Sensitivity of Photoreceptor-Derived Cell Line (661W) to Baculoviral p35, Z-VAD.FMK, and Fas-Associated Death Domain

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Purpose. Rod, cone, cone–rod, and macular dystrophies eventually bring about the death of cone photoreceptor cells. The present study explores means of inhibiting apoptosis in addition to inducing a specific apoptotic pathway within a photoreceptor-derived cell line.

Methods. Retinal cell culture of murine 661W photoreceptor-derived cells was used to assess the effect of both a synthetic peptide inhibitor of caspases (benzyloxycarbonyl-Val-Ala-DL-Asp-[Ome] fluoromethylketone [Z-VAD.FMK]) and a natural inhibitor, baculoviral p35. In addition, the effect of transfection of Fas-associated death domain (FADD), a cellular protein implicated in receptor-induced apoptosis, was assessed. Assays were performed by transient transfection of cell cultures, and results were recorded by cell counting, Western blot, and spectrophotometry.

Results. Western blot analysis and chromogenic caspase substrate cleavage analysis confirmed the activation of caspases within 661W cells. At a concentration of 80 μM, Z-VAD.FMK, 72.36% ± 0.93% of 661W cells survived cytotoxic insult compared with 6.99% ± 1.35% of control cells. Transient transfection of 1200 ng baculoviral p35 conferred a protection of 75.30% ± 4.23%, compared with 19.61% ±1.84% of control cells, and it was additionally observed that as little as 50 ng transfection of FADD was capable of inducing the death of 53.21% ± 1.33% of cells in 661W cultures.

Conclusions. Apoptotic cell death in 661W cells is caspase dependent and may be inhibited with both a synthetic and natural inhibitor of caspase function. Furthermore, 661W cells are highly sensitive to the FADD protein, which may suggest a number of novel therapeutic approaches to halt photoreceptor cell apoptosis. (*Invest Ophthalmol Vis Sci.* 2002;43: 3583–3589)

Rod and cone photoreceptor cells of the mammalian retina represent fully differentiated photoreactive neurons derived from the internal neuroepithelium of the optic cup.[1,2] Within the entire human retina, rods outnumber cones by approximately 20:1[3]; however, it is the loss of cones in patients with retinal degenerative disorders, such as cone dystrophies, cone–rod degenerations, macular dystrophies, and retinitis pigmentosa (RP), that is frequently the source of the more severe functional, social, and psychological stress associated with the later stages of the disorders. The loss of cone cell function has obvious medical consequences, and it is therefore of significant interest to investigate the means by which cone photoreceptor longevity may be extended. In addition, although several degenerative retinopathies may originate from mutations within rod photoreceptor cells, the real and immediate clinical difficulty for such patients is tantamount to a cone cell dystrophy. Consequently, studies of cone photoreceptor cells may provide critical information and potential novel lines of intervention for the treatment of a range of retinal disorders.

661W cells represent a genetically engineered dispersed cell culture used in the course of these studies for the investigation of apoptotic cell death and its inhibition. Originally transformed and kindly provided by Muayyad Al-Ubaidi (University of Illinois, Chicago, IL), the 661W cell line was cloned from retinal tumors of a transgenic mouse line expressing the SV-40 T antigen under the control of the interphotoreceptor retinal binding protein promoter (IRBP).[4]

Despite their highly transformed state, 661W cells have been shown to express several markers of photoreceptor cells and to be sensitive to oxidative stress similar to observations recorded in normal retinal photoreceptor cells.[5–7]

Apoptosis, in the context of retinal degeneration, has been well established,[8–10] and several studies in animal systems in which antiapoptotic approaches were taken have met varying degrees of success.[11–16] Despite this, there are relatively few insights into the molecular pathways of apoptotic cell death that occurs within the retina of experimental animal models.

In this study, two potentially antiapoptotic agents, benzyloxycarbonyl-Val-Ala-DL-Asp- [Ome] fluoromethylketone (Z-VAD.FMK)[17] and baculoviral p35[18] were used to investigate their capacity to interfere with the process of cell death. Z-VAD.FMK is a synthetic peptide inhibitor of the caspase family of proteases responsible for mediating apoptotic cell death, whereas p35 is a natural inhibitor of caspase function originally isolated through its ability to prevent apoptosis in virus-infected insect cells.[18] Each of these agents has been shown to be a successful inhibitor of cell death in a diverse range of contexts.[19–23]

There are several distinct pathways through which apoptosis may be triggered, and the identification of such pathways in operation in cells may reveal therapeutically relevant targets.[24] One of the best characterized membrane receptor pathways leading to apoptosis is the Fas/CD95/APO-1 system.[25] Fas and Fas ligand play important roles in the downregulation of the immune response, in the killing of virally infected and cancerous cells, and in the killing of inflammatory cells at immune-privileged sites such as the eye, brain, placenta, ovary, and testis.[26,27] Fas ligand is a homotrimeric molecule that may bind the Fas receptor, causing the clustering of the receptors' death domains. The clustering facilitates the recruitment of an adaptor protein called Fas-associated death domain (FADD) which itself contains a death-effector domain (DED) that in turn binds caspase-8 directly.[28] The oligomerization of caspase-8 may then drive its own activation by self-cleavage followed by the activation of downstream effector caspases such as procaspase-9 amplifying the signal and committing the cell to an apoptotic

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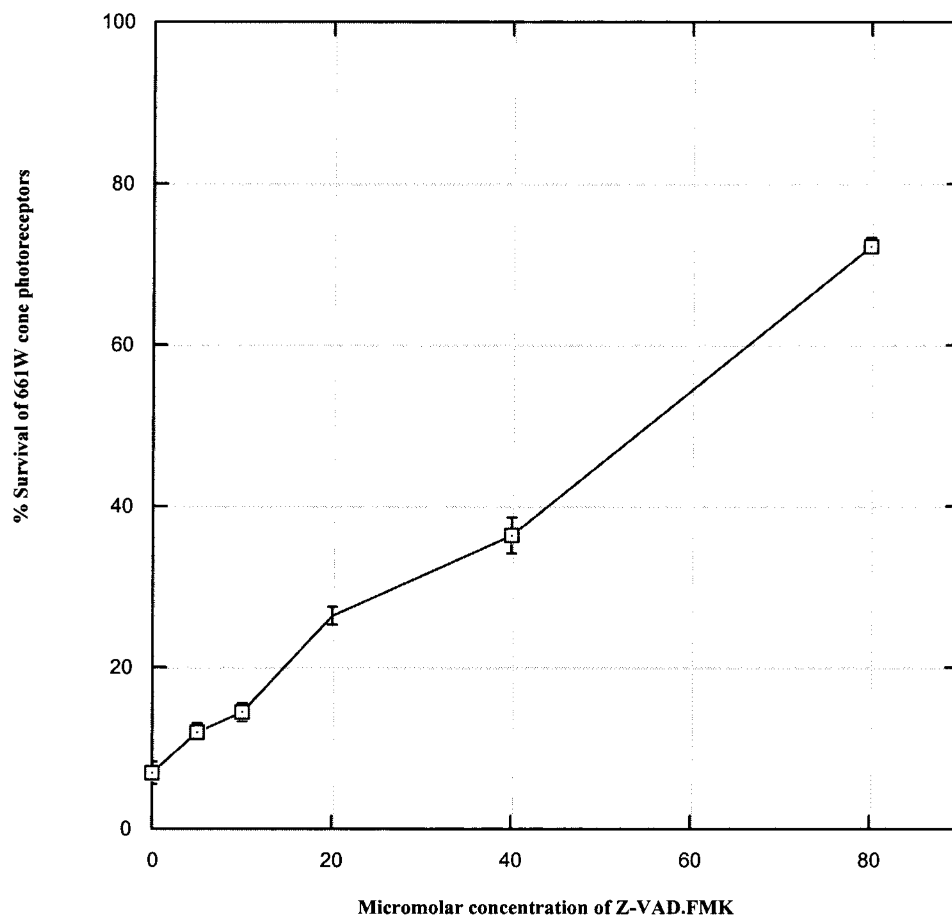

Figure 1. Percentage survival of 661W cone photoreceptors after cytotoxic insult with 2.5 μM daunorubicin.

fate.[28] Transfection of 661W cells with ectopically expressed FADD should indicate the susceptibility of this cell line to ligand-induced cell death.

Materials and Methods

Culturing and Passaging

661W cells grow readily, with a doubling time of approximately 24 hours, in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) fetal calf serum (FCS) and 2 mM L-glutamine. Cultures were maintained in a sterile humidified environment at 37°C in 95% O_2 and 5% CO_2. In most assays we used cultures seeded in a six-well plate at a concentration of 1×10^5 cells in a volume of 3 mL growth media expanded to approximately 85% to 90% confluence.

Inhibition of 661W Cell Death with Z-VAD.FMK

To test the potential effects of Z-VAD.FMK, cultures of 661W cells were prepared as described, seeded at 1×10^5, and allowed to proliferate to approximately 85% confluence. Medium was removed, and cells were washed twice in PBS before incubation with 2× Z-VAD.FMK (double the relevant concentration, to allow for subsequent dilution with daunorubicin) at the appropriate concentration. For each Z-VAD.FMK concentration (0, 5, 10, 20, 40, and 80 μM) three cultures of 661W cells were tested in duplicate (i.e., six wells in total). After a 1-hour preincubation with 2× Z-VAD.FMK, cells were diluted in medium containing 2 × 2.5 μM daunorubicin (double the relevant concentration to allow for volume present) and allowed to incubate overnight before plates were scored for live versus dead cells.

Western Blot Analysis of Caspase-3 in 661W Cell Lysates

Cultures of 661W cells were exposed to a variety of established cytotoxic agents, including 50 μM cycloheximide, 2.5 μM daunorubicin, 200 μM etoposide, 50 ng/μL TNFα, and 90 seconds of UV irradiation and were harvested after 18 hours. A standard protein assay (Bio-Rad, Herts, UK) was used to determine protein concentrations in samples of control and apoptotic 661W cells. Cells were harvested in 200 μL lysis buffer (150 mM NaCl; 20 mM Tris [pH 7.5] 1% Triton-X-100 with protease cocktail inhibitor: 1 mM phenylmethylsulfonyl fluoride [PMSF], 0.1 μg leupeptin, and 2 μg/mL aprotinin) and rested on ice for 5 minutes before appropriate dilution in 1× SDS buffer. β-Mercaptoethanol (5%) and 1 μL bromophenol blue (1% wt/vol) was added, samples were boiled for 5 minutes and electrophoresed on an 8% to 15% SDS polyacrylamide denaturing gel at 100 mV and 40 mA for 2 hours, followed by transfer to PVDF membranes at 40 mA overnight. Blots were blocked in 5% TBS Tween-milk (5% dried milk powder; 10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.05% Tween) and incubated with the appropriate primary antibody for 1.5 hours, washed in TBST (10 mM Tris-HCl [pH 8.0] 150 mM NaCl, 0.05% Tween) and incubated in 5% TBS Tween-milk with the appropriate secondary horseradish peroxidase (HRP)–labeled antibody for 1 hour. Blots were washed three times in TBST, and antibody labeling was detected by enhanced chemiluminescent substrate detection (Pierce Biochemicals, Rockford, IL) of HRP, with 500 μL chemiluminescent substrate (Luminol; Pierce) mixed with 500 μL stable peroxide solution and incubation with blots for 5 minutes at room temperature before autoradiography.

Ac-DEVD-pNA Chromogenic Substrate Cleavage Assays in 661W Cells

To induce cell death for chromogenic substrate cleavage assays, daunorubicin (bright red) was replaced with etoposide (colorless) to avoid absorbance interference in spectrophotometric analysis. After cytotoxic insult with 200 μM etoposide, samples of 661W cells at intervals of 0.5, 3, 6, 9, and 12 hours were washed in serum-free medium, trypsinized, and centrifuged at 1094*g* for 5 minutes at 4°C. Cells were resuspended in 50 μL lysis buffer (150 mM NaCl, 20 mM Tris [pH 7.5] and 1% Triton-X-100 with protease cocktail inhibitor: 1 mM PMSF, 0.1 μg leupeptin, and 2 μg/mL aprotinin) rested on ice for 10 minutes, and added to 50 μL 2× protease reaction buffer (100 mM HEPES [pH 7.4] 150 mM NaCl, 0.2% 3-([3-cholamidopropyl]dimethylammonio-2-hydroxy-1-propanesulfonate [CHAPS] with dithiothreitol [DTT] added separately) and 10 μL of 10 × 10 mM Ac-Asp-Glu-Val-Asp-*p*-nitroaniline (Ac-DEVD-pNA; Bachem Chemicals, Heidelberg, Germany) chromogenic substrate. Samples were incubated at 37°C for 60 minutes followed by the addition of 900 μL ice-cold H_2O before spectrophotometric analysis.

Transfection of 661W Cells with β-Galactosidase, Baculoviral p35, and FADD

Cells were transiently transfected using commercially available lipid-based transfection reagents (Lipofectamine-Plus; GibcoBRL, Paisley, Scotland, UK). Transfection assays were performed with three constructs, using the pCDNA3.0 vector (Invitrogen, San Diego, CA)—cytomegalovirus (CMV) promoter–driven β-galactosidase, CMV promoter–driven p35, and CMV promoter–driven FADD—using stock concentrations of plasmid constructs at 500 ng/μL. Cells were cultured to approximately 1×10^6, washed in PBS, and incubated for 3 hours in 0.8 mL serum-free medium at a ratio of 1:3:12 μL transfection mix (DNA, Lipofectamine, Lipofectamine-Plus; GibcoBRL), according to the manufacturer's instructions. Transfection efficiencies were determined by conducting a simple assay to detect the activity of β-galactosidase.

Results

Inhibition of 661W Cell Death by Z-VAD.FMK

The results (Fig. 1) demonstrated that at the lowest concentration of Z-VAD.FMK (5 μM) there was a significant effect on the survival of 661W cells undergoing apoptotic cell death induced by cytotoxic insult. The survival percentage at 5 μM nearly doubled that of the control (6.99% ± 1.35% for the control vs. 12.01% ± 0.98% for cells incubated with 5 μM Z-VAD.FMK). The concentration of Z-VAD.FMK required to inhibit apoptotic cell death by 50% in vitro was between 40 and 80 μM. At the highest concentration of 80 μM Z-VAD.FMK, 72.36% ± 0.93% of 661W cells were protected from apoptotic cell death. It was additionally observed that the protection provided by Z-VAD.FMK at 20 and 40 μM was readily detectable by the unaided eye. The potency of apoptosis inhibition by preincubation with Z-VAD.FMK strongly implicates the presence and involvement of caspases in the programmed cell death of 661W cells in this in vitro system.

Western Blot Analysis of Caspase Activation in 661W Cells Undergoing Apoptotic Cell Death

An important indicator of apoptotic cell death is the processing of inactive caspase zymogens to their active conformations. Figure 2 demonstrates the disappearance of the intact inactive 32-kDa caspase-3 zymogen from samples induced to undergo apoptotic cell death, whereas in the control samples, the unprocessed caspase-3 protein is clearly present.

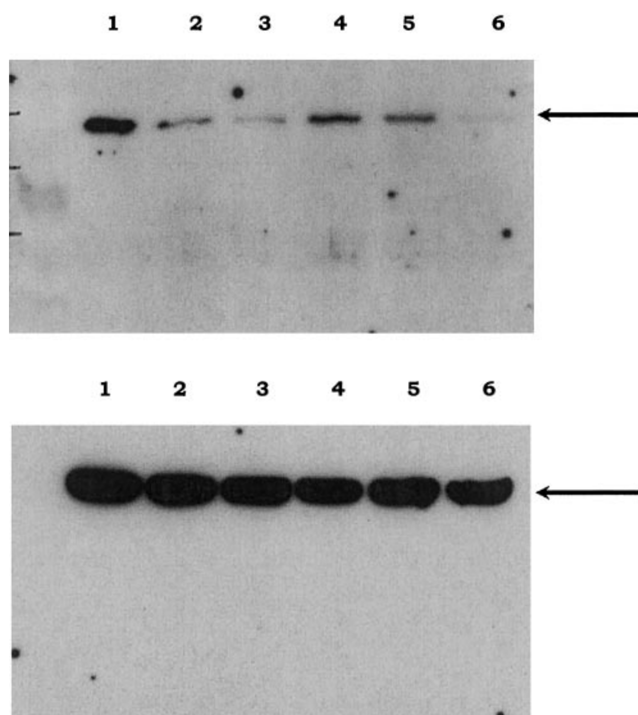

Figure 2. Western blot analysis on cytosolic fractions of 661W cone photoreceptors after cytotoxic insult with a variety of standard cell death agents. *Lane 1*: control; *lane 2*: cycloheximide (50 μM); *lane 3*: daunorubicin (2.5 μM); *lane 4*: etoposide (200 μM); *lane 5*: TNFα; *lane 6*: UV irradiation. *Top*: probed with murine anti-caspase3 antibody (dilution 1:500). *Arrow*: 32 kDa. *Bottom*: duplicate blot probed with murine anti-actin antibody (dilution 1:2000). *Arrow*: 50 kDa.

Detection of Caspase-3 Activity in 661W Cells by a Chromogenic Ac-DEVD-pNA Cleavage Assay

Additional confirmation of caspase-3 processing within 661W cells was obtained by exposing cell lysates to a chromogenic tetrapeptide substrate comprising the preferred target amino acid sequence of caspase-3. Figure 3 shows the increase over time of Ac-DEVD-pNA cleavage. Spectrophotometric analysis of samples incubated for 60 minutes with the chromogenic tetrapeptide substrate show an increase from 0.0843 ± 0.0015 to 0.1717 ± 0.0031 arbitrary units (AU) when assayed at a wavelength of 400 nm. The hydrolysis of Ac-DEVD-pNA was almost entirely inhibited by the presence of 50 μM Z-VAD-.FMK, as may be observed from the values recorded when Z-VAD.FMK was added to the system. Spectrophotometric analysis revealed a signal detection ranging from 0.0857 ± 0.0032 to 0.0967 ± 0.0021 AU, which is comparable to control samples in which no substrate cleavage was detected. These results clearly indicate the presence and activity of caspase-specific proteases and the inhibition of such activity with a highly specific caspase inhibitor, Z-VAD.FMK.

Transfection of the CMV-Driven p35 Gene, a Pancaspase Inhibitor, into 661W Cells

Resistance of 661W cells to daunorubicin-induced cell death was tested by quantifying cell survival in cultures transfected with p35 DNA, compared with vector-transfected cultures alone. Ectopic expression of the p35 gene in a variety of cell types has indicated a strong protective effect from apoptotic cell death. Various concentrations of the p35 gene were transfected into 661W cells. On average (calculated across the three concentrations of p35 transfected) the presence of p35 in

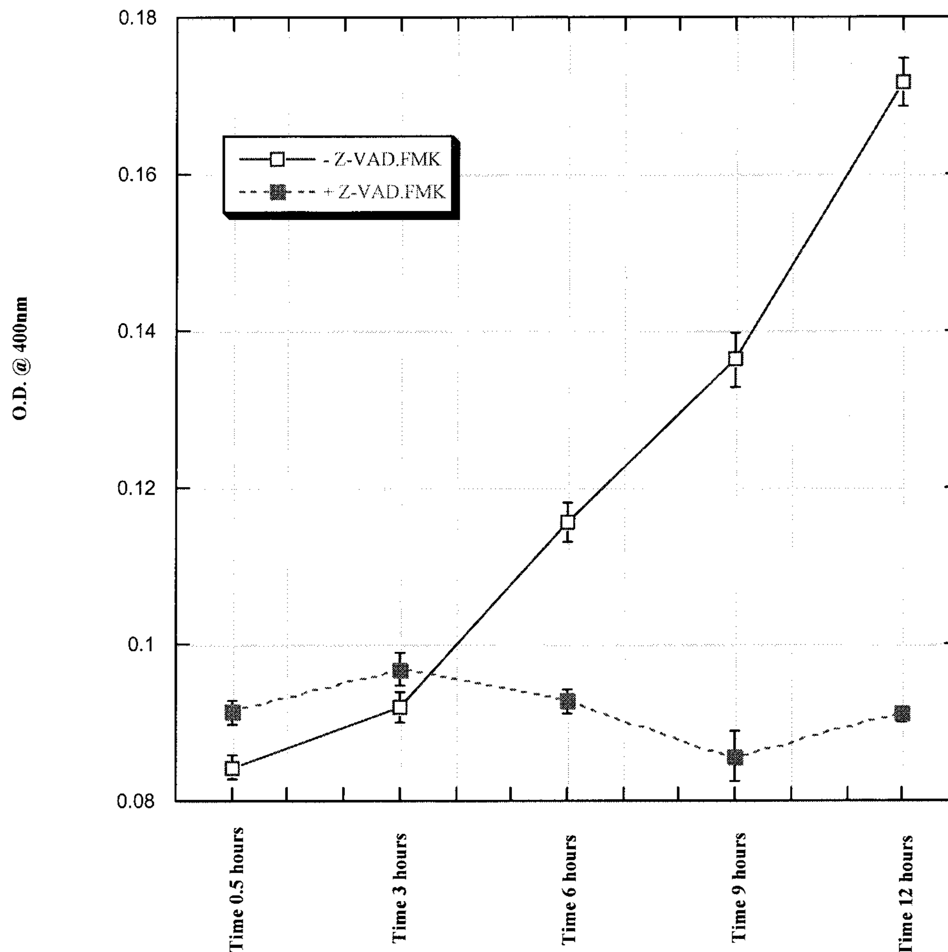

FIGURE 3. Ac-DEVD-pNA assay, with and without 50 μM Z-VAD.FMK, performed on cytosolic fractions of apoptotic 661W cone photoreceptors. O.D., optical density.

661W cells provided a percentage survival of 69.41% ± 5.61%, in comparison with cultures transfected with vector alone at 19.61% ± 1.84% survival. This represents a rescue from apoptotic cell death beyond that observed in control cultures of more than 49.80%, providing a significant level of protection from cytotoxic insult. p35 is known to act as an irreversible inhibitor (or very slowly reversible) of caspase-1, -3, -6, -7, -8, and -10, preventing the cleavage of key infrastructural cell components in addition to suppressing the amplification of caspase signaling.[29] These results of 64.15% ± 1.8% survival with 400 ng p35, 68.77% ± 1.55% with 600 ng p35, and 75.30% ± 4.23% with 1200 ng p35 (Fig. 4), indicate a positive protective effect deriving from the presence of the p35 transgene.

Transfection of 661W Cells with CMV-Driven FADD

To determine whether the caspase-8–specific pathway of apoptotic cell death in 661W cells could be driven by genetic transfection, cells were transfected with various amounts of the FADD gene construct under the control of the CMV promoter. As the results indicate (Fig. 5), FADD was a potent inducer of cell death in 661W cells. A significant potency was observed, even at concentrations as low as 50 ng FADD DNA. The incidence of apoptotic cell death in the 661W cells ranged from 53.21% ± 1.33% when transfected with 50 ng FADD to 89.92% ± 0.8%, when transfected with 1200 ng of FADD DNA. These results suggest that the caspase-8–mediated cell death pathway is intact and functional within 661W cells.

DISCUSSION

Caspase-3 has been consistently demonstrated to orchestrate a key role in the deconstruction of mammalian cells undergoing apoptotic cell death.[24] A number of studies[30,31] have suggested that apoptotic cell death in the retina may be mediated by a caspase-independent mode of cell death, although, in contrast, other reports have suggested that caspases are active.[32] Because such findings would carry significant implications for the design, delivery, and timing of therapeutic intervention, it would be of value to determine the status of caspases within 661W cells and to establish whether such cell death machinery is present and intact functionally.

Z-VAD.FMK is a synthetic caspase substrate designed on the basis of preferred amino acid sequence structure with an aspartate residue in the P1 position.[33,34] In studies conducted with 661W cells a concentration as low as 5 μM Z-VAD.FMK was shown to have a beneficial effect (12.01% ± 0.98% survival) on the percentage of 661W cells capable of resisting apoptotic cell death as induced by the addition of 2.5 μM daunorubicin. At a concentration of 80 μM Z-VAD.FMK, 661W cells demonstrated a resistance to cytotoxic insult of 72.36% ± 0.93%. These results indicate that caspases are active in this photoreceptor-derived cell line, that their action may be inhibited, and that the dose-dependent nature of Z-VAD.FMK inhibition suggests that the tetrapeptide substrate behaves as a competitive decoy in this system. Furthermore, the cell-permeable nature of this tetrapeptide inhibitor supports previous studies,[32] demonstrating that peptide inhibition of caspase

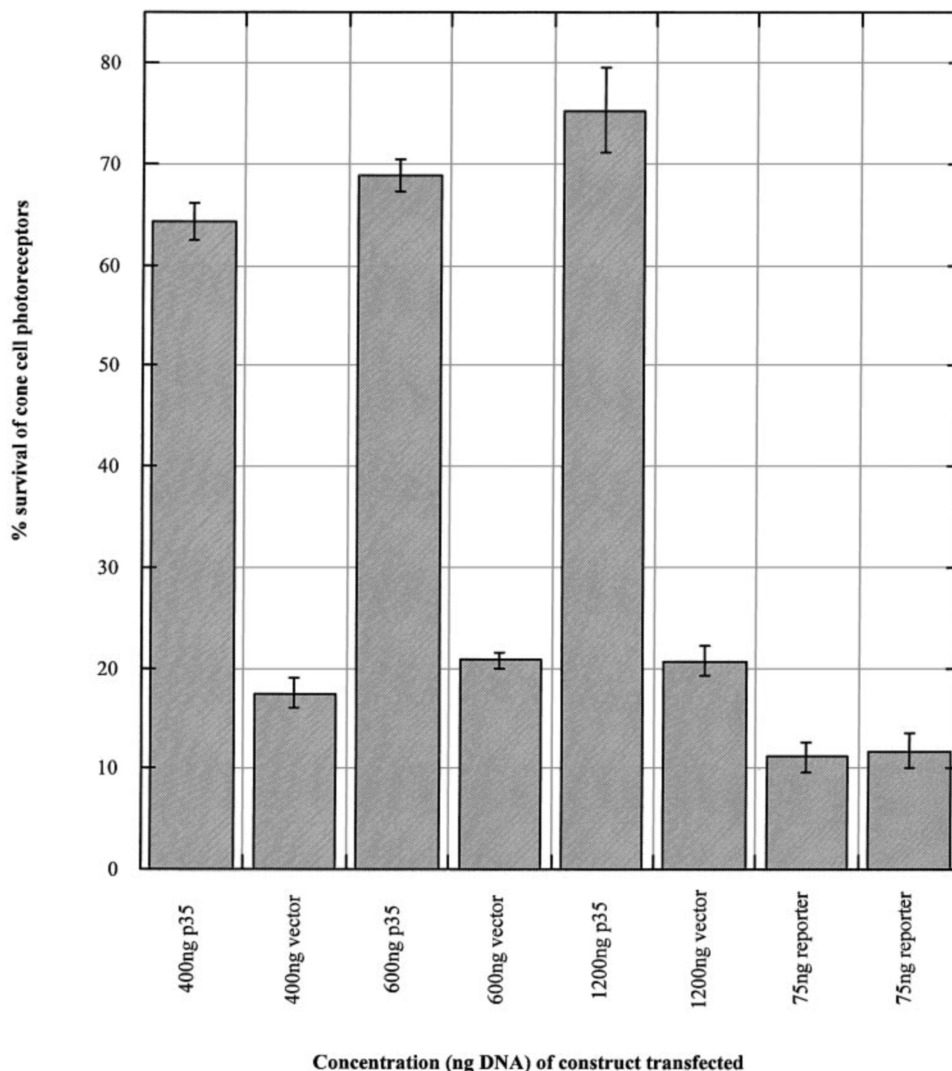

FIGURE 4. 661W cone photoreceptor cell rescue from apoptotic cell death by p35 transfection after cytosolic insult.

activity may provide similarly valuable protection within an animal system if delivered intravitreously or subretinally.

To determine directly the presence and activity of caspase-3 in 661W cells two specific experiments were performed. The first experiments involved the preparation of cytosolic fractions from 661W cell cultures followed by electrophoretic migration and detection with a commercially available murine caspase-3 antibody. Procaspase-3 exists as a 32-kDa zymogen in the cytosol of healthy cells and, when stimulated to die by apoptosis, is cleaved to 17- and 12-kDa subunits. As may be observed in Figure 2, the control sample in lane 1 shows a completely intact 32-kDa fragment in comparison with adjacent lanes that exhibit various degrees of disappearance of the 32-kDa intact fragment. There was no detection of the p17 or p12 fragments, possibly because of proteolytic degradation. To confirm the suggestion of this observation, a second series of experiments were performed in which the ability of cytosolic fractions (prepared from control and apoptotic induced 661W cells) to cleave a chromogenic substrate specific to caspase-3 cleavage was assessed. Ac-DEVD-pNA is an artificial chromogenic peptide substrate that replicates the preferred cleavage amino acid sequence for caspase-3. Cleavage of the peptide is detected by the hydrolytic release of pNA and is subsequently detected spectrophotometrically at a wavelength of 400 nm. As may be observed in Figure 3, there was a significant increase in the detection of the chromogenic cleavage products in 661W samples induced to undergo apoptotic cell death, compared with control samples. The signal detected increased from 0.0843 ± 0.0013 AU in the control sample to 0.1717 ± 0.0031 AU in samples assayed 12 hours after cytotoxic insult. This represents an increase in activity of 98%, strongly indicating the presence of functional activated caspases within 661W cells. Additional experiments performed under precisely equivalent conditions, but including 50 μM Z-VAD.FMK, demonstrated the reverse result—that is, a detection of the chromogenic signal comparable to that observed in controls receiving no cytotoxic insult. These results, when combined, suggest that in 661W cells induced to undergo apoptotic cell death through the addition of 2.5 μM daunorubicin, caspases are present and activated in addition to being capable of inhibition by the caspase antagonist Z-VAD.FMK.

Despite the highly proliferative state of 661W cells expression of photoreceptor-specific markers in the 661W cell line has been observed.[5–7] Consequently, such cell cultures may provide a highly useful system for testing hypotheses before in vivo (animal) testing. If similar apoptotic pathways are in operation within photoreceptors carrying a mutation associated with a retinal degenerative phenotype, then it may be possible to interfere in the course of such degeneration by inhibiting the function of the caspase family of proteases. Although this in itself does not address the primary dysfunction characteristic of RP in vivo it does present the potential to slow photorecep-

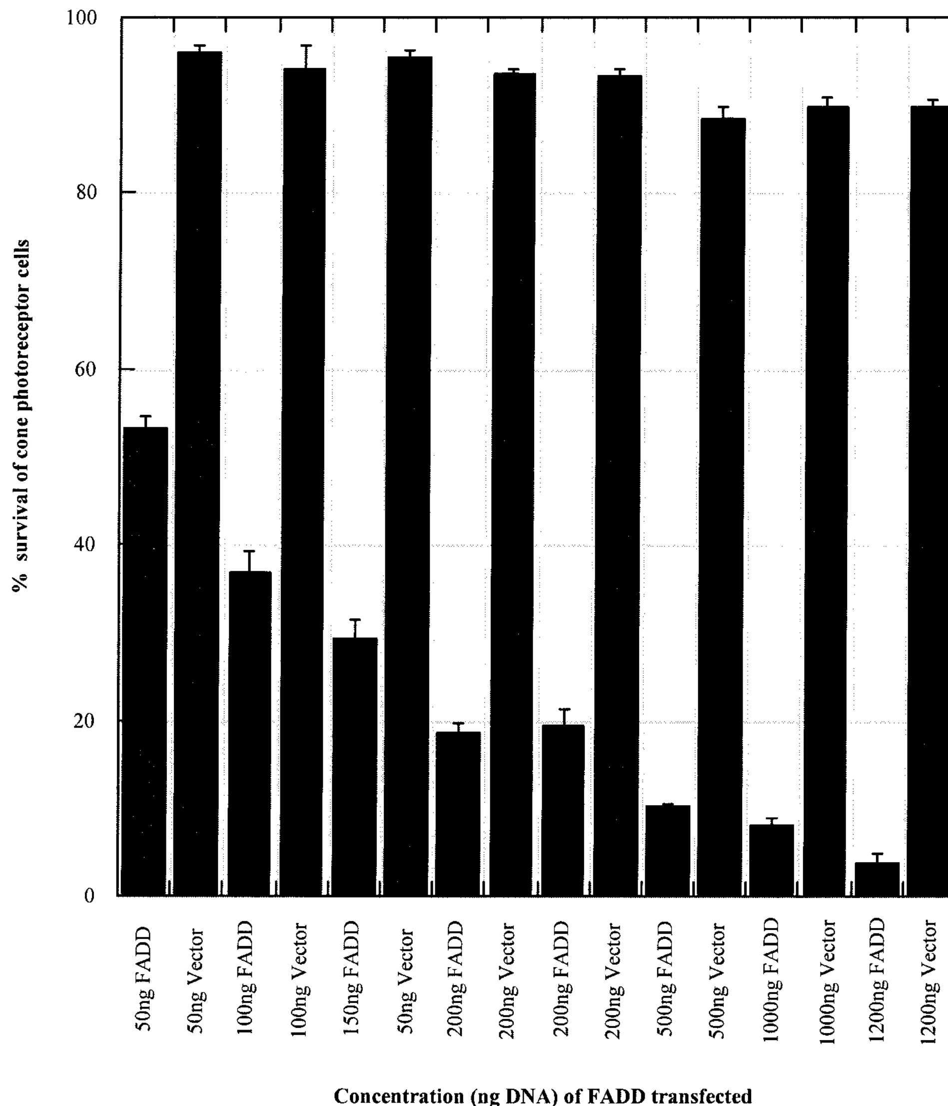

FIGURE 5. Percentage survival of 661W cone photoreceptors after transfection with CMV-driven FADD and empty pCDNA3.0 vector.

tor cell loss in the retina. It is also of note that the rescue of cone cell function would represent a significant advance to subjects loosing daytime vision as a consequence of an unrelated mutation in genes of the rod photoreceptor cell population.

To determine whether apoptotic 661W cells were susceptible to the pancaspase inhibitor p35, a series of transfection experiments were designed to observe cell survival after successful transfection. In this study, a p35 construct under the control of the CMV promoter in the pCDNA3.0 vector was transfected into 661W cells with a commercially available lipid-based transfection system. After cytotoxic insult 24 hours after transfection with the p35 gene, it can be seen from Figure 4 that there was a significant rescue effect mediated by the irreversible inhibition of executioner caspases within the 661W cells. The transfection of 400, 600, and 1200 ng of CMV-driven p35 demonstrated a cell rescue effect of 64.15% $\pm$ 1.8%, 68.77% $\pm$ 1.55%, and 75.3% $\pm$ 4.23% survival respectively, providing an average resistance for 661W cells to 2.5 μM daunorubicin of 69.41% $\pm$ 5.61%. This data, obtained from a mammalian photoreceptor-derived cell line, is consistent with previous studies[35] in which a p35 transgenic *Drosophila* carrying an RP mutation was shown to have functional rescue of photoreceptor cells in comparison to retinal degenerative control subjects. In addition, given the highly specific nature of p35, the inhibition of 661W cell apoptosis provides further evidence of the involvement of caspase proteases in the 661W cell line. p35 protects from apoptotic cell death by a complex formation between the enzyme and the inhibitor, which is thought to involve cleavage of the p35 substrate.[29,36] Detailed biochemical studies of p35 have revealed strong inhibition of caspase-1, -3, -6, -7, and -10 but have demonstrated little or no effect on 12 unrelated serine or cysteine proteases, indicating a highly specific affinity of p35 for the caspase family of proteases.[29] The antiapoptotic effect provided by p35 activity within 661W cells provides a strong rationale for attempting to deliver such a construct to animal models known to undergo degenerative retinopathy, with the purpose of testing the potential therapeutic benefit of p35 in vivo.

Finally, the extreme sensitivity of 661W cells to transient transfection with a construct expressing FADD may be highly significant in the development of novel therapeutic approaches to preventing photoreceptor apoptosis. For example, ribozymes targeting the downstream executioners of apoptosis such as caspase-8 mRNA may be highly effective in extending cone cell longevity which, similar to p35, may either afford a functional rescue on its own[35] or provide an opportunity in which other strategies, such as gene correction of the primary genetic mutation, may be attempted. Although preventing photoreceptor cell death alone may not address the primary cause

of many retinal dystrophies it may represent a critical step in maintaining a cell population pending the introduction of other therapeutic strategies.

The protection of 661W cells from apoptotic cell death, as mediated by the transient transfection of p35, although specific to retinal dystrophy, nevertheless may carry wider implications for the potential abrogation of cell death in a range of diverse neurodegenerative disorders.[37] Modulation of apoptosis provides opportunities for novel therapeutic development for many disorders in which an apoptotic process contributes to the disease's progress. The present study represents an initial exploration of the potential therapeutic benefit to be gained from modulating key regulators of apoptotic cell death in the context of a mammalian photoreceptor-derived cell line and, more broadly, in the context of retinal degenerative disorders.

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