A Cell Surface Glycoprotein Expressed by Colorectal Carcinomas Including Poorly Differentiated, Noncarcinoembryonic Antigen-producing Colorectal Tumors

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ABSTRACT

A monoclonal antibody to a cell surface glycoprotein on human colorectal carcinomas was raised using the undifferentiated colon carcinoma cell line MIP 101 as the immunogen. This antibody, ND4, is an IgG2a which does not cross-react with carcinoembryonic antigen (CEA), nonspecific cross-reacting antigen, or blood group substances A, B, and H. Immunoprecipitation using lysates of cells grown in [35S]methionine or [14C]glucosamine and lysates of cells surface labeled with 125I showed binding to a cell surface glycoprotein with a molecular weight of approximately 160,000. Indirect immunofluorescence showed binding to the cell surface of 14 of 15 human colorectal carcinoma cell lines including six of six that do not secrete CEA. Two of seven human noncolorectal carcinoma lines and one of six nonhuman cell lines also bound antibody. Immunoperoxidase staining of formalin-fixed tissues showed prominent antibody binding with 19 of 33 (58%) human colorectal carcinomas, including five of five poorly differentiated tumors, five of 43 (12%) normal colonic mucosal biopsies, and one of 17 (6%) normal noncolonic tissues. One of 11 (9%) noncolonic tumors, a gastric adenocarcinoma, stained with ND4. Preliminary data obtained by a nonquantitative nitrocellulose dot-immunoassay have tentatively identified this glycoprotein in the serum of 15 of 37 (41%) patients with colorectal cancer. Three of the 15 patients had early stage disease and normal CEA levels (<2.5 ng/ml). Three patients had circulating antigen detectable preoperatively but not after tumor resection. Only one of 11 (9%) sera samples from normal subjects was positive. The characteristics of ND4 suggest that it may be of value in monitoring patients with colorectal carcinomas who do not have plasma CEA elevations. It may also be of value in the differential diagnosis of metastatic, poorly differentiated adenocarcinomas of unknown primary origin.

INTRODUCTION

Gastrointestinal cancers share an expanding inventory of tumor-associated antigens (1–17). Some have been identified as receptors, oncogene products, or altered blood group substances (9, 16, 17). The functional properties of a few of these macromolecules that are associated with transformed cells have been defined (7, 16) and may have clinical or biological significance. For example, overexpression of a certain cell surface glycoprotein on some transformed cells has been associated with drug resistance to chemotherapeutic agents (7).

With two exceptions (Me 491 and H-type 2), tumor-associated antigens described to date are found primarily on differentiated colorectal carcinomas but are progressively lost or not found at all on the more poorly differentiated tumors (17). CEA, CA19-9, and the TAG-72 antigens are not usually found in the circulation of patients with poorly differentiated tumors and tend not to be shed into the supernatants of cultures of poorly differentiated colon cancer cell lines (1, 2, 18). Sensitive assays may find evidence of certain of these antigen epitopes in the cells of poorly differentiated tumors, but the immunoreactive antigen does not appear on the cell membrane and is not shed into the patient’s plasma.

There is evidence for altered processing and secretion of glycoprotein macromolecules by poorly differentiated colorectal cancer cells when compared to the better differentiated cell (19–22). For example, the extent of posttranslational processing and secretion of laminin and other basement membrane components may be related to tumor differentiation (19). However, phenotypic markers of differentiation pathways of the gastrointestinal epithelial cell have not been elucidated as have markers for hematopoietic cells. In contrast to the well-characterized series of antibodies which define differentiation in lymphoid cells, there are no markers which are characteristic of the primitive or undifferentiated cells of the colorectal epithelium maturation pathway. Indeed the pathway(s) itself is still uncertain. Thus characterization of the differentiation of colorectal cancer is still completely dependent upon morphological grading of tumor structure and architecture.

New markers expressed by poorly differentiated colon cancers could provide useful serodiagnostic and immunohistological reagents for a population of colorectal cancer patients where none now exists. We have designed immunization strategies to produce reagents that would identify macromolecules on poorly differentiated and potentially more immature or primitive gastrointestinal epithelial cells. We report the results of immunization using a non-CEA producing, poorly differentiated colorectal cancer cell line MIP 101 (23) to raise mouse monoclonal antibodies. Hybridomas were selected if antibody bound to poorly differentiated colorectal cell line targets. One such monoclonal antibody, ND4, was found to bind to a cell surface glycoprotein on a wide spectrum of colorectal carcinomas including those of poorly differentiated morphology. This hybridoma has been stabilized and subcloned. The resultant monoclonal antibody has been used to characterize its antigen epitope and to identify the clinical expression of the antigen in patients’ tumors.

MATERIALS AND METHODS

Hybridoma Production

BALB/c mice were immunized with MIP 101 cells. Five × 10⁶ cells were injected on each occasion. The first three injections were given i.p., and a fourth injection was given 2 wk later i.v. Cells were injected with complete Freund’s adjuvant on the first occasion, incomplete adjuvant on the second and third occasions, and no adjuvant on the last occasion. Serum obtained prior to the last injection showed prominent binding by solid phase enzyme immunonassay to the immunogen (MIP 101 cells). Three days after the last injection the mouse with the best response was sacrificed, and hybridomas were produced by fusion of spleen cells with NS-1 myeloma cells (American Type Culture
Binding to Cell Lines in Vitro

Binding studies were performed using indirect immunofluorescence with rhodamine-conjugated goat antimouse mixed IgGs on formalin-fixed cells. Cells were viewed using a Zeiss epifluorescent microscope. Twenty-eight different cell lines were examined, including 15 human colorectal carcinoma lines, 9 producing CEA and 6 not producing CEA, 7 human carcinoma lines not of colorectal origin, and 6 nonhuman cell lines (Tables 1 and 2). Ascites from an IgG2a producing mouse myeloma line UPC-10 (Sigma, St. Louis, MO) was used as a negative control antibody. Binding to cell lines were graded --, +, or ++ dependent on degree of fluorescence. All studies were done on three different occasions and were viewed by two independent observers. Cell lines were obtained from several sources as shown in Tables 1 and 2.

Immunohistopathology

Surgical specimens were obtained from the New England Deaconess Hospital and from the files of the Mallory Institute of Pathology. The specimens were fixed in 10% buffered formalin, paraffin embedded, processed routinely, and stained with hematoxylin and eosin. The degree of differentiation of colonic adenocarcinomas was assessed on the basis of conventional histopathological criteria, i.e., the formation of gland-like structures by tumor cells. Tumors were classified as poorly differentiated if less than one-third of the tumor area exhibited gland formation or if gland formation was not apparent but mucin production was used in all subsequent investigations.

Antibody Typing

The ND4 ascites was screened for antibody isotype by Ouchterlony double diffusion in agar plates against anti-IgM, anti-IgG, anti-IgG1, anti-IgG2a, anti-IgG2b, and anti-IgG3 antibodies (Cappell, Malvern, PA). The ascites was diluted 1:10 with PBS and placed in the center well, and the other antibodies were placed in the surrounding wells and used undiluted. Plates were incubated at 24°C for 24 h and were then inspected for the presence of precipitating bands.

Antigen Identification

Antigen identification was performed by immunoprecipitation of lysates of MIP 101 cells which had been radiolabeled by four different procedures. Cells were metabolically labeled overnight with [35S]methionine or [3H]glucosamine (New England Nuclear/Dupont, Wilmington, DE) (25). Cell surfaces of live MIP 101 cells were labeled with 125I using the lactoperoxidase procedure (26). The labeled cells were lysed in RIPA buffer to which 1 mM PMSF was added omitting the 1% deoxycholate (5). Preswollen Protein A-Sepharose was incubated with goat anti-mouse whole serum immunoglobulin (Cappell, Malvern, PA). Forty μl of this complex were placed in each of 3 plastic Eppendorf tubes. To the first tube 5 μl of ND4 ascites were added. To the second tube 5 μl of UPC-10 (Sigma, St. Louis, MO) were added. To the third tube 100 μl of radiolabeled MIP 101 cell lysate and 10 μl of normal mouse serum were added. The tubes were incubated overnight at 4°C with continuous agitation. The third tube was centrifuged, and half of the supernatant was added to each of the first 2 tubes to allow binding of the preclared, radiolabeled cell lysate to the antibody-Protein A complexes. This was incubated for 1 h at 4°C, centrifuged, and washed 4 times with RIPA buffer and then reacted with SDS-polyacrylamide gel electrophoresis on 10% gels followed by autoradiography of the dried gels with Kodak X-Omat film at -80°C. For the [35S]methionine- and [3H]glucosamine-labeled cells the gels were soaked in Enhance (New England Nuclear/DuPont, Wilmington, DE) for 45 min and then washed in distilled water for 30 min prior to drying and exposure to X-ray film.

Effect of Trypsin

Ninety-six-well, sterile, ELISA, microtiter plates were seeded with MIP 101 cells and grown until confluent in 50% Dulbecco's modified Eagle's medium (GIBCO Laboratories, Grand Island, NY) and 50% RPMI 1640 supplemented with 10% calf serum (MA Bioproducts, Walkersville, MD). Plates were incubated at 37°C in 5% CO2 at 100% humidity. The cells were fixed with 40% formaldehyde at room temperature for 15 min and rinsed twice with PBS. Two lines of wells were incubated with 1% trypsin (Gibco, Grand Island, NY) at 37°C for 2 h, and 2 lines were incubated with PBS. All wells were examined by light microscopy to ensure that cells had not detached from the microtiter plate. The plates were then rinsed with PBS, and both trypsin-treated and PBS-treated wells were incubated for 1 h at 37°C with ND4 in serial dilutions from 1:1,000 to 1:1,000,000 in PBS. The plates were washed and incubated at 37°C with peroxidase-conjugated goat anti-mouse immunoglobulin (Hyclone, Logan, UT) diluted 1:1,000 with PBS. The plates were developed in o-phenylene diamine (1 mg/ml) in 0.01 M sodium citrate buffer adjusted to pH 5.0 with citric acid, to which 30 μl of 30% hydrogen peroxide/10 ml were added immediately prior to use. Absorbances were read at 492 nm using a Bio-Rad enzyme immunoassay reader. Absorbance was plotted against ND4 dilution for both trypsin-treated and PBS-treated cells.
Cross-Reactivity with Other Antigens

Purified CEA, NCA, and α1-acid glycoprotein, blood group antigens obtained from saliva of patients of AB and O blood types were bound to wells of ELISA microtiter plates. After blocking with 3% bovine serum albumin in Tris-buffered saline for 1 h at 37°C, the wells were incubated with ND4 and antibodies to A, B, and H antigens as positive controls for the saliva-derived antigens. Subsequent reactions were carried out as described above for the ELISA procedure. Saliva antigens were prepared by centrifuging saliva at 1000 rpm for 10 min and diluting the supernatant 1:1 with 0.1 M bicarbonate buffer, pH 9.6.

Antigen Detection in Ascites and Serum

Ascites from 2 patients with colorectal cancer, serum from 37 patients with colorectal cancer, and serum from 11 normal subjects were assayed for the presence of ND4 antigen using a nitrocellulose "dot" immunoassay (5). Nine of the patients had early stage disease (Dukes B2 or earlier). Five-μl "dots" of 1:5 dilutions of ascites or serum in PBS were placed on a nitrocellulose membrane. After allowing these to air dry, nonspecific binding sites on the membrane were blocked with a mixture of 4 parts reconstituted dried milk (Carnation, Los Angeles, CA), 1 part liquid fish gelatin (Norland Products, New Brunswick, NJ), and 2 parts goat serum (Hazelton/Dutchland, Inc., Denver, PA) at 24°C for 1 h with continuous agitation. The membrane was incubated with a 1:100 dilution of ND4 ascites at 4°C overnight, washed with PBS, and incubated for 2 h at room temperature with sheep anti-mouse IgG (Cappell, Malvern, PA) that had been absorbed with polymerized normal human serum and polymerized human IgG and diluted 1:200 in PBS containing 1% fish gelatin. The reaction was developed with a solution of 4-chloronaphthol (30 mg dissolved in 10 ml of methanol and 50 ml of PBS) to which 100 μl of 30% hydrogen peroxide was added immediately prior to use.

RESULTS

After fusion of MIP 101-immunized splenocytes and ELISA screening of numerous clones, a stable subclone that secreted an antibody that bound to the immunizing tissue culture cell line was selected. The antibody was a monoclonal that formed precipitating bands with anti-IgG and anti-IgG2a antibodies by Ouchterlony double diffusion.

Binding to Cell Lines in Vitro. After formalin fixation, indirect immunofluorescence of formalin-fixed cells showed binding to 14 of 15 human colorectal carcinoma cell lines. Prominent (+++) binding was seen on 12 of 15 (80%) (Table 1; Fig. 1). Prominent binding was also seen on all of the 6 non-CEA-producing colorectal carcinoma cell lines. Two of 7 human noncolorectal carcinoma lines bound antibody, the MCF7 breast cells had prominent binding (+++), and an adrenal carcinoma cell line CCL 105 had weaker binding (+). One of 6 nonhuman lines (MB49) showed moderate binding of the antibody (Table 2).

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Immunohistopathology (Table 3). ND4 showed prominent staining (+++) in 19 of the 33 (58%) colorectal adenocarcinomas studied with the immunoperoxidase technique, including 5 of the 6 poorly differentiated adenocarcinomas (Fig. 2, a and b). An additional 4 moderately differentiated adenocarcinomas

Table 1: Binding of ND4 antibody to colorectal carcinoma cell lines by indirect immunofluorescence of formalin-fixed cells

Table 2: Binding of ND4 antibody to cells not of colorectal origin by indirect immunofluorescence of formalin-fixed cells

Table 3: Prominent binding of ND4 antibody to human tissues in paraffin sections by avidin-biotin-amplified immunoperoxidase staining

Fig. 1. Indirect immunofluorescence showing cell surface binding of ND4 to MIP 101, an undifferentiated colorectal carcinoma cell line.
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Fig. 2. A, immunoperoxidase staining of a moderately differentiated adenocarcinoma of the colon by monoclonal antibody ND4. B, immunoperoxidase staining of a poorly differentiated adenocarcinoma of the colon by monoclonal antibody ND4. C, immunoperoxidase staining of normal colonic mucosa by monoclonal antibody ND4. Normal colon stains only rarely, but when staining is observed, it is confined to the surface mucosa as seen above.

showed weak staining with ND4 (+). The pattern of staining was predominantly diffuse with the majority of tumor cells positive. In tumors which were morphologically heterogeneous, for example, predominantly moderately differentiated tumors with poorly differentiated foci, the antibody stained all components of the tumor. Ten of the 33 tumors (30%), including 9 moderately differentiated adenocarcinomas and 1 poorly differentiated, showed no staining with the antibody. The one poorly differentiated tumor that failed to stain had the signet ring cell morphology. When staining in tumor sections and histologically normal-appearing mucosa were compared in the 9 matched pairs, 3 tumors were strongly positive with equally intense positivity in the normal-appearing mucosa, while 3 negative tumors were also associated with strongly positive normal-appearing mucosa. In two cases the tumors showed weak positivity while the normal mucosa showed intense staining. In the case of the signet ring cell carcinoma in which the tumor showed no staining, the normal-appearing mucosa was intensely positive.

Five of the 43 (12%) normal colonic mucosal biopsies showed prominent staining (+++), while 24 (56%) showed weak positivity (+). ND4-positive cells were situated predominantly on the surface of the mucosa and in the superficial portion of the crypts of Lieberkühn (Fig. 2C). Minimal positivity was seen at the base of the crypts. Immunoperoxidase staining of fetal tissues showed strong staining (+++) by ND4 in all epithelial-derived tissues except lung. No mesenchymal tissues, including brain or thymus, showed any staining. Among the normal noncolonic tissues obtained as surgical specimens from adult patients, prominent staining was seen only in the squamous mucosa of the esophagus. Sections of normal breast (3 cases examined), kidney (2), pancreas, liver, lung, salivary gland, endometrium, and the mesothelium from a hernia sac showed no staining. Weak focal positivity (+) was seen in the mucosa of the stomach (2), gallbladder, and ileum. No staining of any hematopoietic cells was detected in the 3 bone marrow particle sections examined. Autopsy sections of brain, heart, prostate, and spleen were also negative.

Antigen Identification. Immunoprecipitation of antigen labeled with 125I, [35S]methionine, or [3H]glucosamine showed a Mr, 160,000 band on SDS-polyacrylamide gel electrophoresis. This band was not precipitated by the control antibody (UPC-10) (Figs. 3 to 5). No reactivity of ND4 to CEA, NCA and acid glycoprotein, or blood group antigens A, B, and H was detected using the ELISA assay. Control antibodies to the blood group antigens and to CEA all produced strongly positive responses, indicating that the antigens had bound to the ELISA plates.

The Western blots of the MIP 101 tumor extracts showed a Mr, 160,000 band which was absent from the controls. No equivalent band could be seen on the blots from the mucosal extract. This was probably due to a lower concentration of ND4 antigen in normal mucosa.

Trypsin treatment of formalin-fixed MIP 101 cells resulted in loss of >90% of the binding of ND4 to the cells as determined by the ELISA assay (Fig. 6).

Antigen Presence in Sera. Semiquantitative nitrocellulose "dot" immunoassays of serum from patients with colorectal cancer have given preliminary data of positive reactions in 15 of 37 patients (41%). Three of these patients had CEA levels of less than 2.5 ng/ml. Three patients had serial dot assays and were shown to be positive prior to resection and negative after resection of the primary colorectal cancers. Of 11 control sera from laboratory volunteers, only 1 was positive by this assay (9%).
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DISCUSSION

Numerous antibodies have been raised to antigens present on or shed by colon carcinomas (5, 6, 10–15). Many recognize various epitopes in the family of CEA molecules (27). Some of these antibodies have been useful in the diagnosis and monitoring of patients with colorectal carcinoma (1–3, 28–30) and potentially for immunotargeting of carcinomas that contain or secrete CEA (31, 32). However, approximately 50% of patients with colorectal cancer undergoing therapy do not have elevated plasma CEA levels. The use of assays incorporating other antibodies to different antigens associated with colorectal cancers (29) has not improved the rate of initial diagnosis or significantly altered the ability to monitor tumor response during therapy when multiple assays are used serially (30, 33).

In patients with moderately to well-differentiated colorectal carcinomas (80 to 90% of all colorectal carcinoma patients), histological characterization of the tumors is strictly morphological. Application of presently available antibodies to epitopes such as CEA and CEA family members does not help define subsets of moderately to well-differentiated colorectal cancer with differing prognosis. Although reports of new markers for precancerous and well-differentiated colon and rectal carcinoma cells proliferate (12–14), prognosis based on these new markers has not been reliable (34).

Application of presently available monoclonal antibodies in tissue staining of a broad array of colorectal carcinomas from poorly differentiated to well differentiated has not increased our understanding of the biological differences that account for morphological variation. We believe that lack of such biological probes identifying the molecular basis for morphological differences in colorectal carcinoma is a function of not having adequately identified markers for purportedly more primitive, non-CEA-producing colorectal cancers. Currently, morphological assessment by light microscopy of cell structure and tumor architecture is the sole basis of determining differentiation.

No clinically useful tumor markers for non-CEA-producing colorectal cancers are available. Though some of the tumor-associated antigens identified over the last decade by monoclonal antibody hybridoma technique have been found on fetal tissues (8, 35), few are preserved on the more poorly differentiated colorectal adenocarcinoma cells that do not express CEA (2, 3, 15). Durrani et al. (15) described three antibodies that stained aneuploid colorectal tumors. Ernst et al. (17) have demonstrated the conservation of a melanoma-associated antigen (ME 491) and a blood group antigen (H-type 2) on a few poorly differentiated primary colon cancers. The remaining 12 antigens in the Ernst study were not expressed in the poorly differentiated primary tumors of the colon or their metastases. Such a lack of clinically useful markers for undifferentiated tumors or poorly differentiated tumors of the colon hampers the ability of the pathologist to diagnose poorly differentiated tumors of the colon from noncolonic tumors, such as large cell lymphoma, that might be potentially more treatable or at least should be treated in a different manner. In addition, patients with poorly differentiated or undifferentiated colorectal carcinomas are uniformly seronegative for CEA, CA 19-9, and other shed colon cancer-associated antigens (1, 2, 18). These poorly differentiated tumors, as a group, are more virulent than better differentiated tumors, often having a higher metastatic proclivity. Serum markers for this subset of carcinoma would be of
The assay was performed as described in the text. •, no treatment; A, following treatment with 1% trypsin at 37°C for 2 h.

Characterization of the antigen recognized by ND4 has shown that it has an approximate molecular weight of 160,000 based on migration of the immunoprecipitates on SDS-polyacrylamide gels. Labeling by both [35S]methionine and [3H]-glucosamine indicates that it is a glycoprotein. Incubation of MIP 101 cells (those used as the immunogen) with trypsin results in inhibition of antibody binding. The loss of activity on incubation of cells with trypsin and the ability to label with 125I by the lactoperoxidase procedure suggest that the epitope is carried as a glycoprotein on the cell surface.

Screening against other known tumor- and colon-associated antigens showed that ND4 did not bind to CEA, NCA, α1-acid glycoprotein, or the blood group substances A, B, and H (data not shown).

Since ND4 defines an epitope that is present not only on colorectal carcinoma but on normal tissues as well as fetal epithelium, this epitope is by definition a differentiation or oncofetal antigen. However, staining patterns using tissue culture reference cells, paired specimens from patients with colon cancer and adjacent normal tissue, and a variety of other solid tumors show a higher degree of specificity for tumors of colonic origin than many other available markers of human colorectal carcinoma. Specifically, the major difference between the ND4-defined epitope and all other presently available colorectal carcinoma markers is the applicability of this marker in the tissue staining of the more poorly differentiated human colorectal carcinomas. Thus ND4 may have clinical use in the immunopathological differential diagnosis of undifferentiated metastatic tumors of unknown primaries and may also increase our understanding of the analogy between gastrointestinal epithelial maturation and transformation.

Eventual use of ND4 marker assays to determine if the antigen is shed into the serum of patients with poorly differentiated or non-CEA-secreting colorectal carcinomas awaits the development of a quantitative radioimmunoassay or ELISA to confirm preliminary results by nitrocellulose “dot” immunosays showing that ND4 may have potential utility as a more broadly applicable serum marker for patients with a wide morphological array of colorectal cancer.

**REFERENCES**


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