Abstract

Angiogenesis is essential for tumor growth and metastasis, and vascular density is known as an independent prognostic factor in several tumor entities. We studied the prognostic relevance of vascular density in colorectal cancer, examining 146 patients treated surgically for cure. Tumor sections were immunostained with JC70, an endothelial cell marker. Microvessel quantification used light microscopy. The slides were scanned at a low magnification, and individual microvessel counts were made on a ×200 field in the area of the most dense neovascularization. Vascular density was found to be 75±27/visual field and to be independent of age, sex, pT and pN categories, tumor recurrence, and overall survival. Overall survival in the subgroup of patients with tumor recurrence was significantly shorter with tumors of greater vessel density (>75) than in those of less vessel density (<75). Multivariate analysis showed microvessel count to be an independent prognostic factor for the overall survival rate of patients with tumor recurrence; among these patients there was also a significant difference in the relapse-free survival rates between the hypovascular and the hypervascular groups. Our findings suggest that the microvessel density of the primary tumor determines the speed of tumor recurrence after metastatic disease has been triggered by other, unknown mechanisms. Although tumor vascularization can be linked to the aggressiveness of colorectal cancer, it has no value as a new prognostic marker in clinical practice.

Key words

Colorectal cancer · Angiogenesis · Vessel density · Prognosis · Immunohistochemistry

Introduction

Tumor growth and metastases require new blood vessel formation [1]. Microvessel quantification has been performed immuno-histochemically in a variety of tumor entities [2]. Most studies show an inverse relationship between angiogenesis and survival in melanoma [3] and adenocarcinoma of the breast [4], lung [5], prostate [6], and stomach [7]. Published findings on colorectal cancer angiogenesis are conflicting, ranging from strong prognostic relevance [8] to none [9]. This study sought to clarify whether microvessel density is a clinically important prognostic factor.

Patients and methods

Patients

This study included 146 patients who underwent curative resection of colorectal cancer at the Department of Surgery, Benjamin Franklin University Hospital, Berlin, and who had a complete follow-up in our Oncology Outpatient Department until death or for a minimum of 5 years. None of the patients had a previous history of malignancy or chemotherapy. The operations were standard colon or rectum resections with regional lymph node dissection. The tumors were classified according to the TNM system of the UICC (1997). Tumor characteristics, including TNM and UICC classification, grading, venous invasion, lymphangiogenesis, and location in the large bowel were collected from the pathology reports. Clinical factors, including age, sex, surgical procedure, chemotherapy after resection, radiation, presence and site of recurrence, and cause of death were obtained from the clinical charts. Of the 146 patients 22 (15%) had UICC stage I disease, 77 (53%) stage II, and 47 (32%) stage III. Patients did not receive chemotherapy before surgery. Radiation was given to 25 patients (17%) before and 14 (9.6%) after surgery. The tumor was localized in the right hemicolon in 29%, the left hemicolon in 19%, and the rectum in 52%.

Immuno-histochemical study for CD31 antigen

Immunohistochemical staining for CD31 antigen

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embedded tumor sections. Sections 1 μm thick were sliced by a microtome, mounted on glass slides, dewaxed in xylene, and rehydrated in ethanol. Dewaxed sections were pretreated with protease (Sigma) in Tris for 25 min at 37°C to retrieve the structure of the antigens in the fixed tumor sections (Figs. 1–3). Blood vessels were visualized by staining endothelial cells for CD31 antigen (monoclonal antibody JC70 [10], Dako) using a standard immunohistochemical APAAP technique; the primary monoclonal antibody binding to the antigen on the surface of the cell is connected to a monoclonal antibody binding the alkaline phosphatase through a third antibody.

Alkaline phosphatase activity was developed with a reagent containing naphthol-AS-biphosphate, N,N-dimethylformamide, newfuchsin, and levamisol, which resulted in an intense red staining of the cells carrying the antigen on their surface. After immunostaining a light hematoxylin counterstain was applied before mounting. Histologically recognizable blood vessels within tissue sections served as internal control for CD31 immunostaining. This step ensured that the entire sequence of steps – fixation, including embedding, deparaffinization, rehydration, and staining – was performed correctly. Negative controls were performed using normal mouse IgG in place of the primary antibody, which helps to evaluate for nonspecific staining which, if present, implies that the APAAP complex is cross-reacting with an endogenous substance, thereby raising doubt concerning the accuracy of the entire assay.

Determination of microvessel density

Microvessel quantification was performed according to an international consensus [11]. Slides were examined at low-power magnification (×40) to identify the areas with the highest density of microvessels. In each case the four most vascularized areas were selected, and the microvessels in a ×200 field (0.781 mm²) of these four areas were counted. The highest of the four ×200 fields were recorded for analysis. Single endothelial cells or small clusters of endothelial cells, with or without a lumen, were considered individual vessels. Microvessel counts were expressed as the absolute number of vessels per ×200 field. The microvessel count was performed by a single investigator (T.S.). Another blinded investigator independently evaluated 20% of randomly chosen cases to exclude investigator-dependent errors.

Statistical analysis

The clinical characteristics of the patients in relation to the microvessel counts were compared by the χ² test. The survival curves were plotted according to the Kaplan-Meier method, and the statistical difference was analyzed using the log rank and Kolmogorov-Smirnov tests. Multivariate analysis was performed by Cox regression analysis. To test the independence of the vessel density as a prognostic factor for the relapse-free survival we performed a stratification analysis for all variables with significant effect in univariate analysis, since the Cox regression method was not applicable for this analysis. A value of P<0.05 was considered statistically significant. The analysis was performed with SPSS software.

Results

Patient outcome

Of the 146 patients 66 (45%) died from progressive disease within 5 years after surgery. The five-year survival rate in UICC disease stage I was 86%, 53% in stage II, and 43% in disease stage III. Cancer recurred locally in 47 (32%), and 46 (31.5%) developed distant metastases. Metastases were located in the liver (40%), lungs (29%),