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Role of phospholipase A_2 (PLA_2) 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 $A_{2\alpha}$ ($cPLA_{2\alpha}$)-mediated pathway. The efficacy of $cPLA_{2\alpha}$ 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 $cPLA_{2\alpha}$ and macrophage inflammatory protein-2 (MIP-2/CXCL2) levels from corneal cells. Moreover, $cPLA_{2\alpha}$ inhibitors, MAFP (Methyl-arachidonyl fluorophosphonate) and AACOCF3 (Arachidonyl trifluoromethyl ketone), significantly reduce $cPLA_{2\alpha}$ and CXCL2 from these cells ($P < 0.05$). Additionally, $cPLA_{2\alpha}$ 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 $cPLA_{2\alpha}$ is involved in MIP-133 induced apoptosis of corneal epithelial cells, polymorphonuclear neutrophil infiltration, and production of CXCL2. Moreover, $cPLA_{2\alpha}$ inhibitors can be used as a therapeutic target in *Acanthamoeba* keratitis.

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., 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., 2003a,b; 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 $cPLA_2$ and arachidonic acid (AA) release (Kirschnek and Gulbins, 2006). Therefore, we hypothesized that $cPLA_2$ is a key mediator of apoptosis of corneal epithelial cells induced by MIP-133.

PLA_2 enzymes are divided into four major families: platelet-activating factor acetylhydrolases (PAF-AH); secreted PLA_2 ($sPLA_2$); Ca^{2+}-independent PLA_2 ($iPLA_2$); and cytosolic Ca^{2+}-dependent PLA_2 ($cPLA_2$). The $cPLA_2$ group includes α, β, γ, δ, ε, and ζ subclasses (Burke and Dennis, 2009; Taketo and Sonoshita, 2002). $cPLA_{2\alpha}$ is the only PLA_2 that exhibits specificity for hydrolysis of *sn*-2 arachidonic acid

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(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). cPLA$_2$ 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$_{2\alpha}$-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$_{2\alpha}$ signaling. We demonstrate that MIP-133 induced apoptosis of Chinese hamster corneal epithelial cells is associated with an increase in cPLA$_{2\alpha}$ activity and involves changes in the levels of cPLA$_{2\alpha}$, CXCL2, and neutrophil infiltration. In addition, *Acanthamoeba castellanii*-infected hamster eyes treated with cPLA$_{2\alpha}$ 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% CO_2 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., 2003a,b), 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 h. Inhibition of cPLA$_{2\alpha}$ was carried out by pre-incubating HCORN cells for 1 h with cPLA$_{2\alpha}$ 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 h. The inhibitors were dissolved in dimethyl sulfoxide (DMSO, a specific solvent of cPLA$_{2\alpha}$ inhibitors and inhibitor control), Fisher Bio-Reagents, Fair Lawn, New Jersey). Cells and supernatants were collected by centrifugation at 2000 × *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$_{2\alpha}$ 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$_{2\alpha}$ (450 bp) 5′-GAGTTTTGGGCGTTTCTGGT-3′ (sense), 5′-ACGGCAGGTTAAATGTGAGC-3′ (anti-sense); CXCL2 (285 bp) 5′-ACCCTGCCAAGGGTTGACTTC-3′ (sense), 5′-GGCACATCAGGTACGATCCAG-3′ (anti-sense); Chinese hamster GAPDH (333 bp) 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$_{2\alpha}$, CXCL2, and GAPDH gene products. All primers were from Integrated DNA Technologies, Inc (Commercial Park, Coralville, Iowa, USA).

2.6. cPLA$_{2\alpha}$ activity in cell lysates

HCORN cells were collected from 24 wells plates at the indicated times after treatments and then centrifuged at 2000 × *g* for 10 min 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$_{2\alpha}$ activity was measured using the PLA$_2$ substrate arachidonoyl thiophosphatidylcholine 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$_{2\alpha}$ 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 cPLA$_2$ assay kit and the protein