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RESEARCH ARTICLE

Primo Vascular System of Murine Melanoma and Heterogeneity of Tissue Oxygenation of the Melanoma

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Abstract
Murine melanoma requires the complex development of lymphatic, vascular, and non-vascular structures. A possible relationship between the primo vascular system (PVS) and the melanoma metastasis has been proposed. In particular, the PVS may be involved in oxygen transport. Vasculogenic-like networks, similar to the PVS, have been found within melanoma tumors, but their functional relationship with the PVS and meridian structures are unclear. Herein, we report on the use of an electrochemical O_2 sensor to study oxygenation levels of melanoma tumors in mice. We consistently found higher tissue oxygenation in specific sites of tumors ($n = 5$). These sites were strongly associated with vascular structures or the PVS. Furthermore, the PVS on the tumor surface was associated with adipose tissue. Our findings suggest that the PVS is involved in the regulation of metastasis.

1. Introduction

A study by Soh et al (2009) [1] suggested that a primo vascular system (PVS) is well developed in lung cancer tumors and may serve as a pathway for cancer metastasis. A similar vasculogenic-like network composed of undifferentiated melanoma cells, known as vasculogenic mimicry [2], has been extensively studied. Although the relationship between the PVS and vascular mimicry is unclear, the importance of non-lymphatic and non-vascular systems in cancer development and treatment has been acknowledged. Soh et al [1] suggested that the PVS may play an important role in transporting materials within the body, possibly serving as a conduit for oxygen transport.

Higher tissue oxygenation has been associated with acupoints on the human hand [3], suggesting that acupuncture meridians have higher metabolic demands. Metabolic demands and hemodynamic signals are strongly correlated [4,5]. In particular, changes of neuronal activation during brain signal processing induce vasodilation, which increases in blood flow tissue oxygenation to the area (i.e., neurovascular coupling). A recent study provided evidence for the

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role nitric oxide release in regulating hemodynamics and tissue oxygenation of the activated brain area using a dual electrochemical sensor able to monitor real-time changes in hemodynamic signals *in vivo* [4].

Cancer involves uncontrolled cell division, which increases metabolic demands. Therefore, perfusion-based *in vivo* imaging techniques such as positron emission tomography and single photon emission tomography are used in cancer diagnosis and treatment. However, these conventional imaging techniques have low spatial and temporal resolution, which may result in a low rate of detection of early cancers. The ability to monitor the hemodynamic changes in cancer tissues may thus improve diagnosis and treatment.

In this study, we characterized the PVS associated with tumors in a mouse model of melanoma by immunocytochemistry. In addition, and tissue oxygenation in melanoma tissues was measured using an electrochemical oxygen sensor to assess the role of the PVS in oxygen transport.

2. Materials and Methods

2.1. Induction of murine melanoma cancer

To generate the mouse model of melanoma, B16BL6 murine melanoma cells (MD Anderson Cancer Center) were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2.

Male C57BL6 mice (4 weeks old, 15—20 g, $n = 5$) were used in this study (Orient Bio Inc). All procedures involving the animals, their care, and surgical procedures followed the Guide for the Care and Use of Laboratory Animals (National Academy Press, 1996). The animals were acclimated at 25°C in a 12-hour light-dark cycle. After a 1-week adjustment period, the B16BL6 cells (1×10^6 cells/100 μl DMEM) were transplanted into the abdomen area.

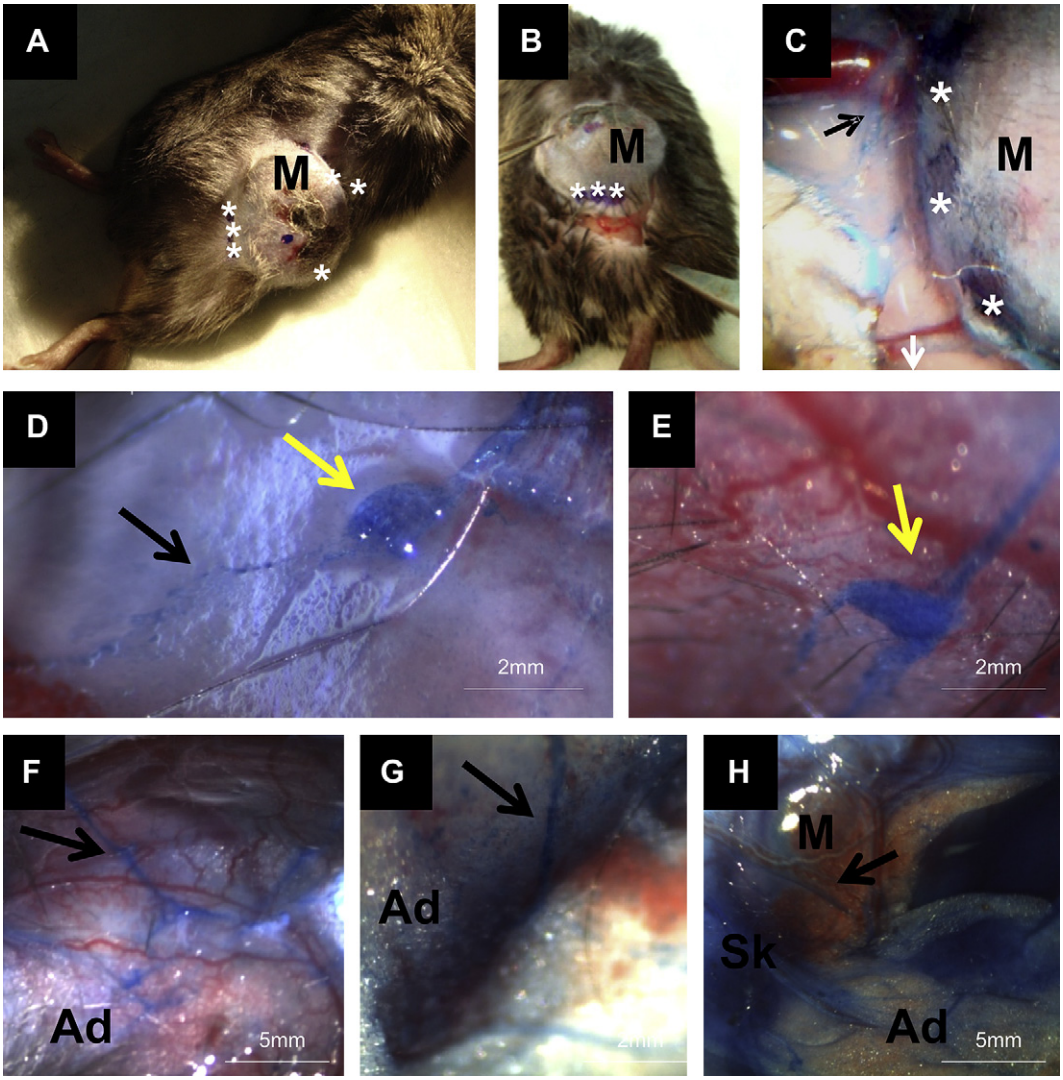


Figure 1 Primo vascular system of a melanoma tumor. (A, B) Tumors in a mouse model of melanoma. White asterisks indicate oxygen sensor measurement points. (C) The primo vascular system is associated with a blood vessel. (D, E) A primo node (yellow arrow) and primo vessel (black arrow) of the primo vascular system. (F, G) Adipose tissue is associated with the primo vascular system on the surface of the tumor. (H) Adipose tissue recruited to the injection site. Ad = adipose tissue, M = melanoma and Sk = skin.

2.2. Investigation of the primo vascular system in melanoma cancer

To visualize the PVS *in vivo*, we stained the area around the melanoma tumor with 2% Trypan Blue and then washed it with saline. Images of the PVS were obtained using a surgical microscope (S6D, Leica, Switzerland) and camera incorporating a charge-coupled device (DFC295, Leica, Switzerland).

2.3. Electrochemical oxygen sensor

High-sensitivity electrochemical oxygen microsensors were fabricated in a manner similar to that previously described for nitric oxide microsensors [1]. The sensor was composed of a platinum disk cathode (diameter 25 μm; Goodfellow Metals Ltd.) and Ag/AgCl anode (127-μm diameter, A-M Systems) covered with a polytetrafluoroethylene gas-permeable membrane (thickness < 19 μm, porosity 50%, pore size 0.05 μm; W. L. Gore & Associates). Currents between the cathode and the anode were recorded as a function of time using a CHI1000A multipotentiostat (CH Instruments Inc., USA) while a potential of -0.6 V relative to the anode was applied to achieve an oxygen reduction reaction. The measured currents were linearly proportional to the oxygen levels in the samples. The sensors were calibrated before and after oxygen measurements using an oxygen standard solution prepared by bubbling de-aerated phosphate-buffered saline (PBS, pH 7.4; Fisher Scientific) with oxygen gas (Dong-A Gas Co, Korea).

2.4. Measuring oxygen in melanoma cancer

The animals were anesthetized with Rompun (Bayer Korea Ltd., Korea) administered by intraperitoneal injection. Body temperature was maintained at a constant level with warm pads, and the anesthesia level and breathing patterns of the mice were closely monitored throughout the experiment. The tumor area was identified by gross inspection, and the skin area was shaved and cleaned with alcohol. The planar oxygen sensor was immersed in the PBS (pH 7.4) solution and carefully positioned about 1 mm above the area of interest. The oxygen microsensors were used to measure the currents responding to the oxygen levels at three anterior-ventral points, three caudal-ventral points, and one to two points in the middle of the tumor (Fig. 1). After the oxygen level had been measured at all points, measurement at the first point was repeated. The measurement positions for all five animals are shown in Fig. 1. Calibration data from a previous study was used to convert the sensor current data to the corresponding oxygen levels.

2.5. Immunocytochemistry

The PVS was analyzed by immunocytochemistry using a polyclonal antibody against the lymphatic vessel endothelial hyaluronan receptor, a specific marker for lymphatic vessels (IgG anti-LYVE-1; Abcam, UK). We collected tissues that included primo vessels and lymphatic vessels near the melanoma tumor. The harvested tissues were embedded in optimal cutting temperature compound and snap-frozen in liquid nitrogen. Tissue cross sections (10 μm thick) were fixed in ice-cold acetone for 5 minutes. The fixed tissues were then washed in PBS and blocked with casein-based blocking solution (CAS-Block; Invitrogen, USA) for 2 hours at 37°C. Then, the tissue slides were incubated with rabbit anti-mouse LYVE-1 (2.5 mg/mL) overnight at 4°C. After

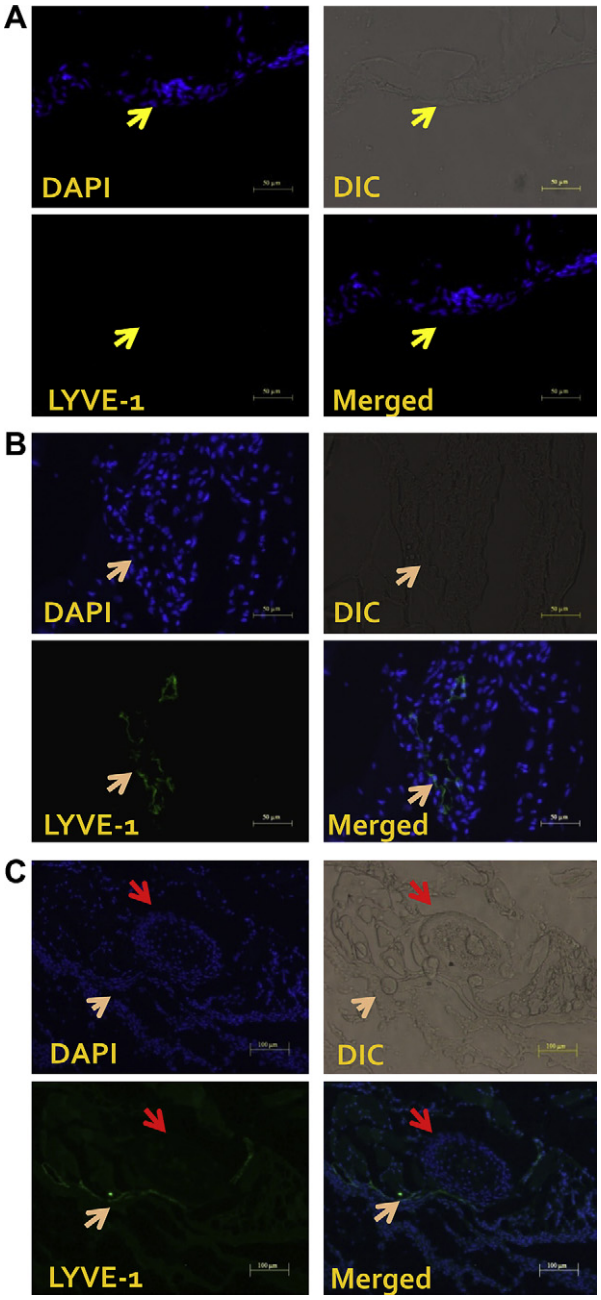


Figure 2 Immunocytohistochemical analysis of the primo vessels, lymphatic vessels, and blood vessels extracted from the murine melanoma tumor: cross sections of (A) primo-vessel (yellow arrow), (B) lymphatic vessel (pink arrow), and (C) blood vessel (red arrow) with a lymphatic vessel (pink arrow). Blue = 4',6-diamidino-2-phenylindole (DAPI), green = lymphatic vessel endothelial hyaluronan receptor (LYVE1), DIC = differential interference contrast microscopy.

washing the slides, they were incubated with goat-anti rabbit IgG conjugated to Alexa Fluor 488 (1:500; Molecular Probes, USA) and counterstained with 4',6-diamidino-2-phenylindole (DAPI to visualize the nucleus. After these procedures, the slides were mounted with antifade reagent (Molecular Probes, USA). A portion of the same sample tissue was treated with DAPI and mounted with antifade reagent. Confocal microscopy (LSM500, Zeiss, Germany) was used to verify the staining of the slides.

2.6. Statistical analysis

For each animal, the relative oxygen levels at points associated with the vasculature or the PVS were compared with oxygen levels at other points by Student's t test (two-tailed); $p < 0.05$ was considered significant.

3. Results

3.1. Primo vascular system of a murine melanoma

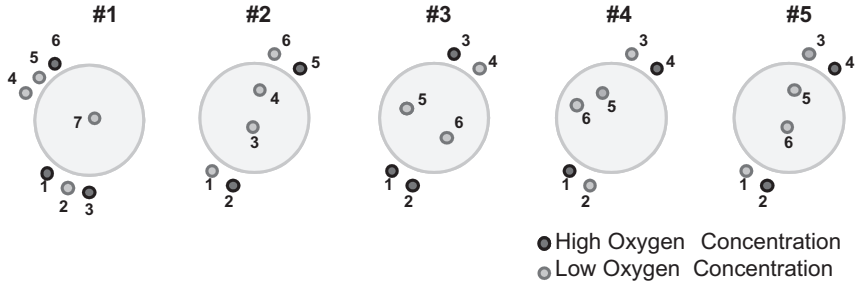
Injection of B16BL6 murine melanoma cells produced a tumor 2 weeks later near the injection site (Fig. 1A and B). The PVS was easily identified near the blood vessel (Fig. 1C) beside the tumor (M) and at the surface of the melanoma (Fig. 1D and E). In particular, an extensive PVS network was found at the junction of the skin and the tumor. The PVS at the melanoma surface contained a primo node (Fig. 1D and E) and web-like structures (Fig. 1F), and was associated with adipose tissue (Fig. 1F and G). We consistently observed well-developed PVS networks alongside adipose tissues in the specimens. In general, adipose tissue was strongly associated with the inoculation site (Fig. 1H).

3.2. Immunostaining with LYVE-1

We investigated the PVS near the melanoma tumor by staining with the lymphatic marker LYVE-1 and the nuclear marker DAPI. The PVS exhibited rod-shaped nuclei (Fig. 2A), but was not stained with LYVE-1, whereas the lymphatic vessel was stained with LYVE-1 and did not have rod-shaped nuclei (Fig. 2B). The PVS characteristics also differed from those of blood vessels, which had round-shaped nuclei (Fig. 2C).

3.3. Measuring oxygen in melanoma cancer

Oxygen was measured at points were selected according to the structure of melanoma cell vasculogenic-like networks



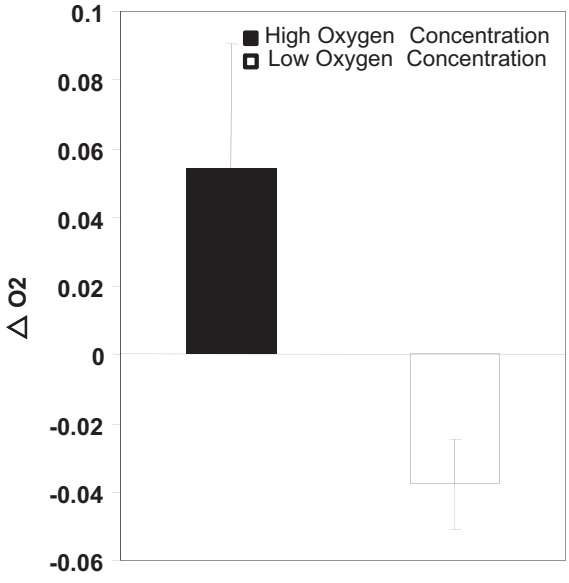


Figure 3 Heterogeneity of tissue oxygenation of murine melanoma cancer. (A) Measurement points for tissue oxygenation of melanoma tumors. Dark gray indicates points associated with the vasculature or primo vascular system, and light gray indicates points not associated with vascular or primo vascular structures. (B) Relative oxygen levels at different points on the melanoma tumor surface.

as indicated by Hendrix et al (2003) [2]. Microsensors were used to record tissue oxygenation levels of the murine melanoma tumor. Baseline levels were obtained by positioning the microsensor near the tumor, and moved to different points, as illustrated in Fig. 3A, while maintaining the sensor's vertical distance from the skin (about 1 mm). Up to seven different points were selected for oxygen measurements in each of the animals (Fig. 3A). Sites in the middle of the tumor were assessed as neither vasculature-related points nor PVS-related points after careful investigation of the vascular and the non-vascular systems. The peripheral tumor regions usually overlapped with the vasculature or the PVS.

Heterogeneity of tissue oxygenation was found in the tumor tissue of all five animals (Fig. 3B). The relative oxygenation level was 0.054 ± 0.036 (ΔO_2) at points that appeared to be associated with either the vasculature or the PVS, and -0.038 ± 0.013 (ΔO_2) at the points that were not associated with the vasculature or the PVS ($p = 0.0124$).

4. Discussion

Melanoma is the most serious type of skin cancer and the most studied cancer model in rodents. Hendrix et al [2] described the ability of melanoma cells to form vasculogenic-like structures. This vasculogenic mimicry is associated with the invasiveness of melanoma cancer. The capillary-like structure of vasculogenic mimicry is similar to that of the PVS described by Soh et al [6], which is believed to play an important role in cell regeneration. In particular, the primo microcells of the PVS may function as embryonic-like stem cells. The presence of circulating very small embryonic-like stem cells was reported by Kucia et al [7]; however, functional similarities between primo microcells and these very small embryonic-like stem cells remain unclear.

Since melanoma development requires high rates of cell proliferation and cell division, it has been suggested that the melanoma may also have a well-developed PVS. Indeed, we found that the PVS was well developed at the melanoma tumor surface, but we did not investigate the presence of the PVS within the tumor. We also noticed that the PVS of a developing tumor was more prominent than that of a mature tumor. The PVS may have developed prior to tumor angiogenesis (Fig. 1D and H). We found a strong association between adipose tissue and the PVS (Fig. 1G and H), suggesting important roles in shaping melanoma development. Various growth factors released by the adipose tissue may stimulate PVS development in cancer tissues [8].

Lee et al [3] recently reported that, compared with sites not associated with acupuncture meridians, oxygen levels were higher at acupuncture points. This difference was thought to be due to the PVS. Their findings were consistent with the notion that the PVS is involved in metabolic processes. Therefore, we investigated oxygenation patterns in melanoma tissues to assess the role of the PVS in regulating material transport, including oxygen.

The heterogeneity of kidney and brain tissues has been reported, suggesting the existence of oxygen pressure fields within biological tissues [9]. This heterogeneity of tissue oxygenation may reflect structural differences in the tumor, as well as in the oxygen pressure field. Sites that exhibited high oxygen levels may be associated with the PVS, although vascular-related sites usually have high oxygen levels. High oxygen levels may be associated with oxygen flux; however, the origin of this oxygen flux remains unclear. In five different animals, we observed similar oxygen concentration patterns. The rostral-ventral and the caudal-ventral areas typically showed high oxygen levels. At these sites, adipose tissue was often found associated with the PVS and sometimes with the vasculature. However, further studies are needed to determine whether the presence of the PVS accounts for this heterogeneity. In addition, the relationship between angiogenesis and the PVS must be elucidated.

In summary, this study reveals the characteristics of the PVS in melanoma tissues, as assessed by biosensor measurements of the oxygenation levels at different locations. These results suggest a role for the PVS in cancer development and metastasis.

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References

1. Yoo JS, Kim HB, Ogay V, Lee BC, Ahn S, Soh KS. Bonghan ducts as possible pathways for cancer metastasis. *J Acupunct Meridian Stud*. 2009;2:118—123.
2. Hendrix MJ, Seftor EA, Hess AR, Seftor RE. Vasculogenic mimicry and tumour-cell plasticity: lessons from melanoma. *Nat Rev Cancer*. 2003;3:411—421.
3. Lee Y, Lee G, Park SS, Jang A, Jhon GJ. Heterogeneity of the oxygen density distribution on skin: relation to the locations of acupuncture points. *J Acupunct Meridian Stud*. 2009;2: 269—272.
4. Park SS, Hong M, Song CK, Jhon GJ, Lee Y, Suh M. Real-time in vivo simultaneous measurements of nitric oxide and oxygen using an amperometric dual microsensor. *Anal Chem*. 2010;82: 7618—7624.
5. Iadecola C. Neurovascular regulation in the normal brain and in Alzheimer's disease. *Nat Rev Neurosci*. 2004;5:347—360.
6. Soh KS. Bonghan circulatory system as an extension of acupuncture meridians. *J Acupunct Meridian Stud*. 2009;2: 93—106.
7. Kucia MJ, Wysoczynski M, Wu W, Zuba-Surma EK, Ratajczak J, Ratajczak MZ. Evidence that very small embryonic-like stem cells are mobilized into peripheral blood. *Stem Cells*. 2008;26: 2083—2092.
8. Baumgärtl H, Zimelka W, Lübbers DW. Evaluation of PO(2) profiles to describe the oxygen pressure field within the tissue. *Comp Biochem Physiol A Mol Integr Physiol*. 2002;132:75—85.
9. Yoo JS, Ayati MH, Kim HB, Zhang WB, Soh KS. Characterization of the primo-vascular system in the abdominal cavity of the lung cancer mouse model and its differences from the lymphatic system. *PLoS ONE*. 2010;5:e9940.