Analysis of Mural Cell Recruitment to Tumor Vessels

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Background—Tumor blood vessels are both structurally and functionally abnormal compared with normal vessels. A limited support of mural cells may contribute to these abnormalities. Here, we characterized mural cell recruitment in 2 mouse tumor models and addressed the question of why tumor vessels fail to recruit a proper coat of mural cells.

Methods and Results—We studied mural cell recruitment to the vasculature of 2 transplantable mouse tumor models, T241 fibrosarcoma and KRIB osteosarcoma. We found that both tumors formed a vessel network with heterogeneous and highly abnormal organization of mural cells. Transplantation of tumors to mice expressing lacZ in mural cells demonstrated that these cells were host-derived. Although tumor vessel endothelium expressed PDGF-B, an embryonic mitogen for mural cells, only very few PDGFRβ-positive cells were found to be associated with the developing tumor vasculature, suggesting a limited pool of recruitable mural cells. We tested whether exogenous mural cells could be recruited to tumor vessels by injecting mixtures of T241 tumor cells and embryonic mesenchymal cells isolated from mice expressing lacZ in mural cells. In the tumors that arose, lacZ-positive cells were efficiently recruited to the tumor vessels.

Conclusions—T241 and KRIB tumors show a similar highly abnormal organization of vessel-associated mural cells. T241 tumor vessels seem highly capable of recruiting exogenously added mural cells. The sparse mural cell coat of tumor vessels may result from a limited pool of mural cells available for recruitment. (Circulation. 2002;105:112-117.)

Key Words: angiogenesis ■ cells ■ pericytes ■ muscle, smooth ■ growth substances

It is widely acknowledged that the growth of most cancers depends on angiogenesis and that inhibition of tumor angiogenesis may provide an efficient strategy to slow down or block tumor growth (see published reviews1–3). In contrast to normal angiogenesis as it occurs during embryonic development, wound healing, and the female estrous cycle, however, angiogenesis in tumors leads to the formation of a poorly organized vasculature characterized by tortuous and leaky vessels unable to support efficient blood flow.14–16 The tumor vasculature appears to be in a continuous state of remodeling, involving simultaneous formation and regression of vessels. A possible contributing factor is excessive production of vascular endothelial growth factor (VEGF)-A by hypoxic tumor cells. Ectopic VEGF-A expression in normal tissues promotes the transient formation of abnormal vessels.6–8 Failure of tumor vessels to recruit a normal coat of mural cells (vascular smooth muscle cells and pericytes) may also contribute to the abnormal characteristics of tumor vessels.9–11 Contact between pericytes and endothelial cells has been suggested to stabilize vessels, promote endothelial survival,12 and inhibit endothelial cell proliferation.13 It is not known why tumor vessels fail to recruit a proper coverage of mural cells. During angiogenic formation of embryonic blood vessels, platelet-derived growth factor (PDGF)-B secreted from endothelial cells stimulates the spreading of PDGF receptor (PDGFR)-β–positive pericytes to the new vascular structures.14–16 Mice lacking PDGF-B or PDGFRβ show a severe deficit in pericytes and develop an abnormal vasculature with many features in common with tumor vessels, such as irregular vessel diameter and increased leakiness.14–16 Here, we analyzed mural cell organization and recruitment in the developing vasculature of 2 transplantable tumor models in mice using a number of markers for such cells. We also cotransplanted tumor cells and genetically tagged mouse embryonic mesenchymal cells to show that the tumor vasculature recruits mural cells efficiently when present. The mural cell deficiency in tumor vessels may result from an inherent inability to properly organize mural cells, but probably also from a limitation to the pool of recruitable mural cells.

Methods

Tumor and MEF Cell Culture and Transplantation

T241 fibrosarcoma, KRIB osteosarcoma, and mouse embryonic fibroblast (MEF) cells (isolated as described by Levêen et al17) were
propagated in DMEM with 10% FCS and standard supplements. For tumor experiments, \(10^6\) T241 cells or mixtures of T241 and MEF cells were suspended in 100 \(\mu\)L PBS and injected subcutaneously on the back of C57Bl6 mice. In some experiments, the MEF cells were prelabeled with the fluorescent dye PKH26 (Sigma) according to the manufacturer’s protocols. After 13 to 14 days, the tumors, 5 to 10 mm in diameter, were removed and processed for histological, immunohistochemical, and in situ RNA hybridization analysis. KRB osteosarcoma cells were injected into the femoral bone of female nu/nu mice, at which site tumors 10 to 15 mm in diameter developed over a 6-week period.

**Tissue Processing and Staining**

For regular microscopy, tumors were fixed in 4% buffered paraformaldehyde, embedded in paraffin, cut at 4-\(\mu\)m thickness, and stained with antibodies against \(\alpha\)-smooth muscle actin (SMA) (clone 1A4) or desmin (clone D33; both from DakoPatts), as described.\(^{15}\) Endothelial cells were stained with a primary antibody against CD31 (PharMingen) and biotinylated rabbit anti-rat IgG (DakoPatts) as a secondary antibody. For confocal microscopy, frozen tumors were fixed in 4% paraformaldehyde, cryoprotected in 30% sucrose, and cut into sections 50 \(\mu\)m thick. For double staining, sections were first incubated with antibody against CD31, followed by washing and incubation with a biotinylated rabbit anti-rat antibody. Sections were then incubated with streptavidin-Alexa568 (Molecular Probes) and FITC-conjugated SMA antibodies (clone 1A4, Sigma). For triple staining, sections were first incubated with antibody against CD31 and \(\beta\)-galactosidase (Abcam), followed by a secondary goat anti-rabbit-Alexa568 antibody. After washing, sections were incubated with biotinylated rabbit anti-rat IgG and finally with streptavidin-Alexa563 (Molecular Probes) and FITC-conjugated SMA antibodies. All steps were performed in PBS containing 1% BSA and 0.5% Tween-20. Images were obtained by scanning specimens at intervals of 0.5 to 1 \(\mu\)mol/L with a Leica TCS-NT microscope and projecting layers to generate a composite image. In situ hybridization and \(\beta\)-galactosidase staining were done as described.\(^{14,15}\)

**mRNA Isolation and Northern Blot Analysis**

Total cellular RNA was isolated from confluent cell cultures with the RNeasy Mini kit (Qiagen). Total RNA (10 \(\mu\)g) was size-fractionated on a denaturing gel and transferred to a BrightStar-Plus membrane with a NorthernMax blotting kit (Ambion). Hybridization with \(^{32}\)P-labeled cDNA probes for GAPDH, PDGF-B, and PDGFR\(\beta\) and PDGFR\(\alpha\) and washings were performed according to the manufacturer’s instructions (Ambion) and standard protocols.

**Vessel Quantification**

Vessel profiles with and without associated SMA-positive (SMA\(^+\)) cells were counted on cryosections 14 \(\mu\)m thick stained for CD31 and SMA. Five random 0.0625-\(\mm^2\) fields at \(\times 400\) magnification were counted in \(\geq 4\) sections of each tumor specimen (T241, \(n = 8\); KRB, \(n = 5\)). Vessel density was expressed as the average number of vessels from all fields counted in each tumor. Vessel profiles associated with lacZ-positive cells in XlacZ4 (\(n = 3\)) and desmin-lacZ (\(n = 3\)) mice and T241:MEF mixtures (\(9:1, n = 4; 5:5, n = 5; \) and \(1:9, n = 3\)) were quantified on sections double-stained for CD31 and \(\beta\)-galactosidase. Five random 0.0625-\(\mm^2\) fields at \(\times 400\) magnification were counted in 3 to 5 sections of each tumor specimen. Association with lacZ-expressing cells was calculated as the ratio between the total number of vessel profiles and the number of profiles with associated lacZ-positive cells.

**Results**

**Tumor Vessels Show Heterogeneous Mural Cell Coverage**

The vasculature was studied in tumors arising from T241 fibrosarcoma cells transplanted to the subcutaneous space of the back skin of C57Bl6 mice and from human KRB osteosarcoma cells orthotopically transplanted to the femoral bone in nude mice. Sections were stained with antibodies against the endothelial marker CD31 to visualize the tumor vasculature (Figure 1, A and B). The osteosarcoma showed a higher vessel density than the fibrosarcoma (140\(\pm\)28 versus 72\(\pm\)27 vessel profiles/\(\mm^2\)). Typically, the tumor vessel profiles of both tumors were highly irregular in their morphology. Antibodies against \(\alpha\)-SMA and desmin were used to visualize mural cells associated with the tumor vessels. Both mural cell markers revealed a heterogeneous and discontinuous endothelial-mural cell association (Figure 1, C through F). In the fibrosarcoma, 56\% of the vessel profiles were associated with SMA\(^+\) cells. In the osteosarcoma, only 5.8\% of the profiles had associated SMA\(^+\) cells. Desmin staining showed a similar picture and was, like SMA, sparser in the osteosarcoma than in the fibrosarcoma. Different regions in the same tumors were compared to see whether mural cell coverage varied between regions with different vascular densities. In Figure 2, plots of vascular densities and mural cell coverage show that the mural cell density was constant over a wide range of vascular densities.

The mural cell coverage index, as calculated above, is influenced by mural cell density, spreading, and shape and therefore provides limited information. To get a better picture of the mural cell organization in tumor vessels, we analyzed 50-\(\mu\)m-thick sections by confocal microscopy. Figure 3 shows images of tumor vessels with appreciable mural cell coverage (A through D) and vessels in immediately neighboring normal tissue (E, F). Each picture represents a stack of 0.5- to 1-\(\mu\)m confocal scans covering 20 to 40 \(\mu\)m of tissue.
thickness. The result shows that the organization of mural cells in tumor vessels and vessels of normal tissue is dramatically different. Regional clustering of SMA⁺ cells is interrupted by large areas of abluminal endothelial surface without SMA⁺ cells or with only thin associated SMA⁺ cytoplasmic processes (Figure 3, A through D, and data not shown). Whereas in the normal vessels, the SMA⁺ cells are preferentially encircling the vessels in a highly ordered fashion (Figure 1, E and F), their organization in the tumor vessels is mostly chaotic. Some mural cells of tumor vessels appear to be loosely associated with the vessels and extend away from the endothelium (Figure 3, C and D, arrows). There are also SMA⁺ cells without apparent vessel association (Figure 3A, arrowheads).

**Mural Cell Recruitment Occurs From the Host**
The analysis above does not reveal whether the mural cells are host- or tumor-derived. This is not a trivial question, because certain tumor cells have been suggested to have the capacity of vascular mimicry, ie, to form vascular structures. In addition, both types of tumor cells used are of mesenchymal origin, and it cannot be ruled out that they may acquire mural cell properties within the tumors. To investigate whether host mural cells are recruited to tumor vessels, we injected T241 cells in desmin-lacZ and XlacZ mice, which both express lacZ in mural cells. In tumors grown in XlacZ mice, lacZ expression was completely restricted to the nuclei of periendothelial cells (Figure 4, B and D). The proportion of tumor vessel profiles with associated XlacZ⁺ nuclei was 10.8%. This was lower than the proportion of profiles positive for SMA (56%), but the figures are nevertheless comparable, because ≈80% of the SMA⁺ profiles seen on thin sections do not contain a nucleus (data not shown). SMA is cytoplasmic, and the mural cells associated with tumor vessels display long and often branched cytoplasmic processes (Figure 3). Double staining revealed that lacZ⁺ cells were invariably SMA⁺ (data not shown). When tumors were transplanted to desmin-lacZ mice (Figure 4, A and C), lacZ⁺ nuclei became associated with 10.6% of the tumor vessel profiles, which is closely similar to the result for XlacZ. Taken together, our results suggest that the vast majority of (or all) tumor vessel mural cells are host-derived in the 2 models analyzed.

**Figure 2.** Relationship between tumor vessel density and mural cell coverage. Number of vessel profiles per microscopic field is plotted against proportion of vessel profiles invested by mural cells in T241 (A) and KRIB (B) tumors. In both tumors, vessel densities vary in different regions. On average, vessel density (black line) is higher in KRIB tumors, but “hot-spot” regions in both tumors show similar densities. Mural cell coverage index (gray line) is different between tumor types but is constant over a range of vascular densities in same tumor type.

**Figure 3.** Organization of mural cells in tumor vessels and normal vessels. Analysis of double stainings of CD31 (red) and SMA (green) by confocal microscopy in T241 (A, C) and KRIB (B, D) tumors and in neighboring normal tissue (E, T241; F, KRIB). A through D show tumor vessels with a high degree of mural cell investment. Many vessel profiles, particularly in KRIB tumors, completely lack mural cells. Arrows, SMA⁺ cells that extend away from vessel. Arrowheads, SMA⁺ cells without vessel association. Bars = 20 μm.
PDGF-B and PDGFRβ Expression

In situ hybridization demonstrated strong expression of PDGF-B by endothelial cells of large and small blood vessels in both tumor types, but not in tumor cells in vivo (Figure 5, A and B, and data not shown) or in vitro (Figure 5F). PDGF-B expression was likewise undetectable in blood vessels of the normal tissues surrounding the tumors (not shown), which is consistent with previous notions that PDGF-B expression is restricted to growing vessels and to proliferating endothelial cells in vitro. PDGF-B expressing endothelial cells were intermingled with nonexpressing cells in the vessel profiles (Figure 5B), indicating that neighboring endothelial cells may have different patterns of gene expression.

At low frequency, tumor vessel profiles were found to be associated with PDGFRβ-positive cells, as detected by in situ hybridization (Figure 5C). These profiles were located mainly at the periphery of the tumor and may represent newly recruited mural cells (or progenitors). The vast majority of vessel profiles, however, lacked PDGFRβ-positive cells (Figure 5D). When found, PDGFRβ-positive cells were tightly associated with, but distinct from, endothelial cells, as revealed by combined PDGFRβ in situ hybridization and CD31 immunohistochemistry (Figure 5E). No expression of PDGFRβ was detected in the KRIB tumor cells in vitro, and only a very weak signal was detected in T241 cells (Figure 5F). In contrast, MEF cultures expressed abundant PDGFRβ mRNA. PDGFRα was expressed in cultured T241 cells but not in KRIB or in MEF cells.

Cointjected Embryonic Cells Become Recruited as Mural Cells by Tumor Endothelium

The abundant expression of PDGF-B mRNA by the tumor endothelium suggests that the limited ability of tumor vessels to recruit a proper coating of mural cells is not caused by lack of PDGF-B. Several other factors with direct regulatory or permissive roles in mural cell recruitment may exist, however, including other growth factors, receptors, intracellular signal transduction molecules, cell adhesion molecules, and extracellular matrix molecules. In principle, any such factor may be deficient in tumor vessels as an underlying cause of the deficient mural cell recruitment. One way to analyze the inherent capacity of tumor vessels to attract mural cells is to add genetically tagged pericytes to tumors and study the efficiency with which they are recruited to tumor vessels. T241 fibrosarcoma cells were therefore mixed with different amounts of MEF cells derived from E12.5 XlacZ4 embryos, and the resulting cell mixtures were injected subcutaneously into C57Bl6 mice. Cell suspensions containing a total of 106 cells of T241:MEF cells in ratios of 9:1, 5:5, and 1:9 were injected. Tumors grew at the same rate irrespective of whether MEF cells were coadded or not, with the exception of the T241:MEF 1:9 ratio, which initially grew at a slower rate, presumably because as little as 105 tumor cells were inoculated. After 14 days, the tumors were removed, fixed, sectioned, and double-stained with X-gal and antibody against CD31 (Figure 6C) or triple fluorescence–stained for lacZ (red), CD31 (blue), and SMA (green) (Figure 6, A and B). These stainings demonstrate XlacZ4+/SMA+ cells in tight association with the tumor vessels. Vessel-associated LacZ−

Figure 4. Mural cells are recruited from host. β-Galactosidase (blue) and CD31 (brown) staining of T241 tumors transplanted to desmin-lacZ (A and C) or XlacZ4 transgenic mice (B and D). LacZ+ cells (only nuclei are stained) found in tumors were always associated with vessels (arrows). Majority of vessel profiles within tumors did not display associated LacZ+ nuclei (arrowheads). Bars: A, C, and D, 30 μm; B, 50 μm.

Figure 5. Expression of PDGF-B and PDGF receptors. In situ hybridization (blue staining) for PDGF-B (A, B) and PDGFRβ (C through E) in T241 tumors. Stained cells were associated with vascular structures. PDGFRβ labels mural cells, as indicated by double in situ hybridization/immunohistochemistry for PDGFRβ (blue) and CD31 (brown) (E). Whereas most vessel profiles were associated with PDGF-B–positive (arrow in B) as well as PDGF-B–negative (arrowhead in B) endothelial cells, most profiles lacked associated PDGFRβ-positive cells (D, arrowheads). E, Northern blot analysis of T241, KRIB, and MEF cells grown in vitro. Bars: A and C through E, 50 μm; B, 200 μm.
recruitment of vascular mural cells. These mutants include components of the transforming growth factor-β, angio-
poietin, and PDGF-B/R signaling pathways. Our previous analysis of PDGF-B- and PDGFRβ-knockout mice revealed many abnormal features of the pericyte-deficient vessels that were similar to tumor vessels, such as variable diameter and increased permeability. The fact that tumor vessels have been reported to be deficiently invested by mural cells therefore attracted our attention.

The vasculature of the 2 tumor models studied here was found to be abnormal in characteristic ways. The mural cell coverage of the abluminal endothelial surface was sparse and heterogeneous and lacked proper organization. Together with other published evidence suggesting that mural cells play a role in stabilizing microvessels and promoting endothelial maturation and survival, this reinforces the notion that lack of (proper arrangement of) mural cells may contribute to the abnormal phenotype of tumor vessels, including the irregular morphology and inefficient blood flow. The apparent inability of tumor vasculature to attract a proper coat of mural cells may give rise to an immature vessel network that depends on a continuous supply of VEGF-A for survival and in which differentiation into quiescent, organized, and functional vessels does not take place.

The strong PDGF-B mRNA expression by the tumor endothelium suggests that the relative shortage of mural cells observed in our tumor models is caused by factors other than deficient PDGF-B expression. The small number of PDGFRβ-positive mural cells found in association with tumor vessels contrasts to the situation in embryos. This may suggest that a limited pool of mural cell progenitor cells is available for recruitment to tumor vessels in the adult mouse. Alternatively, competent mural progenitors are present but fail to be recruited properly because necessary factors other than PDGF-B are missing. The ability of tumor vessels to recruit exogenously added mural cells (or their progenitors) was therefore assessed in tumors derived from mixtures of T241 cells and XlacZ4 MEF cells. Although only a small proportion of the MEF cells were XlacZ4 in vitro and in vivo, the majority of the lacZ+ cells were specifically recruited to the tumor vessels. This demonstrates that tumor vessels are highly capable of attracting mural cells.

The issue of mural cell recruitment to new blood vessels in the adult organism has relevance beyond the question of tumor vasculature and its abnormal features. In therapeutic angiogenesis, the goal is to produce stable and functional vessels. VEGF expression in vivo produces an angiogenic response, but the vessels formed are morphologically abnormal, poorly functional, and transient. This may be connected to an insufficient recruitment of mural cells. Addition of factors with the potential of expanding the pool of mural cell progenitors or addition of ectopic mural cell progenitors in conjunction with proangiogenic therapy may therefore be worth considering.

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