Lamin Cleavage: A Reliable Marker for Studying Staurosporine-Induced Apoptosis in Corneal Tissue

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Purpose. The aims of this study were to identify a robust apoptosis marker suitable for both quantification and back-to-back analyses of programmed cell death and to define specific upstream targets for apoptosis in corneal cells.

Methods. Apoptotic cleavage of initiator caspases and their downstream targets such as lamin A and poly-ADP ribose polymerase was investigated in human corneal endothelial cells (HCEC-12), keratocytes (HCK), epithelial cells (HCEp), and full-thickness corneas using Western blotting and confocal microscopy following apoptosis induction with staurosporine. We specifically focused on nuclear lamins, which have important structural and regulatory functions in the cell nucleus.

Results. The cleavage of lamin A in HCEC-12 was significantly increased following apoptotic induction compared with HCK. More importantly, lamin A cleavage was detected in a dose-dependent manner in full-thickness corneal tissue by both Western blot analysis and fluorescence microscopy. Our study also demonstrates that HCEp show approximately three-fold increase in caspase 6 cleavage compared with endothelial cells or keratocytes. The presence of cleaved caspase 9 was lower in endothelial cells compared with epithelial cells and keratocytes.

Conclusions. We successfully established lamin A cleavage as a quantifiable marker of apoptosis in both corneal cells and tissue. Quantification of lamin A cleavage by Western blotting followed by a back-to-back analysis with fluorescence microscopy was studied for the first time in the experimental (donor) corneal tissue. Screening of downstream apoptosis proteins and establishing cell type-specific protocols allowed us to identify possible targets (caspases, Apaf-1, etc.) for protective therapeutic approaches.

Keywords: apoptosis, lamins, corneal endothelial cells, corneal epithelial cells

Apoptosis is one of the major stabilization processes in vital tissues. There are numerous ways to induce, control, and inhibit apoptosis in cells. Furthermore, cell type-specific pathways in apoptosis induction and control have been described.1–3 During storage of donor corneas, apoptotic cell death dramatically reduces the life span of donor tissue and its availability for transplantation. Apoptosis in different human corneal layers has been studied in various corneal cell lines and corneal tissue under different conditions: Cultivated donor cornea tissue was examined both during hypothermic and during organ culture conditions, identifying apoptosis in all corneal layers.4,5 This indicated that the three major cell types present in the corneal tissue undergo apoptosis when subjected to unfavorable conditions. Additionally, it has been reported that corneal endothelial apoptosis is influenced by apoptotic processes of the keratocytes.6 To understand the molecular mechanisms of apoptosis in cornea, different chemical apoptosis inducers have been used, such as staurosporine or camptothecin in human corneal endothelial cells, in human corneal epithelial cells (HCEp), and in cultivated corneal keratocytes.7–9 Similarly, apoptosis of corneal keratocytes has been shown to affect wound healing and tissue organization. Furthermore, its role in corneal allograft rejection in the mouse model and cryopreservation of primary keratocytes has been investigated.6,10–12 Apoptosis-related studies have been performed in different experimental models such as human and animal cell lines, primary cells, murine corneal tissue, and experimental human tissue.6,9,10,13 However, the overall majority of these studies dealt only with a small number of proteins involved in the apoptosis pathway. Also, the scope of the investigation was often limited to a single cell type such as endothelium or epithelium. Additionally, many apoptosis-related studies in whole human corneal tissue (donor corneas) have mostly involved the detection of DNA fragmentation via the TUNEL assay, one of the major cell death-related readouts. DNA fragmentation is believed to be one of the most global readouts for cell death. However, this assay was reported to have serious drawbacks, including unspecific or false-positive staining.14 Furthermore, as DNA fragmentation is not always required for a cell to enter apoptosis, it is not necessarily detectable at all stages.15 Considering this, it is crucial to obtain a precise overview about apoptotic processes in all three major layers of the cornea (especially during storage). For a strategic development of an antiapoptotic approach of human corneal grafts, a major readout of ongoing apoptosis is needed, starting in cell lines with focus toward lamellar and full-thickness corneal grafts. For a specific target validation, certain proteins of the respective cell type-specific apoptosis pathways in the tissue...
must be analyzed. To date, there is no systematic study investigating downstream factors of apoptosis in all three major cell types of a cornea. In this study, we followed a bottom-to-top agenda (Fig. 1) and analyzed different intermediate and downstream proteins of the apoptosis pathways in corneal endothelial, stromal, and epithelial cells to identify a reliable and global apoptosis marker.

**MATERIALS AND METHODS**

**Culture of Corneal Cells**

A human corneal endothelial cell line (HCEC-12, DMSZ No. ACC-646) was purchased from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). The cells were grown in culture medium containing MEM (Gibco, Paisley, United Kingdom) with 5% fetal bovine serum (Sera plus, Aidenbach, Germany) and 50 μg/mL gentamycin (Life Technologies, Grand Island, NY, USA).

**Induction of Apoptosis in Cells**

In this study, many intermediate and downstream proteins of the apoptosis pathways were analyzed in different corneal cell types and in the whole corneal tissue. The proteins analyzed included Apaf-1, initiator and effector caspases (Caspase-3, -6, -7, and -9), and downstream nuclear targets of activated caspases such as poly-ADP ribose polymerase (PARP) and lamins (Fig. 1). The three different corneal cell types were seeded in six-well plates at 90% to 95% confluency 1 day prior to apoptosis induction. Apoptosis was induced by treating the cells with 2.5 μM staurosporine (Alfa Aesar, Tewksbury, MA, USA) for a period of 4 hours.

**Induction of Apoptosis in Corneal Tissue**

Human corneas (research tissue) with intact corneal layers that could not be used for transplantation were obtained from eye banks (Sightlife, LionEye, FoB). The age of the donors was between 45 and 78 years, and the storage time was between 10 and 15 days. The corneas were equilibrated overnight in the Biochrome I medium (Biochrome, Berlin, Germany) containing 2% FCS. The corneas were cut into pieces (quartered) and then treated with different concentrations of staurosporine for apoptosis induction. The corneal pieces were then analyzed by Western blotting or by confocal microscopy. The experiment was performed three times.
**Western Blotting of Corneal Cells**

After induction of apoptosis, the cells were washed three times with PBS (PanBiotech). Cells were lysed using 2X Laemml buffer (120 mM Tris-HCl, pH 6.8, 4% SDS, and 20% glycerol). Cell lysates were further subjected to sonication (10 cycles, 80 amp) followed by centrifugation at 20,817g for 20 minutes. The supernatants were taken for further analysis. The protein concentration of the lysates was determined using the Protein 660-nm assay containing the ionic detergent compatibility reagent (Thermo Fischer Scientific, Rockford, IL, USA). The protein samples were then subjected to electrophoresis on 12% SDS-acrylamide gels followed by transfer to polyvinylidene difluoride (PVDF) membranes (Roche Diagnostics GmbH, Mannheim, Germany). The membranes were then incubated with enhanced chemiluminescence detection reagent (Amersham ECL Prime; GE Healthcare Life Sciences, Buckinghamshire, United Kingdom) and visualized using LAS-5000 mini imaging system (Fujifilm, Düsseldorf, Germany). The primary antibodies used for detection included Apaf-1 (CST 8969), Caspase-9 (CST 9508), Caspase-3 (CST 9665), cleaved Caspase-6 (CST 9761), Caspase-7 (CST 12827), PARP (CST 9532), lamin A/C (CST 4777), cleaved lamin A (CST 2036), lamin B1 (CST 12586), and β-actin (CST 8457). The secondary antibodies used included anti-rabbit (CST 7074) and anti-mouse (CST 7076) HRP-conjugated secondary antibodies (Cell Signaling Technology, Danvers, MA, USA). The band intensities of the target proteins were normalized to the loading control. Quantification of the cleaved caspase-3 bands showed that amounts of proteins and their cleaved parts were analyzed using ImageJ (National Institutes of Health, Bethesda, MD, USA).

**Western Blotting of Tissue Lysates**

After apoptosis induction, the tissue was cut into small pieces. The pieces were then lysed with ceramic beads using the Percelys homogenizer (Rockville, MD, USA) in the presence of 2X Laemml buffer. After tissue lysis, the lysate was subjected to sonication followed by centrifugation at 20,817g for 20 minutes. The supernatant was taken for further analysis. The protein concentration of the lysates was determined using the Protein 660-nm assay containing the ionic detergent compatibility reagent (Thermo Fischer Scientific, Rockford, IL, USA). The protein samples were then subjected to electrophoresis on 12% SDS-acrylamide gels followed by transfer to polyvinylidene difluoride (PVDF) membranes (Roche Diagnostics GmbH, Mannheim, Germany). The membranes were then incubated with enhanced chemiluminescence detection reagent (Amersham ECL Prime; GE Healthcare Life Sciences, Buckinghamshire, United Kingdom) and visualized using LAS-5000 mini imaging system (Fujifilm, Düsseldorf, Germany). The primary antibodies used for detection included Apaf-1 (CST 8969), Caspase-9 (CST 9508), Caspase-3 (CST 9665), cleaved Caspase-6 (CST 9761), Caspase-7 (CST 12827), PARP (CST 9532), lamin A/C (CST 4777), cleaved lamin A (CST 2036), lamin B1 (CST 12586), and β-actin (CST 8457). The secondary antibodies used included anti-rabbit (CST 7074) and anti-mouse (CST 7076) HRP-conjugated secondary antibodies. All the antibodies were purchased from Cell Signaling Technology and were used with the recommended dilutions following the manufacturers’ protocols. β-Actin was used as a loading control. The band intensities of target proteins were normalized to the loading control. Quantification of target protein bands was performed using ImageJ (National Institutes of Health, Bethesda, MD, USA).

**Staining of Corneal Tissue for Apoptosis-Related Proteins**

Apoptosis was induced in quartered (whole cornea cut into four quarters) corneal tissue samples for 4 hours using different concentrations of staurosporine. The corneas were washed three times in PBS to remove the medium. The corneal pieces were fixed with 0.5% paraformaldehyde for 30 minutes at 4°C. Following fixation, the corneal pieces were incubated in blocking buffer (1X PBS with 5% BSA and 0.3% Triton X-100) for 60 minutes. The corneal pieces were incubated with primary antibodies diluted in antibody dilution buffer (1X PBS with 1% BSA and 0.3% Triton X-100) overnight at 4°C. Following incubation with primary antibodies, the corneal pieces were washed three times with 1X PBS and incubated with respective fluorochrome-conjugated secondary antibodies at room temperature for 2 hours. The pieces were then stained with To-pro-3 (T3605; Life Technologies, Carlsbad, CA, USA) for 50 minutes. The corneal pieces were mounted on glass slide with Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). The samples were analyzed by confocal microscopy (Zeiss LSM 780; Carl Zeiss GmbH, Jena, Germany). The primary antibodies used were ZO-1 (cat. no. 61-7300; Thermo Fischer Scientific) at 1:200 and cleaved lamin A at 1:100 (Cell Signaling Technology). The secondary antibodies used included anti-mouse Alexa-405 and anti-rabbit Alexa-555. To-pro-3 iodide was used for nuclear staining (1:1000).

**Statistical Analysis**

All data are reported as mean with SD. Statistical analysis was performed using GraphPad Prism (Version 6.01) for Windows (GraphPad Software, San Diego, CA, USA). Multiple comparisons were performed using the 1-way ANOVA test. *P* < 0.05 was considered statistically significant.

**RESULTS**

**Analysis of Apoptosis Pathway–Related Proteins in Different Corneal Cells**

**Apoptosome.** Different apoptosis-related proteins were analyzed in HCEC-12, HCK, and HCEp cells, thus representing the major cell types of the cornea. During apoptosis, different apoptosome complexes are activated. This activation leads to an intrinsic cleavage of the respective caspases, and the active forms can be detected in the cell lysates treated with the inducer. Amounts of proteins and their cleaved parts were analyzed via Western blot analysis for untreated and staurosporine-treated cell lysates to represent apoptotic effects. The cytochrome C–dependent apoptosome, namely Apaf-1, and Caspase-9 were investigated first. On apoptosis induction, cytochrome C is released from the mitochondria, which along with dATP causes the dimerization of Apaf-1, which then leads to processing of pro-Caspase-9 to form the active form. Apaf-1 protein levels were found to be similar in the untreated samples. However, in staurosporine-treated samples, the level of this protein was reduced (Fig. 2A). Cleaved Apaf-1 product was also observed in staurosporine-treated samples of all the three cell types. Caspase-9 was activated following apoptotic stimulus. Activated caspase-9 was significantly increased in all the three cell types after apoptosis induction (Fig. 2B). The reason for the reduced Apaf-1 level can be attributed to proteolytic cleavage of the protein following apoptosis induction.16 Degraded Apaf-1 product was observed in all the three cell types.

**Caspase Cascade.** Following caspase-9 apoptosome activation, downstream activation of effector Caspases 3, 6, and 7 was analyzed. As can be seen in the Western blot image in Figure 3A (left), pro-Caspase-3 was detected in both treated and untreated lysates of all three cell types, whereas the active form of caspase-3 was observed in the treated samples. Quantification of the cleaved caspase-3 bands showed that the presence of active caspase-3 was significantly higher in case of HCK and HCEp cells compared with HCEC-12 cells (Fig. 3A, right). As presented in Figure 3B, analysis of cleaved Caspase-6 in the cell lysates showed a significant increase in the presence of active Caspase-6 in all the three cell types. The levels of cleaved Caspase-6 were found to be similar for
endothelial cells and keratocytes; however, the highest levels of this protein were detected in epithelial cells. Similarly, active Caspase-7 was detected in all treated cell lysates, and the levels of this protein were found to be similar in all the three cell types (Fig. 3C). The presence of cleaved effector caspases is a clear indication of apoptosis induction.

**Nuclear Downstream Targets.** Downstream targets of the activated effector caspases chosen for further investigation were nuclear proteins lamins (proteins from lamin family) and PARP. The lamin family members investigated in our study included lamin A/C and B. As demonstrated in Figure 4A, base levels of lamin A (74 kDa) and lamin C (65 kDa) as detected by lamin A/C antibody in untreated cells did not reveal any cell type-specific variation. Cleaved lamin A/C bands were observed in the staurosporine-treated cell lysates. Joint quantification of cleaved lamin A/C bands revealed that the cleavage was much more pronounced in HCEp cells, which was highly significant (Fig. 4A, right). When using antibody for only cleaved lamin A (CST 2036), cleaved lamin A bands (28 kDa) were only detected in the staurosporine-treated samples (Fig. 4B). The quantification of these bands showed that the presence of cleaved lamin A was highest in HCEC-12 cells compared with the HCK and HCEp cells. Analysis of cell lysates for lamin B1 (Fig. 4C) showed increased levels of lamin B1 cleavage in all three cell types. Differences in this effect between the cell types were not found to be statistically significant.

As can be seen in Figure 4D, the base level of the whole PARP (116 kDa) protein was similar in all the three cell types. Cleaved PARP bands (89 kDa) were clearly observed in treated samples for all these cell types. Quantification of cleaved PARP bands was carried out. However, due to a high variation of PARP protein levels, this increase of cleaved PARP was not found to be statistically significant for any of the cell types.

**Lamin A Cleavage as Marker for Apoptotic Cell Death in Corneal Tissue**

Following our results and published data on apoptotic activation in corneal cells, we analyzed whole tissue lysates for the presence of cleavage of all caspases mentioned above, PARP, lamin A/C, and lamin B1 (data not shown). Interestingly, only the analysis of lamin A cleavage turned out to deliver a stable, quantifiable signal. To investigate the dose-dependent behavior of this marker, we tested a range of staurosporine doses. Whereas 1 μM staurosporine led to a 3-fold increase of cleaved lamin A, 5 μM staurosporine led to a 14-fold increase of lamin cleavage compared with untreated controls (Fig. 5A). Therefore, the cleavage of lamin A followed an almost linear dose-dependent relation in tissue extracts, leading to a five-fold increase of lamin cleavage at a five-fold higher concentration of staurosporine. As can be seen in Figure 5C, this enrichment of apoptosis-induced lamin A cleavage product could be confirmed by fluorescence microscopy (Fig. 5). Other signs of ongoing apoptosis, such as loss of tight junction integrity or chromatin condensation, were also clearly detectable.

**DISCUSSION**

Apoptotic cell death has been investigated by several groups in different corneal cells and tissue to understand its role in endothelial cell loss during storage, pathogenesis of Fuchs dystrophy, failure of corneal allografts, cytotoxicity of drugs used to treat ocular ailments, etc. These studies
were performed using various experimental models that included human origin corneal cell lines, primary cells (endothelial, epithelial cells, or keratocytes), and donor corneal tissue; corneal cells and tissue from other animals such as mouse, rat, bovine, porcine have been used. Most previous studies in this regard have been confined to the analysis of apoptosis in single cell type focusing either on endothelial or epithelial cells or keratocytes. Also, only a few of the well-known intermediate and downstream targets of apoptosis pathways have been studied, including Caspase-3 and PARP.

In this study, we analyzed different apoptosis-related proteins in corneal endothelial and epithelial cells, as well as in keratocytes both in vitro and ex vivo in corneal tissue. In addition to the detection of different apoptosis-related proteins as potential readouts, the other important aspect of this study was to quantify the presence of these proteins in cells and whole tissue as direct indicators of the extent of apoptosis in different corneal cell types. We focused our analysis on caspase-dependent mitochondrial cell death pathway proteins.

We studied the most relevant apoptosis pathways from initiator caspases down to lamin cleavage, marking the very end of apoptosis (Fig. 1). The caspases are central to the apoptosis pathways as the caspase cascade is responsible for the biochemical and morphologic changes associated with both intrinsic and extrinsic apoptosis pathways. An important step prior to caspase activation is formation of the apoptosome. After apoptosis induction, cytochrome c is released from the mitochondria, which binds to Apaf-1 and forms the apoptosome, which in turn leads to activation of Caspase-9. In our study, we analyzed the proteins constituting the apoptosome, Apaf-1, and Caspase-9. Apaf-1 was observed in all the three cell types; however, the levels of Apaf-1 were found to be decreased in the staurosporine-treated samples. Interestingly, these reduced levels of Apaf-1 also corresponded with the presence of cleaved Apaf-1 product in the treated cell lysates. This cleaved Apaf-1 protein has been reported in some of the previous studies. The activated form of Caspase-9 was detected in the cell lysates. Following the activation of initiator caspases, the effector caspases are activated, and this results in the degradation of further downstream targets. Three effector caspases (3, 6, and 7) were analyzed in our study; these
executioner caspases have been shown to have specific and nonredundant functions in the apoptosis process using Jurkat cell lysates.\textsuperscript{23,24} As a result of this analysis, some of the findings from former studies could be confirmed and expanded in our work. The study on human corneal endothelial cells showing the presence of activated Caspase-3 and cleaved PARP in staurosporine-treated cells is in line with our results; the authors focused on the clarification whether endothelial cell death is indeed an apoptotic event.\textsuperscript{9} Similarly Caspase-3 activation after apoptosis induction with staurosporine has been investigated in the corneal epithelial cell line by fluorometric assay. In other study concerning failure of corneal allografts in a mouse model active Caspase-3 was analyzed in cultured keratocytes and in mouse corneal tissue by Western blotting.\textsuperscript{6,8} Our results showed the increased presence of activated Caspase-3 in the staurosporine-treated cell lysates of all the three cell types. The presence of activated Caspase-3 was highest in HCEp cells, and quantification of cleaved PARP.

**Figure 4.** Cleavage of downstream targets by activated effector caspases. Figure represents analysis of cleaved lamin A/C (A), cleaved lamin A (B), cleaved lamin B1 (C), and cleaved PARP (D) in untreated (STS−; white bars) and staurosporine-treated (STS+; black bars) cell lysates from HCEC-12, HCK, and HCEp cells. Results are expressed as mean ± SD. *P < 0.05; **P < 0.001 (n = 3).
Caspase-3 bands showed that the results were significant across cell types (Fig. 3A, right). The role of Caspases-3 and -9 has been widely studied with respect to apoptosis execution in corneal cells and tissue. However, the other effector caspases -6 and -7 have not been investigated in corneal cells, so we analyzed these caspases, which play an important role in the execution phase of apoptosis. Caspases-6 and -7 have been shown to have specific downstream targets. Lamin A has been reported to be a highly specific substrate for Caspase-6. In experiments with Hela and Jurkat nuclei, it was conclusively shown that when lamin A is present, it must be cleaved by Caspase-6 for the efficient culmination of the nuclear apoptosis process. Our results clearly showed the presence of cleaved Caspase-6 in all studied cell types, and it was significantly higher for HCEp cells compared with the other two cell types (Fig. 3B, left and right). The downstream nuclear targets of activated Caspase-6, which are lamins, were evidently detected in the HCEC-12, HCEp, and HCK cell lysates. The presence of cleaved lamin A/C products was significantly higher for HCEp cells compared with HCEC-12 and HCK cells, pointing toward the specificity of lamin for caspase-6. The cleavage product of another nuclear target, PARP, which is a substrate for Caspase-3 and -7, was clearly detected in the staurosporine-treated cell lysates; however, on quantification, these results were not statistically significant.

Moreover, we went a step further and performed dose-dependent apoptosis induction with staurosporine in corneal tissue and quantified the extent of lamin A/C cleavage in the tissue lysates with the cleaved lamin A antibody. The staurosporine-treated tissue lysates showed a concentration-dependent increase in the presence of cleaved lamin A compared with untreated (only dimethyl sulfoxide) samples. To our knowledge, this is the first time a comprehensive quantification of apoptotic markers is presented both in vitro and ex vivo identifying lamin A cleavage as a crucial end point marker of apoptosis in corneal cells. Considering the drawbacks associated with the TUNEL assay for analyzing apoptosis, it is important to have an alternate marker that would be helpful in quantifying the apoptotic effect especially in the
tissue. The presented upstream analyses contain data for future targets of antiapoptotic approaches.

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