Relationship of the Expression of the Multidrug Resistance Gene Product (P-Glycoprotein) in Human Colon Carcinoma to Local Tumor Aggressiveness and Lymph Node Metastasis


ABSTRACT

P-glycoprotein mediates classic multidrug resistance by functioning as an efflux pump that excretes lipophilic chemotherapeutic drugs from cancer cells. We now report an association of P-glycoprotein in colon carcinomas with another tumor property, i.e., enhancement of local tumor aggressiveness. P-glycoprotein was detected with monoclonal antibody immunohistochemistry in 65 of 95 primary colon adenocarcinomas, which were stage B1 or greater. In all but 1 of the 95 cases, solitary invading carcinoma cells were present at the leading edge of the tumor. This subpopulation of invasive carcinoma cells expressed P-glycoprotein (P-Gp+) in 47 of the 95 surgically resected colon specimens. Cases were grouped on the basis of the presence (Group 1, 47 cases) or absence (Group 2, 48 cases) of P-Gp+ invasive carcinoma cells. There was a significantly greater incidence of vessel invasion (P < 0.001) and lymph node metastases (P < 0.01) in Group 1 cases. Groups 1 and 2 did not differ with respect to tumor size, depth of invasion of the bowel wall, histological grade, maximum tumor size, mitotic index, mucin production, or presence of perineural invasion (P > 0.1). Our findings indicate that P-Gp+ invasive colon cancer cells may have an increased potential for dissemination, suggesting that P-glycoprotein may influence cell behavior.

INTRODUCTION

P-glycoprotein functions as an energy-driven membrane transporter that enables cancer cells to circumvent the lethal effects of “natural” lipophilic drugs (1–4). Whereas the focus of attention of cancer investigators with an interest in P-glycoprotein has been, in large measure, on the role of P-glycoprotein in intrinsic and acquired anticancer drug resistance, considerations of the sequence and membrane disposition of P-glycoprotein (3, 5) and certain properties of P-glycoprotein-rich epithelial cells in tissue culture (5, 6) support a hypothesis that P-glycoprotein expression may also influence the biological behavior of cancers in vivo (6–8).

We have followed up on these leads in this immunohistochemical study on surgical specimens obtained from 95 patients with previously untreated invasive colon carcinomas. A large subgroup of patients had P-Gp-3 invasive carcinoma cells. These patients were characterized by a high frequency of vascular invasion and lymph node metastases. Our data indicate that the presence of P-Gp+ invasive carcinoma cells in the connective tissue surrounding a tumor has important implications for the clinical and laboratory assessment of colon carcinomas. Furthermore, we suggest that P-Gp+ invasive carcinoma cells may be an important novel target for chemotherapy.

MATERIALS AND METHODS

Patient Materials. Patient materials were obtained from the Surgical Pathology Laboratory at Rush-Presbyterian-St. Luke’s Medical Center (Chicago, IL). We analyzed paraffin sections from surgical pathology specimens of 95 patients with primary colon adenocarcinoma invading into the muscularis propria or beyond. None of the patients had received prior chemotherapy or radiotherapy. All carcinomas were from colon proximal to the rectosigmoid colon. The carcinomas were from 46 men and 49 women including 71 whites, 23 blacks, and 1 Hispanic. The ages of the patients ranged from 27 to 101 years with a mean age of 72.5 years.

Specimens were removed at surgery, fixed in 10% neutral buffered formalin, and processed for routine surgical pathology evaluation. Paraffin blocks of histologically normal tissue taken from proximal and distal resection margins served as controls.

P-Glycoprotein Immunohistochemistry. Two unrelated anti-P-glycoprotein MAb were used for immunostaining of P-glycoprotein in tissue sections (9). MAb JSB-1 is a murine monoclonal antibody, subclass IgG1, developed by Schepel et al. (10) and Van der Valk et al. (11) which binds a cytoplasmic epitope of P-glycoprotein and was purchased from SANBIO (Uden, The Netherlands). MAb JSB-1 was diluted in PBS plus 1% bovine serum albumin at 1:160 for staining of paraffin sections. MAb C219 was obtained from Centocor Diagnostics (Malvern, PA). MAB C219 is a murine monoclonal antibody, subclass IgG2a, which binds a highly conserved cytoplasmic epitope of P-glycoprotein. It reacts with the protein products of both the MDR1 and MDR2 human genes (12, 13). In most slide-processing runs, MAb C219 was diluted in PBS plus 1% bovine serum albumin at 1:200 for paraffin sections. Reciprocal blocking experiments using JSB-1 and C219 have shown that these two anti-P-glycoprotein monoclonal antibodies react with different, mutually exclusive cytoplasmic epitopes of P-glycoprotein (11). Keratin was stained in carcinoma cells using MAB AE1/AE3 at a dilution of 1:1000 (Boehringer, Indianapolis, IN) to verify that isolated MAB C219- and JSB-1-positive cells in connective tissue stroma were epithelial in origin and not stromal cells or macrophages (14). Lack of staining with antimyosin antibodies excluded cross-reactivity with myosin isoforms as the explanation for MAb C219 and JSB-1 reactivity in P-Gp+ invasive cells (15). Staining with mucicarmine and Alcian blue did not colocalize with the MAb C219 or JSB-1 reaction products showing that positivity was unrelated to the distribution of mucins.

As described in detail elsewhere, immunohistochemistry was performed using the Vectastain Elite ABC procedure and reagents (Vector, Burlingame, CA) (16). Endogenous peroxidase activity was blocked in MAB JSB-1 and C219 preparations by incubation of slides in 0.3% H2O2 in absolute methanol for 30 min at room temperature. Each slide was incubated for 20 min at 20°C with normal horse serum (1.0%). Primary antibody, either JSB-1 or C219, was incubated on the tissue
section overnight at 4°C. Slides were equilibrated to room temperature and then rinsed three times in PBS. Slides were incubated in biotinylated horse anti-mouse IgG at 1:200 with 1.5% normal horse serum for 30 min at room temperature. Slides were rinsed three times in PBS and incubated for 30 min at room temperature in the avidin-biotin horseradish peroxidase macromolecular complex. After rinsing in PBS, slides were incubated for 6 min in 0.05% 3,3' diaminobenzidine tetrahydrochloride dihydrate (Aldrich Chemical Co., Inc., Milwaukee, WI) in PBS with 5 ml of 0.03% H2O2. Slides were rinsed in deionized water and then counterstained with hematoxylin, dehydrated, cleared, and mounted in Permount. A negative control slide repeated all these steps excluding the primary antibody and substituting an irrelevant, isotype-matched, monoclonal antibody. KB epidemoid carcinoma cell lines expressing known levels of the P-glycoprotein served as positive controls (17–19). As additional controls, MAB C219 blocking runs were performed on frozen and paraffin tissue sections using the P-glycoprotein reactive epitope peptide sequence VQVEALDKAREGRTC, generously supplied by Drs. Ling and Georges (Toronto, Ontario, Canada) (13).

Histopathology. Tissue sections were reviewed, graded, and scored for immunoreactivity by a pathologist blinded to clinical outcome. Paraffin sections stained with hematoxylin and eosin were used to assess morphological features including grade, stage, perineural invasion, vessel invasion, mitoses, and mucin production (20, 21). Tumors were staged according to the Astler-Coller modification of Dukes' classification: A, mucosa and submucosa; B1, into muscularis; B2, through muscularis; C1 and C2, as B1 and B2, with positive lymph nodes (22, 23). All cases in this study were stage B1 or greater. For each case, scoring of anti-P-glycoprotein MAB reactivity was based on the evaluation of paraffin tissue sections immunostained with MAB C219. MAB JSB-1 was used as a second indicator of sites of anti-P-glycoprotein immunoreactivity (24). Adjacent tissue sections from 40 paraffin blocks of colon carcinoma and 20 paraffin blocks of histologically normal colon were stained in parallel with MAB JSB-1 and MAB C219. Representative blocks of P-glycoprotein positive and P-glycoprotein negative cases were included. Each MAB produced identical patterns of both immunoreactivity and cytolocalization.

The extent of anti-P-glycoprotein reactivity was scored as negative when all tumor cells lacked immunostaining, 1+ when less than 5% of tumor cells were immunostained, 2+ when 5 to 25% of tumor cells were immunostained, and 3+ when more than 25% of tumor cells were immunostained.

Data Analyses. Test sensitivity for the detection of tumor P-Gp expression as an indicator of lymph node metastases was defined as

\[
\text{TP} \equiv \frac{TP}{TP + FN}
\]

and test predictive value was defined as

\[
\frac{TP}{TP + FN}
\]

where TP is a true positive, FP is a false positive, and FN is a false negative test result (25).

The \chi^2 test or Fisher's exact test were used to evaluate the statistical significance of differences among groups. Fisher's exact test was used where there were six or fewer items in a group.

RESULTS

Histopathology. Two histologically distinct tumor components were identified in invasive colonic adenocarcinomas: the major tumor mass (primary tumor; Fig. 1, A, B, and D); and invasive individual cancer cells and small solid cancer cell nests, often at the periphery or leading edge of the tumor (invasive cells; Fig. 1, C and E). The major tumor mass was defined as that component of the primary tumor consisting of large islands of malignant glands and extending out from the luminal surface of the colon. Glands were the predominant component in the well-differentiated and moderately well-differentiated tumors and were present focally in the more poorly differentiated tumors. Operationally, invasive cancer cells were defined as single cancer cells or small cancer cell nests isolated within the connective tissue stroma in the vicinity of the deep edge of the tumor (Fig. 1, C and E). Keratin immunohistochemical staining confirmed the epithelial origin of these single cells and multicell nests in the tumor connective tissue stroma.

Anti-P-Glycoprotein Immunohistochemistry. Anti-P-glycoprotein immunostaining (P-Gp+) of colon carcinomas was typically heterogeneous but was most commonly observed at specific locations within tumor specimens. The most intense P-Gp+ immunostaining was often at the tumor-stroma interface and in deeply invasive carcinoma cells (Fig. 1, B and D).

We previously described three patterns of MAB JSB-1 and C219 immunocytolocalization that occur in normal human colon and colonic adenocarcinomas, i.e., luminal, Golgi, and diffuse cytoplasmic immunostaining (16). All three patterns were identified in malignant glands stained in paraffin sections with either MAB JSB-1 or C219. Generally, the same staining patterns were produced in frozen sections.

In P-Gp+ invasive carcinoma cells, granular and diffuse cytoplasmic staining were common. Plasma membrane staining was difficult to demonstrate in paraffin sections. Patterns of mucicarmine and Alcian blue staining for mucins were distinctly different from patterns of MAB C219 and JSB-1 immunostaining for P-glycoprotein.

Prevalence of Anti-P-Glycoprotein Immunostaining. MAB C219 immunostaining was present in the major tumor mass in 65 of 95 patients (Fig. 2). MAB C219 positivity was present in the subpopulation of invading carcinoma cells in 47 cases and absent in 48 cases. Forty-five of the 65 cases with MAB C219 positivity in the major tumor mass had MAB C219 reactivity in the invading carcinoma cells. MAB C219-positive invading cancer cells were observed in the absence of MAB C219 immunostaining in the major tumor mass in two cases. MAB C219 immunostaining was present in sections from paraffin blocks of histopathologically normal epithelium taken from the resection margins in 27 of 86 patients. Available paraffin blocks of normal epithelium were inadequate for immunoperoxidase studies in the other nine cases.

Correlation of P-Glycoprotein Immunostaining with Lymph Node Metastases. Lymph node metastases were present in 37 of the 95 cases. Lymph node metastases were present in 28 of the 65 cases with P-Gp+ primary tumors and in 9 of 30 cases with P-Gp-negative primary tumors (Figs. 2 and 3). This difference was not significant. However, there was a significant correlation between the presence of P-Gp+ invasive cells and lymph node metastases (P = 0.0091). Whereas 25 of the 47 Group 1 patients (i.e., patients with P-Gp+ invasive cells) had lymph node metastases, only 12 of the 48 Group 2 patients (i.e., patients either without P-Gp+ cells or lacking any single tumor cells or cell nests at the leading edge of the tumor) had lymph node metastases.

The mean numbers of lymph nodes dissected in the surgical pathology specimens of Group 1 and Group 2 patients were not statistically different (P > 0.1). However, Group 1 patients had larger numbers of lymph nodes containing metastasis. In the surgical pathology specimens of 13 of 47 Group 1 patients, 4 or more lymph nodes contained metastatic colon carcinoma. Five of 48 surgical pathology specimens of Group 2 patients had 4 or more lymph nodes containing metastatic tumor. This
Fig. 1. Immunohistochemical demonstration of MAb C219 reactivity in human colon cancers. Paraffin sections, hematoxylin counterstain, × 400. A, colon carcinoma, Astler-Coller stage B2. MAb C219 immunostaining is heterogeneous within the main tumor mass. It is limited to supranuclear Golgi staining in several malignant glands (right). B, colon carcinoma, Astler-Coller stage C2. Nests of MAb C219 immunoreactive carcinoma cells (arrows) appear to be budding into the tumor connective tissue stroma. Although some MAb C219 reactivity is present in the main tumor mass (top), highest levels are present in the nests at the invasive front. C, colon carcinoma, Astler-Coller stage C2. MAb C219 immunostaining is strongest at the tumor invasive front. Individual cells budding into the connective tissue are highly immunoreactive (single arrows), as are small nests of tumor cells embedded in the connective tissue (double arrows). Deeper sections confirmed that the small nests were separated from the main tumor mass. D, colon carcinoma, Astler-Coller stage C1. Malignant gland with strong MAb C219 immunostaining in a supranuclear/Golgi distribution (top) and in a perinuclear distribution at the site of tentacular invasion into the connective tissue stroma (bottom). The perinuclear granular MAb C219 immunostaining may correspond to sites of Golgi stack membranes distributed throughout the cytoplasm of invading carcinoma cells. The most intense immunostaining is at the tip of the invading column of tumor cells (arrows). E, colon carcinoma, Astler-Coller stage B2. Nests of MAb C219-stained carcinoma cells embedded within the tumor connective tissue. The immunostaining is granular and cytoplasmic in distribution. Plasma membrane staining is not apparent.

difference was statistically significant ($P = 0.032$).

We evaluated the determination of P-Gp expression by primary colon carcinomas as a potential laboratory test for detecting the presence of lymph node metastases. Sensitivities of P-Gp expression by primary tumors and invasive cells for predicting lymph node metastases were 75.7 and 67.6%, respectively. The predictive values of P-Gp expression by primary tumors and invasive cells for predicting lymph node metastases were 43.1 and 53.2%, respectively.

Quantitative Analysis of MAb C219 Expression in Lymph Nodes. Quantitative studies showed that lymph node metastases tended to recapitulate the extent of expression of P-glycoprotein in the primary tumor. In 24 of the 37 cases with lymph node metastases, paraffin blocks of lymph nodes were available for
were entirely negative by MAb C219 immunostaining. It is both the major tumor mass and the invasive carcinoma cells. One P-Gp-negative primary tumor (†) did not have histologically identifiable invasive solitary carcinoma cells and cell nests at the deep edge of the tumor.

Physiological functions in normal organs have been hypothesized to serve additional unrelated roles in normal cells (6, 16). Some early studies suggested an inverse correlation between P-glycoprotein expression and malignant phenotype in experimental systems (7).

The processes of tumor invasion and metastasis involve complex changes in cell-cell and cell-substratum interactions (44–46). Glycoproteins play central roles in these processes (47). For example, in colon carcinomas, Fearon et al. (48) recently described a gene that was absent or altered in 70% of cases. The sequence of this gene showed that it coded for a member of the immunoglobulin superfamily related to neural cell adhesion molecules. A loss of this adhesion molecule may be a factor in the release of tumor cells from the constraints of normal cell-cell adhesion, thus promoting early events in the metastatic cascade when invasive carcinoma cells separate from the main tumor mass (49). Furthermore, increased homotypic and heterotypic adhesion may potentiate the formation and propagation of tumor thrombi within blood vessels and the colonization of distant organs by metastasizing tumor cells downstream in the metastatic cascade (50, 51).

Although the adhesion profiles of P-glycoproteins have not been examined in detail, several recent observations raised the possibility that P-glycoprotein may influence cell adhesion and, as a result of this, the processes involved in cancer dissemination. We found that in epidermoid carcinoma cells in tissue culture (16, 17), P-glycoprotein expression was accompanied by increased homotypic adhesion. Grogan et al. (52) recently observed a relationship between the hyperexpressions of both P-glycoprotein and neural cell adhesion molecules in myeloma plasma cells in tissue culture. These cells showed increased homotypic adhesion as well. It remains to be shown whether this increased adhesiveness is mechanistically related to P-glycoprotein, neural cell adhesion molecules, or both and whether the hyperexpression of these molecules can be related to the biological behavior of myeloma plasma cells in situ. Other lines of evidence suggesting that P-glycoprotein per se might influence cancer dissemination include preliminary observations of targeting of P-glycoprotein to adhesion plaques and in vitro evidence of enhancement of cell locomotion in P-glycoprotein-hyperexpressing cells (5). On the other hand, we recognize that P-glycoprotein may be a marker rather than a cause of tumor aggressiveness. This is supported by our observation in the current study that the staining of P-glycoprotein in lymph nodes tended to correlate with the staining in the main tumor mass, although greater anti-P-glycoprotein immunostaining was noted in lymph nodes in at least some cases.

This study describes a new association between P-Gp expression in invasive cells and increased metastases to lymph nodes. We used human colon carcinoma as a model because this is a common type of cancer, depth of invasion can be readily assessed by histopathology, and lymph nodes are routinely removed at surgery for evaluation of tumor dissemination (53). Prevalences of lymph node metastasis as well as numbers of lymph nodes containing colon cancer cells served as measures of the metastatic potential of the primary tumor.

In colon carcinoma, we found statistically significant associations between P-glycoprotein expression in a specific subpopulation of cancer cells and a high prevalence of vessel invasion and lymph node metastases. The subpopulation was defined operationally on the basis of location of the cancer cells at the periphery of the primary tumor and the positive immunoreac-

4 J. B. Ashman and R. S. Weinstein, unpublished observations.

Univariate Analysis of Correlates of Invasive Carcinoma Cell Anti-P-Glycoprotein Immunostaining. Table 1 summarizes the results of an analysis of the relationship of the reactivity of invading carcinoma cells with MAb C219 to additional patient parameters and tumor characteristics. No significant differences were found in patients’ age, sex, tumor size, grade, stage, mitotic index, or several other parameters (Table 1). There was, however, a strong correlation with vessel invasion.

**DISCUSSION**

P-glycoprotein is a Mc 170,000 membrane integral protein that mediates classic multidrug resistance in cancer cells by functioning as an energy-driven efflux pump (1–4, 27). P-glycoprotein is expressed in many different types of tumors in humans (6, 28–32) and in certain normal organs (11, 33–38). Physiological functions in normal organs have been hypothesized and may include roles involving the cellular detoxification of lipophilic xenobiotic compounds in kidney, liver, and intesti-
cinoma cells and the high prevalence of vessel invasion and could also contribute to the dissemination of P-glycoprotein molecule. A'-linked carbohydrate at this locus adds to the P-glycoprotein monomer (57, 58). Freeze-fracture studies have indicated that up to 70% of integral membrane protein may be inserted of large quantities of P-glycoprotein into the plasma membrane may influence tumor cell interactions with effector cells.

Our findings on P-Gp+ invasive cells have important implications for clinical oncology. With respect to the identification of P-glycoprotein-positive and -negative carcinomas, the localized expression of P-glycoprotein at the invasion front predicts that the location of tissue sampling could affect P-glycoprotein testing on solid tumor samples (8, 21). In this study, we found that P-glycoprotein expression was exclusively in the deep half of the tumor without expression of P-glycoprotein, detectable by immunohistochemistry, within the superficial fraction of the tumor. This heterogeneity was extreme in two tumors in which anti-P-glycoprotein staining was exclusively in invading carcinoma cells some distance away from the main tumor mass. Tissue sampling, using small superficial biopsies or fine needle aspirates, could miss P-glycoprotein-rich cancer cells at the periphery of the tumor.

Another finding with important diagnostic implications was that P-glycoprotein expression in lymph node metastases can be at variance with P-glycoprotein expression in the primary tumor. Although our results show that lymph node metastases tend to recapitulate patterns and intensities of expression of P-glycoprotein in the primary colon carcinoma, there were notable exceptions. In one-half of our cases characterized by P-glycoprotein expression in lymph node metastases, the presence of P-Gp-positive cells was predictive of vessel invasion (P < 0.001) and lymph node metastases (P < 0.01). Although the prevalences of liver metastases are different, this was not statistically significant (N.S.).

Table 1

<table>
<thead>
<tr>
<th>Prognostic factor</th>
<th>P</th>
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<tr>
<td>Vessel invasion</td>
<td>0.0001</td>
</tr>
<tr>
<td>Presence of lymph node metastases</td>
<td>0.0091</td>
</tr>
<tr>
<td>Number of lymph node metastases</td>
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<tr>
<td>Histological grade</td>
<td>0.1825</td>
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<tr>
<td>Liver metastases</td>
<td>0.2929</td>
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<tr>
<td>Depth of invasion</td>
<td>0.3246</td>
</tr>
<tr>
<td>Mitosis (&gt;=2/hpf)*</td>
<td>0.3520</td>
</tr>
<tr>
<td>Perineural invasion</td>
<td>0.4005</td>
</tr>
<tr>
<td>Age</td>
<td>0.4703</td>
</tr>
<tr>
<td>Mucinous component</td>
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<tr>
<td>Fibrosis</td>
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<td>Necrosis</td>
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<tr>
<td>Size</td>
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* hpf, high power field.

Modifications of the cell membrane glycocalyx resulting from activity of these cells with anti-P-glycoprotein MAbs (i.e., defining Group 1 patients). This occurrence of P-glycoprotein-rich colon carcinoma cells at the edge of the neoplasms confirms previous observations of isolated high MDR1 mRNA expressors, as identified by MDR1 mRNA in situ hybridization, in this location (8).

We are currently exploring mechanisms by which P-glycoprotein may potentiate tumor dissemination. Our studies using P-glycoprotein-rich cell lines suggest that enhancement of tumor cell locomotion and alterations in cell-cell adhesion by P-glycoprotein expression may be contributing factors. However, there are many other plausible explanations for the strong association between P-glycoprotein expression in invading carcinoma cells and the high prevalence of vessel invasion and lymph node metastases (6, 47, 54). The influence of P-glycoprotein on the attachment of tumor cells to the extracellular matrix, a process mediated by various glycoproteins such as laminin, vitronectin, and fibronectin, remains to be explored (55, 56). Alterations in immunological regulation of tumor cells could also contribute to the dissemination of P-glycoprotein-positive carcinoma cells. There is a single glycosylation locus in the extracellular region of the NH2-terminal half of the P-glycoprotein molecule. N-linked carbohydrate at this locus adds a molecular weight of approximately 30,000 (i.e., approximately 300 saccharide units) to the molecular weight of the P-glycoprotein monomer (57, 58). Freeze-fracture studies have indicated that up to 70% of integral membrane protein may be P-glycoprotein in highly drug-resistant cultured cells (59, 60).
P-glycoprotein expression in invasive cancer cells remains to be examined in relation to survival in order to assess its potential value for estimating prognosis (20, 53, 61, 62).

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