Characterization of Daunorubicin-Induced Apoptosis in Retinal Pigment Epithelial Cells: Modulation by CD95L

Arno Hueber,1 Michael Weller,2 Gerhard Welsandt,1 Norbert Kociok,1 Bernd Kirchhof,1 and Peter Esser1

PURPOSE. To characterize daunorubicin-induced cell death in cultured human retinal pigment epithelial (RPE) cells and its modulation by CD95 ligand (CD95L).

METHODS. In situ DNA end labeling and an ELISA for histone-associated DNA fragments were used to assess apoptosis. CD95 and CD95L expression were examined by immunohistochemistry, flow cytometry, immunoblot, and RT-PCR. Cell death was measured by crystal violet staining. YVAD and DEVD-aminomethylcoumarin (AMC) cleavage was used to measure caspase-1 and -3-like activity. Total RNA and protein synthesis was measured by incorporation level of [3H]-leucine and [3H]-uridine.

RESULTS. RPE cells expressed both CD95 and CD95L, but only CD95 was expressed at the cell surface. Daunorubicin-induced RPE cell apoptosis was associated with enhanced CD95 and CD95L expression. Enhanced CD95L expression was epiphenomenal to the death process, evidenced by the fact that neutralizing CD95 antibodies failed to modulate daunorubicin cytotoxicity. In contrast, the cytotoxic effects of daunorubicin were synergistically enhanced by exogenous CD95L. Synergy appeared to involve enhanced caspase-3-like activity as well as daunorubicin-mediated inhibition of RNA synthesis.

CONCLUSIONS. Apoptosis has been shown to be an important factor in the control of specific cell populations. The synergistic activity of an antiproliferative agent, daunorubicin, and a cytokine, CD95L, induces apoptosis in RPE cells. Such approaches provide a means to reduce the concentration of chemotherapeutic agents with a small therapeutic window owing to retinal toxicity, such as daunorubicin, in the adjuvant therapy of proliferative vitreoretinopathy. (Invest Ophthalmol Vis Sci. 2003;44:2851-2857) DOI:10.1167/iovs.02-1178

Proliferative vitreoretinopathy (PVR) remains the leading cause for the failure of retinal reattachment surgery. PVR is a multistage disease process characterized by the uncontrolled growth of cells at the vitreoretinal interface, eventually leading to formation of contractile membranes with subsequent tractional detachment and severe impairment of vision.1 Over more than two decades, investigators have focused on the cellular components of surgically removed epiretinal membranes and the general idea has emerged, that besides releasing the tractional forces on the retina by means of surgical intervention, inhibition of cellular reproliferation remains a primary target in the treatment of PVR. In pursuit of this goal, early pharmacologic intervention in the course of the disease has been proposed, and several chemotherapeutic substances have been applied intraocularly for efficient reduction of cellular migration and proliferation. The anthracycline, daunorubicin, is one of the commonly used drugs to reduce postoperative reproliferation by intraoperative infusion after vitrectomy,2 but the overall success rates have not been satisfactory.3 As with other agents, doses of daunorubicin, that would probably be required for a stronger antiproliferative effect, cannot be used because of retinal toxicity.4 Moreover, repetitive treatments with daunorubicin are likely to fail because daunorubicin induces a multidrug resistant phenotype in vivo.5

The pharmacologic effect of daunorubicin is generally thought to result from drug-induced DNA damage mediated by quinone-generated redox activity, intercalation-induced distortion of the double helix, or stabilization of the cleavable complex formed between DNA and topoisomerase II. Cellular responses to daunorubicin are regulated by multiple signaling events, including a sphingomyelinase-initiated sphingomyelin ceramide pathway, mitogen-activated kinase, and stress-activated protein/c-Jun N-terminal kinase activation, transcription factors such as nuclear factor κB, and the CD95/CD95L system.6

CD95L is a cytotoxic cytokine that mediates apoptosis through CD95, a cell surface transmembrane protein triggering a killing cascade.7 CD95L is expressed in vivo in mice in corneal epithelium, endothelium, iris, and ciliary body throughout the retina.8 Soluble forms of CD95L were detected in ocular fluids,9,10 and the CD95/CD95L system seems to be one factor of the immune privilege of the eye.9,10

Here we examined the possible synergy of daunorubicin and CD95L in the control of RPE cell proliferation. We selected these cells for our study because they are found in surgically removed epiretinal membranes and contribute significantly to epiretinal membrane formation in PVR.11

MATERIALS AND METHODS

Materials

Daunorubicin was obtained from Sigma-Aldrich (St. Louis, MO), DEVDrho from Bimol (Hamburg, Germany); and YVAD-fmk, YVAD-aminomethylcoumarin (AMC), and DEVDeamc from Bachem (Heidelberg, Germany). Marine soluble CD95L was obtained from CD95L cDNA transfected marine N2A neuroblastoma cell.12 CD95L blocking antibody (NOK-1) was from BD PharMingen (Heidelberg, Germany).

Cell Culture Methods and Viability Assays

Human RPE cells were prepared from eyes used as donors for transplantation of the cornea and cultured in DMEM containing 1 g/L glucose (Life Technologies, Karlsruhe, Germany) and 10% FCS.13 RPE cell origin was confirmed by positive cytokeratin immunocytochemical analysis. Given the number of eyes available at our department, the
quantity of fresh RPE cells that can be obtained in primary cultures is
not sufficient for the large-scale assays performed in this study. There-
fore, the cells were passaged to increase the RPE cell yield per eye.
Fourth-passage cells were used for most experiments. After exposure
to CD95L or daunorubicin, the cultures were monitored closely by
phase-contrast microscopy. Viability was assessed by crystal violet
staining.13

**Immunohistochemistry**

Immunohistochemistry was performed as previously described.14
Briefly, the cells were fixed for 10 minutes in acetone. After incubation
for 1 hour with mouse monoclonal CD95 antibody (F22120, 1 µg/mL;
BD Transduction Laboratory, Lexington, KY) or rabbit polyclonal anti-
CD95L antibody (sc-957, 1 µg/mL; Santa Cruz Biotechnology, Santa
Cruz, CA) diluted in PBS with BSA (5 g/L; mouse IgG or rabbit serum
served as controls), two washes in PBS, Cy2-conjugated goat anti-rabbit
IgG or goat anti-mouse IgG (1:200, 50 minutes; Jackson Immunore-
search, West Grove, PA) were used for detection. Nuclei were coun-
terstained with Hoechst dye H33342. Alternatively, biotinylated sec-
ondary goat anti-mouse and goat anti-rabbit antibodies (AB2, Detection
Kit Code No.: K 5001; Dako, Hamburg, Germany) and mounting in
antifade medium (ABC Kit Standard PK-6100; Vectastain; Vector Lab-
atories, Burlingame, CA) using diaminobenzidine for staining were
used for detection.

**Flow Cytometry**

To assess CD95 and CD95L expression at the cell surface, the cells
were rinsed in cold PBS, incubated for 3 minutes in trypsin at 37°C
and harvested into complete medium containing 10% FCS. The cells
were centrifuged, resuspended (10⁶ cells per tube) in flow cytometry buffer
(PBS/1% BSA/0.01% sodium acide), and labeled for 30 minutes at 4°C
with 2 µg/mL FITC-conjugated CD95 antibody UB-2 (Immunotech,
Marseille, France) or nonspecific FITC-conjugated mouse IgG1 (2 µg/ mL;
Sigma-Aldrich) as a control. Alternatively, the cells were blocked
for 20 minutes in 10% goat serum in flow cytometry buffer before
labeling with 2 µg/mL rabbit polyclonal anti-CD95L antibody (sc-957;
Santa Cruz Biotechnology) at 4°C for 60 minutes. Isotype controls were
incubated with 2 µg/mL nonspecific rabbit IgG. After washing in flow
cytometry buffer, cells were incubated for 30 minutes at 4°C in FITC-
conjugated anti-rabbit IgG1 (Sigma-Aldrich) and analyzed on a
flow cytometer (FacsCalibur; BD Biosciences, Heidelberg, Germany).
The specific fluorescence index (SFI) was calculated as the ratio of
the mean fluorescence values obtained with the specific antibody and
the control antibody.

**Reverse Transcription Polymerase Chain Reaction**

Total RNA from human RPE cells from keratoplasty donor eyes and
cultured cells of passage P7 were prepared by using extraction reagent
(TRI Reagent; Sigma-Aldrich). The RNA was reverse transcribed with a
preamplification system for first-strand cDNA synthesis (Superscript;
Life Technologies, GibcoBRL, Grand Island, NY) with oligo(dT)₁₂₋₁₈
primers. We selected primers for human CD95L (AC: U08137: 161,
position 263: 5’-CCG CCA CCA CTG CCT CCA CTA-3’ and 162, posi-
tion 750: 5’-TCT TCC CCT CCA TCA TCA CCA-3’) and primers for
human glyceraldehyde-3-phosphate dehydrogenase (GAPDH, AC:
M33197: 247, position 285: 5’-ATC TTC CAG GAG CGA GAT CC-3’
and 248, position 769: 5’-ACC ACT GAC ACG TTG GCA GT-3’).15
Aliquots of the diluted cDNAs corresponding to 62.5 ng of initial total RNA
were mixed with PCR buffer (Quagen, Hilden, Germany) containing
Tris-HCl (pH 8.7 at 20°C), (NH₄)₂SO₄, 1.5 mM MgCl₂, 1 U polymerase (HotStar-
Taq Quagen), 0.2 mM of each dNTP and 0.2 µM of each specific primer
in a volume of 50 µL. The PCR cycle parameters were 40 cycles at
95°C, 57°C, and 72°C for 1 minute each. The amplified PCR product
was sequenced by terminator cycle sequencing.

**Apoptosis Assay**

DNA breaks were detected on a single-cell level by in situ DNA end
labeling (TUNEL).13 The cells were equilibrated in TT buffer (30 mM

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**FIGURE 1.** CD95L and CD95 were detected in RPE cells in vitro.
CD95L (A) and CD95 (B) expression in cultured human RPE cells were
assessed by immunocytochemistry. Original magnification, ×400. (A,
B, insets) Negative controls. Specific labeling appears in green; the
blue nuclear staining in the control insets resulted from the H33342
counterstain. CD95L (C) and CD95 (D) protein levels at the cell surface
were measured by flow cytometry. Open peak: unspecific signal; solid
peak: specific signal. CD95L mRNA (508 bp) expression was assessed
by RT-PCR (E) in freshly isolated human RPE cells (Eye) or passage-7-
cultured RPE cells (P7). GAPDH (503 bp) served as a reference mRNA
(C, control without cDNA).
Figure 2. Daunorubicin induced apoptosis in human RPE cells. Human RPE cells were untreated (A) or exposed to daunorubicin at 2 (B) or 20 μM (C) for 24 hours. Apoptotic changes were visualized by in situ DNA end labeling in adherent (A) and detached RPE (C) cells, but not in untreated control cultures (A). (A–C, insets) Samples developed without cobalt chloride. Magnification, ×200. (D) Cytoplasmic histone-associated DNA fragments were quantified by cell death detection ELISA. Data are expressed as mean ± SD of optical density at 405 nm (n = 5).

Tris [pH 7.2] and 140 mM sodium cacodylate, treated with terminal transferase (TT, 0.25 U/μL; Roche Molecular Biochemicals, Mannheim, Germany) and biotin-dUTP (20 μM, Roche Molecular Biochemicals) in TT buffer containing 1 mM cobalt chloride for 60 to 90 minutes at 37°C, washed in 2× SSC (300 mM NaCl, 30 mM sodium citrate) for 15 minutes, rinsed twice in H2O, blocked for 10 minutes with 2% BSA in PBS, treated with streptavidin-alkaline phosphatase diluted 1:500 in PBS, rinsed with ice-cold PBS, they were lysed with 0.1% SDS (0.5 mL/well) for 30 minutes at room temperature. The plates were washed using ice-cold PBS (two times) and incubated for 90 minutes at room temperature. The plates were rinsed with distilled-deionized water (two times) to remove unincorporated, acid-soluble label. After lysis with 1 mL 0.1 N NaOH overnight at room temperature, 0.5 mL of the lysate was mixed with 5 mL scintillation cocktail. The pellet (trichloroacetic acid–precipitable fraction) was washed three times with 6% trichloroacetic acid and dissolved in 0.5 mL 0.1 N NaOH. The radioactivity of the precipitated proteins was measured after addition of 5 mL scintillation cocktail.

CD95 was labeled using horseradish peroxidase–conjugated sheep anti-mouse antibody (1:5000; Amersham, Braunschweig, Germany). The polyclonal rabbit antibody to CD95L was labeled using horseradish peroxidase–conjugated swine anti-rabbit antibody (1:3000, Sigma-Aldrich).

Measurement of Caspase Activity

After stimulation as indicated, the cells were incubated in lysis buffer (25 mM Tris-HCl [pH 8.0], 60 mM NaCl, 2.5 mM EDTA, 0.25% NP-40) for 10 minutes. Then the fluorogenic caspase substrates Ac-DEVD-amc or Ac-YVAD-amc (20 μM) were added and the fluorescence determined in 15-minute intervals using 560 nm excitation and 480 nm emission wave lengths (CytoFluor 4000; PerSeptive Biosystems, Wiesbaden, Germany).17

Determination of Total RNA and Protein Synthesis

The cells were pulse-labeled for 1 hour with 0.5 μCi/mL (5.6±[3H]) uridine (specific activity: 40 Ci/mmol; Amersham) to determine RNA synthesis. The cells were washed using ice-cold PBS (two times) and ice-cold 6% trichloroacetic acid (two times) to remove unincorporated, acid-soluble label. After lysis with 1 mL 0.1 N NaOH overnight at room temperature, 0.5 mL of the lysate was mixed with 5 mL scintillation cocktail and counted in a liquid scintillation counter. For the determination of protein synthesis, the cells were pulse labeled during the last hour of incubation with 1 μCi/mL (+4.5±[3H]) leucine (specific activity: 167 Ci/mmol; Amersham). After cells were washed three times with ice-cold PBS, they were lysed with 0.1% SDS (0.5 mL/well) for 30 minutes at 37°C. Proteins were precipitated by addition of ice-cold 15% trichloroacetic acid (0.5 mL/well) and pelleted by centrifugation (15,000 rpm, 10 minutes, 4°C). The supernatant (trichloroacetic acid–soluble fraction) was counted in a liquid scintillation counter after addition of 5 mL scintillation cocktail. The pellet (trichloroacetic acid–precipitable fraction) was washed three times with 6% trichloroacetic acid and dissolved in 0.5 mL 0.1 N NaOH. The radioactivity of the precipitated proteins was measured after addition of 5 mL scintillation cocktail.

Immunoblot Analysis

Protein studies were performed as previously described.16 Soluble cellular proteins (20 μg per lane) were separated on 12% to 15% SDS-PAGE gels and electroblotted onto nitrocellulose. Equal loading was ascertained by Ponceau S staining. The primary antibodies were anti-CD95 (F22120; BD Transduction Laboratory) and anti-CD95L (sc-957; Santa Cruz Biotechnology). The monoclonal mouse antibody to CD95 was labeled using horseradish peroxidase–conjugated sheep anti-mouse antibody (1:5000; Amersham, Braunschweig, Germany). The polyclonal rabbit antibody to CD95L was labeled using horseradish peroxidase–conjugated swine anti-rabbit antibody (1:3000, Sigma-Aldrich).
**Statistical Analysis**

Data are from experiments performed at least three times with similar results. Synergy was assessed by the fractional product method of Webb (described in Refs. 18, 19). Here, multiplication of survival percentages after exposure to one of two agents alone yields a theoretical predicted effect, assuming that both agents act independently. If the measured value of survival is lower than predicted, synergy is assumed; if survival is higher than predicted, there is antagonism.

**RESULTS**

**Expression of CD95L and CD95**

Immunohistochemistry revealed the expression of CD95L (Fig. 1A) and CD95 (Fig. 1B) in cultured human RPE cells. Immunoreactivity appeared to be diffusely cytoplasmic and not particularly membrane-selective for either antigen. Flow cytometry of nonpermeabilized RPE cells revealed no significant expression of CD95L (SFI = 1.1, Fig. 1C), but strong expression of CD95 (SFI = 5.1, Fig. 1D), at the cell surface. Given the unexpected result of cytoplasmic CD95L expression, but no CD95L expression at the cell surface, we confirmed its expression in RPE cells by immunoblot at the protein level (data not shown, see also Fig. 3A) as well as by RT-PCR at mRNA level (Fig. 1E). The amplified sequence was verified to represent CD95L by DNA sequencing.

**Daunorubicin Induces Apoptosis in Human RPE Cells In Vitro**

The next experiments were designed to evaluate whether daunorubicin-induced cytotoxicity of human RPE cells involves the induction of apoptosis. Figure 2 demonstrates that the exposure of RPE cells to daunorubicin resulted in apoptosis as defined by in situ DNA end labeling and quantification of histone-associated DNA fragments. Twenty-four hours after exposure to daunorubicin (20 μM), in situ DNA end labeling revealed DNA breaks in some of the adherent RPE cells (Fig. 2B) as well as in all RPE cells that had detached from the monolayer as a consequence of the daunorubicin exposure (Fig. 2C). No labeling was seen in untreated RPE cell cultures (Fig. 2A). Negative control samples, developed without cobalt chloride, the cofactor for terminal transferase, did not display any staining (Fig. 2A, B, C, inserts in the upper right corner). Further, daunorubicin treatment for 24 hours induced a concentration-dependent enrichment of histone-associated DNA fragments compared with vehicle-treated control cells (Fig. 2D).

**Daunorubicin-Induced Apoptosis Is Associated with Altered CD95L and CD95 Expression**

Immunoblot analysis revealed that daunorubicin enhanced the cellular levels of CD95L and CD95 (Fig. 3A, 3B). These findings were confirmed by immunohistochemistry in cultured human RPE for CD95L (Figs. 3C, 3D) and CD95 (Figs. 3E, 3F) that were untreated (Figs. 3D, 3F) or treated with daunorubicin (Figs. 3C, 3E). We next asked whether endogenous CD95L, upregulated by daunorubicin, mediated daunorubicin-induced cell death. However, neutralizing CD95L antibodies (NOK-1) failed to modulate daunorubicin cytotoxicity, suggesting that the modulation of CD95L expression by daunorubicin was epiphenomenal to the death process (Fig. 3G).

**Daunorubicin-Induced Apoptosis of Human RPE Cells: Potentiation by CD95L**

The exposure of RPE cells to daunorubicin for 24 hours resulted in a concentration-dependent cytotoxicity with an EC50 of approximately 10 μM (Fig. 4A). Coexposure to CD95L significantly enhanced daunorubicin-induced cell death. CD95L (A) or CD95 (B) expression were measured by immunoblot in cultured human RPE cells that were untreated (lane 1) or treated with 2 (lane 2) or 20 μM (lane 3) daunorubicin for 24 hours. CD95L (C, D) or CD95 (E, F) expression were assessed immunohistochemically in cultured human RPE cells that were untreated (D, F) or treated with 20 μM daunorubicin (C, E) for 24 hours. No counterstain was performed. (G) RPE cells were treated with 10 or 40 μM daunorubicin (DAU) in the presence of 50 μg/mL NOK-1 antibody or 50 μg/mL control IgG.

**FIGURE 3.** Daunorubicin enhanced CD95L and CD95 protein levels in human RPE cells, but CD95L did not mediate daunorubicin-induced cell death. CD95L (A) or CD95 (B) expression were measured by immunoblot in cultured human RPE cells that were untreated (lane 1) or treated with 2 (lane 2) or 20 μM (lane 3) daunorubicin for 24 hours. CD95L (C, D) or CD95 (E, F) expression were assessed immunohistochemically in cultured human RPE cells that were untreated (D, F) or treated with 20 μM daunorubicin (C, E) for 24 hours. No counterstain was performed. (G) RPE cells were treated with 10 or 40 μM daunorubicin (DAU) in the presence of 50 μg/mL NOK-1 antibody or 50 μg/mL control IgG.
Inhibition of RNA synthesis by actinomycin D or of protein synthesis by cycloheximide greatly potentiates CD95-apoptosis in many tumor cell types. We accordingly asked whether the synergy of daunorubicin and CD95L involved an actinomycin D-like or cycloheximide-like effect. Figure 4 shows, indeed, that daunorubicin strongly inhibited RNA synthesis at concentrations acting in synergy with CD95L to induce cell death (Fig. 4A) whereas protein synthesis was unaffected by daunorubicin.

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**Discussion**

We report that human RPE cells, a major population of the cells thought to contribute to vitreoretinal proliferative diseases such as PVR, undergo apoptosis after treatment with daunorubicin in vitro as assessed by TUNEL labeling and DNA fragmentation (Fig. 2). The potential role of death ligand/receptor, e.g., CD95L/CD95, interactions in mediating the cytotoxic effects of cancer chemotherapy has remained controversial. The EC50 concentration (10 μM) after 24 hours of daunorubicin treatment in vitro (Fig. 4A) was within the range of the intraoperatively used daunorubicin infusion (13.3 μM for 10 minutes) to prevent reproliferation in PVR, although the duration of cellular exposure to such drug concentrations after the end of the infusion in vivo is uncertain. The EC50 concentration (10 μM) after 24 hours of daunorubicin treatment in vitro (Fig. 4A) was within the range of the intraoperatively used daunorubicin infusion (13.3 μM for 10 minutes) to prevent reproliferation in PVR, although the duration of cellular exposure to such drug concentrations after the end of the infusion in vivo is uncertain.

The synergistic effects of daunorubicin and CD95L suggest a combined immunotherapy to be a promising approach for the regulation of intravitreal cellular outgrowth in proliferative vitreoretinal disorders and could lead to a reduction of intravitreal daunorubicin with similar or stronger cell proliferation inhibiting effects, but less retinal toxicity. Future studies should concentrate on practicable approaches toward this goal. These studies should include application of synergistically acting proapoptotic substance combinations to reduce the small therapeutic window between antiproliferative action and retinal toxicity of intravitreal monotherapy.

**Acknowledgments**

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**References**


13. Hueber A, Esser P, Heimann K, Kociolek N, Winter S, Weller M. The topoisomerase I inhibitors, camptothecin, and daunorubicin-induced inhibition of RNA synthesis (Fig. 4), given that the inhibition of RNA synthesis is a classic pathway to sensitize for CD95-mediated apoptosis. The cell cycle regulatory protein, p21, is a candidate mediator of protection from death receptor-apoptosis, with a short half-life, as assessed under conditions in which RNA and protein synthesis are inhibited.

14. Hueber A, Esser P, Heimann K. Intraocular RPE membranes of intraocular proliferative disorders (PVR and PDR). The upregulation of CD95 in response to daunorubicin treatment (Figs. 3B, 3E) has been described for other cell types. Synergy between different drugs and CD95L has been described for some neoplastic cell types including neuroblastoma cells, HL60 leukemic cells, and malignant glioma cells. The probable mechanism mediating the synergy of daunorubicin and CD95L in RPE cells was daunorubicin-induced inhibition of RNA synthesis (Fig. 4), given that the inhibition of RNA synthesis is a classic pathway to sensitize for CD95-mediated apoptosis. The cell cycle regulatory protein, p21, is a candidate mediator of protection from death receptor-apoptosis, with a short half-life, as assessed under conditions in which RNA and protein synthesis are inhibited. Accordingly, at least in naive RPE cells, CD95L expression was only cytoplasmic, whereas cell surface expression would be required to transduce a death signal.


