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In vivo Analysis of Tumor Vascularization in the Rat

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By using transparent chambers in rats, we have directly observed tumor-induced neovascularization in the early stage and the formation of intricate networks in Yoshida rat ascites hepatoma AH109A and Sato lung carcinoma at high magnification. We counted branching point numbers per unit area in the microvascular network with and without tumors in order to clarify the sites from which new vascular sprouts originate. Branching point number per unit area in normal tissue was $13.6 \pm 7.4/0.1$ mm^2 in the field near a terminal arteriole, and $12.9 \pm 7.3/0.1$ mm^2 in the field distant from a terminal arteriole. There was no significant difference between these two fields in the normal vascular network. On the other hand, in the tumor vascular network, the branching point number in the field near a terminal arteriole was $50.4 \pm 12.6/0.1$ mm^2, and $30.1 \pm 11.5/0.1$ mm^2 in the field distant from a terminal arteriole. The difference is highly significant ($P < 0.001$). The frequency with which new capillaries originated from veins and venules was very low. We concluded from these results that the position from which tumor vessels originated was usually the terminal portion of a terminal arteriole.

Key words: Transparent chamber — Tumor vessel — Neovascularization process — Starting vessel

Tumor blood flow is one of the major determinants of effectiveness in cancer chemotherapy.[1,2] The delivery of anticancer drugs to tumor tissues is dependent on the flow rate of circulating blood in the tumor. In 1977 Suzuki *et al.*[3,4] found that elevation of blood pressure by continuous venous infusion of angiotensin II produces an approximate 6-fold increase in tumor blood flow, and that this increase is selective and specific to tumor vessels as long as the mean arterial blood pressure is kept under 150 mmHg. A unique cancer chemotherapy based on this functional characteristic of tumor microcirculation affords optimum therapeutic results in both animal experiments[1,3,4] and clinical trials.[5,6] In order to elucidate the mechanism of increased tumor blood flow under angiotensin II-induced hypertension, it is necessary to analyze not only the regulatory mechanisms of tumor blood supply but also the architecture of the tumor vascular system and the modes of vascular connections between normal and tumor tissue.

There have been many reports on morphological studies of tumor vascular systems, using a micro-angiographic method[7] or vital microscopic observation techniques.[8–12] Most of them, however, lay stress on the difference of vascular density between normal and tumor tissue. On the other hand, there are very few reports describing observations of the process of tumor vascular system formation at high magnification. Accordingly, there remain many unknown points concerning the initial stage of tumor-induced neovascularization.

The purpose of this study was to solve the following two problems related to tumor angioarchitecture in the earliest stage: 1) Where does the tumor recruit vessels? and 2) How is the tumor vascular network, which has high vascular density, constructed? In order to solve these problems, we used a transparent chamber that was more stable and of higher resolving power than those previously used and made prolonged microscopic observations of the process of tumor vessel formation *in vivo*.

MATERIALS AND METHODS

Animal and tumors Male Donryu rats (Nippon Rat Co., Urawa), weighing 160–180 g each, were used in this experiment. The tumors were Yoshida rat ascites hepatomas, AH109A and AH272, which have been maintained in our laboratory by successive intraperitoneal transplantation and Sato lung carcinoma (SLC), maintained by subcutaneous transplantation.

Chamber insertion and tumor transplantation Our chamber is shown in Fig.1. The construction of the chamber and surgical techniques for installation were detailed in our previous papers.[13,14] This chamber is based on the "sandwich chamber" developed by Yamaura *et al.*[15] and consists of a pair of quartz glass plates that cover the thin subcutaneous tissue of the rat dorsum.[14] Our chamber is simple, sturdy and stable because of the use of a light-weight metal frame, and gives excellent optical resolution. Surgical operation for chamber insertion takes only 10 min to perform. The rat carrying our chamber is not apparently under stress. To observe the developing vascular system in the tumor, we transplanted a small fragment (about 0.1 mm^3) of solid tumor from a donor rat onto the tissue within the chamber. The mean thickness of subcutaneous tissue within the transparent chamber was about 110 μm. The implanted piece of tumor grew in a sheet-like fashion within the chamber.

Observation and photography Vital microscopic observation and photographic recording were carried out at selected areas in subcutaneous tissues of chamber-installed animals with or without tumors. Rats with the transparent chamber were anesthetized with an intramuscular injection of pentobarbital sodium (30 mg/kg) and placed on a heated stage at 37°C for microscopic observation. Observations were made through a light microscope (BHS-323; Olympus Co., Tokyo) equipped with long-working-distance objectives (×20 or 40) (ULWD CDPlan 20 or 40; Olympus Co.,) and an ocular (×10). The condenser used was a long-working-distance lens (Ultra-long-woking-distance condenser; Nikon Co., Tokyo). The tissue was transilluminated by a 100 W halogen lamp equipped with a heat-absorbing filter.

Normal subcutis and tumor tissue within the chamber were photographed through the microscope on both instant color film (FP-100; Fuji Photo Film Co., Ltd., Tokyo) and black and white film (Type 667; Nippon Polaroid Co., Tokyo).[16] Data presented were based on observations and measurements of about 400 rats.

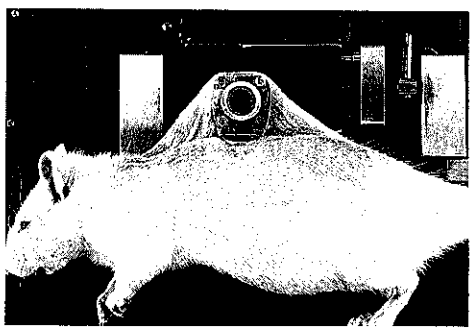


Fig. 1. A rat with a transparent chamber transplanted into the dorsal skin flap.

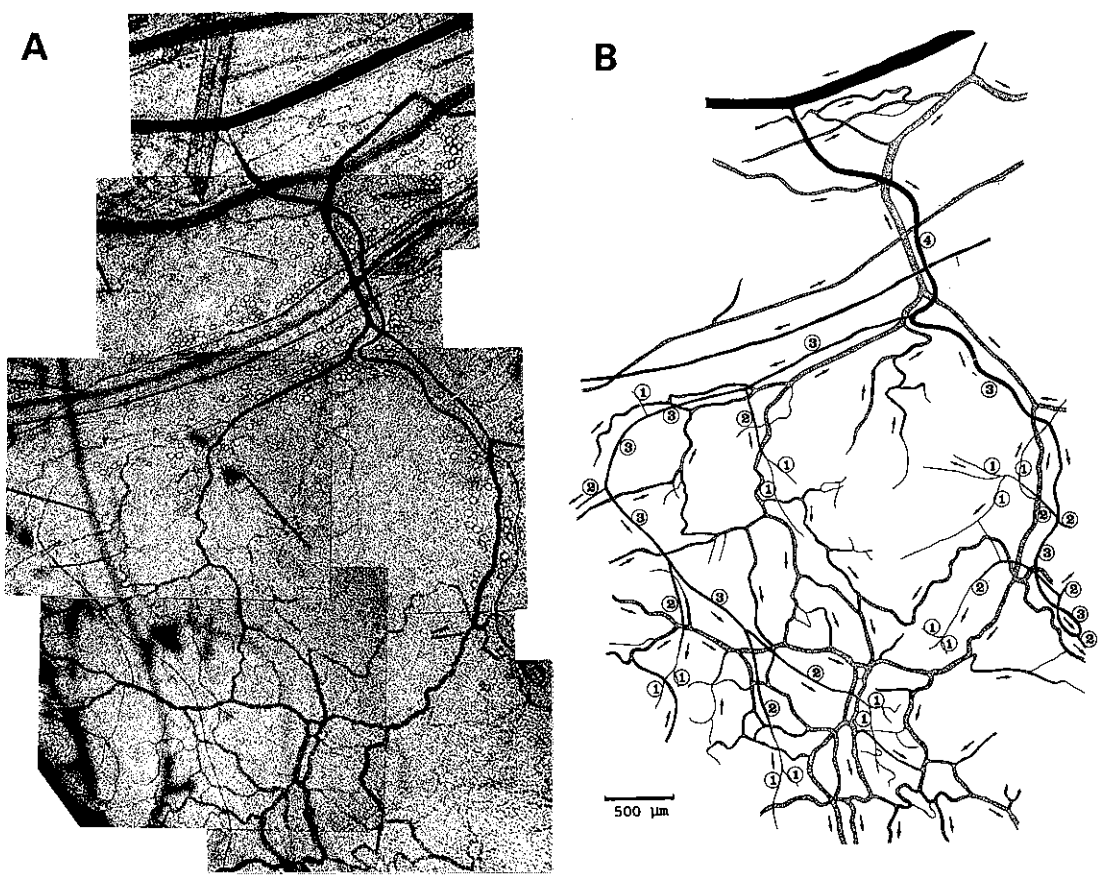


Fig. 2. Microphotographic montage of microvasculature in the rat subcutaneous tissue within a transparent chamber (A). Each print was made through a 40 power microscope. The line drawing shows a reconstruction of the vessels made by tracing the photomontage (B). Most of the true capillaries are not apparent on the photographs at this magnification (×40). Blackened vessels: arterioles, Shaded vessels: venules. Arrows: flow direction. Numbers: vessel orders according to Strahler's nomenclature.

Trace of vascular pattern in subcutaneous tissue Serial photographs were taken at a low magnification (objective 4×, ocular 10×) and individual prints were assembled into a montage. A transparent vinyl sheet was placed over the photomontage. The arterioles, the venules and a part of the true capillaries were traced onto the overlays. The photomontage of the subcutaneous vascular pattern and the line drawing tracing it are shown in Fig. 2, in which arterioles, venules and a part of the true capillaries are drawn. The microscopic blood vessels under observation were categorized following the nomenclature of Wiedeman,[17] as follows. The arterial vessel that marks the entrance into the microcirculation is called an arteriole. The terminal arteriole is easily identified as the last vessel of the arteriolar distribution because it terminates in a capillary network. The capillary vessels are also easily identified because they are pure endothelial tubes. Postcapillary venules are slightly larger than capillaries and are usually devoid of any smooth muscle. They join to form venules.

Most of the true capillaries constituting the fine network were difficult to distinguish at a low magnification.

Traces of normal and tumor capillary network The samples selected for this experiment were subcutaneous tissues less than 100 μm in thickness between two transparent glass discs. Photography and reconstruction of true capillaries were performed in the same way as the analysis of the vascular pattern, except for the use of a 40× objective. After the capillary network had been traced onto an overlay, detailed microscopic observation

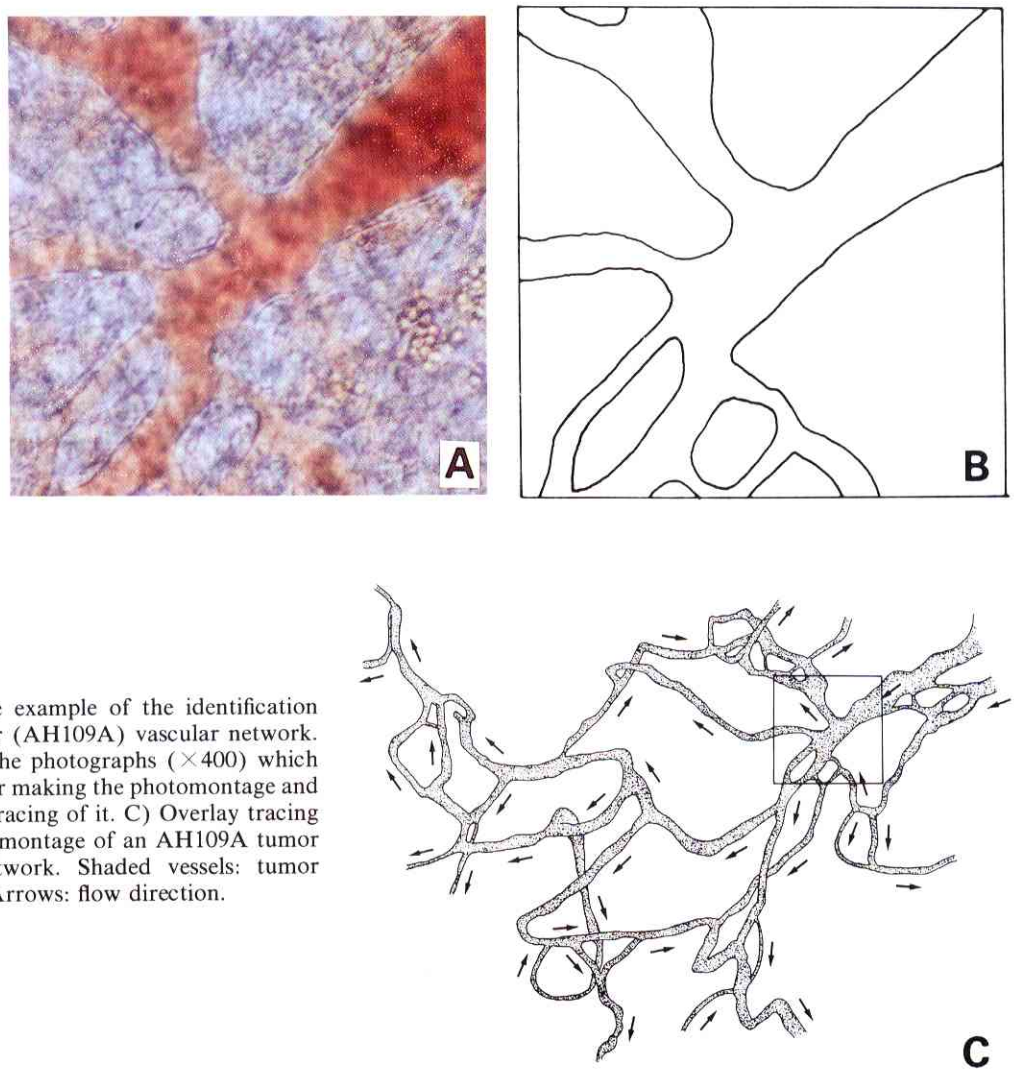


Fig. 3. One example of the identification of the tumor (AH109A) vascular network. A) One of the photographs (×400) which were used for making the photomontage and B) overlay tracing of it. C) Overlay tracing of the photomontage of an AH109A tumor vascular network. Shaded vessels: tumor capillaries. Arrows: flow direction.

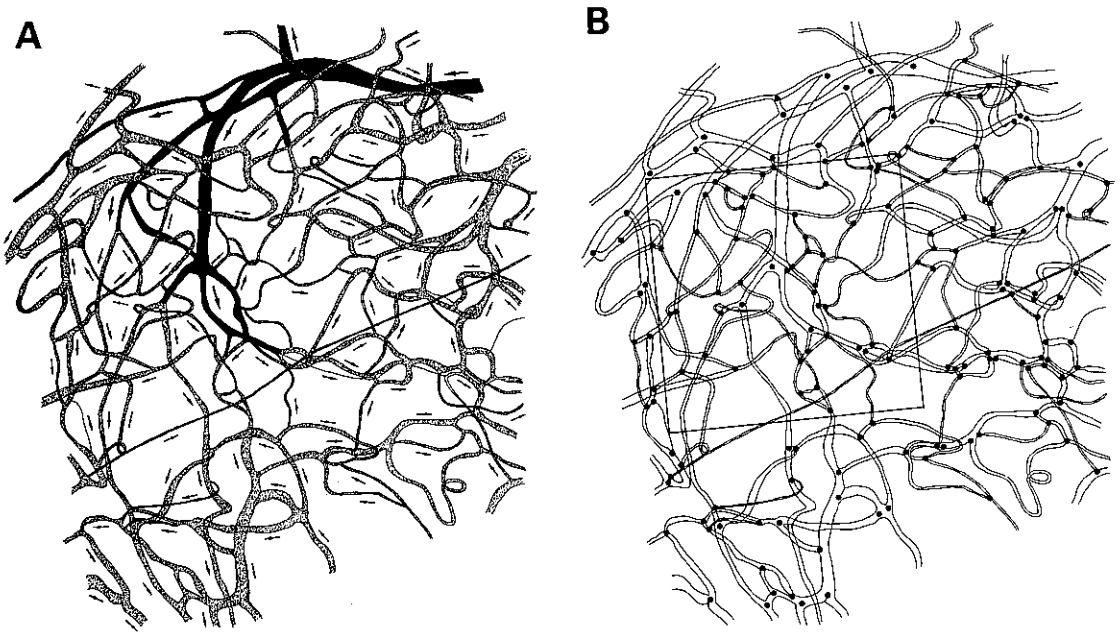


Fig. 4. Branching point counting per unit area in microvascular network. A) Overlay tracing of photomontage of an AH109A tumor vascular network. Blackened vessels: terminal portion of a terminal arteriole ("starting vessel"). Shaded vessels: tumor capillaries. B) Closed circles are vascular branching points. The area (0.32×0.32 mm^2) used for morphometric analysis of branching point number per unit area has been outlined.

was performed to distinguish between overlap of two vessels and ramification of a vessel. A trace sample of the earliest vascular network in AH109A is shown in Fig. 3.

Method of arteriole ordering In the rat subcutis, arteries, arterioles, terminal arterioles and a part of the capillaries could be ordered according to Strahler's nomenclature[18] which has been extensively used in various applications such as rivers, trees and vessels. Strahler's method is as follows. When two segments of the same order join, the parent segment is assigned the next higher order. If two daughter segments which have different orders are joined, the parent segment retains the higher of the two orders. In our experiments capillaries branching off originally from terminal arterioles classified by Wiedeman[17] were assigned order 1. Once those capillaries were defined as order 1, terminal arterioles were assigned to order 2. The numbers in Fig. 2 are vessel orders of arterioles in the rat subcutis, using this nomenclature.

Count of branching point numbers in capillary network Branching point numbers per unit area (0.1 mm^2) in the capillary network were counted on the photomontage overlays (Fig. 4). In normal tissue, both the area adjacent to a terminal arteriole according to Wiedeman's classification[17] and the area more than 200 μm from a terminal arteriole were selected for counting. In tumor tissue two kinds of areas which corresponded to the areas defined in the normal capillary network were also selected.

Serial change of branching points in venule A venule in the vicinity of the growing tumor was photographed at a high magnification ($400\times$) and all its branches were traced onto the overlay. Each branch was numbered in order. This process was carried out every 24 h until the venule was finally enveloped in the tumor and tracing was no longer possible because of disappearance of vessels. On the basis of the tracing data, the fate of each branch of the venule was investigated serially.

Statistical analysis To compare differences of numbers of capillary nodes per unit area between normal and tumor tissue, Student's t test was employed. Differences were regarded as statistically significant if $P<0.05$.

RESULTS

Vascular pattern of normal subcutaneous tissue within the chamber If the surgical operation for implanting a transparent chamber in rat dorsal skin flap was carried out skillfully, the vascular bed of the preformed layer recovered from injury to its original architecture within a few days. In many cases we could observe the vascular pattern in normal subcutaneous tissue from 3 days to two months after chamber installation.

In the subcutaneous tissue within the transparent chamber, arterioles progressively arose from arteries or larger arterioles and finally terminal arterioles produced approximately three capillaries at, or close to, the ends. That is, true capillaries originated as continuations of terminal arterioles. True capillaries bifurcated into branches of equal significance in the distribution of blood. Central channels (thoroughfare channels), similar to those described by Chambers and Zweifach[19] in rat mesenteries, were seldom found in rat subcutaneous tissues. In the capillaries receiving blood from two feeding terminal arterioles, the direction of blood flow constantly changed with modification of the pressure balance between these two terminal arterioles. Capillaries whose blood flow had stopped for several hours were also observed. Although small arteries, arterioles and veins continued to occupy the same relative positions and retained approximately the same form for two months, true capillaries were much more labile.

The early stage of tumor-induced neovascularization The usual time for the establishment of new tumor vessels was between 2 and 7 days. Most of the inflammation vessels due to chamber insertion disappeared within 3–4 days after operation. Almost all grafts formed new vessels by the 7th days after implantation, but neovascularization before the 4th day was seldom found in AH272 and SLC. On the other hand, most of the AH109A implants acquired their first vessels on the 3rd day. Once the implant became vascularized, it could grow exponentially (K. Hori *et al.*, unpublished data).

Development of tumor vessels always initiated from the preexisting microvascular bed. The sites where tumor vessels originated were frequently identical, regardless of the kind of transplanted tumors. Those sites were the terminal points of vessels of order 2, according to Strahler's nomenclature, corresponding to the ends of terminal arterioles. A terminal arteriole itself markedly elongates and dilates with tumor growth. We would like to refer to this terminal arteriole modified by the tumor as a "starting vessel" for initiation of tumor angiogenesis.

To prove that sprouts of tumor capillaries originated from the "starting vessels," we measured branching point numbers per unit area both in the fields adjacent to a terminal arteriole and in the fields more than 200 μm apart from a terminal arteriole in normal and tumor tissue (Fig. 5). Branching point number per unit area in normal tissue was $13.6 \pm 7.4/0.1$ mm^2 (n=30) in the field near a terminal arteriole, and $12.9 \pm 7.3/0.1$ mm^2 (n=77) in the field apart from a terminal arteriole. There was no significant difference between the two fields. On the other hand, in the tumor vascular network branching point number per unit area was much greater than that in the normal network ($P<0.001$). Branching point number in the field adjacent to a terminal arteriole [$50.4 \pm 12.6/0.1$ mm^2 (n=35)] was greater than that in the field apart from a terminal arteriole [$30.1 \pm 11.5/0.1$ mm^2 (n=48)($P<0.001$)]. One example of the process of AH109A tumor vascular formation is shown in Fig. 6. Sprouts of newly formed vessels originate at the arterial ends of host capillaries, where the blood velocity and vessel pressure are relatively high. Preexisting vessels were also modified and eventually incorporated into the tumor vascular network. Both sprouts of vessels and modification of preexisting vessels proceeded simultaneously as the tumor increased in size.

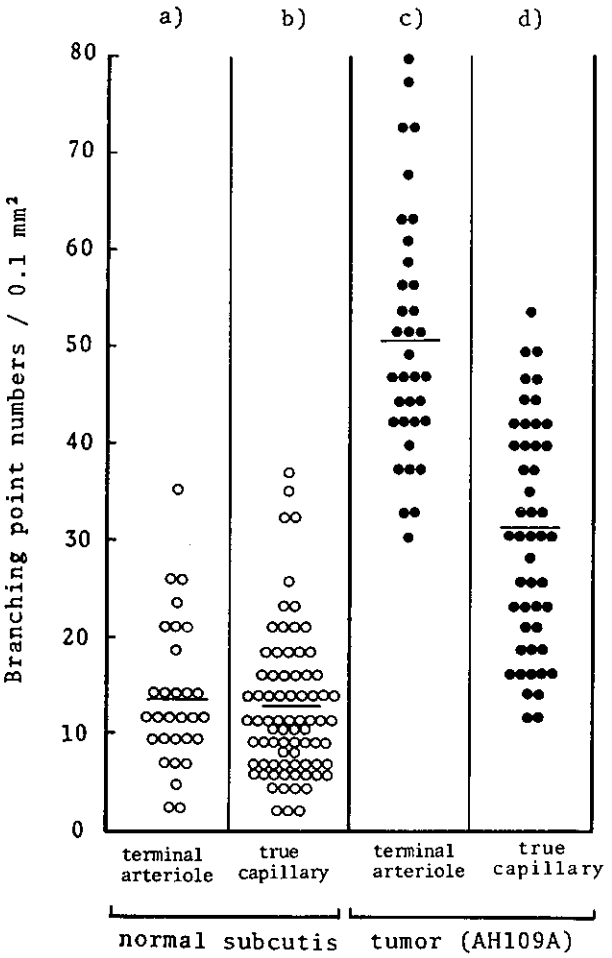


Fig. 5. Branching point numbers per unit area in the microvascular network. Open circles, normal subcutis; closed circles, tumor (AH109A). The ordinate shows branching point numbers per 0.1 mm^2. The abscissa indicates position in the microvascular network; a) field adjacent to a terminal arteriole in normal subcutaneous tissue, b) field more than 200 μm from a terminal arteriole in normal subcutaneous tissue, c) field adjacent to a terminal arteriole in tumor ("starting vessel"), d) field more than 200 μm from a starting vessel.

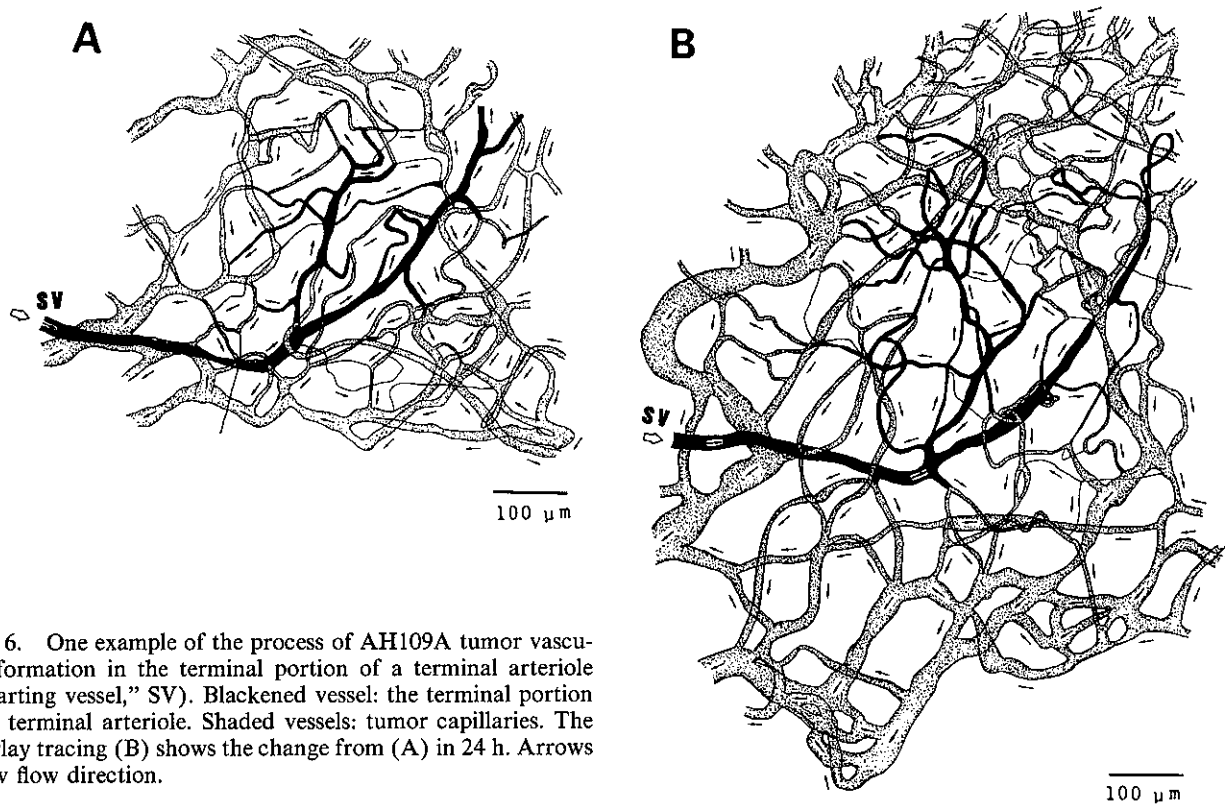


Fig. 6. One example of the process of AH109A tumor vascular formation in the terminal portion of a terminal arteriole ("starting vessel," SV). Blackened vessel: the terminal portion of a terminal arteriole. Shaded vessels: tumor capillaries. The overlay tracing (B) shows the change from (A) in 24 h. Arrows show flow direction.

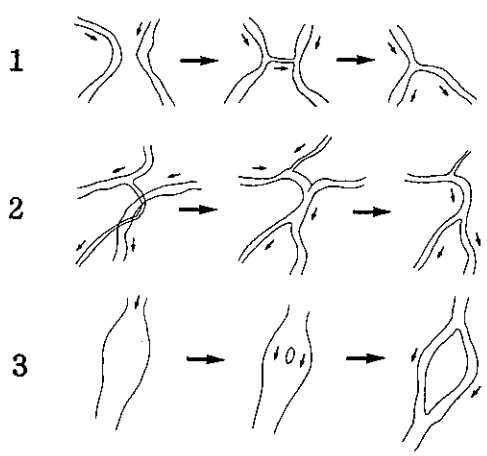


Fig. 7. Modes of branching point formation in tumor vascular network. 1, sprout; 2, cross-connection; 3, split. The example of each mode given is one which was traced in actual cases.

Branching point formation in tumor vascular network An intricate vascular network is one of the morphological characteristics of tumors. There are many vascular branching points in the network, as mentioned above. Vascular branching points in the tumors arise in an apparently haphazard way. There were three modes of branching point formation, i.e., sprouting, cross-connecting and splitting (Fig. 7). Sprouting occurred as mentioned above. Cross-connection was often observed in the process of modification of true capillaries and postcapillary venules, and splitting was dominantly observed at the late stage of tumor-induced neovascularization.

Modification of preexisting vascular system due to tumor growth The preexisting microcirculation system contiguous to the tumor also changed shape with the progress of tumor growth. All of the vessels which constituted the preexisting vascular network were eventually involved in the growing tumor, and changed into tumor vessels. In particular, postcapillary venules and venules became distended, and the vascular length increased. When we observed the neovascularization process within our chamber at a low magnification, new blood vessels looked as if they had arisen predominantly from the postcapillary venules and the venules (Fig. 8). However, most of these vessels that became prominent with tumor growth were not newly evolved, but modified from the preexisting postcapillary venules or venules. The fre-

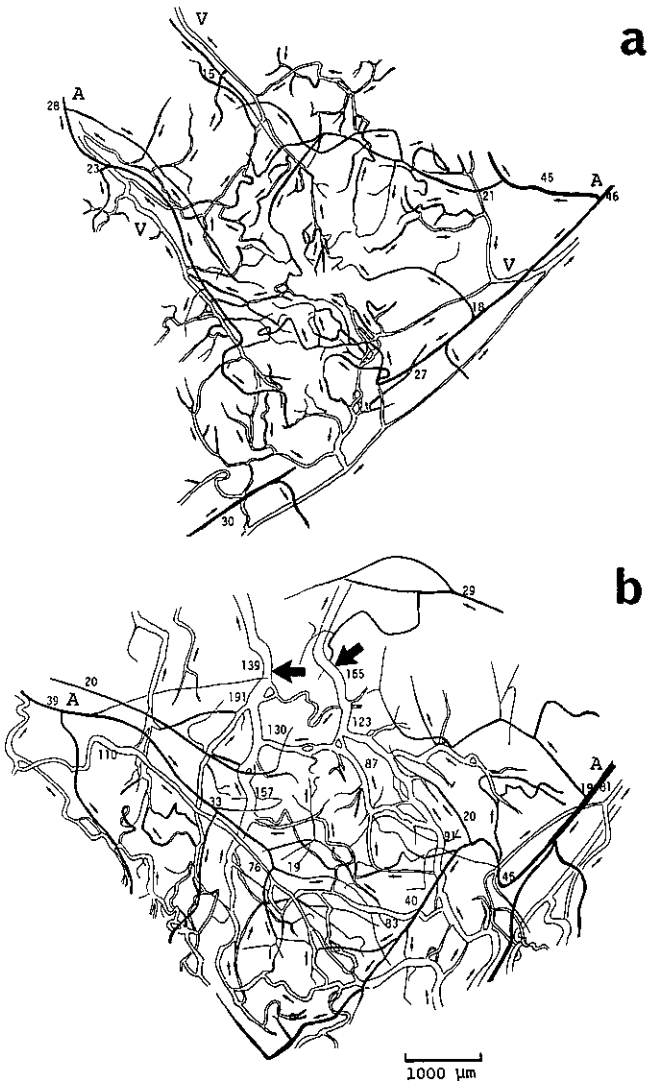


Fig. 8. Modification of preexisting vascular system. (a) is the preexisting vascular network on the 8th day after tumor transplantation, and (b) is on the 12th day. Most of the true capillaries are not apparent in the photograph at this magnification (×40). A, arterioles (blackened vessels); V, venules and small veins (solid-outlined vessels); number, vessel diameter (μm); small arrows, flow direction. Each preexisting vessel was modified progressively by the tumor and the entire vascular network changed continuously. Vessels designated by large arrows are modified postcapillary venules or venules. These modified vessels look as if they had sprouted from venous vessels.

quency of sprouts originating from venules was very low in all of the 15 cases we observed. A typical process of disappearance of a venule near the periphery of a growing tumor is illustrated in Fig. 9. Vascular nodes A and B (shown in Fig. 9) are newly formed ones. The majority of the venules were compressed by the growing tumor and eventually disappeared. In some venules, the vascular diameter increased from about 30 μm to 200 μm as the tumor grew in size.

Arteries remained as they had been before tumor implantation. However, arteriolar vessels enlarged geometrically while retaining their original shapes with the increase in size of the tumor vascular network.

The late stage of tumor-induced neovascularization When the process of tumor-induced neovascularization advanced further, the wall of each tumor vessel became thinner and could not be distinguished in some places. The blood velocity in such a vessel became slower. The direction of blood flow was unstable and often changed even with a slight change of the animal's posture. Blood flow in tumor vessels was restricted as the pressure increased, probably due to increasing tissue pressure,[20] and several areas entered a low-flow state or no-flow state and looked as if they were avascular. Excessive enlargement of the tumor vascular network seems to be another reason for the appearance of such a no-flow area. We observed that tumor blood flows run into these no-flow areas every few hours.[21] Some of these areas, however, eventually became necrotic. Angiotensin-induced hypertension brought about a marked increase in tumor blood flow even in these no-flow areas.[22]

Arteries and arterioles maintained blood flow and retained vasoconstractile response to vasopressors even when the tumor-induced neovascularization process was progressing markedly. At the final stage, however, arterial vessels showed dilatation and could no longer react to vasocontrictors. The color of the blood stream in arterial vessels became the same as that in venous vessels with tumor growth. That seems to imply that the circulating blood altered from the state of being fully oxygenated to that of being hypoxic. Sometimes a new loop was formed between two terminal arterioles. In this terminal arteriolar loop, we observed that blood often flowed in opposite directions, depending on slight changes of pressure balance between the two arterioles. The vessels which derived from arterioles were also compressed by the growing tumor, and eventually the adjacent areas became necrotic.

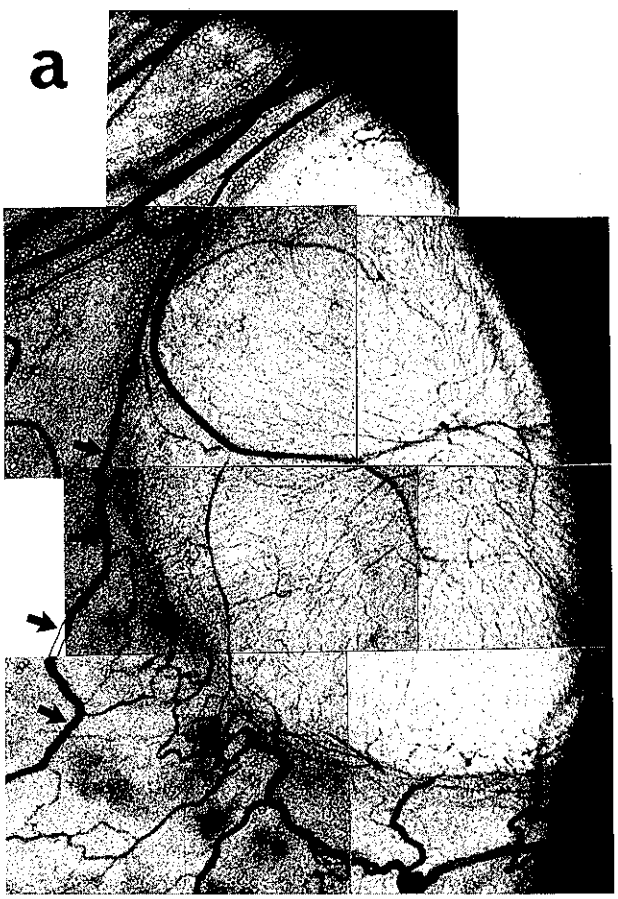
DISCUSSION

Where does a tumor recruit vessels? Our observations show that new blood vessels in tumor tissue originate from sprouting of the end point of preexisting terminal arterioles. There are some papers reporting that in the earliest stage the tumor vascular bed is both supplied and drained by venules and in the later stage an arterial supply is provided.[23-25] In general, it has been considered

that tumor vascular sprouts mainly occurred from postcapillary venules and venules. However, if tumor vessels originate from postcapillary venules or venules, it is impossible to explain why tumor blood flow is increased significantly even in the early stage by angiotensin II, that affects arterial vessels.

There are two problems with reports that tumor vessels originate from vessels in the venular side. The first problem is that those findings are based on vital microscopic observation carried out at low magnification (×40). It is difficult to distinguish a new capillary with a diameter of less than 10 μm at that magnification. When tumor growth within the transparent chamber was observed with a 40 power microscope, it appeared as if numerous sprouts originated from the host venules and grew toward the center of the tumor implant. However, we have observed at high magnification that almost all of those vessels were from post capillary venules modified by the tumor.

The second problem is that there are very few reports which analyze the vessel geometry and network branching pattern in normal tissue prior to tumor implantation. Therefore, identification of vessel types is ambiguous. Vascular diameter itself in a point of time, particularly during the tumor neovascularization process, is not a sufficiently precise criterion for classifying each vessel. Identification of vessel types should be based primarily on the location of the vessel in the vascular bed as a whole. In the tumor neovascularization process, the preexisting vessels are progressively modified by the existence of the tumor and are eventually incorporated into the tumor as the frame of the tumor vascular network. Furthermore, the entire vasculature of a growing tumor



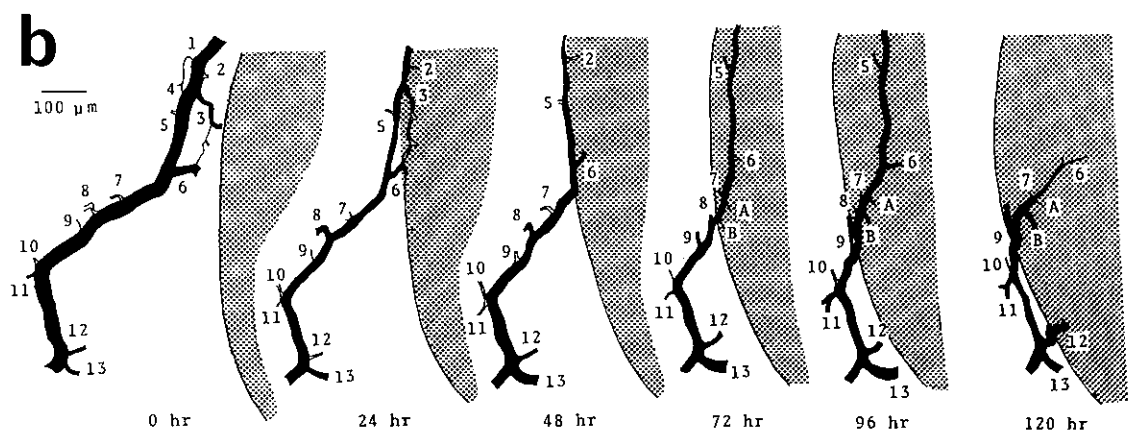


Fig. 9. Modification of venule near the periphery of the growing tumor. a: tumor (SLC) within the transparent chamber. Each print was made with a 40 power magnification and the prints were assembled into a photomontage. A venule designated by arrows was photographed at high magnification (×400) and its branching points numbered in order as shown in b. b: daily changes of venular branching points. Vascular branching points A and B are newly formed ones. However, the majority of branching points were compressed by the growing tumor.

is subject to continuous change. Ide *et al.*[8] reported in 1939 that the host microvasculature is modified and involved in the vascularization of tumors. In recent years, it has been reported that small arteries adjacent to a tumor nest are also modified as the tumor grows.[26] This is the reason why it is difficult to use vessels as landmarks. When tumor vascularization was active, we had to observe the process frequently. If we did not do so, we could not trace the morphological change of each vessel.

In order to overcome these two problems, we fully analyzed the vascular pattern of normal subcutaneous tissue within our transparent chamber and observed the complete process of tumor-induced neovascularization at high magnification (400×). We observed at 3-h intervals, when new formation of vessels was most rapid, and when changes were slow, at daily intervals.

In the rat subcutaneous tissue, the vascular pattern seemed to be the same as those in the hamster cheek pouch reported by Lutz and Fulton,[27] and in the bat wing reported by Nicoll and Webb.[28] The branching order of the vessels in subcutaneous tissue could be numbered according to Strahler's nomenclature.[18] When a tumor was transplanted into the chamber, tumor vessels always sprouted briskly from the end point of vessels with branching order 2. Such a vessel is equivalent to a terminal arteriole. The position where tumor vessels originated from the preexisting vascular system was identical in 3 kinds of transplantable tumors, although they were different from each other in character. This fact implies that the reactions of tumor vessels to vasoactive drugs would also be identical, idependent of the type of tumor.

Eddy *et al.*[29] observed that a number of capillaries of various calibers arose from relatively short host arterioles, and they named this the "fountain" type of tumor blood supply. By measuring the branching point number per unit area of vascular systems, we demonstrated quantitatively that the areas where the fountain-type structures originate are in the center of the tumor vascular formation. In that area, blood velocity and vascular pressure are relatively high (K. Hori *et al.*, unpublished data). On the other hand, the frequency of new capillaries originating from veins and venules was very low. Furthermore, we can confirm Thoma's law[30] that the increase of blood pressure in capillary areas leads to formation of new capillaries, although the idea that induction of tumor vessels is caused by tumor angiogenesis factors[31, 32] has already been established as an experimental fact.

How is the tumor vascular network constructed? Next, we clarified how tumor vascular branching points are formed. The formation of intricate networks in the tumor occurred easily in an apparently haphazard way. We observed three different modes of branching point formation: sprouting, cross-connecting and splitting. New capillary sprouting seemed to occur only when two different vessels approached each other as if they were in contact. Although Folkman and Klagsbrun[32] describe in their recent review of the sequential events of capillary growth *in vivo* that the migrating endothelial cells elongate and align with one another to create a solid sprout and then two hollow sprouts join at their tips to form a loop, after which blood flow begins, we have never observed such a process in any of our 400 transparent chambers. The mode of blood vessel formation in the cornea (used by Gimbrone *et al.*[25]), which has no preexisting vessels, may be different from that in other vascularized tissues. Tumor vascular formation by the utilization of preexisting vessels occurs more easily than that from sprouting. When two tumor vessels crossed each other, the point of intersection easily fused into a new branching in a short time, and one vessel often separated into two vessels. Thus, tumor vessels seem to be more reactive than we had thought.

We believe that our findings in this report offer important morphological information for analyzing microhemodynamics in tumors and the mechanisms of tumor blood flow as modified by several vasoactive agents. We are now investigating how endothelial cells proliferate when preexisting vessels are modified. In a separate paper we will report the changes in vessel pressure of the "starting vessel" brought about by vasoactive drugs or by tumor growth.

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