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CANCER-ASSOCIATED GLYCOPROTEINS DEFINED BY A MONOCLONAL ANTIBODY, MLS 128, RECOGNIZING THE Tn ANTIGEN

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SUMMARY: A murine monoclonal antibody, MLS 128, recognizing the Tn antigen, was established and used for characterization of glycoproteins expressing the Tn antigen. The Tn antigen was expressed on three polypeptide chains with molecular weights of 250k, 210k and 150k daltons. LS 180 cells were labeled with ^{3}H-glucosamine or ^{35}S-sulfate metabolically, and then the immunoprecipitate derived from the cell lysate was subjected to SDS-PAGE followed by fluorography. It was revealed that these Tn antigen glycoproteins were produced through the processing of a high molecular weight precursor.

The carbohydrate moieties of the Tn antigen glycoproteins labeled with ^{3}H-glucosamine were released with alkaline-borohydride, and the released sugars were examined by gel filtration and paper chromatography. The carbohydrates predominantly consisted of GalNAc and sialyl GalNAc (>90%), with a nearly equal distribution.

Various cancer cells produce high molecular weight glycoproteins (mucins) with a large number of O-linked carbohydrate chains attached. It has been shown that mucins are often cancer-associated antigens recognized by a variety of monoclonal antibodies. These monoclonal antibodies are useful for cancer diagnosis and for monitoring of cancer progression (1, 2). For cancer-associated mucins, alterations in glycosylation have been observed, of which incomplete glycosylation occurs most frequently (1). In particular, some core structures of mucin-type glycoproteins, such as Galβ1→3GalNAc-Ser/Thr (T) (3) and GalNAc-

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Abbreviation: PMSF, phenylmethylsulfonylfluoride.

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Ser/Thr (Tn) (4,5), and sialylated T or Tn structures (6-8) are known to be cancer-associated antigens.

MLS 128, which was established by immunizing mice with cultured LS 180 cells, only reacted with mucin-type glycoproteins, i.e., not with serum-type glycoproteins. MLS 128 was identified as an anti-Tn antibody as it agglutinated Tn erythrocytes, and its binding to mucin glycoproteins such as cancer cell surface glycoproteins, or ovine submaxillary mucin (OSM) was inhibited competitively by NCC-LU-35 and CA 3239, which are known to recognize the Tn antigen (9).

Recently, we investigated the epitopic structure for MLS 128 and found that MLS 128 bound preferentially to a cluster structure of GalNAc-Ser/Thr including (GalNAc)-Ser-(GalNAc)-Thr-(GalNAc)-Thr (10). We now report the characterization of MLS 128 antigens on LS 180 cells.

MATERIALS AND METHODS

Materials. MLS 128 was prepared as described previously (9). Sepharose CL 4B, protein A and protein A-Sepharose were from Pharmacia, Uppsala. ^{3}H-Glucosamine, ^{35}S-sulfate and ^{3}H-ENHANCE were purchased from New England Nuclear, Boston. LS 180 cells, a human colorectal carcinoma cell line, were obtained from the American Type Culture Collection, Rockville.

SDS-PAGE and Western blotting. SDS-polyacrylamide slab gel electrophoresis was carried out using the buffer system of Laemmli with a 6 % gel (11). Gels for fluorography were treated with ^{3}H-ENHANCE, dried and then exposed to Kodak X-Omat AR film with an intensifying screen. Proteins separated by SDS-PAGE were transferred to a Zeta-probe membrane at 4 volts/cm for 20 hr. The remaining surface of the membrane was blocked by incubation with 5 % bovine serum albumin in phosphate-buffered saline (PBS) at 50°C for 10 h. The membrane was incubated with MLS 128 (10 μg/ml) at 4°C for 20 h. After washing with 0.15 M NaCl and 0.05 % Tween 20 in 10 mM Tris-HCl, pH 7.4, the membrane was incubated with protein A-peroxidase at room temperature for 1 h. The membrane, washed with the above buffer again, was visualized with 0.03 % diaminoazobenzene and 0.003 % H_2O_2 in 15 mM phosphate buffer, pH 6.8.

Metabolic labeling. Cells ($1x10^7$) were labeled with ^{3}H-glucosamine-HCl (10 μCi/ml) in MEM containing glucose in a one-tenth amount of complete MEM for 20 h. For pulse labeling, the cells were incubated for 5 h in the same medium and then chased in complete MEM. For ^{35}S-sulfate pulse-labeling, the cells were preincubated for 1 h in sulfate-free medium, labeled with ^{35}S-sulfate (25 μCi/ml) and then chased in sulfate-containing MEM. Harvested cells were washed with PBS, and then solubilized with 1 % Triton X-100, 0.2 M NaCl, 1 mM PMSF, in 50 mM Tris-HCl buffer, pH 7.4, and then the lysates were centrifuged at 105,000 x g for 1 h. Each supernatant thus obtained was used for immunoprecipitation. The antigen on the cell surface was detected according to the method of Spiro et al. (12). Cells labeled with ^{3}H-glucosamine for 5 h were dispersed and then incubated with the antibody at 4°C for 2 h. Excess antibody was washed off with PBS,

and then the cells were solubilized as described above and the lysates subjected to immunoprecipitation.

Immunoaffinity chromatography. MLS 128-protein A-Sepharose was prepared by the method of Schneider et al. (13). The affinity column (0.5 x 6 cm) was equilibrated with 0.2 % Triton X-100, 0.1 M NaCl, in 20 mM Tris-HCl buffer, pH 7.8. The cells were solubilized with the same solution and then the lysates were centrifuged as described above. The supernatant was applied to the immunoaffinity column. After extensive washing with the same solution, the column was eluted with 2 M guanidine thiocyanate, 0.1 M NaCl, 0.2 % Triton X-100, in 20 mM Tris-HCl buffer, pH 7.8.

Alkaline borohydride treatment. Alkaline borohydride treatment was carried out according to the method of Carlson (14). The released oligosaccharides were fractionated on a Sephadex G-15 column. Paper chromatography was performed as described previously (15).

RESULTS

SDS-PAGE and Western blotting of Glycoproteins with Tn-antigen. The LS 180 cell lysate was subjected to SDS-PAGE followed by transfer to a Zeta-probe membrane. The antigen was detected by protein A-peroxidase staining. The Tn antigen was found to be expressed on three polypeptide chains with molecular weights of 250k, 210k and 150k daltons (Fig. 1). To investigate the mutual relationship of these mucins with the Tn antigen, we performed pulse-chase analyses of LS 180 cells which had been metabolically labeled with ^{3}H-glucosamine-HCl (Fig. 2) or ^{35}S-

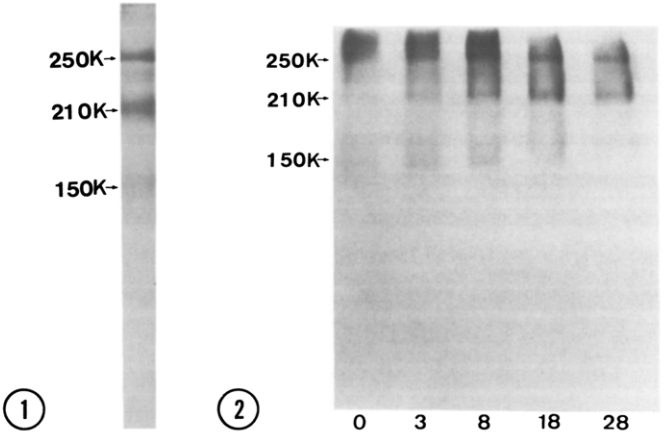


Fig. 1. The Tn antigen glycoproteins. An LS 180 cell lysate (150 µg protein) was subjected to SDS-PAGE followed by transfer to a Zeta-probe membrane. Tn antigens were detected by successive incubation with MLS 128 and protein A-peroxidase, as described under MATERIALS AND METHODS.

Fig. 2. Processing of the Tn antigen glycoproteins. LS 180 cells (1 x 10^{7}) were pulse-labeled with ^{3}H-glucosamine for 5 hr and then chased for various periods, as indicated in the figure. The immunoprecipitates obtained from the lysates were subjected to SDS-PAGE followed by fluorography.

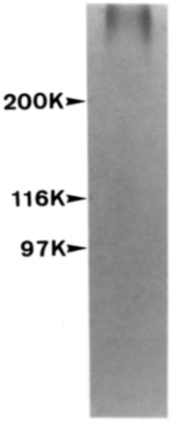
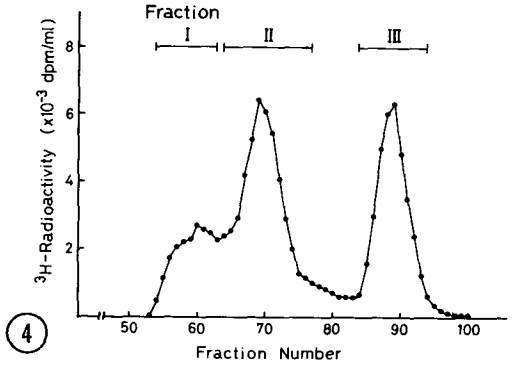


Fig. 3. The Tn antigen glycoproteins on the cell surface. LS 180 cells ($1x10^7$) were pulse-labeled with ^{3}H-glucosamine for 5 h. After washing with PBS, the cells were incubated with MLS 128 at 4°C for 1 h. Excessive antibody was washed off with PBS. From the lysate, an immunoprecipitate was obtained by adding protein A-Sepharose. The immunoprecipitate was subjected to SDS-PAGE followed by fluorography.

sulfate. When cells were pulse-labeled with ^{3}H-glucosamine-HCl, the earliest species detected comprised a diffuse band corresponding to a very high molecular weight (>250k daltons). When chased for 3 h, faint bands of 250k, 210k and 150k daltons could be observed. Thereafter the 250k and 210k bands became more obvious, whereas the 150k one became fainter. On labeling with ^{35}S-sulfate, similar changes were observed on chasing (data not shown). Thus, it appeared that the high molecular weight mucin with the Tn antigen was processed into the three molecular species. Then, cells were pulse-labeled with ^{3}H-glucosamine-HCl for 5 h. The glycoproteins carrying the Tn antigen on the cell surface were detected immunochemically. As shown in Fig. 3, a non-processed glycoprotein with the highest molecular weight was observed, suggesting that the processing occurred on the cell surface, not during intracellular transport.

Carbohydrate moieties of the Tn antigen glycoproteins. LS 180 cells were labeled with ^{3}H-glucosamine-HCl for 20 h. The Tn antigen glycoproteins were isolated by immunoaffinity chromatography. About 6 % of the radioactivity was recovered in the eluted fraction. The glycoproteins were treated with alkaline borohydride. The released oligosaccharides were fractionated on Sephadex G-15. As shown in Fig. 4, the oligosaccharides were separated into three fractions, I, II and III. Fractions II and III were identified as NeuAcα2→6GalNAcol and GalNAcol, respectively, on descending paper chromatography (Fig. 5). The molar



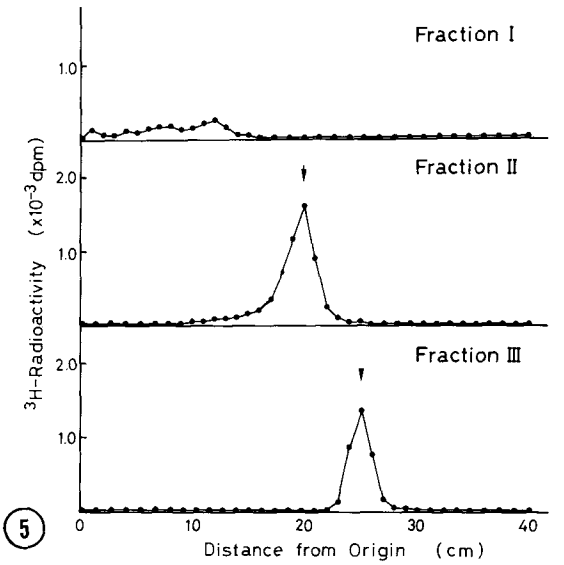


Fig. 4. Gel filtration of oligosaccharides prepared from the Tn antigen glycoproteins by alkali-borohydride treatment. Tn antigen glycoproteins isolated as described in MATERIALS AND METHODS were treated with 0.05 M NaOH and 1 M $NaBH_4$ at 45°C for 16 h, followed by gel filtration on a Sephadex G-15 column (1.0 x 110 cm). Fractions of 0.78 ml were collected and the radioactivity was determined.

Fig. 5. Paper chromatography of oligosaccharides. Oligosaccharide fractions I, II and III, indicated in Fig. 4, were applied on Toyo No. 51A paper, followed by development with ethyl acetate:pyridine:acetic acid:water (5:5:1:3, v/v) for 20 h. The arrow and arrowhead indicate the migration positions of ^{3}H-NeuAcα2→6GalNAcol and ^{3}H-GalNAcol, respectively.

ratios of O-linked carbohydrates were calculated as described previously (15). The percentages of Fractions I, II (NeuAcα2→6GalNAcol) and III (GalNAcol) were 7, 44 and 49 %, respectively.

DISCUSSION

In a previous study, we demonstrated that MLS 128, which was raised against LS 180 cells, reacted with mucin-type glycoproteins from LS 180 cells and OSM. Subsequent studies have shown that MLS 128 is a characteristic antibody recognizing the Tn antigen (9), and that the essential structure of the Tn antigen is a cluster of GalNAc-Ser/Thr, involving (GalNAc)-Ser-(GalNAc)-Thr-(GalNAc)-Thr (10). This paper describes the characterization of glycoproteins carrying the Tn antigen using MLS 128.

Western blotting analysis showed that glycoproteins with molecular weights of 250k, 210k and 150k daltons had the epitopic structure of the Tn antigen. To determine the relationship

between the three molecular species detected by MLS 128, we performed pulse-chase experiments, using ^{3}H-glucosamine or ^{35}S-sulfate to label carbohydrate moieties. Although Johnson et al. reported that the high molecular weight mucin defined by monoclonal antibody B72.3 was sensitive to mechanical shearing and degraded to lower molecular weight forms (16), our results clearly indicate that the present three molecular species were produced through processing, not through mechanical shearing. It appears likely that the processing of the high molecular weight mucin occurred after its transport into plasma membrane because the pulse-labeled antigen on the cell surface was not processed. The molecules on the cell surface may be degraded by proteases secreted by cells or by intracellular proteases if recycled in the cells.

In a previous paper (15), we reported the isolation of glycoproteins expressing the sialyl-Tn antigen using MLS 102, and showed that their carbohydrate chains were composed of O-linked NeuAcα2→6GalNAc (56 %) and O-linked GalNAc (25 %). A similar estimation was carried out for the carbohydrate chains of the Tn antigen glycoproteins. It was revealed that the percentages of O-linked NeuAcα2→6GalNAc and GalNAc were 44 and 49 %, respectively. Compared with the glycoproteins defined by MLS 102, the MLS 128 antigen was characterized by a higher content of O-linked GalNAc and a lower content of longer carbohydrate chain, as illustrated in Fig. 6. They seem to be typical cancer-associated glycoproteins produced through aberrant and incomplete glycosyla-

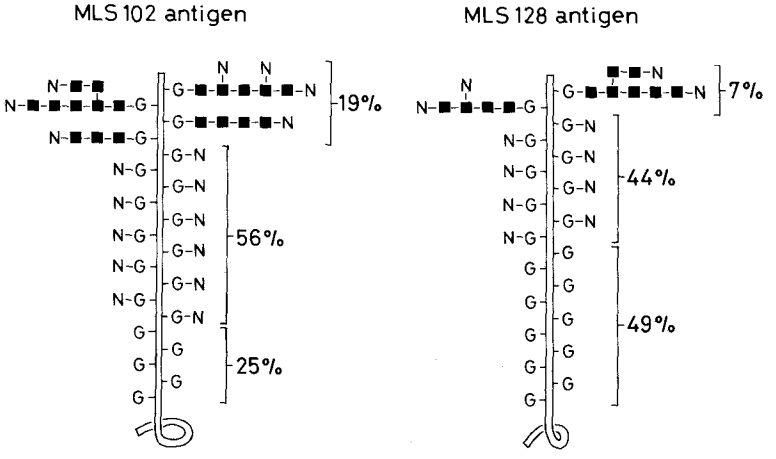


Fig. 6. Proposed gross molecular structures of the MLS 128 antigen. The structure of the MLS 102 antigen is cited from our previous paper (15). G: GalNAc; N: NeuAc; ■:any monosaccharide constituting linear or branched oligosaccharide chains.

tion. Furthermore, it should be noted that some of the long carbohydrate chains contain sulfate groups.

The Tn antigen glycoproteins contained considerable amounts of O-linked sialyl GalNAc in addition to O-linked GalNAc, suggesting that they share two antigens, i.e., the Tn and sialyl Tn antigens on the same polypeptide chain. In fact, an immunohistochemical study demonstrated that the majority of colon cancers expressed both the Tn and sialyl Tn antigens (17).

It has been believed that the structures of the sialyl Tn and Tn antigens are NeuAcα2→6GalNAc-Ser/Thr and GalNAc-Ser/Thr, respectively. However, we recently showed that the essential antigenic structure of sialyl Tn is a cluster structure of the disaccharide (7). In addition, we found that (GalNAc)-Ser-(GalNAc)-Thr-(GalNAc)-Thr is essential for the epitopic structure of the Tn antigen (10). Thus, it appears likely that even if NeuAcα2→6GalNAc-Ser/Thr or GalNAc-Ser/Thr exists, the existence does not necessarily mean the expression of the sialyl Tn or Tn antigenecity.

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