Scale-Invariant Behavior and Vascular Network Formation in Normal and Tumor Tissue

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Tumor vascular networks look different from normal vascular networks, but the mechanisms underlying these differences are not known. By studying the scale-invariant behavior of normal and tumor vascular networks we show that vascular networks exhibit three classes of fractal behavior. Tumor networks display percolationlike scaling. Normal arteriovenous networks display diffusion-limited scaling, and normal capillary networks are compact structures. The mechanisms responsible for these differences are suggested using a growth model.

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The process of vascular network formation (angiogenesis) has been extensively researched over the last two decades [1]. Our understanding of angiogenesis on the molecular level has been significantly enhanced as at least 25 endogenous molecules stimulating or suppressing angiogenesis have been identified [2]. How the effects of these molecules come together to determine the shape of vascular networks, however, is not known. In particular, it is unclear why tumor vascular networks appear so different from normal vascular networks [3], although presumably the same growth factors and inhibitors are involved in their formation. Furthermore, even under normal conditions it is unclear how a space-filling capillary network evolves, since angiogenesis is driven by growth factor diffusion and hence diffusion-limited structures are to be expected. Knowledge of the determinants of vascular network growth and shape formation would be useful in designing interventions that modify angiogenesis.

Fractal analysis has been applied sporadically to the study of vascular networks with little meaningful results. However, measurement of the scale-invariant properties of vascular networks is potentially useful in revealing the determinants of vascular network formation because different statistical growth processes yield structures with different fractal dimensions. In particular, fractal analysis can separate Laplacian, local, and compact growth processes [4]. In view of these potential benefits we carried out the first systematic analysis of the scale-invariant properties of different types of vascular networks.

We measured fractal dimensions of a variety of normal and tumor vascular networks grown in mice bearing dorsal skinfold chambers [5]. Two symmetrical titanium frames were implanted so as to sandwich the extended double layer of skin perpendicular to the animal's back, thus creating a transparent quasi-two-dimensional compartment. In one circular area of 15 mm in diameter, one layer of skin was completely removed and the remaining layer was covered with a coverslip incorporated into the frames. After a 24 h recovery period, 2 μl of a dense tumor cell suspension from cell culture ($\sim 2 \times 10^5$ cells) were inoculated onto the striated muscle of the transparent window area [5]. Nude mice were used to grow human colon adenocarcinoma LS174T. C3H mice were used for Sa1 murine sarcoma, SCC7 murine squamous cell carcinoma, and MCaIV murine mammary carcinoma cells. For quantification of normal vascular networks two preparations were used. First, the normal subcutaneous vascular networks in nude mice dorsal chamber preparations with no intervention were studied. Second, whole femora from newborn nude mice were implanted onto the upper tissue layer of the chamber in nude mice dorsal chamber preparations [6] and the bone-induced vascular networks were observed. Images of vascular networks were obtained when tumors were approximately 4 mm in diameter (9–16 day old) using an intravital microscope [5,6]. Images were analyzed and converted into a binary skeletonized form.

A skeletonized image of each type of vascular network is shown in Fig. 1. Fractal dimensions of the skeletonized images were measured using the box-counting and sandbox algorithms [7]. Fractal dimension measurements of normal arteriovenous subcutaneous vascular

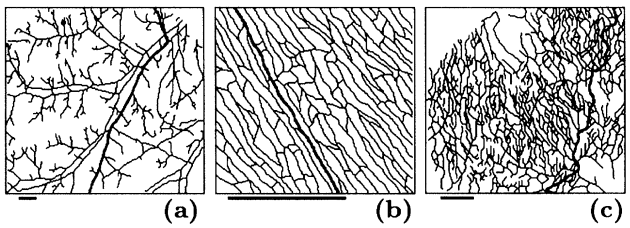

FIG. 1. Typical skeletonized images of the three observed classes of vascular networks. (a) Normal subcutaneous arteriovenous network; (b) normal subcutaneous capillary network; (c) LS174T tumor network. The minimum path is in bold. The bars are 500 μm.

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networks ($n = 12$) yielded $d_{box} = 1.70 \pm 0.03$ and $d_{sand} = 1.70 \pm 0.03$; normal bone-induced arteriovenous networks ($n = 10$) yielded $d_{box} = 1.65 \pm 0.04$ and $d_{sand} = 1.66 \pm 0.05$. In normal subcutaneous capillary networks ($n = 12$) the fractal dimension was $d_{box} = 1.99 \pm 0.01$ and $d_{sand} = 1.97 \pm 0.01$. In tumors, where arteries, capillaries, and veins cannot be distinctly classified [3], measurements of LS174T vascular networks ($n = 12$) in nude mice yielded $d_{box} = 1.88 \pm 0.04$ and $d_{sand} = 1.89 \pm 0.04$. To test the generalizability of these results, fractal dimensions were measured in C3H mice implanted with three other tumor lines ($n = 3$ for each). All results fell within the same range of fractal measurements as in the LS174T tumors (Fig. 2). We characterized these vascular networks further by calculating the minimum-path dimension [8] d_{min}, which can be considered a measure of the efficiency of propagation through the network (in biological terms, it measures tortuosity of the vessels in the network). In this context, we hypothesized that, due to their tortuosity, tumor networks would have a higher d_{min} than normal networks. In support of our hypothesis we found the minimum-path dimension of normal arteriovenous networks to be $d_{min} = 0.99 \pm 0.02$; normal capillary networks $d_{min} = 1.00 \pm 0.02$; and tumor networks $d_{min} = 1.10 \pm 0.04$, significantly higher ($p < 0.0001$) than normal networks.

The measurements in the normal arteriovenous networks are in concert with the fractal dimensions of two-dimensional diffusion-limited aggregates ($d_f = 1.71$ and $d_{min} = 1.00$) [4,9] and with previously published fractal measurements of retinal arterial and venous networks [10,11]. These results seem consistent with the accepted view of the angiogenic process, where growth factors initially diffuse from hypoxic regions and induce growth.

However, experimentally, angiogenesis does not seem to occur on the artery-vein level. Rather, vascular growth occurs at the capillary or postcapillary level. Vascularization studies [12,13] have shown that newly formed capillaries grow in a compact mesh and not in a treelike structure, contrary to the expected structure in diffusion-limited growth. Our measurements corroborated these observations by showing that the normal subcutaneous striated muscle capillary bed is a compact structure. Therefore, there seems to be a contradiction between the accepted view of growth by chemotaxis (gradient-sensitive growth) and the compact structure of the normal capillary bed.

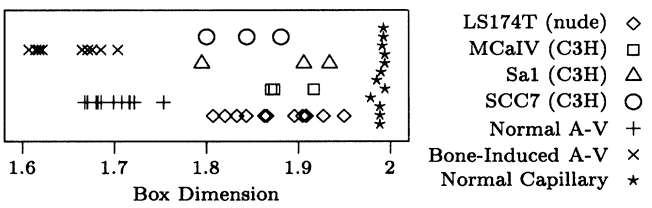

FIG. 2. Fractal dimension of the observed vascular networks as measured with the box-counting algorithm. Box-size range in which networks displayed scale-invariant behavior was approximately 50–900 μm. Results with the sandbox algorithm were similar.

To explain this apparent contradiction we need a mechanism that causes growth factor concentration to increase throughout the growth perimeter, thus "masking" the diffusion field and promoting more uniform gradients and growth. The source of growth factors near the growth perimeter could either be the hypoxic tissue [14] or the growing structure itself. If the source is solely the hypoxic tissue (i.e., points far away from the growing structure), high growth factor concentrations near the growth perimeter could be achieved provided that the rate of growth factor reception or removal at the growth perimeter is slow compared to the diffusion rate. This is tantamount to a *low interaction probability* between growth factors and the growing structure [15]. If, however, the main source of growth factors near the growth perimeter is the growing structure itself, then at each growth site we have a *local amplification* of growth factor that then propagates to neighboring sites. Biologically, local amplification of growth factor levels can be achieved by the autocrine release of growth factors [16]. It is tantamount to a process where a growth event is accompanied by an increasing uniformity of growth probability in sites neighboring the growth site.

We compared both hypotheses for capillary network formation by constructing a simple growth model. The growth model incorporated diffusion of growth factor and vascular growth in response to growth factor reception, in the presence of either a low interaction probability or local amplification. The growth model was implemented according to the following rules. Growth begins at a single central seed. Growth factor "particles" diffuse from points removed at least some minimum distance from the structure. When a "particle" hits the growing structure it is taken up with a preset probability p_i. All uptaken particles are recorded as "hits" within a fixed time period. At the end of the period growth occurs at all hit sites. If there is local amplification, F additional particles are released at each growth site. F is the "local amplification factor." The model was implemented on a 128×128 square lattice with periodic boundary conditions.

Model results (Fig. 3) show that either the low interaction probability or the local amplification mechanisms, if strong enough, can lead to the formation of a compact capillary network. However, when one compares the growth time per unit mass and the growth efficiency (defined as the total amount of growth factor originating from the hypoxic tissue divided by the mass of the final structure) of both processes (Fig. 4), one sees that growth in the presence of local amplification is an order of magnitude faster and more efficient than growth in the presence of a low interaction probability. Thus, it seems reasonable

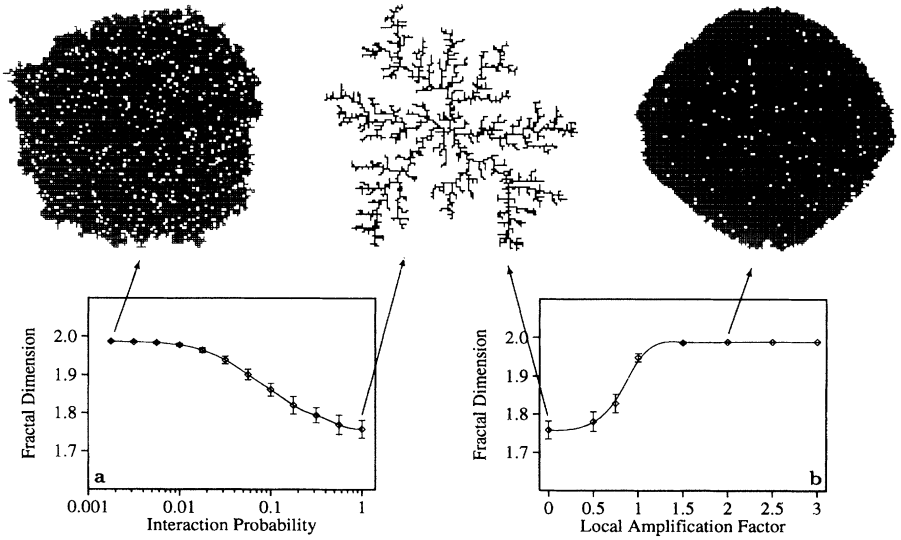

FIG. 3. Effects of (a) low interaction probability and (b) local amplification on fractal dimension.

to suggest that local amplification, which corresponds to biological autocrine mechanisms of growth factor release, is a possible key determinant of the observed compact shape of normal capillary networks.

The fractal measurements in tumor vasculature show that the tumor vessels do not form a compact structure, but are consistent with measurements for the critical percolation cluster ($d_f = 1.896$ and $d_{\min} = 1.13$) [17,18]. This observation represents the first evidence for a biological growth process whose likely determinants are local properties. Tumor vascular networks, like percolation clusters, are characterized by loops and voids of many different length scales (see Fig. 1 and Ref. [19]). Percolation being a local growth process [4] suggests that there exists some local property that determines tumor capillary growth. This local property is hypothesized to be extracellular matrix (or substrate) inhomogeneity, which has two biological foundations: (a) tumor tissue does not possess the regular periodic geometry formed by cells in normal tissue, and has different phenotypic subpopulations; (b) extracellular matrix is a larger and more heterogeneous component of tumor tissue than normal tissue [20,21].

In order to test this hypothesis we modified the previous model so that a randomly selected subset of all sites becomes inaccessible to growth. Each lattice site is assigned a random number R in the range [0,1]. Growth occurs at all particle reception sites where $R < T$, where T is a preset number in the range [0,1] and represents the fraction of lattice sites that are accessible to growth. If no site with $R < T$ is available for growth, growth occurs at the site with the lowest R ($R > T$).

The model results (Fig. 5) emphasize two points. First, an increasing local amplification factor (F) is required to achieve a given fractal dimension as T decreases. Theoretically, below the site-percolation threshold for a square lattice ($T < 0.6$), a compact structure cannot be achieved even as $F \to \infty$. In reality, F is finite and not every

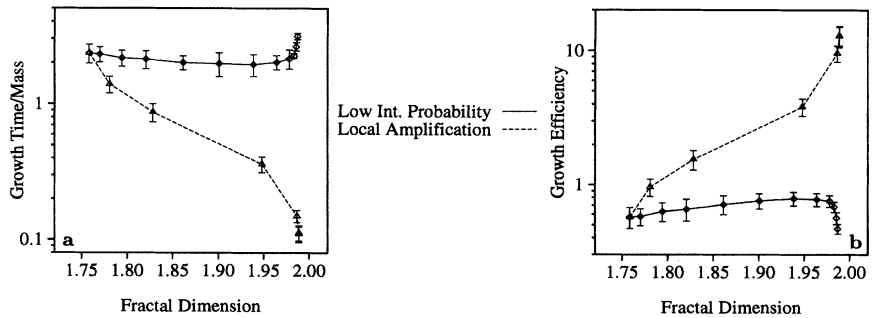

FIG. 4. Comparison of the local amplification and the low interaction probability mechanisms: (a) growth time per unit mass and (b) growth efficiency. Clearly, growth with the local amplification mechanism is both faster and more efficient as the fractal dimension nears 2.0.

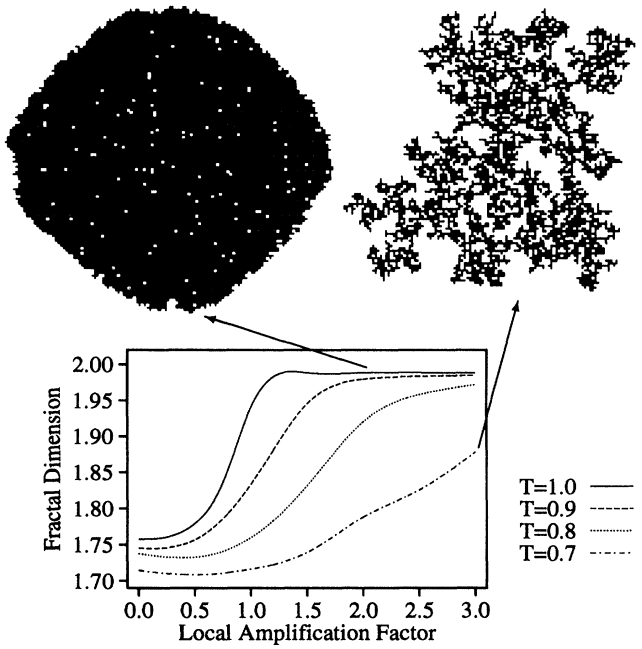

FIG. 5. Effect of substrate inhomogeneity on fractal dimension. The different curves represent different degrees of inhomogeneity as represented by the accessibility parameter T. Error bars were omitted for clarity. Note that the line with $T = 1$ is the same line as in Fig. 3(b).

lattice site is necessarily explored at each growth cycle so that for $F = 3$, for example, $T \leq 0.7$ is sufficient to ensure a percolation-cluster type of vasculature. Second, we see that without local amplification, the structure remains diffusion limited with $d_f \approx 1.75$, relatively insensitive to variations in T. This observation further emphasizes the importance of the local amplification mechanism in normal and tumor vascular network formation. Figure 5 demonstrates that in the presence of local amplification and more than 20% inaccessibility, a noncompact structure reminiscent of tumor vasculature is obtained.

These results support our hypothesis that extracellular matrix inhomogeneity in tumors is indeed responsible for the architecture of tumor vasculature. These results imply that in order to modify tumor vasculature significantly, the underlying substrate properties must also be modified. Recent in vivo studies [22] on the control of angiogenesis by manipulation of the local composition of the extracellular matrix support this conclusion.

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