

とが書かれています。今、少しずつ相談というのが行われているようでございます。

私も相談の中を見る機会があったのですが、やはり相談をするときに、可能な限り具体的なデータを持って相談することが必要だと思います。例えば、重要工程を変えるけれども、それが軽微か一変かという相談の時にどう変更するかも分からないし、だいたいどういう製剤をつくるかもあんまり判然としないようなケースもあります。「製剤」とだけ書かれていて、それが注射剤なのかタブレットなのかも分からない状態で議論をすれば、恐らく規制当局は最悪のケースを想定せざるを得なくなってしまうのだと思います。そういう形で相談を受けますと、やはり極めて厳しい判断が出てしまうかなというように思います。

できるだけその時々々のデータ、それが完璧なデータはもちろんないかもしれないのですが、とともに、やはりメーカーが「これは軽微だろう」と思うのであれば、「こうこうだから、これは軽微だ」というそういった主張も含めてやはり相談をするというようなことが必要になると私は思います。もともとこの制度では、軽微・一変の判断というのはまず申請者がして、それを承認申請することになっており、そのところは相談についても同じだろうと思っております。

スライド35

スライド35、36、37、38、39をご覧ください。

最後にマスターファイルについて簡単にお話をしたいと思います。

マスターファイルは、ここに書いたように、原薬データの開示をめぐる製剤メーカー・原薬メーカーのトラブルの回避を目的として、原薬メーカーのデータを製剤メーカーを通さずに審査当局に提示する手段です。従って、審査当局の側からしてみると、出てきた最終的なデータセットは、マスターファイルを使おうが使まいが同じデータセットでなければいけないということになります。

それから、承認申請というのは最終的にデータを示すだけではなくて、データを基に承認申請事項というようなものも示すわけですから、それについても全く同じ承認申請事項の提示が審査当局になされなければいけないということになります。

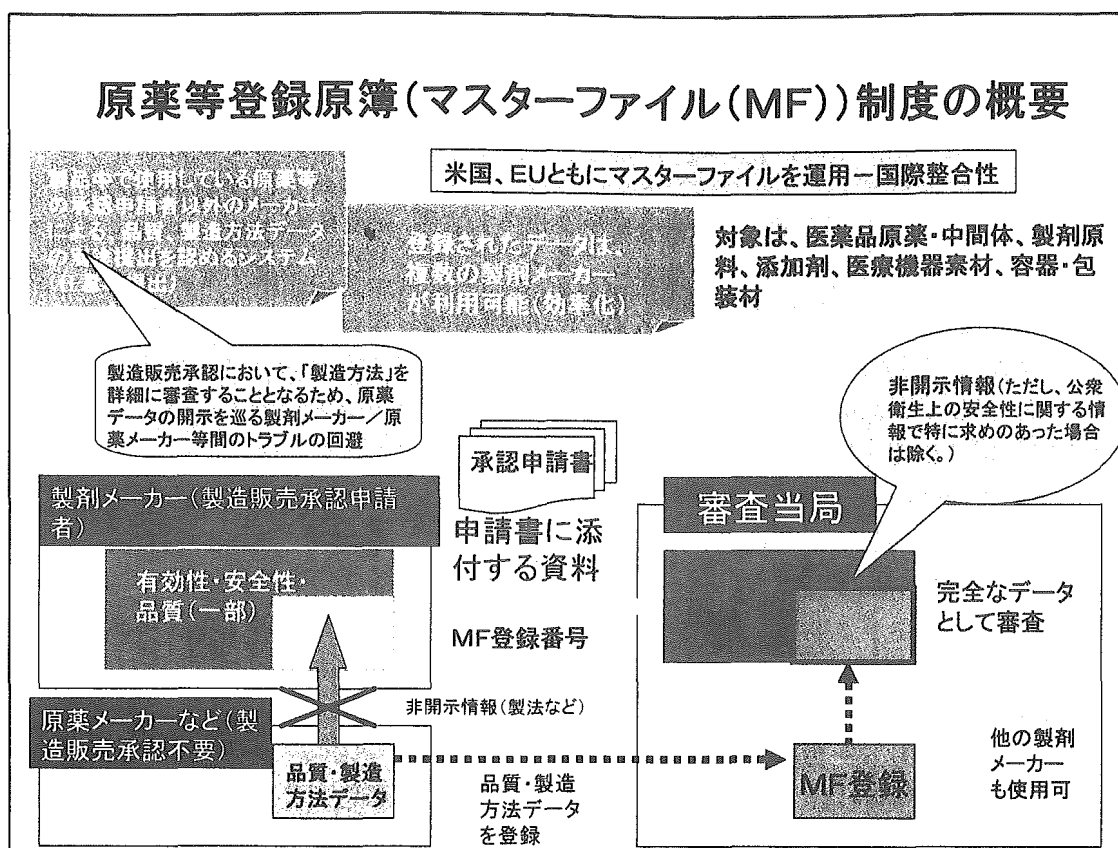
スライド40をご覧ください。

全く同じ事項が最終的に審査当局に提示されなければならないということで、左はそのマスターファイルを使わない場合、右は使う場合ですが、両者は基本的には同じでなければいけません。マスターファイルを使わない場合はCTDの第3部があって、第2部があって、そして承認申請書があるわけです。同じように、マス

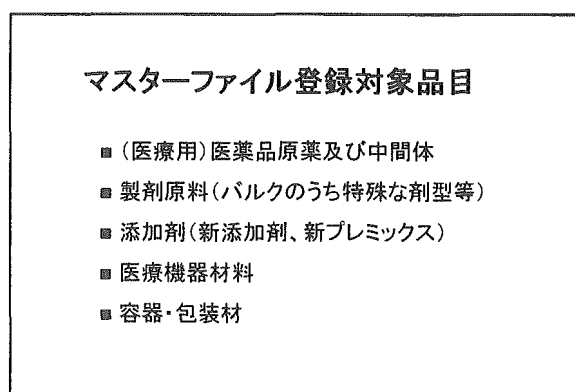
原薬等登録原簿(マスターファイル(MF))制度の概要

- 製品中で使用している原薬等の承認申請者以外のメーカーによる、品質、製造方法データの別途提出を認めるシステム(任意の提出)
 - 製造販売承認において、「製造方法」を詳細に審査することとなるため、原薬データの開示を巡る製剤メーカー／原薬メーカー等間のトラブルの回避
- 登録されたデータは、複数の製剤メーカーが利用可能(効率化)

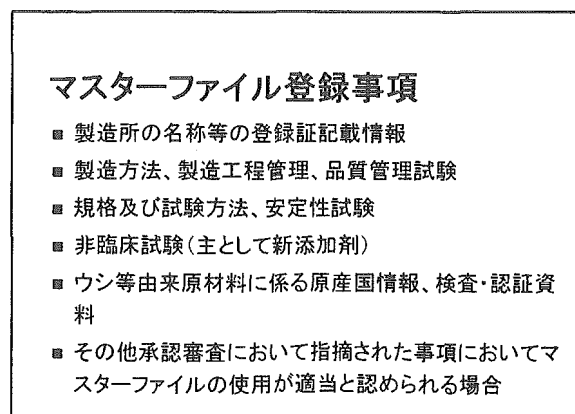
スライド36



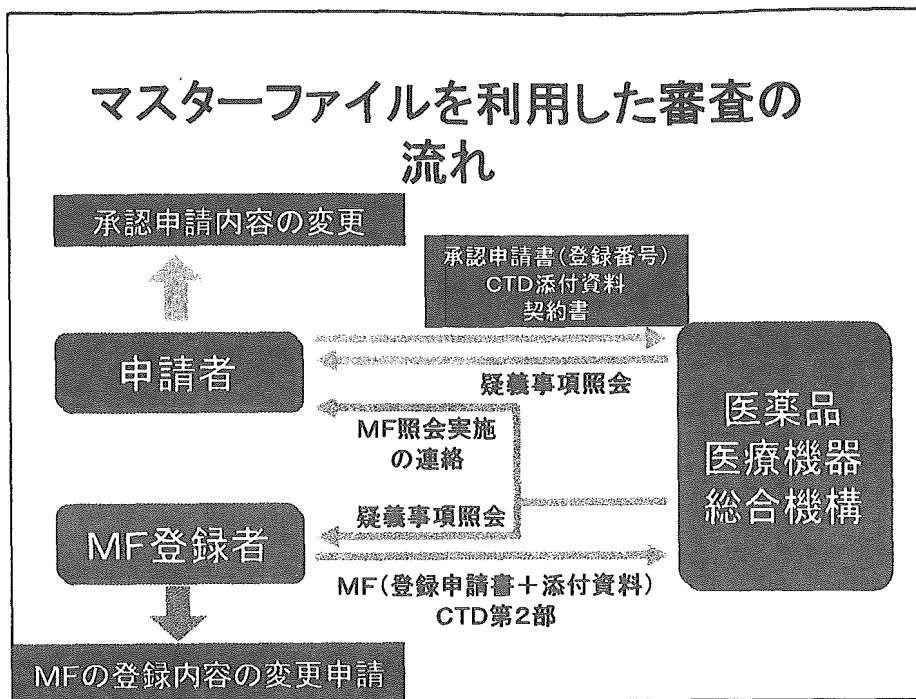
スライド37



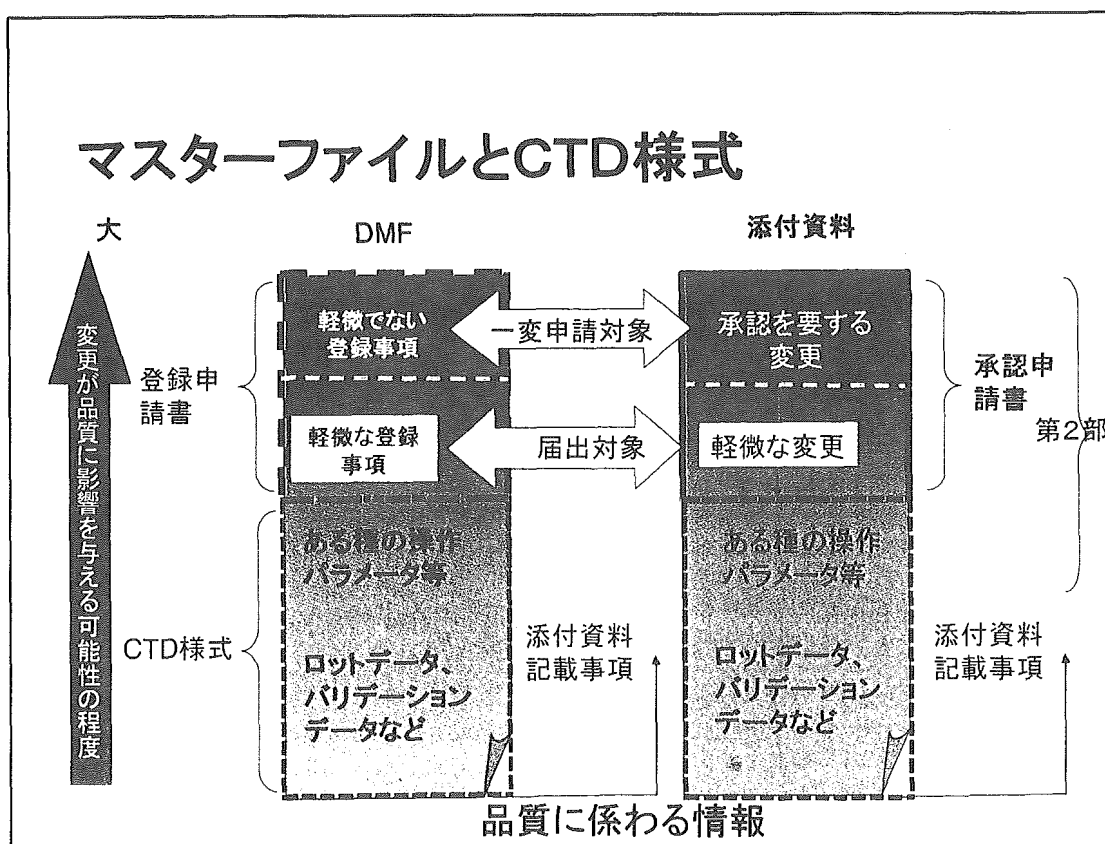
スライド38



スライド39



スライド40



ターファイルを使う場合は、マスターファイルのデータの部分があって、登録事項がある。この登録事項というのはその登録申請書です。

登録申請書に登録したい事項が書かれるのですが、それも一変対象になる部分、即ち軽微でない登録事項と軽微な登録事項がある。ここの関係は承認申請書と全く一致しますので、誤解しないようにして下さい。

カーにデータを開示しないで規制当局にデータを渡すことができると言いますが、やはり最低限必要なデータは原薬メーカーから製剤メーカーに渡らなければいけないということです。そのために承認申請者への情報開示ということが義務づけられています。

それから、変更するときには事前に通知するという事は極めて大事です。

スライド41をご覧下さい。

ただ、マスターファイルの登録者が製剤メー

スライド41

スライド42をご覧下さい。

どのようなことを開示しなければいけないか

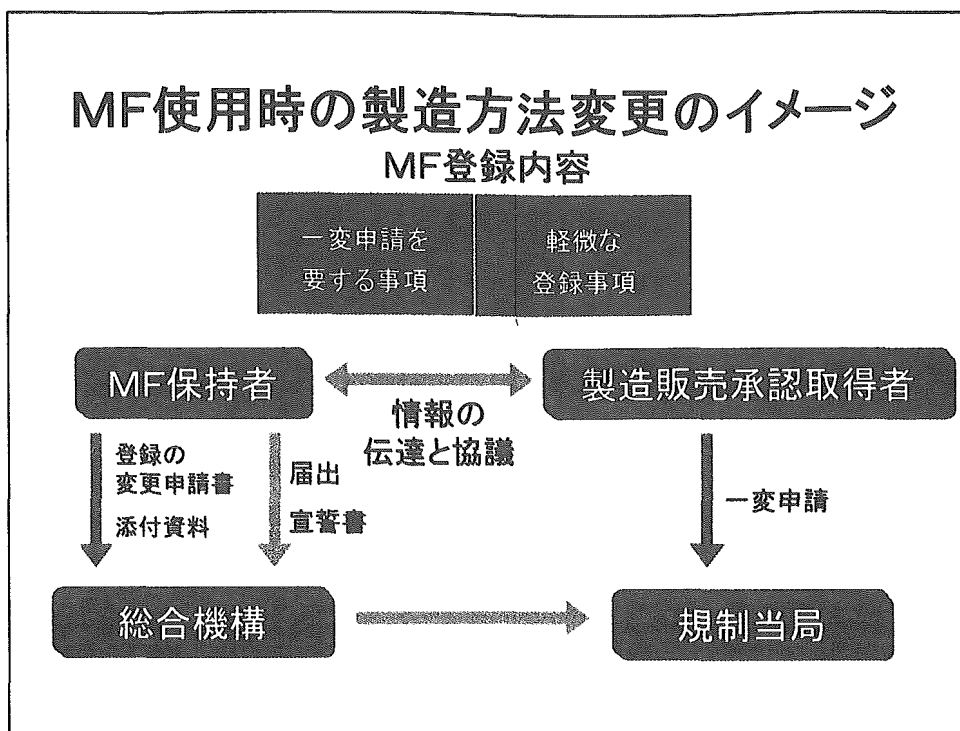
承認申請者への情報の開示

- MF登録者が承認申請者に対して登録の変更に
ついて事前に通知する責務を負うものとする。
- 既に当該MFを利用して承認を得ている品目がある場合は、当該品目の承認取得者に対しても
通知する責務があることは言うまでもない。

スライド42

MF登録内容の開示(製造方法及び製造工程管理)

資料として登録できるデータの範囲		申請者への開示
3.3.S.2.1	製造業者	●
3.3.S.2.2	製造方法及びプロセス・コントロール (製造フローとその説明、工程管理など)	▲
3.3.S.2.3	原材料の管理	×
3.3.S.2.4	重要工程及び重要中間体の管理	×
3.3.S.2.5	プロセスバリデーション/プロセス評価	×
3.3.S.2.6	製造工程の開発の経緯	×



という例がマスターファイルの通知に出ていますが、規格及び試験方法は開示を必要とする部分ですが、製造工程については重要工程とかプロセスバリデーションとかそういったことは開示の必要はありません。ただし、製造方法の概略のプロセスについては「▲」になっているというのは、これは部分的に開示するというようなことであります。

スライド43をご覧ください。

これはマスターファイルを利用しているときの製造方法変更手続きのイメージです。マスターファイル保持者が何か変えようというときに「一変を要する事項か、軽微な事項か」で手法が異なります。いずれにせよ、「変える」ということについては、マスターファイル保持者は、実際にそれを使って製剤をつくっている業者に対して情報をきちっと伝達することが必要です。それが一変の対象であれば、製造販売取得業者は規制当局に一変申請をする。申請が認められて初めてマスターファイル保持者の登録変更が認められるということになります。届出の場合は、この届出の内容が製造販売承認書に書

かれていない、単にマスターファイル記載事項であれば、これはマスターファイルの届出だけでいいということになりますが、その場合についてもやはりまずこのところで情報がきちんと製造業者に伝達され、変更されたということが製造業者が分かるということが極めて重要です。

原薬の製法の影響というのは、本来、製剤の品質にどう影響するかということで最終的には判断されるべきことであります。このところは十分な注意をして頂きたいというように思います。

以上で私の説明を終わりますけれども、去年の7月と6月に大阪と京都でお話をさせて頂く機会があって、そのときにもいろいろな質問をお受けしました。「まだこれから考える部分がいっぱいあります」というようにお答えした部分もあるのですが、それから半年間研究班の活動をしてきて、やはり完全に詰まったわけではありません。これからいろいろな活動をまだやって、相談活動の支援も含めていろいろな活動をやっていくつもりではおります。今後ともよろしく願いいたします。

Expression of Annexin A3 in Primary Cultured Parenchymal Rat Hepatocytes and Inhibition of DNA Synthesis by Suppression of Annexin A3 Expression Using RNA Interference

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Annexin A3 is a member of the lipocortin/annexin family, which binds to phospholipids and membranes in a Ca²⁺-dependent manner. Although annexin A3 has various functions *in vitro*, its cellular significance is completely unknown. Annexin A3 is not found in rat liver *in vivo*. In the present study, we investigated the expression of annexin A3 in primary cultured parenchymal rat hepatocytes. Annexin A3 protein was detected in 48-h, but not 2.5-h, cultured hepatocytes using Western blot analysis. The annexin A3 level further increased after an additional 24 h of culture. Annexin A3 mRNA was not detected in 2.5-h cultured hepatocytes but was detected 22 h after the start of culture by RT-PCR analysis, reaching a maximum value after 48 h of culture. To define the role of Annexin A3 in DNA synthesis, RNA interference was used to reduce annexin III gene expression in hepatocytes. The transfection of small interfering RNAs targeting annexin A3 in the hepatocytes reduced the corresponding mRNA and protein expression by approximately 80% and more than 90%, respectively, at 24 h after transfection. In the annexin A3 small interfering RNAs-transfected cells, DNA synthesis, as assessed by [³H]thymidine incorporation, decreased by approximately 70% not only in the control cultures, but also in the hepatocyte growth factor- or epidermal growth factor-treated cells. These findings show that annexin A3 is expressed in primary cultured parenchymal rat hepatocytes and that the suppression of annexin A3 expression using RNA interference inhibits DNA synthesis.

Key words annexin A3; RNAi; DNA synthesis; primary cultured hepatocyte; hepatocyte growth factor (HGF); epidermal growth factor (EGF)

Annexin (Anx) A3 is also called “lipocortin 3” or “placental anticoagulant protein 3” (PAP-III)¹⁾ and is a member of the lipocortin/annexin family, which binds to phospholipids and membranes in a Ca²⁺-dependent manner.^{2–4)} AnxA3 has been shown to have anticoagulant and anti-phospholipase A₂ properties *in vitro*⁵⁾ and to promote the Ca²⁺-dependent aggregation of isolated specific granules from human neutrophils.⁶⁾ Although the physiological functions of other annexins have been recently clarified in knock-out and transgenic models,^{7–14)} the functions of AnxA3 are completely unknown.¹⁵⁾

Recently, we found that AnxA3 protein and its mRNA are not expressed in isolated parenchymal rat hepatocytes.^{16,17)} Consistent with these findings, AnxA3 protein and its mRNA are not detectable by Western blot analysis and Northern blot analysis in rat liver.^{18–21)} However, there have been no reports on the behavior of AnxA3 in primary cultured parenchymal rat hepatocytes. In the present study, we investigated the expression and function of AnxA3 in cultured parenchymal rat hepatocytes.

MATERIALS AND METHODS

Materials Recombinant human hepatocyte growth factor (HGF) was purchased from R&D systems (Minneapolis, MN, U.S.A.). Mouse epidermal growth factor (EGF) was purchased from Wako (Osaka, Japan). [³H]thymidine (79.9 Ci/mmol) was purchased from PerkinElmer (Boston, MA, U.S.A.). Rabbit anti-human ANXA3 antibody serum

was a generous gift from Dr. F. Russo-Marie and Dr. C. Raguenness-Nicol.

Cell Isolation and Monolayer Cultures Parenchymal hepatocytes were isolated from adult male Wistar rats, weighing 180–200 g, by *in situ* perfusion of the liver with collagenase.²²⁾ All animal care and procedure protocols were approved by the institutional care committee. The cells were then suspended at a density of 2.5 × 10⁵ cells/ml in Williams E medium (WE) containing 5% fetal bovine serum and 1 nM insulin and cultured at a density of 0.5 × 10⁵ cells/cm² in a 6 cm dish and a 48-well microplate precoated with collagen type-1 AC in a humidified chamber at 37 °C in 5% CO₂ and 30% O₂ in air. Cells plated in the 6-cm dish and 48-well microplate were used to prepare total cellular extracts or total RNA and to measure DNA synthesis, respectively. After 2.5 h of culture, the medium was replaced with a serum- and hormone-free medium containing aprotinin (1 µg/ml).

Western Blot Analysis Cell lysates were prepared using a modification of a previously described method.²³⁾ The cells were washed with phosphate-buffered saline (PBS) followed by buffer A (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, and 10 mM EDTA). The cells were then harvested after the addition of 20 µl of buffer A. The cells were suspended, shaken for 15 min at room temperature, and sonicated five times for 15 s each time while in an ice bath after the addition of 1/5 [v/v] of 5 × buffer A containing 2.5% Triton X-100 and 1/100 [v/v] of a protease inhibitor cocktail (SIGMA). After centrifugation at 100000 × g, the cytosolic fraction (about 25 µg) was subjected to sodium dodecyl sulfate-polyacrylamide gel

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electrophoresis on a 10% gel and electroblotted to a PVDF membrane (GVHP; Millipore). After blocking the membrane with 5% skimmed milk, a Western blot analysis was performed using rabbit anti-human AnxA3 antibody serum at a dilution of 1:18000; detection was performed using the ECL detection system (Amersham Bioscience).

Reverse Transcription Polymerase Chain Reaction Analysis Total RNA was extracted from the cells using Trizol reagent (Invitrogen) according to the manufacturer's protocols. Approximately 3 μ g of RNA per sample was reverse-transcribed using the THERMOSCRIPT™ RT-PCR System (Invitrogen) and oligo(dT)₂₀ in a final volume of 40 μ l, according to the manufacturer's protocols. Subsequently, 1 μ l of cDNA was polymerase chain reaction (PCR)-amplified using the THERMOSCRIPT™ RT-PCR System (Invitrogen) in a final volume of 20 μ l per reaction, according to the manufacturer's protocols, for 14–23 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 60 °C, and polymerization for 1 min at 72 °C using Anx AIII or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA specific primers under linear conditions. The PCR products were separated on a 2% agarose gel, stained with SYBR Green I, and visualized and analyzed with a FluorImager 595 (Amersham Bioscience). A computer assisted-analyzer was used to quantitatively analyze the signals, and the signals were normalized to the signal of a house keeping gene, the gene coding GAPDH. The sequences of the AnxA3 primers were as follows: 5'-CAAATTCACCGAGATCCTGT-3' and 5'-TGCTGGAGTGCTGTACGAAA-3'. The sequences of the GAPDH primers were as follows: 5'-ACCACAGTCCATGCCATCAC-3' and 5'-TCCACCACCTGTTGCTGTA-3'.²⁴⁾ The PCR product specificity was confirmed by DNA sequence analysis using an ABI Prism 377 DNA Sequencer (Applied Biosystems, Foster City, CA, U.S.A.).

Preparation and Transfection of Small Interfering RNAs Targeting AnxA3 Small interfering RNAs (siRNAs) targeting rat AnxA3 were designed according to the guidelines of the "Dharmacon siDESIGN Center" (www.dharmacon.com) and obtained from Dharmacon Research (Lafayette) in annealed and lyophilized forms. The target sequences were localized at positions, 493 and 690 bps downstream of the start codon. The sequences of each siRNA pair were as follows: AnxA3 siRNA 1, 5'-GAG ACG AAA GCC UGA AAG UdTdT-3' and ACU UUC AGG CUU UCG UCU cdTdT-3'; ANXA3 siRNA 2, 5'-GGA GAA UUA UCU GGG CAU UdTdT-3' and AAU GCC CAG AUA AUUCUC cdTdT-3; and control siRNA, 5'-ACU CUA UCU GCA CGC UGA CUU-3' and 5'-P G UCA GCG UGC AGA UAG AGU UU-3'. No homology between any relevant mammalian gene and the control siRNA was observed. These siRNAs were dissolved in an RNase-free solution provided by Dharmacon Research at a concentration of 20 μ M. After 20 h of cell culture, the medium was replaced with WE containing aprotinin (1 μ g/ml) immediately prior to transfection. Transfection with siRNA was performed using SiFactor (B-bridge), according to the user guidelines. Sixty microliters of both AnxA3 siRNA 1 and 2 were diluted with OPTI-MEM (Invitrogen) to a final volume of 400 μ l. Sixty-four microliters of SiFactor was also diluted in OPTI-MEM to a final volume of 400 μ l, then suspended and incubated at room temperature for 5 min. Next, the diluted siRNA was com-

bined with SiFactor, and the mixture was incubated at room temperature to allow the siRNA-SiFactor complex to form. Eight hundred microliters of the siRNA-SiFactor complex was added to the cultures (6-cm dish). For the 48-well plates, the siRNA-SiFactor complex was prepared as described above except that the volume of each solution per well was scaled down to 1/16.

Measurement of [³H]thymidine Incorporation After 20 h of culture, the medium was replaced with hormone-free medium containing aprotinin (1 μ g/ml) and 0.1% bovine serum albumin (BSA), and EGF (2 ng/ml) or HGF (20 ng/ml) was added. After 1 h, 50 μ l of siRNA-SiFactor complex, prepared as described above, was added to the wells. After another 24 h, [³H]thymidine (0.626 μ Ci) and thymidine (676.6 ng) were added, and 10 μ g/ml of aphidicolin was added to some wells at the same time. The cells were then cultured for another 24 h. [³H]thymidine incorporation was measured as described previously.²⁵⁾ The difference between the radioactivity in the hot-trichloroacetic acid soluble fraction with and without aphidicolin was calculated as dpm/mg protein. Cell protein was measured using a previously described method,²⁶⁾ with BSA used as a standard.

RESULTS

Expression of AnxA3 during Culture At first, we investigated the expression of AnxA3 in primary cultured parenchymal rat hepatocytes. AnxA3 protein was not detected by Western blot analysis 2.5 and 24 h after the start of culture but was detected after 48 h of culture (Fig. 1A). The level after 72 h of culture was approximately 1.6-fold higher than that after 48 h of culture (Fig. 1B). AnxA3 mRNA was not detected by reverse transcription (RT)-PCR in cultured hepatocytes after 2.5 h of culture but was significantly detected after 22 h of culture (Fig. 2A), reaching a maximum value after 48 h of culture (Fig. 2B). These results indicate

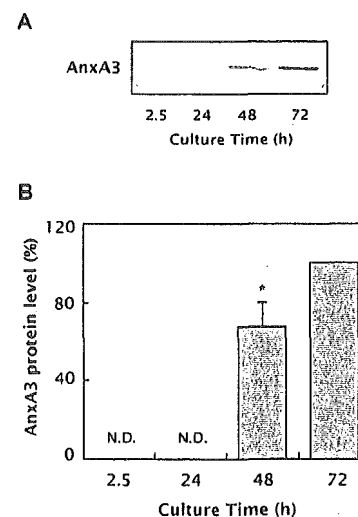


Fig. 1. Expression of AnxA3 Protein during Culture

(A) The data shown are representative of the Western blot analysis results. Cells lysates were prepared from the cells at the indicated times and used for the Western blot analysis. (B) The intensity of each band was quantified, and the results are shown relative to the value of cells cultured for 72 h. The data are expressed as the mean \pm S.D. of 3 experiments. * $p < 0.01$, compared with the value of cells cultured for 72 h. N.D., not detected.

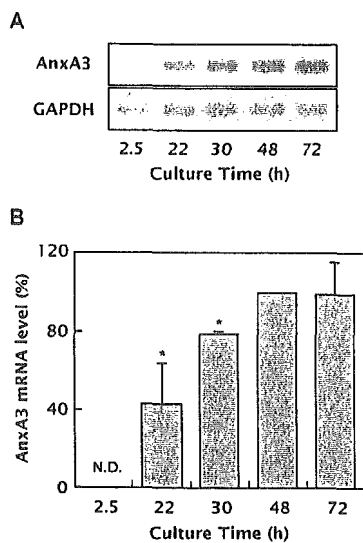


Fig. 2. Increase in AnxA3 mRNA Level during Culture

(A) The data shown are representative of the RT-PCR analysis results. Total RNA was prepared from the cells at the indicated times and used for the RT-PCR analysis. (B) The intensity of each band was quantified, and the results are shown relative to the value of cells cultured for 48 h. The data are expressed as the mean \pm S.D. of 3 experiments. * $p < 0.01$, compared with the value of cells cultured for 48 h. N.D., not detected.

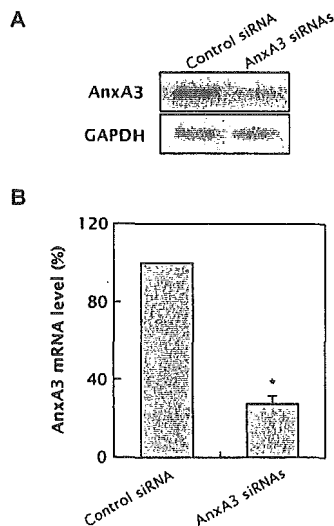


Fig. 3. Suppression of Increase in AnxA3 mRNA Level during Culture with RNAi

(A) The data shown are representative of the RT-PCR analysis results. Total RNA was prepared from the cells 1 d after siRNA transfection and used for the RT-PCR analysis. (B) The intensity of each band was quantified, and the results are shown relative to the value of cells transfected with control siRNA. The data are expressed as the mean \pm S.D. of 3 experiments. * $p < 0.01$, compared with the value of cells transfected with control siRNA.

that the expression of AnxA3 is regulated by its mRNA level.

Suppression of AnxA3 Expression Using RNA Interference Next, we attempted to suppress AnxA3 expression by RNA interference (RNAi) to examine the role of ANXA3 in the cultured hepatocytes. AnxA3 mRNA expression was markedly reduced by treatment with AnxA3 siRNAs, compared with the expression after treatment with control siRNA, 1 d after the transfection (Fig. 3A), with an inhibition of approximately 80% (Fig. 3B). Furthermore, the AnxA3

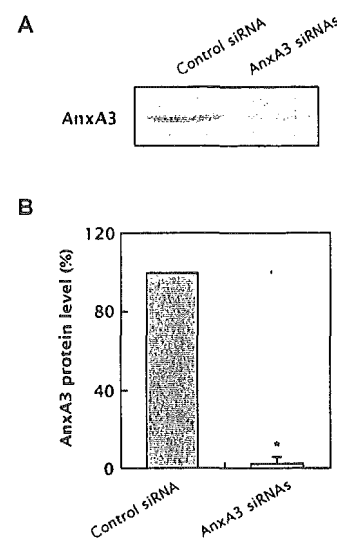


Fig. 4. Suppression of AnxA3 Protein Expression during Culture with RNAi

(A) The data shown are representative of the Western blot analysis results. Cells lysates were prepared from the cells 1 d after siRNA transfection and used for the Western blot analysis. (B) The intensity of each band was quantified, and the results are shown relative to the value of cells transfected with control siRNA. The data are expressed as the mean \pm S.D. of 3 experiments. * $p < 0.01$, compared with the value of cells transfected with control siRNA.

protein level was also reduced by the treatment with AnxA3 siRNAs compared with the level after treatment with control siRNA (Fig. 4A), with an inhibition of more than 95% (Fig. 4B). On the other hand, the control siRNA had almost no effect on AnxA3 protein and mRNA levels compared with those treated with SiFactor alone (data not shown). Neither the control nor AnxA3 siRNAs caused any cytotoxic effects, as observed microscopically or by the quantification of the total amount of protein in each sample (data not shown). These results indicate that AnxA3 siRNAs efficiently and specifically, inhibit the expression of AnxA3 in primary cultured parenchymal rat hepatocytes.

Inhibition of DNA Synthesis by Suppression of AnxA3 Expression Using RNAi Finally, we examined the role of AnxA3 in DNA synthesis by suppressing AnxA3 expression using RNAi. EGF (2 ng/ml) and HGF (20 ng/ml) stimulated DNA synthesis by approximately 7-fold and 9-fold, respectively in hepatocytes treated with control siRNA (Fig. 5). The stimulations were inhibited to approximately 70% by treatment with AnxA3 siRNAs. Similar results were also obtained in the control cells, whereas the control siRNA had almost no effect on DNA synthesis, compared with the effect in cells treated with SiFactor alone (data not shown).

DISCUSSION

In the present study, we showed for the first time that AnxA3 is expressed in cultured parenchymal rat hepatocytes and that the inhibition of AnxA3 expression by RNAi resulted in a significant inhibition of DNA synthesis, suggesting that the expression of AnxA3 is necessary for DNA synthesis in primary cultured parenchymal rat hepatocytes.

Hepatocytes placed under culture conditions, are known to acquire a growth potential characterized by the enhancement of DNA synthesis, which is caused by several growth

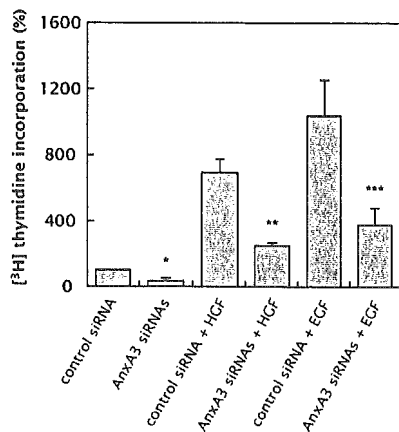


Fig. 5. Inhibition of DNA Synthesis by RNAi

The results are shown relative to the value of control cultured cells transfected with control siRNA. The data are expressed as the mean ± S.D. of duplicate wells in 3 experiments. **p* < 0.01, compared with the value of control cultured cells transfected with control siRNA. ***p* < 0.01, compared with the value of cells cultured in the presence of HGF and transfected with control siRNA. ****p* < 0.01, compared with the value of cells cultured in the presence of EGF and transfected with control siRNA. The mean ± S.D. of [3H]thymidine incorporation in the control cultured cells transfected with control siRNA was $9.72 \times 10^4 \pm 0.68 \times 10^4$ dpm/mg protein.

factors.^{27,28)} Our present findings suggest that the expression of AnxA3 is partly necessary for hepatocytes to acquire a growth potential under culture conditions. In fact, the enhanced expression of AnxA3 has been observed in hepatocellular carcinoma cell lines.²⁹⁾ In addition, we discovered that the enhanced expression of Anx3 was observed in the proliferative hepatocytes after carbon tetrachloride-induced rat liver damage (unpublished observations).

As for other annexins, several findings concerning the relation of AnxA1 to hepatocyte growth has been reported as described below. The suppression of AnxA1 expression using antisense technology inhibited proliferation in a mouse hepatocyte cell line.³⁰⁾ AnxA1 increased in the proliferative hepatocytes after carbon tetrachloride-induced rat liver damage or a partial hepatectomy and in hepatocellular carcinoma tissue.^{31,32)}

Although the mechanism of action of AnxA3 on DNA synthesis is presently uncertain, the target of AnxA3 may be a common signal transduction pathway, and not necessarily a constitutive or growth factor-mediated one, because the suppression of AnxA3 expression using RNAi not only inhibited the control of DNA synthesis, but also EGF- or HGF-stimulated DNA synthesis, almost to a similar level. In this respect, the findings described below may be relevant to speculations on the mechanism of action of AnxA3 on DNA synthesis. The growth factor-mediated enhancement of hepatocyte growth consists of several signal transduction pathways.³³⁾ The activation of cytosolic phospholipase A₂ (cPLA₂) by MAP kinase liberates arachidonic acid from phospholipids and is followed by the generation of prostaglandins, mediators of DNA synthesis, via cyclooxygenase. Interestingly, the suppression of AnxA1 expression using antisense technology inhibited cPLA₂ activity in a mouse hepatocyte cell line.³⁰⁾ This report suggests that cPLA₂ must be phosphorylated by AnxA1 to become active. Additional evidence suggests that AnxA1 (275–346 aa), the region responsible for phospholipid binding is necessary for the interaction between AnxA1 and cPLA₂.³⁴⁾ Further study

is required to clarify the mechanism of action of AnxA3, including the possibility that AnxA3 positively modulates cPLA₂ activity, as in the case of AnxA1.

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Site-specific glycosylation analysis of human apolipoprotein B100 using LC/ESI MS/MS

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Human apolipoprotein B100 (apoB100) has 19 potential *N*-glycosylation sites, and 16 asparagine residues were reported to be occupied by high-mannose type, hybrid type, and monoantennary and biantennary complex type oligosaccharides. In the present study, a site-specific glycosylation analysis of apoB100 was carried out using reversed-phase high-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry (LC/ESI MS/MS). ApoB100 was reduced, carboxymethylated, and then digested by trypsin or chymotrypsin. The complex mixture of peptides and glycopeptides was subjected to LC/ESI MS/MS, where product ion spectra of the molecular ions were acquired data-dependently. The glycopeptide ions were extracted and confirmed by the presence of carbohydrate-specific fragment ions, such as *m/z* 204 (HexNAc) and 366 (HexHexNAc), in the product ion spectra. The peptide moiety of glycopeptide was determined by the presence of the *b*- and *y*-series ions derived from its amino acid sequence in the product ion spectrum, and the oligosaccharide moiety was deduced from the calculated molecular mass of the oligosaccharide. The heterogeneity of carbohydrate structures at 17 glycosylation sites was determined using this methodology. Our data showed that Asn2212, not previously identified as a site of glycosylation, could be glycosylated. It was also revealed that Asn158, 1341, 1350, 3309, and 3331 were occupied by high-mannose type oligosaccharides, and Asn 956, 1496, 2212, 2752, 2955, 3074, 3197, 3438, 3868, 4210, and 4404 were predominantly occupied by mono- or disialylated oligosaccharides. Asn3384, the nearest *N*-glycosylation site to the LDL-receptor binding site (amino acids 3359–3369), was occupied by a variety of oligosaccharides, including high-mannose, hybrid, and complex types. These results are useful for understanding the structure of LDL particles and oligosaccharide function in LDL-receptor ligand binding.

Key words: apolipoprotein B100/glycopeptide/liquid chromatography electrospray mass spectrometry/product ion spectrum/*N*-linked oligosaccharide

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Introduction

Low-density lipoprotein (LDL) is the main cholesterol carrier in human plasma, and a high serum level of LDL is involved in the development of atherosclerosis. LDL is originally secreted as very low-density lipoprotein (VLDL). VLDL is converted to LDL and then removed from the circulation. Apolipoprotein B100 (apoB100) is the only protein component of LDL and is the ligand recognized by the LDL receptor. The amino acid sequence of human apoB100 has been deduced by analysis of the apoB100 cDNA sequence (Chen *et al.*, 1986; Knott *et al.*, 1986; Law *et al.*, 1986; Yang *et al.*, 1986). Mature apoB100 consists of 4536 amino acids, and its molecular weight has been calculated to be 513 kDa. ApoB100 has 19 potential *N*-glycosylation sites (Asn-X-Ser/Thr), of which 16 asparagine residues are found to be glycosylated (Yang *et al.*, 1989). The carbohydrate moieties were linked to asparagine residues at the following 16 positions: 158, 956, 1341, 1350, 1496, 2752, 2955, 3074, 3197, 3309, 3331, 3384, 3438, 3868, 4210, and 4404. The carbohydrate structures of the *N*-linked sugar chains of human apoB100 were reported to be high-mannose, hybrid, and mono- and disialylated complex type oligosaccharides (Garner *et al.*, 2001; Taniguchi *et al.*, 1979).

The role of carbohydrate moieties of apoB100 has been investigated by several laboratories. The *N*-linked oligosaccharides at the amino terminus of human apoB100 are important for the assembly and secretion of VLDL (Vukmirica *et al.*, 2002). Seven of the *N*-glycans are predicted to occur close to the LDL-receptor binding region of apoB100 and seem to have an important role (Yang *et al.*, 1986, 1989). The carbohydrate composition of apoB100, particularly sialylation, has been considered to contribute to the atherogenic properties of LDL. However, Shireman and Fisher (1979) reported that they do not appear to play a significant role in the binding of apoB100 to the LDL receptor. Furthermore, the distribution and diversity of human apoB100 oligosaccharides isolated from normolipidemic, hypercholesterolemic, and hypertriglyceridemic diabetic subjects were highly conserved even when characterized in LDL subfractions (Garner *et al.*, 2001). The potential function of apoB100 carbohydrates posthepatic secretion is not well understood. Glycoproteins have a variety of sugar chains at each glycosylation site. Because of the individual functions at each site, a comparison of glycosylation among various sites is important. Therefore, to investigate the role of carbohydrate moieties of apoB100, we attempted to determine the carbohydrate heterogeneity site-specifically.

To determine the site-specific carbohydrate heterogeneity of glycoproteins, the glycoprotein must be digested into

peptides and glycopeptides, and then both the peptide and sugar chain of each glycopeptide must be analyzed. One of the most effective techniques for mapping proteolytic fragments of glycoproteins is liquid chromatography (LC) coupled with electrospray ionization (ESI) mass spectrometry (MS) (Carr et al., 1993; Duffin et al., 1992; Kawasaki et al., 2004; Ling et al., 1991). The specific detection of glycopeptides can be achieved by monitoring specific diagnostic sugar oxonium ions, such as m/z 204 (HexNAc) and 366 (HexHexNAc) produced by cone voltage fragmentation, or by precursor ion scanning (Carr et al., 1993; Duffin et al., 1992). However, when many *N*-glycosylation sites are present within a glycoprotein, the chromatogram becomes extremely complex and assignment of the glycopeptide ions is very difficult.

We present here an alternative strategy for the site-specific glycosylation analysis of a peptide and glycopeptide mixture using LC/ESI MS/MS, where we acquired the product ion spectrum for all significant molecular ions in a data-dependent manner. Product ion spectra of molecular ions allow the specific detection of glycopeptides from a complex mixture of peptides based on the presence of diagnostic sugar oxonium ions of oligosaccharides. Furthermore, this

method allows confirmation of the amino acid sequence of a glycopeptide by the presence of b- and y-series fragment ions of the peptide. Using this method, we identified one previously unidentified *N*-glycosylated site of ApoB100 and determined the oligosaccharide heterogeneity of each of 17 *N*-glycosylation sites. Our findings provide information on the structure of apoB100 that will be useful to future studies on the structure, function, and metabolism of plasma LDL.

Results

Enzyme digestion

To determine the oligosaccharide heterogeneity at each glycosylation site, reduced and carboxymethylated apoB100 was digested into peptides and glycopeptides. Table I shows the amino acid sequences of the tryptic or chymotryptic peptides, including the putative *N*-glycosylation sites. The putative glycosylation sites were numbered (G1–19). Boldface indicates the previously reported *N*-glycosylation sites (G2–6 and G9–19). When apoB100 is digested by trypsin, potential *N*-glycosylation sites, Asn1341 (G4) and Asn1350 (G5), belong to the same peptide. Because chymotrypsin

Table I. The amino acid sequences of the tryptic or chymotryptic peptides including the putative *N*-glycosylation sites in apoB100

<i>N</i> -glycosylation site ^a		Tryptic digests	Chymotryptic digests		
Residue	ID	Sequence	Theoretical mass ^b	Sequence	Theoretical mass ^b
Asn ⁷	G1	EEEMLEN ⁷ VSLVCPK	1677.8	EN ⁷ VSL	560.3
Asn ¹⁵⁸	G2	QVLF ¹⁵⁸ LDTVYGN ¹⁵⁸ CSTHFTVK	2229.1	GN ¹⁵⁸ CSTHF	822.3
Asn ⁹⁵⁶	G3	QVFPGLN ⁹⁵⁶ YCTSGAYSN ⁹⁵⁶ ASSTDSASYPLTGDTR	3550.5	SN ⁹⁵⁶ ASSTDSASY	1088.4
Asn ¹³⁴¹	G4	LYQLQVPLLGVL ¹³⁴¹ DLSTNVYSNLYN ¹³⁴¹	4692.3	N ¹³⁴¹ W	318.1
Asn ¹³⁵⁰	G5	WSASYSGGN ¹³⁵⁰ TSTDHFSLR		SGGN ¹³⁵⁰ TSTDHF	1021.4
Asn ¹⁴⁹⁶	G6	FN ¹⁴⁹⁶ SSYLQGTNQTGR	1684.8	N ¹⁴⁹⁶ SSY	469.2
Asn ²²¹²	G7	TIHDLHLFIENIDFN ²²¹² K	1968.0	N ²²¹² KSGSSTASW	1023.5
Asn ²⁵³³	G8	N ²⁵³³ LTDFAEQYSIQDWAK	1928.9	AAKN ²⁵³³ L	515.3
Asn ²⁷⁵²	G9	IQSPLFTLDANADIGN ²⁷⁵² GTTSANEAGIAASITAK	3231.6	DANADIGN ²⁷⁵² GTTSANEAGIAASITAKGESKL	2846.4
Asn ²⁹⁵⁵	G10	VNQN ²⁹⁵⁵ LVYESGSLN ²⁹⁵⁵ FSK	1797.9	N ²⁹⁵⁵ F	279.1
Asn ³⁰⁷⁴	G11	YNQN ³⁰⁷⁴ FSAGNNENIMEAHVINGEANLD FLNIPLTIPEMR	4359.1	NQN ³⁰⁷⁴ F	521.2
Asn ³¹⁹⁷	G12	SYN ³¹⁹⁷ ETK	740.3	N ³¹⁹⁷ ETKIF	878.5
Asn ³³⁰⁹	G13	ELCTISHIFIPAMGN ³³⁰⁹ ITYDFSFK	2704.3	IPAMGN ³³⁰⁹ ITY	978.5
Asn ³³³¹	G14	SSVITLNTNAELFN ³³³¹ QSDIVAHLSSSSVIDALQYK	3864.0	N ³³³¹ QSDIVAHL	995.5
Asn ³³⁸⁴	G15	FVEGSHN ³³⁸⁴ STVSLTTK	1605.8	VEGSHN ³³⁸⁴ STVSL	1128.5
Asn ³⁴³⁸	G16	YDFN ³⁴³⁸ SSMLYSTAK	1525.7	N ³⁴³⁸ SSML	550.2
Asn ³⁸⁶⁸	G17	FEVDSPVYN ³⁸⁶⁸ ATWSASLK	1912.9	N ³⁸⁶⁸ ATW	490.2
Asn ⁴²¹⁰	G18	VHN ⁴²¹⁰ GSEILFSYFQDLVITLPPFLR	2836.5	SKVHN ⁴²¹⁰ GSEIL	1082.6
Asn ⁴⁴⁰⁴	G19	DFHSEYIVSASN ⁴⁴⁰⁴ FTSQLSSQVEQFLHR	3155.5	IVSASN ⁴⁴⁰⁴ F	736.4

Human apoB100 amino acid sequence (NP_000375, apolipoprotein B [gi:4502153]) was obtained from the NCBI database (www.ncbi.nlm.nih.gov/pubmed). Boldface indicates previously reported *N*-glycosylation sites. Cystein residue was carboxymethylated, and carboxymethylated cystein was underscored.

^aPotential *N*-glycosylation sites were identified with the consensus sequence NXS/T, where X is any amino acid except P.

^bMonoisotopic mass value.

can cleave apoB100 into glycopeptides containing one glycosylation site, we attempted to analyze both proteolytic fragments from trypsin digestion and chymotrypsin digestion to identify the site-specific glycosylation.

LC/ESI MS/MS analysis of tryptic digest of apoB100

The schema of a site-specific glycosylation analysis of apoB100 is shown in Figure 1. A mixture of peptides and glycopeptides was subjected to LC/ESI MS/MS with a reversed-phase column. Figure 2A shows a total ion chromatogram (TIC) of a time-of-flight (TOF) MS scan for the full scan m/z 1000–2000. When double or higher charged molecular ions were detected, the product ion spectrum was automatically acquired. Figure 2B shows a TIC of the product ion scan. The collision energy at the second quadrupole for the product ion scan was adjusted from 50 to 80 eV depending on the size and charge of the precursor ion. Under these conditions, peptide precursor ions produced b- and y-series fragment ions derived from its amino acid sequence (data not shown), and glycopeptide precursor ions produced abundant carbohydrate-specific ions, m/z 204, 186, 168, and 366 (described later). The intensity of ions at m/z 204.05–204.15 (HexNAc, 204.08) in each product ion scan are illustrated in Figure 2C. The extracted ion chromatogram at m/z 204 (Figure 2C) and 366 (data not shown) provides useful information on the selection of glycopeptide precursor ions. The product ion spectra of glycopeptides show a very characteristic pattern (see later figures). There were intense oligosaccharide-derived peaks of m/z 204 (HexNAc), 366 (HexHexNAc), 186 (HexNAc-H₂O), and 168 (HexNAc-2H₂O), and if present, 163 (Hex), 292 (Neu5Ac), and 274 (Neu5Ac-H₂O). Therefore, we can very easily distinguish the glycopeptide precursor ions from peptide ions. As expected, many parent ions having 204 and

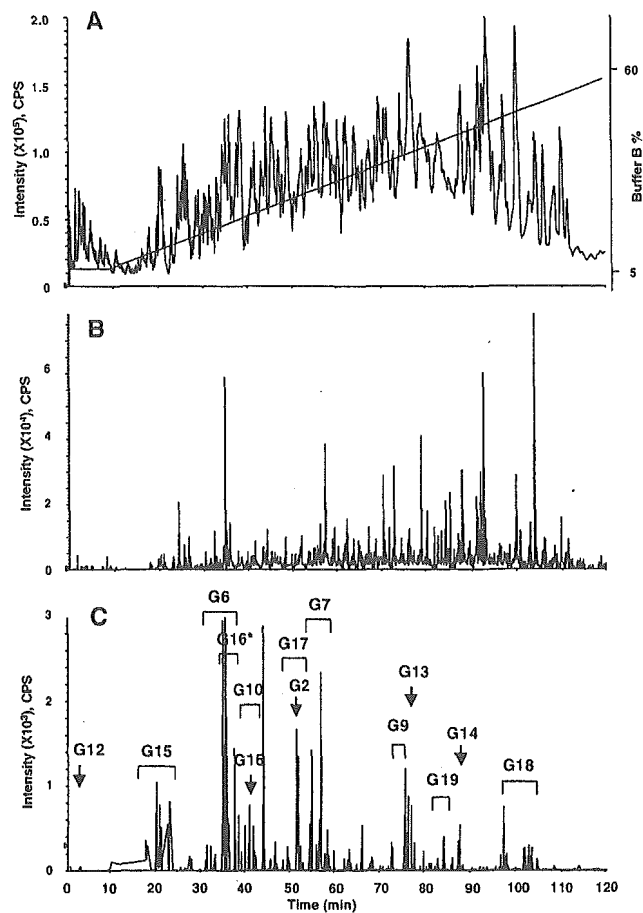


Fig. 2. LC/ESI MS/MS of tryptic digest of apolipoprotein B100. TIC of the TOF MS scan for the full scan m/z 1000–2000 and the HPLC gradient are indicated (A). TIC of the product ion scan data-dependently acquired (B). Extracted ion chromatogram at m/z 204 of product ion spectra (C). Arrows and brackets denote glycopeptide fraction and *N*-glycosylation site ID. G16* was found to be oxidized at a methionine residue.

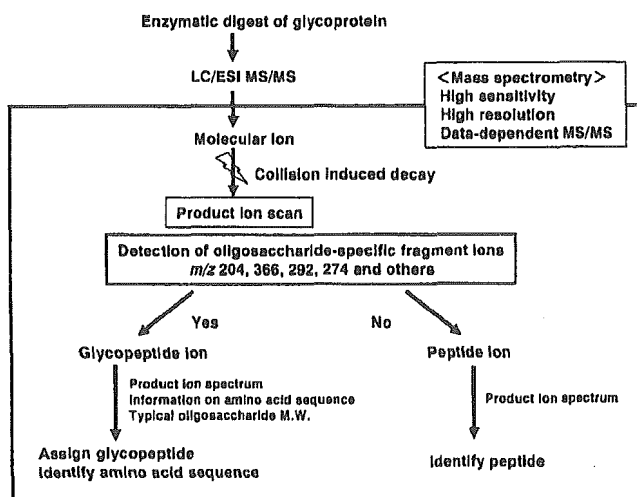


Fig. 1. Schema of site-specific glycosylation analysis. Glycoprotein was digested into peptides and glycopeptides containing only one glycosylation site. Only LC/ESI MS/MS was used. Data-dependent MS/MS acquisition was used to differentiate glycopeptide ions from peptide ions and identify the amino acid sequence of the glycopeptides. The oligosaccharide structure was deduced based on the calculated oligosaccharide molecular weight.

366 fragment ions in the product ion spectrum were detected, and most of these precursor ions were found as glycopeptides.

The glycopeptides were assigned based on an examination of product ion spectra using the information on the peptides containing a putative *N*-glycosylation site. Figures 3A, 3B, and 3C show the product ion spectra of 1412.1 (+2) at 18 min, 1160.4 (+3) at 20 min and 1271.1 (+3) at 22 min for the glycopeptides. There were intense carbohydrate B⁺ ions such as m/z 204 (HexNAc), 366 (HexHexNAc), and 186 (HexNAc-H₂O) and other weak peaks in the product ion spectra. These product ion spectra were very similar to each other (Figure 3A, 3B, and 3C). Careful examination of these product ion spectra for the glycopeptides revealed that several fragment ions were consistent with b- and y-series fragment ions derived from the peptide FVEGSHNSTVSLTTK (residue 3378–3392). The deduced b- and y-series fragment ions of the peptide FVEGSHNSTVSLTTK were listed, and the fragment ions detected in the product ion spectrum of 1160.4 (+3) are underscored in the table (Figure 3D). The molecular ions of the peptide (m/z 1606) and

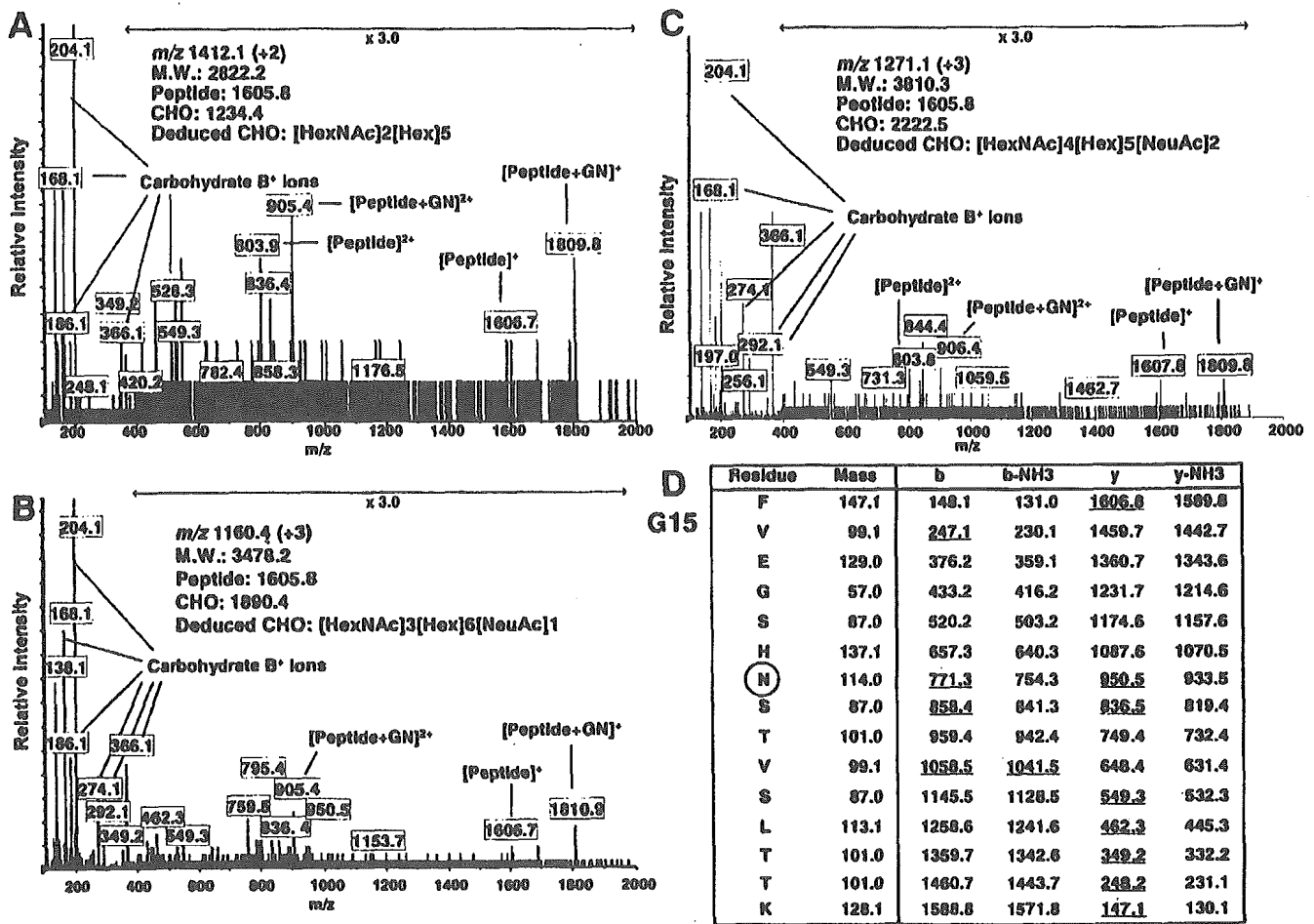


Fig. 3. Product ion spectra of the *N*-glycosylated peptides containing Asn3384 (G15). Product ion scan of m/z 1412.1 (+2) (A), 1160.4 (+3) (B), and 1271.1 (+3) (C) at 18, 20, and 22 min, respectively. These spectra show a characteristic fragmentation pattern with abundant carbohydrate-diagnostic oxonium ions at 163, 168, 186, 204, and 366 and very similar patterns to each other. The oxonium ions at m/z 292 (Neu5Ac) and 274 (Neu5A-H₂O) were observed in the peptides having sialylated oligosaccharide (B) and (C). Several fragment ions are consistent with the b- and y-series fragment ions derived from the peptide FVEGSHNSTVSLTTK (residue 3378–3392). [Peptide]⁺ and [peptide+GlcNAc]⁺ ions were also detected. (D) shows m/z of the proposed b- and y-series fragment ions of the peptide and the fragment ions detected in Figure 3B are underscored. Based on the calculated oligosaccharide mass, the deduced oligosaccharide structure was presented. GN, *N*-acetylglucosamine.

peptide + GlcNAc (m/z 1809) were also detected in the product ion spectra (Figure 3A, 3B, and 3C). These results suggest that these glycopeptides have the same peptide, FVEGSHNSTVSLTTK, including the *N*-glycosylation site Asn3384 (G15). Carbohydrate molecular weight was calculated by subtracting the theoretical molecular weight of the peptide (1605.8) from the calculated molecular weight of the glycopeptide and adding the molecular weight of H₂O (18.0). The oligosaccharide structure was deduced based on the molecular weight and previously reported oligosaccharides of apoB100. The presence of product ions at m/z 274 (Neu5Ac-H₂O) and 292 (Neu5Ac) suggested that those at m/z 1160.4 (+3) and 1271.1 (+3) were glycopeptide ions having sialylated oligosaccharides. Thus the carbohydrate compositions, [HexNAc]₂[Hex]₅, [HexNAc]₃[Hex]₆[Neu5Ac]₁, and [HexNAc]₄[Hex]₅[Neu5Ac]₂, were deduced from the carbohydrate molecular weights, 1234.4, 1890.4, and 2222.5, respectively.

Figures 4 shows the product ion spectra of 1294.8 (+3) at 55 min and 1152.7 (+3) at 35 min for other glycopeptides. There are intense carbohydrate B⁺ ions in the product ion spectra. Several ions consisting of b- and y-series fragment ions from the peptide TIHDLHLFIENIDHNK (residue 2198–2213) were found in the product ion spectrum of 1294.8 (+3) (Figure 4A), and detected ions are underscored in the table. The molecular ions of the peptide (m/z 1968.9) were also detected in the product ion spectra. The carbohydrate molecular weight was calculated from the molecular weight of the peptide, 1968.0, and the molecular weight of the glycopeptide, 3881.4. Carbohydrate composition was deduced from the carbohydrate molecular weight (1931.4) and presence of Neu5Ac. Thus, the peptide moiety TIHDLHLFIENIDHNK and carbohydrate composition [HexNAc]₄[Hex]₅[Neu5Ac]₁ were suggested.

Many ions in the product ion spectrum of 1152.7 (+3) were consistent with the b- and y-series fragment ions

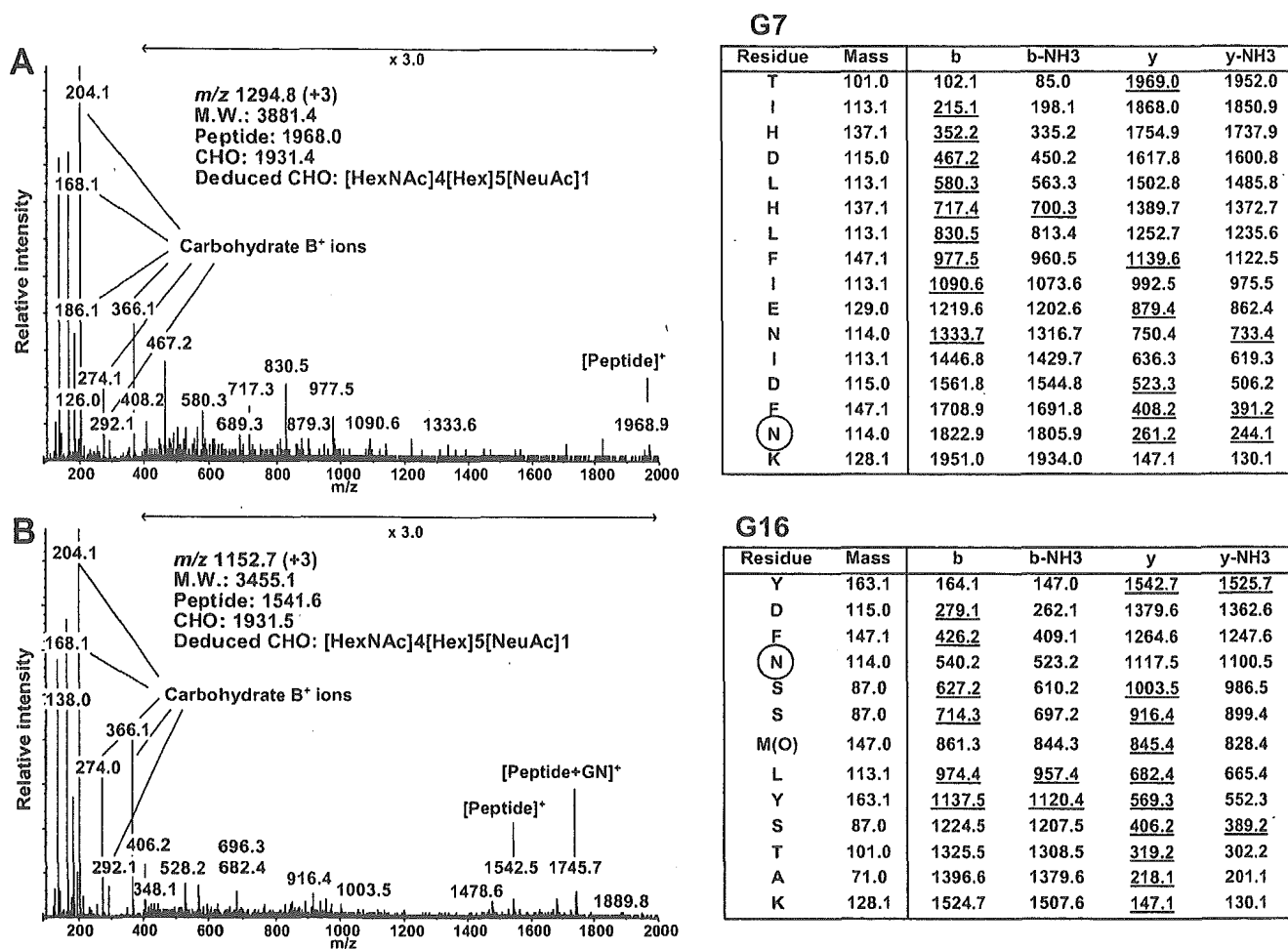


Fig. 4. Product ion spectra of the tryptic *N*-glycosylated peptides of apoB100. (A) Product ion spectrum of m/z 1294.8 (+3) at 55 min for the glycopeptide containing Asn2212 (G7). Several ions are consistent with the b- and y-series fragment ions derived from the peptide TIHDLHLFIENIDFNK (residue 2198–2213). Table shows m/z of the proposed b- and y-series fragment ions and the detected ions are underscored. (B) Product ion spectrum of m/z 1152.7 (+3) at 35 min for the glycopeptide containing Asn3438 (G16) with oxidized methionine. The methionine residue at 3441 was considered oxidized. Several ions are consistent with the b- and y-series fragment ions derived from the peptide YDFNSSM(O)LYSTAK (residue 3435–3447). Table shows m/z of the proposed b- and y-series fragment ions and the detected ions are underscored. M(O), oxidized methionine.

derived from the peptide YSFNSSMLYSTAK (Figure 4B). However, the deduced peptide ion m/z at 1526.7 and peptide + GlcNAc at 1729.8 were not detected. The difference of 203 between the product ions at m/z 1542.5 and 1745.7 suggests that the molecular weight of the peptide moiety may be 1541.5, and an increase in mass of 16 Da suggests that the methionine residue of YSFNSSMLYSTAK (residue 3435–3447, molecular weight 1525.7) was oxidized. The deduced b- and y-series fragment ions of the peptide, YSFNSSMLYSTAK, with the oxidized methionine are listed and detected peptide fragment ions are underscored. Thus, the product ions at m/z 1542.5 and 1745.7 were considered the peptide and peptide + GlcNAc ions, respectively. Our method identified unexpected oxidation of methionine residue (Figure 4B). The carbohydrate molecular weight was calculated, and the carbohydrate composition, [HexNAc]₄[Hex]₅[Neu5Ac]₁, was deduced from the carbohydrate molecular weight, 1931.4, and presence of Neu5Ac.

Results of site-specific glycosylation analysis from tryptic digest are summarized in Table II. To avoid misassignment, only ions that were confirmed as glycopeptides by the product ion spectra or coeluting ions with glycopeptides were listed. We determined 13 of the 19 potential *N*-glycosylation sites and the oligosaccharide heterogeneity at each site in a site-specific glycosylation analysis of the tryptic digest of apoB100. The type of oligosaccharide was deduced based on the oligosaccharide composition. Glycopeptides containing *N*-glycosylation sites Asn7, 956, 1341, 1350, 2533, and 3074 (G1, 3, 4, 5, 8, and 11) could not be detected. The relative peak intensity does not accurately express the relative amount of glycoforms, because of the different ionization efficiency of each glycoform, different detection sensitivity at m/z , and simultaneous acquisition of MS and MS/MS spectra. However, the relative peak intensity of each glycopeptide would provide an indication of the distribution in glycoforms.

Table II. Site-specific glycosylation analysis of the tryptic digest of apoB100 using LC/ESI MS/MS

Glycosylation site ID	Retention time (min)	Peptide theoretical MW ^a	Glycopeptides			Oligosaccharide			Relative peak intensity (%) ^b	Composition ^c	Deduced Type ^d
			Charge	Calculated MW ^a	Calculated MW ^a	Theoretical MW ^a					
G1	—	1677.8	—	—	—	—	—	—	—	—	
G2	51	2229.1	—	—	1882.7	1882.7	1882.6	7	[HexNAc]2[Hex]9	High mannose	
	51	2229.1	—	4093.8	1720.6	1720.6	1720.6	13	[HexNAc]2[Hex]8	High mannose	
	51	2229.1	—	3931.7	1538.4	1538.5	1538.5	22	[HexNAc]2[Hex]7	High mannose	
	51	2229.1	—	3769.5	1396.6	1396.5	1396.5	13	[HexNAc]2[Hex]6	High mannose	
	51	2229.1	—	3607.6	1234.4	1234.4	1234.4	100	[HexNAc]2[Hex]5	High mannose	
	51	2229.1	—	3445.5	1234.4	1234.4	1234.4	—	—	—	
G3	—	3530.5	—	—	—	—	—	—	—	—	
G4, G5	—	4692.3	—	—	—	—	—	—	—	—	
G6	33	1684.8	—	—	1234.4	1234.4	1234.4	15	[HexNAc]2[Hex]5	High mannose	
	34	1684.8	—	2901.2	1931.4	1931.4	1931.7	100	[HexNAc]4[Hex]5[Neu5Ac]1	Biantennary complex	
	34	1684.8	—	3598.2	1931.4	1931.4	1931.4	23	[HexNAc]4[Hex]4[Neu5Ac]1	Biantennary complex	
	35	1684.8	—	3598.2	1769.4	1769.4	1769.6	11	[HexNAc]3[Hex]4[Neu5Ac]1	Hybrid	
	35	1684.8	—	3436.2	1769.4	1769.6	1769.6	5	[HexNAc]3[Hex]5[Neu5Ac]1	Hybrid	
	35	1684.8	—	3233.1	1566.3	1566.3	1566.6	3	[HexNAc]3[Hex]6[Neu5Ac]1	Hybrid	
	35	1684.8	—	3395.4	1728.6	1728.6	1728.6	3	[HexNAc]3[Hex]7[Neu5Ac]1	Hybrid	
	37	1684.8	—	3557.4	1890.6	1890.6	1890.7	24	[HexNAc]4[Hex]5[Neu5Ac]2	Biantennary complex	
	37	1684.8	—	3719.4	2052.6	2052.6	2052.7	66	[HexNAc]4[Hex]5[Neu5Ac]1	Biantennary complex	
	37	1684.8	—	3889.2	2222.4	2222.4	2222.8	100	[HexNAc]4[Hex]5[Neu5Ac]2	Biantennary complex	
G7	54	1968.0	—	3889.4	2222.6	2222.6	1931.7	—	—	—	
	58	1968.0	—	3881.4	1931.4	1931.4	1931.7	—	—	—	
G8	58	1968.0	—	4172.8	2222.8	2222.8	2222.8	—	—	—	
	58	1968.0	—	4172.7	2222.7	2222.7	2222.8	—	—	—	
G9	—	1928.9	—	—	—	—	—	—	—	—	
G10	73	3231.6	—	—	2224.0	2224.0	2222.8	—	[HexNAc]4[Hex]5[Neu5Ac]2	Biantennary complex	
	40	1797.9	—	—	1931.4	1931.4	1931.7	41	[HexNAc]4[Hex]5[Neu5Ac]1	Biantennary complex	
	41	1797.9	—	—	1931.4	1931.4	1931.5	—	—	—	

Table II. continued

Glycosylation site ID	Retention time (min)	Peptide theoretical MW ^a	Glycopeptides			Oligosaccharide			Relative peak intensity (%) ^b	Composition ^c	Deduced Type ^c
			<i>m/z</i>	Charge	Calculated MW ^a	Calculated MW ^a	Theoretical MW ^a				
G11	43	1797.9	1001.6	+4	4002.4	2222.5	2222.8	100	[HexNAc]4[Hex]5[Neu5Ac]2	Biantennary complex	
	43	1797.9	1335.1	+3	4002.3	2222.4	—	—	—	—	
	—	4359.1	—	—	—	—	—	—	—	—	
	2	740.3	1473.6	+2	2945.2	2222.9	2222.8	—	[HexNAc]4[Hex]5[Neu5Ac]2	Biantennary complex	
	75	2704.3	1102.7	+4	4406.8	1720.5	1720.6	22	[HexNAc]2[Hex]8	High mannose	
	75	2704.3	1470.0	+3	4406.9	1720.6	—	—	—	—	
	76	2704.3	1062.2	+4	4244.8	1558.5	1558.5	54	[HexNAc]2[Hex]7	High mannose	
	76	2704.3	1415.9	+3	4244.7	1558.4	—	—	—	—	
	76	2704.3	1021.6	+4	4082.4	1396.1	1396.5	100	[HexNAc]2[Hex]6	High mannose	
	76	2704.3	1361.9	+3	4082.7	1396.4	—	—	—	—	
	76	2704.3	1307.9	+3	3920.7	1234.4	1234.4	50	[HexNAc]2[Hex]5	High mannose	
	88	3864.0	1146.7	+5	5728.7	1882.7	1882.6	—	[HexNAc]2[Hex]9	High mannose	
	88	3864.0	1433.1	+4	5728.3	1882.3	—	—	—	—	
G15	17	1605.8	1063.4	+3	3187.2	1599.4	1599.6	8	[HexNAc]3[Hex]6	Hybrid	
	17	1605.8	1077.0	+3	3228.0	1640.2	1640.6	20	[HexNAc]4[Hex]5	Biantennary complex	
	17	1605.8	1009.4	+3	3025.2	1437.4	1437.5	20	[HexNAc]3[Hex]5	Hybrid	
	18	1605.8	1513.6	+2	3025.3	1437.5	—	—	—	—	
	18	1605.8	1023.0	+3	3066.0	1478.2	1478.5	13	[HexNAc]4[Hex]4	Biantennary complex	
	18	1605.8	1412.1	+2	2822.2	1234.4	1234.4	4	[HexNAc]2[Hex]5	High mannose	
	20	1605.8	1174.1	+3	3519.3	1931.5	1931.7	100	[HexNAc]4[Hex]5[Neu5Ac]1	Biantennary complex	
	20	1605.8	1760.7	+2	3519.4	1931.6	—	—	—	—	
	20	1605.8	1160.4	+3	3478.2	1890.4	1890.7	10	[HexNAc]3[Hex]6[Neu5Ac]1	Hybrid	
	20	1605.8	1106.4	+3	3316.2	1728.4	1728.6	94	[HexNAc]3[Hex]5[Neu5Ac]1	Hybrid	
	20	1605.8	1659.2	+2	3316.4	1728.6	—	—	—	—	
	20	1605.8	1222.8	+3	3665.4	2077.6	2077.7	4	[HexNAc]4[Hex]5[Neu5Ac]1[Fuc]1	Biantennary complex	
	20	1605.8	1052.4	+3	3154.2	1566.4	1566.6	42	[HexNAc]3[Hex]4[Neu5Ac]1	Monoantennary complex	
G16	20	1605.8	1578.2	+2	3154.4	1566.6	—	—	—	—	
	21	1605.8	1120.0	+3	3357.0	1769.2	1769.6	23	[HexNAc]4[Hex]4[Neu5Ac]1	Biantennary complex	
	22	1605.8	1271.1	+3	3810.3	2222.5	2222.8	49	[HexNAc]4[Hex]5[Neu5Ac]2	Biantennary complex	
	42	1525.7	1147.4	+3	3439.2	1931.5	1931.7	—	[HexNAc]4[Hex]5[Neu5Ac]1	Biantennary complex	
	42	1525.7	1720.7	+2	3439.3	1931.6	—	—	—	—	
	35	1541.7*	1152.7	+3	3455.1	1931.4	1931.7	100	[HexNAc]4[Hex]5[Neu5Ac]1	Biantennary complex	

Table II. continued

Glycosylation site ID	Retention time (min)	Peptide theoretical MW ^a	Glycopeptides			Oligosaccharide			Deduced Type ^e		
			m/z	Charge	Calculated MW ^a	Calculated MW ^a	Theoretical MW ^a	Relative peak intensity (%) ^b		Composition ^c	
G17	35	1541.7*	1728.6	+2	3455.2	1931.5	1769.4	1769.6	9	[HexNAc]4[Hex]4[Neu5Ac]1	Biantennary complex
	36	1541.7*	1098.7	+3	3293.1	1769.4	1769.5	1566.6	18	[HexNAc]3[Hex]4[Neu5Ac]1	Monoantennary complex
	36	1541.7*	1647.6	+2	3293.2	1566.5	2222.7	2222.8	19	[HexNAc]4[Hex]5[Neu5Ac]2	Biantennary complex
	36	1541.7*	1546.1	+2	3090.2	1566.5	1931.6	1931.7	100	[HexNAc]4[Hex]5[Neu5Ac]1	Biantennary complex
	38	1541.7*	1249.8	+3	3746.4	1931.6	1931.7	2587.9	6	[HexNAc]5[Hex]6[Neu5Ac]2	Triantennary complex
	51	1912.9	1912.9	1914.3	+2	3826.6	1931.7	2222.7	39	[HexNAc]4[Hex]5[Neu5Ac]2	Biantennary complex
	54	1912.9	1912.9	1495.3	+3	4482.9	2588.0	2222.8	100	[HexNAc]4[Hex]5[Neu5Ac]1	Biantennary complex
	54	1912.9	1912.9	1030.4	+4	4117.6	2222.7	1931.7	10	[HexNAc]4[Hex]4[Neu5Ac]1	Biantennary complex
	54	1912.9	1912.9	1373.5	+3	4117.5	2222.6	1769.6	64	[HexNAc]4[Hex]5[Neu5Ac]2	Biantennary complex
	98	2836.5	2836.5	1188.5	+4	4750.2	1931.7	2222.8	79	[HexNAc]4[Hex]5[Neu5Ac]1	Biantennary complex
G18	98	2836.5	1584.4	+3	4750.1	1931.7	1769.6	1931.7	100	[HexNAc]4[Hex]5[Neu5Ac]1	Biantennary complex
	98	2836.5	1148.0	+4	4588.1	1769.6	2222.8	2222.8	64	[HexNAc]4[Hex]5[Neu5Ac]2	Biantennary complex
	102	2836.5	1009.3	+5	5041.3	2222.8	2223.7	1931.7	79	[HexNAc]4[Hex]5[Neu5Ac]1	Biantennary complex
	102	2836.5	1261.6	+4	5042.2	2223.0	2223.0	2222.8	100	[HexNAc]4[Hex]5[Neu5Ac]2	Biantennary complex
	103	2836.5	1681.5	+3	5041.5	2223.0	1931.5	1931.7	79	[HexNAc]4[Hex]5[Neu5Ac]1	Biantennary complex
	83	3155.5	1014.8	+5	5069.0	1931.5	2223.0	2222.8	100	[HexNAc]4[Hex]5[Neu5Ac]2	Biantennary complex
	86	3155.5	1073.1	+5	5360.5	2223.0	2223.0	2222.8	100	[HexNAc]4[Hex]5[Neu5Ac]2	Biantennary complex

^aMonoisotopic mass value.

^bRelative peak intensity was calculated by comparing same charge state glycopeptide ions. The intensity of glycoform with maximum intensity at each glycosylation site was considered as 100%.

^cThe oligosaccharide composition and type were deduced from its composition.

*The glycopeptides including G16 were found to be oxidized at methionine residue.

When each product ion spectrum of the peptide ions in this LC/ESI MS/MS analysis was identified by the computer program Mascot, the sequence coverage of apoB100 was 38%. The ions, m/z 1177.9 (+3) at 64 min, 1289.0 (+3) at 91 min, and 1053.2 (+3) at 84 min, were identified as TIHDLHLFIENIDFN²²¹²KSGSSTASWIQNVDTK containing the potential *N*-glycosylation site Asn2212 (G7), SSVITLNTNAELFN³³³¹QSDIVAHLLSSSSVIDALQYK containing Asn3331 (G14), and DFHSEYIVSASN⁴⁴⁰⁴FTSQLSSQVEQFLHR containing Asn4404 (G19), respectively (data not shown). These results indicate that some parts of these glycosylation sites were not glycosylated. There were many unexplained peptides and glycopeptides in the digest (data not shown). This may be due to the unexpected digestion or nonspecific cleavage of apolipoprotein B100 as well as the multiple isoforms of the proteins.

LC/ESI MS/MS analysis of chymotryptic digest of apoB100

To determine the carbohydrate at undetected glycosylation sites in the tryptic digest including Asn1341 and 1350 (G4 and G5), which belong to the same tryptic peptide, the chymotrypsin digest was analyzed by LC/ESI MS/MS using the same methodology. Figure 5A shows a TIC of the TOF MS scan for the full scan m/z 700–2000. The collision energy was adjusted at 40–80 eV depending on the precursor ions. A TIC of the product ion scan and extracted ion chromatogram at m/z 204.05–204.15 (HexNAc) are presented in Figure 5B and 5C, respectively.

Figure 6A shows the product ion spectrum of 768.4 (+2) at 14 min for the chymotryptic glycopeptide. The carbohydrate B⁺ ions, y1 and b2 ions of peptide NW (residue 1341–1342), and peptide + GlcNAc ion were found in the product ion spectrum. The carbohydrate composition, [HexNAc]₂[Hex]₅, was deduced from the calculated carbohydrate molecular ion, 1234.6. Figure 6B shows the product ion spectrum of 1444.1 (+2) at 9 min for the glycopeptide. The carbohydrate B⁺ ions, peptide and peptide + GlcNAc ions, and peptide fragment ions from the peptide SGGNT-STDHF (residue 1347–1356) were detected in the product ion spectrum. Carbohydrate molecular weight, 1882.8, was calculated and the oligosaccharide composition, [HexNAc]₂[Hex]₆, was deduced from the molecular weight. The peptide fragment ions were also detected in the product ion spectrum for the chymotryptic glycopeptides as tryptic glycopeptides. The peptide and peptide + GlcNAc ions were detected in product ion spectra. These ions helped us determine the peptide moiety of the glycopeptide ion.

Results of the site-specific analysis of glycosylation of the chymotryptic digest are summarized in Table III. The oligosaccharide heterogeneity at each of 13 *N*-glycosylation sites was determined by LC/ESI MS/MS from the chymotryptic digest of apoB100 (Table III).

Carbohydrate diversity of each site

Results for the tryptic digest and chymotryptic digest of apoB100 are listed in Table IV. The oligosaccharide composition and type were deduced based on the molecular weight and previously reported oligosaccharide structures of apoB100. No information on glycosylation at Asn7 and 2533 (G1 and 8) was obtained from the analysis of the

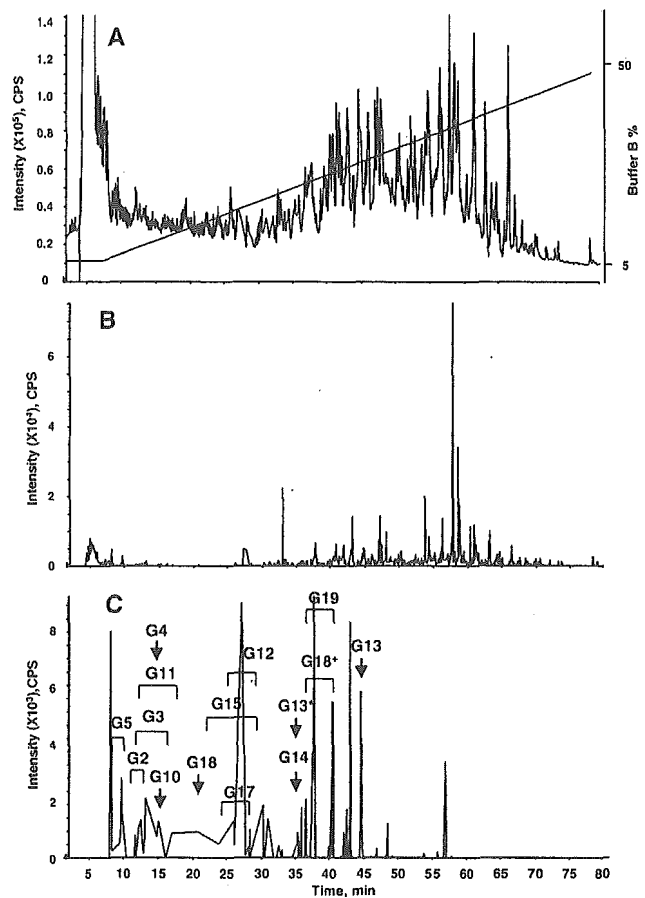


Fig. 5. LC/ESI MS/MS of chymotryptic digest of apolipoprotein B100. TIC of TOF MS scan for the full scan m/z 700–2000 and the HPLC gradient are indicated (A). TIC of the product ion scan data-dependently acquired (B). Extract ion chromatogram at m/z 204 of product ion spectra (C). Arrows and brackets denote glycopeptide fraction and *N*-glycosylation site ID. G13* was found to be oxidized at a methionine residue. G18* was found as missed cleaved glycopeptides.

tryptic or chymotryptic digest. When the tryptic digest of apoB100 was analyzed by LC/ESI MS/MS with the MS range m/z 400–2000, the sequence coverage of apoB100 was 41% and tryptic peptides containing Asn7, 2212, 2533, or 2955 (G1, 7, 8, or 10) were detected (data not shown). Together with the result of LC/ESI MS/MS with the MS range m/z 1000–2000, Asn7 and 2533 (G1 and 8) were not glycosylated or glycosylated only under detection sensitivity, and Asn2212, 2955, 3331, and 4404 (G7, 10, 14, and 19) were partially glycosylated. These findings indicate that 17 of 19 potential *N*-glycosylation sites in apoB100 were glycosylated.

The most heterogeneous oligosaccharides were found at Asn3384 (G15). Asn3384 possessed neutral or monosialylated hybrid and monoantennary complex type and mono- or disialylated biantennary complex type oligosaccharides as well as one high-mannose type oligosaccharide. Asn158, 1341, 1350, 3309, and 3331 (G2, 4, 5, 13, and 14) were occupied by high-mannose type oligosaccharides, whereas Asn956, 1496, 2212, 2752, 2955, 3074, 3197, 3438, 3868,

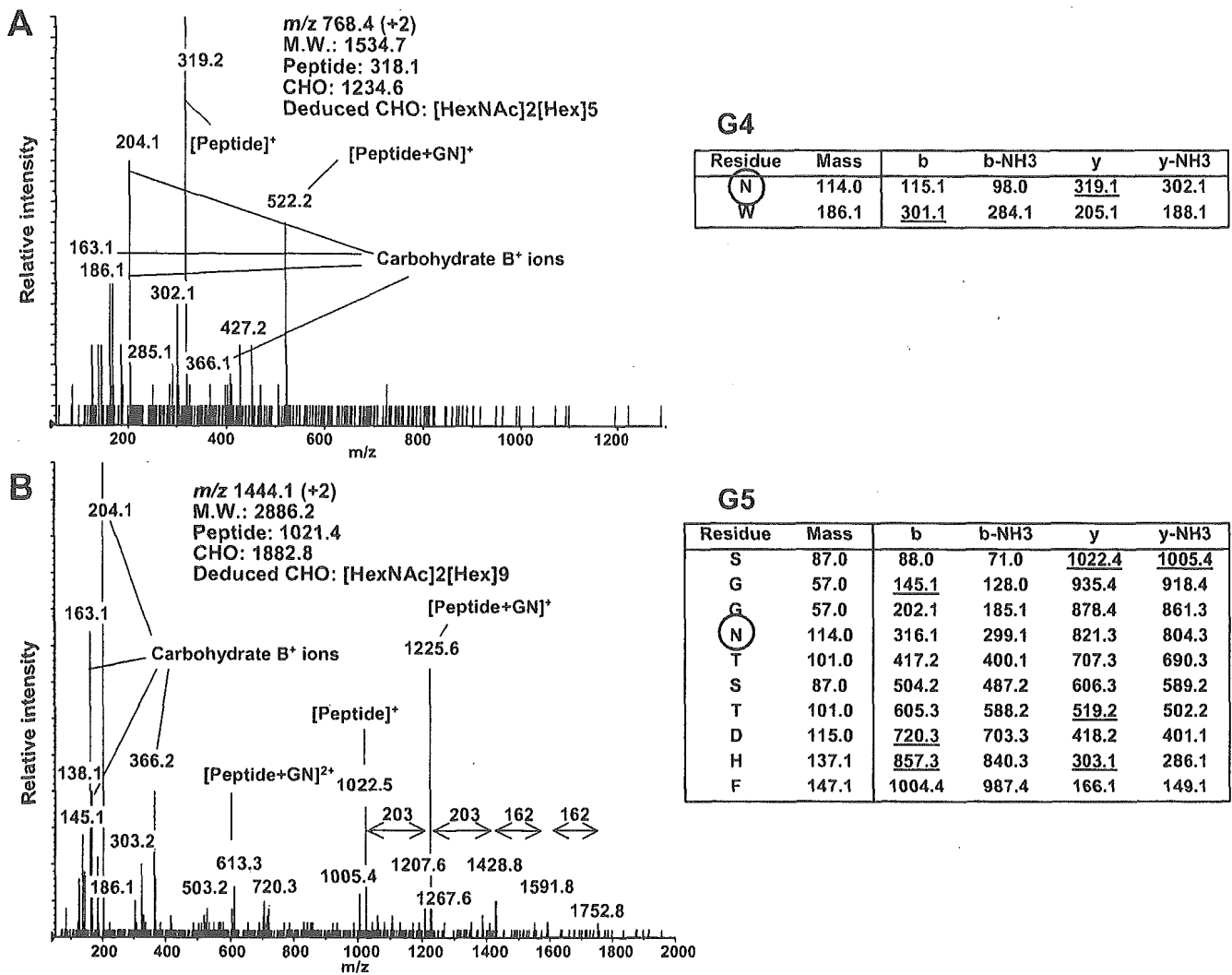


Fig. 6. Product ion spectra of the chymotryptic *N*-glycosylated peptides of apoB100. (A) Product ion spectrum of m/z 768.4 (+2) at 14 min for the glycopeptide consisting of residues 1341–1342. Peptide b and y ions of the peptide NW were found. Table shows m/z of the proposed b- and y-series fragment ions, and the detected ions are underscored. (B) Product ion spectrum of m/z 1444.1 (+2) at 9 min for the glycopeptide consisting of residues 1347–1356. Several ions are consistent with the b- and y-series fragment ions derived from the peptide SGGNTSTDHF. Table shows m/z of the proposed b- and y-series fragment ions, and the detected ions are underscored.

4210, and 4404 (G3, 6, 7, 9–12, and 16–19) were predominantly occupied by monosialylated or disialylated biantennary complex type oligosaccharides (Figure 7).

Discussion

Although the role of the carbohydrate structures in LDL and/or apoB100 has been examined in several studies (Attie *et al.*, 1979; Filipovic *et al.*, 1979; Fujioka *et al.*, 2000; Orekhov *et al.*, 1989; Shireman and Fisher, 1979), it is still unknown. It is necessary to elucidate the diversity of the oligosaccharides at each *N*-glycosylation site. This is the first report on the characterization of *N*-linked oligosaccharides in apoB100 at each glycosylation site. The protein was initially carboxymethylated and digested with an enzyme

(trypsin or chymotrypsin), and then the complex mixtures of peptides and glycopeptides were subjected to LC/ESI MS/MS analysis. Product ion scan of each precursor ion was carried out in a data-dependent manner. The glycopeptide molecular ions were easily distinguished from peptide ions by the presence of carbohydrate-related oxonium ions, such as m/z 204 (HexNAc), 186 (HexNAc-H₂O), 168 (HexNAc-H₂O), 366 (HexHexNAc), and others in product ion spectra. Furthermore, product ion spectra provided information for the elucidation of the amino acid sequence of the glycopeptides.

The oligosaccharide structure could be deduced based on the calculated molecular weight of the oligosaccharide moiety. The glycopeptide precursor ion was assigned using three strategies. (1) By comparing the product ions of the glycopeptide with the expected fragment ions derived from