Figure 4. MS^{1-4} spectra of oligosaccharide II by ESI-MS^n: (A) MS^1 spectrum; (B) MS^2 spectrum of $[M+Na]^+$ at m/z 954.4; (C) MS^3 spectrum of $[Gal(Fuc)GlcNAc+Na]^+$ at m/z 534.2 detected in MS^2; and (D) MS^4 spectrum of $[GalGlcNAc+Na]^+$ at m/z 388.1 detected in MS^3.

Figure 5. Proposed method for the Le^x-specific detection by ESI-MS^n.

Figure 6 shows the MS^{2-4} spectra of Le^x-asialotriantennary (oligosaccharide III). The oligosaccharide sequence can be confirmed by product ions generated by MS^2 (Fig. 6(A)). In addition to the defucosylated ion, many B- and Y-series ions, including the sodiated ion at m/z 534 corresponding to $[Gal(Fuc)GlcNAc+Na]^+$, were generated by MS^2. MS^3 of the sodiated $B_{2\alpha'}$ ion at m/z 534 yielded the sodiated $Y_{5\alpha'''}$ ion at m/z 388 as the most intense ion (Fig. 6(B)). MS^4 of the $Y_{5\alpha'''}$ ion at m/z 388 predictably generated the $^{3,5}A_2$ ion (m/z 259) (Fig. 6(C)). These results indicate that MS^3 of the sodiated ion at m/z 534, followed by MS^4 of the sodiated ion at m/z 388, can be used for the detection of the Le^x-diagnostic motif even in large and complicated N-linked oligosaccharides.

Specific detection and structural elucidation of N-linked Le^x-oligosaccharides in murine kidney by LC/ESI-MS^n

A sequential scan consisting of a full MS^1 scan, data-dependent MS^2 scan, MS^3 scan of the ion at m/z 534, and MS^4 scan of the ion at m/z 388 was applied to the specific detection and structural elucidation of Le^x-oligosaccharides in the murine kidney. In order to separate the many different oligosaccharides, including isomers, we used a LC/ITMS system equipped with a GCC. The N-linked oligosaccharides were released by PNGase F from the carboxymethylated proteins in the murine kidney soluble fraction. To improve the ionization efficiency and sensitivity,[25,26] the oligosaccharides were pyridylaminated, and the PA-oligosaccharides were subjected to LC/ESI-MS^n with a sequential scan. Sodiated ions were generated by a post-column reaction with 10 μM NaCl solution (2 μL/min). Oligosaccharides that yielded the

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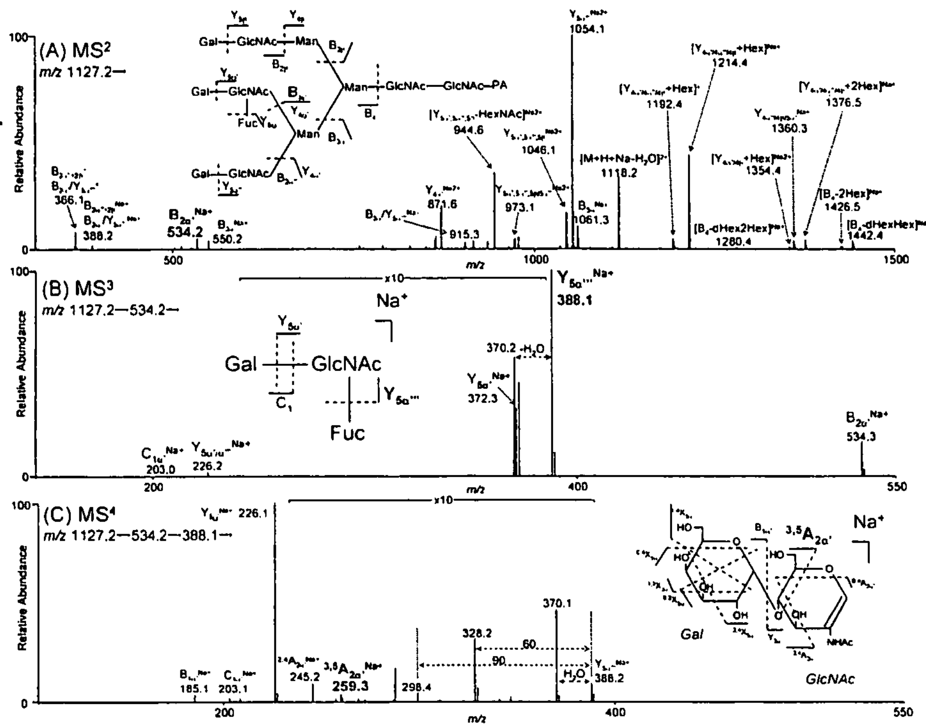

Figure 6. MS$^{2-4}$ spectra of oligosaccharide III by ESI-MS^n: (A) MS^2 spectrum of $[M+H+Na]^{2+}$ at *m/z* 1127.2; (B) MS^3 spectrum of [Gal(Fuc)GlcNAc+Na]$^+$ at *m/z* 534.2 detected in MS^2; and (C) MS^4 spectrum of [GalGlcNAc+Na]$^+$ at *m/z* 388.1 detected in MS^3.

diagnostic ions by MS$^{1-4}$ scans were presumed to be those of Le^x-oligosaccharides, and their detailed structures were elucidated by their data-dependent MS^2 spectra.

Figure 7(A) shows the total ion current (TIC) profile obtained by the full MS^1 scan of PA-labeled oligosaccharides from the murine kidney. Structures of major oligosaccharides a–i were deduced from the masses of the sodiated molecular ions measured by FTMS together with the B/Y ions generated by CID MS^2 (Table 1). Figures 7(B)–7(D) show mass chromatograms at *m*/*z* 534, 388 and 259, respectively, detected by MS$^{2-4}$, respectively. These chromatograms revealed that at least five kinds of oligosaccharides contain the Le^x-motif (a, b, e, f and h). Based on the masses, they were assigned to fucosylated oligosaccharides consisting of $dHex_3Hex_5HexNAc_5$ (a and f), $dHex_2Hex_5HexNAc_5$ (b), $dHexHex_4HexNAc_5$ (e), and $dHex_2Hex_4HexNAc_5$ (h) (abbreviations used here are: dHex, deoxyhexose; Hex, hexose; HexNAc, *N*-acetylhexosamine).

As an example of structural elucidation, we show the MS$^{2-4}$ spectra of oligosaccharide f in Fig. 8. In the MS^2 spectrum, we can observe the product ion [dHex$_2$HexHexNAc+Na]$^+$ at *m*/*z* 680, which can be assigned to either the Lewis b (Le^b)- or Lewis y (Le^y)-motif. As shown in Fig. 1, Le^b- and Le^y-motifs contain Le^a and Le^x as partial structures, respectively. The generation of Le^x-diagnostic ions suggests the attachment of the Le^y-motif to oligosaccharide f. Furthermore, product ions at *m*/*z* 1036 and 446 prove the linkage of GlcNAc at β-mannose in the trimannosyl core structure and fucosylation of the reducing terminal GlcNAc, respectively. Based on these characteristic ions, oligosaccharide f can be assigned to the bisected and fucosylated biantennary bearing the

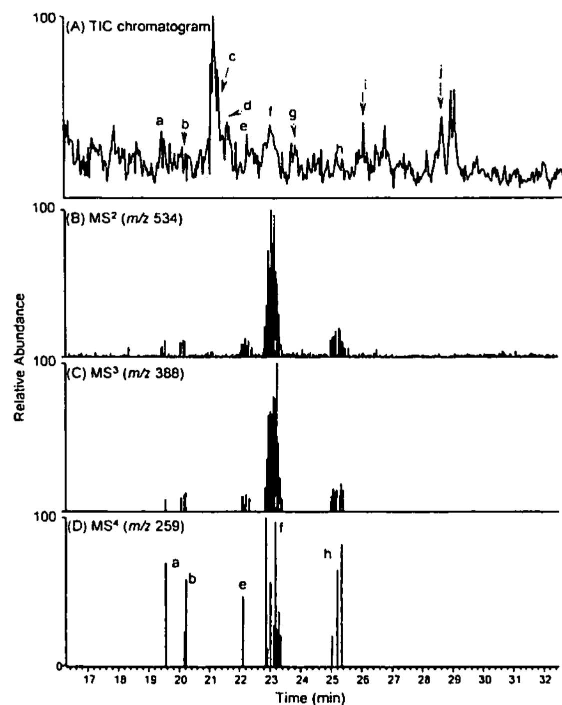

Figure 7. Specific detection of *N*-linked Le^x-oligosaccharides in murine kidney by LC/ESI-MS^n: (A) total ion chromatogram obtained by MS^1; (B) mass chromatogram of [dHexHexNAc+Na]$^+$ at *m/z* 534 detected in MS^2; (C) mass chromatogram of [HexHexNAc+Na]$^+$ at *m/z* 388 detected in MS^3; and (D) mass chromatogram of the cross-ring fragment at *m/z* 259 detected in MS^4.

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Table 1. Sugar composition and deduced structure of *N*-linked oligosaccharide from murine kidney

Sugar No.	Composition[a]	Deduced structure	Lewis type
a	$dHex_3Hex_5HexNAc_5$		Le^y
b	$dHex_2Hex_5HexNAc_5$		Le^x
c	$Hex_8HexNAc_2$		
d	$Hex_9HexNAc_2$		
e	$dHexHex_4HexNAc_5$		Le^x
f	$dHex_3Hex_5HexNAc_5$		Le^y
g	$Hex_6HexNAc_2$		
h	$dHex_2Hex_4HexNAc_5$		Le^x
i	$Hex_7HexNAc_2$		
j	$Hex_5HexNAc_2$		

[a]Fuc, fucose; Hex, hexose; HexNAc, *N*-acetylhexosamine.
O Gal; o Man; ■ GlcNAc; △ Fuc.

Le^y-motif. The Le^y structure in oligosaccharide f was confirmed by an extra LC/MS^2 run without post-column reaction with NaCl. Figure 9 shows the MS^2 spectrum of $[M+H+NH_4]^{2+}$ at *m*/*z* 1189.4. Attachment of the Le^y-motif was proved by the generation of the product ion at *m*/*z* 658 corresponding to $[dHex_2HexHexNAc]^+$.

Other oligosaccharides were assigned to bisected biantennary forms bearing Le^y (oligosaccharide a) and those bearing Le^x (oligosaccharides b, e and h) motifs. In addition to the previously reported Le^x-oligosaccharides,[20] we also detected the presence of Le^y-oligosaccharides in the murine kidney.

CONCLUSIONS

We found that the cross-ring fragment ion at *m*/*z* 259, which can be used for distinction from positional isomers, was generated from Le^x-oligosaccharides by MS^4 of $[GalGlcNAc+Na]^+$ at *m*/*z* 388, which was generated by MS^3 of $[Gal(Fuc)GlcNAc+Na]^+$ at *m*/*z* 534. Then, we successfully detected and elucidated the Le^x- and Le^y-oligosaccharides in the complex mixture using a sequential scan consisting of full MS^1, data-dependent MS^2, MS^3 of the sodiated ion at *m*/*z* 534, and MS^4 of the sodiated ion at *m*/*z* 388.

The Le^x structure is associated with various biological events as oligosaccharide ligands. So far, the detection and structural analyses of Le^x-oligosaccharides have required complicated and time-consuming processes, such as exoglycosidase digestions, sugar mapping,[27] the use of lectins and immunological methods. The mass spectrometric method proposed here would enable the rapid and easy detection of the Le^x-motif and subsequent structural elucidation of

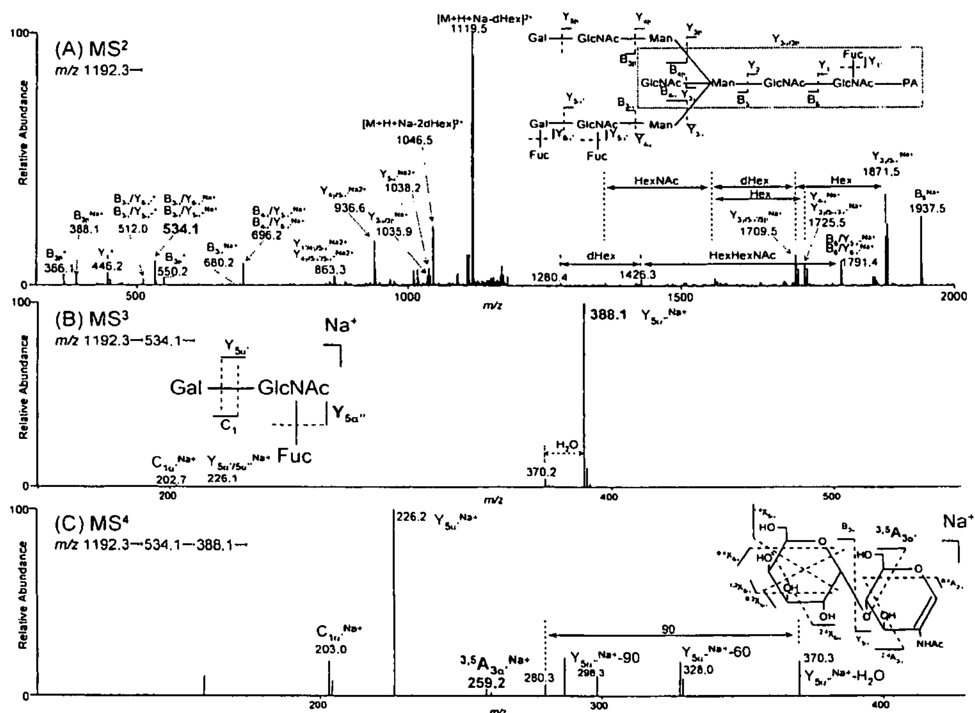

Figure 8. MS$^{2-4}$ spectra of oligosaccharide f by LC/ESI-MS^n: (A) MS^2 spectrum of $[M+H+Na]^{2+}$ at *m/z* 1192.3; (B) MS^3 spectrum of $[dHexHexNAc+Na]^+$ at *m/z* 534.1 detected in MS^2; and (C) MS^4 spectrum of $[HexHexNAc+Na]^+$ at *m/z* 388.1 detected in MS^3.

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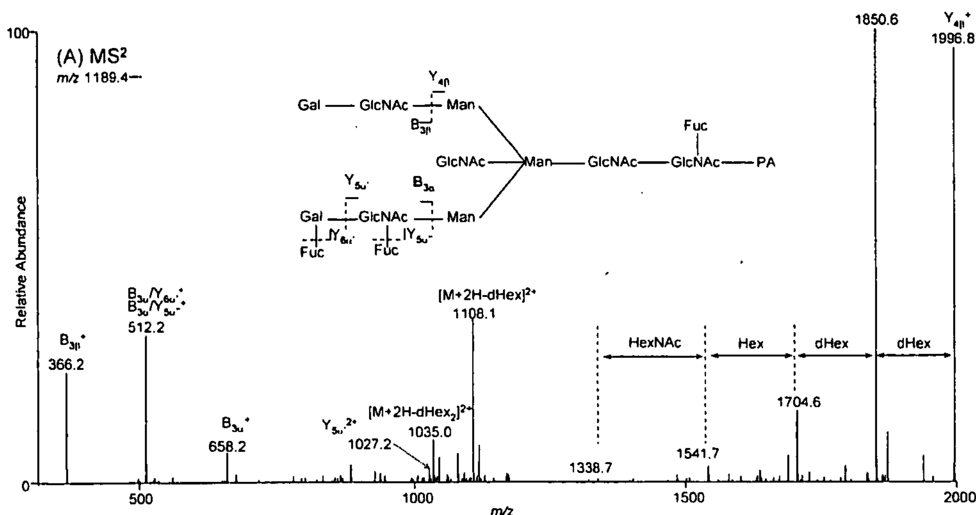

Figure 9. MS^2 spectrum of oligosaccharide f by LC/ESI-MS^n: precursor ion, $[M+H+NH_4]^{2+}$ at *m/z* 1189.9.

Le^x-oligosaccharides in biological samples. Our method, based on a sequential scan for the structure-characteristic ions, may be applicable to the analyses of oligosaccharides carrying other partial motifs, such as sialyl Le^x and sulfated sugar.

Acknowledgements

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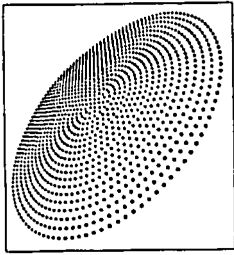

特集●Vol.34 No.4 微量糖鎖分析の現状と将来

LC/MSを用いたグライコーム解析

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Key words：LC/MS、グライコーム、グライコミクス、糖タンパク質、糖ペプチド、糖鎖

はじめに

細胞・組織に発現している全タンパク質（プロテオーム）を系統的・網羅的に解析することによって生命現象を解き明かそうとするプロテオミクスに高い関心が集まっている[1]。さらに最近では、細胞内タンパク質の主な翻訳後修飾の一つである糖鎖が、タンパク質の機能調節等を介して様々な疾患や発生・分化等に深く関わっていることが明らかになってきたことから[2-8]、細胞・組織発現糖タンパク質やその糖鎖部分の構造・機能を解析しようとするグライコミクスへの関心も高まっている[9,10]。

プロテオミクスの基盤的技術である質量分析法（MS）は、グライコミクスにおいても、糖タンパク質や糖鎖の構造特性解析のための有用なツールとして期待されている[11,12]。しかし、糖タンパク質は複数の糖鎖結合部位に様々な糖鎖が結合した不均一な集合体であることや、糖鎖が結合することによってMSにおけるイオン化効率が低下するなどの問題があるため、プロテオミクスの手法をそのまま利用できない場合が多い。そこで、レクチンや各種液体クロマトグラフィー（LC）など、糖鎖生物学分野で従来から利用されてきた糖鎖構造解析技術と、MSやデータベースを中心としたプロテオミクスの技術を組み合わせた様々なグライコーム解析技術の開発が進められている[13,14]。中でもLCとMSをオンラインで結んだLC/MSは、イオン化を妨害する物質を除去したり、不均一な糖鎖混合物を分離しながら、直接質量分析を行うことが可能な分析技術で、簡便・迅速なグライコーム解析法として優れている。本稿では、LC/MSを利用したグライコーム解析例をいくつか紹介する。

1．LC/MSによる細胞糖鎖の解析

疾患や発生・分化等に伴う糖鎖構造や糖鎖分布の微細な変化を見つけ出すには、糖タン

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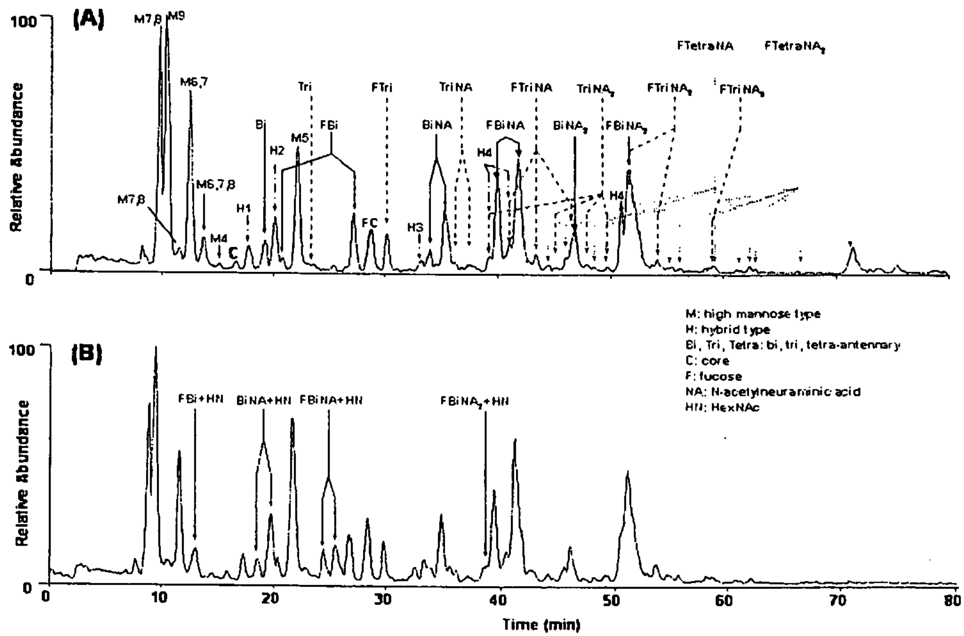

図 1 (A) CH O細胞、及び(B) GnT-III遺伝子導入CHO細胞の糖鎖プロファイル

サンプル：CHO細胞(1×10^{7}) 膜画分から*N*-グリカナーゼによって切り出した糖鎖を$NaBH_4$で還元した
LC/MS：カラム, グラファイトカーボンカラム (0.2 x 150 mm)；溶離液A, 5 mM 酢酸アンモニウム/2 % アセトニトリル; 溶離液B, 5 mM 酢酸アンモニウム/80 %アセトニトリル; グラジエント, B液 10-45 % (90 分)；流速, 2 μ l/min; MS, TSQ-7000 (サーモエレクトロン)

パク質から切り出した糖鎖を LC/MS を用いて解析する糖鎖プロファイリングが適している。構造糖鎖生物学分野ではこれまでに、糖鎖誘導体化とLCを組み合わせた様々な分離技術が開発されている[15-17]。これらをオンライン MS と組み合わせることによって、糖鎖不均一性の高い試料の解析が容易になる[18, 19]。筆者らは、糖タンパク質から*N*結合型糖鎖を酵素的に切り出し、還元末端を $NaBH_4$ で還元した後、親水性物質に対する吸着能の高いグラファイトカーボンカラムを用いて LC/MS (GCC-LC/MS) を行う糖鎖プロファイリング法を開発している[20-23]。以下に GCC-LC/MS を用いて細胞発現糖タンパク質の糖鎖を解析した例を2つ紹介する。

1.1 糖鎖プロファイリング

図1A は CHO 細胞の膜画分から*N*結合型糖鎖を酵素的に切り出した後、$NaBH_4$ で還元し、 GCC-LC/MS 操置で分析して得られた結果をトータルイオンクロマトグラム (TIC) として表したもので、糖鎖の分布（プロファイル）を示している。各ピークの糖鎖構造は、MS によって測定された質量を基に推定された単糖組成より、高マンノース型、及び2本鎖を中心とした複合型シアロ糖鎖であると推定された[24]。**図1B** は、CHO細胞に *N*-アセチルグルコサミン転移酵素 Ⅲ (GnT-Ⅲ) 遺伝子を導入した CHO 細胞の糖鎖プロファイルである。GnT-Ⅲ はトリマンノシルコアの β1-4Man に GlcNAc を付加させる酵素である。GnT-Ⅲ が導入された細胞には複数の新しい糖鎖が出現していることがわかる。これらは質量から、CHO 細胞に結合している 2本鎖糖鎖に *N*-アセチルヘキソサミン (HexNAc) が1分子 (203Da) 付加した糖鎖であることが確認され、GnT-Ⅲ によって生じた GlcNAc 付加糖鎖と推定された。このように糖鎖プロファイリングは、サンプル間の糖鎖の構造や分布を比較する方法として優れ、糖鎖生合成経路に起きた変化や、その変化に伴って生じた糖鎖の構造解析に利用できると期待される。

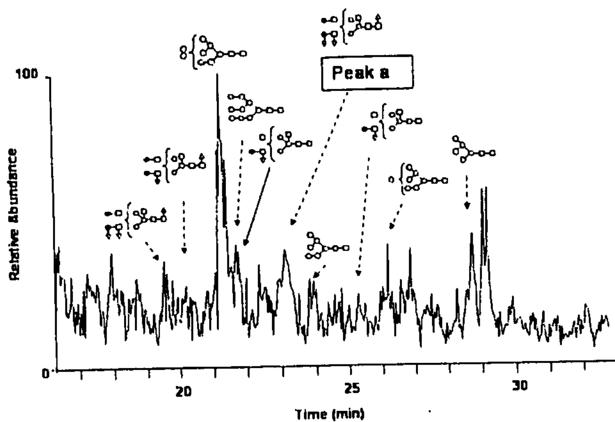

図2　マウス腎臓の糖鎖プロファイル

サンプル：腎臓(20μgタンパク質)の膜を含む画分からN-グリカナーゼによって切り出した糖鎖を$NaBH_4$で還元した
LC/MS：カラム及び溶離液, 図1に準ずる; グラジエント, B液 5-45 % (60 分); 流速, 2μl/min; MS, LTQ (サーモエレクトロン)
●, Gal; ○, Man; □, GalNAc; △, Fuc

1.2　糖鎖配列解析

糖鎖の配列や結合様式は、MS を繰り返す多段階 MS (MS^n) によって、ある程度決定することができる[25-27]。**図2** は、マウス腎臓の膜画分から切り出した N結合型糖鎖を 2-アミノピリジンで誘導体化し、LC/MS による糖鎖プロファイリングを行ったものである。主な糖鎖は質量から、高マンノース型糖鎖、及び複数のフコースが結合した複合型糖鎖と推定された。各糖鎖の配列は、MS^n により決定した[28]。一例として、**図3** にフコシル糖鎖ピーク a の $MS^{2\text{-}4}$ スペクトルを示す。MS^2 によって [ヘキソース(Hex)-HexNAc-Fuc + Na]$^+$ (m/z 534)、及び [Hex-HexNAc-Fuc_2 + Na]$^+$ (m/z 680) が生じたことから、ピーク a にはルイス b (Le^b, Fucα 1-2Gal β 1-3(Fuc α 1-4)GlcNAc)、またはその異性体ルイス y (Le^y, Fuc α 1-2Gal β 1-4(Fuc α 1-3)GlcNAc) 構造が存在することが示唆された(**図3A**)。そこで、m/z 534 を前駆イオンとし

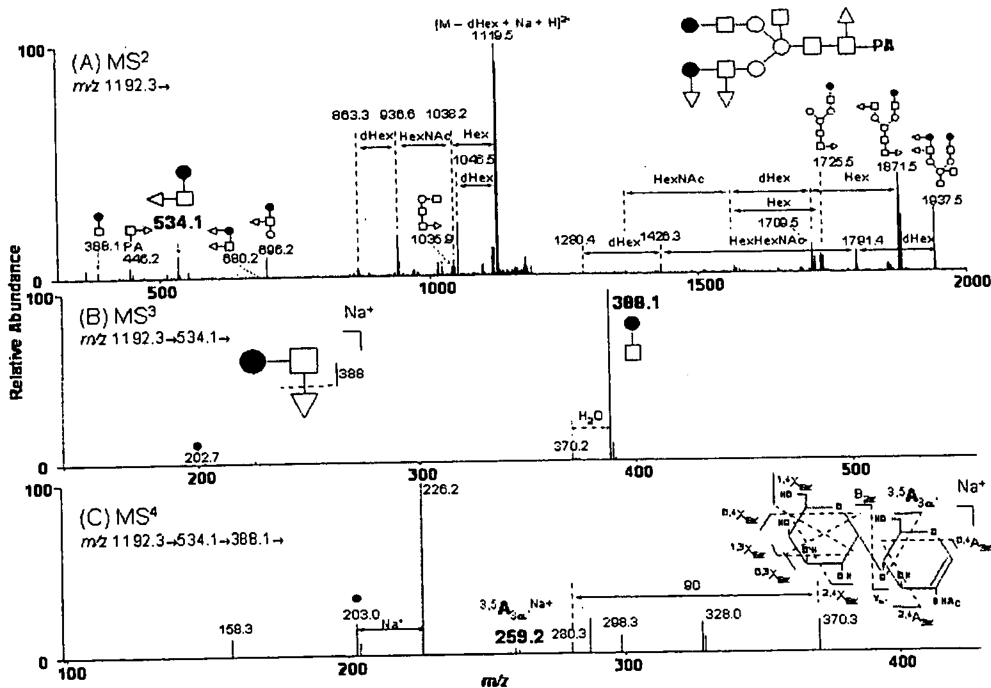

図3　図2中のピークaの (A) MS^2 (前駆イオン：m/z 1192.3)、(B) MS^3 (前駆イオン：m/z 534)、及び (C) MS^4 (前駆イオン：m/z 388) スペクトル

て MS^3 を行ったところ、フコースが開裂した [Hex-HexNAc + Na]$^+$ (*m/z* 388) が検出された (**図3B**)。つぎに *m/z* 388 を前駆イオンとして MS^4 を行ったところ、環開裂した GlcNAc の 4位炭素原子に Gal が結合したイオン (*m/z* 259) が検出されたことから、この部分構造は Le^y と決定された(**図3C**)。さらに、他の糖鎖のプロダクトイオンを解析した結果、マウス腎臓に結合しているフコシル糖鎖は Le^x 及び Le^y 糖鎖であることが明らかとなった。Le^x は SSEA-1 糖鎖としても知られる糖鎖エピトープで、マウス ES 細胞に多く発現していることが知られ、ES 細胞の分化状態のモニタリングに利用されている糖鎖である[29]。また、シアル酸が結合したシアリル Le^x はヒト腫瘍マーカーとして利用されており[30]、マウス腎臓の主な糖鎖が Le^x 糖鎖であったことは興味深い。

2. LC/MSによる糖ペプチド解析

タンパク質から糖鎖を切り離すと、糖鎖とタンパク質間の結合に関する情報が失われてしまうので、細胞・組織中の任意の糖タンパク質糖鎖の構造特性解析は糖鎖を切り離さずに行う。膜糖タンパク質などは不溶性または高分子量タンパク質であることが多いので、還元アルキル化した後、トリプシン、Lys-C、Glu-C、または Asp-N 等で消化してから分析するのが一般的である。MS において、ペプチドに比べて糖ペプチドのイオン化効率が悪いために、ペプチドが混在すると糖ペプチドのマススペクトルが得られにくいという問題があるが、LC/MS によってペプチドを除きながら質量測定を行えば、良好な糖ペプチドのマススペクトルを得ることができる[31-33]。糖タンパク質消化物の LC/MS では複雑なクロマトグラムが得られることが多いが、MS^n やインソースフラグメンテーションによって生じた糖鎖に特徴的なイオン、例えば、$HexNAc^+$ (*m/z* 204) や $Hex\text{-}HexNAc^+$ (*m/z* 366) などを利用することによって、糖ペプチドの MS^2 スペクトルを選び出すことができる[32, 34]。選び出した糖ペプチドの MS^2 スペクトルには、ペプチドやペプチドに GlcNAc が結合したイオンが検出されている場合が多く、これらのイオンを基にペプチドと糖鎖部分の構造を決定する[32, 33]。

2.1 糖タンパク質の網羅的解析

図4A は、アルブミンをある程度除去したヒト血清のトリプシン消化物 0.02 μl 相当を、C18カラムを用いた LC/MS^2 装置で分析して得られた MS^1 の TIC である。血清は様々なタンパク質の混合物であるので、非常に多くのペプチドが検出されているが、MS^2 によって生じた $HexNAc^+$ (*m/z* 204) を指標として、糖ペプチドのMS^2スペクトルを選び出した (**図4B**)。選び出した糖ペプチドのペプチド配列と糖鎖構造は、MS^2 スペクトルを基に決定した。一例として**図5** にピーク b の MS^2 スペクトルを示す。*m/z* 1442 に検出されている [ペプチド + GlcNAc + 2H]$^{2+}$、及びペプチド由来のフラグメント (b, yイオン) から、この糖ペプチドはハ

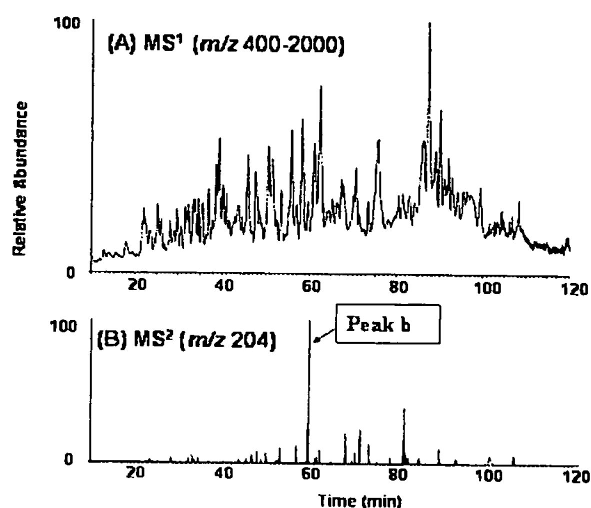

図4 (A) アルブミン除去ヒト血清トリプシン消化物のLC/MSによって得られたTIC、(B)LC/MS/MSによって生じた*m/z* 204イオンのマスクロマトグラム
LC/MS : カラム, C18 (0.2 x 50 mm); 溶離液A, 0.1 % ギ酸-2 %アセトニトリル; 溶離液B, 0.1 % ギ酸-90 %アセトニトリル; グラジエント, B液 5-50% (120 分) ; 流速, 2 μl/min; MS, QSTAR (アプライドバイオシステムズ)

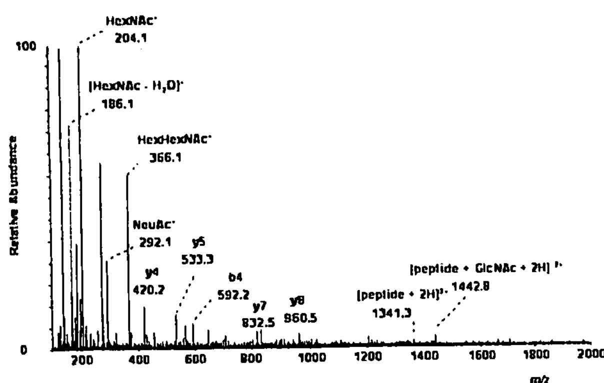

図5　図4B中のピークbのMS²スペクトル

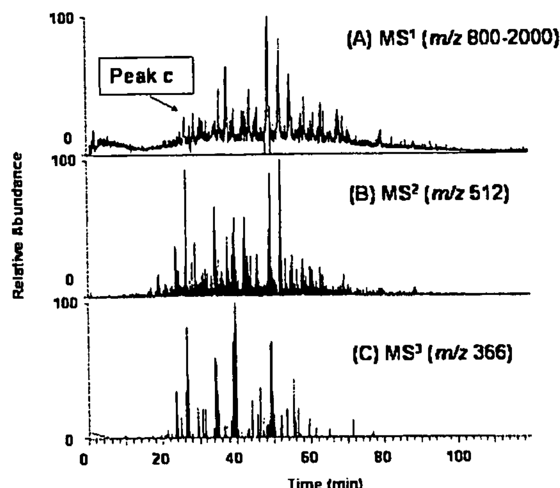

図6　(A)マウス腎臓トリプシン消化物由来フコシル糖ペプチドのTIC、(B)データ依存的MS²によって生じた*m/z* 512イオンのマスクロマトグラム、(C) MS³(前駆イオン：*m/z* 512)によって生じた*m/z* 366イオンのマスクロマトグラム

サンプル：マウス腎臓膜画分トリプシン消化物のAALアフィニティークロマトグラフィー吸着画分
LC/MS：カラム及び溶離液，図4に準ずる；MS，LTQ

プトグロビンの Met120-Lys143 と推定された。また、糖鎖構造は、糖ペプチドの分子量とペプチド部分の分子量の差からジシアロ２本鎖糖鎖と推定された。この方法により、ハプトグロビンの他、血清中のトランスフェリン由来糖ペプチドなども解析することができた。

2.2　糖鎖構造選択的糖タンパク質解析

ペプチド混合物の中からすべての糖ペプチドを選び出す場合は、糖鎖にほぼ共通して存在するHexNAc⁺ を利用するが、任意の糖鎖を有する糖ペプチドのみを選び出す場合は、その構造に特徴的なイオンを利用する。例えば、マウス腎臓から前述した Le^x 結合ペプチドを見つけだす場合は、Le^x に相当する Hex-(Fuc)HexNAc⁺ (*m/z* 512) 及び Hex-HexNAc⁺ (*m/z* 366) を指標とすればよい。**図6** は、マウス腎臓膜画分をトリプシン消化し、フコースを認識する AAL レクチンアフィニティークロマトグラフィーによりフコシル糖ペプチドを回収した後、C18 カラムを用いて LC/MS[2,3] を行った結果である。MS^1 では複雑なクロマトグラムが得られたが (**図6A**)、MS^2 によって Galβ1-4(Fucα1-3)GlcNAc⁺ を生じ (**図6B**)、さらに MS^3 によって Galβ1-4GlcNAc⁺ を生じたペプチドを Le^x 結合糖ペプチドとして選別した (**図6C**)。選び出した糖ペプチドの糖鎖構造は、別途、強度の高いイオンを前駆イオンとして自動的に MS^n を行うデータ依存的 MS^n によって解析した。**図7** はピーク c に溶出された Le^x 結合糖ペプチドのデータ依存的 MS^2 及び MS^3 スペクトルである。フラグメントパターンからこの糖ペプチドは、**図7** に示すような Le^x 部分構造を 2分子有する糖鎖であることが明らかになった。さらに、MS^2 で生じた [peptide + GlcNAc+ 2H]$^{2+}$ (*m/z* 906) を前駆イオンとして MS^3 を行った後、プロテオミクスで利用されているデータベース検索を行ったところ、この糖ペプチドはガンマーグルタミルトランスフェラーゼの LHNQLLPN*TTTVEK (*糖鎖結合位置) と推定された。マウスガンマーグルタミルトランスフェラーゼに Le^x 糖鎖が結合していることは、木幡らによって報告されている[35]。このように、これまでは糖タンパク質を特定してから糖鎖を解析するのが一般的であったが、LC/MS^n とタンパク質データベース検索を利用することによって、任意の糖鎖構造からタンパク質を特定することが可能となってきた。

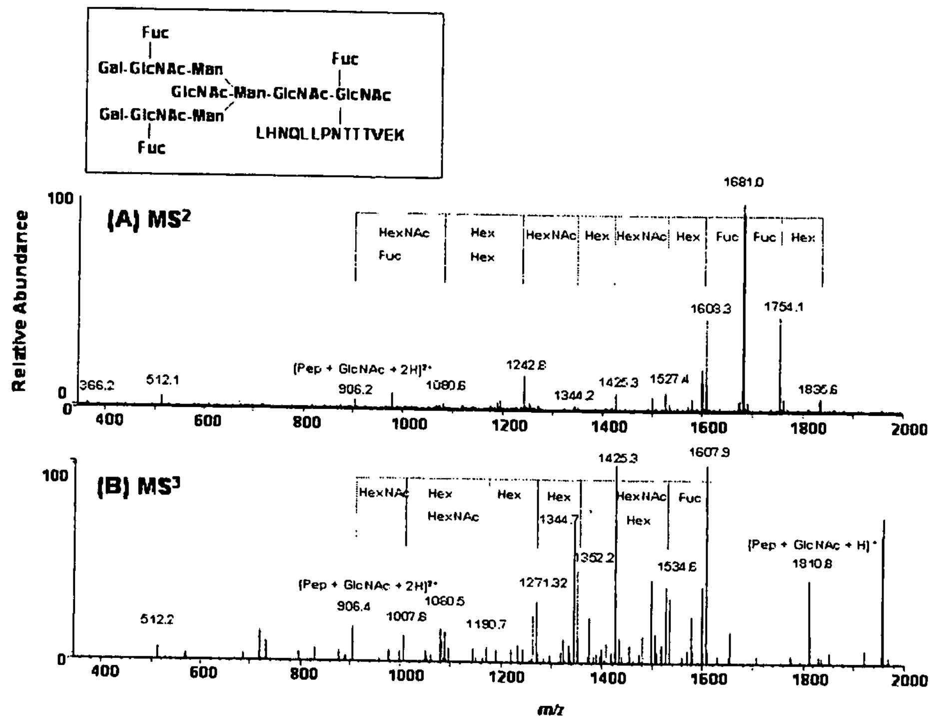

図7　図6中のピークcの位置に溶出された糖ペプチドの（A）データ依存的MS^2、及び（B）データ依存的MS^3スペクトル

3. 電気泳動法とLC/MSを用いた糖タンパク質の構造特性解析

細胞内糖タンパク質をインタクトのままで扱う場合は、不溶性糖タンパク質や、複雑な混合物中の糖タンパク質の分離に優れた電気泳動を利用するのが効果的である[36, 37]。電気泳動を利用したグライコーム解析例を2つ示す。

3.1 レクチンブロットと LC/MS による糖タンパク質の同定

はじめに 2 次元電気泳動とレクチンを利用することによって、任意の糖鎖構造を持つコアタンパク質を特定した例を示す。**図8A** は、前述の GnT-Ⅲ 遺伝子導入 CHO 細胞の 2 次元電気泳動図である。PHA-E_4 レクチン染色を行い、GnT-Ⅲ によって GlcNAc が付加されたタンパク質の位置を特定した（**図8B**）。別に展開した泳動ゲルからレクチンで染まった位置に相当するスポットを切り出し、ゲル内トリプシン消化、ペプチド抽出、LC/MS、及びタンパク質データベース検索を行った結果、このタンパク質はインテグリン $\alpha 3$ と同定された[24]。GnT-Ⅲ は癌細胞転移抑制に関与していることが知られている酵素で[38-40]、そのターゲットタンパク質として細胞接着に関与しているインテグリンが同定されたのは興味深い[41]。

3.2 LC/MS^n によるゲル内糖タンパク質の構造特性解析

つぎに、LC/MS を利用してゲル内糖タンパク質の同定、及び部位特異的糖鎖解析を行った例を示す。**図9A**はマウス脳の膜画分から調製したGPI アンカー型タンパク質群を SDS-PAGE で展開し、クーマシー染色したものである。20-23kDa に表れているタンパク質は、SDS による抽出、トリプシン消化、LC/MS^n（**図9B**）、及びデータベース検索の結果、免疫グロブリンスーパーファミリーに属する Thy-1 と同定された。**図9C** はインソースフラグメンテーションによって生じた m/z 204 のイオンのマスクロマトグラムで、糖ペプチドの溶出位置を示している。各糖ペプチドの糖鎖と

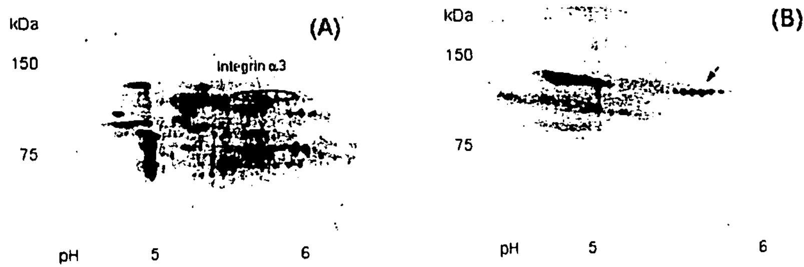

図8　GnT-III 遺伝子導入CHO 細胞の膜を含む画分の2次元電気泳動図
(A)CYPRO Orange 染色、(B)PHA-E_4レクチン染色

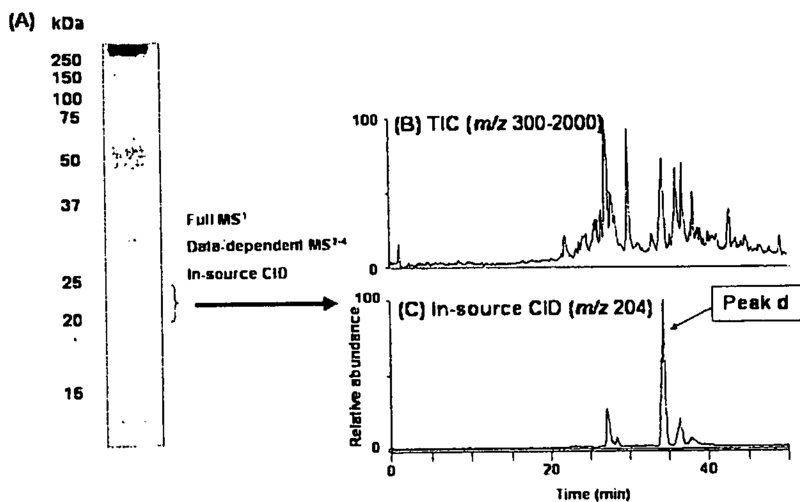

図9　(A)マウス脳由来GPIアンカー型タンパク質のSDS-PAGE、(B)20-23kDaタンパク質トリプシン消化物のLC/MS、及び(C)インソースフラグメンテーションによって生じた*m/z* 204イオンのマスクロマトグラム

サンプル：20-23kDaに泳動されたタンパク質を1％SDSで抽出しトリプシン消化した
LC/MS：図6に準ずる

ペプチドは MS^2 及び MS^3 により決定した。一例として**図10** に、**図9C** のピークd のデータ依存的 MS^2 及び MS^3 スペクトルを示す。フラグメントパターンからそれぞれ糖鎖配列、及びペプチド配列を図のように推定することができた。同様にすべての糖ペプチドの $MS^{2,3}$ スペクトルを解析することによって、Thy-1 の Asn23, 74, 及び 94 に結合している*N*結合型糖鎖の構造を明らかにすることができた[33]。現在では、クーマシー染色される程度の糖タンパク質から、かなりの糖鎖構造情報を得ることが可能となってきた。

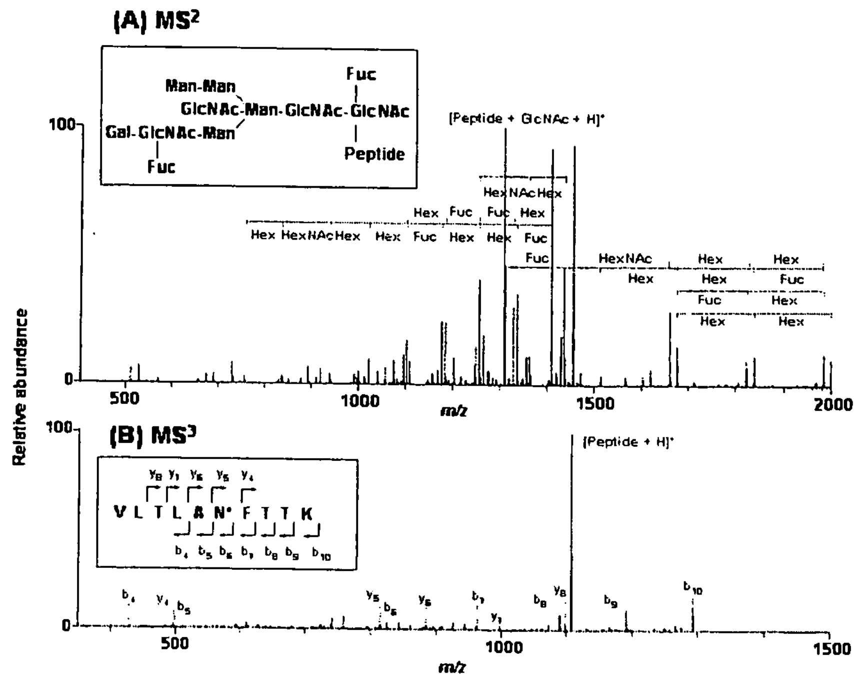

図10　図9中のピークdの(A)データ依存的MS²スペクトル、及び(B)データ依存的MS³スペクトル

おわりに

LC/MSとデータベース検索を基盤技術とするプロテオミクスの手法を糖鎖生物学分野に導入することによって、これまで「解析困難」と考えられていた細胞内糖タンパク質の構造特性が、誰にでも簡単に明らかにできるようになってきた。今後、これらのグライコーム解析技術が、診断や治療法の開発を目的とした疾患関連糖鎖・糖タンパク質の研究に貢献できるようになるものと期待される。

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LC/MS in glycomics

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Adenovirus Vector-Mediated Gene Transfer into Stem Cells

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Abstract: Stem cells, including embryonic stem (ES) cells, mesenchymal stem cells (MSCs), and hematopoietic stem cells (HSCs), are defined by their capacity for self-renewal and multilineage differentiation. Efficient gene transfer into stem cells is essential for the basic research in developmental biology and for therapeutic applications in gene-modified regenerative medicine. Adenovirus (Ad) vectors, based on Ad type 5, can efficiently and transiently introduce the exogenous gene into many cell types via the primary receptor, coxsackievirus, and adenovirus receptor (CAR). However, some kinds of stem cells, such as MSCs and HSCs, cannot be efficiently transduced with conventional Ad vectors based on Ad serotype 5 (Ad5), because of the lack of CAR expression. To overcome this problem, fiber-modified Ad vectors and an Ad vector based on another serotype of Ad have been developed. Here, we review the advances in the development of Ad vectors suitable for stem cells and discuss their application in basic biology and clinical medicine.

Keywords: Adenovirus; stem cell; gene therapy; regenerative medicine; review

Introduction

Adenovirus (Ad) is a nonenveloped virus containing an icosahedral protein capsid with a diameter of approximately 80 nm. At least 51 serotypes of human Ad have been identified and classified into six different subgroups (A–F), many of which are associated with respiratory, gastrointestinal, or ocular diseases. Of them, Ad serotype 5 (Ad5) and Ad serotype 2, both belonging to subgroup C, have been the most extensively studied for use as vectors in gene therapy applications. Ad capsids consist of three major protein components: the hexon, the penton base, and the fiber. Hexon proteins comprise each geometrical face of the capsid, while penton bases associate with fiber proteins to form penton capsomer complexes at each of the 12 vertices (Figure 1A). The two components of the penton capsomer, the fiber and penton base, interact with distinct cell surface receptors during the entry of Ad into susceptible cells. Fiber proteins consist of three distinct domains: the tail, the shaft, and the knob. Each domain has distinct functions in host cell infection. The amino-terminal tail anchors the fiber to the Ad capsid through association with the penton base.[1] The shaft extends away from the virion surface and, in Ad5, is composed of 22 pseudorepeats of 15 amino acids in a triple-β-spiral conformation.[2] By extending the knob away from the virion, the shaft facilitates its interaction with the host

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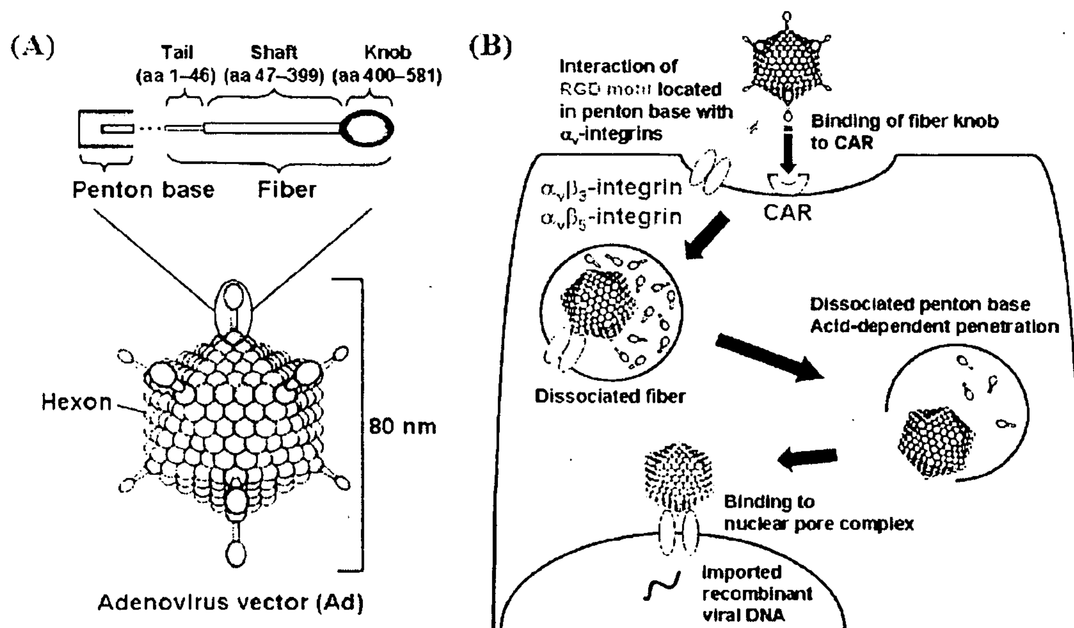

Figure 1. Structure and gene transduction pathway of the Ad vector. (A) The double-stranded virus genome is packaged within an icosahedral protein capsid. Hexon proteins comprise each geometrical face of the capsid, while penton bases associate with fiber proteins to form penton capsomer complexes at each of the 12 vertices. The fiber is composed of the tail, shaft, and knob domain. (B) The Ad vector binds to CAR following internalization in the cells and releases the viral DNA into the nuclei.

receptor [1] The trimeric subunits of the carboxyl C-terminal knob domain are responsible for binding to the host's primary cellular receptor.[3,4]

Human Ad5 contains a linear, approximately 36 kb, double-stranded DNA genome encoding more than 70 gene products. The viral genome contains five early transcription units (E1A, E1B, E2, E3, and E4), two early delayed (intermediate) transcription units (pIX and IVa2), and five late units (L1−L5), which mostly encode structural proteins for the capsid and internal core. Inverted terminal repeats (ITRs) at the end of the viral genome function as replication origins. The E1A gene is the first transcription unit to be activated shortly after infection and is essential to the activation of other promoters and the replication of the viral genome. In the first-generation Ad vectors, the E1 (E1A and E1B) gene is deleted and the virus propagated in E1-transcomplementing cell lines, such as 293,[5] 911,[6] or PER.C6 cells.[7] The E3 region-encoded proteins modulate the host defense but are not required for viral replication in vitro; thus, the E3 region is often deleted to enlarge the packagable size limit for foreign genes. Since up to 3.2 and 3.1 kb of the E1 and E3 regions, respectively, can be deleted[8] and approximately 105% of the wild-type genome can be packaged into the virus without affecting the viral growth rate and titer,[9] E1/E3-deleted Ad vectors allow the packaging of approximately 8.1−8.2 kb of foreign genes.[8]

The coxsackievirus and adenovirus receptor (CAR), which is a broadly distributed type I membrane protein, has been identified as the primary receptor for Ad of subgroups A and C−F.[10−12] The entry of Ad5 into cells is initiated by the

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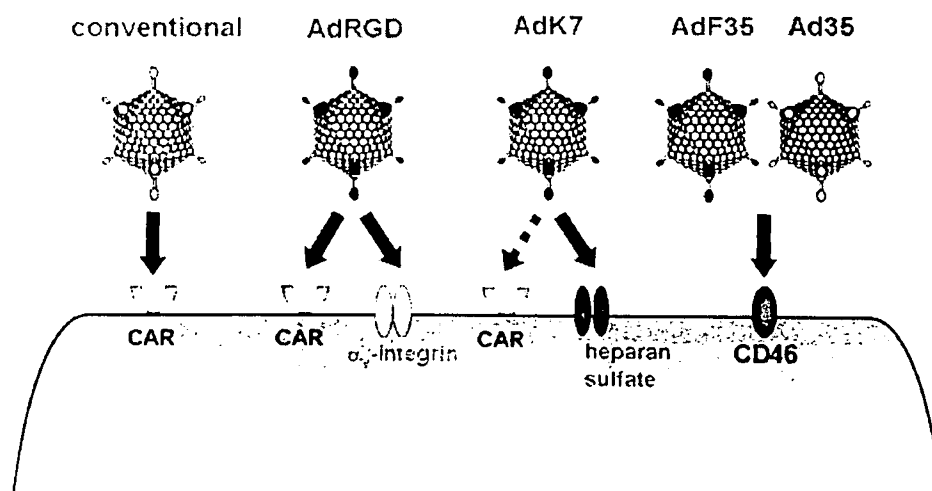

Figure 2. Characteristics of gene delivery by various types of Ad vectors. The conventional Ad vector infects via CAR. The AdRGD vector contains a RGD peptide motif in the HI loop of the fiber knob and infects via αv integrin as well as CAR. The AdK7 vector contains a polylysine peptide in the C-terminus of the fiber knob and infects via heparan sulfate as well as CAR. It is uncertain whether the AdK7 vector infects via CAR. The Ad35 and AdF35 vectors, which contain a fiber protein derived from the Ad5 fiber tail and the Ad35 fiber knob and shaft, infect via CD46.

attachment of fiber on the surface of the capsid to the CAR on the cell surface (Figure 2). The affinity of the RGD (Arg-Gly-Asp) peptide at the penton base of the Ad5 capsid for the cell surface molecules of the integrin family, such as $\alpha_v\beta_5$, $\alpha_v\beta_3$, $\alpha_5\beta_1$, and $\alpha_v\beta_1$, aids in the internalization of Ad5 into the cell.[13-15] Furthermore, heparan sulfate glycosaminoglycans have also been reported to serve as primary attachment sites for Ad2 and Ad5.[16] The abundant expression of these receptors in various cells determines the wide tropism of Ad vectors. Internalized Ad reaches the endosomal pathway and avoids lysosomal degradation (Figure 1B). Inside the endosome, a stepwise disassembly program takes place, allowing the Ad to release its genome into the nucleus. During this process, the pH of the endosome decreases, leading to the release of the fiber from the virion and the dissociation of the penton base.[17] The resulting endosome rupture allows viral DNA to escape from inside the degraded capsid and to enter the nucleus (Figure 1B). During this process, the terminal protein plays a crucial role in translocating the Ad genome into the nucleus. This uncoating process of the Ad starts immediately after internalization and ends 40 min after infection with the translocation of the Ad into the nucleus. As early as 60 min after infection, the Ad begins to transcribe its genome in the host cell.[18]

Although Ad vectors mediate extremely high transduction efficiency, gene transfer with Ad vectors is less efficient in some kinds of cells, such as mesenchymal stem cells (MSCs), hematopoietic stem cells (HSCs), dendritic cells, T cells, smooth muscle cells, skeletal muscle cells, and others because of the scarcity of CAR on their cell surfaces. Modification of the Ad fiber proteins has been used to successfully overcome this obstacle.[19,20] One is constructed by the addition of foreign peptides to the HI loop or C-terminus of the fiber knob of an Ad vector.[21-25] Enhanced gene transfer has been

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114 reported, on the basis of the use of mutant fiber proteins 115 containing either an RGD peptide (AdRGD vector)[21-26] or 116 a stretch of lysine residues [K7 (KKKKKKK) peptide] 117 (AdK7 vector),[21,25,26] which target αv integrins or heparin 118 sulfates on the cell surface, respectively (Figure 2). Altered 119 vector tropism was reported with the substitution of the Ad5 120 fiber protein with that of Ad belonging to subgroup B, such 121 as Ad types 3, 11, and 35.[27-31] These fiber-modified Ad 122 vectors infect cells via CD46, CD80, and CD86, which have 123 recently been identified as the cellular receptors of Ad 124 belonging to subgroup B (Figure 2).[32-36] Mercier et al. described the creation of a chimeric Ad vector encoding the 125 reovirus attachment protein σ1, which targets cells expressing 126 junctional adhesion molecule 1.[37] 127

Several groups have developed an Ad vector from the 128 entire Ad type 35 (Ad35) or Ad type 11 (Ad11) and have 129 demonstrated that the Ad35 and Ad11 vectors exhibit higher 130 transduction efficiencies into hematopoietic progenitor and 131 dendritic cells compared with the conventional Ad5 vector 132 (Figure 2).[38-43] As other approaches to changing the vector 133 tropism, modification of the Ad vector with the antibodies, 134 the fusion protein composed of CAR and the cell binding 135 domain, cationic lipid, or macromolecules has been re- 136 ported.[19,20] Here, we highlight the genetic manipulations of 137 stem cells by the Ad vector and fiber-modified Ad vector 138 for basic research and therapeutic usage. Recent advances 139 in Ad vector-mediated gene transfer into stem cells, such as 140 embryonic stem (ES) cells, mesenchymal stem cells (MSCs), 141 and hematopoietic stem cells (HSCs), will be discussed. 142

Gene Transfer into Stem Cells 143

Stem cells are defined as cells which possess the abilities 144 of self-renewal and multilineage differentiation. Stem cells 145 have been isolated from a wide variety of tissues, and in 146 general, their differentiation potential may reflect the local 147 environment. They lack tissue-specific characteristics but 148 under the influence of appropriate signals can differentiate 149 into specialized cells with a phenotype distinct from that of 150 their precursor. Gene therapy applications that target stem 151

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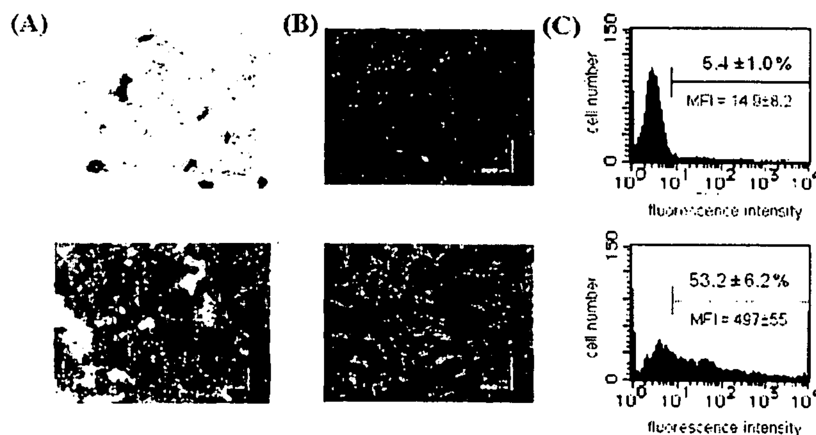

Figure 3. Improved transduction efficiency in the stem cells by the optimized Ad vectors. (A) mES cells were transduced with the LacZ-expressing conventional Ad5 vector containing the CMV promoter (top) or EF-1α promoter (bottom). (B) hMSCs were transduced with the LacZ-expressing Ad5 vector (top) or AdK7 vector (bottom). Both vectors have the CA promoter. (C) Human CD34+ cells were transduced with the GFP-expressing Ad5 vector (top) or Ad35 vector (bottom). Both vectors have the CMV promoter. MFI is the mean fluorescence intensity.

choice of a promoter is important for the efficient expression of exogenous genes in mES cells (Figure 3A). In the transient expression system using a cationic liposome−plasmid complex, the EF-1α (elongation factor 1α) and CA promoter (β-actin promoter/CMV enhancer) were shown to be highly active in mES cells while the CMV promoter was inactive.[62] More recently, we reported that the Ad vector containing the EF-1α or CA promoter has mediated the efficient expression of the reporter gene in mES cells, whereas the Ad vector containing the Rous sarcoma virus (RSV) or the CMV promoter has exhibited little expression.[63] Because CAR was highly expressed in mES cells but not in feeder cells,[64] the Ad vector could be a powerful tool for the genetic manipulation of mES cells when an appropriate promoter is used. To date, although we have no idea about the expression of CAR in hES cells, the Ad vector was reported to mediate the reporter gene expression in both mES cells and hES cells,[64] suggesting that hES cells may also express CAR on their cell surfaces.

As a result of the comparative analysis of mES cells transduced with various types of fiber-modified Ad vectors, the conventional Ad vector exhibited highly efficient and specific transduction, whereas the AdRGD and AdK7 vectors transduced mES cells and feeder cells (embryonic fibroblasts) to the same degree.[63] Therefore, the conventional Ad vector containing the EF-1α or CA promoter should be appropriate when only ES cells are transduced. In turn, the AdRGD or AdK7 vector is adequate when both ES cells and feeder cells are transduced.

The conventional Ad vector containing the EF-1α promoter was applied for the transduction of functional genes. It is well-known that the activation of signal transducer and activator of transcription 3 (STAT3) is essential for leukemia inhibitory factor (LIF)-mediated mES cell self-renewal, and the inhibition of LIF/STAT3 signaling leads to either apoptosis or differentiation.[65] It is also known that transcription factor Nanog maintains the pluripotency of mES cells in a manner that is independent of LIF/STAT3 signaling.[66,67] Ad vector-mediated STAT3F (STAT3 dominant-negative mutant) transduction strongly promoted mES cells to cell differentiation into three germ layers without any nonspecific toxicity.[63] The co-infection of the STAT3F-expressing Ad vector and the Nanog-expressing Ad vector showed that the differentiation suppressing ability of Nanog negated the differentiation promoting function of STAT3F and that mES cells maintained their undifferentiated state.[63] Thus, the differentiation of ES cells could be controlled by the transduction of differentiation-key regulator genes with the Ad vector. ES cells might differentiate into hematopoietic progenitor, pancreatic β cells, or neurons by the Ad vector-mediated introduction of HoxB4,[68,69] Pax4,[70] or nuclear receptor-related 1,[71] respectively.

Gene Transfer into Mesenchymal Stem Cells. MSCs, which reside within the stromal compartment of bone

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152 cells offer great potential for the treatment of many kinds of 153 diseases. Despite this promise, clinical success has been 154 limited by poor rates of gene transfer and poor levels of gene 155 expression. Therefore, an efficient gene delivery system 156 needs to be developed for stem cell gene therapy.

157 **Gene Transfer into Embryonic Stem Cells.** ES cells are 158 pluripotent cell lines derived from the inner cell mass of the 159 developing blastocyst.[44-46] With the establishment of human 160 ES (hES) cells, they have been used as a renewable source 161 of transplantable tiss e-specific stem cells.[47-49] ES cells 162 differentiate spontaneously in vitro in a random manner into 163 a mixture of differentiated cells. The protocols for the 164 differentiation of ES cells enriched for a specific lineage have 165 been developed in both the mouse ES (mES)[50,51] cell and 166 hES cell systems,[52,53] although the differentiated cells are 167 still relatively heterogeneous. Therefore, further research is 168 needed to allow controlled directed differentiation of ES cells into pure cultures of committed cells. One of the most 169 powerful techniques for controlled differentiation is genetic 170 manipulation. Electroporation methods,[54] retroviral vec- 171 tors,[55,56] lentiviral vectors,[57-59] and a supertransfection 172 method based on a replication system using the polyoma 173 replication origin and large T antigen[60] have been used for 174 exogenous gene expression in ES cells, although lentiviral 175 vectors have been shown to be ineffective at expressing 176 exogenous genes in mES cells, but not in hES cells.[57,59] In 177 plasmid-based systems such as eletroporation and super- 178 transfection methods, stable cell lines are generated by 179 selection using a drug resistance gene. All these methods 180 mediate long-term constitutive gene expression, although a 181 long-term gene expression system such as that as described 182 above may be problematic for use in therapeutic applications, 183 because the gene is continuously expressed even after cell 184 differentiation. There is thus a need for efficient vector 185 systems for transient expression. 186

The Ad vector has been thought to be inappropriate for 187 gene transfer into ES cells.[61] It has been reported that the 188 retrovirus vector preferentially transduced ES cells, while 189 the Ad vector containing the cytomegalovirus (CMV) 190 promoter preferentially transduced embryonic fibroblasts as 191 feeders in the ES culture.[61] However, it was found that the 192

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