Tumor Vascularity: A Histological Measure of Angiogenesis and Hypoxia

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ABSTRACT

In this study we sought to clarify the relationship between tumor vascularity, hypoxia, and angiogenesis in human cervix tumors. Two hypotheses were established: first, that measurement of tumor vascularity can provide a histological assessment of both hypoxia and angiogenesis; and second, that expression of angiogenesis-related proteins will provide a surrogate measure of tumor hypoxia. To test the first hypothesis, we studied the prognostic significance of tumor vascularity measured as both intercapillary distance (ICD; thought to reflect tumor oxygenation) and microvessel density (MVD; the hotspot method that provides a histological assessment of tumor angiogenesis). The relationship was also examined of tumor hypoxia, measured using an Eppendorf 

INTRODUCTION

There is a long history of measuring tumor vascularity to provide prognostic information. In the 1960s, Kolstad (1) showed that cervix tumors with low ICDs had low oxygen levels and were more likely to recur after radiotherapy than tumors with short ICDs. There followed several independent reports showing an adverse prognosis in poorly vascularized tumors (2, 3). In recent years, however, attention has focused on measuring vascularity to provide a histological assessment of tumor angiogenesis. The method of choice is counting MVD in areas of neoangiogenesis or vascular hotspots (4). Many studies have now shown that well-vascularized tumors with high MVD respond poorly to treatment in a variety of tumor types including carcinoma of the cervix (5, 6). These apparently contradictory findings suggest that the method of scoring vascularity may provide a histological assessment of both hypoxia and angiogenesis, and that method selection is important.

Our interest in tumor vascularity stems from a need to find an alternative to using an Eppendorf 

PATIENTS AND METHODS

Patients. The work comprised retrospective and prospective studies. Both of the studies were carried out with local ethical approval, and all of the patients gave prior informed consent. The retrospective study involved patients treated at the Christie Hospital between 1987 and 1992. Random cervical punch biopsies were taken prior to the initiation of treatment. All of the patients had bulky advanced disease and received radiation therapy with curative intent. Treatment was given according to the standard techniques and dosage of the Manchester School (12). Patients were reviewed quarterly in the 1st year, twice yearly for the next 2 years, and annually thereafter. Local recurrence (i.e., within the radiation field) was identified by clinical and radiological investigation. Histological confirmation of recurrence was documented when possible. The median follow-up time in surviving patients was 76 months (range, 24–115 months). The prospective series involved patients treated in 1997 and 1998.

Tumor Vascularity. For the retrospective series of patients, formalin-fixed, paraffin-embedded 4-μm thick sections were prepared from pretreatment biopsy specimens. The sections were stained with anti- von Willebrand factor (Dako) as described elsewhere (6, 13). Briefly, sections were trypsinized for 20 min at 37°C in 0.05 m Tris-HCl containing 0.01% trypsin and 0.01% calcium chloride. After washing, the samples were incubated in 10% normal swine serum for 10 min, followed by rabbit antihuman von Willebrand factor (1:500), biotinylated swine antirabbit antibody (1:400) for 30 min, and streptavidin biotin complex/horseradish peroxidase for 30 min. All of the sera and avidin reagents were obtained from Dako.

For the prospective series of patients, 4-μm sections were microwaved on high power for 25 min and incubated with 10% normal rabbit serum for 10 min. They were then stained with a mixture of antibodies to CD31 (Dako M7165) and CD34 (Dako M0823) both 1:70 in TBS for 16 h at 4°C. After washing in TBS, the sections were incubated at room temperature with goat antimonouse immunoglobulin, 1:50 in TBS for 30 min. After further washing in TBS, the sections were incubated with mouse alkaline phosphatase-antialkaline phosphatase complex, 1:50 in TBS for 30 min. The last two steps were then repeated for 10 min each to enhance the level of staining. Color was developed for 15 min with an alkaline

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[3] To whom requests for reprints should be addressed, at CRC Experimental Radiation Oncology Group, Paterson Institute, Christie Hospital NHS Trust, Wilmslow Road, Manchester, M20 4BX, United Kingdom. Phone: 0044-(0)161-446-3200; Fax: 0044-(0)161-446-3199; E-mail: cwesl@picc.man.ac.uk.

[4] The abbreviations used are: ICD, intercapillary distance; MVD, microvessel density; HP5, percentage of Eppendorf values less than 5 mm Hg; VEGF, vascular endothelial growth factor; PD-ECGF, platelet-derived endothelial cell growth factor; TBS, Tris-buffered saline; RR, relative risk; CI, confidence interval.

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phosphatase substrate kit (Vector Red; Vector Laboratories, Burlingame, CA) that included levamisole to inhibit endogenous alkaline phosphatase.

**Measuring MVD and ICD.** Tumor vascularity was assessed without prior knowledge of patient outcome using two methods. First, a semi-automatic image analysis system was used to score ICD (13). A cross-hair, in the eyepiece of a microscope was superimposed over the field of view and the center positioned over a vessel. Using an image analysis system, the distance between the central vessel and a vessel in each of the four quadrants was measured. To avoid bias, the nearest vessel looking clockwise to counterclockwise was chosen. Measurements were fed directly into a computer, and mean values were used. Adjacent fields of the whole tumor section were assessed at ×120 (field size, 0.554 mm²) avoiding areas of gross hemorrhage or necrosis. Second, tumor sections were analyzed using the vascular hotspot technique to obtain MVD (14). Sections were scanned at low power to determine areas of highest vascular density. Within this region, individual microvessels were counted in three separate random fields at high power (0.142-mm² field size). The mean vessel count from the three fields was used. A single countable microvessel was defined as any endothelial cell or group of cells that was clearly separate from other vessels, stroma, or tumor cells without the necessity of a vessel lumen or RBC within the lumen. Areas of gross hemorrhage and necrosis were avoided.

**Expression of VEGF and PD-ECGF.** Sections 4-μm thick were immunostained using a Dako EnVision system. After 25 min microwaving in 1 liter of 0.05 mM Tris-HCl containing 0.001 mM EDTA (pH 8.5), sections were stained with an anti-VEGF rabbit polyclonal antibody (Santa Cruz Biotechnology) at 1:200 dilution. A substrate-chromogen solution containing 3,3'-diaminobenzidine was used to visualize staining. For PD-ECGF staining, no antigen retrieval was required, and the monoclonal mouse antibody supernatant was supplied as a gift from Professor Adrian Harris (John Radcliffe Hospital, Oxford, England). For both VEGF and PD-ECGF, sections were scored using an arbitrary semiquantitative system. Both the intensity and the proportion of tumor cells stained for the whole biopsy were evaluated and sections placed into one of four categories: 0, no or occasional cell stained; 1, weak intensity, 0–24% of cells stained; 2, moderate intensity, 25–75% of cells stained; 3, strong intensity, 75–100% of cells stained.

**Eppendorf Measurements of Hypoxia.** In the prospective series of patients Eppendorf measurements were made immediately prior to taking biopsies at the time of examination under anesthesia for staging purposes. The method has been described in detail elsewhere (15). Measurements were performed with the patient under general anesthesia maintained using propofol infusion and nitrous oxide.

**Results**

**Scoring Reproducibility.** In the retrospective series of patients, one individual (D. P. W.) scored ICD and another (R. A. C.) MVD. The data were obtained independently several years apart. There were 142 patients for whom ICD data were obtained and 107 patients for whom MVD data were obtained. Fewer sections were available for scoring MVD because some blocks no longer contained sufficient biopsy material to take additional sections. Both of the methods for scoring vascularity were validated for intra- and interobserver reproducibility (Table 1). Intertumor variability was greater than interobserver heterogeneity, and by using ANOVA, significant differences were detected between tumors (P < 0.001 for both). The mean ± SD and median values for ICD were 167 ± 56 μm and 165 μm (range, 47–339 μm), respectively. The mean ± SD and median values for MVD were 11 ± 6 and 10 (range, 2–32).

**Log-Rank Analyses.** Patients with short versus long tumor ICDs had a significantly better level of local control (Fig. 1). Five-year local control rates were 83 and 63%, respectively. Stratifying ICD data by quartiles showed a significance for trend (P = 0.019). Patients with low versus high tumor MVD had a significantly better level of local control (Fig. 2). Five-year local control levels were 84 and 67%, respectively. Stratifying MVD data by quartiles showed a significance for trend (P = 0.048). Table 2 summarizes univariable analyses of outcome data for the biological and clinical parameters available for the 107 patients for whom all of the data were available. In the smaller subset of data, ICD did not show prognostic significance.

**Multivariate Analyses.** A Cox multivariate analysis for local control was carried out incorporating disease stage, grade, patient age, MVD, and ICD. MVD emerged as the most important prognostic variable (RR, 3.29; 95% CI, 1.5–7.3; P = 0.002), and after allowing for MVD, only ICD provided independent prognostic information (RR, 6.1; 95% CI, 1.3–18.2; P = 0.011). Fig. 3 illustrates the independence of ICD and MVD data in a bivariable analysis including ICD and MVD. Patients with short ICD and low MVD had a good prognosis (86% local control) compared with patients with long ICD and high MVD (53% local control). In a bivariable analysis including the 107 patients, MVD was a significant prognostic factor for local control after allowing for ICD (P = 0.019), and ICD had borderline significance after allowing for MVD (P = 0.056). In bivariable analysis, both MVD (P = 0.046) and ICD (P = 0.018) were significant prognostic factors for local control after allowing for disease stage.

**Hypoxia and Expression of Angiogenesis Markers.** In a prospective series of 38 patients, there was a significant correlation between HP5 and ICD but not MVD (Fig. 4). No relationship was seen between ICD and MVD. Patients with short ICD and low MVD had a significantly better level of local control (Fig. 1). Five-year local control rates were 83 and 63%, respectively. Stratifying ICD by quartiles showed a significance for trend (P = 0.048). Table 2 summarizes univariable analyses of outcome data for the biological and clinical parameters available for the 107 patients for whom all of the data were available. In the smaller subset of data, ICD did not show prognostic significance.

**Table 1 Correlation coefficients a for scoring reproducibility**

<table>
<thead>
<tr>
<th></th>
<th>MVD</th>
<th>ICD</th>
<th>VEGF</th>
<th>PD-ECGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intraobserver</td>
<td>0.84</td>
<td>0.74</td>
<td>0.80</td>
<td>0.82</td>
</tr>
<tr>
<td>Interobserver</td>
<td>0.88</td>
<td>0.56</td>
<td>0.93</td>
<td>0.95</td>
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</tbody>
</table>

*P < 0.01 for all.

Fig. 1. Local control in relation to tumor vascularity measured as ICD. ICD was measured on Factor VIII-stained formalin-fixed sections. Whole sections were scored to give ~160 measurements per tumor, from which median values were recorded. The median (top) and quartiles (bottom) of the median values from 146 tumors were used to stratify patients. Tumors with long ICDs had low vascularity. Numbers on each arm, the numbers of events and patients.
PD-ECGF was highly reproducible within and between observers (Table 1). The expression levels of neither VEGF nor PD-ECGF correlated with HP5 (Fig. 5).

**DISCUSSION**

These data illustrate the importance of method when scoring tumor vascularity. The use of two different techniques on the same cervix tumor sections provided independent prognostic information. Scoring MVD in vascular hotspots is the most widely used method and was validated as a histological assessment of angiogenesis (4). As seen here and in many other studies on a variety of tumor types including cervix carcinoma, MVD can provide significant prognostic information with high vascularity associated with poor treatment outcome. There are, however, a large number of methods used to score tumor vascularity. This can be illustrated by comparing studies on cervix tumors. Distance to the closest microvessel scored over the whole tumor can also provide a histological assessment of angiogenesis (16), but the method is inferior to the MVD hotspot technique (6). The area of endothelial cell staining and microvessel perimeter within hotspots also relates to angiogenesis (5). However, scoring the percentage of blood vessels within a tumor (13), vascular density per unit tissue volume (17), the proportion of vascular elements in tumor stroma (2), and ICD (18) relates to hypoxia. In all these cases, good vascularity indicates either well-oxygenated tumors or a good prognosis. In contrast with a histological assessment of angiogenesis, however, there is no consensus on which vascularity end point will provide the best histological assessment of tumor hypoxia. In this study, we confirmed the work of Kolstad (1) in finding a correlation between ICD and both tumor oxygenation and treatment outcome. Measurement of ICD, however, is less reproducible than scoring MVD, and ICD was prognostic for local control but not for survival. Therefore, ICD is inferior assessment of angiogenesis (16), but the method is inferior to the MVD hotspot technique (6). The area of endothelial cell staining and microvessel perimeter within hotspots also relates to angiogenesis (5). However, scoring the percentage of blood vessels within a tumor (13), vascular density per unit tissue volume (17), the proportion of vascular elements in tumor stroma (2), and ICD (18) relates to hypoxia. In all these cases, good vascularity indicates either well-oxygenated tumors or a good prognosis. In contrast with a histological assessment of angiogenesis, however, there is no consensus on which vascularity end point will provide the best histological assessment of tumor hypoxia. In this study, we confirmed the work of Kolstad (1) in finding a correlation between ICD and both tumor oxygenation and treatment outcome. Measurement of ICD, however, is less reproducible than scoring MVD, and ICD was prognostic for local control but not for survival. Therefore, ICD is inferior

**Fig. 2.** Local control in relation to tumor vascularity measured as MVD. The number of vessels was scored in three high-power fields in the area of highest vascular density of CD31/34-stained formalin-fixed tumor sections. The median (top) and quartile (bottom) values from 107 tumors were used to stratify patients. Tumors with low MVD had low vascularity. Numbers on each arm, the numbers of events and patients.

**Fig. 3.** Bivariable analysis of local control in relation to tumor vascularity measured as MVD after stratifying for tumor vascularity measured as ICD. Parameters are described in legends for Figs. 1 and 2. Numbers on each arm, the numbers of events and patients.

**Fig. 4.** The relationship of hypoxic fraction measured using an Eppendorf pO2 histograph with MVD (top) and ICD (bottom) in 38 cervical carcinoma. Hypoxic fraction was measured as the percentage of values less than 5 mm Hg (HP5) from ~160 measurements made in four tracks within a tumor.

**Table 2** Univariable analyses for 107 cervical carcinoma

<table>
<thead>
<tr>
<th>Survival</th>
<th>Local control</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICD</td>
<td></td>
</tr>
<tr>
<td>&lt;165 μm</td>
<td>24/54</td>
</tr>
<tr>
<td>&gt;165 μm</td>
<td>24/53</td>
</tr>
<tr>
<td>MVD</td>
<td></td>
</tr>
<tr>
<td>&lt;10</td>
<td>20/55</td>
</tr>
<tr>
<td>&gt;10</td>
<td>28/52</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>7/44</td>
</tr>
<tr>
<td>II</td>
<td>20/41</td>
</tr>
<tr>
<td>III</td>
<td>11/22</td>
</tr>
<tr>
<td>Grade</td>
<td></td>
</tr>
<tr>
<td>Well</td>
<td>7/17</td>
</tr>
<tr>
<td>Moderate</td>
<td>25/58</td>
</tr>
<tr>
<td>Poor</td>
<td>11/24</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>2/8</td>
</tr>
<tr>
<td>Age</td>
<td></td>
</tr>
<tr>
<td>&lt;50 yr</td>
<td>13/53</td>
</tr>
<tr>
<td>≥50 yr</td>
<td>13/54</td>
</tr>
</tbody>
</table>

* n = number of events/number of patients in each group.
to an Eppendorf pO2 histogram in providing prognostic information. It may be that the effectiveness of the Eppendorf approach relates to measuring predominantly acute hypoxia because there is evidence that the level of acute rather than chronic hypoxia limits treatment success (19–21). If acute is more important than chronic hypoxia, then the question of interest is how can vascularity be measured to reflect acute hypoxia. It may be that vessel diameter should be measured. In experimental tumors, large vessel diameter was shown to correlate with low interstitial fluid pressure (22), and in human cervix tumors, interstitial fluid pressure is higher in hypoxic tumors (23). Additionally/alternatively, vascular perfusion should be measured (24). There is clearly a need for a consensus on which method of scoring vascularity can provide a histological assessment of hypoxia, and this area of research warrants further study.

There is interest in angiogenesis as a marker for hypoxia. However, we found no relationship between Eppendorf measurements of hypoxia and either MVD or the expression of hypoxia-inducible angiogenic proteins. The lack of correlation between hypoxia and VEGF expression is consistent with the results from a study showing no relationship between VEGF expression and hypoxia measured using pimonidazole staining in cervix tumors (25). Although the relationship between hypoxia and angiogenesis is likely to be important in tumorgenesis (26, 27) and the transition from carcinoma in situ to invasive disease, the results reported in this paper suggest that there is no relationship between hypoxia and angiogenesis in advanced cervix carcinoma. This is possibly because of the involvement of other mutations in advanced disease leading to increased angiogenesis and the expression of angiogenic proteins. Therefore, measurement of angiogenesis and markers of angiogenesis cannot be used as surrogate measures of tumor hypoxia. However, because measures of angiogenesis and hypoxia can provide independent prognostic information, they can be combined to highlight large differences in treatment outcome probabilities (Fig. 3).

In conclusion, this study illustrates that measurement of tumor vascularity can provide different biological information that is dependent on the method used. It is, therefore, important that studies measuring vascularity should include an appropriate definition. There is a need for a consensus on the best way of obtaining a vascularity assessment of hypoxia. Our work showed that ICD provides only a weak measure of hypoxia. It may be that either other methods will be superior or histological measurements of vascularity will not provide a surrogate measure of hypoxia.
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