Vascularization of Carcinomas of the Esophagus and Its Correlation with Tumor Proliferation

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ABSTRACT

Vascularization and tumor cell proliferation were analyzed in 33 resected human squamous cell carcinomas of the esophagus using the endothelium-specific antibody BW 200 and the proliferation-associated antibody Ki-67. Vascular parameters (relative capillary volume, relative total vessel volume, vascular surface area, and vascular length) as well as the percentage of proliferating tumor cells (Ki-67 index) were evaluated on frozen sections by a morphometric method. Vascular parameters of the normal mucosa exceeded those of tumors significantly, by a factor of 1.4–2.3. The mean distance between tumor capillaries and the onset of necrosis was 92 ± 34 μm. Global vascular density did not correlate with TNM stage, tumor diameter, or overall tumor proliferation (mean Ki-67 index, 35.1%; range, 14.2–64.1%). However, a significant negative correlation existed between the percentage of proliferating tumor cells per tumor cord and the intercapillary distance between capillaries located at the edges of these cords. This observation points to the fact that the esophageal cancers were composed of multiple tumor cords and that each of these cords possessed its own supply capillaries at the base of the cord. The sum of these “supply units” thus constitutes an esophageal cancer. The intercapillary distance may reflect the oxygenation status of tumor cells, which cannot be predicted on the basis of tumor staging or grading.

INTRODUCTION

The progressive growth of malignant tumor cells is possible only after an adequate tumor blood supply has been created by the incorporation of pre-existing host vessels into the tumor tissue and by the formation of new tumor microvessels. The vascular network in human cancers is essential for supplying nutrients and for removing waste products. In addition, the efficacy of radiotherapy depends on the local oxygen concentration determined by the tumor blood flow. The delivery of chemotherapeutic agents is governed by the vascular supply of tumors (1, 2).

Vascularization also has an influence on tumor cell proliferation. Tumor cells in the vicinity of blood vessels have a higher growth rate, which decreases with increasing distance from the blood vessels (3–5).

The majority of vascularization data have been collected in fast-growing rodent tumors. There is, however, relatively little information in the literature by which one can judge the relevance of the experimental tumor systems to the corresponding situation in human primary tumors. To our knowledge, data concerning the vascular supply of esophageal cancer have not been communicated in the literature. We therefore analyzed the vascular supply of human esophageal cancers and its relationship to clinicopathological parameters and to tumor cell proliferation in situ.

MATERIALS AND METHODS

Tumor Specimens. After surgical resection 33 squamous cell carcinomas of the esophagus (29 men, 4 women; age, 45–81 years) were routinely processed for conventional histological assessment. Tumor staging was performed according to the revised UICC classification of 1987. Nineteen tumors were moderately differentiated (grade G2), 11 tumors were poorly differentiated (grade G3), and three tumors were undifferentiated (grade G4). In two tumors, insufficient clinical material was present for the exact staging of distant metastases. Tumor diameters were measured for each tumor after resection.

Tumor Vascularization. Cryostat sections were cut from a representative transverse tumor section. Sections of esophageal cancers and of normal mucosa were air-dried and fixed in acetone. For the immunohistochemical detection of vascular endothelium the monoclonal antibody BW 200 was used. This antibody detects an epitope restricted to human endothelial cells (6). Sections were incubated in turn for 30 min with the BW 200 antibody (diluted 1:15; Behring, Marburg, Germany), peroxidase-conjugated rabbit anti-mouse immunoglobulin (diluted 1:80; Dakopatts, Copenhagen, Denmark), and peroxidase-conjugated swine anti-rabbit immunoglobulin (diluted 1:80; Dakopatts) (7). The slides were developed with diaminobenzidine-hydrogen peroxide. Sections were counterstained with Mayer’s hemalum. Negative controls were performed by substituting phosphate-buffered saline or mouse IgG (Sigma Chemical Co., St. Louis, MO) for the primary antibody.

Tumor Cell Proliferation. Nuclei of proliferating cells were stained with the monoclonal antibody Ki-67, which detects a proliferation-associated nuclear antigen (8). Tumor sections were incubated for 60 min with a 1:10 dilution of the antibody Ki-67 (Dakopatts), as detailed previously (9).

Morphometry. To obtain the vascular parameters by stereological analysis, sections were scanned in a regular manner (10). In normal tissues, vessel density was evaluated in the mucosa and submucosa. A microscope with an eyepiece graticule showing a regular arrangement of 25 crosses was used (total magnification, ×400). Eighty fields were counted in each section. In eight tumors analysis of vascularity was carried out in three different sections, taken from central, intermediate, and peripheral parts of the tumors.

The percentage of vascular volume per tumor (capillary volume and total vessel volume) was quantified with the point-counting method (7, 11). A point was counted if a cross fell on a vessel. The number of coincidences with vessels was recorded. Vessels with a diameter of <10 μm were defined as capillaries. The proportion of the tumor occupied by blood vessels was thus calculated: vascular volume (%) = coincidences/points counted × 100. Vascular surface area (mm²/mm³) and vascular length per unit volume of tissue (mm/mm³) were calculated by measuring the diameters of 100 vessels/tumor (7).

By scanning the whole tumor section the percentage of proliferating tumor cells was also assessed by point counting. A Ki-67 index was defined as the number of tumor cells with positive immunostaining divided by the total number of tumor cells counted per section (9).

Correlation between Tumor Proliferation and Vascularization. Global tumor proliferation (assessed by the Ki-67 index of the whole tumor) was correlated with the global vascular density. In addition, tumor cell proliferation was evaluated separately in tumor cords. In these cords (n = 47), every tumor cell was assessed. Because some tumor cords contained necrotic areas, proliferation in tumor cords was expressed as Ki-67 indexarea³ (12 mm)(necrosis), the number of cells cannot be counted reliably. Cord areas were measured on photographs.

Tumor cords were surrounded circularly by capillaries located at the edge of the cords. Cell proliferation per cord was correlated with the mean intercapillary distance between capillaries opposing each other at the edges of the cords. The distances were measured at least four positions around a given cord, on photographs taken from the corresponding cords. The distance between the onset of necrosis and the capillaries was determined for each of these cords containing necrosis.
**Statistical Analysis.** Values are expressed as arithmetic means ± 1 SD. Statistical analysis was performed by linear regression analysis and Student’s t test for unpaired data. Statistical analysis of the regional variations of tumor vascularization was performed by the method of Aherne et al. (12).

**RESULTS**

In the normal mucosa, the capillaries were preferentially located below the basal membrane of the esophageal epithelium. In esophageal carcinomas, tumor cells were organized in cords surrounded by the stroma containing the vessels. Use of the monoclonal antibody BW 200 allowed easy unequivocal identification of capillaries and small vessels (Figs. 1 and 2). The regular vascular architecture seen in normal tissues was abolished in tumors in favor of an irregular pattern. In the vicinity of the edge of the tumor cords, capillaries were present surrounding the cords circularly. In larger cords, necrosis developed in the central parts. The intercapillary distance was smaller in cords without necrosis (244 ± 120 μm) than in cords with necrosis (344 ± 129 μm) (P = 0.03). The mean distance between capillaries and the onset of necrosis was 92 ± 34 μm, with a maximum of 169 μm.

Vascular parameters (relative capillary volume, relative total vessel volume, vascular surface area, and vascular length) in tumors were significantly lower than those in the corresponding normal tissues, by a factor ranging from 1.4 to 2.3 (Table 1). The total vessel volume correlated significantly with vascular surface area (r = 0.92) and length (r = 0.68) (P < 0.001). In general, considerable tumor-to-tumor variability in vascular density was noted. Total vessel volume ranged from 1.1 to 7.2% and capillary volume from 0.4 to 5.0%. This observation was also reflected by the higher coefficient of variation of vascular parameters in the malignant tissues, in comparison to the normal mucosa.

To study the regional heterogeneity of vascularization, samples from eight tumors were subdivided into peripheral, intermediate, and central regions and analyzed separately. Although a marked spatial variability of the vascular supply was noted in some of these tumors, statistical analysis failed to detect significant regional differences. It must be taken into account that the tumor-to-tumor variations in vascular density were more pronounced than intratumor heterogeneity. Whereas the relative total vessel volume among different tumors reached a 6.5-fold maximum difference, the variations within the tumors were smaller (maximum, 3.8-fold).

Global tumor vascular supply was not influenced by the patients’ age or gender, tumor location, or tumor diameter. The relationship between global tumor vascular density and clinicopathological data is given in Table 2. These parameters had no statistically significant influence on the extent of tumor vascularization.

Proliferating tumor cells were easily identified by nuclear immunostaining with the antibody Ki-67 (Fig. 2). Global tumor proliferation assessed by the Ki-67 index ranged from 14.2 to 64.1% (mean, 35.1%). Proliferation was independent of clinicopathological parameters such as patients’ age, patients’ gender, and tumor location, differentiation, and TNM stage. Statistical analysis failed to detect a correlation between the Ki-67 index of the whole tumor and global tumor vascular density.

In tumor cords, cell proliferation often decreased with increasing distance to the capillaries. Because some tumor cords contained necrotic areas, cord areas were measured on photographs to calculate Ki-67 indexAREA. Linear regression analysis revealed a highly significant correlation between the logarithm of the Ki-67 indexAREA and the distance between the capillaries surrounding the cords (r = -0.472; P < 0.001) (Fig. 3).

**DISCUSSION**

Because the effects of radiotherapy and chemotherapy depend, at least in part, on the local oxygen concentration and on the vascular supply, the vascular density and its influence on tumor proliferation were analyzed in human esophageal cancer. The present study demonstrates for the first time that the vascular supply of esophageal carcinomas is lower than that in the corresponding normal mucosa and that tumor cell proliferation does not depend on global vascular density but on the microcirculation at the base of the tumor cords.

Our values reported here for vascular volume, surface area, and length are in accordance with those reported for large transplantable adenocarcinomas (13), rat colon carcinomas (4), human melanoma xenografts (14), human gastric cancers (15), and human melanomas (16). Comparable differences in vascular density between normal and
malignant tissues were noted by Mlynek et al. (17). Similar intercapillary distances were observed in carcinomas of the cervix uteri (18, 19).

Although a variety of techniques have been proposed for investigations of the vascular supply, precise data on the vascularization of Table 1. Capillary volume, total vessel volume, and vascular surface area and length in normal and carcinomatous tissue of the esophagus determined with the monoclonal antibody BW 200

<table>
<thead>
<tr>
<th></th>
<th>Esophageal carcinomas</th>
<th>Normal esophageal mucosa</th>
</tr>
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<tbody>
<tr>
<td>Capillary volume (%)</td>
<td>2.0 ± 1.0*</td>
<td>3.3 ± 0.7</td>
</tr>
<tr>
<td>Total vessel volume (%)</td>
<td>3.2 ± 1.1</td>
<td>4.5 ± 1.1</td>
</tr>
<tr>
<td>Vascular surface area (mm²/mm³)</td>
<td>3.2 ± 1.5</td>
<td>5.9 ± 2.2</td>
</tr>
<tr>
<td>Vascular length (mm/mm³)</td>
<td>45 ± 20</td>
<td>105 ± 58</td>
</tr>
</tbody>
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* Mean, ±SD. Vascular parameters in carcinomas were significantly lower than in the normal esophageal mucosa.

human cancers remain sparse. Morphometric analysis of tumor vascularization does not necessarily allow judgments concerning functional aspects of the tumor blood supply. However, indirect and direct evidence exists that results of morphological analysis can be taken as an approximation of the oxygenation status of tumors. The differences in vascular parameters between normal and neoplastic esophageal tissue are comparable to the ratios of the pO₂ values in various normal tissues and human tumors. The pO₂ value is about 2.5-fold lower in squamous cell cancers than in normal tissues (2). Studies of xenografts grown in nude mice demonstrated that the extent of vascularization derived from morphometric analysis paralleled the growth rate of tumors (14) and the volume fraction of necrosis (20). During the course of external irradiation the intercapillary distance decreased progressively in cervical cancers, suggesting improved oxygenation (18). The polarographic measurements by Kolstad (19) of the dimin-
significantly with the mean intercapillary distance \((r = -0.472; P < 0.001)\).  

O, cords

The magnitude of the oxygen content of RBC in capillaries correlated with tissue oxygen tension in cervical cancer paralleled the gradual increase in the intercapillary distance during neoplastic progression.  

human oral cavity (21). The observed mean distance from the capillaries to the necrotic tissue in esophageal tumors compares well with the calculated estimates of the oxygen diffusion length (1). Therefore, it can be concluded that the results of morphological analysis might be of the global vascular density can be explained by the growth of tumor cords surrounded by the stroma. Each of these tumors possessed its own supply capillaries located at the base of the cords. Tumor cell proliferation was influenced by the capillaries located at the edge of the cords. This statement can be deduced from the observation that tumor proliferation often decreased with increasing distance to the surrounding capillaries and from the significant correlation between the intercapillary distance and the Ki-67 index-

Table 2 Relationship between vascular density (means ± 1 standard deviation) and T-stage, N-stage, and M-stage as well as tumor grading. The global vascular density of esophageal carcinomas did not correlate with T-, N-, M-, TNM-stage or tumor differentiation.

<table>
<thead>
<tr>
<th>Capillary volume %</th>
<th>Total vessel volume (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1/2 (n = 8)</td>
<td>2.3 ± 1.4*</td>
</tr>
<tr>
<td>T3 (n = 25)</td>
<td>1.9 ± 0.8</td>
</tr>
<tr>
<td>N0 (n = 28)</td>
<td>2.3 ± 1.3</td>
</tr>
<tr>
<td>N1 (n = 25)</td>
<td>2.0 ± 0.9</td>
</tr>
<tr>
<td>M0 (n = 25)</td>
<td>2.1 ± 1.0</td>
</tr>
<tr>
<td>M1 (n = 6)</td>
<td>1.8 ± 0.8</td>
</tr>
<tr>
<td>Stage III (n = 11)</td>
<td>2.1 ± 1.2</td>
</tr>
<tr>
<td>Stage III/IV (n = 22)</td>
<td>2.0 ± 3.1</td>
</tr>
<tr>
<td>G2 (n = 19)</td>
<td>2.1 ± 1.1</td>
</tr>
<tr>
<td>G3/4 (n = 14)</td>
<td>2.0 ± 0.7</td>
</tr>
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* Parenthesis, number of tumors/group; G, tumor grading.  

\(a\) Mean ± SD.

Fig. 3. Cell proliferation in tumor cords, expressed as Ki-67 index, correlated significantly with the mean intercapillary distance \((r = -0.472; P < 0.001)\). ○, cords without necrosis; ▲, cords with necrosis.

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also showed a strong positive correlation with the number of small vessels at the base of the tumor, but not with the total number of blood vessels within the tumors (16). The spatial structure of the esophageal carcinomas also explains why tumor vascularization was independent of pathohistological parameters and tumor diameters. In breast and cervical cancers also the oxygenation pattern did not correlate either with the histopathological stage or with clinically relevant parameters (22, 23).

The thickness of a tumor cord is the result of the extent of oxygen availability and oxygen consumption. The scatter in the regression analysis (cell proliferation versus intercapillary distance) can be explained by the fact that the \(pO_2\) of a tumor cell not only is limited by the distance from the cell to the closest blood vessel but also is influenced by physiological factors such as the intravascular \(pO_2\), the cellular oxygen consumption rate, and the oxygen diffusion coefficient of intervening cells. Oxygen consumption rates in tumors have been shown to be highly variable (2).

Our findings imply that commonly used pathohistological classifications of esophageal carcinomas do not allow any conclusions concerning the proliferation and oxygenation status of tumors and that the microcirculation at the base of tumor cords has an important influence on cell proliferation. Analysis of cervical and nasopharyngeal carcinomas yielded a statistically significant correlation between vascular density and survival time, as well as between intercapillary distance and local recurrence after radiotherapy (18, 19, 24, 25). Analyses of intercapillary distances and vascular density in human tumors therefore seem to allow predictions concerning the radiosensitivity of tumors. However, in esophageal cancer prospective studies remain to be carried out.

REFERENCES


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