Immunoperoxidase Localization of a High-Molecular-Weight Mucin Recognized by Monoclonal Antibody 1D3

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ABSTRACT

The distribution of an antigen recognized by murine monoclonal antibody 1D3 (Bhattacharya, M., Chatterjee, S. K., Barlow, J. J., and Fuji, H. Cancer Res., 42: 1650-1654, 1982) was investigated in various types of human malignant and normal adult tissues by indirect immunoperoxidase assay in fixed paraffin-embedded sections. One hundred percent of ovarian mucinous cystadenocarcinomas expressed high levels of the antigen with intense staining of 80 to 100% of the tumor area, thus confirming our previous finding with radioimmunoassay and absorption analyses. About 51% of colonic carcinomas, 33% of gastric carcinomas, and 22% of pancreatic carcinomas were also positive for this high-molecular-weight mucoprotein antigen. All other ovarian and nonovarian carcinomas tested including carcinoma of lung, breast, endometrium, cervix, and prostate were not stained by 1D3. In addition, sarcomas, melanomas, and lymphomas also did not express any detectable level of the antigen. When surveyed against various normal adult tissues, 1D3 had reactivity limited to the colon. Normal colon, however, exhibited reduced staining intensities compared to tumors or to the apparently normal colon adjacent to tumors. The antigen thus appears to be a colorectal tissue-specific antigen showing increased levels in ovarian mucinous cystadenocarcinomas and in some gastrointestinal tumors. 1D3 antigen is a potential tumor marker for mucinous ovarian and colonic tumors.

INTRODUCTION

Murine monoclonal antibody 1D3 was derived by immunizing BALB/c female mice with human ovarian cystadenocarcinoma extract. In preliminary studies, the antibody had a very restricted distribution in that it reacted only with mucinous cystadenocarcinomas of the ovary and with fetal intestinal tissue, but otherwise showed little cross-reactivity (11). 1D3 did not appear to be related to previous antibodies in the heteroantisera against ovarian tumor antigens that we (6-8) or others (21, 23, 25, 33) had developed earlier. The antigenic determinant recognized by 1D3 was unrelated to carcinoembryonic antigen, normal colonic mucosal glycoprotein, normal human serum components, or ABO blood group materials (11). We examined the antibody by an indirect solid-phase radioimmunoassay and absorption analyses using tissue extracts. These studies gave no information on the cellular distribution of 1D3 or whether or not the antibody had the ability to react with single cells in whole-tissue specimens. In this report, we pursue this line of investigation and describe the tissue distribution of 1D3 antigen by a sensitive and reliable immunohistochemical assay, namely, the immunoperoxidase technique. A variety of human malignant and normal tissues was examined in fixed paraffin-embedded sections by indirect IP assay.

MATERIALS AND METHODS

Antibodies. Monoclonal IgG1 antibody secreted by clone 1D3 was obtained after fusion of spleen cells from BALB/c mice immunized with human ovarian cystadenocarcinoma extract with the mouse myeloma cell line P3/NS1/1-Ag4. Details concerning the immunization, fusion, cloning, and screening procedures have been reported previously (11). In mucinous ovarian cystadenocarcinomas, 1D3 reacts with a high-molecular-weight (Mr > 10^6) mucin-type glycoprotein having a low isoelectric point (details to be published elsewhere). As a control, mouse myeloma P3 of IgG1 isotype was used. One other monoclonal IgG1 antibody 7A10 which recognized a widely distributed human cancer-associated glycoprotein (the M, 48,000 glycoprotein) (9) was used as a positive control for some specimens. Undiluted spent tissue culture medium from 1D3, P3, and 7A10 were used as a source of antibody in the IP assay.

Specimens. Paraffin-embedded blocks of tissues were obtained from the Pathology Departments of Our Lady of Victory Hospital and Roswell Park Memorial Institute, Buffalo, NY. The blocks ranged in age from recent to 12 years old. Freshly obtained autopsy specimens of normal tissues and tumors were also used. Specimens were usually fixed immediately in 10% buffered formalin or Lillie's solution (glacial acetic acid:40% formalin:95% ethanol (1:2:4:75)). The antigen under study was found previously to be well conserved with fixation and paraffin-embedding procedure. Blocks were cut into 5-μm sections and collected on slides pretreated with ovalbumin and glycerin. The sections were air dried at room temperature. The tumor specimens analyzed in this study contained virtually all grades of tumor, i.e., well, moderately, and poorly differentiated.

Indirect IP Assay. The IP assay was performed by a modification of the procedure described previously (9). After deparaffinizing the sections with xylene (2 changes for 10 min each) and hydrating the sections (absolute ethanol, 2 changes for 10 min each; 95% ethanol, 10 min; 70% ethanol, 10 min; 50% ethanol, 10 min; 20% ethanol, 10 min; PBS, 10 min), the endogenous peroxidase activity was inhibited by treating with 0.3% H2O2 in absolute methanol for 15 min. The tissue sections were then washed with PBS (3 changes for 5 min each) and pretreated with 10% normal rabbit sera in PBS containing 1% BSA for 10 min. The slides were then successively incubated for 1 h at room temperature with 200 μl of undiluted culture supernatant containing mouse monoclonal antibody, peroxidase-conjugated rabbit antibodies against mouse immunoglobulin, preabsorbed with human immunoglobulin (1:30 dilution; Accurate Chemical Corp., Hicksville, NY) with 10-min rinses in PBS:BSA between all steps. The slides were then treated with 0.06% diaminobenzidine with 0.01% H2O2 (Sigma Chemical Co., St. Louis, MO) in PBS:BSA for 5 min, counterstained with hematoxylin, dehydrated, and mounted.

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positive and the other one negative for the expression of the 1D3 antigen, were serially sectioned and included as internal standards for each separate lot of staining procedures. The 1D3 antibody used for all staining reactions was in antibody excess as determined previously by serial dilution of culture supernatant. Increasing the length of antibody incubation to 18 h at 4 °C did not have any effect on the sensitivity of the assay.

Scoring of Slides. 1D3 incubated slides were compared to control P3-incubated slides. A very strong, deep, crisp brown staining was scored as + + +, a moderate staining was scored as + +, and a weak staining was +, and no staining was negative (−) in Tables 1 to 3. If cells showing staining reaction were present in small clusters on the background of negative cells, the reaction was described as focal. The percentage of positive area was estimated from the total tumoral area in the section.

Neuraminidase Treatment. The IP procedure was begun as usual, but after pretreatment with 10% normal rabbit sera and before incubation with the monoclonal antibody 1D3, the tissue sections were incubated at 37 °C for 2 h with various concentrations of type VI Clostridial neuraminidase (Sigma) as described in detail by Atkinson et al. (2). After neuraminidase treatment, the slides were washed, treated with monoclonal antibody, and processed as described before.

RESULTS

Expression of 1D3 Antigen in Human Ovarian Tissues and Tumors. The results of immunoperoxidase staining of normal adult ovaries, benign diseased ovaries, and ovarian malignant tumors have been presented in Table 1. Normal ovarian tissue components including stroma and the outer epithelial layer (single row of columnar cells) failed to show any detectable level of the antigen (Fig. 1a). In addition, none of the benign ovarian lesions tested was positive. It was of interest that 3 of 3 benign mucinous cystadenomas were also negative (Fig. 1b), but the examination of a larger number of specimens will be needed to determine whether this finding is consistent.

A variety of ovarian tumors of different histological types was checked by IP assay. Assessment of a group of 62 ovarian epithelial tumors consisting of such tumor types as serous (Fig. 2d), mucinous, endometrioid, and clear cell carcinomas and poorly differentiated adenocarcinomas revealed that 1D3 reacted only with mucinous cystadenocarcinomas. Fourteen of 14 mucinous cystadenocarcinomas were positive. One of 2 mucinous tumors of borderline cancer was also positive for the expression of the antigen. Mucinous ovarian carcinomas displayed high intensity of staining with 80 to 100% of tumoral areas being positive. In papillary mucinous tumors, the staining was localized to the apical luminal surface of cells and in the mucins secreted by these cells (Fig. 1c). Mucinous tumors which exhibited a glandular morphology had staining both at the apical membrane and cytoplasm of cells (Fig. 1d). Serial sections stained in parallel with control P3 antibody were always negative (Fig. 1e). Some mucinous ovarian tumors, characterized by gelatinous ascites and scattered mucin lakes with very scanty cellular elements, had deep brown staining in the mucinous material (Fig. 2a). In one case of mixed serous and mucinous cystadenocarcinoma, staining was restricted to the mucinous area only (Fig. 2b).

The one mucinous tumor of borderline cancer that did not express the antigen belonged to the pure endocervical type according to the classification of Fenoglio et al. (18, 19). It has been suggested that ovarian mucinous tumors of borderline cancer contain 2 types of epithelium, i.e., endocervical-like and intestine-like epithelium, whereas the fully malignant tumors are solely composed of intestinal-type epithelium. The endocervical-like epithelium contains only neutral mucin which is similar to the mucin found in normal cervical epithelium, whereas the intestinal-like epithelium contains both neutral and acidic mucin (16). The mucin recognized by 1D3 is an acidic mucin* which might explain its absence in the pure endocervical-type mucinous tumor of borderline cancer (Fig. 2c).

Other ovarian tumors tested such as dysgerminoma, endodermal sinus tumor, immature malignant teratoma, Brenner tumor, granulosa and granulosa-theca cell tumor, etc., did not express any detectable level of the antigen. In most ovarian cases studied, tissue blocks from common metastatic sites such as omentum, anterior abdominal wall, small and large intestine, and uterus were also analyzed by IP assay (data not included in Table 1) along with the primary tumor. In general, if a primary tumor were positive for 1D3 antigen, the metastatic lesions were also positive.

IP Staining of Nonovarian Tumors. A variety of tumors was tested by IP staining with 1D3 antibody (Table 2). Of 39 colorectal carcinoma specimens examined 20 were found to have detectable antigen by this procedure. In 4 specimens, a focal staining pattern was observed, while in others, 50 to 90% of tumor cells were stained (Fig. 3, a, c, and d). Of 18 cases having adjacent normal mucosa, all 18 showed intense staining reaction in the...
epithelium of normal mucosa and goblet cells (Fig. 4c), despite the fact that 8 of the tumors were negative (Fig. 3b). We also examined metastatic lesions from some primary colorectal tumors to such sites as omentum, ovary, small intestine, and lymph nodes. For example, the tumor specimen in Fig. 3a was from the primary well- to moderately well-differentiated colonic tumor of a patient who after 3 years had metastasis to the ovary (Fig. 3e). The intensity of staining reaction was even greater in the ovary than the colonic primary. Similarly, Fig. 3f represents the metastatic lesion from the poorly differentiated colonic carcinoma in Fig. 3d. The section in Fig. 3f had almost complete replacement of the ovarian parenchyma by tumor composed of nests and sheets of cells which showed intense granular staining pattern. Thus, the 1D3 antigen was found to be present in metastatic lesions of colorectal cancer also.

Within the gastrointestinal cancer group, 4 of the 12 gastrointestinal carcinomas and 2 of 9 pancreatic carcinomas were positive. The staining of tumor cells was strong and apical in gastrointestinal carcinomas but weak and diffuse in pancreatic carcinomas (Fig. 4f). The normal epithelium at these sites (stomach and pancreas) was consistently negative for the expression of the antigen (Fig. 4, d and e). It was noteworthy that adenocarcinomas of lung, breast, endometrium, cervix, and prostate, some of which also produce mucin, were not stained by 1D3. A variety of other tumors (Table 2) including melanoma, sarcoma, and lymphoma were also negative for the expression of the antigen in this assay. Many blocks with negative staining for 1D3 have demonstrated positive staining with another mouse monoclonal antibody 7A10 (details to be published elsewhere).

Immunoperoxidase Staining of Normal Tissues. The reactivity of 1D3 with normal adult tissues has been summarized in Table 3. The tissue blocks of most of the organs were obtained from autopsy of heart attack victims without any other known disease. Gynecological specimens were also obtained during surgery (oophorectomies and hysterectomies). In addition, normal tissues adjacent and distal to tumors were also analyzed, but these data were not included in the numbers of Table 3 (except for normal colonic mucosa adjacent to tumor). Of all the normal tissues examined, 1D3 reacted with the colon. But the intensity of the reaction was very weak as compared to that of tumors or normal colon adjacent to tumor (Fig. 4, a to c). The staining was localized in the epithelium of intestinal mucosa and goblet cells. All other normal epithelium, including bronchial glands in normal lung, alveolar epithelium, small biliary ducts of normal liver, tubules and collecting ducts of kidney, epithelial layer of fallopian tube, endometrial glands, and other specimens listed in Table 3, did not show any detectable level of the antigen. Normal spleen, bone marrow, and lymph nodes also did not show any reactivity with 1D3.

Neuraminidase Treatment of Tissue Blocks. The antigen recognized by 1D3 was not sensitive to neuraminidase treatment (0.01 to 0.5 unit of neuraminidase) as was judged from the intensity of the staining reaction which remained unchanged before and after treatment with neuraminidase in the IP assay (data not shown).

**DISCUSSION**

This report further demonstrates the restricted distribution of 1D3 antigen in an in vivo tissue distribution study using the
The 1D$_3$ antigen is probably a mucoprotein as judged from its high molecular weight, its localization in the mucous epithelium and in the adherent mucinous material secreted by these cells, and its presence in mucinous, but not in serous ovarian cystadenocarcinomas. Unlike the gastrointestinal cancer-associated antigen shown by Magnani et al. (27), the 1D$_3$ antigen was not a glycolipid. Several investigators have described antigens with similar physicochemical characteristics and tissue localization as 1D$_3$ antigen using heteroantisera. Gold (22) reported a colonic mucoprotein antigen specific for colonic tumors which cross-reacted with only one of 5 ovarian mucinous cystadenocarcinomas. Colonic mucoprotein antigen was not detectable in any gastric or pancreatic tumors analyzed. Pant et al. (31) described a colon-specific antigen which was produced in increased amounts in some ovarian mucinous and gastrointestinal tumors. Colon-specific antigen, however, had a much lower molecular weight ($M$, 70,000 to 110,000) than 1D$_3$ antigen. DeBoer et al. (16) found 2 antigens, small intestine mucin antigen and large intestine mucin antigen in some mucinous ovarian tumors. But small intestine mucin antigen was absent from normal colon, and large-intestine mucin antigen was not detectable in ovarian mucinous cystadenocarcinomas. Recently, Bara et al. (4) have described several gastrointestinal antigens designated as "M" antigens which cross-react with mucinous ovarian cysts. 1D$_3$ antigen could be similar to one of them. It is, however, very difficult to compare the results reported by the above investigators using absorbed polyclonal heteroantisera with the data obtained in this study using a monoclonal antibody. The use of monoclonal antibodies should help greatly in standardizing results and comparing findings between laboratories. The pattern of staining reported here with 1D$_3$ antibody is distinct from the reactivity of previously described monoclonal antibodies which recognize antigens expressed by ovarian tumors (1, 9, 10, 14, 17, 24).

The 1D$_3$ antigen appears to be a truly colon-specific antigen which is produced ectopically in mucinous ovarian tumors and some gastrointestinal tumors. It is not very unusual for tumors to express "inappropriate" antigens which are not present in the normal tissue from which they are derived (28). On extensive testing by immunohistochemical techniques, they are usually found elsewhere in the body. The presence of an intestinal-like epithelium in mucinous tumors of the ovary has been suggested by several authors using different approaches (12, 16, 18, 19, 29, 30, 32). The behavior of the epithelium of mucinous ovarian tumors in many respects mimics that of gastrointestinal epithelium. So it is not too surprising to see the presence of 1D$_3$ antigen in intestinal-type epithelium of mucinous ovarian cystadenocarcinomas. Since the antigen is not detectable in serous or any other ovarian tumors, it could be a potential marker for mucinous ovarian cancer. 1D$_3$ antibody also could be of additional help in the histological subclassification of ovarian tumors. The sensitivity and reliability of the IP staining procedure will precisely distinguish mucinous-type differentiation from others. Since 1D$_3$ antigen was also expressed in ovarian mucinous cystadenocarcinoma metastases, the antibody could be a powerful reagent for immunotherapy of these tumors.

The presence of 1D$_3$ antigen in some gastric and pancreatic tumors may also represent intestinal-type differentiation of those tumors. Lauren (26) classified gastric tumors into 2 major types, intestinal and diffuse. However, gastric carcinomas are frequently polymorphous showing both patterns, and sometimes a correct classification is difficult. The presence or absence of 1D$_3$ antigen could be another criterion for more precisely defining these tumors. However, a systemic investigation using a larger number of gastric and pancreatic tumor specimens would be needed to determine whether this antigen could be used as a good marker for intestinal-type differentiation.

Colorectal carcinomas displayed a range of staining patterns and a great degree of antigenic heterogeneity. The staining was apical in gland-like structures and was granular (cytoplasmic) in less differentiated areas. About one-half of the colonic tumors tested showed detectable levels of the antigen. The antigen-positive tumors in many instances were histologically indistinguishable from the tumors lacking antigen expression. It was noteworthy that in all cases examined, the nonneoplastic colonic mucosa adjacent to tumors was always brightly stained despite the fact that some of the tumors were unstained. Mucous modification of such cells has already been observed by ultrastructural analysis and other means (3, 5, 13, 20). Dawson and Filipe (15) have shown that abnormal goblet cells of colonic mucosa produce mainly sialomucins as compared to true normal goblet cells which produce mainly sulfomucins. Our immunohistological studies showed another difference between normal and peritumoral colonic mucosa with regard to the presence of 1D$_3$ antigen. Whether the mucin recognized by monoclonal antibody 1D$_3$ in normal, peritumoral, and malignant colonic mucosa is the same or different will be the subject of further analysis.

A possibly important application of this IP assay might be to separate colorectal adenocarcinomas into 2 major groups based on the presence or absence of 1D$_3$ antigen and determine if there are differences with regard to various aspects of biological behavior. Large retrospective studies using IP assay with fixed-tissue sections can now be conducted to see if there is a correlation with the clinical response and prognosis of the colorectal cancer patients and the expression of 1D$_3$ antigen.

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REFERENCES

Fig. 1. IP staining of fixed and paraffin-embedded tissue sections with monoclonal antibody 1D3 and control P3. a, normal ovary. No staining in the outer epithelial layer (Ep) or stroma (N) with 1D3, b, benign mucinous cystadenoma of the ovary. No staining in the benign epithelium (Ep), cyst (C), or stroma (N) with 1D3. c, papillary mucinous cystadenocarcinoma of ovary (Ca). Arrows, surface-related immunoperoxidase staining. Note deep staining in secreted mucinous material with 1D3. d, mucinous cystadenoma of ovary (M). Arrows, surface-related immunoreaction product. Note diffuse cytoplasmatic staining throughout the tumor specimen with 1D3. e, serial section of d stained with control P3. No staining. Counterstained with hematoxylin, x 240.

Fig. 2. IP staining of fixed and paraffin-embedded tissue sections with monoclonal antibody 1D3 and control P3. a, mucinous cystadenocarcinoma of ovary characterized by gelatinous material with very scanty cellular elements, stained with 1D3. Arrows, mucin lakes and gelatinous material. Note the sharp contrast between immunoreactive areas (dark reaction) and the immunonegative stroma surrounding them. b, mixed serous and mucinous cystadenocarcinoma of ovary stained with 1D3. Arrows, surface-related staining in the areas with mucinous differentiation. c, papillary adenocarcinoma of colon stained with 1D3. Arrows, endocervical-type epithelium. There was no staining in the epithelium, cyst (C), or stroma (N). d, ovarian serous papillary adenocarcinoma stained with 1D3. No detectable staining in the tumor (Ca). Counterstained with hematoxylin, x 240.

Fig. 3. IP staining of fixed and paraffin-embedded tissue sections with monoclonal antibody 1D3 and control P3. a, primary well- to moderately well-differentiated colonic adenocarcinoma stained with 1D3. Tumor cells show apical reaction (arrows) on the apex of epithelium and diffuse cytoplasmatic reaction in some other areas. b, moderately differentiated primary colonic adenocarcinoma stained with 1D3. There was no reactivity with tumor (Ca) or stroma (N). c, moderately differentiated primary rectal carcinoma (Ca) stained with 1D3. Note very delicate surface-related staining (arrows) in carcinoma and deep staining of inspissated material. d, poorly differentiated primary colonic adenocarcinoma stained with 1D3. Note intense granular staining of tumor cells (Ca), while the adjacent normal epithelium (NE) was also positive. e, metastatic adenocarcinoma to ovary (from the same patient as in a) stained with 1D3. Arrows, very intense surface-related immunoperoxidase staining. The mucinous material within the small cysts was also deeply stained. f, metastatic colonic carcinoma to ovary (from the same patient as in d) stained with 1D3. Note intense granular staining of tumor cells (Ca). Counterstained with hematoxylin, x 240.

Fig. 4. IP staining of fixed and paraffin-embedded tissue sections with monoclonal antibody 1D3 and control P3. a, normal colonic mucosa (M) stained with 1D3. Note weak staining in mucosal epithelium and goblet cells. b, normal colonic mucosa (M) adjacent to tumor stained with 1D3. Intense staining in mucosal epithelium and goblet cells. Note the contrast between b and c. d, normal stomach mucosa (M) stained with 1D3. No staining. e, normal pancreas stained with 1D3. There was no staining in the acinar cells (Ac) or the duct (D). f, metastatic pancreatic adenocarcinoma (Ca) to omentum stained with 1D3. Arrows, diffuse staining of tumor cells. Counterstained with hematoxylin, x 240.
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