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Research article

The effects of ripasudil (K-115), a Rho kinase inhibitor, on activation of human conjunctival fibroblasts

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ABSTRACT

The most common cause of glaucoma surgery failure is scar formation induced by activation of wound-healing responses and resultant fibrosis at the surgical site. We investigated the effects of ripasudil, a Rho kinase inhibitor, on activation of human conjunctival fibroblasts (HConF). HConF were pretreated with different concentrations of ripasudil for 1 h before addition of transforming growth factor (TGF)-β2, followed by incubation for 48 h. TGF-β2-treated fibroblasts exhibited a significant increase in expression of α-smooth muscle actin (α-SMA), a marker of fibroblast-to-myofibroblast differentiation, and this increase was significantly suppressed, in a dose-dependent manner, by pretreatment with ripasudil. Ripasudil pretreatment also significantly attenuated TGF-β2-induced fibronectin production and collagen gel contraction. TGF-β2 increased both the number of viable cells and the number of cells in the G2/M phase of the cell cycle; these effects were attenuated by pretreatment with ripasudil. In addition, we explored the effects of ripasudil on stimulation of HConF by activated macrophages. Human monocytic cell line THP-1 cells were differentiated into M1 or M2 macrophage-like cells, and HConF were treated with conditioned media derived from these macrophages in the presence or absence of ripasudil. Conditioned medium from M2 macrophage-like cells induced a significant increase in α-SMA expression, viable cell numbers, and gel contraction, all of which were significantly suppressed by ripasudil. Thus, overall, ripasudil attenuated activation of human conjunctival fibroblasts. Ripasudil may be of therapeutic utility, preventing excessive scarring after glaucoma filtration surgery.

1. Introduction

Glaucoma is the second leading global cause of blindness (Quigley and Broman, 2006) and filtering surgery is currently one of the most effective treatments to lower intraocular pressure (IOP) (Desai et al., 2011). The most common cause of filtering surgery failure is scarring of the surgical field (Khaw et al., 2001; Chang et al., 2000). Although antimetabolites remarkably improve surgical outcomes, these materials are associated with high incidences of vision-threatening complications including late-onset infection and hypotonic maculopathy (Chang et al., 2000). Currently, no postoperative medication directly targets fibrosis, and the development of safe and effective therapeutic modalities inhibiting fibrosis development after filtration surgery is an important target of glaucoma research. Throughout wound healing, fibroblasts are activated by a myriad of peptides including cytokines and growth factors, among which are transforming growth factor (TGF)-β, platelet-derived growth factor (PDGF), connective tissue growth factor (CTGF), fibroblast growth factor (FGF), a variety of interleukins, and members of the matrix metalloproteinase (MMP). The inflammatory mediators are produced principally by activated inflammatory cells (including macrophages, mast cells, lymphocytes, and leukocytes) recruited to the surgical site. Transforming growth factor (TGF)-β is one of the central mediators (Cordeiro, 2002) that plays critical roles in wound healing through SMAD-dependent and SMAD-independent signaling pathways. Activated fibroblasts differentiate into myofibroblasts, marked by de novo expression of α-smooth muscle actin (α-SMA), development of a high-level contractile capacity, and abundant extracellular matrix (ECM) production (Wynn, 2008).

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It has been hypothesized that preoperative topical antiglaucoma medications are risk factors for surgical failure (Broadway et al., 1994; Berthold and Pfeiffer, 2006). Several studies have investigated the effects of antiglaucoma drugs on subconjunctival fibroblasts. Prostaglandin $F_{2\alpha}$ analogs, which are currently the most widely prescribed first-line treatments, increased proliferation of rabbit subconjunctival fibroblasts (Lark et al., 1999) and induced collagen gel contraction by human Tenon fibroblasts (Liu et al., 2008). Also, prolonged use of antiglaucoma medications, including timolol, epinephrine, and pilocarpine, induced recruitment of inflammatory cells (including macrophages) and increased fibroblast numbers in the conjunctiva (Young et al., 1990; Sherwood et al., 1989). These observations suggest that antiglaucoma drugs may negatively influence subconjunctival wound healing by enhancing the proliferation, contractility, and inflammatory activities of fibroblasts.

Rho, a Ras homolog of small GTPase, modulates formation of the actin cytoskeleton (Narumiya, 1996), and Rho-associated kinase (ROCK) inhibitors potently lower IOP by decreasing the outflow resistance via the trabecular meshwork and Schlemm's canal cells (Honjo et al., 2001; Rao et al., 2001; Koga et al., 2006; Kameda et al., 2012). Ripasudil (K-115), a selective ROCK inhibitor, is a promising emerging antiglaucoma drug, and was thus approved by the Japanese administrative authority in 2014. Although ROCK inhibitors exhibit antifibrotic effects in various cells of other organs, including the lung, kidney, and heart (Shimizu et al., 2001; Nagatoya et al., 2002; Hartmann et al., 2015), the effects of the drug on the conjunctiva remain poorly understood. Therefore, in this study, we explored the effects of ripasudil on the activation of human conjunctival fibroblasts (HConF), such as differentiation into myofibroblasts, increased ECM production, and enhanced cell contractility; the effects of ripasudil on the TGF-β-induced proliferative response, and associated alterations in the cell cycle, of conjunctival fibroblasts; and whether ripasudil inhibited activation of conjunctival fibroblasts induced by conditioned media from cultures of two subtypes of activated macrophages.

2. Materials and methods

2.1. Cell culture and treatment

Two lots (lot numbers 3072 and 5965) of primary HConF were obtained from ScienCell Research Laboratories (Carlsbad, CA). According to the manufacturer, HConF were isolated from conjunctiva of human eyes, dissected, and digested with enzymes. HConF cultures were grown in Fibroblast Medium (ScienCell) supplemented with 5% fetal bovine serum (FBS; ScienCell), fibroblast growth supplement (FGS; ScienCell), and Penicillin (100 U/ml)/Streptomycin (100 μg/mL) solution (ScienCell). Cells at passage 6 or 7 were used in all experiments. The morphology and markers of fibroblasts (fibroblast-specific protein 1 (FSP1); 1:200; Abcam, Cambridge, UK) and vimentin (1:250; Abcam) were assessed to confirm that cells were not transformed during the passages (data not shown). The medium was changed to serum-free Dulbecco's modified Eagle's medium (DMEM; WAKO Pure Chemical Industries, Osaka, Japan) for 24 h prior to each treatment. To trigger myofibroblast differentiation, HConF were treated with 5 ng/mL TGF-β2 (R&D Systems, Minneapolis, MN) for 48 h. Prior to addition of TGF-β2, the cells were treated with different concentrations of ripasudil (K-115; 0, 25, or 50 μM) (Kowa Company, Ltd, Nagoya, Japan). The human monocytic cell line THP-1 (JCRB0112.1) was purchased from the JCRB Cell Bank (Osaka, Japan) and cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Thermo Fisher Scientific, Waltham, MA) supplemented with 5% FBS (Sigma-Aldrich, St. Louis, MO) and penicillin (100 U/ml)/streptomycin (100 μg/mL) (Thermo Fisher Scientific). THP-1 cells were differentiated and polarized using the classical stimuli (Tjiu et al., 2009; Ishimoto et al., 2014), with minor modifications. Briefly, THP-1 cells were treated with 320 nM phorbol myristate acetate (PMA; Sigma-Aldrich) for 36 h. Subsequently, for macrophage M1 polarization, PMA-treated cells were additionally treated for 12 h with 100 ng/mL lipopolysaccharide (LPS; InvivoGen, San Diego, CA) and 20 ng/mL interferon (IFN)-γ (R&D). For M2 polarization, PMA-treated cells were additionally treated for 12 h with 20 ng/mL interleukin (IL)-4 (R&D) and 20 ng/mL IL-13 (R&D). The PMA-containing media were removed and the cells were washed three times with phosphate-buffered saline (PBS). Conditioned medium, based on RPMI 1640, was collected after 24 h of culture of serum-starved M1 or M2 macrophage-like cells and added to HConF in the presence (or absence) of 50 μM ripasudil. We measured cytokine production by M1- and M2-polarized macrophage-like cells using high-sensitivity Luminex® bead-based multiplex screening assays (R&D), and confirmed that the cells exhibited the distinct profiles described in a previous report (Ishimoto et al., 2014) (data not shown).

2.2. Western blotting

Cells were lysed and the lysates were treated as previously described (Fujimoto et al., 2012). Equal amounts of total protein were subjected to SDS-PAGE in Novex Bis-Tris protein gels or Tris-acetate protein gels (Life Technologies), and transferred to polyvinylidene difluoride (PVDF) membranes (GE Healthcare, Little Chalfont, Buckinghamshire, UK). After blocking, the membranes were probed with primary antibodies, and finally with appropriate secondary antibodies. The primary antibodies used were anti-α-SMA (1:1000; Sigma-Aldrich), anti-fibronectin (1:1000; Abcam), and anti-type I collagen (1:1000; Abcam). The secondary antibodies were horseradish peroxidase (HRP)-linked anti-rabbit (1:2500; Cell Signaling, Danvers, MA) and sheep anti-mouse (1:20,000; GE Healthcare) antibodies. The ECL-prime Western blotting detection reagent (GE Healthcare) was used to visualize protein bands. Digital images were obtained using a luminescent image analyzer (LAS-4000; Fujifilm, Tokyo, Japan). The band densities of each sample were normalized to that of β-actin.

2.3. Immunocytochemistry

Immunocytochemistry was performed as described previously (Fujimoto et al., 2012) with minor modifications. Briefly, cells were fixed, permeabilized, and incubated with anti-α-SMA antibody (1:400; Sigma-Aldrich) or anti-phospho-Histone H3 (Ser10) antibody (1:1500; Cell Signaling). The cells were then probed with a goat anti-mouse IgG secondary antibody conjugated with Alexa Fluor 488 (1:1000; Thermo Fisher Scientific) or a similarly conjugated goat anti-rabbit IgG secondary antibody (1:1000; Thermo Fischer Scientific). Phalloidin-TRITC (1:200; Sigma-Aldrich) was used to stain the F-actin cytoskeleton. Cells were counterstained with DAPI or Hoechst 33342 (Dojindo Laboratories, Kumamoto, Japan) and observed under a fluorescence microscope (model BZ 700; Keyence, Osaka, Japan).

2.4. Collagen gel contraction assay

We used an *in vitro* collagen contraction model to measure contractile activity. The assay was performed as described previously (Koga et al., 2006; Nakamura et al., 2002), with minor modifications. Briefly, cultured HConF (1.1×10^6 cells/mL) were added to a mixture of type I collagen (Nitta-Gelatin, Osaka, Japan), 10 × DMEM (Sigma-Aldrich) and reconstitution buffer (Nitta-Gelatin). Aliquots of the mixture were poured into wells of a bovine