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Abnormalities in Pericytes on Blood Vessels and Endothelial Sprouts in Tumors

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Endothelial cells of tumor vessels have well-documented alterations, but it is less clear whether pericytes on these vessels are abnormal or even absent. Here we report that α-smooth muscle actin (α-SMA) and desmin-immunoreactive pericytes were present on >97% of blood vessels viewed by confocal microscopy in 100-μm-thick sections of three different spontaneous or implanted tumors in mice. However, the cells had multiple abnormalities. Unlike pericytes on capillaries in normal pancreatic islets, which had desmin but not α-SMA immunoreactivity, pericytes on capillary-size vessels in insulinomas in RIP-Tag2 transgenic mice expressed both desmin and α-SMA. Furthermore, pericytes in RIP-Tag2 tumors, as well as those in MCa-IV breast carcinomas and Lewis lung carcinomas, had an abnormally loose association with endothelial cells and extended cytoplasmic processes deep into the tumor tissue. α-SMA-positive pericytes also covered 73% of endothelial sprouts in RIP-Tag2 tumors and 92% of sprouts in the other tumors. Indeed, pericyte sleeves were significantly longer than the CD31-immunoreactive endothelial cell sprouts themselves in all three types of tumors. All three tumors also contained α-SMA-positive myofibroblasts that resembled pericytes but were not associated with blood vessels. We conclude that pericytes are present on most tumor vessels but have multiple abnormalities, including altered expression of marker proteins. In contrast to some previous studies, the almost ubiquitous presence of pericytes on tumor vessels found in the present study may be attributed to our use of both desmin and α-SMA as markers and 100-μm-thick tissue sections. The association of pericytes with endothelial sprouts raises the possibility of an involvement in sprout growth or retraction in tumors. *(Am J Pathol 2002, 160:985–1000)*

Blood vessels in tumors are recognized as a clinically important therapeutic target.[1] The abnormalities of tumor vessels provide the potential for targeting these vessels without destroying the normal vasculature.[2,3] Tumor vessels are recognized as dynamic, both in terms of the formation of new vessels by angiogenesis and the remodeling of existing vessels.[4,5] Tumor vessels also express novel molecules that can serve as selective targets for therapeutic agents,[6,7] and have structural and functional abnormalities, such as leakiness, that are important for the accessibility of drugs to cancer cells or other cellular constituents of tumors.[8–10]

With the increasing promise of vascular targeting in cancer, a thorough understanding of the cellular structure and function of tumor vessels becomes even more important, as this information is key to interpreting the effects of anti-angiogenic agents. The endothelial cells of tumor vessels have been studied at the tissue, cellular, and molecular level, both historically and recently,[10–17] but less is known about the structure and function of pericytes on tumor vessels.[18–22]

Pericytes, also known as Rouget cells, periendothelial cells, or mural cells, are adventitial cells located within the basement membrane of capillaries and postcapillary venules.[23] Because of their multiple cytoplasmic processes, distinctive cytoskeletal elements, and envelopment of endothelial cells, pericytes are generally considered to be contractile cells that stabilize vessel walls and participate in the regulation of blood flow in the microcirculation.[24–26] Pericytes may also influence endothelial permeability, proliferation, survival, migration, and maturation.[27–29] Blood vessels of mouse embryos lacking platelet-derived growth factor-B (PDGF-B) or its receptor (PDGF receptor-β, PDGFR-β) do not have pericytes or vascular smooth muscle cells and develop microaneurysms and vessel leakiness before the animals die in late gestation.[30,31]

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Pericytes were initially recognized by their distinctive shape and location, but they are now most commonly identified by molecular markers such as α-smooth muscle actin (α-SMA), nonmuscle myosin, tropomyosin, desmin, nestin, PDGFR-β, aminopeptidase A, aminopeptidase N (CD13), sulfatide, or high-molecular weight melanoma-associated antigen (NG2).[18,26,31–36] The expression of these markers varies with the type of vessel. Pericytes on normal capillaries typically express desmin but not α-SMA, whereas normal venular pericytes express both molecules.[26] Marker expression can also vary in different organs and with pathological conditions.[18,34,35,37] Because no single commonly used marker identifies all pericytes with certainty, there may be problems with identifying pericytes in pathological conditions such as cancer when the cells change their expression of marker proteins.[18,34,37]

Little is known about the variability of marker expression by pericytes in tumors because most studies have used a single marker, usually α-SMA or desmin, and have equated lack of immunoreactivity with lack of pericytes.[21,22,38,39] Published reports suggest that the amount of pericyte coverage on vessels in different tumors ranges from extensive[18,20] to little or none.[21,22,40] Some of these differences may be explained by differences in pericyte marker expression among tumors. However, others are likely to result from differences in the markers used to identify pericytes or differences in section thickness, where partial pericyte coverage was missed in thin histological sections.

One reason for determining whether pericytes are a consistent feature of tumor vessels is to explore whether they participate in angiogenesis by guiding newly formed blood vessels through their association with endothelial sprouts.[41,42] Another reason is to examine the suggestion that the absence of pericytes sensitizes tumor vessels to withdrawal of vascular endothelial growth factor (VEGF).[21]

In the present study, we compared the amount of pericyte coverage of tumor vessels, as determined by immunoreactivity of two markers, α-SMA and desmin, with that of normal vessels. We also questioned whether the relationship of pericytes to endothelial cells in tumors differs from that in normal tissues and examined the relationship of pericytes to endothelial sprouts. We compared three tumors in mice: spontaneous pancreatic tumors in RIP-Tag2 mice, implanted MCa-IV mouse mammary carcinomas, and Lewis lung carcinomas. Pericytes and endothelial cells were co-localized in immunohistochemically stained 100-μm-thick sections and examined by confocal microscopy. A preliminary description of this work has been reported.[43]

Materials and Methods

Animals and Preparation of Tumors

Spontaneous pancreatic islet cell tumors were studied in RIP-Tag2 transgenic mice with a C57BL/6 background. In these mice, expression of the SV40 virus T antigen is driven by the rat insulin promoter.[44] Mice expressing the viral oncogene were identified by genotyping tail-tip DNA by the polymerase chain reaction, and tumors were studied when the mice reached 10 weeks of age.[44] Implanted MCa-IV mouse mammary carcinomas[9] and Lewis lung carcinomas (American Type Culture Collection, Rockville, MD) were studied in syngeneic male C3H and C57BL/6 mice, respectively (25 to 30 g body weight).[10] Two-mm cubes of tumor were implanted under the dorsal skin, and the tumors were examined 10 to 20 days later when they had reached a diameter of 8 to 12 mm. Mice were housed under barrier conditions in the animal care facility at University of California at San Francisco. All of the experimental procedures were approved by the University of California at San Francisco Committee on Animal Research.

Lectin Staining and Perfusion Fixation of Vasculature

Mice were anesthetized with ketamine (87 mg/kg) plus xylazine (13 mg/kg) injected intramuscularly. In some animals, fluorescein isothiocyanate (FITC)-labeled *Lycopersicon esculentum* lectin (100 μg in 100 μl of 0.9% NaCl; Vector Laboratories, Burlingame, CA) was injected into the femoral vein and allowed to circulate for 3 minutes before perfusion of fixative.[10] The chest was opened rapidly, and the vasculature was perfused for 3 minutes at a pressure of 120 mmHg with fixative [4% paraformaldehyde in 0.1 mol/L phosphate-buffered saline (PBS), pH 7.4] from a 18-gauge cannula inserted into the aorta via an incision in the left ventricle.[10] The fixative was not preceded by a saline rinse. The right atrium was incised to create a route for the fixative to escape. After removal, tissues were stored in fixative at 4°C until they were processed for immunohistochemistry.

Immunohistochemistry and Imaging

Specimens were rinsed several times with PBS, embedded in 10% agarose, and cut with a Vibratome or infiltrated overnight with 30% sucrose, frozen, and cut with a cryostat. Sections 100 μm in thickness were incubated at room temperature for 12 to 15 hours in a mixture of anti-mouse CD31 (PECAM-1, clone MEC 13.3 rat monoclonal, 1:500; Pharmingen, San Diego, CA) antibody for endothelial cell identification plus anti-α-SMA (Cy3-conjugated mouse monoclonal, clone 1A4, 1:1000; Sigma Chemical Co., St. Louis, MO) or anti-desmin (rabbit polyclonal 1:2000; DAKO Corp., Carpinteria, CA) antibody for pericyte identification. Antibodies were diluted with PBS containing 0.01% thimerosal as an anti-bacterial and 0.3% Triton X-100 to improve penetration of 100-μm sections. After several rinses with PBS, specimens were incubated for 6 hours at room temperature with a goat anti-rat or goat anti-rabbit secondary antibody labeled with FITC or Cy5 for CD31 staining or with Cy3 for desmin staining (antibodies from Jackson ImmunoResearch, West Grove, PA). After mounting in Vectashield (Vector Laboratories, Burlingame, CA), specimens were examined with a Zeiss Axiophot fluorescence microscope and a Zeiss LSM 410 laser-scanning confocal microscope. Confocal images were stored as digital files, viewed with Photoshop (Adobe,

Mountain View, CA), and printed on a Fujix Pictography 3000 color printer (Fuji Photo Film Inc., Elmsford, NY).

Transmission Electron Microscopy

Tumors, fixed by vascular perfusion of 0.5% glutaraldehyde and 1% paraformaldehyde in 0.075 mol/L sodium cacodylate buffer, pH 7.4, were removed, immersed in 2.5% glutaraldehyde in cacodylate buffer for a minimum of 2 hours, and embedded in 10% agarose.[10] Sections 100 μm in thickness were cut with a Vibratome. Specimens measuring $\sim 1 \times 3$ mm were cut from the sections, treated with OsO_4 and uranyl acetate, dehydrated, and embedded in epoxy resin.[45] Sections 0.5 μm in thickness were stained with toluidine blue for light microscopy, and sections 50 to 100 nm in thickness were stained with lead citrate and examined with a Zeiss EM-10 electron microscope.

Morphometric Measurements

Morphometric measurements of blood vessels were made on images obtained from 100-μm-thick sections of four specimens ($n = 4$ mice) from each of the three tumors and normal tissues, unless designated otherwise. Regions of necrosis were avoided. The sections were double-stained for CD31 and α-SMA immunoreactivities. Real-time video images were viewed or digital images were captured with a Zeiss Axiophot microscope equipped with single and dual filters for FITC and Cy3 and a low-light, three-chip CoolCam CCD camera (Sci-Measure Analytical Systems, Atlanta, GA). Measurements were made using image analysis software developed for this purpose in our laboratory.[45] The proportion of vessels covered by pericytes was determined for 50 vessels in each specimen. Pericytes were considered present if α-SMA or desmin immunoreactivity was visible anywhere around the vessel perimeter. The number, length, and pericyte coverage of endothelial sprouts were determined in the same specimens. Sprouts were identified as tapered CD31-immunoreactive processes that extended away from the main axis of a vessel and ended abruptly. For all of the sprouts identified on vessels in each specimen (10 to 27 sprouts were analyzed per 50 vessels in each specimen), the lengths of the CD31-immunoreactive endothelial cell strands (sprout length) and the surrounding α-SMA immunoreactive pericyte sleeve (pericyte sleeve length) were measured. In addition, for 10 sprouts from each specimen that had FITC-lectin staining *in vivo* ($n = 2$ mice for each type of tumor), the lengths of the lectin-stained lumen (lumen length) and the associated CD31-immunoreactive endothelial cell strands were measured. The extent of pericyte coverage on vessels was determined on 15 properly cross-sectioned vessels in each specimen by measuring the proportion of CD31-positive vessel perimeter covered by α-SMA-immunoreactive cells. Values are expressed as means ± SEM (SEM). The significance of differences between means was assessed by analysis of variance followed by the Bonferroni/Dunn test ($P < 0.05$).

Results

α-SMA and Desmin Immunoreactivity of Pericytes

Normal Pericytes

In normal pancreatic acini and islets, α-SMA-immunoreactive cells were abundant on arterioles and venules but not on capillaries (Figure 1A). By comparison, desmin immunoreactive cells were present on all segments of the microvasculature, as shown by the extensive co-localization of desmin and CD31 immunoreactivities (Figure 1B). In normal pancreatic islets, α-SMA immunoreactivity co-localized with desmin immunoreactivity on arterioles and venules but not on capillaries where the adventitial cells had only desmin immunoreactivity (Figure 1; C, D, and E). As capillaries predominated in the pancreas, most of the vessels were enveloped by cells that had desmin immunoreactivity but lacked α-SMA immunoreactivity (Figure 1, A and B).

Pericytes in Tumors

The distribution of α-SMA-immunoreactive cells in islet cell tumors was very different from that in normal islets. In tumors in RIP-Tag2 mice, α-SMA and desmin immunoreactivities had essentially identical distributions (Figure 2, A and D). The two markers were also co-localized on most vessels in implanted MCa-IV breast carcinomas and Lewis lung carcinomas (Figure 2; B, E, and F).

Examination of α-SMA immunoreactivity in RIP-Tag2 mice, which had islet cell tumors at various stages of development, showed the transformation in expression of this marker from the normal to neoplastic state. The number of α-SMA-immunoreactive cells increased progressively as tumors enlarged (Figure 3A). Double staining for α-SMA and desmin showed that most adventitial cells on blood vessels in the smallest RIP-Tag2 tumors (hyperplastic islets) expressed desmin but not α-SMA (Figure 3B), whereas those in large tumors expressed both markers (Figure 3C). Most vessels in MCa-IV breast carcinomas and Lewis lung carcinomas were enveloped by cells with both α-SMA and desmin immunoreactivities within 7 days of implantation (data not shown).

Morphology of Pericytes

Pericytes on Normal Vessels

In the normal pancreas, arterioles were completely covered by circumferentially arranged, α-SMA-immunoreactive smooth muscle cells (Figure 4, A and B). Venules >50 μm in diameter were almost completely covered by α-SMA-positive cells that had a more irregular shape and looser packing than smooth muscle cells on arterioles (Figure 4A). Venules <50 μm in diameter were covered by pericytes that had α-SMA immunoreactivity, were irregular in shape and orientation, had multiple cytoplasmic processes, and incompletely covered the endothelium (Figure 4B). Capillaries in the pancreas had pericytes that were immunoreactive for desmin but not

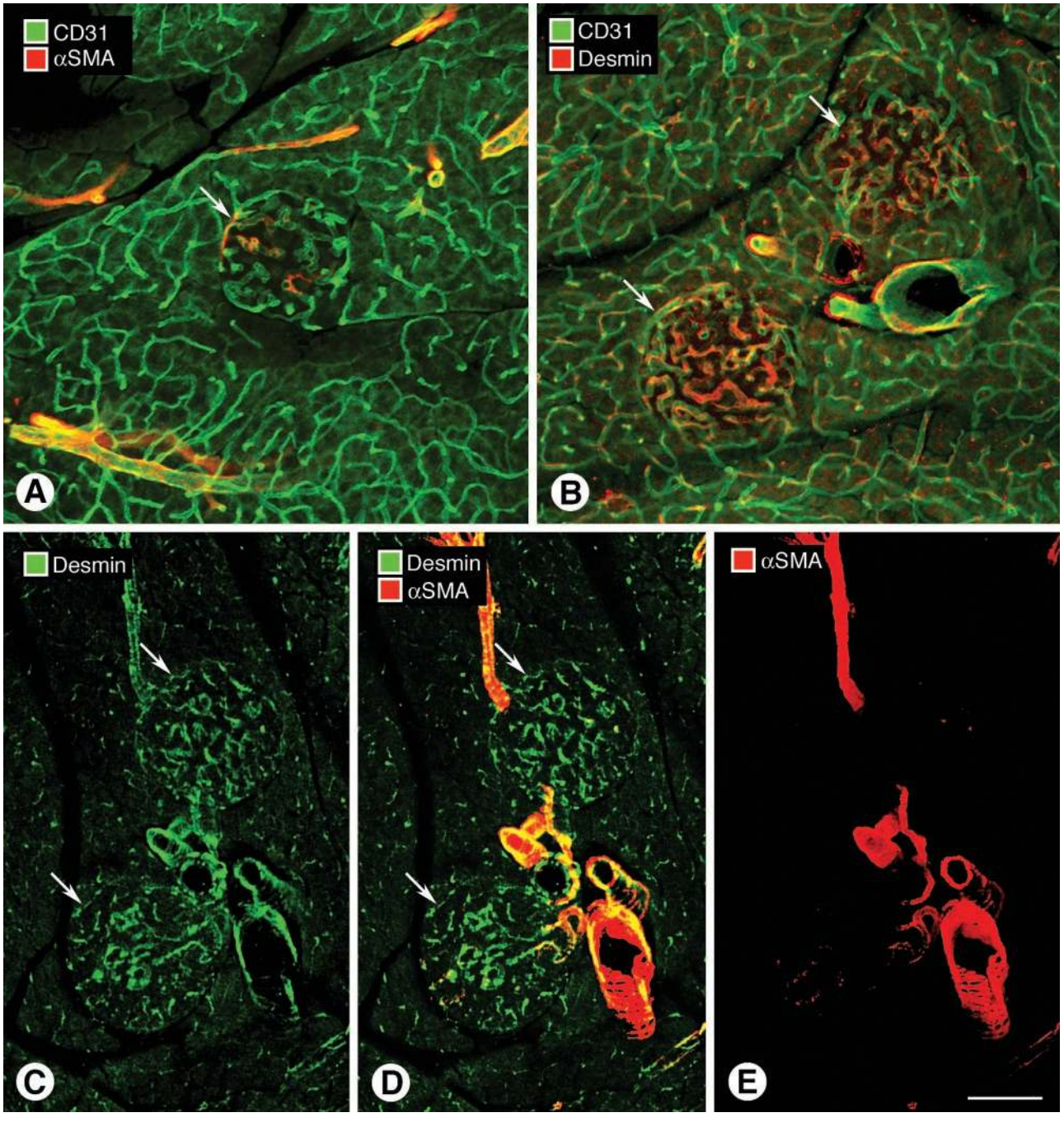


Figure 1. Comparison of α-SMA and desmin immunoreactivities of blood vessels in normal pancreas. **A** and **B:** Vessels in pancreatic islets (**arrows**) and acini stained for CD31 and α-SMA or desmin immunoreactivity. Arterioles and venules but not capillaries have α-SMA-positive adventitial cells (**A**), whereas desmin immunoreactive cells are present on most vessels in islets (**B**). **C–E:** Desmin-immunoreactive cells are associated with most vessels including capillaries in islets (**C** and **D**, **arrows**), but α-SMA-positive cells are restricted to arterioles and venules (**D** and **E**). Scale bar in **E** applies to all figures. Bar lengths: 100 μm (**A** and **B**); 120 μm (**C–E**).

α-SMA (Figure 4C). Unlike those on venules, pericytes on capillaries had multiple long, branched cytoplasmic processes that projected along the longitudinal axis of the vessel and covered only a small proportion of the endothelial surface (Figure 4C).

On all blood vessels in the normal pancreas, pericytes or smooth muscle cells were tightly associated with the underlying endothelial cells, and no cellular processes projected away from the vessel wall into the surrounding tissue (Figure 4; A, B, and C).

Abnormalities of Pericytes on Tumor Vessels

Blood vessels in the three types of tumors we examined had multiple abnormalities, and most did not have the typical morphological features and sequential hierar-

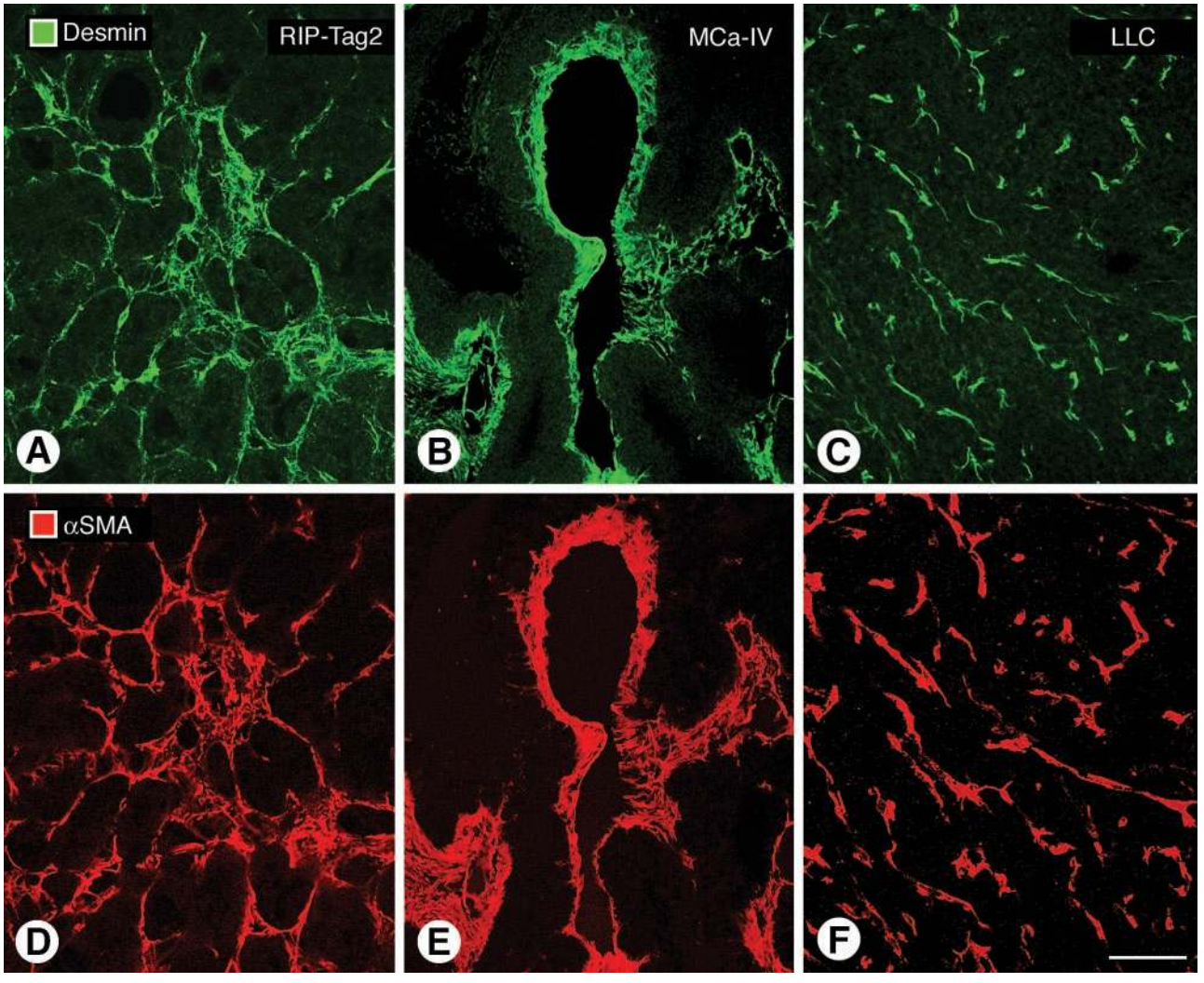


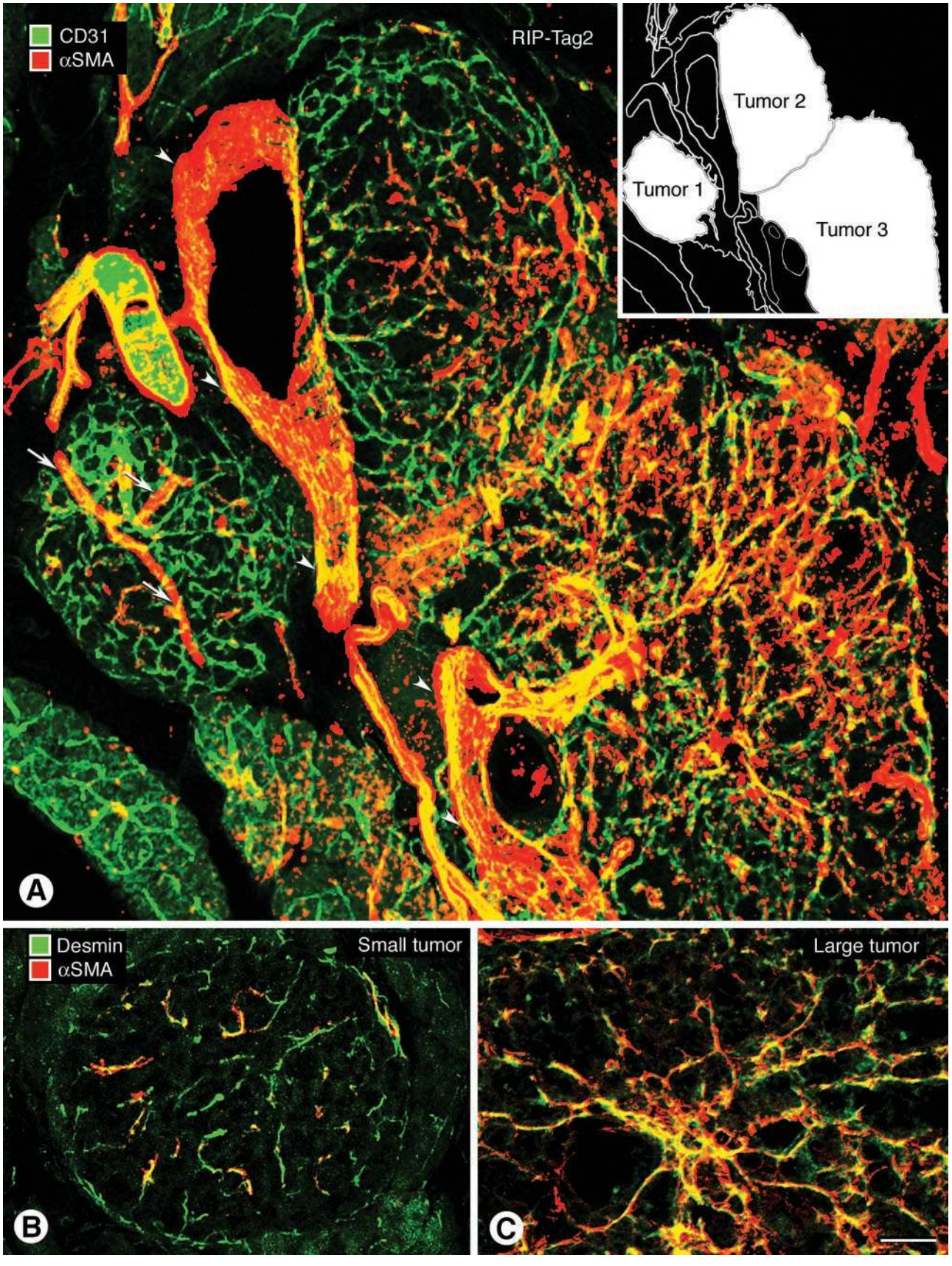
Figure 2. Double staining for desmin (**A–C**) and α-SMA (**D–F**) immunoreactivity of vessels in 100-μm sections of three types of tumors. **A** and **D:** Large pancreatic islet cell tumor in RIP-Tag2 transgenic mouse. **B** and **E:** Implanted MCa-IV mammary carcinoma. **C** and **F:** Implanted Lewis lung carcinoma (LLC). Unlike their different distributions on normal vessels, desmin and α-SMA are mostly co-localized in all three tumors. Scale bar in **F** applies to all figures. Bar length, 100 μm.

chy of arterioles, capillaries, or venules. Pericytes on tumor vessels viewed by confocal microscopy were conspicuously abnormal in shape and had an abnormal association with endothelial cells. These cells resembled the pericytes on small venules more than those on capillaries, but were irregularly scattered over the endothelium and had bizarre cytoplasmic processes (Figure 4; D to H). Pericyte processes projected along the abluminal surface of the endothelium or away from the vessel wall into the tumor parenchyma (Figure 4F). Some pericyte processes contacted one another within the tumor parenchyma (Figure 4G). Unlike the tight association of pericytes and endothelial cells in normal vessels (Figure 4; A, B, and C), on tumor vessels there were abnormal separations between the α-SMA and CD31-positive cells, indicating that pericytes were loosely associated with endothelium (Figure 4; D, E, and F). Some pericytes overlapped one another (Figure 4, G and H).

Ultrastructural observations were consistent with the findings made by confocal microscopy (Figure 5; A to D). Pericytes examined by electron microscopy had cytoplasmic processes that extended away from the vessel wall toward the tumor parenchyma (Figure 5, A and B). In addition, pericytes seemed to be loosely associated with the endothelium (Figure 5, C and D). Some pericytes overlapped other pericytes (Figure 5, A and D).

Differences in Vessel Size and Pericyte Coverage among Tumors

The vascular architecture was strikingly different in the three types of tumors we examined. RIP-Tag2 tumors had uniformly small, capillary-size blood vessels (Figure 6A), with a mean diameter of 8 μm (Table 1). In MCa-IV tumors, capillary-size vessels intermingled with extremely large vessels (Figure 6B). Vessel diameters in MCa-IV tumors ranged from 8 to 294 μm; the mean vessel diameter of 45 μm was fivefold that for RIP-Tag2 tumors (Table 1). The size of vessels in Lewis lung carcinomas (Figure 6, C and D), mean diameter of 31 μm, was intermediate between the other two tumors (Table 1).


CD31
αSMA
RIP-Tag2
Tumor 2
Tumor 1
Tumor 3
A
Desmin
αSMA
Small tumor
B
Large tumor
C

Despite the differences in vascular architecture, α-SMA-positive pericytes were present on at least 97% of the vessels in all three tumors (Figure 7). The smallest to the largest vessels had extensive pericyte coverage. By comparison, α-SMA-immunoreactive cells were found on only 22% of vessels, predominately capillaries, in normal pancreatic islets (Figure 1, A and B; Figure 7). Approximately half of the vessel surface was covered by α-SMA-positive pericytes in RIP-Tag2 tumors and Lewis lung carcinomas, but 80% of the vessel surface was covered by pericytes in MCa-IV tumors (Table 1).

The density of blood vessels at the tumor periphery differed between the implanted tumors and the RIP-Tag2 tumors. In Lewis lung carcinomas (Figure 6D) and MCa-IV carcinomas, the density of vessels at the interface of the tumor and the surrounding tissue was unusually high. Densely packed vessels at the tumor surface were accompanied by correspondingly numerous α-SMA-positive cells (Figure 6D). In contrast, the density of blood vessels and α-SMA-positive cells was relatively uniform throughout RIP-Tag2 tumors (Figure 3A).

Myofibroblasts in Tumors

All three tumors contained cells that were immunoreactive for both α-SMA and desmin but had no apparent association with blood vessels (Figure 6; A to D). These cells, which were continuous with and morphologically similar to pericytes on blood vessels, fit the characteristics of myofibroblasts (Figure 4G and Figure 6; A to D). Myofibroblasts were more abundant at the periphery of Lewis lung carcinomas (Figure 6D) and MCa-IV carcinomas but not RIP-Tag2 tumors.

Pericytes on Endothelial Sprouts

Identification of Endothelial Sprouts in Tumors

CD31-immunoreactive sprouts were found on 24 to 33% of blood vessel segments examined in 100-μm sections of the three types of tumors (Table 2). Sprouts, which projected away from the endothelium, were broadest at their base and tapered progressively to blind endings (Figure 8; A to E). Sprouts projected into the tumor parenchyma a distance of 3 to 69 μm (Table 2). The sprouts in MCa-IV carcinomas averaged approximately twice as long as those in RIP-Tag2 tumors. Fluorescent *L. esculentum* lectin, injected into the bloodstream, bound uniformly to the luminal surface of tumor vessels but stained only the proximal portion of sprouts (Figure 8, A and B). On average, the lectin-stained segment was 20 to 35% of the total length of the CD31-positive sprouts (Figure 9, Table 2), suggesting that the distal two-thirds of the sprouts did not have a lumen.

Pericytes on Endothelial Sprouts in Tumors

Examination of double-labeled sections revealed that α-SMA-immunoreactive pericytes were closely associated with most of the CD31-immunoreactive sprouts in tumors. Measurements showed that pericytes formed sleeves on 73 to 92% of the sprouts in the three types of tumors (Table 2). Interestingly, the α-SMA-positive pericyte sleeves were consistently longer than the CD31-positive sprouts (Figure 8, C and E). The length of pericyte sleeves was from 38% (Lewis lung tumors) to 80% (RIP-Tag2 tumors) longer than the sprouts themselves (Figure 9, Table 2).

Discussion

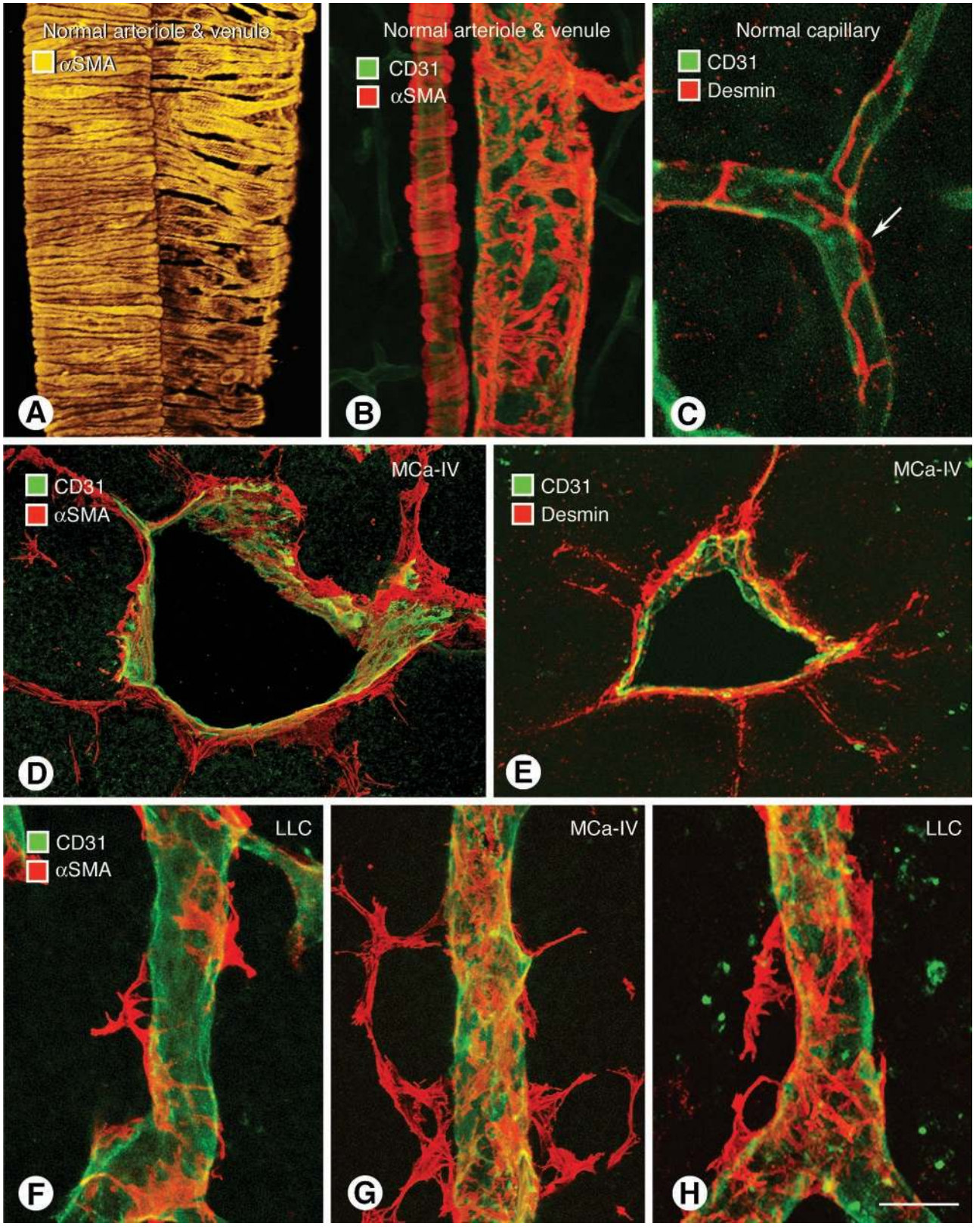
By using confocal microscopy to analyze the three-dimensional structure of blood vessels in three different types of mouse tumors stained immunohistochemically for CD31, α-SMA, and desmin, we identified multiple distinctive abnormalities of pericytes. Unlike those on corresponding normal vessels, pericytes on tumor vessels uniformly expressed α-SMA on capillary-size vessels, were loosely associated with endothelial cells, had cytoplasmic processes that projected into the tumor parenchyma, and formed a sleeve around endothelial sprouts that was longer than the sprouts themselves. These abnormalities are illustrated schematically in Figure 10.

α-SMA and Desmin as Molecular Markers of Pericytes

We found that pericytes on normal capillaries in the pancreas had desmin immunoreactivity but lacked α-SMA, whereas smooth muscle cells on arterioles and pericytes on venules were immunoreactive for both. This finding matches what is known about the microvascular beds of the mesentery.[26] In contrast, >97% of blood vessels in the three tumors we examined, including vessels the size of capillaries, had abundant pericytes with both α-SMA and desmin immunoreactivities. Pericytes expressing α-SMA as well as desmin enveloped tumor vessels of all size, shape, and configuration. Tumor vessels were not classified as arterioles, capillaries, or venules because they did not have the structural characteristics and hierarchy that would make this classification meaningful.

The uniform co-localization of α-SMA and desmin indicates that both are consistent markers of pericytes in the tumors we examined. Abundant α-SMA-positive pericytes are also present in glioblastoma multiforme,[20] but other types of tumors have been reported to have vari-

Figure 3. Increasing α-SMA expression in pericytes during tumor progression in RIP-Tag2 mice. **A:** In small tumor (**left center** and tumor 1 in **inset**) α-SMA-positive cells are present on arterioles (**arrows**) but are sparse on capillary-size vessels. In larger tumors (**top center** and tumor 2 in **inset**; **bottom right** and tumor 3 in **inset**) α-SMA-positive cells are more abundant on vessels of all size. CD31-immunoreactive endothelial cells (green). **B** and **C:** Double staining for desmin and α-SMA immunoreactivity. Pericytes on most vessels in small RIP-Tag2 tumor (**B**), which is a hyperplastic islet, resemble those in normal islet by expressing desmin but not α-SMA, but corresponding cells in a larger tumor (**C**) have both desmin and α-SMA immunoreactivities. **Arrowheads** mark large vein between the tumors. Scale bar in **C** applies to all figures. Bar lengths: 85 μm (**A**); 60 μm (**B** and **C**).


Normal arteriole & venule
αSMA
A
Normal arteriole & venule
CD31
αSMA
B
Normal capillary
CD31
Desmin
C
CD31
αSMA
MCa-IV
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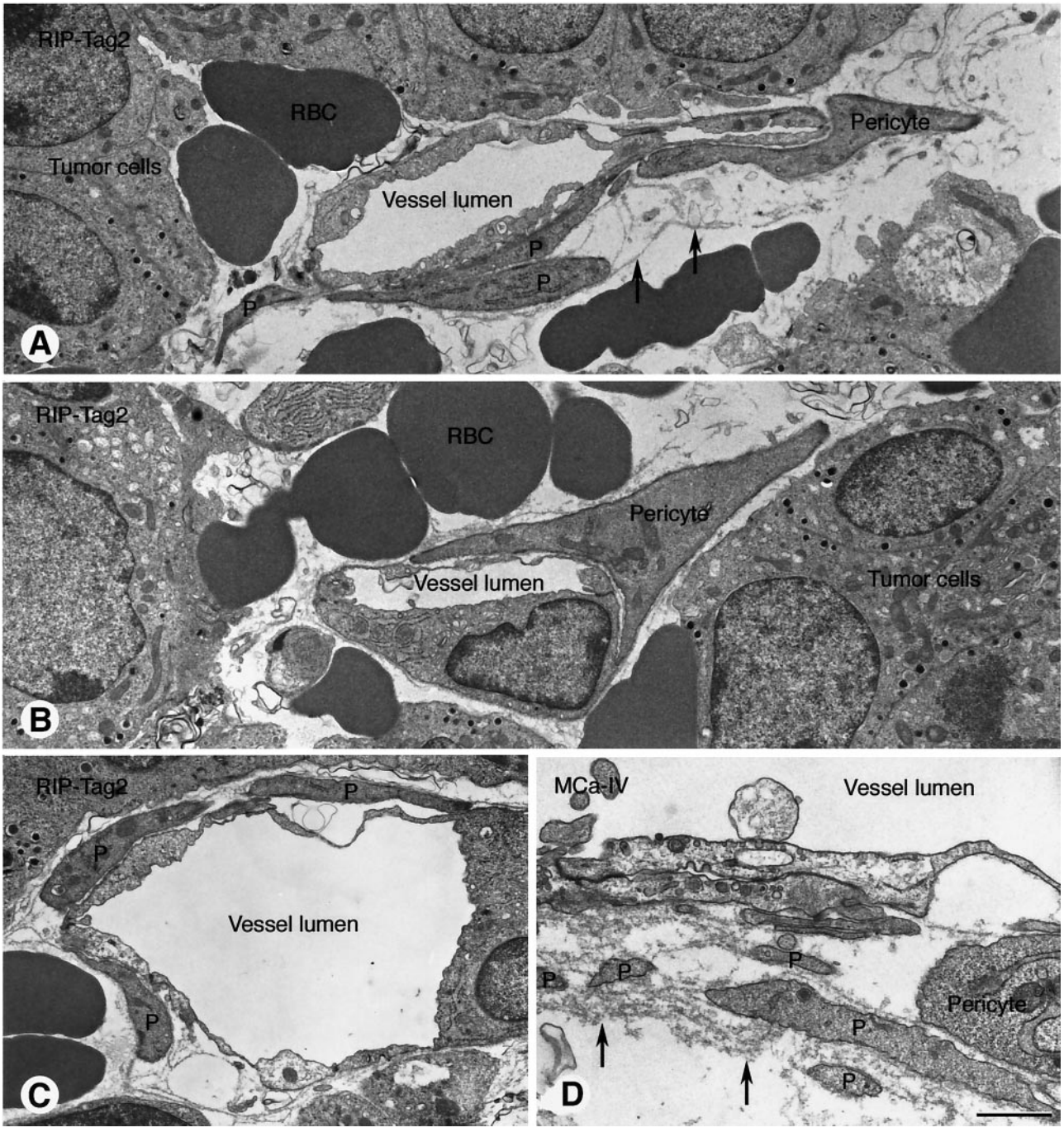


Figure 5. Transmission electron micrographs showing abnormal pericytes on blood vessels in RIP-Tag2 tumors (**A–C**) and MCa-IV carcinoma (**D**). RBC, extravasated erythrocytes. **A** and **B:** Irregularly shaped pericyte processes (P) near a tumor vessel with a loose basement membrane (**arrows**). **B:** Pericyte process directed away from a capillary-size tumor vessel. **C:** Pericyte processes (P) loosely associated with the endothelium of tumor vessel. **D:** Pericyte processes (P) within loose, multilayered basement membrane (**arrows**) of tumor vessel. Scale bar in **D** applies to all figures. Bar lengths: 2 μm (**A–C**); 1 μm (**D**).

Figure 4. Morphology of adventitial cells on blood vessels in normal pancreas and in tumors. **A:** α-SMA-immunoreactive smooth muscle cells are circumferentially oriented and closely spaced on a larger arteriole (**left**) and venule (**right**) in normal pancreas, but are more uniformly shaped and closely packed on the arteriole. **B:** Smooth muscle cells on smaller arteriole (**left**) and pericytes on smaller venule (**right**) in normal pancreas stained for CD31 and α-SMA. The arteriole has regularly and circumferentially arranged smooth muscle cells, whereas the venule has irregularly arranged pericytes with multiple cytoplasmic processes that incompletely cover the vessel wall. **C:** Pericyte (**arrow**) on normal capillary, stained for CD31 and desmin, is positioned along the vessel axis. Most of the pericyte processes are oriented longitudinally. **D–H:** Pericytes on tumor vessels stained for CD31 (green) and α-SMA (red) are irregularly arranged, loosely associated with the endothelium, have cytoplasmic processes projecting in multiple directions, and have some similarities to venular pericytes. **D** and **E:** Unlike normal pericytes, some pericytes in MCa-IV carcinoma project away from the endothelium and into the tumor parenchyma. This feature is evident both with α-SMA and desmin immunoreactivity. **F:** Lewis lung carcinoma showing the loose association of pericytes with the endothelium of tumor vessels. **G:** Pericytes contacting one another near a vessel in a MCa-IV carcinoma. Pericytes also contact α-SMA-positive stromal cells that are apparently not associated with this vessel. **H:** Some pericytes in this Lewis lung tumor overlap other pericytes. Scale bar in **H** applies to all figures. Bar lengths: 35 μm (**A**, **F–H**); 30 μm (**B**); 15 μm (**C**); 80 μm (**D** and **E**).

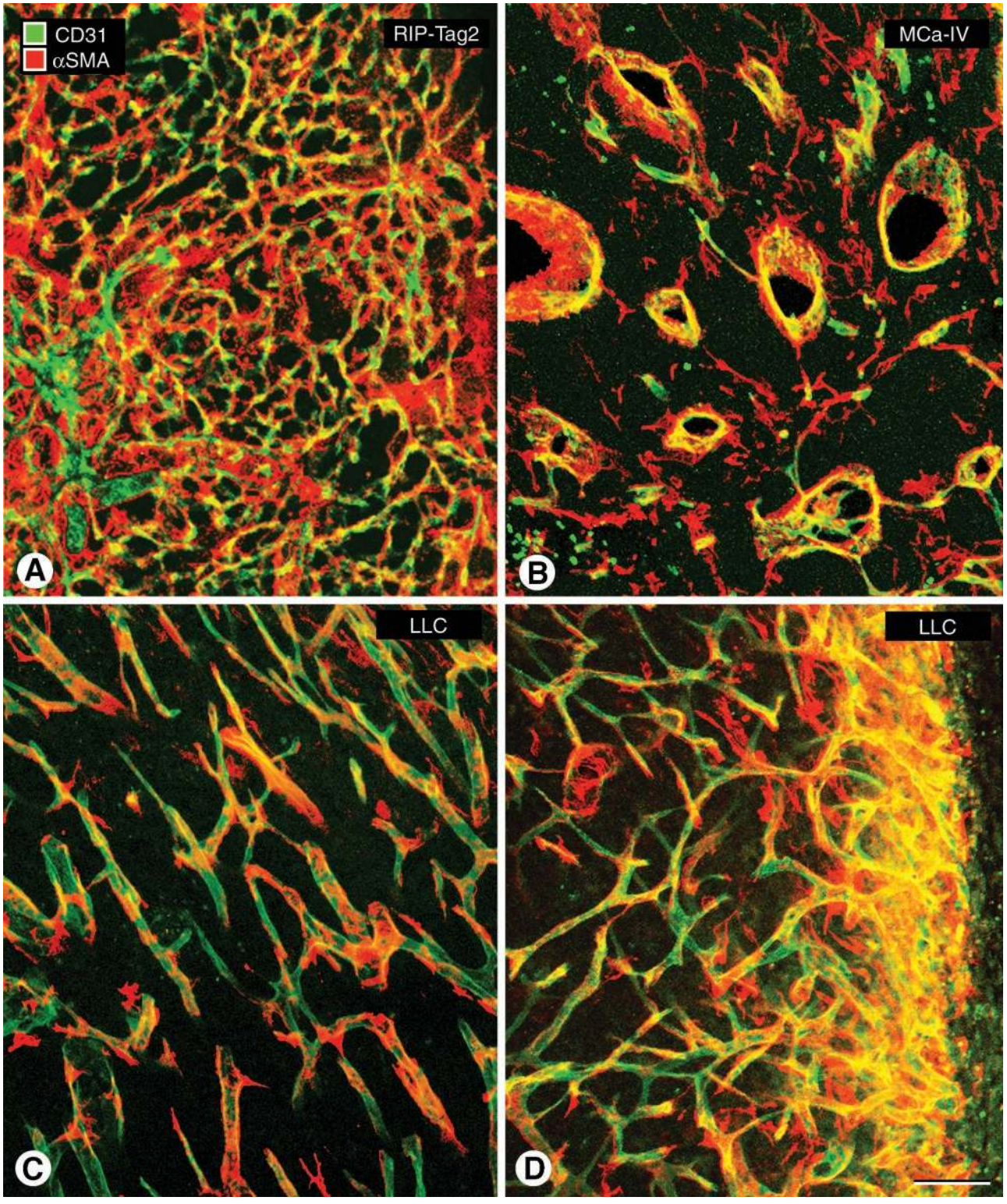


Figure 6. Distribution of α-SMA-immunoreactive cells (red) in three types of tumors. Some α-SMA-positive cells are pericytes on blood vessels marked by CD31 immunoreactivity (green), whereas others have no such association and are presumed to be myofibroblasts. **A:** RIP-Tag2 tumor: most vessels are the size of capillaries. Almost all are accompanied by α-SMA-positive cells. **B:** MCa-IV mammary carcinoma: vessels vary in size over a broad range and contain the largest vessels of the three tumors. Some α-SMA-positive cells surround blood vessels; others are located in the stroma between vessels. **C:** Lewis lung carcinoma (LLC): nearly all vessels are at least partially covered by α-SMA-positive cells. **D:** Lewis lung carcinoma: vessels and α-SMA-positive cells are especially densely packed at the periphery of the tumor. Some α-SMA-positive cells surround blood vessels; others do not. Scale bar in **D** applies to all figures. Bar length, 100 μm.

Table 1. Diameter and Amount of α-SMA-Immunoreactive Pericyte Coverage of Tumor Vessels*

	RIP-Tag2 pancreatic islet tumors	MCa-IV mammary carcinomas	Lewis lung carcinomas
Blood vessel diameter (range)	8 ± 1 μm† (5–16 μm)	45 ± 6 μm (8–294 μm)	31 ± 2 μm (5–154 μm)
Extent of α-SMA pericyte coverage (% of blood vessel surface)	56 ± 1%	80 ± 2%‡	51 ± 4%

*Vessel diameter and fraction of vessel perimeter covered by pericytes were measured in 15 tumor vessels cut perpendicular to their longitudinal axis in 100-μm-thick sections, double-stained for α-SMA and CD31 immunoreactivities, from each mouse ($n = 4$ mice per group). Measurements were made on 1-μm optical sections obtained by confocal microscopy.
†Vessels in RIP-Tag2 tumors were significantly smaller than those in MCa-IV and Lewis lung tumors ($P < 0.05$).
‡Pericyte coverage of vessels in MCa-IV tumors was significantly greater than in RIP-Tag2 and Lewis lung tumors ($P < 0.05$).

able proportions of α-SMA-positive pericytes.[18,19,22] There are also reports that certain tumors have few or no α-SMA-positive pericytes.[21] Pericytes in ovarian carcinoma uniformly express both α-SMA and NG2, but some tumors have a larger proportion of pericytes that express NG2 than α-SMA,[18,19] indicating that a lack of α-SMA immunoreactivity does not necessarily mean a lack of pericytes.

Methods of tissue preparation also can influence the apparent number of pericytes on tumor vessels. Our study of three-dimensional confocal images of 100-μm sections revealed that, despite the high incidence of vessels with pericytes, on average from 30 to 50% of the endothelial surface had no pericyte coverage. Pericytes have been reported to be abundant in conventional histological sections of some types of human tumors,[20,46] but uncovered regions could be misinterpreted in thin histological sections as vessels lacking pericytes.

The abundance of pericytes on tumor vessels examined in the present study—in contrast to what has been reported in some previous studies—can be explained in part by the use of both desmin and α-SMA as immunohistochemical markers in combination with 100-μm-thick tissue sections. Differences among tumors may also be a factor.

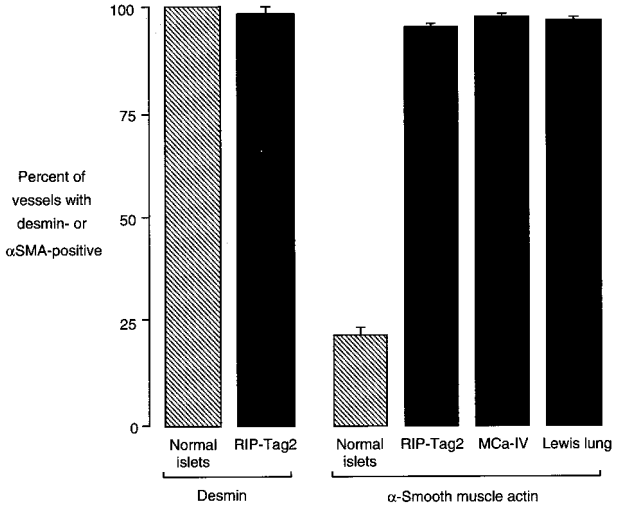


Figure 7. Bar graph comparing the percentage of blood vessels covered by desmin in normal pancreatic islets and islet cell tumors in RIP-Tag2 mice and by α-SMA-immunoreactive pericytes in normal islets, islet cell tumors in RIP-Tag2 mice, MCa-IV breast carcinomas, and Lewis lung carcinomas. Frequency of tumor vessels with pericytes was analyzed on 50 blood vessels in 100-μm sections double-stained for CD31 and desmin or α-SMA immunoreactivities of a tumor in each mouse ($n = 4$ mice for each type of tumor). One hundred percent of blood vessels in normal islets were covered by desmin-positive pericytes.

The uniform presence of α-SMA-positive pericytes on the vasculature of RIP-Tag2 tumors reinforces the distinction between blood vessels and the prominent collections of extravascular erythrocytes (blood lakes) in these tumors.[10] Such collections of erythrocytes have been considered by some as examples of tumor cell-lined vascular channels.[47] However, blood vessels in RIP-Tag2 tumors are composed of endothelial cells and pericytes, whereas blood lakes in these tumors are lined only by tumor cells.[10] Despite their appearance, blood lakes seem not to be connected to the bloodstream and are not channels for flowing blood.[10] Thus, the erythrocytes in blood lakes are stagnant.

Changes in Pericytes during Tumor Progression

Islet cell tumors arise asynchronously in RIP-Tag2 mice.[44] Multiple tumors are present in each mouse, and these tumors are at different stages of development. This feature of the RIP-Tag2 model led to our observation of the loss of heterogeneity of the pericyte population during tumor progression. During tumorigenesis, pericytes with the capillary phenotype (desmin-positive and α-SMA-negative) were replaced by or transformed into pericytes with the venular phenotype (desmin-positive and α-SMA-positive) even though capillary-size vessels continued to predominate in RIP-Tag2 tumors. The lack of α-SMA-positive pericytes in smaller tumors may also be a reflection of an anti-angiogenic action of larger tumors in the same mouse, representing the inhibition of tumor growth by tumor mass.[48] MCa-IV carcinomas and Lewis lung carcinomas had mixtures of small and large vessels, but α-SMA and desmin immunoreactivities were co-localized on all vessels regardless of their size. Thus, the change in the phenotype of pericytes was not simply a reflection of vessel enlargement during tumor development. Consistent with these observations, the number of α-SMA-positive Ito cells on liver sinusoids, which are considered equivalent to pericytes, increases in metastatic liver cancer.[49]

During tumorigenesis, alterations in the tissue microenvironment, including both the extracellular matrix and soluble factors, are likely to contribute to the phenotypic transformation of pericytes in tumors. Pericytes from human brain capillaries begin to express α-SMA when grown in culture in the presence of transforming growth

Table 2. Pericyte Coverage of Endothelial Sprouts on Tumor Vessels*

	RIP-Tag2 pancreatic islet tumors	MCa-IV mammary carcinomas	Lewis lung carcinomas
% of vessel segments with sprouts (sprouts per 50 vessel segments)	24 ±4% (13)	33 ±5% (20)	31 ±5% (17)
% of sprouts with sleeve of α-SMA pericytes	73 ±2%	92 ±2%	92 ±1%
Length of CD31 endothelial sprouts (range of sprout lengths)	15 ±1 μm (3–46 μm)	29 ±4 μm (3–69 μm)	23 ±4 μm (5–56 μm)
Length of lectin-stained lumen of sprouts (% of endothelial sprout length)	20%	28%	35%
Length of pericyte sleeve around sprouts as (% of endothelial sprout length)	180%	162%	138%

*Sprouts, identified as thin, tapered, blind-ended CD31-positive projections away from the main axis of vessels, were analyzed on 50 blood vessels in 100-μm sections double-stained for α-SMA and CD31 immunoreactivities from a tumor in each mouse (n = 4 mice per group). Pericytes were identified by α-SMA immunoreactivity. The length of the lectin-stained lumen of sprouts was measured in 10 sprouts for each tumor in preparations stained with lectin *in vivo* and then stained for CD31 immunoreactivity (n = 2 mice per group).

factor-β 1.[50] Similarly, pericytes on retinal capillaries express α-SMA *in vitro* but not *in vivo*.[26]

Morphological Abnormalities of Pericytes in Tumors

Pericytes in the tumors we examined had some features in common with pericytes on venules but were morphologically very different from smooth muscle cells on arterioles. Nonetheless, pericytes on tumor vessels had structural abnormalities that clearly distinguished them from pericytes on venules and, indeed, from adventitial cells on any part of the normal microcirculation (Figure 10).

One abnormality we observed was that pericytes on tumor vessels were loosely associated with endothelial cells, with wide spaces separating some regions of the two types of cells. Pericytes also had cytoplasmic processes that penetrated deep into the tumor parenchyma, a feature not found on normal vessels. Some confocal images gave the impression that pericytes are actively moving within tumors. Published ultrastructural observations provide additional clues that pericytes are activated under pathological conditions. Pericytes on inflamed vessels may become amoeboid, plump, and mitotically active.[51,52]

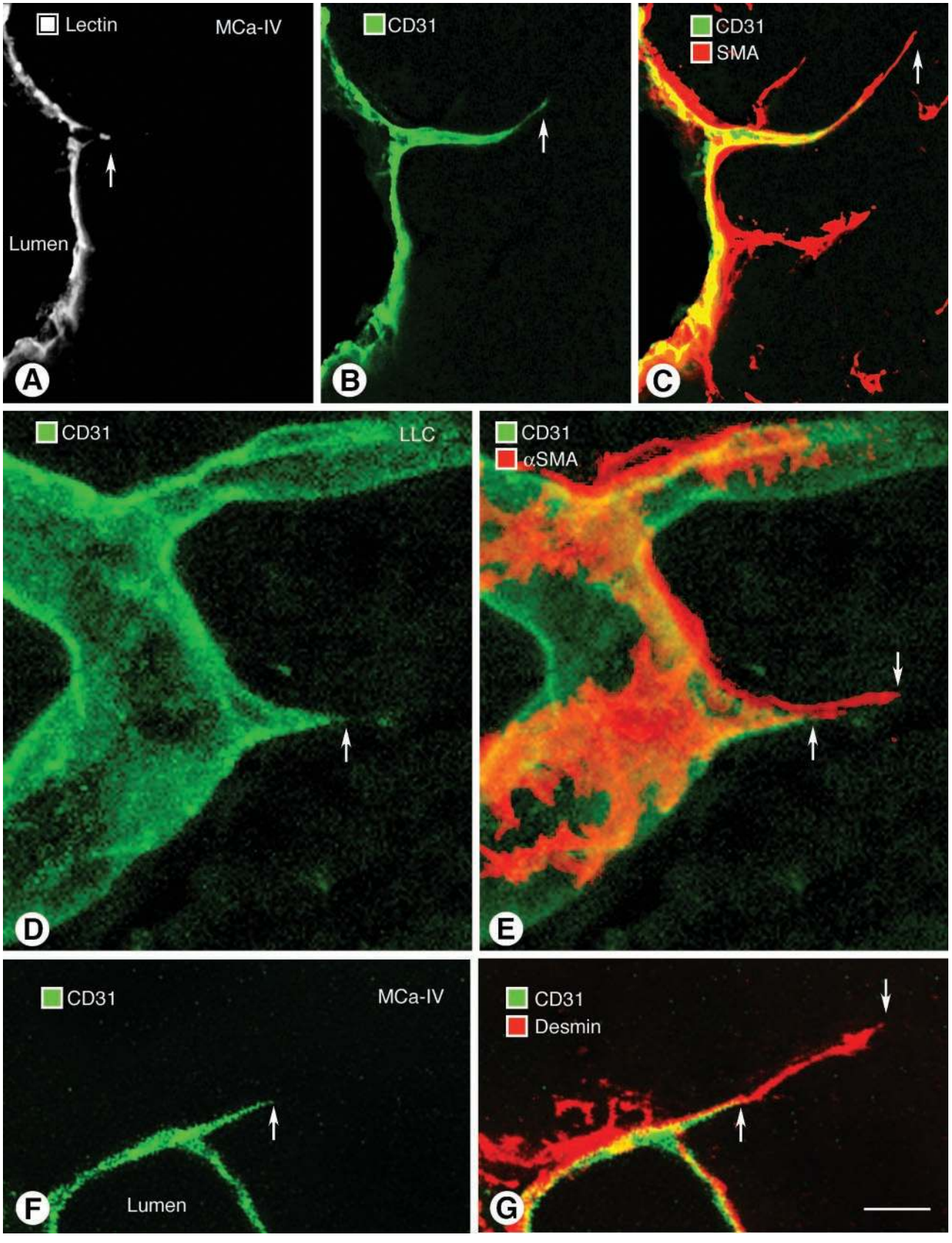
Another abnormality was the amount of pericyte coverage on capillary-size vessels in tumors. Nearly all of the vessels in the three tumors we examined were covered with pericytes. However, the extent of coverage ranged from an average of ~50% in RIP-Tag2 tumors and Lewis lung carcinomas to as much as 80% in MCa-IV breast carcinomas. Similarly, the pericyte coverage of vessels in human cerebellar hemangioblastomas has been reported to be 69%.[46] These values for tumors are higher than those for most normal capillaries, where the amount of pericyte coverage, expressed as a proportion of endothelial surface, has been estimated as 11% in cardiac muscle, 21% in skeletal muscle, 22 to 30% in brain, and 41% in retina.[53] Instead, the values for tumors fit better with those for normal venules, estimated to be 81% in skeletal muscle.[53]

Further, basement membrane had an abnormally loose association with pericytes and endothelial cells in the three tumors we examined, and in some regions was multilayered or extended beyond the pericytes into the tumor parenchyma (unpublished observations). Basement membrane on blood vessels in these tumors was continuous except for scattered discontinuities smaller than a few micrometers.

The abnormal relationship of pericytes with endothelial cells may alter the influence of pericytes on the endothelium and contribute to the leakiness of tumor vessels[9,10] and explain the sensitivity of tumor vessels to VEGF withdrawal.[21]

An important next step will be to address the question of whether pericytes on blood vessels in human tumors have abnormalities similar to those observed in mouse tumors. However, answering this seemingly straightforward question is likely to be a challenge. Our study of three mouse tumors took advantage of the many attributes of experimental tumors, including well-defined growth conditions, uniform age and genetic background of the host, multiple stages of tumorigenesis, and no previous treatment, along with the technical advantages of using vascular perfusion and other conditions of optimal tissue preservation. Because corresponding studies of pericytes in human tumors cannot readily be performed under such idealized conditions, the immunohistochemical and morphological properties of pericytes in human tumors are likely to depend on many variables, including the tumor's histological type, grade, stage, anatomical location, age, treatment history, and fixation conditions. The heterogeneity of pericyte coverage of vessels in different types of human cancer is one mani-

Figure 8. Endothelial sprouts on tumor vessels. **A–C:** Sprout on vessel in MCa-IV carcinoma stained by intravenous injection of FITC-*L. esculentum* lectin followed by staining of 100-μm sections for CD31 and α-SMA immunoreactivities. **A:** Only the proximal portion of the sprout is made visible by the fluorescent lectin (**arrow**), suggesting that the distal part of the sprout has no lumen. **B:** Same region showing the slender endothelial sprout with a tapered, blind ending (**arrow**) after CD31 staining. **C:** Same region showing a pericyte sleeve around and beyond (**arrow**) the end of the sprout. **D** and **E:** Blood vessel in Lewis lung carcinoma showing CD31-stained endothelial cells (**D**) and α-SMA-positive pericytes (**E**) with cytoplasmic processes that extend beyond the end of an endothelial sprout (**arrows**). **F** and **G:** Endothelial sprout in MCa-IV carcinoma accompanied by desmin-positive pericyte continuing beyond the sprout itself (**arrows**). Scale bar in **G** applies to all figures. Bar length: 25 μm (**A–C**); 10 μm (**D–G**).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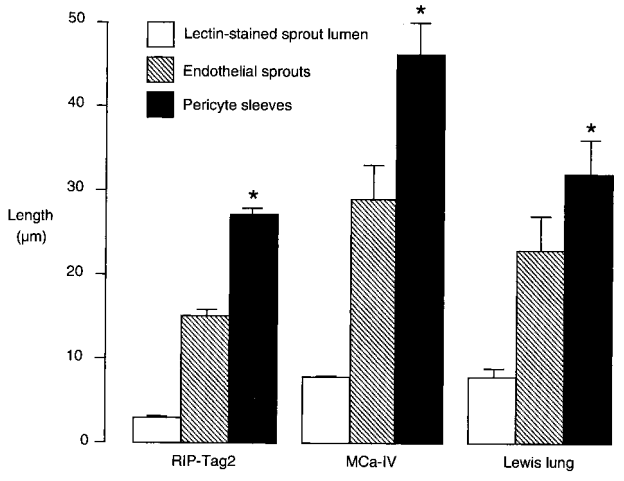

Figure 9. Bar graph comparing the lengths of three components of endothelial sprouts in tumors: sprout lumen visualized by lectin staining, endothelial sprouts visualized by CD31 immunoreactivity, and pericyte sleeves on the sprouts visualized by α-SMA immunoreactivity. The sprout lumen was only 20 to 35% of the length of the sprouts, whereas the pericyte sleeves covering the sprouts were significantly longer than the sprouts in all three tumors (*, $P < 0.05$).

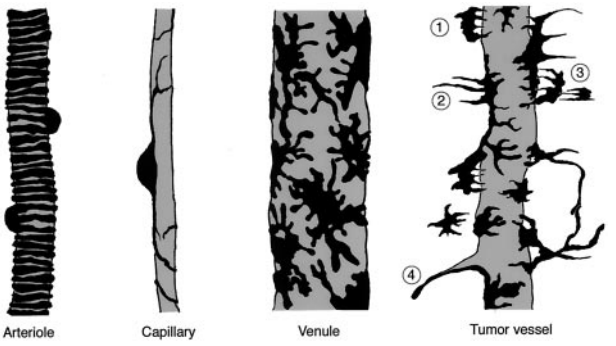

Figure 10. Schematic drawing comparing normal adventitial (periendothelial or mural) cells on an arteriole, capillary, and venule in the pancreas with abnormal pericytes on a tumor vessel representative of those found in RIP-Tag2 tumors, MCa-IV breast carcinomas, and Lewis lung carcinomas. Smooth muscle cells on arterioles were uniformly shaped, circumferentially arranged, closely packed, and tightly associated with the endothelium. Pericytes on capillaries were oriented longitudinally along the vessel, had long thin processes, were tightly positioned next to the endothelium, and covered only a small proportion of the vessel surface. Pericytes on venules had an irregular shape, close association with endothelial cells, and covered much of the vessel surface. By comparison, pericytes in the tumors we examined (**1**) were loosely associated with the endothelium, (**2**) had processes that extended away from the vessel wall, (**3**) in some cases overlaid other pericytes, and (**4**) accompanied endothelial sprouts, and even extended beyond the ends of the sprouts.

festation of these variables.[22] Future studies that examine pericyte abnormalities in human tumors should take these issues into account.

Pericytes Associated with Endothelial Sprouts in Tumors

CD31-positive endothelial sprouts arising from the wall of blood vessels were found in all three tumors we examined. The CD31 immunoreactivity, origin from endothelial cells, incomplete lumen, and blind termination fit the identification of the structures as sprouts. Because the *L. esculentum* lectin circulating in the bloodstream stained only the proximal third of the sprouts, it is likely that the distal portion did not have a lumen.

Nearly all of the sprouts in the tumors we examined were covered by sleeves of pericytes. The sleeve extended well beyond the end of most endothelial sprouts. The leading position of the pericyte sleeve may reflect a role of these cells in sprout growth and retraction.[54,55] Pericytes regularly accompany endothelial sprouts on growing blood vessels, seem to be involved in the earliest stages of sprout formation, may determine the location of sprout formation, and guide the outgrowth of sprouts.[41,42] In the ovary, pericytes are among the first cells to invade newly vascularized corpora lutea,[56] and pericytes in tumors proliferate early in angiogenesis.[26] Observations made in the present study are consistent with reports that pericytes are located on blood vessels at the growing front of tumors where angiogenesis is most active.[18–20,57] Pericytes are found on newly formed vessels in other conditions as well.[19,41] As to the source of the pericytes, although we did not assess pericyte migration, the cells accompanying sprouts could have come from pericytes on the parent vessels or have been α-SMA-positive stromal cells recruited to the sprouts.

If pericytes play a significant role in blood vessel growth in tumors, these cells would be a potential target in anti-angiogenic therapy. In this regard, the apparent dependence of pericytes on PDGFR-β signaling[30,31] raises the likelihood that inhibitors of these tyrosine kinase receptors may disrupt angiogenesis in tumors.[58]

Pericytes may promote angiogenesis by secreting basic fibroblast growth factor[59,60] or VEGF.[61] Some VEGF-expressing stromal cells near endothelial sprouts in tumors[62] may indeed be pericytes. Pericytes may also be associated with regressing sprouts, and may signal blood vessel degeneration and undergo apoptosis before endothelial cells.[63,64]

Origin and Function of Pericytes in Tumors

Pericytes or their precursors migrate into tumors with the developing vasculature. Studies of implanted tumors have shown that desmin-positive cells accumulate initially at the interface of the tumor and host tissue and later around new blood vessels.[65] A large proportion of stromal cells in some tumors express smooth muscle proteins and are potential pericyte precursors.[66] We found that cells expressing α-SMA and desmin were abundant at the tumor-host tissue interface of MCa-IV carcinomas and Lewis lung carcinomas. Some of these cells were closely associated with blood vessels, but others were not.

Stromal cells that express α-SMA and desmin but are not associated with blood vessels are usually designated myofibroblasts. These cells are abundant in many tumors.[67] Fibroblasts co-cultured with tumor cells can differentiate into myofibroblasts.[67] The stromal (mesenchymal) origin of pericytes is well documented,[26] and myofibroblasts are likely to contribute. Because pericytes may migrate away from the basement membrane and

become extramural pericytes under some conditions,[26] pericytes and myofibroblasts may be interconvertible.

Conclusions

Pericytes are abundant on blood vessels in the three different mouse tumors we examined. The pericytes in tumors, unlike those on normal vessels, uniformly express both α-SMA and desmin even on capillary-size vessels. This abnormal expression pattern appears as the tumors enlarge. Pericytes in tumors also have multiple structural abnormalities, including a loose association with endothelial cells and cytoplasmic processes that invade the tumor parenchyma, which may make the vessels sensitive to VEGF inhibitors. Similarities of pericytes in spontaneous tumors and implanted tumors suggest that the abnormalities are common in neoplasms. Pericytes form sleeves around endothelial sprouts that arise from tumor vessels, may participate in blood vessel growth, and are a potential target in anti-angiogenic therapy.

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