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

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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-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 adjunct 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 traction retinal detachment and severe impairment of vision. 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, but the overall success rates have not been satisfactory. As with other agents, doses of daunorubicin, that would probably be required for a stronger antiproliferative effect, cannot be used because of retinal toxicity. Moreover, repetitive treatments with daunorubicin are likely to fail because daunorubicin induces a multidrug resistant phenotype in vivo.

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.

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

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.

MATERIALS AND METHODS

Materials

Daunorubicin was obtained from Sigma-Aldrich (St. Louis, MO); DEVD-cho from Bionol (Hamburg, Germany); and YVAD-fmk, YVAD-amc and DEVD-amc from Bachem (Heidelberg, Germany). Marine soluble CD95L was obtained from CD95L cDNA transfected murine N2A neuroblastoma cells; 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. 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. Therefore, 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 ImmunoResearch, West Grove, PA) were used for detection. Nuclei were counterstained with Hoechst dye H33342. Alternatively, biotinylated secondary 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 Laboratories, 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 acetate), 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, 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 IgG₁. 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 premultiplication system for first-strand cDNA synthesis (Superscript; Life Technologies, GibcoBRL, Grand Island, NY) with oligo(dT)₁₂₁₈ primers. We selected primers for human CD95L (AC: U08137: 161, position 263: 5’-CCG CCA CCA CTG CCT CCA CTA-3’ and 162, position 750: 5’TCT TCC CCT CCA TCA TCA CCA-3’) and primers for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH, AC: M35319: 247, position 286: 5’-ATC TTC CAG GAG CGA GAT CC-3’ and 248, position 769: 5’-ACC ACT GAC ACG TTG GCA GTG-3’). 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, 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).15 The cells were equilibrated in TT buffer (30 mM

![Figure 1](image-url)
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, and developed using nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate (both from Sigma-Aldrich) as substrates. The slides were not counterstained. The specificity of dUTP incorporation was ascertained by omitting cobalt chloride from the reaction. Thymus served as the positive control.

**Cell Death Detection ELISA**

DNA fragmentation was quantified by an immunoassay for histone-associated DNA fragments (Roche Molecular Biochemicals). RPE cells were trypsinized and centrifuged. The pellet was resuspended in lysis buffer for 30 minutes at 4°C. The lystate was centrifuged at 15,000 rpm for 10 minutes, and the supernatant was carefully removed. The supernatants were matched for initial cell counts. ELISA plates were coated with monoclonal antibody (clone H11-4) to cytoplasmic histone-associated DNA fragments. Triplicate samples were incubated for 90 minutes at room temperature. The plates were rinsed and incubated with peroxidase-coupled DNA antibody for 90 minutes at room temperature. The plates were rinsed two times in H2O, and blocked for 10 minutes with 2% BSA in PBS, treated with streptavidin-alkaline phosphatase diluted 1:500 in PBS, and incubated for 90 minutes at room temperature. The plates were rinsed twice in H2O, and developed with terminal transferase (TT, 0.25 U/mL, 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, and developed with nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate (both from Sigma-Aldrich) as substrates. The slides were not counterstained. The specificity of dUTP incorporation was ascertained by omitting cobalt chloride from the reaction. Thymus served as the positive control.

**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 antirabbit 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% NP40) 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, Weisbaden, Germany).17

**Determination of Total RNA and Protein Synthesis**

The cells were pulse-labeled for 1 hour with 0.5 μCi/mL (5.6×106 Ci/mmol; Amersham) 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 lystate 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 (4.5×106 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 (13,000 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.
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 of 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. To confirm that there was synergy of daunorubicin and CD95L, we used the fractional product method of Webb. The asterisks in Figure 4A indicate combinations of daunorubicin and CD95L which resulted in synergy exceeding 15%. Maximal synergy with 80 U/mL CD95L was 33% ± 9%, and 28% ± 8% with 40 U/mL CD95L. We next examined whether the synergy of daunorubicin and CD95L could be verified at the level of caspase activation. Neither daunorubicin nor CD95L induced caspase-1–like YVAD-amc–cleaving activity. A cytosolic extract prepared from THP-1 cells (4 mg/mL; Amsbio, Wiesbaden, Germany) was used as positive control for YVAD-amc–cleaving activity (Fig. 4B). Caspase-3–like DEVD-amc–cleaving activity was detected after CD95L treatment starting at 40 U/mL, but was not detectable after daunorubicin treatment (Figs. 4B, 4C). Caspase-3–like DEVD-amc cleaving activity induced by CD95L was markedly enhanced by daunorubicin (Fig. 4D). Inhibition of caspase-3 with DEVD-cho (100 μM) or ZVAD-fmk (100 μM) did protect slightly against the cell death induced by combinations of CD95L and daunorubicin (Fig. 4E).
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 4F 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.
We report that human RPE cells, a major population of the cells thought to contribute to vitreoretinal proliferative dis- eases such as PVR,1,14 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 mediat- ing the cytotoxic effects of cancer chemotherapy has re- mained controversial.20 The upregulation of CD95L in RPE cells after daunorubicin treatment demonstrated in the current study (Fig. 3) was epiphenomenal to the death process, because neutralizing antibodies to CD95L were not protec- tive (Fig. 3G) but caused daunorubicin-induced cell death, in contrast to exogenous CD95L-induced cell death, did not involve caspase-3 activation.

Previously, we have shown the sensitivity of RPE cells to exogenous CD95L,1,21 observed CD95 expression in surgically obtained PVR specimens in vivo,22 and reported synerg- ergetic cytotoxic activity for the combination of CD95L and the topoisomerase I inhibitor, camptothecin.15 When daunorubicin was combined with CD95L in the present study, strong synergy was found as well (Fig. 4A), which was also observed in cultures of cardiac myocytes23 and leukemic blasts.24 The upregulation of CD95 in response to daunorubicin treatment (Figs. 3B, 3E) has been described for other cell types.24 Synergy between different drugs and CD95L has been described for some neoplastic cell types including neuroblastoma cells,25 HL60 leukemic cells,26 and malignant glioma cells.18 The probable mechanism mediat- ing 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.16 The cell cycle regulatory protein, p21, is one 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.27

Since RPE cells express CD95 and CD95L (Figs. 1, 3) without undergoing suicidal or fratricidal apoptosis, these cells may express specific endogenous inhibitors of apopto- sis. A nonlethal coexpression of CD95 and CD95L was also found in glioma cell lines.28 Alternatively, at least in naive RPE cells, CD95L expression was only cytoplasmic, whereas cell surface expression would be required to transduce a death signal.

The clinical perspectives of the data presented herein are still 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,3 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 sug- gest a combined immunochemotherapy to be a promising approach for the regulation of intravitreal cellular out- growth 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 be- tween antiproliferative action and retinal toxicity of intra- vitreal monotherapy.

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References


