Inhibitory Effect of Bevacizumab on the Angiogenesis and Growth of Retinoblastoma

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Objective: To evaluate the potential effect of bevacizumab, a monoclonal antibody against vascular endothelial growth factor (VEGF), on the angiogenesis and tumor growth of retinoblastoma in vitro and in vivo.

Methods: The antiangiogenic effects of bevacizumab were evaluated in a coculture of a Y-79 human retinoblastoma cell line and a human umbilical vein endothelial cell line by means of a cell proliferation assay kit and a VEGF enzyme-linked immunosorbent assay. The Y-79 xenotransplanted nude mice were treated with bevacizumab intraperitoneally twice weekly for 4 weeks, during which each tumor was measured once a week. The mice were then euthanized, and the weight of each tumor and its microvessel density were determined via CD34 immunohistochemical staining.

Results: The mean (standard error of the mean) increased human umbilical vein endothelial cell proliferation, when cocultured with Y-79 (156% [1%]), was suppressed 58% (5%) by the blockage of VEGF induced by bevacizumab. By causing a 2-fold reduction in microvesSEL density in the Y-79 xenograft model, bevacizumab induced a 75% reduction in the growth of the retinoblastomas without producing significant systemic toxicity.

Conclusions and Clinical Relevance: Treatment with bevacizumab suppressed the angiogenesis and growth of retinoblastoma in vitro and in vivo. Bevacizumab is likely to be of benefit in the treatment of retinoblastoma.

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RETNBLASTOMA, WHICH IS the most common primary intraocular malignancy of childhood, occurs in 1 of 15,000 live births.1,2 Timely diagnosis and treatment have contributed to an improvement in patient survival of up to 95% or more.3 Current treatment modalities, such as external beam radiotherapy and chemotherapy, have focused on increased globe conservation.4,5 Because of concerns about the significant morbidity and potential mortality associated with current therapies in the treatment of retinoblastoma, newer therapeutic modalities are being investigated.6,7

Vascularity is critical to the survival of a solid tumor mass, and angiogenesis is essential to continuing and rapid tumor growth.8-11 Vascular endothelial growth factor (VEGF), a diffusible glycoprotein produced by healthy and neoplastic cells, is an important regulator of physiologic and pathologic angiogenesis. Preclinical studies have shown that a murine anti-human monoclonal antibody that blocks human VEGF–induced proliferation of murine endothelial cells can inhibit the growth of a human tumor xenograft; a humanized variant of that antibody (bevacizumab [Avastin; Genentech, Inc, South San Francisco, California]) is being used to treat colon cancer and is undergoing evaluation in clinical trials as a treatment for other types of cancer.12,13

Retinoblastoma is a solid, well-vascularized tumor that is dependent on its vascular supply.14 Vascular endothelial growth factor is highly expressed in patients with a retinoblastoma.15-17 The aim of this study was to evaluate the effects of a humanized variant of the antibody bevacizumab on the angiogenesis and tumor growth of retinoblastoma in vitro by means of a tumor-endothelial coculture system and in vivo with a retinoblastoma xenograft model.

METHODS

CELL LINES

The Y-79 human retinoblastoma cell line and culture medium were obtained from American Type Culture Collection (Manassas, Virginia). The cells, which were grown in a suspension culture in Roswell Park Memorial Institute (RPMI) Buffalo, New York medium containing 20% fetal calf serum, 100 U/mL of penicillin, and 100 µg/mL of streptomycin, were kept at a concentration of $2.5 \times 10^3$ cells/mL. To test VEGF expression in normoxic and hypoxic conditions, the cells were
in the culture medium that were incubated for 3 days served as
throughout the well of the tissue culture plate. Untreated cells
directly to the cells to ensure a uniform concentration of the drug
days. A solution of the drug mixed with the medium was added
cells were seeded into the medium. The cocultured Y-79 cells
normoxic chamber (n=3). CO2 indicates carbon dioxide; N2, nitrogen.
medium of Y-79 cells incubated at 37°C in a hypoxic chamber and in a
analyzed with an enzyme-linked immunosorbent assay kit in a culture
proliferation. The mean (standard error of the mean) expression of VEGF was

tems, Minneapolis, Minnesota) according to the manufacturer's
say (Quantikine Human VEGF Immunoassay kit; R&D Sys-
Oregon). The presence of VEGF protein secreted into the me-
chamber, HUVECs were plated onto 6-well tissue culture plates
bovine serum. To establish tumor endothelial cells in an in vitro
culture medium supplemented with 10 ng/mL of human recombinant
epidermal growth factor, 1 µg/mL of hydrocortisone, 50 µg/mL
gentamicin, 12 µg/mL of bovine brain extract, and 10% fetal
bovine serum. To establish tumor endothelial cells in an in vitro
coculture, HUVECs were plated onto 6-well tissue culture plates
(5 × 10^4 cells/well) and were incubated at 37°C in 5% carbon di-
oxide. After incubation, the HUVECs adhered strongly to the floor
of the wells, and the medium was exchanged for a mixed me-
dium of both Y-79 cells and HUVECs, after which 5 × 10^4 Y-79
cells were seeded into the medium. The cocultured Y-79 cells and
HUVECs were treated with 0.01-mg/mL bevacizumab for 3
days. A solution of the drug mixed with the medium was added
directly to the cells to ensure a uniform concentration of the drug
throughout the well of the tissue culture plate. Untreated cells
in the culture medium that were incubated for 3 days served as
controls.

CELL PROLIFERATION
AND VEGF ASSAY

Cell proliferation was assessed with the CyQUANT NF cell pro-
liferation kit (Invitrogen Molecular Probes, Eugene, Oregon)
according to the manufacturer's instructions. Calibration curves
for each cell line showed that the fluorescence reading was lin-
erly related to the cell number (data not shown). The cell num-
ers were determined before and 3 days after exposure to be-
vacizumab. To produce a nuclear stain in the coculture, the
cultures were washed 3 times with phosphate-buffered saline.
The HUVECs were fixed with 4% paraformaldehyde and were
then stained with Hoechst 33342 (Molecular Probes, Eugene,
Oregon). The presence of VEGF protein secreted into the me-
dium was determined by enzyme-linked immunosorbent as-
say (QuantiKine Human VEGF Immunoassay kit; R&D Sys-
tems, Minneapolis, Minnesota) according to the manufacturer's
instructions.

TREATMENT

Intrapertoneal injections of bevacizumab were repeated twice
weekly for 4 weeks. The body weight of each mouse and the
size of its tumor were measured in 2 dimensions with a digital
caliper once weekly for 4 weeks. The volume of each tumor
was calculated using its measured dimension and the formula
(M×N)/2, where variable M equals major length and N equals
minor length. At the completion of the experiment, the mice were
killed in a carbon dioxide chamber, and the weight of each
cocultured tumor was determined.

IMMUNOSTAINING OF TISSUES

The tumor specimens were fixed in Bouin fluid (Polysciences,
Inc, Warrington, Pennsylvania) and embedded in paraffin. Tissue
sections of 4 µm were cut. The paraffin-embedded tissue
sections were deparaffinized in xylene and rehydrated in
alcohol. The sections were incubated in sodium citrate buffer
to unmask antigens and epitopes and were incubated in 3%
hydrogen peroxide to block endogenous peroxidase activity.
Each section was incubated with normal rabbit serum for
30 minutes at room temperature, after which a 1:30 dilution
of rat antimouse CD31 antibody (sc-18917; Santa Cruz Bio-
technology, Santa Cruz, California) was applied to the sec-
tions; they were then incubated overnight at 4°C. After incu-
bation with biotinylated rabbit antirat IgG (Vectastain ABC
kit; Vector Laboratories, Burlingame, California) for 1 hour at
room temperature, each section was rinsed in phosphate-
buffered saline and incubated in the avidin-biotin peroxidase
complex for 13 minutes at 37°C. The peroxidase was visual-
ized with 3,3-diaminobenzidine tetrahydrochloride solution.
Microvessel density was assessed with the random field
method. Immunostained slides were scanned at the original
magnification ×200 in 20 randomly selected fields. Image-
Pro Plus (version 4.0 Software; Media Cybernetics Inc, Silver
Spring, Maryland) was used for the computerized quantifica-
tion of the immunoassayed vascular structures. Diaminoben-
zidine tetrahydrochloride–positive pixels were selectively
detected, and the area of selected pixels was calculated with
that program.

STATISTICAL ANALYSIS

All data were expressed as the mean (standard error of mean).
Differences in means between the treatment groups were ex-

Figure 1. Vascular endothelial growth factor (VEGF) expression in Y-79–cell
proliferation. The mean (standard error of the mean) expression of VEGF was
analyzed with an enzyme-linked immunosorbent assay kit in a culture
medium of Y-79 cells incubated at 37°C in a hypoxic chamber and in a
normoxic chamber (n=3). CO2 indicates carbon dioxide; N2, nitrogen.
Levels of VEGF assayed in the culture medium of the Y-79 cells increased in both normoxic and hypoxic conditions (Figure 1). Although VEGF levels in the medium of Y-79 cells increased more under a hypoxic condition, the media level of VEGF after 3 days under a normoxic condition was already high enough to induce endothelial proliferation. Because prolonged exposure to hypoxia itself may have variable effects on the proliferation of HUVECs, we decided to coculture Y-79 and HUVECs under normoxic conditions for 3 days.

Secretoned VEGF was minimal in the HUVEC monoculture; however, it was increased in the HUVEC and Y-79 cell coculture system. When HUVECs were cocultured with Y-79 cells, the growth of HUVECs increased to 156% (1%) higher than that of the HUVECs in the monoculture, an increase that corresponds to the increase in VEGF levels (P < .05). While 0.01-mg/mL bevacizumab completely blocked the increase in VEGF levels and suppressed the increased growth of HUVECs to 58% (5%) of the initial cell count (P < .05) (Figure 2A and B), bevacizumab had no effect on the growth of Y-79 cells or HUVECs (Figure 2C). Higher doses of bevacizumab (0.02 and 0.04 mg/mL) were also tested; neither dose affected the viability of Y-79 cells or HUVECs, but both completely blocked the increase in VEGF levels (data not shown). Because 0.01 mg/mL was maximally effective and safe, we used this dose in the following experiments.

Figure 2. A-I, The effects of vascular endothelial growth factor (VEGF) and bevacizumab on cocultured Y-79 cells and human umbilical vein endothelial cells (HUVECs). Cells were cultured for 3 days with or without 0.01-mg/mL bevacizumab. A, VEGF in the coculture medium with or without bevacizumab exposure. B, Cell proliferation of HUVEC cocultured with Y-79 cells with or without bevacizumab exposure. Cell viability of HUVECs is expressed as the mean (standard error of the mean) percentage of initial HUVEC count (n = 3). Asterisk indicates a statistically significant difference from the control group (P < .05). Dagger indicates a statistically significant difference from the group not treated with bevacizumab (P < .05). C, The effects of bevacizumab on the growth of Y-79 cells and HUVECs. D, Phase-contrast microscopy and E, fluorescent microscopy of HUVECs. F, Phase-contrast microscopy and G, fluorescent microscopy of HUVECs cocultured with Y-79 cells. H, Phase-contrast microscopy and I, fluorescent microscopy of HUVECs cocultured with Y-79 cells with exposure to bevacizumab. Scale bar, 100 µm.
Using phase-contrast microscopy and fluorescent microscopy with Hoechst 33528, we obtained photographs of the HUVECs in coculture with Y-79 with or without exposure to bevacizumab. These photographs show results similar to those of our cell viability assay (Figures 2D-2I).

In all mice that received bevacizumab, treatment was tolerable. Data regarding the rate of each animal’s growth are shown in Figure 3A. Weight gain was similar in the control and bevacizumab-treated groups. The final tumor sizes were as follows: 427 (88) mm³ in mice that received 100 µg of bevacizumab, 606 (117) mm³ in mice that received 50 µg of bevacizumab, and 1028 (300) mm³ in those that received 10 µg of bevacizumab (Figure 3B). The tumors in mice that received 50 µg or 100 µg of bevacizumab were statistically significantly smaller than the tumors in the control group, which were 1480 (46) mm³ in size (P < .05). The differences in tumor size between the groups became more pronounced as treatment continued. The final tumor weight after treatment for 4 weeks was 0.15 (0.03) g in mice that received 100 µg of bevacizumab, 0.22 (0.05) g in mice that received 50 µg of bevacizumab, and 0.54 (0.17) g in mice that received 10 µg of bevacizumab (Figure 3C). The final tumor weight in mice that received 50 µg or 100 µg of bevacizumab was statistically significantly less than the final tumor weight of the control group, which was 0.86 (0.07) g (Figure 3C) (P < .05).

Blood vessels were more abundant in the tumor tissue from the control group than in tissue from bevacizumab-treated mice (Figure 4A-F). There was a statistically significant reduction of up to 50% of microvessel density in the tumor sections after 4 weeks of bevacizumab treatment (Figure 4G).

**COMMENT**

The goal of our study was to investigate the inhibitory effect of bevacizumab, an anti-VEGF monoclonal antibody, on the angiogenesis and tumor growth of retinoblastoma. Our results showed a remarkable antiangiogenic effect on retinoblastomas in vitro and in vivo and reduced tumor growth, perhaps caused by the inhibition of VEGF in vivo.

Our in vitro experiments showed that Y-79 human retinoblastoma cells expressed VEGF in both hypoxic and normoxic conditions. It is well known that VEGF is expressed in a wide variety of tumors, including retinoblastomas, and that VEGF is essential for tumor growth.14-17,22 It is also known that (1) VEGF messenger ribonucleic acid is expressed in neoplastic cells, but little such expression occurs in endothelial cells, and (2) VEGF, which is secreted from neoplastic cells, influences nearby endothelial cells and functions as a paracrine mediator.22,23 These findings are consistent in a retinoblastoma cell line showing increased HUVEC proliferation, which correlated with increased VEGF in a coculture of HUVECs and Y-79 cells. Furthermore, this increased HUVEC proliferation was statistically significantly suppressed by the anti-VEGF activity of bevacizumab. Bevacizumab had no effect on the growth of Y-79 cells or HUVECs.

Although we didn’t specifically check all potential adverse effects of bevacizumab, the animals tolerated this therapy relatively well, showing normal growth without any visible serious adverse effects such as external bleeding, and demonstrated a suppression of tumor...
growth for the total treatment period, an effect that persisted as the duration of treatment increased. Various human tumor xenografts differ in their sensitivity to anti-VEGF antibody treatment. Kim and colleagues reported reductions of about 95%, 75%, and 70% in the final weight of rhabdomyosarcoma, glioblastoma multiforme, and leiomyosarcoma xenografts, respectively, after 4 weeks of treatment with an anti-VEGF antibody. The more than 75% reduction in the final weight of retinoblastomas in our bevacizumab treatment group indicates that retinoblastoma may be a good potential target for antiangiogenic therapy.

Immunohistochemical analysis confirmed that this effect is achieved in part by antiangiogenic mechanisms. The number of tumor vessels in the bevacizumab treatment group was statistically significantly less than in the control group; this suggests that antiangiogenic and antitumor effects may result from blocking VEGF, because bevacizumab did not affect tumor growth. Previous reports have suggested that VEGF has an important role in the growth of retinoblastomas and that blocking VEGF may be effective in suppressing tumor growth.16,24-27 Recently, Jia and colleagues reported that the suppressed angiogenesis and tumor growth of retinoblastoma was caused by VEGF-targeted ribonucleic acid interference. There are many ways of inhibiting VEGF signaling, such as the use of an anti-VEGF antibody, VEGF traps, and VEGF receptor protein–tyrosinase kinase inhibitors. Many of these modalities, which induce different levels of suppression in various types of tumors, are also under investigation.28,29 We demonstrated a suppression in angiogenesis and growth of retinoblastoma after treatment with bevacizumab, which has been approved as a first-line therapy for the treatment of advanced colorectal cancer and is thus the first specific inhibitor of angiogenesis that is clinically available. Additionally, bevacizumab is currently under investigation or used in various ophthalmologic diseases, including neovascular age-related macular degeneration and diabetic macular edema.30,31

The dose of bevacizumab administered in this study was identical to that administered to patients.32 The adverse effects of systemic bevacizumab treatment in adult patients, such as nose bleeding, hypertension, proteinuria, neutropenia, diarrhea, and delayed wound healing, among others, are already well known; however, much less is known about the effects of this agent in pediatric patients. Although bevacizumab has been investigated in the treatment of other tumors (including neuroblastoma and Ewing sarcoma) in children, further study of the safety of that drug in pediatric patients may be warranted.33-35 Because retinoblastoma rarely metastasizes, and most of the patients with that type of tumor are children, intravitreal administration may be advantageous. To investigate the effects of locally administered bevacizumab on retinoblastoma, additional studies of the local administration of this agent in transgenic mice are needed.

In our study, the angiogenesis and tumor growth of retinoblastoma were not suppressed completely. There is experimental and clinical evidence of the synergistic effect of bevacizumab in combination with chemotherapy.32,36,37 Jockovich and colleagues reported on the efficiency of carboplatin plus an antiangiogenic treatment in a retinoblastoma model. In addition to its use as a monotherapy, bevacizumab may ultimately have an important role as an adjuvant to current antineoplastic regimens such as chemotherapy, external beam radiotherapy, and local laser ablative therapy.

In summary, we found that by reducing the vasculature necessary for tumor growth, bevacizumab caused a 75% reduction in the growth of retinoblastoma in a well-documented experimental model without producing significant systemic toxicity. We confirmed this effect of bevacizumab on cocultured HUVECs and Y-79 cells in vitro.

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Figure 4. A-E, The effect of bevacizumab on microvessel density of retinoblastoma. A, Photograph of a retinoblastoma treated with phosphate-buffered saline (PBS) and stained with hematoxylin. Arrows indicate vessels. B, Photograph of a retinoblastoma treated with PBS and stained with CD34. C, Computerized image of a diaminobenzidine (DAB)–positive field corresponding to the CD34-positive region in a retinoblastoma treated with PBS. Scale bar, 200 µm. D, Photograph of a retinoblastoma treated with bevacizumab and stained with hematoxylin. Arrows indicate vessels. E, Photograph of a retinoblastoma treated with bevacizumab and stained with CD34. F, Computerized image of a DAB-positive field corresponding to the CD34-positive region in a retinoblastoma treated with bevacizumab. G, Comparison of the quantification of microvessel density; asterisk indicates a statistically significant difference from the control group (P<.05).

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Endothelial Area, Percentage of Tumor Area
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REFERENCES