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Early Keratocyte Apoptosis after Epithelial Scrape Injury in the Human Cornea

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Abstract

Animal studies in mice, rats, rabbits, pigs and hens demonstrated that anterior keratocytes undergo programmed cell death or apoptosis after corneal epithelial injury. Many other wound healing changes subsequently follow the keratocyte apoptosis reponse. This study evaluated early keratocyte apoptosis after corneal epithelial scrape injury in human eyes scheduled for enucleation for malignancy. Two eyes had corneal epithelial scrape one hour prior to the enucleation and another eye served as a control and had no corneal scrape prior to enucleation. One additional eye was enucleated, washed with balanced salt solution, and then had the corneal epithelium scraped one hour prior to processing for analysis. Apoptosis was identified by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay and confirmed by transmission electronic microscopy (TEM). Anterior keratocyte apoptosis was detected in the three corneas that had epithelial scrape injury, but not in the control unwounded cornea. This study confirmed that keratocyte apoptosis is also an early response to corneal epithelial injury in humans and showed that tears are not essential for keratocyte apoptosis to occur in response to epithelial injury.

Keywords

apoptosis; keratocyte; corneal wound healing; epithelial scrape; human

Studies in primates and other species observed that keratocytes in the anterior stroma apparently disappear immediately following epithelial scrape injury (Dohlman et al., 1968; Campos et al. 1994; Nakayasu, 1988). Wilson and coworkers (1996) demonstrated that this phenomenon is mediated by apoptosis or programmed cell death. Apoptosis is a controlled, gentle form of cell death that leads to the formation of apoptotic bodies that contain the cellular contents of the dying cell, including lysosomal enzymes or other intracellular components that would damage surrounding cells and tissue. These apoptotic bodies diffuse into the tissue and are absorbed by other living cells. Thus, apoptosis causes minimal damage to the surrounding cells and tissue and has a critical role in tissue development, homeostasis, response to infection, and wound healing (Arends and Wyllie, 1991; Wilson and Kim, 1998; Wyllie et al., 1980; Wilson, Chaurasia and Medeiros, 2007). The anterior keratocyte apoptosis response that occurs after epithelial injury in the cornea has been hypothesized to function as a mechanism to limit

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the extension of viruses such as herpes simplex that infect the corneal epithelium but have the capacity to infect keratocytes, endothelial cells, and other cell types that would allow the viral infection to extend into the eye and brain (Wilson, et al., 1997). Thus, it has been demonstrated that defective keratocyte apoptosis in Stat 1 null mice is associated with more aggressive herpes simplex keratitis (Mohan et al., 2000b).

Several studies have suggested the Fas/Fas ligand system and cytokines that regulate Fas/Fas ligand are involved in regulating early keratocyte apoptosis (Mohan et al., 1997; Mohan et al., 1998; Mohan et al., 2000a; Wilson et al., 1996). Triggers that injure the epithelium initiate these cytokine-death apparatus activation pathways.

Early keratocyte apoptosis has been reported in animal models after many different types of epithelial injury, including mechanical scrape, incisions, photorefractive keratectomy (PRK), laser in situ keratomileusis (LASIK) and infection such as herpes simplex keratitis (Gao et al, 1997; Helena et al., 1998; Martínez-García et al., 2006; Mohan et al., 2003; Netto at al., 2005; Wilson et al., 1997; Wilson, et al., 2003). Virtually any epithelial injury can induce keratocyte apoptosis. For example, even mild epithelial damage from pressing a plastic ring against the cornea will trigger superficial stromal keratocyte apoptosis (Helena et al., 1998; Wilson, 1998).

The location and level of early keratocyte apoptosis in the cornea is related to the type of epithelial injury (Helena et al., 1998; Wilson, 1997). For example, photorefractive keratectomy (PRK) and laser insitu keratomeliusis (LASIK) incite different levels of early apoptosis and at different locations (Mohan, et al., 2003). Interestingly, the intensities of the subsequent wound healing events — for example, keratocyte proliferation and myofibroblast transformation — are proportional to the early apoptotic response (Mohan et al., 2003). Moreover, there is also biological variability in this response between the eyes of different animals after the same type of stimuli (Mohan et al., 2003; Netto at al., 2005). This observation is of clinical significance because these differences relate the differences in outcomes between different eyes after the same surgical procedures (Dupps and Wilson, 2006).

Animal studies in mice, rats, rabbits, pigs and hens demonstrated that anterior keratocytes undergo programmed cell death or after epithelial scrape injury (Wilson, et al., 1996; Mohan, et al., 2003; Martinez-Garcia, et al., 2006). There are no studies determining whether keratocyte apoptosis occurs in human eyes that have corneal epithelial injury. In this study, we evaluated early keratocyte apoptosis after epithelial scrape in human corneas in a unique model involving patients with normal corneas and anterior segment scheduled for enucleation or exenteration of the eye because of choroidal melanoma or orbital malignancy. These experiments were approved by the Human Subjects Division of the Institutional Review Board of the University of Washington, Seattle, WA (HSD 99-2341-A 02) and by the Ethics Commission for Research Projects in Humans from the University of São Paulo (CAPPesq – 704/02) and informed consent was obtained from each patient.

Four subjects consented to participate in this study. Two eyes had corneal epithelial scraping with a #64 Beaver Blade over the entire central cornea, sparing only 0.5 mm at the limbus, one hour before enucleation (female, age 51 and male, age 56). One eye (male, age 41) had epithelial scrape performed after enucleation and profuse washing of the globe with balanced salt solution (BSS) to remove tears. After epithelial scraping, this eye was maintained for one hour in a humidified chamber at 37° C before excision of the corneal-scleral button. One eye (female, age 46) had no epithelial scrape and served as a control. The corneal-scleral buttons were removed using 0.12 forceps and sharp Westcott scissors immediately after enucleation, with the exception of the eye that was maintained in a humidified chamber. Immediately after the excision of the corneal-scleral buttons, each was partially fixed in 4% paraformaldehyde (PFA)

for 4 hours before being bisected. Half of each cornea was then cryofixed in Optimal Cutting Temperature (OCT) compound (Tissue-Tek, Torrance, CA) within a 24 mm×24 mm×5 mm mould (Fisher, Pittsburgh, PA, USA for TUNEL assay and immunohistochemistry (IHC) and half was fixed for transmission electron microscopy (TEM).

The frozen tissue blocks were stored at −85 °C until sectioning was performed. Central corneal sections (7 μm thick) were cut with a cryostat (HM 505M, Micron GmbH, Walldorf, Germany). Sections were placed on 25 mm×75 mm×1 mm microscope slides (Superfrost Plus, Fisher) and tissue sections were fixed in acetone at −20 °C for 2 minutes, dried at room temperature for 5 minutes, and then placed in balanced salt solution. A fluorescence-based TUNEL assay was performed according to the manufacturer's instructions (ApopTag, Cat No; S7165; Intergen, Purchase, NY, USA). Positive (rabbit cornea 4 hours after epithelial scrape) and negative (rabbit cornea, unwounded) control slides were included in each assay. Photographs were obtained with a Nikon E600 fluorescent microscope (Melville, NY, USA).

Tissue for TEM was fixed in 2% paraformaldehyde and 2% glutaraldehyde in a vehicle of 1.3 sodium phosphate buffer containing 0.05% $MgCl_2$–$6H_2O$, pH 7.3, at 4°C for 24 hours and then washed twice with the buffered fixative vehicle for 15 minutes at room temperature. Specimens were then stored at 4°C in the fixative vehicle until they were processed. A 1.5 mm strip was removed from the center of each half corneal-scleral button fixed for TEM. The strips were again bisected and the fragments were placed in the primary fixative vehicle prior to secondary fixation. Secondary fixation was performed in 1% OsO_4 in 1.0 phosphate buffer, pH 7.3, for 45 minutes at room temperature, followed by three washings in the phosphate-buffered fixative vehicle and dehydration in a graded ethanol series. The transition from 100% ethanol to epoxy was mediated by two changes of propylene oxide. An epoxy medium of Spurr's formulation (Spurr, 1969) was used for infiltration and embedding. The fragments were mounted in flat molds and hardened at −70°C for 24 hr before sectioning. Both 1 μm thick light microscopic sections and ultra-thin TEM sections were cut. The light microscopy sections were stained with 50% modified Richardson's stain (1% methylene blue and 1% azure II in 1% sodium borate solution diluted 1:1 with 1 dibasic sodium phosphate solution at pH 8.5) and the TEM sections were mounted on polyvinyl butyral-coated grids (Pioloform, Sigma, St Louis, MO, USA) (Robards and Wilson, 1993) and stained with saturated aqueous uranyl acetate and Reynolds lead citrate (Reynolds, 1963). TEM was performed with a model PW6020, CM10 transmission electron microscope, Philips Electronics N.V. (Eindhoven, The Netherlands).

Superficial keratocyte apoptosis was detected by the TUNEL assay under the wounded area in all scraped corneas, including the case scraped after enucleation and washing with BSS to remove tears, although there was variability in the depth that the apoptosis extended into the stroma from the anterior surface in different corneas. The TUNEL assay was negative in the control cornea, as it was in the stromal beneath intact epithelium in scraped corneas (not shown). Keratocyte apoptosis (chromatin condensation, formation of apoptotic bodies, intracellular blebbing and cellular shrinkage) was detected by transmission electron microscopy in the anterior stroma of in all scraped corneas, including the one scraped after enucleation and washing with BSS to remove tears (Figure 1B).

This study confirms keratocyte apoptosis occurs in the anterior sub-epithelial stroma immediately after epithelial scrape in human corneas, as has been reported in other animals, including mouse, rabbit and pigs. The time point after scraping used in this study was one hour, which is similar to the time course in other species. This study also confirms that tears are not essential for keratocyte apoptosis to occur after epithelial scrape injury, which is in agreement with a previous study in mice and rabbits (Mohan RR, Mohan, RR, Ambrósio Jr R, Wilson SE, Activation of keratocyte apoptosis in response to epithelial scrape injury does not require tears. Association for Research in Vision and Ophthalmology (ARVO) Annual Meeting, May,

2002. Program No. 1679). This supports the hypothesis that the stromal apoptosis response is mediated locally by modulators derived from the injured corneal epithelium and keratocytes (Mohan et al., 1997; Mohan et al., 2000a).

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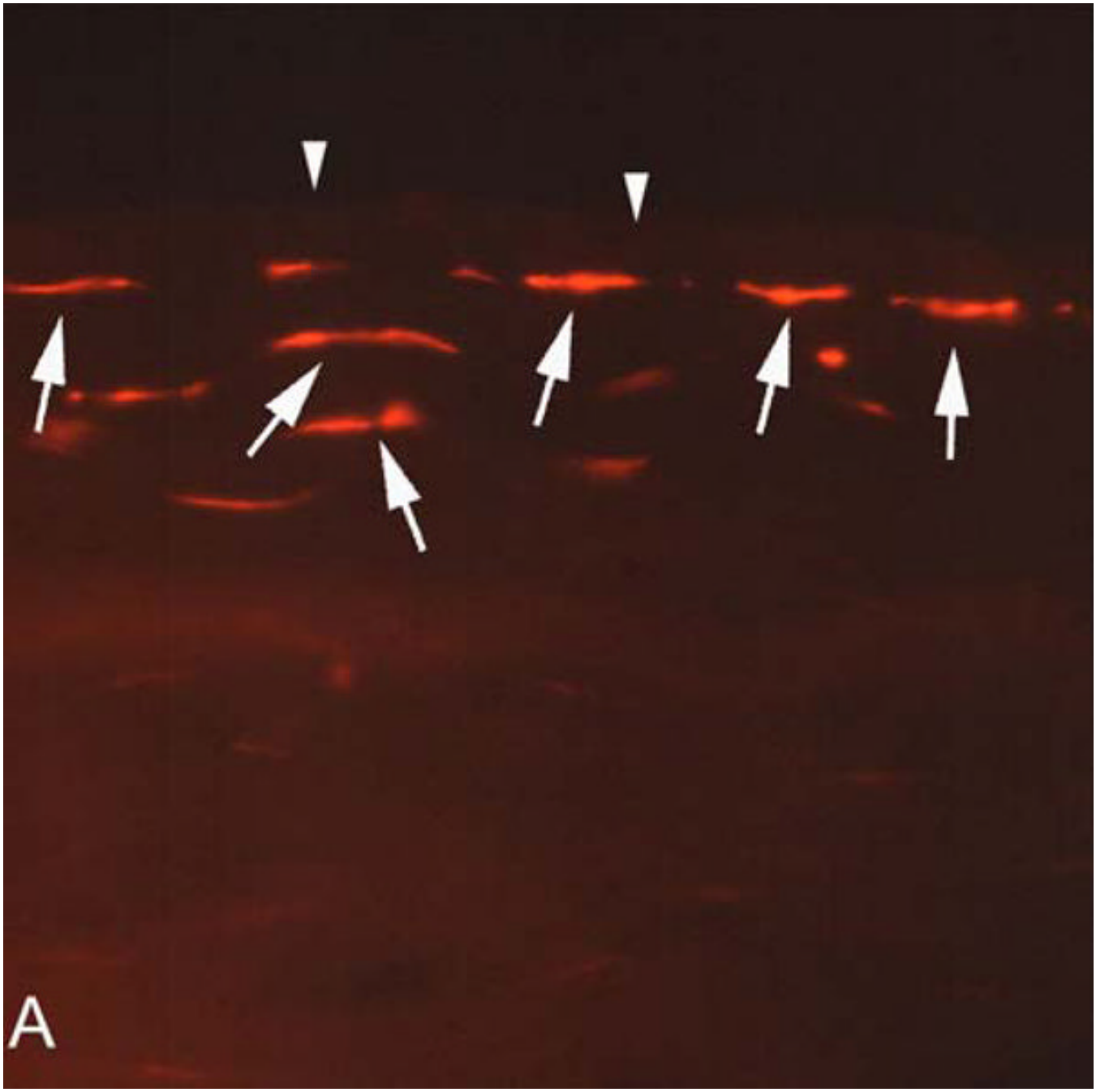

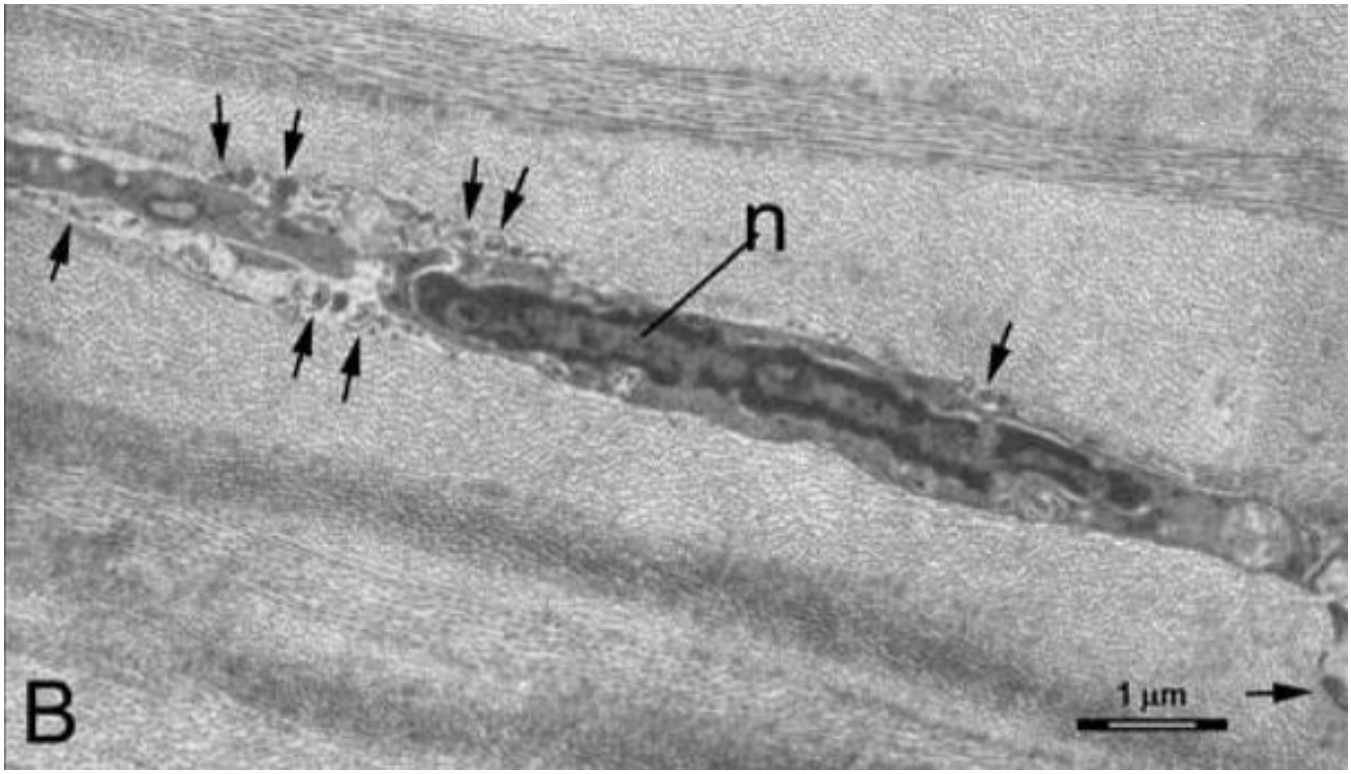

Figure 1.

A. Keratocyte apoptosis detected in human corneas one hour after epithelial scrape injury prior to enucleation for a choroidal melanoma. TUNEL-positive keratocytes are detected (arrows) in the anterior stroma beneath Bowman's layer (arrowheads). Magnification 400X. B. Transmission electron microscopy in a human cornea one hour after enucleation, washing with balanced salt solution, and epithelial scrape with a scalpel. Note condensation of the chromatin in the nucleus (n), cell shrinkage and formation of apoptotic bodies (arrows). Magnification 7,500X.