Expression of Vascular Endothelial Growth Factor in Retinoblastoma

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Objectives: To investigate the immunohistochemical expression of vascular endothelial growth factor (VEGF) and to determine its possible association with tumor differentiation status, optic nerve and/or choroidal invasion, anterior chamber invasion, vitreous seeding, and basophilic staining of the vascular walls.

Methods: A retrospective study was performed to identify the expression of VEGF in 47 of 129 consecutive patients with retinoblastoma treated at the Ocular Pathology Laboratory of the Anatomy and Pathology Institute of the Central University of Venezuela in Caracas from January 1, 2000, through December 31, 2007.

Results: A positive correlation between VEGF staining intensity and time of progression and mitotic and apoptotic indexes was observed. However, no correlation was found between VEGF expression and other prognostic factors in this malignant neoplasm, including tumor stage as assessed by the Grabowski and Abramson classification.

Conclusions: Although the isolated characterization of VEGF in retinoblastoma is not grounds for this protein to be considered a prognostic factor, its association with mitotic and apoptotic indexes suggests it may play a role in the progression of this disease. Thus, therapeutic targeting of VEGF in retinoblastoma may be an effective strategy to reduce tumor progression.


Retinoblastoma is the most common intraocular tumor in children: it represents 3% of the malignant neoplasms that occur in patients younger than 15 years1,2 and less than 1% in all pediatric groups.3 Retinoblastoma is a rare tumor with an incidence that varies from 1 per 15,000 to 1 per 34,000 live births.1 In the United States, retinoblastoma has an incidence of 11 new cases per million children younger than 5 years.4 Retinoblastoma is a congenital neoplasm derived from primitive neuroectodermal cells with retinal differentiation.5 In poorly developed countries, this malignant neoplasm is typically diagnosed at advanced stages, between 16 months and 2 years of age.4

The retinoblastoma gene is a tumor suppressor located on the long arm of chromosome 13. Retinoblastoma development requires loss or inactivation of both alleles.6,7 Retinoblastoma has distinctive histopathologic characteristics: presence of sleeves surrounded by viable cells and necrotic tissue peripherally; small, round malignant cells with retinal differentiation, in some cases fleurettes; and Flexner-Wintersteiner and Homer-Wright rosettes. The term Homer-Wright rosettes denotes less-differentiated tumors.5 Poor prognosis of this malignant neoplasm is associated with invasion of the optic nerve and meninges and choroidal involvement.5

The importance of angiogenesis in tumor growth has been known for decades, and recently several key advances have been made in the development of antiangiogenic therapies.9 Nevertheless, few studies have investigated the relationship between tumor angiogenesis and the prognosis of retinoblastoma. The purposes of this retrospective study were to investigate the immunohistochemical expression of vascular endothelial growth factor (VEGF) and to determine its possible association with known clinical-histopathologic characteristics.
METHODS

PATIENTS

A large series of patients treated at the Ocular Pathology Laboratory of the Anatomy and Pathology Institute of the Central University of Venezuela in Caracas was reviewed, from which 47 patients were selected for further immunohistochemical analysis. This series represented patients with disease from each of the 6 stages (Table 1) based on the Grabowski and Abramson classification. Histopathologic reports were reviewed to obtain the following data: age, sex, time between initial symptoms and enucleation, and pathologic staging.

TISSUE SAMPLES

All samples were previously fixed in 10% buffered formalin, dehydrated with alcohol solutions and xylene, and paraffin embedded. The cut sections were 4 µm thick and were stained with hematoxylin-eosin, periodic acid–Schiff, and Gomori's trichrome stain (original magnifications, ×100, ×100, and ×120, respectively).

MICROSCOPIC CLASSIFICATION

Two independent pathologists (M.E.O. and C. Areán) reviewed the slides by light microscopy and reported the pattern of tumor growth (endophytic, exophytic, mixed, necrotic, or spontaneous regression), cell differentiation status (well differentiated: foci of Flexner-Wintersteiner rosettes or fleurettes; poorly differentiated: absence of these structures; moderately differentiated: isolated Homer-Wright rosettes), optic nerve and choroidal invasion (according to the Khelfaoui protocol), anterior chamber invasion, presence of vitreous seeding, and vascular basophilic staining.

IMMUNOHISTOCHEMICAL STUDY

Immunostaining was performed on specimens from the 47 selected patients according to conventional protocols, using the streptavidin-biotin method. Sections (4 µm) of formalin-fixed, paraffin-embedded tissues mounted on poly-L-lysine-coated slides were deparaffinized in xylene and rehydrated through serial baths of alcohol and water. The slides were then placed in a target retrieval solution with high pH (pH 9.9), and antigen retrieval was performed overnight at 40°C. The hydrated sections were then treated with a 3% hydrogen perox-
ide solution for 15 minutes to eliminate endogenous peroxide activity and washed in Tris-buffered saline (pH 7.6) for 5 minutes. The primary antibody used in this study, for 1 hour, was a polyclonal antibody to VEGF (A-20; Santa Cruz Biotechnology Inc, Santa Cruz, California).

The monoclonal antibody–treated slides were then rinsed in a phosphate-buffered saline solution for 5 minutes and incubated with secondary antibody (Biotinylated Link Universal, DakoCytomation LSAB + System-HRP; Dako North America Inc, Carpin teria, California) for 20 minutes and streptavidin–horseradish peroxidase for 20 minutes. Intermittent washing between steps was performed with Tris-buffered saline (pH 7.6), and the color was developed with freshly prepared solution of 3,3′-diaminobenzidine tetrahydrochloride for 10 minutes, posteriorly contrasted with Meyer hematoxylin-eosin (1 dip). Healthy renal tissue was used as a control, with vessels that showed strong VEGF immunoreactivity. Negative controls included the omission of the primary antibody and substitution with nonimmune serum.

IMMUNOSTAINING ANALYSIS AND STATISTICAL ANALYSIS

For immunostaining analysis, tumors were scored by assessment of the proportion and intensity of stained tumor cells and then scored by a semiquantitative method. The presence of cytoplasmic staining was considered a positive finding. Positively stained cells were counted in 10 randomly selected fields under a magnification of ×400. Intensity was graded as negative, weak, moderate, or intense, and the percentage of stained cells was classified as less than or equal to 50.0%, 51.0% through 75.0%, or more than 75.0%.

For statistical analysis of the results, we calculated frequency and percentage for nominal and ordinal variables. Data were analyzed for statistical significance using the Pearson $\chi^2$ test and the Pearson product-moment correlation coefficient ($P<.05$ was considered significant).

RESULTS

Clinicohistopathologic data for the 129 patients in the study are given in Table 1. There were more male than female patients (1:0.82). The most common symptoms recorded were leukocoria, strabismus, diminished visual acuity, and orbital mass.

A mixed-type pattern of growth was seen in 110 patients, and the endophytic pattern was seen in 13 patients. Other patterns (regression, exophytic, and diffuse) were seen infrequently (Figure 1A and B).
According to the histopathologic results, poorly differentiated tumors were the most frequent type (81 patients), followed by moderately differentiated (24 patients) and well differentiated (16 patients) (Figure 1C and D). Anterior chamber invasion was observed in 24.8% of the patients, whereas 48.8% of the patients had massive choroidal invasion. Compromise of the sclera was seen in 11.5% of intrascleral cells (stage IV) and 8.6% of extrascleral cells (stage V), respectively. Assessment of optic nerve invasion yielded no remarkable differences among the 4 groups. Most patients (84.5%) showed vitreous seeding (Figure 2).

The mitotic index was variable among the patients; 38.0% of patients had 11 to 20 mitoses per 10 high-power fields (HPF), 22.0% had more than 20 mitotic figures per 10 HPF, and 20.0% had 6 to 10 mitoses in 10 HPF. Evaluating the apoptotic index, 30.0% of patients had 41 to 60 apoptotic bodies per 10 HPF, and 19.0% had an apoptotic index of 21 to 40 per 10 HPF.

The immunoreactivity of VEGF was positive in 43 of 44 patients (Table 2 and Figure 3). Three of the 47 patients studied could not be evaluated because of extensive necrosis. No statistically significant correlation was found between VEGF immunostaining and many established retinoblastoma prognostic factors, such as optic nerve or choroidal invasion, anterior chamber invasion, vitreous seeding, or basophilic staining on the vascular walls. In our series, the lack of follow-up prevents the possibility of establish-

<table>
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<th>Staining</th>
<th>No. (%) of Patients</th>
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<tr>
<td>Intensity</td>
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<tr>
<td>Negative</td>
<td>1 (2)</td>
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<tr>
<td>Weak</td>
<td>7 (16)</td>
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<tr>
<td>Moderate</td>
<td>13 (30)</td>
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<tr>
<td>Intense</td>
<td>23 (52)</td>
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<tr>
<td>Extent, %</td>
<td></td>
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<tr>
<td>≤50</td>
<td>5 (12)</td>
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<tr>
<td>51-75</td>
<td>14 (33)</td>
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<tr>
<td>76-100</td>
<td>24 (56)</td>
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Figure 2. Retinoblastoma. A, Vessel surrounded by viable neoplastic cells that form a perivascular sleeve (hematoxylin-eosin, original magnification ×200). B, Tumor extrascleral invasion (Gomori trichrome, original magnification ×120). C, Postlaminar invasion (Gomori trichrome, original magnification ×100). D, Massive invasion of the choroid (hematoxylin-eosin, original magnification ×320).
ing a correlation between VEGF immunostaining and prognosis, even though this variable was not related to the stage of the disease, according to the Grabowski and Abramson classification. Using the coefficient correlation test, we found a positive correlation between VEGF immunostaining intensity and the number of apoptotic bodies \((P = .03)\); the higher the apoptotic index, the stronger the intensity of VEGF staining (Figure 4). A relation was found between mitotic index and immunostaining intensity according to the \(\chi^2\) test \((P = .003)\) (Figure 4). In addition, a relation was found between the intensity of VEGF staining and an independent variable: time with symptoms before the enucleation, also known as the interval onset of symptoms and enucleation \((P = .03)\) (Figure 5).

**COMMENT**

Retinoblastoma is the most common intraocular tumor in childhood, with an annual mean incidence of 1 in 18 000 live births and a mortality rate that varies widely across the globe. In well-developed countries, retinoblastoma is rarely a life-threatening condition because of early diagnosis, but in underdeveloped and developing countries, clinical diagnosis is made in advanced stages, and the mortality rate remains high. In our study, patients had a mean age at diagnosis of 29 months. This value is similar to those from other Latin American and Asian series (32, 31, and 26 months in Venezuela, Mexico, and Taiwan, respectively). The interval between the first symptoms and enucleation ranged from 15 days to 44 months, with a slightly higher number of patients in the 0-month to 6-month group. Most of our patients had poorly differentiated tumors, similar to the work of Biswas et al.

Marback et al measured the relative vascular area of the tumor in retinoblastomas and compared their results with optic nerve and choroidal invasion. They indicated that the relative vascular area of the tumor seems to be a promising prognostic marker for metastatic disease and should be evaluated in a large sample of eyes with retinoblastoma.

Previous studies have reported that angiogenic potential in retinoblastoma correlates with invasive growth and metastasis and that these 2 factors are associated with poor prognosis. These tumors, however, appear to depend on a heterogeneous vasculature composed of angiogenic neovessels and pericyte-committed mature vasculature, the latter composed of endothelial cells dependent on angiogenic factors such as VEGF.

It is known that VEGF messenger RNA is expressed in retinoblastoma neoplastic cells, but little of such ex-
pression occurs in tumor endothelial cells, and that VEGF, which is secreted from neoplastic cells, influences nearby endothelial cells and functions as a paracrine mediator. In vitro studies have shown that VEGF stimulates endothelial cell division and migration, and its production is induced by hypoxia. This factor has been implicated in cellular proliferation of gastrointestinal carcinomas and in other types of cancer.

In our series, 98% of the patients tested positive for retinoblastoma by VEGF immunostaining, of which most had diffuse and strong staining. Of the 7 patients with well-differentiated retinoblastoma, 2 showed focal and weak VEGF staining, whereas the other 5 had strong and diffuse staining. These results differ from those of Kerimog˘glu et al, who measured angiogenesis (microvessel density) and found higher levels in poorly differentiated retinoblastomas.

We found a statistically significant relation between the interval from the onset of the symptoms to the enucleation and VEGF immunostaining intensity. This finding indicates that the intensity of the immunostaining depends on the time between the onset of symptoms and enucleation. This could suggest that tumors with delayed diagnosis and treatment could have more angiogenic potential and may be more prone to dissemination.

High mitotic and apoptotic indexes reflect high proliferative activity in any tumor. Kerimog˘glu et al have suggested that the apoptotic index could be an important metastatic predictor for retinoblastoma. Most of our patients had high mitotic and apoptotic indexes, and we found a statistically significant correlation between VEGF immunostaining intensity and the apoptotic index (Figure 4). Moreover, a relation between VEGF immunostaining intensity and percentage of staining with the mitotic index was also found. With respect to the apoptotic index, it has been demonstrated that deletion of the retinoblastoma gene produces apoptosis rather than tumor formation because the loss of the retinoblastoma gene triggers a p53-mediated apoptotic response.

No correlation was found between VEGF immunostaining and tumor staging. This lack of correlation implies that all retinoblastomas, not just those limited to retina and cases with choroidal or optic nerve invasion, have the capacity to produce angiogenic factors and by extension induce angiogenesis. Marback et al have suggested that retinoblastomas with numerous blood vessels (measured with the relative vascular area of the tumor) are those at an advanced stage of disease. Although the isolated characterization of VEGF in retinoblastoma should not be taken as a prognostic factor, its association with the apoptotic index suggests a role for this protein in the progression of this disease.

Our data suggest that the use of anti-VEGF therapies in retinoblastoma may be efficacious in targeting immature neovessels within the tumor. This approach, coupled
with a strategy to treat pericyte-committed mature tumor vasculature, may be effective in the management of this disease.

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REFERENCES