Vascularization of Melanoma by Mobilization and Remodeling of Preexisting Latent Vessels to Patency

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Abstract

Tumors must manipulate the host vasculature to provide a blood supply adequate for their proliferation. Although tumors may arise as avascular masses, there is increasing evidence that some tumors begin to proliferate by first co-opting preexisting host blood vessels. By fluorescent vascular imaging, we provide evidence that the vasculature in orthotopically implanted melanoma arises from a preexisting red cell–deficient vascular network that remodels to patency to accommodate the requirements of the expanding tumor mass. Topical application of vascular endothelial growth factor to vascular beds generated immediate and robust vascular transitions that were morphologically similar to tumor-induced transitions. Nω-nitro-arginine, a nitric oxide inhibitor, significantly inhibited the growth of a syngeneic K1735M2 melanoma by reducing blood supply to the tumor by a mechanism independent of endothelial cell proliferation. These findings suggest that tumor-induced remodeling of red cell–deficient vessels to patency contributes to tumor vascularization and growth. (Cancer Res 2005; 65(3); 913-8)

Introduction

The growth and dissemination of tumors depends on the establishment of an adequate blood supply to provide nutrients sufficient for their proliferation. Although it is widely accepted that development of a neovasculature through various angiogenic mechanisms is critical for tumor development (1–3), there is increasing evidence that co-option of preexisting vascular beds by the proliferating tumor plays an important role in the early stages of tumor growth in highly vascularized tissues (4–6). Typically, these tumors expand along the capillaries to produce multiple diffuse lesions within the parenchyma (7).

In many tissues, a large fraction of the capillary beds remain closed to the flow of red cells for long periods due to contraction of precapillary sphincters. This typically occurs in muscle where exercise-induced hypoxia (decreased pO₂) stimulates their relaxation and induces vessel dilation to provide a large reserve flow capacity and red cell flux that accommodates the increased oxygen demand of the working tissue (8). Because relaxation of precapillary sphincters and contractile tone of capillary pericytes is under the control of local metabolic factors (9–12), we hypothesized that tumor growth is partly dependent on a similar mechanism. Indeed, proliferating tumors produce factors that effect endothelial cell migration, proliferation, and vessel dilation (13–15).

To define the contribution of preexisting vasculature to tumor growth, we mapped resident patent (normal hematocrit) and latent (red cell–deficient) vessels in normal mouse skin by imaging fluorescein-conjugated, albumin-perfused blood vessels. The topography of the premapped vasculature was then monitored in live animals after inoculation of melanoma cells. In parallel, endothelial cell proliferation within the tumor was estimated by the proliferation-dependent incorporation of BrdUrd into CD31-positive endothelial cells. We show that normal skin has a substantial red cell–deficient vascular network that the tumor mobilizes to patency. Blocking this process with a nitric oxide inhibitor inhibited tumor growth.

Materials and Methods

Animals, Cells, and Reagents. Specific pathogen-free female C3H/HeN and nu/nu mice were purchased from the National Cancer Institute-Frederick Cancer Research Facility (Frederick, MA). K1735M2 melanoma cells were derived from a spontaneous lung metastases produced from parental K1735 cells originally induced in C3H/HeN mice by UVB radiation (16). K1735M2 cells were transfected with pEGFPC1 (Clontech Laboratories, Inc., Palo Alto, CA) using FuGene VI transfection reagents (Roche Molecular Biochemicals, Indianapolis, IN) according to the protocol of the manufacturer. Cells were harvested after 48 hours by trypsinization and subcultured at a ratio of 1:15 in G418-containing medium (800 μg/mL). Clones that expressed high-intensity green fluorescent protein were pooled and used for the in vivo studies. Fluorescein-conjugated albumin (FITC-SA) was prepared by incubating human serum albumin (12.5 mg/mL) with FITC (1 mg/mL) in 0.1 mol/L sodium carbonate (pH 9.0) for 8 hours at 4°C. Unreacted FITC was removed by dialysis or by column chromatography. Quench curves were generated by plotting the fluorescence intensity of FITC-SA solutions in the absence (I₁₁) and presence (I₂₂) of the indicated concentrations of red cells, where:

\[%Quench = 100 \left( \frac{I_{11} - I_{22}}{I_{11}} \right) \]

Immunofluorescence. To monitor endothelial cell proliferation within tumors, animals were injected with BrdUrd (1 mg) 2 hours before tumor harvest. Acetone-fixed frozen sections (5 μm) were sequentially incubated with rat anti-mouse CD31 (PharMingen, San Diego, CA) and Texas red–conjugated goat anti-rat antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). For BrdUrd staining, the thin sections were postfixed with paraformaldehyde (4%), permeabilized with 0.2% Triton X-100/N HCl, and stained with monoclonal BrdUrd antibody (Becton Dickinson Immunocytometry, San Jose, CA) followed by Alexa 488–conjugated goat anti-mouse IgG (Molecular Probes, Eugene, OR). Fluorescence microscopy was done with an epifluorescence microscope equipped with a mercury vapor lamp and appropriate narrow bandpass excitation and emission filters (Ludl Electronic Products, Hawthorne, NY).

Intravitral Microscopy. Mice were anesthetized by metofane inhalation for imaging and recording purposes. Metofane inhalation did not affect blood vessel topography. Normal (pre-tumor) nude mouse ears were mapped with FITC-SA under both bright-field and fluorescent illumination.
To accurately determine the location and topography of the inoculated tumor cells at the initial stage of growth, green fluorescent protein–expressing K1735 melanoma cells (5 × 10⁴ cells in 5-10 μL of saline) were injected into the subcutis of the right ear. Left ears were injected with saline alone and served as individual controls for each mouse. Tumor-dependent vascular alterations were imaged in live mice using a fluorescence stereo microscope (Leica model L212) equipped with narrow bandpass excitation and emission filters mounted in a selectable filter wheel (Ludl Electronic Products). Real-time images were directly captured with an Evolution MP camera (Media Cybernetics, Inc., Silver Spring, MA) or by frame capture from videotaped images. Image analyses were independently carried out in five mice. The images presented are typical and consistent with results obtained in every animal.

**Determination of Tumor Blood Volumes.** Tumor-bearing mice were injected i.v. with 0.1 mL of 40% packed syngeneic RBC labeled with ⁵¹Cr (1.0 mCi/mL packed red cells for 10 minutes at 37°C). After ~10 minutes, 5 to 10 μL of tail vein blood were collected, the mice were killed, and tumors were removed. Radiation in the blood and tumors was quantified by scintillation counting. Tumor blood volume was determined from tumor-associated radiation per gram of tissue standardized from counts per minute per microliter of blood calculated from the tail vein sample.

**N-nitro-L-arginine Treatment, Tumor Growth, and Vascular Permeability (Miles) Assay.** N-nitro-L-arginine (N-NLA) was given to mice ad libitum through the drinking water (1 mg/mL) beginning on the day of tumor inoculation. The Miles assay (17) was done on N-NLA-treated or untreated mice. Briefly, mice were injected i.d. with vascular endothelial growth factor (VEGF; 50 μL at 50 ng/mL). After 10 minutes, mice were injected i.v. with 0.2 mL of 0.5% Evans blue dye (Sigma, St. Louis, MO) in PBS. Dye release at the VEGF injection site was recorded 30 minutes later. Tumor growth was monitored in C3H/HeN mice injected s.c. with 2 × 10⁵ K1735M2 melanoma cells. The mice were randomized into groups of 10 mice each that received drinking water with and without N-NLA. Tumor size was estimated by measuring two perpendicular diameters with calipers every week. Tumor volumes were calculated by 0.56 × a × b², where a and b are the long and short diameters, respectively.

**Statistical Analysis.** Data were expressed as mean ± SD. ANOVA and Student's t test were used for data analysis.

**Results**

**Differentiation ofLatent and Patent Blood Vessels In vivo.** To address the potential role of latent vasculature in supporting the establishment and progression of tumors, a model system that differentiates between preexisting latent (closed) and patent (open) vessels in live animals was developed. The protocol takes advantage of the ability of red cells to quench fluorescein fluorescence through the inner filter effects of hemoglobin. The technique is based on observations that the extent of fluorescence quenching is dependent on hematocrit (percentage of packed red cell volume) and independent of fluorophore concentration. In principle, therefore, concentrations of fluorophores can be selected such that latent (red cell–deficient) vessels fluoresce and patent (red cell–rich) vessels do not. Thus, fluorescence detection of blood vessels with increasing red cell content requires increasing concentration of fluorophore (Fig. 1B). To test this, anesthetized mice were injected with...
increasing concentrations of FITC-SA. Fluorescence microscopy of ears in live nude mice injected with low concentrations of FITC-SA revealed the presence of a large number of small fluorescent vessels (Fig. 1D) that were mostly not visible under bright-field illumination (Fig. 1C). Conversely, red blood vessels visible under bright field were mostly nonfluorescent and appeared as negative images. Figure 1E shows that injection of an additional aliquot of fluorophore breached the quenching threshold of the large red cell–filled vessels and became fluorescent. Thus, the fluorescent-visible vessels at low concentrations of FITC-SA contained very low concentrations of red cells. Taken together, these data indicate that normal tissue contains a network of red cell–deficient blood vessels that can be differentiated from patent vessels in live animals by carefully controlling the concentration of fluorophore in vivo.

Tumor-Induced Mobilization and Remodeling of Red Cell–Deficient Vasculature. After mapping the preexisting vascular network in the ears of nu/nu mice (Fig. 2A and B), green fluorescent protein–expressing K1735M2 melanoma cells were injected into the subcutis of the mapped area. Tumor growth and vascular patency were assessed daily by monitoring green fluorescent protein expression and red blood vessels, respectively. Vessels near the injection site became dilated within 1 day but returned to normal size within 48 hours (attributed to a moderate inflammatory reaction at the site of injection). From day 5, however, a second vascular response resulted in the appearance of previously invisible red blood vessels that duplicated the pattern of the mapped preexisting fluorescent vascular bed. The origin of these vessels was traced to the preexisting large red feeder vessel (Fig. 2A versus Fig. 2C). From day 7, the vessels became more dilated and tortuous and appeared to separate from each other at the tumor core (Fig. 2D-F). By day 13, some of the vessels became less visible and appeared to be engulfed or compressed by the outgrowing tumor (Fig. 2E). As the tumor expanded more, it appeared to carry the peripheral vasculature with it by stretching and pushing the vessels ahead of its leading edge (Fig. 2E and F). This process led to an increase in the density of interweaving vessels at the tumor margin. It seems, therefore, that the continuous proliferation of the tumor dramatically changed both the topography and the patency of the preexisting latent vascular network. Importantly, daily monitoring of the dynamic changes in the pattern of tumor blood vessels revealed that the new bright-field visible red blood vessels could be traced back to the corresponding previously mapped latent vessels present in the tissue before inoculation of the tumor cells. Control ears (left) of the same mice injected with identical volumes of inoculate without tumor cells showed some vascular dilation at the site of injection that resolved within 48 hours. This observation suggests that tumors can acquire a blood supply by co-opting latent vascular beds that are progressively mobilized to patency.

VEGF-Induced Angiogenesis-Like Vascular Remodeling. The data presented above suggest that tumor-dependent alterations in blood vessel function result in the influx of red cells that contributes

![Figure 2](image-url)
to tumor growth. Because VEGF is an important tumor angiogenic factor that also induces persistent vasodilation and increased vasopermeability (13–15), we tested whether the vascular alterations observed in the tumor system could be reproduced with VEGF alone. VEGF-induced vascular alterations were monitored in real time by imaging blood vessel hemodynamics in mesenteric vascular beds. Mice were anesthetized, and the mesentery was draped on the specimen stage of a dissecting microscope. Areas of interest were treated by topically applying VEGF. Sequential bright-field and fluorescent images captured for 1 hour showed that the blood vessels began to dilate, elongate, and form tortuous tubes within 10 minutes after the addition of VEGF. Examination of time-matched, bright-field and fluorescent images revealed new red blood vessels (Fig. 3C) that were visible only under fluorescence illumination before the addition of VEGF (Fig. 3A versus Fig. 3B) and, on reaching patency, could be seen under bright-field and fluorescent illumination as red-positive (Fig. 3C) and fluorescent-negative (Fig. 3D) images, respectively. The topography of the blood vessels was unchanged on treatment with diluent (0.1% bovine serum albumin in saline) alone.

**Inhibition of Tumor Growth with N-NLA.** The data presented above raise the possibility that tumor growth can be sustained, at least in part, by specifically increasing the availability of patent blood vessels to tumors by a vasodilation-dependent mechanism. If this is indeed the case, then one would expect that inhibition of blood vessel dilation and concomitant latent to patent vascular transitions would significantly reduce tumor growth rate. To test this, tumor-bearing mice were treated with N-NLA, a potent nitric oxide inhibitor (18). Administration of N-NLA through the drinking water decreased K1735M2 tumor growth by ~90% (P < 0.01; Fig. 4A) and reduced tumor blood volume by 28% (9.15 ± 4.50 µL/g tumor for control versus 6.60 ± 1.26 µL/g tumor for N-NLA; P < 0.05). Treatment with N-NLA also inhibited VEGF-induced vascular permeability (Fig. 4B). To determine whether N-NLA-dependent decrease in tumor size and blood volume was due to inhibition of endothelial cell proliferation, tumor thin sections from BrdUrd-injected control and N-NLA-treated mice were analyzed for BrdUrd-positive endothelial cells. Rigorous comparisons among groups revealed that whereas N-NLA decreased tumor size, a small but significant increase in the fraction of BrdUrd-labeled, CD31-positive endothelial cells in the tumor periphery was observed (2.2 ± 0.4% versus 5.4 ± 0.6% for control and N-NLA-treated animals, respectively; P < 0.05). This increase might reflect compensatory endothelial cell proliferation-mediated angiogenesis in the presence of N-NLA-dependent inhibition of blood vessel dilation.

**Endothelial Cell Proliferation during Tumor Development.** Because inhibition of tumor growth by N-NLA appeared to occur through an endothelial cell proliferation-independent pathway, we further analyzed the potential contribution of proliferating endothelial cells to tumor growth. K1735M2 mouse melanoma cells (2 × 10^5) were implanted into the subcutis of syngeneic C3H/HeN mice, and tumor together with adjacent tissue was collected at days 1, 2, 3, 5, 7, 13, and 23. Frozen sections were prepared, and the tissue was stained for CD31 (an endothelial cell label) and BrdUrd (a proliferating cell label). Table 1 shows that the fraction of BrdUrd-labeled endothelial cell in the center of small tumors (<7 days; <2 mm diameter) decreased as tumor size increased. In larger tumors (>13 days; >5 mm diameter), BrdUrd-labeled endothelial cell were usually found only within 1 mm of the tumor periphery. In contrast, regardless of tumor size, about one third of tumor cells at the tumor margin were BrdUrd positive.

**Discussion**

It is widely accepted that small tumors remain dormant in the absence of angiogenesis and that tumor growth can be suppressed by inhibiting angiogenesis (19). Although development
of angiogenesis inhibitors focuses on inhibition of tumor-induced
endothelial cell proliferation that leads to angiogenic sprouting,
evidence that tumor growth can be sustained by endothelial cell
proliferation-independent mechanisms of vascular expansion is
accumulating (6, 20). Because proangiogenic factors, like VEGF (6)
and basic fibroblast growth factor (10), are also potent blood
vessel dilators, it is possible that some antiangiogenic therapies
might be affected by mechanisms other than abrogation
of endothelial cell proliferation. Because the tumor blood vessels
are present in the dermis before inoculation of tumor, the data
presented here provide evidence for a mechanism in which
preexisting plasma-perfused but red cell–poor vessels are co-
opted by tumor that topographically and functionally remodels it
to patency in a manner that is morphologically indistinguishable
from endothelial cells proliferation-dependent angiogenesis. The
significant divergence in the fraction of proliferating endothelial
cells and proliferating tumor observed in all stages of tumor
growth (Table 1) further suggests that endothelial cell prolifera-
tion-mediated vascular expansion alone cannot accommodate the
vascular demands imposed by the proliferating tumor. In
principle, tumor growth can be accommodated in the absence
of significant proliferation-dependent angiogenesis through VEGF-
mediated endothelial cell mobilization and remodeling that
characteristically lead to increases in cell surface area and
vascular dilation and lengthening (up to >3 times the original
size) without membrane synthesis (21). Nitric oxide is the primary
vasodilatory mediator (22, 23), and competitive inhibition of nitric
oxide synthase with L-arginine analogues results in decreased
tumor growth rates (18) because of decreased tumor blood flow
(23, 24).

Taken together, these data support a model in which a
proliferating tumor modifies preexisting capillary beds to facilitate
the flow of RBC in a manner analogous to the “on-demand”
opening of gated latent blood vessels in muscle. This likely
proceeds through several steps that include relaxation of
precapillary sphincters and dilation of blood vessels by tumor-
secreted factors (e.g., VEGF/VPF; refs. 13, 25–27). Unlike the
concept that tumor growth is dependent on ingrowth of a new
vasculature, the data presented here show orthotopically implanted
melanoma grow by co-opting and mobilizing a latent vasculature
that is morphologically similar to angiogenic sprouting. This model
predicts, therefore, that tumor expansion depends on the unabated
radial intrusion of tumor into preexisting latent vascular beds that
topographically and functionally remodel to accommodate the
requirements of the enlarging tumor mass.

### Table 1. BrdUrd labeling of endothelial cells and tumor cells during tumor development

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<th>Time post-implantation (d)</th>
<th>% of Endothelial cells</th>
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NOTE: K1735M2 cells (2 × 103) were injected s.c. into the flank of C3H/HeN mice. The injection was marked, and tumors together with adjacent tissue were collected 2 hours after i.v. injection of BrdUrd (1 mg) at the indicated days and stained for CD31 and BrdUrd. Periphery: <1 mm from the tumor margin. Mean ± SD of the ratio of BrdUrd positive to total number of endothelial cells and tumor cells in each sample determined from measurements in 5 to 10 random 0.159-mm2 fields. Because palpable tumors were not present until day 5, tissue was collected from the marked injection site.
Acknowledgments


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References

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