Glycoconjugate Journal (1994) **11**:262–265

Coexpression of cancer-associated carbohydrate antigens, Tn and sialyl Tn

HIROSHI NAKADA, MIZUE INOUE, NOBUHIRO TANAKA and IKUO YAMASHINA*

Department of Biotechnology, Faculty of Engineering, Kyoto Sangyo University, Kita-ku, Kyoto 603, Japan

Received 2 May 1994, revised 23 May 1994

The expression of cancer-associated antigens, Tn and sialyl Tn, was examined using monoclonal antibodies, MLS 128 and MLS 102, recognizing these two antigens, respectively. A cell lysate from a human carcinoma cell line, LS 180 cells, was analysed by Western blotting using these two antibodies. Three glycoprotein bands were discernible with each antibody, of which two, corresponding to 250 and 210 kDa, were reactive with both the antibodies. LS 180 cells were metabolically labelled with ^{3}H-glucosamine and then the lysate from these cells was applied to two immunoaffinity columns. Sixty-five per cent of the Tn antigenic glycoproteins, based on radioactivity, bound to the MLS 102 affinity column. On the other hand, 45% of the sialyl Tn antigenic glycoproteins bound to the MLS 128 affinity column. These results indicate that some Tn and sialyl Tn antigens were expressed on the same polypeptide chains.

The presence of non-sialylated GalNAc residues on the polypeptide chain with many Sia-GalNAc residues appears to be due to the incapability of three consecutive moieties of GalNAc-Ser/Thr to accept sialic acid.

Keywords: Tn antigen; sialyl Tn antigen; coexpression; monoclonal antibodies

Abbreviations: PSMF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; GalNAc, *N*-acetylgalactosamine; Sia, sialic acid.

Various carcinoma cells produce high molecular weight mucins containing a large number of *O*-linked carbohydrate chains (*O*-glycans). Incomplete glycosylation is frequently observed in *O*-glycans [1]. In particular, some core structures of *O*-linked carbohydrate chains, such as Galβ1 → 3GalNAc-Ser/Thr(T) [2] and GalNAc-Ser/Thr(Tn) [3, 4], and sialylated core structures, such as NeuAcα2 →6GalNAc-Ser/Thr(Sialyl Tn) [5–7], are known to be cancer-associated antigens.

MLS 128 and MLS 102, which were established by immunizing mice with cultured LS 180 cells, recognized Tn and sialyl Tn, respectively [5, 8]. It has been reported that the majority of colon cancers and Tn erythrocytes expressed both the Tn and sialyl Tn antigens [9, 10]. We have shown the expression of the Tn and sialyl Tn antigens on the same polypeptide chains in LS 180 cells. It was further shown that the Tn antigens are incapable of accepting sialic acid from CMP-sialic acid in the presence of sialyltransferase.

* To whom correspondence should be addressed.

0282-0080 © 1994 Chapman & Hall

Materials and methods

Materials

MLS 128 and MLS 102 were prepared as described previously [5, 8]. Sepharose CL 6B, protein A and protein A-Sepharose were from Pharmacia. ^{3}H-Glucosamine-HCl was purchased from New England Nuclear. GalNAcα2,6-sialyltransferase, a product of gene cloning [11], was a gift from Dr S. Tsuji, The Institute of Physical and Chemical Research (RIKEN).

Cell culture and metabolic labelling

LS 180 cells, a human colorectal carcinoma cell line, were obtained from the American Type Culture Collection (Rockville) and cultured in Eagle's MEM supplemented with 10% fetal calf serum. Some of the cells were labelled with ^{3}H-glucosamine-HCl in MEM containing one-tenth of the glucose in the standard MEM for 20 h. The harvested cells were washed with phosphate-buffered saline, and then solubilized with 50 mM Tris-HCl buffer, pH 7.4, containing

1% Triton X-100, 0.2 M NaCl an 1 mM PMSF, and centrifuged at 105000 × **g** for 1 h. The supernatant from unlabelled cells was used for analysis by Western blotting and immunoprecipitation, and that from labelled cells for analysis by immunoaffinity chromatography.

Immunoprecipitation

MLS 128 was added to the cell lysate from LS 180 cells. After incubation at 4 °C for 2 h, protein A-Sepharose was added, followed by incubation for an additional 2 h. The immunoprecipitate was extensively washed with the solubilizing solution as described above. The glycoproteins bound to MLS 128 were freed from the antibody by boiling in 50 mM Tris-HCl buffer, pH 7.5, containing 1% sodium dodecyl sulfate (SDS).

Western blotting

SDS-polyacrylamide (SDS-PAGE) slab gel electrophoresis (6% gel) was performed using the buffer system of Laemmli [12]. Proteins separated by SDS-PAGE were transferred to a Zeta-probe membrane at 4 V cm^{-1} for 20 h. The additional surface was blocked by incubation with 5% bovine serum albumin in phosphate-buffered saline at 50 °C for 10 h. The membrane was incubated with monoclonal antibody at 4 °C for 20 h. After washing with 10 mM Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl and 0.05% Tween 20, the membrane was incubated with protein A-peroxidase at room temperature for 1 h. The membrane, after another wash with the above buffer, was treated with 0.03% diaminoazobenzene and 0.003% H_2O_2 in 15 mM phosphate buffer, pH 6.8, for visualization.

Affinity chromatography

Columns containing MLS 128 or MLS 102 coupled to protein A-Sepharose were prepared according to the method of Schneider *et al.* [13]. The cell lysate was applied to these columns (0.5 × 5 cm). After washing with the solubilizing solution, each column was eluted with 10 mM Tris-HCl buffer, pH 7.8, containing 0.2% Triton X-100 and 2 M guanidine thiocyanate. The eluate, after dialysis against 10 mM Tris-HCl buffer, pH 7.8, containing 0.2% Triton X-100, was further applied to another immunoaffinity column and the procedure was repeated once more.

Enzymatic sialylation

A mucin-type glycopeptide preparation (G50-I from LS 180 cells [14]) was coated on a plastic plate. CMP-[^{3}H]-sialic acid (0.5 nmol) and the sialyltransferase (1.5 × 10^{-6} U) were added and incubated up to 24 h. The reaction mixture was discarded and the binding of MLS 128 was determined using ^{125}I-labelled Protein A. Radioactivity due to sialic acid incorporated into the glycopeptide was determined after alkali treatment to release the carbohydrates.

Results

SDS-PAGE and Western blotting

The cell lysate from LS 180 cells was subjected to SDS-PAGE followed by transfer to a Zeta-probe membrane for Western blotting. The Tn antigen was expressed on three glycoprotein bands corresponding to molecular sizes of 250, 210 and 150 kDa (Fig. 1, lane b), whereas the sialyl Tn antigen was expressed on glycoprotein bands corresponding to 250, 210 and 170 kDa (Fig. 1, lane a), suggesting that the 250 and 210 kDa bands coexpressed the Tn and sialyl Tn antigens.

The coexpression of the two antigens could be further shown as follows. The LS 180 cell lysate was treated with either MLS 128 or with MLS 102 to yield the immunoprecipitates. The precipitates were subjected to SDS-PAGE followed by Western blotting using the antibodies, MLS 102 for the MLS 128-precipitate and MLS 128 for the MLS 102-precipitate. Bands of 250 and 210 kDa were detected for the two precipitates, thus identifying these bands as glycoproteins expressing both the Tn and sialyl Tn antigens.

Immunoaffinity chromatography

Glycoproteins carrying the Tn and sialyl Tn antigens were then subjected to analytical affinity chromatography. For this, the cell lysate from the ^{3}H-glucosamine labelled cells was divided into two aliquots. As shown in Fig. 2 (top), one was first applied to the MLS 128 immunoaffinity column and eluted as described under Materials and methods. About 6% of the radioactivity was eluted with the buffer

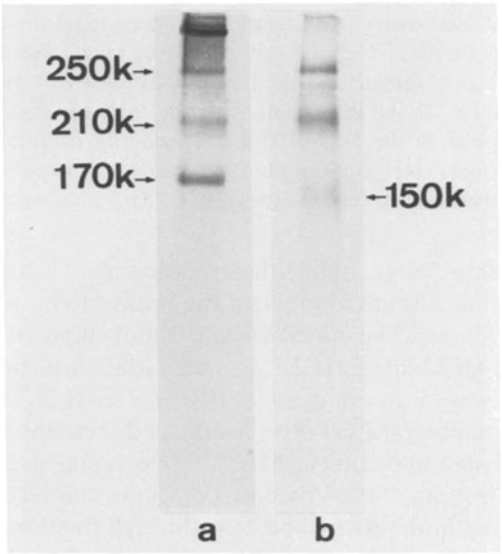

Figure 1. Western blotting analysis of glycoproteins carrying the Tn and/or sialyl Tn antigens. The LS 180 cell lysate (150 μg protein) was subjected to SDS-PAGE followed by transfer by a Zeta-probe membrane. Antigens were detected by successive incubations with monoclonal antibodies (a: MLS 102, b: MLS 128) and protein A-peroxidase, as described under Materials and methods.

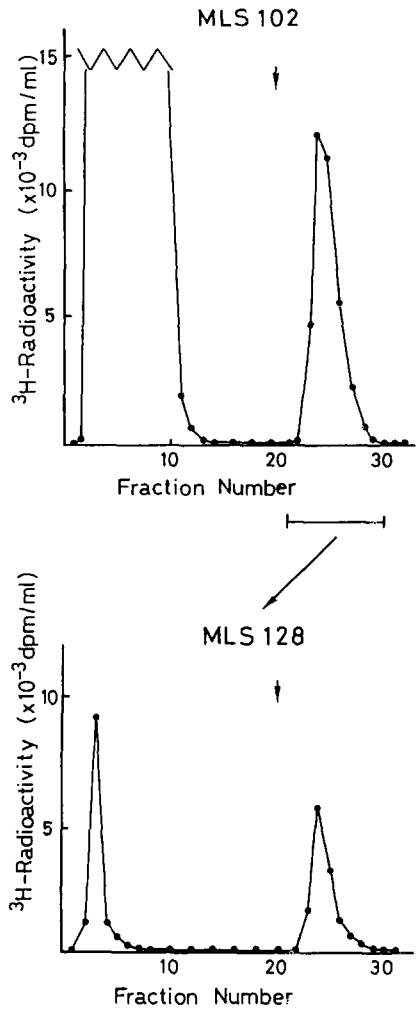

Figure 2. Successive immunoaffinity chromatography (MLS 128 followed by MLS 102). LS 180 cells were metabolically labelled with ^{3}H glucosamine and the lysate from these cells was applied to the MLS 128 immunoaffinity column (top), and then the eluate was applied to the MLS 102 immunoaffinity column (bottom). Equilibration and elution were carried out as described under Materials and methods. Fractions of 0.73 ml were collected.

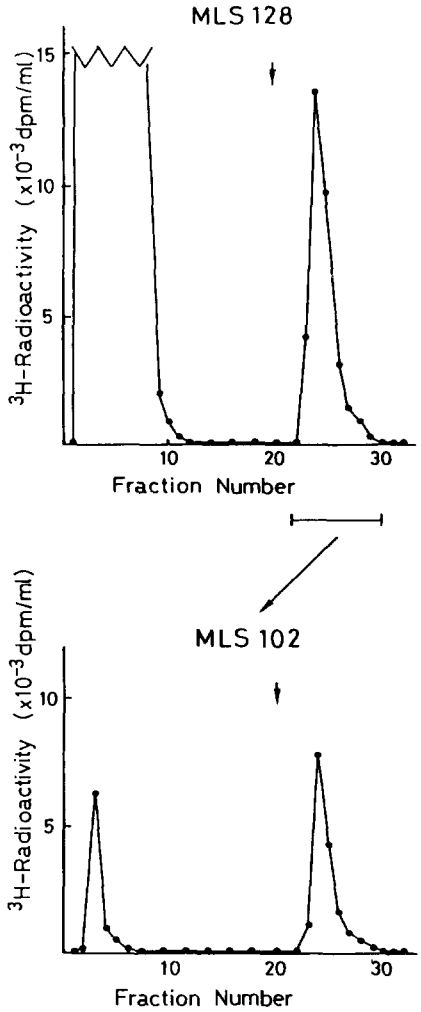

Figure 3. Successive immunoaffinity chromatography (MLS 102 followed by MLS 128). Two immunoaffinity chromatographies were performed as in Fig. 2, except in the reverse order. The eluate from the MLS 102 immunoaffinity column (top) was applied to the MLS 128 immunoaffinity column (bottom).

containing 2 M guanidine thiocyanate (arrow). It should be noted that a small amount of the radioactivity (much less than 6%) could be eluted when ethanolamine, pH 11.5, or glycine-HCl buffer, pH 2.3, was used (data not shown). The eluate was dialysed against 10 mM Tris-HCl buffer, pH 7.5, containing 0.2% Triton X-100, and then applied to the second immunoaffinity (MLS 102) column, as shown in Fig. 2 (bottom). Sixty-five and 35% of the radioactivity were recovered in the eluate and pass-through fractions, respectively, indicating that 65% of the antigenic glycoproteins, in terms of the ^{3}H-glucosamine radioactivity, coexpress the sialyl Tn antigen.

The other portion of the cell lysate was applied to the two immunoaffinity columns in the reverse order, as shown in Fig. 3. About 7% of the radioactivity was recovered in the first eluate. Forty-five per cent of the radioactivity of the sialyl Tn antigenic glycoproteins bound to the MLS 128 affinity column.

Enzymatic sialylation

To see whether three consecutive non-sialylated GalNAc residues (the essential Tn antigen epitope) could accept sialic acid residue from CMP-sialic acid catalysed by α2,6 sialyltransferase, the glycopeptide, G50-I from LS-180 cells, previously shown to carry the Tn epitope, was incubated with the sialyltransferase. The incorporation was marginal and the amount of bound anti-Tn antibody (MLS 128) showed no change during the incubation. As a positive control for this sialylation, asialo G50-I was used. About 700 dpm of ^{3}H was incorporated into G50-I at the plateau of the reaction whereas 20 000 dpm was incorporated into asialo G50-I and the incorporation was still progressive.

Discussion

We have recently reported that the essential epitopic structure of the Tn antigen is a cluster composed of three or four consecutive sequences of GalNAc-Ser/Thr [15, 16]. For the sialyl Tn antigen, a similar cluster structure has also been proposed, but the essential epitopic structure has not yet been definitely identified [6]. If any glycoproteins carry both the Tn and sialyl Tn antigens, they should comprise cluster structures of GalNAc-Ser/Thr and Sia-GalNAc-Ser/Thr at different positions on the polypeptide chain. Separate non-clustered GalNAc-Ser/Thr or Sia-GalNAc-Ser/Thr moieties exhibit essentially no reactivity with anti-Tn or anti-sialyl Tn antibodies, respectively. A glycoprotein preparation isolated by immunoaffinity chromatography using MLS 128 had the following *O*-glycan composition, GalNAc (49%), NeuAcα2,6GalNAc (44%), and longer oligosaccharide chains (7%) as reported previously [16]. This composition meets the compositional requisites for the Tn and sialyl Tn antigens.

Analytical immunoaffinity chromatography has revealed that 35% of the MLS 128-bound glycoproteins and 55% of the MLS 102-bound glycoproteins are specific to one antibody, no coexpression occurring in these fractions (Figs 2 and 3). From the analysis of the immunoprecipitates, it was concluded that the 170 kDa band carries only the sialyl Tn antigen and the 150 kDa band only the Tn antigen (Fig. 1).

In vitro sialylation was carried out. To help to reveal the mechanism for the synthesis of the Tn and sialyl Tn antigens on the same polypeptide chain. Results indicated that the Tn antigen was not susceptible to *in vitro* sialylation. This means that the Tn antigen, a unique cluster structure composed of $(GalNAc\text{-}Ser/Thr)_{3-4}$, cannot be recognized by the sialyltransferase. Whether this incapability of sialylation is due to the primary structure (amino acid sequence surrounding the Tn antigen) or to steric hindrance due to clustering remains to be investigated.

Acknowledgements

This work was supported in part by a Grant-in-Aid for Scientific Research and a Grant-in-Aid for Scientific Research on Priority Areas, No. 05274107, from the Ministry of Education, Science and Culture of Japan, by the Fugaku Trust for Medicinal Research, and by a Grant-in-Aid from the Sankyo Foundation of Life Science.

References

1. Hakimori S (1985) *Cancer Res* **45**:2405–14.
2. Stein R, Chen S, Grossman W, Goldenberg M (1989) *Cancer Res* **49**:32–7.
3. Hirohashi S, Clausen H, Yamada T, Shimosato Y, Hakomori S (1985) *Proc Natl Acad Sci USA* **82**:7039–43.
4. Takahashi HK, Metoki R, Hakomori S (1988) *Cancer Res* **48**:4361–67.
5. Kurosaka A, Fukui S, Kitagawa H, Nakada H, Numata Y, Funakoshi I, Kawasaki T, Yamashina I (1987) *FEBS Lett* **215**:137–39.
6. Kurosaka A, Kitagawa H, Fukui S, Numata Y, Nakada H, Funakoshi I, Kawasaki T, Ogawa T, Iijima H, Yamashina I (1988) *J Biol Chem* **263**:8724–26.
7. Kjeldsen T, Clausen H, Hirohashi S, Ogawa T, Iijima H, Hakomori S (1988) *Cancer Res* **48**:2214–20.
8. Numata Y, Nakada H, Fukui S, Kitagawa H, Ozaki K, Inoue M, Kawasaki T, Funakoshi I, Yamashina I (1990) *Biochem Biophys Res Commun* **170**:981–5.
9. Itzkowitz SH, Yuan M, Montgomery CK, Kjeldsen T, Takahashi HK, Bigbee WL, Kim YS (1989) *Cancer Res* **49**:197–204.
10. Kjeldsen T, Hakomori S, Springer GF, Desai P, Harris T, Clausen H (1989) *Vox Sang* **57**:81–7.
11. Kurosawa N, Hamamoto T, Lee YC, Nakaoka Y, Kojima N, Tsuji S (1994) *J Biol Chem* **269**:1402–9.
12. Laemmli UK (1970) *Nature (Lond.)* **227**:680–5.
13. Schneider C, Newman RA, Sutherland DR, Asser U, Greaves MF (1982) *J Biol Chem* **257**:10766–69.
14. Nakada H, Numata Y, Inoue M, Tanaka N, Kitagawa H, Funakoshi I, Fukui S, Yamashina I (1991) *J Biol Chem* **266**:12402–5.
15. Nakada H, Inoue M, Numata Y, Tanaka N, Funakoshi I, Fukui S, Mellors A, Yamashina I (1993) *Proc Natl Acad Sci USA* **90**:2495–9.
16. Nakada H, Inoue M, Numata Y, Tanaka N, Funakoshi I, Kitagawa H, Fukui S, Yamashina I (1992) *Biochem Biophys Res Commun* **187**:217–24.