Ocular Fibroblast Diversity: Implications for Inflammation and Ocular Wound Healing

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PURPOSE. Various ocular and orbital tissues differ in their manifestations of inflammation, although the reasons for this are unclear. Such differences may be due to behaviors exhibited by resident cell types, including fibroblasts. Fibroblasts mediate immune function and produce inflammatory mediators. Chronic stimulation of ocular fibroblasts can lead to prolonged inflammation and, in turn, to impaired vision and blindness. Interleukin (IL)-1 β , which is produced by various cells during inflammation, is a potent activator of fibroblasts and inducer of the expression of inflammatory mediators. The hypothesis for this study was that human fibroblasts derived from distinct ocular tissues differ in their responses to IL-1 β and that variations in the IL-1 signaling pathway account for these differences.

METHODS. Human fibroblasts were isolated from the lacrimal gland, cornea, and Tenon's capsule and treated with IL-1 β in vitro. Cytokine and prostaglandin (PG)E₂ production were measured by ELISA and EIA. Cyclooxygenase (Cox)-2 expression was detected by Western blot. Components of the IL-1 signaling pathway were detected by flow cytometry, ELISA, Western blot, and immunofluorescence.

RESULTS. Cytokine and PGE₂ production and Cox-2 expression were greatest in corneal fibroblasts. VEGF production was greatest in Tenon's capsule fibroblasts. Variations in IL-1 receptor and receptor antagonist expression, I κ B α degradation and p65 nuclear translocation, however, did not account for these differences, but overexpression of the NF- κ B member RelB dampened Cox-2 expression in all three fibroblast types.

CONCLUSIONS. The results highlight the inherent differences between ocular fibroblast strains and provide crucial insight into novel, tissue-specific treatments for ocular inflammation

and disease, such as RelB overexpression. (*Invest Ophthalmol Vis Sci.* 2011;52:4859–4865) DOI:10.1167/iovs.10-7066

Ocular inflammation can occur as a consequence of disease, injury, and surgery.^{1–4} Inflammation, the body's normal response to tissue damage, is typically associated with an increase in the production of proinflammatory mediators,^{5–7} and persistent inflammation in the eye can lead to impaired vision and blindness.^{4,8,9} However, the different ocular tissues may vary in their sensitivities to inflammatory stimuli.^{2,4,8} These sensitivities may be mediated in part by the cell types present at the site of the inflammation, such as fibroblasts.^{10–12}

In addition to providing structural support, fibroblasts play crucial roles in both inflammation and wound healing.^{11,12} They act as sentinel cells of the immune system by producing proinflammatory mediators in response to injury and inflammatory stimuli.^{11,12} Interleukin (IL)-1 β , a proinflammatory cytokine produced by epithelial cells and macrophages,⁹ increases fibroblast production of proinflammatory cytokines and chemokines, such as monocyte chemoattractant protein (MCP)-1 and IL-6 and -8, which promote immune cell infiltration,^{11,13} and vascular endothelial growth factor (VEGF), which promotes blood vessel formation.¹⁴ IL-1 β also increases expression of the enzyme cyclooxygenase (Cox)-2 and production of its proinflammatory metabolite, prostaglandin (PG)E₂, in fibroblasts.^{10,12} These proinflammatory mediators are critical in the pathogenesis of several ocular abnormalities, including corneal scarring, dry eye syndrome and postglaucoma filtration scarring.^{2–4,8,9} Activation of corneal fibroblasts has also been linked to vernal keratoconjunctivitis.¹⁵

Although fibroblasts were once thought to be a relatively homogeneous cell type, it is now known that they vary in morphology and function depending on their tissue of origin.^{12,13} To date, few studies have compared primary human fibroblasts from different ocular compartments regarding their response to proinflammatory signals or their ability to produce inflammatory mediators. Herein, we compared three primary fibroblast strains derived from three different human ocular (corneal and Tenon's capsule) or ocular adnexal (lacrimal gland) tissues after exposure to IL-1 β . Our results show that fibroblasts derived from the cornea are far more responsive than those derived from either the lacrimal gland or Tenon's capsule and that expression and activity of members of the IL-1 β and NF- κ B pathways vary among fibroblast types. Moreover, manipulation of NF- κ B through overexpression of the NF- κ B family member RelB dampens IL-1 β -induced Cox-2 expression. Recognizing the differences inherent to fibroblasts subtypes may allow for the development of novel, targeted therapeutic strategies against inflammation-associated eye diseases.

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MATERIALS AND METHODS

Reagents

Recombinant human IL-1 β was obtained from R&D Systems (201-LB; Minneapolis, MN). The RelB recombinant adenovirus was obtained from Cell Biolabs (San Diego, CA) and the DL-70 control recombinant adenovirus containing an empty vector was used as previously described.¹⁶

Cell Culture

Human lacrimal gland, corneal, and Tenon's capsule tissues were obtained anonymously from patients under informed, written consent, with the approval of the Institutional Review Board of the University of Rochester and in compliance with the Declaration of Helsinki. All patients at the Flaum Eye Institute were undergoing various ophthalmic procedures that did not affect these tissues, and, to our knowledge, the tissue obtained was not involved in the patients' disease processes. Nine strains of lacrimal gland fibroblasts, 11 of corneal fibroblasts, and 5 of Tenon's capsule fibroblasts were developed from these tissues. No more than one strain or type of fibroblast was collected from any one donor. These sites were chosen because they are all ocular tissues for which inflammation and scarring can be directly linked to clinically relevant human diseases. Lacrimal gland tissue was minced and cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories, Logan, UT) and a penicillin/streptomycin cocktail (P/S; Invitrogen). Corneal fibroblasts were isolated as previously described¹⁷ and suspended in DMEM supplemented with 20% HEPES and P/S. Tenon's capsule tissue was minced and cultured in minimum essential medium (MEM; Invitrogen) supplemented with 10% FBS, 2 mM L-glutamine, and P/S. Fibroblasts were characterized by their adherent morphology, expression of vimentin and collagen (types I and III), and absence of CD45, factor VIII, and cytokeratin. Further, all fibroblast types were shown to be greater than 90% positive for Thy-1 (CD90) as assessed by flow cytometry (data not shown). Three representative strains of each fibroblast type were used in the experiments detailed herein. All cells were used at as early a passage as possible (between passages 3 and 6) and at a similar passage number.

ELISA and EIA

Cytokines and PGE₂ were detected in the supernatants of fibroblasts plated in vitro at 100,000 cells/well in six-well plates. To detect IL-6, IL-8, and MCP-1, we coated 96-well plates with antibodies targeting human IL-6 (554543; BD PharMingen, San Diego, CA), IL-8 (M-801; Endogen), or MCP-1 (MAB679; R&D Systems) in 1 M NaHCO₃ and blocked with 5% BSA in PBS. Supernatants were incubated in their respective wells overnight, after which biotinylated antibodies targeting IL-6 (554546; BD PharMingen), IL-8 (M-802-B; Endogen), or MCP-1 (BAF279; R&D Systems) were added. Streptavidin-conjugated alkaline phosphatase (1:1000 in 5% BSA/PBS) was then added to each well for 1 hour, and IL-6 and -8 were detected by developing with ρ NPP/DEA developing solution (172-1063; Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Optical density readings were taken at a 405-nm wavelength with a 610-nm reference filter. VEGF and IL-1RA were detected in the supernatants with ELISA kits according to the manufacturer's instructions (DuoSet; DY293B and DY280, respectively; R&D Systems). PGE₂ was detected in the supernatants by competitive EIA, as previously described.¹⁸

ELISpot Analysis

Multiscreen 96-well filtration plates (Millipore, Bedford, MA) were coated with antibodies targeting human IL-6 (554543; BD PharMingen) or IL-8 (M-801; Endogen) in 1 M NaHCO₃ and blocked with 10% FBS/MEM for 1 hour at 37°C. Fibroblasts were seeded into the wells at 100 cells/well and incubated in 1% FBS/MEM with or without IL-1 β for

6 hours. Biotinylated antibodies targeting IL-6 (554546; BD PharMingen) or IL-8 (M-802-B; Endogen) were added to their respective wells in 5% BSA/PBS, and the plates were incubated overnight. Streptavidin-conjugated alkaline phosphatase (1:1000 in 5% BSA/PBS) was then added to each well for 1 hour, and IL-6 and -8 were detected by developing with BCIP/NBT chromogen liquid substrate (Sigma-Aldrich, St. Louis, MO) for 5 minutes. The plates were then washed with water and dried, and the filters were transferred onto a pressure-sensitive, 96-well plate reader with an 8-well punch (ELISpot; Costar, Corning, NY). Spot intensity and frequency were calculated with gel-analysis software (Quantity One 1D; Bio-Rad).

Western Blot Analysis

The cells were homogenized in an SDS lysis buffer containing a protease inhibitor cocktail (Sigma-Aldrich) and were centrifuged to remove debris. Total protein concentration was determined with a bicinchoninic acid (BCA) detection assay (Pierce Rockford, IL). Five to 20 μ g of protein was separated by 12% SDS-PAGE, transferred onto PVDF membrane (Immobilon-P; Millipore), and blocked with 10% nonfat dry milk in 0.1% Tween 20 in PBS. Antibodies targeting Cox-2 (160112; Cayman Chemical, Ann Arbor, MI), I κ B α (9936; Cell Signaling Technology, Danvers, MA), RelB (sc-226; Santa Cruz Biotechnologies, Santa Cruz, CA), GAPDH (sc-32,233; Santa Cruz), β -tubulin (sc-53,140; Santa Cruz), and total actin (CP01; Calbiochem, San Diego, CA) were diluted according to the manufacturers' instructions. HRP-conjugated secondary antibodies were obtained from Jackson ImmunoResearch (West Grove, PA). Protein was visualized using a Western chemiluminescent HRP substrate (Immobilon; Millipore) and developed on classic blue-sensitive x-ray film (Laboratory Product Sales, Rochester, NY). Densitometric analysis was performed with imaging software (Kodak 1D; Eastman Kodak, Rochester, NY).

Flow Cytometry

Fibroblasts were fixed with 4% paraformaldehyde in PBS for 10 minutes and blocked with 10 mg/mL BSA in PBS containing 0.2% Triton X-100. IL-1RI was targeted with a carboxyfluorescein (CFS)-conjugated anti-human IL-1RI antibody (FAB269F; R&D Systems). IL-1RII was targeted with an anti-human IL-1RII antibody (311202; BioLegend, San Diego, CA) and subsequently labeled with an FITC-conjugated secondary antibody (115-096-062; Jackson ImmunoResearch Laboratories). CFS and FITC intensity were detected by flow cytometry (FACSCanto; BD Biosciences, San Jose, CA) and analyzed (FlowJo; Tree Star, Ashland, OR).

Immunofluorescent Microscopy

Fibroblasts were plated in eight-well chamber slides and serum starved overnight before treatment. Afterward, the cells were fixed with 100% methanol at -20°C for 10 minutes, and the slides were blocked with 5% goat serum and 1% BSA in 0.2% Triton X-100/TBS. The NF- κ B family member p65 was targeted with a rabbit polyclonal antibody (sc-372; Santa Cruz Biotechnologies), detected with an Alexa Fluor 488 goat anti-rabbit antibody (A11001; Invitrogen), and visualized via confocal microscopy at 60 $\times$ magnification.

Statistical Analysis

Results are presented as the mean $\pm$ SD from triplicate samples. A two-tailed Student's *t*-test was performed (Prism ver. 4.0; GraphPad Software, San Diego, CA) *P* < 0.05 indicated significance.

RESULTS

IL-1 β Increases Proinflammatory Cytokine and VEGF Production in Ocular Fibroblasts

Fibroblasts exposed to IL-1 β , a known inducer of inflammation, produce proinflammatory mediators such as IL-6, IL-8, and MCP-1 and growth factors such as VEGF.^{11,13,14} To investigate

FIGURE 1. IL-1 β increased proinflammatory cytokine and VEGF production in ocular fibroblasts. Fibroblasts isolated from the lacrimal gland, cornea and Tenon's capsule were treated with IL-1 β at 10 ng/mL for 24 hours, after which IL-6 (A), IL-8 (B), MCP-1 (C), and VEGF (D) were detected in the supernatants by ELISA. Production of IL-6, IL-8, and MCP-1 was increased most dramatically in corneal fibroblasts, whereas VEGF production was increased most noticeably in Tenon's capsule fibroblasts. * $P < 0.05$, IL-1 β -treated versus vehicle-treated fibroblasts. † $P < 0.05$, IL-1 β -treated lacrimal gland or Tenon's capsule fibroblasts versus IL-1 β -treated corneal fibroblasts. ‡ $P < 0.05$, IL-1 β -treated lacrimal gland or corneal fibroblasts versus IL-1 β -treated Tenon's capsule fibroblasts ($n = 3$ per group).

possible differences in the inflammatory response between fibroblast populations from different ocular compartments, fibroblasts isolated from the cornea, lacrimal gland, and Tenon's capsule were treated in vitro with IL-1 β . As seen in Figure 1, IL-1 β caused a significant increase ($P < 0.05$) in the production of IL-6, IL-8, MCP-1, and VEGF in all three fibroblast types. The

increase was significantly more robust in corneal fibroblasts than in those derived from either Tenon's capsule or the lacrimal gland (Figs. 1A–C). VEGF production was also increased in all three fibroblast types, but was significantly higher in Tenon's capsule, relative to corneal or lacrimal gland fibroblasts (Fig. 1D).

Enumeration of the Frequency of IL-6 and -8 Production by Individual Fibroblasts

The increase in cytokine production induced by IL-1 β could be due to a change in the cytokine production per cell or in a cytokine-producing subpopulation. To distinguish between these possibilities, fibroblasts isolated from the cornea, lacrimal gland, and Tenon's capsule were treated with IL-1 β at 10 ng/mL for 24 hours, and IL-6 and -8 production were detected by the plate reader (ELISpot; Corning; Fig. 2A). Cytokine production by individual cells was measured by spot intensity (Figs. 2B, 2C), and the number of cytokine-producing cells was measured by spot frequency (Figs. 2D, 2E). IL-6 spot intensity increased significantly in lacrimal gland and corneal fibroblasts, but not in Tenon's capsule fibroblasts, after IL-1 β treatment (Fig. 2B). IL-8 spot intensity was significantly increased in corneal, rather than lacrimal gland or Tenon's capsule, fibroblasts (Fig. 2C). IL-6 spot intensity was also significantly greater in IL-1 β -treated corneal fibroblasts relative to IL-1 β -treated lacrimal gland and Tenon's capsule fibroblasts. IL-6 spot frequency was significantly increased in IL-1 β -treated lacrimal gland and corneal fibroblasts, but not in Tenon's capsule fibroblasts (Fig. 2D). IL-8 spot frequency increased significantly in IL-1 β -treated lacrimal gland and Tenon's capsule fibroblasts, but not in corneal fibroblasts (Fig. 2E). These data suggest that the increased production of IL-6 and -8 shown in Figure 1A is due to both an increase in the number of cytokine-producing cells and an increase in cytokine production by individual cells.

IL-1 β -Induced Cox-2 Expression and PGE₂ Production Are Greater in Corneal Than in Lacrimal Gland and Tenon's Capsule Fibroblasts

To further characterize the inflammatory response of the different ocular fibroblast populations, IL-1 β -induced Cox-2 expression was measured in all three fibroblast types. IL-1 β is a known inducer of Cox-2, and Cox-2 is the proinflammatory enzyme responsible for PGE₂ production.^{10,12} Consistent with the IL-1 β -induced proinflammatory cytokine production shown in Figure 1, Cox-2 expression was increased in all three fibroblast types, with the highest expression seen in corneal fibroblasts (Fig. 3A; results from fibroblasts from two different

FIGURE 2. Enumeration of the frequency of IL-6 and -8 production by individual fibroblasts. Lacrimal gland, cornea, and Tenon's capsule fibroblasts were treated with IL-1 β (10 ng/mL) for 6 hours, and IL-6 and -8 production was measured with a plate reader. (A) Representative images of IL-6 and -8 results. (B, C) Quantitation of IL-6 and -8 spot intensity. (D, E) IL-6 and -8 spot frequency expressed as the number of spots per 100 cells. * $P < 0.05$, IL-1 β -treated versus vehicle-treated fibroblasts. † $P < 0.05$, IL-1 β -treated lacrimal gland or Tenon's capsule fibroblasts versus IL-1 β -treated corneal fibroblasts ($n = 3$ per group).

FIGURE 3. IL-1 β -induced Cox-2 expression and PGE₂ production were greater in corneal than in lacrimal gland and Tenon's capsule fibroblasts. Fibroblasts isolated from the lacrimal gland, cornea, and Tenon's capsule were treated with IL-1 β at 10 ng/mL for 24 hours, and Cox-2 expression was detected in whole cell lysates by Western blot (A). Results from fibroblasts from two different subjects are shown. GAPDH is included as a housekeeping protein. PGE₂ was detected in the supernatants of these cultures by EIA (B). * $P < 0.05$ IL-1 β -treated versus vehicle-treated fibroblasts ($n = 3$ per group).

subjects are shown). Of note, PGE₂ production, measured in the supernatants by EIA, was also significantly increased in the corneal fibroblasts, but not in the lacrimal gland- or Tenon's capsule-derived fibroblasts (Fig. 3B; $P < 0.05$).

IL-1 β -Treated Ocular Fibroblasts Do Not Exhibit Differences in IL-1 Receptor Expression and IL-1 Receptor Antagonist Production

To establish a mechanism accounting for the observed differences in IL-1 β -induced inflammatory mediator production, critical components of the IL-1 β signaling pathway were examined. IL-1 β signals through either of two transmembrane surface receptors, IL-1 receptor types I and II (IL-1RI and

IL-1RII).¹⁹ IL-1RI binding regulates expression of its respective genes, whereas IL-1RII binding is antagonistic.¹⁹ Since corneal fibroblasts appeared to be more sensitive to IL-1 β exposure than lacrimal gland or Tenon's capsule fibroblasts in terms of IL-6, IL-8, and MCP-1 production, we hypothesized that corneal fibroblasts would display increased surface expression of IL-1RI and/or decreased surface expression of IL-1RII. Interestingly, flow cytometry indicated that, although these receptors were expressed in all three fibroblast strains, expression of both receptors was significantly higher in lacrimal gland fibroblasts than in either of the other two cell types (Figs. 4A, 4B). These data highlight the diversity among fibroblasts derived from different tissues, but do not account for the increased sensitivity of corneal fibroblasts to IL-1 β observed in Figure 1.

To investigate the possibility that variations in production of IL-1 receptor antagonist (IL-1RA), a competitive inhibitor of IL-1 β binding,²⁰ account for the differences in IL-1 β -induced proinflammatory mediator production, we measured IL-1RA levels in the supernatants of untreated and IL-1 β -treated fibroblasts. Intriguingly, only IL-1 β -treated Tenon's capsule fibroblasts produce significant quantities of this molecule (Fig. 4C). These data suggest therefore that IL-1RA expression may mediate production of IL-6, IL-8, and MCP-1 by Tenon's capsule fibroblasts via IL-1 β .

IL-1 β -Treated Ocular Fibroblasts Do Not Exhibit Differences in p65 Nuclear Translocation and I κ B α Degradation

Since differences in IL-1 receptor and receptor antagonist expression did not systematically account for the observed differences in proinflammatory mediator production among the three fibroblast types, nuclear translocation of the NF- κ B family member p65 was examined. NF- κ B is a complex signaling pathway involved in the production of inflammatory mediators, including IL-6, IL-8, Cox-2, and PGE₂, and is integral to cell proliferation and survival.^{21–25} Activation of this pathway by inflammatory stimuli such as IL-1 β promotes degradation of the inhibitor of NF- κ B α (I κ B α) and nuclear translocation of the NF- κ B family member p65.²³ The increased production of IL-6, IL-8, and MCP-1 displayed by corneal fibroblasts, compared to lacrimal gland and Tenon's capsule fibroblasts, may therefore be associated with increased I κ B α degradation and p65 nuclear translocation. Nuclear translocation of p65 was detected in all three fibroblast types by immunofluorescence microscopy after IL-1 β treatment (Fig. 5A), and expression of I κ B α was detected by Western blot (Fig. 5B). IL-1 β dramatically increased p65 nuclear translocation and induced I κ B α degrada-

FIGURE 4. IL-1 β -treated ocular fibroblasts did not exhibit differences in IL-1 receptor expression and IL-1 receptor antagonist production. Fibroblasts were treated with IL-1 β for 24 hours at 10 ng/mL, after which cell surface expression of IL-1RI and -II were measured by flow cytometry (A, B), and IL-1RA production was measured in the supernatants by ELISA (C). IL-1RI and -II expression were higher in lacrimal gland fibroblasts, whereas IL-1RA production was notably higher in Tenon's capsule fibroblasts. Flow cytometry measurements are presented as a percentage of the maximum. * $P < 0.05$ IL-1 β -treated versus vehicle-treated fibroblasts ($n = 3$ per group).

FIGURE 5. IL-1 β -treated ocular fibroblasts did not exhibit differences in p65 nuclear translocation and I κ B α degradation. Fibroblasts were treated with IL-1 β (10 ng/mL) for either 20 minutes or 6 hours, after which p65 nuclear translocation was detected by immunofluorescent microscopy (A; 20 minutes only), and I κ B α expression was detected by Western blot (B). p65 nuclear localization and I κ B α expression or phosphorylation did not vary dramatically among the fibroblast types.

tion after 20 minutes in all three fibroblast types. Thus, p65 translocation 20 minutes after stimulation is insufficient to explain the increased production of IL-6, IL-8, and MCP-1 after IL-1 β treatment in corneal, compared with lacrimal gland and Tenon's capsule, fibroblasts.

Overexpression of the NF- κ B Family Member RelB Dampens IL-1 β -Induced Cox-2 Expression

Although p65 activity does not appear to account for the differential sensitivities of the various fibroblast types to IL-1 β , other NF- κ B family members may be involved. NF- κ B is generally thought to be proinflammatory,^{22–24} but RelB, an understudied NF- κ B protein, has been shown to have anti-inflammatory properties.^{6,26–28} RelB deficiency promotes proinflammatory mediator production, and reconstitution of RelB expression dampens these effects.^{6,26–28} We therefore hypothesized that overexpression of RelB could dampen IL-1 β -induced inflammatory mediator production in these fibroblasts. To increase RelB expression, each type of fibroblast was infected with an adenovirus containing the human RelB gene for 24 hours before IL-1 β exposure. Cox-2, which was detected in whole cell lysates by Western blot, was dramatically reduced in all three fibroblast strains after RelB overexpression (Fig. 6). These data indicate that modulation of RelB expression affects proinflammatory mediator production in ocular fibroblasts and suggest that production of these mediators may be regulated by an NF- κ B-dependent mechanism.

DISCUSSION

Ocular inflammation may result from disease, injury, or surgery, and the consequences of this vary according to the

tissue(s) affected.^{1–4} Inflammation of the lacrimal gland, for example, may result in aqueous-tear-deficient dry eye syndrome,^{2,4} whereas corneal inflammation may lead to corneal opacification and decreased vision.^{8,9,29} Since the effects of inflammation vary depending on the affected tissue, there are likely to be differences in the sensitivities of these tissues to inflammatory stimuli. Such differences may arise through variations among particular cell types, such as fibroblasts, which can vary greatly in their biosynthetic capabilities.^{11,12}

Fibroblasts, once considered to be relatively passive structural cells, are now recognized to regulate inflammation through the production of cytokines, chemokines, and lipid mediators.^{11,12} The sensitivity of a tissue to inflammatory stimuli is most likely determined, at least in part, by the regional type of fibroblast.^{13,30} In this study, we compared the responses of fibroblasts isolated from the cornea, lacrimal gland, and Tenon's capsule, to the proinflammatory cytokine, IL-1 β . We found that, after IL-1 β stimulation, corneal fibroblasts exhibit heightened proinflammatory cytokine and PGE₂ production relative to lacrimal gland or Tenon's capsule fibroblasts (Figs. 1–3). Importantly, the cornea protects the eye from the external environment, but because of its location and lack of vasculature, it is particularly susceptible to wounding and infection.^{8,9,31} Bacteria can induce blindness by infecting the cornea.³² This tissue may therefore be more sensitive to inflammatory stimuli, and increased fibroblast activity would maintain normal function of the cornea by increasing repair of the epithelium and elimination of infection through the recruitment of immune cells. While infection is also undesirable in the lacrimal gland and Tenon's capsule, it is less likely to occur in these tissues, so less robust fibroblast activity is sufficient. Tenon's capsule fibroblasts, however, exhibited heightened VEGF production after IL-1 β treatment compared to corneal and lacrimal gland fibroblasts. Tenon's capsule is a thin membrane that envelops the eye which simultaneously isolates the eye from the rest of the body and forms the cavity within which the eye can move.³³ The rear of Tenon's capsule is perforated to allow blood vessels and nerves access to eye,³³ and so it is possible that cells within Tenon's capsule release VEGF, a growth factor that induces blood vessel formation, to

FIGURE 6. Overexpression of the NF- κ B family member RelB dampened IL-1 β -induced Cox-2 expression. Fibroblasts were infected with either a control or RelB recombinant adenovirus at 10,000 MOI for 24 hours, after which they were exposed to IL-1 β at 10 ng/mL for 24 hours. Cox-2 and RelB expression were detected in whole cell lysates by Western blot. IL-1 β -induced Cox-2 expression was reduced in all three types of ocular fibroblasts after RelB overexpression (red boxes). Basal Cox-2 expression was also decreased after RelB overexpression in corneal fibroblasts. All samples from each blot were transposed from different locations on the same gel, but are shown separated by white space.

increase blood supply to ocular tissues. Interestingly, as seen in Figure 2, the increased production of IL-6 and -8 by all three fibroblast types was due to increases in both the number of cytokine-producing cells and the cytokine production by individual cells. IL-1 β is a powerful inflammatory stimulus, and so it is likely that this cytokine would increase inflammatory mediator production in an increased number of cells in these regions. Subsets of cells within these populations may also exhibit a heightened sensitivity to inflammatory stimuli, which would account for the increased cytokine production relative to adjacent cells.

IL-1 β is a critical mediator of inflammation in ocular tissues, including the cornea and lacrimal gland.^{2,4,9,13} Studies have indicated that IL-1 β levels are elevated in the lacrimal glands of mice affected by aqueous-tear-deficient dry eye syndrome.^{2,4} Expression of IL-1 β is also increased during aging, as it has been found in higher quantities in the lacrimal glands of mice exceeding 12 months of age.^{4,34} Increased fibrosis and signs of atrophy have also been found in the lacrimal glands of aged mice.^{35,36} These findings encouraged us to use this cytokine in our study. Although each type of ocular fibroblast exhibited differences in IL-1 β -induced production of inflammatory mediators and of VEGF, we did not see differences in the expression of either IL-1RI or II (Figs. 4A, 4B), in the production of IL-1RA (Fig. 4C), in nuclear translocation of the NF- κ B subunit p65 (Fig. 5A), or in the degradation of I κ B α (Fig. 5B). The mechanisms regulating the observed differences in the production of the aforementioned mediators therefore remains unclear.

While NF- κ B signaling is typically characterized by I κ B α degradation and p65 translocation,²³ other members of this pathway are key regulators of inflammation. The NF- κ B member RelB regulates chemokine production in mouse kidney fibroblasts and human macrophages,^{28,37} and RelB-deficient mice exhibit severe multiorgan inflammation.²⁷ We have also shown that a decrease in RelB expression promotes inflammatory mediator production in mouse lung fibroblasts and that reconstitution of RelB expression dampens these effects.²⁶ Since RelB regulates the inflammatory response, we hypothesized that overexpression of RelB would dampen ocular fibroblasts' response to IL-1 β . To test this hypothesis, we infected all three ocular fibroblast types with a recombinant adenovirus containing the human RelB gene, and we found that RelB overexpression dramatically dampened IL-1 β -induced Cox-2 expression (Fig. 6). These data suggest that IL-1 β -induced inflammatory mediator production is regulated by an NF- κ B-dependent mechanism and that manipulation of the NF- κ B pathway through RelB overexpression may be a novel therapeutic strategy against inflammation in the eye.

Recognizing the extent of fibroblast heterogeneity among ocular tissues is crucial to understanding the signaling pathways underlying these phenotypic differences and will provide critical insight into the development of various ocular diseases. Our findings indicate that overexpression of the NF- κ B protein RelB dampens IL-1 β -induced inflammatory mediator production in these fibroblasts. Moreover, abnormally low RelB expression may promote inflammatory mediator production in affected tissues. Further recognition and characterization of these differences will also allow us to develop targeted, tissue-specific therapies against diseases of the eye.

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