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Apoptosis in the Initiation, Modulation and Termination of the Corneal Wound Healing Response

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Abstract

Stromal keratocyte apoptosis has been well-characterized as an early initiating event of the corneal wound healing response, triggering subsequent cellular processes that include bone marrow-derived cell infiltration, proliferation and migration of residual keratocyte cells, and, in some circumstances, generation of myofibroblast cells. Recent studies, however, have suggested a more general role for apoptosis in the overall stromal wound healing response that includes modulation and termination functions. This review article highlights, and ties together, recent studies that have demonstrated the important role apoptosis likely plays in the weeks to months following an initial insult to the cornea—depending on the type and extent of corneal injury.

Keywords

apoptosis; corneal wound healing; keratocytes; stroma; bone marrow-derived cells; myofibroblasts

1. Introduction

Immediately after epithelial insult, keratocyte cells underlying the area of injury undergo apoptosis or programmed cell death (Wilson, et al., 1996; Dupps and Wilson, 2006), an involutional and controlled form of death in which there is limited release of intracellular contents such as enzymes, chemokines and other components that could directly damage surrounding structures and cells and promote infiltration of excessive numbers of inflammatory cells with the potential to further damage the tissue. The type of epithelial injury dictates the location and extent of this early keratocyte apoptosis response (Helena, et al., 1998). Thus, extensive debridement of the epithelium over the central cornea triggers widespread keratocyte apoptosis within the anterior stroma underlying the epithelial injury. Conversely, incisional injuries from a blade or microkeratome stimulate apoptosis at the site of the epithelial and stromal penetration. Even epithelial pressure from a poorly fit contact lens may trigger limited superficial stromal keratocyte apoptosis (Wilson, 1998).

Keratocyte apoptosis is an exceedingly rare event in the normal uninjured cornea. Thus, in studies that included unwounded control corneas in rabbits (Helena, et al., 1998, Mohan, et al.,

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2003, Szentmary, 2005), mice (Wilson, et al., 1997), or humans (Kim, et al., 1999), almost no apoptotic keratocytes or other stromal cells were noted in control corneas, even when dozens of tissue sections were examined. Apoptotic keratocytes may, however, be noted away from sites of epithelial injury in keratoconus corneas (Kim, et al., 1999), leading to the hypothesis that abnormally high levels of ongoing keratocyte apoptosis could play a role in the pathophysiology of this ectatic corneal disease (Kim, et al., 1999; Chwa, et al., 2006). Abnormally high levels of keratocyte apoptosis have also been associated with the pathophysiology of aniridia (Ramaesh, et al., 2006). Thus, it appears that stromal apoptosis is tightly controlled during homeostasis in the absence of corneal injury or disease. However, once corneal injury occurs—whether mechanical, infectious, or chemical—stromal apoptosis becomes an important component of the wound healing response. Stromal apoptosis occurs immediately following corneal injury and, depending on the type and extent of injury, may persist in the tissue for months or even years.

Stromal cells that undergo apoptosis following injury vary depending on the type of injury, extent of injury and the time following injury. This review focuses on the identity of stromal cells undergoing apoptosis and the function of the apoptosis response at different time points after corneal injury. To facilitate discussion, apoptosis responses will be divided into the early phase (detected minutes to a few hours after injury), intermediate phase (hours to weeks after injury) and the late phase (occurring weeks to months, or even years, after injury).

2. Early phase apoptosis

Immediately following any sort of epithelial injury, stromal keratocytes underlying the epithelial injury undergo rapid keratocyte apoptosis (Wilson, et al., 1996). It is possible that other stromal cells such as Langerhans' cells, nerves and a few resident and circulating inflammatory cells could also be caught up in the wave of apoptosis, but there is no conclusive evidence one way or the other. In species with thin corneas, such as the mouse, one occasionally notes corneal endothelial cells that also appear to undergo apoptosis in response to extensive corneal epithelial injury, such as scrape (Wilson, et al., 1996).

The injury precipitating programmed keratocyte death can be produced by epithelial scrape (Fig. 1), incisional injuries from scalpel blades or microkeratomes, or even significant pressure of a contact lens on the epithelial surface (Wilson, et al., 1996; Wilson, 1998; Helena, et al., 1998; Mohan, et al., 2003). Most commonly, apoptosis is detected using the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) assay, and with this assay the labeling peaks at approximately four hours after epithelial injury (Wilson, et al., 1996). However, using transmission electron microscopy, it can be noted that chromatin condensation, cell shrinkage, and budding of apoptotic bodies begins immediately after epithelial injury (Wilson, et al., 1996). It cannot be emphasized enough how important it is to confirm TUNEL assay results using another method when studying a new system if at all possible. Unfortunately, under some circumstances, the TUNEL assay also labels cells undergoing necrosis where there is random degradation of deoxyribonucleic acid (DNA). There are two important examples of this that have been noted in our laboratory. The first is when TUNEL labeling of cells in the anterior stroma after epithelial scrape injury or epithelial scrape with photorefractive keratectomy continue to be noted for a week or more after the injury even though transmission electron microscopy shows that by a few days after injury almost all the cells that are continuing to die are undergoing necrosis (Mohan, et al., 2003). The second example is when the femtosecond laser is used to make a lamellar cut in the stroma, without injury to the epithelium, keratocytes surrounding the cut label with the TUNEL assay and are found to be dying only by necrosis when the tissue is studied with transmission electron microscopy (Netto, et al., in press). Thus, confirmation of TUNEL results in a particular system is critical. The gold standard for this remains transmission electron microscopy. There is some

hope that other methods, such as immunocytochemical detection of activated components of the apoptosis cascade, such as activated caspase-3, will supplant the laborious transmission electron microscopy method. However, to date, we have not been convinced of the reliability of these newer methods and, therefore, do not depend on them when working with a new model in which apoptosis has not been previously verified by characteristic cellular morphology detected using transmission electron microscopy.

What is the mechanism(s) that triggers keratocyte apoptosis following corneal epithelial injury? Any mechanism proposed must account for interesting observations that have been made regarding the process. First, it is not necessary to remove the epithelium to trigger keratocyte apoptosis. Pressure on a contact lens can trigger underlying anterior stromal keratocyte apoptosis without removal of the epithelium (Wilson, 1998). Second, a lamellar cut through the peripheral epithelium and into the central corneal stroma, such as the incision produced by a microkeratome, triggers keratocyte apoptosis anterior and posterior to the lamellar cut across the diameter of the cornea, far removed from the site of epithelial injury, even if the resulting flap is not lifted (Helena, et al., 1998). We believe that these observations suggest that keratocyte apoptosis is triggered by soluble mediators released from injured corneal epithelial cells themselves. A large body of work has suggested that interleukin-1 (Wilson, et al. 1996) and tumor necrosis factor α (Mohan, et al., 2000b) released from injured epithelial cells are involved in mediating keratocyte apoptosis, probably via more specific cell death pathways such as those that involve Fas and Fas ligand (Wilson, et al., 1996; Mohan, et al., 1997).

There has been work suggesting that the tear film contains mediators that can trigger keratocyte apoptosis (Zhao, Nagasaki, and Maurice, 2001; Zhao and Nagasaki, 2003). It makes sense that modulators that can induce keratocyte apoptosis would be present in tears since the tears continuously bathe the corneal epithelium. However, the rabbit or mouse eye can be removed from the animal and bathed extensively before epithelial scrape and anterior stromal keratocyte apoptosis still occurs in response to the injury (Mohan R.R., Mohan R.R., Ambrósio R. Jr., Wilson S.E. Activation of keratocyte apoptosis in response to epithelial scrape injury does not require tears. Association for Research in Vision and Ophthalmology (ARVO) Annual Meeting, Program No. 1679, May, 2002) and, therefore, tears are not required for induction of keratocyte apoptosis.

What is the function of such the elaborate early keratocyte apoptosis system that has been identified in the corneas of all species examined, including mouse, rabbit, pig, and human (Ambrosio R. Jr., Wilson S.E., unpublished data, 2002)? We hypothesized that the early apoptosis response to epithelial injury serves as an initial defense mechanism against posterior extension of infectious organisms, such as herpes simplex and adenovirus, that initially infect the epithelium, but have the capacity to extend to the keratocytes, corneal endothelium, retina, and even into the central nervous system (Wilson, et al., 1997). Once a cornea infection begins, it takes several hours for immune cells to mobilize into the cornea to fight the infectious organism. Rapid death of underlying keratocytes in response to viral injury to the epithelium sets up a firebreak of sorts to retard extension of the virus into the deeper cornea where it gains access to the endothelium, and the retina and central nervous system beyond. Consistent with this hypothesis, studies in stat 1 null mice defective in apoptosis (Mohan, et al., 2000a) found that mutant mice, but not control normal mice, were susceptible to virus extension into the central nervous system when the cornea was infected with herpes simplex virus (Hill J.M. and Wilson S.E., unpublished data, 2001). Such an infection would represent a major threat to the survival of the organism and there would likely be a selective advantage to having an effective early response.

3. Intermediate phase apoptosis (*and necrosis*)

The early apoptosis process in the cornea is relatively rapid in eliminating cells—with cells that die by this process disappearing completely from the stroma within minutes to hours after injury (Wilson, et al., 1996), similar to the rate of completion of apoptosis of cells in other organs (Kerr, Wyllie and Currie, 1972). What, then, are the cells that continue to die in the anterior stroma for a week (Fig. 2), or even longer, following epithelial scrape injury or epithelial injury that is a part of photorefractive keratectomy (Mohan, et al., 2003), for example? Some of these cells are likely bone marrow-derived cells that are attracted into the corneal stroma after injury by cytokines and chemokines released directly by the injured corneal epithelium or induced in keratocytes following injury (Wilson, et al., 2004, Carlson, et al., 2006). Many of these cells die by apoptosis but others clearly die by necrosis, confirmed by transmission electron microscopy (Mohan, et al., 2003), and it is unclear how the fate of a particular cell is determined following the initial wave of keratocyte death that seemingly occurs exclusively by apoptosis. Other cells that could die during the interval from a few hours to a week or more after epithelial injury (Fig. 3), include migrating corneal fibroblasts derived from mitosis of residual keratocytes, progenitor cells to myofibroblasts that are currently detectable only once they express α -smooth muscle actin, and potentially other uncharacterized cell types that participate in the corneal wound healing response. Little work has been directed towards characterizing these other cells that die following corneal epithelial injury and detailed knowledge in this area would likely augment our understanding of the overall wound healing process.

What is the function of this intermediate phase apoptosis response? We hypothesize that it serves to down-regulate the ongoing wound healing response and terminate the inflammatory response. For example, it may serve to down-regulate and terminate the inflammatory response of infiltrating bone marrow-derived cells to the corneal injury that, if left unchecked, could further damage the corneal stroma and lead to augmented corneal opacity and loss of corneal transparency. It could also serve to eliminate progenitor cells to myofibroblasts if a surveillance system were present to determine when injury to the cornea was insufficient to require the presence of these contractile factories of extracellular matrix involved in wound healing. There is no information about whether or not such a system exists, but if so, it could help explain why the generation of mature myofibroblasts is highly dependent on the level of corneal injury (Moller-Pedersen, et al., 1998; Mohan, et al., 2003). Thus, in the rabbit or human eye, when a -9 diopter photorefractive keratectomy is performed, a high proportion of the eyes generate subepithelial myofibroblasts and associated opacity or haze. However, when a -4.5 diopter photorefractive keratectomy is performed, using an otherwise identical procedure, few corneas generate significant myofibroblasts or haze. Some of this difference is clearly related to surface irregularity and resulting structural and functional defects of the regenerated basement membrane that functions to limit epithelial transforming growth factor β , and possibly other cytokines, necessary for myofibroblast differentiation in the corneal stroma (Jester, et al., 2002; Stramer and Fini, 2004; Netto, et al., 2006), however, there are clearly other factors involved, too, since myofibroblasts and haze can develop in some corneas with lower levels of injury, even when the surface is aggressively smoothed. Perhaps defects in this proposed surveillance system accounts for the emergence of myofibroblasts in rare patients who develop haze following low PRK corrections.

The mechanisms involved in triggering the intermediate phase of apoptosis in bone marrow-derived cells, keratocyte progeny and, potentially, myofibroblast progenitor cells, are completely unknown. Some of the response could be passive. For example, bone marrow cells in the corneal stroma could undergo apoptosis (or possibly necrosis) after a period of time removed from cytokine signals that maintain cell viability present in blood (Rodriguez-Tarduchy, Collins and Lopez-Rivas, 1990). However, more active processes could be present

to remove cells that are not needed, perhaps modulated by cytokines derived from the healing epithelial or stromal cells.

4. Late phase apoptosis

In the months to years following corneal injury or surgery, cell death related to the original insult may be detected in the stroma, especially in corneas with stromal haze (Mohan, et al., 2003; Netto, et al., 2006). These cells are presumed to undergo apoptosis since they are detected by the TUNEL assay, but it has not been possible to confirm this with transmission electron microscopy due to the very small numbers of these cells that are present. In order to appreciate the mechanisms involved in this much slower “wave” of late anterior stromal cell death, one must have a basic understanding of the biology of the myofibroblast cell involved.

Jester and coworkers (1999) demonstrated that myofibroblasts themselves make a major contribution to the corneal opacity associated with haze. Thus, these cells are opaque relative to keratocytes due to down regulation of corneal crystallins. In addition, myofibroblasts lay down considerable irregular matrix that is opaque, in contrast to the highly regular structure of the matrix in the normal transparent cornea.

Recent animal studies have conclusively demonstrated the key contribution of corneal epithelial basement membrane structure and function in determining whether myofibroblasts develop in a particular cornea (Netto, et al., 2006). Thus, functional or structural defects in the regenerated basement membrane following surgical procedures like photorefractive keratectomy, injuries like corneal abrasions, disorders such as herpes simplex infection, or possibly as a result of genetic abnormalities, lead to the generation of haze—probably as a result of ongoing penetration of transforming growth factor β and other epithelium-derived cytokines into the anterior stroma...cytokines that are critical to differentiation and persistence of myofibroblasts (Jester, et al., 2002; Netto, et al., 2006).

Over the past decade, it has been thought that myofibroblasts develop from the progeny of keratocytes, and there is considerable laboratory evidence to support this assertion (Masur, et al., 1996; Jester, et al., 1999; Maltseva, et al., 2001). However, in other organs myofibroblasts are derived from bone marrow-derived cells (Schmitt-Graff, et al., 1994; Direkze, et al., 2003; Hashimoto, et al., 2004). It is our hypothesis that corneal myofibroblasts can be derived from both sources, perhaps explaining the clinical observation that corneal haze in some patients is topical steroid-responsive, while in the majority of patients corneal haze is steroid-resistant. If our hypothesis is correct, then in the former patients a significant proportion of the myofibroblast population may originate from bone marrow-derived cells, while in the latter, myofibroblasts of keratocyte lineage predominate. Conclusive evidence for bone marrow-derived cell origin of corneal myofibroblasts has been elusive, even using chimeric mice with green fluorescent protein (GFP)-labeled bone marrow-derived cells, likely due to technical difficulties with the duration of expression of the FGP relative to the timing of myofibroblast development in the cornea.

Late haze clears spontaneously over one to three years in the majority of patients in which it develops. Recent studies have demonstrated that myofibroblasts associated with haze in a particular cornea undergo cell death detected with the TUNEL assay (Fig. 4) at differing time points from approximately one month to many months, or even years, following the original injury (Mohan, et al., 2003; Netto, et al., 2006). This is consistent with apoptosis being thought to be the primary mechanism of myofibroblast disappearance in other organs (Desmouliere, et al., 1995; Iredale, et al., 1998; Gabbiani, 2003; Darby and Hewitson, 2007). This doesn't exclude the possibility that some myofibroblasts may undergo transdifferentiation back to corneal fibroblasts or keratocytes, as has been demonstrated in vitro (Maltseva, et al., 2001).

What is the precipitating factor involved in late myofibroblast apoptosis in corneas with haze? We speculate that repair of corneal epithelial basement membrane over time, with restoration of basement membrane structure and barrier function, leads to stromal withdrawal of epithelial-derived transforming growth factor β , and possibly other cytokines, necessary for maintenance of myofibroblast viability, and the cells, therefore, undergo apoptosis. Following the death of the myofibroblasts, keratocytes then re-enter the subepithelial stroma and reabsorb irregular matrix materials and restore stromal transparency. It follows from this proposed mechanism that persistence of defects in basement membrane structure and function likely underlies the perseverance of haze in rare cases where there is no resolution over time. Studies by He and Bazan (2006) have suggested a more active role for platelet-activating factor (PAF) and tumor necrosis factor α in the elimination of myofibroblasts. This mechanism could certainly have a role in the control of early myofibroblast development, but exactly how this mechanism would contribute to myofibroblast death months to years after the injury is difficult to explain.

5. Conclusions

Apoptosis of keratocytes, bone marrow-derived cells, myofibroblasts, and, possibly, other cell types is important in the initiation, modulation and termination of the corneal wound healing response following corneal injury, infections and surgery.

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Fig. 1. Superficial keratocyte apoptosis (red label indicated by arrows) at four hours after epithelial scrape and -9 diopter photorefractive keratectomy injury in a rabbit cornea detected with a fluorescent TUNEL assay. Intact nuclei of residual keratocytes are labeled blue with DAPI. Magnification 600X

Fig. 2.

Two TUNEL-positive cells in the rabbit corneal stroma at 1 week after -9 diopter PRK. The number of TUNEL-positive cells in the corneal stroma at one week varies between individual corneas. These cells could be keratocytes, corneal fibroblasts, myofibroblast progenitors, bone marrow-derived cells, or other cell types. Magnification 400X.

Fig. 3. Keratocytes in the rabbit corneal stroma undergoing mitosis (arrows) detected by immunocytochemistry for the mitosis-specific antigen Ki-67 at 24 hours after corneal epithelial scrape and -9D PRK. Cells in the newly regenerated epithelium are also undergoing mitosis. Magnification 600X.

Fig. 4. TUNEL-positive myofibroblast cells in the subepithelial stroma at one month after -9D PRK. The cells are double-stained with the TUNEL assay and immunocytochemistry for α -smooth muscle actin. Magnification 400X.