Purinergic Signaling in Corneal Wound Healing: A Tale of 2 Receptors

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Abstract

Nucleotide release and purinergic signaling make up the earliest response to corneal injury and are vital for proper wound healing. In this study, we review the importance of nucleotide release in the injury response and focus on the contribution of 2 receptors that mediate purinergic signaling, P2Y2 and P2X7. These receptors mediate the early response to injury and activate downstream signaling to promote cytoskeletal rearrangement and cell migration. The contribution of corneal nerves to the purinergic injury response is also discussed. Finally, we look at implications of altered purinergic signaling in diabetic wound healing and important targets for future research.

Keywords: purinergic signaling, P2Y2, P2X7, wound healing, cornea

THE CELLULAR RESPONSE to injury and subsequent wound healing is a complex signaling process that involves changes in cellular adhesion, migration, and proliferation, as well as alterations in matrix composition, with the ultimate goal of restoring tissue to an unwounded state. Understanding the wound healing process in the cornea is particularly important, as complete regeneration is necessary to maintain a transparent cornea and clear vision. Over the past 2 decades, research has progressed in determining the signaling factors involved in response to corneal injury and the downstream signaling cascades that effect wound repair. In addition to growth factors, nucleotides have been shown to be essential signaling factors in the corneal wound response. Interestingly, nucleotides were historically thought of solely as the genetic and transcriptional material in living cells. In 1970, Burnstock et al. first described nucleotides as neurotransmitters.¹ Nucleotides have since been widely recognized for their role in regulating autocrine and paracrine signaling molecules in addition to their role in nucleic acids as reviewed by Burnstock.² Our goal in this chapter is to focus on the current state of understanding regarding the importance of purinergic signaling in response to epithelial injury in the cornea.

Identification of ATP as a Signaling Mediator of Corneal Epithelial Injury Response

In the cornea, nucleotides are responsible for cell–cell communication, especially in response to injury. In 2001, Klepeis et al. showed that factors released from a wound resulted in the immediate mobilization of calcium waves away from the site of injury. The calcium wound response was rapid,

initiated within milliseconds after injury, and transient, as the wave dissipated within minutes. The authors demonstrated that the wound-induced calcium wave could propagate over an acellular region, demonstrating that gap junctions were not responsible for the initial calcium wave. In addition, the authors showed that extracellular levels of adenosine triphosphate (ATP) were elevated upon injury and that the calcium wave could be inhibited with apyrase, an ectonucleotidase.³ ATP was subsequently quantified at micromolar concentrations after injury.^{4,5} Subsequent studies identified several purinergic receptors expressed in the epithelium and further demonstrated that injury-induced ATP release was necessary for calcium mobilization, signal transduction, and cell migration.^{6–8}

Stimulation of epithelial cells with individual nucleotides has revealed that the nucleotide triphosphates ATP and uridine triphosphate (UTP) elicited strong calcium responses and subsequent signaling in corneal epithelial cells.^{3,6,7} Adenosine diphosphate (ADP) and uridine diphosphate (UDP) can elicit weak calcium responses and promote cell migration, but their release after injury and their role in wound healing *in vivo* is not well known. Adenosine does not appear to play a significant role in corneal epithelial response to injury as it neither induces a calcium wave nor causes activation of ERK or paxillin.⁶ The role of ectonucleotidases in wound healing has not been well studied in the cornea. However, nonhydrolyzable forms of ATP have elicited similar calcium mobilization and migration responses,^{5,6,8} suggesting that nucleotide triphosphates are the predominant mediators of the wound response.

While activation of the epidermal growth factor receptor (EGFR) has been shown to be an important component of

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TABLE 1. PHOSPHORYLATION RESIDUES DOWNSTREAM OF EGFR THAT ARE ACTIVATED BY ATP STIMULATION, BUT NOT BY EGF (DATA FROM KEHASSE ET AL.¹⁸)

Residues activated by ATP, but not by EGF

PKC (pY-313)
Src (pY-421)
MAPK-1 (pT-185, pT-190)
MAPK-12 (pT-183, pY-185)
MAPK-13 (pT-180, pY-182)
Paxillin (pY-88, pS-91, pS-85/90, pY-118)
FAK (pY-861)

ATP, adenosine triphosphate; FAK, focal adhesion kinase; MAPK, mitogen activated protein kinase; PKC, protein kinase C.

wound healing,^{9–11} data indicate that wound-induced EGFR signaling is dependent upon nucleotide release. For example, apyrase treatment to degrade nucleotides resulted in a loss of wound-induced ERK1/2 activation,⁷ and also resulted in a loss of cell migration.⁸ Nucleotides such as ATP have been shown to transactivate the EGFR through matrix metalloprotease protein (MMP)-mediated cleavage of heparin binding-EGF, as MMP inhibitors, TIMP-3, and GM6001, as well as an HB-EGF inhibitor, CRM197, inhibited wound-induced EGFR activation.⁴ EGFR transactivation through injury or ATP stimulation results in a phosphorylation profile that is distinct from that induced by EGF itself (Table 1).

Together, these studies show that released nucleotide triphosphates, such as ATP, are the primary mediators of the corneal response to injury, and that nucleotides mediate downstream activation of EGFR. P2 purinoreceptors bind nucleotide di- and triphosphates as their ligands and, therefore, mediate the nucleotide-induced wound response in the corneal epithelium. There are 2 distinct families of P2 purinoreceptors, the 7-transmembrane P2Y family and the trimeric ion channel P2X family.¹² Research has shown that 2 prominent purinoreceptors, P2Y2 and P2X7, are involved in mediating the corneal epithelial wound response.^{13,14}

Role of the P2Y2 GPCR and EGFR in Corneal Epithelial Wound Healing

P2Y purinoreceptors are G-protein-coupled receptors that signal through different G α subunits depending on the receptor.^{15,16} Most relevant to corneal wound-induced calcium wave, the P2Y2, P2Y4, and P2Y6 signal through G α_q , which results in activation of phospholipase C gamma (PLC γ). PLC γ activation results in the cleavage of phosphoinositol 4,5-bisphosphate (PIP₂) to form the effector molecules diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP₃). IP₃ activates release of intracellular calcium stores by binding to its receptors in the endoplasmic reticulum and sarcoplasmic reticulum.¹⁷ The PLC γ signaling cascade is required for the wound-induced calcium wave, as the PLC γ inhibitor U73122 blocks the calcium wave in corneal epithelial cells.³

Although major contributions occur from the P2X family of receptors as will be described later, the P2Y2 receptor has been identified as the mediator of the calcium wave after injury in corneal epithelial cells among the the G α_q -signaling P2Y receptors. The transient calcium mobilization that was first identified using UTP, a potent agonist for P2Y2, evokes a similar calcium response to ATP. In comparison, other P2Y-specific agonists evoke little to no cal-

cium response in corneal epithelial cells, including UDP, a selective agonist of P2Y6.^{6,8} Knockdown of P2Y2 by siRNA, but not the related P2Y4, abrogates the wound-induced calcium wave and cell migration after injury.¹³

P2Y2 activation and the resulting intracellular calcium signaling are required to transactivate the EGF receptor in corneal epithelial injury. Reactive blue 2 (RB2), a general P2Y inhibitor, decreased wound-induced EGFR activation and ERK1/2 activation.⁴ More specifically, wound-mediated EGFR activation was ablated by siRNA knockdown of P2Y2.¹⁸ EGFR is the main downstream effector of P2Y2-mediated cell migration, as the inhibitor, AG1478, abrogated UTP-stimulated corneal epithelial cell migration.¹⁹ Furthermore, mutation of different C-terminal EGFR phosphorylation sites revealed that tyrosine 1068 and tyrosine 1086 were required for UTP-stimulated directional migration wound closure.¹⁹

As mentioned before, injury and nucleotide stimulation result in transactivation of EGFR through PKC and MMP-mediated cleavage of HG-EGF. It is not surprising that P2Y2 mediates this effect, as other G protein-coupled receptors in other cell types have also been reported to transactivate EGFR through the same pathway.²⁰

This nucleotide-mediated transactivation results in differential activation compared with EGF-stimulated EGFR signaling. In corneal epithelial cells, immunoprecipitation of the EGF receptor shows that wounded and UTP-stimulated EGFR does not complex with Shc or Grb2 adaptor proteins, and UTP-stimulated EGFR is internalized and recycled at a faster rate than EGF stimulation.¹⁹ Since Grb2 binding leads to Cbl-mediated ubiquitination of EGFR,²¹ these data suggest that nucleotide-stimulated EGFR activation leads to enhanced recycling and less degradation of the receptor. Mass spectrometry analysis of phosphopeptides revealed that Cbl is activated upon EGF treatment, but not ATP stimulation, supporting this hypothesis.¹⁸

The P2Y2-stimulated pathways and EGF-stimulated signaling were investigated further in a nonbiased way using stable isotope-labeled amino acids in cell culture (SILAC), where P2Y2 knockdown and cell stimulation were followed by mass spectrometry analysis of phosphopeptides.¹⁸ The results show that activation of residues on paxillin (y118), focal adhesion kinase (FAK; y861), and Src (y421 and y466) are dependent upon P2Y2 purinoreceptor. This altered receptor trafficking and downstream signaling may be the result of HB-EGF-mediated versus EGF-mediated EGFR activation, or of EGFR-independent signaling downstream of P2Y2, or both.

In addition to ATP and UTP, P2Y2 receptors can be activated by dinucleotides such as diadenosine P1, P4 tetraphosphate (Ap₄A) or P1, P5 pentaphosphate (Ap₅A).^{22,23} Dinucleotides are present in tear fluid and play a role in maintaining corneal epithelial hydration.²⁴ Although their presence in tears demonstrates another potential role for nucleotide signaling in the cornea, their role in wound healing remains unclear. Dinucleotides can stimulate cell migration,²⁵ but their release in response to injury has not been shown. For a more thorough review of dinucleotides in the eye, refer to a recent review by Guzman-Aranguez et al.²⁶

The P2X7 Ion Channel in Corneal Epithelial Wound Healing

Although the importance of P2Y2 in corneal wound healing is well established, it may not be the only purinergic receptor

involved in the epithelial wound response. Knockdown of P2Y2 did not completely ablate cell migration.¹³ Additionally, knockdown of P2Y2 or depletion of intracellular calcium stores by BAPTA or thapsigargin did not eliminate the wound-induced calcium response in cells adjacent to the wound, suggesting that a calcium ion channel may play a role in the wound response. In fact, recent experiments demonstrate that the intracellular calcium level increases and there is minimal lamellipodial ruffling in the presence of thapsigargin. While P2X agonists α,β -meATP and β,γ -meATP have no effect on cell migration or the calcium response,⁷ the P2X7 agonist BzATP does stimulate cell migration.^{8,14}

P2X7 is a trimeric ion channel that allows influx of extracellular calcium in response to activation by ATP.^{27–29} P2X7 is unique among P2X receptors as it has a long C-terminal cytoplasmic tail that is ~200 amino acids longer than any other P2X family member.^{29,30} Denlinger et al. identified several putative binding motifs on the P2X7 C-terminal tail, including a Src homology 3-binding motif, a TNF α receptor-associated death domain (TRADD)-like region, and a lipopolysaccharide (LPS)-binding site similar to that on LPS-binding protein.³¹ Additionally, motifs with homology to mycobacteria HMW3 and *Caenorhabditis elegans* C18H2.1 were identified.³¹ However, the significance of these putative domains in corneal wound healing is unknown.

Given the uniqueness of the C-terminal tail, it is not surprising that unique phenotypic responses have been associated with P2X7, including large pore formation,^{30,32} mature IL-1 β release through NALP3 inflammasome activation,^{33–35} and

pannexin-1 association.^{35,36} In the corneal epithelium, however, large pore formation does not occur and P2X7 stimulation by BzATP promotes epithelial cell migration.¹⁴ Furthermore, inhibition of P2X7 delayed epithelial migration *in vitro* and *ex vivo*, demonstrating that it plays a role in the corneal epithelial wound response.³⁷

Recently, it was shown that P2X7 localized at the leading edge of the migrating epithelium in *ex vivo* organ culture, and this localization did not occur in presence of the irreversible inhibitor of P2X7, oxidized ATP.³⁷ Experiments using live cell imaging and fluorescently expressed cytoskeletal proteins demonstrated that inhibition of P2X7 decreased actin reorganization and focal adhesion turnover at the leading edge. These data suggest that the critical activity of P2X7 after injury occurs at the leading edge and that change in localization of the receptor may precede other events. Additional experiments demonstrated that inhibition of P2X7 did not alter the propagation of the wound-induced calcium wave, but did significantly alter the amplitude and duration of the wave.³⁷ Together, these indicate that P2X7 is necessary for proper cytoskeletal reorganization during migration after injury and plays a role distinct from P2Y2.

A Tale of 2 Receptors

Currently, investigators hypothesize that both P2Y2 and P2X7 purinoreceptors play a role in the nucleotide-mediated wound response in the corneal epithelium. While both

FIG. 1. P2X7 and P2Y2 purinoreceptor responses to nucleotides released upon injury. P2X7 (green) and P2Y2 (blue) have distinct, complementary responses to injury as well as shared responses (yellow). The full nature of these downstream effects is still only partially understood, as is the effects of corneal nerves and glutamate release on the wound response. Color images available online at www.liebertpub.com/jop

initiate a calcium wave and activate cell migration pathways, data also support distinct roles for each receptor (Fig. 1).

Previously, we discussed that knockdown of P2Y2 decreases injury-induced calcium propagation, while cells adjacent to the wound still respond.¹³ Calcium store depletion experiments support these observations, as calcium propagation is decreased, but the wound-adjacent cells still respond.³⁸ These data suggest that P2X7 may drive the calcium response in cells adjacent to the wound. P2X7 inhibition did not affect the rate or distance of calcium wave travel, but significantly altered the level of intracellular calcium in cells adjacent to the site of injury.³⁷ Together, these data indicate that P2Y2 and P2X7 play complementary roles in the calcium response to epithelial injury (Fig. 1).

In addition to the calcium response, data suggest distinct roles for these receptors during epithelial cell migration after injury. *P2Y2* mRNA levels increase when migration in cell culture is detected, whereas *P2X7* mRNA levels drop significantly after corneal epithelial injury in both organ culture and *in vitro* models.³⁷ In each of these models, there is inverse regulation of *P2Y2* and *P2X7* mRNA (Fig. 2). Furthermore, we recently demonstrated that when *P2X7* is

knocked down with siRNA, there is an increase in *P2Y2* mRNA. It is likely that other complementary roles occur in cell migration and wound healing as well. One example is that activation of P2Y2 leads to activation of specific tyrosine residues of FAK and paxillin, whereas loss of P2X7 results in slower FA turnover and delayed migration. However, the phosphoproteomic profile is not known for P2X7 at this time.

Nucleotides: Cotransmitters Are Released from Corneal Nerves with Other Transmitters in Response to Injury

While nucleotide-mediated epithelial cell-cell communication is necessary for the normal corneal wound healing response, the contribution of corneal nerves must be considered. The cornea is the most densely innervated tissue of the body. The sensory nerves in the cornea originate from a trigeminal branch and enter the cornea from the limbal periphery. These nerves form a dense spiral in the subbasal plexus, and electron microscopy has revealed that the nerves penetrate the basal epithelial cells.^{39,40}

The role of nucleotides in corneal nerves has been studied in primary nerve cultures isolated from the trigeminal bundle. Injury induced a calcium response that could be inhibited by apyrase, demonstrating that corneal nerves release nucleotides in response to injury.⁴¹ Interestingly, injury to primary nerves induced a secondary calcium peak that was not inhibited with apyrase. This second peak could be inhibited with NMDA receptor inhibitors. Both ATP and glutamate were detected in media of cultured corneal nerves after scratch wounding, and both stimulated a calcium response in nerves. In contrast, neither kainate nor AMPA elicited a calcium response from corneal nerves.⁴¹ Finally, initial and secondary calcium peaks were also detected in corneal epithelial cells when stimulated with wound media from corneal nerve cultures. These data indicate that *in vivo*, corneal nerves contribute to the epithelial wound response with nucleotide release as well as other factors.⁴¹

The mechanism of wound-induced glutamate release from corneal nerves has not been determined. However, ATP is a known activator of glutamate release in neurons in other systems.^{42–45} Since primary nerves isolated from the trigeminal bundle express the full repertoire of P2X and P2Y receptors,⁴¹ it would be interesting to see if wound-induced glutamate release from corneal nerves is dependent on upstream nucleotides and purinergic signaling. Overall, purinergic signaling from corneal nerves is a vital part of the corneal wound response.

In addition to mediating the corneal wound response, corneal nerves may be important in the maintenance of corneal health through constant signals to the basal epithelium. These signals may be nucleotides, or nucleotide-stimulated glutamatergic agonists, and may play a role in promoting proper basal adhesion and prevention of recurrent epithelial erosion.

Altered Nucleotide Signaling May Underlie Diabetic Wound Healing Defects

Long-term hypoxic conditions in the cornea can result from diabetes, with the severity worsening as the disease progresses.^{46,47} Recent work suggests that one of the consequences of corneal hypoxia is altered nucleotide signaling. Lee et al. showed that hypoxia can alter nucleotide signaling

FIG. 2. P2Y2 and P2X7 mRNA are reciprocally regulated after epithelial injury in rat organ culture and human cell culture. (A) RNA from rat organ cultured corneal epithelium was collected at indicated times after debridement injury. (B) RNA from HCLE cells was collected at indicated times after scratch wounded injury. *P2Y2* and *P2X7* mRNA relative expression was determined by quantitative real-time PCR using verified TaqMan probes (ABI) and normalized to 18S rRNA. Figure updated from Kehasse et al.¹⁸ and Minns et al.³⁷ with additional data points.

in a number of different ways: by decreasing the levels of ATP released from both nerves and corneal epithelial cells, by affecting intracellular calcium stores, by affecting the purinergic receptor response, and by altering gap junction-mediated communication. As a result of defective nucleotide signaling in hypoxic conditions, both activation of the focal adhesion protein paxillin and rate of migration was reduced.³⁸

Interestingly, P2X7 expression is elevated in human diabetic corneal epithelium.¹⁴ A number of diabetic animal models such as streptozotocin-treated rats and diet-induced obese mice have displayed similar increases in P2X7. In both of these models, wound healing is impaired. However, the attenuation in the wound healing after 22 weeks on the high-fat diet appears to follow the changes in the epithelial and stromal nerves that occur as rapidly as 15 weeks after inception of the diet. The effects of P2X7 also increase and the nature of altered purinergic signaling in the manifestation of diabetes is under active investigation.

Interestingly, P2X7 has been shown to mediate inflammation and cell death in oligodendrocytes⁴⁸ and central nerves.^{49,50} Diabetic patients demonstrate a significant loss of corneal nerves,⁵¹ and neuropathic corneas have been shown to be more susceptible to recurrent corneal erosions.⁵² We hypothesize that purinergic receptor misregulation contributes to diabetic nerve loss, and could be a potential therapeutic target.

Future Perspectives on Nucleotide-Dependent Signaling in Corneal Wound Healing

One of the most important goals for purinergic signaling research in the future is to better understand the relationship and signaling between the P2X7, P2Y2, and EGFR in corneal epithelial wound healing. It is clear that nucleotide-mediated signaling is critical in the corneal epithelial wound response, not just in mediating the intercellular calcium wave and EGFR transactivation, but in activation of focal adhesion proteins and promotion of cytoskeletal rearrangements for cell migration. Current data suggest that there are both unique and distinct pathways activated by the different receptors.

P2Y2 stimulation (diasiquol and INS645) has been shown to improve tear secretion and is approved in Japan for dry eye treatment. Stimulation of P2Y2 by UTP or Ap₄A has been shown to increase cell migration *in vitro*⁸ and *in vivo*,²⁵ and could potentially improve the wound healing response in patients. Our studies indicate that P2Y2 stimulation supplemented with a P2X7 agonist such as BzATP would be even more effective at improving wound healing outcomes.

Forty-five years after Dr. Burnstock first reported purinergic neurotransmitters, the role of nucleotides as signal transduction mediators has been well accepted. However, the complex web of purinergic signaling and receptor interplay has only begun to be untangled. A more complete understanding of nucleotide release and purinergic receptor signaling will shed new light on the mechanism of pathologies in wound healing, in the cornea and beyond.

Acknowledgments

This study is supported by N.I.H. grants EY06000 and EY06000S, The New England Corneal Transplant Fund (V.T.-R.), and a departmental grant from Massachusetts Lions Eye Research. The authors would like to thank Celeste B. Rich and Jenna Meyer for critical reading of the article.

Author Disclosure Statement

No competing financial interests exist.

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Received: February 3, 2016

Accepted: April 22, 2016

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