

Comparative Morphometric Study of Tumor Vasculature in Human Squamous Cell Carcinomas and Their Xenotransplants in Athymic Nude Mice¹

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ABSTRACT

When human tumor xenotransplants into nude mice are used as experimental models, it is important to know whether their microvascular anatomy is rather host or tumor specific. Therefore a morphometric comparison of the vascular network in human squamous cell carcinomas and their xenotransplants was carried out. Biopsies were taken from surgical specimens of three squamous cell carcinomas of the oral cavity. Part of the material was processed for histology and the rest was cut into 1-mm³ cubes and transplanted s.c. into the lateral thorax of athymic nude mice [NCr/Sed(nu/nu)]. The microvascular architecture of original tumors and of three first, one second, and one late generation xenografts was compared. Capillaries were identified in original human tumors by anti-factor VIII staining and in xenografts with antilaminin staining. The median distances between interphase tumor cells and blood vessels were determined and were found to be much longer in original human tumors than in xenografts, ranging from 81 μ m to 99 μ m and 53 μ m to 65 μ m, respectively. However, the characteristic qualitative histology of tumors appeared to be preserved in xenotransplants. Analysis of the topographic distribution of mitotic figures revealed that in both original tumors and xenotransplants proliferation of tumor cells was concentrated around blood vessels. Again, vascular distances in original tumors were significantly longer than in xenotransplants. In addition, xenotransplants into nude mice from a long passaged cell line from a human pharyngeal squamous cell carcinoma, FaDu, was investigated. FaDu showed a rarefaction of the capillary network with increasing tumor volume, but a constant median distance of mitotic figures from blood vessels.

In conclusion, the pattern of spatial distribution of proliferating tumor cells as well as differentiation characteristics appear to be retained in xenograft tumors, but the density of the vascular system is host specific. This has to be taken into account when physiological parameters of blood supply are studied in xenotransplanted tumors.

INTRODUCTION

Research into the response of tumor tissue to radiation and or drugs has to consider the micrometabolic milieu of the tumor cells (1-4). The concentration of each of the metabolites, e.g., oxygen, glucose, lactate, phosphonucleotides, etc., is dependent upon the adequacy of the nutrient perfusion. Accordingly the vascular anatomy in tumor tissue is an important subject for study. Much of the research conducted to date has employed isografts of rodent tumors. However, little attention has been paid to differences in the vascular anatomy of human and rodent tumors. Human tumor xenografts in athymic mice are studied with the goal to utilize experimental systems which resemble the spontaneous tumors in patients more closely than do isogenic mouse tumors (5). The vascular system and stroma in xenograft tumors are known to be host derived (6). It is important to understand the vascular anatomy in the xenograft and its difference from the original tumor in humans and their role in the observed responses (7).

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In the present study the vascular anatomy of three first and one second generation xenotransplant of human SCC³ were compared to the original human tumors and to late generation human SCC xenografts (FaDu). The distribution of distances between tumor cells and blood vessels was measured in histological slides and was used as parameter to describe the vascular system.

MATERIALS AND METHODS

Human Tumors. From surgical specimens of three oral SCC several biopsies were taken. One tumor originated from the floor of mouth and root of tongue (EC), one from the tonsillar fossa (JH), and the third biopsy was taken from a metastatic lesion in extranodal soft tissue (RB).

Xenotransplants. Part of the biopsy material was cut into 1-mm³ cubes under sterile conditions; these were then incubated for 1 h with 1% streptomycin in Hanks' medium. Ten cubes of each tumor were transplanted s.c. into the backs of five nude mice of the NCr/Sed(nu/nu) strain. The mice were bred and maintained in a defined flora, pathogen-free mouse colony (5, 8) and were whole body irradiated with 6 Gy before transplantation. When tumors reached a size of 100-400 mg the animals were sacrificed and the tumors excised for histological examination. For each tumor line four tumors of the first generation xenotransplants were studied, except for JH, where only two tumors were available. The tumor take rate was only 20-40%; however, some of the transplanted tissue may have contained only stroma and not tumor cells. From one tumor (EC) seven xenotransplants arose. Of these, four were used for morphometric analysis and three were processed for retransplantation. Another four tumors of the second generation xenotransplants were studied.

In addition another tumor xenograft line originating from an oral SCC, FaDu, was studied. This cell line had been long established *in vitro* and was obtained from the American Type Culture Collection, Rockville, MD. Tumor fragments were transplanted s.c. into the leg and a wide range of tumor volumes of 40-560 mg was examined.

For xenograft tumors a smaller volume range of 80-400 mg was available.

Histology. The remaining biopsy material was embedded into paraffin and was cut into 4- μ m sections for histological evaluation. Vascular endothelial cells were labeled with antifactor VIII immunoperoxidase staining.

When a xenograft tumor was excised, a slice of 0.7-mm thickness was cut by a pair of parallel-fixed razor blades through the center of the tumor. The diameter of the tumor slices examined varied according to the tumor volume from about 4.5 to 11.5 mm. The slice was fixed for 40 min in buffered formalin and embedded into glycolmethacrylate as previously described (9).

Since stroma and vasculature in xenotransplants are host derived, the immunohistochemical methods routinely used for staining of endothelial cells in human tissues are not applicable. The best compromise between well-preserved morphology and sensitive staining was achieved by using antilaminin immunoperoxidase staining on glycolmethacrylate-embedded tissue. It gives reproducible staining with little background reaction and can be performed on glycolmethacrylate sections which allow to control section thickness very precisely. Antilaminin does not stain endothelial cells but the accompanying basal membrane. However, tumors have been reported to contain laminin positive tumor

³ The abbreviation used was: SCC, squamous cell carcinoma.

cells (10) or blood vessels without basal membranes (11). Therefore the sensitivity and specificity of antilaminin for blood vessels in the particular tumors used in this study was verified using a monoclonal antibody specific for mouse endothelial cells (MECA-20; obtained from A. M. Duijvestijn, University of Limburg, The Netherlands) (12). In consecutive serial sections corresponding tumor areas were examined and showed the same structures to be positive for MECA-20 and antilaminin.

Morphometry. From the original human tumors, two to three biopsies were embedded for histology and from each biopsy one section was counted. From each of the embedded central slices of xenograft tumors, two sections taken at a distance of approximately 50 μm from each other were examined.

The distance of a tumor cell to the closest blood vessel limits the exchange of substrates. Therefore these distances were directly measured in histological sections. Absolute values of diffusion distances cannot easily be derived from these two-dimensional measurements. However they do allow relative statements, that is, comparison between tumors. Comparison of cumulative distributions of distances between tumor cells and blood vessels allows some inference on the existence of major differences in the architectural pattern of the vascular network.

Morphometric analysis was performed using a Zeiss microscope equipped with a camera lucida which allowed superimposition of drawings to the microscopic field of vision. At a magnification of $\times 200$, the distribution of distances between tumor cells and blood vessels was determined. A transparency with a point grid, with 40 points/ mm^2 , was attached to the lower side of the slide. For each tumor cell which happened to be located at a grid point, the distance to the closest blood vessel was estimated by projecting a set of concentric circles into the 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. Thus the distance between two consecutive circles was 24 μm . In each tumor section as many measurements were carried out as grid points fell on tumor cells, which was 150–350 in transplanted tumors and 300–600 in original human tumors.

In order to estimate the spatial distribution of proliferating tumor cells with respect to blood vessels, the distances of 50 mitotic figures to the closest blood vessel were measured in each tumor. The mitotic figures were randomly selected for measurement as described above.

From the obtained distributions median distances between interphase tumor cells or mitotic figures and blood vessels were calculated and compared. The absolute values of these median distances refer to the two-dimensional section plane.

In FaDu the size of necrotic areas was measured by the help of a magnetic tablet and compared to the total tumor section area.

Data Analysis. Cumulative distributions of distances between tumor cells and blood vessels were compared by the Kolmogorov-Smirnoff test, medians by Student's *t* test.

RESULTS

Qualitative Histology. The spontaneous tumor EC was a well-differentiated SCC with clearly defined tumor cell nests that were scattered in the surrounding submucosal tissue and displayed central keratinization (Fig. 1). The first generation xenotransplants did not form epithelial pearls, but in some areas tumor cells were arranged in a circular pattern with intercellular bridges in the outer zone, reminiscent of the tumor cell nests present in the original tumor (Fig. 1). The second generation xenografts showed a somewhat intermediate histology, displaying keratinization of individual tumor cells as well as epithelial pearls (Fig. 2).

Tumor JH was poorly differentiated, invading normal tissue in unstructured narrow strands. Neither the original tumor nor the xenotransplants did show any keratinization or intercellular bridges. In the transplants a marked nuclear pleomorphism was noted (Fig. 3).

The metastatic lesion RB was fairly well differentiated, form-

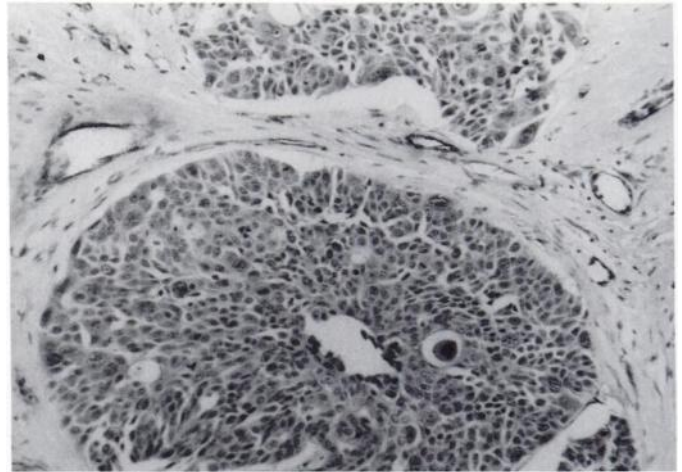


Fig. 1. Microphotographs of original tumor EC showing a tumor cell nest surrounded by dark capillaries and stroma. Magnification, $\times 50$.

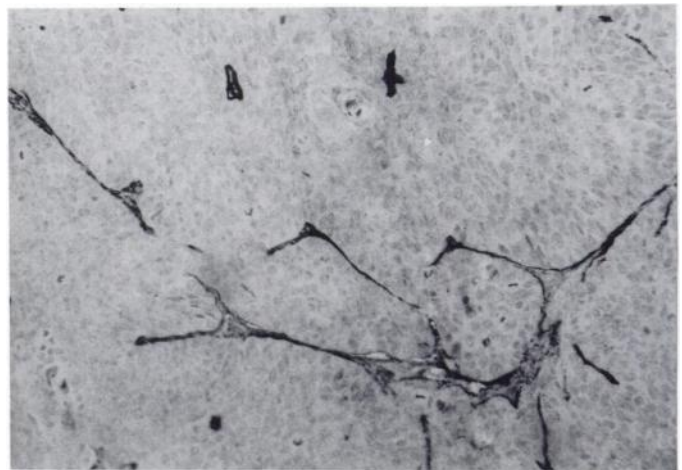


Fig. 2. Microphotograph of the second generation xenotransplant of EC with keratinization of individual tumor cells. Magnification, $\times 50$.

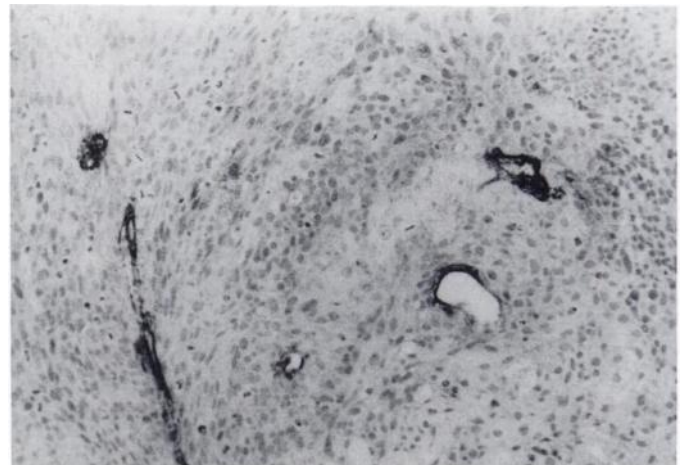


Fig. 3. Micrograph of the first generation xenotransplant of JH. Note the nuclear polymorphism of tumor cells. Magnification, $\times 50$.

ing distinct cell nests with gradual keratinization towards the center. However, no intercellular bridges were detectable. In xenotransplants an extensive keratinization involving the whole center of the tumors was found.

FaDu was quite similar to the first generation of EC showing a spiral arrangement of tumor cells, intercellular bridges and,

in addition, keratinization of individual cells. Thus the main histological tumor cell characteristics of the original human tumors appear to be preserved in their xenotransplants.

In the original tumors EC and RB tumor cell nests were clearly defined and largely avascular (Fig. 1). Blood supply came from the well-vascularized submucosal stroma surrounding tumor cell nests. In the original tumor JH there was no clear separation between stroma and tumor cell nests and blood vessels could not be ascribed to either compartment.

Xenotransplants contained no significant stromal tissue components except blood vessels. The blood vessels appeared to be randomly distributed; vessels of grossly atypical morphology, e.g., like sinusoids, were not observed in any of the tumors examined.

Morphometry. Qualitative histological examination did not reveal any systematic differences in the vascular density of viable tumor tissue between central and peripheral areas of tumor slices. However, no quantitative comparison was made. In Fig. 4 the cumulative distributions of distances between tumor cells and blood vessels for the original tumor and first two generation xenotransplants of EC are shown. Points represent average values for the respective generation. The two generations of xenograft tumors show nearly identical distributions which, compared to the original tumor, are shifted towards shorter distances. The differences between original tumor and first or second generation xenograft are statistically significant with $p < 0.01$. The curves are nearly parallel suggesting a quantitative difference that can be sufficiently described by comparison of median distances between tumor cells and blood vessels. Vascular distances in FaDu were even shorter than in early generation xenotransplants ($p < 0.05$); however the difference between FaDu and EC early generation xenotransplants was smaller than between original tumor EC and xenotransplant ($p < 0.01$).

In Fig. 5a the average of the median distances are given for all tumors evaluated. The values for the three original human tumors are within a relatively small range of 81–99 μm and are longer than for corresponding xenograft tumors ranging from 53–63 μm with $p < 0.01$. The smallest difference between original tumor and xenograft is found in the poorly differentiated tumor JH and the largest in the well differentiated tumor EC.

Similar distributions of distances can be measured for mitotic figures. Fig. 5b shows the median distances of mitotic figures to the closest blood vessel. In all SCCs they are less than half of the median distances of interphase tumor cells. This means proliferation is concentrated around blood vessels. Again the median distances for different SCC xenograft tumors were very

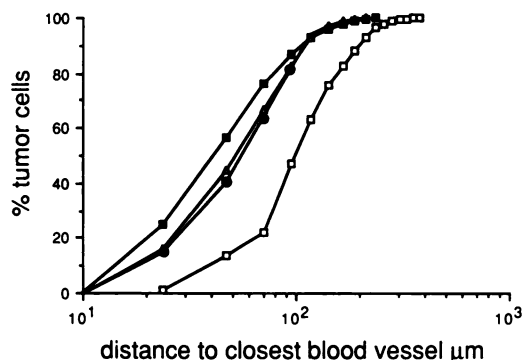


Fig. 4. Cumulative distribution of distances between tumor cells and blood vessels for the original tumor EC ($\square$), first ($\triangle$) and second ($\bullet$) generation xenotransplant of EC and late generation xenotransplant of FaDu ($\blacksquare$).

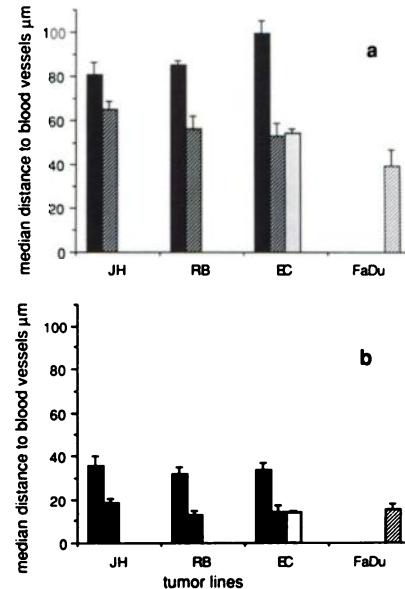


Fig. 5. Averages and standard deviations of median distances between interphase tumor cells (a) or mitotic figures (b) and blood vessels for all tumor lines investigated. Black columns stand for original human tumors, dark hatched columns for first generation human tumor xenografts, light hatched column for EC second generation xenograft, and striped columns for late generation xenografts of FaDu.

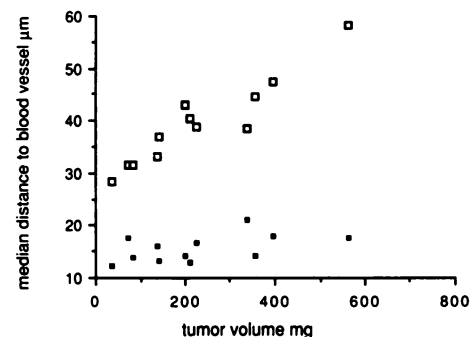


Fig. 6. Variation of median distances between interphase tumor cells ($\square$) or mitotic figures ($\blacksquare$) of FaDu with tumor volume. Each point represents the average count of two sections from one tumor.

similar ranging from 13 to 19 μm , but were significantly smaller than in the original tumors ranging from 32 to 36 μm ($p < 0.01$).

The vascular anatomy as well as the spatial distribution of proliferating cells appear to be identical in first and second generation xenografts of EC, although there were distinct differences in qualitative histology.

As a possible factor of influence, tumor volume was studied in FaDu over a wide range. Fig. 6 illustrates that the median distance of interphase tumor cells to blood vessels increases with tumor volume, while mitotic figures are always located in close proximity to blood vessels, independent of tumor volume. Thus the quiescent inner part of avascular tumor areas increases in relative proportion with tumor volume. The large range of tumor volumes studied therefore explains the relatively large standard deviation of the average median distance plotted in Fig. 5a. A similar relationship is suggested for EC; however, the number of tumors available did not allow meaningful analysis.

In FaDu, percentage of section area of necrosis appeared to increase with tumor volume. In human tumors and early generation xenografts analysis of necrosis was difficult because keratinizing as well as necrotic areas were present. If both are

taken together, there is no correlation between the amount of nonviable tumor tissue in original human tumors and xenografts. Meaningful separate analysis was not possible because both foci of necrosis and keratinization are easily lost from tissue sections during processing.

DISCUSSION

In the present study blood vessels were identified by antilaminin staining of basal membranes or antifactor VIII staining of endothelial cells and the distribution of distances of tumor cells from blood vessels was chosen as quantitative parameter to describe the vascular network. The effectiveness of chemo- or radiation therapy is a function of the availability of drugs or oxygen to tumor cells. The geometrical factor limiting the pO_2 of a tumor cell is its distance to the closest supplying blood vessel. Spatial distribution of blood vessels may be inhomogeneous within as well as between tumors. If blood vessels occur in clusters the proportion of tissue volume occupied by vasculature (volume density) may be high although there may be avascular areas containing tumor cells that are far away from blood supply and possibly hypoxic. In that case measurement of volume density of blood vessels or intercapillary distances may give irrelevant information. Furthermore, the vessel diameters apparent in a histological section are determined by the accidental physiological situation in the individual animal at the time of sacrifice. Without perfusion fixation at defined pressures any morphometric parameter that is a function of vessel diameter or volume cannot be expected to give reproducible and comparable results. Therefore the distances between tumor cells and blood vessels were directly measured in the present study. Although vessel diameter is an important determinant of blood flow and hence of vascular function, it was not considered in our study. In tumors, caliber irregularities along single vessels (11) complicate the physiology of blood flow as well as meaningful analysis of vessel diameters. A classification of vessels based on analysis of vessel wall components alone would also be difficult to interpret in tumors because tumors are known to contain large sinusoidal, atypical vessels (11) without smooth muscle cells. Therefore analysis of vessel wall components in tumors does not allow conclusions on vessel caliber or functional properties. Distributions of distances between tumor cells and blood vessel are a function of total vessel length as well as of the spatial distribution of vessels and seemed to be the best compromise between reliable and meaningful measurement.

Morphometric analysis revealed a more dense vascular network of first and second generation xenotransplants of three oral SCC than their parent human tumors. The median distances between blood vessels and interphase tumor cells were nearly twice as long in the spontaneous human SCC. The variations of median distances between different human tumors or between xenografts of different origin were comparatively small. FaDu, a late generation human SCC xenograft, was even better vascularized than first and second generation xenotransplants. Further, the relationship between blood vessels and neoplastic cells was observed to be modified by transplantation. In xenotransplants tumor cells appear in contact with the wall of small blood vessels, whereas in spontaneous human tumors (EC, RB) cell nests are separated from the vasculature by connective tissue. Thus, the vascular anatomy of xenotransplanted tumors appears to be more host specific than tumor specific.

These observations are not in accordance with the conclu-

sions drawn by Solesvik *et al.* (13) in a study on vascular anatomy of five different human melanoma xenograft lines. Differences between cell lines were interpreted as indirect proof of tumor specificity of vascular anatomy, but xenografts were not compared to the spontaneous human melanomas.

However, in other experimental situations vascular anatomy was shown to be tumor specific. Vascular morphology in tumors arising from isotransplantation of astrocytoma spheroids to different sites, *e.g.*, brain and skeletal muscle, was identical but was quite different from normal brain (14). Shubik *et al.* (15) as well as Kraus *et al.* (16) reported that the spatial patterns of vessels were characteristic for tumors of different histological origin.

In contrast to vascular anatomy, morphological characteristics of tumor cells such as keratinization, formation of epithelial pearls, and intercellular bridges appeared to be retained after xenotransplantation in our experiments. Similar conclusions were drawn by Tilgen *et al.* (17) who studied two SCC cell lines in culture and as xenografts. Even after long periods *in vitro* and many passages *in vivo* as well as after reimplantation of cultured cells into nude mice, each tumor line preserved its morphological and ultrastructural characteristics.

In the present study mitotic figures of SCC were concentrated in close proximity to blood vessels. Their median distance to blood vessels was less than half the distance of interphase tumor cells in spontaneous SCCs as well as in xenograft SCCs. Thus the pattern of spatial distribution of proliferating tumor cells was retained in xenotransplants.

Proliferation of tumor cells has been found to be a function of the availability of substrates in other isotransplanted mouse tumors (18) as well as in tumor spheroids (19). One might therefore speculate that the comparatively dense capillary network of xenograft tumors is functionally less effective, resulting in a steeper gradient of oxygen concentration with distance from a blood vessel than in human tumors. Human oral SCCs in particular appear to be largely supplied by the abundant normal submucosal vasculature. In contrary, the microvascular of experimental tumors probably consists mainly of newly formed capillaries. Such tumor-induced vessels are known to have a different wall structure and diameter and hence different hemodynamic properties (11). Further, the vascular density in the primary tumor in humans and in xenografts is likely to be strongly influenced by the dissociation curve for oxygen from hemoglobin. The pO_2 50 values for mouse and human hemoglobin are 41 and 25 mmHg, respectively (20). This suggests that anatomically identical vascular networks would result in a less effective tissue oxygenation in mice.

Vaupel *et al.* (7) reported a similar tumor blood flow in human breast cancer and in human breast cancer xenografts in nude rats, concluding that xenografts are a good model to study tumor blood flow experimentally. However, even if nearly identical in unperturbed blood flow, the two vascular networks are so different structurally that a different blood flow regulation and physiology in human and xenograft tumors is most likely. In that case the clinical relevance of experiments to modify tumor blood flow would have to be considered particularly carefully.

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