Blood Vessel Maturation in Retinoblastoma Tumors: Spatial Distribution of Neovessels and Mature Vessels and Its Impact on Ocular Treatment

Yolanda Piña, Hinda Boutrid, Amy Scheffler, Sander Dubovy, William Feuer, Maria-Elena Jockovich, and Timothy G. Murray

PURPOSE. The purposes of this study were to evaluate the spatial distribution of neovessels versus mature vessels in both human retinoblastoma (RB) and LHBETATAG tumors, assess similarities and differences between the animal model and the human RB specimens, and determine whether vessel maturation is associated with risk factors for metastasis.

METHODS. Immunohistochemical analyses were performed on human (n = 10) and LHBETATAG (n = 11) enucleation specimens to evaluate the spatial distribution of neovessels and mature vessels. In human RB, vessel maturation was correlated with treatment history and metastatic risk factors.

RESULTS. In human RB, the percentage of neovessels was higher in the periphery of the tumor than in the center (P = 0.021). This finding was mostly attributed to the distribution of large-caliber vessels (i.e., neovessels were higher in the periphery for large [P = 0.050] and medium [P = 0.032] caliber vessels; and mature vessels were higher in the center for large-caliber vessels [P = 0.032]). In this small series, vessel maturation did not correlate with risk for metastasis. Similar results were observed in LHBETATAG tumors. The percentage of large-caliber neovessels was higher in the periphery than in the center (P = 0.038).

CONCLUSIONS. There is a spatially distributed, heterogeneous vessel population containing neovessels and mature vessels in advanced RB disease. There is a significantly higher concentration of mature, large-caliber vessels in the center of tumors that is similar in human RB and LHBETATAG retinal tumors. From these data the authors hypothesize that tumor vessel maturation in RB initiates in central regions of the tumor and radiates toward the periphery. (Invest Ophthalmol Vis Sci. 2009;50: 1020–1024) DOI:10.1167/iovs.08-2654

Retinoblastoma (RB) is the most common primary intraocular malignancy in children.1,2 Significant advances in screening and treatment during the past century have led to greater than 95% long-term survival rates in the United States. The high survival rates have led to a laboratory focus on local tumor control and globe conservation with preservation of sight. However, current treatments can result in significant local and systemic complications.3–5 Enucleation, due to advanced disease with persistent vitreous seeding, is performed in approximately 20% of the patients with intraocular RB.6,7

Although most patients in the United States present with intraocular disease, recurrent orbital RB has been reported in both low-risk (9.5%) and high-risk (13.6%) cases.7 Several factors have been associated with metastatic RB including optic nerve (ON) involvement,3,5,8 extrascleral extension,7 anterior chamber seeding, choroidal involvement,4 and ciliary body infiltration.9 Of these factors, extrascleral extension and ON involvement to the surgical margin present a higher risk for metastatic disease.10,11

Since angiogenesis has been significantly associated with tumor proliferation and metastasis, several antiangiogenic treatments have been developed to target intraocular tumors.12–16 However, results in previous studies suggest that antiangiogenic therapy inhibits tumor growth, with almost no impact on tumor regression.17 Using the LHBETATAG mouse model of RB, we have shown that antiangiogenic therapy significantly reduces retinal tumor burden with minimal associated toxicities.18 We have further shown that antiangiogenic therapy targets primarily areas with high angiogenic activity, while having little to no effect in established mature blood vessels.19 Thus, the presence and spatial distribution of mature blood vessels in ocular tumors may affect the efficacy of antiangiogenic therapies and may dictate the therapeutic strategies used.

The purposes of this study were to evaluate the spatial distribution of neovessels versus mature vessels in both human RB and LHBETATAG retinal tumors; assess similarities and differences between the LHBETATAG mouse model and the human RB specimens and determine the applicability of this model for human preclinical studies; and determine whether vessel maturation is associated with risk factors for metastasis in human RB.

MATERIALS AND METHODS

LHBETATAG Mouse Model Tissue Specimens

The study protocol was approved by the University of Miami Institutional Animal Care and Use Review Board Committee and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The LHBETATAG transgenic mouse model used in this study has been characterized previously.20 LHBETATAG retinal tumor samples were obtained from enucleations performed on 11 transgenic mouse model animals at 16 weeks of age.

Human Tissue Specimens

This study was conducted in compliance with the tenets of the Declaration of Helsinki and the University of Miami Institutional Research Board. Informed consent was obtained from the parents of the subjects after they were given a complete explanation of the nature and possible consequences of the study. Human RB tissue samples were obtained from enucleations performed at the Bascom Palmer Eye Institute between May 13, 2005, and November 6, 2007, on 10 patients (eight girls and two boys; median age, 18 months; age range, 7–36 months).

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Risk Factors for Metastatic Retinoblastoma

The following clinical factors were considered to be indicators of poor prognosis and were correlated with the presence and distribution of mature tumor vessels in human specimens: ON involvement,4,5,8 extrascleral extension,7 anterior chamber seeding; 4 presented with choroidal involvement; and 4 received treatment before enucleation.

Procedures

Hematoxylin and Eosin. Enucleation specimens were fixed with 10% formalin, embedded in paraffin, and sectioned (8 μm). Slides were stained with hematoxylin and eosin (H&E) in tissues previously bleached. Tumor morphology was analyzed by H&E staining. In mouse tumors only-caliber vessels using standard immunohistochemical analyses were performed. Total vessels were detected with Alexa Fluor 568 conjugated lectin (*Bandetra simplicifolia*, a panendothelial binding agent; 1:1000; Invitrogen, Carlsbad, CA)21 and anti-collagen type IV (1:3000; Sigma-Aldrich, St. Louis, MO). Mature vessels were detected with α-smooth muscle actin (α-smma) Cy3 conjugate (1:3000; Sigma-Aldrich), which specifically binds to pericytes.22 Neovessels were detected with anti-endoglin (CD105 Wi, 1:500; Abcam, Cambridge, MA), which has been shown to have specificity for endothelial cells (ECs) undergoing angiogenesis.7,23 Alexa Fluor 568 goat anti-mouse and 488 donkey anti-mouse were used as secondary antibodies for anti-collagen type IV and endoglin, respectively (1:500; Invitrogen, Carlsbad, CA). Omission of the primary antibody (secondary only) was used as a negative control for nonspecific binding. Cell nuclei were stained for 5 minutes with 4’6’ diaminido-2-phenylindole (DAPI, 1:5000; Invitrogen, Carlsbad, CA).

Immunohistochemistry. Tumor samples were frozen in OCT immediately after enucleation and serially sectioned (8 μm). Slides were fixed with methanol for 10 minutes (~20°C) and immunohistochemical analyses were performed. Total vessels were detected with Alexa Fluor 568 conjugated lectin (*Bandetra simplicifolia*, a panendothelial binding agent; 1:1000; Invitrogen, Carlsbad, CA)21 and anti-collagen type IV (1:3000; Sigma-Aldrich, St. Louis, MO). Mature vessels were detected with α-smooth muscle actin (α-smma) Cy3 conjugate (1:3000; Sigma-Aldrich), which specifically binds to pericytes.22 Neovessels were detected with anti-endoglin (CD105 Wi, 1:500; Abcam, Cambridge, MA), which has been shown to have specificity for endothelial cells (ECs) undergoing angiogenesis.7,23 Alexa Fluor 568 goat anti-mouse and 488 donkey anti-mouse were used as secondary antibodies for anti-collagen type IV and endoglin, respectively (1:500; Invitrogen, Carlsbad, CA). Omission of the primary antibody (secondary only) was used as a negative control for nonspecific binding. Cell nuclei were stained for 5 minutes with 4’6’ diaminido-2-phenylindole (DAPI, 1:5000; Invitrogen, Carlsbad, CA).

Vessel Count. A vessel count for neovessels and mature vessels was performed in a masked fashion in the designated regions of the tumors by two investigators. Vessel count was performed for small (i.e., <11 μm in diameter for both human tumors and LHβETA TAG retinal tumors), medium (i.e., 11 to 16 μm in diameter for human tumors only), and large (i.e., >16 μm in diameter for human tumors, and ≥11 for LHβETA TAG retinal tumors)-caliber vessels using standard photographs of the vessels (see Fig. 1). The vessel counts of the two examiners showed excellent interobserver reliability and were averaged for subsequent analyses. Vessel density was defined as the number of vessels present per high-power field.

Three variables were measured for both human RB and LHβETA TAG mouse RB: (1) degree of vessel maturation (neovessels versus mature blood vessels); (2) vessel location (center versus periphery); (3) vessel caliber (small versus large). The three variables above were statistically correlated with treatment history and risk factors for poor prognosis in human RB tumors only. For the LHβETA TAG mouse RB tumors, four tumor areas were able to be analyzed: center, margin, base, and apex. For the human tumors, only center and periphery were assessable,

### Table 1. Vessel Counts in Human RB Tumors

<table>
<thead>
<tr>
<th>Vessel Size</th>
<th>Location</th>
<th>Neo</th>
<th>Mature</th>
<th>P (Neo vs. Mature)</th>
<th>P (Periphery vs. Center)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large</td>
<td>Periphery</td>
<td>0.550 (±0.433)</td>
<td>0.193 (±0.164)</td>
<td>0.050</td>
<td>0.013 (0.037)</td>
</tr>
<tr>
<td></td>
<td>Center</td>
<td>0.158 (±0.231)</td>
<td>0.600 (±0.486)</td>
<td>0.037</td>
<td>0.025 (0.074)</td>
</tr>
<tr>
<td>Medium</td>
<td>Periphery</td>
<td>0.866 (±0.340)</td>
<td>0.543 (±0.341)</td>
<td>0.032</td>
<td>0.094 (0.305)</td>
</tr>
<tr>
<td></td>
<td>Center</td>
<td>0.492 (±0.320)</td>
<td>0.975 (±0.691)</td>
<td>0.094</td>
<td>0.025 (0.074)</td>
</tr>
<tr>
<td>Small</td>
<td>Periphery</td>
<td>3.036 (±1.194)</td>
<td>3.107 (±1.805)</td>
<td>0.925</td>
<td>0.094 (0.305)</td>
</tr>
<tr>
<td></td>
<td>Center</td>
<td>3.075 (±2.598)</td>
<td>3.600 (±2.070)</td>
<td>0.482</td>
<td>0.025 (0.074)</td>
</tr>
</tbody>
</table>

Data are the mean (±SD). Repeated-measures ANOVA found a significant interaction between vessel maturity and location (P = 0.021). Paired t-tests indicate differences in counts between neo- and mature vessels and between periphery and center, especially for large vessels.

* Two-sided paired t-test. Bold probabilities are statistically significant.

FIGURE 1. Standard photograph used to measure the different caliber vessels. Small-, medium-, and large-caliber vessels were differentially analyzed for both mature vessels (red) and neovessels (green).
neovessels by vessel size and location. The most substantial difference was seen in the large vessels.

**Correlation with Risk Factors**

No significant associations were found between tumor vessel maturation and either treatment history ($P = 0.33$) or metastatic risk factors (i.e., there were no cases with ON involvement, extrascleral extension, or ciliary body infiltration; anterior chamber seeding, $P = 0.20$; and choroidal involvement, $P = 0.94$).

**LH>BETA>TAG Retinal Tumors**

LH>BETA>TAG retinal tumors provide an intact morphology and tumor orientation that can be used to analyze differences between the three peripheral areas of the tumor (i.e., base, margin, and apex) and compare them to the center. Table 2 presents the peripheral data. There were no significant differences between measurements made among different areas in the periphery, either in counts (all $P > 0.8$) or when the number of neovessels was expressed as a percentage of total vessels in each location (all $P \geq 0.58$; Table 2).

Since the three peripheral areas did not differ, we combined them for comparison to the central area of the tumor (Table 3). There was a significant difference between neovessels and mature vessels ($P = 0.047$) and a borderline significant interaction between vessel maturity and vessel size ($P = 0.054$) for vessel count. There was a borderline significant interaction effect between vessel size and location for the average percent of neovessels ($P = 0.063$, see details in Fig. 3) confirming results found for the human data. In LH>BETA>TAG retinal tumors there is a greater difference in the percentage distribution of large-caliber neovessels between peripheral and central locations ($P = 0.038$, paired $t$-test) than small neovessels ($P = 0.49$, paired $t$-test; Fig. 3).

**DISCUSSION**

It has been widely recognized that tumor growth is contingent on the process of angiogenesis, the formation of neovessels from preexisting blood vessels. The process of angiogenesis involves a chain of events and regulatory factors that may represent targets for therapy. Although angiogenic activity is associated with tumor proliferation and metastasis, vessel maturation also plays an essential role in the later stages of tumor development, having an impact on the efficacy of angiogenic treatment modalities. The use of antiangiogenic agents may inhibit the formation of neovessels, but has no effect on mature vessels. During earlier stages of tumor development, angiogenic stimuli are essential for the formation of ECs; without these stimuli, ECs undergo apoptosis and regress. On the other hand, during later stages of tumor development, mature vessels become stabilized by pericytes and are no longer dependent on angiogenic stimuli. The endogenous withdrawal of angiogenic stimuli in the tumor microenvironment chronologically coincides with the stabilization of blood vessels.

**Table 2. Vessel Counts in LH>BETA>TAG, Retinal Tumors**

<table>
<thead>
<tr>
<th>Vessel Size</th>
<th>Location</th>
<th>Neovessels</th>
<th>Mature Vessels</th>
<th>Percent Neovessels*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Periph:margin</td>
<td>3.227 (±1.694)</td>
<td>1.955 (±1.722)</td>
<td>65 (±16)</td>
</tr>
<tr>
<td></td>
<td>Periph:base</td>
<td>3.091 (±1.700)</td>
<td>2.273 (±1.603)</td>
<td>61 (±20)</td>
</tr>
<tr>
<td></td>
<td>Periph:apex</td>
<td>3.182 (±1.401)</td>
<td>2.455 (±2.067)</td>
<td>61 (±18)</td>
</tr>
<tr>
<td>Small</td>
<td>Periph:margin</td>
<td>17.82 (±11.63)</td>
<td>9.977 (±9.910)</td>
<td>65 (±12)</td>
</tr>
<tr>
<td></td>
<td>Periph:base</td>
<td>16.82 (±14.84)</td>
<td>11.09 (±14.04)</td>
<td>60 (±19)</td>
</tr>
<tr>
<td></td>
<td>Periph:apex</td>
<td>17.14 (±15.01)</td>
<td>8.545 (±15.16)</td>
<td>68 (±25)</td>
</tr>
</tbody>
</table>

Data are the mean (±SD). Repeated measures analysis of variance found no significant differences ($P = 0.67$) between the three peripheral areas with respect to counts ($P_{location} = 0.92$; $P_{location+size} = 0.82$; $P_{location+size+interaction} = 0.88$).

* Mean percentage of neovessels is not necessarily equal to the percentage of mean neovessels.
by pericytes. Mature blood vessels remain intact for months, regardless of angiogenic factor depletion. Recruited pericytes and smooth muscle cells form a protective barrier that stabilizes blood vessels and reduces apoptotic events, causing blood vessels to be more resistant to antiangiogenic treatments. Thus, the heterogeneity and quantity of mature blood vessels present in tumors may limit the efficacy of vessel targeting therapy.

In this study, we observed in both human RB and LHβETATAG retinal tumors considerable differences in the spatial distribution of angiogenic versus mature vasculature. In human RB, the degree of vessel maturation is very heterogeneous; however, a trend of angiogenic activity (density of neovessels) predominates in peripheral areas of the tumor, whereas mature, pericyte-associated vessels are most prominent in the center. This trend was observed as a factor of degree of maturation (neovessel density versus mature vessel density) and vessel location (periphery versus center) (Table 1). These findings were mainly obtained for large-caliber vessels, which are more established than small-caliber vessels and thus are most representative of the degree of vessel maturation within the tumor.

Moreover, in human RB specimens, the association of treatment history and risk factors (i.e., ON involvement, extracranial extension, anterior chamber seeding, and ciliary body infiltration) with vessel maturation was assessed. No association was found for any of the variables analyzed. ON involvement to the surgical margin and extracranial extension present a higher risk for metastatic disease. In our series, there were no patients with these higher risk features on histopathology, and this most likely accounted for the lack of statistical association with vessel maturation.

Using the LHβETATAG RB mouse model, we previously showed a trend of pericyte recruitment during tumor progression. A linearly significant increase in pericyte recruitment is observed during later stages of tumor development, making LHβETATAG retinal tumors ideal to study vessel maturation in advanced disease. Although human RB tumor samples for research do not afford us the opportunity to assess spatial orientation within the globe, LHβETATAG retinal tumors provide an intact morphology that allows for the analysis of four different tumor areas (i.e., center, margin, base, and apex).

We previously reported data that support a spatial maturational pattern of tumor development in uveal melanoma. The data allowed us to propose a model for the spatial development of vessel maturation, which shows a significant, linear increase of angiogenic vasculature from basal regions to leading edges of the tumor (Fig. 4B). Conversely, in the present study we hypothesized that angiogenic vasculature in RB tumors would be most prevalent in the peripheral edges of the tumor (i.e., margin, base, apex), whereas mature vessels would be most predominant in the center (Fig. 4A). Results obtained in the present study support our hypothesis. The spatial distribution of neovessels and mature vessels in retinal tumors of LHβETATAG mice present a different developmental pattern than that found in melanoma samples. Since the ratios of neovessels to total vessel density by tumor location in the peripheral areas (i.e., margin, base, apex) were similar (Table 2), the three areas were averaged. Similar to the developmental maturational pattern found in human RB tumors, LHβETATAG retinal tumors have a significant increase in the ratio of neovessels to total vasculature, from central areas of the tumor to peripheral, leading edges of the tumor for large-caliber, pericyte-stabilized vessels (Fig. 5). The model presented in Figure 4 provides a description of spatial tumor vessel maturation associated with progressive tumor development.

**TABLE 3.** Vessel Counts in LHβETATAG Retinal Tumors Compared between Central and Peripheral Locations

<table>
<thead>
<tr>
<th>Vessel Size Location</th>
<th>Neovessels</th>
<th>Mature Vessels</th>
<th>Total Vasculature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large</td>
<td>Periphery</td>
<td>3.164 (± 1.233)</td>
<td>2.230 (± 1.457)</td>
</tr>
<tr>
<td></td>
<td>Center</td>
<td>2.955 (± 1.293)</td>
<td>3.257 (± 2.279)</td>
</tr>
<tr>
<td>Small</td>
<td>Periphery</td>
<td>17.261 (± 10.734)</td>
<td>9.868 (± 12.841)</td>
</tr>
<tr>
<td></td>
<td>Center</td>
<td>16.23 (± 12.66)</td>
<td>6.182 (± 4.173)</td>
</tr>
</tbody>
</table>

Data are expressed as the mean (± SD). Repeated-measures analysis of variance demonstrates a significant difference between neovessels and mature vessels (P = 0.047), and a borderline significant interaction between vessel maturity and vessel size (P = 0.054).

**FIGURE 3.** Spatial distribution of the average percentage of neovessels in LHβETATAG retinal tumors. The average percentage of neovessels over total vasculature by vessel caliber (large and small) and location (periphery and center). Repeated measures analysis of variance of percent neovessels found a borderline significant interaction between vessel size and location (P = 0.063). Two-tailed t-tests between central and peripheral areas yielded the probabilities for each caliber vessel represented in figure. The average percentage of neovessels is not necessarily equal to the percentage of mean neovessels.

**FIGURE 4.** Model of vessel maturation in retinoblastoma and uveal melanoma. (A) The developmental pattern of vessel maturation in LHβETATAG retinal tumors shows a radial increase of the percentage of neovessels to total vasculature, from the center of the tumor to peripheral edges of the tumor (i.e., margin, base, and apex). (B) As previously shown, the developmental pattern of vessel maturation in uveal melanoma tumors shows a linear increase of the percentage of neovessels to total vasculature, from the basal areas of the tumor (adjacent to the retina) to the apical, leading edges of the tumor. Part B reproduced with permission from Piña Y, Cebulla CM, Murray TG, et al. Blood vessel maturation in human uveal melanoma: spatial distribution of neovessels and mature vasculature. *Ophtalmic Res.* In press. © 2008 S. Karger AG, Basel, Switzerland.
Data obtained in the present study suggest that LH(BETA)TAG retinal tumors are similar in vasculature to human RBs. Thus, the study further substantiates similarities between the two diseases and validates the use of the LH(BETA)TAG mouse model for preclinical studies to measure the efficacy of vessel-targeting therapy.

RB tumors present with significant angiogenic vasculature making the tumor an ideal target for vascular targeting therapy. Mature vessels and their close association with pericytes have recently gained attention as significant contributors to tumor angiogenesis; thus, they are also useful as potential new targets for vessel targeting treatment. The data presented herein indicate that in fact there is a heterogeneous vessel population in RB containing both neovessels and mature vessels in advanced disease. In particular, there is a significantly higher concentration of mature, large-caliber vessels in the center of the tumors. From these data we hypothesize that tumor vessel maturation in RB initiates in central regions of the tumor and radiates toward the periphery (Fig. 4A).

The heterogeneity and spatial distribution of the vasculature is clinically significant since blood vessel maturation may limit antiangiogenic treatments that mainly target immature vasculature. An ideal vessel targeting treatment would thus include agents that target neovessels as well as mature vessels (e.g., pericyte targeting agents17). This integrated strategy could provide a comprehensive therapeutic approach for the treatment of RB. Furthermore, given our finding of the presence of mature vessels in the center of the tumor, an integrated strategy is more likely to successfully treat advanced intraocular disease than current unimodal approaches.

References