な製品を開発するといったストラテジーがよく取られており，このために早期に臨床試験を実施し，ヒトでの有効性や安全性に最も優れた製品を早い時期に選択することが開発を効率よく進める上で有用と考えられている．従って，本稿で考察しようとしている臨床開発初期における製品群の品質・安全性確保の合理的手法は，従来のバイオ医薬品はもとより，これから開発が進む非天然型のバイオ医薬品を効率的に開発するために重要であると言える.

2.1 バイオ医薬品の製法と品質特性

バイオ医薬品の承認申請時に必要な品質・安全性・有効性に関するデータについては，品質特性解析，非臨床試験，臨床試験に関連するICHガイドライン[2~8]を始め様々な国内指針や基準[9, 10]が出されている．これらのガイドラインや基準は承認申請時ばかりでなく，バイオ医薬品開発におけるロードマップとしての役割も果たしている．例えば細胞バンクの樹立，特性解析，製造工程全体での恒常性評価，管理方法などについてはICHガイドライン（Q5A，Q5D）を参照することが可能であり，宿主細胞の選択，マスターセルバンク（MCB）／ワーキングセルバンク（WCB）を樹立した際の特性解析，製造期間を通じての細胞特性の評価などにこれらのガイドラインが準用されている．しかし，ガイドラインに記載されている内容はあくまでも承認申請時に必要となるデータに関するものであり，開発段階で求められるデータについては記載されていない.

バイオ医薬品の品質・安全性確保のためには，品質特性をどのようにとらえるかがまず第一に重要である．臨床開発初期では全ての特性解析データが得られていない場合も多いと考えられるが，複数の候補品から開発品を絞り込む様な開発戦略をとる場合に，多くの候補品はドロップアウトしていくことになるため，全ての候補品についてどこまで品質特性を明らかにしておくべきかが製品開発の迅速化につながる重要なポイントである．以下に，バイオ医薬品の特性と臨床開発初期における品質・安全性確保において特に配慮すべき点を述べる.

化学薬品と異なり，バイオ医薬品は非常に複雑な高分子であり，目的タンパク質やその関連物質からなる不均一性を持つことから，有効成分に関しては不均一性の解析をどの程度行っておくべきかが重要なポイントとなる．抗体医薬品などの糖タンパク質製品では糖鎖の不均一性や特定の糖鎖構造が有効性や安全性に大きく影響することもあり，不均一性を含めた糖鎖構造を明らかにすることが品質特性のみならず体内動態や生物活性への影響を予測するために非常に重要である．従って，糖鎖構造が有効性・安全性に影響する製品では，開発初期においても安全性や有効性に関連する特定の構造の糖鎖の存在や存在比率を明らかにしておく必要がある.

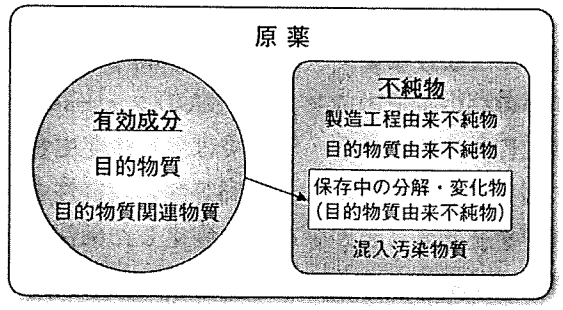
また，バイオ医薬品の品質特性は，目的タンパク質のみならず目的物質関連物質や不純物も含めてとらえる必要がある（Fig. 2）．目的物質関連物質は，製品中に存在する目的物質の分子変化体で，同等の生物活性があり，製品の安全性及び有効性に悪影響を及ぼさないものとされている．非臨床試験や生物活性の解析を通じて一定の評価はされているものではあるが，臨床試験の結果を踏まえてその判断の妥当性を評価する必要が出てくる場合もある．目的物質関連物質の構造等に関する詳細なデータについては治験開始前までに必ずしも取得しておく必要はないかもしれない．一方，目的物質由来不純物は生物活性や有効性，安全性に関して目的物質に匹敵する特性を持たないものであり，含量等の評価が必要である.

さらに，バイオ医薬品は，製造に生きた細胞を用いること，また生物由来原料を用いていることから，感染性物質の存在や混入を否定することが安全性確保の上から非常に重要である．ウイルス

等の感染性物質は微量でも重大な安全性上の問題を生じさせる可能性があることから，被験者の安全性確保のため治験初期の製品であっても，十分な解析を行いその存在を否定しておかなくてはならない．ただし，必ずしも複数のロットを用いた検討である必要は少なく，開発初期では治験に用いるロットについての試験が実施されていればよいと考えられる．また，製品の安定性に関しては，最低限目的とする臨床開発期間での安定性を担保するデータが得られていればよいと考えられる．

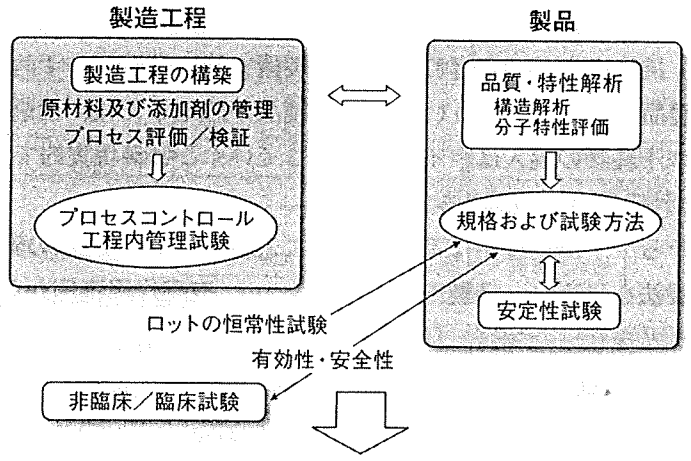
バイオ医薬品の品質・安全性確保のためには品質特性解析のみならず，頑健性の高い製造方法の構築と，品質の恒常性を担保するための適切な工程管理法の確立も重要である（Fig. 3）．これは，1）培養工程の変動が組換えタンパク質産生細胞の特性に影響するなど，製造方法の違いにより糖鎖などの不均一性が大きく変化する可能性があること，2）バイオ医薬品のような複雑な高分子製品ではロットごとに品質特性の全てを明らかにすることが困難であること，3）特に，各ロットに含まれる不純物の全てを試験することは現実的でない上，微量でも免疫原性に影響を与える可能性があるなどの理由による．従って，製造工程の中で特に品質に影響を与えると考えられる重要工程では，重要中間体を設定して工程内管理試験を行うことが

Fig. 2 Drug substance of biotechnological products



目的物質関連物質 …… 目的物質の分子変化体のうち目的物質に匹敵する特性を持つもの
目的物質由来不純物 …… 目的物質の分子変化体のうち目的物質に匹敵する特性を持たないもの
（例：前駆体，切断体，脱アミド体，ジスルフィド結合ミスマッチ体，酸化体，凝集体）
製造工程由来不純物 …… 細胞基材，細胞培養液，抽出・分離・加工・精製工程に由来する不純物
（例：宿主細胞由来タンパク質，核酸，血清由来成分，抗生物質，クロマトグラフ用担体）
混入汚染物質 …… 製造工程に本来存在しないはずの外来性物質
（例：外来性の化学物質，生化学的な物質，ウイルス等の微生物類）

Fig. 3 Elements for ensuring product quality and consistency of biotechnological products



製造工程の管理＋最終製品の規格・試験法による品質の一定性確保