Soluble Factor in Normal Tissues That Stimulates High-Molecular-Weight Sialoglycoprotein Production by Human Colon Carcinoma Cells

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ABSTRACT

The stimulation of high molecular weight sialoglycoprotein synthesis by a soluble factor derived from normal colon tissues was studied in vitro with human colon carcinoma cell lines, HT-29 P and a metastatic variant HT-29 LMM. The synthesis of all three high-molecular-weight sialoglycoproteins (approximate M, 900,000, 740,000, and 450,000) by HT-29 P cells or HT-29 LMM cells growing in vitro was enhanced by supplementing the culture medium with a conditioned medium of fresh human colon organ culture. Changes were detected by polyclaralamide gel electrophoresis of lysates from [3H]glucosamine-labeled cells on 3% gels followed by fluorography, or by electrophoresis of lysates from unlabeled cells followed by incubation with [125I]-labeled wheat germ agglutinin and autoradiography. No changes were detected in the major protein components or in glycoproteins at M, < 200,000 as revealed by polyclaralamide gel electrophoresis. The treated cells did not change their growth rate or morphology. The connective tissue portions of the colon tissues were apparently responsible for the production of this stimulatory substance. The stimulatory activity was preserved at 56°C but was inactivated by heating at 100°C. The substance was eluted from a Sephadryl S-200 column at a position between the elution positions of ovalbumin and trypsinogen. The colon carcinoma cells treated with the conditioned medium and producing increased amounts of high-molecular-weight sialoglycoproteins were less sensitive to the cytolytic effects of recombinant interleukin 2-activated human peripheral blood lymphocytes than untreated cells were. The treated colon carcinoma cells induced stronger platelet aggregation than their untreated counterparts did. Therefore, this substance may represent one of the normal host tissue factors that can influence and modulate malignant behavior of carcinoma cells growing in vivo.

INTRODUCTION

Changes in the production and structures of glycoconjugates upon malignant transformation have been described for a wide variety of cells (1–5). In colon epithelial cells, changes in the carbohydrate chains on mucin-like, very high-molecular-weight glycoprotein molecules during carcinogenesis have been reported since the 1960s (6, 7). A variety of antigenic molecules associated with normal and malignant human colon epithelium were reported as mucin-like, high-molecular-weight glycoproteins or mucin associated, but the epitope structures and specificities of the corresponding antibodies were not clear (8–12). Furthermore, the biochemical basis for changes in the expression of such tumor-associated antigens was not well understood. Particularly, the effects of host microenvironments on the production of these molecules were not elucidated. Mucins are major products of various epithelial cells and are defined by their large molecular size and high content of serine/threonine-linked carbohydrate chains. Secreted mucins are believed to function as protective molecules and as lubricants on tissue surfaces. Despite previous attempts by several laboratories to classify and characterize colorectal mucins, it remained unclear whether a specific biological function is associated with unique carbohydrate structures in these mucins (10, 13–22). Our recent studies utilizing pathological specimens of colorectal carcinoma suggested that at least four mucin-like glycoproteins with different carbohydrate chains were independently regulated and either directly or inversely correlated with the metastatic potential of these malignant tumors (23–27). Thus, these changes were apparently associated with the progression of colon carcinoma to the metastatic phenotype, but not simply with malignant transformation. Determining a positive correlation between the expression of high molecular weight sialoglycoproteins and the progression of colorectal carcinoma to the metastatic phenotype as we demonstrated seemed to be particularly significant because increased production of sialoglyconjugates has been implicated in a variety of experimental metastases in rodents (28–34).

Two important basic biological questions remained unanswered after these studies. The first was what molecular and cellular mechanisms are involved in the increased expression of sialoglycoproteins during the progression of colorectal carcinoma to the metastatic phenotype. This question is not simple because the amount of sialoglycoproteins produced by colon carcinoma cells growing in vivo appears to be influenced by the cellular origin of the carcinoma as well as by microenvironmental factors (26). The second question was whether sialoglycoproteins account for the metastatic behavior of colon carcinoma cells. In experimental animal models, various cell surface characteristics have been proposed as being potentially involved in metastatic phenotypes. For example, sialylation of cell surface adhesion molecules may modulate the interaction of tumor cells with extracellular matrices (35). The effects of cell surface glycosylation on invasion of embryonic muscle tissues by tumor cells have also been reported (36). Furthermore, sialoglycoconjugates on tumor cell surfaces are known to interfere with the cytotoxic effects of lymphocytes through nonspecific or specific mechanisms (37–41). In rat renal carcinoma, sialoglycoconjugates are proposed to play an important role in tumor cell-induced platelet aggregation (42). Mouse colon carcinoma cells that were treated with an inhibitor of sialyltransferase showed significant reduction in their ability to induce platelet aggregation (43).

We describe a soluble factor from normal colon tissues that appears to stimulate the production of cell-associated high-molecular-weight sialoglycoproteins by colon carcinoma cells in vitro. The present study is carried out with organ culture-conditioned media because we did not find a similar stimulatory factor when an aqueous extract of normal colon tissues or

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partial fractionation products of the extract were tested at various concentration. The results should eventually lead to answers to important questions regarding the function and regulation of carcinoma mucins.

MATERIALS AND METHODS

Preparation of Conditioned Media from Normal Colon Tissues. Human colon tissues used in this study were derived from sigmoid or left colon tissue resected during surgery for colon carcinoma. Normal mucosa at least 5 cm from the carcinoma was used. For the preparation of NCCM, the colon epithelium with attached submucosal connective tissues and muscularis propria was made free of fat tissues. Fragments of tissue (approximately 1.0 x 0.3 cm) were then rinsed five times with gentle agitation and decantation with DPBS supplemented with 50 units/ml penicillin, 50 μg/ml streptomycin, and 1.25 μg/ml amphotericin B (Fungizone; Sigma, St. Louis, MO). The tissue fragments were then incubated with DME/F12 supplemented with antibiotics as described for DPBS. The incubation volume was 10 ml of medium/3.3 g of tissue. After a 48-h incubation at 37°C in a humidified atmosphere with 5% carbon dioxide in the supernatants were removed. The supernatants were either centrifuged at 5000 x g or filtered through Whatman No. 1 filter paper to remove insoluble materials and then filter sterilized with 0.2-μm nitrocellulose filter membranes.

Conditioned media from different portions of normal colon tissues were obtained as follows. One tissue fragment free of fat (approximately 2 x 6 cm) was prepared from normal colon wall tissue, cut into three portions of equal size, and rinsed with DPBS containing antibiotics as described above. One portion was incubated whole in DME/F12. The second was cut between epithelial cell layer and muscularis through the connective tissue portion, and yielded a muscularis tissue with attached connective tissues and epithelial cell layers with attached connective tissues. These tissue fragments were rinsed with antibiotics again and incubated separately. The third portion was scraped with a glass slide to obtain epithelial cells and to prepare epithelial cell-conditioned media.

Cultures of Human Colon Carcinoma Cells. HT-29 P cells and HT-29 LMM cells (obtained from Dr. I. J. Fidler, The University of Texas M.D. Anderson Cancer Center, variant cell lines of HT-29; see Refs. 26 and 44 for details) were used to examine the effect of NCCM on sialoglycoprotein production. The cells were seeded at 5 x 10^5 cells/10-cm plastic tissue culture dish in 10 ml of DME/F12 with 10% FBS. One day later, the culture media were further supplemented with 0.5 ml of NCCM (treated or fractionated), other test substances, or incubation medium containing FBS for control. The substances tested included serially diluted TGF-β1 (R & D Systems, Minneapolis, MN) or IL-6 (Genzyme, Boston, MA). In another experiment, various concentrations of a polyclonal antibody against TGF-β1 or IL-6 (R & D Systems) were included. In some experiments, NCCM-treated and untreated cells were also incubated with [3H]glucosamine at a final concentration of 5 μCi/ml for 24 h before harvesting to metabolically label cellular glycoproteins. [3H]Selenomethionine at a final concentration of 50 nCi/ml and 10 μCi/ml of [3H]histidine were used for metabolic labeling of polypeptides.

Growth Rate and Morphology of HT-29 Cells Grown in Presence or Absence of Normal Colon-conditioned Media. During the incubation of HT-29 P cells and HT-29 LMM cells with NCCM, the number of cells in cultures were estimated with direct cell counting on a cell counter after detachment by trypsin treatment. The effects of NCCM on the proliferation rate of these cells were also determined by a crystal violet staining method (45) by using 96-well micro tissue culture plates. In this experiment, the cells were seeded at 5 x 10^4 cells/well in the absence or presence of 5% NCCM. The cells were fixed in 0.05% glutaraldehyde at days 1, 2, 3, 4, and 5, stained with 0.1% crystal violet, and rinsed repeatedly with water. The relative number of the cells were estimated by measuring absorbance at 590 nm after being dissolving in 1% SDS. The morphology of these cells during incubation with NCCM was examined under a phase contrast microscope.

Electrophoretic Analysis of Colon Carcinoma Cell Lysates. Cellular glycoproteins were extracted with non-ionic detergent and treated with SDS and 2-mercaptoethanol as previously described (31). Electrophoretic analysis of high-molecular-weight sialoglycoproteins was performed by using 3% polyacrylamide gels according to previously established methods (25, 26). Sialoglycoproteins were identified by direct binding of [125I]WGA to polycrylamide gels. Lysates from [3H]glucosamine-labeled and [3H]histidine-labeled cells were analyzed by autoradiography with Enhance (DuPont NEN, Wilmington, DE). Lysates from [35Se]selenomethionine-labeled cells were directly autoradiographed after electrophoretic separation.

Quantitation of Changes in High-Molecular-Weight Sialoglycoprotein Production. In most of the experiments, the relative amounts of high-molecular-weight sialoglycoprotein production were estimated by the binding of [125I]WGA (10 μg/ml) to the M, 740,000 sialoglycoprotein on 3% polyacrylamide gels. This was achieved by removing and counting the radioactivity associated with the appropriate area of the gels. Alternatively, densitometry scanning of the autoradiographs was used for estimating the relative amounts of the M, 740,000 sialoglycoprotein.

Treatments and Fractionation of Normal Colon-conditioned Media. The heat stability of the stimulatory activity for sialoglycoprotein production by colon carcinoma cells was tested by heating NCCM at 56, 60, or 100°C for 30 or 60 min. To determine the size of the substance in NCCM responsible for the stimulatory activity, fractionation by gel filtration chromatography was carried out. NCCM (1 ml) was fractionated on a Sephacryl S-200 column (0.8 x 20 cm) equilibrated with DME/F12 medium, and 1-ml fractions were collected. To further study the biochemical nature of this stimulatory activity, 1 ml of NCCM was applied to a 1-ml column of Con A-Sepharose (Pharmacia). The pass-through fraction, which was eluted with DPBS, and another fraction, which bound to Con A-Sepharose and was eluted with 0.5 M α-methylmannoside, were pooled separately. These fractions were dialyzed against water, lyophilized, and reconstituted in DME/F12 medium.

Cytolytic Assays. NCCM-treated and untreated HT-29 P cells and HT-29 LMM cells were labeled with 13Cr by incubation with 100 μCi/ml of sodium [13Cr]chromate for 2 h (46) and then compared for their sensitivity to lymphokine-activated killer lymphocytes. The cells were washed extensively, detached from the dish, and then suspended in DME/F12 at a final concentration of 5 x 10^5 cells/ml. Fresh human peripheral blood lymphocytes were obtained from healthy donors and subjected to Ficol-Hypaque centrifugation and depletion of plastic- and nylon-adherent cells. The cells were cultured for 4 days in the presence of 100 units/ml of recombinant interleukin 2 (Cetus Corp., Emeryville, CA) and were then harvested, washed by centrifugation, and resuspended in RPMI 1640 medium containing 5% heat-inactivated human AB serum (Hazelton, Kansas City, MO), 300 mg/ml of L-glutamine, and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (lymphokine-activated killer lymphocytes). Large granular lymphocytes from fresh blood were separated by Percoll (Pharmacia, Uppsala, Sweden) discontinuous gradient centrifugation and were used as natural killer cells. Supernatants from NCCM were used to incubate U-bottomed 96-well plates to test the sensitivity of the cells to the cytotoxic effects of lymphokine-activated killer lymphocytes as well as natural killer cells. In these assays, serially diluted effector cells (1.25 x 10^4 to 4 x 10^5 cells/100 μl) were added with target cells (5 x 10^5 cells/100 μl), centrifuged at 500 rpm for 5 min, and incubated for 4 h at 37°C under humidified conditions in the presence of 5% carbon dioxide and 95% air. At the end of the assay, the plates were centrifuged, and the supernatants were harvested, and the percentage of specific lysis was calculated by the formula:

\[
\frac{cpm_{	ext{maximum}} - cpm_{	ext{spontaneously released}}}{cpm_{	ext{with effector cells}} - cpm_{	ext{spontaneously released}}} \times 100
\]

All determinations were performed in quadruplicate.

* The abbreviations used are: NCCM, normal colon conditioned medium; DPBS, Dulbecco’s phosphate-buffered saline; DME/F12, 1:1 mixture of Dulbecco’s minimal essential medium and Ham’s F-12 medium; FBS, fetal bovine serum; Con A, concanavalin A; TGF-β, transforming growth factor β; WGA, wheat germ agglutinin; SDS, sodium dodecyl sulfate.
Platelet Aggregation Assays. Platelets were prepared from freshly drawn heparinized blood from normal healthy donors. A suspension of platelets at a concentration of $4 \times 10^9$/ml in Hanks' balanced salt solution was placed in an aggregometer and preincubated for 15 min at 37°C. An aggregation reaction was initiated by adding $5 \times 10^5$ untreated or NCCM-treated cells suspended in 0.1 ml of Hanks' balanced salt solution. The degree of light scattering was determined by measuring the absorbance at 550 nm (47).

RESULTS

Increase in Levels of High-Molecular-Weight Sialoglycoproteins in Colon Carcinoma Cells Induced by NCCM in Vitro. Because we had previously observed that a greater amount of high-molecular-weight sialoglycoproteins was associated with HT-29 P cells and HT-29 LMM cells growing in vivo in nude mice than with the same cells growing in vitro, we attempted to mimic the alterations in the sialoglycoproteins in cell cultures. The cells grown in the absence or presence of NCCM were extracted with non-ionic detergent solution (lysis buffer) and the extracts were separated by polyacrylamide gel electrophoresis in the presence of SDS. High-molecular-weight sialoglycoproteins were examined after they were incubated with $^{125}$I-WGA and autoradiographed as previously described (25, 26). Fig. 1 shows electrophoretic profiles of high-molecular-weight sialoglycoproteins of HT-29 P and HT-29 LMM cells incubated with 5% NCCM for 4 days. An increase in the binding of $^{125}$I-WGA to high-molecular-weight glycoproteins ($M_r \approx 900,000, 740,000, \text{ and } 450,000$) was seen in NCCM-treated cells. No significant changes were observed in WGA binding to the components with $M_r < 200,000$. A slight change seen on $M_r 540,000$ component was not reproducible. Fig. 2, left, shows similar changes as detected by the incorporation of $[^3H]$glucosamine into HT-29 LMM cells. The incorporation of $[^3H]$glucosamine into the $M_r 190,000$ component did not significantly change after NCCM treatment. As seen in Fig. 2, center and right, incorporation of $[^3H]$threonine or $[^75]$Seleno-Methionine into components with molecular weights ranging between 400,000 and 1,000,000 was very low relative to that to components $M_r < 200,000$. The incorporation did not seem to significantly change after NCCM treatment, but this was not conclusive because the levels of the incorporation were below the ranges of detection by these methods. No alterations were detected in the profiles of proteins with molecular weights as low as 30,000, as revealed by Coomassie blue staining or by the incorporation of $[^3H]$threonine or $[^75]$Seleno-Methionine (data not shown). Mild acid treatment (50 mM sulfuric acid treatment at 80°C for 60 min) of glycoproteins in polyacrylamide gels prior to incubation with WGA eliminated WGA reactivity from all the components, indicating that the WGA-identified molecules are sialoglycoproteins. The intensity of the bands revealed by fluorography based on $[^3H]$glucosamine incorporation was not significantly altered after mild acid treatment (data not shown), indicating that the increase in WGA reactivity and $[^3H]$glucosamine incorporation seen in NCCM-treated cells was not simply due to enhanced sialylation. The electrophoretic migration distance of high-molecular-weight sialoglycoproteins did not change after treatment with NCCM. At this time, it is not clear whether the changes in WGA binding or $[^3H]$glucosamine incorporation is due to increased production of entire sialoglycoprotein molecules or increased glycosylation.

Growth Rate and Morphology of HT-29 Cells Grown in Presence or Absence of Normal Colon-conditioned Media. There was no detectable change in the growth rate of these cells during incubation in NCCM, and the saturation densities of these cells grown in the presence or absence of NCCM were very similar.

![Fig. 1. Results of electrophoretic analysis in the presence of SDS on 3% polyacrylamide gels of lysates of HT-29 P cells (Lanes 1 and 2) and HT-29 LMM cells (Lanes 3 and 4) grown in the absence (Lanes 1 and 3) or presence (Lanes 2 and 4) of 5% NCCM for 4 days. The molecular weights (ordinate) of the major bands were estimated from the positions of intact laminin and subunits of laminin as described previously (25, 26). Autoradiograms of the gels incubated with $^{125}$I-WGA, rinsed, and dried are shown.](cancerres.aacrjournals.org/article-pdf/34/24/3298/32357352.pdf)

![Fig. 2. Results of electrophoretic analysis in the presence of SDS on 3% polyacrylamide gels of lysates of HT-29 LMM cells grown in the absence or presence of 5% NCCM for 4 days. The molecular weights (ordinate) of the major bands were estimated from the positions of intact laminin and subunits of laminin as described previously (25, 26). Left, autoradiograms of metabolically $[^3H]$glucosamine-labeled components. Center, autoradiograms of metabolically $[^3H]$threonine-labeled components. Right, autoradiograms of metabolically $[^75]$Seleno-Methionine-labeled components.](cancerres.aacrjournals.org/article-pdf/34/24/3298/32357352.pdf)
Although these cells in culture always have heterogeneous morphology, the proportion of cells with flat appearances, compared with other cells that were tightly associated with each other, did not appear to differ between untreated and NCCM-treated cells (data not shown).

Time Course and Reversibility of Effects of Normal Colon-conditioned Media. Fig. 3 shows the time course of the increase of sialoglycoproteins as revealed by WGA binding to electrophoretically separated HT-29 LMM cell lysates. The effect of NCCM can be seen as early as 24 h after addition and reaches a plateau at days 2–3. The effects of NCCM on WGA binding to high-molecular-weight sialoglycoproteins were completely reversed after a 3-day incubation of HT-29 LMM cells in the absence of NCCM (Fig. 4).

Partial Characterization of a Soluble Factor That Stimulates Production of High-Molecular-Weight Sialoglycoproteins. The sialoglycoprotein stimulatory activity in NCCM was stable after it was heated at 56°C for 30 min but was inactivated by treatment at 80°C for 60 min (Fig. 5). The activity was maintained after freezing and thawing and after lyophilization. More than 70% of the stimulating activity was found in the unbound fraction after Con A-Sepharose affinity chromatography. Heparin-agarose did not retain the activity. NCCM was subjected to gel filtration on Sephacryl S-200 and eluted with culture media. The fractions obtained were tested for their stimulatory activity on sialoglycoprotein production by HT-29 LMM cells.

The fraction containing the activity was eluted from the column at a position between ovalbumin ($M_\text{r} \approx 45,000$) and trypsinogen ($M_\text{r} \approx 25,000$), approximately corresponding to $M_\text{r} \approx 30,000$ (data not shown).
Cells Producing the Factor and Their Viability. Previously we have shown that cultures of normal human colon tissues maintain their metabolic activity such as production of sulfated mucins and production and secretion of collagenolytic enzymes for at least 48 h (24, 48). Therefore, we prepared conditioned media from normal colon tissues after a 48-h incubation. We further tested whether the cells in normal colon tissues maintain their metabolism during this incubation period. When protein profiles with metabolically incorporated [3H]threonine at periods of 0-24 h, 24-48 h, and 48-72 h were analyzed and compared by polyacrylamide gel electrophoresis, difference was not seen as judged by Coomassie brilliant blue staining or by fluorography (data not shown). Histological observation of the tissues at days 0, 1, and 2 indicated that connective tissues and muscularis remained intact during the incubation period, although some damage was seen in epithelial cells.

To identify the tissue compartments producing this stimulatory factor for sialoglycoprotein production by colon carcinoma cells, we separated normal colonic mucosa into the epithelial cell layer and the connective tissues with muscularis propria. Epithelial cells with submucosa were also prepared. We then obtained conditioned media from the cultures of these tissue segments and compared them for their ability to stimulate sialoglycoprotein production. The results shown in Fig. 6 indicate that a higher concentration of this soluble factor seemed to be produced and secreted from tissue fragments containing exposed connective tissues. Thus, we tested whether a conditioned medium of human colon fibroblasts elicited activity similar to that of NCCM. Human colon fibroblast cells CCD-18Co (ATCC-CRL 1459) from the American Type Culture Collection, were grown in DME/F12 with 10% FBS. The conditioned media of these cells at a concentration as low as 1% showed similar effects on sialoglycoprotein production, suggesting that fibroblasts are the cells responsible for the production of the soluble factor in NCCM.

Could Effects of Normal Colon-conditioned Media be Explained by Previously Known Soluble Mediators? Because fibroblasts are known to produce TGF-β and IL-6 which have apparent molecular weights within the range of 25,000 to 45,000, we examined whether these known soluble mediators had similar biological effects on HT-29 P cells and HT-29 LMM cells as those seen with NCCM. TGF-β1 (0.1-10 μg/ml) did not have any effect on the binding of WGA to high-molecular-weight sialoglycoproteins. Neutralizing polyclonal antibodies against TGF-β1 + β2 did not change the effects of NCCM. Human IL-6 (5-5000 units/ml) did not have significant effects on high-molecular-weight sialoglycoprotein production, as revealed by 125I-WGA binding after electrophoretic separation.

Interaction of Activated Lymphocytes with Colon Carcinoma Cells Cultured in Presence or Absence of Normal Colon-conditioned Media. We hypothesized that the increased sialoglycoproteins would influence and modulate the interaction of colon carcinoma cells with activated lymphocytes. The NCCM-treated and untreated cells previously labeled with sodium 197Co (ATCC-CRL 1459) from the American Type Culture Collection, were grown in DME/F12 with 10% FBS. The conditioned media of these cells at a concentration as low as 1% showed similar effects on sialoglycoprotein production, suggesting that fibroblasts are the cells responsible for the production of the soluble factor in NCCM.

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Induction of Platelet Aggregation by Colon Carcinoma Cells Cultured in Presence or Absence of Normal Colon-conditioned Media. It has been reported that metastatic mouse colon carcinoma cells possess the capacity to induce strong platelet aggregation and that tumor cell surface sialic acid might be important in this process (43). Therefore, we hypothesized that HT-29 P and HT-29 LMM human colon carcinoma cells producing different amounts of high-molecular-weight sialoglycoproteins might have different platelet-aggregating activities.

Untreated and NCCM-treated cells were mixed with suspensions of human platelets, and the changes in light transmission under constant gentle mixing were monitored. As shown in Fig. 8, the rate of platelet aggregation induced by NCCM-treated
cells and HT-29 LMM cells was apparently stimulated when sialoglycoproteins by human colon carcinoma HT-29 P cells was significantly higher than that induced by untreated scattering than untreated HT-29 P cells did. It was further augmented by NCCM transmission induced by untreated and NCCM-treated HT-29 P cells. HT-29 retic separation of the cell lysates. Production in vitro of all of the binding of 125I-WGA to high-molecular-weight components coproteins in human colon carcinoma cells were revealed by production is shown in vitro using culture-conditioned media the present report, such a factor which stimulates sialoglycoprotein production compared with the synthesis of other coprotein production. NCCM-treated cells did not secrete the M, 900,000 sialoglycoprotein that were either treated or not treated with NCCM were examined for the sensitivity of the sugar chains to alkaline degradation, and the results strongly suggested that these high-molecular-weight sialoglycoproteins are mucin-like molecules. A similar stimulatory effect was seen on other human colon carcinoma cell lines such as HCC 1410, DiFi, and KM12C.

We have also tested other cellular and molecular changes in the NCCM-treated HT-29 P cells and HT-29 LMM cells. No changes were seen in the growth rate or the morphological appearance of the cells under the same conditions. These findings indicated that these cells were not undergoing differentiation toward goblet-like cells or any other cells, in contrast with the differentiation seen in other sublines of HT-29 cells grown in glucose-depleted medium (49). Foss et al. (50) have reported that protein kinase activity associated with src<sup>pp60</sup> is much lower in colon carcinoma cells expressing differentiated phenotypes than those with undifferentiated phenotypes. However, the levels of phosphorylation of src<sup>pp60</sup> and epidermal growth factor receptor did not change after NCCM treatment of HT-29 P cells or HT-29 LMM cells. Furthermore, the increase in sialoglycoprotein production was reversible. These results strongly suggest that the effect of NCCM is not due to selective growth of a carcinoma cell subpopulation expressing higher contents of high-molecular-weight sialoglycoproteins or to irreversible differentiation. However, whether other cellular changes may be associated with increased sialoglycoprotein production induced by NCCM remains unknown.

Our initial experiments were performed with NCCM, conditioned media of organ-cultured colon tissues containing epithelial cells, connective tissues, and muscularis. To examine the metabolic activity of the tissue during such incubations, metabolic labeling of the tissues with [3H]threonine, followed by electrophoretic analysis of the lysate of the tissues was performed. The results of this experiment and morphological examinations demonstrated that the tissues maintained their metabolic activity and their original morphology during the 48-h incubation period. To identify which portion of the tissue produced and secreted the active substance responsible for the stimulation of high-molecular-weight sialoglycoprotein production, we obtained conditioned media from different portions of large bowel wall tissues, and tested their ability to induce the change in 125I-WGA binding to high-molecular-weight sialoglycoproteins produced by HT-29 LMM cells. The results suggested that the cells within connective tissues were responsible for the production of this factor. Furthermore, we found that fibroblasts are probably the cells that produce this factor in normal colon tissues. Substances in serum were apparently incorporation, the observed changes may be due to increased glycosylation or to increased synthesis of whole glycoprotein molecules. Increased sialylation does not account for the decreased incorporation of [3H]glucosamine because a very similar difference in the radioactivity between untreated and NCCM-treated cells was observed after specific removal of sialic acid from these sialoglycoproteins by mild acid hydrolysis. Structural characterization of purified sialoglycoproteins synthesized in the presence or absence of NCCM along with identification and quantitation of the core polypeptide can answer the questions about the biochemical basis of the stimulation of sialoglycoprotein synthesis caused by NCCM treatment. Nevertheless, this provides a unique system with which we can elucidate the regulation of high-molecular-weight sialoglycoprotein production by colon carcinoma cells and the biological functions of these molecules. [3H]Glucosamine-labeled high-molecular -weight sialoglycoproteins that were either treated or not treated with NCCM are mucin-like molecules. A similar stimulatory effect was seen on other human colon carcinoma cell lines such as HCC 1410, DiFi, and KM12C.

Fig. 8. Time course of platelet aggregation as indicated by increase of light transmission induced by untreated and NCCM-treated HT-29 P cells. HT-29 LMM cells induced more intense platelet aggregation as shown by higher light scattering than untreated HT-29 P cells did. It was further augmented by NCCM treatment (data not shown).

DISCUSSION

We previously showed that the production of high-molecular-weight sialoglycoproteins by human colon carcinoma HT-29 P cells and HT-29 LMM cells was apparently stimulated when the cells were grown in vivo in nude mice (26). We hypothesized that the levels of high-molecular-weight sialoglycoproteins on colon carcinoma cells were influenced by the intrinsic nature of the tumor cells (clonal variation) as well as by host microenvironmental factors that have not yet been characterized. In the present report, such a factor which stimulates sialoglycoprotein production is shown in vitro using culture-conditioned media of normal colon tissues. We obtained NCCM by incubating fresh human colon tissues in culture. The changes in sialoglycoproteins in human colon carcinoma cells were revealed by the binding of 125I-WGA to high-molecular-weight components after electrophoretic separation of the cell lysates or by metabolic incorporation of [3H]glucosamine followed by electrophoretic separation of the cell lysates. Production in vitro of all of the three high-molecular-weight sialoglycoproteins (approximate M, 900,000, 740,000, and 450,000) was enhanced by the addition of this conditioned medium to the culture, whereas differences in the production of glycoproteins with M, < 200,000 or in total protein profiles were not detected. These results suggested that the conditioned media from human colon tissues specifically enhanced high-molecular-weight sialoglycoprotein production compared with the synthesis of other glycosylated and nonglycosylated proteins. NCCM-treated cells secreted an increased amount of a M, 900,000 sialoglycoprotein but did not secrete the M, 740,000 or M, 450,000 components. This may explain why the M, 900,000 component appeared to be predominant in tumors grown in vivo, which maintained secretion products (26).

Because the increase in high-molecular-weight sialoglycoprotein production was detected by means of increased sialylated carbohydrate chains, i.e., 125I-WGA binding or [3H]glucosamine
necessary for fibroblasts to produce and/or secrete the factor in vitro. Normal colon tissues did not require serum for the production of the factor, probably because the active principle in the serum was endogenously provided.

The sialoglycoprotein-stimulatory activity in NCCM was relatively stable after mild heating or freeze-thawing. Preliminary fractionation and characterization experiments suggested that the factor has a molecular weight of 30,000 and is likely to be a protein or glycoprotein. Therefore, we have tentatively named the factor mucomodulin, referring to its ability to modulate mucin synthesis. Based upon our finding that this molecule is produced by fibroblasts, we have tested whether known polypeptides produced by normal fibroblasts such as TGF-β and IL-6 possess a similar activity and have concluded that these substances did not stimulate the production of high-molecular-weight sialoglycoproteins by colon carcinoma cells. Therefore, the factor(s) may specifically regulate cell-cell interaction by changing the architecture of the cell surface. Our observation is unique because the effect seems very specific to mucin-like high-molecular-weight sialoglycoproteins. Rearick et al. (51) reported that mucin production by cultured rabbit tracheal cells was stimulated by mouse 3T3 fibroblast-conditioned media in the presence of retinoic acid. In the human colon carcinoma experimental systems we used, the effects of retinoic acid were inhibitory against high-molecular-weight sialoglycoprotein production.

Examination of a large number of fresh tumor specimens previously showed that WGA-binding high-molecular-weight sialoglycoproteins are often associated with advanced stages of colorectal carcinoma with high metastatic potential (26). Therefore, the cellular mechanisms for the regulation of these molecules may have direct relevance to the progression and malignant behavior of human colorectal cancer. Many experimental and clinical studies have demonstrated that the biological behavior of tumor cells directly isolated from tumor tissues was different from that of cells growing in culture. Sialoglycoproteins and stimulators of sialoglycoprotein production may mediate such alterations. Endogenous factors that stimulate the production of sialoglycoproteins have not been documented previously. We assessed whether colon carcinoma cells treated with NCCM was altered in their sensitivity to natural immune effectors, such as natural killer lymphocytes and lymphokine-activated killer lymphocytes. The cells expressing increased sialoglycoproteins were shown to be much less sensitive to cytolysis by human lymphokine-activated killer lymphocytes suggesting that the higher levels of cell surface sialoglycoprotein resulted in increased chances of survival and metastatic colonization of colon carcinoma cells.

The importance of sialoglycoconjugates in immune effector cell recognition has been suggested for various tumor cells. For example, Dennis and Laferté (37) demonstrated that WGA-resistant MDAY leukemia-like mouse cells lacked cell surface sialoglycoproteins and were more sensitive to natural killer cell-mediated cytolysis. They showed that nonsialylated sugar chains can be recognized by syngeneic natural killer lymphocytes. A similar finding was reported by Ahrens and Ankel using Chinese hamster ovary cells (52). In contrast, Van Rinsum et al. (53) found that sialylated carbohydrate chains were crucial structures in a post-binding event in natural killer lymphocyte-mediated cytolysis. Fukuta et al. (40) demonstrated that cytolysis of melanoma mediated by human anomalous killer cell (similar or equivalent to lymphokine-activated killer cells) was blocked by a monoclonal antibody specific for disialoganglioside GD2 [GalNAc 1-4[NeuAc 2-8NeuAc 2-3]Gal 1-4Glc 1-ceramide], and Altevogt et al. (54) showed in the ESB/ Eb T-lymphoma system that the terminal N-acetylgalactosamines recognized by some lectins (soybean agglutinin and Vicia villosa agglutinin) are concealed by sialylation on the surface of ESB cells highly metastatic to the liver. In the stimulation of mouse thymocyte proliferation by allogeneic B-lymphoma cells, sialic acid on the surface of the tumor cells serves as a negative signal reducing thymocyte response (39). Similar concealing effects of high-molecular-weight mucin-like glycoproteins were already reported in mouse and rat mammary carcinoma systems (38, 41). Our study demonstrated that the expression of high-molecular-weight mucin-like sialoglycoproteins was induced by normal tissue-derived factors and was associated with reduced sensitivity of human colon carcinoma cells to lymphokine-activated killer activity. We examined the incidence of conjugate formation of activated lymphocytes with HT-29 P and HT-29 LMM target cells (55, 56), based on the hypothesis that a greater amount of high-molecular-weight sialoglycoproteins concealed the sites on the tumor cells required for recognition by lymphokine-activated killer lymphocytes. A smaller proportion of lymphocytes adhered to NCCM-treated cells than to untreated cells. In contrast, only a small difference was found in the proportion of dead conjugated target cells among effector-target conjugates detected by trypan blue dye exclusion tests in semisolid agar. These results suggested that high-molecular-weight sialoglycoproteins apparently influence tumor cell-lymphocyte conjugations. A more systematic approach is necessary to prove this hypothesis.

The other well-known biological activity of tumor cell surface sialoglycoproteins is their ability to induce platelet aggregation. Pearlstein et al. (42, 57) reported that a substance that induces platelet aggregation present in extracts of renal carcinoma and other cells can be destroyed by sialidase treatment. Kijima-Suda et al. (43) demonstrated that the amount of mouse colon carcinoma cell surface sialoglycoconjugates decreased after treatment of the cells with an inhibitor of sialyltransferase and that there was a concomitant decrease in platelet aggregation and in metastatic potential. Later, they suggested that mucin-type sugar chains are important for the induction of platelet aggregation (58). Our results clearly demonstrated that the ability of human colon carcinoma cells to induce platelet aggregation was enhanced by prior treatment of the cells with NCCM. It is plausible, therefore, that the changes in platelet aggregation by colon carcinoma cells can be attributed to the increase in sialoglycoproteins. Thus, an induction of high-molecular-weight sialoglycoprotein production probably provides colorectal carcinoma cells with an enhanced capacity to metastasize by means of their abilities to evade immune defense mechanisms and to form emboli with platelets. Purification and characterization of this substance, which we are tentatively calling mucomodulin, will greatly facilitate future studies of tumor cell-host cell interaction in metastasis formation.

REFERENCES

MUCOMODULIN EFFECTS ON COLON CARCINOMA CELLS


Soluble Factor in Normal Tissues That Stimulates High-Molecular-Weight Sialoglycoprotein Production by Human Colon Carcinoma Cells

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