Angiogenesis in Colorectal Tumors: Microvessel Quantitation in Adenomas and Carcinomas with Clinicopathological Correlations

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ABSTRACT

Angiogenesis is a crucial step in tumor growth and progression. Its quantitation by microvessel counting is of prognostic value in several types of malignancies. Scarce data are available on angiogenesis in gastrointestinal tumors. We studied 36 adenomas and 178 large bowel carcinomas to evaluate the onset of angiogenesis in colorectal tumorigenesis and to assess the prognostic significance of microvessel quantitation. Endothelial cells were immunostained with an anti-CD31 mAb; in each case three microscopic fields (× 200) with the highest number of microvessels were counted: the average value of the three fields was used to evaluate the significance of microvessel density (MVD). MVD of normal mucosa (41 cases) served as controls. MVD was 42 ± 10 in the normal mucosa, 64 ± 10 in adenomas, and 115 ± 39 in carcinomas (normal versus adenomas, P < 0.001; adenomas versus carcinomas, P < 0.0001). The transition zone adjacent to carcinomas displayed intermediate levels of MVD (89 ± 23; P < 0.001 versus adenomas; P < 0.001 versus carcinomas). High MVDs were not associated with metastases, disease stage, and patient survival. The data indicate that angiogenesis is an early, critical step in colorectal tumorigenesis. MVD, however, does not provide significant prognostic information in colorectal cancer patients.

INTRODUCTION

Angiogenesis, the physiological process of new blood vessel formation, is essential in tissue development, reproduction, and wound healing (1). Unregulated angiogenesis, however, plays a critical role in several diseases, including diabetes, arthritis, and neoplasia (1). Tumor growth, after reaching the size of about 1–2 mm³, is strictly dependent on angiogenesis (2). Angiogenesis also contributes to the metastatic process, facilitating shedding of tumor cells into the blood vessels (3).

In human tumors, the evidence that angiogenesis, quantitated by microvessel counting, could be related to metastases and patient survival was initially reported for cutaneous melanomas (4), and subsequently for tumors of several other organs, including breast, lung, head and neck, prostate, and testis (5–9). Furthermore, the extent of neoangiogenesis is of independent prognostic value in invasive breast carcinoma (10–13) and in brain tumors (14).

The exact time when angiogenic clones appear within a tumor remains to be clearly defined. Experimental data suggest that angiogenesis may occur as early as at the transition from hyperplasia to neoplasia (15). In human tumors, the presence of highly neovascularized premalignant, or in situ, lesions also suggests that angiogenesis may occur early in tumor progression. Indeed neovascularization has been observed in breast intraductal carcinoma (5, 10, 16) and in cervical intraepithelial neoplasia (17).

Data on tumor angiogenesis and its clinical and prognostic implications have been obtained in several malignancies of parenchymal organs, whereas tumors of the gastrointestinal tract have not been investigated extensively thus far. Invasiveness in the latter tumors occurs through the different layers of the intestinal wall, and it is not known whether this unidirectional growth pathway (at least for its clinically significant implications) is dependent on neovascularization. In two studies focusing on gastric and rectal carcinoma, elevated microvessel counts have been correlated with the presence of metastases and/or poor prognosis (18, 19).

We investigated immunohistochemically a retrospective series of 36 adenomas and 178 carcinomas of the large bowel using an anti-CD31 mAb to visualize endothelial cells. We then quantitated MVD in the normal mucosa, benign and malignant tumors, and peritumoral tissues.

The purposes of our investigation were: (a) to determine whether angiogenesis can be documented in colorectal tumor progression and the time of its onset; and (b) to assess whether the quantitation of microvessels can be correlated to tumor aggressiveness and provide prognostic information.

MATERIALS AND METHODS

Thirty-six adenomas were obtained from the files of the Department of Pathology of the San Paolo Hospital (Milan, Italy), including endoscopically excised lesions and surgical resection specimens.

For colorectal adenocarcinomas the study population included 178 patients who were treated surgically at the Lahey Clinic Medical Center (Burlington, MA) between 1982 and 1984. The 178 patients are a subgroup of a previously published study of 206 patients in which we evaluated immunohistochemically p53 accumulation (20). In brief, to be included in the study, patients had to meet the following criteria: (a) no history of previous malignancies (excluding skin carcinomas); (b) surgical resection margins negative for tumor; (c) no preoperative treatment; and (d) no perforation of the bowel by the tumor. Among the 178 carcinomas, transitional mucosa, adjacent to but not infiltrated by the tumors, was available for evaluation in 94 cases. Normal colonic mucosa at a distance of at least 10 cm from the invasive tumors was investigated in 41 cases.

The original slides and pathology reports were reviewed to confirm the pathological grading and staging according to the American Joint Committee for Staging of Cancer (21). Patient age, sex, and tumor location in the large bowel were also evaluated. Patients were followed up for at least 5 years, and their survival and clinical status were obtained from the tumor registry or contact with the patients’ physician or both.

Immunohistochemistry. For the immunohistochemical localization of blood vessels, sections were stained with an anti-CD31 mAb (clone JC/70; Dakopatts, Glostrup, Denmark). Dewaxed sections were rehydrated and treated with 0.4% pepsin in 0.01 N HCl for 30 min at 37°C and with 5% normal horse serum for 20 min at room temperature before being subsequently incubated with the following: (a) JC/70 mAb diluted 1:15 in PBS overnight at 4°C; (b) biotinylated rabbit

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2 The abbreviations used are: MVD, microvessel density; VEGF, vascular endothelial growth factor.
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antimouse immunoglobulin serum (Vector Laboratories, Inc., Burlingame, CA) diluted 1:200 for 30 min at room temperature; and (c) alkaline phosphatase-labeled streptavidin (Dakopatts) diluted 1:100 for 30 min at room temperature. Alkaline phosphatase activity was developed with the McGeady reagent (nitro blue tetrazolium and bromo-chloro-indolyl phosphate) containing 1 mM levamisole for 1 h at room temperature.

Histologically recognizable blood vessels within tissue sections served as internal control for CD31 immunostaining. The intensity of staining of internal controls was also used to test whether different fixation times (usually shorter for biopsies and longer for surgical resection specimens) affected CD31 immunoreactivity. The intensity of staining of blood vessels in small biopsies was similar to the one observed in large specimens.

Microvessel quantitation was performed according to Bosari et al. (10). Briefly, slides were examined at low-power magnification (× 40) to identify the areas with the highest density of microvessels. In each case, the three most vascularized areas were selected, and the microvessels in a ×200 field (field diameter, 0.9 mm) of these three areas were counted. The highest and the average counts of the three ×200 fields were recorded for analysis. In the adenomas, counts were evaluated in three ×200 fields except for seven cases: in five cases only two fields and in two cases only one field could be evaluated due to the small size of the specimens. For the adenocarcinomas, microvessels were separately evaluated within the tumor and in the transitional mucosa. The MVD evaluation of normal mucosa controls was performed in three random fields.

Single endothelial cells or small clusters of endothelial cells, with or without a lumen, were considered individual vessels. Vessels of a caliber larger than approximately eight red blood cells and vessels with a thick muscular wall, ranging from none to less than 2% of the overall vessel count in the microscopic fields investigated, were excluded from the final count. The microvessel count was performed in all cases by a single pathologist (P. B.); 20% of the cases, randomly chosen, were independently evaluated by another pathologist (S. B.). There were no significant differences in microvessel counts obtained by the two investigators. Microvessel counts were expressed as the absolute number of vessels per ×200 field.

**p53 Aberrations.** p53 accumulation and its intracellular compartmentalization were determined using immunocytochemistry. p53 gene mutations were detected using single-strand conformation polymorphism analysis. Details of the techniques and the results of the investigation have been previously reported (20, 22).

**DNA Ploidy Status.** DNA ploidy was determined by image analysis using the CAS 200 (Cell Analysis System Inc., Elmhurst, IL) image analyzer and software. The technique and results have been published previously (23, 24).

**Statistical Analysis.** Data were analyzed using for each case both the highest microvessel count and the average of the three highest fields. Since the results did not show differences among the two types of microvessel counts, only the average value of three fields for each case is reported. Data are reported as mean ± SD; for simplicity, numbers have been rounded.

Statistical differences between variables were analyzed with the Mann-Whitney U test or ANOVA, as appropriate. Contingency tables were analyzed using Fisher’s exact test or the χ² test. Survival distributions were calculated using the Kaplan-Meier product-limit method. The statistical significance of differences between distribution was analyzed using the method of Mantel-Cox. The analysis was performed with BMDP statistical software (BMDP Statistical Software, Los Angeles, CA) and with SAS statistical software (SAS Inst., Cary, NC).

Survival analysis of the 178 patients included in the current study confirmed the previously reported results (20, 24) obtained in the larger series of 206 patients.

**RESULTS**

**Angiogenesis in Tumor Progression.** The use of anti-CD31 mAb provided reliable and selective immunostaining of endothelial cells. Tumor cells, as well as other normal tissue constituents, were consistently unstained.

Microvessel counts in the normal mucosa were 42 ± 10, which was the lowest level of MVD in our investigation. In the adenomas microvessel counts were 64 ± 10 (normal mucosa versus adenomas, P < 0.001). No differences in MVD were seen comparing adenomas with mild to moderate to severe dysplasia. Adenocarcinomas exhibited the highest number of vessels: in the overall series counts were 115 ± 39 (P < 0.0001 versus adenomas). The transitional mucosa displayed intermediate levels of MVD between adenomas and adenocarcinomas (89 ± 23; P < 0.001 versus adenomas and P < 0.001 versus adenocarcinomas).

These results are summarized in Table 1, and examples of vessel staining in normal mucosa and adenocarcinoma with high MVD are depicted in Fig. 1.

**Angiogenesis and Clinicopathological Correlations.** MVD of colorectal carcinomas showed no association with the patients’ age and sex (data not shown).

The evaluation of MVD according to the tumor stage is shown in Table 2: no differences in microvessel counts were demonstrated among different stages. Similarly, the tumors that did not have regional lymph node or distant metastases displayed microvessel counts comparable to the tumors that had already metastasized (stage I + II versus stage III + IV, 115 ± 39 versus 114 ± 38).

MVD was analyzed in relation to other clinicopathological variables, including tumor differentiation, DNA ploidy status, and p53 nuclear and cytoplasmic accumulation (Table 3). Microvessel counts were not correlated to any of the parameters analyzed.

The prognostic significance of MVD was finally evaluated in both the overall series and individual disease stages using the Kaplan-Meier product-limit method. Tumors with low (<115) and high (≥115) microvessel counts were compared. No differences in overall and disease-free survival could be demonstrated. The overall survival curves for the entire patient population are shown in Fig. 2. To evaluate further the significance of MVD on overall and disease-free survival, the patients were stratified by quintiles according to MVD values. No significant differences in survival were observed; indeed, patients within the lowest quintile (MVD < 81) displayed a survival rate similar to the patients in the highest quintile (MVD > 143; data not shown). Multivariate analysis also failed to reveal a significant prognostic role for the microvessel counts.

**DISCUSSION**

The current study shows that neovascularization is invariably present in colorectal adenocarcinomas, irrespective of the different pathological disease stages. Furthermore, microvessel counts are significantly increased in adenomas compared to normal colonic mucosa. These data suggest that angiogenesis is stimulated early in colorectal tumorigenesis and reaches a maximum level in adenocarcinomas. New blood vessel formation is maintained thereafter. MVD, however, is not related to the presence of local and/or distant metastasis and to patient survival.

Although the onset of angiogenesis may vary in different types of tumors, it is expected to take place relatively early in tumor growth (2). Indeed, experimental observations support this hypothesis: using a transgenic mouse model in which pancreatic β cells progress from normality to hyperplasia to neoplasia, it was demonstrated that angio-

### Table 1 MVD and tumor progression

<table>
<thead>
<tr>
<th>Tumor Type</th>
<th>No. of cases</th>
<th>MVD (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal mucosa</td>
<td>41</td>
<td>42 ± 10</td>
</tr>
<tr>
<td>Adenoma</td>
<td>36</td>
<td>64 ± 10</td>
</tr>
<tr>
<td>Transitional mucosa</td>
<td>94</td>
<td>89 ± 23</td>
</tr>
<tr>
<td>Carcinoma</td>
<td>178</td>
<td>115 ± 39</td>
</tr>
</tbody>
</table>

* Statistics (Mann-Whitney U test): normal mucosa versus adenomas, P < 0.001; adenomas versus carcinomas, P < 0.0001; adenomas versus transitional mucosa, P < 0.001; transitional mucosa versus carcinoma, P < 0.001.
Angiogenesis occurs as early as the transition from hyperplasia to neoplasia (15). Our observation that MVD is increased in adenomas of the large bowel is consistent with the hypothesis that angiogenesis is stimulated even before the occurrence of the overt malignant phenotype. However, since MVD is related to metastases and patient survival in several types of carcinomas, including breast, lung, prostate, and head and neck carcinomas (6–8, 10–13), it would appear that angiogenesis is progressively stimulated during tumor progression.

In contrast, our data in malignant tumors arising in the large bowel demonstrate that in this organ angiogenesis is equally stimulated in all pathological disease stages. These findings suggest that even small, superficially invasive, stage I carcinomas are capable of eliciting neovascularization of the same extent as seen in tumors presenting with distant metastases. Indeed, the MVD of stage I adenocarcinomas was already more than twice that of the normal colonic mucosa.

Our results contrast with the data reported by Maeda et al. (18) in gastric carcinoma. These authors found a significant association between high MVD counts and the presence of metastases; in addition, univariate and multivariate survival analysis showed a borderline significance for MVD. Interestingly, the MVD values per × 200 fields reported by these investigators were much lower than the ones reported in the literature using either factor VIII-related antigen or CD31 antibodies. Saclarides et al. (19) evaluated angiogenesis in 48 rectal carcinomas and reported a correlation between MVD, the depth of invasion within the bowel wall, and shorter survival. However, no statistically significant MVD differences were detected comparing tumors with and without metastases. Additional studies will be needed to further clarify the levels of MVD in gastrointestinal tumorigenesis and tumor progression, as well as its clinical significance.

Angiogenesis, although always crucial for solid tumor growth, may...
be stimulated in different manners, both quantitatively and qualitatively, in tumors arising in different organs. In organs like the breast or the prostate, tumors can grow by infiltrating in all directions the surrounding tissues. Not uncommonly, human tumor growth and invasion in solid organs takes place with either single cells or small glands that might be effectively nourished by the preexisting circulation. Only subsequently, after having reached a considerable volume, is the process of angiogenesis stepped up. Conversely, tumors arising from the surface of the gastrointestinal tract can grow, in an invasive and clinically significant manner, only through the bowel wall. It is therefore possible that the threshold volume required for angiogenesis could be smaller.

The metastatic process depends on several factors, including angiogenesis (25, 26): some of them have been related to poor survival in colorectal cancer patients. For instance, overexpression of cathepsin B, which can degrade several extracellular matrix components, has been linked to tumor progression and shortened patient survival (27). Furthermore, several oncogenes and tumor suppressor genes are commonly altered in colorectal carcinomas, particularly frequent are mutations in K-ras and p53. Several reports have linked aberrations of K-ras and p53 to poor prognosis (20, 28–30). It is therefore likely that angiogenesis is necessary for tumor cell growth and allows carcinomas to increase in volume, but that other mechanisms play a critical role in determining tumor aggressiveness and the ability to metastasize to regional lymph nodes and to distant sites.

Liotta et al. (3) proposed that metastases are related to the overall tumoral vascular surface area: an increased vascular surface area would constitute an easier target for tumor cells in the process of escaping from the original site and entering the circulation. The relationship between MVD and the overall intratumoral vascular surface area, however, is poorly understood. An increased MVD may reflect a larger surface area but the size of the tumor should also be taken into account. Quantitation of MVD represents a two-dimensional analysis and may be a poor indicator of vascular surface area in cases in which there are many highly vascularized fields: in such cases the tumor size would also be a critical determinant of the vascular surface area. In our series, MVDs of stage I colorectal carcinomas were similar to the ones with more advanced stage. The latter, however, being larger tumors, are more likely to have an increased overall vascular surface area, thus explaining the higher incidence of metastases.

Interestingly, the transitional mucosa displayed levels of MVD which were significantly higher than those found in colonic mucosa distant from the tumors. The MVD of tissues surrounding the carcinomas, however, was significantly less than that within the malignant tissues. These results suggest that angiogenesis is maximally stimulated within the tumors by angiogenic mediators that are also capable of diffusing to adjacent tissues and determining the increase in microvessels.

Several angiogenic factors have been described (for review, see Refs. 1 and 31). Some of these have been detected in colorectal tumors: Li et al. (32), using in situ hybridization, showed angiogenic and basic fibroblast growth factor gene expression in tumors arising in the gastrointestinal tract, including colorectal carcinomas. Brown et al. (33) have demonstrated overexpression of VEGF and its cognate receptors, flt-1 and kdr, in colorectal carcinomas and other gastrointestinal tumors. Therefore, it is clear that these carcinomas can produce several mediators that have been shown to stimulate endothelial cell proliferation and migration. The end result of the activation of angiogenesis in colorectal cancer is demonstrated by the increased MVD found in these tumors. The VEGF121 and VEGF165 splicing variants are secreted proteins that may explain the increased neovascularization in tissues adjacent to carcinomas.

We were especially interested in evaluating the possible relationship between angiogenesis and aberrations of the p53 tumor suppressor gene product, since experimental evidence suggest that p53 may modulate angiogenesis. Indeed, it has been shown that mutant p53, but not wild-type p53, can stimulate VEGF synthesis via protein kinase C activation (34). Furthermore, wild-type p53 can regulate the production of thrombospordin, which is an angiogenic inhibitor (35). In our series, however, we were unable to demonstrate any relationship between p53 accumulation and MVD. Additionally, analysis of p53 mutations in 126 of these patients (22) confirmed the lack of correlation between p53 gene status and MVD. Similar findings have been also reported in invasive breast carcinomas (13, 36). The discrepancy from in vitro and in vivo studies may depend on the multiple genetic and functional alterations displayed by tumors in vivo, including the interactions with the extracellular matrix and the likely involvement of multiple tumoral and extratumoral mediators and growth factors.

The results of our study suggest that angiogenesis is a critical and early step in colorectal tumor progression. Quantitation of MVD, however, does not provide prognostic information.

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REFERENCES


Fig. 2. Kaplan-Meier survival curves for the overall series according to MVD. Patients with tumors displaying MVD values of 115 or more (○) are compared with patients with carcinomas showing MVD values of less than 115 (●).
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