Distinctive Features of Angiogenesis and Lymphangiogenesis Determine Their Functionality during De novo Tumor Development

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Abstract

Blood and lymphatic vasculature are essential components of all organs, responsible for maintaining organ fluid dynamics and tissue homeostasis. Although both vessel systems are composed of similar lineages of endothelial cells whose crude functions include fluid and cell transport, each system also possesses distinctive physiologic properties, enabling their distinctive functions in tissues. The role of hemogenous vasculature and development of angiogenic blood vessels during cancer development is well established; however, the role of lymphangiogenesis and structural/functional alterations occurring within lymphatic vessels during cancer development are incompletely understood. To assess premalignant versus malignant alterations in blood and lymphatic vasculature associated with squamous epithelial skin carcinogenesis, we assessed architectural and functional features of both vascular systems using a mouse model of de novo carcinoma development. We report that, as vasculature acquires angiogenic and/or lymphangiogenic properties, angiogenic blood vessels become leaky in premalignant tissue and at peripheries of carcinomas, where enlarged lymphatic capillaries efficiently drain increased tissue fluid, thereby maintaining tissue hemodynamics. In contrast, central regions of carcinomas exhibit elevated tissue fluid levels, compressed lymphatic lumina, and decreased vascular leakage, thus indicating impaired hemodynamics within solid tumors. Together, these data support the notion that therapeutic delivery of anticancer agents is best realized in premalignant tissues and/or at the peripheries of solid tumors where hemodynamic forces support drug delivery. Strategies to normalize intratumoral hemodynamics would therefore enhance therapeutic delivery to otherwise poorly accessible central regions of solid tumors. [Cancer Res 2007;67(11):5211–20]

Introduction

Blood and lymphatic vessels comprise two interdependent vascular networks in all tissues. Whereas blood vessels deliver blood cells, plasma proteins, and oxygen to tissues, lymphatics, composed of lymph-forming capillaries and collecting vessels, continually regulate interstitial fluid pressure by draining interstitial fluid and debris to maintain tissue homeostasis. When tissues are acutely damaged, activation of both vascular systems occurs as part of innate repair programs; once complete, both systems return to their homeostatic states. In contrast, sustained activation of one or both vascular networks is associated with some chronic disorders, such as rheumatoid arthritis (1) and psoriasis (2, 3), and contributes to disease pathogenesis. Cancer development is similarly associated with chronic activation of blood vasculature (i.e., angiogenesis) in premalignant and malignant tissues (4). Activation of angiogenic vasculature in premalignant tissue is characterized by increased proliferation of vascular endothelial cells (VEC) and sprouting of new immature leaky blood vessels from preexisting vascular beds (5). Increased leakage of plasma proteins from immature angiogenic vessels leads to increased interstitial fluid content, lymph formation, and drainage via lymphatic vessels back into the blood circulation (6). High interstitial fluid pressure (IFP), which forms a barrier to transcapillary transport, results in inefficient delivery of therapeutic drugs from vasculature into tumor stroma (7). It has been postulated that high IFP common to many solid tumors, is in part a result of inefficient clearance of tissue fluid by lymphatic vessels (7). This postulate is supported by histochemical studies evaluating lymphatic architecture and diminished lumen diameters in archival human carcinomas and murine xenograft tumors (8–12). Studies evaluating functional changes in the status of lymphatic endothelial cells (LEC) and/or lymphatic vessels compared with VECs and blood vessels in premalignant tissues have not been well described.

To critically examine distinctive versus common physiologic and functional properties of blood versus lymphatic vasculature that accompany and/or contribute to cancer development, we used a transgenic mouse model of de novo epithelial squamous cell carcinoma (e.g., K14-HPV16 mice; ref. 13). HPV16 mice progress through well-defined premalignant stages before de novo carcinoma development, mirroring histopathologic stages observed during human cervical carcinogenesis (14). HPV16 mice develop hyperplastic skin lesions with 100% penetrance by 1 month of age that focally progress to dysplasia by 3 to 6 months (13). Precursor dysplasias undergo malignant conversion into varying grades of squamous cell carcinoma (SCC) in skin in 50% of mice that metastasize to regional lymph nodes with a 30% frequency (13, 15). Angiogenic vasculature is first evident in premalignant hyperplasias, development of which is linked to infiltration of innate immune cells (e.g., mast cells, granulocytes, and macrophages; refs. 15–18). Using this model, we assessed molecular, histopathologic, and functional variables of blood and lymphatic vasculature to reveal their distinctive physiologic properties at each stage of neoplastic development.

Materials and Methods

Animal Husbandry, Genotype, and Histopathologic Analyses

All animals were maintained within the University of California at San Francisco (UCSF) Laboratory for Animal Care barrier facility according to

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Institutional Animal Care and Use Committee procedures. HPV16 transgenic mice (19), preparation of tissue sections (ear skin) for histologic examination, and characterization of neoplastic stages based on H&E histopathology and cytokeratin intermediate filament expression have been described previously (13, 15, 20). Paraffin-embedded tissue sections were fixed by immersion in 10% neutral-buffered formalin, dehydrated through graded ethanol and xylene, embedded in paraffin, cut with a Leica 2135 microtome into 5-μm-thick sections, and histopathologically examined following H&E staining and immunoreactivity of keratin intermediate filaments. Hyperplastic lesions were identified by a 2-fold increase in epidermal thickness and an intact granular cell layer with keratohyalin granules. Dysplastic lesions were characterized based on basal and spinous cell layers with hyperchromatic nuclei representing greater than half of the total epidermal thickness and incomplete terminal differentiation of keratinocytes. SCC was identified by abundance of abnormal mitotic figures and an invasive loss of integrity in epithelial basement membrane with clear development of malignant cell clusters proliferating in the dermis. SCCs were graded as has been described previously (17).

Flow Cytometry

Single cell suspensions were prepared from ear (n = 4) and tumor (n = 7) tissue as described previously (21). Cells were incubated for 10 min at 4°C with rat anti-mouse CD61/CD32 monoclonal antibody (mAb; BD Biosciences) at a 1:200 dilution in PBS/bovine serum albumin (BSA) to prevent nonspecific antibody binding. Subsequently, cells were washed and incubated for 20 min with phycoerythrin-conjugated anti-mouse CD31 (1:200; eBioscience) and anti-mouse podoplanin hybridoma supernatant (1:10; BD Biosciences) was added to discriminate between viable and dead cells. Data acquisition was done on a FACSCalibur using CellQuestPro software. Tumors were analyzed with a FACSCalibur using a CellQuestPro software (Tree Star, Inc.). Data shown represent the mean ± SE. P values of <0.05 were considered to be statistically significant.

Immunohistochemistry

Immunohistochemistry on paraffin-embedded tissue sections. Tissue sections were deparaffinized, briefly washed in PBS, and blocked for 15 min in blocking buffer (5.0% normal goat serum/0.5% BSA/PBS). Primary antibodies were diluted in 0.5× blocking buffer: rat anti-mouse CD31 (1:50; PharMingen), Syrian hamster anti-mouse podoplanin (1:200, clone 8.1.1; Developmental Studies Hybridoma Bank, University of Iowa), guinea pig anti-mouse cytokeratin pan (1:100; Progen Biotech GmbH), and rabbit anti-mouse lymphatic vesicle endothelial receptor-1 (LYVE-1: 1:200; Upstate). Sections were incubated with primary antibody overnight at 4°C in a humidified chamber followed by three brief washes in PBS and subsequently incubated with secondary antibodies Alexa Fluor 488–conjugated anti-rat antibody (1:500; Molecular Probes), Alexa Fluor 633–conjugated anti-guinea pig antibody (1:500; Molecular Probes), Alexa Fluor 594–conjugated anti-hamster antibody (1:500; Molecular Probes), or Alexa Fluor 546–conjugated anti-rabbit antibody (1:500; Molecular Probes) for 2.0 h at room temperature in a humidified chamber. After five 3-min washes in PBS, the fluorescently stained sections were subjected to a nuclear staining with SYTO62 (0.7 μmol/L in H2O; Molecular Probes) for 10 min and then mounted in ProLong Gold mounting medium. Fluorescence was visualized using a laser scanning confocal microscope LSM510 META and analyzed with a Zeiss LSM Image Examiner. A minimum of 100 endothelial cell nuclei in control (n = 4), premalignant ear (n = 4), and tumor tissue (n = 7) was identified in tissue sections based on their morphologic protrusion into the vessel lumen, and the percentage of proliferating nuclei was determined.

Fluorescent Angiography and Immunohistochemistry of Whole-Mount Ear Tissue and Thick Frozen Tumor Sections

Whole-mounted ear tissue (n = 3 per time point) was prepared as described previously (23). Briefly, mice were anesthetized with a 2% isofluorane/98% oxygen mixture and 100 μL of FITC-conjugated L. esculentum (tomato) lectin (2.0 mg/mL) were injected into the tail vein and allowed to circulate for 3.0 min followed by cardiac perfusion with 35 mL of PBS-buffered 4% paraformaldehyde (pH 7.4; ref. 22). Ears were harvested, ventral and dorsal aspects were separated, and cartilage was removed from ventral aspects, which were then immersion fixed in PBS-buffered 4% paraformaldehyde (pH 7.4) overnight at 4°C and subjected to the following staining procedure under exclusion of light: Ventral aspects of ears were rinsed briefly in PBS containing 0.3% Triton X-100 (PBS-T) and blocked in PBS-T containing 3% goat serum overnight at 4°C. After five 3-min washes in PBS, the fluorescently stained sections were subjected to a nuclear staining with SYTO62 diluted (0.7 μmol/L in H2O; Molecular Probes) for 10 min and then mounted in ProLong Gold mounting medium. Fluorescence was visualized using a laser scanning confocal microscope LSM510 META and analyzed with a Zeiss LSM Image Examiner. A minimum of 100 endothelial cell nuclei in control (n = 4), premalignant ear (n = 4), and tumor tissue (n = 7) was identified in tissue sections based on their morphologic protrusion into the vessel lumen, and the percentage of proliferating nuclei was determined.

Intravitral Perfusion of Lymphatics

Intravitral perfusion of lymphatics was modified from what has been described by Nagy et al. (24). Mice were anesthetized with a 2% isofluorane/98% oxygen mixture and cradled in a transparent acrylic resin mold.
Ears \( (n = 6 \text{ per time point}) \) were mounted flat on a resin support, held in place by silicone vacuum grease, and viewed in a dissecting microscope (Olympus). Colloidal carbon (Higgins non-waterproof black drawing ink; Sanford) was diluted 1:1 in PBS, filtered through a 0.22-μm filter, and injected i.d. through a prepulled borosilicate glass micropipette with an inner diameter of 1.0 μm (World Precision Instruments) attached to a 100 μL Hamilton syringe (World Precision Instruments). The micropipette was repeatedly injected into the dermis of the peripheral ear until a lymphatic vessel was entered. Colloidal carbon was then slowly injected. For lymphatic vessel diameter analysis, ear tissue \( (n = 3 \text{ per time point}) \) was subsequently harvested and fixed overnight in acetone at 4°C. After fixation, the tissue was cleared in toluene 48 h at room temperature and mounted in Permount (Fisher Scientific). For photography, a digital camera (Nikon Coolpix 950) was used and the images were analyzed using Openlab software (Improvision). For in vivo analysis of colloidal carbon clearance, images were captured using a LUMAR microscope (Carl Zeiss MicroImaging) within 1.0 min of injection and again after 20 min. Both ears of negative littermate (−LM) and 4-month HPV16 mice \( (n = 3 \text{ each}) \) were analyzed.

**Miles Assay**

Mice were anesthetized with a 2% isofluorane/98% oxygen mixture, and Evans blue dye (30 mg/kg in 100 μL PBS; Sigma-Aldrich) was injected into the tail vein. In some experiments, after 1 min, 30 μL of 5% mustard oil (phenyl isothiocyanate, 98%; Sigma-Aldrich) diluted in mineral oil (Sigma-Aldrich) or mineral oil as control were applied to the dorsal and ventral
surfaces of the ear; the application process was repeated 15 min later. Evans blue dye was allowed to circulate for 30 min. Anesthetized mice were subsequently cardiac perfused with 1% paraformaldehyde in 0.05 mol/L citrate buffer (pH 3.5). Ears (n = 6 per time point; n = 3 per mineral oil/mustard oil treatment) were removed, blotted dry, and weighed. Tumors (n = 4) were dissected and cut into seven pieces representing six peripheral sections and center. Premalignant nontumor tissue was derived from neck, chest, and abdomen of non–tumor-bearing HPV16 animals. Evans blue dye was extracted from ears, nontumor tissue, and tumor pieces in 1.0 mL formamide for 48 h at 60°C and measured spectrophotolectrically at 610 nm in a SpectraMax 340 (Molecular Devices). Data are expressed as mean ± SE. P values of <0.05 were considered to be statistically significant.

**Tissue Fluid Determination**

Mice were euthanized, and ears (n = 6 per time point) and/or tumors (n = 4) were removed and weighed (wet weight). Tumors were dissected into six peripheral sections and tumor centers. Premalignant nontumor tissue was derived from neck, chest, and abdomen of non–tumor-bearing HPV16 animals. Tissue was snap frozen, lyophilized, and reweighed (dry weight). The difference between wet and dry weight reflects fluid tissue component, although dry weight reflects solid tissue component. Data are expressed as mean ± SE. P values of <0.05 were considered to be statistically significant.

**In vivo High Molecular Dextran Injection**

Functionality of initial lymphatics was measured as uptake and drainage of interstitial fluid containing high molecular weight (2,000,000 Da) dextran.

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**Figure 2.** VEC and LEC proliferation in premalignant and carcinoma tissue.  
A. quiescent and proliferating (red staining) endothelial cell nuclei (solid arrows) in blood (green staining) and lymphatic (yellow staining) vessels in premalignant and malignant (white staining for cytokeratin-positive keratinocytes; open arrow) tissue. Bar, 50 μm. e, epidermis; d, dermis.  
B. quantitative analysis of proliferating VECs and LECs in –LM, premalignant, and carcinoma tissue. Proliferating LECs were identified in the periphery and center of well-differentiated grade 1 SCCs (SCC-I) but limited to periphery of less-differentiated grade 2 SCCs. Absence of open lumen lymphatic vessels SCC-II centers precluded analysis of LECs in that locale. *, P < 0.05, two-tailed unpaired nonparametric Mann-Whitney U test. Dashed line, basement membrane. Blue staining, SYTO62-nuclear counterstain.
conjugated to the fluorophore tetramethylrhodamine isothiocyanate (TRITC; 10 mg/mL in PBS; Sigma) following i.d. injection. Drainage of TRITC-dextran was monitored in 
\( /C_0 \) LM (\( n=6 \)) and 4-month HPV16 (\( n=6 \)) ears over 20 min using a LUMAR microscope.

**Results**

**Endothelial composition of blood and lymphatic vasculature during neoplastic progression.** To evaluate the endothelial cell composition of premalignant versus malignant tissues (skin) during squamous carcinogenesis, we determined the relative percentage of endothelial cells at each stage of neoplastic progression in HPV16 mice, compared with wild-type negative littermate (–LM) controls, by assessing expression of the pan-endothelial cell adhesion molecule CD31 (25), present on both VECs and LECs, albeit at varying levels of expression (26), using flow cytometry of single cell suspensions (Fig. 1A). We found a progressive increase in the relative percentage of CD31⁺ endothelial cells at each distinct premalignant stage (Fig. 1A). Carcinomas on the other hand contained a lower percentage of endothelial cells by comparison with premalignant tissues.
(Fig. 1A), although tumor vasculature possessed angiogenic characteristics (16).

To determine the hematogenous versus lymphatic composition of these endothelial cell populations, we further used flow cytometry of single cell suspensions and quantitatively examined endothelial cells expressing CD31 and the mucin-type transmembrane glycoprotein podoplanin (Fig. 1B). Histochemically, podoplanin-positive endothelial cells are also LYVE-1 positive (Fig. 1C) and thus allow distinction between CD31+/podoplanin− VECs from CD31+/podoplanin+ LECs. The average difference in CD31 signal intensity between CD31+/podoplanin− VECs and CD31+/podoplanin+ LECs was 28%. A progressive increase in both VEC and LEC populations was found during premalignant progression; however, at the tumor stage, VECs represented a larger proportion of the total CD31+ cells (Fig. 1B).

To determine the degree to which VECs and LECs exhibited proliferative behavior at each neoplastic stage, we evaluated endothelial cell proliferation in blood and lymphatic vessels as a function of BrdUrd incorporation. BrdUrd analysis of endothelial cells was limited to vessels with open lumina so as to distinguish BrdUrd-positive endothelial cells [whose nuclei protrude into vessel lumina (Fig. 2A)] from BrdUrd-positive mural cells (whose nuclei protrude toward interstitia). BrdUrd-positive CD31+/LYVE-1− VECs and CD31+/LYVE-1+ LECs were not observed in skin sections of −LM mice, reflecting the quiescent nature of endothelial cells in homeostatic tissue (Fig. 2B). In contrast, BrdUrd-positive VECs and LECs were readily identified throughout premalignant progression albeit with distinct profiles (Fig. 2A and B). Whereas BrdUrd-positive VEC percentages steadily increased at each distinct premalignant stage (Fig. 2B), percentages of premalignant BrdUrd-positive LECs remained constant, albeit higher than in −LM skin (Fig. 2B). In carcinomas, open lumen blood and lymphatic vessels possessed distinct BrdUrd-positive endothelial cell distributions dependent on (a) SCC grade (lower-grade, well-differentiated SCC-I versus higher-grade, moderately-differentiated SCC-II) and (b) SCC location (tumor center

Figure 4. Functional interdependence of blood and lymphatic vessels during neoplastic progression. A, plasma protein extravasation and blood vessel leakiness in −LM, premalignant, and tumor tissue as assessed by Miles assay. B, quantification of tissue fluid in −LM, premalignant, and tumor tissue. C, induction of acute vascular leakage following mustard oil (MO) application in ear tissue of −LM and dysplastic HPV16 mice results in significantly increased vascular leakage, 16.5-fold ($P < 0.0001$, two-tailed unpaired nonparametric Mann-Whitney $U$ test) and 3.5-fold ($P = 0.0043$, two-tailed unpaired nonparametric Mann-Whitney $U$ test), respectively. MinO, mineral oil. $D$, mustard oil–induced leakage results in significantly increased tissue fluid in −LM controls (8.0%) and premalignant tissue (8.0%). *, $P < 0.05$, two-tailed unpaired nonparametric Mann-Whitney $U$ test.
Increased vessel diameters significantly increased at each premalignant stage histopathologically staged neoplastic lesions, we found that blood in both tumor centers and periphery (Fig. 3). Using fluorescent angiography to assess blood vessel diameter in both tumor centers and periphery (Fig. 3A) at each neoplastic stage (Fig. 3). Preexisting vessels, we assessed blood and lymphatic vessel alternately only resulted in increased girth (diameter) of increased vessel densities and sprouting of new vessels or proliferation of endothelial cells in blood or lymphatic vessels yielded increased vessel diameters in SCC-I and SCC-II carcinomas in SCC-I and SCC-II carcinomas and in centers of SCC-I (Fig. 2A and B). In the center of SCC-IIs, open lumens were only found in blood vessels and not in lymphatic vessels (Fig. 2B). Analysis of proliferating endothelial cells in vessels with open lumens in carcinomas revealed an increase in both BrdUrd-positive VECs and LECs (Fig. 2B) compared with precursor premalignant lesions. VECs and LECs both evidenced increased presence of BrdUrd-positive endothelial cells in tumor centers and peripheries of SCC-I (Fig. 2A and B); however, in SCC-II, proliferating VECs were found in tumor centers and in the periphery, whereas proliferating LECs were limited to tumor periphery (Fig. 2A and B).

**Blood and lymphatic vessel density and diameter during neoplastic progression.** To determine whether increased proliferation of endothelial cells in blood or lymphatic vessels yielded increased vessel densities and sprouting of new vessels or alternatively only resulted in increased girth (diameter) of preexisting vessels, we assessed blood and lymphatic vessel diameters and density (number of vessels per random high-power field of vision) at each neoplastic stage (Fig. 3). Using fluorescent angiography to assess blood vessel diameter in SCC-I and SCC-II carcinomas in SCC-I and SCC-II carcinomas and in both tumor centers and periphery (Fig. 3A). Assessment of LYVE-1-negative lymphatic capillary diameter similarly revealed a progressive increase during premalignant progression, albeit less dramatic compared with blood vessels (Fig. 3A). Because collecting lymphatic vessels express LYVE-1 at low levels, we used intralymphatic perfusion of colloidal carbon to assess their diameter in SCC-I and SCC-II carcinomas in SCC-I and SCC-II carcinomas and in both tumor centers and periphery (Fig. 3A). In SCC- and SCC-IIs, blood vessel diameter was similar in both tumor centers and periphery (Fig. 3A). In SCC-I and SCC-II carcinomas, blood vessel diameter was similar in both tumor centers and periphery (Fig. 3A). Because.Exists of Evans blue tracer in hyperplastic and dysplastic HPV16 tissue compared with LM skin (Fig. 4A). In SCC-I and SCC-II carcinomas, blood vessel diameter was similar in both tumor centers and periphery (Fig. 3A). Because.Exists of Evans blue tracer in hyperplastic and dysplastic HPV16 tissue compared with LM skin (Fig. 4A). In SCC-I and SCC-II carcinomas, blood vessel diameter was similar in both tumor centers and periphery (Fig. 3A). Because.Exists of Evans blue tracer in hyperplastic and dysplastic HPV16 tissue compared with LM skin (Fig. 4A). In spite of the increased density and diameter of perfused, thus functional blood vessels in tumor tissue (Fig. 3A and C), we found decreased vascular leakage in carcinomas where distinct extravasation characteristics were found in tumor centers versus periphery (Fig. 4A). To examine whether increased extravasation of plasma proteins resulted in increased tissue fluid content, we evaluated the
relationship between blood vessel leakage (Fig. 4A) and lymphatic drainage by determining tissue fluid amounts at each neoplastic stage (Fig. 4B) and found that tissue fluid in premalignant HPV16 ear tissue was significantly higher than −LM tissue and further increased in tumor peripheries, with the central regions of SCCs having the highest fluid content (Fig. 4B). Because a major function of lymphatics is to drain excess tissue fluid, one interpretation of these data would be that the drainage capacity of premalignant lymphatics had been exceeded due to lymphatic dysfunction perhaps reflected by tissue fluid accumulation. To access this hypothesis, we further induced vascular leakage acutely by topical application of mustard oil (compared with mineral oil) and assessed Evans blue dye leakage into dysplastic HPV16 ear skin versus −LM. As expected, mustard oil application further increased leakage of plasma proteins out of blood vessels in both −LM and HPV16 skin, 16.5- and 3.5-fold, respectively (Fig. 4C); however, these increases resulted in similar accumulations of tissue fluid content in −LM (8%) and dysplastic HPV16 (8%) tissue (Fig. 4D), indicating that clearance of tissue fluid by premalignant lymphatics is not at maximum capacity. Thus, whereas lymphatics in acutely activated homeostatic tissue are more efficient at clearing excess tissue fluid, functional lymphatics in premalignant tissue maintain tissue fluid dynamics to a similar degree.

To directly assess lymphatic functionality in premalignant tissue, we i.d. injected high molecular weight, fluorescently labeled dextran (TRITC-dextran) tracer and monitored uptake and drainage kinetics by lymphatic capillaries over time in −LM and dysplastic ear skin (Fig. 5A). Lymphatic capillaries were rapidly labeled by tracer in both control and HPV16 ears, indicating rapid uptake. Twenty minutes following injection, tracer-containing capillaries were detectable, indicating continuous lymph formation, thus showing their functional status.

Functionality of collecting lymphatic vessels in premalignant tissue was also evaluated following intralymphatic injection of colloidal carbon (Fig. 5B). Colloidal carbon is excluded by lymphatic capillaries during lymph formation due to its large molecular weight.4 Direct injection of colloidal carbon into dermal lymphatics of −LM and dysplastic ear skin revealed efficient drainage of tracer from collecting lymphatic vessels in both tissues within 20 min (Fig. 5B), thus showing their similar functionality. Together, these analyses reveal functionality of lymphatic capillaries and collecting vessels in premalignant tissues based on their ability to efficiently clear tracer and fluid (Fig. 5A and B) through their open lumina (Fig. 3A and B). Direct analysis of lymphatic functionality in tumor tissue was hampered by technical difficulties doing direct intralymphatic colloidal carbon injection as well as analysis of interstitial drainage using smaller molecular weight dyes (colorimetric or fluorescent) readily taken up by functional lymphatics. Despite these failed attempts to analyze lymphatic functionality in tumor tissue, the implication that open luminal status is associated with vessel functionality in premalignant tissue, in combination with our assessment of tissue fluid content in tumor centers versus tumor periphery (Fig. 4B), leads us to conclude that lymphatics with open lumina in premalignant tissue, at peripheries of SCCs and centers of grade 1 SCCs (Fig. 3C), retain functionality where they efficiently clear interstitial fluid, as opposed to tumor centers of less-differentiated SCCs where lymphatic lumens are predominantly compressed and exhibit diminished fluid drainage capacity (Fig. 3C, bottom right), a characteristic feature of poorly differentiated carcinomas (28–30).

Discussion

Data presented herein indicate that blood and lymphatic vasculature undergo distinct physiologic and architectural alterations during de novo neoplastic progression accompanying carcinoma development. Whereas endothelial cells of each network acquire proliferative capabilities as neoplasia ensues, resultant phenotypes of blood versus lymphatic vessels are distinct. Whereas blood vessels undergo classic angiogenic changes (e.g., increased endothelial cell proliferation, vessel diameter, density, and leakage), lymphatic vessels evidence no alterations to support the notion that sprouting of new vessels occurs. Instead, the increased level of proliferating LECs present in lymphatic vessels enhances diameter of vessels that efficiently clear interstitial fluid in premalignant tissues and peripheries of carcinomas, but not in their centers, areas where interstitial fluid content remains high. Together, these studies show that neoplasia-associated angiogenesis, as opposed to lymphangiogenesis, is a distinct vascular process even when initiated by similar physiologic stimuli.

Endothelial cell proliferation. Endothelial cells in quiescent tissues divide approximately once every 2 to 3 years if unstimulated (31). During cancer development, however, it is well established that local tissue levels of the proangiogenic factor vascular endothelial growth factor (VEGF)-A increase and, as a result, enhance proliferation of VECs and sprouting of new blood vessels from preexisting vascular beds (5). In contrast, lymphangiogenesis (e.g., induction of LEC proliferation) in human cancers (32, 33) correlates instead with increased tissue levels of VEGF-C and VEGF-D (34–36). Accordingly, exogenous expression of VEGF-C or VEGF-D in experimental murine tumor xenograft models induces LEC proliferation and subsequent lymphangiogenesis (37, 38), whereas exogenous VEGF-A expression induces not only an angiogenic response but also lymphangiogenesis in nontumor (24) and tumor tissue (39). It is not clear, however, if LEC responses to increased VEGF-A levels represent a direct intrinsic response of LECs or, instead, an indirect physiologic response resulting from increased tissue fluid due to increased leakage of plasma proteins from newly formed immature angiogenic blood vessels.

In this study, we found evidence of angiogenesis as well as lymphangiogenesis in premalignant tissues of HPV16 mice and in emergent malignant carcinomas. Patterns of LEC proliferation varied from that observed with VECs, thus indicating distinct responses between the two endothelial cell subpopulations to proangiogenic and/or prolymphangiogenic growth factors present in their microenvironment. Whereas VEGF-A mRNA levels have been reported to remain constant during premalignant angiogenesis in a UV-induced murine skin carcinoma model (40), enhanced VEC proliferation in HPV16 mice is paralleled by progressive increases in VEGF-A mRNA expression (41) and protein levels (42). About LEC proliferation, our analyses indicate that, although LECs in premalignant tissue evidence enhanced proliferative status compared with homeostatic tissue, their proliferation does not significantly increase until malignant conversion occurs. Because we have observed increased mRNA levels of VEGF-C in fully malignant carcinomas (data not shown), our interpretation of these data is that LECs respond modestly to increased VEGF-A levels in premalignant tissue but more are responding to increased

4 J. Nagy, personal communication.

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microenvironmental stress and need to clear fluid from premalignant tissue resultant from leaky immature angiogenic blood vessels.

**Vascular sprouting**, VEGF-A is a major proangiogenic factor well documented to induce VEC proliferation and vascular sprouting (43). Sprouting of the lymphatic vasculature, however, depends on VEGF-C in experimental chick chorioallantoic membrane assays, wound healing, and embryonic development (44–46). Recent studies in murine tumor models (transgenic and xenograft models) provide evidence that increased levels of VEGF-C and/or VEGF-D promote tumor lymphangiogenesis, including lymphatic vessel sprouting (47–50). In contrast to these experimental tumor models, no increase in lymphatic vessel density was observed in HPV16 tissues, indicating that LEC proliferation in a de novo tumor model lacking exogenous expression of VEGF-C and/or VEGF-D does not result in lymphatic vessel sprouting but instead contributed to increased diameter (girth) of existing lymphatics to enable rapid clearance of tissue fluid resulting from increased leakage of plasma proteins from angiogenic blood vessels.

**Tissue fluid dynamics in premalignant and SCC tissue.** Blood and lymphatic vessels are functionally interdependent vascular systems (6) that together regulate tissue fluid dynamics and influence tissue homeostasis. In a UV-induced skin carcinogenesis model, Hagendoorn et al. (40) revealed alterations in blood vasculature, architectural changes, and impairment of lymphatic vessels along with increased IFP in premalignant tissues and thus hypothesized that blood and lymphatic vasculature lose their functional interdependence during premalignant progression. Our data, however, indicate that, during squamous carcinogenesis in HPV16 mice, the two vessel systems retain a functional interrelationship in premalignant tissue and in well-differentiated carcinomas as evidenced by increased blood vessel leakage accompanied by retained ability to clear fluid, albeit in tissue with higher tissue fluid content than normal.

**Lymphatic vessel functionality.** We observed that intratumoral lymphatic vessel functionality inversely correlates with SCC grade. In the published literature, there are contradicting reports on the existence of intratumoral lymphatics in human (9, 10, 32, 33, 51) and experimental rodent (37, 38, 40, 49, 52) tumors, where only peripheral lymphatic structures are hypothesized to retain functionality, whereas intratumoral lymphatic structures were rendered nonfunctional due to compression by rapidly proliferating tumor cells (52). Similarly, we found functional lymphatic vessels at tumor peripheries but also observed open lumen functional lymphatic vessels in centers of low-grade SCCs. Our interpretation of these observations is that functional properties of higher-grade, less-differentiated carcinomas correlate with, and is effected by, limited lymphatic vessel functionality that, in turn, likely regulates tumor cell physiology and/or behavior. In summary, by examining proliferation, diameter, density, and functionality of blood versus lymphatic vasculature during de novo cancer development, we have revealed unique features of each vasculature that illuminates their interdependency during premalignant progression as opposed to their independence in higher-grade carcinomas. Maintenance of the interdependence between blood and lymphatic systems during premalignancy and in low-grade carcinomas enables efficient fluid transport from leaky blood vasculature via interstitial draining, open lumen lymphatic vessels, which would result in minimizing IFP. The closed nature of lymphatic vessels in higher-grade carcinomas would exclude this and support elevated IFPs common to less-differentiated carcinomas. The implication of these findings supports the notion that delivery of anticancer therapeutics would best be realized in premalignant tissue, in situ carcinomas, or carcinomas displaying well-differentiated characteristics, where tissue hemodynamics better support drug delivery.

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