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THE CHARACTERISTIC STRUCTURAL FEATURES OF THE BLOOD VESSELS OF THE LEWIS LUNG CARCINOMA
(A Light Microscopic and Scanning Electron Microscopic Study)

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Abstract

Vascular corrosion casts of Lewis lung carcinomas (LLC) grown subcutaneously in C57BL/6-mice are correlated with histological sections and with tumor tissue prepared for scanning electron microscopy (SEM). By making low, medium and high pressure cast preparations we studied the influence of perfusion and injection pressure on the resulting cast sample.

Three types of vascular proliferations are distinguishable in LLC: 1) Small globular outgrowths on sinusoidal dilated tumor capillaries, caused by proliferation of their endothelial cells. 2) New sprouts on surrounding host vessels, invading the small, still avascular implant. 3) Superficially located, centrifugally running sprouts in peripheral regions of large tumors. They invade the surrounding host tissue.

Vascular sprouts are of venous origin, have a fragmentary endothelium and are rather "leaky" if casted.

High pressure preparations of large tumors reveal central avascular cavities surrounded by centripetally running, compressed and blind ending tumor vessels.

Irrespective of the applied injection pressure, the casts always exhibit extravasal channels caused by degeneration of the endothelium of central tumor vessels.

We show that SEM of vascular corrosion casts combined with histology not only demonstrates such contrary processes as the development of tumor blood vessels and the simultaneously occurring vascular degeneration, but also elucidates all other morphological characteristics of the tumor vascular system.

Key words: Angiogenesis, vasoproliferation, vascular degeneration, vascular compression, lacunas, vascular corrosion casts, vasodilation, capillary sprouts
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Introduction

Following a previous study on the gross three-dimensional organisation of the vascular bed of the Lewis lung carcinoma (LLC) (26) we now present a detailed morphological analysis of the characteristic structural features of the tumor blood vessels.

The fact that this rapidly growing murine tumor regularly spreads into the blood circulatory system, which then causes pulmonary metastases (33-36), indicates that the structure of the tumor blood vessels is decisive - among other factors - for the lethal course of this disease.

It is well known that tumors are able to produce substances, which cause the adjacent host blood vessels to form new growing sprouts (17, 19, 45). Folkman et al. (1971) (15) were the first to isolate an angiogenic fraction from malignant cells. According to them this tumor angiogenesis factor (TAF) is synthetized by and secreted from the tumor cells. For expansion most tumors need additional blood vessels. Therefore during tumor treatment the tumor growth may be blocked by inhibition of the angiogenesis (30, 38, 42, 18). In tumor diagnosis, on the other hand, angiogenesis may serve as an early marker for a malignant transformation (3).

Simultaneously, however, enlarging tumors frequently suffer from blood vascular degeneration, which causes wide-spread necrosis of the central tumor tissue. The simultaneous occurrence of such opposite processes is a very remarkable characteristic of tumor vascular systems and results in the spatial distribution of the viable tumor tissue being similar to that of the functioning vascular bed.

Summarizing our present knowledge, the tumor vascular system is important not only for the tumor metabolism and tumor growth but also for tumor diagnosis and tumor therapy.

The aim of this study is: 1) to clarify which criteria must be taken into account in interpreting the casted structures, and 2) to demonstrate the potentials this method opens to visualize and classify angiogenesis and vascular degeneration more precisely and even at the microvascular level. Special attention will be

Table 1: Some relevant rheological data for low, medium and high pressure preparations.

	tyrode rinsing		resin injection		
preparation	hydrostatic pressure (mmHg)	duration (min)	influx-rate (ml/min)	duration (min)	total amount of resin (ml)
low pressure	37 - 60	5	0.2	12	2.4
medium pressure	60 - 80	10	0.5	11	5.5
high pressure	95 - 111	15	0.85	10	8.5

given to distinguish clearly between structures caused by vasoproliferation and those caused by vessel regression.

Materials and Methods

The vascularization of LLC was studied in 53 C57BL/6-mice. A suspension containing 2.5 - 5 x 10^5 tumor cells was injected subcutaneously (s.c.) into the axillary region of the animals.

Most tumors were prepared for vascular corrosion casts. A few remaining tumors, however, were used for correlative histological studies, which were done by scanning electron microscopy (SEM) of critical-point-dried (CPD) tumor tissue blocks or by light microscopy (LM) of tissue sections. Animals used for these studies were fixed either by perfusion and immersion or merely by immersion in the fixative. Cast preparations and histological preparations were made from tumors of different growth stages. According to the applied perfusion pressure and rates of resin-influx - three distinct types of cast preparations could be distinguished (table 1).

Further details of the methods used for tumor transplantation, for vascular casting and for preparing tumor tissue blocks for SEM or for LM are described elsewhere (26).

Results

The blood vessels of LLCs exhibit 4 characteristic features: 1) dilated blood vessels with globular outpouchings, 2) blind ending blood vessels, 3) blood channels and lacunas, and 4) extravasal structures.

Blood vessels with globular outpouchings and blind ending blood vessels were studied preferentially on high pressure cast preparations.

Globular outpouchings

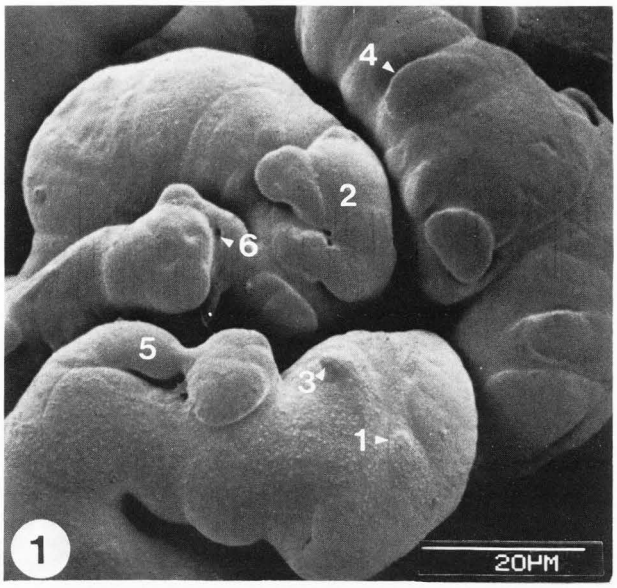
Casts of sinusoidal dilated basal tumor vessels show many globular outpouchings (23 - 25). Originally we thought that these outpouchings are formed by random bulging of the vessel walls. Later on, however, systematic studies allowed us to classify these structures into distinct categories, which could be combined by several ways of development. Thus each structure represents a particular stage of a dynamic process, which is characterized by a multiple anastomosing of two or more outgrowing pouches and by a special type of vasodilation. While conventional, reactive distension of normal blood vessels occurs solely by neuromuscular reflexes, this type of "active" vasodilation is managed - similar to the forming of new anastomoses - by endothelial proliferation and vascular fusion. This "active" vessel widening is explained by an extensive fusion between the parent vessel and a closely neighbouring approximated sprout. The formerly separate sprout thus becomes incorporated into the distended lumen. These processes occur during the entire period of tumor growth, and they represent the first vascular reactions induced by a tumor implant. Thus they establish the 1st phase of tumor angiogenesis.

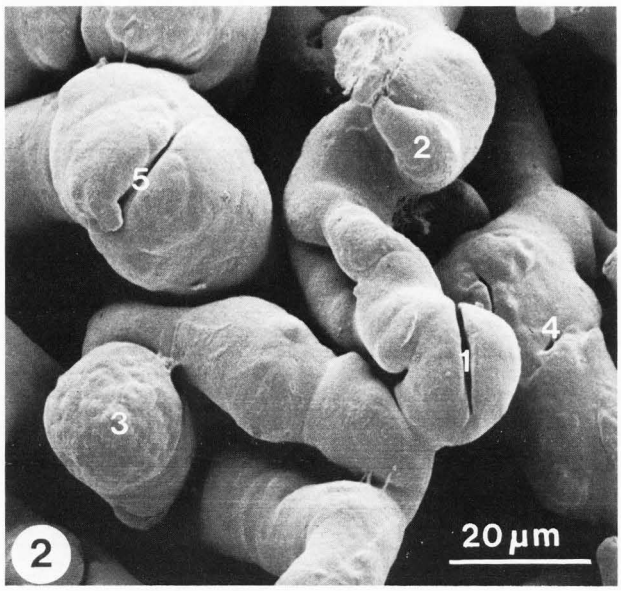
Detailed examinations on vascular corrosion casts reveal that in one case the tumor vessel first forms a small and inconspicuous furrow at its surface (Fig. 1, No. 1 and diagram 1a). Abbreviations for figs. 1-22: VCC ... vascular corrosion cast, HS ... histological section, ST ... section thickness, HE ... hematoxylin-eosin, LM ... light microscopy, SEM ... scanning electron microscopy, CPD ... critical-point-dried tumor tissue Then a semicircular or incompletely circular furrow develops by irregular growth of the bulge and separates a small flap from its parent vessel (Fig. 2, No. 1 and diagram 1b). This flap then continues to enlarge (Fig. 1, No. 2 and diagram 1c).

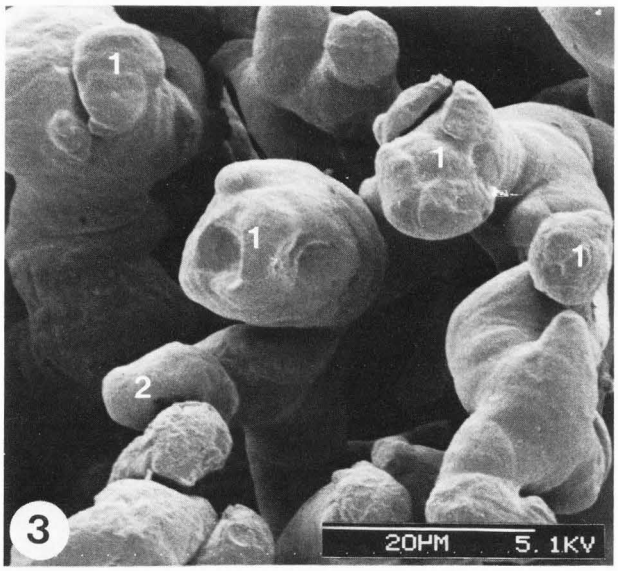
In another case the vasoproliferation first starts with the development of a small and pointed elevation at the vascular surface (Fig. 1, No. 3 and diagram 1d). Thereafter this structure elongates (Fig. 2, No. 2 and diagram 1e). and forms a blind ending vascular sprout with a distinct endothelial cell border line at its surface (diagram 1f).

In a third situation a small groove separates two slight bulges (Fig. 1, No. 4 and diagram 1g), which continue to extend to form dome-shaped, fungiform or pedunculate vascular protuberances (Fig. 3, No. 2 and Fig. 2, No. 3 and diagram 1h and 1i). During further growth the terminal globular swellings diminish (Fig. 3, No. 3 and Fig. 4, No. 2 and diagram 1j) and frequently secondary sprouts develop - sometimes even before the (primary) parent sprout is canalized by fusion with another vessel (diagram 1j).

The different types of evolution of tumor blood vessels presented here is only a simplified description of a very dynamic and complex process. Transition from one model course of vascular development into another one is likely







Figs. 1 - 3: Vascular proliferations on dilated tumor vessels at the tumor base (1st phase of tumor angiogenesis).
Fig. 1: 1 ... small furrow, 2 ... large flap, 3 ... small, tapered bulge, 4 ... small furrow between two slight bulges, 5 ... bow-like collateral, 6 ... small hole, VCC. SEM.
Fig. 2: 1 ... deep, incomplete circular furrow separating a small flap, 2 ... slightly curved, tapered structure, 3 ... dome-shaped to fungiform bulge, 4 ... small hole, 5 ... deep furrow, VCC. SEM.
Fig. 3: 1 ... dome-shaped to fungiform bulges, 2 ... capillary sprout without terminal bulge, VCC. SEM.

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to occur.

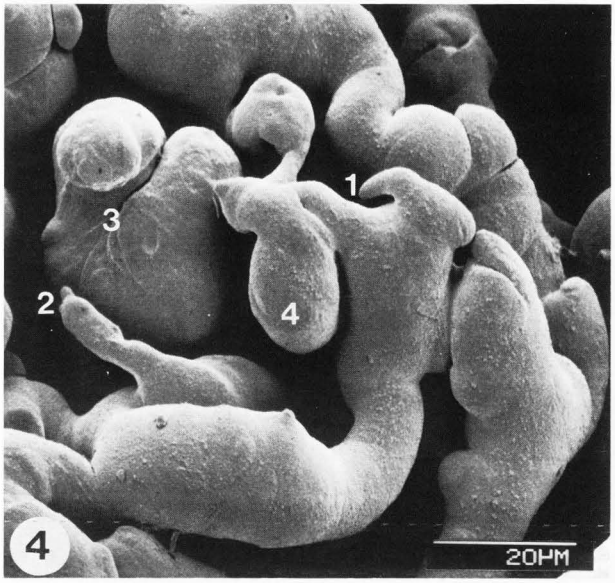
By studying the spatial relation between the individual vascular structures one may trace the different stages and variants of the formation of anastomoses. Two or more vascular bulges, for example, may lean towards each other (Fig. 5, No. 1 and diagram 1k) to fuse at a later time. The sprouts often contact other vessels or sprouts of other vessels and thus form real anastomoses. Some sprouts, however, fuse with their parent vessels thus giving way to collateral shunting (Fig. 1, No. 5 and diagram 1l). If the collateral branch takes a diameter similar to that of the parent vessel, a vascular circle is formed, which encloses a tiny central cavity (Fig. 5, No. 2). Subsequent reduction of this central space (Fig. 1, No. 6 and Fig. 2, No. 4) transforms the vascular circle into an excessive dilated sinusoid, which has lost the central hole. Preceding a fusion bulges of a vessel frequently approximate and form a narrow cleft between each other (Fig. 2, No. 5 and Fig. 4, No. 3).

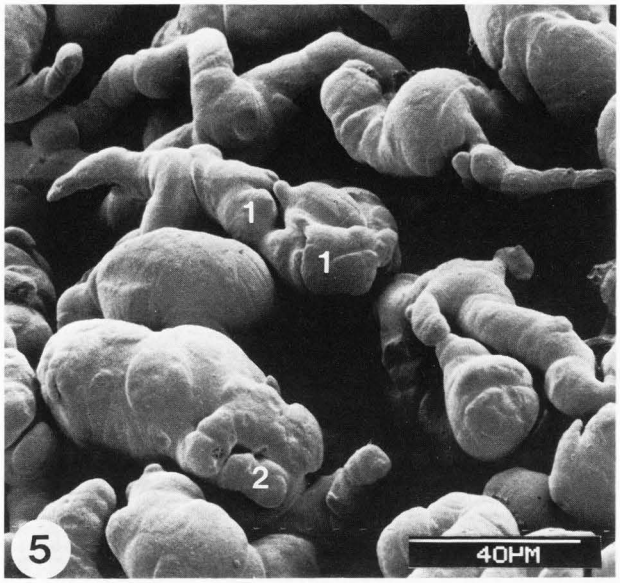
Several reports describe globular outpouchings at the surface of casts of tumor blood vessels (8, 23, 25, 27, 28, 44, 46, 55, 57-59). However, there is no study - to our knowledge - dealing in detail with their development and sprouting.

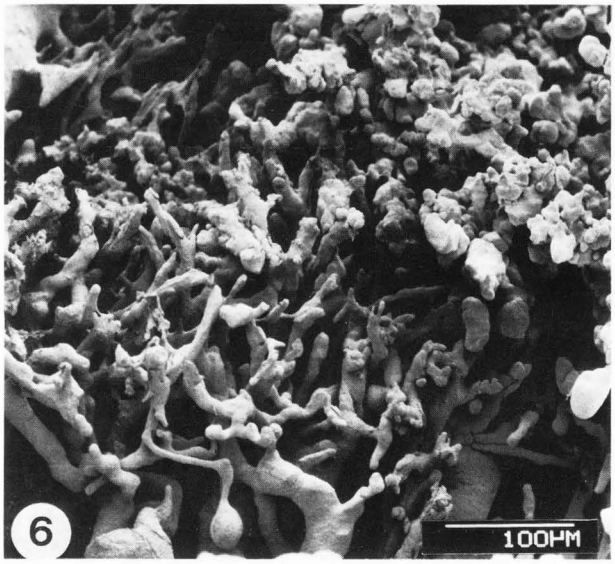
Blind ending blood vessels

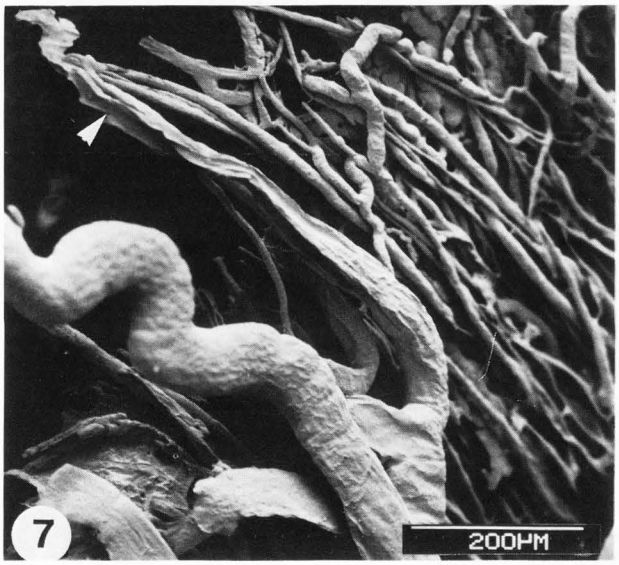
At the third or fourth day after tumor transplantation blind ending vascular sprouts emerge from surrounding venous host vessels. They cover a central avascular space, which represents the small avascular implant (Fig. 6). By subsequent elongation the centripetally arranged sprouts grow radially into the center of the central cavity. They form an initial, venous microcirculation and thus establish the second phase of tumor angiogenesis.

At about the 8th day of growth the tumor vascular system takes the shape of a hollow sphere with a central cavity and a peripheral basket-like plexus (26). Simultaneously the peripheral envelope vessels start to form centrifugally running vascular sprouts, which









Figs. 4 - 5: Vascular proliferations on dilated tumor vessels at the tumor base (1st phase of tumor angiogenesis).
Fig. 4: 1 ... slightly curved, tapered structure, 2 ... capillary sprout without terminal bulge, 3 ... narrow cleft, 4 ... endothelial nuclear imprint, VCC. SEM.
Fig. 5: 1 ... complex arrangement of several bulges, 2 ... ring-like anastomose, VCC. SEM.
Fig. 6: A 4 day old tumor. Centripetally running vascular sprouts emerge from surrounding venous host vessels and vascularize the implant (2nd phase of tumor angiogenesis). Note the resin leakage. VCC. SEM.
Fig. 7: Apical tumor periphery. Venous vessels of the "tumor vascular envelope" with a wrinkled, concave, surface (arrowhead). VCC. SEM.

grow out of the tumor and invade the surrounding host tissue. Similarly to the centripetally (radially) arranged vascular growings these sprouts end with rounded tips or pass into terminal extravasations. In contrast to the initial tumor vessels, however, the centrifugal sprouting is summarized as third phase of tumor angiogenesis.

The occurrence of blind ending tumor vessels is a common feature in vascular corrosion casts of malignant tissues. They are not confined to any special tumor region. However, according to the underlying mechanisms causing such structures, they may be classified into two separate groups. While blind ending sprouts are produced by endothelial proliferation, flattened and tapering casts of various size represent

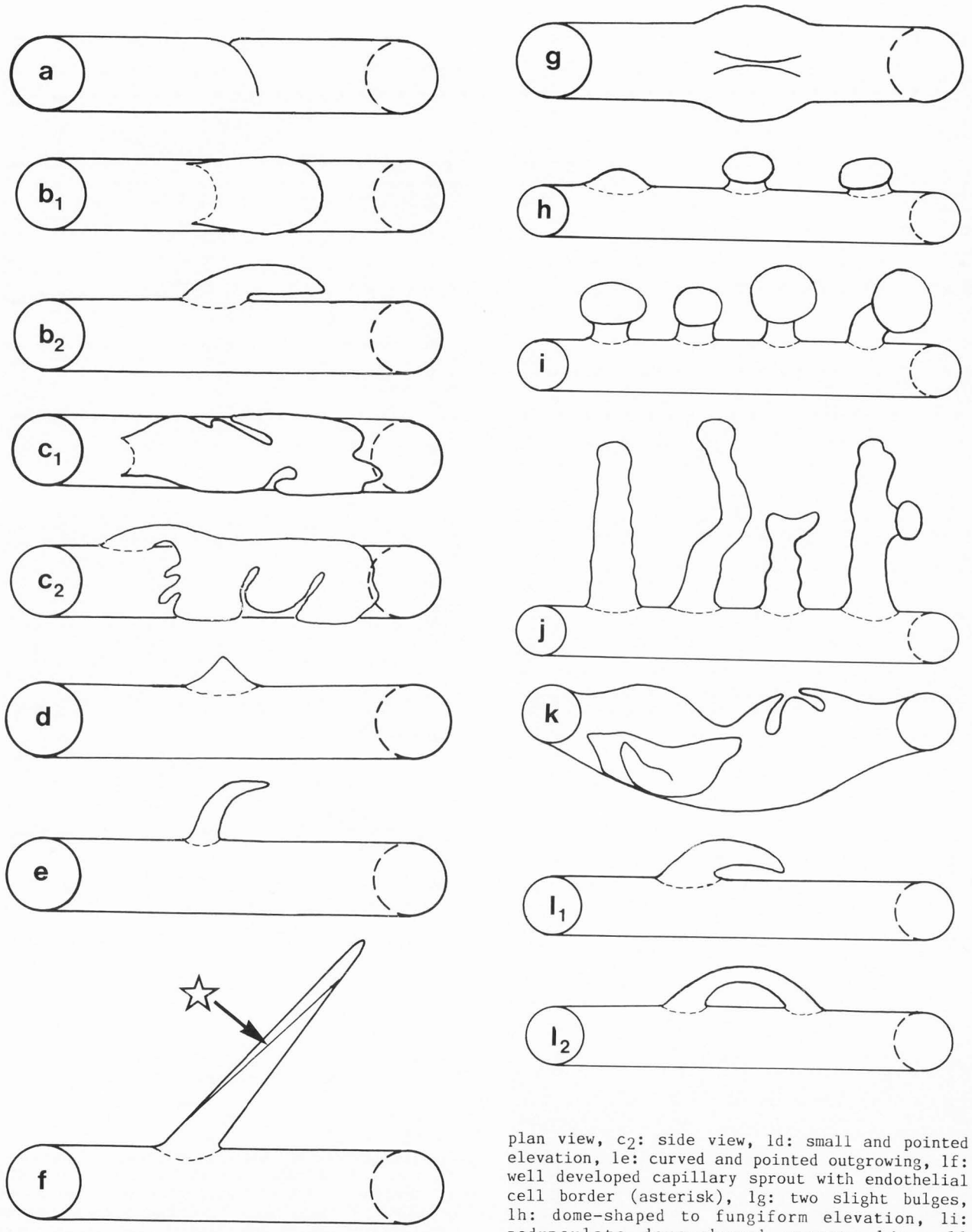
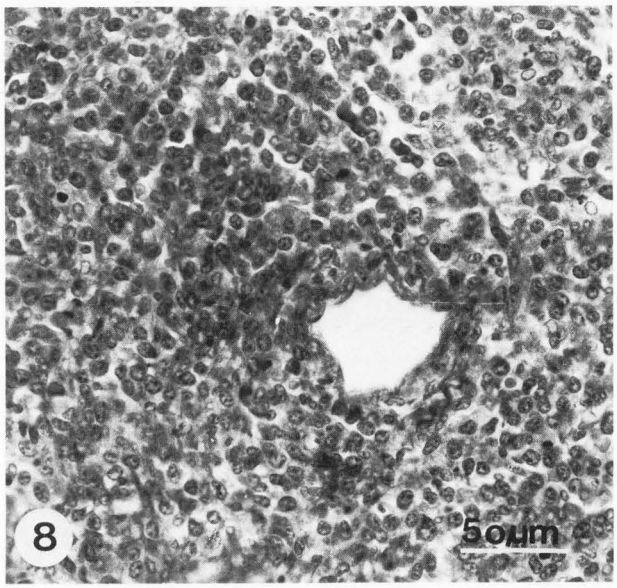
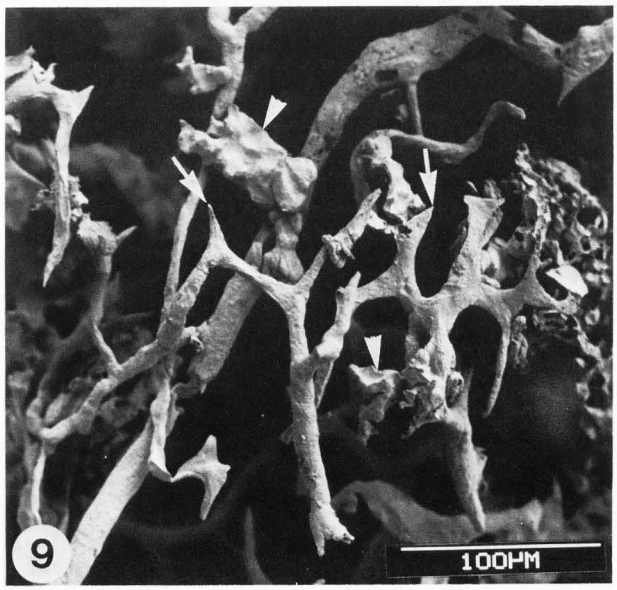


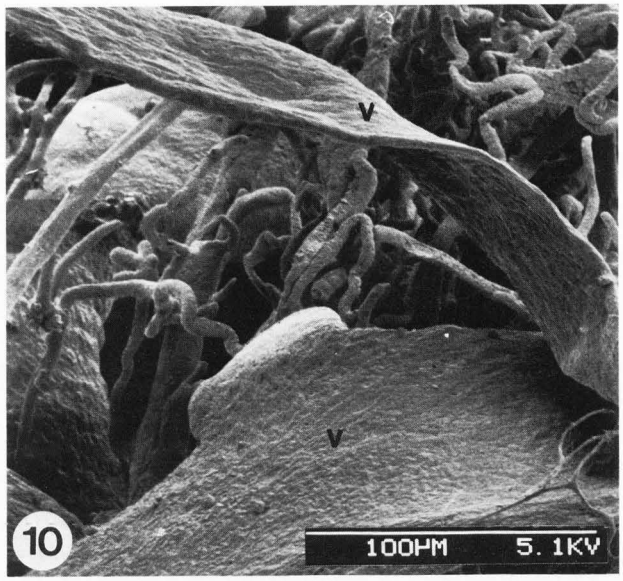
DIAGRAM 1: Several different possibilities of the development of capillary sprouts on dilated basal tumor vessels: 1a: small furrow, 1b: small flap, b_1: plan view, b_2: side view, 1c: large flap, c_1: plan view, c_2: side view, 1d: small and pointed elevation, 1e: curved and pointed outgrowing, 1f: well developed capillary sprout with endothelial cell border (asterisk), 1g: two slight bulges, 1h: dome-shaped to fungiform elevation, 1i: pedunculate dome-shaped sprout, 1j: well developed capillary sprout; frequently with a secondary sprout as shown in diagram 1h or 1i, 1k: complex arrangement of several sprouts, 1l: formation of collaterals above l_1: before the fusion, below l_2: after the fusion.

compressed vessels.

By continued growth of the tumor cell cords the tissue pressure increases and causes multiple occlusion of the tumor blood vessels. Most casts of such vessels exhibit a concave or wrinkled surface (Fig. 7), which is easily correlated with delta-shaped figures in histological sections (Fig. 8). Small, tapered tumor capillaries of the irregular plexus at the interior zone of the basket-like vascular envelope, (Fig. 9) and muscle capillaries incorporated near the tumor margin, are not the only vessels which are affected by such narrowing forces. Even large arteries and veins become flattened and clamped.







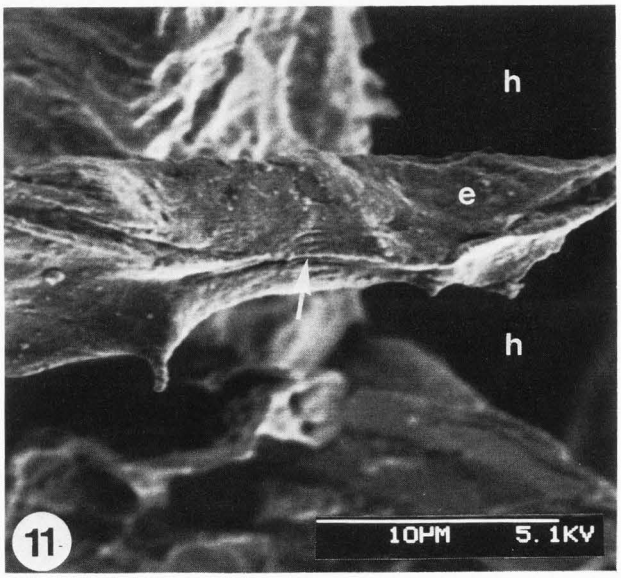
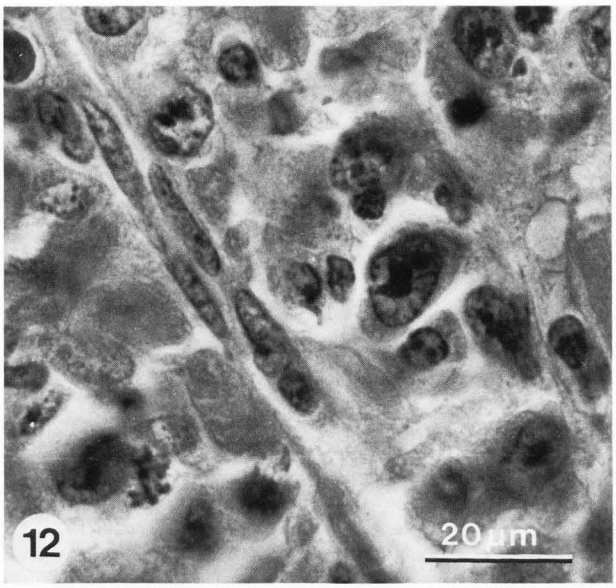


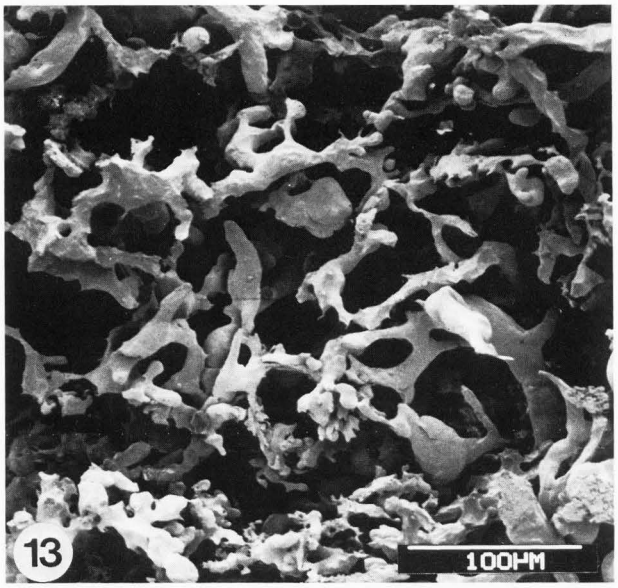
Fig. 8: Blood vessel with "delta-shaped" cross-section surrounded by the tumor tissue. HS. ST 7 µm. AZAN. LM.

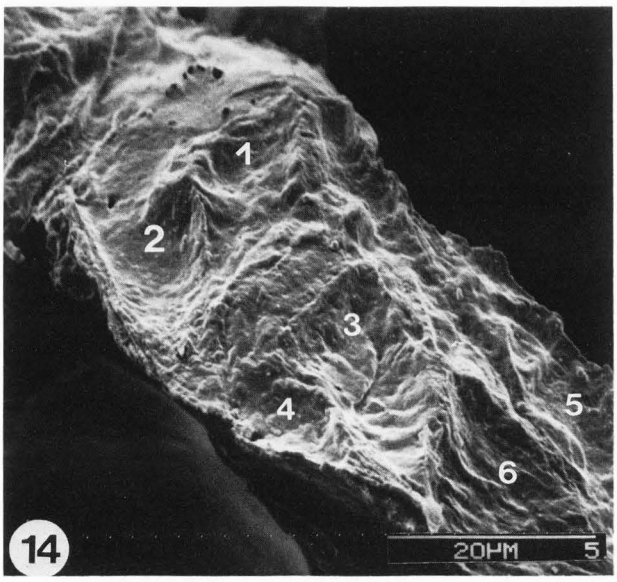
Fig. 9: Near the central, avascular cavity. Irregular formed plexus with tapered vessels (arrows) and with extravasal structures (arrowheads). VCC. SEM.

Fig. 10: Tumor base. Extremely flattened, venous vessel (v). VCC. SEM.

Fig. 11: A tapered tumor vessel projects into the central, avascular cavity (h). Note: the endothelial cell nuclear imprint (e) at the end of the tapering vessel. A longitudinal furrow (arrow) represents the border line between two adjacent endothelial cells. VCC. SEM.







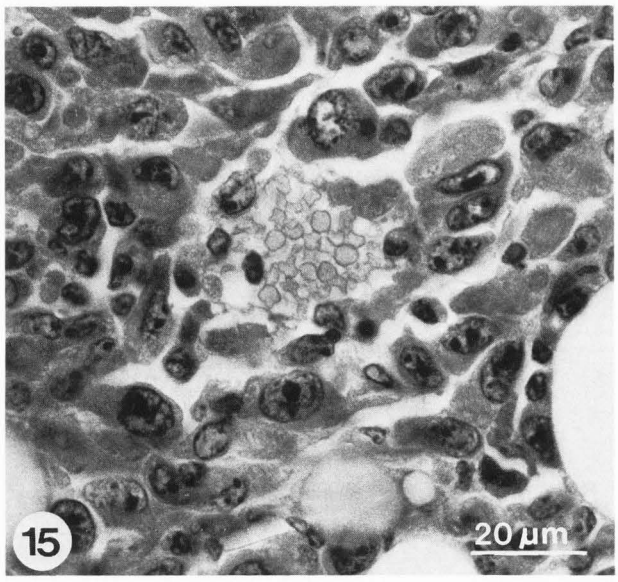
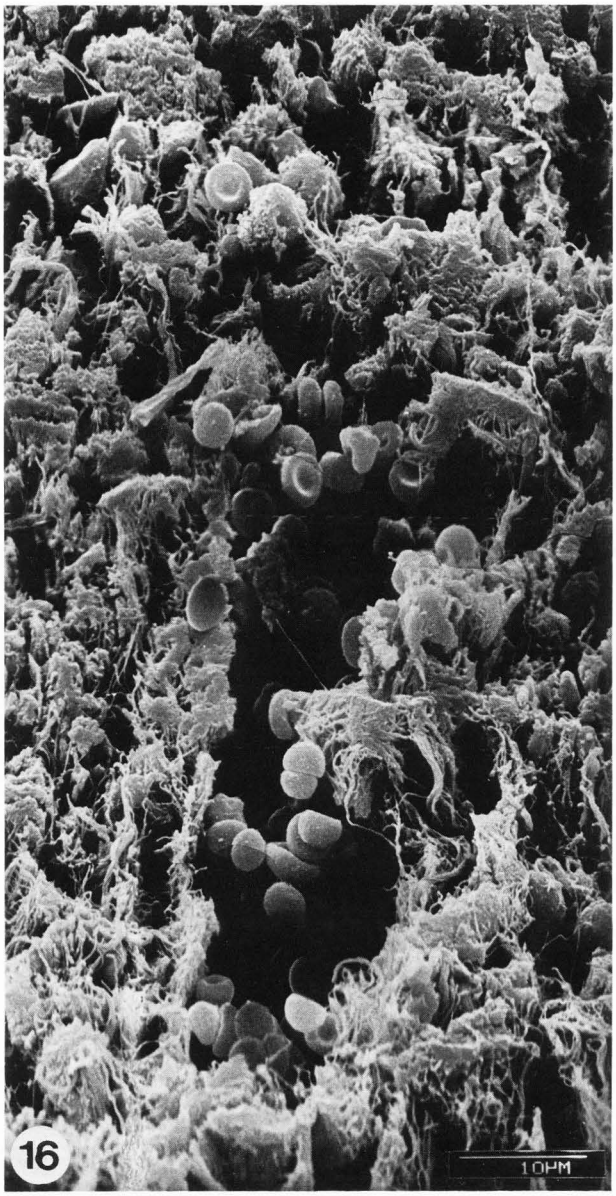
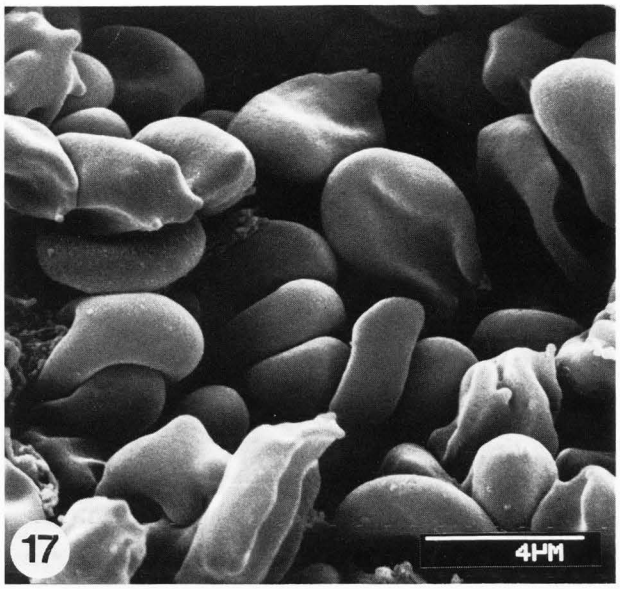


Fig. 12: Tumor center. Collapsed capillary. Fixation by immersion. HS. ST 5 µm. HE. LM.
Fig. 13: Interior region of the "tumor vascular envelope" near the central cavity. Plexus-like arrangement of lacunary replicae. VCC. SEM.
Fig. 14: Interior region of the "tumor vascular envelope" near the central cavity. Casted blood channel with tumor cell imprints (1 - 6). VCC. SEM.
Fig. 15: Near the central cavity. Blood channel without any endothelium. Fixation by immersion. HS. ST 5 µm. HE. LM.

This is shown very convincingly in Fig. 10. Simultaneously many casts of tumor blood vessels reveal marked endothelial invaginations and pleatings. In high pressure preparations tapering tumor vessels projecting into the central cavity are frequently in direct contact with extravasal structures (Fig. 9). This proves, together with the occurrence of imprints of endothelial cell nuclei at the tip of the pointed sprouts (Fig. 11), that the present structures represent true vascular formations, which are not caused by incomplete filling of the vascular bed. Casts of such blood vessels sometimes reveal diagonal grooves at their surfaces resembling endothelial cell border lines known from casts of normal blood vessels (Fig. 11).

Histological sections of tumors, fixed by immersion and by perfusion, reveal collapsed, central blood vessels, which correlate very well with the structures found in vascular corrosion





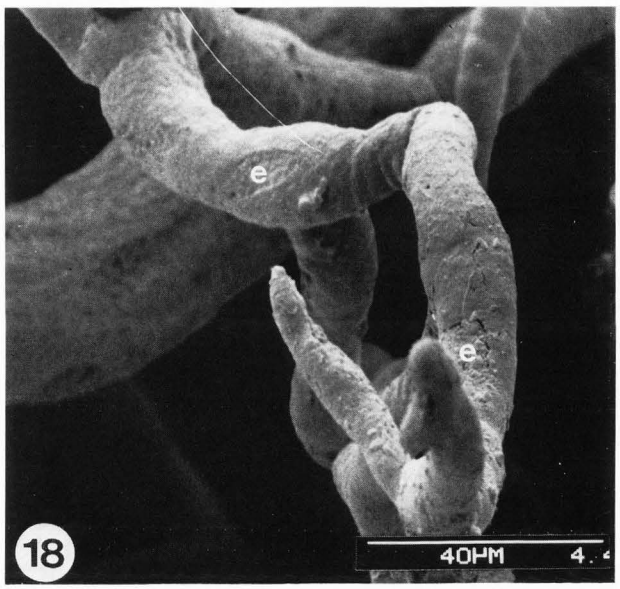


Fig. 16: "Tumor vascular envelope". Extravasal red blood cells in the interstitial space of the tumor tissue. Fixation by immersion. CPD. SEM.
Fig. 17: Extravasal red blood cells are packed to "rouleaux-columns". CPD. SEM.
Fig. 18: "Tumor vascular envelope". Imprints of endothelial cell nuclei at the surface of a casted capillary sprout. VCC. SEM.

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casts (Fig. 12).

The following results were obtained from low and medium pressure preparations.

Blood channels and lacunas

Near the central avascular cavity the inner lying areas of the tumor vascular envelope are formed by the irregularly arranged vascular plexus. They are composed of apparently normal casts with longitudinal course and of irregularly shaped blood channels and lacunas. These make multiple contacts with the normal casts and frequently end in plump saccules (Fig. 13). Some of these channels traverse the central cavity.

Detailed studies reveal symmetrically arranged pits at the surface of the casted channels. According to their size and shape they probably represent imprints of lining tumor cells (Fig. 14). This indicates that many tumor blood vessels own a fragmentary endothelium. If endothelial regression proceeds, the blood space is lined exclusively by tumor cells. This becomes evident from correlative histological studies done by LM of tissue sections (Fig. 15) or by SEM of CPD specimens (Fig. 16).

Extravasal structures

Irrespective of the applied perfusion pressure vascular corrosion casts of tumors consistently reveal extravasal structures. They may be viewed as a characteristic feature of tumor vascular systems. This opinion is supported by correlative histological methods. LM of tissue sections and SEM of CPD tumor tissue blocks either demonstrate extravasal, pale red blood cells lying tightly packed between the tumor cells or show loose arrangements and "rouleaux"-formations of interstitial red blood cells (Fig. 17). From vascular corrosion casts it becomes evident that the vascular system has multiple contacts to the interstitial space. Thus casts of endothelialized tumor blood vessels usually run directly into extravasal compartments (Fig. 9) forming there masses of typically rounded and segmented structures (Fig. 6).

Discussion

In a previous report we have given a survey of the angioarchitecture of the LLC (26). Now we have performed a detailed analysis of the basic and characteristic structural features of the tumor vessels. Special attention is given to the implications between the vascular abnormalities and the behavior of the malignant tissue.

According to the literature and to our findings, excessive vasodilation is not caused merely by conventional reflex mechanisms but arises from endothelial proliferation. Diagram 2 demonstrates some possible variations of vascular fusion and dilation.

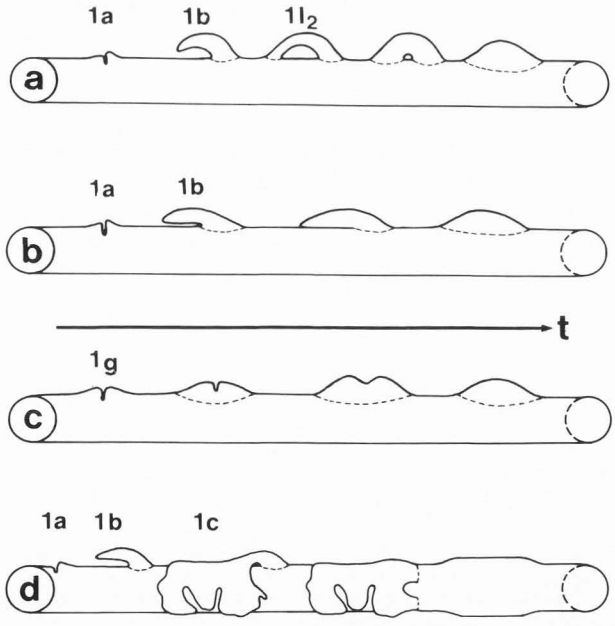


DIAGRAM 2: Variations of the fusion and dilation processes on basal tumor vessels (a-d). In accordance with the diagrams 1a - 11 the numbers designate several types of capillary sprouts. t ... time

Summarizing our results we are able to demonstrate a generalized survey of several possible mechanisms for vasodilation, anastomosing and collateral shunting, all having been originally caused by endothelial proliferation (diagram 3).

DIAGRAM 3: Generalized presentation of the different courses of development of angiogenic outgrowths (see also diagrams 1a - 11) with regard to the individual variations of vasodilation (diagrams 2a - 2d).

I) 1a → 1b → collateral (11) or dilation (2a)
II) 1a → 1b → dilation (2b)
III) 1a → 1b → 1c → flap enlargement → partly or complete fusion with the stem vessel → collateral (11) or dilation (2d)
IV) 1d → 1e → 1f → anastomose or collateral (11)
V) 1g → 1h → 1i → 1j → anastomose or collateral (11)
VI) 1g → 1b + 1b → collateral (11) or dilation (2c)
VII) 1g → 1b + 1h → collateral (11) or dilation (2c)
VIII) 1g → 1h + 1h → collateral (11) or dilation (2c)

However, endothelial cell multiplication additionally causes a striking vascular elongation of the affected vessels (10, 51). During growth the tumor proceeds to displace the vascular system. The increasing disproportion between the actual vessel length and the space available for the blood vessels therefore induces the development of many endothelial invaginations and causes the tumor vessels to take a highly tortuous and irregular course. They are arranged in tightly packed pads and glomeruloids with minimal intervascular distances.

Since the sprouts of the 1st phase of tumor angiogenesis are not capable of traversing long distances, the initial tumor vascular system is formed just by centripetally arranged, venous sprouts of the 2nd phase of tumor angiogenesis. A similar situation was reported by Schoefl (1963) (51), who studied the ultrastructural vascular changes occurring during wound healing. According to this author, elongation of capillary loops by intercalation of new endothelial cells represents a slow vascular regeneration, which is induced only by a weak stimulus, but capillary sprouts invading the injured region are caused by massive stimuli and represent intense vasoproliferation.

During the 3rd phase of tumor angiogenesis centrifugally arranged sprouts grow out and prepare the surrounding host tissue for further invasion and expansion of the tumor.

Kligerman and Henel (1961) (37) were the first, to recognise that the initial tumor vascular bed is of venous origin.

According to Reinhold and Van Den Berg-Blok (1984) (48) the angiogenic active substances produced by the tumor cells and secreted into the interstitial space are drained by the

interstitial fluid stream. Therefore, they may at first reach the venous side of the vascular tree inducing there the first angiogenic processes. This would explain the different resistance of arteries and veins against tumor induced vascular changes. Arterial sprouting, for instance, was never seen.

Many authors have reported on the leaky nature of the capillary sprouts (11, 51, 63). Van Den Brenk et al. (1977) (4) consider the occurrence of large gaps in the endothelial lining of capillary sprouts as a significant characteristic of angiogenic processes in tumors. This is in line with our findings. Fig. 6 comes from a medium pressure cast preparation so the injection pressure may not have been too high. Nevertheless radial sprouts of the second phase are seen in close contact to extravasal structures. This indicates that the blood vessels of the LLC have fragmentary endothelia.

According to Clark and Clark (1939) (6) and Van Den Brenk et al. (1977) (4) the development of capillary sprouts first starts with the formation of a small lumen in the activated endothelial cell. It is formed most probably by secretory activity of the endothelial cell (7) and originally it does not have any contact to the intravascular lumen. Sholley et al. (1984) (53) states that vascular sprouting may occur without endothelial proliferation too.

Ausprunk and Folkman (1977) (1), however, think that the first response to a tumor-induced angiogenic stimulus is a migration of the activated endothelial cell towards the stimulus. This cell attraction is apparently directed by a gradient of concentration and represents a sort of positive chemotaxis. Autoradiographic studies undertaken by these authors have shown that it is only the subsequent formation of interendothelial gaps which represents the mitotic stimulus. The outgrowing sprouts - their tips being formed by solid cords of endothelial cells without any lumen (51) - follow the way of least resistance. This means that their growth direction is determined in part by the structure of the surrounding tissue.

In making and analysing vascular corrosion casts of regeneration vascular systems it is most important (similar to other injection methods) to ascertain that structures which are considered to be tumor-specific, do not merely represent artifacts caused by the preparation. This is achieved most effectively by proper preparation and a critical assessment of the casts.

Anesthesia was controlled in order to avoid cardiac arrest; and sufficient heparinization of the blood prevented intravascular coagulation during the preparation. By exact adjustment of different values of perfusion pressure and rate of resin injection it was possible to study their influence on the organization of the resulting vascular cast. Studies on capillary sprouting were preferentially performed on high pressure cast preparations.

Prior to the preparation the anesthetized mice were mounted on special stages (26), to prevent obliteration of superficial blood vessels caused by external pressure to the tumor.

Since most of the casted muscle capillaries show a straight course, which is characteristic for relaxed muscles, the injected resin should not cause spasms of the s.c. muscles, which in turn would prevent sufficient filling of the blood vessels (26).

For the discrimination between incompletely filled blood vessels and real vascular structures, it is useful to make a detailed morphological analysis of the structure in question and to assess the extent of resin filling in the surrounding area. The following situations are indicative for capillary sprouts: 1) close spatial relations between blind ending casts and extravasal structures (Fig. 6); 2) endothelial cell nuclear impressions during the course or at the tip of the blind ending cast (Fig. 18) (27, 41). 3) "plastic strips" (Fig. 19) or extravasal structures during the course, or 4) at the tip of the blind ending cast (Fig. 20).

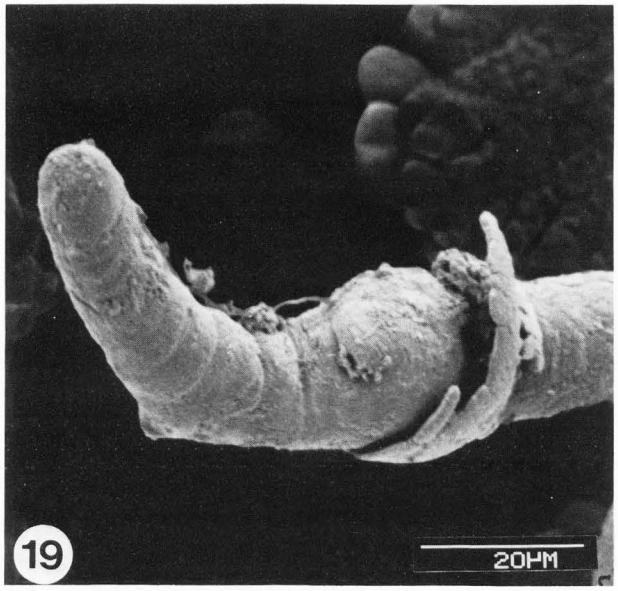
However, these situations are to be contrasted with the following features, which prove that such blind ending structures are caused by incomplete resin filling: 1) Blind ending vessels with a diameter of more than 25 μm. 2) Small spherical structures at the tip or along the course of a blind ending cast (Fig. 21). They are caused by a contact, during polymerization, between the hydrophobic resin and a hydrophilic fluid. 3) Blind ending casts branching from contracted arterioles. They may obstruct the resin influx into the capillary bed. 4) Broken vascular casts. They are identified very easily by their shared edges.

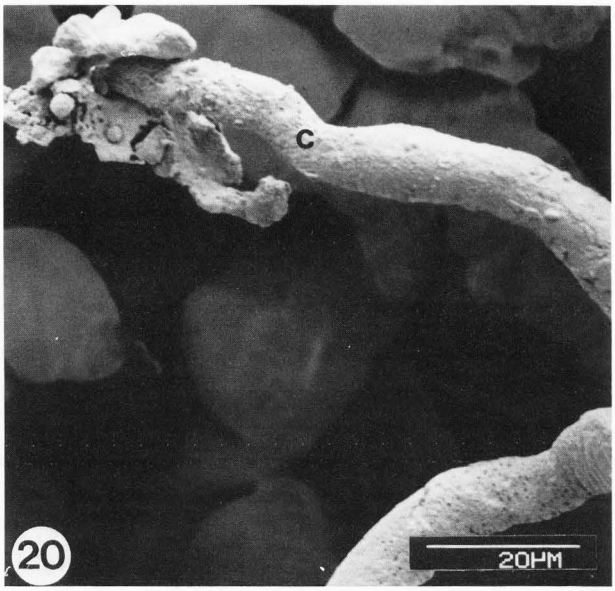
A recent study on the microvascular bed of the hamster melanotic melanoma (29) shows very convincingly that non-endothelial cells are also involved in the angiogenic processes. This is in line with results from Ausprunk and Folkman (1977) (1), which show that angiogenic stimuli also enhance ^{3}H-thymidine-incorporation into pericytes and fibroblasts. In malignant tissues apparently both methods of endothelial genesis - the angioblastic and the mesenchymal - are used.

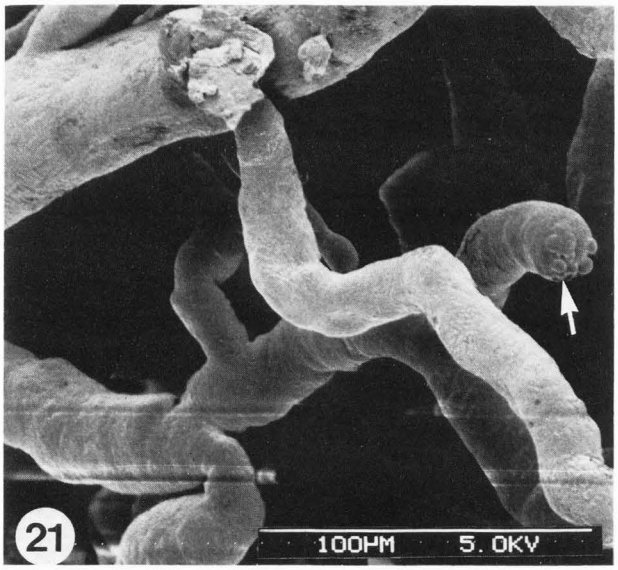
However, the question as to how close vascular reactions induced by other processes, such as wound healing or myocardial infarction resemble those occurring in tumors, is still a subject of controversy.

Activated endothelial cells have many luminal and abluminal projections (61) and the nucleus lies often in abluminal cell sectors (62). The luminal projections are used for metabolic exchange, while the abluminal ones represent migratory cell activity, which is also reflected in a high structural variability of the contact zones between adjacent endothelial cells (51). The variable shape of the surface of the casted sprouts is caused, therefore, by the variable differentiation of the surface of the proliferating endothelium.

Folkman et al., in 1971 (15), were the first, to isolate an angiogenic active fraction from tumor cells (tumor angiogenesis factor = TAF). It did not represent a single substance, however, but was composed of a mixture of molecules, which are common to every cell (RNA,







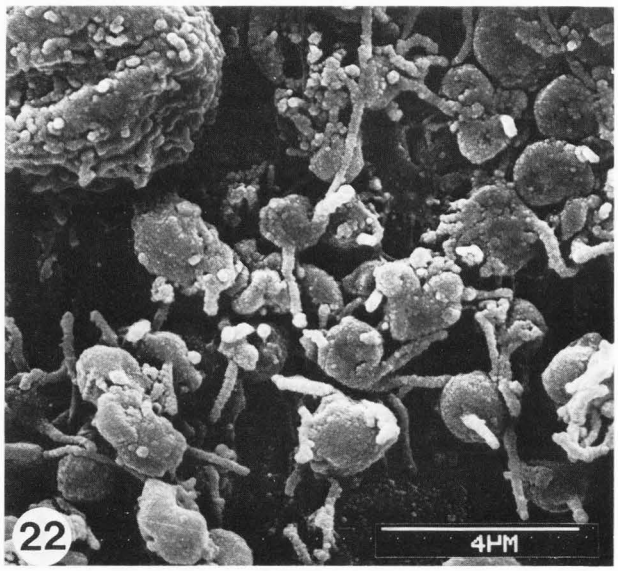


Fig. 19: "Tumor vascular envelope". "Plastic strips" at the cast of a capillary sprout. VCC. SEM.
Fig. 20: "Tumor vascular envelope". Extravasation at the tip of a capillary (c) sprout. VCC. SEM.
Fig. 21: "Tumor vascular envelope". Incompletely filled, blind ending, wide tumor blood vessel with spherical structures at the surface (arrow). VCC. SEM.
Fig. 22: Activated, "dendritic" thrombocytes in the interstitial space of the tumor tissue. CPD. SEM.

proteins, carbohydrates and presumably a small quantity of lipids). Presently two TAF's are purified and their amino acid sequences are reported (12, 14, 40, 52, 56). Several recent reports reveal that angiogenesis may occur in many different situations (21, 22, 39, 65). Reinhold and Van Den Berg-Blok (1984) (48) think that accumulated lactic acid, high pCO_2, low pH and low pO_2 as well as substances deliberated during necrotic cell lysis could induce reactions similar to those caused by TAF.

Leakage of blood through the fragmentary endothelium of the sprouts is followed by extravasal coagulation, so fibrin threads surround the growing sprouts. Thompson and Campbell (1982) (60) stress that fibrin and fibrin degradation products are effective in inducing angiogenesis.

It is believed that plasminogen activator, which converts plasminogen to plasmin and thus controls fibrinolysis and tissue degradation, is a decisive stimulator of both vasoproliferation and tumor invasion. Skriver et al. (1984) (54) could demonstrate that the cells of the LLC produce plasminogen activator, which is most

accumulated in peripheral regions with invasive growth. According to Christman et al. (1977) (5) a close relationship exists between the expression of plasminogen activator and the malignancy of the tumor.

Irrespective of the fact that the LLC does not exhibit any histoincompatibility to the host (32, 36) we have found many lymphocytes and macrophages lying in the tumor and in the adjacent host tissue. According to Benacerraf and Unanue (1982) (2) no tumor is absolutely non-immunogenic to the host.

Simultaneously, most tumors carry leucocytes and macrophages. Today there is increasing evidence that activated macrophages and lymphocytes (inflammatory cells) induce capillary proliferation (16) and Heparin seems to play a regulatory function (20).

It was relatively easy to distinguish casts of large compressed vessels from those of small capillary sprouts. Casts of compressed capillaries, on the other hand, could be identified by their characteristically tapered shape frequently exhibiting an endothelial cell nuclear imprint and/or an endothelial cell border line near their tip (Fig. 11) and by their specific location in the irregularly arranged plexus bordering the central avascular space (Fig. 9). In tumors which have developed a central cavity already, vascular sprouting is confined to peripheral and basal regions. Compressed capillaries, therefore, may not be confused with capillary sprouts. Supporting histological studies on both immersion-fixed (Fig. 12) and perfusion-fixed tumor reveal that the collapse of the tumor vessels is not caused by a circulatory breakdown at death of the animals.

Wiig et al. (1981) (64) estimated the maximal interstitial tissue pressure in a rat mammary carcinoma (23 mm Hg). By correlating this value with the mean pressure in tumor capillaries (23.8 mm Hg) and tumor venules (9.7 mm Hg) they showed that compression of tumor vessels is an inevitable occurrence. In the tumor the perfusion pressure is therefore determined by the difference between the arteriolar pressure and the tissue pressure (22 mm Hg). By comparing this pressure with the value for the normal skin (40 mm Hg) it becomes evident that the tumor blood flow must be very slow.

The interstitial pressure increases not merely in response to the tumor cell proliferation but also due to the development of an interstitial edema, caused by the increased permeability of the tumor blood vessels. After circulatory arrest the edema recedes and the mean tissue pressure drops from 11.2 mm Hg to 5.9 mm Hg (64). This is a further indication that the vascular compression does not represent a post mortem vessel collapse.

However, vascular degeneration may also occur by endothelial cell lysis (13, 29), which may cause a wide-spread loss of the sinusoidal walls. According to several studies, a substance called I.C.R.F. 159 may prevent this endothelial degeneration (31, 43, 49, 50, 66).

Van Den Brenk et al. (1977) (4), however, do not believe that there is any endothelial degeneration. They think that extravasal blood is caused exclusively by the leakage through the capillary sprouts. However, this hypothesis may be disproved. During our studies we have gained sufficient morphological evidence to demonstrate a chronological course for the endothelial degeneration (26). In comparing the results from Hammersen et al. (1983) (29) with the present findings a surprising interrelation between "active" vasodilation and endothelial degeneration arises. According to them intraendothelial vacuoles of 4 - 5 µm diameter develop, which then open into the distending vascular lumen thus leaving a thinned-out endothelium. This agrees with the observations of outgrowing globular pouches, which in their initial phase have diameters of less than 5 µm.

Blood channels are bordered exclusively by tumor cells. They contain loosely packed red blood cells indicating that the channels are still perfused. Blood lacunas and extravasal structures, on the other hand, are filled with interstitial red blood cells of pale colour, which are tightly packed between the tumor cells. Apparently this static blood has lost contact with the circulation. Efficient metastasis may only occur in perfused circulatory areas ("giant capillaries", sinusoids, blood channels).

According to Poggi et al. (1977) (47) the animals suffer from a microangiopathic, hemolytic anemia. This is supported by the pale colour of static red blood cells indicating a loss of haemoglobin.

Several features, such as the aggregation and "rouleaux-formation" of red blood cells (Fig. 17) and the extravasal occurrence of activated platelets (Fig. 22) indicate haemostasis and coagulation. Experimental and human tumors often show irregular and contradictory haemostatic reactions, which may range from massive bleeding to intravascular coagulation. This is explained, at least in part, by two different activities of the tumor cells. According to Curatolo et al. (1979) (9) the cells of the LLC produce a procoagulant, which is able to activate the coagulation factor X directly. The same cells, however, may also produce fibrinolytically active plasminogen activators. Poggi et al. (1977) (47) have found that intramuscularly growing LLCs do not exhibit marked signs of intravascular coagulation.

Casts of blood vessels, which are obstructed by an intravasal blood clot, would not be confined to any special tumor region. However, the casts of vascular sprouts and compressed vessels had a very regular and individual pattern of distribution. This and the facts that the animals were heparinized before starting the preparation and that they were perfused with high pressure ascertains that the presented blind ending casts are not caused by thrombosis or embolism.

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Discussion with Reviewers

K.C. Hodde: Has anybody done carbon labelling studies of the very early vascular changes after tumor implantation such as described by Majno and colleagues (J. Biophys. Biochem. Cytol. 11, 571-626 (1961)?

Authors: Yes, there is one paper (43) dealing in part with carbon black (Pelikan ink) labelling in the LLC in order to demonstrate the vascular structures of this tumor.

K.C. Hodde: What could be the reason for the exclusive venous origin of the phase 2 tumor vascularization?

Authors: There are two reasons: 1) Angiogenic substances secreted by the tumor cells into the interstitial fluid reach the venous side of the microcirculation (48) and 2) Arterial walls are very compact and thus are resistant to angiogenic stimuli.

K.C. Hodde: Are the centrifugal sprouts (phase 3) also just of venous origin?

Authors: In elucidating the origin of tumor vascular sprouts one has to realize that the major constituents of the tumor vascular bed are formed by veins, venules and sinusoids, which frequently carry oxygen-deprived blood and so may be characterized as venous vessels too. If in LLC true capillaries would make up a larger part of the tumor vascular bed, capillary sprouting would be a frequent feature too. What can be stated is that arterial sprouting did not occur in our specimens.

K.C. Hodde: Do you think that the stimuli for centrifugal and centripetal sprouting are identical?

Authors: We think that the stimuli are the same for both, but our studies are not able to give evidence for this suggestion.

K.C. Hodde: Do you think that fully filled blind endings can occur because the plastic can push out the fluid in the vessel through the leaky endothelium but is too viscous to pass through itself?

Authors: Yes, we think so. Expulsion of rinsing fluid to us seems essential for a complete filling of blind ending vessels. This expulsion seems to be facilitated by the leaky nature of the sprouts.

J.G. Walmsley: Do the "leaky" vessels cause different parts of the vessels to be exposed to different pressures during casting and result in different "flattened, collapsed and bulging" vessels?

Authors: It could occur that in "leaky" vessels parts of the vessels are exposed to different pressures during casting, but we consider these pressure differences as too minimal to cause flattening, collapsing or bulging of tumor vessels during the casting procedure. These processes are obviously caused by the growth pattern of the tumor itself.

J.G. Walmsley: Do these structures occur in non-tumor situations during vascularization or angiogenesis?

Authors: So far sprouting is also reported to occur in wound healing and in inflammation.