Experimental Eye Research 94 (2012) 136–145

Contents lists available at SciVerse ScienceDirect

Experimental Eye Research

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

Electrophilic PPARγ ligands inhibit corneal fibroblast to myofibroblast differentiation *in vitro*: A potentially novel therapy for corneal scarring

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ARTICLE INFO

Article history:
Received 22 March 2011
Accepted in revised form 27 November 2011
Available online 8 December 2011

Keywords:
corneal scarring
myofibroblast differentiation

ABSTRACT

A critical component of corneal scarring is the TGFβ-induced differentiation of corneal keratocytes into myofibroblasts. Inhibitors of this differentiation are potentially therapeutic for corneal scarring. In this study, we tested the relative effectiveness and mechanisms of action of two electrophilic peroxisome proliferator-activated receptor gamma (PPARγ) ligands: cyano-3,12-dioxolean-1,9-dien-28-oic acid-methyl ester (CDDO-Me) and 15-deoxy-$\Delta^{-12,14}$-prostaglandin J_2 (15d-PGJ_2) for inhibiting TGFβ-induced myofibroblast differentiation *in vitro*. TGFβ was used to induce myofibroblast differentiation in cultured, primary human corneal fibroblasts. CDDO-Me and 15d-PGJ_2 were added to cultures to test their ability to inhibit this process. Myofibroblast differentiation was assessed by measuring the expression of myofibroblast-specific proteins (αSMA, collagen I, and fibronectin) and mRNA (αSMA and collagen III). The role of PPARγ in the inhibition of myofibroblast differentiation by these agents was tested in genetically and pharmacologically manipulated cells. Finally, we assayed the importance of electrophilicity in the actions of these agents on TGFβ-induced αSMA expression via Western blotting and immunofluorescence. Both electrophilic PPARγ ligands (CDDO-Me and 15d-PGJ_2) potently inhibited TGFβ-induced myofibroblast differentiation, but PPARγ was only partially required for inhibition of myofibroblast differentiation by either agent. Electrophilic PPARγ ligands were able to inhibit myofibroblast differentiation more potently than non-electrophilic PPARγ ligands, suggesting an important role of electrophilicity in this process. CDDO-Me and 15d-PGJ_2 are strong inhibitors of TGFβ-induced corneal fibroblast to myofibroblast differentiation *in vitro*, suggesting this class of agents as potential novel therapies for corneal scarring warranting further study in pre-clinical animal models.

1. Introduction

Corneal scarring, the second most common cause of blindness in the world, can be due to ocular infections (Whitcher et al., 2001), trauma (Farjo et al., 2009), and surgeries (Marchini et al., 2006; Saini et al., 2003; Wilson et al., 2001). Safe and efficacious medical therapies for the treatment of corneal scarring are limited.

Keratocytes are transparent cells of the corneal stroma that are involved in corneal wound healing and scarring (Fini and Stramer, 2005; Fini, 1999; Jalbert et al., 2003; Muller et al., 1995). Corneal wounds result in the release of several cytokines and chemokines that drive keratocyte activation, migration, and differentiation into fibroblasts and myofibroblasts (West-Mays and Dwivedi, 2006). Corneal myofibroblasts provide wound contraction and produce extracellular matrix molecules (ECMs), such as Type I and III collagen (Funderburgh et al., 2001) and fibronectin (FN) (Welch et al., 1990), which are important in regenerative wound repair (Jester et al., 1995; West-Mays and Dwivedi, 2006). However, myofibroblasts also contribute to corneal haze and corneal shape change through decreased crystallin production (Jester et al., 1999; Jester, 2008) and excessive and disordered production of collagen and other extracellular matrix molecules (Marchini et al., 2006; Netto et al., 2006; Pal-Ghosh et al., 2004; Saini et al., 2003; Sosne et al., 2002; Wilson et al., 2001). Both haze and changes in corneal shape decrease visual acuity (Marchini et al., 2006; Netto et al., 2006; Pal-Ghosh

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doi:10.1016/j.exer.2011.11.018

et al., 2004; Saini et al., 2003; Sosne et al., 2002; West-Mays and Dwivedi, 2006; Wilson et al., 2001).

Differentiation of keratocytes to myofibroblasts *in vitro* and *in vivo* is driven primarily by transforming growth factor beta (TGFβ) (Jester et al., 1996), a cytokine released by corneal epithelial cells, corneal fibroblasts, the lacrimal gland, and conjunctival cells under inflammatory conditions (Buehren et al., 2008; Wilson et al., 1992). Netto et al. (2006) demonstrated that by damaging the corneal epithelial basement membrane, penetrating keratectomy in mice causes the release of TGFβ into the corneal stroma, stimulating keratocyte to myofibroblast differentiation. While factors other than TGFβ are involved in the corneal scarring phenomena, including platelet-derived growth factor (PDGF) (Kaur et al., 2009a, 2009b) and integrin signaling (Jester et al., 2002), pharmaceutical inhibitors of TGFβ have been shown to decrease myofibroblast differentiation and corneal opacification in several animal models of corneal scarring (Buehren et al., 2008; Moller-Pedersen et al., 1998; Jester et al., 1997).

PPARγ ligands have anti-fibrotic properties and have been studied as agents capable of inhibiting TGFβ-induced myofibroblast differentiation in different tissues (Ferguson et al., 2009; Galli et al., 2002; Kawai et al., 2009), including the cornea (Pan et al., 2009, 2010). PPARγ gene transfer decreased corneal opacification in an alkali burn mouse model of corneal scarring (Saika et al., 2007). A non-electrophilic PPARγ ligand, pioglitazone, was found to inhibit TGFβ-induced keratocyte to myofibroblast differentiation *in vitro* (Pan et al., 2010). However, a recent *in vitro* study found two electrophilic peroxisome proliferator-activated receptor gamma (PPARγ) ligands, cyano-3,12-dioxolean-1,9-dien-28-oic acid (CDDO) and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2), to inhibit TGFβ-induced lung myofibroblast differentiation more potently than non-electrophilic PPARγ ligands (Ferguson et al., 2009). 15d-PGJ_2 (Kliewer et al., 1995), CDDO, and CDDO derivatives bind PPARγ with high affinity (Wang et al., 2000). Once a PPARγ ligand binds to PPARγ, the latter forms a heterodimer with the retinoid X receptor (RXR) and its ligand (Ferguson et al., 2009; Liby et al., 2007; Rizzo and Fiorucci, 2006). The heterodimer then translocates to the nucleus and interacts with PPAR response elements (PPRE), leading to PPARγ-induced gene transcription (Ferguson et al., 2009; Liby et al., 2007; Rizzo and Fiorucci, 2006). Electrophilic PPARγ ligands have α/β-unsaturated ketone rings with electrophilic carbons (Brookes et al., 2007; Ferguson et al., 2009; Shi and Greaney, 2005). These electrophilic carbons are highly susceptible to Michael addition reactions (Shi and Greaney, 2005), which enables them to form covalent bonds with intracellular nucleophiles, such as thiol groups or cysteine residues (Brookes et al., 2007; Chintharlapalli et al., 2005; Ferguson et al., 2009; Ray et al., 2006; Straus et al., 2000; Suh et al., 1999). For this reason, electrophilic PPARγ ligands, such as CDDO (Brookes et al., 2007; Chintharlapalli et al., 2005; Ferguson et al., 2009; Suh et al., 1999) and 15d-PGJ_2 (Ray et al., 2006; Straus et al., 2000), can also exert PPARγ-independent effects.

The present experiments examine the ability of two electrophilic PPARγ ligands, a CDDO derivative, CDDO-methyl ester (-Me), and 15d-PGJ_2 to inhibit differentiation of corneal fibroblasts to myofibroblasts *in vitro*. The anti-scarring properties of these agents have been studied in fibroblasts in the lung (Ferguson et al., 2009). However, due to the heterogeneity of fibroblasts between tissues (Smith et al., 2002), this study is an essential first step towards assessing the potential effectiveness of these molecules for treating corneal scarring. CDDO in particular has already been used systemically in Phase I and II clinical trials as a chemotherapeutic agent (Dezube et al., 2007). Since it was shown to have a favorable safety profile when administered systemically in humans (Dezube et al., 2007), this significantly increases its translational potential for a topical application such as eye drops in the treatment of corneal scarring.

2. Methods

2.1. Cells and reagents

Human corneal fibroblast cell strains were derived from anatomically normal donor rim corneal tissue donated to the Rochester/Finger Lakes Eye and Tissue Bank. Human tissue was obtained and handled in accordance with the tenets of the Declaration of Helsinki. The cells derived from these explants were isolated and then cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Sigma Aldrich, St. Louis, MO), gentamicin (0.05 mg/ml; Invitrogen, Carlsbad, CA), amphotericin (0.25 mg/ml; Invitrogen), and 25 mM HEPES at pH 7.4 at 37 °C in 7% CO_2. The cells were morphologically consistent with fibroblasts, expressing vimentin, but not CD45, factor VIII, or cytokeratin. Cells from three different patient strains were used at passages 4–10. The PPARγ ligands, 15d-PGJ_2 (Biomol, Plymouth Meeting, PA), 9,10-dihydro-15-deoxy-$\Delta^{12,14}$-PGJ_2 (CAY10410; Cayman Pharmaceuticals, Ann Arbor, MI), rosiglitazone (Cayman Pharmaceuticals), CDDO-Me (a kind gift from Dr. Michael Sporn, Dartmouth University), were prepared as 10 mM stocks in dimethyl sulfoxide (DMSO) and added to cell cultures to the final concentration indicated. GW9662 (Cayman Pharmaceuticals), an irreversible, small-molecule PPARγ antagonist, was prepared in the same manner. This molecule has been used by us (Burgess et al., 2005; Feldon et al., 2006; Ferguson et al., 2009) and others (Fu et al., 2001; Mix et al., 2004) and been shown to block PPARγ activity. Recombinant human TGFβ1 (#240B) was purchased from R&D systems (Minneapolis, MN).

2.2. Cell viability assay

The calcein AM/EthD-I Live/Dead Viability/Cytotoxicity Kit (Molecular Probes, Eugene, OR) was used to assess cell viability. Calcein AM specifically stains live cells via their intracellular esterase activity, and EthD-I specifically stains dead cells that have lost plasma membrane integrity. Corneal fibroblasts were plated in 25 ml flasks (Greiner Bio-One) in 10% FBS media and were either untreated, treated with TGFβ alone, or with PPARγ ligands with or without TGFβ for 72 h. PPARγ ligands were added 30 min prior to TGFβ. Prior to harvesting the cells, one of corneal fibroblast samples were killed using 0.1% saponin treatment for 15 min. At harvest, cells were trypsinized and washed with PBS twice and then stained with 1 μM calcein AM and 8 μM EthD-1 for 10 min. Samples were analyzed on a FACSCanto II (BD Biosciences). The live cell gate was set based on cells staining with calcein AM in the untreated corneal fibroblast culture. The dead cell gate was set based on cells staining with EthD-1 in the 0.1% saponin treated culture. Treated samples were analyzed using these gates to assess percent of live and dead cells.

2.3. Western blots for αSMA, fibronectin, collagen I

Primary human corneal fibroblasts were plated in 60 or 100 mm dishes (Falcon/Becton Dickson, Franklin Lakes, NJ) or 6 well plates (Falcon/Becton) in 10% FBS media and were either untreated, treated with TGFβ alone, or treated with PPARγ ligands with or without TGFβ for 72 h. PPARγ ligands were added 30 min prior to TGFβ. For certain experiments, the fibroblasts were infected with an adenovirus vector expressing a dominant negative PPARγ or an empty vector for 24 h before treatment with TGFβ and PPARγ ligands. In other experiments, the fibroblasts were treated with