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Lens epithelial cells express CD95 and CD95 ligand treatment induces cell death and DNA fragmentation *in vitro*

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PURPOSE. *Despite advances in intraocular lens design and material, posterior capsule opacification remains one of the major problems in modern cataract surgery. Therefore, the use of antiproliferative agents has been advocated. CD95 ligand (CD95L, Fas, Apo-1) is a death ligand that triggers apoptosis in susceptible target cells. Apoptosis allows for the safe disposal of cells without damaging the surrounding tissue. The goal of this study was to characterize and evaluate CD95L-induced cell death in cultured lens epithelial cells (LEC).*
METHODS. *Expression of CD95 in untreated porcine LEC was investigated by flow cyto metry. Cell death after CD95L or CD95 agonistic antibody treatment was assessed by crystal violet assay and DNA fragmentation was measured by comet assay.*
RESULTS. *The presence of CD95 was observed in LEC. CD95L treatment resulted in a time- and concentration-dependent killing of LEC, which was synergistically enhanced by the addition of cyclohexamide. CD95L treatment induced DNA fragmentation.*
CONCLUSIONS. *The present study confirms the use of apoptosis-inducing CD95L in the inhibition of LEC proliferation. Further studies are needed before clinical application of CD95L to inhibit posterior capsule opacification will be feasible. (Eur J Ophthalmol 2003; 13: 241-5)*

KEY WORDS. *Lens epithelial cells, CD95 ligand, Apoptosis*

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INTRODUCTION

Proliferation of lens epithelial cells (LEC) following cataract surgery leads to posterior capsule opacification (PCO). Clinically, PCO results in reduced vision and increased glare. Advances in intraocular lens design and material have been shown to reduce the rate of PCO (1), but it remains one of the major problems in modern cataract surgery.

Researchers in the field of cancer treatment have focused on apoptosis induction to eliminate fast-growing tissues. Apoptosis is also called programmed cell death and allows for the safe disposal of cells without damaging the surrounding tissue and was also reported to occur after lens extraction in rabbits (2). CD95L is a cytotoxic cytokine that mediates apoptosis through CD95, a cell surface transmembrane protein triggering a caspase-dependent killing cascade in susceptible target cells (3).

The goal of this study was to investigate the induction of apoptosis in LEC as a possible new pathway for treatment procedures to inhibit PCO after cataract surgery. Therefore, we characterized and evaluated CD95L-induced cell death in cultured LEC.

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METHODS

Materials

Murine soluble CD95L was obtained from CD95L cDNA transfected murine N2A neuroblastoma cells (4). We also used a CD95 agonistic antibody (clone CH-11) from AMSbio (Frankfurt, Germany).

Cell culture

LEC were obtained from porcine eyes within 2 hours postmortem. Five- to ten-millimeter diameter fragments of posterior capsule were cultured as previously described (5). Cells were kept in Minimum Essential Medium Eagle (Sigma, Taufkirchen, Germany) with addition of 15% FCS (Boehringer, Mannheim, Germany), 50 µg/ml gentamycin (Sigma), and 2.5 µg/ml amphotericin B (Sigma). Cells were passaged when confluent. The experiments were done with cells of passage P3 to P6.

Flow cytometry

Expression of CD95 on cultured LEC was investigated by flow cytometry as described (6). Briefly, cells were rinsed in cold phosphate buffered saline (PBS), incubated for 3 minutes at 37°C in trypsin, harvested into complete medium containing 15% FCS, centrifuged, resuspended (10^6 cells per tube) in flow cytometry buffer (PBS/1% BSA/0.01% sodium acid), 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 IgG_1 (2 µg/ml; Sigma). A total of 10,000 cells were analyzed using a FacsCalibur flow cytometer (Becton Dickinson, Heidelberg, Germany). The specific fluorescence index (SFI) was calculated as the ratio of the mean fluorescence values obtained with the specific antibody and the unspecific control.

Viability assay

Plates were laminated with 0.1 mg/ml poly-L-lysin (Sigma) for 2 hours at 37°C and washed with PBS. After exposure to combinations of CD95L or CD95 agonistic antibody and cyclohexamide (CHX) (Sigma), the cultures were monitored closely by phase contrast microscopy. Viability was assessed by crystal violet staining as described (7). Briefly, surviving cells were stained with 0.5% crystal violet in 20% methanol/PBS for 20 minutes. Plates were washed extensively under running tap water and air dried. Bound crystal violet was solved in 0.01 M sodium citrate/50% ethanol/PBS and optical density values were determined in an enzyme-linked immunosorbent assay reader at 550 nm.

DNA fragmentation assay

For the comet assay, LEC were detached with trypsin, pelleted, and 1000 cells/µl dispersed in 0.75% low melting agarose at 37°C, and layered on partly frosted microscope slides in 20-µl aliquots. The slides were kept at a plain metal plate on ice for 3 minutes to allow the solidification of agarose and then immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl; pH 10, 1% N-lauroylsarcosine sodium salt, 1% Triton-X 100) for 1 hour (8). The slides were removed from lysis solution and placed on a horizontal gel electrophoresis unit in 1x TAE-buffer (40 mM Tris, 20 mM sodium acetate, 2 mM EDTA; pH 8.0). Electrophoresis was conducted for 20 minutes at 30 V (1.2 V/cm). The slides were stained with 2.5 µg/ml propidium iodide for 30 minutes following observation using a Zeiss epifluorescent microscope and photographically documented on 400 ASA black and white film.

Statistical analysis

Data are from experiments performed at least three times with similar results. Synergy was assessed by the fractional product method of Webb (9). Here, multiplication of survival percentages after exposure to one of two agents alone yields a theoretical predicted effect, assuming that both agents acted independently. If the measured value of survival is lower than predicted, synergy is assumed; if survival is higher than predicted, antagonism is assumed.

RESULTS

We confirmed the expression of CD95 in cultured porcine LEC. Flow cytometry revealed a constitutive expression of CD95 with a SFI of 1.60 (Fig. 1).

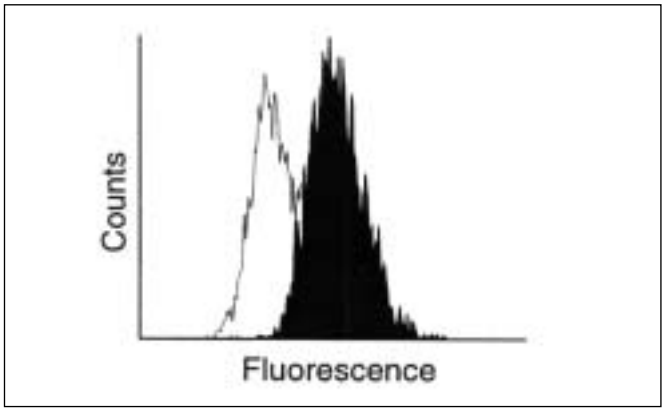

Fig. 1 - *Lens epithelial cells (LEC) express CD95. Untreated LEC were analyzed by flow cytometry for the presence of CD95. The open line corresponds to the unspecific signal and the solid black line corresponds to the specific signal.*

Next, we analyzed sensitivity of LEC to CD95L and CD95 agonistic antibody. LEC were incubated with increasing concentrations of CD95L or CD95 agonistic antibody (Fig. 2). For treatment with CD95 agonistic antibody alone, there was no significant killing effect, even after a 22 hour treatment. The killing effect of CD95 agonistic antibody in combination with CHX was time dependent, but not concentration dependent in the chosen dose range (0.2 and 1.0 µg/ml). For treatment with CD95L, the killing effect started as early as 4 hours after treatment. When exposure time or CD95L concentration was increased, a stronger killing effect was found. The combination of CD95L or CD95 agonistic antibody with CHX resulted in strong synergy according to Webb (9). In Figure 2, synergy of more than 30% for combinations of CD95 ligand or CD95 agonistic antibody and CHX is labeled by asterisks (*). The strongest synergy detected was 45.6% for a 10 hour treatment with 160 U/ml CD95L and CHX.

To further characterize the killing effect of CD95L, we conducted a comet assay after CD95L treatment and fragmented DNA appeared as prominent comets (Fig. 3).

DISCUSSION

PCO is the most common complication after cataract surgery. PCO occurs in approximately 25% of patients after undergoing cataract surgery (10); in about 50% of these patients with PCO, Nd:YAG laser treatment is

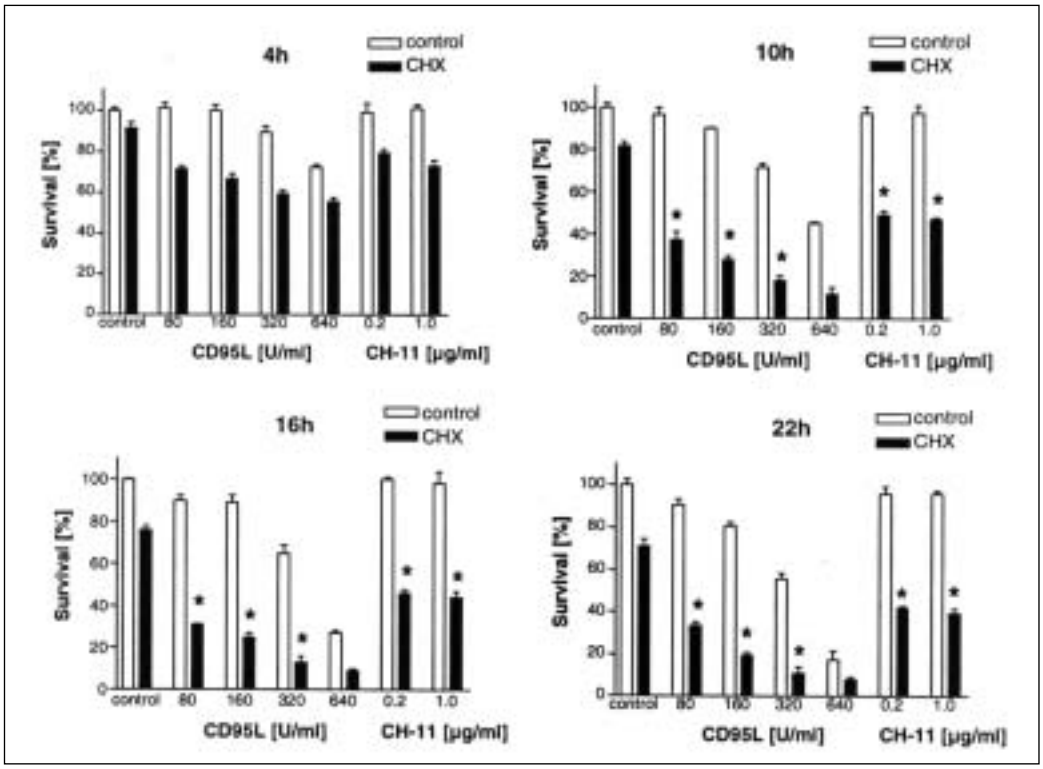

Fig. 2 - *CD95L induces lens epithelial cell (LEC) death. LEC were treated with increasing concentrations of CD95L or CD95 agonistic antibody in the presence or absence of cyclohexamide (CHX, 1 µg/ml) for various lengths of time. SEM (standard error of the mean) is indicated.*

performed to treat the PCO (11). For an overall higher success rate of cataract operations, enhanced prevention and treatment of PCO is necessary.

Several drugs have been used to prevent PCO after cataract surgery and inhibition of LEC proliferation was found *in vitro* (12, 13) and in animal studies (14-16); however, clinical application of these substances has not yet been carried out, mostly because of toxic side effects to other intraocular structures.

CD95 is the best-characterized member of the tumor necrosis factor receptor superfamily and triggers apoptosis in susceptible target cells. Downstream caspase activation leads to cleaving of various intracellular substances and to genomic DNA fragmentation and cell membrane blebbing. CD95 seems to be ubiquitously expressed in most tissues, but the CD95L surface protein expression is limited (17). *In vivo* CD95L was found to be expressed in corneal epithelium, endothelium, iris, ciliary body, and throughout the retina in the eye of mice (18) and soluble forms of CD95L were detected in all ocular fluids (19, 20). The CD95-CD95L system seems to be one important factor in immune-regulating processes, including the immune privilege of the eye (18).

Previous studies have used reverse transcriptase polymerase chain reaction to identify the mRNA of CD95 in LEC (21, 22). An actual CD95 protein expression has not yet been proven, to our knowledge. Using flow cytometry we could identify CD95 at the protein level (Fig. 1).

Nishi et al (22) found evidence of apoptosis after CD95 agonistic antibody treatment of LEC using a nuclear staining. We further evaluated this effect by the use of CD95 agonistic antibody and CD95L itself. We found a concentration- and time-dependent killing of LEC after CD95L treatment (Fig. 2). Because inhibition of protein synthesis sensitizes various cell types to CD95-mediated apoptosis (23), these experiments were performed in the absence or presence of CHX. Inhibition of protein synthesis showed a strong synergy. The synergy for LEC is comparable to our results for retinal pigment epithelial cells, where a sensitizing to CD95-mediated apoptosis (7, 24) was observed. Consistent with the findings of Nishi et al (22) the proapoptotic action of the CD95 agonistic antibody seems to be independent of the different concentrations used in the experiment.

Using the comet assay we could detect fragmented DNA, as we found prominent tails after CD95L treatment (Fig. 3b) compared to untreated controls (Fig. 3a). This fragmented DNA could be a sign for apoptosis, because CD95L is known to induce apoptosis in sensitive cells.

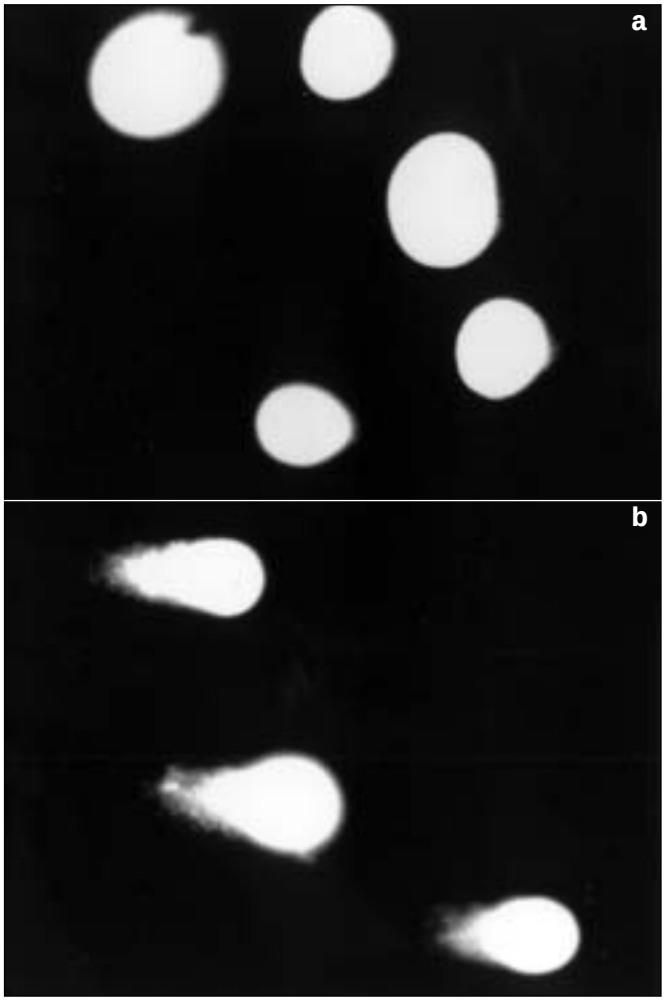

Fig. 3 - *CD95L treatment induces DNA fragmentation. Lens epithelial cells were untreated* **a)** *or treated with 320 U/ml CD95L* **b)** *for 24 hours. A comet assay was conducted to visualize DNA fragments.*

Because systemic application of CD95L leads to liver damage (25), local application was necessary. CD95 and CD95L are physiologically present in structures surrounding the eye. We believe toxicity of externally administrated additional CD95L to ocular tissues is limited, but to our knowledge this has not yet been examined. The present study confirms the utility of CD95L in the inhibition of LEC proliferation.

Further studies are needed before a clinical application of CD95L will be possible. Coating of intraocular lenses with substances like CD95L could limit toxic side effects to other ocular structures and may demonstrate a feasible way of inhibiting PCO.

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