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Role of phospholipase A₂ (PLA₂) inhibitors in attenuating apoptosis of the corneal epithelial cells and mitigation of *Acanthamoeba* keratitis

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Abstract

The aim of this study is to determine if the mannose-induced protein (MIP-133) from *Acanthamoeba castellanii* trophozoites induces apoptosis of corneal epithelial cells through a cytosolic phospholipase A_{2α} (cPLA_{2α})-mediated pathway. The efficacy of cPLA_{2α} inhibitors to provide protection against *Acanthamoeba* keratitis was examined *in vivo*. Chinese hamster corneal epithelial (HCORN) cells were incubated with or without MIP-133. MIP-133 induces significant increase in cPLA_{2α} and macrophage inflammatory protein-2 (MIP-2/CXCL2) levels from corneal cells. Moreover, cPLA_{2α} inhibitors, MAFP (Methyl-arachidonyl fluorophosphonate) and AACOCF₃ (Arachidonyl trifluoromethyl ketone), significantly reduce cPLA_{2α} and CXCL2 from these cells ($P < 0.05$). Additionally, cPLA_{2α} inhibitors significantly inhibit MIP-133-induced apoptosis in HCORN cells ($P < 0.05$). Subconjunctival injection of purified MIP-133 in Chinese hamster eyes induced cytopathic effects resulting in corneal ulceration. Animals infected with *A. castellanii*-laden contact lenses and treated with AACOCF₃ and CAY10650, showed significantly less severe keratitis as compared with control animals. Collectively, the results indicate that cPLA_{2α} is involved in MIP-133 induced apoptosis of corneal epithelial cells, polymorphonuclear neutrophil infiltration, and production of CXCL2. Moreover, cPLA_{2α} inhibitors can be used as a therapeutic target in *Acanthamoeba* keratitis.

Keywords

Acanthamoeba; keratitis; mannose-induced protein; corneal epithelial cells; cytosolic phospholipase A_{2α}; macrophage inflammatory protein-2; Chinese hamster

1. Introduction

The trophozoites of several species of *Acanthamoeba* are the causative agents of *Acanthamoeba* keratitis (AK), a sight threatening disease (McCulley et al., 1995; Visvesvara and Stehr-Green, 1990). Treatment of AK is very demanding, consisting of hourly applications of brolene, polyhexamethylene biguanide, 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 transplantation (Alizadeh et al.,

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1996). Two important mechanisms of the pathogenesis of AK shed light on the biology of *Acanthamoeba*; first, its trophozoites bind to the corneal surface by mannose binding protein (MBP) which induces cytopathic effects (Yang et al., 1997) and second, the binding of *Acanthamoeba* to corneal epithelial cells induces release of mannose-induced protein (MIP-133) which affects the subsequent steps in the pathogenic cascade of AK (Alizadeh et al., 2005; Hurt et al., 2003; Leher et al., 1998; Niederkorn et al., 1999; Yang et al., 1997). We have shown that Fas receptor is not involved in MIP-133 induced apoptosis (Tripathi et al., 2012). Although *Acanthamoeba* demonstrates contact-dependent pathogenesis (Siddiqui and Khan, 2012), the host intracellular signaling pathways and the molecular mechanisms associated with MIP-133-mediated corneal epithelial cells' cytotoxicity have not been determined. Similar to *Acanthamoeba*'s contact-dependent mechanism, *Pseudomonas aeruginosa* induces apoptosis in human lung fibroblasts and human conjunctiva epithelial cell lines through the activation of cPLA₂ and arachidonic acid (AA) release (Kirschnek and Gulbins, 2006). Therefore, we hypothesized that cPLA₂ is a key mediator of apoptosis of corneal epithelial cells induced by MIP-133.

PLA₂ enzymes are divided into four major families: platelet-activating factor acetylhydrolases (PAF-AH); secreted PLA₂ (sPLA₂); Ca²⁺-independent PLA₂ (iPLA₂); and cytosolic Ca²⁺-dependent PLA₂ (cPLA₂). The cPLA₂ group includes α , β , γ , δ , ϵ , and ζ subclasses (Burke and Dennis, 2009; Taketo and Sonoshita, 2002). cPLA_{2 α} is the only PLA₂ that exhibits specificity for hydrolysis of *sn*-2 arachidonic acid (AA) from phospholipids. AA is metabolized via the cyclooxygenase (COX) or lipoxygenase (LOX) pathways leading to the production of bioactive eicosanoids in response to a wide variety of extracellular stimuli (Hirabayashi and Shimizu, 2000). cPLA₂ signaling has been shown to be involved in the expression of macrophage inflammatory protein (MIP)-2 [Chemokine (C-X-C motif) ligand 2 (CXCL2)] through cPLA_{2 α} -12/15-LOX pathway (Kuwata et al., 2005). CXCL2 has a chemotactic effect on neutrophils and induces neutrophil infiltration (Hurt et al., 2001). In addition, macrophages and neutrophils are the first-line of defense in active AK infections; elimination of these cell types drastically alters the outcome of AK (Hurt et al., 2001). However, the signaling pathways involved in attracting macrophages and neutrophils into the infected cornea in AK are not completely understood.

The present study addresses the role of MIP-133 in the induction of apoptosis, and examines the role of CXCL2 and neutrophil infiltration both *in vitro* and *in vivo* by cPLA_{2 α} signaling. We demonstrate that MIP-133 induced apoptosis of Chinese hamster corneal epithelial cells is associated with an increase in cPLA_{2 α} activity and involves changes in the levels of cPLA_{2 α} , CXCL2, and neutrophil infiltration. In addition, *A. castellanii*-infected hamster eyes treated with cPLA_{2 α} inhibitors show less severe keratitis as compared with control groups.

2. Methods and Materials

2.1. Amoebae and cell lines

Acanthamoeba castellanii (ATCC 30868), isolated from a human cornea, was obtained from the American Type Culture Collection (ATCC), Manassas, Va. Amoebae were grown as axenic cultures in peptone-yeast extract-glucose at 35°C with constant agitation on a shaker incubator set at 125 rpm (Visvesvara et al., 1983). Chinese hamster corneal epithelial cells (HCORN) were immortalized with human papillomavirus E6 and E7 genes, as previously described (Leher et al., 1998) and cultured in complete minimum essential medium (MEM; BioWhittaker®, Lonza Walkersville, MD, USA) containing 1% L-glutamine, 1% penicillin, streptomycin, amphotericin B, 1% sodium pyruvate (BioWhittaker®, Lonza Walkersville, MD, USA), and 10% fetal calf serum (FCS, HyClone Laboratories, Inc., Logan, Utah), respectively at 37°C in a humidified 5% CO₂ atmosphere.

2.2. Animals

Chinese hamsters were purchased from Cytogen Research and Development, Inc., West Roxbury, MA, USA. All animals used were from 4 to 6 weeks of age and all corneas were examined before experimentation to exclude animals with preexisting corneal defects. All procedures were performed on the left eyes. The right eyes were not manipulated. Animals were handled in accordance with the Association of Research in Vision and Ophthalmology “Statement on the Use of Animals in Ophthalmic and Vision Research” (<http://www.arvo.org/animalst.htm>).

2.3. Isolation of MIP-133

The MIP-133 protein was isolated by fast liquid pressure chromatography (FPLC) and characterized by Western Blot as stated previously (Hurt et al., 2003), and protein concentrations were determined by bicinchoninic acid (BCA) protein assay (Smith et al., 1985).

2.4. Cell cultures and treatment experiments

HCORN cells were cultured in 24 wells plates at ~90% confluence in MEM and incubated with or without MIP-133 at doses of 7.5, 15, and 50 µg/ml for 6, 12, and 24 hrs. Inhibition of cPLA_{2α} was carried out by pre-incubating HCORN cells for 1 hr with cPLA_{2α} inhibitors [10 µM of Methyl-arachidonyl fluorophosphonate (Kirschnek and Gulbins, 2006); MAFP (Cayman Chemical Company, Ann Arbor, Michigan, USA) or 20 µM of Arachidonyl trifluoromethyl ketone (Kirschnek and Gulbins, 2006; Panupinthu et al., 2007); AACOCF3 (Enzo Life Sciences, Inc., Farmingdale, NY, USA)] and inactive inhibitor control [20 µM of Arachidonyl methyl ketone (AACOCH₃), BIOMOL Research Laboratories, Inc., Plymouth Meeting, PA] with or without 15 µg/ml of MIP-133 for 24 hrs. The inhibitors were dissolved in dimethyl sulfoxide (DMSO, a specific solvent of cPLA_{2α} inhibitors and inhibitor control), Fisher BioReagents, Fair Lawn, New Jersey). Cells and supernatants were collected by centrifugation at 2,000×g for 10 min at 4°C.

2.5. Isolation of RNA and reverse transcriptase-PCR

HCORN cells were collected from 24 wells plates at the indicated times after treatments. The total cellular RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The concentration of total RNA and RNA integrity were determined by the absorbance at 260 nm and 280 nm, also by agarose gel electrophoresis, respectively. cDNA was synthesized from 2 µg of total RNA by RT-PCR using random primers (High capacity cDNA Reverse Transcription kit; Applied Biosystems, Foster City, CA). PCR was performed using Ampli Taq Gold PCR Master Mix (Applied Biosystems, Foster City, CA). The amplification profile included one cycle of initial denaturation at 94°C for 5 min, 40 cycles for cPLA_{2α} of denaturation at 94°C for 1 min, primer annealing at 60°C for 1 min, and extension at 72°C for 1 min, and subsequently, one cycle of final extension at 72°C for 10 min. The mRNA expression of each gene was normalized to that of GAPDH as the internal control. The oligonucleotide primers used were: cPLA_{2α} (450bp) 5'-GAGTTTGGGCGTTTCTGGT-3' (sense), 5'-ACGGCAGGTTAAATGTGAGC-3' (anti-sense); CXCL2 (285bp) 5'-ACCCTGCCAAGGGTTGACTTC-3' (sense), 5'-GGCACATCAGGTACGATCCAG-3' (anti-sense); Chinese hamster GAPDH (333bp) available in the UniProtKB/Swiss-Prot database (<http://www.uniprot.org/uniprot/P17244>) 5'-CAAGTTCAAAGGCACAGTCAA-3' (sense), 5'-GTGAAGACGCCAGTAGATTCC-3' (anti-sense). All primers were verified by BLAST (Basic Local Alignment Search Tool, in the public domain, <http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Search of the National Center for Biotechnology Information (NCBI) demonstrated that these primers amplify mouse/rat

cPLA_{2α}, CXCL2, and GAPDH gene products. All primers were from Integrated DNA Technologies, Inc (Commercial Park, Coralville, Iowa, USA).

2.6. cPLA_{2α} activity in cell lysates

HCORN cells were collected from 24 wells plates at the indicated times after treatments and then centrifuged at 2,000×g for 10 minutes at 4°C. The cell pellets were washed with phosphate buffered saline (PBS), homogenized in 1 ml of cold PBS containing 50 mM HEPES (Mediatech, Inc., Manassas, VA), pH 7.4, with 1 mM EDTA (VWR International, LLC, West Chester, PA). Cell lysates were collected by centrifugation at 10,000×g for 15 min at 4°C. cPLA_{2α} activity was measured using the PLA₂ substrate arachidonoyl thio-phosphatidylcholine and the protocol recommended by the manufacturer (Cayman Chemical Company, Ann Arbor, MI). The absorbance was measured at 414 nm in an ELISA reader (Gen5 1.10, BioTek Instruments Inc., Winooski, Vermont). cPLA_{2α} activity was expressed in nanomoles/milligram of protein/minute determined from the extinction coefficient of 5,5'-dithio-bis-(2-nitrobenzoic acid) (DNTB) as instructed by the cPLA₂ assay kit and the protein content in the supernatants was analyzed by BCA protein assay (Smith et al., 1985). Sensitivity of assay is 3.5 – 42 nmol/min/ml without sample dilution.

2.7. Apoptosis assessment by Cellular DNA fragmentation assay

Apoptosis was determined by Cellular DNA fragmentation assay (Cell Death Detection ELISA^{plus} kit; Roch Diagnostics GmbH, Mannheim, Germany) as described previously (Bonfoco et al., 1995). In brief, HCORN cells (1×10^5) were cultured in 200 μl MEM and then preincubated for 1 hr with cPLA_{2α} inhibitors (10 μM of MAFP or 20 μM of AACOCF₃), 20 μM of AACOCH₃ (inhibitor control) and then incubated for 24 hrs with or without 15 μg/ml of MIP-133. Cells were centrifuged for 10 min at 200×g and the cell pellets were incubated with lysis buffer supplied by the manufacturer for 30 min at room temperature. 20 μl of the supernatant (cytoplasmic fraction) was used in the Cellular DNA Fragmentation ELISA following the manufacturer's standard protocol. The absorbance was measured at 405 nm and 490 nm (reference wavelength) using a microplate reader (Gen5 1.10, BioTek Instruments Inc., Winooski, Vermont). Signals in the wells containing the substrate only were subtracted as background. The results were expressed in Absorbance [$A_{405 \text{ nm}} - A_{490 \text{ nm}}$]. Cells treated with DMSO or cPLA_{2α} inhibitors served as control.

2.8. Enzyme-linked immunosorbent assay (ELISA)

CXCL2 was quantified from cell supernatants and cell lysates using ELISA. Briefly, cell culture supernatants and cell lysates were collected at the indicated times after treatments and centrifuged to remove cell debris. Total protein concentrations of each supernatant and lysate were determined by BCA protein assay (Smith et al., 1985). Level of CXCL2 was determined by ELISA test kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. The absorbance was measured at 450 nm using an ELISA reader. The minimum detectable dose of CXCL2 was >1.5 pg/ml. The results were expressed in pg/mg of CXCL2.

2.9. Contact lens preparation

Contact lenses were prepared as stated previously (Alizadeh et al., 2007). Lenses were placed in sterile 96-well microtiter plates (Costar, Cambridge, Mass.) and incubated with 1×10^6 *A. castellanii* trophozoites at 35°C for 24 hours. Attachment of amoebae to the lenses was verified microscopically before infection.

2.10. Subconjunctival injection of MIP-133

Chinese hamsters were anesthetized as described previously (Alizadeh et al., 2007). Panels of Chinese hamsters ($n = 8/\text{group}$) were injected subconjunctivally with MIP-133 at dosage $40\mu\text{g}/40\mu\text{l}$ per four quadrants in the eye encircling the entire conjunctiva. Control animals ($n = 8/\text{group}$) received subconjunctival injection of bovine serum albumin (BSA). Eyes were removed 3 days after infection and used for histopathological examination.

2.11. In vivo treatment of animals with cPLA_{2α} inhibitor

Chinese hamsters ($n = 9/\text{group}$) were infected with parasite-laden contact lenses as described previously (Alizadeh et al., 2007). cPLA_{2α} inhibitor (AACOCF3 and CAY10650; Cayman Chemical Company, Ann Arbor, MI) $50\mu\text{g}/5\mu\text{l}$ was injected with topical eye-drop under the contact lens of an infected cornea three times a day for 6 days and topically on days 7 to 20 postinfection. We observed that repeated topical application of MAFP induced inflammation and edema on the eyelids, therefore, another cPLA_{2α} inhibitor (CAY10650) is used for *in vivo* study. As a control infected animals were treated with $50\mu\text{g}/5\mu\text{l}$ of BSA. Lenses were removed 7 days postinfection, and corneas were scored for clinical severity for the period indicated. Each graph line represents the mean severity of the observed time points. The results shown are representative of three separate experiments. The infections were scored on a scale of 0 to 5 based on the following parameters: corneal infiltration, corneal neovascularization and corneal ulceration. The pathology was recorded as: 0 = no pathology, 1 = <10% of the cornea involved, 2 = 10 to 25% involved, 3 = 25 to 50% involved, 4 = 50 to 75% involved, and 5 = 75 to 100% involved, as described previously (Alizadeh et al., 2007). Any animals receiving a score of at least 1.0 for any parameter were scored as infected.

2.12. Clinical pathology of infected cornea treated with cPLA_{2α} inhibitor

Chinese hamsters ($n = 6/\text{group}$) were infected with parasite-laden contact lenses and treated with cPLA_{2α} inhibitors (AACOCF3 and CAY10650) $50\mu\text{g}/5\mu\text{l}$ was injected with topical eye-drop under the contact lens of an infected cornea three times a day for 6 days and topically on days 7 to 14 postinfection. Animals were anesthetized and sacrificed 15 days after application of cPLA_{2α} inhibitors. Each animal right eye (uninfected and untreated) was used as negative control. As control, uninfected eyes of animals ($n = 6/\text{group}$) were treated with cPLA_{2α} inhibitors. Eyes were removed and used for histopathological examination.

2.13. Histological examination

Treated and untreated eyes were removed and stored in 10% Carson's formalin for 24 hours. Specimens were then embedded in paraffin, cut into $4\mu\text{m}$ sections using a Reichert Histostat rotary microtome (Reichert Scientific Instruments, Buffalo, NY), and placed on polysine hydrobromide-precoated slides (Polysciences, Warrington, Pa.). Sections were stained with hematoxylin and eosin, covered with a coverslip, and examined by light microscopy. Pictures were taken by camera-enhanced light microscopy (BX50; Olympus Optical, Tokyo, Japan).

2.13. Statistics

All experiments were performed in triplicate and results are presented as mean $\pm$ SEM. Differences between multiple groups were determined by a repeated measures (subjects within groups) two-way analysis of variance (ANOVA) followed by the Bonferroni post hoc test. When appropriate, unpaired Student's *t*-test was performed to determine the significance in the difference between two mean values. While Clinical severity scores were analyzed by the Mann-Whitney's U test. In all analysis, $P < 0.05$ was considered as statistically significant.

3. Results

3.1. MIP-133 induces up-regulation of cPLA_{2α} mRNA expression and cPLA_{2α} activity in HCORN cells

To determine if MIP-133 activates phospholipids on corneal epithelial cells, HCORN cells were treated with different doses of MIP-133 and the expression of cPLA_{2α} was detected by RT-PCR. MIP-133 induced up-regulation of cPLA_{2α} mRNA expression at doses of 7.5, 15, and 50 μg/ml after 6, 12, and 24 hrs treatments. cPLA_{2α} mRNA expression was significantly increased at the doses of 15 and 50 μg/ml after 6 hrs treatments and also at the dose of 15 μg/ml after 24 hrs treatments. Two-way ANOVA followed by the Bonferroni post hoc test indicated a significant effect for treatment [$F_{(1,8)} = 269.32$, $P = 0.0001$], for time [$F_{(2,16)} = 6.80$, $P = 0.007$] and for interaction (treatment $\times$ time) [$F_{(6,16)} = 0.511$, $P = 0.791$] (Fig. 1A). cPLA_{2α} enzyme activity was examined in cell lysates. All three doses of MIP-133 induced significant increase of cPLA_{2α} activity at 6, 12, and 24 hrs after incubation. cPLA_{2α} activity was the highest observed at the 15 μg/ml of MIP-133. Two-way ANOVA followed by the Bonferroni post hoc test indicated a significant effect for treatment [$F_{(1,8)} = 447.549$, $P = 0.0001$], for time [$F_{(2,16)} = 109.68$, $P = 0.0001$] and for interaction (treatment $\times$ time) [$F_{(6,16)} = 27.54$, $P = 0.0001$] (Fig. 1B); therefore, we chose 15 μg/ml concentration of MIP-133 for the inhibitor experiments. In addition, cPLA₂ enzyme activity was examined in purified MIP-133 protein however no cPLA₂ enzyme activity was detected in purified MIP-133 protein (data not shown). The results demonstrate that MIP-133 induced up-regulation of cPLA_{2α} at mRNA levels and its enzyme activity.

3.2. Up-regulation of cPLA_{2α} by MIP-133 is diminished by cPLA_{2α} inhibitors in HCORN cells

To confirm that MIP-133 activates the cPLA_{2α} pathway in HCORN cells, the effect of various cPLA_{2α} inhibitors on cPLA_{2α} activity was determined by enzyme assays. AACOCF₃ and MAFP are known inhibitors of cPLA_{2α}. The results showed that the level of cPLA_{2α} activity increased significantly ($P < 0.05$) after MIP-133 treatment when compared to untreated cells. Moreover, AACOCF₃ and MAFP alone or in combination significantly inhibited MIP-133-induced cPLA_{2α} activity ($P < 0.05$). The combination of inhibitors did not have an additive effect on inhibition of cPLA_{2α} activity, most likely because each inhibitor alone reduced cPLA_{2α} activity to basal levels. In contrast, the inactive control compound AACOCH₃ did not block cPLA_{2α} activity induced by MIP-133. Moreover, DMSO, AACOCH₃, AACOCF₃, and MAFP without MIP-133 showed cPLA_{2α} activity similar to untreated cells (Fig. 2). These results indicate that MIP-133 activates cPLA_{2α} enzyme activity in HCORN cells, which is blocked by pre-incubation with cPLA_{2α} inhibitors.

3.3. cPLA_{2α} inhibitors reduce MIP-133 induced apoptosis in HCORN cells

We aimed to gain insight into the functional role of cPLA_{2α} activity in HCORN cells upon MIP-133 stimulation. Therefore, the effect of various cPLA_{2α} inhibitors on HCORN cell was examined by Cellular DNA fragmentation ELISA. Treatment of HCORN cells with MIP-133 induced significant ($P < 0.05$) DNA fragmentation in corneal cells as compared to untreated control cells (Fig. 3). cPLA_{2α} inhibitors, AACOCF₃, and MAFP significantly ($P < 0.05$) inhibited this MIP-133 induced DNA fragmentation. In contrast, the inactive control compound AACOCH₃ did not block DNA fragmentation induced by MIP-133. DMSO had no effect on DNA fragmentation in corneal epithelial cells. The results indicate that cPLA_{2α} is involved in MIP-133 mediated apoptosis of HCORN cells (Fig. 3).

3.4. MIP-133 is involved in pathogenesis of *Acanthamoeba* keratitis

The pathogenic property of MIP-133 was assessed by subconjunctival injection of the purified MIP-133 protein in Chinese hamsters. To this end, we observed that MIP-133 induced severe keratitis resulting in corneal ulceration and, in some cases perforation of the cornea. In contrast, BSA treated eyes exhibited only mild edema and the corneas were clear within 3 days after injection (Fig. 4A). The histopathological features of the corneas treated with MIP-133 consisted of epithelial ulceration, focal thickening, and polymorphonuclear neutrophil (PMN) exocytosis. Stromal changes included lamellar connective tissue disruption, PMN infiltration, neovascularization, and thickening. Few inflammatory cells were present under the corneal endothelium of corneal section from control (BSA-treated animals (Fig. 4B). These results suggest that MIP-133 plays an important role in the pathogenicity of *Acanthamoeba* keratitis by inducing cytolysis of the corneal cells and stimulating the accumulation of PMN in the cornea.

3.5. MIP-133 induces up-regulation of chemokine CXCL2 in HCORN cells

We hypothesized that MIP-133 interacts with the corneal epithelial cells through activation of the cPLA_{2α} pathway, leading to the production of chemokine CXCL2 and recruitment of neutrophils into the cornea. To determine the expression and production of CXCL2 in cultured corneal cells, cells and supernatants were collected at indicated times after MIP-133 stimulation. The cells and supernatants were subjected to RT-PCR and ELISA for detection of CXCL2 (Fig. 5A and 5B). Un-stimulated HCORN cells constitutively expressed low level of CXCL2 mRNA and protein production. The addition of 7.5, 15, and 50 µg/ml of MIP-133 increased significant mRNA and protein expression of CXCL2 at all three doses and all three time points (6, 12, and 24 hrs). Two-way ANOVA followed by the Bonferroni post hoc test indicated a significant effect of CXCL2 mRNA expression for treatment [$F_{(1,8)} = 1280.73$, $P = 0.0001$], for time [$F_{(2,16)} = 28.32$, $P = 0.0001$] and for interaction (treatment $\times$ time) [$F_{(6,16)} = 37.01$, $P = 0.0001$] (Fig. 5A). Also, two-way ANOVA followed by the Bonferroni post hoc test indicated a significant effect of CXCL2 protein expression for treatment [$F_{(1,8)} = 1725.84$, $P = 0.0001$], for time [$F_{(2,16)} = 102.05$, $P = 0.0001$] and for interaction (treatment $\times$ time) [$F_{(6,16)} = 31.41$, $P = 0.0001$] (Fig. 5B). These results demonstrate that HCORN cells secreted significantly higher quantities of CXCL2 after MIP-133 stimulation.

3.6. cPLA_{2α} inhibitors diminish MIP-133-induced chemokine CXCL2 production

To determine whether cPLA_{2α} is involved in MIP-133-induced chemokine release from HCORN cells; the effect of various cPLA_{2α} inhibitors on production of CXCL2 was tested *in vitro*. HCORN cells were pre-incubated with either MAFP or AACOCF3 and then incubated with 15 µg/ml of MIP-133. Chemokine CXCL2 production was measured by ELISA 24 hrs after MIP-133 stimulation. AACOCH3 was used as an inactive control inhibitor and DMSO was used as a control of inhibitors' solvent. CXCL2 production was increased significantly ($P < 0.05$) after MIP-133 stimulation (Fig. 6). MAFP and AACOCF3 significantly inhibited MIP-133-induced chemokine CXCL2 production ($P < 0.05$). However, the combination of both MAFP and AACOCF3 did not have an additive effect on inhibition of CXCL2 production, most likely because each inhibitor alone reduced cytokine expression to basal levels. Moreover, AACOCF3, MAFP, AACOCH3, and DMSO without MIP-133 did not show significant effect on CXCL2 production when compared to untreated cells. In contrast, the inactive control compound AACOCH3 did not block CXCL2 production induced by MIP-133. These results indicate that cPLA_{2α} is involved MIP-133-induced chemokine CXCL2 production in HCORN cells.

3.7. cPLA_{2α} inhibitor mitigates the pathogenesis of *Acanthamoeba* keratitis in vivo

We hypothesized that blocking cPLA_{2α} activity through the administration of cPLA_{2α} inhibitor would reduce the severity of the clinical course of the keratitis and hasten the onset of resolution. Treatment with the AACOCF3 and CAY10650 had a profound effect on the severity and chronicity of keratitis (Fig. 7). In addition, hamsters treated with AACOCF3 had significantly less severe keratitis as compared with CAY10650 group. These results indicate that a cPLA_{2α} inhibitor can be used as a therapeutic target in *Acanthamoeba* keratitis (AK).

3.8. Histopathological examination of infected cornea treated with cPLA_{2α} inhibitor

Whole eyes were collected from infected animals treated with AACOCF3, CAY10650, and BSA or uninfected untreated control on day 10 postinfection for histopathologic analysis. The histopathologic features of the eyes from BSA-treated Chinese hamsters were markedly and consistently more severe than those of untreated controls, AACOCF3, and CAY10650 treated animals. Histopathologic changes were found only in those eyes with clinically present gross lesions. The BSA treated group was characterized by focal epithelial ulceration and PMN crust formation (Fig. 8). The corneal stroma of this group contained marked lamellar connective tissue disruption, thickening, and neovascularized. Severe PMNs cell infiltration, and small lymphocytes were evident in this group of animals. By contrast, CAY10650 treated groups had very mild inflammation and very few PMNs infiltration in the corneal stroma. Corneal epithelium was normal and mild stroma swelling was observed in the corneal stroma. It should be noted that the cornea from animals treated with topical application of AACOCF3 had very mild inflammation, however, AACOCF3 treated corneal section is similar to the corneal section from control untreated animals. Corneal section of control untreated showed few inflammatory cells under the corneal endothelium. The overall histopathologic changes were very mild to moderate for AACOCF3 and CAY10650 group, respectively; the clinical and histologic character of these lesions was consistent with that of untreated Chinese hamsters at this time point. However, the inflammatory responses in the BSA treated group were substantially more severe than either AACOCF3- and CAY10650-treated or untreated Chinese hamsters (Fig. 8). The histologic features of the eyes from uninfected animals treated with AACOCF3 and CAY10650 was similar to that of control untreated eyes (data not shown).

4. Discussion

The pathogenesis and pathophysiology of *Acanthamoeba* keratitis (AK) are extensively studied (Nieder Korn et al., 1999). Several lines of evidence suggest that *Acanthamoeba* expresses MBP on their cell surface, which facilitates adhesion of amoebae to the mannose-6-phosphate receptor on the corneal epithelium; this interaction induces *Acanthamoeba* to secrete the cytopathic protease MIP-133, which causes a sequential cascade of apoptosis via a caspase-3-dependent pathway (Alizadeh et al., 2005; Hurt et al., 2003; Leher et al., 1998; Nieder Korn et al., 1999; Yang et al., 1997). We demonstrated that unlike “amoebapores”, the *Entamoeba histolytica* cytolytic peptides, MIP-133 does not perforate the lipid bilayers to cause cell death (Hurt et al., 2003). Moreover, Fas/Fas ligand interactions are not involved in MIP-133 induced apoptosis of corneal cells because MIP-133 was able to induce apoptosis in corneal cells of Fas receptor deficient mice (*lpr/lpr*) (Tripathi et al., 2012). The mechanism by which MIP-133 interacts with the corneal epithelial cell surface to lead to apoptosis is not completely understood. The main goal of this study was to determine the cytopathic signaling of MIP-133 upon interaction with phospholipids on HCORN cells *in vitro* and in the Chinese hamster model of AK *in vivo*. We hypothesized that MIP-133 interacts with phospholipids on HCORN cells to activate cPLA_{2α} and employs cPLA_{2α} pathways to induce apoptosis of corneal epithelial cells, CXCL2 production, and neutrophil

infiltration. We have shown that specific inhibitors of cPLA_{2α} enzymes therapeutically block MIP-133 induced cPLA_{2α} activity and mitigate AK. The results presented herein provide evidence for the key role of cPLA_{2α} signaling in MIP-133-induced pathogenesis of AK. The results indicated that MIP-133 induces the expression of cPLA_{2α} enzyme in HCORN cells in a dose and time-dependent manner. In addition, cPLA_{2α} inhibitors AACOCF3 and MAFP inhibit the MIP-133-induced cPLA_{2α} activation, suggesting that MIP-133 alters the biphasic modification of phospholipids on the cell surface and triggers activation of the cPLA_{2α} pathway. Several studies have suggested that phospholipases are important virulence factors in a wide range of microbial pathogens including bacteria, protozoa, and fungi (Ghannoum, 2000; Istivan and Coloe, 2006; Ivanovska, 2003). It is possible that induction of apoptosis and cPLA_{2α} release is due to MIP-133 expressing cPLA_{2α} activity. However, no cPLA_{2α} activity is detected in purified MIP-133 *in vitro* (data not shown). Other studies have shown that PLA₂ activities could be involved in mediating apoptosis following *Pseudomonas aeruginosa* infection (Kirschnek and Gulbins, 2006; Taketo and Sonoshita, 2002). Apoptosis is classified as early or late apoptosis on the basis of whether the parasite attacks the host cell initially causing death, or if the host cells death occurs later during the infection. It is possible that early apoptosis could favor the penetration of the parasite; late apoptosis also has benefits towards the parasite as it may prevent the detrimental effects of the inflammatory response (Bienvenu et al., 2010). This mechanism is in accordance with earlier findings of the multifaceted process of the pathogenesis of AK. These include binding of *Acanthamoeba* trophozoites to corneal epithelium secretes MIP-133 which mediates apoptosis of corneal epithelial cells, facilitating the desquamation of the corneal epithelium, the penetration of Bowman's membrane, and the dissolution of the stromal matrix leading to early and late apoptosis (Clarke and Niederkorn, 2006; Hurt et al., 2003; Visvesvara et al., 1983). The detailed mechanisms of PLA₂ signaling is involved in apoptosis of corneal epithelial cells during AK needs to be elucidated. *In vitro* inhibitors targeting the cPLA_{2α} enzyme significantly inhibited apoptotic cell death in the presence of MIP-133. Moreover, treatment with specific cPLA_{2α} inhibitors (AACOCF3 and CAY10650) significantly protected animals against *Acanthamoeba* infection *in vivo*, suggesting that the pharmacological manipulation of the cPLA_{2α} activity could be an interesting therapeutic approach. Histologic features of the cornea from AACOCF3 and CAY10650 treated group confirm the efficacy of cPLA_{2α} inhibitors treatment. The results showed that inflammatory responses in the BSA-treated group were substantially more severe than either AACOCF3- and CAY10650-treated or untreated Chinese hamsters. We envision that cPLA_{2α} inhibitors not only inhibits the inflammatory responses during initial infection but prolong application of cPLA_{2α} inhibitors in clinical setting may affect inflammatory responses induced by proliferating trophozoites during the infection and the trophozoites that emerge from cysts, if amoeba encysted in the cornea. It has been shown that corneal lesions are heavily infiltrated with neutrophils in *Acanthamoeba* and *Pseudomonas aeruginosa* keratitis (Clarke and Niederkorn, 2006; Kernacki et al., 2000a). Neutrophils are recruited to the cornea in response to the chemokine CXCL2, which is secreted by corneal cells (Kernacki et al., 2000b; Kwon and Hazlett, 1997). We have shown that corneal infections with *Acanthamoeba* trophozoites induce production of CXCL2, which promotes the recruitment of neutrophils to the infected cornea (Hurt et al., 2001). However, present studies indicate that inhibitors of cPLA_{2α} significantly inhibited production of CXCL2 in HCORN cells. The enhanced production of CXCL2 and its inhibition by cPLA_{2α} specific inhibitors suggests that cPLA_{2α} plays an important role in controlling chemokine production. Histological evaluation of AK lesions in both human and experimental animals showed large numbers of neutrophils in the cornea (He et al., 1992; Larkin and Easty, 1991; Mathers et al., 1987; Yan et al., 1998). The activation of cPLA_{2α} may have both beneficial and detrimental inflammatory effects on the cornea, depending on the effectiveness and duration of the host inflammatory response. There is critical balance between generating a successful inflammatory response to eliminate the microorganism and

an excessive inflammatory response that can result in corneal scarring and blindness. *In vivo* injection of MIP-133 in Chinese hamster corneas showed severe keratitis resulting in corneal ulceration and perforation. Moreover, histopathology of corneas showed epithelial ulceration, focal thickening, PMN infiltration, and neovascularization. Previous findings showed the importance of neutrophil infiltration and production of CXCL2 in the innate immune system and demonstrated its controlling role in AK (Hurt et al., 2001). There are lots of factors which are involved in pathogenicity of AK (Nieder Korn et al., 1999; Clarke and Nieder Korn, 2006; Panjwani, 2010). However, our data indicate that there is correlation between upregulation of cPLA_{2α} induced by MIP-133 in the corneal epithelial cells and destruction of corneal tissue. Since the specific inhibitors of cPLA_{2α} block the apoptosis of corneal epithelial cells *in vitro* and mitigate inflammatory responses in *Acanthamoeba* infection *in vivo*. We concluded that cPLA_{2α} pathway is involved in destruction of corneal tissues and inflammatory response.

5. Conclusions

The present studies demonstrate a novel corneal epithelial cell response to *Acanthamoeba*-cytopathic protease MIP-133, which occurs through a cPLA_{2α} signaling cascade. Identification of the components of this cPLA_{2α} pathway may facilitate the development of more effective therapeutic strategies in AK.

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- MIP-133 induces significant increase in cPLA_{2α} level in corneal epithelial cells.
- cPLA_{2α} inhibitors significantly reduce cPLA_{2α} and CXCL2 from these cells.
- cPLA_{2α} inhibitors significantly inhibit MIP-133-induced apoptosis in corneal cells.
- cPLA_{2α} inhibitors reduced severity of *Acanthamoeba* keratitis *in vivo*.
- Corneal epithelial cell response to MIP-133, through a cPLA_{2α} signaling cascade.

Fig. 1. MIP-133 induces cytosolic phospholipase A_{2α} (cPLA_{2α}) up-regulation in HCORN cells. HCORN cells were treated with MIP-133 at doses 7.5, 15, and 50 μg/ml for 6, 12, and 24 hrs following which cells were processed for total RNA isolation and RT-PCR. The amount of mRNA expression was quantified by densitometry of bands in comparison to the Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (**1A**). HCORN cells were homogenized and then cell lysates were used to measure cPLA_{2α} enzyme analysis (**1B**). The data are mean ± SEM of three independent experiments. *Asterisk* indicates *P* values < 0.05 were obtained by repeated measures (subjects within groups) two-way analysis of variance (ANOVA) followed by Bonferroni post hoc test.

Fig. 2.

Effect of cytosolic phospholipase A_{2α} (cPLA_{2α}) inhibitors (AACOCF3 and MAFP) on MIP-133 induced cPLA_{2α} enzyme activity in HCORN cells. HCORN cells were pre-incubated for 1 hr with cPLA_{2α} inhibitors (20 μM AACOCF3 or 10 μM MAFP) or an inactive control compound (20 μM AACOCH3) or DMSO (5 μL solvent of inhibitors and its control compound), and then incubated with or without 15 μg/ml of MIP-133 for 24 hrs. Induction of cPLA_{2α} enzyme activity was examined by cPLA₂ assay kit. The data are mean ± SEM of three independent experiments (**P* < 0.05). *P* values were obtained by unpaired Student's *t*-test. The inactive control compound AACOCH3 did not block cPLA_{2α} enzyme activity induced by MIP-133. Moreover, DMSO, AACOCH3, AACOCF3, and MAFP without MIP-133 showed cPLA_{2α} activity similar to untreated cells.

Fig. 3.

MIP-133 induces apoptosis is blocked by cytosolic phospholipase A_{2α} (cPLA_{2α}) inhibitors in HCORN cells were pre-incubated for 1 hr with cPLA_{2α} inhibitors (20 μM AACOCF3 or 10 μM MAFP) or an inactive control compound (20 μM AACOCH3) or DMSO (5 μL solvent of inhibitors and its control compound), and then incubated with or without 15 μg/ml MIP-133 for 24 hrs. Apoptosis was examined by Cellular DNA Fragmentation Assay (Cell Death Apoptosis ELISA^{plus} Assay Kit). MIP-133-induced apoptosis is confirmed by the positive control provided in the Cell Death Apoptosis Kit. The data are mean ± SEM of three independent experiments (**P* < 0.05 and). *P* values were obtained by unpaired Student's *t*-test. The inactive control compound AACOCH3 did not block apoptosis induced by MIP-133. Moreover, DMSO, AACOCH3, AACOCF3, and MAFP without MIP-133 showed cPLA_{2α} activity similar to untreated cells.

Fig. 4. MIP-133 protein is involved in pathogenesis of *Acanthamoeba* keratitis. Representative photographs of Chinese hamster eyes 3 days after subconjunctival injection of MIP-133 (40 μ g/40 μ l) or BSA. MIP-133 induced severe keratitis resulting in corneal ulceration and increased corneal infiltration (**4A**). The histopathological features of the infected corneas included epithelial ulceration, focal thickening, PMN infiltration, neovascularization, corneal thickening when compared with corneal section from control, BSA treated animals (**4B**). PMN cells (small arrows), and blood vessels (thick arrows) were present in the stroma. Few inflammatory cells (asterisks) were also observed under the corneal endothelium from control, BSA treated animals.

Fig. 5. MIP-133 induces up-regulation of CXCL2 in HCORN cells. HCORN cells were exposed to MIP-133 at indicated doses and times, and were then processed for total RNA isolation and RT-PCR analysis. The amount of mRNA expression was quantified by densitometry of bands in comparison to GAPDH (**5A**). Supernatants were collected from harvested cells and then processed for protein estimation by ELISA (**5B**). The data is mean $\pm$ SEM of three independent experiments. Asterisk indicates P values < 0.05 were obtained by repeated measures (subjects within groups) two-way analysis of variance (ANOVA) followed by Bonferroni post hoc test.

Fig. 6.

MIP-133 induces the up-regulation of chemokine CXCL2 and cPLA_{2α} inhibitors diminish its production in HCORN cells. Inhibition of cPLA_{2α} involved pre-incubating HCORN cells for 1 hr with cPLA_{2α} inhibitors (20 μM AACOCF3 or 10 μM MAFP) or an inactive control compound (20 μM AACOCH3) or DMSO (5 μL solvent of AACOCF3, MAFP, and AACOCH3), and then incubated with or without 15 μg/ml of MIP-133 for 24 hrs. MIP-2 production was examined by ELISA. The data shown represents mean ± SEM of three independent experiments (**P* < 0.05 and). *P* values were obtained by unpaired Student's *t*-test. AACOCF3, MAFP, AACOCH3, and DMSO without MIP-133 did not show significant effect on CXCL2 production when compared to untreated cells. The inactive control compound AACOCH3 did not block CXCL2 production induced by MIP-133.

Fig. 7.

Chinese hamsters ($n = 9/\text{group}$) were infected with parasite-laden contact lenses as described earlier. cPLA_{2α} inhibitors (AACOCF3 and CAY10650) 50 $\mu\text{g}/5\mu\text{l}$ was injected under the contact lens of an infected cornea three times a day for 6 days and topically on days 7 to 20 post-infection. As a control infected animals were treated with 50 $\mu\text{g}/5\mu\text{l}$ of BSA. Lenses were removed 7 days post-infection, and corneas were scored for clinical severity for the period indicated. Each graph line represents the mean $\pm$ SEM of clinical severity of nine animals for the observed time points. The results shown are representative of three separate experiments. Asterisk indicates P values < 0.05 compared with infected untreated group.

Fig. 8.

Photomicrograph of corneas from a Chinese hamster infected with *Acanthamoeba* trophozoites-laden contact lenses. cPLA_{2α} inhibitors (AACOCF3 and CAY10650) 50 μg/5 μl was injected under the contact lens of an infected cornea three times a day for 6 days and topically from 7 to 14 days post-infection. As a control infected animals were treated with 50 μg/5 μl of BSA. Lenses were removed 7 days post-infection. Animals were anesthetized and sacrificed 15 days after application of cPLA_{2α} inhibitors. (A) Cornea from control uninfected animal. (B) Cornea from infected animal, the corneal stroma of this group contained marked lamellar connective tissue disruption, extensive stroma swelling and neovascularized. Severe PMN cells and lymphocytes infiltration were present. (C) Cornea from infected animal treated with AACOCF3 had very mild inflammation. Which is similar to the corneal section from control, untreated animals. (D). Cornea from animals treated with CAY10650 had very mild inflammation and few PMN cells infiltration. PMN cells (small arrows), and blood vessels (thick arrows) were present in the corneal stroma. Few inflammatory cells (asterisks) were also observed under the corneal endothelium from control, BSA treated animals.