Photodynamic Therapy in Retinoblastoma: Effects of Verteporfin on Retinoblastoma Cell Lines

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PURPOSE. In contrast to the excellent survival rates of the malignant childhood tumor retinoblastoma (RB), morbidity is high in patients with this disease because of the enucleation or loss of retinal areas caused by current bulb-saving therapies. The authors aimed to preclinically assess the effects of phototherapy using second-generation photochemotherapeutics as a prerequisite to develop a promising therapeutic alternative. This therapy implies intravenous application of a photosensitizer activated locally by light of the appropriate wavelength. Activation leads to the formation of free radicals, vascular occlusion, and death of affected cells in the area of irradiation. The photosensitizer verteporfin is approved for the therapy of neovascularizations, such as age-related maculopathy.

METHODS. The uptake of verteporfin in RB cell lines was investigated. Established RB cell lines, an RB subline resistant to etoposide, and dissociated cells from a primary RB were incubated with verteporfin and irradiated with activating laser light. Proliferation was measured at different time points after application.

RESULTS. All five RB cell lines investigated incorporated verteporfin, and nanomolar concentrations were sufficient for effective killing. At lower doses, surviving cells started to proliferate again after several days, but verteporfin 50 ng/mL and 100 J/cm2 were sufficient for irreversible killing. High verteporfin concentrations caused cell death with little to no irradiation. Etoposide-resistant cells and primary tumor cells had a comparable susceptibility to photodynamic therapy (PDT) as established parental cell lines.

CONCLUSIONS. PDT using verteporfin efficiently kills chemotherapy-resistant and nonresistant retinoblastoma cell lines and primary tumor cells in vitro, and it warrants further preclinical evaluation as a therapeutic option for the treatment of retinoblastoma. (Invest Ophthalmol Vis Sci. 2008;49:3158–3163) DOI:10.1167/iovs.07-1016

The common malignant childhood tumor retinoblastoma (RB) is treated by photocoagulation, cryotherapy, or irradiation. Other methods are used as additions or alternatives to combat special problems, such as additional chemotherapy in the case of hematogenous spreading or involvement of the central nervous system. In some cases, local treatment such as photocoagulation or cryotherapy follows primary chemoreduction. Most large tumors must be enucleated.

The greater than 95% survival rate contrasts the high morbidity caused by therapy. All current therapeutic options lead to the loss of retinal areas if the eye bulb can be saved. Additionally, many patients with hereditary retinoblastoma develop monocular secondary tumors after chemotherapy or irradiation. New therapeutic insights must be identified to reduce patient morbidity.

In photodynamic therapy (PDT), a primarily nontoxic photosensitizer is administered intravenously. Subsequent activation of the dye by low-energy, nonthermal laser light leads to the formation of reactive oxygen species that cause oxidative stress and cell death within the immediate vicinity. PDT is of interest for organs, including the brain, bronchial system, and intestine, that must retain microscopic tissue structure to maintain functionality. By focal activation of the photosensitizer, it is possible to selectively treat the tumor. The photosensitive substance is more enriched in the tumor than in the surrounding tissue because of the higher metabolic activity, more permeable vessels, and lower lymphatic drain of the tumor. The antitumor effect of PDT results from the combination of direct cytotoxicity and vascular obliteration. Success rates from 70% to 95% have been described based on tumor type.

PDT was first attempted on retinoblastoma cell lines using hematoporphyrin and white light. A sensitizing period of at least 3.5 hours, 20 μM photosensitizer, and 6 μW/mm2 irradiation were required to induce cell death in Y-79 cells. PDT using hematoporphyrin derivative (HPD) was used against human retinoblastoma xenografts in the anterior eye chamber of athymic nude mice and resulted in marked tumor cytotoxicity. Amelanotic melanoma was heterotransplanted to the iris in rabbits and treated with HPD. HPD accumulated only in vascularized structures of the eye, in the photoreceptor cell outer segments of the retina, and in the sclera in low concentrations. High-dose PDT did not damage lens, cornea, aqueous, or vitreous but led to permanent, nonprogressive acute retinal damage. Using this first generation of photosensitizers, the benefit of PDT was negligible considering the severe side effects.

The second-generation photosensitizer verteporfin (benzoporphyrin derivative monoaacid ring A), a chlorin-type molecule, has been approved by the United States Food and Drug Administration for treating ocular tumors, neovascularization (as in age-related maculopathy [ARM]), and other macular diseases. Worldwide, more than 200,000 patients have already been treated with verteporfin. It efficiently absorbs light of 680- to 695-nm wavelengths and is able to penetrate blood, melanine, and fibrotic tissue. A nonthermal diode laser is the most suitable light source for verteporfin PDT, eliminating the possibility of thermal damage to eye structures. Verteporfin is encapsulated in liposomes, thus increasing stability and reducing renal and hepatic elimination. Liposomal packaging makes verteporfin lipophilic and able to bind LDL in the blood plasma. LDL-bound verteporfin enters cells through the
LDL receptor by receptor-mediated endocytosis. Fast-growing cells, such as tumor cells and neovascularizations, accumulate more verteporfin than normal cells due to selective extravasation because of leaky vasculature, providing high target specificity and negligible adverse effects to normal tissues. Verteoporf

MATERIALS AND METHODS

Cell Lines

Retinoblastoma cell lines Y-79 and WERI-Rb1 were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). RB247C3, RB355, and RB383 were kindly provided by B. Gallic (Hospital for Sick Children, Toronto, Canada). All cell lines were cultivated in RB medium (DMEM supplemented with 15% FBS, 4 mM l-glutamine, 50 μM β-mercaptoethanol, 10 μg insulin/mL, and penicillin/streptomycin) at 37°C in 10% CO2 and 80% humidity.

Chemotherapy-Resistant Cell Lines

Chemotherapy-resistant subclones were produced by consecutively increasing the concentration of a chemotherapeutic drug in the culture medium over time until the IC50 of the subclone was at least 10-fold higher than that of the parental cell line. A subclone of the WERI-Rb1 cell line resistant to etoposide was used in PDT experiments.

Primary Tumors

Primary tumor material was isolated from the eye bulb using a syringe immediately after enucleation and passed through a 40 μm nylon cell strainer. Cells were washed, resuspended in an appropriate volume of RB medium, and immediately used for PDT.

Western Blotting

Retinoblastoma cells were washed three times with PBS and lysed in ice-cold NP-40 buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% NP-40) containing inhibitor (Complete Protease Inhibitor Cocktail; Roche, Basel, Switzerland). Lysates were cleared by centrifugation, separated (50 mg total protein/lane) on a polyacrylamide gel system (NuPAGE BisTRIS; Invitrogen, Carlsbad, CA) gel (4%–12% polyacrylamide gradient), and electroblotted onto nitrocellulose. Filters were blocked with 5% nonfat dry milk powder in PBS, then incubated with the anti-LDL receptor monoclonal antibody (Oncogene, San Diego, CA) diluted 1:500 for 16 hours at 4°C. After washing 3 × 10 minutes with PBS containing 0.02% Tween-20, blots were incubated for 30 minutes with anti-mouse immunoglobulin (Dako, Glostrup, Denmark) diluted 1:1000 and with streptavidin-biotin-horseradish peroxidase complex (Amersham, Piscataway, NJ) for 1 hour at room temperature. Immunocomplexes were detected with enhanced chemiluminescence and film (ECL Plus kit and ECL Hyperfilm; Amersham).

Demonstration of Cellular Uptake

For microscopic demonstration of cellular uptake, 2 μg/mL verteporfin was added to the culture medium. The intensity of the green color within the cells was observed using bright-field microscopy.

PDT and Cell Proliferation Assay

The previously described MTT assay for proliferation was modified to avoid problems with aggregation of retinoblastoma cell lines cultured in 96-well plates. For each treatment group, 104 cells were seeded in a volume of 15 mL into 9-cm Petri dishes. Verteporfin (Visudyne; Novartis, Basel, Switzerland) was added to each treatment group in the appropriate concentration. Cells were incubated for 1 hour, washed twice with 15 mL RB medium, resuspended in 5 mL RB medium, and transferred to a 12-well plate. PDT was carried out (Visulas 690; Carl Zeiss, Oberkochen, Germany) with a spot size of 5 mm at 689 ± 3 nm. Higher light doses were achieved using longer irradiation times. After the indicated treatment of cells, 100 μL aliquots were transferred to a 24-well plate together with 900 μL RB medium and were incubated for 24, 48, or 72 hours. Cells were incubated for 1 hour after the addition of 200 μL MTT solution (6 mg/mL in PBS) and were solubilized by the addition of 1 mL stop solution (10% SDS, 5% acetic acid in dimethyl sulfoxide). Absorbance at 570 nm was measured using a universal plate reader (EM800; Bio-Tek Instruments, Bad Friedrichshall, Germany). Each experiment was performed at least in triplicate.

RESULTS

Internalization of Verteporfin by Retinoblastoma Cell Lines

We assessed whether verteporfin would be taken up by the retinoblastoma cells once it reached the tumor. Verteporfin circulates in the blood bound to low-density lipoprotein (LDL), and the LDL receptor must be present for its cellular uptake. The LDL receptor was expressed in every retinoblastoma cell line investigated using Western blotting of whole cell homogenates (Fig. 1). Because of the intensely green color of verteporfin, cellular uptake could be directly visualized using bright-field microscopy. All retinoblastoma cell lines investigated were intensely stained a few minutes after high doses of verteporfin were added to the culture medium. Representative results are shown for the cell lines RB247C3 and WERI-Rb1 (laser scanning microscopy) in Figure 2.}

Verteporfin PDT of Established Retinoblastoma Cell Lines

To preclinically test the efficiency of verteporfin for the treatment of retinoblastoma, we first defined the optimal combination of verteporfin concentration and laser light dosage sufficient to markedly decrease retinoblastoma cell proliferation. Established retinoblastoma cell lines were treated with 0 to 1000 ng verteporfin/mL. Verteporfin concentrations that were most probably the result of the activation of a portion of the verteporfin molecules by residual room lighting. Cells continued to proliferate in the presence of 10 ng/mL verteporfin, even after irradiation with the highest light dose of 100 J/cm2. Verteporfin concentrations lower than 50 ng/mL in combination with a range of laser light energy densities were also tested for their effect on cell proliferation over a period of 72 hours (Fig. 4). All cells were killed by 100 J/cm2 light after
treatment with 50 ng/mL verteporfin, whereas untreated control cell proliferation decreased only slightly.

To determine the light dose sufficient to maximally reduce cell proliferation, cells were treated with 50 ng/mL verteporfin and were exposed to up to 250 J/cm² doses of laser light. Cell proliferation was measured after 72 hours. The proliferation of treated cells was reduced by approximately 20% even without laser irradiation in comparison with untreated cells, indicating that dimmed room lighting during handling had some effect on verteporfin-treated cells (Fig. 5). Treatment with 50 J/cm² decreased proliferation to approximately 36%, and 100 J/cm² laser light or more killed 100% of the treated cells. No cell proliferation was detected 72 hours after treatment with 250 J/cm² laser light.

We investigated the potential long-term effects of PDT to determine whether the few remaining cells eventually die or resume proliferation and whether verteporfin-treated nonirradiated cells experience irreparable damage. Regardless of the primary inhibition of proliferation, verteporfin-treated nonirradiated cells grew as nontreated control cells until the cell density reached critical levels at day 15 and proliferation decreased (Fig. 6). Cells irradiated with 50 J/cm² resumed proliferation after approximately 10 days, whereas 100 J/cm² led to the death of all cells.

To investigate the susceptibility of different established retinoblastoma cell lines to PDT, we also examined the proliferation of WERI-Rb1, Y-79, RB247C3, RB355, and RB383 72 hours after irradiation with 0, 50, and 100 J/cm² and treatment with 50 ng/mL verteporfin. In all cases, proliferation decreased markedly in a dose-dependent manner (Fig. 7).

Verteporfin PDT in Chemoresistance and Primary Tumor Cells

A major problem in the therapy of retinoblastoma is chemotherapy resistance. Multidrug resistance-associated proteins such as lung resistance protein and P-glycoprotein are known to be expressed in retinoblastoma, and verteporfin could potentially be actively transported out of the cells. We assessed the effect of PDT using verteporfin on resistant retinoblastoma cells to test whether this treatment would be as potent as against nonresistant retinoblastomas. We created a subline of the etoposide-sensitive WERI-Rb1 cell line through long-term culture in the presence of increasing etoposide concentrations. This subline is resistant to 10 times the etoposide IC₅₀ concentration for the parental cell line. Both the sensitive parental cell line and the resistant subline were exposed to 0, 50, and 100 J/cm² after treatment with 50 ng/mL verteporfin. Both cell lines showed marked decreases of cell proliferation after PDT (Fig. 8), and there was no significant difference in the response of etoposide-sensitive and -resistant WERI-Rb1 cells to PDT ($P = 0.916$). Thus, at least in this case, chemotherapy resistance did not influence the efficacy of PDT.

Another factor potentially influencing the susceptibility to PDT in comparison with primary tumor cells is the long-term culture of established retinoblastoma cell lines. Therefore, tumor cells isolated immediately after enucleation were treated with 50 ng/mL verteporfin and irradiated with 0, 50, and 100 J/cm². Proliferation was markedly reduced in a dose-dependent manner after 72 hours compared with untreated control cells (Fig. 9).

Discussion

We show here that drug uptake and treatment sensitivity as essential prerequisites for successful treatment of retinoblastoma with PDT using verteporfin are fulfilled. As shown in the first reports demonstrating the feasibility of fluorescence angiography for retinoblastoma, retinoblastomas are sufficiently vascularized as a prerequisite for treatment with intravenous applicable drugs, which are dependent on a local
Thus, verteporfin may principally reach the tumor through the vessels and has the potential to cause effects similar to those of age-related macular degeneration (AMD) treatment.

Because verteporfin, like fluorescein, may leak from tumor vessels, we suggest that PDT using verteporfin has an effect on tumor cells and tumor capillaries. Compared with retinoblastoma cells in culture, PDT may be even more effective in tumors that are essentially dependent on blood vessels for nutrition, comparable to an antiangiogenic effect of drugs in other tumors.

The LDL receptor as a prerequisite for verteporfin internalization is expressed in all retinoblastoma cell lines investigated, as demonstrated by Western blotting (Fig. 1). This receptor shows increased expression on some malignant and proliferating cells and is a good target for LDL in combination with therapeutic drugs. Expression of the LDL receptor in the retina was demonstrated in retinal pigment epithelium and another potential receptor for verteporfin, the LDL receptor-related protein 6 (LRP6), in photoreceptor cells. LDL has previously been used as an endogenous carrier of the photosensitizer chlorin E6 in the retinoblastoma cell line Y-79, where receptor-mediated uptake was demonstrated by satisability and competitive inhibition using free LDL. Covalent binding to LDL increased the uptake of chlorin E6 by a factor of 4 to 5.

A verteporfin concentration of 50 ng/mL and a light dose of 100 J/cm² caused irreversible damage in all retinoblastoma cell lines tested here (Fig. 7). Interestingly, in previous reports, much higher concentrations of verteporfin were used (up to 2500 ng/mL) in combination with much lower energy densities (1.2–3.6 J/cm²). Our results indicate that lower verteporfin concentrations and higher energy densities lead to the formation of a sufficient number of free radicals, causing irreversible cell damage with fewer side effects in the surrounding tissue.

In the clinical setting proposed by the supplier, 6 mg/m²...
Verteporfin is used to treat AMD. This should also be a suitable concentration for the treatment of retinoblastoma.

Verteporfin has been used worldwide to treat over 200,000 patients with AMD since 2002. One of its benefits is the absence of severe side effects. The low risk for severe photosensitivity reactions makes it necessary for the patient to avoid sunlight for up to 48 hours. Our results with retinoblastoma cells treated at suboptimal verteporfin PDT doses suggest that therapy of retinoblastomas may not irreversibly kill all the tumor cells. For this reason, multiple treatments may be necessary to completely eradicate retinoblastoma.

Chemotherapy resistance is a major problem in retinoblastoma therapy. Expression of multidrug resistance-related proteins could enable retinoblastoma cells to secrete verteporfin through ABC transporter activity or similar mechanisms after uptake by LDL receptors. The etoposide-resistant WERI-Rb1 subline was as susceptible to verteporfin PDT as the parental etoposide-sensitive cell line (Fig. 8). These results also suggest the efficacy of verteporfin PDT against chemotherapy-resistant retinoblastoma cells.

Genomic alterations occurring in cells that are maintained in long-term culture may result in phenotypic differences of cell lines in comparison with the primary tumor cells. We tested the efficiency of verteporfin PDT on a primary tumor directly after enucleation to estimate whether PDT treatment would be as efficient as in established retinoblastoma cell lines. We did not detect any difference in the efficacy of verteporfin PDT in primary retinoblastoma cells compared with established retinoblastoma cell lines (Fig. 9).

In a first clinical trial in 1986, five retinoblastoma patients were treated with the first-generation photosensitizer HPD using an argon laser photocoagulator. High doses of photosensitizer (2.5 mg/kg) and light (2 mm spot size, 200 mW, 10 minutes) were required because activated HPD was not effectively causing cell death. The excessive irradiation resulted in vitreous hemorrhaging and retinal damage in this study. Between 1987 and 1990, Winther et al. published a series of PDT experiments using retinoblastoma-like tumor cells growing under cell culture conditions or in the vitreous of rat eyes. They used EXP-5 cells established from a tumor induced by human adenovirus serotype-12 in rat retina. The hematoporphyrin derivatofolin II and red light were used in these experiments. PDT efficiently killed the retinoblastoma-like cells. However, it also resulted in severe vascular damage. Taken together, the preclinical and clinical uses of first-generation photosensitizers were characterized by several disadvantages: they required long incubation times (1–5 days) before irradiation and high doses of drug (2.5 mg/kg body weight) and light; PDT treatments produced severe side effects, including vascular damage, leukocyte infiltration of the conjunctiva and cornea, edema, and damage to the photoreceptor cells in the retina; and only one third of the treated tumors could be controlled in vivo.

Novel second-generation photosensitizers, such as verteporfin, are characterized by better systemic tolerance and more favorable activation wavelengths. Encapsulation of the modified benzoporphyrin ring system into liposomes and modifications for cellular uptake by way of the LDL receptor enable more selective targeting to tumor cells. All these advantages reduce the necessary drug dose, incubation, and irradiation times. Combined with advances in laser technology, verteporfin PDT should be a realistic alternative to treat retinoblastoma.

**Acknowledgments**

The authors thank Kathy Astrahantseff for critically reading the manuscript.

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