Photodynamic Therapy Using Lu-Tex Induces Apoptosis In Vitro, and Its Effect Is Potentiated by Angiostatin in Retinal Capillary Endothelial Cells

Reem Z. Renno,1 François C. Delori,2 Robin A. Holzer,1 Evangelos S. Gragoudas,1 and Joan W. Miller1

PURPOSE. To examine the effect of combining angiostatin with photodynamic therapy (PDT) using Lutetium Texaphyrin (Lu-Tex; Alcon, Fort Worth, TX) as a photosensitizer in bovine retinal capillary endothelial (BRCE) and retinal pigment epithelial (RPE) cells and to determine the mode of PDT-induced cell death in these cell lines.

METHODS. Cultured BRCE and RPE cells were incubated with angiostatin (500 ng/ml) for 18 hours and subjected to Lu-Tex/PDT, using treatment parameters previously optimized (5 µg/ml Lu-Tex for 30 minutes followed by timed irradiation at 732 nm). Cellular survival was assessed after a 1-week cellular proliferation. Data were analyzed using Student’s t-test. Caspase 3 activity was monitored in cells after PDT using a fluorogenic substrate, (Asp-Glu-Val-Asp)-AFC (7-amino-4-trifluoromethyl coumarin) [DEVD-AFC], of caspase 3. After PDT, expression of Bcl-2, Bcl-xL, Bax, and Bak was also examined in cell lysates by Western blot analysis.

RESULTS. A synergistic cytotoxic effect of angiostatin and Lu-Tex/PDT was observed in BRCE cells at all fluences used (5, 10, and 20 J/cm²; P ≤ 0.05). These findings applied only if angiostatin was delivered before PDT. No such interactive killing effect was observed in RPE cells. Caspase 3 activity was elevated within 10 minutes of PDT in BRCE and RPE cells and was fluence dependent. Differential modulation of Bcl-2 family members was observed after PDT in BRCE and RPE cells.

CONCLUSIONS. The combination of angiostatin and Lu-Tex/PDT potentiates the cytotoxic effect of Lu-Tex/PDT on BRCE but not on RPE cells. This may provide a strategy to increase the selectivity of PDT in damaging capillary endothelial cells with less damage to RPE cells. Lu-Tex/PDT induces rapid caspase-dependent apoptosis in BRCE and RPE cells. Furthermore, Lu-Tex/PDT induces apoptosis through selective modulation of members of the Bcl-2 family and differs between BRCE and RPE cells. (Invest Ophthalmol Vis Sci. 2000;41:3963–3971)

A ge-related macular degeneration (AMD) is the leading cause of severe vision loss in people aged more than 65 years in Western countries.1–3 Choroidal neovascularization (CNV) occurs in 15% of patients with AMD but accounts for 80% of severe vision loss due to AMD.4,5 Photodynamic therapy (PDT) is showing promising results as a new modality for CNV.6–9 PDT involves the systemic administration of a photosensitizer dye that accumulates in proliferating tissues such as tumors and newly formed vessels. It is followed by irradiation of the target tissue with low-intensity, nonthermal light at a wavelength corresponding to the absorption peak of the dye.10 Excitation of the dye leads to the formation of singlet oxygen and free radicals—better known as reactive oxygen species (ROS)—causing photochemical damage to the target tissue.11

Preclinical studies using PDT for the treatment of CNV have demonstrated that, with the proper treatment parameters of photosensitizer dose, laser light dose, and timing of irradiation, relative selective damage to experimental CNV can be achieved, sparing retinal vessels and large choroidal vessels and with minimal changes in the neurosensory retina.12–15 However, in clinical studies, fluorescein leakage appeared in at least a portion of the CNV by 1 to 3 months of treatment, and increasing photosensitizer or light doses did not prevent the recurrence. This could also lead to undesirable nonselective damage to retinal vessels.6 Several multicenter phase 3 trials are under way to study repeated PDT, applied every 3 months. The interim data look promising, showing decreased rates of moderate vision loss.8 The necessity for repeated PDT can nevertheless be expected to lead to cumulative damage to the retinal pigment epithelium (RPE) and choriocapillaris, which may lead to progressive treatment-related vision loss.

Angiostatin, a proteolytic fragment of plasminogen that was first isolated from the serum and urine of tumor-bearing

From the 1Laser Laboratory, Retina Service, Massachusetts Eye and Ear Infirmary; and the 2Schepens Eye Research Institute, Harvard Medical School, Boston.

Supported by the Massachusetts Lions Eye Research Fund, The Foundation Fighting Blindness, and the Macula Society Research Funds.

Submitted for publication April 17, 2000; revised July 11, 2000; accepted July 19, 2000.

Commercial relationships policy: P (JWM, ESG); N (all others).

The Massachusetts Eye and Ear Infirmary is an owner of a patent covering the use of verteporfin. Should the Massachusetts Eye and Ear Infirmary receive royalties or other financial remuneration related to that patent, JWM and ESG would receive a share of same in accordance with the Massachusetts Eye and Ear Infirmary’s institutional Patent Policy and Procedures, which include royalty-sharing provisions.

Corresponding author: Joan W. Miller, Laser Research Laboratory, Retina Service, Massachusetts Eye and Ear Infirmary, 243 Charles Street, Boston, MA 02114. jwmiller@meei.harvard.edu

Copyright © Association for Research in Vision and Ophthalmology

3963
mice, inhibits angiogenesis. In vitro and in vivo studies have shown that radiation and angiostatin have combined cytotoxic effects on endothelial cells, and the combination of those two components has produced no increased toxicity to normal tissue. These results provide support for further investigation of the effect of combining photodynamic therapy with angiostatin to improve CNV closure without damaging normal tissues. We tested whether angiostatin potentiates PDT-induced bovine retinal capillary endothelial (BRCE) cell damage, by inhibiting proliferation or by other means, without affecting the RPE. If this could be achieved, the combination of angiostatin and PDT might provide increased selectivity in damaging the targeted CNV with less damage to the RPE.

Intracellular events associated with photosensitizers and their subsequent activation with light are currently not well understood. PDT induces cell death by apoptosis in several cell lines, and we wanted to characterize the mechanism of PDT-induced cell death in cell lines relevant to CNV. Lutetium Tøxaphyrin (Lu-Tex) is a new generation photosensitizer currently in clinical trial for the treatment of CNV, because of its favorable characteristics for clinical use, including absorption at 732 nm permitting deep tissue penetration and rapid clearance. Lu-Tex/PDT appears to induce tumor involution in the murine EMT6 sarcoma model by a mixture of apoptosis and necrosis. However, because PDT-induced apoptosis appears to be a function of the photosensitizer, cell line, and severity of treatment conditions, these findings cannot be extended to CNV.

Apoptosis involves the activation of a genetically determined programmed cell suicide that results in a morphologically distinct form of cell death characterized by cell shrinkage, nuclear condensation, DNA fragmentation, membrane reorganization, and blebbing. It has been suggested that apoptosis is associated with the generation of ROS and that the product of the bcl-2 gene protects against apoptosis by inhibiting the generation of ROS. Bcl-2 belongs to a growing family of apoptosis-regulating gene products, which may be either antagonists (Bcl-2, Bcl-xL) or death agonists (Bax, Bak). Control of cell death appears to be regulated by these interactions and by constitutive activities of the various family members. It is known that several apoptotic pathways coexist in mammalian cells that are preferentially activated in a stimulus-, stage-, and context-specific and cell-type manner. A proper understanding of the specific mechanism(s) involved in Lu-Tex/PDT-induced cytotoxicity in cells of relevance to CNV may permit interventions that enhance the selectivity and effectiveness of this modality.

Previously, we reported the characterization of an in vitro system for the study of Lu-Tex/PDT’s effect in cell lines of relevance to CNV treatment: BRCE cells and human RPE cells (Renno et al., unpublished data, May 1999). In the present study, the same system was used to investigate the possibility of an interactive cytotoxic effect of human angiostatin and Lu-Tex/PDT selective to BRCE as a means to reduce the cytotoxic effect of PDT on RPE cells. In the second part of the study, the mode of Lu-Tex/PDT-induced cell death was investigated in BRCE and RPE cell lines. In view of the special relationship among Bcl-2, PDT, and ROS, we also analyzed the constitutive expression of Bcl-2, Bcl-xL, Bax, and Bak in BRCE and RPE cells and determined their modulation after PDT.

### MATERIALS AND METHODS

#### Cell Culture

BRCE cells (kindly provided by Patricia A. D’Amore, Schepens Eye Research Institute, Boston, MA) and human RPE cells (generous donation of Anthony P. Adamis, Massachusetts Eye and Ear Infirmary, Boston) were grown at 37°C in 5% CO₂ in Dulbecco’s modified Eagle’s medium (DMEM; Sigma, St. Louis, MO), 5% heat-inactivated fetal bovine serum (FBS; Gibco, Grand Island, NY), supplemented with 1-glutamine, penicillin, and streptomycin (Gibco).

#### Photosensitizer

Lutetium-Tøxaphyrin (Lu-Tex, motexafin lutetium, PCI 0123) was supplied by Alcon Research (Fort Worth, TX) as a stock solution of 2 mg/ml stable in the dark at 4°C and was used according to the manufacturer’s guidelines.

#### Photodynamic Treatment of Cell Cultures

Cells were plated at a density of 10⁵ in DMEM with 5% FBS and incubated (37°C in 5%CO₂) for 24 hours. The medium was removed and replaced by 3 µg/ml Lu-Tex in DMEM plus 5% FBS. Thirty minutes later, the cultures were exposed to timed irradiation using an argon/dye photocoagulator at 732 nm and laser delivery system (model 920; Coherent, Palo Alto, CA). Irradiance was delivered at a rate of 10 mW/cm² to give a total dose of 5 to 20 J/cm², and irradiation time ranged from 7 to 28 minutes, respectively. After irradiation, the medium was removed and replaced with complete medium. Cultures were photographed at various times after Lu-Tex/PDT using a 16 × 0.32 numeric aperture on a phase-contrast inverted microscope (Diaphot; Nikon, Melville, NY).

#### Proliferation Assay

BRCE and RPE cells were plated at a density of 10⁵ in DMEM with 5% FBS and incubated at 37°C in 5% CO₂. After 18 hours, recombinant human angiostatin (Calbiochem, La Jolla, CA) was added at a concentration of 500 ng/ml. Eighteen hours later, medium was removed and replaced by 3 µg/ml Lu-Tex in complete medium. Thirty minutes later, cells were treated with Lu-Tex/PDT at various light doses, as described. Cultures were returned to the incubator for 7 days, after which cells were dispersed in trypsin and counted in a masked fashion, and the surviving fraction was determined. Results are reported as the mean of triplicate experiments ± SD.

#### Preparation of Cell Lysates and Protein Determination

At various times after administration of Lu-Tex/PDT, 10⁶ cells were collected by centrifugation, and the washed cell pellet was resuspended in 500 µl ice-cold lysis buffer (pH 7.5) containing 10 mM Tris, 130 mM NaCl, 1% Triton X-100, 10 mM NaF, 10 mM NaPi, 10 mM NaPPi, 16 µg/ml benzamidine, 10 µg/ml phenanthroline, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 10 µg/ml pepstatin, and 4 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, hydrochloride (AEBSF). Cellular lysates were stored in aliquots at −84°C for later protease activity assay or Western blot analysis. A protein assay (Coomassie Plus; Pierce, Rockford, IL) with bovine serum albumin (BSA) standard was used to assay protein concentration in cell extract.
Protease Activity

Aliquots containing 50 μg of cellular protein were incubated with 14 μm (final concentration) N-acetyl(Asp-Glu-Val-Asp)-AFC(7-amino-4-trifluoromethyl coumarin) [Ac-DEVD-AFC] (PharMingen; San Diego, CA) in 1 ml protease assay buffer (pH 7.2), containing 20 mM piperazine-N,N′-bis(2-ethanesulfonic acid; PIPES), 100 mM NaCl, 10 mM dithiothreitol, 1 mM EDTA, 0.1% (wt/vol) 3-[1-cholamidopropyl]dimethylammonio)-2-hydroxy-1-propanesulfonate [CHAPS], and 10% sucrose, at 37°C for 1 hour. Fluorescence was measured using a spectrofluorometer (λ excitation, 400 nm; λ emission, 505 nm; model MPF-44A; Perkin-Elmer, Norwalk, CT). Cellular protein served as the blank. Results were compared with a standard curve constructed with AFC (Sigma).

Protein Electrophoresis and Western Blot Analysis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) of proteins was performed with 12% SDS-polyacrylamide gels. All samples were boiled in denaturing sample buffer, and equal amounts of proteins were loaded per lane. Proteins were separated at room temperature under reducing conditions at 120 V. Western blot transfer of separated proteins was performed at room temperature, using polyvinylidene fluoride membranes at 50 mA for 1 hour. To verify equal protein loading, blots were stained with 0.1% ponceau red (Sigma) diluted in 5% acetic acid. Afterward, blots were blocked for 1 hour in Tris-buffered saline (TBS; 10 mM Tris-HCl [pH 7.5] and 150 mM NaCl) containing 5% nonfat dried milk. Next, the membranes were probed with an appropriate dilution (1:250-1:1000) of primary antibody in TBS containing 2.5% nonfat dried milk for 1 hour 30 minutes. Mouse polyclonal antibodies against Bcl-2, Bcl-xL, Bax, and Bak were purchased from PharMingen. After incubation with primary antibody, the blots were washed for 30 minutes with frequent changes of TBS, blocked in 1% nonfat dried milk in TBS for 30 minutes, and incubated in a peroxidase-coupled secondary antibody for 1 hour in TBS containing 1% nonfat dried milk. The blots were washed for 1 hour with frequent changes of TBST (TBS + 0.1% Tween). Immunoblot analysis was performed using enhanced chemiluminescence plus Western blot detection reagents (Amersham Pharmacia Biotec, Piscataway, NJ) followed by exposure to x-ray film (ML; Eastman Kodak, Rochester, NY).

Statistical Analysis

Data for all experiments were analyzed using Student’s t-test with the level of significance set at P ≤ 0.05.

RESULTS

Effect of Combined Angiostatin and Lu-Tex/PDT: BRCE

To assess the effect of combining angiostatin to Lu-Tex/PDT on BRCE cell survival, cells were pretreated for 18 hours with 500 ng/ml angiostatin after which cells were treated with Lu-Tex/PDT at various fluences. Cellular survival was measured by a 1-week cellular proliferation assay. A 1-week interval was chosen rather than a shorter interval to better distinguish the lasting cytotoxic effect of the combination of angiostatin/PDT versus the short-term angiostatic effect that angiostatin exerts on the cells during the incubation period. Before testing the combination of angiostatin and Lu-Tex/PDT, we demonstrated that human angiostatin targets BRCE cells. When exposed to angiostatin alone, the proliferation assay demonstrated a 12.61% killing of BRCE cells at the angiostatin dose used. It was also observed that pre-exposing BRCE cells to angiostatin did not interfere with the subsequent cellular uptake of Lu-Tex (data not shown). More important, results showed a synergistic cytotoxic effect of angiostatin and Lu-Tex/PDT on BRCE cells at all fluences used (5, 10, and 20 J/cm²), consistently exceeding the cytotoxicity resulting from Lu-Tex/PDT alone, angiostatin alone, or the arithmetic sum of their respective toxicities (Fig. 1a). Controls consisted of cells exposed to light only, because no dark toxicity was observed at the concentration of Lu-Tex used. Furthermore, it was observed that angiostatin was not effective in potentiating the effect of Lu-Tex/PDT if delivered after PDT (Table 1).

Effect of Combined Angiostatin and Lu-Tex/PDT: RPE

In contrast to BRCE cells, no cytotoxicity was observed when human RPE cells were treated with human angiostatin, and no interactive killing was observed after exposure to angiostatin and Lu-Tex/PDT (Fig. 1b, Table 1). When combined with angiostatin, Lu-Tex/PDT had a lethal dose (LD100) of 20 J/cm² for BRCE cells, whereas Lu-Tex/PDT alone required 40 J/cm² to achieve the same effect on RPE cells. Our previous studies have shown that at fluences of 20 and 40 J/cm² RPE cell survival is 43% and 21%, respectively (Renno et al., unpublished data, May 1999).

Cellular Morphology after Treatment

Although studies have shown that cells appear severely damaged immediately after PDT (Renno et al., unpublished data, May 1999), 1 week after PDT, some cells had disappeared, whereas those that remained had regained their spindle shape and their ability to attach (Figs. 2b, 2c). However, in BRCE cells that were first primed with angiostatin followed by PDT, widespread and massive cell death was observed at 1 week. Only remnants and densely refractive bodies of dying cells were seen floating in the medium (Fig. 2c). Particles were recovered and placed in fresh complete medium, but none showed any sign of reattachment or proliferation onto a new dish. It was concluded that the combination of angiostatin and Lu-Tex/PDT was lethal to BRCE cells under the conditions used. Control BRCE and RPE cells that were treated with angiostatin alone for 18 hours continued to proliferate and reached confluence (Figs. 2a, 2d). No additive effect of angiostatin to Lu-Tex/PDT was observed in RPE cells. Cells that were subjected to Lu-Tex/PDT alone or angiostatin + Lu-Tex/PDT appeared unchanged, as evidenced by the morphology (Figs. 2e, 2f).

Caspase 3-like Activation after Lu-Tex/PDT

To investigate the role of apoptosis in Lu-Tex/PDT–mediated cell death in BRCE and RPE cells, the activation of caspase 3-like (DEVD-ase) protease was monitored, as a hallmark of apoptosis. The kinetics of activation were measured spectrofluorometrically by assaying the hydrolysis of a substrate that can be cleaved only by the caspase 3-like protease family members (Ac-DEVD-AFC). Figure 3 illustrates the time course of Ac-
FIGURE 1. BRCE and RPE cell survival after Lu-Tex/PDT + angiostatin.
In vitro survival of (a) BRCE cells and (b) RPE cells on exposure to Lu-Tex/PDT in the presence of angiostatin. Cells were plated and exposed to angiostatin 18 hours before Lu-Tex/PDT. A 1-week proliferation assay was used to determine the surviving fraction. Data represent the mean of triplicate experiments ± SD.

TABLE 1. Summary of Cellular Survival (%) as a Function of Treatment

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Lu-Tex/PDT*</th>
<th>Angiostatin</th>
<th>Angiostatin Followed by Lu-Tex/PDT</th>
<th>Lu-Tex/PDT Followed by Angiostatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>BRCE</td>
<td>79.13 ± 4.05 (5)</td>
<td>87.39 ± 5.76</td>
<td>55.22 ± 3.65</td>
<td>77.61 ± 3.52</td>
</tr>
<tr>
<td></td>
<td>53.17 ± 0.32 (10)</td>
<td></td>
<td>38.11 ± 2.50</td>
<td>67.16 ± 3.20</td>
</tr>
<tr>
<td></td>
<td>33.34 ± 2.26 (20)</td>
<td></td>
<td>0.90 ± 0.52</td>
<td>32.97 ± 2.20</td>
</tr>
<tr>
<td>RPE</td>
<td>94.55 ± 1.60 (5)</td>
<td>99.09 ± 0.8</td>
<td>91.84 ± 7.97</td>
<td></td>
</tr>
<tr>
<td></td>
<td>59.59 ± 5.56 (10)</td>
<td></td>
<td>56.84 ± 6.61</td>
<td></td>
</tr>
<tr>
<td></td>
<td>53.47 ± 5.18 (20)</td>
<td></td>
<td>45.83 ± 5.51</td>
<td></td>
</tr>
</tbody>
</table>

The interactive in vitro antiendothelial effect of combined treatment with angiostatin and Lu-Tex/PDT are greater than additive when compared with the sum of expected effects of each treatment alone. The potentiation of Lu-Tex/PDT’s effect on BRCE cells was effective with pre-exposure to angiostatin only. No effect of angiostatin was observed on RPE cells. Data are mean percentage of cellular survival ± SD.

*Fluences in parentheses are expressed in joules per square centimeter.
DEVD-AFC cleavage after Lu-Tex/PDT at three different light doses in BRCE and RPE cells. Results show a rapid elevation of caspase 3-like activity immediately after Lu-Tex/PDT—as early as 10 minutes after Lu-Tex/PDT and peaking at 40 minutes—in both BRCE and RPE cells and at all doses used. Clearly, the rate of entry into apoptosis was time and dose dependent in each cell line. However, the amount of caspase 3-like activation was always significantly higher in BRCE cells than in RPE cells. Furthermore, whereas at 10 and 20 J/cm² the amount of caspase 3-like activation was increased by approximately 50% in BRCE cells compared with RPE cells, at 40 J/cm² (equivalent to the LD₁₀₀ for BRCE cells), the levels in BRCE cells were five times those in RPE cells.

Caspase 3-like Activation after Angiostatin + Lu-Tex/PDT
To examine the effect of combining angiostatin and Lu-Tex/PDT on DEVD-ase activation in BRCE cells, cells were treated with angiostatin alone, Lu-Tex/PDT alone, and angiostatin + Lu-Tex/PDT, after which caspase 3-like activity was assayed as described. Fluences of 20 and 40 J/cm² were used, corresponding to an LD₁₀₀ of combination angiostatin + Lu-Tex/PDT and Lu-Tex/PDT alone, respectively. Results demonstrated that the combination of angiostatin + Lu-Tex/PDT induced a statistically significant increase of caspase 3-like activity compared with Lu-Tex/PDT alone, when using a fluence of 20 J/cm² (Fig. 4). However, although both Lu-Tex/PDT (40 J/cm²) and the combination of angiostatin + Lu-Tex/PDT (20 J/cm²) resulted in 100% lethality to BRCE cells, Lu-Tex/PDT (40 J/cm²) resulted in increased levels of caspase 3-like activity compared with angiostatin + Lu-Tex/PDT (20 J/cm²). As in the case of BRCE cells treated with Lu-Tex/PDT alone, the rate of entry into apoptosis of BRCE cells treated with combination of angiostatin + Lu-Tex/PDT was time dependent. Nevertheless, the time courses differed significantly, in that the induction of caspase 3-like activation occurred abruptly and more rapidly as a result of angiostatin + Lu-Tex/PDT, peaking at 30 minutes and reaching minimum levels at 90 minutes after treatment.

Modulation of Bcl-2 Family Members after Lu-Tex/PDT
To evaluate the expression of Bcl-2 family members in BRCE and RPE cells after Lu-Tex/PDT, BRCE and RPE cells were subjected to Lu-Tex/PDT, and resultant cellular lysates were subjected to Western blot analysis for detection of the antiapoptotic Bcl-2, Bcl-xL, and proapoptotic Bax and Bak. Results showed a differential expression of members of Bcl-2 family in BRCE and RPE cells: Bcl-2 and Bax were detected in BRCE cells, whereas Bcl-xL and Bak were detected in RPE cells (Table 2). After Lu-Tex/PDT at LD₅₀, downregulation of Bcl-2 and upregulation of Bax was observed in BRCE cells, resulting in an increase in the ratio of Bax to Bcl-2 protein (Fig. 5a). In RPE cells, there was an upregulation of both Bcl-xL and Bak up to 4 hours after PDT, after which Bcl-xL levels reached a plateau, and Bak level started to decline (Fig. 5b). Furthermore, our results demonstrated that the upregulation of Bax in BRCE cells was dose dependent; however, the upregulation of its proapoptotic counterpart Bak in RPE cells exhibited dose dependence only until 20 J/cm², after which it began to decline (Fig. 5c).

DISCUSSION
The promising results witnessed with PDT for the treatment of CNV along with some observed side effects sustained by the RPE in the course of treatment, prompted us to seek different strategies to improve the efficacy and selectivity of PDT to CNV. One such strategy was to investigate a role for angiostatin as a potential adjuvant of Lu-Tex/PDT because of its established property as a specific inducer of quiescence in certain endothelial cell lines. Another approach was to investigate the mode of Lu-Tex/PDT–induced cytotoxicity in BRCE and RPE cells as a preliminary step for the design of treatments that might help modulate specifically these effects at the cellular level.

Our data showed a specific antiproliferative effect of angiostatin on retinal capillary endothelial cells as demonstrated by the reduction in cell number in a 1-week proliferation assay. In contrast, no effect of angiostatin was observed on RPE. Thus, our work adds BRCE cells to the list of endothelial cell lines already known to be specifically targeted by angiostatin: bovine adrenal cortex microvascular, bovine adrenal cortex capillary, bovine aortic, human umbilical vein, and human dermal microvascular endothelium. In our study, BRCE cells were used as a representative capillary endothelial line of the posterior segment to test the antiangiogenic effect of angiostatin, because angiostatin does not seem to rely on specific cell surface antigen recognition to exert its action on the endothelium. Therefore, it seems reasonable to assume that angiostatin would have similar effects on the choriocapillaris...
Kinetics of caspase 3-like activation after Lu-Tex/PDT in BRCE and RPE cells. BRCE and RPE cells were exposed to Lu-Tex/PDT at fluences of (a) 10, (b) 20, and (c) 40 J/cm². At the indicated times thereafter, cells were collected and lysed. Aliquots (50 µg of protein) were incubated with Ac-DEVD-AFC at 37°C for 30 minutes. The amount of fluorochrome released was determined by comparison with an AFC standard curve in lysis buffer. Data represent the means from three independent experiments.
and retinal and choroidal neovascular endothelium. Moreover, in culture many of the differences between the choriocapillaris and retinal capillary endothelium are lost. Because angiostatin has a cytostatic rather than cytocidal effect, it could be expected it to have a selective effect on proliferating versus resting endothelium. In addition, tissue culture is thought to more closely represent proliferating tissue such as CNV than resting tissue. The finding that angiostatin induced apoptosis in BRCE cells suggests that cell death may contribute to the overall reduction of cell number; however, little is known concerning the exact antiangiogenic mechanism of angiostatin.

Our in vitro studies showed that Lu-Tex/PDT and angiostatin had combined cytotoxic effects on retinal capillary endothelial cells but not pigment epithelial cells. However, when angiostatin were administered after PDT, the combination did not potentiate the effects of PDT. The efficacy of a photosensitizer is intimately related to its subcellular distribution. Although angiostatin did not affect the intracellular incorporation of Lu-Tex, this does not exclude the possibility that it may induce a redistribution of the dye to subcellular compartments whereby its potency of action is enhanced. In the combination of angiostatin before Lu-Tex/PDT, a fluence of 20 J/cm² sufficed to achieve nearly 100% mortality of BRCE cells. In the absence of angiostatin, a light dose of 40 J/cm² would be required to achieve this level of cytotoxicity. At the light dose of 20 J/cm², RPE cells survival after PDT was improved by 20%. The results of our experiments thus support the potential of combining angiostatin with Lu-Tex/PDT to improve CNV eradication and decrease deleterious effects on the RPE cells. Work is currently under way in our laboratory to test the combination of angiostatin and PDT in small animal models of laser-induced CNV.

In our study, Lu-Tex/PDT induced caspase 3-like activation in both BRCE and RPE cells in a dose- and time-dependent manner.

**Table 2.** Summary of Immunodetection of Bcl2 Family Members in BRCE and RPE Cells

<table>
<thead>
<tr>
<th>Bcl2 Family Member</th>
<th>Cell Line</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BRCE</td>
</tr>
<tr>
<td>Bcl2</td>
<td>+</td>
</tr>
<tr>
<td>Bcl-xL</td>
<td>−</td>
</tr>
<tr>
<td>Bax</td>
<td>+</td>
</tr>
<tr>
<td>Bak</td>
<td>−</td>
</tr>
</tbody>
</table>

Detectable (+) or undetectable (−).

**Figure 4.** Caspase 3-like activity in BRCE cells after angiostatin + Lu-Tex/PDT versus Lu-Tex/PDT alone. BRCE cells were exposed to angiostatin (500 ng/ml) alone, Lu-Tex/PDT (20 J/cm², 40 J/cm²) alone, and angiostatin + Lu-Tex/PDT. At the indicated times thereafter, cells were collected and lysed. Aliquots (50 µg of protein) were incubated with Ac-DEVD-AFC at 37°C for 30 minutes. The amount of fluorochrome released was determined by comparison with an AFC standard curve in lysis buffer. Data represent the means from three independent experiments.

**Figure 5.** Expression of Bcl-2, Bcl-xL, Bax, and Bak in BRCE and RPE cells after Lu-Tex/PDT. (a) BRCE and (b) RPE cells were treated with the 50% lethal dose (LD₅₀) of Lu-Tex/PDT. At the indicated time points after PDT, whole cell extracts were obtained and analyzed by SDS-PAGE followed by Western blot analysis using antibodies to Bcl-2, Bcl-xL, Bax, and Bak. In BRCE cells, upregulation of Bax and downregulation of Bcl-2 were observed over 12 hours. In RPE cells, upregulation of Bcl-xL was observed along with peak upregulation of Bak up to 4 hours followed by its progressive decline. (c) After incremental doses of PDT, BRCE and RPE cellular lysates were obtained at 4 hours after treatment and analyzed by SDS-PAGE followed by Western blot analysis using antibodies to Bcl-2, Bcl-xL, Bax, and Bak. In BRCE cells, Bak was upregulated in a dose-dependent fashion. In RPE cells, the level of Bak plateaued at a fluence of 20 J/cm².
fashion, suggesting that apoptosis is a mediator of Lu-Tex/PDT cytotoxicity in these cell lines. Furthermore, our data indicate that Lu-Tex/PDT induced apoptosis in BRCE cells through the 
modulation of Bcl-2 and Bax in a dose- and time-dependent fashion and in RPE cells through the modulation of Bcl-xL and Bak. However, Lu-Tex/PDT may cause alternative death modes as was shown when tested in vivo in the murine EMT6 sarcoma model, and based on the evidence that photofrin/PDT induces apoptosis or necrosis in a monkey kidney cell line (CV1) depending on the incubation protocol. Therefore, in vivo confirmation of such a finding is required in CNV models.

The time course of caspase 3 activation after PDT, as noted by other investigators, varies according to cell lines and photosensitizers, ranging from minutes to hours: less than 10 minutes for LY-R, 20 minutes for BRCE and RPE cells, and hours for Hela cells. However, unlike other reports, the kinetics in our study in BRCE and RPE cells were constant when the PDT light dose was varied. Furthermore, whereas the magnitude of DEVD-ase activity was 50% higher in BRCE versus RPE cells at flucences of 10 and 20 J/cm², it nearly exceeded 500% at LD₁₀₀ (40 J/cm²); this however does not necessarily correlate with the number of apoptotic cells involved. The possible explanations include the fact that individual intracellular levels of caspase 3-like are unknown, as is the threshold of DEVD-ase activation required for cellular death. Yet, at all times after PDT, there was an upregulation of the antiapoptotic Bcl-xL levels in RPE cells. Concomitantly, at 4 hours after treatment, the levels of the proapoptotic Bak started declining after its initial upregulation. Furthermore, after incremental PDT doses, the proapoptotic Bak was upregulated in RPE cells until 20 J/cm² after which Bak levels started declining despite an increase of PDT dose to 40 J/cm². It is thus conceivable to think of a protective survival response being mounted in RPE cells at these lethal doses to counteract the apoptotic trigger. Such a hypothesis is further supported by the histologic evidence of RPE cell recovery after PDT in vivo and by reports from other investigators that overexpression of antiapoptotic Bcl-2 family members renders cells partially resistant to PDT and inhibits the activation of caspase-3 after PDT. Reversibly, antisense Bcl-2 retrovirus increases the cells' sensitivity to PDT.

The present data show that the combination of angiotatin and Lu-Tex/PDT in BRCE cells resulted in an increase in DEVD-ase activity compared with the same dose of Lu-Tex/PDT applied alone. This suggests that the potentiating action of angiotatin on the effect of Lu-Tex/PDT in BRCE cells proceeds through apoptosis. Even if angiotatin induces a subcellular localization of Lu-Tex, such redistribution remains confined to cellular compartments (mitochondria, lysosomes, and melanosomes) where their mode of action ensues through apoptosis. However, the time course of caspase 3-like activity for angiotatin + Lu-Tex/PDT differed from that of Lu-Tex/PDT alone, in that it proceeded faster without latency and peaked as soon as 20 minutes after Lu-Tex/PDT. An explanation for the latter could that the apoptotic cascade was already primed by preincubation with angiotatin first, and thus the application of Lu-Tex/PDT benefited from an already lowered threshold of activation to rapidly amplify the apoptotic response. However, this does not exclude the possibility of the interplay of more than one apoptotic pathway, especially because PDT is known to initiate cytotoxicity through the generation of ROS, whereas angiotatin was recently shown to act on human endothelial cells by binding to the α-subunit of adenosine triphosphate (ATP) synthase present on the cell surface. Furthermore, whereas angiostatin + Lu-Tex/PDT (20 J/cm²) resulted in a 100% lethality of BRCE cells as did Lu-Tex/PDT (40 J/cm²) alone, the levels of DEVD-ase activation were significantly higher in the former regimen. This supports the hypothesis that Lu-Tex/PDT and angiotatin + Lu-Tex/PDT operate through different apoptotic pathways in BRCE cells.

In summary, in our study angiotatin exhibited an antiproliferative effect on BRCE cells and had no notable effect on RPE cells. Angiotatin combined with Lu-Tex/PDT potentiated cytotoxicity in BRCE cells. Lu-Tex/PDT induced rapid caspase-dependent apoptosis in BRCE and RPE cells. Furthermore, Lu-Tex/PDT induced apoptosis through the selective and differential modulation of members of the Bcl-2 family in BRCE and RPE cells.

References


50. Lu-Tex PDT with Angiostatin in BRCE 3971