A novel population of local pericyte precursor cells in tumor stroma that require Notch signaling for differentiation

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ABSTRACT

Pericytes are perivascular support cells, the origin of which in tumor tissue is not clear. Recently, we identified a Tie1$^+$ precursor cell that differentiates into vascular smooth muscle, in a Notch-dependent manner. To understand the involvement of Notch in the ontogeny of tumor pericytes we used a novel flow immunophenotyping strategy to define CD146$^+$/CD45$^-$/CD31$^{-/lo}$ pericytes in the tumor stroma. This strategy combined with *ex vivo* co-culture experiments identified a novel pericyte progenitor cell population defined as Sca1^{hi}/CD146$^-$/CD45$^-$/CD31$^-$. The differentiation of these progenitor cells was stimulated by co-culture with endothelial cells. Overexpression of the Notch ligand Jagged1 in endothelial cells further stimulated the differentiation of Sca1^{hi}/CD146$^-$/CD45$^-$/CD31$^-$ cells into pericytes, while inhibition of Notch signaling with a γ-secretase inhibitor reduced this differentiation. However, Notch inhibition specifically in Tie1-expressing cells did not change the abundance of pericytes in tumors, suggesting that the pericyte precursor is distinct from the vascular smooth muscle cell precursor. Transplant experiments showed that the bone marrow contributes minimally to tumor pericytes. Immunophenotyping revealed that Sca1^{hi}/CD146$^-$/CD45$^-$/CD31$^-$ cells have greater potential to differentiate into pericytes and have increased expression of classic mesenchymal stem cell markers (CD13, CD44, Nt5e and Thy-1) compared to Sca1$^{-/lo}$/CD146$^-$/CD45$^-$/CD31$^-$ cells. Our results suggest that a local Sca1^{hi}/CD146$^-$/CD45$^-$/CD31$^-$ pericyte progenitor resides in the tumor microenvironment and requires Notch signaling for differentiation into mature pericytes.

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Introduction

Tumors require the formation of new blood vessels to supply oxygen and nutrients to grow beyond a certain volume (Weis and Cheresh, 2011). Endothelial cells line the interior of blood vessels and are surrounded by mural cells (e.g. vascular smooth muscle cells (VSMC) and pericytes) that maintain endothelial stability and control vascular tone. Pericytes are a type of perivascular cell associated with the microvasculature (Armulik et al., 2011). In contrast to healthy microvasculature from adult tissues or developing embryos, pericytes from tumor capillaries are loosely associated with blood vessels (Morikawa et al., 2002). Pericyte coverage is generally deficient in the tumor microvasculature (Morikawa et al., 2002), and poor vascular coverage is associated with worse prognosis (De Bock et al., 2011; O'Keeffe et al., 2008; Stefansson et al., 2006; Yonenaga et al., 2005). The mechanism responsible for these defects is not clear, but recent clinical and preclinical animal model studies suggest that improved vascular wall pericyte investment results in decreased leakiness and thus normalized blood flow, decreased metastasis, improved drug delivery and inflammatory cell infiltration (De Bock et al., 2011). Therefore, a better understanding of pericyte function could lead to therapeutic strategies to normalize tumor vasculature.

Morphologic criteria, including close apposition with endothelial cells in microvessels, are the most reliable current criteria to define pericytes (Armulik et al., 2011). However, these require high-definition imaging, which does not allow for functional studies. Further, the requirement for close apposition to endothelial cells is not a reliable criterion in remodeling vasculature, especially in tumors where investment of pericytes is aberrant, e.g. loosely associated with the vascular wall (Morikawa et al., 2002). More commonly, markers such as α-SMA, NG2, Desmin and PDGFRβ are used for pericyte identification, but no single molecular marker is a *bona fide* marker that distinguishes pericytes from other mesenchymal cells such as the VSMC present in

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larger blood vessels or fibroblasts, which can also have a perivascular location and express pericyte markers (e.g. α-SMA and PDGFRβ). Moreover, these surface markers are labile and expression varies during the differentiation of pericytes. Therefore, it is crucial to develop new approaches to identify and isolate pericytes from tumors.

The ontogeny of pericytes is complex as various cellular sources have been reported and these cells appear to provide different contributions depending on the animal model and specific study (Armulik et al., 2011). During tumor neovascularization, bone marrow has been reported to be a source of pericytes (Du et al., 2008; Rajantie et al., 2004; Song et al., 2005). However, the level of contribution of bone marrow (marrow)-derived pericytes to the tumor vasculature remains unclear due to the lack of consensus markers for these cells. In fact, earlier studies suggest that pericytes in tumors are recruited from a local immature mesenchymal cell (Abramsson et al., 2002; De Palma et al., 2005). Recently, we identified a Tie1$^+$/CD31^{dim}/VE-cadherin$^-$/CD45$^-$ local precursor for VSMC that is recruited to the vascular wall during embryonic and adult arteriogenesis (Chang et al., 2012a). Whether pericytes of the tumor vasculature are derived from the same precursor that gives rise to VSMC during arteriogenesis remains to be determined.

Notch signaling regulates various cell functions during development of adult metazoans. The Notch family is composed of four heterodimeric transmembrane receptors and five ligands (Kopan and Ilagan, 2009). Ligand-mediated Notch activation results in cleavage of the Notch receptor and translocation of the intracellular domain to the nucleus, where transcription of Notch-regulated genes is induced. Notch signaling regulates blood vessel patterning during normal and pathological angiogenesis by regulating endothelial cell sprouting and proliferation (Benedito et al., 2009; Eilken and Adams, 2010; Gridley, 2010). Notch signaling is also important for regulating maturation of mural cells and their recruitment to blood vessels (Sainson and Harris, 2008). In this context, the interaction between the Notch ligand Jagged1 on endothelial cells and the receptor Notch3 in VSMC appears to be critical as demonstrated in retinal angiogenesis (Domenga et al., 2004; Liu et al., 2009; Liu et al., 2010a). A role for the Notch ligand Dll4 has been also suggested for the differentiation of mural cells in different adult neovascularization models (Patel et al., 2006; Schadler et al., 2010; Stewart et al., 2011). Notch is also an important regulator for the expansion/maintenance of various stem/progenitor cells (Liu et al., 2010b). However, the role of Notch signaling in vascular progenitor cell expansion/maintenance remains poorly understood. Recently we have shown that Tie1 precursor cells differentiate into mature VSMC with a requirement for Notch signaling (Chang et al., 2012a). Whether Notch signaling is also required for pericyte maturation remains an open question. Indeed, in the absence of clear pericyte precursor markers, this question is difficult to answer.

Here we define a set of cell surface markers that distinguish tumor pericytes from other vascular cells, and identify a precursor source for these cells from the local tumor microenvironment. Importantly, we also show that the differentiation of these pericyte precursor cells is regulated by Notch signaling.

Materials and methods

Cells and animal models

Animal studies were approved by the University of British Columbia Animal Care Committee in conformity with guidelines of the Canadian Council for Animal Care. Tie1-tTA and TetOS-dnMAML-GFP were described previously, and the dnMAML construct was shown to be specific for Notch inhibition (Chang et al., 2012a; Chang et al., 2012b). For Tie1-tTA × TetOS-dnMAML-GFP double transgenic (Tg) and littermate controls, doxycycline (Dox) (100 μg/ml), which induces dnMAML-GFP expression, was added to the drinking water from breeding, changed every other day and withdrawn 7 days before tumor implantation. Pep3B-CFP and W41 were bred in-house and C57/Bl6-GFP were purchased from Jackson Laboratories. Lewis lung carcinoma (LLC) and B16F10 melanoma parental cells were from ATCC and not authenticated. All cell lines were cultured in DMEM 10% calf serum supplemented with glutamine and penicillin–streptomycin and routinely tested for mycoplasma contamination. LLC or B16F10 overexpressing the human Jagged-1 (Jag1) or empty-vector controls were generated using the retroviral vector MSCV-pac-Jag1 and MSCV-pac, respectively. HUVEC, HMEC, HMEC-Vector and HMEC-Jag1 were cultured and prepared as described previously (Noseda et al., 2004).

Bone marrow transplant

W41 were sublethally irradiated the day before injection by the tail vein with 5×10^5 marrow cells flushed from femurs and tibias of Pep3B-CFP or Pep3B-GFP mice (Benz et al., 2012). In some experiments, marrow reconstituted mice were generated with GFP-tagged marrow from transduction with the retroviral vector MSCV-IRES-GFP as described previously (Larrivee et al., 2005). Chimerism of transplanted mice was analyzed at 8 weeks post-irradiation by FACS with the CD45 (LCA) antibody and CFP or GFP fluorescence. Tumors were implanted in chimeras at a minimum of 8 weeks post-irradiation.

Tumor implantation, harvest, digestion and preparation for FACS

Animals were anesthetized with isoflurane and injected dorsally with LLC or B16F10 with 1×10^6 and tumors were grown for 2 weeks. Animals were euthanized by CO_2 inhalation before tumor extraction. The tumor was cut with a razor blade in 1 mm³ pieces and digested for 30 min at 37 °C with gentle agitation in DMEM containing 20% fetal bovine serum (FBS), 1 mg/ml of collagenase I, II and IV (Sigma, St. Louis, MO) and DNase I (50 μg /ml). The following steps were performed on ice. Cells were filtered through a 100 μm mesh and resuspended with red blood cell lysis solution according to the manufacturer's instructions (Stem Cell Tech., Vancouver). Cells were next washed in PBS with 2% FBS (PBS–FBS) and resuspended in blocking solution (PBS–FBS with FC blocker rat anti-CD16/CD32, BD Biosciences) for 30 min with at 1×10^7 cells/ml. Cells were next mixed with one volume of diluted antibody for 30–60 min, washed with PBS–FBS and resuspended in PBS–FBS with 1 μg/ml DAPI (4′,6-diamidino-2-phenylindole) before FACS. The following antibodies were used for FACS: CD31-Alexa 647 (clone 390), CD31-PerCP-efluor 710 (clone 390), CD146-PE (clone ME-9 F1), CD45-APC-Cy7 (clone 30-F11) and Sca1-PE-Cy7 (clone D7). All antibodies were rat IgG and used at 1:200 dilution and purchased from BD, Biosciences (Palo Alto, CA), eBiosciences (San Diego, CA) or Biolegend (San Diego, CA). Cells were analyzed and sorted with an Influx cell sorter (BD Biosciences, San Diego, CA). Cells were sorted with high purity into PBS–FBS and

Fig. 1. Pericytes present a CD146$^+$/CD45$^-$/CD31$^{-/lo}$ signature in the tumor stroma. (A) Flow-sorting strategy to isolate pericytes in the tumor stroma. Larger panels are from YFP-expressing tumors stained for CD45, CD31 and CD146, grown in mice constitutively expressing CFP. The numbers in the rightmost panel (CD31/CD146 dotplot) indicate the FACS-sorted cell populations: 1) CD146$^-$/CD45$^-$/CD31$^-$, 2) CD146$^+$/CD45$^-$/CD31$^{-/lo}$, and 3) CD146$^+$/CD45$^-$/CD31$^+$. Upper inset graphs show the flow analysis from unstained naïve LLC tumor implanted in wild-type mice and used to define background staining gates. (B) RT-qPCR analysis of endothelial, pericyte and fibroblast markers from tumor stromal cells sorted populations described in A. The histograms show fold-change in expression relative to the bulk tumor cells. The values indicate mean ± SD, $n = 4$ independent experiments. C) Desmin immunofluorescence and Hoechst staining of freshly sorted stromal CD146$^+$/CD45$^-$/CD31$^{-/lo}$ cells from LLC tumors. Tumors were grown in mice ubiquitously expressing GFP. The histogram shows the quantification of Desmin$^+$/Hoechst$^+$ from total Hoechst$^+$ cells in the bulk tumor cells and indicated sorted cell populations. D) RT-qPCR analysis of pericytes and fibroblast markers from stromal cell-sorted populations from B16F10 tumors. Tumors were grown in mouse ubiquitously expressing CFP. The sorting strategy used was similar to LLC tumors as described in A. The histograms show the fold-change of expression relative to the bulk tumor cells. The values indicate mean ± SD, $n = 3$ mice.