A New Mechanism for Pillar Formation during Tumor-Induced Intussusceptive Angiogenesis

Inverse Sprouting

Sándor Paku,* Katalin Dezső,* Edina Bugyik,*
József Tóvári,[†] József Tímár,[‡] Péter Nagy,*
Viktoria Laszlo,[§] Walter Klepetko,[§] and
Balázs Döme^{§¶}

From the 1st Institute of Pathology and Experimental Cancer Research and the 2nd Department of Pathology,[‡] Semmelweis University, Budapest, Hungary; the Department of Experimental Pharmacology,[†] National Institute of Oncology, Budapest, Hungary; the Department of Thoracic Surgery,[§] Translational Thoracic Oncology Laboratory, Medical University of Vienna, Vienna, Austria; and the Department of Tumor Biology,[¶] National Koranyi Institute of Pulmonology, Budapest, Hungary*

One of the hallmarks of intussusceptive angiogenesis is the development of intraluminal connective tissue pillars. The exact mechanism of pillar formation has not yet been elucidated. By using electron and confocal microscopy, we observed intraluminal nascent pillars that contain a collagen bundle covered by endothelial cells (ECs) in the vasculature of experimental tumors. We proposed a new mechanism for the development of these pillars. First, intraluminal endothelial bridges are formed. Second, localized dissolution of the basement membrane occurs and a bridging EC attaches to a collagen bundle in the underlying connective tissue. A pulling force is then exerted by the actin cytoskeleton of the ECs via specific attachment points, which contain vinculin, to the collagen bundle, resulting in suction and subsequent transport of the collagen bundle into and through the vessel lumen. Third, the pillar matures through the immigration of connective tissue cells and the deposition of new collagenous connective tissue. The proposed simple mechanism generates a connection between the processes of endothelial bridging and intussusceptive angiogenesis and identifies the source of the force behind pillar formation. Moreover, it ensures the rapid formation of pillars from pre-existing building blocks and the maintenance of EC polarity. To describe it, we

coined the term inverse sprouting. (*Am J Pathol* 2011, 179:1573–1585; DOI: 10.1016/j.ajpath.2011.05.033)

Angiogenesis is the formation of new blood vessels from pre-existing ones. Several different forms exist,¹ but so far endothelial sprouting^{2,3} and intussusceptive angiogenesis^{4–8} have been investigated the most intensively.

Endothelial sprouting is characterized by the parallel migration of capillary bud endothelial cells (ECs). During this process, proliferating ECs maintain their basal-luminal polarity and form a slit-like lumen that is continuous with the lumen of the so-called mother vessel. Basement membrane material is deposited continuously by the sprout ECs, whereas only the tip of the growing bud is in contact with the collagenous connective tissue matrix. As the final step, proliferating pericytes of the mother vessel migrate along the basement membrane of the sprout, resulting in the maturation of the new vessel.²

In contrast to endothelial sprouting, the other major angiogenic mechanism, intussusceptive microvascular growth, or intussusceptive angiogenesis, which has been described in a wide variety of normal and pathological conditions, is faster and does not depend primarily on EC proliferation. The most characteristic feature of intussusceptive angiogenesis is the insertion of connective tissue columns, called tissue pillars, into the lumen and the subsequent growth of these pillars, resulting in partitioning of the vessel lumen and the consequent increase in the density of the given capillary network. According to

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Address reprint requests to Sándor Paku, Ph.D., 1st Institute of Pathology and Experimental Cancer Research, Semmelweis University, 1085 Üllői út 26, Budapest, Hungary. E-mail: paku@korp1.sote.hu.

the current view, the development of tissue pillars is preceded by the formation of vessel wall folds or the protrusions of the opposite points of the vessel wall into the lumen.^{4–8} However, the origin of the force generating these invaginations has not yet been clarified. Although it is believed that perivascular cells or pericytes may play a role in this initial step by exerting a pushing force on the vessel wall, this concept is questionable because the structure of the cellular cytoskeleton allows only pulling forces at high strength, whereas pushing forces are several hundredfold lower in magnitude.^{9,10}

Another phenomenon thought to be different from intussusceptive angiogenesis, but also leading to vascular division, was described as well. This process is characterized by the development of intraluminal bridges formed by ECs, followed by the development of connective tissue by an unknown mechanism within these bridges.^{11–13}

Based on our observation of the vascularization of s.c. growing tumors in mice, we present herein the detailed mechanism of intraluminal pillar formation, which offers a rationale for the puzzles previously discussed and incorporates the previous two concepts.

Materials and Methods

Animals and Tumor Lines

The C38 mouse colorectal carcinoma line was maintained by serial s.c. transplantations in C57Bl/6 mice, as previously described.^{14,15} Tumor tissue was cut into cubes measuring 5 × 5 × 5 mm. Animals were anesthetized with ketamine, 80 mg/kg, and xylazine, 12 mg/kg (Sigma Chemical Co, St Louis, MO); one piece of tumor tissue was transplanted into the back of each mouse. Animals were sacrificed 3 weeks after tumor inoculation. For analysis of immunofluorescence labeling with monoclonal antibodies, the tumors were transplanted into mice with severe combined immunodeficiency to reduce non-specific staining.

HT25 human colon carcinoma cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (Sigma Chemical Co). The s.c. tumors were produced by injecting 2 × 10⁶ tumor cells into the back of anesthetized male mice with severe combined immunodeficiency, as previously described.¹⁶ Animals were sacrificed 4 weeks after tumor inoculation.

Electron Microscopy and 3D Reconstruction of Semithin Sections

Preparation of tumor samples for electron microscopy was performed as previously described.¹⁴ In brief, the anesthetized animals (three mice for each tumor line) were perfused via the left ventricle with PBS for 10 minutes and with 4% paraformaldehyde and 1% glutaraldehyde in PBS (pH 7.2) for 15 minutes at room temperature. The s.c. tumors were removed, cut into 1 × 2-mm pieces, and immersed in the same fixative for an additional 2 hours. The pieces were post-fixed in 1% OsO₄, 0.5% K-ferrocya-

nide in PBS for 2 hours, dehydrated in a graded series of acetone, and embedded in Spurr's mixture.

A total of 8 to 10 serial semithin sections were cut, stained by 0.5% toluidine blue (pH 8.5), and analyzed for the presence of pillars. The structures identified on the last semithin section were followed backward to ensure that they represented pillars and were not simply vessel bifurcations or other structures. Areas of interest were trimmed out by comparing the structures on the cut surface of the tissue blocks with the semithin sections and then serially sectioned by an RMC MT-7 ultramicrotome (Research and Manufacturing Co, Tucson, AZ). The sections were placed on thin bar grids, stained with 2% uranyl acetate and lead citrate, and analyzed using a Philips CM10 electron microscope (Eindhoven, The Netherlands). Pillars cut lengthwise were also examined during analysis of serial ultrathin sections. In this case, the entire thickness of the pillar was available for analysis at the ultrastructural level.

Serial semithin sections were captured by an Olympus DP50 camera (Olympus, Tokyo, Japan). Digitized images were transferred to the BioVis3D software program (BioVis3D, Montevideo, Uruguay). Three-dimensional (3D) reconstructions were performed using color contouring to highlight the recreated structures.

Immunofluorescence Analysis

Frozen sections (15-μm thick) were fixed in methanol and were incubated at room temperature (for 1 hour) with a mixture of the following primary antibodies: monoclonal anti-mouse CD31 (dilution, 1:100; catalogue no. 01951D; Pharmingen, San Diego, CA), polyclonal anti-collagen I (dilution, 1:100; catalogue no. AB765P; Chemicon, Billerica, MA), monoclonal anti-vinculin (dilution, 1:100; catalogue no. V4505; Sigma Chemical Co), monoclonal anti-integrin α-1 (dilution, 1:20; catalogue no. 555001; BD Pharmingen, San Jose, CA), polyclonal anti-integrin α-1 (dilution, 1:50; catalogue no. sc-10728; Santa-Cruz Biotechnology, Santa-Cruz, CA), monoclonal anti-integrin α-2 (dilution, 1:100; catalogue no. 108901; Biolegend, San Diego), polyclonal anti-integrin α-2 (dilution, 1:200; catalogue no. AB1936; Millipore, Billerica, MA), polyclonal anti-integrin α-11 (dilution, 1:100; catalogue no. sc-98740; Santa-Cruz Biotechnology), and monoclonal anti-mouse CD29 (dilution, 1:100; catalogue no. 550531; Pharmingen). After washing, appropriate secondary antibodies conjugated with fluorescein isothiocyanate, tetra rhodamine isothiocyanate, or Cy5 were used (all from Jackson ImmunoResearch Inc., Suffolk, UK). The vinculin and integrin α-2 signals were amplified by using an appropriate biotinylated secondary antibody (dilution, 1:200; Vector Laboratories, Burlingame, CA), followed by streptavidin fluorescein isothiocyanate (Jackson ImmunoResearch Inc.). To analyze the localization of actin filaments within the pillars, the sections were reacted with phalloidin-tetra rhodamine isothiocyanate (dilution, 1:500; catalogue no. P1951; Sigma Chemical Co).

Sections were scanned by eye for the presence of pillars using a ×100 objective. Only pillars running parallel and lying completely within the sectioning plane

were analyzed by a Bio-Rad MRC 1024 confocal microscope (Bio-Rad, Richmond, CA). For 3D reconstructions, 30 to 40 optical sections were generated.

Determination of the Size of Collagen Bundles

The size of the collagen bundles was determined at the ultrastructural level in the peritumoral connective tissue and within the pillars. Measurements were made on digitalized electron micrographs (original magnification, $\times 1500$ to $\times 7000$) taken from s.c. tumors of both cancer cell lines using Olympus Quick Photo Micro software (Olympus). In the peritumoral connective tissue, collagen bundles tightly packed with collagen fibers were chosen randomly (>250). In cross sections, the smallest diameter of the bundle was measured. After their identification in semithin sections, >50 pillars were analyzed at the ultrastructural level. Only pillars exclusively containing collagen fibers, but no pillars with connective tissue cells, were chosen. The total thickness of the pillars (including ECs) was also measured.

In Vivo Treatments

To study the effects of angiogenesis-modulating agents on tumor vascularization and pillar formation, groups of six mice bearing C38 tumors received recombinant human erythropoietin α (rHuEPO, epoetinum α ; Janssen-Cilag, Shaffhausen, Switzerland),¹⁶ PTK787/ZK22854 (vatalanib; Novartis/Schering AG, Berlin, Germany)¹⁷ (obtained from Selleck Chemicals LLC, Houston, TX), or the vehicle as a control. In mice treated with vatalanib, tumors were allowed to grow for 12 days before treatment. Then, mice were treated orally with 100 mg/kg vatalanib (PTK787/ZK22854, dissolved in water containing 5% dimethyl sulfoxide and 1% Tween-80) for 4 days, as in a previous study.¹⁸ Mice treated with rHuEPO were given 150 IU/kg in physiological salt solution three times per week i.p. from day 1 after tumor inoculation, as previously described.¹⁶ The mice in all groups were sacrificed on day 17, and tumors were removed, weighed, and frozen.

CD31-labeled frozen sections were scanned by eye using a $\times 100$ objective to determine the number of pillars within the entire section. The total area of the sections was determined using Olympus Quick Photo Micro software. To determine the area fraction of CD31-positive blood vessels in tumor sections, two to three confocal images were taken from each tumor section using a $\times 4$ objective (area, 11.3 mm^2). The micrographs were analyzed using ImageJ software (NIH, Bethesda, MD). Results are expressed as the number of pillars per squared millimeter of tumor tissue or microvessel area.

Analysis of Skin Wounds

Animals were anesthetized and shaved. A 1-cm-long full-thickness incision was made in the dorsal skin of C57 black mice. The wounds were partially closed by a single nylon suture. The mice were euthanized on days 3, 5, 7, and 10 after wounding. Two mice were sacrificed at each time point. The wounds and the surrounding intact skin, measuring $2 \times 2 \text{ mm}$, were removed and cut further into

$1 \times 2\text{-mm}$ pieces, with the long axis running perpendicular to the wound. These pieces were fixed and embedded for electron microscopy, as previously described. Eighty-six tissue blocks were semithin sectioned and analyzed (total area, approximately 250 mm^2) for the presence of pillars using a $\times 63$ objective.

Statistical Analysis

Statistical analysis was performed using the Student's *t*-test.

Results

Development of Intraluminal Connective Tissue Pillars

Intussusceptive angiogenesis was observed in s.c. tumors of both cancer cell lines. This type of angiogenesis was the main means of new vessel formation. Endothelial sprouting with characteristic slit-like lumen-containing capillaries² was scarcely detected. Intussusception was mainly detected in angiogenic hot spots peritumorally, but it also occurred within the tumor mass. The first step of intussusception is thought to be the development of protrusions or infoldings of the vessel wall within the lumen.⁶ We analyzed 89 infolds sharply intruding into the vessel lumens in >172 serially sectioned areas (semithin sections) altogether. None of these structures projected into the lumen by themselves. By tracing them over several serial sections, we found that each capillary infold was connected to a different part of the vessel lumen (on the opposite or the same side). These infolds proved to be pillars, part of blind-ending lumens or simple vessel ramifications (Figure 1; see also Supplemental Figure S1 at <http://ajp.amjpathol.org>). In areas of intensive intussusception, proliferating ECs (Figure 1, H and L; see also Supplemental Figure S1H at <http://ajp.amjpathol.org>) and intraluminal endothelial bridges were frequently observed (Figure 1, H–M and K–O; and Figure 2, A–E). These bridges either were simple EC processes projecting into the vessel lumen and attaching to the endothelial tube in a different position (Figure 2F) or were formed by the participation of cellular processes of different ECs (Figure 2G). However, the most characteristic phenomenon of this type of tumor-induced intussusceptive angiogenesis was the development of transluminal pillars containing tightly packed collagen fibers covered by ECs (Figure 2H). The pillars either spanned the vessel lumen or originated and terminated on the same side of the vessel (Figures 1 and 3). The diameters of these collagen bundles did not differ significantly from those within the peritumoral connective tissue (Table 1). The overall diameter (including the EC layer) of the pillars corresponded well with those observed earlier in other studies ($2.5 \mu\text{m}$).⁴ The fibers were oriented parallel to the axis of the pillars (Figure 3, B–D) and were covered by several ECs. However, the basement membrane under these cells was generally absent (Figures 2H and 3G). Moreover, neither pericytes nor other cells were present in

Figure 1. Light microscopic appearance and 3D reconstruction of endothelial bridges and pillars. **A–O:** 0.5- μm thick serial semithin sections were cut over a distance of 10 μm in an area of intensive intussusceptive angiogenesis. Serial sections $>10 \mu\text{m}$. Two pillars are visible. One starts in **A** (section 2, **large arrow**), runs through the lumen, and joins the vessel wall in **H** (section 13). There is a sharp protrusion of the vessel wall in **I** (**large arrow**, section 15). The other pillar (**small arrow**) starts in **C** (section 6), reaches the other side of the lumen in **L** (section 19), makes a turn, and joins the same side of the vessel in **N** (section 21). Two endothelial bridges are also visible. One (**large arrowhead**) is positioned between the opposite sides of the vessel lumen (**J–O**); the other smaller one (**small arrowhead**) starts and ends on the same side of the vessel (**H–M**). There is a proliferating EC (**asterisk**) in **H** and **L**. Scale bar = 20 μm . **P** and **Q:** 3D reconstruction of 25 semithin sections represented in **A–O**. The vessel wall and the surrounding tissue are indicated in red, pillars separated from the vessel wall and containing collagen bundles are indicated in green, and endothelial bridges are indicated in yellow. **P** and **Q** are different views from above to show the localization of both endothelial bridges.

these small nascent pillars. Along the pillars, cut parallel to their axis, high electron density areas could be observed in the membrane of the ECs, suggesting specific adhesion between the ECs and the collagen bundle. In accordance with this observation, immuno-

fluorescence analysis revealed vinculin-containing adhesion spots along the pillars (Figure 4, A–D). However, although immunolabeling with antibodies against integrin α -1, α -2, or α -11 demonstrated high α -2 and α -11 expression levels in the pericapillary connective tissue,

Figure 2. 3D and ultrastructure of endothelial bridges and transcapillary pillars. **A–D:** Serial semithin sections of an endothelial bridge. The bridge (arrows) is present only in two consecutive sections. The light blue staining (collagen core) within the bridge is absent. Scale bar = 20 μ m. **E:** 3D reconstruction of 13 semithin sections. The endothelial bridge and the nuclear area of the ECs are highlighted in yellow, and the vessel wall and the surrounding tissue are shown in red. **F:** An endothelial bridge formed by a single cellular process (arrow). The process is attached to the EC itself (arrowhead). Scale bar = 1 μ m. **G:** Endothelial bridges formed by cellular processes of several ECs. Arrows point at intercellular junctions. The collagen bundle is located close to the vessel (arrowhead). Scale bar = 5 μ m. **H:** Cross section of a transluminal pillar. The pillar is formed by a collagen bundle tightly packed with fibers and by two covering ECs. Arrows point at interendothelial junctions. Some basement membrane material is visible below the ECs at the upper part of the pillar (arrowhead). Scale bar = 1 μ m.

these collagen-binding integrin subunits were either occasionally present as small dots at a low density at the abluminal surface of pillar-forming ECs (as in the case of α -2 labeling; see Supplemental Figure S2, A and B, at <http://ajp.amjpathol.org>) or totally absent (as in the case of α -1 or α -11 labeling; see Supplemental Figure S2, E and F, at <http://ajp.amjpathol.org>). Nevertheless, in more developed pillars, we could detect large integrin α -2–containing adhesion spots (see Supplemental Figure S2, C and D, at <http://ajp.amjpathol.org>). Staining for integrin β -1 showed no specific localization of this subunit that was distributed evenly under the ECs of the vessel and pillars (data not shown).

The part of the cell body of the ECs that formed the pillars frequently contained a high density of microfilaments, excluding all other cellular organelles (Figure 3, G and H). These microfilaments were generally not in a parallel arrangement; rather, they formed a mesh. The presence of polymerized actin within the ECs of the pillars was also confirmed by phalloidin staining (Figure 4, E and F). The microfilaments were attached to the membrane through specific structures that appeared as dots (approximately 50 nm, Figure 5A) when the plane of the section ran parallel to the membrane and as tiny rods (<200 nm, Figure 5B) when the plane of the section was perpendicular to it. The adhesion spots were arranged in a regular manner along individual collagen fibers (Figures 3G and 5A) and were connected to each other by

microfilaments (Figure 5B). In these attachment regions, the collagen fibers were in close contact with the plasma membrane of the EC (Figure 5C). Confocal and electron microscopic analysis of serial sections of the pillars and extensive light revealed that, in a small portion of the pillars, the collagen bundles did not span the whole length of the pillar. In these cases, as in the intraluminal endothelial bridges previously described, the rest of the pillar was composed only of ECs (Figures 6 and 7, A and B; see also Supplemental Figure S3 at <http://ajp.amjpathol.org>). Collagen bundles situated in the nascent pillars extended into the connective tissue (Figure 7, C and D). Maturing pillars, into which cellular processes extended, or larger pillars containing pericytes and other connective tissue cells were also present in the vessel lumens (Figure 7, E and F).

Effects of Angiogenesis-Modulating Agents on Tumor Capillary Parameters and Pillar Formation

In the next set of experiments, we also studied whether rHuEPO (which recently induced intussusceptive angiogenesis in the chick embryo chorioallantoic membrane assay)¹⁹ or the anti-angiogenic drug vatalanib (an oral small-molecule multitargeted tyrosine kinase inhibitor that blocks all known vascular endothelial

Figure 3. Ultrastructure of the pillar. **A–E:** Serial ultrathin sections (approximately 100 nm) of a nascent pillar (**A**, section 3; **B**, section 14; **C**, section 20; **D**, section 22; **E**, section 36). The pillar was present in 22 sections. The collagen bundle is densely packed with fibers and oriented parallel to the axis of the pillar (**B–D**). **A–E:** The collagen bundle does not extend into the connective tissue (**up**). **E:** In contrast, the collagen bundle is still visible (**arrow**) and runs with a sharp change in direction along the circumference of the vessel (**down**). This suggests that the putative direction of the collagen bundle transport is from right to left. Apparently, the bundle just reached the left side of the vessel. In **A**, an **arrow** points at a small process of the EC, indicating the location of the pillar. The body parts of the ECs in contact with the collagen bundle show homogeneous staining because of the high microfilament content (**arrowheads** in **B**). Scale bar = 2 μ m. **F–H:** High-power micrographs showing details of the pillar region (**F**, section 1; **G**, section 14; **G inset**, section 10; **H**, section 36). **F** represents the first section that suggests the presence of a pillar. The small cellular process (**arrow**) is densely packed within a meshwork of microfilaments. Microfilaments are also present under the plasma membrane in the left part of the cell. The **large arrowhead** points at an area containing intermediate filaments, whereas small **arrowheads** point at microtubules. Scale bar = 0.5 μ m (**F**). **G:** Details of **B**. The cell body above the collagen bundle contains many microfilaments but no other organelles. The high electron density of the plasma membrane is cut at a low angle. Adhesion spots are also visible (**small arrowheads**). A basement membrane is lacking in the area where the EC faces the collagen bundle (**small arrows**). However, it is present in other areas of the vessel wall (**large arrow**). **Inset:** An adhesion area where the plasma membrane is cut at a low angle. The adhesion spots are situated exactly and regularly above the collagen fibers (**small arrowheads**). Scale bar = 0.5 μ m (**G** and **inset**). **H:** The collagen bundle (cut perpendicularly) of the pillar extends outside into the connective tissue and runs around the circumference of the vessel. Although adhesion spots are not visible, a basement membrane is not present at the area where the EC faces the collagen bundle. Many microfilaments are present in this body part (**arrowheads**) of the EC. An intact basement membrane is present under the other parts of the EC (**arrows**). Scale bar = 1 μ m.

growth factor receptors, with additional activity against platelet-derived growth factor receptor and c-kit)¹⁷ could affect pillar formation in our C38 colon carcinoma model system.

In C38 tumors treated with rHuEPO, pillar densities of tumor sections were significantly increased compared

with untreated controls (Table 2). In accordance with our previous findings,¹⁶ a tendency toward an increased tumor microvessel surface in mice treated with rHuEPO was also observed. However, when pillar density was calculated for the area of tumor microvessels, the difference in pillar densities between tumors in the rHuEPO-

Table 1. Collagen Bundle Diameters in the Peritumoral Connective Tissue and in Transcapillary Pillars

Value	Diameter (μ m)		
	Collagen bundles within the peritumoral connective tissue (n = 260)	Collagen bundles within the pillars (n = 55)	Pillars (including endothelium) (n = 55)
Mean $\pm$ SD	1.61 $\pm$ 0.9	1.72 $\pm$ 1.0*	2.5 $\pm$ 1.1
Range	0.3–5.9	0.4–5.0	0.8–5.8

*The diameters of the collagen bundles of the connective tissue and the pillars did not differ significantly ($P = 0.4$).

Figure 4. Immunofluorescent labeling of pillars. **A–D:** Vessel with pillar (**arrow**) stained for CD31 (**A**, red), vinculin (**B**, green), and collagen I (**C**, blue). The pictures show horizontal views of 35 optical sections. CD31 and vinculin are present mainly on the two sides of the pillar, whereas collagen is positioned in the middle. **D** shows the merged picture. **Inset:** One optical section showing that vinculin is present in small spots along the periphery of the pillar (green). There is a high density of adhesions in the connective tissue. Black areas (except for the vessel lumen) correspond to tumor tissue. Scale bar = 20 μ m (**A–D**). **E** and **F:** Vessel with pillar (**arrow**) stained for CD31 (green) and phalloidin (red). Phalloidin staining, representing filamentous actin, colocalizes with CD31 staining at the sides of the pillar. Myofibroblasts outside of the vessel are also stained by phalloidin–tetra rhodamine isothiocyanate. Scale bar = 20 μ m (**E** and **F**).

treated and control groups remained statistically nonsignificant (Table 2).

In mice treated with vatalanib, significantly decreased tumor burdens and a tendency toward reduced microvessel areas were observed. Moreover, tumor samples in the vatalanib group had significantly more pillars/tumor microvessel areas than those in the control group (Table 2).

Vascularization of Skin Wounds

To elucidate the process of pillar formation in conditions other than tumor-induced angiogenesis, we also analyzed full-thickness cutaneous incision wounds (3, 5, 7, and 10 days after wounding) for the presence of pillars. However, endothelial sprouting was the characteristic mechanism of angiogenesis in the healing wounds

(mostly 5 and 7 days after wounding); we failed to detect any signs of pillar formation (data not shown).

Discussion

Although the formation of transluminal pillars^{4–8} is considered the most characteristic feature of intussusceptive microvascular growth, the exact mechanism of this process has yet to be completely clarified. By investigating the vascularization of experimental tumors growing in mice, we present herein the putative sequence of steps of transluminal pillar development during intussusceptive angiogenesis (Figure 8). First, transluminal endothelial bridges are formed. Second, collagen bundles adjacent to the vessel are seized by the abluminal side of a bridge-forming EC. The force exerted by the actin cytoskeleton of the EC through specific vinculin-containing attachment

Figure 5. The ultrastructure of the adhesion region. **A** and **B**: Serial sections of an adhesion region. **A**: Adhesions (appearing as dots, **arrowheads**) are arranged into rows and spaced regularly (the sectioning plane is parallel to the plasma membrane of the EC). There is a high density of microfilaments in this region. **B**: When the sectioning plane is perpendicular to the plasma membrane of the EC, the same adhesions appear as rods (**arrowheads**). Microfilaments connect the rods to each other (**arrows**). Scale bar = 0.5 μm (**A** and **B**). **C**: Cross section of a pillar at an adhesion region. The collagen fibers are in close contact with the plasma membrane of the EC. Only one rod is visible (**arrowhead**), which faces a collagen fiber. The plasma membrane is thickened. Scale bar = 0.5 μm .

points on the collagen bundle pulls the pillar into and through the vessel lumen. Finally, maturation of these nascent pillars occurs via the migration of pericytes and myofibroblasts into the collagen core of the pillar and the deposition of additional collagenous connective tissue by these cells.

The sequence of events during intussusceptive angiogenesis was analyzed in detail in the chicken chorioallantoic membrane and in developing lung tissue, and it was concluded that the appearance of collagen bundles is the last step of pillar formation.^{4,5} In these models, protrusion of the vascular wall into the lumen, interendothelial adherence, and perforation of the endothelial bilayer by reorganization of the interendothelial junctions were the first events. These events were promptly followed by the appearance of perivascular cells within the pillar. Interestingly, although transluminal pillars were formed under different conditions in our study (ie, during tumor vascularization because there were no signs of pillar formation in healing cutaneous wounds), the initial size of pillars (approximately 2.5 μm) was remarkably similar to that observed in nontumorous conditions (<2.5 μm).^{4,5}

Intussusceptive angiogenesis, which results in high local vascular density, is initiated by rapid nascent pillar formation, followed by slow pillar enlargement (a noninvasive process for which extensive connective tissue synthesis is required). Therefore, one reason for the lack of intussusceptive angiogenesis in skin wounds could be that neither pillar development nor the intussusceptive angiogenesis itself is an invasive process; thus, both are not suitable to vascularize initially avascular spaces (such as a fibrin clot). Also, during intussusception in nontumorous tissues, the area covered by the vasculature can be increased solely by the collagenous matrix deposited by the connective tissue cells immigrating into the pillars. In tumor tissues, the invading tumor mass incorporates and occupies the newly formed vasculature (including the developing pillars). Tumor cells are able to both incorporate into the pillars and contribute to their growth; therefore, they help to dilute the newly formed capillary network. In contrast, during wound healing, the

fibrin clot (an existing avascular extracellular matrix) is invaded/occupied by sprouting vessels. This can occur rapidly (within days) and is necessary to supply the incoming collagen-synthesizing cells with nutrients. Another reason for the difference in the presence of intussusceptive angiogenesis between skin wounds and tumors could be that a sustained angiogenic stimuli elicited by tumors (often referred to as never-healing wounds)¹² is necessary to induce/maintain intussusceptive angiogenesis.

In the mechanism proposed herein, the formation of transluminal endothelial bridges is immediately followed by the appearance of a collagen bundle within the pillar. This collagen bundle may serve as a highway for later immigration of other cells (eg, pericytes and myofibroblasts) into the pillar. The deposition of an additional extracellular matrix by these cells can result in the enlargement of the pillar.

The driving force behind the formation of the protrusion in the vessel lumen during intussusceptive microvascular growth remains elusive. Although it is believed to be exerted by perivascular cells, such as pericytes or myofibroblasts,^{4–6} a cell's pushing force (which can be exerted only through filopodia or lamellipodia) is only in the 10 to 100 pN range.⁹ In contrast, contractile forces generated by cells lie in the several hundred nanonormal range.¹⁰ According to our model, no extraluminal force is necessary for the formation of the pillar. Slender EC processes floating in the lumen can contact other parts of the endothelial tube (probably to the same side initially). Additional growth of cytoplasmic processes of ECs can result in the repositioning of this initial contact to reach farther parts of the lumen. This may be followed by the formation of endothelial bridges consisting of several ECs. The contractile force exerted by the microfilaments present at a high density within the ECs forming the bridge may be strong enough to pull a collagen bundle into and through the lumen. The highly edematous and loosely organized peritumoral connective tissue might allow this process. However, the presence of adhesions at a high density on the myofibroblasts of the pericapillary

Figure 6. Pillar development. **A–H:** Developing pillar partially filled with a collagen bundle. Small **arrows** mark the part of the pillar consisting only of ECs. The bridge is attached to the vessel wall at two positions (**A** and **C**). The collagen core of the pillar (light blue staining) is discernible first on **D** (small **arrowhead**). The pillar increases in thickness during its further run before it joins the vessel wall (small **arrowheads**). Large **arrows** mark a twin pillar that joins the vessel wall on **D**. The large **arrowhead** points to a mature connective tissue post that joins the vessel wall on **C** and blends into it on the following sections. Scale bar = 20 μm (**A–I**). **I:** 3D reconstruction of 30 semithin sections represented in **A–H**. The vessel wall and the surrounding tissue are shown in red, pillars separated from the vessel wall and containing a collagen bundle are shown in green, and the endothelial bridges are shown in yellow. The structure marked by the small **arrow** and **arrowhead** in yellow and green, respectively, indicates that the collagen bundle does not fill the whole length of the pillar. **J–M:** Serial ultrathin sections of a pillar partially filled with a collagen bundle. The sections represent the transitional area where the pillar continues in a short endothelial bridge. The tiny collagen bundle (**arrows** in **J** and **K**) contains only approximately 20 individual collagen fibers. The pillar is sealed by endothelial junctions (**arrowheads** on **J**). The body part of the ECs in the vicinity of the collagen bundles (**J**) contains microfilaments at a high density. The collagen bundle is absent in **L** and **M**. Numerous interendothelial junctions are visible in **L** (**arrowheads**), suggesting that more ECs of the bridge have joined each other in this region. These junctions are absent in **M**, suggesting that the bridge terminates in a single EC. This implies that the other ECs of the bridge have not yet reached this side of the vessel. Scale bar = 1 μm (**J–M**).

connective tissue suggests that the collagen matrix is under tension, through either indirect (ie, fibronectin fibrils) or direct attachment of these cells to the collagen bundles. These attachments may counteract the movement of the collagen bundles. Although we do not have direct evidence for the movement of the collagen bundles, the observed similarity between the diameter of the collagen bundles within the pillar and within the connective tissue, and, moreover, the discovery of collagen bundles extending only halfway into the lumen (while their other end extended into the connective tissue) have led us to conclude that pre-existing collagen bundles are

transferred by these ECs through the lumen. The observation that collagen bundles are transferred in a hand-over-hand cycle in the case of fibroblasts *in vitro* supports this hypothesis. This process was dependent on integrin α -2/ β -1-mediated adhesion and on the contractile activity of the actomyosin cytoskeleton.²⁰ However, in our case, the adhesion receptor responsible for the binding of collagen I in the pillar remains unknown. We could not detect integrin α -1 or α -11 expression levels, and integrin α -2 was present only occasionally at a low density, which did not correlate with the number of adhesion spots containing vinculin within the pillar. This calls into question

Figure 7. Pillar development and maturation. **A** and **B**: Horizontal view of 33 optical sections. Double labeling for CD31 (green) and collagen I (red). **A**: Collagen labeling. **B**: Merged red and green channels. The collagen bundle extends only halfway into the vessel lumen within the developing pillar (**arrow**). Another pillar (**arrowhead**) is completely filled with collagen. Scale bar = 20 μ m (**B**). **C** and **D**: Horizontal view of 32 optical sections. Double labeling for CD31 (red) and collagen (blue). **C**: Collagen labeling. **D**: Merged red and blue channels. The collagen bundle originating from the pericapillary connective tissue spans the entire length of the pillar (**arrow**). Scale bar = 20 μ m (**D**). **E**: Base of a maturing pillar. A connective tissue cell extends a process (**arrow**) toward the original collagen core of the pillar (**arrowhead**). Scale bar = 1 μ m. **F**: Mature pillar containing numerous cells and collagen bundles. The basement membrane is visible on the right side of the pillar under the endothelium (**arrow**). Flat cells under the ECs (**arrowheads**) are presumably pericytes, although they are not covered by a basement membrane. Scale bar = 1 μ m.

the role of these integrin subunits in the transport of the collagen bundle. Nevertheless, the presence of adhesion spots containing large α -2 subunits may be the consequence of the maturation process during which myofibroblasts migrate into the pillar. In these cells, integrin α -2 was observed at a high density in the connective tissue surrounding the vessels. Despite the lack of collagen I-binding α subunits within the pillar, integrin β -1 was evenly distributed along the vessel and the pillars, suggesting

that this integrin is paired with laminin-binding α subunits at the basal surface of the ECs.

Electron-dense adhesion sites containing vinculin were observed along the pillars, indicating that the ECs are attached firmly to the collagen bundle. The adhesion spots were unique in structure because they were placed regularly along individual collagen fibers, extended >100 nm from the membrane into the cytoplasm, and were connected to microfilaments. Recently, a strikingly similar

Table 2. Effect of Angiogenesis-Modulating Agents on Tumor Growth and Pillar Densities

Variable	Treatments		
	Control	rHuEPO	Vatalanib
C38 colon carcinoma s.c. tumor weight (g)	2.73 ± 0.72	2.77 ± 1.12	1.46 ± 0.82*
Relative vessel area (%vessel/tumor area)	1.47 ± 0.32	1.75 ± 0.28	1.21 ± 0.14
No. of pillars/vessel area (no./mm ²)	21 ± 3	25 ± 5	32 ± 6*
No. of pillars/tumor area (no./mm ²)	0.31 ± 0.04	0.43 ± 0.06*	0.38 ± 0.08

Results are expressed as mean ± SD.

**P* < 0.05.

structure was discovered during the analysis of the ultra-structural architecture of focal adhesions in *in vitro* cultured cells. Patla et al²¹ found that the membrane-cytoskeleton interaction within focal adhesions is mediated through vinculin-containing particles located at the cell membrane and attached to actin fibers. Their observation strongly supports our idea that the transluminal transport of the collagen bundle is mediated by the force exerted by the actin cytoskeleton via the adhesion spots (ie, particles).

The finding that collagen fibers touch the plasma membrane of the ECs suggests that ECs adhere directly to the collagen bundle rather than attach to other extracellular matrix elements, such as the basement membrane or fibronectin fibrils. This latter extracellular matrix component is involved in the formation of fibronexus junctions,

which are thought to be responsible for force transmission by myofibroblasts and ECs.^{22,23}

In a recent study,²⁴ the tyrosine kinase inhibitor vatalanib (PTK787/ZK22854) delayed the intussusceptive-dependent maturation of the vascular network in the developing chicken chorioallantoic membrane. In contrast, other researchers¹⁹ reported that rHuEPO can induce intussusceptive angiogenesis in the same angiogenesis assay. Therefore, we also decided to study whether these angiogenesis-modulating molecules have an effect on pillar formation in our C38 tumor model. We found that rHuEPO treatment resulted in a significant increase in intratumoral pillar numbers. However, possibly because of the concomitant increase in intratumoral capillary surface (a phenomenon that corresponds with our previous observations on rHuEPO's effects on tumor capillaries),¹⁶

Figure 8. Schematic representation of the putative process of pillar formation. **A:** Simple endothelial bridges are formed. **B:** Bridges can reposition themselves, and more ECs may be involved in bridge formation. On the abluminal side of the ECs that form the bridge, the basement membrane is locally disrupted. An EC from the bridge adheres to a nearby collagen bundle. **C and D:** The collagen bundle, seized firmly through specific adhesion sites, is transferred through the lumen by the bridge-forming ECs as they exert a pulling force on it. **Arrows** indicate the direction of this force. **E:** The collagen bundle reaches the other side of the lumen. The interendothelial junctions are reorganized, ensuring that the luminal and basal polarity of the ECs is maintained during the process. **F:** The collagen bundle is transferred into the connective tissue on the other side of the vessel. A nascent pillar, consisting only of a collagen bundle and the covering ECs, is established. Pillar maturation and enlargement occur through the immigration of fibroblasts/myofibroblasts and pericytes into the pillar and through the subsequent extracellular matrix deposition performed by these cells.

this difference remained nonsignificant when pillar densities were calculated for intratumoral microvessel areas. Thus, these results do not unequivocally indicate that rHuEPO induces pillar formation; they may only suggest that more capillary surface was provided for pillar formation. However, in our experiments with vatalanib, we found a significant increase in pillar densities defined for the microvessel areas in C38 tumors, suggesting an activity for this drug similar to that reported in another earlier study by the previously mentioned researchers.¹⁸ In this study, their group observed a switch from endothelial sprouting to intussusceptive angiogenesis after treatment of mammary carcinoma allografts with vatalanib. Altogether, both our results and theirs support the general notion that inhibition of just a single tumor vascularization mechanism can trigger alternative ones. This can help tumors to develop resistance to anti-angiogenic treatments.^{1,25}

The main limitation of this study is the lack of direct *in vivo* evidence for collagen bundle movement. However, each of the available real-time imaging techniques has serious shortcomings/confounding factors that could hamper its use in studying *in vivo* pillar formation in tumorous conditions. Red blood cell flow makes it impossible to detect an unstained transluminal collagen bundle using common phase-contrast microscopy (S. Paku, unpublished data). The obscuring effect of the blood stream would also exist when using confocal reflection imaging.²⁶ The only way to overcome this problem would be to detect harmonic signals generated during multiphoton excitation. However, although this method highlights unstained fibrillar collagen,^{27,28} the penetration depth of the second harmonic signal is limited by the light-scattering effect of turbid media (to approximately 200 μm),²⁹ such as the peritumoral s.c. tissue. An additional key problem with *in vivo* pillar imaging is that the equipment should be focused on an object that does not exist at the beginning of the observation period (ie, the probability that a collagen bundle will move in front of a high-power objective is extremely low). Nevertheless, the confocal and ultrastructural evidence we have presented strongly suggests that the observed collagen bundles are transferred through the lumen by the bridge-forming ECs. The evidence includes the following: i) the adhesion sites along the pillars, ii) the dense actin filament network within the pillars, iii) the similarity between pillar and connective tissue collagen bundle diameters, and iv) collagen bundles extending only halfway into the lumen with their other end extended into the connective tissue.

In summary, this study reports the detailed mechanism of connective tissue pillar formation during tumor-induced intussusceptive angiogenesis. This new mechanism of pillar formation can also be termed inverse sprouting. During the normal sprouting process, ECs maintain their polarity, migrate surrounded by connective tissue, and form a slit-like lumen.² During pillar formation, ECs are surrounded by the vessel lumen and the connective tissue is situated inside the sprout. As in normal sprouting, ECs involved in inverse sprouting also maintain their polarity. Meanwhile, the complete EC coverage of the collagen bundle ensures that the collagen core of

the pillar is not in contact with blood elements during the process. These results provide a better understanding of this type of angiogenesis and may also represent a new piece to the puzzle of cancer therapy via angiogenesis inhibition.

References

1. Dome B, Hendrix MJ, Paku S, Tovari J, Timar J: Alternative vascularization mechanisms in cancer: pathology and therapeutic implications. *Am J Pathol* 2007, 170:1–15
2. Paku S, Paweletz N: First steps of tumor-related angiogenesis. *Lab Invest* 1991, 65:334–346
3. van Hinsbergh VW, Koolwijk P: Endothelial sprouting and angiogenesis: matrix metalloproteinases in the lead. *Cardiovasc Res* 2008, 78:203–212
4. Makanya AN, Hlushchuk R, Djonov VG: Intussusceptive angiogenesis and its role in vascular morphogenesis, patterning, and remodeling. *Angiogenesis* 2009, 12:113–123
5. Djonov V, Baum O, Burri PH: Vascular remodeling by intussusceptive angiogenesis. *Cell Tissue Res* 2003, 314:107–117
6. Burri PH, Hlushchuk R, Djonov V: Intussusceptive angiogenesis: its emergence, its characteristics, and its significance. *Dev Dyn* 2004, 231:474–488
7. Patan S, Tanda S, Roberge S, Jones RC, Jain RK, Munn LL: Vascular morphogenesis and remodeling in a human tumor xenograft: blood vessel formation and growth after ovariectomy and tumor implantation. *Circ Res* 2001, 89:732–739
8. Patan S, Munn LL, Jain RK: Intussusceptive microvascular growth in a human colon adenocarcinoma xenograft: a novel mechanism of tumor angiogenesis. *Microvasc Res* 1996, 51:260–272
9. Cojoc D, Difato F, Ferrari E, Shahapure RB, Laishram J, Righi M, Di Fabrizio EM, Torre V: Properties of the force exerted by filopodia and lamellipodia and the involvement of cytoskeletal components. *PLoS One* 2007, 2:e1072
10. Burton K, Park JH, Taylor DL: Keratocytes generate traction forces in two phases. *Mol Biol Cell* 1999, 10:3745–3769
11. Nagy JA, Chang SH, Dvorak AM, Dvorak HF: Why are tumour blood vessels abnormal and why is it important to know? *Br J Cancer* 2009, 100:865–869
12. Dvorak HF: Rous-Whipple Award Lecture: how tumors make bad blood vessels and stroma. *Am J Pathol* 2003, 162:1747–1757
13. Zhou A, Egginton S, Hudlická O, Brown MD: Internal division of capillaries in rat skeletal muscle in response to chronic vasodilator treatment with alpha1-antagonist prazosin. *Cell Tissue Res* 1998, 293:293–303
14. Paku S, Kopper L, Nagy P: Development of the vasculature in “pushing-type” liver metastases of an experimental colorectal cancer. *Int J Cancer* 2005, 115:893–902
15. Dezso K, Bugyik E, Papp V, László V, Dóme B, Tóvári J, Timár J, Nagy P, Paku S: Development of arterial blood supply in experimental liver metastases. *Am J Pathol* 2009, 175:835–843
16. Tóvári J, Gilly R, Rásó E, Paku S, Bereczky B, Varga N, Vágó A, Timár J: Recombinant human erythropoietin alpha targets intratumoral blood vessels, improving chemotherapy in human xenograft models. *Cancer Res* 2005, 65:7186–7193
17. Wood JM, Bold G, Buchdunger E, Cozens R, Ferrari S, Frei J, Hofmann F, Mestan J, Mett H, O'Reilly T, Persohn E, Rösel J, Schnell C, Stover D, Theuer A, Towbin H, Wenger F, Woods-Cook K, Menrad A, Siemeister G, Schirner M, Thierauch KH, Schneider MR, Dreys J, Martiny-Baron G, Totzke F: PTK787/ZK 222584, a novel and potent inhibitor of vascular endothelial growth factor receptor tyrosine kinases, impairs vascular endothelial growth factor-induced responses and tumor growth after oral administration. *Cancer Res* 2000, 60:2178–2189
18. Hlushchuk R, Riesterer O, Baum O, Wood J, Gruber G, Pruschy M, Djonov V: Tumor recovery by angiogenic switch from sprouting to intussusceptive angiogenesis after treatment with PTK787/ZK222584 or ionizing radiation. *Am J Pathol* 2008, 173:1173–1185
19. Crivellato E, Nico B, Vacca A, Djonov V, Presta M, Ribatti D: Recombinant human erythropoietin induces intussusceptive microvascular growth in vivo. *Leukemia* 2004, 18:331–336

20. Meshel AS, Wei Q, Adelstein RS, Sheetz MP: Basic mechanism of three-dimensional collagen fibre transport by fibroblasts. *Nat Cell Biol* 2005, 7:157–164
21. Patla I, Volberg T, Elad N, Hirschfeld-Warneken V, Grashoff C, Fässler R, Spatz JP, Geiger B, Medalia O: Dissecting the molecular architecture of integrin adhesion sites by cryo-electron tomography. *Nat Cell Biol* 2010, 12:909–915
22. Eyden B: The myofibroblast: phenotypic characterization as a prerequisite to understanding its functions in translational medicine. *J Cell Mol Med* 2008, 12:22–37
23. Eyden B: Fibronexus junctions associated with in vivo human endothelium. *Ultrastruct Pathol* 2009, 33:28–32
24. Baum O, Suter F, Gerber B, Tschanz SA, Bueggy R, Blank F, Hlushchuk R, Djonov V: VEGF-A promotes intussusceptive angiogenesis in the developing chicken chorioallantoic membrane. *Microcirculation* 2010, 17:447–457
25. Hillen F, Griffioen AW: Tumour vascularization: sprouting angiogenesis and beyond. *Cancer Metastasis Rev* 2007, 26:489–502
26. Friedl P, Maaser K, Klein CE, Niggemann B, Krohne G, Zanker KS: Migration of highly aggressive MV3 melanoma cells in 3-dimensional collagen lattices results in local matrix reorganization and shedding of alpha2 and beta1 integrins and CD44. *Cancer Res* 1997, 57:2061–2070
27. Provenzano PP, Eliceiri KW, Keely PJ: Multiphoton microscopy and fluorescence lifetime imaging microscopy (FLIM) to monitor metastasis and the tumor microenvironment. *Clin Exp Metastasis* 2009, 26:357–370
28. Brown E, McKee T, diTomaso E, Pluen A, Seed B, Boucher Y, Jain RK: Dynamic imaging of collagen and its modulation in tumors in vivo using second-harmonic generation. *Nat Med* 2003, 9:796–800
29. Balu M, Baldacchini T, Carter J, Krasieva TB, Zadayan R, Tromberg BJ: Effect of excitation wavelength on penetration depth in nonlinear optical microscopy of turbid media. *J Biomed Opt* 2009, 14:010508