medium (NISSUI Co. Ltd., Tokyo, Japan) containing 10% fetal calf serum (FCS). The human osteosarcoma cell line HOS [51] and CD4-transduced human glioma cell line U87/CD4 [52,53], as well as NP-2/CD4 [49], NP-2/CD4/CCR5 [49], NP-2/CD4/CXCR4 [49], and NP-2/CD4/FPRL1 (see below) cells were cultured in Eagle's minimum essential medium (NISSUI Co., Inc., Tokyo, Japan) supplemented with 10% FCS. The human embryonal kidney cell line 293T [54], human hepatoblastoma cell line HepG2 [55], human hepatoma cell line Huh7, and human astrocytoma cell line U251MG [56] were maintained in Dulbecco's modified Eagle minimum essential medium (NISSUI Co., Inc., Tokyo, Japan) supplemented with 10% FCS. Brain-derived fibroblast-like BT-20/N cells [53,57], derived from the surgically dissected human brain tissue of a patient with glioma and thought to originate from brain blood vessels, were cultured in RPMI 1640 medium containing 10% FCS, endothelial cell growth supplements (BD Bioscience, Medford, MA) (10 μg/ml), and epidermal growth factor (10 ng/ml). Peripheral blood lymphocytes (PBLs) were isolated from healthy blood donors as previously described [58]. PBLs were stimulated with phytohemmagglutinin (PHA) and cultured in RPMI 1640 medium containing 10% FCS and recombinant IL-2 (100 U/ml).

Virus strains

Cell line-adapted R5-X4 HIV-1 strains (GUN-1WT [57], GUN-4WT [59], and GUN-7WT [59]), GPR1-X4 HIV-1 variants (GUN-1V [57], GUN-4V [59], and GUN-7V [59]), an X4 HIV-1 strain (IIIB [60]), R5 HIV-1 strains (SF162 [61] and Ba-L [62]), HIV-2 strains (CBL23 [63], GH-1 [64], ROD/B [65], and SBL6669 [66]), and SIV strains (mac251 [67] and mndGB-1 [68]) were used. All of these HIV-1 strains are classified as subtype B based on their amino acid sequences of the Env protein [57-62]. The culture supernatants of C8166 cells infected with HIV/SIV strains except SF162, Ba-L, and mac251 strains, were harvested as viral stocks when cytopathicity was microscopically observed. SF162, Ba-L, and mac251 strains were propagated in C8166/CCR5 cells as previously described [49]. Primary HIV-1 isolates were propagated in PBLs and used in this study. Their origins, subtypes, and Genbank accession numbers of the *env* genes are described below. AG204 (Vietnum, subtype AE, Genbank accession number AB044003), AG206 (Vietnum, subtype AE, AB044005), AG208 (Vietnum, subtype AE, AB044007), HCM303 (Vietnum, subtype AE, AB044020), HCM305 (Vietnum, subtype AE, AB044022), HCM308 (Vietnum, subtype AE, AB044024), HCM309 (Vietnum, subtype AE, AB044025), and HCM342 (Vietnum, subtype AE, AB044034), mIDU101 (Myanmar, subtype C, AB097871), mSTD104 (Myanmar, subtype C, unpublished).

PCR primers

Oligonucleotide primers were synthesized (Proligo K. K., Tokyo, Japan) to detect the expression of mRNA for CD4, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), CCR5, CXCR4, GPR1, or FPRL1 by reverse transcription (RT)-PCR. Each gene name, followed by each sense and antisense primer name, their nucleotide sequences and positions in the open reading frame [DDBJ/EMBL/Genbank accession number] are described below. CD4: CD4CN, 5'-ATGAACCGGGGAGTCCCTTTTAGGCACTTG-3' (sense: from the 1st to the 30th position, i. e. 1st–30th); and CD4CR, 5'-TCAAATGGGGCTACATGTCTTCTGAAACCG-3' (anti-sense: 1,039th–1,068th) [DDBJ/EMBL/Genbank accession number, NM000616]. GAPDH: G3PDHN, 5'-TGAAGGGTCGGAGTCAACGGATTTGGT-3' (sense, 11th–36th); and GAPDHR, 5'-TAGACGGCAGGTCAGGTCCACCAC-3' (antisense, 724th–747th) [BT006893]. PCR primers used to detect GPCR cDNA are as follows: CCR5: CCR5CN, 5'-ATGGATTATCAAGTGTCAAGTCCAATCTAT-3' (sense, 1st–30th); and CCR5CR, 5'-TCACAAGCCCACAGATATTTCCTGCTCCCC-3' (antisense, 1,001st–1,030th) [NM000579]. CXCR4: CXCR4CN, 5'-ATGGAGGGGATCAGTATATACACTTCAGAT-3' (sense: 1st–30th); and CXCR4CR, 5'-TTAGCTGGAGTGAAAACTTGAAGACTCAGA-3' (antisense, 979–1,008th) [NM003467]. FPRL1: FPRL1CN, 5'-ATGGAAACCAACTTCTCCACTCCTCTGAAT-3' (sense, 1st–30th); and FPRL1CR, 5'-TCACATTGCCTGTAACTCAGTCTCTGCAGG-3' (antisense, 1,027rd–1,056nd) [M76672]. GPR1: GPR1CN, 5'-ATGGAAGATTTGGAGGAAACATTATTTGAA-3' (sense, 1st–30th); and GPR1CR, 5'-TTATTGAGCTGTTTCCAGGAGACACAGATT-3' (antisense, 1,039th–1,068th) [U13666].

Detection of GPCR mRNA

Total RNA was isolated from human cells using an RNA extraction kit, SepaGene (Sanko-Junyaku Inc., Tokyo, Japan), in accordance with the manufacturer's protocol. cDNA for the total cellular RNA was constructed as described elsewhere [49]. mRNA expression for CCR5, CD4, CXCR4, GPR1, FPRL1, and GAPDH was detected by PCR of cDNA preparations using the sense and antisense primer pairs described above [49]. Amplified cDNA was detected by 1% (w/v) agarose gel electrophoresis.

Cloning of the FPRL1 gene

A DNA fragment encoding the entire open reading frame (ORF) of the FPRL1 gene was amplified by RT-PCR using the FPRL1-specific primers, FPRL1CN and FPRL1CR, and cDNA constructed from the total RNA isolated from C8166 cells. The ORF DNA of the FPRL1 gene was cloned into the TA-cloning plasmid pDrive (QIAGEN K. K., Tokyo, Japan) and the plasmid obtained was designated pDrive/FPRL1. The DNA fragment containing FPRL1 ORF was isolated from the pDrive/FPRL1 plasmid by *Eco*RI

digestion and subcloned into the expression plasmid pCX-bsr [69]. The FPRL1 plasmid obtained was designated pCX-puro/FPRL1. The cloned FPRL1 gene was sequenced and found to be 100% homologous in terms of amino acid sequences to the reported gene [M76672] [30].

Establishment of FPRL1-expressing cells
An FPRL1-expressing cell line was established as follows. The plasmid harboring the receptor gene for ecotropic murine leukemia virus (MuLV) and hygromycin-resistance gene was transfected into NP-2/CD4 cells and hygromycin-resistant cells were selected as reported previously [16]. BOSC23 cells [70] were transfected with the pCX-bsr/FPRL1 plasmid and ecotropic MuLV pseudotype was produced from the cells. NP-2/CD4 cells were infected with the ecotropic MuLV pseudotype produced by BOSC23 cells. The blasticidin-resistant NP-2/CD4 cells were selected through cultivation in medium containing blasticidin (10 μg/ml) (CALBIOCHEM, San Diego, CA) for two weeks. Surviving cells were designated NP-2/CD4/FPRL1. NP-2/CD4/CCR5, NP-2/CD4/CXCR4, and NP-2/CD4/GPR1 cells were established previously [16,23]. The expressions of mRNAs for CCR5, CXCR4, GPR1, and FPRL1 genes in these cells were detected by RT-PCR using cDNA and the PCR primers prepared as described above.

Infection assay
NP-2/CD4, NP-2/CD4/CCR5, NP-2/CD4/CXCR4, NP-2/CD4/FPRL1 and NP-2/CD4/GPR1 cells (5×10^4) were seeded into 24-well culture plates 24 h prior to viral inoculation. These cells were exposed to HIV/SIV in an amount of virus corresponding to 1×10^4 cpm of the reverse transcriptase activity as previously described [71]. After incubation for two hours, the cells were washed three times with E-MEM containing 10% FCS and then cultured in 500 μl of fresh medium at 37°C. The cells were passaged every two days.

Determination of the effects of an anti-CD4 monoclonal antibody, GPCR ligands, and tyrosine sulfation on HIV-1 infection
To determine CD4 dependency of HIV infection, NP-2/CD4/CCR5 and NP-2/CD4/FPRL1 cells were cultured in E-MEM containing a serially diluted anti-CD4 monoclonal antibody (MoAb), Nu-TH/I (Nichirei Inc., Tokyo, Japan), at 37°C for two hours. The cells were incubated in E-MEM with or without Nu-TH/I MoAb at 37°C for two hours and then exposed to HIV-1 in an amount corresponding to 1×10^4 cpm of RT activity. After removing the inocula, the cells were incubated at 37°C in E-MEM containing 10% (v/v) FCS for four days.

To investigate the effects of ligands on infection of cells with HIV/SIV strains, NP-2/CD4/CCR5 or NP-2/CD4/FPRL1 cells were incubated in E-MEM containing RANTES (100 μg/ml) (BIOCARTA US, San Diego, CA) or forymyl-Met-Leu-Phe (fMLF) peptide (100 μg/ml) (WAKO Junyaku, Inc., Tokyo, Japan) at 37°C for three hours. Then, the cells were exposed to HIV-1 as described above.

CCR5 and FPRL1 contain several tyrosine residues in their NTRs and ECLs, but only NTRs harbor the signal sequence for tyrosine sulfation [73]. To clarify the effect of tyrosine sulfation in NTR of CCR5 and FPRL1, NP-2/CD4/CCR5 and NP-2/CD4/FPRL1 cells were incubated in E-MEM containing an inhibitor of tyrosine sulfation, $NaClO_3$ (10 mM), for 48 hours and then inoculated with viruses.

Detection of HIV-/SIV-infected cells
The susceptibilities of the cells described above to HIV/SIV were determined by indirect immunofluorescence assay (IFA), which detects HIV-1-, HIV-2-, or SIV-antigens expressed in infected cells, as previously reported [71]. A pool of sera derived from HIV-1-infected humans or SIVmac-infected macaques was used as the first antibody [16,49]. Infection was checked on days 2, 4, and 6 after inoculation.

Phylogenetic analysis
The multiple alignment of the amino acid sequences of 20 CKRs and 16 GPCRs closely related to CKRs and their phylogenetic tree was constructed using the ClustalW program [72]. GPCR names and their abbreviations in the protein database SWISS PROT were as follows: type-1 angiotensin II receptor AG2R [DDBJ/EMBL/Genbank accession number, M91464], type-2 angitensin II receptor AG22 [U20860], type-1B angiotensin II receotor AG2S [D13814], apelin receptor APJ [U03642], B1 bradykinin B1 receptor BRB1 [U12512], B2 bradykinin receptor BRB2 [M88714], C3a anaphylatoxin receptor C3AR [U28488], C5a anaphylatoxin receptor C5AR [X58674], C5a anaphylatoxin receptor C5L2 [B038237], CC-CKR CCR1 [NM001295], CC-CKR CCR2b [NM000648], CC-CKR CCR3 [NM001837], CC-CKR CCR4 [NM005508], CC-CKR CCR5 [NM000579], CC-CKR CCR6 [NM004367], CC-CKR CCR7 [NM001838], CC-CKR CCR8 [NM005201], CC-CKR CCR9 [NM006641], CC-CKR CCR10 [NM016602], CC-CKR CCR11 [AF110640], CX3C-CKR CX3CR1 [NM001337], CXC-CKR CXCR1 [NM000634], CXC-CKR CXCR2 [NM001557], CXC-CKR CXCR3 [NM001504], CXC-CKR CXCR4 [NM003467], CXC-CKR CXCR5 [NM001716], CXC-CKR CXCR6 [NM006564], CC-CKR D6 [NM001296], formylpeptide receptor 1 FPRL1 [M76672], formylpeptide receptor 2 [M37128], FPRL1-related receptor FPR1 [M76673], proteinase-activated receptor 1 PAR1 [M62424], protease-activated receptor 2 PAR2 [Z49993], protease-activated receptor 3 PAR3 [U92971], and protease-activated receptor 4 PAR4 [AF080214].

Authors' contributions

NS conceived and designed this study, NS also carried out the molecular genetic and virological studies, and drafted the manuscript, AT carried out establishment of cell lines, AO and TM carried out biochemical studies. AH, CA, SK, TO, and YT participated in virus preparation and their characterization, HH played important roles in coordination of this study and helped to draft the manuscript.

Additional material

Additional file 1

Table 1. HIV/SIV coreceptors and formylpeptide receptors, and amino acid sequences of their NTRs.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1742-4690-5-52-S1.ppt]

Additional file 2

Table 2. Use of GPCRs as coreceptors by HIV-1, HIV-2, and SIV.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1742-4690-5-52-S2.ppt]

Additional file 3

Table 3. FPRL1 use and amino acid sequences of the V3 domain of HIV-1 strains.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1742-4690-5-52-S3.ppt]

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Detection of human papillomavirus type 56 in Bowen's disease involving the nail matrix

A. Shimizu, A. Tamura, M. Abe, S. Motegi, Y. Nagai, O. Ishikawa, Y. Nakatani,* Y. Yamamoto,† H. Uezato† and H. Hoshino*

Department of Dermatology and *Department of Virology and Preventive Medicine, Gunma University Graduate School of Medicine, 3-39-22 Showa-machi, Maebashi, Gunma 371-8511, Japan

†Department of Dermatology, School of Medicine, University of the Ryukyus, Okinawa, Japan

Correspondence
Akira Shimizu.
E-mail: ashimizu@med.gunma-u.ac.jp

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Bowen's disease, human papillomavirus type 56, longitudinal melanonychia, matrix, nail, polymerase chain reaction

Conflicts of interest
None declared.

Summary

Background As Bowen's disease of the nail apparatus is quite rare, there have been only a few reports on the prevalence of human papillomavirus (HPV) infection in this condition.

Objectives The purpose of this study was to clarify the association of HPV with this disease involving the nail apparatus.

Methods Five patients with Bowen's disease of the nail apparatus were investigated clinically, virologically and histologically. Total DNAs extracted from excised skin lesions were analysed using polymerase chain reaction (PCR) for the presence of HPV DNA and the amplified products were subjected to DNA sequence analyses. Histological localization of HPV DNA was examined by *in situ* hybridization.

Results In three of five patients, HPV was detected by PCR amplification, and subsequent sequence analyses of the PCR products showed the sequences of HPV type 56. A common clinical feature of the three HPV-positive patients was longitudinal melanonychia. In contrast, the two HPV-negative patients presented with a convex nail deformity and a periungual ulcerative lesion. In two of three positive cases, there was a silent point mutation in the L1 gene of each HPV. In the remaining one case, the nucleotide sequence was consistent with the consensus sequence of HPV 56. Sequence analyses of the E6 gene revealed the infection of different variants of HPV 56 among the three cases. The viral genomes were located in keratinocyte nuclei upon *in situ* hybridization.

Conclusions HPV 56 may be involved in the carcinogenesis of Bowen's disease affecting the nail matrix with longitudinal pigmentation.

Bowen's disease is a squamous cell carcinoma *in situ* of the skin and mucocutaneous regions.[1] If untreated, invasive carcinoma capable of metastasis may develop in 3–5% of patients with Bowen's disease.[2] In recent studies, several types of human papillomavirus (HPV) DNA have been identified in the lesions of Bowen's disease.[3–12] Most HPV-positive lesions in Bowen's disease are localized in the genital region or distal extremities, and HPV type 16 has been frequently detected.[10–12]

Bowen's disease arising at areas other than the nail apparatus usually manifests as an erythematous, pigmented patch with scale and erosion. The nail apparatus consists of six main components: the nail matrix, nail plate, cuticular system, nail bed, anchoring system of ligaments, and nail folds. As Bowen's disease develops in the epithelium, Bowen's disease of the nail apparatus occurs within the epithelium of the nail matrix, nail bed or nail fold.

Probably because Bowen's disease arising from the nail apparatus is quite rare, there have been only a few reports investigating the prevalence of HPV infection in this condition.[3–8] In the present study, we performed a clinicopathological examination and DNA analyses of five cases of Bowen's disease that had developed in the nail apparatus to clarify the involvement of HPV infection in these cases.

Materials and methods

Patients and specimens

Surgically resected specimens from five patients with Bowen's disease of the nail apparatus, treated between January 1997 and December 2005, were used for this study. The tissue specimens, fixed in 10% neutralized buffered formalin and

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embedded in paraffin wax, were used for routine histopathological examination. Informed consent was obtained from the subjects.

Polymerase chain reaction amplification with consensus sequence primers and sequence analysis

Formalin-fixed and paraffin-embedded samples were cut into 10-μm sections. DNA was extracted from them using Dexpat® (Takara, Kyoto, Japan) according to the manufacturer's instructions. HPV polymerase chain reaction (PCR) was performed with two sets of consensus sequence primers, L1C1/L1C2[13] and GP5+/6+,[14] which locate in the L1 open reading frame of the HPV genome. The predicted sizes of amplified DNA using the L1C1/L1C2 and GP5+/6+ primers are about 250 and 140 bp, respectively. These two primer pairs have been reported to amplify mainly mucosal HPVs.[13,14] The PCR conditions were described previously.[13,14] Briefly, to detect viral DNA by PCR, an 18-μL reaction mixture, containing 1× *Ex Taq* buffer, 2 mmol L^{-1} $MgCl_2$, each deoxyribonucleotide triphosphate at 200 nmol L^{-1}, 60 ng sense and antisense PCR primers and 5 U *TaKaRa Ex Taq*® (Takara) at a final concentration, was added to 2 μL DNA extracts. The PCR protocol employed for the primer pair L1C1/L1C2 was 40 cycles under the following conditions: denaturation at 95 °C for 1·5 min, annealing at 48 °C for 1·5 min and extension at 72 °C for 2 min. For the primer pair GP5+/6+, the PCR protocol was 40 cycles under the following conditions: denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min. To assess the DNA quality of each sample, the human β-globin gene was also amplified. Each PCR product was separated by electrophoresis in 2% agarose gels, stained with ethidium bromide, and visualized under ultraviolet (UV) radiation. The PCR products were purified and cloned into a pGEM®-T Easy vector (Promega, Madison, WI, U.S.A.) and transfected into *Escherichia coli*. The PCR products cloned into the vectors were sequenced with an automated sequencer. At least six colonies were selected for each PCR product and subjected to DNA sequencing. All sequencing data obtained from each colony were identical. We checked for contamination by including in-between sectioning of paraffin blocks with other skin tissue, and subsequent PCR analysis of isolated DNA from these negative control sections.

Polymerase chain reaction amplification with human papillomavirus type 56-specific primers and sequence analysis

As the amplified PCR products were consistent with HPV type 56, we further performed PCR with two sets of HPV 56-specific primers, HPV56 120F/307R and HPV56 285F/510R, which locate in the E6 open reading frame of the HPV genome. The sequences of the primers as follows: HPV56 120F, 5′-AATCCACAGGAACGTCCACC-3′; HPV56 307R, 5′-CATACTCTGCACACTGCATAAGG-3′; HPV56 285F, 5′-CCTTATGCAGTGTGCAGAGTATG-3′; HPV56 510R, 5′-CGGTCCAACCATGTGCTATTAG-3′. The predicted sizes of amplified DNA using the HPV56 120F/307R and HPV56 285F/510R primers are 187 and 225 bp, respectively. The PCR conditions were the same as those used with the L1C1/L1C2 and GP5+/6+ primers. The PCR protocol employed for the primer pairs HPV56 120F/307R and HPV56 285F/510R was 40 cycles under the following conditions: denaturation at 95 °C for 1·5 min, annealing at 48 °C for 1 min and extension at 72 °C for 2 min. Each PCR product was separated by electrophoresis in 2% agarose gels, stained with ethidium bromide, and visualized under UV radiation. The PCR products were purified and cloned into a pGEM®-T Easy vector and transfected into *E. coli*. The PCR products cloned into the vectors were sequenced with an automated sequencer.

In situ hybridization

The catalysed signal amplification method (GenPoint System; Dako, Kyoto, Japan) was used for *in situ* hybridization according to the manufacturer's instructions. Formalin-fixed, paraffin-embedded sections from patients 1, 3, 4 and 5 were used, while in patient 2, frozen specimens were used. Briefly, for patients 1 and 4, formalin-fixed, paraffin-embedded 4-μm tissue sections mounted on silanized slides were deparaffinized; for patient 2, cryostat 4-μm tissue sections were mounted on silanized slides without deparaffinization. Each sample was pretreated in buffer (Target retrieval solution; Dako) at 95 °C for 40 min, digested with 4 μg mL^{-1} proteinase K at room temperature for 10 min, incubated in 0·3% hydrogen peroxide in methanol for 20 min, and then air dried. After heat denaturation at 90 °C for 5 min in hybridization solution mixed with biotinylated high-risk HPV probe cocktail (GenPoint HPV; Dako), that contains HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68 DNA, the slides were incubated at 37 °C overnight. Then, the slides were washed with 0·1 × saline sodium citrate at 50 °C for 20 min. Following treatment with horseradish peroxidase-conjugated streptavidin (Dako), the biotinyl tyramide amplification reaction was conducted. Then, the substrates were exposed to streptavidin conjugated with horseradish peroxidase. The sections were incubated with 3′-diaminobenzidine chromogen substrate and counterstained with haematoxylin.

Results

Clinical features

We examined five lesions of Bowen's disease involving the nail apparatus of five Japanese patients (three men and two women; mean age 49 years, range 29–68). The clinical characteristics of the five lesions were diverse (Table 1). Four lesions were located on the toes and one lesion on the finger. Patients 1, 2 and 4 presented with longitudinal melanonychia with partial nail plate destruction (Figs 1a, 2a). Convex nail deformity with yellowish discoloration was observed in

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Table 1 Summary of clinical features and human papillomavirus (HPV) analyses of Bowen's disease of the nail apparatus

Patient	Age (years)/sex	Site	Clinical appearance	HPV type	Variants	In situ hybridization
1	34/M	Left II toe	LM, PND	56 (P)	ED2162, ED2232, OK315, OK42	+
2	68/M	Right I finger	LM, PND	56	Reference sequence, BR5868	+
3	63/M	Right IV toe	Ulcer, PND	–	–	–
4	29/F	Left I toe	LM, PND	56 (P)	ED2162, ED2232, OK315, OK42	+
5	51/F	Left II toe	Nail deformity	–	–	–

LM, longitudinal melanonychia; PND, partial nail plate destruction; P, polymorphism; ED, Edinburgh; OK, Oklahoma; BR, Brazil.

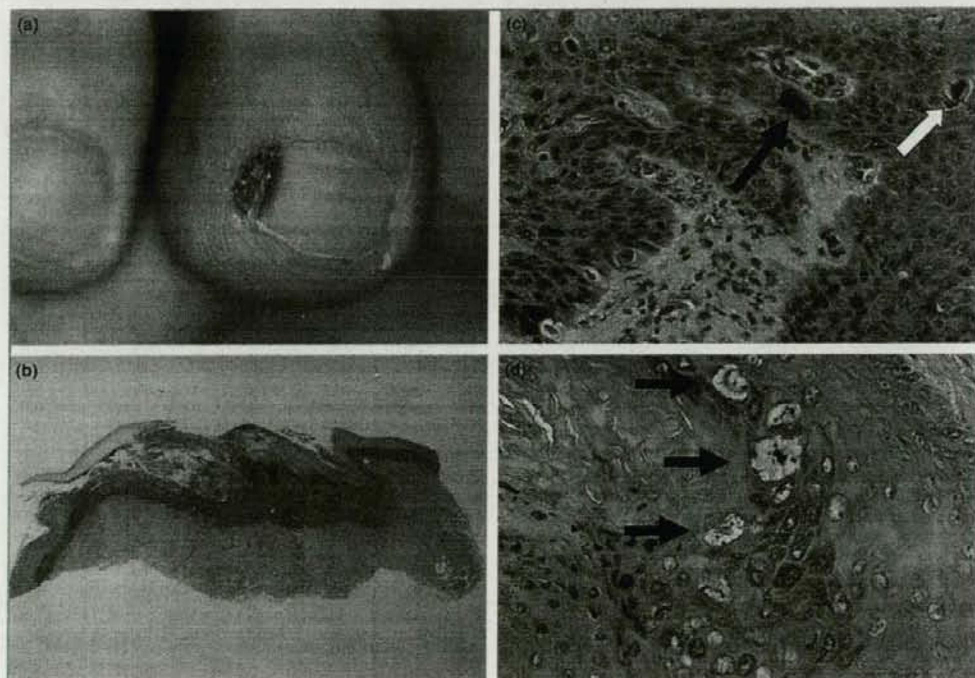

Fig 1. Patient 1: clinical appearance and histopathology. (a) Clinical appearance. Longitudinal melanonychia with partial nail plate destruction in the left second toenail. (b) Histological specimen of the tumour. A low-power view showing marked hyperkeratosis and acanthosis with nail plate destruction. (c) A high-power view of the same section. The black arrow shows a clumping cell, which is characteristic for Bowen's disease. The white arrow shows a dyskeratotic cell. (d) A high-power view of the same section. Atypical keratinocytes with koilocytosis (indicated by arrows) are present in the stratum granulosum. Haematoxylin and eosin; original magnification: (a) × 20; (b, c) × 200.

patient 5, and periungual ulceration was observed in patient 3. None of the patients had a history of arsenic exposure, radiation therapy, or immunosuppressive therapy.

Histopathology

Despite the different clinical features, all the lesions showed essentially the same histopathological changes. The epithelium was irregularly thickened (Figs 1b, 2b). Throughout the epithelium, the cells lay in disarray, resulting in a 'windblown' appearance.[15] Highly atypical keratinocytes with large, irregularly shaped nuclei, including 'clumping cells', a characteristic of Bowen's disease, proliferated within the epithelium (Figs 1c, 2c). Keratinocytes with koilocytosis, which are frequently seen in HPV-infected lesions,[16] were seen in some areas (Figs 1d, 2d).

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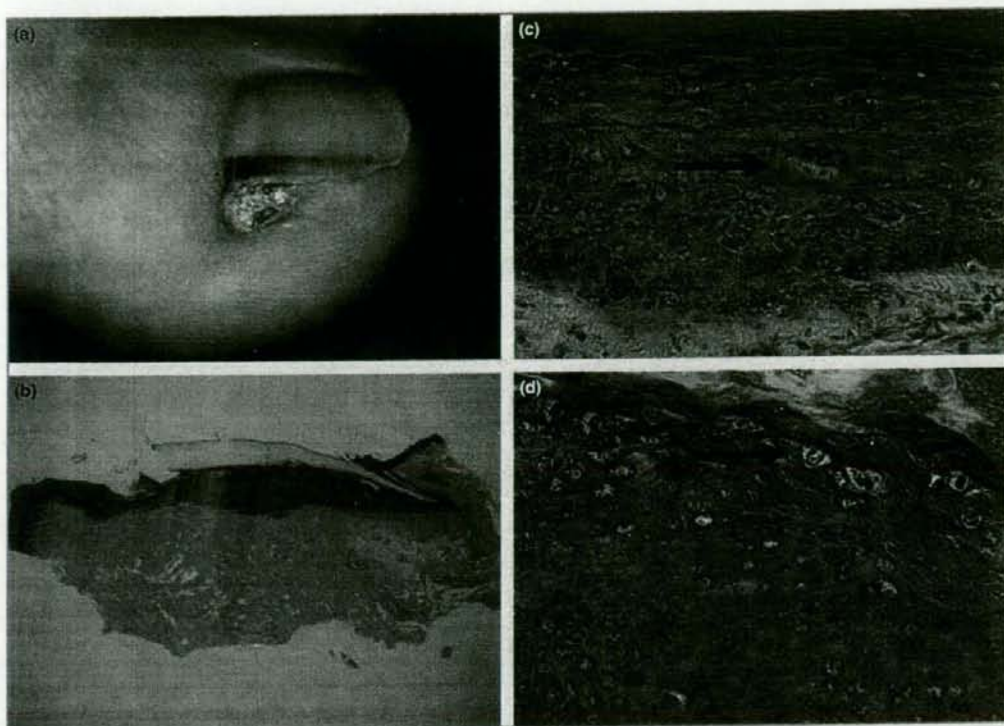

Fig 2. Patient 4: clinical appearance and histopathology. (a) Clinical appearance. Longitudinal melanonychia with partial nail plate destruction in the left first toe. (b) Histological specimen of the tumour. A low-power view showing acanthosis in the nail bed and matrix. (c) A high-power view of the same section. The epithelium contained many atypical cells including clumping cells (arrow). (d) A high-power view of the same section showing atypical cells arranged irregularly in the nail matrix. The arrow indicates a keratinocyte with koilocytosis. Haematoxylin and eosin; original magnification: (a) × 20; (b, c) × 200.

Polymerase chain reaction amplification with consensus sequence primers and sequence analysis

When DNA isolated from these five lesions of Bowen's disease was subjected to PCR using the L1C1/L1C2 primers, HPV DNAs were successfully amplified in patients 1, 2 and 4 (Fig. 3a). The PCR products from these three patients were subjected to DNA sequencing which revealed that their sequences corresponded to the L1 gene of HPV 56. The sequences of patients 1 and 4 showed a substitution of A for G at the 5680th position, which is predicted to be translationally silent (Val→Val). The remaining patient, patient 2, gave a sequence identical to the consensus L1 sequence (Fig. 3b). As a control, DNA extracted from a lesion of bowenoid papulosis was used. The sequence of HPV type 16 had been amplified previously from this sample. A PCR band was seen at the expected position of 252 bp. By sequencing, it was confirmed that this sample harboured HPV type 16 (data not shown) but not HPV 56.

Furthermore, HPV DNAs were also successfully amplified using the GP5+/6+ primers (Fig. 3a). PCR bands were obtained from patients 1, 2 and 4. The PCR products were subjected to DNA sequencing, which revealed that their sequences corresponded to the L1 gene of HPV 56. In the bowenoid papulosis sample, a PCR band was seen at the expected position of 142 bp and the sequences were consistent with HPV type 16 (data not shown). These findings exclude the possibility of laboratory contamination of HPV 56 DNA.

Polymerase chain reaction amplification with human papillomavirus type 56-specific primers and sequence analysis

Because the amplified PCR products were consistent with HPV 56, we performed further PCR with two sets of HPV-56 specific primers, HPV56 120F/307R and HPV56 285F/510R, which locate in the E6 open reading frame of the HPV genome. When DNA isolated from these five lesions of Bowen's disease was subjected to PCR using the HPV56 120F/307R primers, HPV DNAs were successfully amplified in patients 1, 2 and 4 (Fig. 4a). The PCR products from these three patients

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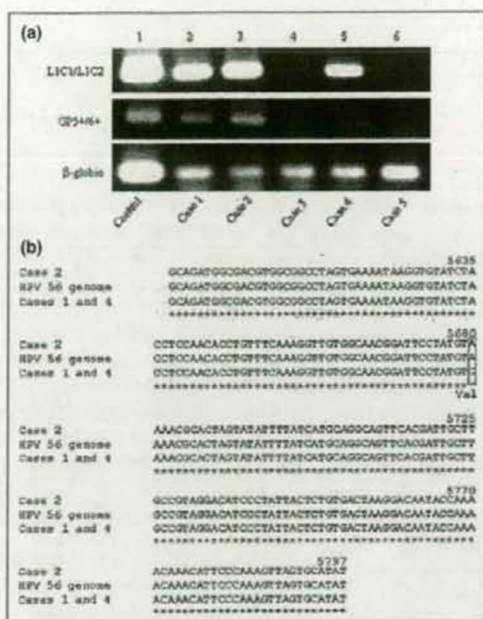

Fig 3. Human papillomavirus (HPV) DNA detection using polymerase chain reaction (PCR) and sequence analysis. (a) Detection of HPV DNA using the two HPV primer pairs, L1C1/L1C2 and GP5+/6+. Lane 1, patient with bowenoid papulosis (control, HPV type 16); lane 2, patient 1; lane 3, patient 2; lane 4, patient 3; lane 5, patient 4; and lane 6, patient 5. Amplification of human β-globin was used as a positive control. In HPV type 16 used as a control, positive bands were seen at 252 bp and 142 bp by using primer pairs L1C1/L1C2 and GP5+/6+, respectively. (b) DNA sequencing of the PCR products obtained with the L1C1/L1C2 primers and their sequence alignment. The consensus sequence of HPV 56 is shown as HPV 56 genome. The sequence obtained from patient 2 was identical to the consensus sequence. The sequences obtained from patients 1 and 4 were identical to each other and differ from the consensus sequence at one position, which is indicated by the boxed letters and the lack of an asterisk in the sequence alignment.

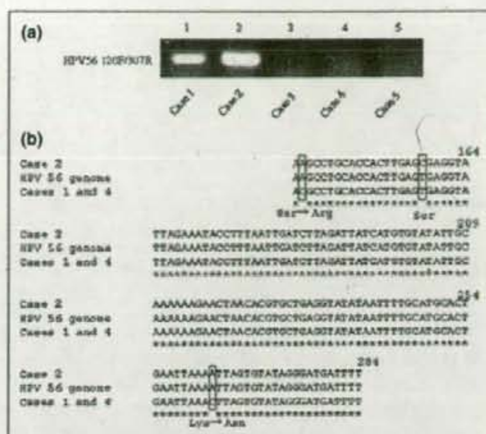

Fig 4. Human papillomavirus (HPV) DNA detection using polymerase chain reaction (PCR) and sequence analysis. (a) Detection of HPV DNA using the HPV 56-specific primer pairs, HPV56 120F/307R. Lane 1, patient 1; lane 2, patient 2; lane 3, patient 3; lane 4, patient 4; and lane 5, patient 5. (b) DNA sequencing of the PCR products obtained with the HPV56 120F/307R primers and their sequence alignment. The consensus sequence of HPV 56 is shown as HPV 56 genome. The sequence obtained from patient 2 was identical to the consensus sequence. The sequences obtained from patients 1 and 4 were identical to each other and differ from the consensus sequence at three positions, as indicated by the boxed letters and the lack of an asterisk in the sequence alignment.

were subjected to DNA sequencing, which revealed that their sequences corresponded to the E6 gene of HPV 56. The sequences of patients 1 and 4 showed two missense mutations and those of patient 2 showed one silent mutation compared with the consensus E6 sequence (Fig. 4b). It was clarified that cases 1 and 4 were compatible with the HPV 56 variants ED2162, ED2232, OK315, HK2555 and OK42 with one point mutation, while case 2 was compatible with the HPV 56 variants BR4114, BR5868 and consensus E6 sequence with one point mutation.

Using primers HPV56 285F/510R, HPV DNAs were successfully amplified in patients 1, 2 and 4 (data not shown). The PCR products from these patients were subjected to DNA sequencing which revealed that their sequences corresponded to the E6 gene of HPV 56. The sequences of patient 1 showed one silent mutation and those of patients 2 and 4 were consistent with the consensus E6 sequence (data not shown). It was clarified that case 1 was compatible with the HPV 56 variants BR5868, ED2162, ED2232, OK315, OK42 and consensus E6 sequence with one point mutation, while cases 2 and 4 were consistent with the HPV 56 variants BR5868, ED2162, ED2232, OK315, OK42 and consensus E6 sequence. Based on the results of PCR using HPV56 120F/307R and HPV56 285F/510R primers, we concluded that HPV 56 in patient 1 was related to ED2162, ED2232, OK315 and OK42 with two mutations, whereas that in patient 4 was also related to them with one mutation. In patient 2, the sequences of HPV 56 were related to BR5868 and consensus E6 sequence with one mutation. According to the phylogenetic evaluation by Prado *et al.*, the variants in patients 1 and 4 were remote from that in patient 2.[17]

In situ hybridization

In situ hybridization showed that the HPV probes hybridized to cells derived from all three specimens from patients 1, 2 and 4, that were positive for HPV by PCR. On the other hand, negative results were obtained for cells derived from the two specimens from patients 3 and 5, which were negative for HPV by PCR. Positive cells with intense nuclear staining were

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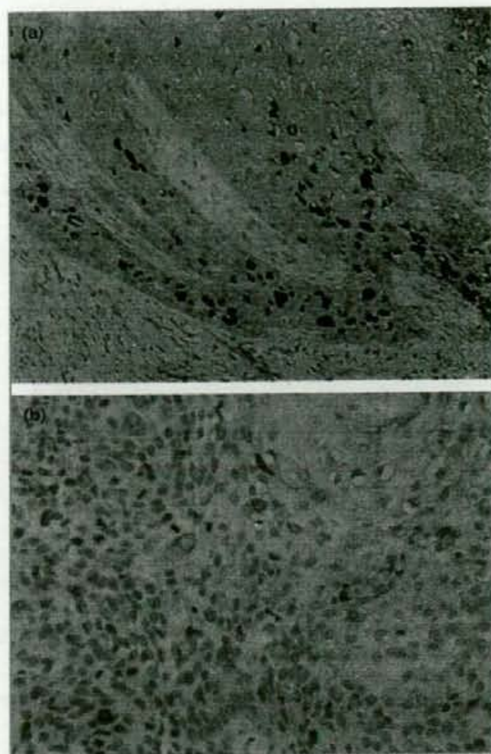

Fig 5. Demonstration of human papillomavirus (HPV) DNA. (a) HPV DNA was detected in the nuclei of the nail matrix epithelium by *in situ* hybridization using the GenPoint HPV (Dako, Kyoto, Japan) system (patient 2). (b) No positive signal was obtained in a polymerase chain reaction-negative sample (patient 3). Haematoxylin counterstaining; original magnification × 100.

found in almost the full thickness of the epithelium of patient 2 (Fig. 5a) and in the upper layers of the epithelium of patients 1 and 4 (data not shown). No positive signal was obtained throughout the epithelium of patients 3 (Fig. 5b) and 5 (data not shown).

Discussion

Bowen's disease is a squamous cell carcinoma *in situ*, often arising at sun-exposed areas of the skin. However, most lesions occur without an apparent cause. The lesions sometimes develop after UV exposure, intake of arsenic, and HPV infection.[10,18,19]

It is well known that HPVs are closely involved with the development of uterine cervical cancer.[20] HPVs have been divided into two major groups: high-risk and low-risk groups for cancer.[21] The high-risk group consists of HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56 and 58, which are often detected in intraepithelial neoplasia and/or invasive cancer. The low-risk group includes HPV types 6, 11, 42, 43 and 44, which are frequently found in benign neoplasms. HPV 56, detected in our patients, belongs to the high-risk group.

Bowen's disease presenting with longitudinal melanonychia is rare: only eight patients have been reported to date.[3,5,8,22–25] Genetic analyses were performed in only three of these patients, and HPV type 16 or 56 was detected in each of these patients.[4,8] However, sequence analyses were not performed in any patients. Bowen's disease of the nail apparatus is rare and, to our knowledge, genetic analyses of nine patients have been reported to date. In these reports, HPV type 16 was detected in six patients[3–6] and each of HPV types 34, 56 and 73 was demonstrated in one patient.[6–8] Interestingly, the HPV 56-positive lesion in a reported patient was clinically similar to our HPV 56-positive lesions, i.e. longitudinal melanonychia.[8] On the other hand, the lesions where other types of HPVs were detected represented different clinical manifestations, e.g. subungual verrucous tumour (type 16),[3] periungual plaques (type 34)[7] or subungual pigmented plaques (type 73).[6] These lesions are known to be clinical features when the nail bed or the nail fold is mainly involved.

HPV 56 was first detected in uterine cervical cancer and was reported as a new type of HPV in 1989.[26] HPV 56 has often been found in invasive cancer of the cervix and intraepithelial neoplasia, in contrast to a low incidence of detection in normal cervical tissues and cervical condylomas. To our knowledge, there have been only three reports of skin lesions in which HPV 56 was detected. One patient had Bowen's disease on the foot,[9] and the second patient had Bowen's disease presenting longitudinal melanonychia described above.[8] In the third patient, HPV 56 was detected in hair follicles in the genital area after treatment of perianal warts caused by HPV 56.[27]

Longitudinal melanonychia represents the presence of a pigmented lesion involving the nail matrix, which generates the nail plate. Therefore, Bowen's disease presenting longitudinal melanonychia indicates the involvement of the nail matrix. In contrast to HPV types 16 and 18, which are strongly linked to Bowen's disease of the genital region or distal extremities, HPV 56 has rarely been detected in these lesions of Bowen's disease. However, four of five reported patients with HPV 56-positive Bowen's disease, including our three patients, developed manifestations in the nail apparatus and clinically exhibited longitudinal melanonychia. Furthermore, in the patient with longitudinal melanonychia in whom HPV type 16 was detected, the procedure of HPV typing was PCR and hybridization and sequence analysis was not performed.[4] By sequencing the E6 gene, we found that some specific variants of HPV 56 might be associated with Bowen's disease of the nail matrix. It was clarified that the variants in patients 1 and 4 were related to ED2162, ED2232, OK315 and OK42, obtained in Edinburgh and Oklahoma, whereas the variants in patient 2 was related to the reference sequence and BR5868, obtained in Brazil. There has been no report on the variants of HPV 56 in Japan. Then, we could not explain why the variants detected in our patients coincided with the variants collected in Europe, U.S.A. and Brazil, far from Japan. We did not ask

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the patients if they had been to those countries. According to the phylogenetic analysis by Prado *et al.*, these two groups are quite remote from each other.[17] From the clinical aspect, patients 1, 2 and 4 showed fundamentally the same features, i.e. longitudinal melanonychia with partial nail plate destruction. These findings suggest that several variants of HPV 56 are involved in the carcinogenesis of Bowen's disease of the nail matrix, which results in longitudinal melanonychia.

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MINIREVIEW

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Mass spectrometric analysis of carbohydrate heterogeneity for the characterization of glycoprotein-based products

糖タンパク質医薬品の特性・品質評価における質量分析法を用いた糖鎖不均一性解析

Nana, Kawasaki*; Satsuki, Itoh; Noritaka, Hashii; Akira, Harazono; Daisuke, Takakura; and Teruhide, Yamaguchi

National Institute of Health Sciences 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501

Tel: 81-03-3700-9074; Fax: 81-03-3700-9084; E-mail: nana@nihs.go.jp

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***Key Words:** liquid chromatography/mass spectrometry, glycoprotein, oligosaccharide, glycopeptide*

Abstract

Analysis of the carbohydrate heterogeneity of glycoprotein-based substances is crucial for establishing the nomenclature and definition of biological substances, ensuring consistency in the quality of these products, comparatively assessing the products obtained after the implementation of changes in the manufacturing process, and developing biosimilar or follow-on biological products. Liquid chromatography/mass spectrometry is recognized as one of the most useful techniques for analyzing the carbohydrate heterogeneity of glycoprotein substances. Here, we demonstrate the utility of LC/MS for analyzing the carbohydrate heterogeneity by using some representative glycoproteins such as tissue-plasminogen activator, a monoclonal antibody, the follicle-stimulating hormone, and human chorionic gonadotropin. Further, we demonstrate that MS-based glycoprotein analysis has potential applications in glycomics.

要 約

糖タンパク質医薬品の糖鎖の不均一性解析は，医薬品の命名・定義，品質の恒常性確保，並びに製造方法変更及びバイオ後続品開発の際の同等性・同質性評価において重要である。液体クロマトグラフィー/質量分析法は，糖タンパク質医薬品の糖鎖不均一性解析法として最も有用性の高い分析法の一つとして知られている。本稿では，組織プラスミノーゲンアクチベータ，モノクローナル抗体，卵胞刺激ホルモン及びヒト絨毛性性腺刺激ホルモンなどの代表的糖タンパク質の分析例を示しながら，糖鎖不均一性解析におけるLC/MSの有用性を概説する。また，質量分析法を用いた糖タンパク質解析法をグライコミクスに応用した例を紹介する。

A. Introduction

Recent advances in the field of biotechnology have enabled the development of various proteins, protein derivatives, and fusion proteins for use as drugs in medicine. Most biotechnological drugs that have been approved in Japan in 2007 are glycoprotein derivatives; these drugs include Alglucosidase Alfa, Bevacizumab, Darbepoetin Alfa, and Idursulfase (1). The carbohydrate moieties present in most glycoprotein derivatives are known to affect the biological activity of these substances. Glycan analysis is therefore considered important for the characterization and evaluation of the quality of drugs.

Analysis of the carbohydrate heterogeneity of glycoproteins is becoming increasingly important. The heterogeneity of glycan moieties is a crucial factor to be

A. はじめに

遺伝子組換え技術の進展に伴い，様々なタンパク質，タンパク質改変体，あるいは融合タンパク質が医薬品として開発されている。2007年に日本で承認されたバイオ医薬品の多くは，アルグルコシダーゼ アルファ，ベバシズマブ，ダルベポエチン アルファ，及びイデュルスルファーゼのような糖タンパク質である(1)。多くの糖タンパク質医薬品の糖鎖が生物活性等に影響を与えることが知られていることから，特性解析や品質評価における糖鎖解析は重要である。

さらに別の側面からも，糖鎖不均一性解析の重要性が認識されるようになってきた。すなわち，糖鎖不均一性は，医薬品を命名・定義する上で重要であるということである。医薬品の名称は，基原や臨床効果に基づき，世界保健機関

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considered for establishing the nomenclature and definition of glycoprotein-based substances. The International Nonproprietary Names (INN) committee of the World Health Organization (WHO) proposes names for new drugs on the basis of the origins and clinical effects of the drugs (2). Different names are proposed for drugs containing different amino acid sequences. Glycoproteins that contain identical amino acid sequences but comprise different glycoforms are distinguished from each other by the use of Greek letters such as alfa and beta. For instance, epoetins, whose amino acid sequences are identical to that of human erythropoietin, are assigned different Greek letters on the basis of differences in their glycoforms. As of 2007, the INN had recognized 9 different epoetins and had assigned them letters from alfa to zeta. The terms "epoetin alfa" and "epoetin beta" are used to refer to epoetins consisting of the alfa and beta glycoforms, respectively; these drugs are marketed as different substances in Japan. However, the degree of glycosylation is known to change during various manufacturing processes; hence, a method for comparative assessment of the degree of glycosylation is required for the development of biosimilar or follow-on biological products as well as for the implementation of changes in the processes adopted for the manufacture of glycoprotein-based products. The establishment of the general criteria to be considered and of a relevant method for analyzing carbohydrate heterogeneity is a high-priority issue related to the manufacture of glycoprotein products in the United States, European Union, and Japan.

The methods most commonly used for analyzing the carbohydrate heterogeneity of glycoprotein substances are high-performance liquid chromatography (HPLC) with fluorescence detection (3), capillary electrophoresis, fluorescence-assisted carbohydrate electrophoresis (FACE) (4), and high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) (5).Recently, mass spectrometry (MS) has gained popularity as a method for analyzing the carbohydrate heterogeneity of glycoproteins (6). Although MS is advantageous in that it can provide structural information in considerable detail, it has limitations with regard to reproducibility and quantification. Investigations focusing on these limitations should be performed in order to extend the applications of MS to the analysis of glycans in glycoprotein substrates. Here, we demonstrate the utility of MS, especially liquid chromatography/electrospray ionization mass spectrometry (LC/ESI-MS) for analyzing the carbohydrate heterogeneity in glycoprotein-based substances. Further, we discuss the applications of MS-based analyses in glycomics.

(WHO) の医薬品国際一般名称 (INN) 委員会が命名する(2)。アミノ酸配列が異なる医薬品には，異なる名称が与えられる。ペプチド部分が同一でグリコフォームが異なる医薬品は，名称の後に alfa や beta などの異なるギリシャ文字が追加されて，区別される。例えば，ヒトエリスロポエチンと同一のアミノ酸配列を持つエポエチンには，グリコフォームの違いで異なるギリシャ文字が与えられており，現在 INN にはアルファからゼータまで 9 種類のエポエチンが収載されている。日本で販売されているエポエチン　アルファ及びエポエチン　ベータは，それぞれアルファグリコフォーム及びベータグリコフォームを持つ異なる医薬品である。しかし，グリコフォームは製造工程が変わると変化するため，製造方法の変更やバイオ後続品の開発にあたっては，糖鎖不均一性の同等性・同質性評価が要求される。そのために，米国，ヨーロッパ及び日本において，糖タンパク質医薬品の糖鎖の不均一性をどの程度解析すべきか等の基本的考え方，並びに解析のための適切な方法を確立することが，バイオ後続品開発における重要課題となっている。

糖タンパク質医薬品の糖鎖の不均一性解析法としてよく利用されている方法は，HPLC/蛍光検出法(3)，キャピラリー電気泳動法，蛍光標識糖鎖電気泳動法 (FACE) (4)及び高速陰イオン交換クロマトグラフィー/パルス電流検出法 (HPAEC-PAD) (5)であろう。さらに最近，質量分析法 (MS) が不均一性解析に利用されるようになってきた(6)。MS は，多くの構造情報を提供できる点で優れているが，定量性や再現性に問題がある。今後，定量性や再現性について新たな手法が導入されれば，糖鎖試験法としての有用性はさらに広がるものと思われる。本稿では，糖タンパク質の糖鎖不均一性解析法としての MS，特に液体クロマトグラフィー/エレクトロスプレーイオン化質量分析法 (LC/ESI-MS) の有用性について概説する。さらに，MS をグライコミクスに応用した例についても紹介する。

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B. LC/MS of proteolytic glycoproteins

Peptide mapping is performed to confirm amino acid sequences and identify posttranslational modifications for the structural characterization of almost all recombinant proteins, and it is used to test the quality of these substances. In recent studies, LC/MS has frequently been employed for peptide mapping. The MS-based peptide mapping of proteolytic glycoproteins enables not only the analysis of amino acid sequences but also the detection of site-specific glycosylation (7–10). As an example of peptide mapping, here, we describe the MS-based peptide mapping that has previously been performed for tissue-plasminogen activator (t-PA) (11), which is an anticoagulant, and for a monoclonal antibody (12).

1) t-PA

t-PA is a single-chain glycoprotein consisting of 527 amino acid residues (molecular weight (MW): approximately 70,000). It contains 3 potential N-glycosylation sites–at Asn117, Asn184, and Asn448–and is attached to Fuc at Thr61 (Fig. 1) (13,14). The order of domains present in this protein from the N-terminal end is as follows: finger, epidermal growth factor (EGF), kringle 1, kringle 2, and catalytic domains. The single polypeptide is converted to a heterodimer through plasmin-mediated cleavage at Arg275-Ile276. t-PA is rapidly cleared from the blood, and high-mannose-type oligosaccharides that

B. 糖タンパク質消化物のLC/MS

ペプチドマッピングは，タンパク質部分の一次構造や翻訳後修飾を確認するための方法として，多くの遺伝子組換えタンパク質医薬品の特性解析及び品質試験に用いられている。最近は，LC/MSがペプチドマッピングによく利用されるようになってきた。LC/MSを用いて糖タンパク質消化物マッピングを行うと，アミノ酸配列だけでなく，部位特異的な糖鎖の不均一性も同時に確認することができる(7–10)。LC/MSを用いたペプチドマッピングの代表例として，以下に，血栓溶解剤である組織プラスミノーゲンアクチベータ（t-PA）(11)とモノクローナル抗体(12)のペプチドマッピングを紹介する。

1) t-PA

t-PAは，527個のアミノ酸残基からなる分子量約7万の1本鎖糖タンパク質で，分子内の3箇所（Asn117, 184, 448）にN結合型糖鎖が，また，Thr61にフコースが結合している(Fig. 1)(13,14)。t-PAは，N末側から，フィンガードメイン，EGFドメイン，クリングル1ドメイン，クリングル2ドメイン，catalyticドメインから構成されている。t-PAは，プラスミンによりArg 275–Ile276が切断されると2本鎖t-PAになる。t-PAは，血中からの消失が早く，肝臓での代謝には，クリングル1ドメイン上のAsn117に結合している高マンノース型糖鎖やEGFドメインが関与していると考えられている(15,16)。

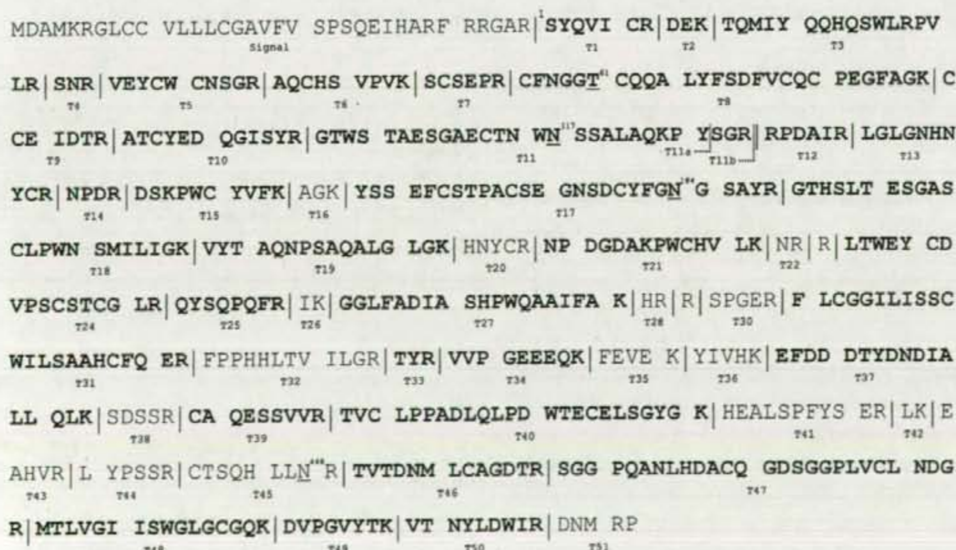

Fig. 1. Amino acid sequences of t-PA and tryptic peptides T1-T51. Boldface type indicates the peptides identified by the database search analysis with the SEQUEST search engine (Thermo Fisher Scientific). Potential N-glycosylation sites are underlined. Thr61 is fucosylated.

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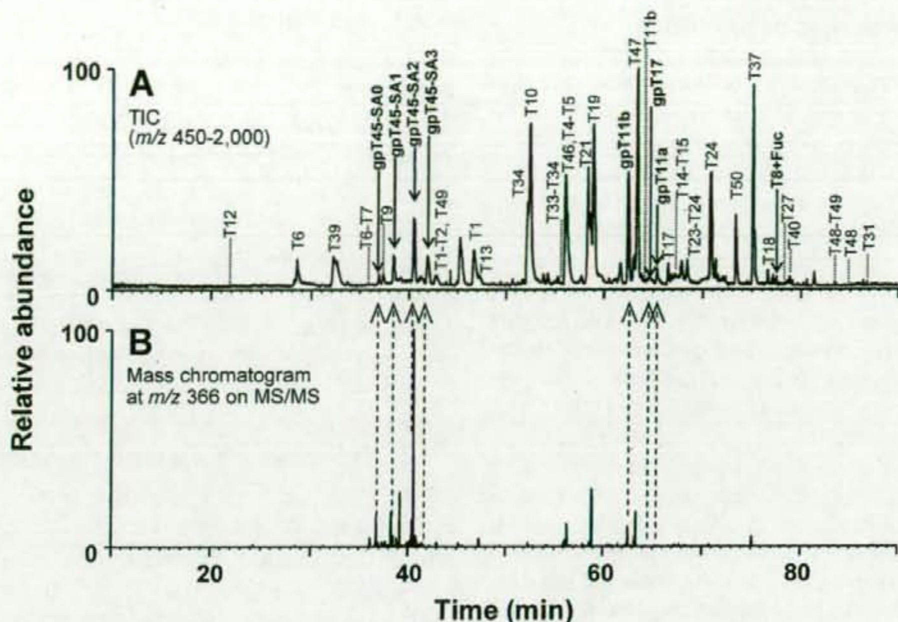

Fig. 2. Mass spectrometric peptide map of tryptic digest of carboxymethylated t-PA from human melanoma cell (Wako Pure Chemical Industries). (A) Total ion chromatogram (TIC) obtained by a single MS scan (m/z 450–2,000) in the positive ion mode. (B) Mass chromatogram at m/z 366 obtained by MS/MS. LC, Paradigm MS4 system (Michrom Bioresources); column, L-column (C18, 150 × 0.075 mm, 3 μm, Chemicals Evaluation and Research Institute); flow rate, 300 nl/min; A buffer, 0.1% formic acid/2% acetonitrile; B buffer, 0.1% formic acid/90% acetonitrile; gradient condition, 2–45% of B (100 min); MS, LTQ-FT (Thermo Fisher Scientific); electrospray voltage, 1.8 kV (positive ion mode).

are present at the Asn117 residues of the kringle 1 and EGF domains are involved in its rapid clearance via the liver (15,16). Site-specific glycosylation analysis as well as amino acid sequencing is important for the characterization of t-PAs. Alteplase, which is marketed worldwide, is a glycoprotein consisting of an amino acid sequence identical to that of human t-PA. Two t-PA analogs (monteplase and pamiteplase) that have been genetically modified in order to prolong their half-life in blood are also marketed in Japan, and 2 other analogs, namely, reteplase and tenecteplase, have been approved in the United States and the European Union (17).

Figure 2A shows a tryptic map of t-PA (derived from human melanoma cells), as observed in a single MS scan. Each peak in the map was identified by running the MS^n data that was acquired in a data-dependent manner through a database search (with 85% amino acid coverage). Fucosylated T8 (T8 + Fuc), non-glycosylated T11b, glycosylated T11b (gpT11b), and non-glycosylated T17 were successfully identified by using the possible

t-PAはアミノ酸配列だけでなく，部位特異的な糖鎖不均一性の確認が必要な医薬品の一つといえよう。現在，国内外で販売されているアルテプラーゼは，ヒトt-PAと同じアミノ酸配列を持つ糖タンパク質医薬品である。さらに，血中での滞留時間を延長させるために，遺伝子工学的にt-PAを改変した医薬品が国内では2品目（モンテプラーゼ，パミテプラーゼ），欧米でも異なる2品目（reteplase, tenecteplase）が販売されている(17)。

Fig. 2Aに，シングルMSスキャンで得られたt-PA（ヒト黒色腫細胞由来）のトリプシン消化物マップを示す。マップ上の各ピークは，データ依存的に取り込まれたMS^{n}データを用いたデータベース検索によって帰属した（アミノ酸配列の85%を確認した）。また，AsnにHexNAc（203 Da），Ser/ThrにdHex（146 Da）が結合している可能性を追加してデータベース検索することによって，Fuc結合T8（T8 + Fuc），糖鎖非結合T11b，糖鎖結合T11b（gpT11b），及び糖鎖非結合T17を同定することができた。データベース検索ではT45を

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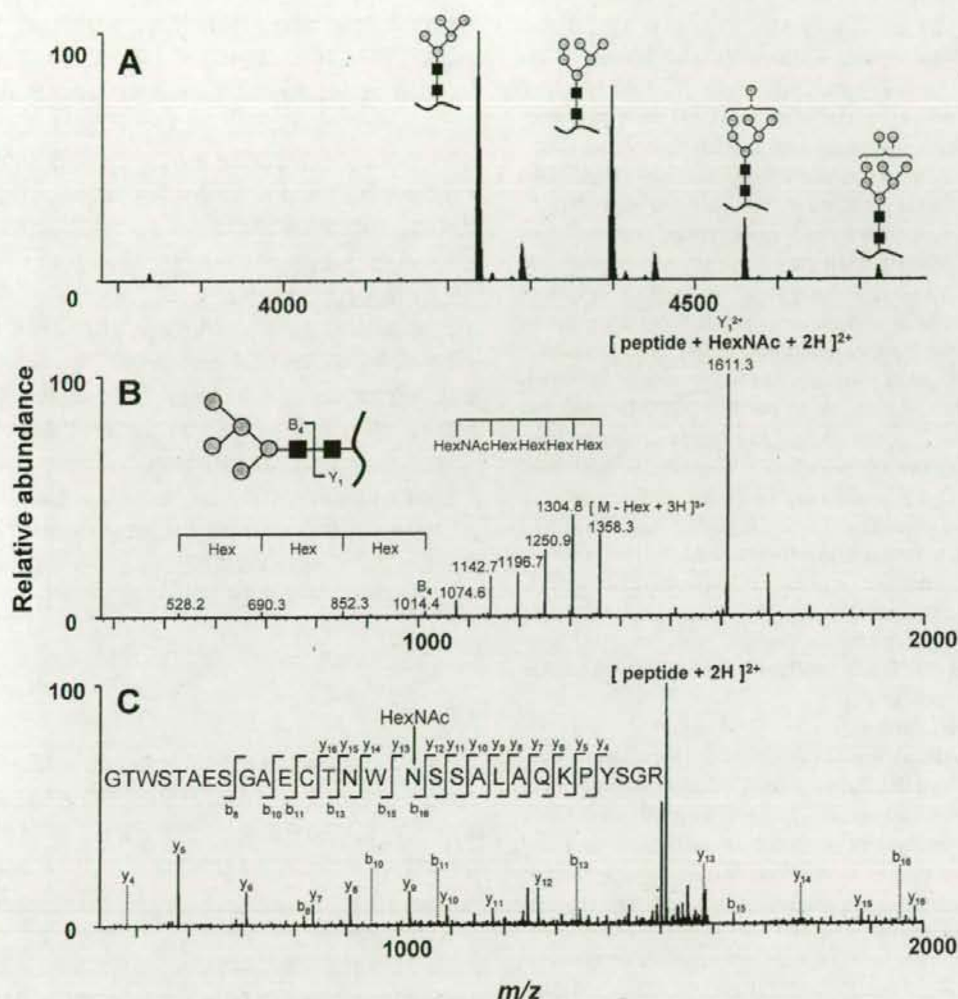

Fig. 3. Mass spectra of glycopeptide T11b. (A) Deconvoluted mass spectrum acquired at elution position of glycopeptides T11b (62.22–62.68 min). (B) MS/MS spectrum acquired from $[M+3H]^{3+}$ (*m/z* 1411.94) as a precursor ion. Carbohydrate structure was deduced from the fragment pattern. (C) MS/MS/MS spectrum acquired from $[peptide+GlcNAc+2H]^{2+}$ (*m/z* 1611.3) as a precursor ion. The peptide sequence and glycosylation site were identified by the database search analysis.

modifications of HexNAc (203 Da) on Asn and dHex (146 Da) on Ser/Thr. The final peptide, i.e., T45, could not be identified by a database search. However, using the glycan-distinctive $Hex_1HexNAc_1^+$ ions (*m/z* 366) that yielded peaks in the MS/MS, the acquisition positions of the glycopeptide MS data were mapped (Fig. 2B). The T45 glycopeptides (gpT45) were separated into 4 peaks based on the number of sialic acid residues present in the glycans (gpT45-SA0-SA3).

Figure 3A shows the deconvoluted mass spectrum

特定できなかったが，MS/MS によって生じた糖鎖に特徴的なイオン $Hex_1HexNAc_1^+$（*m/z* 366）を用いることによって，糖ペプチドの MS データが取り込まれている位置を特定することができた（Fig. 2B）。糖ペプチド T45（gpT45）は，シアル酸結合数によって異なる 4 つのピークに分離されていた（gpT45–SA0–SA3）。

Fig. 3A は，糖ペプチド gpT11b のマススペクトルをデコンボリューションしたものである。主なイオンの計算質量から，高マンノース型糖鎖（Man-5, -6, -7 及び -8）の結合が

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of gpT11b. The linkages of Man-5, Man-6, Man-7, and Man-8 were deduced from the calculated masses of the predominant ions. Figure 3B shows the MS/MS spectrum acquired using $[M+3H]^{3+}$ (*m/z* 1411.9) as a precursor ion; this spectrum suggests that the attachment of Man-5 is determined by the product ions. MS/MS reveals that peptide fragments attached to the reducing-end GlcNAc residue generally arise from N-glycosylated peptides. Further, MS/MS/MS reveals that these peptide-related ions yield b- and y-ions via cleavage of the peptide backbone (18). The peptide sequence can be deduced by running these fragments through a database search. The ion at *m/z* 1611.3 in Fig. 3B can be assigned to $[\text{peptide}+\text{GlcNAc}+2H]^{2+}$ of T11b. As shown in the MS/MS/MS spectrum obtained using the peptide-related ion as a precursor (Fig. 3C), the sequence of peptide T11b could be confirmed from the b- and y-ions arising from the peptide region.

Thus, through the peptide mapping by LC/MS/MS, it was confirmed that Asn117, 184, and 448 of t-PA were attached to high-mannose-type oligosaccharides, monosialylated, biantennary oligosaccharide, and bi- and triantennary oligosaccharides containing 0–3 NeuAcs, respectively. These results were consistent with those in previous reports.

2) Monoclonal antibody

Monoclonal antibody-derived biological substances are being developed globally, and as of November 2007, the INN had named 144 monoclonal antibodies. Monoclonal antibodies produced in cells such as CHO cells by biotechnological techniques are now being used as drugs. These antibodies are categorized on the basis of their origin as murine, chimeric, humanized, and human-derived antibodies. In 2007, 3 chimeric antibodies (Basiliximab, Infliximab, and Rituximab) and 5 humanized ones (Tocilizumab, Bevacizumab, Palivizumab, Trastuzumab, and Gemtuzumab Ozogamicin) were approved in Japan. The CH_2 domain in the H-chain of IgGs is commonly attached to an N-linked oligosaccharide. Since the antibody-dependent cell-mediated cytotoxicity (ADCC) of most antitumor drugs derived from IgG1 is influenced by core fucosylation in the N-linked oligosaccharide attached to the CH_2 domain (19), it is necessary to analyze the carbohydrate heterogeneity and to establish a test for glycan characterization in order to apply monoclonal antibody-based drugs. The test for glycan characterization could be replaced with an *in vivo* assay if the glycan profile is found to be strongly associated with the *in vivo* activity of the drug. The establishment of a quantitative and qualitative method for glycan characterization that can be used as an alternative to animal experiments is crucial for the development of glycoprotein-based drugs.

推定された。Fig. 3Bは，$[M+3H]^{3+}$（*m/z* 1411.9）を前駆イオンとして得られたMS/MSスペクトルで，生じたフラグメントから，Man-5であることが確認された。N結合型糖鎖付加ペプチドのMS/MSでは，ペプチドに還元末端GlcNAcが結合したイオンが生じることが多い。これらのペプチド関連イオンを前駆イオンとしてさらにMS/MS/MSを行うと，ペプチド部分が開裂してb-及びy-イオンが生じる(18)。これらのフラグメントを使ってデータベース検索すると，ペプチドの配列を推定することができる。Fig. 3Bでは，*m/z* 1611.3のイオンをT11bの$[\text{peptide}+\text{GlcNAc}+2H]^{2+}$と帰属することができる。Fig. 3Cは，このイオンを前駆イオンとして得られたMS/MS/MSスペクトルである。ペプチド部分から生じたb-及びy-イオンから，ペプチドT11bの配列を確認できる。

以上のようにLC/MS/MSを用いたペプチドマッピングによって，t-PAのAsn117, 184, 及び448にそれぞれ高マンノース型糖鎖，モノシアリル2本鎖型糖鎖，及びシアル酸を0–3個含む2及び3本鎖型糖鎖が結合していることが確認された。この結果は，既知の結果とよく一致している。

2) モノクローナル抗体

モノクローナル抗体を基原とする医薬品の開発が世界中で進められており，2007年11月現在，INNに144品目が収載されている。現在医薬品として用いられているモノクローナル抗体は，遺伝子組換え技術によってCHO細胞などを用いて製造されたものである。モノクローナル抗体は基原によって，マウス抗体，キメラ抗体，ヒト化抗体，及びヒト抗体などに分類される。現在国内では，キメラ抗体3品目（バシリキシマブ，リツキシマブ，インフリキシマブ）及びヒト化抗体5品目（トシリズマブ，ベバシズマブ，パリシズマブ，トラツズマブ，ゲムツズマブオゾガマイシン）が承認されている。IgGのH鎖のCH_2ドメインには共通してN結合型糖鎖が結合している。この位置に結合しているN結合型糖鎖のコアフコースの有無は，IgG1を基原とする抗腫瘍薬の抗体依存性細胞傷害活性（ADCC活性）に影響を及ぼすことから(19)，このようなモノクローナル抗体の承認申請にあたっては，糖鎖不均一性解析と糖鎖試験法の設定が必要になるだろう。特に，糖鎖試験の結果と*in vivo*活性試験の間に相関がある場合は，糖鎖試験法を*in vivo*活性試験におきかえられる可能性があり，動物実験代替法として，糖鎖を定性的かつ定量的に分析する試験法を設定することは重要であろう。

Fig. 4Aは，あるモノクローナル抗体製剤のトリプシン消化物マップである。32分前後に検出されているピークがH鎖

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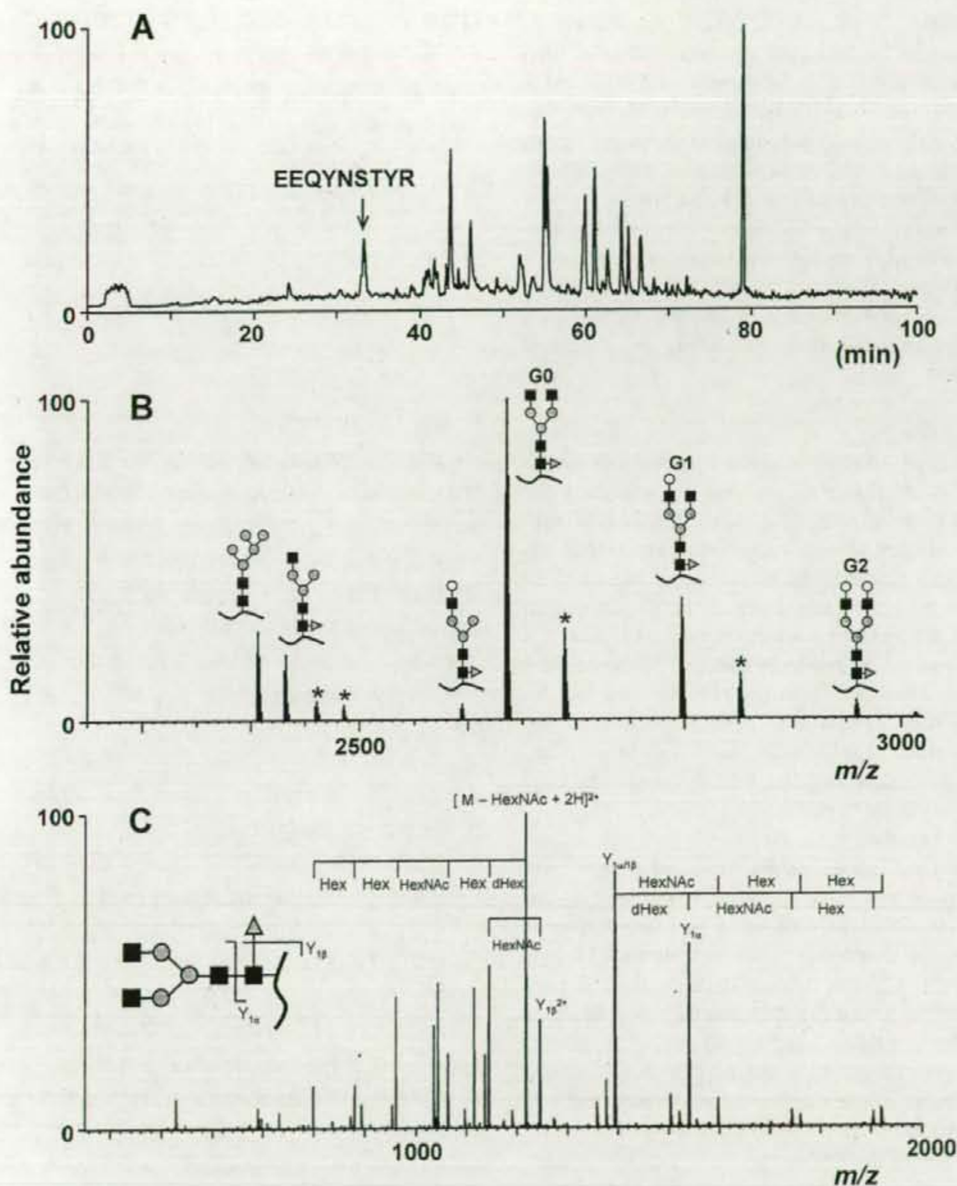

Fig. 4. Mass spectrometric peptide map of tryptic digest of the carboxymethylated monoclonal antibody. (A) TIC obtained by single MS scan (m/z 450–2,000) in the positive ion mode. (B) Deconvoluted mass spectrum at the elution position of the glycopeptides (32.57–33.66 min). * Non-glycopeptide signal (C) Representative MS/MS spectrum acquired from $[M+2H]^{2+}$ (m/z 1318.04) as a precursor ion.
Analytical condition: see Fig. 2.

Figure 4A shows the tryptic map of a commercially available monoclonal antibody-based drug. The peak detected at 32 min was assigned to the glycopeptide derived

に由来する糖ペプチドのピークと帰属された。このペプチドに結合している糖鎖は，デコンボリューションしたマススペクトル上のプロトン化分子の計算質量から，Man-5及び2本

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from the H-chain. On the basis of the calculated masses of the protonated molecules in the deconvoluted mass spectrum, the glycans attached to the peptide were predicted to be Man-5 and biantennary oligosaccharides (Fig. 4B). The structure of each glycan was deduced from the corresponding peak in the MS/MS spectra that were acquired using individual protonated molecules as precursors. The glycan distribution in IgG, which was determined by performing LC/MS/MS, was identical to that reported previously (20). Figure 4C shows the MS/MS spectrum obtained for agalactosyl biantennary oligosaccharides as a representative spectrum that was used for structural characterization.

C. Glycan profiling

Profiling of the glycans released from glycoproteins is employed as an alternative method for analyzing the carbohydrate heterogeneity of glycoprotein-derived substances. This method provides additional information regarding the heterogeneity and structural details of these substances. It is useful for evaluating the consistency in the degree of glycosylation among product batches, for comparatively assessing products obtained by implementing changes in the manufacturing process, and for developing biosimilar or follow-on biological products. We demonstrate the applicability of LC/MS using monoclonal antibodies and the follicle-stimulating hormone (FSH) to glycan profiling.

1) Monoclonal antibody

N-linked oligosaccharides released from the monoclonal antibody-based product described in the previous section were analyzed using an LC/MS system equipped with a graphitized carbon column (21-23). Figure 5 shows the glycan profile, as determined by performing a single MS scan for the glycans. It is difficult to distinguish the peptides attached to different glycan isomers by performing MS-based peptide mapping because glycopeptide isomers are coeluted. In contrast, LC permits separation of the liberated glycans on the basis of their subtle structural differences. Thus, LC/MS of glycans provides additional information regarding the carbohydrate heterogeneity of glycoproteins, including isomers. On the basis of the masses of the protonated molecules identified and the fragment peaks arising in the MS/MS spectra, the carbohydrate structures responsible for the peaks were predicted as shown in the image inset in Fig. 5. Table 1 shows the relative peak areas of the glycans, as were calculated by glycan profiling, using single MS scans of the released glycans (Fig. 5) and glycopeptides (Fig. 4B). These peak ratios were found to be almost identical, with the exception of Man-5, whose intensity determined

鎖糖鎖であることが示唆された（Fig. 4B）。検出された各糖ペプチドの糖鎖部分の構造は，各プロトン化分子を前駆イオンとしたMS/MSより推定した。本分析により得られた糖鎖の分布は，既に報告されているIgGの糖鎖の分布とよく一致していた(20)。代表的スペクトルと構造帰属例として，アガラクトシル2本鎖のMS/MSスペクトルをFig. 4Cに示す。

C. 糖鎖プロファイリング

糖タンパク質性医薬品の糖鎖不均一性解析法としてよく利用されるもう一つの方法は，タンパク質から切り出した糖鎖のプロファイリングである。この方法は，糖鎖の不均一性や詳細構造に関する多くの情報が得られることから，ロット間の糖鎖の恒常性評価や，製造方法変更もしくはバイオ後続品開発における同等性・同質性評価法として有用である。糖鎖プロファイリングにLC/MSを用いた例として，モノクローナル抗体と卵胞刺激ホルモン（FSH）を分析した例を紹介する。

1）モノクローナル抗体

前項のペプチドマッピングで使用した市販のモノクローナル抗体製剤から，N結合型糖鎖を切り出し，グラファイトカーボンカラムを接続したLC/MSシステムを用いて分析した(21-23)。Fig. 5は，シングルMSスキャンで得られた糖鎖プロファイルである。糖ペプチドのLC/MSでは，糖鎖異性体が結合したペプチドはほぼ同じ位置に溶出されるので，異性体同士を区別することは難しいが，遊離糖鎖のLC/MSでは，糖鎖異性体は構造上のわずかな違いで分離されるので，異性体を含む多くの不均一性情報を得ることができる。Fig. 5に見られる各ピークの糖鎖構造は，シングルMSで得られたプロトン化分子の質量とMS/MSで得られたフラグメントから図中の構造のように推定された。Table 1は，糖ペプチドのシングルMSで得られたプロトン化分子のピーク面積比と(Fig. 4B)，糖鎖のLC/MSで得られた各糖鎖のピーク面積比(Fig. 5)を比較した結果である。Man-5が結合したペプチドのピーク面積が高く観測されたことを除き，おおよそ一致していた。

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