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Electrophoretic Analysis of Four High Molecular Weight Sialoglycoproteins Produced by Metastatic Human Colon Carcinoma Cells

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We have found that polyacrylamide gel electrophoresis in 3% gels in the presence of sodium dodecyl sulfate is suitable for the separation of cellular glycoproteins having molecular weights ranging from 200,000 to 1,000,000. The gels secured on a rigid support (Gelbond) allow blotting techniques with lectins and antibodies for the detection of glycoproteins. Using these methods we have separated lysates of HT-29 human colon carcinoma cells and detected at least four distinct high molecular weight sialoglycoproteins having molecular weights of 900,000, 740,000, 560,000, and 450,000. The expression of the 900,000 component, as revealed by wheat germ agglutinin binding, was much higher in a subline of HT-29 cells established from liver metastases in a nude mouse than it was in the parental cells. The relative intensity of wheat germ agglutinin binding to these four sialoglycoprotein components differs depending upon their growth phase in vitro. These glycoproteins were also detectable by the binding of peanut agglutinin, provided the glycoproteins were previously treated in the gels with mild acid to remove the sialic acid from their carbohydrate chains, suggesting that mucin-type carbohydrate chains are present on these glycoproteins. The same set of glycoproteins can be detected by metabolic labeling of the cells with [^{3}H]glucosamine in tissue culture. Very similar glycoprotein profiles are revealed by metabolic labeling of fresh colon carcinoma tissues with [^{3}H]glucosamine in vitro.

Key words: colon cancer, metastasis, mucins, electrophoresis, lectins

Evidence from a number of studies indicates that carcinomas of various origins produce very high molecular weight, highly glycosylated glycoproteins. These so-called mucins have been found associated with mammary, pancreatic, colonic, ovarian, and other carcinoma cells and tumor tissues of human origin [1–6]. They were also found in the circulating blood of patients with carcinoma [7–11]. In many cases, specific monoclonal antibodies generated against carcinoma cells or purified glycolipid antigens were shown to recognize antigenic epitopes expressed on the mucin carbohydrate chains.

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However, biochemical characterization of the mucin molecules has been hindered by their large size and heterogeneity. By conventional polyacrylamide gel electrophoretic separation in the presence of sodium dodecyl sulfate ($NaDodSO_4$) according to Laemmli [12], mucins stay at the top of the running gels. Although separation of human urinary mucin into multiple components was achieved by using 5–15% gradient polyacrylamide gels, their molecular weights were unknown [13,14]. The other method of electrophoretically separating high molecular weight glycoproteins was performed in 1% agarose [3,15]. The separated glycoproteins have to be transferred before immunochemical reactivity is tested because agarose gels are fragile, but the efficiency of transfer may not be identical among different high molecular weight glycoproteins. Agarose-polyacrylamid hybrid gels or large-pore polyacrylamide gels have similar fragility problems [16,17].

In this report, we present a relatively simple and convenient electrophoretic method of separating high molecular weight glycoproteins, suitable for immunochemical staining, by using 3% polyacrylamide gels cast on Gelbond PAG. Using this method, we found that HT-29 human colon carcinoma cells and colon carcinoma tissues produce at least four different wheat germ agglutinin (WGA)-binding sialoglycoproteins having molecular weights ranging from 450,000 to 900,000.

MATERIALS AND METHODS

Cells

HT-29 human colon carcinoma cells (designated as HT-29 P), were grown in plastic tissue culture dishes as previously described [18]. A variant cell line, HT-29 LMM, was established from liver metastases in a nude mouse inoculated intrasplenically with HT-29 P cells. When reinjected into nude mice, HT-29 LMM cells produced a greater number of liver metastases than did HT-29 P cells [18]. The growth medium used was a 1:1 mixture of Dulbecco's modified minimum essential medium and Ham's F-12 medium (Gibco, Grand Island, NY), supplemented with 10% fetal bovine serum (Hyclone, Logan, UT). The cells were subcultured before the culture became confluent by brief treatment with 0.25% trypsin and 2 mM EDTA solution (Gibco).

Tumor Tissue

One of the colon carcinoma tissues used in this study was derived from a 69-year-old woman who had a moderately differentiated adenocarcinoma of the sigmoid colon. This specimen was used for extraction of glycoproteins for lectin-blotting analysis. The other primary carcinoma tissue specimen used was derived from a 57-year-old woman who had a moderately differentiated adenocarcinoma at the splenic flexure of the colon. This specimen was used for metabolic labeling of the glycoproteins as described below. The specimens were obtained from the luminal edge of the tumors where the content of host-derived cells and necrotic tumor cells was presumably minimal [19,20].

Metabolic Labeling of HT-29 Cells and Tumor Tissues With [^{3}H]Glucosamine

HT-29 P or HT-29 LMM cells (10^6 cells) were seeded in a 10-cm plastic tissue culture dish containing 10 ml medium and incubated under humidified conditions in the presence of 5% CO_2 and 95% air. On the third day, 6-[^{3}H]glucosamine (35 Ci/mmole)

was added to the culture to give a final concentration 10 μCi/ml. An additional incubation was performed for 48 hr in the continuous presence of 6-[^{3}H]glucosamine. A 500-mg tumor tissue specimen was minced into small pieces with a scalpel blade and incubated for 48 hr with 5 ml of the same medium containing 50 unit/ml penicillin, 50 μg/ml streptomycin, 1.25 μg/ml amphotericin B, and 50 μCi/ml 6-[^{3}H]glucosamine (35 Ci/mmole)[16]. During this period, the original morphology was not always preserved, but tissue necrosis was minimal [18,20].

Extraction of Cells and Tissues

Extracts of cultured cells were prepared after detaching them from the culture dish as previously described. The cells were washed with 5 mM Tris-HCl, 0.25 M sucrose, 50 μM $CaCl_2$, and 50 μM phenylmethylsulfonylfluoride, pH 7.4, and then lysed with the same buffer containing 0.5% Nonidet P-40 by suspending 2×10^7 cells/ml at 4°C for 1 hr [19]. The supernatant was collected after centrifugation at 13,000g, mixed with 6% $NaDodSO_4$, 3% 2-mercaptoethanol, 1.5 mM EDTA, 30% glycerin, and 187.5 mM Tris-HCl buffer, pH 6.8, in a 2:1 ratio, and heated at 100°C for 5 min. The minced tumor tissues were extracted with the same Nonidet P-40 lysis buffer (100 mg wet tissue/667 μl) with brief ultrasonication [19]. After centrifugation at 13,000g, the supernatant was mixed with 333 μl of Tris-HCl buffer (pH 6.8) containing 6% $NaDodSO_4$ and 3% 2-mercaptoethanol as described above [19,21].

Electrophoresis

Polyacrylamide gel electrophoresis was performed in 1.5-mm-thick 3% running gels (13.5 cm long) with 2.5% stacking gels (1.5 cm long) using a BioRad Protean II electrophoresis unit. The gels were polymerized on Gelbond PAG (FMC Corp. Rockland, ME), which was previously attached on one of the glass plates with two-sided adhesive tape. We also prepared 2.1% polyacrylamide/0.83% agarose composite gels with 2.1% polyacrylamide stacking gels according to Perret et al. [16] and used these for comparison. Electrophoresis was performed under constant current (35 mA/1.5 mm thick, 16-cm-wide slab gel) for 4 hr. Laminin purified from Engelbreth-Holm-Swam sarcoma grown in C57BL/6 mice was used as a molecular weight marker [22]. Gels were either stained with 0.1% Coomassie brilliant blue in 10% acetic acid and 25% isopropanol or fixed in 10% acetic acid and 25% isopropanol.

Detection of Lectin-Binding Glycoproteins

In this study we used ^{125}I-labeled wheat germ agglutinin (WGA: approximately 50 cpm/ng) and ^{125}I-labeled *Arachis hypogaea* (peanut) agglutinin (PNA: approximately 30 cpm/ng) as lectin probes for the detection of glycoproteins in polyacrylamide gels [23]. In some cases, the glycoproteins separated by electrophoresis were treated with mild acid (50 mM sulfuric acid 80°C for 1 hr) in situ to remove terminal sialic acid [22]. Untreated or treated gels were washed extensively with 25 mM Tris-HCl, 0.125 M sodium chloride pH 7.3, and incubated with ^{125}I-labeled lectins (5 μg/ml) diluted in 25 mM Tris-HCl and 0.125 M NaCl pH 7.3, containing 1% bovine serum albumin (BSA). After incubation for 4 hr, the gels were washed repeatedly with the same buffer without BSA until the radioactivity of the wash fell below 100 cpm/ml. The gels were air dried after soaking in 2.5% glycerin and were processed for autoradiography as previously described [19–21].

RESULTS

Sialoglycoprotein Profiles Revealed by Lectin Binding

As shown in Figure 1, autoradiography revealed the presence of ^{125}I-WGA-binding glycoproteins from the HT-29 LMM human colon carcinoma cell lysates that were separated in the presence of $NaDodSO_4$ by electrophoresis on 6 or 3% polyacrylamide gels as well as polyacrylamide/agarose composite gels. Resolution of high molecular weight components was much improved by using 3% gels rather than 6% gels. Agarose/acrylamide gels also have good separation, though these gels were extremely fragile and not useful for subsequent blotting analysis. On the 3% gels, five components with apparent molecular weights higher than 180,000 were detected. The molecular weights of these components were estimated as shown, based on their migration relative to laminin [22]. As shown in Figure 2, the expression of the molecular weight 900,000 component was less in HT-29 P than in the metastic variant cell line (HT-29 LMM). The intensities of WGA binding to the other components (M_r 190,000, 450,000, 560,000, and 740,000) were almost identical between the two cell lines. All the WGA-reactive components were presumably sialoglycoproteins because WGA did not bind to them if the gels were pretreated with mild acid to remove sialic acid [23]. After removal of sialic acid from the glycoproteins, they reacted with ^{125}I-PNA (Fig. 2). This PNA reactivity suggested that T-antigen is present in mucin-type carbohydrate chains on these glycoproteins [24]. To test whether the trypsin treatment used for harvesting the cells influenced the glycoprotein profiles, HT-29 P and LMM cells were scraped from the tissue culture dish and analyzed by the same method by using ^{125}I-WGA to detect the glycoproteins. The migration positions and the relative intensity of WGA binding to these glycoproteins were identical to those of the cells detached by trypsin/EDTA treatment (data not shown).

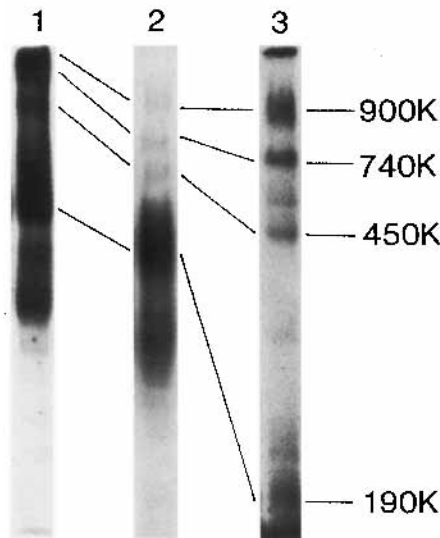

Fig. 1. Comparison of WGA-binding glycoproteins produced by HT-29 LMM cells separated by electrophoresis on polyacrylamide gels in the presence of sodium dodecyl sulfate. **Lane 1**, 6% polyacrylamide gels; **lane 2**, 2.1% polyacrylamide/0.83% agarose composite gels; **lane 3**, 3% polyacrylamide gels. The positions of the corresponding WGA-binding glycoproteins are indicated.

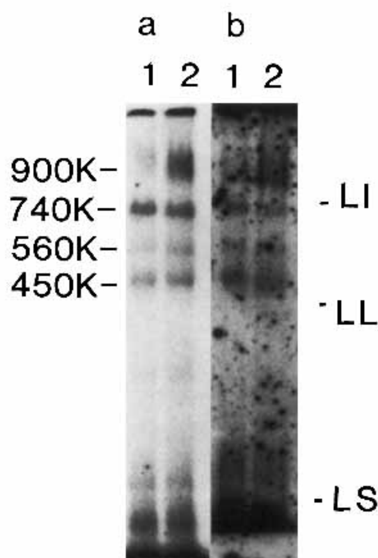

Fig. 2. High molecular weight glycoprotein profiles of cultured HT-29 human colon carcinoma cells resolved on 3% polyacrylamide gels after electrophoretic separation in the presence of $NaDodSO_4$ followed by staining with ^{125}I-labeled lectins. **Lane 1:** 50 μl of HT-29 P cell lysate prepared as described in Materials and Methods. **Lane 2:** 50 μl of HT-29 LMM cell lysate. **a:** Gels were stained with ^{125}T-WGA and the binding was detected by autoradiography. **b:** Gels were treated with 50 mM sulfuric acid at 80°C for 1 hr and stained with ^{125}I-PNA; binding was detected by autoradiography. Purified laminin was labeled with ^{125}I by using the chloramine-T method and used as a set of molecular weight markers (LI: Intact molecule, approximate M_r 800,000; LL: large subunit; approximate M_r 440,000; LS: small subunit, approximate M_r 220,000).

Separation of Glucosamine-Labeled Sialoglycoproteins

Lysates of glucosamine-labeled HT-29 cells were analyzed on 3% polyacrylamide gels. In the high molecular weight region, the WGA-binding sialoglycoproteins were the only components that metabolically incorporated glucosamine, except for one WGA-nonreactive high molecular weight component (>1,000,000). Glucosamine incorporation into a 900,000 molecular weight component was significantly higher in HT-29 LMM cells than it was in a comparable glycoprotein from HT-29 P cells. Interestingly, the components having molecular weight >1,000,000, 900,000, 740,000, and 560,000 were also revealed in tumor tissues by metabolic labeling with [^{3}H]glucosamine (Fig. 3a, lane 4). However, WGA staining failed to reveal similar discrete glycoprotein components after electrophoretic separation of tumor tissue extracts (Fig. 3b, lane 2), indicating that electrophoretic profiles of colonic mucins accumulated in vivo and those which incorporated ^{3}H-glucosamine in vitro were different.

Growth-Phase-Dependent Changes in the Relative Amount of High Molecular Weight Sialoglycoproteins

HT-29 P and LMM cells were plated at 2 × 10^6 cells/10 cm dish. After 1, 3, 6, and 9 days the cells were harvested by trypsin/EDTA treatment, counted, lysed, and processed for electrophoretic analysis as described in Materials and Methods. Figure 4 shows the number of cells at each time point, indicating the growth phase of the cultures

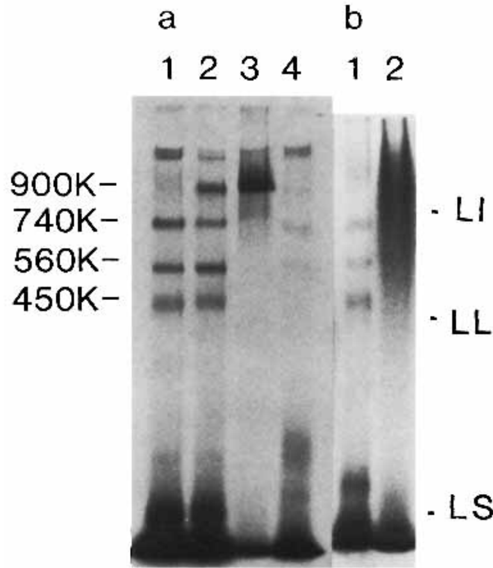

Fig. 3. Comparison of high molecular weight glycoprotein profiles of HT-29 human colon carcinoma cells grown in tissue culture and human colon carcinoma tissue minced and incubated in vitro. **a:** Cells or tissues incubated in the presence of [^{3}H]glucosamine for 48 hr. Polyacrylamide gel electrophoresis was carried out on 3% gels in the presence of $NaDodSO_4$ as in Figure 1. **Lane 1:** 50 μl of HT-29 P cell lysate. **Lane 2:** 50 μl of HT-29 LMM cell lysate. **Lane 3:** 50 μl of lysate from normal colonic mucosa adjacent to the carcinoma used in lane 4. **Lane 4:** 50 μl of lysate from a moderately differentiated adenocarcinoma of the colon. See text regarding the conditions for the preparation of these lysates. The labeled glycoprotein components were detected by autoradiography after treatment of the gels with Enhance (NEN-DuPont, Wilmington, DE). **b:** Comparison of high molecular weight glycoprotein profiles of HT-29 P human colon carcinoma cells (**lane 1**) and tissue extracts (**lane 2**) revealed by ^{125}I-WGA binding. Laminin was used as a set of molecular weight markers as described in the legend for Figure 1.

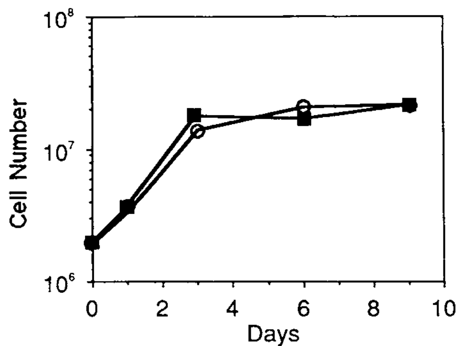

Fig. 4. Comparison of the growth rates of HT-29 P (○) cells and HT-29 LMM (■) cells in culture. Cells were harvested and counted before being processed for electrophoretic analysis.

at the time the cells were harvested. There was no difference between the growth rate of HT-29 P cells and that of HT-29 LMM cells. Changes in the profiles of WGA-binding sialoglycoproteins are shown in Figure 5. The data demonstrate that the production of high molecular weight sialoglycoproteins by HT-29 P and LMM cells was greatly influenced by the cell's growth phase in tissue culture. Production of sialoglycoproteins having molecular weights of 900,000, 740,000, and 450,000 were higher in the rapidly growing cells, whereas production of the M_r 540,000 sialoglycoprotein was higher in the senescent cells. Production of M_r 220,000 and M_r 190,000 sialoglycoprotein components stayed at the same level irrespective of the growth phase of the cells. The difference between HT-29 P and HT-29 LMM in the expression of the M_r 900,000 component was more prominent during their growing phase.

DISCUSSION

We have been attempting to determine biochemical characteristics of colorectal carcinoma cells and tissues that have higher metastatic potential. The significance of such an approach is based on our previous finding that the size of primary colorectal carcinoma—that is, tumor burden—and growth rate have no relationship to metastatic potential [25]. Biochemical analyses were performed on (1) primary colorectal carcinoma tissues from patients at various disease stages, (2) adjacent normal mucosa, and (3) tumor tissues from distant metastases, focusing on the properties known to be associated with metastatic tumor cells in experimental animal models. We have recently shown that there are specific changes in the expression of at least three different types of large molecular weight glycoproteins [18–20]. We are interested in separating and analyzing these glycoproteins in detail to determine their structures and biological functions.

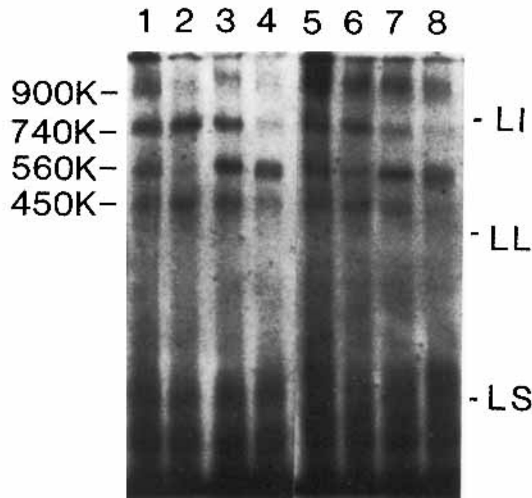

Fig. 5. Changes in the production of WGA-binding high molecular weight sialoglycoproteins during the growth of HT-29 P cells (**lanes 1–4**) and HT-29 LMM cells (**lanes 5–8**) in culture. Lanes 1 and 5, 1 day after subculture. Lanes 2 and 6, 3 days after subculture, when the cells were not confluent. Lanes 3 and 7, 6 days after subculture, at this point the cultures were confluent. Lanes 4 and 8, 9 days after subculture, when the cultures were overconfluent. The amount of lysate loaded on the gels, conditions for the electrophoresis, and conditions for the staining with ^{125}I-WGA are the same as in Figure 2.

In this report, we would like to emphasize three points. First, we have developed an electrophoretic technique to separate and detect glycoproteins having very large relative molecular mass. Using this technique, we were able to separate at least four different high molecular weight sialoglycoproteins (M_4: 900,000, 740,000, 560,000, and 450,000) produced by HT-29 cells. Second, production of these high molecular weight glycoproteins was greatly influenced by the growth phase of the cells in tissue culture. The expression of components having molecular weights of 900,000, 740,000, and 450,000 decreased when the cells reached confluency, whereas the production of a M_r 560,000 component increased. Third, as partially described previously [18], the expression of 900,000 component was significantly higher in HT-29 LMM cells than in the parental HT-29 cells. This difference was more prominent in log-phase than stationary-phase cells.

Since the discovery of "epiglycanin" on the surface of mouse mammary carcinoma cells by Jeanloz and co-workers [26], increasing evidence has indicated the usefulness of these very high molecular weight glycoproteins as tumor cell markers [1–11,15]. There is also evidence to suggest a role for these molecules as suppressors of specific and nonspecific host immune defenses [27,28]. The lack of a convenient separation technique for glycoproteins in the molecular weight range greater than 200,000 has prevented structural and functional studies from being carried out on molecules in heterogeneous mucin preparations. Our method, using 3% polyacrylamide gels bound to Gelbond, enabled us to separate glycoproteins having molecular weight higher than 400,000 up to approximately 1,000,000 according to their size. This gel preparation was rigid enough to allow immunoblotting with lectins and antibodies and to tolerate chemical modification of the glycoprotein carbohydrate chains in the gels after electrophoretic separation [20].

[^{3}H]glucosamine labeled molecules from HT-29 cells have profiles similar to those revealed by WGA binding, except that the former technique reveals an additional component with an approximate molecular weight 1,000,000. This component might be hyaluronic acid, because it had no reactivity with WGA and did not incorporate [^{35}S]sulfate (Yamori and Irimura, unpublished observation). These discrete high molecular weight glycoproteins were also detected by metabolic labeling of human colon carcinoma tissues. However, ^{125}I-WGA binding to the carcinoma tissue preparation after polyacrylamide gel electorphoresis revealed a very broad band. This pattern was apparently not due to distortion during electrophoretic separation but to the characteristics of the WGA-reactive glycoproteins in the tumor tissues. The sialoglycoprotein molecules produced in vitro during a 48-hr incubation period are likely to have more uniform size, whereas the high molecular weight sialoglycoproteins accumulated during the growth of the tumor could have been modified or partially degraded.

We do not know at this moment whether these high molecular weight sialoglycoproteins are actually mucin-like. Their reactivity with PNA after removal of sialic acid suggested that the galactosyl-N-acetylglucosamine sequence was present. When we incubated the cells scraped from the colonic mucosa adjacent to the carcinoma with [^{3}H]glucosamine, most of the radioactivity in the high molecular weight region was incorporated into an M_r 900,000 component (see Fig. 3a). We purified molecular weight 900,000 component from [^{3}H]glucosamine-labeled normal mucosa. A significant portion of the radioactive residues was released from this material by alkaline reduction (incubation in 0.6 M $NaBH_4$/0.2 M NaOH at 25°C for 48 hr). These results strongly suggested that the M_r 900,000 sialoglycoprotein was a mucin-like molecule.

It has been well known that microenvironmental factors in vivo or in vitro influence the metastatic potentials of tumor cells. The results shown in this paper suggest that cell contact or microenvironmental factors may influence the production of high molecular weight sialoglycoproteins which possibly determine the tumor cells' biological behavior related to metastasis.

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