Mechanisms of staurosporine induced apoptosis in a human corneal endothelial cell line

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Background: Apoptosis very probably plays a key part in endothelial cell loss during corneal storage in organ culture as well as hypothermic storage. However, the mechanisms underlying endothelial apoptosis are poorly understood. The response of a human corneal endothelial cell (HCEC) line to staurosporine, a known inducer of apoptosis, was investigated to gain insights into the intracellular modulators that participate in endothelial cell death.

Methods: Immortalised HCECs were studied after 3, 6, 12, and 24 hours of incubation with 0.2 μ M staurosporine. Cell shedding was monitored. Hoechst 33342 fluorescent DNA staining combined with propidium iodide was used for apoptosis/necrosis quantification and morphological examination. The caspase-3 active form was assessed using western blot, proteolytic activity detection, and immunocytochemistry. The cleaved form of poly(ADP-ribose) polymerase (PARP) was assessed using immunocytochemistry and western blot. The ultrastructural features of cells were screened after 12 hours with staurosporine or vehicle.

Results: The specific apoptotic nature of staurosporine induced HCEC death was confirmed. The ultrastructural features of staurosporine treated cells were typical of apoptosis. HCEC shedding and DNA condensation increased with time. Caspase-3 activity was detected as early as 3 hours after exposure with staurosporine, peaking at 12 hours of incubation. The presence of cleaved PARP after 3 hours confirmed caspase-3 activation.

Conclusions: These data suggest strongly that HCEC cell death induced by staurosporine is apoptosis. The main consequence of HCEC apoptosis is shedding. Staurosporine induced apoptosis of endothelial cells involves activation of caspase-3, and could be a useful model to study strategies of cell death inhibition.

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Apoptosis is one of the most fundamental biological processes in mammals, in which individual cells die by activating an intrinsic suicide mechanism. Over the past decade, it has become evident that a family of cysteine proteases, so far comprising 14 members,¹ related to interleukin-1 β converting enzyme (ICE) and termed caspases,² plays a crucial part in apoptosis. After activation, caspases cleave their specific substrate proteins after aspartic acid residues. Some so called "downstream" caspases thus cleave numerous targets that are essential for cell survival. For example, caspase-3, which is one of the main downstream caspases,^{3,4} cleaves, among other targets, poly(ADP-ribose) polymerase (PARP), which is normally responsible for DNA repair.⁵ PARP cleavage is thus one of the hallmarks of caspase-3 activation.

Activation of apoptosis in human corneal endothelial cells (HCECs) was recently highlighted during hypothermic storage of corneas⁶ and in organ culture.^{7,8} Moreover, excessive apoptosis seems to be implicated in the pathogenesis of Fuchs' dystrophy.^{9,10} However, the molecular mechanisms responsible for human corneal endothelial apoptosis remain largely unknown. Only the implication of caspase-3 has been suggested in immunohistochemical tests by Albon.⁷ Analysis of the intracellular mechanisms of endothelial apoptosis in a whole human cornea is difficult for several reasons. Firstly, these cells are particularly well protected against in vivo cell death in normal conditions, since physiological loss is only about 0.6% per year in adults.¹¹ Moreover, the monolayer structure of the endothelium hampers histological observation, and also allows rapid shedding of altered cells,¹² which makes concurrent observation of a large number of cells at the same stage of cell death unlikely. In vitro, unmodified HCEC

cultures derived from adult donors provide only a limited number of cells. They quickly dedifferentiate, lose their morphological characteristics, and lead to reproducibility problems.^{13–16} This limits the use of such cultures for techniques requiring large quantities of cells, and justifies study on a cell line to develop an in vitro model of endothelial apoptosis.

Apoptosis of cultured human endothelial cells was induced by the mycotoxin staurosporine, which has been shown to induce apoptosis in a wide variety of cell types.^{17,18} Many important mechanisms involved in apoptosis have been demonstrated in staurosporine induced apoptosis models.^{18,19} The intracellular signalling pathways of staurosporine triggered apoptosis are however not fully known, and depend on cell type. While there seem to be phases common to all staurosporine induced apoptosis,¹⁷ this one can however include caspase dependent^{20,21} or caspase independent^{22–24} phases, whose relative importance varies according to cell type.

The aim of this study was to establish a model of staurosporine induced apoptosis of a human corneal endothelial cell line, and to explore whether caspase-3 is involved in this model of cell death.

MATERIALS AND METHODS

Cell line and treatment by staurosporine

The HCEC line was obtained after transfection with the coding gene for the large T protein (LT) of the oncogenic DNA simian virus 40 (SV40).²⁵ This line reproduced the morphological and functional characteristics of normal endothelium.^{25,26} The cells were cultured in 25 cm² dishes (Becton Dickinson Falcon,

Figure 1 Progressive shedding of human corneal endothelial cells induced by 0.2 μM staurosporine after 3, 6, 12, and 24 hours (B, C, D, and E respectively), compared with untreated cells (A) or cells incubated for 24 hours with the vehicle (F). Scale bar = 100 μm .

Bedford, MA, USA) for the cell counts and western blot; in Lab-Tek four well culture chambers (Nalge Nunc, Naperville, IL, USA) for the immunocytochemistry; and in Nunclon six well dishes (Nalge Nunc) for double staining with Hoechst 33342/propidium iodide and transmission electron microscopy (TEM). The culture medium composition has been detailed elsewhere¹⁴ and the cells were generally used for experiments 4–5 days after subculture, when their density reached about 3500 cells/ mm^2 , a level close to that of the normal human cornea. The culture medium was renewed 24 hours before each experiment. The cells were treated by incubation with 0.2 μM staurosporine (Roche Diagnostics, Meylan, France) in dimethyl sulfoxide (DMSO) (Sigma) for 3, 6, 12, or 24 hours. This concentration was chosen after a preliminary dose-effect study of 0.05, 0.1, 0.2, 0.5, and 1 μM concentrations (data not shown). The 0.2 μM concentration offered the best compromise between necrosis and apoptosis during a period of 24 hours. The cells incubated with the vehicle were the control.

Quantification of cell shedding rate

After incubation with staurosporine or the vehicle, the floating cells were retrieved and counted with the same haemocytom-

eter. The adherent cells were detached by incubation for 3 minutes with trypsin, and counted in the same way. The shedding rate corresponded to the ratio $100 \times \text{floating cells}/(\text{floating} + \text{adherent cells})$. Each count was done twice and the results were averaged. For each incubation period, three separate cultures were counted.

Immunocytochemistry

Immediately after incubation with staurosporine, supernatant was gently removed in order to respect fragile cells. Culture chambers were then removed and slides were dried at 37°C for 30 minutes, followed by acetone fixation for 10 minutes at room temperature (RT). Immunocytochemistry was performed using a classic ABC technique described elsewhere.²⁷ Primary antibodies were polyclonal rabbit anti-human active caspase-3 (19 and 17 kDa) (1/100 dilution; Cell Signaling, New England Biolabs, Beverly, MA, USA) or cleaved PARP (89 kDa) (1/100 dilution; Cell Signaling). Controls consisted of replacement of primary monoclonal antibody by an irrelevant antibody of the same isotype. Slides were examined using $\times 20$ magnification. Cells were considered stained if any diffuse reddish cytoplasmic staining could be identified.

Quantification of apoptotic/necrotic cells

After incubation with staurosporine, double staining was performed without fixation, directly on living cells, as follows. Bis-benzimidazole Hoechst 33342 (H) (Sigma) and propidium iodide (PI) (Sigma) were added to the culture medium at the final concentration of 1 $\mu\text{g}/\text{ml}$ for each reagent and incubated for 15 minutes at 37°C in the dark. Medium containing floating cells was gently removed and cells were observed using fluorescence microscopy (Diaplan, Leitz, Wetzlar, Germany) with two filters (DAPI fluorescent filter, excitation 340–380 nm, barrier filter 430 nm; and rhodamine filter, excitation 530–560 nm, barrier filter 580 nm) with $\times 40$ magnification. Three separate experiments were performed. For each experiment and each incubation time, three microscopic fields were photographed with a 1600 ISO film (Fuji, Elmsford, NY, USA) and results were averaged. For each field, one photograph of H and one of PI were taken for comparison. Images were digitised for subsequent analysis using a free image analysis software (University of Texas Health Science Centre at San Antonio, ImageTool, from [ftp://maxrad6.uthscsa](http://maxrad6.uthscsa)). Cells displaying a highly condensed nucleus with bright blue coloration were considered H+. PI+ cells and the total number of adherent cells were also counted. During counting,

Figure 2 Percentage of shedding of endothelial cells after exposure to staurosporine. Staurosporine induced the progressive shedding of endothelial cells. In our model, 0.2 μM staurosporine triggered the shedding of nearly half the cells within 24 hours, which appeared to be sufficient to study large number of cells engaged in cell death but without excessive cell toxicity related to a too high dose. Results expressed the mean (SD) of each experiment done in triplicate.

Figure 3 Ultrastructural features of untreated corneal endothelial cells (A) compared with 0.2 μ M staurosporine treated cells for 12 hours (B). Morphological changes observed in treated cells were typical of apoptosis and comprised cell shrinking and chromatin condensation at the periphery of the nucleus. Asterisks indicated the nuclei. Scale bar: 2 μ m.

particular care was taken not to overestimate the number of apoptotic cells by incorrectly counting too small elements corresponding to apoptotic bodies.²⁸ After superposition of H and PI images of the same field using Photoshop software v5.0 LE (Adobe Systems Inc, San Jose, CA, USA), double stained H+/PI+ cells were counted. Apoptotic cells were defined as H+/PI- cells and necrotic cells as PI+ cells.

Transmission electron microscopy

The culture medium was gently removed and cells were fixed in 2% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.4) and processed for conventional TEM. Adherent cells were rinsed in sodium cacodylate buffer, postfixed in 1% osmium tetroxide, prestained with uranyl acetate, and embedded in Spurr (Electron Microscopy Sciences, Fort Washington, PA, USA). Ultrathin sections were cut and conventionally stained with uranyl acetate and lead citrate, and examined with a Hitachi H-800 electron microscope (Hitachi, Tokyo, Japan).

Whole cell extracts

After incubation with staurosporine, adherent cells were detached from the culture dish by gentle scraping. The cells, including spontaneous floating and scraped cells, was washed twice with PBS. A pellet of approximately 10^7 cells was resuspended in 200 μ l of lysis buffer. For western blot analysis the buffer consisted of CHAPS 0.1%, 2 mM EDTA, 50 mM PIPES/KOH (pH 6.5), 20 mg/ml leupeptin, 10 mg/ml pepstatin A, 10 mg/ml aprotinin, 5 mM DTT, 1 mM PMSF for caspase-3 detection; and 20 mM TRIS (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.5% NP40, 2.5 mM sodium pyrophosphate, 1 mM β -glycerol phosphate, 1 mM Na_2VO_4 , 1 μ g/ml leupeptin, 1 mM PMSF for cleaved PARP detection. For caspase-3 activity assay, the extraction buffer consisted of 50 mM HEPES, 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 1 mM EDTA, 10% glycerol, pH 7.4. All reagents were from Sigma. In all cases, the cell pellet was homogenised. Cells were lysed by

four cycles of freezing in liquid nitrogen for 30 seconds and thawing at 37°C for 1 minute. After centrifugation at 17 000 *g* for 45 minutes at 4°C, the resulting supernatant was used as the soluble cytosolic fraction and stored at -80°C in multiple aliquots. Cellular protein contents were determined using the DC Bio-Rad assay kit according to the manufacturer's instructions (Bio-Rad, Hercules, CA, USA).

Western blot for caspase-3 and PARP

Forty μ g of total cell extract per lane were run on 7% and 12.5% precast SDS polyacrylamide gels (Bio-Rad) and electroblotted to nitrocellulose membranes (Bio-Rad). The protein blots were blocked with 5% milk in TRIS buffered saline (TBS; 10 mM TRIS (pH 8.0), 150 mM NaCl) for 2 hours at RT. Each blot was then incubated overnight with either a rabbit monoclonal anti caspase-3, recognising both the proenzyme 32 kDa and the cleaved 19/17 kDa forms (1/500 dilution; Becton Dickinson Pharmingen, Franklin Lakes, NJ, USA), or rabbit polyclonal anti-cleaved caspase-3 (1/1000 dilution; recognising only the 19 kDa inactive cleaved and 17 kDa active cleaved form of caspase-3; Cell Signaling), or rabbit polyclonal anti-PARP (116 kDa) and anti-cleaved PARP (89 kDa) (both at 1/1000 dilution; Cell Signaling) antibodies. Blots were then incubated for 45 minutes with a horseradish peroxidase conjugated secondary antibody (anti-rabbit IgG; Amersham, Arlington Heights, IL, USA) at a dilution of 1/5000 followed by revelation with an enhanced chemiluminescence detection kit (ECL, New England Biolab).

Caspase-3 activity assay

Caspase-3 protease activity was measured by cleavage of the fluorogenic substrate Ac-DEVD-AMC (Acetyl-Asp-Glu-Val-Asp-7-amino-4 methylcoumarin) (Calbiochem, Nottingham, UK) that mimics the known cleavage site of PARP (DEVD/G), for which caspase-3 shows the highest affinity.³ Fluorescence

Figure 4 Double staining of cells with (A) Hoechst 33342 (H) and (B) propidium iodide (PI) distinguished between the typical features of apoptosis (fragmented bright H+/PI-) (arrowheads), necrosis (H-/PI+ or weak H+/PI+) (asterisk) and rare mitosis (H+/PI-) (arrow). Superposition (C) of the two images allowed detection of double stained cells probably indicating late apoptosis. Here, an example of staining after 6 hours' incubation with 0.2 μ M staurosporine (original magnification $\times 40$)

Figure 5 Quantification of apoptosis and necrosis and caspase-3 activity after incubation with 0.2 μ M staurosporine for 0–24 hours. Double staining with Hoechst 33342 (H) and propidium iodide (PI) was applied on adherent cells. Apoptotic cells were defined as cells with bright blue nuclei (H+) and intact membranes (PI–). All PI+ nuclei were deemed necrotic. Apoptosis increased during the first 12 hours. Afterwards, necrosis (true necrosis and/or late apoptosis) was the main phenomenon. Bars represent the mean (SD) of the three separate experiments and were expressed as percentages of, respectively, apoptotic and necrotic cells among the remaining adherent cells. For caspase-3 activity, the bars represent the mean (SD) of a triplicate. Caspase-3 activity started at 3 hours and peaked at 6–12 hours.

was measured in a Kontron fluorometer (Kontron Instruments, Everett, WA, USA) using an excitation wavelength of 380 nm and an emission wavelength of 460 nm. Aliquots containing 100 μ g of cytosolic proteins were incubated with 100 μ M of fluorogenic substrate for 15 minutes at RT in a 1 ml final assay buffer containing 100 mM Hepes, 100 mM NaCl, 10 mM DTT, 1 mM EDTA, 0.1% CHAPS, 10% glycerol, pH 7.4. Fluorescence was then measured each minute for 8 minutes. The amount of fluorochrome released was determined by comparison to a 0–800 pmol standard curve prepared in the same buffer. Caspase-3 activity was finally defined in pmol/min/mg of proteins. Each measurement was done twice and averaged. For each incubation time with staurosporine, three separate assays were performed.

RESULTS

Cell shedding rate induced by staurosporine

Changes in cytoplasmic morphology were soon observed with, in particular, moderate cell shrinkage. These changes were not displayed by cells incubated with the vehicle. Shedding of cells from the culture plate was observed from 3 hours. The floating cells were small and highly refringent. Shedding increased with time, involving about half the cells at 24 hours (Figs 1 and 2). At this stage, nearly all still adherent cells showed major morphological changes such as rounding or membrane blebbing.

Morphological characterisation of endothelial apoptosis and apoptosis rate

Staurosporine treated cells displayed condensed rounding cytoplasm, intact organelles and membranes, condensed nucleus with chromatin packed at the periphery. Apoptotic cells lost contact with adjacent cells (Fig 3).

H/PI double staining allowing apoptotic cells to be distinguished from necrotic cells was applied to cells still adherent after incubation with staurosporine. H+/PI– endothelial cells with bright blue nuclei displayed morphological features characteristic of apoptotic cell death. Compared with untreated cells, cells in the presence of staurosporine shrank, retracted, and the cytoplasm became condensed. Cells had

typical highly condensed chromatin and/or fragmented nuclei (Fig 4). The apoptosis rate seemed to increase sharply, peaking at 12 hours at 38.4% (SD 8.0), while the necrosis rate seemed to remain moderate: 8.7% (SD 3.9) (Fig 5). At 24 hours, the necrotic cell rate reached 31.6% (SD 4.3).

Figure 6 Western blot analysis of procaspase-3 (A), cleaved caspase-3 (B), poly (ADP-ribose) polymerase (PARP) (C), and cleaved PARP (D) expression in human corneal endothelial cells incubated with 0.2 μ M staurosporine for 3, 6, 12, and 24 hours. Ct: control (untreated cells). Each lane was loaded with 40 μ g of protein (total cell extract). Blots were probed with monoclonal antibody to human pro-caspase-3 (32 kDa), or polyclonal antibodies against cleaved caspase-3 (19 and 17 kDa), PARP (116 kDa), or cleaved PARP (89 kDa).

Figure 7 Immunostaining of cleaved caspase-3 (A, B) and cleaved poly (ADP-ribose) polymerase (PARP) (C, D). (A) and (C) were untreated cells and (B) and (D) were stained after 12 hours' incubation with 0.2 μ M staurosporine. This time corresponded to the peak of caspase-3 activity. Normal culture exhibited very few positive cells. At 12 hours, numerous cells displayed a strong cytoplasmic red staining either for cleaved caspase-3 or for cleaved PARP. Positive cells had a condensed nucleus, a shrunken cytoplasm, and membranes blebbing consistent with morphological features of apoptosis.

Caspase-3 activation

Western blot revealed a decrease of the pro-caspase-3 (32 kDa), mainly from 6 hours, and the appearance from 3 hours of the cleaved inactive and active fragments of 19 kDa and 17 kDa respectively (Fig 6). In parallel, fluorimetric assay detected the proteolytic caspase-3 activity from 3 hours, peaking at 12 hours (Fig 5). Cleaved PARP, an indirect indicator of caspase-3 activation, also appeared from 3 hours (Fig 6). With all these techniques, signs of caspase-3 activity decreased between 12 and 24 hours.

The number of positive immunostained cells for cleaved caspase-3 and PARP grew with time (data not shown) and involved cells whose morphology corresponded to apoptosis, with a shrunken nucleus and retracted or sometimes blebbing cytoplasm (Fig 7).

DISCUSSION

Based on morphological and biochemical criteria, staurosporine induced apoptosis in a human corneal endothelial cell line has been clearly demonstrated in this study. Exposure of an HCEC line to 0.2 μ M staurosporine triggers apoptosis in nearly 40% of the cells after 12 hours, rapidly activates caspase-3, and induces PARP cleavage. These molecular events are associated to cellular modifications such as a progressive shedding of HCEC cells, increased DNA condensation/fragmentation, and finally apoptotic body formation.

For regular and reproducible toxicity testing, well characterised human cell lines are needed that retain the appropriate features of the original tissue. Lines are commonly used in other areas of ophthalmology such as studies of epithelial apoptosis in corneas²⁹ or conjunctiva.³⁰ The most common method of immortalising a cell is transfection by the coding gene for the LT protein of SV40.³¹ Such human corneal endothelial cell lines were obtained in the past decade.^{25 26 32–35} Even though extrapolation to non-immortalised cells must take account of possible interactions of the LT protein with the apoptotic machinery,^{36–41} staurosporine induced apoptosis seems to be a useful cell model for studying the intracellular signalling pathways of HCEC apoptosis. Moreover, with the concentrations and incubation times used in our study and in similar studies in the literature, staurosporine is a preferential inducer of apoptosis in many cell models.^{17 18 20 23 42–46}

We observed that endothelial apoptosis was accompanied by substantial shedding of nearly half the cells in 24 hours. Relatively early detachment from their basal membrane is characteristic of apoptosis of monolayer adherent cells⁴⁷ and is called anokis.⁴⁸ Shedding of dead HCECs into the anterior chamber is a long known phenomenon.¹² In a recent study we showed that, during cornea storage in organ culture, this shedding is very probably linked to apoptosis.^{8 27} Indeed, disturbed contact between the adhering cells and the basal membrane in itself stimulates apoptosis.^{49–51} It was reported very recently that one of the mechanisms of staurosporine induced apoptosis of vascular endothelial cells involved dephosphorylation of focal adhesion kinase (FAK), a protein involved in cell/extracellular matrix adhesion and in the transduction of survival signals.⁵² For this reason, further studies will be required on adhesion/apoptosis association within the corneal endothelium, in particular via the focal adhesion complex and FAK. Albon's work⁷ and our own⁸ found, during prolonged organ culture storage, most apoptotic cells in the corneal folds, where contact between cells and Descemet membrane is disturbed. Likewise, the triggering of endothelial apoptosis in Fuchs' dystrophy⁹ could be related to changes in contact between the cells and an abnormal Descemet membrane.

In our model, the maximum apoptosis rate was reached at 12 hours, suggesting that cell resistance mechanisms are effective in the first hours. Antiapoptotic molecules such as Bcl-2, and certain heat shock proteins expressed in native HCECs^{27 46} could play this part temporarily, but the persistent apoptotic stimulus probably exceeds their protective ability. Moreover, the increase in the "necrosis" rate at 24 hours, identified by nucleus staining with PI, may correspond partly to a final phase of apoptosis (so called "late apoptosis"), when the plasmic membrane may become permeable.^{28 53 54}

Caspase activation during apoptosis results in the cleavage of critical cellular substrates, including PARP, so precipitating the dramatic morphological changes of apoptosis. We have shown that staurosporine induced HCEC apoptosis is accompanied by caspase-3 activation and concurrent PARP cleavage, as in many other cell models using this inducer.^{18 20 21 23 55} The role of caspases in HCEC apoptosis had until now only been suspected. Wilson showed the presence of the caspase-1 messenger RNA in HCECs.³⁶ Albon, using immunohistochemistry

on organ cultured corneas, showed the presence of active caspase-3 in the endothelial cells located in the folds, where there are most apoptotic cells.⁷ Conversely, in similar conditions Crewe found only few cells expressing active caspase-3. It should be stressed however that this author chose to ignore the endothelial folds.³⁷ Our in vitro model is therefore fully coherent with previous research, and for the first time highlights caspase-3 activity that is effective during endothelial apoptosis. The implication of caspases, and of caspase-3 in particular, offers the prospect of using specific synthetic inhibitors when accelerated apoptosis is observed, as in the ex vivo storage of corneas. This sequence must be verified on non-immortalised cells and for other stimuli that may exist during cornea storage: accumulation of free radicals and lack of specific growth factors, the latter circumstance being especially likely in serum free media.

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