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Resolvin E1 analog RX-10045 0.1% reduces corneal stromal haze in rabbits when applied topically after PRK

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Purpose: To perform a masked study to determine whether resolvin E1 (RvE1), a lipid-derived immunomodulator, could regulate the development of corneal haze and opacity-related myofibroblasts after opacity-generating high correction photorefractive keratectomy (PRK) in rabbits.

Methods: Three groups of eight rabbits each were included in the study. Nine diopter (D) PRK for myopia was performed in each test cornea, and the eyes were treated with 30 µl of topical solution every 4 h (six times a day) for 5 days starting immediately after PRK. Group 1 was treated with 0.1% RX-10045, a prodrug of an RvE1 analog; group 2 was treated with 0.01% RX-10045; and group 3 was treated with vehicle control solution. At 1 month after PRK, haze was graded at the slit-lamp by a masked observer. Immunohistochemistry for α-smooth muscle actin (SMA) was performed on the central cornea of each test eye to determine the anterior stromal myofibroblast density.

Results: Corneal opacity was significantly lower in the 0.1% RX-10045 group, but not the 0.01% RX-10045 group, compared to the vehicle control group (p=0.029), at 1 month after −9.0D PRK. At 1 month after −9.0D PRK, SMA+ myofibroblast densities in the anterior stroma were not statistically significantly different among the three groups, although a trend toward lower myofibroblast generation was noted in the 0.1% RX-10045 group.

Conclusions: Topical 0.1% RX-10045, a prodrug of an RvE1 analog, reduces corneal opacity after haze-generating PRK in rabbits. Further studies are needed to determine the precise points at which RvE1 decreases corneal opacity after injury.

Corneal injury, surgery, or infection often results in the loss of stromal transparency [1]. Corneal myofibroblast generation associated with a decrease in the expression of corneal crystallins by these cells and production of abnormal extracellular matrix have been identified as important biologic events that lead to corneal opacity (clinically referred to as haze) during corneal wound healing [2-4].

Myofibroblasts are fibroblastic cells that are generated from keratocyte-derived and bone marrow–derived precursor cells [5-7]. Epithelial–stromal interactions modulate the generation of corneal myofibroblasts and the development of stromal opacity [8]. Myofibroblast development and persistence appear to occur when structural and functional defects in the regenerated epithelial basement membrane facilitate the penetration of transforming growth factor beta (TGF-β) and platelet-derived growth factor (PDGF) from the epithelium into the anterior stroma at sufficient levels required for ongoing receptor activation in precursor cells [4,9,10]. TGF-β promotes myofibroblast development [11] and suppresses interleukin (IL)-1-mediated apoptosis of myofibroblasts and their precursors [9,12].

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Resolvins belong to a novel class of lipid-derived endogenous molecules that have potent immunomodulatory properties and have been shown to regulate the resolution phase of an active immune response [13,14]. These modulators are derived from omega-3 polyunsaturated fatty acids (PUFA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), and can be categorized in E-series (from EPA) or D-series (from DHA) [13,15]. Resolvin E1 was the first resolvin described in inflammatory exudates of acute inflammation in mice (5,12,18R-trihydroxy-EPA) [16]. Since then, resolvin E1 has been identified as a critical anti-inflammatory mediator in humans [17] and rabbits [18]. In 2005, the molecule's complete stereochemical assignment was established as 5S,12R,18R-trihydroxy-6Z,8E,10E,14Z,16E-EPA [17].

Some RvE1 effects are related to decreased polymorphonuclear neutrophil infiltration [14], increased macrophage phagocytosis of apoptotic neutrophils, and inhibition of the host tissue inflammatory response [19]. Resolvins have been shown to be effective in a range of experimental models of inflammatory diseases, including pneumonitis [20], colitis [21], and periodontitis [18], as well as eye disorders such as retinal angiogenesis [22], dry eye [23], and herpes simplex virus-induced ocular inflammation [24]. In addition, RvE1 was investigated in a human corneal epithelial cell wound model in vitro and was found to promote wound closure and to reduce cytokine and chemokine release [25]. The purpose

of this current study was to evaluate the effect of a novel topical formulation of a resolvin E1 analog [RX-10045; (5S,8E,10E,12R)-isopropyl 5,12-hydroxypentadeca-8,10-dien-6,14-diynoate] on haze and myofibroblast generation after haze-producing corneal injury (photorefractive keratectomy; PRK) in rabbits since prior studies have suggested resolvins can reduce myofibroblast generation.

METHODS

Resolvin RX-10045: RX-10045 is an isopropyl ester prodrug of the resolvin E1 analog, RX-10008. The prodrug (RX-10045) very rapidly hydrolyzes to its active acid form (RX-10008) in biologic matrices (data on file at Auven Therapeutics). RX-10045 was formulated at a concentration of 0.01% and 0.1% in a novel biocompatible preservative-free vehicle that contains mixed polymeric micelles comprising hydrogenated castor oil-40 and octoxynol-40 to help keep RX-10045 in aqueous solution. The 0.1% concentration was the highest dosage possible without using components not on the Food and Drug Administration list of approved components while maintaining the stability of the drug. The vehicle solution contained mixed polymeric micelles that contained no drug. Other additives included common excipients to maintain desirable tonicity, osmolality, and a buffering capacity of around pH 5.5. Prepared as an aqueous nanomicellar formulation, the solution is intended to increase the ocular and periocular tissue concentrations of the drug (RX-10008) after ocular administration. Vials of formulated RX-10045 and vehicle were shipped in masked fashion by Auven Therapeutics (New York, NY) and stored at 4 °C until use.

Animals, surgery, and drug application groups: All animals were treated in accordance with the tenets of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the Animal Control Committee at the Cleveland Clinic approved these studies. Anesthesia was achieved with intramuscular injection of ketamine hydrochloride (30 mg/kg) and xylazine hydrochloride (5 mg/kg). In addition, topical proparacaine hydrochloride 1% (Alcon, Fort Worth, TX) was applied to each eye just before surgery.

Twenty-four 12- to 15-week-old female New Zealand white rabbits weighing 2.5–3.0 kg each were included in this study. Haze-generating injury was performed in one cornea of each rabbit with a 9 mm diameter epithelial scrape and application of −9.0D PRK with a VISX S4 IR excimer laser (AMO, Irvine, CA), as previously described [3]. One eye of each rabbit selected at random was treated with masked aliquots of the test solutions. The key to masking was held by a neutral party and was not disclosed to the investigators until the haze and myofibroblast density data were finalized and tabulated for all rabbits. The three groups with eight rabbits each were as follows: Group 1 received 30 µl of 0.1% RX-10045 per application. Group 2 received 30 µl of 0.01% RX-10045 per application. Group 3 received 30 µl of vehicle solution per application.

All drops in each group were given with a Pipetman (Gilson, Inc., Middleton, WI) with sterile tips every 4 h beginning immediately after PRK surgery and for 5 days (period of time that the drug can reach the corneal stroma without blockage by the corneal epithelial barrier function). The corneal epithelium had closed before the last application of the test drug in all rabbits. All rabbits were provided with acetaminophen in drinking water at a concentration of 6 mg/ml post-procedure until the epithelium healed at 3 to 5 days after surgery.

Biomicroscopic grading of corneal haze: Treated animal corneas were evaluated at the slit-lamp (Haag-Streit BM900, Haag-Streit, Switzerland) while under general anesthesia and photographed at 1 month after PRK surgery. Corneal opacity (haze) was graded according to the system reported by Fantes et al. [26]: grade 0 for a completely clear cornea, grade 0.5 for trace haze seen with careful oblique illumination with slit-lamp biomicroscopy, grade 1 for more prominent haze not interfering with the visibility of fine iris details, grade 2 for mild obscuration of iris details, grade 3 for moderate obscuration of the iris and lens, and grade 4 for complete opacification of the stroma in the area of the ablation.

Cornea collection, fixation, and sectioning: Rabbits were euthanized at 1 month after PRK, the point of the peak of myofibroblast generation and haze formation in rabbits after PRK [3], with an intravenous Beuthanasia overdose (100 mg/kg), and the corneoscleral rim was collected without manipulation of the cornea using 0.12 forceps and sharp Westcott scissors. The corneoscleral rims were immediately embedded in liquid optimum cutting temperature (OCT) compound (Sakura Finetek, Torrance, CA) within a 24 mm × 24 mm × 5 mm mold (Fisher, Pittsburgh, PA). Specimens were centered within the mold so that the block could be bisected and transverse sections cut from the center of the cornea. Frozen tissue blocks were stored at −80 °C until sectioning was performed. Central corneal sections (7 µm thick) were cut with a cryostat (HM 505M, Micron GmbH, Walldorf, Germany). Sections were placed on 25 mm × 75 mm × 1 mm microscope slides (Superfrost Plus, Fisher) and maintained frozen at −80 °C until staining was performed.

Immunohistochemistry: Immunohistochemical staining was performed on tissue sections as previously described [6]. Briefly, sections from the central cornea were stained for α-smooth muscle actin (α-SMA) using a monoclonal mouse

Table 1. Masked corneal opacity grading at the slit lamp at 4 weeks after surgery

Grade	Group 1 0.1% Resolvin	Group 2 0.01% Resolvin	Group 3 Vehicle Control
Grade 0			
Grade 0.5	3	1	
Grade 1	2	2	1
Grade 2	2	3	4
Grade 3	1	2	3
Grade 4			
Mean ± SD	0.9±0.9	1.8±0.9	2.3±0.7

Each value represents the number of corneas in the group with the indicated level of opacity graded by the method of Fantes et al., 1990.

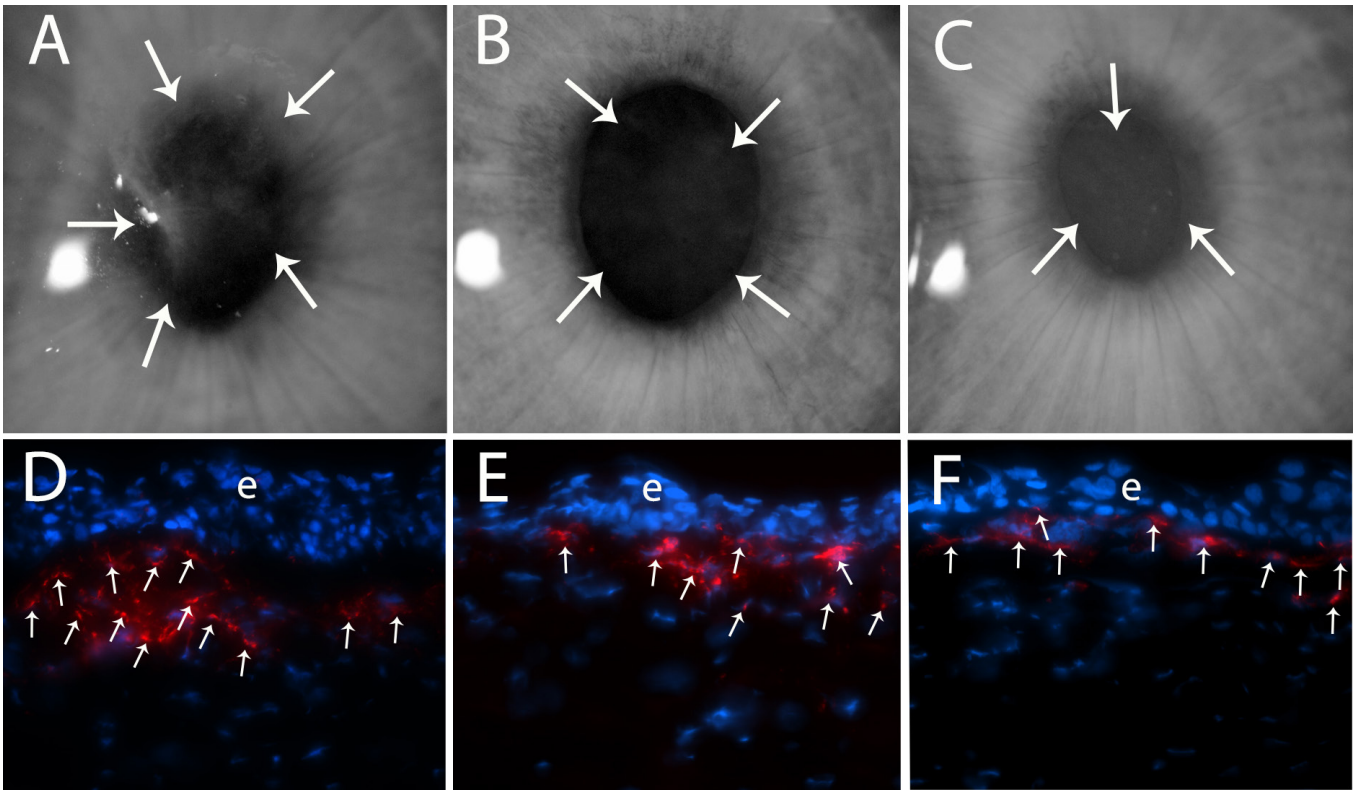

Figure 1. Representative slit-lamp photographs of sub-epithelial haze and immunohistochemistry for myofibroblast marker alpha-smooth muscle actin in rabbits. **A**–**C**: Representative slit-lamp photographs of subepithelial haze in rabbits. **A**: Moderate to severe (grade 3) haze (arrows) was noted in a vehicle control group cornea. **B**: Grade 2 subepithelial haze was observed in a rabbit eye that was treated with 0.01% resolvin E1. **C**: Faint subepithelial grade 0.5 haze was seen in a rabbit treated with 0.1% resolvin E1. Magnification 10X. **D**–**F**: Representative images of immunohistochemical staining for the α-smooth muscle actin (SMA) marker for myofibroblasts in the central cornea of rabbit eyes in groups treated with (**D**) vehicle control solution, (**E**) 0.01% resolvin E1, and (**F**) 0.1% resolvin E1 at 4 weeks after −9D PRK. Cell nuclei are stained blue with 4',6-diamidino-2-phenylindole (DAPI) and SMA+ cells are stained red (arrows). e=epithelium. Magnification 400X.

Table 2. Alpha-smooth muscle actin+ myofibroblast density in the anterior stroma (SMA+/400X field) at 1 month after surgery.

Rabbit	Group 1 0.1% Resolvin	Group 2 0.01% Resolvin	Group 3 Vehicle Control
1	7	11	25
2	1	6	14
3	0	27	15
4	1	49	29
5	10	32	19
6	52	10	0
7	14	33	50
8	28	1	9
Mean ± SD	14±18	21±17	20±15

Each value represents the average of seven non-overlapping 400X fields for each cornea. The mean ± the standard deviation is provided for each group.

anti-human smooth muscle actin clone1A4 (Cat. # M0851, Dako, Carpinteria, CA). The antibody was used on the sections at 1:50 dilution in 1% bovine serum albumin (BSA) and incubated at room temperature for 90 min. Sections were washed with 1X PBS (160 mM NaCl, 7.6 mM Na_2HPO_4, 2 mM NaH_2PO_4, pH 7.4) and then incubated at room temperature for 60 min in Alexa Fluor 568 (Cat. # A11031, Invitrogen, Carlsbad, CA) secondary antibody and goat anti-mouse immunoglobulin G (IgG; H + L; Red) diluted 1:100 in PBS before washing with PBS three times. Immunocytochemical controls were performed by substituting mouse non-specific IgG1 for the primary antibody. Coverslips were mounted with Vectashield containing 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories Inc., Burlingame, CA) to allow visualization of all nuclei in the tissue sections. The sections were viewed and photographed with a Leica DM5000 microscope equipped with Q-Imaging Retiga 4000RV (Surrey, Canada) camera and ImagePro software. Immunohistochemistry (IHC) was performed at least three times on sections from each cornea to ensure consistent results.

Quantification of cells: SMA+ cells were counted real-time under a microscope to delineate individual cells for accurate quantification and not from photographic images. SMA+ myofibroblast densities were performed by counting the number of cells per 400X field tangent to the epithelial basement membrane in the central cornea and averaging the counts for seven non-overlapping fields—as previously described [4]. Each count was the mean of values from five separate sections from each treated cornea.

Statistical analysis: Data processing and analysis were performed with SPSS version 20.0 software (SPSS, Inc., Chicago, IL). Means and standard deviations of SMA+ myofibroblast density were determined for each group. Comparisons between opacity grades and SMA+ cell counting in the treatment groups were evaluated using the Mann–Whitney U test. A p value less than 0.05 was considered statistically significant.

RESULTS

Effect of resolvin E1 on corneal opacity (haze) evaluated with slit-lamp biomicroscopy: Corneal opacity was significantly lower in the 0.1% RX-10045 group compared to the vehicle control group (p=0.029) at 1 month after −9.0D PRK (Figure 1). There was no significant difference in opacity between the 0.1% RX-10045 group and the 0.01% RX-10045 group (p=0.15) or between the 0.01% RX-10045 group and the vehicle control group (p=0.19). The masked opacity grade for each rabbit cornea is shown in Table 1.

Effect of resolvin E1 on stromal myofibroblast density: SMA+ myofibroblast cell densities (cells per 400X microscopic field) at 1 month after −9.0D PRK were not significantly different between the 0.1% RX-10045 group and the vehicle control group (p=0.17), the 0.01% RX-10045 group and the vehicle control group (p=0.48), or the 0.1% RX-10045 group and the 0.01% RX-10045 group (p=0.19). The SMA+ myofibroblast density in each cornea, as well as the mean and standard deviation in each group, is provided in Table 2.

There appeared to be outliers in each group (corneas with 50 or more SMA+ cells/400X field and corneas with virtually no SMA+ cells). Therefore, the data were also analyzed after the highest and lowest SMA+ cell values were excluded in each group. With these exclusions, the difference between the 0.1% RX-10045 group and the vehicle control group nearly reached statistical significance (10±10 cells/400X field versus

18±7 cells/400X field, respectively, p=0.054). Similarly, a stronger trend toward a significant difference was noted between the 0.1% RX-10045 group and the 0.01% RX-10045 group (10±10 cells/400X field versus 20±12 cells/400X field, respectively, p=0.11). There was no difference between the 0.01% RX-10045 group and the vehicle control group (20±12 cells/400X field versus 18±7 cells/400X field, respectively, p=0.47).

DISCUSSION

Resolvins have been characterized as agonists of inflammatory resolution in many ocular and non-ocular inflammatory disorders [15,24]. The present masked study found that topical 0.1% RX-10045 given immediately after laser application and every 4 h for 5 days after opacity-inducing PRK in rabbits decreases haze generation in the cornea at 1 month after surgery. There was also a trend toward reduced corneal myofibroblast generation that may have reached significance if more eyes had been included in the study. Li et al. [23] found that resolvin E1 decreased α-SMA+ cell generation in a murine dry eye model. That study also found that resolvin E1 attenuated the influx of cells of macrophage lineage, which suggests that the effect of this regulator may be mediated via differentiation of bone marrow-derived fibrocytes from macrophage precursors or the subsequent differentiation of fibrocytes into myofibroblasts [27-31]. Corneal myofibroblasts may be derived from bone marrow–derived precursors and keratocyte-derived precursors [6,32]. We hypothesize that the RX-10045 produces its effect on haze by blocking myofibroblast generation from bone marrow–derived precursors without a substantial effect on myofibroblast generation from keratocyte-derived precursors. If this is the case, then treatment with a compound (or compounds) that modulates development of myofibroblasts from both precursor types will be needed to further decrease opacity and SMA+ myofibroblast generation after haze-inducing injury.

It is encouraging that the RX-10045 effect on corneal haze after PRK was achieved with topical application. In a prior study of PRM-151, a recombinant form of human pentraxin-2 (PTX-2—also referred to as serum amyloid P), that inhibits differentiation of circulating monocytes into fibrocytes and profibrotic macrophages, myofibroblast generation after haze-inducing PRK was modulated only by subconjunctival injection of the compound, not topical application [33]. Presumably, this occurred because of inadequate penetration of PRM-151 into the stroma after topical application. The current study suggests that RvE1 penetrates the cornea after topical application—at least when an epithelial defect associated with PRK is present. In the rabbit PRK model used in this study, the rabbit epithelium closed approximately 4 days after surgery.

Additional studies are needed to determinate the precise point(s) in myofibroblast precursor recruitment or development at which RvE1 decreases corneal opacity generation. Understanding the exact pathways of action of resolvin RX-10045 will likely yield a better understanding of the mechanisms involved in myofibroblast development and corneal opacity generation after haze-generating corneal injury.

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