Delayed Development of Limbal Stem Cell Deficiency Following Chemical Injury—Pathogenesis and Therapeutic Strategies

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Abstract

Limbal epithelial stem cell deficiency (LSCD) occurs as a result of damage to the limbal epithelial stem cells (ESC) population. It may derive from direct destructive loss of the ESC (common chemical burn), and/or from dysfunction of the SC niche, leading to delayed death of the cells. This review focuses on delayed-onset LSCD, induced by antineoplastic chemicals, such as mitomycin C, 5-fluorouracil, and mustards, in terms of pathogenesis and management. These agents are used in ocular surface chemotherapy, in ocular surgery procedures, and as warfare agents, and target proliferating cells as slow-cycling cells, such as the ESC, are relatively resistant. Although the mechanism of the delayed loss of ESC is not entirely clear, we have shown, in the rabbit model, pathologic alterations in the limbal stroma, following the application of sulfur mustard, suggesting that dysfunction of the niche triggers the death of the SC later on. The absence of direct cytotoxic effects of these agents on the ESC, indicates a therapeutic window for prevention of the delayed LSCD.

Homeostasis of corneal epithelium is essential for the maintenance of healthy ocular surface as well as for corneal transparency and accurate vision. Continuous renewal of corneal epithelium is provided by a population of adult stem/progenitor cells residing in the limbus, the transitional zone between the vascular conjunctiva, and the avascular transparent cornea.[1–7] At the limbus, the corneal epithelial stem cells (ESC) reside within the basal layer of the epithelium. Although no single specific marker is available to identify stem cells (SC), a series of markers are used to characterize them. These include the expression of ABCG2, p63, or Notch-1, the absence of differentiation markers, such as CK3 and connexin-43, as well as morphologic criteria, such as small cell size (6–7 um) or high nucleus to cytoplasm ratio.[8–14] The limbus differs from the central cornea in the organization of the epithelium and in the composition of the basement membrane and stroma. The distinctive characteristics of the two tissues are thought to play a role in the regulation of their respective populations of epithelial cells.[15,16]

Limbal ESC require a special environment to retain their SC properties. The environment is provided by the SC niche in which signaling from adjacent cells, as well as properties of the basal membrane, are believed to play a role in the maintenance of their 'stemness.'[7,12,14,17–19] The cells in the niche have been suggested to regulate the preservation, proliferation, and differentiation of the ESC by producing specific matrix components and secreting growth factors and signaling molecules in a tightly regulated spatial and temporal pattern.[20,21] Consistently, the extracellular matrix composition of the limbus differs significantly from that of cornea and conjunctiva and specific cell surface receptors and adhesion molecules appear to mediate limbal ESC anchorage to their niche.

When limbal SC are depleted below a certain threshold, clinical signs of limbal epithelial stem cell deficiency (LSCD) appear, causing gradual vision loss.

LSCD occurs as a result of disease or damage to the limbal ESC population. Deficiency can arise from injuries, including chemical or thermal burns, and through diseases, such as Stevens Johnson syndrome and aniridia.[22] It could be focal or diffuse depending on the extent of limbal involvement with underlying disease process. Due to the damage in the limbus, the barrier between the vascular conjunctiva and the avascular cornea is impaired and conjunctival epithelial cells migrate toward the corneal surface, accompanied by ingrowth of blood vessels. The clinical signs of LSCD, resulted from conjunctivalization of the cornea, include persistent epithelial defects, corneal vascularization, and chronic stromal inflammation leading to functional impairment and visual loss. Diagnosis of LSCD is based on the symptomatic hallmarks and is supported by identification of conjunctival goblet cells in the cornea, using impression cytology. Recently, the diagnosis of LSCD was supported by *in vivo* confocal microscopy.[19,23] Typical characteristics of LSCD are shown in *Figure 1*.

Figure 1: Characteristics of LSCD Following Sulfur Mustard Chemical Injury in a Rabbit Model
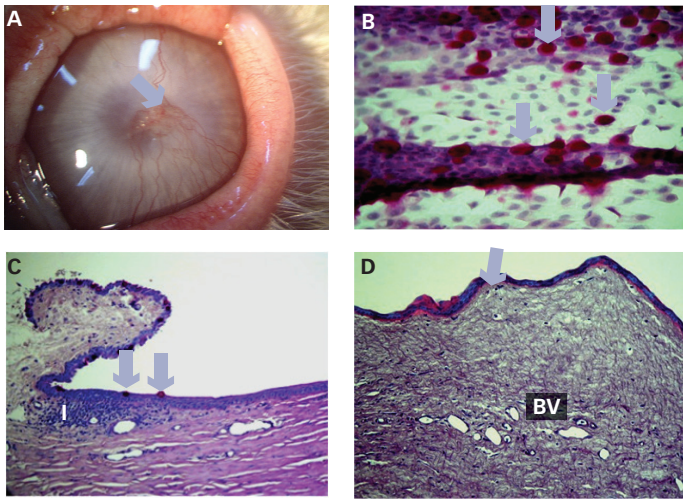
A: Slit-lamp photograph of neovascularized (arrow) inflamed cornea; B: Diagnosis of limbal stem cell deficiency (LSCD) by impression cytology, showing conjunctival epithelium including goblet cells (arrows) in corneal tissue; C: Histologic section of the limbus showing focal inflammation (I) and migration of goblet cells (arrows) from the conjunctiva towards the cornea; D: Histologic section of the central cornea demonstrating irregular thin epithelium (arrow), stromal edema, and blood vessels (BV). B–D Periodic acid-Schiff (PAS) staining.

LSCD may derive from destructive loss of limbal SC, and/or from dysfunction of the microenvironment of LSC, the SC niche, leading to insufficient support and death of the ESC.[5,24,25] Chemical and thermal burns are the most common cause of a direct destruction of limbal SC. In contrast to the immediate loss of SC following a primary destruction, a gradual loss of the SC population with time characterizes the second category. The appearance of the LSCD symptoms in this case is delayed and takes months to years after the initial insult. Neurotrophic keratopathy and chronic limbitis are examples of delayed onset LSCD, deriving from poor nutritional supply of neuronal trophic factors, essential for the maintenance of the epithelium,[26] and secretion of undesirable cytokines in the limbus in chronic limbitis.[1,2,24]

The present article focuses on delayed-onset LSCD induced by chemical agents. This less-familiar type of LSCD will be discussed in terms of pathogenesis and management.

LSCD in Chemical Burns

The term ocular chemical burn usually relates to alkali or acid insults to the ocular surface, characterized by a significant immediate destruction of the anterior segment of the eye.[27] The severity of a chemical burn is dependent on the anion or cation concentration (pH) of the solution, duration of contact, volume of solution, and the solution's penetrability. Agents such as hydroxide bases of ammonium, sodium, potassium, and calcium are the most common etiologic factors due to their ability to penetrate deep into the ocular tissue. In this type of insult, the damage is immediate and is associated with persistent or recurrent epithelial defects, neovascularization, and chronic inflammation. In this case, the epithelial damage is indiscriminate of cell type and both differentiated and undifferentiated cells, including ESC are damaged. The severe damage of both limbus and central cornea implies on the severe pathology and the management of LSCD in this case.

In addition to the classic chemicals there is a group of agents that are associated with delayed appearance of LSCD. These chemicals are known as antiproliferative drugs and are used in ocular surface tumor therapy and in specific procedures of ocular surgery. The cytotoxic metabolites, mitomycin C (MMC), 5-fluorouracil (5-FU), and mustards are included.

Delayed-onset LSCD Following Chemical Injury

The use of topical MMC or 5-FU for the treatment of ocular surface neoplasia as a sole or adjuvant treatment has been well described and it is considered as a safe chemotherapy with only transient side effects that are dose dependent.[28–32] However, in a retrospective study, Lichtinger et al.[33] reported on a relatively high incidence (23 %) of LSCD occurring after prolonged treatment with topical MMC.

Another case of delayed-onset LSCD following MMC or 5-FU is in glaucoma surgery. A subconjunctival injection of MMC or 5-FU is common for modulation of wound healing after filtration surgery (trabeculectomy). However, a known late side effect of this treatment is the development of delayed LSCD years after.[34–36] Indeed, due to these findings, it has been suggested that this treatment should be reconsider in routine antiglaucomatous surgery. For the best of our knowledge, the mechanism for the delayed LSCD following MMC or 5-FU has not been studied as yet.

Mustards are another group of toxic alkylating agents,[37,38] preferentially targeting proliferating cells, and therefore are used in chemotherapy. Analogs of nitrogen mustard, such as mechlorethamine, cyclophosphamide, and melphalan, are used for treatment of a wide range of cancers. Ocular injuries following exposure to nitrogen mustard were previously described, including delayed corneal pathology with the clinical symptoms of LSCD.[37,39,40]

Sulfur mustard (SM) is a potent toxic agent that reacts with many tissue components and preferentially targets dividing cells of surface epithelia.[37,41,42] The development of delayed LSCD following SM was described in details in the literature in experimental models[43,44] and in human victims.[45–48]

Although the mechanism of the delayed-onset LSCD is still not entirely clear, recent studies on the pathogenesis of SM-induced ocular lesions increase our knowledge on the pathogenesis of this unique type of LSCD and may be helpful in understanding the mechanism underlying the injuries of other chemicals belonging to this category.

Mechanism of the Delayed-onset LSCD

Similar to 5-FU, SM is a reactive alkylating agent preferentially targeting dividing cells. Ocular injuries following exposure to SM are characterized by an acute phase that is expressed clinically by corneal erosions and inflammation of the anterior segment that may be followed (after clinically quiescent period) by delayed irreversible LSCD.[49,50] We have shown that corneal ESC were not damaged directly and immediately by SM. On the contrary, they were preserved during the acute phase following exposure,[51] unlike the proliferating epithelial cells in the central cornea that were damaged initially. The SM cytotoxic damage that was observed in the central corneal epithelium reflects the well-known effect of the alkylating agent on proliferating cells.[37,42] Using various markers to identify the limbal SC, we have shown that the SC were not damaged primarily by SM. On the contrary, due to the central corneal erosions they became active and proliferated as under normal healing (see *Figure 2 A–B*).[51]

In this respect, Tseng et al.[25] have shown that slow-cycling cells were more resistant to 5-FU than proliferating cells, thus strengthening our findings that slow-cycling cells are less affected by alkylating agents.

Parallel to the late appearance of LSCD following MMC and 5-FU, a delayed decrease in the number of SC following SM exposure was observed by our group (see *Figure 2 C–D*). The loss of SC occurred after healing of the acute injuries, associated with the clinical manifestation of LSCD.[51] In our rabbit model it takes weeks, but in humans the late LSCD is developed years after the initial insult.[49]

The effects of the above-mentioned chemicals resemble the late effects of radiation. The onset of corneal changes after radiation therapy varies from a few days to years and LSCD may appear even without acute corneal signs of radiation-induced toxicity. It has been suggested that the delay in appearance of LSCD following radiation may be a consequence of the special regulation of SC mitotic activity so that damage to DNA only manifests when SC replicate to maintain the SC pool.[52,53] According to this assumption, the late loss of SC derives from primary molecular event within the SC that is expressed clinically weeks to years later.

On the other hand, the loss of SC may develop secondary to corneal injury response (e.g. edema, inflammation) and dysfunction of the SC niche. Supporting this hypothesis, we have shown pathologic alterations in the limbal stroma of eyes developing the delayed injury, such as degeneration of corneal nerves and chronic inflammation.[54] Consistent with these findings, anti-inflammatory drugs administered before the development of LSCD postponed and reduced the severity of the delayed SM-induced ocular injury.[55] Interestingly, recent studies demonstrated the dependence of corneal stem/progenitor cells on ocular surface innervation.[56] Still, we currently do not have enough evidence to distinguish between these two putative mechanisms.

Although the pathologic mechanism of the delayed injury following radiation and chemical alkylating agents is still not clear, as well as the susceptibility of part of the exposed eyes, we proposed that the delayed LSCD may derive indirectly from dysfunction of the niche that initiates their gradual death.[51,54]

Nevertheless, the delayed loss of the SC offers a window of opportunity for therapy targeted to prevent the death of ESC and facilitate their survival.

Management of the Delayed-onset LSCD

Generally, the delayed onset LSCD represents a milder form of the disease since, in most cases, limbal damage is partial and may resolve spontaneously. Indeed, some cases of LSCD after topical MMC and 5-FU and following radiation therapy recovered spontaneously without medication.[57]

A characteristic of the disease that may facilitate successful therapy is the delayed loss of limbal ESC. The apparent absence of a primary cytotoxic effect on the SC points toward the presence of a therapeutic window for intervention, before the appearance of the severe clinical symptoms. Therefore, clinicians should be aware of the risk of future LSCD and should be proactive in early diagnosis and preventative measures. The preventive medication depends on the pathologic mechanism (if known) and on predisposing symptoms. As chronic inflammation is involved

Figure 2: Preservation of Limbal Stem Cells During the Acute Phase (A–B, Arrows) and their Delayed Loss (C–D) Following Sulfur Mustard Exposure
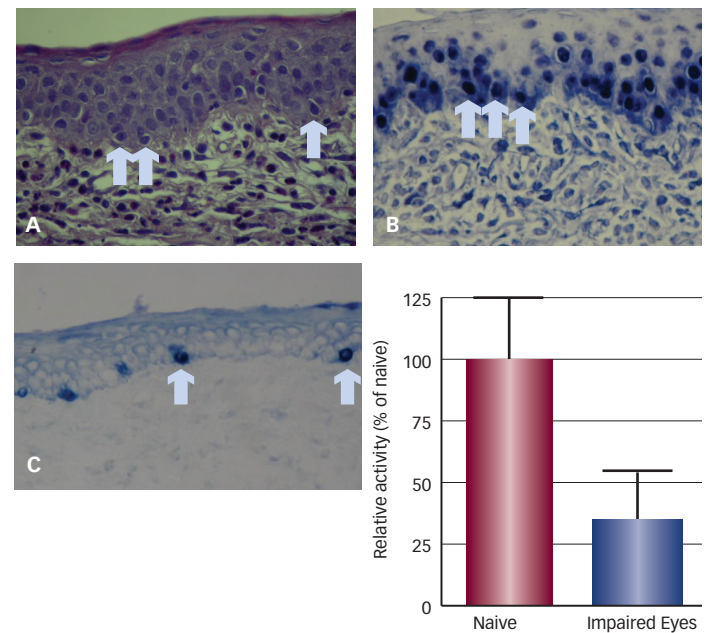
The preservation of limbal stem cells (arrows) during the acute phase following sulfur mustard exposure (A–B) and the delayed loss of stem cells in 'clinically impaired' rabbit eyes, developing limbal stem cell deficiency (LSCD) 4 weeks after exposure (C–D), shown by hematoxylin and eosin (H&E) histology (A), p63 immunohistochemistry (B–C), and reverse transcription polymerase chain reaction (RT-PCR) of limbal ABCG2 (which is a member of the ATP binding cassette [ABC] family of cell surface transport proteins) messenger RNA levels, respectively.

in most cases, then a postexposure prophylactic treatment with anti-inflammatory drugs would probably be beneficial in reducing the rate of LSCD, as indeed was shown by our group for SM keratopathy in the rabbit model.[55,58]

It is important to distinguish between total and partial LSCD. For mild symptoms, conservative management with preservative-free artificial tears, due to tear dysfunction, may be sufficient. Also, ointment and/or medical use contact lenses may be appropriate. Topical corticosteroids may be useful to both minimize discomfort and to control the inflammatory component of LSCs and may help in the regression of corneal neovascularization.[58] Anti-vascular endothelial growth factor (anti-VEGF) agents such as bevacizumab may also prove beneficial in the management of corneal neovascularization, secondary to LSCD; however, early anti-VEGF therapy may impair the SC (our unpublished data). Amniotic membrane transplantation alone without SC was sufficient for treatment of LSCD, in patients receiving 5-FU after glaucoma filtering surgery.[35]

Severe cases may require surgical intervention with LSC transplantation.[59–61] The kerato-limbal tissue may be taken from autologous, allogenic, or cadaveric sources. Since 1997, when cultured autologous limbal epithelial cell implants were used successfully, transplantation of cultivated epithelial SC has become a treatment of choice for LSCD patients.[62] Novel biofunctional scaffolds to enhance SC expansion and transplantation efficacy are becoming available.[63] Standardization of culture conditions and development of xenobiotic-free culture systems, as well as the identification of the required niche components is under progress. In

summary, the pathogenesis and management of the delayed-onset LSCD following the cytotoxic agents MMC, 5-FU, and mustards differ from the common chemical burns. While further studies are needed to shed light on the dominant factors underlying the delayed pathology, the management may be based mostly on a postexposure preventive therapy before the appearance of the clinical signs and on early diagnosis of the disease. ■

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