

Influence of microenvironments on microcirculation patterns and tumor invasion-related protein expression in melanoma

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Abstract. This study aimed to investigate the influence of different microenvironments on melanoma microcirculation patterns, invasiveness and metastatic behavior. Sixty C57BL/6J mice were randomly divided into two groups with 30 mice per group. Melanoma B16 cells were injected into the subretinal space and groin area of mice synchronously. The number of each type of microcirculation pattern was counted. Invasion and metastasis were observed. Epithelial cell kinase (EphA2), matrix metalloproteinase (MMP)-2 and -9 expression and their mRNA levels were detected by immunohistochemical staining and real-time PCR and compared between the two groups. Five invasions and six lung metastases were found in the subretinal group while no invasion and metastasis were found in the groin group. The number of vasculogenic mimicry (VM) was significantly higher in the subretinal group ($P=0.000$). However, no significant difference in the numbers of mosaic and endothelium-dependent vessels was observed between the two groups ($P=0.076$ and 0.146 , respectively). EphA2, MMP-2 and MMP-9 expression was significantly higher in the subretinal group. The mRNA levels of EphA2, MMP-2 and MMP-9 were slightly higher in the subretinal tumors ($P=0.002$, 0.001 and 0.001 , respectively). In conclusion, this experimental paradigm can be a powerful one in which to investigate tumor-microenvironment interactions in melanoma. Tumor cells in the intraocular microenvironment had increased EphA2 expression which induced the formation of VM channels. Moreover, expression of MMP-2 and -9 in tumor tissue was increased to enhance the invasiveness and metastatic behavior.

Introduction

The concept of 'vasculogenic mimicry' (VM) was introduced to describe the unique ability of highly invasive tumor cells to form capillary-like structures (CLS) and a matrix-rich patterned network. Melanoma cells have the ability to mimic endothelial cells and form VM channels (1,2). VM channels connect with endothelium-dependent vessels to create a network that provides for tumor growth, invasion and metastasis (3). Although the molecular mechanism of VM channel formation is still unclear (4), it is possible that a change in microenvironment induces VM channel formation directly to enhance the behavior of tumor metastasis (5). A poor intraocular microenvironment can activate some invasion- and metastasis-associated tumor genes, enabling cells to become more invasive for survival (6,7). Epithelial cell kinase (EphA2) plays critical and diverse roles in regulating cell adhesion, migration and proliferation. It was revealed that increased expression of EphA2 in aggressive melanoma cells plays an important role in mediating VM. Matrix metalloproteinases (MMPs) can degrade many components of the extracellular matrix (ECM) and basement membrane. They can contribute to cancer growth, invasion and angiogenesis (8). Upon immunohistochemical staining, MMP-2 and -9 were found to co-localize with VM, and they are thought to play an important role in VM formation (5,9,10). In this study, we hypothesized that the local tumor microenvironment affects the formation of VM channels, the number of tumor metastases and expression of EphA2, MMP-2 and MMP-9 in xenografted and orthotopic melanoma.

Materials and methods

Animals and cell line. Sixty C57BL/6J mice, 30 males and 30 females, were used in this study. They included 6- to 8-week-old black mice weighing 20-25 g, purchased from the Experimental Animal Institute of the Chinese Academy of Medical Science (Beijing). All 60 mice were randomly divided into two groups with 30 mice per group.

A suspension of B16 cells (1×10^5 cells/mouse) was injected into the left groin area and the right subretinal space. The skin of the left groin area in each mouse was sterilized with

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75% alcohol, and a 0.1-ml cell suspension (1×10^6 cells/ml) was injected into this area. The right eyes of the mice received microscopically guided subretinal injections of 0.004 ml of a cell suspension (4×10^8 cells/ml). By the 14th day, the engrafted tumors were palpated, and the mice were sacrificed. Some fresh tumor masses were collected for real-time PCR and some were fixed and embedded followed by immunohistochemical staining.

Immunohistochemical staining. The tumors were removed, fixed with formalin and embedded in paraffin. Then the tissue was cut into 4- μ m sections to be placed on slides, dried overnight at 65°C and deparaffinized in xylene. The sections were rehydrated through graded alcohols into water. Endogenous peroxidase was blocked with 3% hydrogen peroxide in 50% methanol for 10 min at room temperature. After rehydrating, the sections were washed with PBS and pretreated with citrate buffer (0.01 M citric acid, pH 6.0) for 20 min at 100°C in a microwave oven. After rinsing with PBS, slides were incubated overnight at 4°C with primary polyclonal antibodies, including the antibodies against EphA2 (Upstate, NY, USA, dilution 1:100), MMP-2 (BA0596) and MMP-9 (BA0573) (both from Boster Biological Technology Ltd., Wuhan, P.R. China, dilution 1:100). The sections were then washed with PBS and incubated with the secondary antibody for 30 min at 37°C. The sections were incubated with HRP-conjugated antibody for 30 min at 37°C after the PBS washes. Visualization was performed using a DAB Kit (DC 10, Boster Biological Technology Ltd.) according to the manufacturer's instructions. The sections were then incubated in 95% alcohol with agitation for 30 min to remove formalin granules. After washing in running water, the sections were incubated in potassium permanganate for 3 h and bleached with 2% oxalic acid for 5 min. The nuclei were counterstained with hematoxylin, followed by dehydration and cover-slip mounting. Appropriate positive and negative controls were included.

Quantitation of VM channels, mosaic vessels (MV) and microvessel density and immunohistochemistry. VM channels, MV and endothelium-dependent vessels in the H&E-stained sections were counted using x400 magnification. Five fields were chosen randomly, and the average blood supply pattern was defined as the number of microvessels or channels in one section. The endothelium-dependent vessels, mosaic vessels and VM channels were counted in every visual field, and the channels lined with melanoma cells, containing red cells, without necrosis or inflammatory cells, were considered as VM. The mean value of the necrotic and each type of microvessel in five fields was the final outcome. Expression of MMP-2, MMP-9 and EphA2 in tumor cells was determined by counting the percentage of positive cells in 100 tumor cells with a cell counter. The counting was carried out as follows. Five microscopic fields in one section were observed under x400 magnification, and positive cells were counted in 100 tumor cells per field. The average percentage of five fields was the final score.

Real-time PCR. Total RNA was extracted with Trizol reagent according to the manufacturer's instructions. Complementary

DNA (cDNA) was synthesized and amplified from total RNA using the Access Real-time PCR system (Takara Biotechnology Co., Ltd., Japan). Real-time PCR primers for MMP-2 and -9 were designed by Primer 5.0 according to the sequences in Gene Bank. The primer sequences used for MMP-2 (gene ID 17390) detection were 5'-GATAACCTG GATGCCGTCGTG-3' (sense) and 5'-CTTCACGCTCTTG AGACTTTGGTTC-3' (antisense) (TM=53.1). The primer sequences used for MMP-9 (gene ID 17395) detection were 5'-GCCCTGGAACCTCACACGACA-3' (sense) and 5'-TTGG AAACCTCACACGCCAGAAG-3' (antisense) (TM=53.1). The forward primer for EphA2 (gene ID 067552) was 5'-CC GTGTGGAAGTACGAAGTCA-3' and the reverse primer was 5'-GGTACGTGGTATCCGGAGCAAG-3' (TM=64). The forward β -actin primer was 5'-CATCCGTAAAGACC TCTATGCCAAC-3', and the reverse primer was 5'-ATGGA GCCACCGATCCACA-3'. The resultant cDNA products of MMP-2, MMP-9, EphA2 and β -actin were 109, 86, 119 and 174 base pairs. Real-time PCR was performed in the Gene AMP PCR System 5700 Sequence Detector (ABI, USA). Every sample was analyzed in triplicate. The CT value (the cycle number at which the fluorescence crosses the threshold) was measured and $2^{-\Delta CT}$ ($\Delta CT = CT_{\text{target genes}} - CT_{\beta\text{-actin}}$) was defined as the quantity of the amplified fragment.

Statistical analyses. All analyses were performed using SPSS 11.5 (SPSS Inc., USA). A P-value <0.05 was defined as statistically significant. A t-test for the two groups was used to analyze the difference in protein expression and the number of VM channels, endothelium-dependent vessels and necrotic cells.

Results

Growth and metastasis in the different melanoma models.

Two weeks after injection, the subretinal masses had enlarged considerably, and a more or less pronounced proptosis was visible in all animals. All mice in the model were sacrificed, and soft black engrafted tumor masses were observed in the right eye (Fig. 1A). In comparison, 14 days after melanoma injection, the subcutaneous engrafted tumors were observed in the left groin area, and rich blood vessels were noted on the surface of the tumors (Fig. 1B). Five tumors in the subretinal group extended into the orbit, and six models of micro-metastases of lungs were found in the subretinal group by gross pathological examination of all internal organs (Fig. 1C) while no adjacent tissue invasion and lung metastasis were found in the groin group, which indicated that melanoma cells in the subretinal group were more invasive than in the groin group. Results from the H&E-stained sections indicated numerous black and brown melanin granules on the tumor micrometastases of the lungs in the subretinal group (Fig. 1D) while no metastasis was found in the groin group. Comparison of tumor metastasis by counting the lung metastases confirmed that mice in the subretinal group carried a significantly greater metastatic burden than mice in the groin group ($t=2.963$, $P=0.009$).

VM channels, mosaic vessels and endothelium-dependent vessels in different microenvironments. There were three

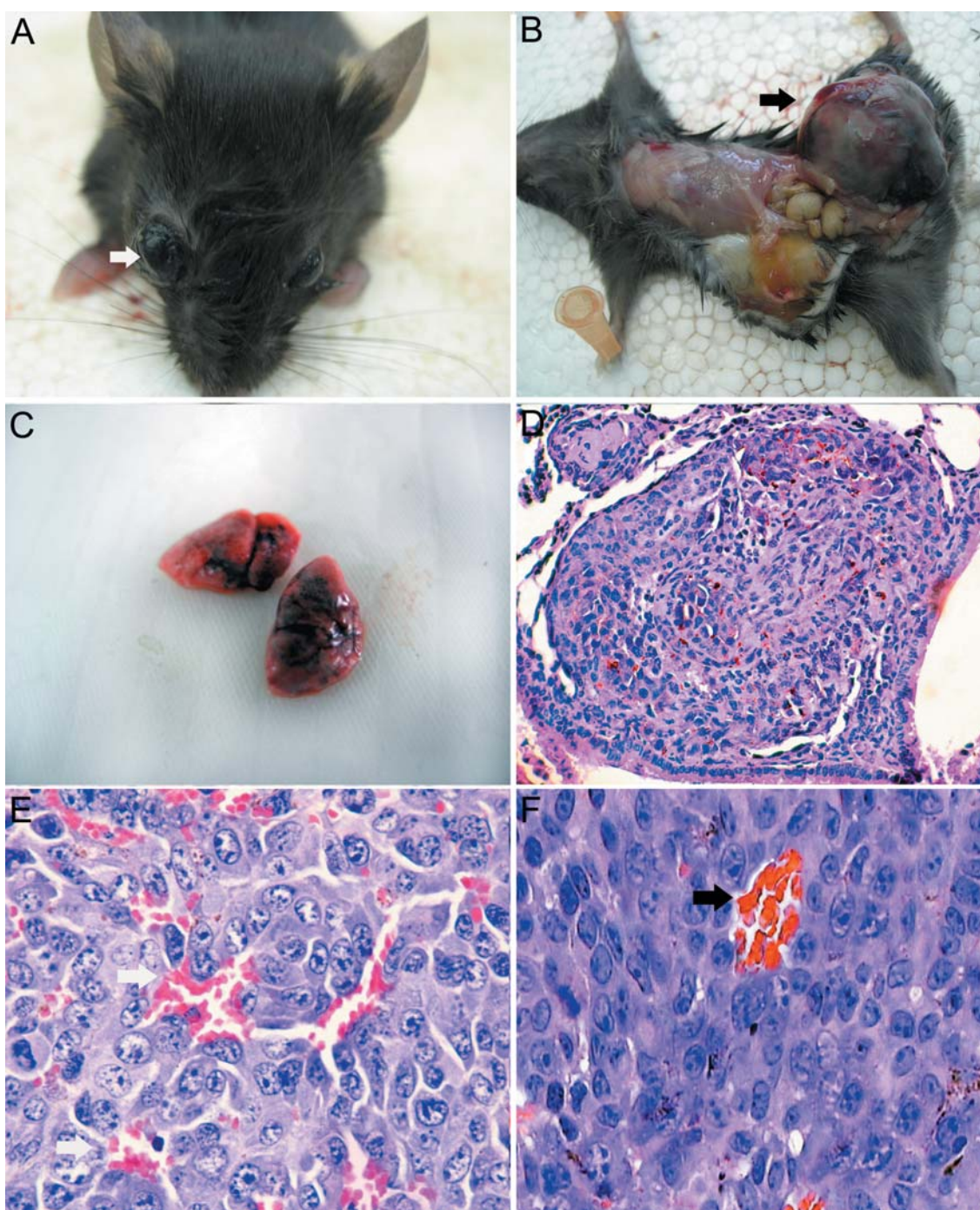


Figure 1. (A) A C57BL/6J mouse bearing B16 melanoma with pronounced proptosis of the right eye (white arrow, tumor). (B) A soft black tumor mass in the groin area and vessels were viewed on the surface of the tumor (black arrow, tumor). (C) Tumor micrometastases of the lung (black, spot-like) which invaded the lung tissues in the subretinal group. (D) Numerous black and brown melanin granules on the tumor micrometastases of the lung (H&E, x200). (E) A large number of VM channels (white arrow) with red blood cells in a tumor from the subretinal group (H&E, x400). (F) Compared with the subretinal group, there were less VM channels (black arrow) in tumors of the groin group.

microcirculation patterns in the engrafted melanoma in different microenvironments from the subretinal space and the groin area. VM channels and mosaic vessels contained red cells, and no necrosis or inflammatory cells were observed around them, indicating that they were functional vessels for melanoma blood supply. An unpaired t-test was performed to analyze the difference in the number of VM channels and endothelium-dependent vessels between subretinal space tumors and those in the groin area. The subretinal tumors had

more VM channels than the groin tumors (Fig. 1E and F). These findings demonstrated that different microenvironments influence VM formation. We observed an abundance of mosaic vessels and endothelium-dependent vessels in the tumors of both the subretinal and groin groups. No significant difference in tumor mosaic vessels and endothelium-dependent vessels was observed between the two groups. The results of H&E staining for VM channels and endothelium-dependent vessels are shown in Fig. 2.

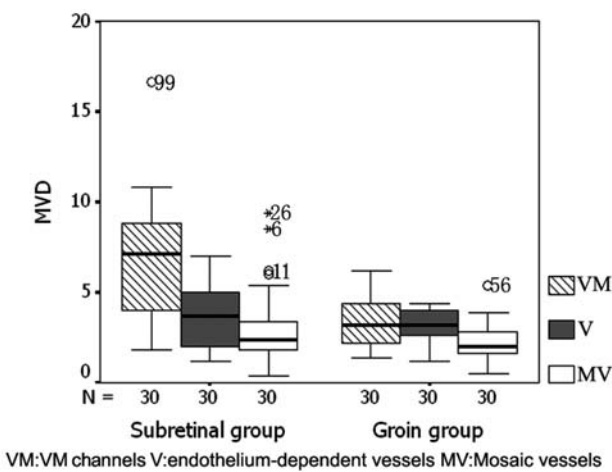


Figure 2. There were more VM channels in melanoma tumors in the subretinal group than in the groin group ($P=0.000$). However, the difference in the number of endothelium-dependent vessels and mosaic vessels between the two groups had no statistical significance ($P=0.146, 0.076$, respectively). The horizontal line inside the box represents the median. The outliers are cases with values between 1.5 and 3 box-lengths from the 75th percentile or the 25th percentile. The extreme values are cases with values >3 box-lengths from the 75th or 25th percentile.

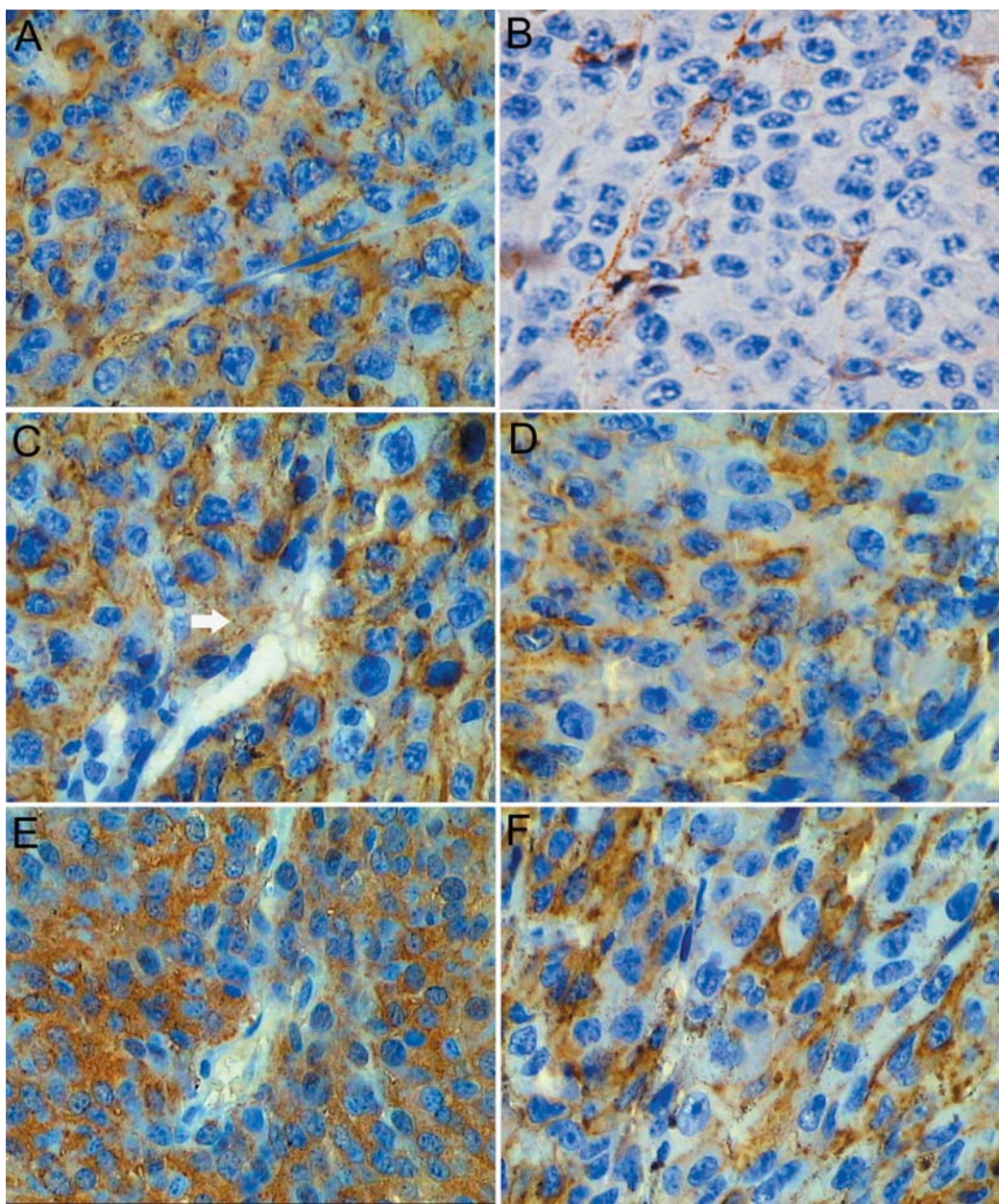


Figure 3. (A) Strong expression of EphA2 in the cytoplasm and membrane of melanoma cells from tumors in the subretinal group (IHC, x400). (B) Compared with the subretinal tumors, there was weak expression of EphA2 in the groin group (IHC, x400). (C) Compared with the groin tumors, MMP-2 was strongly positive in melanoma in the subretinal group, especially around the vessel (white arrow) (IHC, x400). (D) Melanoma cells in the groin tumors expressed MMP-2 weakly and infrequently (IHC, x400). (E and F) MMP-9-positive melanoma cells in the two groups. The percentage of positive cells and staining intensity were stronger in the subretinal group (E) than in the groin group (F) (IHC, x400).

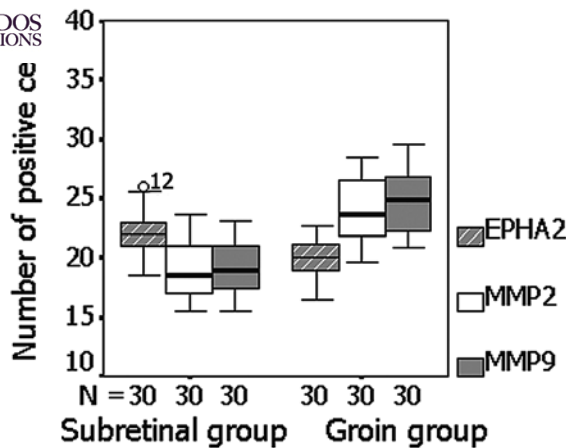


Figure 4. The mRNA levels of EphA2, MMP-2 and MMP-9 were higher in the subretinal group than those in the groin group. The CT value of EphA2, MMP-2 and MMP-9 in the subretinal group was lower than that in the groin group ($P=0.002$, 0.001 , and 0.001 , respectively). The horizontal line inside the box represents the median. The outliers are cases with values between 1.5 and 3 box-lengths from the 75th percentile or the 25th percentile. The extreme values are cases with values >3 box-lengths from the 75th or the 25th percentile.

Expression of EphA2, MMP-2 and MMP-9 in the different microenvironments. Immunohistochemical staining showed expression of EphA2 in the cytoplasm of melanoma cells. Melanoma growing in the subretinal group expressed more EphA2 than that in the groin group (Fig. 3A and B; and data not shown). There was strong staining for MMP-2 and -9 in the cytoplasm of tumor cells. Compared with melanoma in the groin area, both MMP-2 and -9 were overexpressed in melanoma in the subretinal group (Fig. 3C-F; and data not shown).

The mRNA level of EphA2, MMP-2 and MMP-9 in the different microenvironments. Real-time PCR results demonstrated that expression of MMP-2 and -9 mRNA in the groin group was decreased compared with the subretinal group. The CT value of EphA2, MMP-2 and MMP-9 in the subretinal group was lower than that in the groin group. There was statistical significance for EphA2, MMP-2 and MMP-9 between the two groups (Fig. 4, $P=0.002$, 0.001 and 0.001 , respectively).

Discussion

Melanoma is a type of malignant and poorly differentiated tumor which is prone to metastasize, and the prognosis of patients is very poor (11). Furthermore, these patients succumb to the disease quickly after diagnosis because of relapse and metastasis, and the exact mechanism is still unknown (12). Tumors require a blood supply for survival, growth and metastasis. There are three blood supply patterns for melanoma, namely VM, mosaic vessels and endothelium-dependent vessels (13-15). The existence of VM has been shown to be related to metastasis and poor prognosis. Therefore, two groups of mice received injections of melanoma cell suspension in the subretinal space and the groin area, respectively, to investigate the mechanism.

Tumor growth, invasiveness and metastasis are regulated by many associated factors, and tumor cells display distinguished growth patterns and invasive ability in different microenvironments (16,17). The environmental factors impacting tumor cell behavior include interstitial fluid pressure in the tumor tissue, pH, oxygen pressure, focal concentration of cytokines, ECM and microvessel density (18-20). We found 6 micrometastases of the lungs in the subretinal group, but no metastases were found in the groin group. The possible reasons can be outlined as follows. The groin area has more space, and its pressure in a mouse is usually low. There is a distensible interspace for tumor growth in the groin area, so the mechanical stress produced by the growth in tumor size increases slowly. In contrast, the structure of the subretinal space is a small cavity. As the tumor proliferates, the intraocular pressure (IOP) increases greatly. Secondary glaucoma occurs with elevated intraocular pressure. So, tumor cells in a high-pressure microenvironment must secrete proteinases to enable continual growth, invasiveness and metastasis. Hence, high interstitial fluid pressure has more influence on tumor invasiveness and microcirculation patterns than other factors (21). Another potential reason is that the choroid is a frequent metastatic site for blood-borne tumors because of its rich blood supply. Vice versa, intraocular tumors may extend into adjacent tissues or may metastasize to distant site organs.

There was a great difference in tumor microcirculation patterns between the two locations. We found that melanoma in the subretinal space extended into the orbit, while tumors in the groin area diffused with no distinct shape. The subretinal tumors had more VM channels than those in the groin group. These findings demonstrated that melanoma in the subretinal space was perfused mainly by VM. When the microcirculation networks formed by endothelium-dependent vessels were not adequate for supplying blood, tumor cells formed VM channels to enhance perfusion. A possible explanation is that high interstitial fluid pressure in the tumor tissue blocked the sprouting of endothelial cells into the tumor from host tissue. VM channels and endothelium-dependent vessels form networks to supply oxygen and nutrition to growing tumor cells, but they play different roles in tumor growth and development in different microenvironments. That is, as the number of VM channels increases, the melanoma invasiveness and metastasis increases (22). This feature suggests that VM channels can satisfy the needs of melanoma growth. In our study, melanoma tumor development in the subretinal group occurred more slowly than that in the control group. This finding may be associated with the effects of hypoxia and ischemia occurring in the melanoma microenvironment (23,24). In this type of microenvironment, tumor cells can secrete certain proteins or up-regulate expression of specific proteins in order to adapt to the poor conditions (25,26).

The potential mechanism involves increased interstitial fluid pressure which leads to ischemia and hypoxia in the tumor. Expression of invasion-associated proteins in tumor cells is then up-regulated to enhance blood perfusion (27,28). This mechanism is an adaptation of tumor cells to the poor intraocular microenvironment. Only tumors cells with the capacity to secrete MMPs are able to survive in an environment with high interstitial fluid pressure. Our results indicate

that, compared with melanoma growing in the groin area, melanoma in the subretinal space had less necrosis, and expression of MMP-2 and -9 was significantly increased. MMP-2 and -9 degrade ECM components and facilitate tumor angiogenesis, invasion and metastasis (29). We showed that tumor cells with positive MMP-2 and -9 expression were mainly distributed in the boundary between melanoma and normal tissue, especially in the tumor cells invading into the orbital cavity and metastasizing into the lung. Elevated IOP may lead to hypoxia and anoxia. Hypoxia may induce melanoma cells to express and also to activate MMP-2 and -9, proteinases associated with tumor invasiveness (9,30). To receive adequate oxygen, melanoma cells invade adjacent tissue and undergo distal metastasis. This hypothesis was supported by the growth pattern of melanoma in our study.

EphA2 is a member of the Eph family of protein receptor tyrosine kinases (31). EphA2 plays important and diverse roles in controlling cell adhesion, migration and invasion. It was previously reported that EphA2 also plays a role in tubular formation by aggressive melanoma cells. Transient knockout of EphA2 expression in aggressive uveal melanoma tumor cells resulted in the inhibition of tubular network formation by these cells (32). Our findings indicated that areas containing patterned tubular networks also showed high levels of EphA2, MMP-2 and MMP-9. It was inferred that they may play an important role in the formation of VM channels. The role of EphA2 in the process of tubular network formation was confirmed, and EphA2 was expressed in aggressive melanoma cells. The treated cells were then assessed for their ability to develop tubular structures when cultured on a three-dimensional matrix (33). These results, coupled with those of Hess and his co-researchers (34), suggest that EphA2 could be the candidate kinase involved in this process. The MMP-2 protein is considered to play a significant role in VM formation in melanoma *in vitro*. EphA2 and VE-cadherin could activate MMP-2 through the PI3K pathway, and the activated MMP-2 could cleave the laminin5 γ 2 chain into 2 segments (laminin5 γ 2x and laminin5 γ 2'), which affects VM formation (34-37). We found expression and activity of MMP-2 and -9 to be correlated with VM formation, and they were more abundant in the subretinal group. We believe that the melanoma cells in the subretinal group secreted and activated MMP-2 and -9, which degraded and remodeled the ECM and then assisted the formation of VM. In this type of microenvironment, tumor cells can secrete certain proteins or up-regulate expression of specific proteins such as EphA2, laminin5 γ 2 and MMPs in order to adapt to hypoxic conditions. Our results demonstrated that different microenvironments affect melanoma cell microcirculation patterns, invasiveness and metastatic behavior. Melanoma cells in an intraocular microenvironment become more aggressive. EphA2, MMP-9 and MMP-2 may play an important role in the formation of VM channels.

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