Role of protease-activated receptors 2 (PAR2) in ocular infections and inflammation

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Protease-activated receptors (PARs) belong to a unique family of G protein-coupled receptors (GPCRs) that are cleaved at an activation site within the N-terminal exodomain by a variety of proteinases, essentially of the serine (Ser) proteinase family. After cleavage, the new N-terminal sequence functions as a tethered ligand, which binds intramolecularly to activate the receptor and initiate signaling. Cell signals induced through the activation of PARs appear to play a significant role in innate and adaptive immune responses of the cornea, which is constantly exposed to proteinases under physiological or pathophysiological conditions. Activation of PARs interferes with all aspects of the corneal physiology such as barrier function, transports, innate and adoptive immune responses, and functions of corneal nerves. It is not known whether the proteinase released from the microorganism can activate PARs and triggers the inflammatory responses. The role of PAR2 expressed by the corneal epithelial cells and activation by serine proteinase released from microorganism is discussed here. Recent evidences suggest that activation of PAR2, by the serine proteinases, play an important role in innate and inflammatory responses of the corneal infection.

Keywords: Protease-activated receptors; PAR1, PAR2; aPA; Acanthamoeba; Keratitis; Inflammation; Corneal epithelial cells

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Introduction

Protease-activated receptors (PARs) belong to a distinctive family of the seven transmembrane G protein-coupled receptors (GPCRs) that are cleaved at an activation site within the N-terminal exodomain by the serine (Ser) proteinase (or serine endopeptidases) family [1-5]. The cleaved new N-terminal sequence acts as a tethered ligand which interacts with the receptor within extracellular loop-2 to activate the receptor and initiates signaling [1-5]. To date, four members of the PARs family have been recognized: PAR1-PAR4 [1-5]. PAR1, PAR3, and PAR4 can be stimulated by thrombin and PAR2 can be activated by trypsin, tissue factor/factor VIIa/factor Xa, neutrophil proteinase 3, mast cell tryptase, and membrane-tethered serine proteinase-1 [1-5]. Activation mechanism of PAR1 through PAR4 by serine proteinases, coupling to intracellular signaling cascades, and their important physiological and pathophysiological functions in chemotaxis, inflammation, platelet aggregation, cell
growth and division, endothelial barrier dysfunction, neuronal cell survival, gastrointestinal, cardiovascular responses, regulation of skin function, thrombosis and vascular remodeling, genetic disorders, cancer, inflammatory diseases, and neurological disorders, have been extensively reviewed [6]. Lang et al. [7] first time demonstrated the PAR1 and PAR2 expression and their functional activity in proinflammatory cytokines secretion in human corneal epithelial (HCE) cells. Expression of PAR1 and PAR2 in HCE cells is a first step to investigate corneal biology by targeting PARs signaling in corneal inflammatory diseases (Acanthamoeba keratitis, bacterial keratitis, and fungal keratitis). Recently, we have demonstrated that Acanthamoeba castellanii trophozoites’ secreted serine protease, Acanthamoeba plasminogen activator (aPA) [8], induces proinflammatory cytokine IL-8 by the activation of PAR2 signaling in HCE cells [9]. This signaling is the first illustration of PAR2 activation by microbial serine proteinase in corneal epithelial cells that triggers the inflammatory response; also this might be a novel mechanistic approach of aPA-induced pathogenesis of Acanthamoeba keratitis. This review briefly focuses the role of Acanthamoeba trophozoites’ secreted serine protease, aPA, in the production of inflammatory mediator by the activation of PAR2 pathway in HCE cells.

Acanthamoeba Keratitis and Pathogenesis

Acanthamoeba keratitis is known as a rare but potentially sight-threatening and painful infectious corneal disease worldwide [10, 11]. It is caused by the ubiquitous free-living pathogenic species of Acanthamoeba [10, 12-15]. Acanthamoeba can be found commonly in soil, air, water, cooling towers, sewage systems, and heating, ventilating, air conditioning (HVAC) systems [16, 17]. Since the first case of Acanthamoeba keratitis reported by Naginton et al. [18] in United Kingdom and shortly thereafter by Jones et al. [19] in United States, the incidence of this disease has been augmented with increasing the number of contact lens (CL) wearers [20] (Table). Recently, the preliminary investigations of Centers for Disease Control and Prevention (CDC) of a national Acanthamoeba keratitis outbreak of 105 patients from 30 states of United States, 2005-2007, showed that enrolled patients had percent corneal symptoms of pain 74, redness 74, sensitivity to light 72, sensation of foreign body 68, increased tearing 56, blurred vision 54, and discharge from eye 19[33]. Thus, severe uneven ocular pain to the clinical signs has long been known as one hallmark of Acanthamoeba keratitis.

Many studies have been conducted on the pathogenesis of Acanthamoeba keratitis [9, 34-62]; however, biology and pathogenic mechanisms of Acanthamoeba are still beginning to emerge. The national outbreak of Acanthamoeba keratitis reported that Acanthamoeba primarily affects contact lens users [33]; however, other risk factors and environmental exposures associated with Acanthamoeba keratitis should be considered. Moreover, an increased incidence of Acanthamoeba keratitis is known as an important cause of amoebic keratitis in non-contact lens wearers. A three-year clinical study conducted between 1999 to 2002 in South India diagnosed thirty three patients with positive Acanthamoeba infection (approximate 1%) out of 3183 enrolled patients with corneal infections [63]. They observed that twenty six out of thirty three patients diagnosed with Acanthamoeba infection were peasants from countryside areas and got corneal injury from mud [63]. Treatments of Acanthamoeba keratitis exist with hourly applications of brolene, polyhexamethylene biguanide (PHMB), and chlorhexidine for several weeks. Even with such therapies, Acanthamoeba species can cause severe damage to the corneal epithelium and stroma, resulting in the need for corneal grafting [12]. Topical steroids are often used to control corneal inflammation and uveitis or is administered after surgery, to prevent the rejection of corneal transplant; however, in vitro exposure of Acanthamoeba cysts to dexamethasone increased trophozoite’s number through excystment and growth [64]. Thus, reactivation of Acanthamoeba keratitis may occur after corneal grafting if residual Acanthamoeba cysts in the host peripheral cornea are exposed to steroids after surgery. Although the biology of Acanthamoeba is not fully explored, Acanthamoeba trophozoites’ secreted serine proteases, mannose-induced protein (MIP-133) by contact-dependent mechanism [14, 15, 52, 65] and Acanthamoeba plasminogen activator (aPA) by contact-independent mechanism [8], (a) Contact-dependent mechanism of the Acanthamoeba keratitis pathogenesis begins when Acanthamoeba trophozoites interact to the corneal surface by mannose binding protein (MBP) [11, 56]. This interaction releases the MIP-133 from A. castellanii trophozoites [15], which interacts with membrane phospholipids on corneal epithelium and triggers arachidonic acid production, pro-inflammatory cytokines (IL-8, IL-6, IL-1β, IFNγ, and CXCL2), apoptosis, and polymorphonuclear neutrophils (PMNs) infiltration that leads to corneal lesion by the activation of cytosolic phospholipase A2a (cPLA2a) pathway; cPLA2a inhibitors (AACOCF3, CAY10650, and MAFP) therapeutically in vitro and in vivo mitigate inflammation and resolved the Acanthamoeba keratitis [60, 61]. (b) Contact-independent mechanism of the Acanthamoeba keratitis pathogenesis involves in the secretion of the Acanthamoeba plasminogen activator (aPA) which has been characterized a serine protease [8]. Acanthamoeba plasminogen activator recently has been revealed to be involved in stimulation of pro-inflammatory cytokine in HCE cells via PAR2 pathway [9].
Acanthamoeba Plasminogen Activator (aPA)

Pathogenic species of Acanthamoeba elaborate proteolytic enzymes, especially plasminogen activator (aPA) in the PYG medium [8, 65] (Figure 1A). aPA characterized as a 40 kDa protease which causes a single band of lysis on fibrinogen-agarose zymographs [8]. Activity of aPA is completely blocked by the diisopropylfluorophosphate (DFP or DIFP) treatment [8], phenylmethanesulfonyl fluoride (PMSF), and 1,10-phenanthroline (1,10-ph) [65], indicating that it is a serine protease (Figure 1B); however, aPA activity is not blocked by amiloride, which is a robust inhibitor of urokinase-type plasminogen activator (uPA) [8]. Additionally, the activity of this enzyme is not blocked by plasminogen activator inhibitor-1 (PAI-1) which is the primary physiological blocker of both uPA and tissue-type plasminogen activator (tPA) [8] (Figure 2). aPA does not cross-react with antibodies specific for uPA or tPA [8], aPA stimulates plasminogen from a number of mammalian species, including human, cow, and pig [8].

Activation of PARs and Role of Pathogen-Derived Proteases in Inflammatory Response by PAR2 Pathway

Proteases cleave PARs’ molecules at a specific activation site on the extracellular N-terminal domain. The released new N-terminal domain of the receptor acts as a tethered ligand which binds to the second extracellular loop of the receptor and triggers intracellular signaling [5]. Stimulation of PAR2 leads to a variety of responses such as activating the secretion of cytokines, prostanoids, and metalloproteases which involves to play critical pathophysiological roles in the inflammatory response in joints, skin and kidney, allergic inflammation of airways, and leukocyte migration [2]. Importantly, P. aeruginosa secretes elastase B (EPa) which potentially silences the role of PAR2 in the respiratory tract and alters the host’s innate defense mechanisms and respiratory tract functions, thus EPa by PAR2 signaling contributes a significant role in the pathogenesis of a disease like cystic fibrosis [66]. Other microorganisms such as Serratia marcescens (gram-negative enteric bacterium), and Porphyromonas gingivalis and Aggregatibacter actinomycetemcomitans (gram-negative bacterium causes periodontitis), activate PAR2 and induce proinflammatory cytokines secretion [67-69] and stimulate inflammatory responses in vitro and in vivo [70, 71].

Role of Plasminogen Activator System in Corneal Biology

Fibrinolytic activity is caused by plasminogen activators. Pandolfi and Astrup [72] demonstrated histochemically that corneal epithelial cells of human and several mammals contain plasminogen activator (PA) which appears firmly bound to cellular structures so that it is only available for interaction when cells have been

Table 1. Worldwide incidence of Acanthamoeba keratitis

<table>
<thead>
<tr>
<th>Geographic Area</th>
<th>Years of Data Collection</th>
<th>Acanthamoeba keratitis cases (For depth review refer to reference[26])</th>
</tr>
</thead>
<tbody>
<tr>
<td>USA</td>
<td>1981-1984</td>
<td>1.36 per million contact lens wearer (CLW)[22]</td>
</tr>
<tr>
<td></td>
<td>1985-1987</td>
<td>1.65-2.01 per million CLW[22]</td>
</tr>
<tr>
<td>England</td>
<td>1992-1996</td>
<td>1.4 per million individual[23]</td>
</tr>
<tr>
<td></td>
<td>1997-1998</td>
<td>19.5 per million CLW[23]</td>
</tr>
<tr>
<td></td>
<td>1998-1999</td>
<td>1.26 per million individual[24]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>31 per million CLW[24]</td>
</tr>
<tr>
<td>Japan</td>
<td>1988-1993</td>
<td>1.3 per million individual[24]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>27 per million CLW[24]</td>
</tr>
<tr>
<td>New-Zealand</td>
<td>1990-1996</td>
<td>89.7% CLW[24]</td>
</tr>
<tr>
<td>Australia</td>
<td>1984-1994</td>
<td>7 cases[27]</td>
</tr>
<tr>
<td>Scotland</td>
<td>1990-1995</td>
<td>130 cases[27,24]</td>
</tr>
<tr>
<td>Hong Kong</td>
<td>1997-1998</td>
<td>149 per million CLW[29]</td>
</tr>
<tr>
<td>Africa</td>
<td>1990-1995</td>
<td>33 per million CLW[29]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>33 per million CLW[30]</td>
</tr>
<tr>
<td>Netherlands</td>
<td>1996</td>
<td>22 cases[31]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.06 per million CLW[31]</td>
</tr>
</tbody>
</table>
injured or have undergone degeneration. Furthermore, Pandolfi and Lantz \cite{73} isolated a 55 kDa keratinase (KK), a fibrinolytic enzyme, from in vitro cultures of cornea and characterized the fibrinolysis activity of KK similar to uPA and tPA. They investigated that KK exhibits a biphasic activity to increase the concentration of epsilon aminocaproic acid (EACA) and 46 kDa, and 35 kDa MW. They showed that rabbit epithelial cells, fibroblasts, and ulcer corneas release uPA-like PA; and human cornea extracts and tears also have PA immunoreactivity with anti-uPA antibodies. Noteworthy, the tPA expressed by the corneal epithelium, endothelium, stroma, and the lens, as well as the aqueous and vitreous humors, dog, calf, and monkey eyes. It suggests that tPA plays an important role in non-fibrinolytic processes, mitosis and cell migration, as well as in the destructive remodeling of the extracellular matrix such as the turnover of collagen and glycoproteins in the corneal stroma and vitreous body \cite{76}. However, Tervo et al. \cite{77} observed a robust immunoreaction for uPA, a weaker reaction for PAI-1, and a very weak tPA-like immunoreaction in the anterior stroma of the wounded cornea. Mirshahi et al. \cite{78} support the findings of Tervo et al. \cite{77} and showed that bovine corneal epithelial cells secrete tPA, uPA, and their inhibitor (PAI-1). This study first time demonstrated the role of PAI-1 in the regulation of active protease which is plasmin, may be involved in the destructive processes or tissue remodeling such as the normal turnover of stromal collagens and glycoproteins.

Berk et al. \cite{79} observed the overexpression of PA system (uPA, the receptor of uPA (uPAR), tPA, and both inhibitors, PAI-1 and PAI-2) in mice corneas during the inflammatory response intracorneally infected with P. aeruginosa. Urokinase PA activity also investigated in the epithelial cell migration during corneal epithelial wound healing, which was significantly inhibited by antibodies to uPA. Thus, the activity of uPA in corneal epithelial wound healing may be facilitated in part by the expression of uPAR on epithelial cell surface \cite{80}. Recently, Sugioka et al. \cite{81} investigated the role of uPA in a murine model of lipopolysaccharide (LPS)-induced corneal inflammation. Their results suggest that uPA promotes LPS-induced leukocyte infiltration in cornea, and that uPA is an important component in LPS-induced corneal inflammatory responses. Thus, PA released from all layers of cornea may play an important role in corneal biology, as for example, in the regulation of vessel permeability, angiogenesis, and in the initiation of PMNs chemotaxis.

Interestingly, PARs are specific targets to initiate protease mediated inflammation \cite{1-3, 82}. Zieske and Bukusoglu \cite{83} demonstrated that protease inhibitors inhibit corneal epithelial migration. Moreover, Lang et al. \cite{7} and Nickel et al. \cite{84} demonstrated that human corneal and conjunctival epithelial cells express functional PAR1 and PAR2. Activation of PAR1 and PAR2 on HCE cells by thrombin and trypsin resulted in the production of proinflammatory cytokines such as IL-6, IL-8, and TNFα \cite{7}. In this regard, PAR1 and PAR2 may be important in inflammatory eye diseases.

**The Role of aPA in Recognition of PAR2, But not**
PAR1 in HCE Cells

We have shown that PAR1 and PAR2 transcripts were constitutively expressed in HCE cells and aPA induced up regulation of PAR2, but not PAR1 transcript in the HCE cells. HCE cells stimulated with PAR2 agonists (SLIGRL-NH2 and AC 55541) and PAR1 agonists (thrombin and TRAP-6) upregulate PAR2 and PAR1 mRNA, respectively. PAR2 antagonist (FSLLRY-NH2), but not PAR1 antagonist (SCH 79797), effectively inhibits PAR2 mRNA expression induced by aPA, suggested that aPA activates PAR2 similar to PAR2 agonist. Likewise, upregulated transcripts of PAR1 and PAR2 by their specific agonists were inhibited by SCH 79797 and FSLLRY-NH2, respectively (Figure 3A and 3B). Furthermore, immunolocalization studies demonstrated that HCE cells stimulated with specific PAR1 and PAR2 agonist activate PAR1 and PAR2 on HCE cell surface. aPA specifically activates PAR2 expression on HCE cell surface, but not PAR1[9]. Thus, taken together, the flow cytometry and immunocytochemistry assessments demonstrated that aPA is a PAR2 activator, but not a PAR1 activator (Figure 4A and 4B) [9]. Immunolocalization findings of our study [9] agree with surface protein expression of PAR1 and PAR2 in whole human cornea [7]; however, in our study PAR1 and PAR2 cell surface expression observed upon stimulation by specific agonists [9] while whole human cornea expressed PAR1 and PAR2 immunoreactivity on the apical cell surface of the most superficial corneal epithelial cells in normal condition [7]. Gene expression and cell surface expression of PAR1 and PAR2, and specific activation of PAR2 by aPA seems to play a critical role in pathophysiology of Acanthamoeba keratitis and fascinates to use PAR2 blockers in vivo to determine therapeutic approach in management of Acanthamoeba keratitis.

The Role of aPA in Proinflammatory Cytokine Production by PAR2 Pathway in HCE Cells

Thrombin and trypsin are the multifunctional serine proteinase and stimulate proinflammatory cytokines by PAR1 and PAR2 pathways, respectively [7, 85-87]. We observed the functional activity of Acanthamoeba serine proteinase, aPA, in HCE cells. aPA upregulated proinflammatory cytokine IL-8 expression by PAR2 pathway, but not by PAR1 pathway. Treatments with aPA, PAR2 agonists (SLIGRL-NH2 and AC 55541), and PAR1
agonists (thrombin and TRAP-6) upregulated the IL-8 mRNA expression and protein production in HCE cells. While PAR2 antagonist, FSLLRY-NH2, inhibited IL-8 mRNA expression and protein production stimulated by aPA, SLIGRL-NH2, and AC 55541. In contrast, PAR1 antagonist (SCH 79797) blocked IL-8 mRNA expression and protein production induced by PAR1-agonists, but not by aPA. These results suggest that aPA activates expression and production of pro-inflammatory cytokine IL-8 in HCE cells by PAR2 pathway, but not by PAR1 pathway [9] (Figure 5). It is unlikely that the IL-8 protein production stimulated by aPA is due to activation of TLR2 and TLR4 receptors on HCE cells. We have shown that PAR2 specific antagonist, FSLLRY-NH2, inhibited IL-8 mRNA expression and protein production in HCE cells activated by aPA. Moreover, aPA stimulated IL-8 mRNA expression and protein production is not inhibited by PAR1-antagonist, SCH 79797, in HCE cells [9]. These findings are in agreement with Lang et al. [7] who demonstrated that trypsin (PAR1 agonist) and SLIGRL-

Figure 3. Protease-activated receptor 2 (PAR2) is upregulated by Acanthamoeba plasminogen activator (aPA) in HCE cells. HCE cells were incubated with aPA (100 µg/ml), PAR1 agonists (thrombin, 10 µM; TRAP-6, 10 µM), and PAR2 agonists (SLIGRL-NH2, 100 µM; AC 55541, 10 µM) for 24 hours. Inhibition of PAR1 and PAR2 involved pre-incubating the HCE cells for 1 hour with the antagonist of PAR1 (SCH 79797, 60 µM) and PAR2 (FSLLRY-NH2, 100 µM) and then incubated with or without aPA, PAR1 agonists, and PAR2 agonists for 24 hours. Total RNA was isolated and assessed using quantitative RT-PCR (qRT-PCR) for mRNA expression of PAR1 (3A) and PAR2 (3B). Reprinted with permission [9]. Copyright held by The Association for Research in Vision and Ophthalmology (ARVO).
NH2 (PAR2 agonist) specifically induced production of pro-inflammatory cytokines (IL-6, IL-8, and TNFα) protein in HCE cells. Our study suggests that aPA is an inflammatory protein which plays a critical role in pathogenesis of *Acanthamoeba* keratitis through PAR2 pathway [9], and suggests further in vivo studies to...
delineate the PAR2 pathway as a specific target to approach Acanthamoeba keratitis related therapy.

Summary: Potential Role of PAR2 Antagonists to Attenuate aPA Induced Proinflammatory Mediators

The potential role of PAR2 antagonist in attenuating proinflammatory cytokine IL-8 induced by Acanthamoeba plasminogen activator, aPA, in human corneal epithelial (HCE) cells, is summarized in figure 6. It is possible that other chemokines/cytokines are involved in inflammatory responses in Acanthamoeba keratitis. We have shown that CXCL2 (IL-8 equivalent in rodents) is the major chemokines that plays a major role in attracting
inflammatory cells such as polymorphonuclear neutrophils (PMNs) at the site of infection in Chinese hamster model of *Acanthamoeba* keratitis [61, 62]. Therefore, to determine PAR2 implication in *Acanthamoeba* keratitis using in vitro model of human corneal epithelial cells, we focused on IL-8 production to explore the functional activity of aPA which effectively inhibited by the use of PAR2 antagonist [9]. Likewise the agonists of PAR2 including trypsin and SLIGRL-NH2, modulate IL-6, IL-8, TNFα in HCE cells [7], aPA can induce other chemokines/cytokines in corneal epithelial cells, warranting further study.

In conclusion, increased expression and activation of PAR2 in HCE cells by aPA exposure is closely associated with inflammatory conditions, suggesting that PAR2 is a relatively novel receptor to contribute critical role in inflammatory process during contact-independent mechanism of *Acanthamoeba* infection. *Acanthamoeba* plasminogen activator induced PAR2 expression and IL-8 modulation at gene and protein levels is specifically inhibited by PAR2 antagonist [9]. Thus, PAR2 antagonists may be therapeutic target to cure *Acanthamoeba* keratitis.

**Future Work**

In order to further evaluate the potential roles of PAR2
in *Acanthamoeba* keratitis, the following experiments should be considered: (i) investigating further expression and production of various inflammatory mediators mediated by aPA through PAR2 pathway; (ii) investigating the effects of various antagonists of PAR2 associated G protein and anti-aPA antibody on expression and function of inflammatory mediators during aPA exposures; (iii) investigating PAR2 associated downstream signaling pathway induced by aPA exposures. More importantly, future studies are warranted to investigate pharmacological approach using the selective PAR2 antagonists and proteinase inhibitors to determine the role of PAR2 in corneal infections and inflammation induced by *Acanthamoeba* infection.

### Conflicting interests

The authors have declared that no competing interests exist.

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