Brief Report

Preparation of asialo-agalacto-glycophorin A for screening of anti-Tn antibodies

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Summary **Oncogenic antigens such as Tn-antigen (GalNAcα-Ser/Thr) are involved in metastatic processes and are associated with a poor prognosis, thus representing excellent targets for cancer intervention. Available anti-Tn antibodies which can be applied for therapeutics or diagonostics are severely limited mostly because the Tn-antigen epitope by itself is too small to be antigenic in addition to the fact that many carbohydrates are self-antigens. To characterize anti-Tn monoclonal antibodies as well as to perform panning and screening for isolation of anti-Tn single chain variable fragments from phage-display libraries, a large quantity of inexpensive Tn-antigens are needed. In this study, thus, glycophorin A which is a highly glycosylated sialoglycoprotein with approximately 12 *O*-glycans was sequentially treated with sialidase and β-galactosidase to remove sialic acid and galactose residues. The resulted product was shown to be an asialo-agalacto-glycophorin A which is reactive to an anti-Tn-antigen antibody. The simple preparation procedures described here would greatly help production and characterization of potentially valuable anti-Tn-antigen antibodies, which can be readily developed for cancer therapeutics and diagnostics.**

Keywords: Glycophorin A, anti-Tn antibodies/Tn-antigen, oligosaccharides, glycotechnology

1. Introduction

Tumor-associated carbohydrate antigens, so-called oncogenic antigens, are involved in metastatic processes and are associated with a poor prognosis, thus representing excellent targets for cancer intervention. Tn-antigen (GalNAcα-Ser/Thr) and T-antigen (Galβ1-3GalNAcα-Ser/Thr) are such antigens associated with carcinomas and are generally masked by covalently linked terminal carbohydrate moieties in normal human tissues but are exposed in most primary and metastatic epithelial malignant tumors (*1*). Two anti-Tn-antigen specific monoclonal antibodies (mAbs) have been independently produced as a result of immunizing mice with either colon cancer cells or primary breast tumors (*2*,*3*). MLS128 is an IgG_3 that recognizes the structure of three consecutive Tn antigens (Tn3) whereas 84D4 is an IgM that recognizes two and three consecutive GalNAcα-Ser/Thr residues (Tn2 and Tn3, respectively) with similar affinity (*4*,*5*).

Our recent studies revealed that MLS128 treatment significantly inhibited colon and breast cancer cell growth by binding to 110-210 kDa glycoproteins on the cell surface, and that MLS128 treatment caused down-regulation of insulin-like growth factor-I receptor and epidermal growth factor receptor in LS180 colon cancer cells, suggesting that MLS128-inhibited cancer cell growth is in part mediated by down-regulation of growth factor receptors (*6*). Based on these findings, human-type antibodies against Tn3, which is the epitope for MLS128, were screened from a phage library displaying human single-chain variable fragments (scFvs) (*7*). For characterization of anti-Tn mAbs as well as panning and screening in isolation of anti-Tn scFvs, synthetic Tn3- or Tn2-peptide and the backbone peptide have been used (*5*,*7*). These peptides are suitable for precise measurements of affinities for either Tn2 or Tn3 epitope, but are rather costly when used for screening of anti-Tn-antigen antibodies. Tn2- and/or Tn3-containing glycoproteins are obviously alternative

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choices for screening purposes. Glycophorin A (GPA) contains major *O*-linked oligosaccharide structures, which have been extensively characterized (*8*). GPA is a highly glycosylated sialoglycoprotein containing approximately 12 *O*-glycans and one *N*-glycan. GPA peptides were previously isolated by HPLC and treated with neuraminidase and β-galactosidase after being immobilized on plastic plates for determination of specificity of MLS128 (*4*). Here, we report a bulk preparation of asialo-agalacto-GPA which can readily be used for screening anti-Tn antibodies.

2. Materials and Methods

2.1. *Materials*

GPA and β-galactosidase from bovine testes were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sialidase from *Arthrobactor ureafacience* was obtained from Nacalai Tesque (Kyoto, Japan). Biotinylated *Arachis hypogaea* (PNA) and *Maackia amurensis* (MAM) lectins were purchased from J-OIL MILLS (Tokyo, Japan). Streptavidin-horseradish peroxidase (streptavidin-HRP) was purchased from GE Healthcare Biosciences (Piscataway, NJ, USA). Anti-mouse IgG-HRP was obtained from Medical & Biological Laboratories (Nagoya, Japan). Anti-mouse secondary antibody labeled with biotin was from Kierkegaard & Perry Lab (Gaithersburg, MD, USA). 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS)/H_2O_2 was from Roche Diagnostics (Mannheim, Germany). Production and characterization of MLS128 were previously described (*2*,*4*).

2.2. *Sialidase treatment of GPA and detection of T-antigen by a lectin*

One mg of GPA was dissolved in 500 μL of distilled water. Four hundred fifty μL containing 0.9 mg of GPA were used for sialidase digestion at 37ºC for 24 h in a final volume of 790 μL of 0.2 M sodium acetate buffer, pH 5.0, containing 0.2 units of sialidase. After 2 h digestion, an additional 0.2 units of the enzyme were added to assure complete digestion. PNA-biotin was used to assess the removal of sialic acid residues from GPA by detection of T-antigen epitopes on GPA (*9*). Briefly, 5 μL were each taken from the reaction mixture at different time points, mixed with 45 μL of 10 mM phosphate buffered-saline, pH 7.4 (PBS), and plated in wells of a 96-well plate. After incubation for 1 h at room temperature, the wells were blocked by incubating with 50 mM Tris-buffered saline, pH 7.4 (TBS) containing 3% BSA at 37ºC for 1 h. To each well, PNA-biotin (50 μL of 5 μg/mL of TBS containing 1% BSA) was allowed to react for 1 h at room temperature. After washing with 200 μL TBS 5 times, the wells were mixed with 50 μL of 1,000-fold diluted streptavidin-HRP. After 1 h incubation at room temperature, the wells were washed with 200 μL TBS 5 times. The bound HRP was detected using ABTS as a substrate. After 30 min incubation in the dark, absorbance at 405 nm was measured using a plate reader (Model 680, Bio-Rad Laboratories, Hercules, CA, USA).

2.3. *β-galactosidase treatment of asialo-GPA and enzyme-linked immunosorbent assay (ELISA)*

The resulting asialo-GPA (~ 0.83 mg) was further treated with 0.1 units of β-galactosidase for 24 h at 37ºC in 3 mL of 50 mM acetate buffer, pH 4.6, containing 0.15 M NaCl. After 2 h incubation, an additional 0.1 units of β-galactosidase were added to assure complete digestion. The resulting asialo-agalacto-GPA solution was aliquoted and stored at −80ºC.

Reactivity of uncovered Tn-antigen epitopes on GPA to MLS128 was determined by ELISA. Briefly, 10 μL (containing ~ 2.8 μg of asialo-agalacto-GPA) before and after 24 h β-galactosidase digestion was placed in wells of a 96-well plate and incubated for 1 h at room temperature. The wells were blocked with 3% BSA/TBS as described above. To the wells, MLS128 (50 μL of 5 μg/mL 1% BSA/PBS) was added and then incubated for 1 h at room temperature. After washing as described above, 50 μL of 1,000-fold diluted anti-mouse IgG-HRP were added, followed by incubation for 1 h at room temperature. The procedures for HRP activity measurement were the same as described above.

2.4. *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting*

Four μg each of GPA, asialo-GPA, and asialo-agalacto-GPA were separated by SDS-PAGE on a 10% gel and stained with Coomassie Brilliant Blue (CBB). The same sets of samples separated by SDS-PAGE were transferred to an Immobilon-P transfer membrane (Millipore Co., Bedford, MA, USA). The membrane was blocked with 5% skim milk in TBS for 1 h at room temperature. After incubation with MLS128 (6.25 μg/mL) for 16 h at 4ºC, bound MLS128 was detected using the biotin-labeled anti-mouse IgG antibody, the Vectastain ABCAmPTM kit, and the Vector substrate kit (Vector Lab., Inc., Burlingame, CA, USA) as previously described (*6*).

3. Results and Discussion

The PNA reactivity of either 2 h- or 24 h-sialidase digested GPA was the same (Figure 1A). The results suggested that under the conditions used, the removal of sialic acid residues from GPA apparently was completed after 2 h digestion. Treatment of this asialo-GPA preparation with β-galactosidase for 24 h resulted

in production of MLS128 reactive asialo-agalacto GPA (Figure 1B). The stepwise removal of sialic acid and galactose residues from GPA was confirmed by SDS-PAGE/CBB-staining analysis (Figure 1C), which clearly showed reduction of apparent molecular sizes of the original, asialo-, and asialo-agalacto-GPAs. The appearance of Tn-antigen epitopes on the asialo-agalacto-GPA was clearly seen as a MLS128-reactive band (Figure 1D, lane 3). In contrast, the original and asialo-GPAs were not detected by MLS128 (Figure 1D, lanes 1 and 2, respectively). The LS180 colon cancer cell lysate (*6*) was included as a positive control (Figures 1C and 1D, lane4). These results demonstrated that Tn3 epitopes on GPA are exposed after treatments with sialidase and β-galactosidase. Assays carried out to examine the reactivity of GPA, asialo-GPA, and asialo-agalacto-GPA to MAM-lectin, PNA-lectin, and MLS128 revealed that sialic acid residues were not detected in asialo- and asialo-agalacto-GPA preparations by MAM-lectin and that Tn3/Tn2 antigens were not detected in BPA and asialo-GPA by MLS128, but that PNA-lectin, which did not bind to GPA as expected, bound to both asialo- and asialo-agalacto-GPA preparations (data not shown). The results obtained with PNA-lectin suggested the presence of T-antigens on the asialo-agalacto-GPA preparation.

Next, to determine optimum conditions for assaying anti-Tn-antigen antibodies, ELISA was carried out using MLS128 under reduced concentrations of either the antibody or the asialo-agalacto-GPA preparation in combination with increased ABTS incubation times. The results indicated that 5 μL of asialo-agalacto-GPA with 0.2 μg/mL of MLS128 (Figure 2A) or 5 μg/mL of MLS128 with < 0.625 μL of asialo-agalacto-GPA (Figure 2B) would be enough to detect the binding activity. Thus, asialo-agalacto-GPA prepared here would allow more than 3,000 assays for screening anti-Tn-antigen antibodies with similar affinity to that of MLS128. For anti-Tn-antigen single-chain antibodies with typically 1/100 less affinity than mAbs, however, 5 μL/well of asialo-agalacto-GPA and 6 h of incubation with ABTS should be tested first. The assay conditions

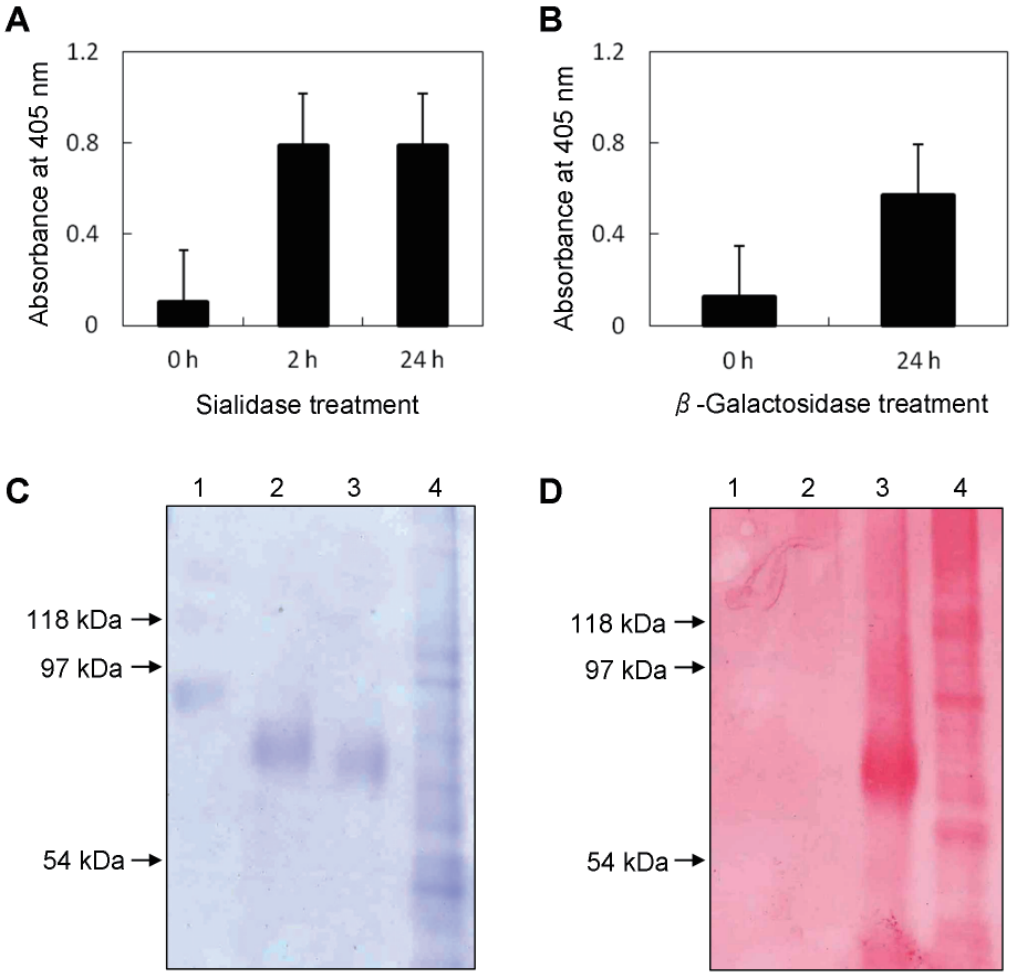

Figure 1. T- and Tn-antigenicity of GPA treated with sialidase (A) and β-galactosidase (B), and molecular size and Tn antigenecity analyzed by SDS-PAGE (C) and Western blotting (D). (A) T-antigen reactivity of GPA after sialidase digestion was monitored by binding to PNA as described in Materials and Methods. **(B)** Tn-antigen reactivity of GPA after β-galactosidase digestion was monitored by ELISA using MLS128 as described in Materials and Methods. **(C)** and **(D)** SDS-PAGE gel stained with CBB and Western blotting with MLS128, respectively. Lanes 1, 2, 3, and 4 contained GPA, asialo-GPA, asialo-agalacto-GPA, and LS180 cell lysate (as a positive control, *Ref. 6*), respectively.

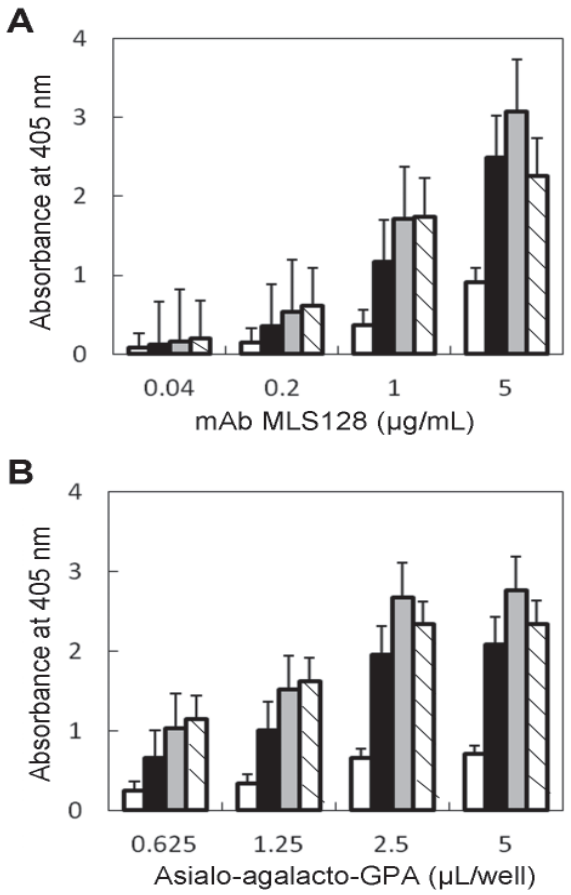

Figure 2. ELISA with various concentrations of MLS128 (A) or asialo-agalacto-GPA (B) in combination with different ABTS incubation times. (A) Five μL of asialo-agalacto-GPA per well were analyzed with 0.04, 0.2, 1, and 5 μg/mL of MLS128. **(B)** Various asialo-agalacto-GPA amounts (0.626, 1.25, 2.5, and 5 μL) were analyzed with 5 μg/mL of MLS128. The reactivity after ABTS incubation times of 15 min (open bar), 1 h (black bar), 2 h (gray bar), or 6 h (hatched bar) is shown.

then need to be optimized for the type of antibodies to be examined. Although enzymes used for digestions remained in the final asialo-agalacto-GPA solution, reproducible ELISA results have been obtained as shown in Figures 1 and 2, which indicated that the antibody-Tn-antigen reaction was not affected by the freed carbohydrates or traces of enzymes under the conditions used.

The simple preparation procedures described here would greatly help production and characterization of potentially valuable anti-Tn-antigen antibodies, which can be readily developed for cancer therapeutics and diagnostics.

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