MINIREVIEW

Corneal Epithelial Wound Healing

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One of the important functions of the cornea is to maintain normal vision by refracting light onto the lens and retina. This property is dependent in part on the ability of the corneal epithelium to undergo continuous renewal. Epithelial renewal is essential because it enables this tissue to act as a barrier that protects the corneal interior from becoming infected by noxious environmental agents. Furthermore, the smooth optical properties of the corneal epithelial surface are sustained through this renewal process. The rate of renewal is dependent on a highly integrated balance between the processes of corneal epithelial proliferation, differentiation, and cell death. One experimental approach to characterize these three aspects of the renewal process has been to study the kinetics and dynamics of corneal re-epithelialization in a wound-healing model. This effort has employed *in vivo* and *in vitro* studies. From such studies it is evident that the appropriate integration and coordination of corneal epithelial proliferation, adhesion, migration, and cell demise is dependent on the actions of a myriad of cytokines. Our goal here is to provide an overview into how these mediators and environmental factors elicit control of cellular proliferation, adhesion, migration, and apoptosis. To this end we review the pertinent literature dealing with the receptor and the cell signaling events that are responsible for mediating cytokine control of corneal epithelial renewal. It is our hope that a better appreciation can be obtained about the complexity of the control processes that are responsible for assuring continuous corneal epithelial renewal in health and disease.

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Normal vision depends on the ability of the cornea to remain transparent and, together with the ocular lens, to appropriately refract impinging light onto the retina. Another function is that the outer corneal epithelial layer acts as a physical barrier in preventing noxious agents from infecting this tissue. This protective function is the result of the high resistance of the tight junctions between its neighboring epithelial cells (1). The maintenance of its epithelial tight functional integrity is essential for the corneal optical properties when the tissue undergoes continuous renewal. The tight junctional integrity is needed for the corneal epithelial barrier function. This function helps in the preservation of corneal deturgescence and transparency (2). The inner endothelial layer is essentially a nondivided monolayer after birth in the human, yet it is able to maintain corneal optical properties and its epithelial tight junctional integrity (3). It elicits net fluid transport outward from the stroma into the anterior chamber, which offsets the natural tendency of the stromal ground substance, e.g., proteoglycans to imbibe fluid and swell. By preventing stromal swelling, corneal clarity is preserved (3, 4). Therefore, corneal transparency and appropriate optical refraction are dependent on the ability of the epithelial layer to undergo continuous renewal and on the endothelial fluid transport activity to maintain stromal thinness.

The epithelium can also mediate some outward fluid transport from the central stromal compartment into the tears. Epithelial fluid transport is vital for the maintenance of normal corneal functions, although it only provides a minor contribution relative to that of the endothelium, even under maximally stimulated conditions (5). However, transport and barrier functions can persist even if renewal is impaired. Like other epithelia, the corneal epithelium undergoes continuous renewal (6). This process can occur provided that there is synchronized control of proliferation in the basal cell layer coupled with differentiation of the

daughter cells as they move into the suprabasal layers and are ultimately sloughed off into the tears. Based on studies performed in cell culture, corneal epithelial cells do undergo apoptosis characterized by activation of specific signaling pathways and nuclear chromatin condensation (7). We have shown *in vivo* that apoptosis can be induced by UV irradiation through the activation of cytokine receptor-linked cellular signaling pathways. Our focus here is to provide evidence that corneal epithelial renewal also depends on cytokine receptor-mediated control of its proliferative capacity, differentiation, and turnover (7–12). Therefore, determining how cytokines mediate control of corneal epithelial growth, differentiation, and apoptosis is relevant to identifying potential strategies for promoting its essential function in vision.

Our focus here is to provide an overview of the mechanisms involved in mediating control of corneal epithelial growth, differentiation, and turnover. We first review pertinent issues regarding corneal homeostasis and we describe the roles of cell migration, adhesion, and proliferation in renewal. The critical roles are considered of a number of cytokines that induce growth, differentiation, and apoptosis. In addition, we discuss some of our emerging evidence indicating how *in vitro* and *in vivo* exposure to environmental levels of UV irradiation induces increases in the incidence of apoptosis.

Roles of Corneal Epithelium in Transparency Maintenance

It has been demonstrated that corneal epithelial renewal is a continuous process that is required to maintain the above mentioned epithelial functions. Should wounding of the corneal epithelial surface occur, breakdown of tight junctional integrity due to loss of the outer limiting epithelial layer may take place. This loss can lead to breakdown of cell membrane permeability and selectivity, which is not restored until after epithelial migration from the periphery has resulted in resurfacing of the denuded corneal surface. A possible outcome of wounding and loss of tight junctional integrity is that the cornea interior becomes vulnerable to infection by invasive pathogens. Such a scenario could also lead to the development of corneal opacity as the result of decreases in endothelial fluid transport, which could increase stromal hydration.

One potential therapeutic approach to hasten recovery of epithelial function after wounding is to stimulate corneal epithelial cell renewal. As wound closure studies have shown that healing can be stimulated by various exogenous cytokines, numerous studies have been targeted towards obtaining a better understanding of the intracellular signaling pathways mediating receptor control of growth, differentiation, and apoptosis. This endeavor could ultimately identify intracellular targets for drug development. Drug modulation of such targets could bypass dysfunctional cytokine receptor-mediated control of growth, differentiation, and apoptosis, which could overcome loss of cytokine-mediated control of these processes. With this outcome, the time needed for recovery of normal corneal function would be decreased.

Importance of Corneal Epithelial Renewal to Function

The two major properties of the corneal epithelium needed for normal vision are to form a smooth refractive surface via its interaction with the tear film and to form a protective tight junctional barrier that prevents decreases in net fluid transport out from the stroma and prevents corneal penetration by pathogens. To provide the epithelial barrier function, epithelial cells are specialized to form tight adherences between one another and to the basal lamellae. The cells from the basal layer migrate into the suprabasal layers and undergo differentiation. The superficial cells possess tight junctions, zonulae occludens, which serve as a semipermeable, highly resistive (12–16 kohms/cm^2) membrane (13). As the superficial differentiated cells are lost, they are replaced by the underlying proliferating basal cell layer. (There are two to three layers of wing cells—the suprabasal cells) Following their emergence, the newly formed cells migrate first from the basal layer into the upper differentiating wing layers and then into the superficial layers. It is of paramount importance that the differentiating cells are able to re-establish the tight junctional physical barrier that protects the cornea interior from noxious agents, e.g., microorganism infections, as the epithelial cell layers are interconnected via desmosomes and communicate with one another via gap junctions, especially in the wing cell layer. Therefore, through the processes of mitosis, migration, and shedding, the cornea epithelium is well maintained.

The human corneal epithelial thickness is about 50 to 52 μm and it is made up of five to seven layers of very regularly arranged differentiating epithelial cells (14–16). As with other epithelia, the renewal of corneal epithelium is maintained by the proliferation and differentiation of stem cells residing in the limbus. The limbal epithelium is the transitional zone of the cornea and conjunctiva and is about 10 to 12 cell layers thick, and contains melanocytes, Langerhan cells, and an underlying network of blood vessels in the limbal stroma. The limbal stroma with its overlying epithelium is arranged in radial fibrovascular elevations called the Palisades of Vogt, which alternate with epithelial rete ridges (17, 18). These palisades are present all around the cornea, but are most defined inferiorly and superiorly. The population density of basal cells is maximal in the palisade region. The centripetal migration is characteristic of limbal basal epithelial cells that undergo cell division and migrate towards the central cornea and upward towards the corneal surface. Meanwhile, once the epithelial cells have left the limbal basal layer, they become differentiated and express keratin 12, the specific corneal epithelial differentiation marker (19). The basal corneal epithelial cells, transient amplifying cells, are capable of undergoing a few cell divisions prior to their upward migration, resulting in their terminal differentiation. Daughter cells of transient

amplifying cells comigrate upward as a pair when they become committed to terminal differentiation. In addition to keratin 12 expression, the differentiating corneal epithelial cells also express connexin 45 or 43, (20) and $\alpha_6\beta_4$ integrin, which participates in the formation of desmosomes and hemidesmosomes via interaction with keratin intermediate filaments (21). Injuries can alter connexin expression and cell communication in healing corneal epithelium.

The basal layer of columnar cells is tightly adherent to an underlying uniform 50-nm-thick basement membrane. The basement membrane is largely composed of type IV collagen, laminin, heparin, proteoglycans, and lesser amounts of fibronectin (FN) and fibrillin. Immunohistochemical studies revealed that the epitope of α1 (IV) chain of type IV collagen is masked in the corneal epithelial basement membrane, whereas the anti-α1 (IV) chain antibody readily labels the limbal epithelial basement membrane (22).

These results are consistent with the notion that the tissue environments are important in the maintenance of the differentiation status of limbal basal and corneal basal epithelial cells. Each basement membrane is divisible into an anterior clear lamina lucida, which provides hemidesmosomal attachments to basal epithelial cells and a posterior dark lamina densa. Anchoring fibrils composed of type VII collagen anchor the lamina densa to localized anchoring plaques located in the underlying stroma and Bowman's membrane (16). Two or three layers of interdigitating, wing, or polygonal cells make up the intermediate layer followed by two layers of small flattened superficial cells. The anterior plasma membrane of the most superficial layer of cells shows numerous microvilli and microplicae, which facilitate transport of metabolites and tear film adhesion (14–16).

Wound Healing Models for Characterizing Epithelial Renewal

To gain insight into how renewal occurs, wound closure models have been employed by many investigators. They involve making a defined central epithelial wound and characterizing the kinetics of the healing response. The healing of epithelial wounds can be divided into several distinct but continuous phases: sliding of superficial cells to cover the denuded surface, cell proliferation, and stratification for re-establishment of multicellular layers. Prior to the onset of these events, there is a lag phase during which the cells alter their metabolic status. The size and depth of the wound and the nature of the injury affects the healing mode and outcome.

Cell Migration. Epithelial migration is initiated by forming lamellipodia, filopodia, rearranging actin filaments, and the complete resurfacing of defects. However, the event that initiates and controls healing is not fully understood. After about 5 hr, cells begin to migrate at a constant rate of 60 to 80 μm/hr until wound closure has been completed (23–26). Spreading corneal epithelial cells normally elaborate cytoplasmic arrays of actin- rich stress fibers, which insert onto the inner surface of the cell membrane at discrete adhesion complexes. It is known that blocking actin polymerization inhibits epithelial cell migration. Proparacaine, a topical ocular anesthetic, inhibits corneal epithelial migration and adhesion partly through alteration of the actin cytoskeleton. Cell migration can be stimulated by cyclic adenosine 3′, 5′–phosphate (cAMP), whose levels increase during migration (27). Of all tissues, the corneal epithelium accumulates the highest levels of acetylcholine. Choline acetylase and cholinesterase activity have been detected. Cholinergic stimulation of the epithelium causes an increase in guanosine 3′, 5′-cyclic phosphate (cGMP) levels, suggesting a possible role of a cholinergic system in the regulation of cell division, cell growth, and wound healing (28–30). In cases of delayed healing, wound/healing promotion with therapeutic agents has been explored. Agents that have selective effects on epithelial growth, migration, adhesion, and differentiation have been identified. They include topically applied growth factors such as epidermal growth factor (EGF), fibroblast growth factor (FGF), transforming growth factor (TGF), keratinocyte growth factor (KGF), hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF), insulin-like growth factor (IGF), interleukins-(IL) 1 and -6, TNF-α, endothelin-1 (ET-1) FN, and retinoids (31–36).

During the lag phase between wounding and the initiation of cell migration, there is at first a great deal of cellular reorganization and protein synthesis. Migration and healing depend on the synthesis of cytoskeletal proteins such as vinculin, actin, talin, and integrin and other cell surface receptors, e.g., CD44, the hyaluronan (HA) receptor. Synthesis of cell surface glycoproteins and glycolipids also increase during wound healing (21, 37–41). Upon epithelial debridement, integrin $\alpha_6\beta_4$ dissociates from desmosomes and hemidesmosomes and evenly distributes on the cellular surface. It may serve as an adherens molecule to extracellular matrix (ECM) instead of a component of desmosomes and hemidesmosomes. The integrin receptor engagement and clustering leads to the formation of focal contacts in which integrins link to intracellular cytoskeletal complexes and bundles of actin filaments (42, 43). Although much is known about the extracellular interactions that occur between integrins and their ligands, very little is known about the intracellular biochemical pathways and the subsequent cellular functions that are controlled and regulated by integrins.

Another potential candidate for mediating dynamic cell adhesion and migration in corneal epithelial wound healing is CD44. CD44 gene transcripts start to increase in epithelial cells surrounding the wound margin 3 hr after wounding and they peak at 18 hr in the basal epithelial cell layers, at which time the epithelia are actively migrating. As the cells begin proliferation after wounding, the density of CD44 mRNA label declines, but is still significantly higher than that in control specimens. The localization of CD44 on cell surfaces during corneal re-epithelialization is con-

sistent with its mRNA expression pattern. In corneas at 18 hr after wounding, CD44 immunoreactivity increases over the entire epithelium from its leading edge to the limbal-corneal border. As with mRNA, cell surface CD44 declines as cells differentiate to reestablish the multilayered epithelium. The expression of CD44 correlates with corneal re-epithelialization, suggesting that CD44 may be involved in cell-cell interactions that provide adhesive strength for the much-stressed epithelial sheet and in the cell-substratum interactions that mediate cell migration during re-epithelialization.

During the corneal epithelial wound healing process, other specific intracellular proteins such as vinculin, keratins, and extracellular matrix proteins such as lumican, collagens, and metalloproteinates (MMPs) are upregulated. (44–49). At the early stage of wound healing, the local formation of fibrin functions as a provisional matrix and chemotractant to support migration and adhesion of corneal epithelial wound healing. Recent studies using plasminogen- and fibrinogen-deficient mice demonstrate that crosslinking of the expanding fibrin network is essential for corneal wound healing (50). Subsequently, FN from the tear film also deposits on the denuded corneal surface and may facilitate cell adhesion and re-epithelialization during healing.

The intermediate filament components cytokeratins 3 and 12 (i.e., K3 and K12, respectively) are regarded as hallmarks of the corneal epithelium since they are selectively expressed in many species. Both K3 and K12 play important roles in the maintenance of corneal epithelial integrity. In K12-deficient animals, the corneal epithelial layer becomes thinner and fragile. Corneal epithelial cells are not able to adhere firmly onto the corneal surface (51). In addition, corneal epithelial wound healing is dependent on reorganization of the extracellular matrix. One contributor to this process is thought to be amyloid precursor-like protein, APLP2. Recently, in cultured cells, isoforms of APLP2 and APP (amyloid precursor protein) were demonstrated to be post-translationally modified by the addition of chondroitin sulfate (CS) (52, 53). CS proteoglycans (CSPGS) are important constituents of the extracellular matrix of mammalian cells and are involved in different aspects of cell adhesion, cell migration, axonal guidance, and wound healing (53–57). Hence, alternatively spliced APLP2 transcripts can give rise to at least four different isoforms and each of these isoforms may have a unique function in epithelial wound healing (48, 49). Furthermore, APLP2 expression was observed in the denuded wound bed immediately adjacent to the leading edge of migratory cells and under the epithelial sheet after wound closure. The wound-induced, basal cell-specific APLP2 expression correlates with epithelial cell migration, suggesting that APLP2 mediates reorganization of the extracellular matrix and dynamic cell-matrix adhesion during re-epithelialization. Remodeling of basement membrane takes place during healing following epithelial debridement in which the epitope of α1(IV) chain can be detected underneath the migrating epithelium. It has been recently demonstrated that the remodeling of basement membrane may also be associated with upregulated expression of MMPs that are proportionate to the size of epithelial defects

FN and HA have been identified as important components of the extracellular matrix, both being absolutely required in various biological phenomena, including wound healing and morphogenesis. Only small amounts of each are present in the stroma of unwounded corneas. However, once corneal epithelium becomes injured, both FN and HA expression appears to be rapidly stimulated in stromal fibroblasts and the wounded area of the cornea, a hallmark for the initiation, ultimately resulting in the activation of the corneal epithelial wound healing mechanisms *in vivo*. Binding of HA to FN may facilitate the induction of allosteric changes in the FN molecule that may stimulate the attachment of corneal epithelial cells to the FN-binding integrins (58). Increases in FN expression have been shown to be modulated both *in vitro* and *in vivo* by various mediators, including cAMP, glucocorticoids, and growth factors such as EGF, TGFβ1, and PDGF (59–63).

Effects of Neural Factors on Epithelial Wound Healing. The cornea is more densely innervated with sensory nerve fibers than any other tissue in the body. In the cornea, dense networks of substance P- (SP) positive nerve fibers have been reported with immunohistochemical techniques (64, 65). SP is a constituent of sensory nerve fibers and has been postulated to mediate various functions such as plasma extravasation, vasodilatation, and mast cell histamine release (66–71). SP also has been assumed to play a key role in ocular and some other tissue neurogenic responses to various stimuli (64, 72, 73). The SP level in the cornea of the adult mouse is reduced 40% by denervation of the trigeminal nerve to the eye (74). In fact, in various types of corneal diseases such as herpetic keratitis, corneal sensitivity is reduced, which often associates with epithelial defects in those anesthetized corneas (75, 76). It is reported that SP and IGF-1 have a permissive effect on the stimulation of rabbit corneal epithelial migration in an organ culture. The addition of either SP or IGF-1 alone did not affect epithelial migration, while the combination of SP and IGF-I stimulated epithelial migration in a dose-dependent fashion. Their interactive effects were nulled by the addition of an SP antagonist or enkephalinase. Among neurotransmitters (vasoactive intestinal peptide, calcitonin gene-related peptide, acetylcholine chloride, norepinephrine, and serotonin) or tachykinins (neurokinin A, neurokinin B, kassinin, eledoisin, and physalaemin), only SP had a permissive effect with IGF-1 on cellular migration. The attachment of the corneal epithelial cells to FN, collagen type IV, and laminin matrices increased after treatment of the cells with SP and IGF-1, but SP or IGF-I by themselves did not affect the attachment of the cells to these extracellular matrix proteins. An NK-1 receptor agonist in place of SP also had a permissive effect in combination with IGF-1 on corneal epithelial

migration, suggesting that this response may be through the NK-1 receptor system. These results suggest that the maintenance of the normal integrity of the corneal epithelium might be regulated by both humoral and neural factors.

Corneal epithelial wound healing is complete once the newly regenerated stratified epithelium firmly anchors itself to the underlying connective tissue. Thus, wound healing is in part dependent on integrins, which are receptors that promote interactions of corneal epithelial cells with their extracellular matrix (77–80).

Glucose Utilization. Corneal epithelial cell migration and proliferation depend on metabolic support provided by glucose in the aqueous humor and epithelial glycogen stores. In the rat cornea, it was reported that expression of glucose transporter 1 (GLUT1) rapidly increased by half fold above its control at 4 hr post-debridement. GLUT1 protein levels continued to increase even after epithelial wound closure (24 hr) and reached the peak of 5.8-fold higher than the control at 2 days post-debridement. The increase in GLUT1 protein levels coincides with enhanced GLUT1 mRNA levels (81–85). The increases in GLUT1 protein expression persist until 24 hr post-wounding. They are localized over the entire basal limbal and corneal epithelial cell membranes. However, after 24 hr, the increased GLUT1 expression level remained elevated in the wound area, whereas it declined to its control level in the limbal basal cells. Interestingly, in addition to its ability to transport glucose, GLUT1 contains a water-filled channel that spans the plasma membrane, which may serve as a water channel (86).

Cytokine-Mediated Control of Proliferation and Differentiation

Wound healing models are used to understand the roles of cytokines in eliciting control of growth, differentiation, and apoptosis. Following epithelial wounding, there is a pause in the natural process of exfoliation. Cells near the wound edge cease to divide for up to 1 day, while those at some distance from the wound undergo an increased rate of cell division. A wave of mitosis moves from the periphery towards the wound and continues until the wound has healed and normal thickness of the epithelium is restored. One approach to developing more appropriate treatments for corneal epithelial damage has been to test the effects of various growth factors on healing (87). Cytokines that have been found in human and rabbit tears include EGF, ET-1, HGF, TNFα, TGFβ-1, TGFβ-2, VEGF, PDGF-BB, IL-6, and bFGF (88–92). A number of them stimulate epithelial growth, whereas others can trigger epithelial cell apoptosis. Most recently, using a bovine corneal epithelium reconstruction (multilayer) culture system, EGF, HGF, and TGFβ1 appear to effectively elicit full-thickness epithelial structure (93). This effect of EGF is consistent with its effectiveness as a mitogen in accelerating epithelial wound closure *in vivo* and *in vitro* (94). As the tears contain the above-mentioned cytokines, it is possible that there are interactions between them that could affect their individual effects on growth, differentiation, and apoptosis.

Effect of EGF on Corneal Epithelial Cell Proliferation. EGF is a 6-kDa polypeptide consisting of 53 amino acids and it contains three intermolecular disulfide bonds that are required for its tertiary structure. Ligand binding causes the EGF receptor to dimerize, which enables the two cytoplasmic domains to cross-phosphorylate on multiple tyrosine autophosphorylation (95). Both EGF and its receptor are expressed in all three major corneal cell types: i.e., epithelium, stroma, and endothelium, which suggests that EGF could have either autocrine- or paracrine-mediated effects (9, 96). It has been reported that EGF facilitates corneal epithelial wound repair by promoting migration and mitosis of the epithelial cells in both *in vivo* and *in vitro* model systems. The mitogenic effect of EGF was initially studied under a variety of conditions and in a variety of cells (36, 97). It is an especially potent mitogen for tissues of ectodermal and endodermal origin (98), and is involved in tissue repair and wound healing (99, 100). In the corneal epithelium, EGF accelerates epithelial healing based on increases in cell replication (proliferation) as measured by rises in DNA content in the regenerating epithelium (101).

A simple organ culture model for assessing the effects of growth factors on corneal re-epithelialization revealed that the application of exogenous EGF resulted in corneal re-epithelialization following wounding. Re-epithelialization occurred in a similar fashion to that observed *in vivo*, i.e., a lag phase followed by migration/proliferation and stratification (102). To examine differential regulation of proliferation in cornea epithelium, a colony growth assay was developed. The presence of EGF enhanced the clonal growth of ocular surface epithelial cells. The cells gradually developed an increasing number of colonies by Day 6. Elimination of EGF from the culture medium abolished most of the colony growth, further indicating EGF's role in cellular proliferation in cultured corneal epithelium (103). Indeed, *in vitro* studies have well established EGF as a potent mitogen for corneal epithelial proliferation

EGF Receptor-Linked Signaling Pathways. In EGF receptor-mediated signaling, transduction can occur by various pathways to affect downstream targets (i.e., nuclear transcription factors). EGF binding to its receptor (EGFR) results in its dimerization, which leads to activation of the tyrosine kinase domains and autophosphorylation of the receptor (104). Upon EGF binding to its receptor, phospholipase C (PLC; a membrane enzyme) binds to phosphotyrosine domains on the EGF receptor and is activated by tyrosine phosphorylation via a G protein (105, 106). In rabbit corneal epithelial cells, EGF receptor stimulation can result in the stimulation of PLC and phospholipase D (PLD). (107). Activated PLC hydrolyzes phosphatidylinositol-4, 5-biphosphate (PIP_2) to yield two active second messengers, diacylglycerol (DAG) and inositol-1,4,5,-

triphosphate (IP_3). (108, 109). Alternatively, PLC activation can result in stimulation of PIP_3 kinase (110). IP_3 and DAG both facilitate activation of protein kinase C (PKC) (108, 111, 112). IP_3 causes release of Ca^{2+} from intracellular stores, thereby activating Ca/calmodulin-dependent protein kinase (CaM kinase) and also facilitating activation of PKC (112). DAG activates PKC. Activated PKC then promotes activation of the mitogen-activated protein (MAP) kinase cascade leading to MAPK activation. (113–115).

Another pathway linked to EGFR receptor activation (i.e., tyrosine autophosphorylation) involves EGFR docking with Src homology domains of adapter proteins. This event leads to activation of Ras, a GTP-binding protein, followed by activation of the MAP kinase pathway. In this sequential chain, Raf is the entry point to the MAPK cascades. They are a superfamily of parallel-linked serine threonine kinases that elicit receptor control of nuclear events occurring in response to various stresses, growth, differentiation, and apoptosis-related cytokines. The limb of this superfamily that primarily responds to growth factor stimulation is the extracellular signal response kinases 1 and 2 (Erk-1 and Erk-2). Raf is also referred to as MAP kinase kinase kinase (MAPKKK) or MEKK and it ultimately phosphorylates MAPK (116). There are two isoforms of MAP kinase, the p44 MAPK (Erk-1) and the p42 MAPK (Erk-2), which are expressed in most cell types. The substrates of MAPK include nuclear transcription factors and non-nuclear substrates such as the protein, serine/threonine kinase p90sk, cytoskeletal proteins. EGF-induced nuclear transcription activation stimulates cell proliferation by initiating G1 progression to the S phase of the cell cycle. Another substrate of MAPK activation is phospholipase A_2 ($cPLA_2$) (117–120). $cPLA_2$ catalyzes the release of arachidonic acid from phospholipids in membranes and is one of the rate-limiting steps in the synthesis of prostaglandins and other eicosanoids. In cultured rabbit corneal endothelial cells, it was shown that the prostaglandin PGE_2 inhibits mitosis (121).

Feedback Effect of EGF-Induced PGE_2 Production on MAP Kinase Activation. The EGF-induced mitogenic response depends on the extent of stimulation of two different signaling pathways linked to EGFR. They are the MAP kinase cascade and another one that elicits increases in PGE_2 levels as a consequence of stimulation of COX-2 and cPLA2. Increases in PGE_2 levels result in stimulation of adenylate cyclase and enhanced levels of cAMP and protein kinase A activity. This chain of events elicits a negative feedback effect on EGF-induced MAP kinase activation at the level of Raf-1. Such feedback is consistent with our findings that the mitogenic response to EGF is inversely related to cellular PGE_2 levels and the amount of PGE_2 in conditioned medium. There is also evidence in vascular endothelial cells, PC12 cells, fibroblasts, and renal mesangial cells that EGF-induced increases in PGE_2 levels can inhibit at the level of Raf-1 EGF-linked MAPK cascade activation and proliferation (122–125). In addition, the EGF-induced mitogenic response may also result from induction of increases in protein kinase C (PKC) activity through activating PLC. Activation of PKC can initially activate Raf-1 in many cell types and subsequently MAPKKK and Erks (i.e., MAPK) (113, 114, 126, 127). As summarized in Figure 1, our data suggest that the level of Raf-1 activation is determined by the balance between EGF-induced stimulation of Ras and cPLA2, which in turn affects the extent of Erk-2 activation and ultimately the size of the mitogenic response (128).

Effect of ET-1 on Corneal Epithelial Cell Proliferation. ET-1 stimulates proliferation and wound healing in a variety of cells including human ovarian carcinoma cells, Swiss 3T3 cells, vascular smooth muscle cells, and epithelial cells of the thymus (129–132). ET-1 has also been shown to stimulate DNA synthesis and proliferation in vascular smooth muscle cells (129), OVCA 433 cells (131), Swiss 3T3 cells (130), and epithelial cells of the thymus (132).

Since ET-1 is also found in human tears and rabbit lacrimal gland, studies were performed examining the effect of ET-1 on corneal epithelial wound healing (90, 133). ET-1 is a potent mitogen for corneal wound healing in rabbits and in cultured bovine corneal cells and it has a permissive effect on the mitogenic response to EGF (134, 135). This effect of ET-1 occurs through ET_A and ET_B receptor-mediated increases in Ca^{2+} levels that are a consequence of calcium-induced calcium release from intracellular stores (136). *In situ* there is suggestive evidence that each of these receptor subtypes may elicit control over different aspects of corneal epithelial renewal. This appears tenable because ET_A and ET_B receptor distribution is not homogeneous. The proliferating basal layer contains much higher levels of ET_A gene expression, whereas in the differentiating suprabasal layers, ET_B expression predominates (134).

In some other tissues, the cell signaling components mediating the mitogenic response to EGF includes activa-

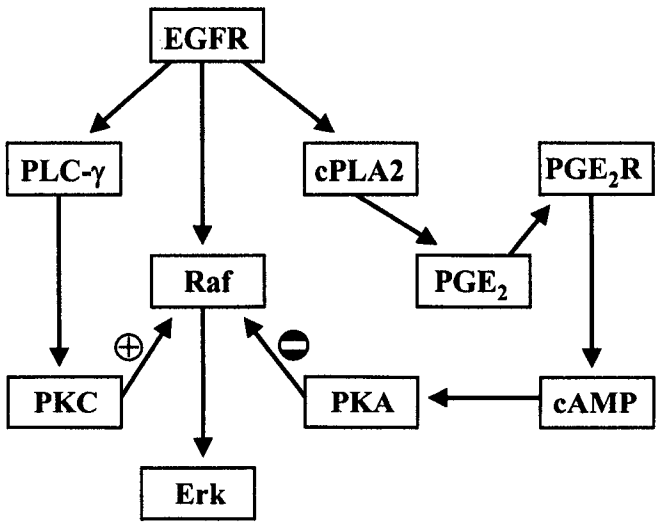

Figure 1. Summary of EGF-induced signaling pathways in RCE cells. Erk, MAP kinase; PGE2R, prostaglandin E2 receptor; PLA, phospholipase A; and Raf, MAPK kinase kinase.

tion of membrane ion transport pathways. These events include stimulation of various calcium channels and, in ML-1 cells, a growth factor-mediated K^+ channel (137–140). We also have preliminary data that in rabbit corneal epithelial cells EGF induces K^+ channel activation. In addition to increases in plasma membrane K^+ channel activity, EGF increases in corneal epithelial cells Na:K:2Cl cotransport activity based on preliminary data obtained from measurements of its effects on bumetanide-sensitive ^{86}Rb influx; intracellular Na^+ concentration, and cell volume with confocal microscopy (P. Reinack unpublished data).

Effect of Other Cytokines on Corneal Epithelial Cell Proliferation. In primary epithelial cultures of human cornea, there is expression of a myriad of cytokine receptors and their ligands (141). Northern hybridization with oligonucleotide and cDNA probes to a total of 25 cytokines and 12 of their receptors revealed cytokines that could be divided into four different expression patterns. Type I: TGF-α, IL-1 β, and PDGF-β are expressed exclusively by cornea epithelial cells, but their respective receptors EGFR and IL-1R are predominantly expressed by fibroblasts. PDGFR-β is exclusively expressed by fibroblasts. Type II: IGF-1, TGF-β1, -β2, LIF, and bFGF and their receptors are expressed by both epithelial cells and fibroblasts. Type III: KGF and HGF and their respective receptors are expressed exclusively by fibroblasts. Type IV: M-CSF, IL-6, and IL-8 are expressed by fibroblasts and/or epithelial cells, but their receptors are not expressed by either epithelial or fibroblast cells. On the other hand, they are expressed by immune or inflammatory cells. These and other cytokines have been found in both human and rabbit tears (88, 133, 142). In preoperative and postoperative analysis of tear fluid following photoreactive keratectomy (PRK), cytokine release rates increased significantly during the first 2 days after PRK and returned to preoperative levels by Day 7 (133). These observations suggest that corneal wounding induces an increased release of several growth modulating cytokines (e.g., PDGF-BD, HGF, TGF-β and TNF-α) that may be involved in wound healing. Many of these cytokines have been linked to the regulation of proliferation in many cell types. PDGF has been shown to have mitogenic effects via a receptor-mediated activation of a phospholipase C-IP3 pathway (143). TGF-β has been shown to promote proliferation and lamellar differentiation of corneal epithelial cells through keratocyte-mediated stimulation (93).

The distribution expression patterns of the above-mentioned cytokine receptors and their ligands by epithelial cells and stromal fibroblasts suggests that epithelial growth control depends on epithelial stromal interactions. This is indicated since in a bovine corneal epithelium reconstruction (multilayer) culture system, EGF, HGF, and TGF-β1 appear to effectively elicit full-thickness structure for the epithelium through their control of proliferation and differentiation (93). EGF has been shown to cause proliferation of embryonic cornea epithelium in organ culture and increased epithelial resurfacing rates in adult rabbit tissue (32, 35, 36).

Corneal Epithelial Cell Apoptosis

The physiological balance between the ability of the corneal epithelium to proliferate and turnover contributes to the maintenance of corneal deturgescence, transparency, and normal vision (7–11). In a clinical setting, disturbances in epithelial renewal can compromise corneal structure and function, which can in turn lead to infection, development of corneal opacity, and loss of vision. Therefore, there have been a number of studies directed towards understanding the role of cytokines in eliciting corneal apoptosis.

Apoptosis-Related Cytokines in Corneal Epithelial Cell and Tears. Some of the cytokines expressed in corneal epithelial cells and in tears during the wound healing process are apoptosis-related. Those found in human and rabbit tears include HGF, TNFα, TGFβ-1, TGFβ-2, VEGF, PDGF-BB, IL-6, bFGF, EGF, and ET-1 (88–92, 142). A number of them stimulate epithelial growth, whereas others can trigger epithelial cell apoptosis. Besides EGF, the cell death-related cytokines include FGF, IL-1α, IL-6, IL-8, and TNFα. It will be of interest to determine in a reconstruction (multilayer) culture system whether there are any dose-dependent interactions between EGF and the above-mentioned cytokines that could modulate their individual effects on growth and apoptosis.

Susceptibility of Corneal Epithelium to UV-Induced Apoptosis. The upper layers of the Earth's atmosphere filter out impinging high-energy UV rays. Nevertheless, some short-wavelength UV light still penetrates through the ozone layer and strikes the Earth. The majority of this impinging UV is in the 320 to 400 nm range (UV-A) and the rest is UV-B (290–320 nm) and UV-C (200–290 nm). There is recent biophysical evidence showing that this impinging UV light penetrates and is differentially absorbed by ocular tissues. Their absorption pattern in the anteroposterior direction follows a wavelength-dependent decade (Fig. 2). In our environment, the corneal epithelial layers are

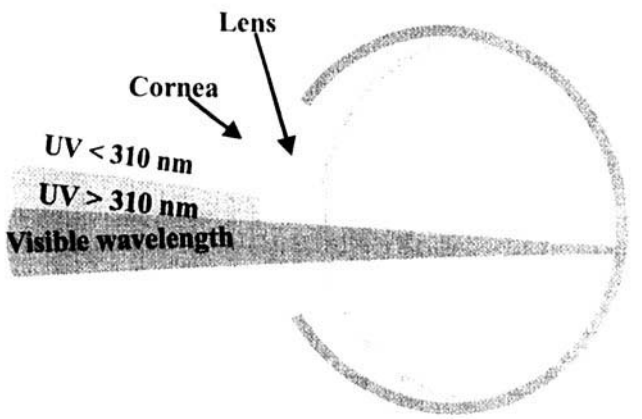

Figure 2. Illustration of UV light absorption by the anterior segment of the eye. UV light with a wavelength shorter than 310 nm is absorbed by the cornea and UV light with a wavelength longer than 310 nm is absorbed by the lens.

more exposed to ambient short-wavelength UV light than any other ocular tissue. The corneal epithelium absorbs UV wavelengths shorter than 310 nm, thereby acting as a filter and protecting the lens and retina from UV-induced damage (144). On the other hand, the lens absorbs most of the UV-irradiation at wavelengths longer than 310 nm. Therefore, these anterior segment tissues only allow light in the visible and infrared wavelength range to reach the retina. In doing so, the corneal epithelium and lens protect the retina from UV irradiation-induced damage (144–146). Due to the absorption profile of most biological tissues, exposure to doses of UV-B and UV-C irradiation is 100- to 100,000-fold more damaging than UV-A. The UV irradiation sufficient to induce corneal epithelial cell death is in the range from 10 to 100 $\mu J/cm^2$. This range covers those previously reported to produce cell death in the intact cornea. It is comparable with that which people are exposed to on a daily basis (147).

UV irradiation-induced corneal epithelial injury can affect corneal epithelial barrier function and can in turn increase susceptibility to infection and the development of corneal opacity. Recently, it was shown that exposure to UV is the leading cause of post-PRK corneal epithelial detachment and death (11, 148–150). In the normal corneal epithelium and endothelium, it has been suggested that aromatic amino acids such as tryptophan are in part responsible for absorbing UV. In addition, ascorbate may effectively absorb UV in the cornea and lens epithelium (144). However, when the system is overwhelmed by too much UV irradiation, it may induce apoptotic damage.

Healing in response to increased UV irradiation induces cell shedding into the tear pool followed by replenishment of cells moving centrally from the limbus and anteriorly from the basal layers of the epithelium. This response is an exaggeration of the normal growth factor-induced cellular and subcellular events mediating continuous corneal epithelial renewal. However, very little is known about how apoptosis-related cytokine receptors are activated by UV irradiation and what the immediate early events are in response to UV irradiation. Even though effective symptomatic relief for UV-related diseases is available, therapeutic options are limited for stimulating corneal re-epithelialization. Therefore, a characterization of the mechanism whereby UV induces corneal epithelial damage has clinical relevance.

Molecular Signaling Events Responding to UV Irradiation. Recent findings in a leukemic cell line indicate that UV irradiation triggers activation within minutes of plasma membrane ion transport pathways, which are linked to intracellular signaling pathways that include the MAPK superfamily cascade (151). $Erk_{1/2}$, JNK/SAPK, and p38 are three parallel MAP kinase pathways that can be activated by UV irradiation (152, 153). Their degree of activation, however, varies. We found after 5 min of exposure to UV irradiation that JNK-1 was strongly activated in cultured rabbit and human corneal epithelial cells. UV-induced MAPK activation in corneal epithelial cells subsequently results in increases in expression of transcription factors AP-1 and NF-κB (154, 155) and of immediate early response genes *c-fos* and *c-jun* (156–158). There is definitive evidence that UV irradiation at wavelengths in the range from 280 to 310 nm induces dose-dependent increases in the expression of apoptosis-related cytokine messenger RNAs (9, 159, 160). It has also been shown that UV irradiation can induce in corneal epithelial cells expression of stress- and apoptosis-related proto-oncogenes *c-Jun* and *c-Fos* (158), as well as increases in p53 and p21 gene expression after 5 to 8 hr and 10 to 12 hr, respectively (161). Other evidence in the intact rabbit cornea that UV irradiation induces apoptosis is that there is TUNEL positive staining, ethidium bromide/acridine orange staining, and DNA laddering (145, 151).

Roles of K^+ Channel Activity in Growth and Apoptosis. K^+ channel activity is widely distributed and involved in maintaining electrophysiological stabilization that is essential for salt and water balance in kidney cells (162) and guard cells (94), insulin release from pancreatic B-cells (163), volume regulation (164), electrical excitability in neurons and cardiac muscle (165), activation of T- and B-lymphocytes (166, 167), and controlling myeloblastic cell development (139). As in some other tissues, we found that in corneal epithelial cells there is a growth factor-regulated 4-aminopyridine- (4-AP) sensitive K^+ channel. Its activation is an early event since EGF-induced MAPK cascade stimulation does not occur in the presence of 4-AP. This channel may be multifunctional since we found with the nystatin-perforated whole cell technique a K^+ current whose amplitude markedly increased upon exposure to UV-C light for as short as 1 min. In the cell-attached mode, exposure to UV-C irradiation (~45–65$\mu J/cm^2$) strongly stimulated K^+ channel activity within 30 sec. As with the EGF-induced K^+ current, this UV-evoked K^+ current was completely blocked by either 2 mM 4-AP or 20 mM tetraethylammonium (TEA). It has been suggested that UV-induced damage in corneal epithelium delays replacement of the surface layer of epithelial cells and wound repair (168). This delay could lead to an increase in susceptibility to viral infection following surgical procedures. Our more definitive evidence that 4-AP-sensitive K^+ channel can initiate apoptosis is that 4-AP did not block the apoptotic-inducing effect of etoposide. This effect of etoposide is consistent with its known inhibition of topoisomerase II activity at the level of the nucleus. On the other hand, in both human and rabbit corneal epithelial cells, suppression of K^+ channel activity with 4-AP protected the cells against UV irradiation-induced DNA fragmentation and nuclear death. We have now also determined in the intact rat cornea that suppression of K^+ channel activity with 4-AP decreases UV- (65 $\mu J/cm^2$) induced apoptosis. Taken together, environmental doses of UV irradiation induce increases in K^+ channel activity, which is an early signaling event resulting in apoptosis.

Conclusion

Studies of corneal wound healing have yielded useful information for our understanding on the roles of cytokine in the maintenance of corneal epithelial functions. It should be noted, however, that our knowledge about the roles of cytokines in corneal wound healing and epithelial homeostasis are largely derived from the expression patterns of ligands and their respective receptors as determined by immunohistochemistry and molecular biology techniques. However, little information is available regarding the precise roles of individual cytokines in corneal morphogenesis during development and homeostasis in adults. Recently, the use of mutant mice created via transgenic and gene-targeting techniques provides unique opportunities to elucidate the significance of cytokines in modeling corneal morphogenesis during development. The mutant mice often manifest mal development of ocular tissues including the corneal epithelium. For example, transgenic mice overexpressing FGF or KGF under the control of the alpha crystalline promoter are characterized by the existence of exocrine glandular epithelium overlying the cornea (169). The defects of mutant mice often occur early during development. Thus, it limits the usefulness of the mutant mice in elucidating the role of cytokines in the maintenance of corneal homeostasis in adult animals . The creation of inducible expression mouse lines using corneal specific promoters, e.g., keratocan (keratocyte-specific) and keratin 12 (corneal epithelium-specific) will allow us to circumvent the pitfalls of transgenic mice that constitutively express cytokines and/or their respective dominant negative mutant receptors.

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