Experimental Eye Research 145 (2016) 10–16

Contents lists available at ScienceDirect

Experimental Eye Research

journal homepage: www.elsevier.com/locate/yexer

Research article

Molecular mechanism of fluoroquinolones modulation on corneal fibroblast motility

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ARTICLE INFO

Article history:
Received 26 May 2015
Received in revised form
20 October 2015
Accepted in revised form 22 October 2015
Available online 10 November 2015

Keywords:
Cell motility
Human corneal fibroblasts
FAK
Paxillin
Fluoroquinolone
Ciprofloxacin
Moxifloxacin
Levofloxacin

ABSTRACT

Topical fluoroquinolones are widely used to prevent ocular infections after ophthalmic surgery. However, they have been shown to affect the corneal cell motility, whose mechanism remains indefinite. The purpose of this study was to investigate how fluoroquinolones affect corneal stromal cell motility. Human corneal fibroblasts (HCFs) were incubated in ciprofloxacin (CIP), levofloxacin (LEV), or moxifloxacin (MOX) at 0, 10, 50, and 100 μg/ml for up to 3 days. Effect of CIP, LEV, or MOX on HCF migration was monitored using migration assay. HCF viability was determined by WST-1 assay. Expression of focal adhesion kinase (FAK), paxillin (PXN), and their phosphorylated forms were analyzed by immunoblotting. Binding affinity between FAK and PXN was determined by co-immunoprecipitation. Our results revealed that CIP and MOX, but not LEV, noticeably retarded HCF migration. HCF proliferation was significantly reduced by CIP (38.2%), LEV (29.5%), and MOX (21.3%), respectively ($p = 0.002$). CIP and MOX suppressed the phosphorylation of PXN at tyrosines (10.2 ± 4.3%, $p < 0.001$; 11.7 ± 2.4%, $p < 0.001$, respectively), including tyrosine 118 (33.3 ± 5.2%, $p < 0.001$; 34.0 ± 4.4%, $p < 0.001$, respectively). CIP and MOX diminished the binding affinity between FAK and PXN (8.2 ± 1.8%, $p < 0.001$; 9.0 ± 4.5%, $p < 0.001$, respectively). Nevertheless, tyrosine dephosphorylation and FAK dissociation of PXN were not found in LEV-treated HCFs. None of these fluoroquinolones affect phosphorylation of FAK-Y397. We conclude that CIP and MOX, but not LEV, might delay corneal fibroblast migration via interfering with recruitment of PXN to focal adhesions and dephosphorylation of PXN at the tyrosines.

1. Introduction

Cornea is the first barrier of the eye and its transparency is critical for a clear vision, the maintenance of which relies largely on corneal stromal cells. Corneal stromal cells loss is inevitable after laser corneal surgeries (Tomas-Juan et al., 2015) and infectious keratitis (Vemuganti et al., 2004). Cell migration from residual stroma contributes to the corneal stromal reformation via secreting growth factors and extracellular matrixes. Motility of the residual stromal cells is thus an important issue in wound healing. Special attention in topical medication selection is needed if they modulate corneal stromal cell migration and repopulation, particularly in cases with pre-existing stromal deficits and compromised ocular surface.

Topical fluoroquinolones, including ciprofloxacin (CIP), levofloxacin (LEV), and moxifloxacin (MOX), have been widely used in the management of ocular infections and prophylactically before and/or after ophthalmic surgery for the lower risk of ocular discomfort and chemical conjunctivitis (Chung et al., 2009). Confirming and limiting fluoroquinolones' effects on host corneal stromal cell migration is thus worthy of exploring. In addition to the well-known antimicrobial activity, fluoroquinolones have antifibrotic efficiency in the cultivated human corneal fibroblasts (HCFs) (Chen et al., 2013) and reduce corneal epithelial cell viability (Tsai et al., 2010). Fluoroquinolones are derived from non-fluorinated nalidixic acid and mainly inhibit two bacterial enzymes, bacterial topoisomerase IV and DNA gyrase, which are selective to bacteria (Pawar et al., 2013). The fluoroquinolones were developed with a broader spectrum of activity by modifying the basic molecular structure (Blondeau, 2004). CIP, a second

Abbreviations: HCFs, human corneal fibroblasts; dpi, day post incubation; FAK, focal adhesion kinase; PXN, paxillin; CIP, ciprofloxacin; MOX, moxifloxacin; LEV, levofloxacin.

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http://dx.doi.org/10.1016/j.exer.2015.10.018
0014-4835/

generation fluoroquinolone, demonstrates both activities against gram-negative and gram-positive pathogens and is still widely used in clinical practice since early 1990s. MOX, a fourth generation fluoroquinolone, improves activity against gram-positive cocci and also retains the potency of levofloxacin, a third generation fluoroquinolone, against gram-negative pathogen (O'Brien et al., 2007). In addition to their antimicrobial activities, fluoroquinolones could modify corneal fibroblast-to-myofibroblast differentiation and potentially reduce corneal scar formation when used adequately (Chen et al., 2013).

Fluoroquinolones have been shown to suppress cell migration in the isolated cells, such as CIP in rat tenocytes (Chang et al., 2012), gemifloxacin in human breast cells (Chen et al., 2014), and MOX in human corneal cells (Bezwada et al., 2008; Oum et al., 2014). As fluoroquinolones are commonly used prophylactically after corneal surgery and in the treatment of corneal ulcer (Sharma et al., 2013), understanding of how fluoroquinolones modify corneal fibroblast migration facilitates treatment selection and the clinical evaluation of treatment results. However, the molecular mechanisms of how fluoroquinolones suppress cell migration were not illustrated.

Recent studies demonstrated several important modulators in corneal cell migration include transglutaminase-2 (Tong et al., 2013) and vitronectin (Chow and Di Girolamo, 2014) in human corneal epithelial cells, intracellular β-actin-related protein (Joseph et al., 2014) and relaxin 2 (Hampel et al., 2012) in HCFs, fibroblast growth factor 2 (Lee and Heur, 2013) and Wnt5a (Lee and Heur, 2014) in human corneal endothelial cells. Turnover of the focal adhesion sites is required for transmission of intracellular motion signals to keep a steady direction of cell migration. Focal complexes are first formed by recruiting several intracellular molecules, including focal adhesion kinase (FAK), PXN, vinculin, and talin (Lawson and Schlaepfer, 2012). Among the recruited molecules, PXN is a critical factor at the cell leading edges by recruitment of active FAK to form a complete dynamic signaling for cell migration (Cheng et al., 2014; Hermann et al., 2015). In addition, autophosphorylation of FAK at tyrosine 397 (FAK-Y397) maintains its own activation in forming the lamellipodial structure (Lawson and Schlaepfer, 2012) and is a key central mediator of integrin signaling to recruit PXN at focal adhesions (Hu et al., 2014). The binding affinity between active FAK and PXN at the lamellipodial protrusion is important in forming complete FAK/PXN complexes for motility regulation (Hu et al., 2014; Le Devedec et al., 2012). However, whether fluoroquinolones modulate the corneal fibroblast migration via modulating the FAK/PXN pathway remains to be determined.

In patients with severe corneal ulcers, breakdown of stromal tissue may cause corneal melt or perforation. Proper antibiotics treatment following corneal wound healing including keratocyte motility are crucial. Herein, we focused on exploring whether and how fluoroquinolones interfere with these migration-related protein expressions. The results of our study will facilitate our understanding of the effects of topically applied fluoroquinolones on wound healing and the development of emerging topical medications.

2. Materials and methods

2.1. Human corneal fibroblasts (HCFs)

Following the tenets of the Declaration of Helsinki, HCFs were isolated from the residual donor cornea rims of the donors after the central corneas were used for penetrating keratoplasty surgery ($n = 6$). All included donor tissues aged between 20 and 70 years and met the inclusion and exclusion prior to participation in this study according to the rule in Eye Bank Association of America standards for human corneal transplantation (EBAA, 2012). Specifically, the inclusion criteria were donors aged between 20 and 70 years old of either sex, no previous ocular trauma and/or definite ocular diseases (such as Down Syndrome or infectious diseases). The exclusion criteria were donors aged less than 20 years old or older than 70 years old, pregnant women, death of unknown cause, the definite ocular diseases, biological dysfunction (such as immediate endothelial failure, donor corneal dystrophy, malignancy, or evidence suggestive of prior refractive surgery). This study was approved by the Institutional Review Board. In laboratory, the residual corneal rims were digested in collagenase (2 mg/ml; Roche Applied Science, Mannheim, Germany)-containing DMEM (Gibco, Grand Island, NY) supplemented with 4.5 mg/ml glucose (Dreier et al., 2013), 10% heat-inactive fetal bovine serum (FBS; Biological industries, Kibbutz Beit Haemek, Israel), 200 unit/ml penicillin G, and 200 μg/ml streptomycin sulfate (Sigma–Aldrich Co., St. Louis, MO) for about 6 h. After spun down as a pellet, the digested cells were resuspended and maintained in DMEM supplemented with 4.5 mg/ml glucose, 10% heat-inactive FBS, 100 unit/ml penicillin G, and 100 μg/ml streptomycin sulfate under a humidified atmosphere containing 5% CO_2 at 37 °C. The cultivated HCFs were used in passages 4 to 10 under DMEM supplemented with indispensable 10% FBS.

2.2. Incubation of fluoroquinolones

To analyze the effect of fluoroquinolones on HCF migration, HCFs were directly and continuously incubated in antibiotics-free DMEM supplemented with 4.5 mg/ml glucose, heat-inactive 10% FBS, and fluoroquinolones at 0, 10, 50, and 100 μg/ml, including CIP (Ciproxin®; Bayer Schering Pharma, Leverkusen AG, Germany), LEV (CRAVIT®; Sanofi-Aventis Deutschland GmbH, Frankfurt am Main, Germany), or MOX (VIGAMOX®; Alcon Laboratory Inc., Fort Worth, TX), until the analyzed time points as described in the following methods.

2.3. Migration assay

HCFs were seeded into each area of the ibidi Culture Inserts (ibidi GmbH, Martinsried, Germany) at 1.0×10^4 cells in antibiotics-free DMEM supplemented with 4.5 mg/ml glucose and heat-inactive 10% FBS according to our previous conditions with appropriate modifications (Chen et al., 2012). They were allowed to attach and grow to a confluence layer under a humidified atmosphere containing 5% CO_2 at 37 °C. The cells were incubated with or without CIP, LEV, or MOX. The migratory cells were directly monitored and captured using a Real-Time Cultured Monitoring System with Multiple-Point Imaging Capture (CCM-MULTI; ASTEC Co., Ltd., Fukuoka, Japan).

2.4. Viability

To determine cell growth rate of fluoroquinolone-incubated HCFs, the cells were evaluated by the formazan-based cell proliferation reagent WST-1 assay (Roche Applied Science, Mannheim, Germany), according to manufacturer's instructions and our previous conditions with appropriate modifications (Ho et al., 2014). Briefly, HCFs were cultured in each well of a 96-well plate and adjusted to 5.0×10^3 cells in 100 μl of culture medium. The medium was refreshed in normal culture medium, CIP-, LEV-, or MOX-containing medium for 0, 1, 2, or 3 days. At the analyzed time points, 10 μl of WST-1 reagent was directly added into each well and incubate at 37 °C for 2 h. Finally, the absorbance of all samples was determined at 450 nm against a reference at 690 nm with a spectrophotometer. Cell viability at the harvest time of the three