数のバンドが確認されたため、次に１M 尿素を添加したバッファーで精製を試みた。尿素を添加しないときと比較し、ピークがシャープになり、またクロマトパターンもシンプルになった。そこでDEAE 樹脂を初期精製用のカラムとして選択し、シグナルが確認されたフラクションを集めて、0.86 M の硫安を添加し、Butyl-S カラムに供した。

　疎水性カラムである HiTrap Butyl-S カラムによる精製のため、まず硫酸アンモニウム（硫安）での塩溶限界を調べた。その結果、20％飽和硫安では沈殿しないものの、30％飽和硫安では沈殿することが確認された。次に 20％飽和硫安（0.86 M）にしたサンプルをカラムにかけ、0％硫安のバッファーでグラジエント溶出を行なったが、HBsAg の溶出は確認されなかった。そこで、１M 尿素、0.86 M 硫安を含むバッファーを用い、0％硫安のバッファーでグラジエント溶出を行なったが、残念ながら HBsAg の溶出は確認されなかった。カラムを洗浄する目的で H2O による溶出を行なったところ、ポリクローナル抗体（ANT-163）を用いたウエスタンブロットでのみ HBsAg のシグナルを確認することが出来た。条件検討をさらに行い、初期バッファー：１M 尿素、１M 硫安、0.2％ Tween-20 を含む 10 mM リン酸ナトリウムバッファー（pH 7.0）、溶出を H2O とすることにより、Butyl-S カラムでの HBsAg 精製が可能となることを確認した。

　次にゲルろ過を検討するため、分子量マーカー（フェリチン、チログロブリン、ブルーデキストラン）を HiPrep 16/60 Sephacryl S-300 HR カラムに供し、それぞれの溶出位置を確認した。次に HiPrep 16/60 Sephacryl S-300 HR カラムに Butyl-S の溶出画分を供した。クロマトパターンはブロードなシングルピークであったが、SDS-PAGE による純度確認を行なったところ、残念ながら全てのフラクションにシグナルが出

ており、また原因不明のタンパク質の混入が観察されたため、ゲルろ過での精製については再検討が必要である。

　次にスケールを５倍とし、カラムサイズも５倍量で検討した。培養液から沈殿させた HBsAg 溶液 80 ml 分を用いて、前処理、DEAE カラム、Butyl-S カラムでの精製を連続して行なってみた。最終的に得られた画分はタンパク質濃度が低く、初期の処理量を増やすことが必要であることが示唆された。そこで、再度サンプルを調製し、同様の精製を行なったところ、最終的に Butyl-S 溶出画分にシグナルが観察されたものの、それ以外の夾雑タンパク質も以前残っており、この方法では精製が難しいと考えられた。

　次に 5L のジャー培養を行ない、硫安添加により粗 HBsAg を回収した。その際、大量調製に合わせて条件の改変を加えた。まず粗 HBsAg の溶解に必要なバッファー量を増加させることで、回収量の向上が可能となった。また培養ロット間によって、酵母の色が変化することがあり、特に赤色を示すと HBsAg の生産量が増加することが示唆された。酵母が赤色を呈する場合、培地中のアデニンが不足している場合が多い。以前にも貧栄養培地で HBsAg の生産が多く見られたが、一方無機塩では酵母が生育しなかったため、培地の条件検討も今後の課題であると考えられた。

　これらを並行して複数ロットで進め、HBsAg の大量調製を試みた。得られた HBsAg について電気泳動を行ない、その純度を確認したが、まだまだ複数のタンパク質のバンドが見られたため、初期カラムを Butyl-S とし、次に DEAE カラムの順番で精製を行なうことにより精製度は高くなった。しかし 50kDa 付近のタンパク質が依然残っており、現在の方法では除くことは困難であると考えられた。次に非還元条件でのウエスタンブロット解析を行なったところ、HBsAg のシグナルが 250 kDa 以上の高分子側

に現れたことから、100 kDa cut off のフィルターを用いて濃縮することを検討した。膜へ吸着が見られたため、膜を濃縮液でよく洗い流して回収することで回収率を向上させたることができた。また依然 50kDa 付近のタンパク質が残っているものの、目的の HBsAg 以外の多くの 100 kDa 以下のタンパク質が除去できたため、フィルター処理が有効であることが示唆された。

D. 考察

今年度は HBs 抗原の精製について様々な条件検討を行った。当初考えていたよりも HBs 抗原の培養液中での挙動が不明な点が多く、精製も困難を極めた。

培養条件については、比較的低栄養源の 1x カザミノ酸-Ura 培地が適していると考えられた。また 120 時間培養まで HBs 抗原の発現の上昇が確認された。さらに長期間の培養についても今後の検討が必要であると考えられた。

培養液中では、HBs 抗原は微粒子またはタンパク質複合体として存在していることが示唆された。通常固液分離を行なう場合は、遠心法またはフィルターを用いる場合が多い。遠心法の場合、酵母菌体自体も 3000 rpm では沈殿するため、効率よい回収のためにはより回転を遅くして菌体との分離を行なうか、あるいは別の分離条件が望ましいと考えられた。またポアサイズ 1 μm のフィルターやグラスフィルター等の処理などを試みたが、酵母菌体と HBs 抗原をきれいに分離することが出来なかった。この点については今後更なる検討が必要である。

次に前処理の方法であるが、過去に報告された pre-S2 を含む HBs 抗原の精製方法として尿素による変性が報告されており、本研究でも尿素やグアニジン塩酸による変性と、その後の透析で HBs 抗原のフィルター処理を可能とした。これによりカラムによる精製が可能となった。

カラム処理では、当初安価な DEAE カラムを初期精製に用い、次に Butyl-S カラム、ゲルろ過の精製行程を検討したが、前処理から脱塩操作なしで精製行程へ持ち込むことが出来る Butyl-S カラムを初期精製に用い、その後 DEAE カラムとフィルター処理を行なうことで HBs 抗原を精製することとした。ゲルろ過については大量精製には不向きであることに加え、今回の精製においては新たな不純物の混入もあったため、使用を中断した。最後にフィルター処理であるが、非還元条件で SDS-PAGE を行なったところほとんどゲルに入らず、泳動されなかったため、かなり高分子の複合体あるいは粒子状になっていると考えられた。従って 100 kDa cut off のフィルターはかなり効果的であった。しかし依然 50 kDa の分子量を有するタンパク質が混入しているため、前処理の段階でもう少し効率よく除く方法を検討することが必要である。またこのタンパク質を同定し、可能であれば遺伝子破壊を行なって混入を抑制することも検討したい。

E. 結論

2 種類の HBs 抗原を酵母で発現し、その培養上清から糖鎖付加された HBs 抗原の精製を試みた。培養条件、前処理法、カラムによる精製、フィルター処理等を行ない、HBs 抗原の精製方法を構築した。

F. 研究発表
1. 論文発表
なし

2. 学会発表
なし

G. 知的財産権の出願・登録状況（予定を含む。）
1. 特許取得
なし

2. 実用新案登録
   なし

3. その他
   なし

Ⅲ.研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Kuno A, Sato T, Shimazaki H, Unno S, Saitou K, Kiyohara K, Sogabe M, Tsuruno C, Takahama Y, Ikehara Y, **Narimatsu H.**	Reconstruction of a robust glycodiagnostic agent supported by multiple lectin-assisted glycan profiling.	Proteomics Clin Appl	7(9-10)	642-647	2013
Kaji H, Ocho M, **Togayachi A, Kuno A,** Sogabe M, Ohkura T, Nozaki H, Angata T, **Chiba Y,** Ozaki H, Hirabayashi J, Tanaka Y, **Mizokami M,** Ikehara Y, **Narimatsu H.**	Glycoproteomic Discovery of Serological Biomarker Candidates for HCV/HBV Infection-associated Liver Fibrosis and Hepatocellular Carcinoma.	J Proteome Res	12(6)	2630-2640	2013
Yoshio S, Kanto T, Kuroda S, Matsubara T, Higashitani K, Kakita N, Ishida H, Hiramatsu N, Nagano H, Sugiyama M, Murata K, Fukuhara T, Matsuura Y, Hayashi N, M **Mizokami M,** Takehara T.	Human blood dendritic cell antigen 3 (BDCA3)(+) dendritic cells are a potent producer of interferon-lambda in response to hepatitis C virus.	Hepatology	57(5)	1705-1715	2013
Korenaga K, **Korenaga M,** Teramoto F, Suzuki T, Nishina S, Sasaki K, Nakashima Y, Tomiyama Y, Yoshioka N, Hara Y, Moriya T, Hino K	Clinical usefulness of non-protein respiratory quotient measurement in non-alcoholic fatty liver disease.	Hepatol Res	43(12)	1284-1294	2013
Murakami S, Takaoka Y, Ashida H, Yamamoto K, **Narimatsu H, Chiba Y.**	Identification and characterization of endo-ß-N-acetylglucosaminidase from methylotrophic yeast Ogataea minuta.	Glycobiology	23(6)	736-744	2013

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Tomimoto K, Fujita Y, Iwaki T, **Chiba Y**, Jigami Y, Nakayama K, Nakajima Y, Abe H.	Protease-deficient Saccharomyces cerevisiae strains for the synthesis of human-compatible glycoproteins.	Biosci Biotechnol Biochem	77(12)	2461-2466	2013
Akkarathamrongsin S, Hacharoen P, Tangkijvanich P, Theamboonlers A, Tanaka Y, **Mizokami M**, Poovorawan Y.	Molecular epidemiology and genetic history of hepatitis C virus subtype 3a infection in Thailand.	Intervirology	56(5)	284-294	2013
Watanabe T, Sugauchi F, Tanaka Y, Matsuura K, Yatsuhashi H, Murakami S, Iijima S, Iio E, Sugiyama M, Shimada T, Kakuni M, Kohara M, **Mizokami M.**	Hepatitis C virus kinetics by administration of pegylated interferon-alpha in human and chimeric mice carrying human hepatocytes with variants of the IL28B gene.	Gut	62(9)	1340-1346	2013
Yotsuyanagi H, Ito K, Yamada N, Takahashi H, Okuse C, Yasuda K, Suzuki M, Moriya K, **Mizokami M,** Miyakawa Y, Koike K.	High levels of hepatitis B virus after the onset of disease lead to chronic infection in patients with acute hepatitis B.	Clin Infect Dis	57(7)	935-942	2013
Tan B, Matsuda A, Zhang Y, **Kuno A, Narimatsu H.**	Multilectin-assisted fractionation for improved single-dot tissue glycome profiling in clinical glycoproteomics.	Mol Biosyst	10(2)	201-205	2014
Ocho M, **Togayachi A,** Iio E, Kaji H, **Kuno A,** Sogabe M, **Korenaga M,** Gotoh M, Tanaka Y, Ikehara Y, **Mizokami M, Narimatsu H.**	Application of a glycoproteomics-based biomarker development method: alteration in glycan structure on colony stimulating factor 1 receptor as a possible glycobiomarker candidate for evaluation of liver cirrhosis.	J Proteome Res	13(3)	1428-1437	2014
Ito K, Yotsuyanagi H, Yatsuhashi H, Karino Y, Takikawa Y, Saito T,	Risk factors for long-term persistence of serum hepatitis B surface antigen following acute	Hepatology	59(1)	89-97	2014

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Arase Y, Imazeki F, Kurosaki M, Umemura T, Ichida T, Toyoda H, **Yoneda M**, Mita E, Yamamoto K, Michitaka K, Maeshiro T, Tanuma J, Tanaka Y, Sugiyama M, Murata K, Masaki N, **Mizokami M.**	hepatitis B virus infection in Japanese adults.				
Murata K, Sugiyama M, Kimura T, Yoshio S, Kanto T, Kirikae I, Saito H, Aoki Y, Hiramine S, Matsui T, **Ito K, Korenaga M,** Imamura M, Masaki N, **Mizokami M.**	Ex vivo induction of IFN-lambda3 by a TLR7 agonist determines response to Peg-IFN/Ribavirin therapy in chronic hepatitis C patients.	J Gastroenterol	49(1)	126-137	2014
Khudayberganova D, Sugiyama M, Masaki N, Nishida N, Mukaide M, Sekler D, Latipov R, Nataliya K, Dildora S, Sharapov S, Usmanova G, Raxmanov M, Musabaev E, **Mizokami M.**	IL28B Polymorphisms and Clinical Implications for Hepatitis C Virus Infection in Uzbekistan.	PLoS One	9(3)	e93011	2014
Nishida N, Sawai H, Kashiwase K, Minami M, Sugiyama M, Seto WK, Yuen MF, Posuwan N, Poovorawan Y, Ahn SH, Han KH, Matsuura K, Tanaka Y, Kurosaki M, Asahina Y, Izumi N, Kang JH, Hige S, Ide T, Yamamoto K, Sakaida I, Murawaki Y, Itoh Y, Tamori A, Orito E, Hiasa Y, Honda M, Kaneko S,	New Susceptibility and Resistance HLA-DP Alleles to HBV-Related Diseases Identified by a Trans-Ethnic Association Study in Asia.	PLoS One	9(2)	e86449	2014

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Mita E, Suzuki K, Hino K, Tanaka E, Mochida S, Watanabe M, Eguchi Y, Masaki N, Murata K, Korenaga M, Mawatari Y, Ohashi J, Kawashima M, Tokunaga K, Mizokami M.					
Toshima T, Shirabe K, Ikegami T, Yoshizumi T, Kuno A, Togayachi A, Gotoh M, Narimatsu H, Korenaga M, Mizokami M, Nishie A, Aishima S, Maehara Y.	A novel serum marker, glycosylated Wisteria floribunda agglutinin-positive Mac-2 binding protein (WFA+-M2BP), for assessing liver fibrosis.	J Gastroenterol			2014 (In Press)

Ⅳ.研究成果の刊行物・別刷

TECHNICAL BRIEF

Reconstruction of a robust glycodiagnostic agent supported by multiple lectin-assisted glycan profiling

Atsushi Kuno[1#], Takashi Sato[1#], Hiroko Shimazaki[1], Sachiko Unno[1], Kozue Saitou[1], Katsue Kiyohara[1], Maki Sogabe[1], Chikayuki Tsuruno[2], Youichi Takahama[2], Yuzuru Ikehara[1], Hisashi Narimatsu[1*]

[1]Research Center for Medical Glycoscience (RCMG), National Institute of Advanced Industrial Science and Technology (AIST), 1-1-1 Umezono, Tsukuba, Ibaraki, 305-8568, Japan.
[2]Immunology & Chemistry Product Engineering, ICH Business Unit, Sysmex Corporation, 4-4-4 Takatsukadai, Nishi-ku, Kobe, Hyogo 651-2271, Japan

#These authors contributed equally to this study.

*Contact Information: Hisashi Narimatsu, Ph.D., M.D.

Research Center for Medical Glycoscience (RCMG), National Institute of

Advanced Industrial Science and Technology (AIST), 1-1-1 Umezono, Tsukuba,

Ibaraki 305-8568, Japan.

Tel: +81-29-861-3200

E-mail: h.narimatsu@aist.go.jp

Statement of clinical relevance

"Glycodiagnosis" is a new paradigm of clinical diagnosis based on quantitation of glyco-alteration of cells and their secreted molecules as a biomarker that reflects disease progression well. To quantitate such proteins bearing disease-specific changes in glycosylation, a lectin–antibody sandwich immunoassay has been proposed for developing glycodiagnosis. This study mostly focused on the construction of a robust calibrator and a standardized parameter (the cutoff index) involved in a glycodiagnostic agent, FastLec-Hepa. This agent has been developed for use in the assessment of disease severity and in evaluating the efficacy of therapy; for instance, monitoring of the degree of liver fibrosis along with antiviral therapy in cases of viral hepatitis. This unique technical approach supported by multiple lectin-assisted glycan profiling is applicable to the development pipeline for a wide variety of glycodiagnostic tools. We believe our approach is capable of revolutionizing the use of glycodiagnosis in clinical medicine and provides a framework for the development of a new generation of biomarker assays.

Abstract

Purpose: *Wisteria floribunda* agglutinin-positive human Mac-2 binding protein (WFA⁺-hM2BP) was recently validated as a liver fibrosis glycobiomarker with a fully automated lectin-antibody sandwich immunoassay. In this study, we supplied recombinant WFA⁺-hM2BP as the standard glycoprotein and the overlaid antibody to enhance the robustness of WFA⁺-hM2BP quantification.

Experimental design: The optimum conditions for producing recombinant WFA⁺-hM2BP were selected by cell glycome analysis based on a lectin microarray. Interlot variability of recombinant WFA⁺-hM2BP was determined using an antibody-overlay lectin microarray. Screening of anti-M2BP monoclonal antibody was completed by incorporating a WFA-antibody sandwich ELISA and an antibody-overlay lectin microarray.

Results: The lectin microarray analysis revealed that human embryonic kidney 293 (HEK293) cells efficiently and stably produced WFA⁺-hM2BP in DMEM containing 10% FCS without any lot variation in the M2BP glycosylation level. A spiking experiment with recombinant WFA⁺-hM2BP was mostly effective for antibody screening. The reconstituted sandwich immunoassay was useful for the continuous quantification and cutoff index (COI) expression of serum

WFA$^+$-hM2BP.

Conclusions and clinical relevance: The multiple use of lectin-assisted glycan profiling enabled us to construct a reliable sandwich assay kit for monitoring liver fibrosis in patients with viral hepatitis. This will assist in the development pipeline for other glycodiagnostic agents.

The close relationship of alterations in glycosylation (glyco-alterations) with the biological phenomena or diseases has long been reported in many studies. The annotation of such glyco-alterations on specific glycoproteins has been challenging for technical reasons and few outputs feasible for clinical use have been proposed [1]. A few excellent examples of diagnostic systems, referred to as "glycodiagnostic agents", have been developed based on highly practical and promising markers such as the ratio of core fucosylation in α-fetoprotein (AFP-L3%) [2]. In the past decade, biomarker development has been pipelined along with the assistance of recent revolutionary progress in proteomic technologies [3] and the pathway for applying glycoprotein biomarkers has been established successively from the discovery phases to verification (see Supporting Information Fig. S1) [4]. Furthermore, various unique systems assisted by advanced technologies such as "lab-on-a-chip" have been proposed for the detection of glycoprotein biomarkers [5, 6]. However, a complete form of the "glycodiagnostic agent" has not yet been developed.

To develop reliable systems toward clinical implementation and therapeutic benefits, glycoprotein biomarkers should be quantified by a sandwich immunoassay based on an anti-glyco-epitope reagent [7], in which lectins are

employed widely as the reagents. The sandwich immunoassay can be classified

unambiguously based on the immobilized substance on the plate: either as an

antibody raised against the protein moiety or as a lectin probing the glyco-epitope

[8]. No matter which system is selected, a complex problem of detection noise will

arise from unfavorable direct interactions between the lectin and antibody, as well

as the abundant serum proteins such as albumin and IgG. However, there is no

versatile method to resolve this, so researchers have been addressing these

problems specifically with regard to the detection of each target molecule [8–12].

In addition, a diagnostic system has to be developed along with the

establishment of a recombinant glycoprotein as a robust calibrator and the cutoff

index (COI) for quality control before commercialization for practical use.

   Here, we introduce our efficient approach to these important tasks based on

glycan profiling including a lectin array analysis (Supporting Information Fig. S1)

[13], which was employed in the development of our novel glycan-based

sandwich immunoassay system for quantifying fibrosis, FastLec-Hepa [8].

   FastLec-Hepa automatically detects the *Wisteria floribunda* agglutinin

(WFA)-positive human Mac-2 binding protein (WFA$^+$-hM2BP), which has been

confirmed as a marker glycoprotein having fibrosis-related glyco-alteration. In

other words, it serves as a M2BP glycosylation isomer (M2BPGi) for estimating

the progression level of fibrosis. We validated the correlation of FastLec-Hepa

counts with the degree of fibrosis using samples from 209 patients with chronic

hepatitis (CH) at two locations and documented its possible clinical utility for

evaluating therapy by quantifying the degree of disease severity. In particular, the

count-dependent monitoring for long-term follow-up (up to 3 years) of severely

affected patients with any antiviral treatments was considered to be effective in

the prevention of liver-related morbidity and mortality such as hepatic

decompensation and hepatocellular carcinoma. To achieve the continuous

monitoring based on the "on-site measurement", the assay must be standardized

and the obtained counts should be normalized with an appropriate COI value.

These might also achieve standardization in measuring disease severity and

planning therapies. From these viewpoints, we decided that our validated

FastLec-Hepa assay could be reconstructed effectively (see the final step of the

developmental pipeline shown in Supporting Information Fig. S1) by creating a

reliable calibrator.

In principle, as FastLec-Hepa monitors the level of fibrosis-related hM2BPGi,

it requires an assay calibrator with dual epitopes: a glycan that is recognized by

WFA and an hM2BP peptide that reacts to an anti-hM2BP monoclonal antibody (mAb). Recombinant proteins for use as assay calibrators are generally produced in bacterial or yeast expression systems because of their high productivity and cost-effectiveness. However, these were not suitable for our purpose as the recombinant proteins produced from bacterial cells had no glycans and those from yeast cells contained only high mannose-type glycans, neither recognized by WFA. In our system, a mammalian cell line exhibiting WFA positivity was needed to produce the recombinant hM2BP (rhM2BP) as the assay calibrator. It was important to select a suitable host cell line for expressing rhM2BP because WFA positivity for hM2BP would depend on the glycosynthetic machinery of each cell line. To select host cells with prominent WFA reactivity, we first conducted a lectin microarray analysis on culture supernatants from two mammalian cell lines (Supporting Information Fig. S2), Chinese hamster ovary (CHO) cells and human embryonic kidney (HEK293) cells, both of which are popular for producing recombinant proteins. Both cell lines were obtained from the American Type Culture Collection (Manassas, VA) and were cultured in DMEM and RPMI-1460 medium (Gibco, Grand Island, NY) without FCS for 48 h. In the lectin microarray analysis under the direct-labeling method with Cy3, the culture supernatant of the

HEK293 cells exhibited higher WFA signals than that of the CHO cells (Fig. 1A). This result indicated that HEK293 cells had the potential to produce glycoproteins with higher WFA reactivity. Regarding the culture conditions, the lectin microarray also showed that DMEM was more suitable for a high WFA response than RPMI-1460 (Fig. 1A). Therefore, we finally established a stable cell line using HEK293 cells as a host.

To confirm the WFA positivity of rhM2BP expressed by HEK293 cells and to determine the interlot variability of production simultaneously, rhM2BP was subsequently expressed in HEK293 cells four times on different days and purified twice from each culture medium (Supporting Information Fig. S3 and Methods). A comparative glycan profiling of rhM2BP with signal intensities of the lectin microarray suggested WFA-positivity for rhM2BP without remarkable difference among the eight purification variants (Fig. 1B). Focusing on the WFA signals showing slight differences in intensity (CV = 15.7%, in the *left* panel of Fig. 1C), which might be attributed to variability in protein concentration, we further qualified the reactivity of each sample against WFA on the lectin microarray using data processing as described previously [12]. In this case, it would be appeared as a quantitative difference in rhM2BP in the level of a terminal Gal/GalNAc

marker of *N*-glycan, one of the WFA ligands [8]. The rate of produced asialo form shows an almost negative correlation with the ratio of sialylation. Therefore, data processing using the 6′-sialyllactosamine-binding lectin from *Sambucus sieboldiana* (SSA) as a normalizer enabled the precise qualification of WFA reactivity. As we expected, the result suggested that there was no qualitative difference among the production lots of rhM2BP in terms of the amount of the WFA ligand (CV = 5.6%, see the *right* panel of Fig 1C). Collectively, we constructed the protocol for production of rhM2BP as the assay calibrator.

We next examined the construction of an anti-rhM2BP mAb optimized for the sandwich immunoassay. Mice were immunized with rhM2BP and 13 productive mAb clones were selected based on their productivity and reactivity against rhM2BP. The mAb produced from each clone was purified (Supporting Information Fig. S4) and then biotinylated. Biotinylated mAb (25 ng per assay) was overlaid on the lectin microarray that had been incubated with rhM2BP overnight. The suitability of each mAb as the detecting antibody was assessed from the signal-to-noise ratio in the lectin–antibody sandwich immunodetection system (Supporting Information Figs S5 and S6). As a result, all mAb clones except for No. 2, with a high signal-to-noise ratio, were selected at this step. After