Photodynamic Therapy Induces Caspase-Dependent Apoptosis in Rat CNV Model

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PURPOSE. To investigate the mechanism of cell death in laser-induced choroidal neovascularization (CNV) after photodynamic therapy (PDT).

METHODS. PDT was performed in Brown-Norway rats using laser light at a wavelength of 689 nm, irradiance of 600 mW/cm², and fluence of 25 J/cm² after intravenous injection of verteporfin at the doses of 3, 6, and 12 mg/mL. Apoptotic cells in CNV were detected by TUNEL assay at 1, 3, 6, 15, and 24 hours after PDT. Caspase activation at 1, 3, 6, 15, and 24 hours after PDT was determined by immunohistochemistry (IHC) with a cleaved caspase-3 or -9 antibody. Akt activity was determined by Western blot and IHC with a phosphorylated-Akt (pAkt) antibody. To investigate the roles of Akt in PDT-induced apoptosis, insulin-like growth factor (IGF)-1, an Akt activator, with or without wortmannin, an inhibitor of PI3K-Akt pathway, was injected into the vitreous before PDT.

RESULTS. The number of TUNEL-positive cells in CNV increased at 3 hours after PDT and peaked at 6 hours, showing a dose dependence of verteporfin. Caspase activation was detected in TUNEL-positive cells. Dephosphorylation of Akt in CNV occurred within 1 hour. IGF-1 significantly activated Akt and suppressed the number of TUNEL-positive cells in CNV, and the effects of IGF-1 were diminished by wortmannin.

CONCLUSIONS. PDT induced caspase-dependent apoptosis in CNV. These results suggest that PDT leads to dephosphorylation of Akt and subsequent activation of the caspase-dependent pathway. Understanding the intracellular signaling mechanisms of apoptosis in PDT may lead to more selective and effective treatment of CNV secondary to age-related macular degeneration. (Invest Ophthalmol Vis Sci. 2007;48: 4741–4747) DOI:10.1167/iovs.06-1534

Materials and Methods

Induction of Choroidal Neovascular Membranes

All experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the guidelines established by the Animal Care Committee of the Massachusetts Eye and Ear Infirmary. Brown-Norway rats were anesthetized with a 0.1 to 0.2 mL of a 50:50 mixture of 100 mg/mL ketamine and 20 mg/mL xylazine. Pupils were dilated with a topical application of 5.0% phenylephrine and 0.8% tropicamide. CNVs were experimen-
tally induced with an argon-dye-pulsed laser at 630 nm (model 920; Coherent Medical Laser, Palo Alto, CA) to disrupt Bruch membrane using a protocol similar to one previously described. A spot size of 100 μm was used, and the power delivered ranged from 130 to 150 mW, applied for 0.1 second. Four lesions were induced for immunohistochemistry and TUNEL assay, and 20 lesions were induced for Western blot analysis in the left eye of each animal. On occasion, the inducing laser burst created an extensive subretinal hemorrhage, and these spots were excluded from further treatment or analysis.

**Photodynamic Therapy**

PDT was performed as previously described. Two weeks after induction of CNV, anesthetized rats were immobilized on a stereotactic frame (Visudynne; Novartis, Basel, Switzerland) and was injected intravenously at doses of 3, 6, and 12 mg/m² 15 minutes before PDT. PDT was performed with a diode laser at a wavelength of 689 nm, irradiance of 600 mW/cm², spot size of 800 μm, and fluence of 25 J/cm² (Visulas; Carl Zeiss Meditec, Dublin, CA). Untreated rats were used as controls.

**Immunohistochemistry**

Eyes for IHC were enucleated at 1, 3, and 6 hours after PDT. Immunohistochemistry was performed as previously reported. The eyes were bisected behind the limbus and placed in 4% paraformaldehyde at 4°C overnight and then cryoprotected with PBS (0.1 M phosphate buffer, pH 7.4, 0.15 M NaCl) containing 20% sucrose. Cryosections (10 μm) were mounted onto slides and incubated with blocking buffer (PBS containing 10% goat serum, 0.5% gelatin, 3% BSA, and 0.2% Tween 20) and then were incubated with a primary antibody against pAkt or cleaved caspase-3 and -9 (dilution 1:100; Cell Signaling Technology, Inc., Beverly, MA). Normal rabbit serum (DAKO, Osaka, Japan) was used as a negative control. The sections were then incubated with fluorescein-conjugated secondary antibody diluted 1:200 in blocking buffer for 1 hour. These included goat anti-rabbit immunoglobulin G conjugated to fluorescent dye (Alexa TM 546; Molecular Probes, Eugene, OR). Sections were mounted with mounting media with DAPI (Vectashield; Vector Laboratories, Burlingame, CA). Photomicrographs were taken with fluorescence microscopy (QFluoro System; Leica Microsystems, Wetzlar, Germany).

Immunofluorescence-positive cells in CNV were counted in the sections cut through the middle of the CNV lesions. The number of DAPI-positive cells in the CNV was defined as the number of total cells in the CNV per section. The rate of pAkt, cleaved caspase-3-positive, or cleaved caspase-9-positive cells in the CNV was measured by dividing the number of cells positive for these markers by the number of total cells in CNV. Cell counting was performed in a masked fashion.

**Terminal dUTP Nick-End Labeling**

TUNEL assay was performed as previously reported and Intergen, Purchase, NY) to detect cell death in CNV induced by PDT. Eyes were obtained at 1, 3, 5, 15, 24, and 48 hours after PDT, and transverse sections with CNV were prepared as mentioned. After two washes with PBS, sections were incubated with TdT enzyme at 37°C for 1 hour. The sections were washed three times in PBS for 1 minute and incubated with anti-digoxigenin conjugate (rhodamine) in a humidified chamber for 30 minutes at room temperature (RT), followed by three rinses with PBS at RT. Sections were mounted with mounting media (Vectashield; Vector Laboratories) including DAPI for nuclear stain. The number of TUNEL positive cells was determined.

The number of TUNEL-positive cells in CNV was counted in the sections that were cut through the middle of the CNV lesions. DAPI-positive staining was used to define the total number of cells in the CNV per section. The rate of TUNEL-positive cells in the CNV was determined by dividing the number of TUNEL-positive cells by the total number of cells in the CNV.

**Western Blot Analysis**

Western blot analysis was performed as described. In brief, after the RPE-choroid complex was sonicated in lysis buffer, 30 μg total protein was used for SDS-PAGE and then was transferred to a polyvinyl fluoride membrane (Bio-Rad, Hercules, CA). After blocking, the membrane was incubated in a blocking buffer containing rabbit anti-pAkt antibody or total Akt antibody (dilution 1:1000; Cell Signaling Technology, Inc.) overnight at 4°C. Chemiluminescence was detected with an alkaline phosphatase-conjugated anti-rabbit IgG (dilution 1:20,000: Promega Corp., Madison, WI) and with a chemiluminescent substrate (CDP-Star; Amersham Pharmacia Biotech, Buckinghamshire, UK). For loading control, the membrane was incubated with mouse β-actin (1:2000; Sigma-Aldrich, St. Louis, MO). The blot was exposed to medical x-ray film (RX-U; Fujifilm, Tokyo, Japan).

**Intravitreous Injection Procedure**

After rats were anesthetized and the pupils dilated, vitreous injections were performed as previously reported. Briefly, a 33-gauge needle tip was connected to the Hamilton syringe and inserted into the vitreous through a sclerotomy site 1 mm posterior to the corneal limbus of the eye. The tip of the needle was placed in the midvitreous, and 2 μL solution was slowly injected into the vitreous under direct observation with an operating microscope. Any subjects showing lens or retinal damage were excluded from the study.

**Experimental Design**

Apoptotic cells in CNV were detected by TUNEL assay at various time points after verteporfin PDT (verteporfin 3 mg/m²—6 and 24 hours; 6 mg/m²—1, 3, 6, 15, and 24 hours; verteporfin 12 mg/m²—1, 3, 6, 15, 24, and 48 hours) (n = 5 in each verteporfin dose at each time point).

Activation of caspase-3 and -9 in CNV was determined by IHC with antibody against cleaved caspase-specific antibodies at various time points (1, 3, 6, 15, and 24 hours) after PDT (verteporfin 12 mg/m²; n = 5 for each time point).

Akt activity in CNV was determined by IHC with a pAkt-specific antibody at various time points (1, 3, 6, 15, and 24 hours) after PDT (verteporfin 12 mg/m²; n = 5 for each time point).

Double staining for caspase-9 and TUNEL was performed at 3 hours after PDT. Double staining for pAkt and TUNEL was performed at 1 and 3 hours after PDT.

To investigate the role of Akt in PDT-induced apoptosis, 5 μg insulin-like growth factor (IGF)-1, an activator of Akt, with or without 12.5 μM wortmannin, an inhibitor of the PI3K-Akt pathway, was injected into the vitreous before PDT (verteporfin 12 mg/m²). IGF-1 was injected 1 hour before PDT, and wortmannin was injected 30 minutes before IGF-1 injection. Activation of Akt in RPE-choroid was determined by Western blot analysis with a pAkt-specific antibody 1 hour after PDT. Apoptotic cells in CNV were detected by TUNEL assay at 6 hours after PDT (n = 5 in each group). In TUNEL assay, 10-μg IGF-1 or 12.5-μM wortmannin injected groups were added.

Table 1 shows verteporfin dose and time after PDT for each aspect of the study.

**Statistical Analysis**

All values are presented as mean ± SD. Paired groups were compared using the paired t-test. For three groups, data were compared by ANOVA followed by post hoc comparisons tested using the Bonferroni procedure (Statview version 5.0; SAS Institute, Cary, NC). P < 0.05 was considered statistically significant.

**RESULTS**

**TUNEL-Positive Cells in CNV after PDT**

PDT was performed 2 weeks after laser-induced CNV. In control rats without PDT, TUNEL-positive cells were not seen in...
CNV. In PDT-treated rats, few TUNEL-positive cells could be recognized 1 hour after PDT. The rate of TUNEL-positive cells per total cells in CNV increased 3 hours after PDT, peaked at 6 hours, and then decreased by 48 hours, showing dose dependence with respect to verteporfin dose (Fig. 1). Six hours after PDT, the rate of positive cells in CNV was 72.5% ± 12.5% at the doses of 12 mg/m².

### Cleaved Caspase-3 and -9 Immunoreactivity

To investigate whether caspase was involved in PDT-induced apoptosis, IHC with anti-cleaved caspase-3 and -9 was performed 1, 3, 6, 15, and 24 hours after PDT. In PDT-treated rats, several cleaved caspase-3–positive cells were recognized 1 hour after PDT. The number of positive cells in CNV peaked at 3 hours and then decreased by 24 hours (Figs. 2A–D). The rate of positive cells in CNV was 19.0% ± 2.4% at 3 hours after PDT. The number of cleaved caspase-9–positive cells in CNV also peaked at 3 hours after PDT and then decreased by 24 hours (Figs. 2E–H). The rate of positive cells in CNV was 26.8% ± 9.6% at 3 hours after PDT. These data suggest that caspases are activated after PDT and are earlier events preceding TUNEL positivity.

<table>
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<tr>
<th>Verteporfin Dose</th>
<th>TUNEL after PDT</th>
<th>IHC</th>
<th>Western Blot Analysis</th>
<th>TUNEL after PDT + Vitreous Injection</th>
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**Table 1. Verteporfin Dose and Time after PDT for Each Phase of the Study**
Double Staining of Cleaved Caspase-9 and TUNEL

To determine whether cleaved caspase-9–positive cells are committed to apoptosis, double staining of cleaved caspase-9 and TUNEL was performed 3 hours after PDT (verteporfin 12 mg/m²). Some cells stained by DAPI in CNV were characterized by the presence of condensed and fragmented nuclei (Fig. 3A, arrows). These cells colocalized with TUNEL-positive signal (Figs. 3B, 3C). Most of the cleaved caspase-9–positive cells were TUNEL positive (Fig. 3E). These results suggest that PDT induced caspase-dependent apoptosis in CNV.

Double Staining of pAkt and TUNEL and Time Course of pAkt Immunoreactivity

To investigate further the signaling pathway after PDT, we examined whether the Akt signaling pathway was activated after PDT and served as an upstream regulator of the caspase pathway. Without PDT treatment, nearly all cells in CNV were immunoreactive for pAkt with no TUNEL positivity (Figs. 4A, 4D, 4G, 4J). One hour after PDT, the number of pAkt-positive cells was significantly reduced ($P < 0.01$; Fig. 4B), and the rate of pAkt-positive cells in CNV was 50.3% ± 16.8% at 1 hour after
PDT, suggesting that Akt was significantly dephosphorylated 1 hour after PDT in CNV. However, at this time point, there were few TUNEL-positive cells in CNV (Fig. 4E). Three hours after PDT, though the number of TUNEL-positive cells increased, the number of pAkt-positive cells was still significantly reduced compared with controls (Fig. 4C). Interestingly, TUNEL signals and pAkt signals were seldom colocalized in cells of the PDT-treated CNV (Fig. 4I). These results strongly suggest that Akt is first dephosphorylated by 1 hour after PDT, followed by activation of caspase-9 and -3 by 3 hours after PDT, with eventual apoptosis. The rate of pAkt-positive cells in CNV reached a minimum (14.2% ± 11.5%) 6 hours after PDT and returned to 74.8% ± 13.8% by 48 hours (Fig. 4J).

**FIGURE 4.** Double staining of pAkt and TUNEL. (A, D, G) Control PDT. (B, E, H) One hour after PDT. (C, F, I) Three hours after PDT. (A) Almost all cells in CNV showed phosphorylated Akt-positive in controls. (D) There are no TUNEL-positive cells in controls. (G) Merged images of pAkt and TUNEL. (B) The number of phosphorylated Akt-positive cells was significantly reduced 1 hour after PDT. (E) There are few TUNEL-positive cells 1 hour after PDT. (H) Merged images of pAkt and TUNEL. (C) The number of pAkt-positive cells was still low 3 hours after PDT. (F) There are some TUNEL-positive cells in CNV at 3 hours after PDT. (I) Merged images of pAkt and TUNEL. pAkt-positive cells in CNV do not stain with TUNEL. (J) Time course of the number of pAkt and TUNEL-positive cells. *P < 0.01 compared with control (pAkt). Dotted line: CNV lesion. Scale bar, 50 μm.

**Western Blot Analysis**

To confirm quantitatively that Akt in RPE-choroid is dephosphorylated after PDT, the RPE–choroid complex was harvested 1 hour after PDT, and protein levels of pAkt and total Akt were examined by Western blot analysis. As shown in Figure 5A, the RPE–choroid complex treated with PDT had a sparser pAkt band (molecular weight, approximately 60 kDa) than controls, whereas the total Akt band showed a similar signal density for the two conditions. Thus, PDT changed the level of phosphorylation but not the amount of Akt protein. To investigate the role of the Akt pathway in PDT-induced apoptosis, we attempted to modify the phosphorylation of Akt in the RPE–choroid complex with intravitreal injection of IGF-1, an activator of Akt. One hour after PDT with IGF-1 injection, PDT-induced dephosphorylation of Akt was significantly suppressed (Fig. 5B). The effect of IGF-1 was diminished by wortmannin, an inhibitor of the PI3K-Akt pathway (Fig. 5B), without affecting the level of Akt protein. These data suggest that PDT-induced Akt dephosphorylation is reversed by intravitreal injection of IGF-1 through PI3K activation.

**FIGURE 5.** Western blot analysis of phospho-Akt. PDT reduced the level of phosphorylation of Akt in RPE-choroid complex, whereas total Akt level was similar between them 1 hour after PDT (A). Injection of IGF-1 (5 μg) activated Akt in RPE-choroid 1 hour after PDT. Effect of IGF-1 on phosphorylation of Akt was diminished by wortmannin (B).

**TUNEL Assay after PDT with Activation of Akt**

Next, we performed TUNEL assay after PDT using the same experimental conditions (Fig. 5). Rates of TUNEL-positive cells in IGF-1–treated rats were 40.9% and 31.4% (5 μg and 10 μg IGF-1, respectively) 6 hours after PDT (Figs. 6B, 6C). They were significantly lower than those of the PDT-only treated group (P < 0.01). The effect of IGF-1 (5 μg) on the rate of TUNEL-positive cells in CNV was significantly diminished by wortmannin (P < 0.05; Figs. 6D, 6E). However, there was no significant difference in the number of TUNEL positive cells between PDT-only treated rats and wortmannin-only injected rats 6 hours after PDT (Fig. 6E). These results suggest that Akt activation reduced apoptosis in CNV after PDT.

**DISCUSSION**

PDT is a clinically effective treatment for AMD; however, the mechanism of PDT-induced cellular apoptosis is largely unknown. With the use of a rat model of PDT for laser-induced CNV, we characterized the signaling pathways leading to cell death after PDT. One hour after PDT, Akt in the cells of CNV was dephosphorylated; this was followed by caspase activation at 3 hours. The number of TUNEL-positive cells increased by 6 hours. Western blot analysis showed that the level of pAkt protein in the RPE–choroid complex was significantly decreased after PDT, indicating dephosphorylation but not degradation of Akt protein. Intravitreal injection of IGF-1 before PDT led to activation of the Akt protein in the RPE–choroid complex and reversed the PDT-induced dephosphorylation of Akt in the CNV through PI3K activation. Furthermore, IGF-1 suppressed the increase in TUNEL-positive cells in CNV after PDT. Thus, we have demonstrated that the suppression of Akt activity and the activation of caspase may play an important role in the death of cells in CNV after PDT.
Phosphorylated Akt strongly suppresses mito-
supported by previous reports that PDT with verteporfin in-
signals in the same cells occurred after PDT. These data are
cells and that colocalization of TUNEL positivity and caspase
Caspase 3, a downstream effector of caspase 9, executes cel-
dria after injury and results in the activation of caspase 9.
Generally, apoptotic cell death pathways are classified as
caspase dependent or caspase independent.22 In the caspase-
dependent pathway, cytochrome c is released from mitochon-
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activation occurred earlier than peak TUNEL positivity in
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supported by previous reports that PDT with verteporfin in-
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Bad.14,23 We did not investigate whether a caspase inhibitor
suppressed PDT-induced cell death in CNV; however, IGF-1, a
hormone and decreased by 48 hours. The variations in time
course may be related to cell- and tissue-specific differences. It is well
known that PDT causes direct activation of free radical-mediated
cell injury and secondary formation of thrombi, leading to
vessel occlusion. We examined TUNEL staining 3 and 7 days
after PDT at the verteporfin doses of 12 mg/m² in considera-
tion of that point. Three days after PDT, there were few
TUNEL-positive cells in CNV like the results 48 hours after PDT. No TUNEL-positive cells were observed in CNV 7 days
after PDT. We speculate that the critical time is up to 2 days
after PDT. Moreover, we have shown that PDT-induced throm-
bus already occurred 24 hours after PDT.17 Although it is
difficult to define which effect induces individual apoptosis,
further studies defining specific cell types that undergo apo-
tosis and the time courses after PDT may clarify that point.

To the best of our knowledge, an apoptosis time course
after verteporfin PDT in rat laser-induced CNV has not been
previously reported. Granville et al.6 reported that DNA frag-
mentation in cultured human aortic smooth muscle cells was
observed by 1 hour after PDT with verteporfin and that more
than 40% of cells exhibited DNA fragmentation by 5 hours after
PDT. In this rat study, the number of TUNEL-positive cells
showed an increase at 3 hours after PDT, peaked at 6 hours,
and decreased by 48 hours. The variations in time course may
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suppressed PDT-induced cell death in CNV; however, IGF-1, a
specific upstream activator of the Akt signaling pathway, sup-
pressed the immunoreactivity of cleaved caspase-9 in CNV
(data not shown) and suppressed TUNEL-positive cells after
PDT (Fig. 6). Taken together, caspase activation appears to be the
central mediator for verteporfin PDT-induced cellular apo-
tosis in CNV.

Many studies have demonstrated that VEGF is expressed in
human and experimental CNV.24,25 VEGF regulates endothelial
cell survival through the PI3K-Akt signaling pathway in angio-
genesis.12,26 On the other hand, phophatase and tensin homologue deleted on chromosome (PTEN) phosphatase is known as a major negative regulator of
the PI3 kinase/Akt signaling pathway.27 PTEN inhibits PI3K-
dependent activation of AKT, and deletion or inactivation of
PTEN results in constitutive AKT activation. Akt plays a critical
role in controlling survival and apoptosis by the balance of
signals between kinase and phosphatase. Schmidt-Erfurth et
al.4 reported that VEGF was detected in endothelial cells of the
choriocapillaris after PDT. Solban et al.28 reported that subcu-
rative PDT induced VEGF in prostate cancer cells, and this
induction may contribute to tumor survival and regrowth and,
therefore, may be responsible for limiting the efficacy of PDT
for tumors. Recently, the suppression of PDT-induced inflam-
mation using steroid appears to improve the therapeutic effi-
cacy of PDT for AMD.29,30 In our study, Akt phosphorylation
was significantly suppressed by PDT within 24 hours; how-
ever, the rate of pAkt-positive cells in CNV returned to
the baseline level by 48 hours. Thus, if PDT for CNV induces
inflammation- and inflammatory-related cytokines such as
VEGF and TNF, it may affect Akt phosphorylation and may lead
in turn to cell survival and to CNV recurrence. Further inves-
tigations are needed to seek the upstream factors that control
PDT-induced dephosphorylation of Akt in CNV.

Our next interest is to define the specific cell types that
undergo apoptosis in CNV after PDT. The main target of PDT
for CNV is endothelial cells. In our other experiment using
human umbilical vascular endothelial cells, p-Akt was dephos-
phorylated and apoptosis was induced after PDT. Hence, we
speculate that vascular endothelial cells in CNV might induce
caspase-dependent apoptosis after PDT. Further investigations
will be needed to define the specific cell types and pathway
that undergo apoptosis in CNV using immunohistochemical
double staining in vivo.

In conclusion, we have begun to delineate the molecular
mechanisms of PDT-induced cellular apoptosis in laser-induced
CNV. Targeting Akt and caspase signaling pathways may im-

**FIGURE 6.** TUNEL staining in CNV 6 hours after PDT (A–D). Rate of TUNEL-positive cells in CNV (E). (A) PDT only. (B) IGF-1 (5 μg) + PDT. (C) IGF-1 (10 μg) + PDT. (D) Wortmannin (12.5 μM) + IGF-1 (5 μg) + PDT. IGF-1 significantly reduced the number of TUNEL-positive cells in CNV, and the effect of IGF-1 was diminished by wortmannin (E). *P < 0.01 compared with PDT-treated rats. †P < 0.05 compared with IGF-1 (5 μg) + PDT-treated rats. Dotted line: CNV lesion. Scale bar, 50 μm.
prove the efficiency of PDT on CNV by increasing cellular apoptosis in CNV cells. These findings may yield new insight into the development of strategies for the treatment of PDT for patients with AMD.

References