Microcirculation patterns in different stages of melanoma growth

SHIWU ZHANG1, HUA GUO1, DANFANG ZHANG1, WENZHI ZHANG3, XIULAN ZHAO2, ZHIYONG REN4 and BAOCUN SUN1,2

1Department of Pathology, TianJin Cancer Hospital, 2Department of Pathology, TianJin Medical University, 3Department of Molecular Pathology, TianJin Huanhu Hospital, TianJin 300060, P.R. China; 4Department of Biochemistry and Molecular Biology, The University of Texas, M.D. Anderson Cancer Center, Houston, TX 77030, USA

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Abstract. Several microcirculation patterns in tumors have been reported, including vasculogenic mimicry (VM), mosaic vessels (MV) and endothelium-dependent vessels. To investigate the sources of blood supply during different tumor stages, we studied the correlation between expression of vasculogenic mimicry (VM), mosaic vessels (MV) and endothelium-dependent vessels in a mouse melanoma xenograft. Sixty C57 mice were divided into 12 groups (5 mice per group) and inoculated with B16 melanoma cells. Eleven days later, the average tumor size was approximately 0.2 to 0.3 cm. From days 11 to 22, one group per day was randomly sacrificed, and the density of vasculogenic mimicry, mosaic vessels and endothelium-dependent vessels was measured in tumor tissue sections. Immunohistochemical dual-staining and electronic microscopy were also used to confirm the vessel types. All three types of microcirculation patterns were observed during tumor development. In the early stage of tumor growth, vasculogenic mimicry is the main pattern of blood supply. As the area of tumor tissue expands and the number of endothelium increase, vasculogenic mimicry is replaced by endothelium-dependent vessels. Mosaic vessels might be the interim state between vasculogenic mimicry and endothelium-dependent vessels. The number of endothelium-dependent vessels correlated with the size of the tumor (r=0.718, P=0.009), while the number of vasculogenic mimicry was inversely correlated (r=0.77, P=0.003). In conclusion, the number of vasculogenic mimicry decreased and the number of endothelial-dependent vessels increased during tumor growth.

Introduction

To maintain development and rapid growth, tumors need a sufficient blood supply (1). Besides the well-studied angiogenesis, recent studies have revealed several new patterns by which tumor tissues nourish themselves, including the pattern of mosaic vessels from both endothelium and tumor cells (2,3), and the pattern of vessels lined exclusively with tumor cells mimicking the presence and function of endothelial cells. This last process is termed vasculogenic mimicry (4,5).

Three patterns of microcirculation were reported to participate in tumor blood supply. However, it is unclear how these patterns are involved in tumor growth. To answer this question, we monitored blood supply patterns during different stages of growth of melanoma xenografts in C57 mice. The results demonstrate that there are specific microcirculation pattern traits in different stages of tumor growth. Vasculogenic mimicry is the dominant blood supply pattern in the early stage characterized by rapid tumor growth (6). When the tumor mass expands, endothelial cells differentiate and proliferate, and the mosaic vessels appear as a transitional pattern. Consequently, endothelium-dependent vessels replace vasculogenic mimicry and mosaic vessels to become the major pattern of blood supply in the late stage of tumor growth (7,8).

Materials and methods

Animals. Sixty C57/6J 6-8 week-old black mice including 30 males and 30 females were purchased from the Animal Base of Union Drug Institute (Beijing). The average weight of these mice was 21 g.

Cell strain. A single cell suspension of B16 malignant melanoma was provided by the Tianjin Cancer Hospital Department of Biochemistry and stored in liquid nitrogen. Before injection, the suspension was incubated for 20-30 sec in a 43°C water bath and centrifuged at 1000 gps for 10 min. The supernatant was absorbed by an asepsis tampon, and the pellet was diluted with 1-2 ml 0.9% NaCl solution to ensure a final cell density of 3-5x10⁶ cells/ml.

Tumor-bearing animal model. All 60 C57BL/6J mice were divided randomly into 12 groups (5 mice per group). A week later, mice were inoculated with a single cell suspension of...
B16 malignant melanoma (the groins of mice were sanitized with an alcohol tampon, and a 0.2 ml single tumor cell suspension was injected subcutaneously). Eleven days after inoculation, a tumor mass with an approximate diameter of 0.2-0.3 cm was observed in more than 90% mice at the site of injection. From days 11 to 22, one group of 5 mice per day was randomly sacrificed.

Immunohistochemistry dual-staining. The antibodies used in this study were mouse monoclonal anti-CD31 (clone JC/70A) and anti-HMB45, both purchased from Sigma Chemical Co., (St. Louis, MO, USA), which were used at dilutions of 1:200 or 1:300, respectively.

The tumors were removed, fixed with formalin and embedded in paraffin. The 4-μm paraffin-embedded tissue sections were mounted onto poly-L-lysine-coated slides. Paraffin sections were routinely deparaffined, and endogenous peroxidase activity was blocked with 3% hydrogen peroxide in 50% methanol for 10 min at room temperature. The sections were rehydrated and washed with PBS, then pretreated with citrate buffer (0.01 M citric acid, pH 6.0) for 20 min at 100°C in a microwave oven. After nonspecific binding sites were blocked using 2% normal goat serum in phosphate-buffered saline (PBS) for 15 min at 37°C, the sections were incubated for 1 h at 37°C with the first primary antibody indicated. The sections were then rinsed with PBS, incubated with the mixture of avidin-alkaline phosphatase and BCIP/NST for 20 min at 37°C. For the second staining procedure, the sections were first incubated in dual-staining intensifier buffer for 30 min, then blocked with serum for 10 min at room temperature and repaired in the microwave oven again. In the second staining, sections were incubated with primary antibody at 4°C overnight. Biotin-labeled secondary antibodies and peroxidase-labeled avidins were added to the sections, which were stained with AEC, air-dried and mounted with neutral gum. It should be cautioned that sections cannot be placed in dimethylbenzene again.

Microvessels density (MVD) counting. According to the standard introduced by Weidner et al (9), capillary vessels and microvessels in the tumor stained with CD31 were counted.

Microvessel density counting standard. A single positively stained endothelial cell can be counted as one MVD (9,10); when the area of a vessel is large (>8 red blood cells), an improved method reported by Tanigawa et al can be used (every length of 40 μm vessel is counted as one MVD) (11).

Vasculogenic mimicry counting standard. The wall of vasculogenic mimicry is lined with tumor cells, and red cells can be found in the vasculogenic mimicry tube, with no necrosis, inflammation cells or red cell leakage (12,13). The counting method is similar to microvessel density counting.

Mosaic vessel counting standard. The vessel wall was lined with both tumor and endothelial cells, and inflammation cells and red cell leakage were not found around the vessels (8). More than 5 microscopic fields in one section were observed, and the average was considered as the MVD. The distance of five eye-field intervals in one section is approximately one microscopic field.

Electron microscopy. Specimens from the 12 groups were randomly selected and fixed with glutaraldehyde. The presence of red blood cells in the channels without endothelium was used as a criterion for authentic vasculogenic mimicry. The presence of red blood cells in the channels with endothelium and tumor cells was used as a criterion for an authentic mosaic vessel. Ultra-thin sections were examined using a JEM-1010 electron microscope.

Results

Vasculogenic mimicry, mosaic vessels and endothelium-dependent vessels all occurred in melanoma xenographs. Eleven days after inoculation, rich blood vessels were seen on the surface of all tumors in every mouse when the skin of the groin was exposed. Some tumors invaded the adjacent normal tissue, and the boundary between tumor and normal tissue was unclear. While splitting the tumor mass, the tumor was found to be very soft (Fig. 1A). The liver, spleen, lung, kidney and brain of each mouse were examined, but no metastasis was found in any of the 60 cases. We observed that necrosis was not detected when the tumor diameter was <1.0 cm, demonstrating that the tumor had a good blood supply. Necrosis began to appear in the tumor when the diameter reached approximately 1.5-2.0 cm at day 21 or so after inoculation.

Rich blood sinusoids were observed in the H&E-stained sections, and a large amount of vasculogenic mimicry and red blood cells were also found. The PAS-positive material can be observed with PAS stain between the red blood cells and tumor cells in some VM (9). Moreover, under an oil microscope, melanin granules were identified in the cytoplasm of tumor cells that formed in the vessels (Fig. 1B and C), which indicated that these cells were melanoma cells. Besides vasculogenic mimicry, mosaic vessels that consist of both tumor and vascular endothelial cells were found in the same tumor mass (Fig. 1C). To confirm the structure of vasculogenic mimicry and mosaic vessels, the sections were dual-stained with both endothelial cell-specific marker (CD31) and melanoma-specific marker (HMB45). As shown in Fig. 1D, brown-stained melanoma cells were found to encircle the vessel and blue-purple red cells were found inside. No purple-stained endothelial cells were found in the vessel wall. In mosaic vessels, both the brown melanoma cells and blue-purple endothelium lined the vessel walls (Fig. 1E). Moreover, the trend of vasculogenic mimicry being replaced by endothelium-dependent vessels can be observed with microscopy. We observed that endothelium crept through the vasculogenic mimicry (Fig. 1F). When samples from the same tumor were observed under an electronic microscope, the structure of vasculogenic mimicry and mosaic vessels were clearly identified (Fig. 2A and B).

We observed that, morphologically, the tumors manifested endothelial cell traits, i.e. patch, girder, crater shapes and some ultrastructure features such as desmosomes and junctional complexes. We simultaneously performed PAS staining and staining for the endothelial cell markers CD31. CD31 is a marker of endothelial cells, and the base membrane is positive for PAS, so CD31 and PAS dual staining (9,10) was used to distinguish VM and endothelial-dependent vessels.
Figure 1. (A) In a C57 mice carrying B16 melanoma, black and brown tumors invaded the muscle of its limb (red arrow, tumor). (B) Vasculogenic mimicry. Encircled tumor cells form a tube structure where red cells were observed. Necrosis, inflammatory cells and red cell leakage were not found (red arrow, vasculogenic mimicry). Original magnification, x400. (C) A mosaic vessel was formed with tumor cells (red arrow) and endothelial cells (yellow arrow). Original magnification, x400. (D) Vasculogenic mimicry. Yellow-brown tumor cells and blue-purple endothelial cells lined the tube structure, as shown with immunohistochemical dual-staining (LAB-SA), original magnification, x1000. (E) Mosaic vessel. The mosaic vessel was formed with tumor cells (red arrow) and endothelial cells (green arrow). Original magnification, x400. (F) The trend of vasculogenesis mimicry (red arrow) being replaced by endothelium-dependent vessels (green arrow) can be observed with microscopy. Original magnification, x400.

Figure 2. (A) The structure of vasculogenic mimicry under an electronic microscope; tumor cells form a vessel-like structure with RBCs inside. Original magnification, x3000. (B) The structure of mosaic vessel under an electronic microscope; tumor cells (red arrow) and endothelial cells (yellow arrow) form a vessel-like structure with RBCs inside. Original magnification, x3000.
The VM channels with walls negative for CD31 confirmed that the channels were not composed of endothelial cells. However, we also observed that some channels were lined with both CD31-positive/PAS positive and CD31-negative/PAS positive cells, that is, mosaic channels composed of tumor cells and endothelial cells. Due to the sufficient blood supply offered by vasculogenic mimicry and mosaic vessels, no necrosis was found in the early tumor stage. At this time, the main microcirculation pattern was endothelium-independent vessels.

The expression of vasculogenic mimicry, mosaic vessels and endothelium-dependent vessels in different stages of tumor growth. To identify the time pattern of tumor blood supply in different stages of melanoma growth, we measured the density of each type of vessel on a daily basis from day 11 after inoculation. In the early stage of melanoma graft formation, tumor blood supply consisted of vasculogenic mimicry, mosaic vessels and endothelium-dependent vessels, among which the number of vasculogenic mimicry was dominant (Fig. 3 and Table I). However, as time passed, the number of mosaic vessels and endothelium-dependent vessels increased. As the tumor volume \( TV = (\text{length} \times \text{width})^2/2 \) expanded, the number of endothelium-dependent vessels highly correlated with tumor volume \( (r=0.718, P=0.009) \), while the number of vasculogenic mimicry inversely correlated with tumor volume \( (r=0.77, P=0.003) \). As shown in Fig. 3, the density of endothelium-dependent vessels increased according to time after day 11, thus becoming the major pattern of tumor blood supply. These data suggested that vasculogenic mimicry is the major pattern of blood supply for tumor growth at early stages, and endothelium-dependent vessels developed in a time-dependent manner.

Discussion

Without blood vessels, a tumor cannot grow beyond a critical size (1-2 mm in diameter). Increased vasculogenesis must occur for tumors to develop and be maintained (14). Microcirculation in the tumor not only provides enough nutrition and oxygen and disposes waste for tumor growth, but also provides the window and pathway for metastasis (15). A sufficient blood supply is essential for tumor growth and invasion in its progression (16). Conventionally, vascular networks are believed to consist of tube structures lined with endothelial cells. However, vasculogenesis is not the only pattern in which tumor cells acquire nutrition. There is evidence suggesting that some highly aggressive tumors have several mechanisms of blood supply, including endothelium-dependent vessels, mosaic vessels and vasculogenic mimicry.

Vasculogenic mimicry was recently reported as a new blood supply by Maniotis et al in highly aggressive malignant melanoma that characteristically had tumor cells, but not endothelial cells, pasted to the surface of the basement

Table I. The average number of vasculogenic mimicry, mosaic vessels and endothelium-dependent vessels varied with time.

<table>
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<th>Day(s)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
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<tr>
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<td>3.70</td>
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TV, tumor volume; VM, vasculogenic mimicry; MV, mosaic vessels; E, endothelium-dependent vessels. \( TV = (\text{length} \times \text{width})^2/2 \)
membrane in tubular structures (5,17). The tumor tissue connects with host vessels for blood supply by vasculogenic mimicry (18). There was no necrosis, inflammation cell or red cell leakage around the vasculogenic mimicry. In the 1950s, it was reported that some types of malignant tumors had mosaic vessels, which is a pattern of blood supply for tumor growth in which the wall of the tube is randomly lined with endothelial and tumor cells. Nevertheless, the mechanism of mosaic vessels is still unclear. Reported theories about the mechanism include: 1) due to endothelial cells dropping from the vessel wall, tumor cells are directly exposed to the blood tube (7); 2) some endothelial cells lose immunological marker activation in tumor progression, cannot be stained and become recessive cells; 3) tumor cells invade and are located in the blood vessel wall, and endothelial cells co-form the structure of blood vessels (8).

Why do tumor cells develop three patterns of microcirculation, given that endothelium-dependent vessels can supply blood and nutrients to the tumor? Endothelium-dependent vessels, which require the recruitment of normal endothelial cells, may not be efficient enough to sustain aggressive tumor growth at the early stage of rapid growth. Some tumor cells, via dedifferentiation and changes in topology, connect with other tumor cells or endothelium and line the wall of the tube (19). Vasculogenic mimicry and mosaic vessels become the sources of blood supply. Tumors that are more self-sufficient and can provide their own microcirculation components would have the greatest growth potential at the early stage of rapid growth. An interesting observation was that VM was most frequently observed in the boundary regions between the tumor and surrounding normal tissues (20). Thus, VM may also play a role in tumor invasion by supplying immediate nutrition. The unique topology of VM distribution is consistent with the notion that specific molecular and cellular modifications occur, which facilitate tumor invasion and growth. Therefore, VM is a poor prognosis indicator in patients with sarcomas and melanomas (21-23).

Three microcirculation patterns exist in the melanoma tissue with certainty, but the relationship between them is unclear. Our animal model inoculated with B16 suggested that the three above-mentioned blood supply patterns are time-dependent in tumor growth. When the diameter of the tumor is <1 mm, the tumor requires nutrients and oxygen by permeation where there are no endothelial cells (13). When the diameter of tumor is >1-2 mm and enters a rapid growth stage, endothelial cells from normal tissue around the tumor cannot meet the needs for tumor growth. Therefore, some tumor cells connect with other tumor cells to line the wall of tube via dedifferentiation and changes in topology, and vasculogenic mimicry becomes the major pattern of blood supply. As the tumor size grows and endothelial cells undergo continual mitosis, some tumor cells of vasculogenic mimicry are replaced by endothelium (20). There is a transition between vasculogenic mimicry and endothelium-dependent vessels, and mosaic vessels become the major blood supply for the tumor at this stage. Finally, when the tumor reaches a size more than 1.5-2 cm, the rate of growth is decreased. At this stage, endothelium-dependent vessels are the major pattern of blood supply. Tumor cell necrosis takes on a patch and lies primarily at the center of tumor tissue. It can be inferred that the three microcirculation patterns of blood supply including vasculogenic mimicry, mosaic vessels and endothelium-dependent vessels may compose a series of stages in tumor growth.

Different tumor cells have different patterns of blood supply in which some can access blood supply by tumor vasculogenesis, and others acquire sufficient oxygen and nutrition by vasculogenic mimicry (24). These patterns are necessary because the tumor must adapt to different internal environments to grow, invade and metastasize (25,26). Vasculogenic mimicry has been found in several different types of cancers such as melanoma, and breast, prostate and ovarian cancer, which are all highly malignant, can easily metastasize and have poor clinic prognosis (27). The adoption of vasculogenic mimicry and mosaic vessels may be a way to adapt to rapid tumor growth. Due to vasculogenic mimicry and mosaic vessels, tumor cells have sufficient blood supply and direct contact with the host circulation. Because of direct contact with the bloodstream, tumor cells have a greater chance to metastasize compared to endothelium-dependent vessels (28). Clinical data show that nearly all patients with high-grade malignant melanoma die of tumor metastasis via the bloodstream. Therapy that only targets angiogenesis may not affect vasculogenic mimicry and mosaic vessels (29,30). Therefore, the existence of an alternative microcirculation poses a major challenge to anti-angiogenesis treatment, and additional forms of therapy are necessary.

Acknowledgements

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References


