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The Vascular System of Xenotransplanted Tumors -Scanning Electron and Light Microscopic Studies-

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Abstract

A widely used model for investigating basic tumor characteristics and different treatment modalities preclinically is the immune-deficient, athymic nude mouse. This model offers many morphological parallelisms to the clinical situation.

The aim of this study is to demonstrate the vascularization pattern of xenotransplanted human melanomas and sarcomas using different methods. Xenotransplanted tumors of 62 congenital thymus-aplastic nude mice were examined ultrastructurally and topographically after corrosion cast and tissue preparation. Quantitative measurements of tumors injected with India ink were carried out to obtain comparable information on the vascular densities in the tumors.

Quantitative measurements showed that there is no zonal, topographic arrangement of the vascular densities. Comparisons of the vascular densities in the centers of tumors with the densities in the periphery showed an extreme heterogeneity in tumor vessel distribution, which does not generally support the idea of a better vascularisation in the tumor periphery.

Neither in the periphery nor in the center of the tumor regular vessels are to be seen consisting of intimal, medial and adventitial layers. Even the largest peripheral vessels, which often take a tortuous course, consist mainly of an endothelial layer and some perivascular connective tissue only. Areas with high vascular densities could be seen just beneath areas almost free of vessels. Besides endothelial-lined vessels numerous irregular, tumor cell-lined sinusoids are visible both in sarcomas and melanomas. The morphology of the vessels found scanning electron microscopically is generally in agreement with many features described transmission electron microscopically.

KEY WORDS: Tumor vascularization, tumor blood flow, tumor growth, xenograft, nude mouse, vascular corrosion cast, ultrastructure, scanning electron microscopy, heterogeneity.

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Introduction

Treatment of advanced soft tissue sarcomas and malignant melanomas still yields disappointing results and is often restricted to symptomatic palliation. A widely used model for investigating basic tumor characteristics and different treatment modalities preclinically is the immune-deficient, athymic nude mouse (Flanagan, 1966). The nude mice are ideally suited for such studies because of their immunological defect (Pantelouris, 1968) .

A wide spectrum of human tumors has been reported to grow successfully after transplantation in these animals (Giovanella et al., 1978 a, b). One of its advantages is the presence of a functional vascular system which is lacking in all in-vitro-assays.

Hirst et al. (1979) and Gabbert et al. (1983) found that tumor cell proliferation is strongly correlated with their position in relation to the blood vessels. It can be assumed that the tumor-inherent vasculature also plays a major role in its sensitivity to therapeutic measures, e.g., radiotherapy and chemotherapy (Revesz and Siracka, 1984; Peters et al., 1986; Jain, 1988). Denekamp (1984) and Rubin et al. (1966 a, b) have suggested the vascular endothelium to be the vulnerable element in tumor therapy.

This is also true for hyperthermia: Van Beuningen and Streffer (1985) have pointed out that a successful treatment by hyperthermia is strongly connected with the tumor blood flow. Numerous studies (e.g., Hill and Denekamp, 1978; Overgaard, 1980; Song, 1978; Song et al. 1980) suggest that the effects of treatment by hyperthermia are directly cytotoxic ones and also affect the vascular system with subsequent necrosis.

Ultrastructural studies of the vascular system are necessary in order to obtain more detailed information on the sites affected by tumor therapy. Grunt et al. (1986 a, b) have already demonstrated conclusively the development and the characteristic structural features of the angioarchitecture of the Lewis lung carcinoma by microcorrosion casting. Walmsley et al. (1987) have tried to define differences of tumor vascularisation in old and young hosts. However, such syste-

matic studies have only been carried out on a few experimental tumors (Illig, 1961; Hammersen et al., 1983; Warren, 1979a, b) but not in the more suitable tumor model in nude mice: Several authors have concluded that this model offers many morphological parallelisms to the clinical situation (Donhuijsen et al., 1988). Furthermore, the individuality of human tumors with respect to the vascular system is maintained in the nude mouse model (Steinberg et al., 1987).

The aim of this study is to demonstrate vascularization patterns of xenotransplanted human melanomas and sarcomas using vascular microcorrosion casting, scanning electron microscopy and morphometry.

Materials and Methods

Animals and Xenotransplantation

For our studies 62 male and female congenital thymus-aplastic nude mice (NMRI-strain), 8-10 weeks old, weighing 22-35 g,were used for transplantation. The animals were kept under specific pathogen-free conditions at a room temperature of 28 +/- 2°C and a relative humidity of 85 +/-5%.

An undifferentiated human amelanotic melanoma and 6 different sarcomas (three leiomyosarcomas, spindle cell sarcoma, neurofibrosarcoma, liposarcoma) from patients were transplanted on 18 and 44 mice, respectively. The surgical specimens were subsequently cut into slices three to four mm in diameter and one mm thick. In each animal, one of these slices was inoculated subcutaneously through a lateral incision at the level of the right anterior axillary line behind the foreleg where the best growth results were previously reported (Budach et al., 1986). The wounds were closed with tiny metal clamps which were removed five days later. Serial passaging, i.e. transplantation from animal to animal, was done in a similar manner. The melanoma and the sarcomas were in the second and 6th-40th passage respectively at the time of transplantation. The tumor volumes were measured daily. The animals were sacrificed 10 to 28 days after transplantation except for 13 sarcomas which were used for long term growth rate measurements.

Scanning Electron Microscopy

The tumor tissue from 9 melanoma and 11 sarcoma-bearing mice was processed for scanning electron microscopy. The animals were thoracotomized in deep pentobarbital anesthesia (Nembutal, 8 mg/100g body weight i. p.) 20 minutes after an i.p. application of heparin (Liquemin, 500 IU/100g body weight). The vascular system was exsanguinated in most cases with up to 40 ml 0.9% NaCl (temperature 38°C) given by means of an olive-tipped cannula inserted into the left ventricle. The mean pressures used for perfusion were between 70-90 mmHg and the solution temperatures ranged from 35-38°C. The animals were then fixed by perfusion at pressures of 75-100 mmHg with 2.5% cacodylate-buffered glutaraldehyde (pH 7.40, 860 mosmol) with a total of 130 ml over a period of five to ten minutes.

After dissection the tumors were cut into pieces of 3 mm length and thickness and postfixed for 6-12 h in 2.5% glutaraldehyde at 4°C. The specimens were then washed with cacodylate-buffer and contrasted in osmic acid solution (Dalton, 1955).They were dehydrated with ascending grades of alcohol and amyl-acetate. The specimens were transferred from the pure amyl-acetate to fluid propane gas in liquid nitrogen. After complete freezing they were broken with a scalpel, retransferred to amyl-acetate, dried in liquid CO_2 by the critical point method, mounted on specimen holders and sputtered with gold in an argon-atmosphere. They were examined with a Stereoscan 180 scanning electron microscope (Cambridge) at an acceleration voltage of 17-25 kV.

Corrosion casting technique

Vascular corrosion casts were made from 9 melanomas and 10 sarcomas mainly as described by Hodde et al. (1980) and Lametschwandtner et al. (1984). After perfusion with saline and fixation as described above up to 35 ml of undiluted Mercox CL-2B (Japan Vilene Comp. Ltd., Japan) mixed with 1.5% catalyzing substance was injected.

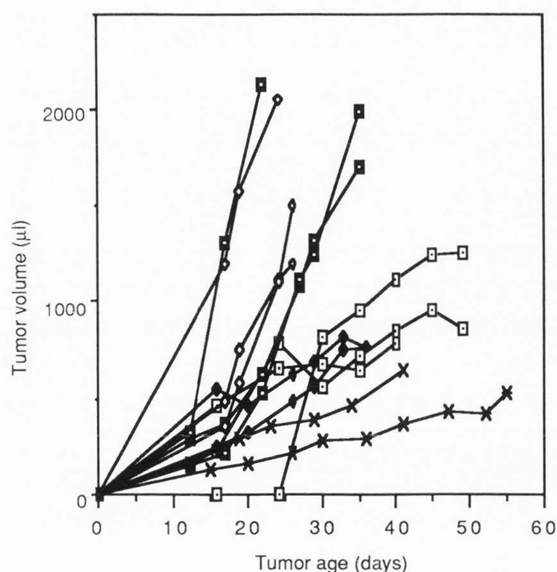
After complete hardening in a water bath at 40°C the tumors were dissected and macerated in 15% KOH at 40°C. After complete maceration the casts were rinsed and frozen in distilled water and some of them cut tangentially. After freeze-drying and mounting on specimen holders they were sputtered with gold in an argon atmosphere and examined with a Stereoscan 180 scanning electron microscope (Cambridge) at an acceleration voltage of 10 kV.

Quantitative Measurements of Tumors Injected with India Ink

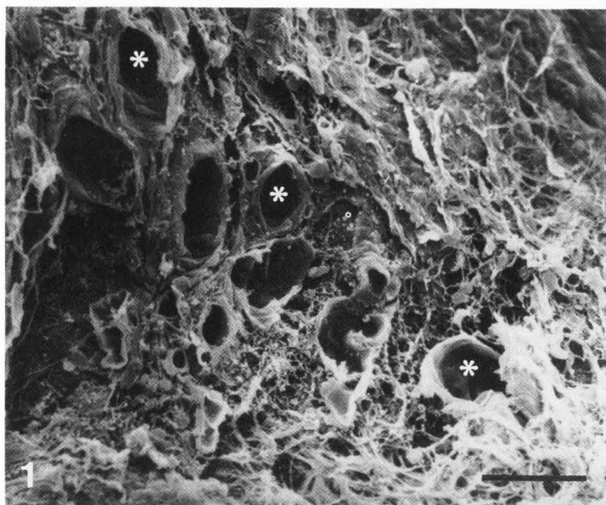
In order to obtain comparable information on the vascular densities in the tumors, 10 sarcomas of four different tumor cell lines were injected with India ink. The animals were thoracotomized in deep pentobarbital anesthesia as described above. After perfusion with saline thirty-five ml of water-proof India ink (temperature 35-38°C) was injected by means of an olive-tipped cannula inserted into the left ventricle as described above. The mean pressure used for perfusion was 70-95 mmHg. After dissection of the tumor, the tissue was frozen and coronally cut into 5 µm thick sections in a cryostat (Leitz 1720 Digital). The sections were stained with hematoxylin and eosin. The densities of the ink-injected vessels were measured with an automatic image analyzing system (Quantimet 970, Cambridge) at an objective magnification of x10. The size of the areas measured was 1.92 mm², which corresponds to about 1,060,000 pixel points after digitalisation. In order to compare peripheral and central vascular densities, two contra-lateral fields as well as a central field was measured in each case. The whole tumor was measured to determine the average vascular density of the total tumor and the arithmetic mean of all individual areas measured was calculated. In this case, up to 40 areas were necessary depending on the size of the tumor.

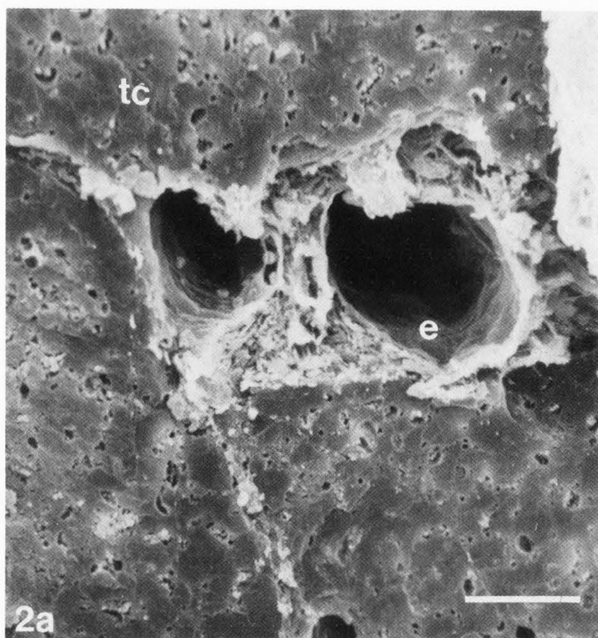
Results

The xenotransplanted tumors grew subcutaneously reaching an average volume of 1.5 ml within two to four weeks. In animals not sacrificed, the tumors reached volumes of up to 15 ml. Even in the same tumor entities extremely different tumor



Graph 1: Growth rates of 13 xenotransplanted melanomas of the same entity on nude mice. Each curve represents one single tumor. Pooled data were deliberately not used since the typical heterogeneity would no longer be recognised in that case.





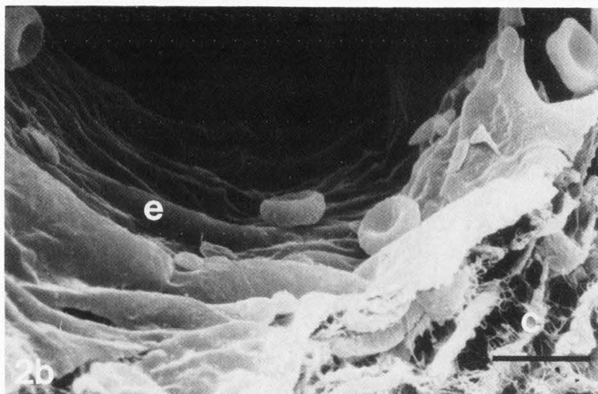


Fig. 1: Surface of a xenotransplanted melanoma after dissection and partial removal of the peritumoral subcutaneous tissue. Note the supplying and draining vessels (*), respectively. Bar=50 µm

Fig. 2a, b: Freeze-broken specimen of an amelanotic melanoma. Main vessels in the tumor periphery consisting mainly of an endothelial layer (e) and some connective tissue (c). tc = tumor cells. Bar in a = 50 µm, bar in b = 5 µm

growth rates could be measured (graph 1). Metastases were never observed in bone, lung, liver, brain, kidney and soft tissues either macroscopically or histologically during this time. This parallels the results of Bastert et al. (1981). Contrary to many experimental tumors, which are characterized by invasive and local destroying growth behaviour, our xenografts grew non-invasively. The subcutaneous tissue formed a capsularlike structure, through which the main supplying and draining vessels pass to and from the tumor (fig. 1). The supplying arteries are largely derived from the lateral thoracic, the external mammarian and the superficial epigastric arteries. Venous drainage takes place by numerous subcutaneous branches preferably into the corresponding veins (for details see Grunt et al., 1986 a).

These arteries and veins, showing normal structural features outside the tumor, lead into intratumoral vessels which are characterized by a widely different architecture. Regular vessels consisting of intimal, medial and adventitial

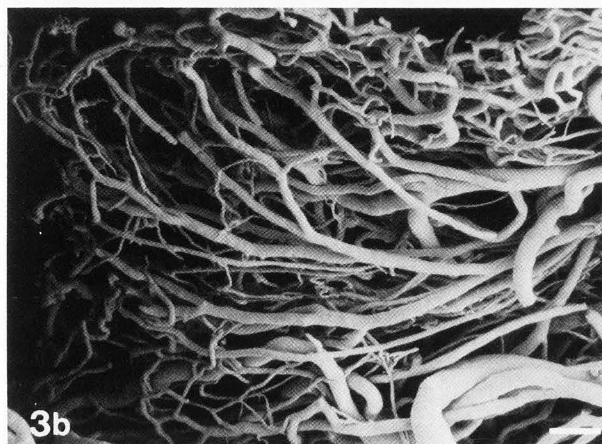


Fig. 3a, b: Corrosion cast specimens of a melanoma. Peripheral vessels derived from subcutaneous vessels with nearly normal branching modes form the "tumor vascular envelope" as described by Grunt et al. (1986a). v = main draining vein. Bars = 100 µm

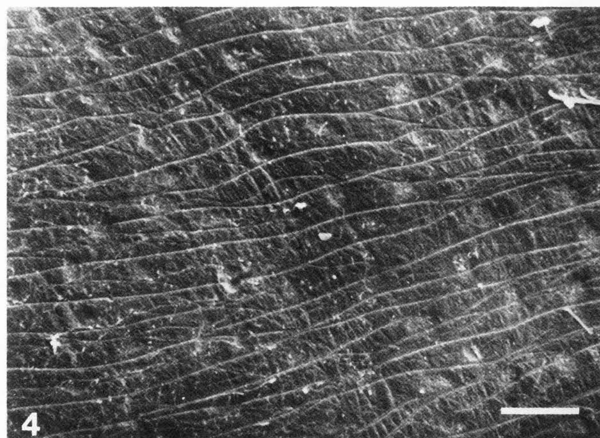


Fig. 4: Impressions of endothelial cell borders and nuclei in the main draining vein. Magnification of fig. 3a. Bar = 30 µm

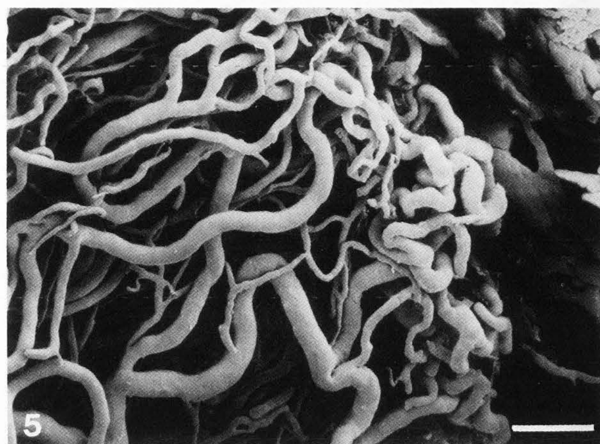


Fig. 5: Tortuous courses of vessels approaching the tumor (soft tissue sarcoma). Bar = 100 µm

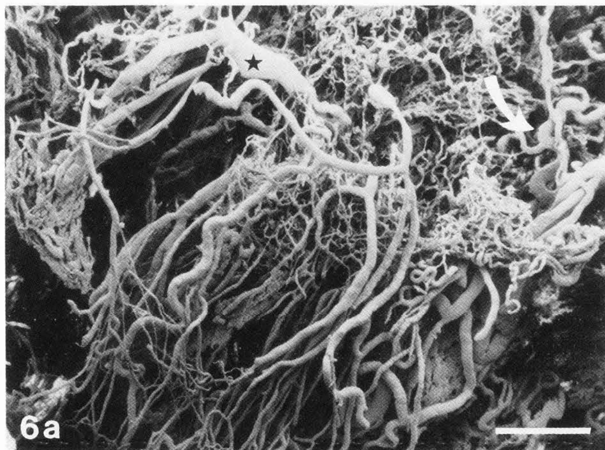
layers are neither to be seen in the periphery nor in the center. Even the largest peripheral vessels with diameters of 50-150 µm consist mainly of an endothelial layer and some perivascular connective tissue only (fig. 2a and b).

Vascular corrosion casts display the course of these vessels: Adjacent to the xenograft they follow normal courses and branching modes (fig. 3a and b), thus partially forming a "vascular envelope" as described by Grunt et al. (1986 a). The luminal surfaces of these vessels show typical endothelial cell impressions (fig. 4). Frequently these vessels take a tortuous course when reaching the tumor tissue (fig. 5), where features such as glomeruloidal arrangement or widening and dilatations -already excellently described by Grunt and coworkers (1986 a, b)-are visible.

In the tumor itself the vascular system has a much more chaotic arrangement. Very often regions with clusters of vessels could be seen just beneath areas almost free of vessels (fig. 6a and b). Both in sarcomas and in melanomas these vessels form a sinusoidal system with numerous blind ends without clearly discernible endothelial cell impressions (fig. 7a and b). The diameters vary between 5 and 50 µm. Thus, they clearly differ from vessels in normal tissues.

Freeze-broken melanomas depict the typically occurring vessels in more detail. Besides endothelial-lined vessels numerous irregular, tumor cell-lined sinusoids corresponding to the casted network of sinuses are visible (fig. 8). Often the endothelium seems to be incomplete and the diffusion distance to the tumor cells shortened (fig. 9). The amount of vessels without or with incomplete endothelium seems to be much higher than the number of structurally real (giant) capillaries. A considerable number of leucocytes and lymphoycytes were found both in the sinusoids and interstitial spaces of necrotic and viable areas (fig. 10).

These different types of vessels also occur in sarcomas (fig. 11 and 12). However, at a first



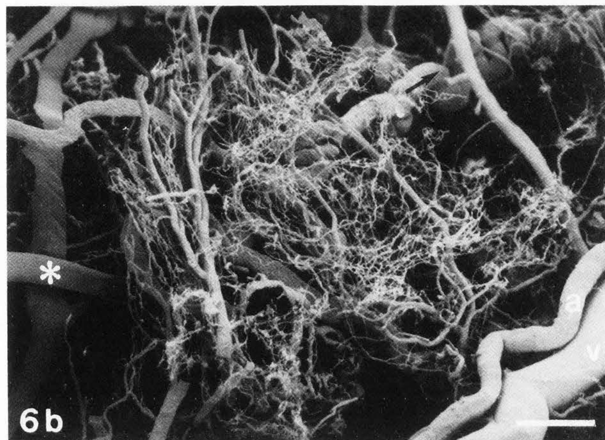
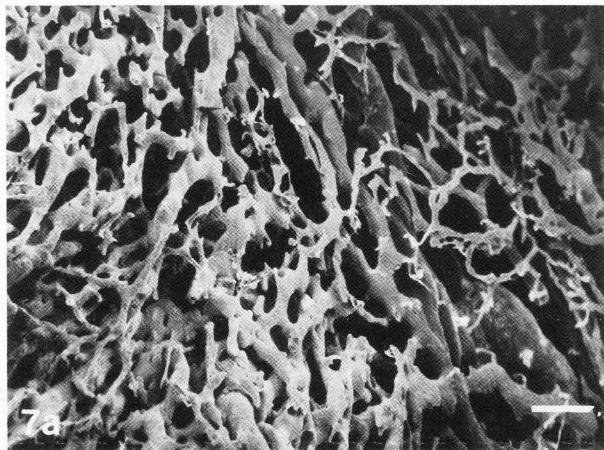


Fig. 6a, b: Corrosion cast specimens demonstrating the heterogeneity in vascular distribution (amelanotic melanoma). Both micrographs were taken from peripheral regions. Note the differences in vascular densities, the absence of vascular hierarchy, tortuous courses (arrow) and flattening of main vessels (*). a = artery, v = vein. Bars = 300 µm



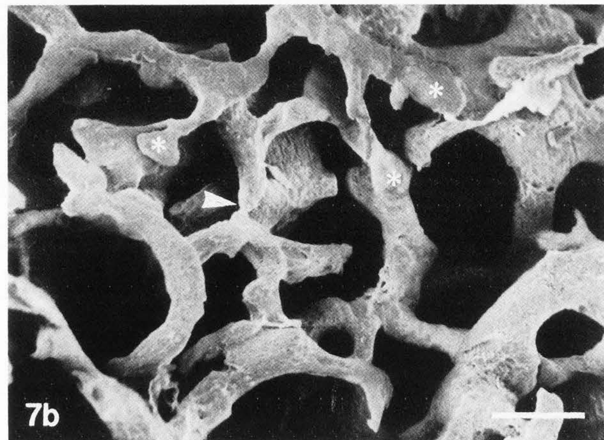


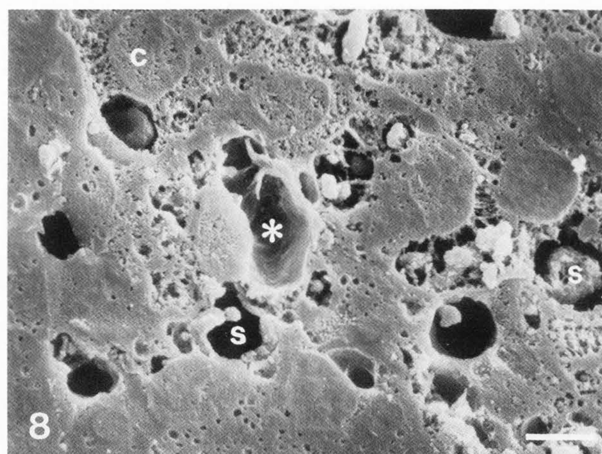
Fig. 7a, b : Vascular corrosion cast of the sinusoidal system in a sarcoma. Note the irregular density and polymorphy. Numerous blind ends (*) and changes in diameter (arrow) are typical features. No endothelial cell imprints are visible. Bar in a = 100 µm, bar in b = 30 µm

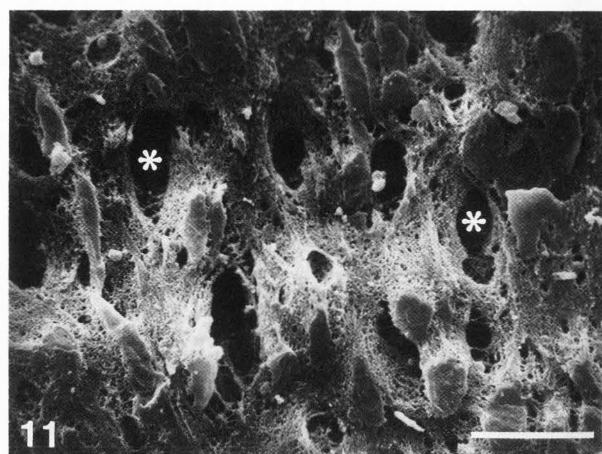
glance there was no evidence for a possible correlation of areas with different cell packing densities in the single xenografts to the vascular densities. However, systematic morphometric investigations are still outstanding.

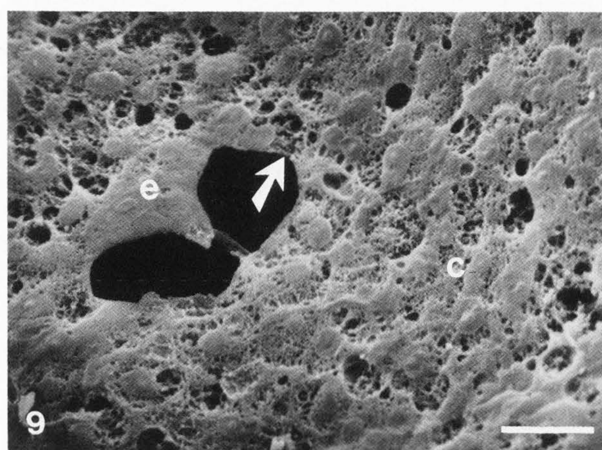
Quantitative measurements from 10 India ink-injected sarcomas suggested that there is no zonal, topographic arrangement of the vascular densities (table 1). Comparisons of the vascular densities in the centers of tumors with the densities in the periphery do not generally support the idea of a better vascularization in the periphery but, rather, an extreme heterogeneity in tumor vessel distribution. The great variation in vascularization in different areas of single tumors parallels the large differences in the average vascular densities in different tumors of the same entities. Thus, this is contrary to the existence of a tumor-specific or tumor-characterizing vascular density.

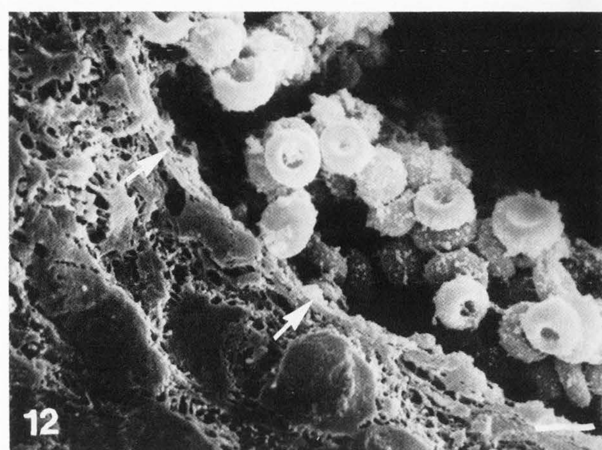
Discussion

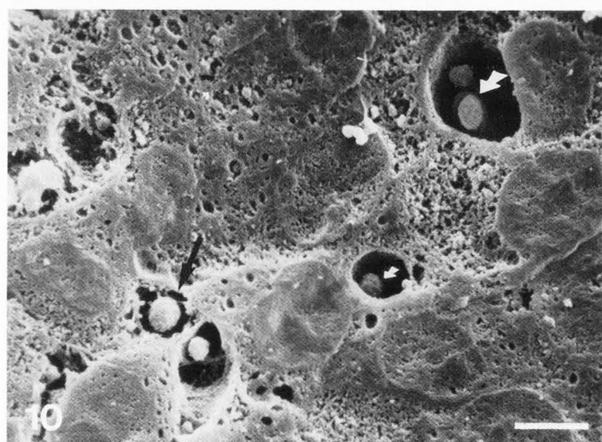
This study was designed to examine the vascular system of xenotransplanted human melanomas and sarcomas. Nearly all concepts underlying the effects of different therapies stress the important role of tumor vascularization and great efforts have been made to define such parameters for predicting tumor growth and curability (Streffer et al., 1986; Peters et al., 1986). The importance of the vascular system in tumor therapy was suggested as early as the last century (Gassmann, 1899). Further studies on the vascular system are necessary despite a sizeable body of literature concerning this problem because most studies have been carried out on a few experimental tumors. Fewer than 30 papers examining the ultrastructure of tumor vessels have been published during the last four years. Systematic studies of the vascular system in primary tumors, which are charac-











Tumor		mean	Peri. 1	Centr.	Peri. 2
Sarcoma	I/1	250	201	209	215
"	I/2	121	73	25	98
"	I/3	167	53	80	12
"	II/1	501	22	750	380
"	II/2	60	-	33	45
"	III/1	100	76	117	58
"	III/2	94	100	107	33
"	IV/1	694	812	926	315
"	IV/2	107	193	56	45
"	IV/3	574	615	413	436

Table 1

terized by heterogeneous growth rates, malignancies and therapy responses (Scherer, 1986) are lacking completely (Rubin 1985; Jain, 1988). The xenotransplanted tumor on nude mice as favoured by many authors shows many parallelisms to the human situation.

This is clear from the growth rates of such xenotransplanted tumors. The data in graph 1 show that tissue from the same tumor shows completely different proliferation rates under similar experimental conditions. Thus, the tumor volume doubling times and comparisons of the vascular densities already indicate an extreme degree of heterogeneity.

This is in agreement with results of some physiological experiments: This heterogeneity even exists in different areas of an individual tumor. Vaupel (1979) and Vaupel et al.(1981) have demonstrated the heterogeneity in pO_2 distribution and Endrich et al. (1982) have shown vari-

Fig. 8: Freeze-broken melanoma with endothelial-lined vessel (*) and numerous tumor cell lined sinusoids (s). c = tumor cells. Bar = 5 µm

Fig. 9: Freeze-broken melanoma showing vessel with presumably incomplete endothelium (arrow). The diffusion distance to the tumor cells seems to be shortened. e = endothelial cell, c = tumor cells. Bar = 20 µm

Fig. 10: Amelanotic melanoma depicting numerous leucocytes (arrows), resp., lymphocytes in the sinusoids and interstitial spaces. Bar = 10 µm

Fig. 11: Freeze-broken sarcoma with loosely packed cells and large sinusoids (*) without endothelial lining. Compare with fig. 7a and b. Bar = 20 µm

Fig. 12: Vessel of a freeze-broken sarcoma. Arrows indicate sparse endothelium with little perivascular connective tissue. Bar = 5 µm

Table 1: Vascular densities of 10 sarcomas with mean values and densities in the centers and two contralateral peripheral regions of each sarcoma. Areas measured were 1.92 mm^2 in size. The mean values were obtained by dividing all tumor vessels by the number of areas, which were determined by the tumor size. Same Roman numerals = same tumor cell line.

ations in the distribution of ischemic areas in tumors. On comparing central and peripheral vascular densities, it becomes evident that the vascular density is not the only determining factor in the development of necroses. Even so, "classical" central necroses have occasionally been seen by us although they cannot be described from our experiments as typical features as suggested by Grunt and coworkers (1986 a). In a previous study with a greater collection of sarcomas on nude mice (Steinberg et al., unpublished data) it could be shown that there is no correlation of necrosis to tumor vascularisation.

The heterogeneity of the vascular distribution by morphometric measurements was also clearly visible in the corrosion cast specimens (fig. 6-9). In the periphery they partially depicted many parallelisms to the "tumor vascular envelope" described by Grunt and co-workers (1986 a). In this connection, it would be interesting to distinguish the possible influence of wound healing processes on the peripheral tumor vascularisation and vice versa as well as on tumor growth. Our results showing no real arteries and veins in the tumor are contrary to those of Warren (1979 a) but, instead, confirm those of Grunt et al. (1986 a) and Walmsley et al. (1987). We believe that the arteries seen by Warren (1979 a) might represent incorporated, preformed vessels from the host animals.

The morphology of the vessels found in our scanning electron microscopic studies generally confirms the results of other authors (Grunt et al, 1986 a, b; Walmsley et al., 1987). This is in agreement with many features described by Hammersen et al. (1983) using transmission electron microscopy, who have found that all vessels of amelanotic melanomas are structurally similar to capillaries showing characteristic gaps in their flat endothelium. In addition, they have found numerous lacunary vessels the walls of which are partially formed by tumor cells. Our own recent studies on the ultrastructure of these vessels (Konerding et al., unpublished data) have also shown that the sinusoids described in this study using scanning electron microscopy are frequently lined by the tumor cells themselves. Fenestrations were very seldomly seen in completely endothelially lined vessels and the basal laminae were rarely developed.

There are several indications that the vascular morphology is characteristic of an individual tumor: Solesvik et al. (1982) have reported significant differences in the vascular structure characterising the respective tumor in five different melanomas in nude mice. Microangiographic examinations of different human tumors on nude mice showed a good correlation of the radiological with the histopathological findings (Kraus et al., 1983). In their review article concerning the evidence for and against a tumor specific vascularity, Vaupel et al. (1986) came to the conclusion that the histological type can modulate, but not dictate the vascular pattern. In addition, they established that up to now it is not possible to identify a tumor from the vascular structure or function.

Blood flow in normal tissues is regulated, among others, by sphincters or contractile cells. There is evidence that, at least conditionally, this also applies to tumor blood flow (Jain, 1988). From physiological experiments both the existence and lack of such regulatory means in human and experimental tumors have been postulated (Peterson, 1983; Appelgren, 1979). Abnormalities in the vascular morphology lead to abnormalities in the functional microcirculation and vice versa (Vaupel, 1979, Vaupel et al., 1987). This is important both for the pathogenesis of tumor hypoxia and for therapeutic measures. Hyperthermia, for instance, initially induces an increase in blood flow and then leads to vascular collapse (Song et al., 1980; Mäntylä et al., 1982). The results of our study cannot explain the mechanisms and further studies need to be carried out in order to do so.

Little is known about the morphological aspects of tumor angiogenesis (Warren, 1979b), although the discovery of the tumor angiogenesis factor (Folkman et al., 1983; Crum et al., 1985) has attracted great interest in this matter. We have not made an attempt to classify the typical features of angiogenesis in this study since this has already been done very carefully by Grunt et al. (1986 b). Moreover, a study of this aspect seems to be more problematic after transplantation of solid tumor slices (see materials and methods) rather than after injection of cells.

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Discussion with Reviewers

J.G. Walmsley: Would the tumors used for this study grow in the mouse which was not immune-deficient? If so, what would you suspect would be the difference in the rate of tumor growth and the type of vascularization?

Authors: Prior to the introduction of the nude mouse model by Flanagan (1966), the experience of numerous groups has shown that it is extremely difficult to transfer human tumor material to non-immune deficient animals. Immune reactions in general prevent the growth of human tumor cells after transplantation.

A. Lametschwandtner: Did the authors try to do any correlation of the vascularity of the tumors with their passage numbers, i.e. might there be a change in growth behavior during passaging?

Authors: The aim of this study was primarily to study the tumor vascular system qualitatively. However, we are aware from our own as yet unpublished results that considerable changes in the parameters measured here such as proliferation behavior, histopathological classification, DNA content, number of micronuclei and growth rate can occur during the course of passaging. According to the studies by Donhuiysen et al. (1988), the degree of differentiation and malignancy can change in both directions. It is therefore likely that the function and morphology of the vascular system will also change in the individual passages. Up to now we have not attempted to find a direct correlation between vascular density and tumor passage number because the known intra- and interindividual heterogeneity will additionally complicate this question.

J.G. Walmsley: Could the necrosis you describe be the result of poor metabolite distribution as a result of afunctional sinuses rather than functioning blood vessels in the center of tumors? Is it possible that the sinuses that you have observed or the India ink filled regions at the center may be totally afunctional?

Authors: The development of necroses is almost certainly a multifactorial process, in which the oxygen supply (Vaupel et al., 1981, 1987), the nutritive supply (Müller-Klieser, 1987, personal communication) through the vascular system (Tannock, 1970; Gabbert et al., 1983) and the effect of specific, necrosis-inducing factors such as the tumor necrosis factor (Old, 1985) play a major role.

From our present morphological investigations we are unable to assess to what extent the described sinusoids are involved in perfusion. Based on physiological studies, Vaupel et al. (1987) have however pointed out that both the

flow rate as well as the overall perfusion of the sinusoids is small and which can lead to stasis and reverse shunts.

J.G. Walmsley: When the animals or tumors were being macerated was there any tendency for the finer vessels or sinuses to fall off the cast?
Authors: Particularly in the case of larger tumors with volumes greater than 1.5 mml, vascular breakages and loss of individual segments were seen due to the lack of hierarchy of the vascular system and the various vascular densities as well as varying diameters. This artifactual problem was taken into consideration in the evaluation of corrosion cast specimens by a thorough search for breakages particularly in the vicinity of avascular regions. In this case, it is possible to differentiate between artificial and de facto intravital avascular regions with sufficient accuracy provided there is a good filling.

J.G. Walmsley: Were any precautions necessary to prevent the loss of cast segments through the use of detergents to reduce surface tension or with a special rinsing technique?
Authors: At the beginning of these studies we realised that the maceration had to be carried out much more carefully because of its own low stability than with corrosion cast specimens of normal vessels. However, the use of detergents to lower the surface tension was dispensed with because the specimens were not air-dried but freeze-dried instead. To prevent vessel breakages during the washings we have, as suggested by H. Ditrich (personal communication, 1987), tried to avoid turbulence as far as possible by introducing several mesh filters with slow flow. The higher quality of the casts justifies the longer time involved.

J.G. Walmsley: Is it not possible to quantify the vascular densities using the freeze-broken tumor tissue?
Authors: This should be quite possible with smaller tumors and adequate preparation. We have, however, favored ink-injection for the described quantification since the vascular densities even in larger specimens could be determined much more quickly by this means. Furthermore, in our opinion it is not permissible and representative to study only small areas in view of the given heterogeneity of the vascular distribution in individual tumors.

J.G. Walmsley: With respect to fig. 4.: How were you able to get impressions of the cell borders? This is typically only achieved by the use of silver staining.
Authors: We assume that this usually not so clearly expressed phenomenon arises in dependence on fixation. We have observed this more often when using very fast (intravital) acting fixation and more rapid injection of the casting medium than with unfixed specimens (Käs et al., 1987).

A. Lametschwandtner: From figs. 2a and b a circular arrangement of endothelium becomes evident. Interestingly in fig. 4 endothelial cell borders are orientated parallel to the longitudinal axis of the vessel. Please comment on that.
Authors: The longitudinal orientation of the endothelia as shown in fig. 4 should more closely reflect the intravital situation. There are reasons to believe that the circular form shown in fig. 2 is due to an artifact of fixation. From our experience (Konerding et al., 1988) in vessel replicas which have circular constrictions there are no endothelial impressions whose axis is orientated vertically to the vessel. This has also been reported by Motti et al. (1987).

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