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A Morphometric Study of the Vascularity of Oral Squamous Cell Carcinomas and its Relation to Outcome of Radiation Therapy

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Abstract—*In 26 patients the vascular morphology of pretreatment biopsies from oral squamous cell carcinomas (scc) was studied. Patients had been treated by radiation alone and followed up for 25–73 months. Vascular endothelial cells were identified using antifactor VIII staining. The distances from 700 randomly selected tumor cells to the closest blood vessel were measured in each tumor. Stepwise logistic regression analysis revealed that tumors with fewer than 21% of the morphologically intact tumour cells located within 48 μm of the closest blood vessel had a better control rate than tumors with a higher proportion of tumor cells within this distance. Using the analysis as a predictive assay, local tumor control was predicted from a single histological section with a sensitivity of 81% and a specificity of 60%. In correctly predicted cases of local control, the average median distance between tumor cells and blood vessels was 105 μm. These tumors formed well differentiated avascular tumor cell nests that were surrounded by stroma and blood vessels. Mitotic figures were observed only in the outer rim of cell nests while the center was often keratinizing or necrotic. In cases of correctly predicted treatment failures the average median distance from intact tumour cells to capillaries was 76 μm. These tumors showed varying degrees of organization and capillaries penetrating tumor cell areas. Clinical outcome of radiation therapy of oral scc appears to be correlated with different patterns of tumor architecture and blood supply.*

INTRODUCTION

HYPOXIA of tumor cells has long been considered to be of fundamental importance in determining radiation response. In animal tumor systems hypoxic cells have been found to cause radioresistance and tumor control rates have been improved effectively with hypoxic-cell radiosensitizers [1]. However, in clinical trials no or very little improvement of local tumor control was attained by radiation with hyperbaric oxygen or misonidazole [2]. The contradictory results of the clinical trials could be caused by heterogeneity of tumors. If only some of the tumors contained a significant hypoxic fraction, the beneficial effect of treatment modifications to overcome tumor hypoxia is diluted and is not statistically detectable in study groups of a realistic size. A method to recognize tumors with radiobiologically effective hypoxia would therefore be helpful.

The pO_2 of a tumor cell is limited by its distance to the closest blood vessel as well as by physiological factors such as the intravascular pO_2, the cellular O_2 consumption rate (QO_2) and oxygen diffusion coefficient of intervening cells or structures. If hypoxic tumor cells are important for radiation therapy, a correlation between the distance of tumor cells from capillaries and the likelihood of local failure would be expected. Indeed there have been reports that intercapillary distances and capillary density may predict for local radiation treatment success in cervix carcinomas [3, 4]. In the present work a different morphometrical criterion was introduced and the distances from tumor cells to the nearest blood vessel were quantified and matched with clinical outcome of radiation therapy. The aim was to find out whether the distance distributions are of prognostic value and may therefore allow a better selection of patients for clinical studies.

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MATERIAL AND METHODS

Patients

Pretreatment biopsies of stage T2 and T3 squamous cell carcinomas (scc) of the tongue and floor of the mouth were studied. Patients had been treated at the Massachusetts General Hospital by radiation alone according to the twice-a-day (BID) treatment program to total tumor doses of 60–81 Gy, as described by Wang [5].

An equal number of patients with local failure and local tumor control were initially chosen. In several cases pretreatment biopsies had been performed at other hospitals and the paraffin blocks were no longer available. Fifteen cases of local tumour control and 11 cases of local failure have been evaluated. The minimum observation time for controlled tumors was 25 months; the median time was 46 months. The selection of cases was done by H.D.S. and the morphometric analysis by S.L. To exclude bias in scoring the histological material, the outcome of radiation therapy was unknown to S.L.

Biopsies and sections

In five cases, two biopsies were taken; usually, only one biopsy specimen was available. The amount of tumor tissue in the biopsy specimen varied greatly. From small biopsies, with a cross section area of tumor tissue of 1.2 mm^2, 4–6 sections were cut at distances of approximately 20 μm. In large biopsies, only two sections at distances of 20–60 μm were analyzed. Needle biopsies were not included in this study.

To find out how well the vascular structure of a tumor is represented by so few sections from a single biopsy, the intratumoral variability was assessed in two tumors. For this purpose biopsies were taken from different regions of the surgical specimen. One tumor was a carcinoma of the larynx, extensively invading adjacent normal tissue. Seven biopsies were taken from different parts of this tumor. The other tumor was a carcinoma of the base of the tongue, which was growing as one solid tumor mass from which three biopsies were taken; each biopsy extended from the periphery to the center of the tumor. Two biopsies from the first tumor were cut in 19 serial sections at 50 μm distances. From the remaining biopsies, 7–9 serial sections were cut at distances of 150 or 200 μm.

Counting error and variation within one section were assessed by counting 10 sections twice.

Staining

In a pilot study, two immunoperoxidase staining techniques of endothelial cells were compared in consecutive sections. Antifactor VIII has been reported to be less sensitive in old paraffin blocks than staining with the *Ulex europaeus* lectin I. *Ulex* has the disadvantage of being less specific, staining also lymphatic endothelial cells, prickle cells in normal stratified epithelium as well as tumor cells of well differentiated squamous cell carcinomas [6].

After dewaxing, endogenous peroxidase activity was blocked by immersion of the tissue in 1% H_2O_2 in methanol. For factor VIII staining, enzyme digestion was achieved with 4% pepsin in 0.1N HCl at 37°C for 8 min. The sections were then incubated with the following solutions in PBS: (1) normal swine serum 1:20 for 15 min, (2) either rabbit antifactor VIII 1:100 for 40 min or *Ulex europaeus* I 1:300 for 30 min followed by rabbit anti-*Ulex* 1:100 for 30 min, (3) swine anti-rabbit 1:20 for 20 min, (4) rabbit peroxidase–antiperoxidase complex 1:80 for 20 min, (5) 0.05% diaminobenzidine and 0.35% H_2O_2 in 0.1M Tris buffer pH 7.4 under microscopic control. The cell nuclei were then counterstained with hematoxylin.

Morphometry

Morphometric analysis was performed using a Zeiss microscope equipped with a camera lucida which allowed superimposition of drawings to the microscope field of vision. At a magnification of ×200, the distribution of distances between tumor cells and blood vessels was determined. A transparency with a point grid, with 40 points per mm^2, was attached to the lower side of the slide. For every tumor cell which happened to be located at a grid point, the distance to the closest blood vessels was estimated by projecting a set of concentric circles into the microscopic field; the chosen tumor cell was moved into the center of the circles and the smallest circle hitting or including a blood vessel was recorded. As projected into the microscopic field, the radius of the smallest circle and the distance between two consecutive circles was 24 μm. In each section as many measurements were taken as grid points fell on tumor cells. The number of measurements per section ranged from 78 to 811, with a mean of 275; an average of 790 measurements were made per tumour.

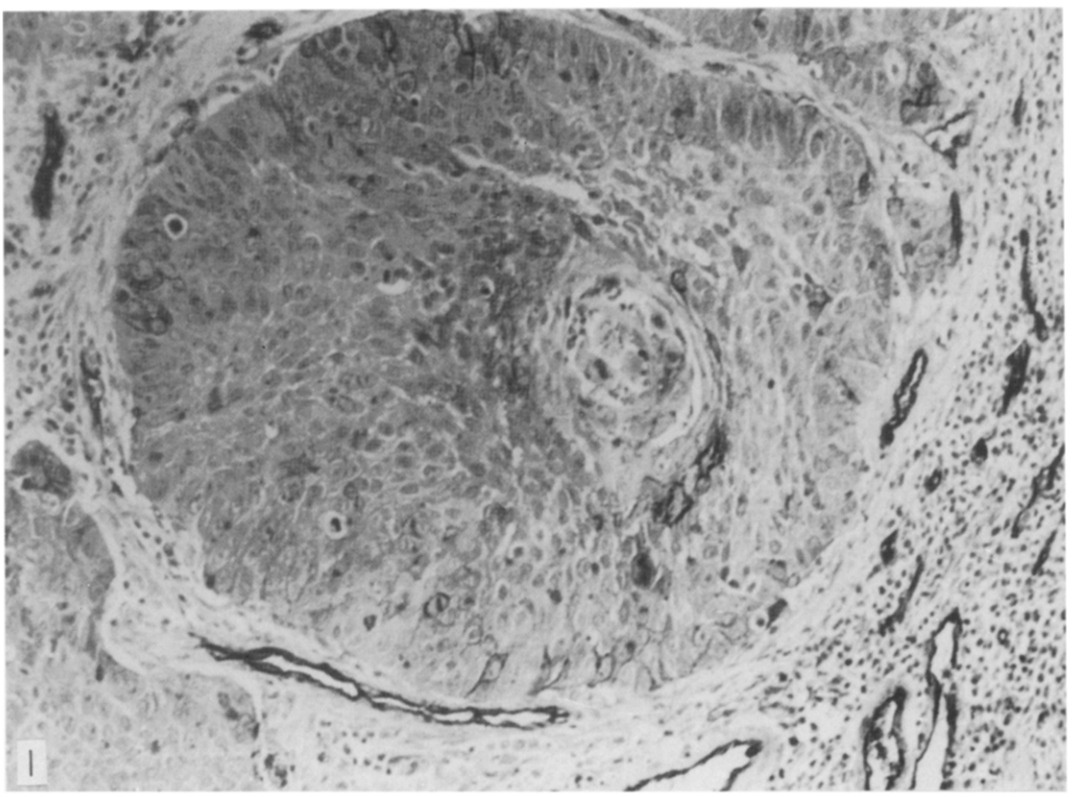
No distinction was made between different subtypes of blood vessels. Most vessels measured were capillaries, some were venules or sinusoids. Veins or arterioles did not occur in close proximity to tumor cells.

Keratinized or necrotic tumor cells were also evaluated, but were recorded separately. The spatial distribution of proliferating tumor cells was estimated by measuring the distance to the closest blood vessel for 25 randomly sampled mitotic figures in each tumor.

Tumor cell differentiation was assessed qualitatively according to the similarity of a tumor to normal stratified epithelium. Particularly the presence or absence of tumor cells resembling prickle cells and of keratinization was noted.

Data analysis

For each tumor section, distances measured between tumor cells and blood vessels were summarized. The proportion of cells closer than 24 μm to a blood vessel (24 μm being the radius of the first and smallest concentric circle) was denoted by x_1 and similarly $x_2, \ldots, x_{22}$ denoted the proportions of cells with the nearest blood vessel at most



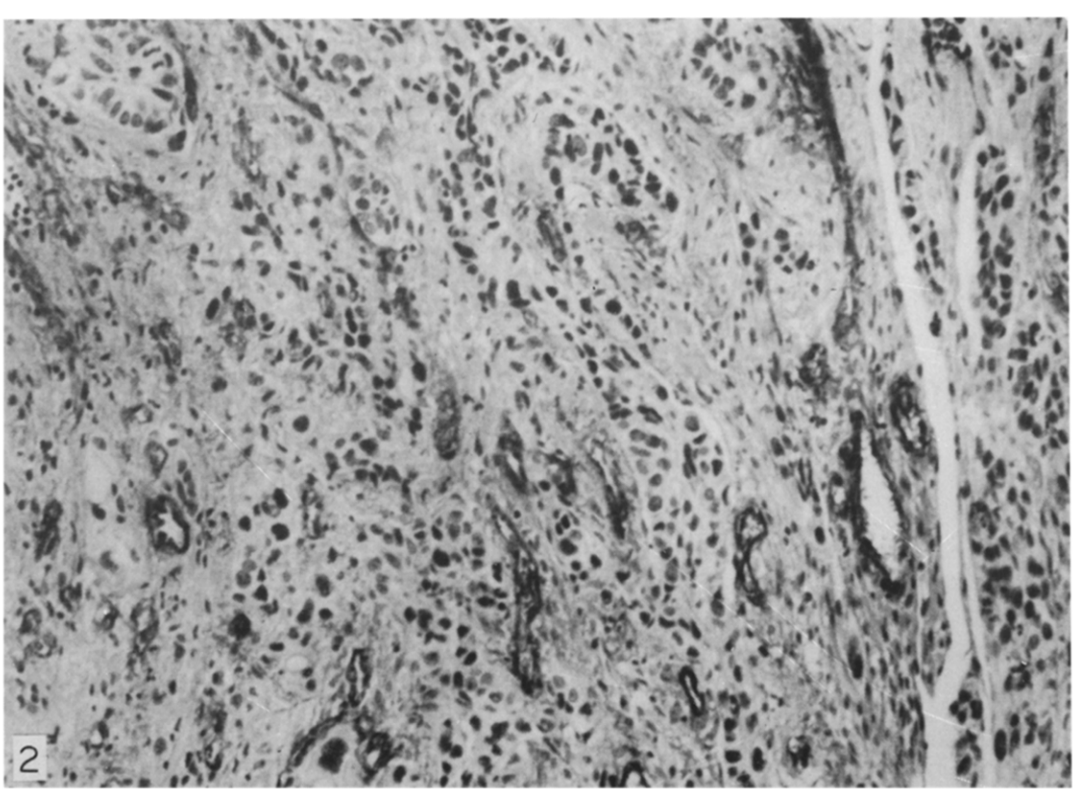


Fig. 1. *Microphotograph showing a highly differentiated, avascular tumor cell nest, surrounded by stroma and by blood vessels (file number* 2). 10.4% *of the morphologically intact cells in this tumor were within* 48 μm *of the nearest blood vessel. Endothelial cells are stained dark. Magnification* × 180.

Fig. 2. *Microphotograph of a poorly differentiated tumor, showing blood vessels not only in stroma, but also in direct contact with tumor cells (file number* 20). 31% *of the tumor cells were within* 48 μm *on the closest blood vessel. Magnification* × 180.

2×24 μm, . . ., 22×24 μm distant repectively. With these variables measured for each section, we used stepwise logistic regression to derive a model for predicting the probability of local control. Since there were only $n = 78$ sections, 22 explanatory variables would have been too many to enter into a stepwise model selection process. Accordingly four subsets with $n/10$ @ 7 or 8 variables were chosen:

(i) $x_1,x_3,x_5,x_7,x_9,x_{11},x_{15},x_{19}$ ('odds)
(ii) $x_2,x_4,x_6,x_8,x_{10},x_{12},x_{16},x_{20}$ ('evens')
(iii) $x_1,x_2,x_3,x_4,x_5,x_6,x_7$ ('lows')
(iv) $x_{13},x_{14},x_{16},x_{17},x_{18},x_{20}$, x_{21},x_{22} ('highs').

The choice of stepwise method was to include all variables of the subset and eliminate the variable with the least predictive power. This elimination was repeated until all variables left in the model were statistically significant and thus no further elimination could take place. To assess the representativeness of one section from a tumor, we investigated the variability of x_2. Using a variance component analysis (PROC VARCOMP in SAS, method = restricted maximum likelihood), we decomposed the variation of x_2 into three components: the first due to variation between biopsies of the same tumor, the second to variation between sections within a biopsy, and the last to variation from counting the same section twice. Since the tumor cells selected for measurement were not identical in the first and second counting, the last component reflects variability within sections as well as counting error.

RESULTS

Staining

Following carefully timed enzyme digestion, anti-factor VIII gave a strong staining of vascular endothelium with little background. It was not influenced by the degree of differentiation of tumors, or by the age of paraffin blocks. *Ulex europaeus* I showed exactly the same staining pattern in corresponding areas of consecutive sections, but in some cases tumor cells were also weakly *Ulex* positive.

Qualitative histology

Fifteen of 26 tumors were very well differentiated. Well defined nests of tumor cells were scattered in the submucosal stroma and were organized in layers very similar to stratified epithelium. The outer rim consisted of cuboidal cells, followed by an area of polygonal cells with prominent intercellular gaps resembling prickle cells and in the center the cells were often keratinized. With the exception of one tumor, these well differentiated cell nests were avascular. Blood supply was provided by the abundant submucosal microvasculature. Figure 1 shows a typical example of this tumor type. Three tumors were growing in well defined and avascular cell nests, but were not organized in layers and consisted of anaplastic cells. Six tumors were poorly differentiated and showed anaplastic tumor cells without architectural structure. They were invading normal tissue in nets or narrow strands of tumor cells or were growing as solid tumor mass. Here capillaries, venules or atypical sinusoidal vessels were distributed throughout the tumor mass and were found in direct contact with tumor cells. This is illustrated in Fig. 2. Table 3 summarizes the qualitative histological characteristics of each tumor.

Table 1. Result of stepwise logistic regression analysis of distances between tumor cells and blood vessels (see Appendix for derivation)

Proportion of tumor cells within 48 μm of the closest blood vessel (x_2)	Odds of local control:failure
11%	7:1
21%	1:1
31%	1:7

Table 2. Clinical result of radiation therapy and prediction of local tumor control by morphometric analysis of distribution of blood vessels

Clinical outcome of radiation therapy	Number of tumors with greater than 21%*	less than 21%*
Local control	3	12
Local failure	7	4

*Proportion of tumor cells located within 48 μm of the closest blood vessel.

Morphometric analysis

The average cumulative distributions of distances between tumor cells and blood vessels for local tumor control and local failure are shown in Fig. 3. For each radius the percentage of tumor cells located within this particular radius of the closest blood vessel is plotted. In treatment failures a higher proportion of tumor cells are located in close proximity to blood vessels than in local controls. With increasing radii the difference between local controls and failures becomes smaller, but not zero. Even for large radii local failures have a larger proportion of tumor cells within that radius of a blood vessel and local controls have more tumor cells still farther away. The largest measured distance of approximately 400 μm corresponds to the diameter of the largest tumor cell nest or the largest viable rim of tumor cell nests.

Stepwise logistic regression yielded a consistent interpretation of the data in the first three subsets ('odds', 'evens', 'lows'). In these cases, models with only one variable were chosen, and that variable

Table 3. Summary of the clinical result of radiation therapy, of the proportion of tumor cells located within 48 μm of the closest blood vessel and of semiquantitative score for tumor differentiation for each case

Patient number	Local tumor control (= y)	Proportion of tumor cells within 48 μm of a blood vessel	Prickle cells	Central keratinization	Blood supply by stromal vessels only
Local controls with less than 21% tumor cells within 48 μm					
8	y	9.6	+	+	+
2	y	10.4	+	+	+
32	y	10.6	+	+	+
31	y	11.5	+	+	+
25	y	13.1	+	+	+
7	y	13.8	−	−	+
6	y	14.2	−	−	+
10	y	15.3	+	+	+
29	y	15.7	±	+	+
5	y	16.2	+	+	+
30	y	16.3	±	+	+
15	y	17.4	+	+	+
Local controls with more than 21% tumor cells within 48 μm					
26	y	21.9	+	+	+
21	y	22.5	−	−	−
19	y	24.2	−	−	−
Local failures with less than 21% tumor cells within 48 μm					
11	n	14.8	±	−	+
18	n	13.4	+	+	−
28	n	15.3	+	+	+
14	n	16.8	+	+	+
Local failures with more than 21% tumor cells within 48 μm					
22	n	19.8	−	−	−
17	n	21.9	−	−	−
16	n	22.1	+	+	+
23	n	23.8	+	+	+
9	n	26.7	−	−	−
20	n	30.1	−	+	−
13	n	33.1	+	+	+

was always very significant ($P < 0.0005$). For the 'evens' and the 'lows' it was x_2, for the 'odds' it was x_3, indicating that the proportion of cells within a small distance of the nearest blood vessel was the greatest determining factor of local tumor control. The resultant model derived from the 'high' subset was a much poorer predictor of local control than the model derived from the other three subsets, and the P-value for the coefficient was barely significant. The final model chosen was the one with x_2 alone, since when x_2 and x_3 were entered (the 'low' subset), x_3 was eliminated in preference to x_2 as the better predictor. The results of the final model are given in Table 1. If less than 21% of morphologically intact tumor cells were within 48 μm from the closest blood vessel, the tumor was likely to be a local control. If more than 21% of tumor cells were found within that radius of a vessel, the tumor was likely to be a local failure (see Appendix for derivation). The P-value for the coefficient of x_2 being zero in the logistic regression is $P < 0.0001$, but this is likely to be slightly optimistic since a selection process was used. The proportion of tumor cells within 48 μm (x_2) of the closest blood vessel predicts local tumor control from one single section with a sensitivity of 81% and a specificity of 50%.

Contradictory predictions for different tissue sections of the same tumor were made in four cases of treatment failure and two cases of local tumor control. In all of them the prediction of treatment failure prevailed, that is the average proportion of tumor cells within 48 μm of the closest blood vessel was larger than 21%. Therefore, combining the information available for each patient 12 of 15 local control and seven of 11 local failures were predicted correctly by the analysis. This is summarized in Table 2.

In Table 3 the qualitative assessment of tumor differentiation and the proportion of tumor cells located within 48 μm from a blood vessel are listed. Ten of the 12 local controls with less than 21% tumor cells within 48 μm of a blood vessel were very well differentiated, although in two cases prickle cell formation was not as obvious as in the others. Similar to the example shown in Fig. 1, these 10 tumors were growing in avascular tumor cell nests,

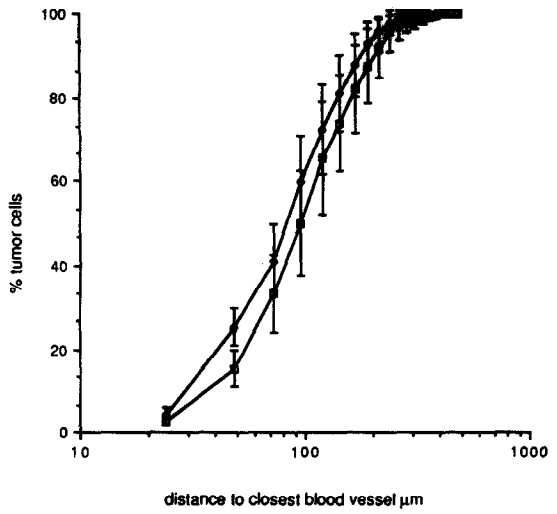


Fig. 3. Cumulative distribution of distances between tumor cells and blood vessels for local controls (open symbols) and for local failures (closed symbols). For each radius the average and standard deviation proportion of tumor cells within this distance from the closest blood vessel is plotted.

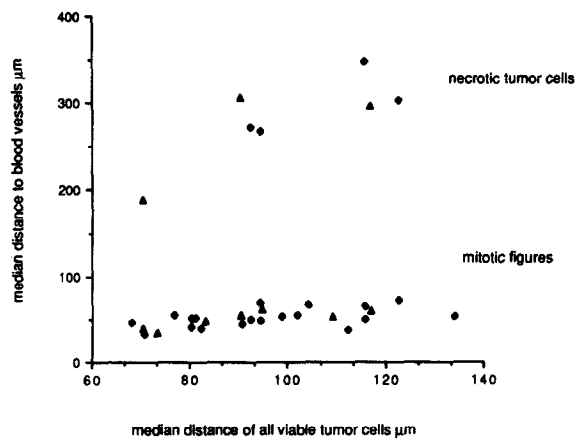


Fig. 4. Relationship between the median distance to the nearest blood vessel of necrotic or mitotic tumor cells and of all morphologically intact tumor cells. ◊ Local controls with ≤21% tumor cells within 48 µm; △ local controls with ≥21% tumor cells with 48 µm; ♦ local failures with ≥21% tumor cells within 48 µm; ▲ local failures with ≤21% tumor cells within 48 µm.

supplied by surrounding stroma vessels. Towards the center, tumor cells were gradually degenerating and keratinizing, so that a clear morphological distinction between viable keratinizing tumor cells and already degenerated tumor cells was very difficult. The group of local treatment failures was very heterogeneous with respect to tumor differentiation. Well differentiated tumors with less than 21% tumor cells within 48 µm had a local control rate of 10/12 (83.3%), whereas those with more than 21% tumor cells within 48 µm had a control rate of only 1/4 (25%). Thus morphometric analysis of vascular density gives more information than qualitative histology alone.

Mitotic figures were located at an average median distance to the closest blood vessel of 44.6 µm. Plotting this median for each tumor against the median distance of all viable tumor cells (Fig. 4), illustrates that these are independent of each other. This means that in large avascular tumor cell nests proliferation is restricted to the outer rim. There was no correlation between tumor control rate or differentiation and distance of mitotic figures. Only for those tumors with more than 21% tumor cells within 48 µm of a blood vessel did the analysis of the spatial distribution of mitotic figures give a significant result. The median distance of mitotic figures was slightly larger in poorly differentiated tumors than in well differentiated tumors (mean 42 µm vs. 33 µm; $P = 0.066$).

Necrotic tumor cells were found farther from blood vessels than most of the viable tumor cells. The median distance of necrotic tumor cells to blood vessels ranged from 142 to 264 µm. Figure 4 shows that it increased with the median distance of viable tumor cells, with a correlation coefficient of 0.8. However, only in seven tumors were the areas of necrosis large enough for analysis.

Intratumoral variability

Table 4 shows the contributions of variability between numerous sections from one biopsy and between different biopsies from one tumor to the overall variability. It is remarkable that for one tumor there was very little variability between different biopsies, while for the other the contribution of variability between biopsies was about the same as within biopsy variability. Note also that the recount variability is nearly the same for both tumors and is considerably smaller than the variation between sections, indicating reliability of the counting method and that this source of variability is not of major concern. Plotting the proportions of tumor cells within 48 µm for consecutive sections (Fig. 5) shows that in both tumors studied there is no systematic trend of these values with section depth. Sections that are only 50 or 150 µm apart from each other differ as much as sections that were very far apart. These results indicate that vascular anatomy of these tumors changes within very small areas, and that one biopsy is reasonably representative of the whole tumor for this parameter.

DISCUSSION

In the present study we found a higher local control rate of oral scc among tumors which were less well vascularized, as judged by pretreatment biopsies. The proportion of tumor cells located within 48 µm of the closest blood vessel was correlated negatively with satisfactory clinical outcome of radiation therapy. This finding is paradoxical in that much of the research in experimental radiation therapy is based upon the idea that local failures

Table 4. Contribution of variability between numerous sections from one biopsy and between different biopsies from one tumor to the overall variability of the proportion of tumor cells within 48 μm from a blood vessel in two tumors

	Tumor 1 (scc larynx)	Tumor 2 (scc floor of mouth)
Biopsy	8.1	1.97
Section within biopsy	17.4	7.6
Recount of section	3.92	3.58

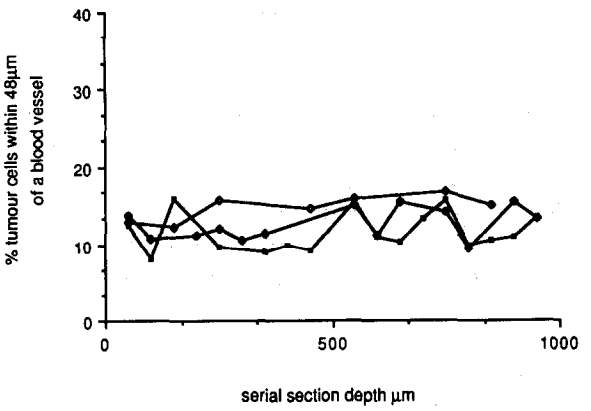


Fig. 5. Variation of the proportion of tumor cells within 48 μm in consecutive serial sections with section depth for three biopsies from one tumor (tumor 1, scc larynx).

are in part due to insufficient vascular supply with resultant hypoxic and hence radioresistant tumor cells.

This concept has been supported by several clinical studies on squamous cell carcinoma of the uterine cervix. Kolstad [7] measured the smallest diameter of the largest avascular area on the surface of cervix squamous cell lesions in pretreatment colpophotographs. In 51 of 105 cases avascular areas with a diameter larger than 400 μm were observed. In the course of 5 years of follow-up, local recurrence in the pelvis was found in 26 of these patients (51%), as compared to 16.7% in the remaining patients.

Siracka *et al.* [3] analyzed pretreatment biopsies of stage I and IIa cervix cancers. They found that the proportion of tissue occupied by vascular elements as well as the proportion of stroma occupied by vessels was significantly larger in the tumors of patients who survived longer than 5 years. Awwad *et al.* [4] studied intercapillary distances in pretreatment biopsies from 47 stage IIb and III squamous cell carcinomas of the cervix. They reported that the mean intercapillary distance as well as the proportion of distances exceeding 300 μm were smaller in patients with local tumor control after 2 years of follow-up than in patients with local recurrence.

Siracka *et al.* performed morphometric analysis after trichrome staining and identified blood vessels as 'redstained erythrocytes with a lumen or a clearly visible lumen'. With this definition, the observation was largely restricted to the fraction of blood vessels which happened to be perfused at the time when the biopsy was taken. In anaplastic tumors even open and plasma-perfused vessels may be hard to recognize without specific endothelial staining. Therefore Awwad *et al.* [4] examined cryostat sections using the alkaline phosphatase activity as a marker of endothelial cells. Venules are not alkaline phosphatase positive [8]. Since many small tumor vessels are atypical, resembling venules or sinusoids rather than capillaries, it is uncertain whether the sensitivity of this staining method is as high in tumors as it is in normal tissue and whether it is independent of the histological grade of the tumor. In both studies not all blood vessels may have been recognized and the vascularity may have been underestimated, particularly in poorly differentiated tumors. Such tumors may be associated with an unfavourable prognosis for reasons other than deficient vascular supply. Thus, the result of a lower local control rate in tumors classified as poorly vascularized may have been biased.

The geometrical factor limiting the pO_2 of a tumor cell is its distance to the closest supplying blood vessel. If blood vessels occur in clusters adjacent to large avascular tumor areas or if the amount of stroma between tumor cells varies greatly in different tumors, measurement of intercapillary distances or of the proportion of tissue occupied by blood vessels may be misleading.

There are several possible explanations for the contradictory results of the present work and the studies on cervix cancer. The different histological and morphological techniques used by Siracka *et al.* and Awwad *et al.* could account for an underestimation of vascularity of poorly differentiated tumors in these studies. But there could also be a true biological difference between squamous cell carcinomas arising from oral or cervix mucosa which is not simulated by different histological techniques. Finally, radiation schedules in cervix and in oral scc were not comparable. The patients studied by

Awwad *et al.* were treated by a combination of external pelvic irradiation, given in daily fractions, and intracavitary irradiation with ^{137}Cs or radium. Oral scc at the MGH have been treated with accelerated hyperfractionation giving two fractions of 1.6 Gy per day, with a 2-week break in the middle. With this treatment schedule repopulation and reoxygenation of tumor cells in the break must be a very important factor determining individual treatment response. Since morphometry separated two different types of architecture that are associated with different spatial distributions of mitotic figures, one may speculate that there are differences in tumor proliferation associated with the different architectural patterns which for this particular treatment schedule, would be as important as hypoxia.

However, the observations of the present study do not contradict the idea that hypoxia of tumor cells is due to insufficient vascularization. As shown in Fig. 3, the group of treatment failures differed from tumor controls mainly in the proportion of cells that were found very close to blood vessels; the proportion of tumor cells found far from blood vessels and potentially hypoxic, was similar in both groups. Tumor cell proliferation was restricted to the close proximity of blood vessels. This confirms experimental results in animal tumors [9, 10] and in tumor spheroids [11] that the highest proliferative activity is found in the best oxygenated areas. Most of the poorly vascularized local controls were very well differentiated tumors, growing in organized tumor cell nests that are gradually keratinizing towards the center. We judge it likely that between obviously nonviable cells in the center and proliferating cells in the rim of such tumor cell nests there could be a region of cytologically intact but non-clonogenic tumor cells. The latter cannot be distinguished morphologically from clonogenic cells. In this study we counted all intact cells. A better local control rate in these tumors could therefore be due to the existence of a well oxygenated clonogenic fraction of tumor cells in close proximity to blood vessels and large areas of hypoxic but non-clonogenic tumor cells. The experimental method employed in the present study only analyzed the distance between cytologically intact cells and the nearest blood vessel. Clonogenicity and cytologically intact cells cannot be assessed histologically.

In all local controls with fewer than 21% of the tumor cells within 48 μm of a blood vessel, tumor cell nests were avascular. Apparently they were supplied entirely with oxygen and nutrients by the microvasculature of the surrounding submucosal stroma. Roughly simplified they could best be described as growing in chords that are surrounded by stroma and vessels. This pattern of vascular supply has been described by Thomlinson *et al.* [12] in bronchial carcinomas. In 160 tumors studied the largest tumor chord without central necrosis had a radius of 200 μm. They have estimated an oxygen diffusion distance of 145 μm for those tumors.

In many cases of local failure, capillaries and atypical small blood vessels that were directly surrounded by tumor cells suggested angiogenesis in these tumors. Ausprunk and Folkman [13] described chords of cells surrounding a central blood vessel as typical morphology of newly formed tumor blood vessels. Oxygen and substrates diffuse centrifugally to the periphery. This second type of blood supply is less efficient and the oxygen diffusion distances have been estimated to be about 75% compared to diffusion from surrounding vessels towards the center of chords [14]. In local failures both types of vascular supply could be found within one tumor and the average median distance between tumor cells and blood vessels was 86% of that in local controls. Considering these functional implications of the different patterns of blood supply observed in each group, it is possible that clonogenic tumor cells in treatment failures were in fact less well oxygenated than in local controls, in spite of a shorter median distance between tumor cells and blood vessels. Therefore our findings do not contradict the clinical observation of a beneficial effect of radiation therapy under hyperbaric oxygen in head and neck squamous cell carcinomas [1].

Measuring the proportion of tumor cells that are located within 48 μm of the closest blood vessel does not give direct information of hypoxia; but it may be useful to distinguish between different patterns of tumor architecture that are apparently associated with different numbers and distributions of clonogenic tumor cells and with types of blood supply of a different functional efficiency.

Further studies should investigate whether contradictory observations in the studies of Awwad *et al.* and Siracka *et al.* and the present study are due to different morphometrical techniques or to a difference in the tumors studied. Different techniques should be compared in the same material, and oral squamous cell cancers should be compared with cervix cancers using identical morphometric methods.

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APPENDIX

The final logistic regression model used to derive Table 1 is most simply expressed in terms of the odds of local control versus local failure

$$\text{logit} = \log \quad \text{odds} = 4 - 0.194\, x_2$$

where x_2 is the proportion of tumor cells with the nearest blood vessel at most 48 μm distant. For example, if $x_2 = 21\%$ the odds of control to failure are 1:1, and for each 10% increase in this proportion the odds decrease by a multiplicative factor of $7.0 = e^{1.94}$, so that when $x_2 = 31\%$, the estimated odds of control to failure are 1:7, and when $x_2 = 11\%$ the odds are 7:1.