Vascular Morphogenesis and Remodeling in a Human Tumor Xenograft

Blood Vessel Formation and Growth After Ovariectomy and Tumor Implantation

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Abstract—To determine mechanisms of blood vessel formation and growth in solid tumors, we used a model in which LS174T human colon adenocarcinomas are grown in the isolated ovarian pedicle of nude mice. Reconstruction of 3500 histological serial sections demonstrated that a new vascular network composed of venous-venous loops of varying sizes grows inside the tumor from the wall of the adjacent main vein. Loops elongate and remodel to establish complex loop systems. The mechanisms of loop formation and remodeling correspond to intussusceptive microvascular growth (IMG). In the tissue surrounding the tumor segmentation, another mechanism of IMG is prevalent in venous vessels. Comparison to vascular morphogenesis in the ovariectomized pedicle not only confirms the existence of corresponding mechanisms in both systems, but also reveals numerous sprouts that are superimposed onto loop systems and pathological deviations of loop formation, remodeling, and segmentation in the tumor. These pathological mechanisms interfere with vessel patency that likely cause heterogenous perfusion and hypoxia thus perpetuating angiogenesis. Blood vessel formation based on IMG was also detected in a large thrombus that completely occluded a part of an ovarian artery branch. (Circ Res. 2001;89:732-739.)

Key Words: angiogenesis • colon adenocarcinoma (LS174T) • endothelial cell • intussusceptive microvascular growth • restenosis

The formation of sprouts in angiogenesis has been widely accepted as a basic mechanism of angiogenesis in wound healing and tumors. Another mechanism of vascular morphogenesis termed intussusceptive microvascular growth (IMG) has been identified in many organs and different species. Intussusception involves growth and remodeling of the vascular system based on partitioning of the vessel lumen by columns of tissue, called tissue pillars or posts (diameter <2.5 μm) and interstitial or intervascular tissue structures (ITs, diameter >2.5 μm). Evidence for IMG in pathological states comes from findings that intussusception is induced by tumor ascites fluid in peritoneal lining tissues. It has also been suggested that the remodeling of vascular branching points during tumor angiogenesis be implemented by IMG. In the first in vivo documentation of IMG in solid tumors, we demonstrated basic mechanisms of its implementation and its coexistence with sprout-like structures. Our data showed that the network architecture in tumors changes on a time scale of minutes based on frequent remodeling by IMG. This might explain intermittent blood flow.

In the present study, we analyzed histological serial sections to reconstruct the architecture of the vascular network of human colon adenocarcinomas (LS174T) transplanted onto the isolated ovarian pedicle of nude mice. Two new mechanisms of blood vessel formation based on IMG were identified: in situ loop formation and remodeling, which give rise to a new vascular network inside the tumor and segmentation that expands and remodels the preexisting network outside the tumor. In comparison with IMG in the healing ovariectomized pedicle, tumor vascularization exhibits pathological variations of these mechanisms and sprout-like structures that are superimposed onto many loop systems.

Materials and Methods

Animals and Tumor Model
Tumors were grown in NCrSed–nu/nu athymic mice, and tumor cells were transplanted to the ovarian pedicle after ovariectomy, as previously described. Two new mechanisms of blood vessel formation based on IMG were identified: in situ loop formation and remodeling, which give rise to a new vascular network inside the tumor and segmentation that expands and remodels the preexisting network outside the tumor. The mice were bred in the animal facility of the Edwin L. Steele Laboratory (Massachusetts General Hospital). The animals were treated according to the National Institutes of Health guidelines.

Perfusion Fixation
Perfusion fixation of the ovarian pedicles was performed, as described. In total, 30 tumors were fixed by this procedure and
partially or totally dissected into sequential serial sections used for reconstruction of the vascular networks. In one tumor, 10 loop systems and around 60 ITSs and tissue pillars were reconstructed from 3,500 serial sections.

**Tissue Preparation for Light Microscopy**

After perfusion, the tumors were removed and postfixed in the same fixative overnight. Specimens were embedded in resin (Historesin, Leica) and paraffin according to manufacturers’ protocols. Serial sections of resin (2 μm thick) and paraffin (3 μm thick) were cut. Resin sections were stained with toluidine blue; paraffin sections were labeled with rat–anti-mouse PECAM antibody (Pharmingen) according to manufacturer’s protocol. The antigen was visualized using the ABC peroxidase technique (Vector). Sections were viewed with an Olympus BX 40 microscope. Arterial and venous vessels were distinguished based on histological criteria.

**Results**

**Time Course of Vascular Morphogenesis and Remodeling**

Vascular morphogenesis and remodeling in the LS174T tumors were studied for 3 weeks, specifically around day 7 after tumor cell implantation, since in this early phase all stages of network formation, growth, and remodeling coexist. Information concerning the onset of the remodeling and growth processes that lead to the formation of new blood vessels and the further development of the vascular network can be found in the online supplementary information (see online data supplement available at http://www.circresaha.org).

**Elementary Loops Originating From the Ovarian Vein**

Elementary loops at varying stages of their formation were detected in the wall of the ovarian vein in all tumors studied (Figure 1AA). Figures 1A through 1L illustrate elementary loops. Elementary (single) loops have the following structure: the venous lumen evacuates around an ITS core located within the intervening vessel wall to isolate a free column of tissue, the ITS, which is then surrounded by the evagination, which forms a short elementary loop (Figures 1B and 1E). The bottom and the top of the ITS remain connected to the venous wall, which forms a fold that projects into the vessel lumen (Figures 1A and 1D and 1C and 1F).

The stages of loop formation and growth are illustrated by three structures of different sizes: a tissue pillar, a small ITS, and a large ITS, in Figures 1G through 1L. This implies that elementary loop formation begins with retraction of the endothelium around tissue pillars. The figure also shows that small loops can be added to each other or to larger ones forming compound loop systems (see below). Interestingly, the large elementary loop depicted in Figures 1G through 1L is not yet completely patent. It thus represents a precursor stage to the type of elementary loop illustrated in Figures 1A through 1C and 1D through 1F. Another ITS depicted in Figures 1D through 1F is also not yet completely separated from the venous wall. However, this structure is already undergoing remodeling by splitting into several parts as indicated by two holes filled with erythrocytes within its center, which, by themselves, form a loop system.

**Systems of Longer Loops Derived From the Ovarian Vein**

 elementary loops can remodel, forming longer and more complex loop systems. Four such systems are shown in Figures 2 through 4. Figures 2 and 3 are photomicrographs of serial sections of loop 1 and a part of loop 4. Figures 4A through 4D give a schematic illustration of all four systems, and online Figure 2 is a computer reconstruction of loop 1. As is evident from the figures, these loop systems frequently form double, triple, and quadruple loops, etc, since they possess multiple connected segments. They are therefore termed compound loop systems. The latter are interconnected, forming a new vascular system inside the tumor.

In the following, the structural details of typical examples of loop systems are presented. They exhibit varying degrees of complexity. Systems 3 and 4 (Figures 4C and 4D) also consist of a more pathological structure, since they have disconnected segments. Additionally, in the tumor, blind-ending sprout-like structures are included with many loop systems.

**Loop 1**

Loop 1 (Figures 2 and 4A and online Figure 2) forms a double-loop system that is supplemented with three blind-ending sprout-like structures and a second short loop containing an ITS (diameter ∼12 μm). It consists of a segment (a) that is derived from the main vein and reconnects to the latter through two other segments, (b) and (c), Figures 2G through 2N and 2P through 2U. One of the connecting segments (b) also links to segment (b) of loop system 4, two intraluminal folds decreasing the patency between these segments (Figures 2L through 2K). Furthermore, segment (a) of loop 1 is connected to loop 2, forming another short loop.

**Loop 2**

Loop 2 (Figure 4B) corresponds to a “quadruple-loop” system composed of five completely connected segments: segment (a) originates from the main vein and reconnects to it via two other segments, (b) and (c), two additional reconnecting segments derive from branch (b). Segment (a) of loop 2 also forms a small “double” loop with two segments of loop 1 and loop 3, respectively. Several blind-ending sprout-like structures are superimposed on the basic segments of this loop system. One tissue pillar (diameter ∼2 μm) and three large ITSs (diameters ∼28, ∼38, and ∼44 μm) were detected.

**Loop 3**

Loop 3 (Figure 4C) corresponds to a disconnected double-loop system. It consists of one blind-ending sprout-like structure (a) and a single long loop (b). (a) connects to loop 2 within its area of origin from the ovarian vein, thus forming an elementary loop. It lies close to single loop (b). If both vessels were connected, they would form a double-loop system (comparable to that illustrated in Figure 4A), but this connection either is not yet established or, if it previously existed, it has been lost.

**Loop 4**

This system (Figure 4D) forms another example of a pathological loop system, consisting of a double-loop system.
disconnected by a single intraluminal cell. It is composed of a complete loop (b-c) derived from the main vein. It also consists of a single segment (a) that originates from the main vein and runs so close to the loop (b-c) that only a single intraluminal cell prevents their connection. Except for the interruption of a single intraluminal cell, this structure resembles that of loop 3, and, if both segments had connected, would resemble the structure of loop 1, thus forming a
double-loop system (Figures 3A through 3D). Additionally, the first part of the loop (b-c) is connected to loop 1 (b), although an intraluminal fold decreases the patency of this connection (Figures 2I through 2K). Loop system 4 also contains a tissue pillar (diameter ≈ 2 μm), an ITS (diameter ≈ 12 μm), and several blind-ending sprout-like structures superimposed onto the basic segments of the loop system.

Incidence Between Loops and Sprouts
For more detail, see the online supplementary information.

Segmentation in the Connective Tissue
Segmentation in the connective tissue surrounding the tumor also displayed a pathological variation, similar to loop formation and remodeling (see online supplementary information).

IMG During the Recanalization Process of a Large Thrombus
In the main ovarian vessels (artery and vein) of some tumors, large thrombi were detected. The present thrombus located in the artery occluded the entire lumen and was undergoing recanalization by IMG (online supplementary information).

Discussion
Vascular Morphogenesis Occurs by Different Mechanisms
Our data demonstrate that vascular morphogenesis in our LS174T human colon adenocarcinoma xenograft starts in venous vessels of all sizes, which is consistent with previous reports on tumor angiogenesis22-23 (online Figures 1A and 1B). Receptors for vascular permeability factor/vascular endothelial growth factor (VPF/VEGF) are expressed predom-
inantly in venules and small veins surrounding tumors where we observed segmentation by IMG (online Figures 4 and 5). Correspondingly, it has recently been reported that VEGF is produced in the LS174T human colon adenocarcinoma xenografts. Additionally, we have shown that large veins close to the tumor respond to the angiogenic stimulus by loop formation, similar to the situation in the ovarian pedicle in tissue repair. This pattern is confirmed by intravital microscopy of the same tumor grown in the dorsal skin fold chamber (online Figure 1C). Although our model consists of a tissue repair component (the ovariectomized pedicle), loop formation from larger marginal veins has also been observed in tumor xenografts grown at other sites and in spontaneously evolved human tumors (Patan et al, unpublished data).

Interestingly, vascular morphogenesis starts in small venous vessels that become surrounded by tumor cell aggregates. This is combined with folding of the wall of the large ovarian vein and early ITS separation reflecting the onset of loop formation (online Figures 1A and 1B). Thus the network architecture at the beginning of blood vessel growth confirms the pattern detected by the more detailed analysis performed on day 7. The alterations of the network architecture over time suggest an increasing fragmentation based on tumor cell infiltration of blood vessels or their compression (online supplementary information). Interestingly, in the healing pedicle, PECAM-positive fibroblasts likely contribute to vessel walls to permit vessel growth at early stages. Later they invade the vessel lumen together with macrophages to induce vessel regression. In malignant tumors, tumor cells might have a similar role that, however, likely causes the observed pathological mechanisms of vessel growth and remodeling at early stages. Later on, the invasion of vessel lumens might increase the tendency to metastasize. (Patan et al, unpublished data).

“In situ loop formation” occurs as observed by intravital microscopy and in the healing pedicle by retraction of the endothelial lining of the lateral wall of the main vein around the core of an ITS. This forms two sheath-like expansions of the lumen that meet at the opposite side (Figures 5A and 5B). The ITS core is composed of extensions of periendothelial cells that encircle a bundle of collagen fibers and is formed in the vessel wall before or on ITS separation. The latter occurs by fusion of opposing endothelial cell membranes lining the evaginations to form a transcellular hole. The ITS remains connected to the lateral venous wall only at its bottom and its top (Figures 1A through 1L).

Thrombus recanalization can also be based on IMG, following another mechanism that includes the deposition of pillar cores between endothelial cells and lumen formation by cell detachment around these cores (online supplementary information).

Loop Remodeling Is Based on IMG and Causes Formation of Complex Loop Systems

Concurrent growth of the ITSs and elongation of the surrounding loop result in expansion of the elementary loops into the tumor (Figure 5C). During this process, elementary loops undergo further remodeling by IMG to form compound
loop systems. Loops become more complex by superposition of additional loops (Figures 1G through 1L) or by division of the ITS in the center of the loop (Figures 1D through 1F). Splitting of ITSs causes formation of additional loop segments between them (see loop systems 1 through 3, which form a very small triple-loop system through which their initial segments are connected, and Figure 5D). These new segments automatically reconnect, directly or indirectly, to the main vein (Figure 5F). An ITS located in the center of a loop can also split asymmetrically to separate a small tissue pillar (Figure 5E). Our ultrastructural analysis demonstrates that ITS splitting is depending on formation of pillar cores within the ITS. Splitting of ITSs has recently been detected by in vivo microscopy in the same tumor transplanted to the dorsal skin fold chamber of SCID mice and in the chicken chorioallantoic membrane (CAM). Pathological Variants of Loop Formation and Remodeling Are Detected in Tumors Although the tumor exhibits loops that are similar to those in the healing pedicle, they are less frequent, and the complexity of compound loop systems is reduced. In tumors, a single cellular extension projecting intraluminally can disconnect a loop system. This is shown by the close apposition but lack of patency between segments a-b in loop 4 (Figures 3A through 3D). The comparison with loop systems in the healing pedicle reveals that loops in tumors, but not in our model of tissue repair, exhibit pathological mechanisms of formation and remodeling.

Pathological Loop Remodeling Formation of intervascular walls that split vessels is an important mechanism of vascular growth and remodeling in embryonic development and is based on IMG (also Patan et al, unpublished data). In this process, a newly formed intraluminal fold connects to the opposite wall of a vessel to divide the lumen. The vessel remains patent, because the fold forms an ITS and does not occlude its entire diameter. In the tumor, insertion of transluminal tissue folds does not always cause formation of free ITSs but can disconnect loops and occlude vascular segments. This results in the formation of two long sprout-like structures that point toward each other (Figures 2I through 2K and 5G). In loop 1, segment (b), the existence of two transluminal folds that bridge the vessel lumen from opposite sides and nearly insert at the opposing vessel wall, supports this interpretation (Figures 2I through 2K). The analysis of serial sections reveals that these folds are part of larger ITSs and subsequently do not form free ITSs. The same mechanism has also been observed in tumor angiogenesis by in vivo microscopy where occlusion of vascular segments by insertion of folds was documented in real time.

Pathological Loop Formation The alternative explanation concerning the pattern of loop 4 would be that separation of the ITS located in the central part of the loop might occur extremely late in the tumor, when the ITS has grown to a large size (Figures 1D through 1F, 1G
through 1L, and 5H). This process of late separation of the central ITS could result in the structures shown in Figures 1D through 1F or 1G through 1L. In these nonpatent elementary loops, the relatively large ITS is still connected to the lateral venous wall in several places, even though the ITS in Figures 1D through 1F already exhibits signs of splitting, which indicates that loop remodeling has started. In the healing pedicle, the existence of completely patent loops suggests, comparable to the findings in the chicken CAM, that separation of the ITS normally occurs rapidly and while it is still small.

The observed pathological deviations of loop formation cause discontinuity of the loop systems that were absent in the healing pedicle. This might explain why the loop systems of the tumor xenografts exhibit an increasing fragmental character as observed over 21 days (online Figure 1F). However, we cannot exclude the possibility that endothelial sprouting occurs in addition to in situ loop formation, although we did not observe single tiny endothelial buds.

The Proposed Mechanisms of Vascular Morphogenesis Have Implications for Its Regulation

The connection of the newly formed vascular segments to the circulation is less well established in the tumor compared with the healing pedicle. This is documented by the existence of disconnected loops or loops that become patent late and also by pathological segmentation (online Figure 5). It characterizes one of the profound differences between blood vessel growth in the tumor xenografts and in the healing pedicle. This fact not only explains why the tumor circulation is heterogeneous, but why hypoxia, which is known to induce apoptosis and necrosis, is more severe and varying on a larger scale in tumors. Hypoxia itself

Figure 5. A through H, Mechanisms of loop formation and remodeling based on IMG. The vessel lumen is shown in red or yellow, respectively, and the surrounding tissue in blue. In situ elementary loop formation in the wall of the main vein based on lumen evagination around an intervening part of the vessel wall, which forms the ITS (A and B). Growth of the elementary loop is achieved by its elongation into the wall of the main vein and surrounding tissue and by growth of the central ITS (C). Loop remodeling causes “loop multiplication.” It occurs by splitting of the central ITS, which leads to the formation of a new segment that reconnects to the main vein (D, indicated by broken line). Loop remodeling can also occur by fold formation from the central ITS or from the lateral wall. The subsequent separation of the tip of the fold forms a free tissue pillar (E, indicated by broken line). Results of loop remodeling: loop remodeling results in the formation of compound loop systems, as double, triple, and quadruple loops (F). Pathological loop formation and remodeling: Sometimes loop patency is reached extremely late when the loop has reached a large size, based on the persistence of one intraluminal cell (see Figures 3 and 4D) (G). Loop remodeling by formation and insertion of intraluminal folds at the opposite vessel wall causes occlusion of the loop and loss of patency at a specific site (see Figures 2I through 2K) (H).
induces VPF/VEGF production and secretion by tumor cells34–36 and upregulation of its receptors.37

It thus appears that vessel growth in a malignant tumor could be a self-perpetuating process driven by hypoxia, whereas in tissue repair hypoxia gradually decreases as new loops form. In the latter, the process of vessel growth is thus clearly terminated causing vessel regression and transformation of the granulation tissue into a scar. In contrast, tumors are “wounds that do not heal.”38 Interestingly, gene expression in endothelial cells in tumor and wound-healing angiogenesis is very similar.39 Differences that cause pathological conditions are thus independent from endothelial gene regulation.

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Online supplementary information

1. Time course of vascular morphogenesis and remodeling in the LS174T human colon adenocarcinoma xenografts

For the present investigation it was important to distinguish between newly formed- and pre-existing vessels. Our criteria were based on a comparison with the normal ovarian pedicle that is surrounding the ovary. Vessel segments that were identified as parts of loop systems that could be traced back to the ovarian vein were considered “newly formed”. Mostly they were located within the tumor tissue. The same applied to sprout-like structures that were superimposed onto loop systems. Vessel segments that were located in the tissue around the tumor were considered “pre-existing”, they often showed signs of growth and remodeling based on IMG, as tissue folds and pillars or ITSs.

Tumor cells migrate from the injected cell mass to form aggregates around small veins which display signs of angiogenesis with tissue folds and pillars starting around 3 days after tumor cell injection. This corresponds to the mechanism of segmentation presented in greater detail below. The main ovarian vein is dilated, and exhibits a few small folds within its wall and a small ITS in its lumen representing early signs of loop formation (online Figures. 1A and B). Around day 7 the tumor aggregates have grown to a single compact tumor mass composed of numerous gland-like structures separated and surrounded by strands of connective tissue. A new vascular network composed of loops is growing from the ovarian vein at the tumor margin into the tumor. Some loop systems are superimposed with sprout-like structures (Figure 1AA, Sections 2 and 3, full text version).
This pattern corresponds to the one produced by the same tumor transplanted to the dorsal skin fold chamber where it is possible to view the tumor and its blood supply from outside. A network of connected loops of different sizes displaying an almost fractal similarity is evolving from a few large venous vessels located at the tumor margin (online Figure 1C).

In the pedicle, smaller venous vessels around the tumor grow by segmentation (Section 5). Importantly, both loop formation and segmentation display pathologic variants that decrease the patency of vessels (sections 2, 3, 4 and 5 full text version). Around day 14 increasing signs of tumor disorganization and infiltration of the surrounding tissue are visible. In many places, tumor cells break through the basement membrane surrounding the tumor glands and migrate in the connective tissue towards blood vessel walls (online Figure 1D). Blood vessels, so far located in the peripheral connective tissue, become surrounded and infiltrated by tumor cells. Additionally, tumor cell migration affects vessels inside the tumor. This can cause bleeding and the transformation of tumor ducts to blood channels and of “tumor glands” to blood lakes (online Figure 1E). Continuation of these processes forms a vascular network increasingly consistent of pathologic features around day 21. The analysis of serial sections reveals many discontinuous blood vessels that have two blind endings and form sausage-like structures. Other vessels are compressed, either by tumor cells or by a surrounding edema (online Figure 1F). Thus loop systems become more fragmental and are superimposed with an increasing number of sprout-like structures. These factors likely promote a heterogenous perfusion pattern in the tumor that displays large variations through time and might further be responsible for the regression of parts of the vascular network followed by apoptosis and necrosis of tumor cells.

2. Quantitative relationship between loops and sprout-like structures
In the five loop systems (loop 1-4 and the small compound system, loop 1-2-3) presented in section 3 we observed 18 loops and 17 sprout-like structures superimposed onto the loop systems. The quantitative relationship is given in online Figure 3. In four other loop systems (loop 6-9) analyzed the relationship was similar 22 loops and 19 sprouts (online Figure 3).

3. Segmentation in the connective tissue surrounding the tumor

Online Figures 4 and 5 illustrate two examples of segmentation which is the main mechanism of vascular morphogenesis in the tissue around the tumor. The lumen of a flat sinus-like vein contains numerous folds, ITSs and tissue pillars (online Figure 4A). These folds are connected in the center of the lumen thus forming intervascular walls that divide the lumen into different segments (online Figure 4B-F). The walls split to give rise to ITSs and tissue pillars. Fourteen vessels out of 41 were undergoing segmentation in the pedicle illustrated. Online Figure 5 shows a similar pattern. However, in this case the newly formed segments form non-perfused, “pockets” with blind endings (online Figures 5A-H).

4. IMG during the recanalization process of a large thrombus

In one tumor, a large thrombus completely occluded a part of a large branch of the ovarian artery (online Figure 6A). The intima was ruptured with bleeding into the media and disorganization of the wall structure. Cells from the media, which was sparse of cellular elements, invaded the intima causing it to grow to several cell layers (online Figures 6A-B). In the intima cores of ITSs and tissue pillars composed of coagulated blood and fibrin were deposited (online Figure 6B). Between these cores, endothelial cells detached from each other to give rise to a new lumen
Based on this mechanism, a ring of connected sinusoidal vessels formed around the thrombus replacing the former intima of the vessel wall (online Figure 6D).

Legends to online Figures

Online Figure 1: Vascular morphogenesis in the ovarian pedicle (day 3-21) (A-B, D-F). Early stage (day 3), tumor cells migrate in the connective tissue of the pedicle (red arrowheads) around small veins that exhibit tissue folds within their lumens (red arrows). A few folds are also visible in the wall of the large ovarian vein (black arrows) and a small ITS within its lumen (black arrowhead). Large dark dots in the tissue correspond to fat cells (A). Tumor cells migrate around smaller veins (arrows) and form tiny aggregates (red asterix, B). Dorsal skin fold chamber of nude mice: view “from top” onto a similar vascular network of connected loops of varying sizes derived form three large veins at the tumor margin (arrows) in a LS174T human colon adenocarcinoma (day 10, C). Day 14, tumor cells become spindle-shaped, lose contact to their neighbor cells, leave the “tumor glands” and migrate into the surrounding connective tissue of the ovarian pedicle (red arrows) towards blood vessels (black asterix). Macrophages (red arrowheads) surround tumor cells (red arrows), erythrocytes and edema are visible (D). Tumor glands form blood lakes filled with blood cells. Single tumor cells become spindle-shaped and break through the basement membrane surrounding the glands (arrows, E). Day 21, anti-PECAM labeling of endothelial cells (brown), hematoxylin counterstaining. Many vessels inside the tumor are compressed by tumor cells (black arrows) or edema (red arrow). Analysis of serial sections shows that numerous vessels are also discontinuous, forming structures with blind endings (black arrowheads), and invaded by tumor cells. Areas with necrotic and apoptotic
tumor cells are visible (red arrowheads, F). Bar = 204 µm, A; 63.75 µm, B; 408 µm, C; 32 µm, D, E; 127.5 µm, F.

**Online Figure 2:** Three dimensional computer reconstruction of photomicrographs of compound loop system, Loop 1 (illustrated from four perspectives, see also Figs. 2 and 4A). The system is depicted in two rotational planes, the anterior and posterior aspects of the 3-dimensional image are indicated as light and dark colored regions. The double images produce a stereo-image when viewed at the right distance. The computer reconstruction of vascular networks from serial sections was performed as described.¹

**Online Figure 3:** Distribution of loops and sprout-like structures that are superimposed onto loop systems in 9 loop systems in the LS174T tumor xenografts on day 7.

**Online Figure 4A-F:** Vessel segmentation (serial sections 5048, 5026, 5018, 5020, 5022, 5024). The lumen of smaller veins outside the tumor is divided by folds that are connected within its center forming intervascular walls (A) in a spoke-like pattern (arrows in C-F). These walls also split to give rise to ITSs and tissue pillars (arrowheads in B, D). Corresponding structures are labeled with the same sign. Bar = 80 µm, A; 40 µm, B; 20 µm, C; 13 µm, D-F.

**Online Figure 5A-H:** Pathological vessel segmentation (serial sections 5012, 5018, 5026, 5028, 5038, 5046, 5080, 5088) resulting in formation of blind ending pocket-like structures. Intraluminal folds connect in the center of the lumen to form large intervascular walls (arrows, A-E) that expand throughout several micrometers of the vertical diameter of the lumen and exclude segments 1-3 from the vessel lumen (D-H). Segment 1 ends between (D) and (E), the end of segment 2 is visible in (F), and segment 3 disappears after (G). Bar = 45 µm.
Online Figure 6A-D: Recanalization of a thrombus that completely occludes a segment of the main artery (serial sections 6384, 6380, 6320, 6636). Folds and ITSs are composed of thrombus matrix (arrows in B, A-B). The intima is several cell layers thick based on cell invasion from the media (arrowhead in A and D) which is devoid of cellular elements. Between these intimal cells new lumens are formed (arrows) with ITS cores in between (C). This leads to formation of a ring of newly formed vessels, which surrounds the thrombus to bypass the stenosis (D). Bar = 80 µm, A,D; 40 µm, B-C.

References:


Incidence between loops and sprout-like structures superimposed onto loop systems