Correlation of Stromal Cells by Morphometric Analysis with Metastatic Behavior of Human Colonic Carcinoma

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INTRODUCTION

There is considerable evidence that the response of the host to colonic carcinoma may be important (1). Ioachim (2) studied a series of human lung cancers and found that different histological types of lung cancers contained quantitatively and qualitatively different cellular infiltrates; for example, “Plasma cells were present in large amounts in squamous cell carcinomas... few or none were seen in other histologic types.” Similarly, the inflammatory or possibly immunological responses to different kinds of tumors originating in different organs are often quite different and probably differ in their prognostic significance. For example, high concentrations of eosinophils infiltrating tumors are a favorable prognostic sign in colonic carcinoma (1, 3, 4), lung carcinoma (5), and gastric carcinoma (4, 6) but are of little or no prognostic significance in carcinoma of the cervix (7). The few quantitative morphometric studies of colonic carcinomas that have been performed have concentrated on single kinds of cells or a limited number of phenotypic markers; these studies include our studies of eosinophils (1, 3) and several subpopulations of macrophages (8) in colonic carcinomas, studies of plasma cells in colonic carcinoma (9, 10) and in contiguous transitional epithelium (11), studies of T-lymphocytes and macrophages recognized by various monoclonal antibodies (12) in “five random fields” in samples “that included the deep margin,” and studies of lymphocytes just outside of the tumor at its periphery (13).

While Russell et al. (14) have suggested that host or inflammatory cells may not be distributed homogeneously within tumors, i.e., may be distributed differently in specific compartments such as the periphery of the tumor, connective tissue bands within tumors, etc., few studies have addressed this possibility quantitatively. Previously, we demonstrated that a compartment of the tumor at least 1 cm remote from the margin (closer to the center of the tumor) is quite different from a compartment of tumor that contains only the peripheral 0.5 cm of tumor with respect to the concentrations of eosinophils (3) and the concentrations of several subpopulations of macrophages (8). In the present study we attempted to increase our understanding of the interrelationships that exist among all the different kinds of host cells in the stromal microenvironments in defined compartments of colonic carcinomas. While a knowledge of the kinds of stromal cells in tumors seems to us to be a very basic point of departure for the study of in situ tumor immunology, we are not aware of any quantitative assessments of total stromal infiltrates in these two compartments of human colonic carcinoma. Before pursuing more detailed characterizations of these different compartments with additional specialized markers, we have quantified cells that can be identified in 2-μm sections of samples from these compartments stained with hematoxylin, eosin, and azure II.

In this study, we wished to ask several questions: (a) what kinds of cells can be quantified in human colonic carcinomas in SCM and SRM without the need for enzyme histochemical or immunohistochemical techniques, (b) are the concentrations of any particular kinds of cells in particular subcompartments of primary colonic carcinomas related to the presence or absence of detectable metastases at the time of the initial colonic resection, and (c) are the concentrations of particular kinds of cells in particular subcompartments related to the concentrations of other specific kinds of cells?

MATERIALS AND METHODS

Tissue. Specimens were obtained by the Tissue Procurement Service of the Comprehensive Cancer Center of the University of Alabama at Birmingham and the Tissue Conservation Core Facility of the Case Western Reserve University Cancer Center as detailed previously (1, 8). In the operating room, specimens were placed in 0.9% NaCl solution in an ice water bath, examined by a pathologist, described, and sampled. All samples were taken as 9-mm-diameter cylinders as described (3). Sections were taken contiguous to the margin consisting of approximately half tumor and half uninvolved colon. Samples remote from the margin were taken at a distance >1 cm from the margin between the tumor and the grossly uninvolved colonic mucosa, i.e., closer to the center of the tumor. The sections were sectioned at intervals of approximately 1 mm, snap frozen, and processed as before (3).

Tissue Processing. Previously, we (15) described a method for the storage of tissue over liquid nitrogen prior to its use embedded in glycol
methacrylate for the demonstration of several enzyme histochemical reactions as detailed by Beckstead et al. (16, 17). Three serial sections, 2 μm thick, were cut from each methacrylate block as before (8, 18, 19).

Tissue Staining. The first serial section was stained with HEA as described (3). The second, serial, 2-μm section was stained for CAE with the method of Beckstead et al. (17) as modified by us previously (15) with a 3-h incubation. The incubation mixture contained 0.04 M sodium fluoride and was changed every hour. The third, serial, 2-μm section was stained with 0.01% azure A, pH 7.0, in 30% ethanol (20). Mast cells stained metachromatically.

Tissue Evaluation. In our initial review of 62 samples (31 SCM and 31 SRM), 8 were rejected because of technical problems. The most common reason for this rejection resulted from our requirement that normal mucosa be visible in the section contiguous to tumor for all SCM. When this was not true because of the way in which the specimen was embedded and/or cut, another sample of SCM from the same patient was taken from the nitrogen freezer, embedded, and used. One section was not used because of extensive areas of tumor necrosis. When a second sample from this patient also showed extensive necrosis, this patient (SRM40-28) was excluded from our study. This was the only patient excluded from our study.

In order to avoid bias in the area selected and to count areas defined to permit multiple observers to obtain data with a high degree of precision, we used the method detailed previously (3) for the enumeration of cells in colon cancers. Twenty consecutive grids (each grid measured 0.158 x 0.158 mm) were counted by the two observers independently to yield an area of 0.5 mm². All areas within the grid were evaluated, with the exception of the lumen of any gland formed by an adenocarcinoma; these often contained necrotic debris with neutrophils and/or macrophages.

After the counts were completed by the observers, the patients' hospital records were reviewed to determine the presence or absence of metastases, noted either grossly by the surgeon or histologically by the pathologist in sampled lymph nodes. The extent of the tumor invasion, the segment of the large intestine occupied, and the degree of differentiation were also obtained.

Statistical Methods. Comparison of the cell counts (average of two observers) between tumors with and without metastases for each patient was the Wilcoxon two-sample rank test (21). The relationship between the concentration of one cell type and the concentration of another cell type was evaluated with Spearman's rank correlation coefficient (21). Comparison of one staining method to another staining method used the Wilcoxon signed rank test (21). Results were declared statistically significant if P < 0.05.

RESULTS

Table 1, data for SRM and SCM, shows the numbers of cells counted as neutrophils, eosinophils, plasma cells, fibrocytes, lymphocytes, unidentified cells, and macrophages as well as percentage of epithelium. With the exceptions noted below, the quantification of cells was accomplished with slides stained with HEA; the criteria for the identification of lymphoid cells were as described by Cottier et al. (22). Neutrophils were quantified by the demonstration of CAE as described below. Comparable numbers (Table 1) of mast cells were observed in stains for CAE and in stains with azure A. While both were bright red, mast cells and neutrophils could be differentiated readily in preparations reacted for the demonstration of CAE because of their differences in size and granularity.

Consistent with our earlier studies (1, 3), higher concentrations of eosinophils were found in primary tumors without metastases as compared to primary tumors with known metastases for both SRM (P = 0.0098) and SCM (P = 0.0224). For SRM, if we consider only the 15 patients with concentrations of eosinophils above 20/mm², we could correctly predict the absence of metastases for 13 of 15 or 87% of the patients (Fig. 1). For SCM, the absence of detectable metastases could be correctly predicted for 12 of 15 or 80% of patients whose SCM contained more than 25 eosinophils/mm² (Fig. 2).

The parameter that differed most between primary tumors with and without detectable metastases was the concentration of plasma cells (P < 0.002) in SRM (Fig. 3). A threshold set at a level of 115 plasma cells/mm² appears to discriminate between patients with and without metastases. Fourteen of 15 patients (93%) whose SRM contained more than 115 plasma cells/mm² were free of detectable metastases. With this parameter one might correctly predict that 14 of the total population of 31 patients whose SRM were examined would be free of metastases. The concentrations of plasma cells in SCM were not as useful as the concentrations of plasma cells in SRM; however, only 1 of 6 patients with more than 400 plasma cells/mm² in SCM had metastases.

Table 1: Quantification of host cells in colonic tumor

<table>
<thead>
<tr>
<th>Data*</th>
<th>NEU*</th>
<th>N(CAE)</th>
<th>EOS</th>
<th>PC</th>
<th>MAC</th>
<th>FIB</th>
<th>LYM</th>
<th>M(CAE)</th>
<th>M(AA)</th>
<th>UN</th>
<th>%EPI</th>
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</thead>
<tbody>
<tr>
<td>Tumor remote from the margin resected from patients with metastases (n = 12)</td>
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<tr>
<td>Mean ± SD</td>
<td>59.2 ± 71.3</td>
<td>81.0 ± 87.0</td>
<td>23.3 ± 55.8</td>
<td>80.7 ± 68.7</td>
<td>0.2 ± 0.4</td>
<td>471.2 ± 213.3</td>
<td>6.0 ± 7.8</td>
<td>1.3 ± 3.4</td>
<td>0.8 ± 2.3</td>
<td>161.1 ± 131.4</td>
<td>35.8 ± 18.1</td>
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<tr>
<td>Range</td>
<td>0-178</td>
<td>16-270</td>
<td>0-196</td>
<td>22-282</td>
<td>0-1</td>
<td>94-594</td>
<td>0-24</td>
<td>0-12</td>
<td>0-8</td>
<td>48-469</td>
<td>5-67</td>
</tr>
<tr>
<td>Tumor remote from the margin resected from patients without metastases (n = 19)</td>
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<tr>
<td>Mean ± SD</td>
<td>82.6 ± 116.9</td>
<td>158.4 ± 199.9</td>
<td>45.8 ± 51.9</td>
<td>319.1 ± 265.0</td>
<td>0.2 ± 0.4</td>
<td>385.5 ± 169.0</td>
<td>6.7 ± 5.4</td>
<td>1.6 ± 5.0</td>
<td>1.6 ± 4.9</td>
<td>253.3 ± 240.2</td>
<td>32.3 ± 21.1</td>
</tr>
<tr>
<td>Range</td>
<td>9-417</td>
<td>14-818</td>
<td>1-174</td>
<td>10-1069</td>
<td>0-1</td>
<td>63-645</td>
<td>0-18</td>
<td>0-21</td>
<td>0-19</td>
<td>29-839</td>
<td>7-47</td>
</tr>
<tr>
<td>P</td>
<td>NS</td>
<td>NS</td>
<td>0.0098</td>
<td>0.0019</td>
<td>NS</td>
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<td>NS</td>
<td>NS</td>
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<td>Tumor contiguous to the margin resected from patients with metastases (n = 13)</td>
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<tr>
<td>Mean ± SD</td>
<td>37.2 ± 28.9</td>
<td>61.4 ± 63.1</td>
<td>22.4 ± 32.4</td>
<td>204.5 ± 190.4</td>
<td>0.5 ± 1.4</td>
<td>289.8 ± 166.7</td>
<td>8.8 ± 19.7</td>
<td>2.6 ± 5.6</td>
<td>1.6 ± 3.6</td>
<td>146.6 ± 145.1</td>
<td>47.3 ± 22.0</td>
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<tr>
<td>Range</td>
<td>3-84</td>
<td>6-230</td>
<td>0-110</td>
<td>28-755</td>
<td>0-5</td>
<td>45-555</td>
<td>0-72</td>
<td>0-20</td>
<td>0-13</td>
<td>15-508</td>
<td>5-82</td>
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<tr>
<td>Tumor contiguous to the margin resected from patients without metastases (n = 18)</td>
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<tr>
<td>Mean ± SD</td>
<td>70.3 ± 145.7</td>
<td>137.8 ± 231.7</td>
<td>64.0 ± 68.0</td>
<td>337.1 ± 290.8</td>
<td>0.5 ± 1.3</td>
<td>319.2 ± 170.4</td>
<td>18.8 ± 55.5</td>
<td>4.5 ± 10.1</td>
<td>4.2 ± 7.2</td>
<td>168.9 ± 108.8</td>
<td>40.6 ± 18.6</td>
</tr>
<tr>
<td>Range</td>
<td>0-637</td>
<td>13-1023</td>
<td>0-235</td>
<td>48-1011</td>
<td>0-5</td>
<td>103-670</td>
<td>0-239</td>
<td>0-35</td>
<td>0-21</td>
<td>46-432</td>
<td>1-66</td>
</tr>
<tr>
<td>P</td>
<td>NS</td>
<td>NS</td>
<td>0.0224</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
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</table>

* Values for each slide were obtained by two independent observers (cells/mm²). The averages of these values for each patient were used to obtain mean ± SD.

* NEU, neutrophils identified in sections stained with HEA; N(CAE), neutrophils identified in sections stained for CAE; EOS, eosinophils; PC, plasma cells; MAC, macrophages; FIB, fibrocytes; LYM, lymphocytes; M(CAE), mast cells identified in sections stained for CAE; M(AA), mast cells identified in section stained with azure A; UN, unidentified cells; %EPI, percentage of tumor composed of epithelial elements. Each of the 20 (0.158- x 0.158-mm) grids was subdivided into 100 squares to facilitate this evaluation; NS, not significant (P > 0.05) by the Wilcoxon two-sample rank test.
complete inhibition of nonspecific esterase with the techniques stained for CAE. In SRM, we counted 73.6 ± 101.0 (SD) red when stained for CAE under conditions that permit the neutrophils to be demonstrated in human tissues to stain bright with the HEA stain, we often saw cells that we suspected were neutrophils but could not identify with certainty. Since neutrophils had been demonstrated in human tissues to stain bright red when stained for CAE under conditions that permit the complete inhibition of nonspecific esterase with the techniques that we used previously (15), neutrophils were counted in sections stained for CAE. In SRM, we counted 73.6 ± 101.0 (SD)

neutrophils/mm² in sections stained with HEA and 128.4 ± 168.0/mm² in sections stained for CAE. In SCM, the corresponding figures were 56.4 ± 112.4 neutrophils/mm² in sections stained with HEA and 105.8 ± 183.0/mm² in sections stained for CAE. Except for the sections from the patient who was excluded from this study, we saw very few areas of necrosis in colonic carcinomas, and these were small. Contrary to our expectation, the infiltration of neutrophils in colonic carcinomas was usually observed in areas that were free of necrosis and was often very thin and diffuse in character.

In some microenvironments, the concentrations of some kinds of cells were related to the concentrations of other kinds of cells. In SCM, the concentration of plasma cells was related to the concentrations of neutrophils as stained for CAE (R = 0.63, P = 0.0001) or with HEA (R = 0.44, P = 0.0127), eosinophils (R = 0.46, P = 0.0085), fibroblasts (R = 0.47, P = 0.0075), and lymphocytes (R = 0.55, P = 0.0014). In SRM, the concentration of plasma cells was related to the concentration of neutrophils stained for CAE (R = 0.38, P = 0.0371), mast cells stained for CAE (R = 0.38, P = 0.0371), eosinophils (R = 0.36, P = 0.0457), and lymphocytes (R = 0.36, P = 0.0442).

Mast cells were not common host cells in human colonic carcinoma. Similar values were obtained when mast cells were quantified with CAE or azure A. When present, they were more common in SCM than in SRM. In SRM, 3 of 31 patients had more than 2 mast cells/mm². Of this group, one had evidence of metatases; 2 did not. For SCM, there were slightly more patients whose tumors contained mast cells; 3 of 4 patients whose tumors contained greater than 10 mast cells/mm² with either stain and 5 of 6 with 5 or more mast cells/mm² identified with azure A were without metatases.

DISCUSSION

Infiltration of various stromal or host cells within and/or surrounding colon carcinoma has generally been thought to be correlated with a good prognosis (1, 3, 4, 8, 13, 23–36). An attempt to quantify all identifiable stromal cells in any human solid tumor seems to us to be a significant goal because: (a) there may be correlations between the concentrations of inflammatory cells in tumors and their behaviors that may be useful both for the prediction of prognosis and for the stratification of patients in clinical trials; (b) most of the numerous studies of the infiltration of tumors by inflammatory cells have been less than rigorously quantitative; and (c) very little is known about the ecology of particular kinds of inflammatory cells in microenvironments in tumors. While the use of enzyme histochemical markers and monoclonal antibodies will almost undoubtedly add much information to that which can be revealed with commonly used histological preparations, it seemed important to establish a baseline with commonly used stains used for the histopathological assessment of tissues. Our two most important new findings in this paper are (a) the strength of the relationship between the concentration of plasma cells in SRM and the presence or absence of detectable metatases and (b) the strong correlation between the concentration of plasma cells and the concentration of neutrophils in SCM.

While we were aware that there have been published, not precisely quantified reports that infiltration by plasma cells is associated with a good prognosis in colonic carcinoma, for us the most surprising finding in our research was the magnitude of the correlation between the absence of detectable metatases and the concentration of plasma cells in SRM. We were also surprised to see that the correlation between the presence or
absence of metastases and the concentration of plasma cells was highly significant (SRM, \( P = 0.0019 \)) towards the center of the tumor but less than significant (\( P = 0.1553 \)) in tumor near the margin (SCM). While others have suggested in a very general vein that the infiltration of tumors by mononuclear cells including plasma cells is associated with a more favorable prognosis in colonic carcinoma, we are not aware of any previous report that would suggest that the plasma cell is more important as an indicator of the presence or absence of metastases than the other cells in these infiltrates in colonic carcinoma.

Spratt and Spjut (24) found that patients with no "inflammatory reaction" around their colorectal carcinomas exhibited a shorter survival than those with a "moderate reaction" who did slightly less well than those with an "intense" reaction. Without attempting to estimate the intensity of the infiltration, Hopker et al. (25) found that a "lymphoplasmocytic infiltration [yes; no] \( P < 0.05 \)" was associated with the absence of metastases in colorectal carcinoma. Zamcheck et al. (28) noted that a "heavy round cell" infiltration was prognostically "useful"; however, his series was small, and there was no reported statistical evaluation of the data. Watt and House (13) found a highly significant difference between the concentrations of the "lymphocyte infiltration at the periphery of colorectal carcinomata" in patients with and without metastases; however, they did not quantify plasma cells or count any cells within the tumor itself. Werkmeister et al. (32) quantified "stromal lymphoreticular cells comprising plasma cells, eosinophils, macrophages, and lymphocytes ..." immediately outside colorectal carcinomas and found that an intense ("exceeding 2000 cells/mm"²) infiltration outside of the tumor was associated with a good prognosis. Svennevig et al. (35) found that the colorectal carcinomas of patients who survived for longer than 5 years contained more mononuclear cells than those of patients who died in less than 5 years; however, "No attempt was made to differentiate between lymphocytes, plasma cells and macrophages."

The functions of plasma cells in human solid tumors have not been studied in detail. This is particularly interesting in view of the facts that (a) plasma cells have been observed frequently in colonic carcinomas (25, 28, 32) and (b) plasma cells and fibroblasts were found by us to be the most common stromal cells in primary human colonic carcinomas that lacked detectable metastases. Berg (37) found plasma cell infiltration to be associated with a longer survival of patients with breast cancer. Ioachim et al. (38) have commented upon the propensity of well-differentiated squamous cell carcinomas of the lung to be infiltrated by plasma cells. Antibodies that react with malignant epithelial cells in lung carcinoma have been recovered from malignant effusions and from the tumors themselves (2, 39). The origins of these antibodies have not been demonstrated; however, one of the many alternative possibilities is that they might be made by the plasma cells in the tumors. Ioachim (40) has discussed the possibility that locally produced antibody could be helpful or deleterious to the patient in his defense against his tumor. Vaage and Pepin (41) suggested that systemic resistance to the MC2 murine tumor may be due to "the very large number of plasma cells found in close association with the tumor."

In addition to the several histopathological studies of inflammatory infiltrates reviewed above and our own studies (1, 3) of eosinophils in these tumors, we shall briefly list other factors that might encourage one to speculate that the immunology of colorectal carcinoma might be exciting. Of the 13 types of tumors treated by Rosenberg (42) with LAK cells and interleukin 2, 8 kinds of tumors showed no responses at all. If one excludes types of tumors for which 4 or fewer patients were studied, colorectal carcinoma is the solid tumor that showed the third highest response rate (4 of 27 patients) to this therapy (Ref. 42, Table 12-9). In a recent, very large study (43) of patients treated for colon cancer with either surgery alone or surgery and BCG, there was a "survival advantage in favor of the BCG-treated group (\( P = 0.03 \))"; however, as described by the authors, "results achieved with BCG administration remain unclear." This lack of clarity relates to their finding that the group treated with surgery alone had more deaths from cardiovascular causes; in addition, even this lack of clarity was a bit unclear since the diagnosis of the recurrence of tumor was not always based on a tissue diagnosis, and the authors do not tell us what proportions of either group of dead patients received postmortem examinations. In addition to the immunotherapeutic studies of lymphokine activated killer cells and BCG, excitement about the immune response to colon cancer is increased by the work of Werkmeister et al. (31). They described the purification of T-lymphocytes from human colon cancer that were cytotoxic for autologous malignant epithelial cells. The tumors that contained "intrinsic-lymphocyte antitumor cytotoxicity" were tumors highly likely (\( P < 0.001 \)) to have significant perivascular lymphocyte cuffing adjacent to the tumor as assessed morphometrically; this cuffing had been shown previously to be a favorable prognostic indicator (29). Werkmeister et al. (31) found that "Twelve of the 18 tumors with cytotoxic E-rosetting lymphocytes and 6 of the other 42 whose intrinsic lymphocytes showed no reactivity were non-metastatic ...".

We have observed a significant association between the absence of metastases and the concentrations of eosinophils and plasma cells in particular compartments of colonic carcinomas. In these same compartments, the concentrations of several kinds of inflammatory cells, e.g., lymphocytes, were correlated with the concentrations of plasma cells and/or eosinophils but not correlated with the presence or absence of metastases. This apparent inconsistency results from the relative strengths of these correlations. While trends are apparent from an examination of the data in Table 1, the number of patients investigated was not sufficient to permit us to establish the presence or absence of a significant relationship between the concentrations of these cells and the presence or absence of metastases.

Our observations should stimulate increased interest in the biology of colon cancer. We can only speculate about the functions of plasma cells, eosinophils, and other inflammatory cells in colon cancer. Similarly, we have no obvious explanation for the described associations between several kinds of stromal cells in specific microenvironments within colon carcinomas.

ACKNOWLEDGMENTS

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