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A novel method for generating corneal haze in anterior stroma of the mouse eye with the excimer laser

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Abstract

Refractive surgery is a popular method used to reduce or eliminate dependence on glasses and contact lenses. Corneal haze is one of the common complications observed after photorefractive keratectmomy (PRK). The objective of this study was to develop an in vivo mouse model that consistently produces moderate to severe corneal haze in the anterior stroma of the mouse cornea after excimer laser treatment to study myofibroblast biology and corneal wound healing in a genetically defined model. Regular- or irregular-phototherapeutic keratectomy (PTK) was performed on black C57BL/6 mice with the Summit Apex excimer laser (Alcon, Ft. Worth, TX). Different numbers of laser pulses (45; ablation depth ~10 μm) were fired on the central cornea, after scraping the epithelium prior to excimer laser ablation. Irregularity was generated by positioning a fine mesh screen in the path of laser after firing 50% of the pulses. Eyes were collected 1, 2, 3 or 4 weeks after the procedure. Haze formation was gauged with slit lamp biomicroscopy. Immunocytochemistry was used to determine number of myofibroblasts in the mouse cornea using antibodies specific for the myofibroblast marker alpha-smooth muscle actin (SMA). The numbers of SMA-positive cells/400X microscopic were determined by counting within the stroma. Statistical analysis was performed using analysis of variance (AVOVA) with the Bonferonni-Dunn adjustment for repeated measures. Regular-PTK with epithelial scrape (Group 3) and irregular-PTK with epithelial scrape (Group 4) in the mouse eyes were performed to produce corneal haze. Eyes collected 4 weeks after regular- or irregular-PTK after epithelial scrape showed 22 ± 6.6 (Group 3) or 34 ± 7.9 (Group 4) SMA-positive cells in the anterior cornea. The difference in the SMA-positive cells detected among the groups was statistically significant ($p < 0.01$). Less than 4 SMA-positive cells were detected in the tissue sections of the mouse eyes collected after 1, 2 or 3 weeks of regular (Group 3) or irregular PTK (Group 4) or controls (Group 1 and 2). The optimized PTK excimer laser conditions developed in this study produces haze selectively in anterior stroma of the mouse cornea immediately beneath the epithelial basement membrane. Irregular PTK performed after epithelial scrape by applying 45 laser pulses was found to be the most effective method to generate myofibroblasts. This PTK technique for inducing haze in mouse cornea *in vivo* provides a useful model for studying wound healing and myofibroblast biology in transgenic mice.

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Keywords

Haze; cornea; phototheraputic keratectomy; stroma; myofibroblasts

1. Introduction

Development of corneal haze is a common complication of photorefractive keratectomy (PRK), especially in eyes undergoing correction for high myopia (Seiler and McDonnell, 1995; Lipshitz et al., 1997; Sakimoto et al., 2006). Mechanical and/or surgical injury to the cornea triggers a wound healing response causing changes in extracellular matrix organization and cellular phenotype and density (Wilson et al., 2001; Jester et al., 1999a; Fini and Stramer, 2005). Numerous studies have shown that injury to the cornea facilitates release of multiple cytokines and growth factors from both corneal cells and the lacrimal glands (Fini, 1999; Mohan et al., 2003; Zieske, 2001; Baldwin and Marshall, 2002). Cytokines that have been shown to play important roles in maintaining corneal transparency and consequently clear vision are platelet-derived growth factor, transforming growth factor beta, fibroblast growth factor, and the interleukins (Jester et al., 1995; Jester et al., 2002; Girard et al., 1991; Masur et al., 1996). Transforming growth factor beta has been shown to have a central role for inducing opacity (haze) in the cornea by promoting trans-differentiation of progenitor cells, including keratocytes and, possibly, bone marrow-derived cells into myofibroblasts (Jester et al., 1997; Masur et al., 1996, Dupps and Wilson, 2006). The precise mechanisms of corneal haze formation are still unclear. However, the basement membrane of the epithelium has been shown to have a central role in modulating myofibroblast generation, and, therefore haze (Netto et al., 2006).

In previous studies, rabbits (Mohan et al., 2003; Wilson et al., 2003; Netto et al., 2006), rats (Power et al., 1995), hens (Martinez-Garcia et al., 2006) or monkeys (Del Pero et al., 1990; Malley et al., 1990) were used to study haze formation. Mice have not been used extensively because mouse corneas are relatively resistant to haze generation following normal PRK. This limitation has restricted the researcher's ability to perform in-depth investigation of genetic factors that may be important in myofibroblast generation. The purpose of this study was to develop a technique that could be used to consistently generate moderate to severe corneal haze in the mouse cornea.

2. Method

2.1. Animals and haze generation

Eight to ten week-old black C57BL/6 mice were used in this study. All procedures in animals were performed in accordance with the tenets of the ARVO Statement for the Use of Animals and approved by the Institutional Animal Care and Use Committee of the Cleveland Clinic, Cleveland, Ohio.

Anesthesia was performed by intramuscular injection of ketamine hydrochloride (130 mg/kg) and xylazine hydrochloride (8.8 mg/kg). In addition, topical proparacaine hydrochloride 1% (Alcon, Ft. Worth, TX, USA) was applied to each eye just before surgery. Euthanasia was performed with an intravenous injection of pentobarbital (100 mg/kg) while the animal was under general anesthesia.

An attempt to induce corneal haze was carried out by performing regular or irregular phototherapeutic keratectomy (PTK) with a 2 mm ablation zone on the central stroma by firing 45 laser pulses (ablation depth ~10 μm) with the Summit Apex excimer laser (Alcon, Ft. Worth, TX) after removing entire epithelium (~4 mm) with #64 surgical blade without injuring the

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limbus. Irregular PTK was performed by positioning a fine mesh screen in the path of the laser after firing 50% of the pulses, as was previously used successfully in the rabbit (Netto et al., 2006). Animals were divided in six groups and only one eye of each animal was used for experiments. Group 1 animals had epithelial scrape, group 2 animals had neither epithelial scrape nor PTK, group 3 animals had regular PTK with epithelial scrape (45 pulses), and group 4 animals had irregular PTK with epithelial scrape (45 pulses). Each group had four time points (1, 2, 3 or 4 weeks), with six eyes at each time point.

2.2. Biomicroscopic grading of corneal haze

The level of opacity (haze) in the cornea was measured with slit lamp after 1, 2, 3 and 4 weeks of PTK following a method reported previously (Fantes et al., 1990), with animals under general anesthesia. Grade 0 was a completely clear cornea; grade 0.5 had trace haze seen with careful oblique illumination with slit-lamp biomicroscopy; grade 1 was more prominent haze not interfering with visibility of fine iris details; grade 2 was mild obscuration of iris details; grade 3 was moderate obscuration of the iris and lens; and grade 4 was complete opacification of the stroma in the area of the ablation. Haze grading was performed in a masked manner by two independent observers.

2.3. Tissue collection

Eyes were removed with 0.12 forceps and sharp Westcott scissors, embedded in liquid OCT compound (Sakura FineTek, Torrance, CA) within a 15 mm × 15 mm × 5 mm mold (Fisher, Pittsburgh, PA) and snap frozen following previously reported methods (Mohan et al, 2003). The frozen tissue blocks were maintained at −85°C. Tissue sections (7 microns) were cut with a cryostat (HM 505M, Micron GmbH, Walldorf, Germany) and maintained frozen at −85°C until staining was performed.

2.4. Immunocytochemistry

Immunofluorescent staining for alpha smooth muscle actin (SMA), a marker for myofibroblasts, was performed using mouse monoclonal antibody for SMA (DAKO, Carpinteria, CA). Tissue sections (7 microns) were incubated at room temperature with the monoclonal antibody for SMA at a 1:50 dilution in 1x PBS for two hours and with secondary antibody Alexa 568 or 488 goat anti-mouse IgG (Invitrogen, Carlsbad, CA) at a dilution of 1:200 for one hour. Coverslips were mounted with Vectashield containing DAPI (Vector Laboratories, Inc.; Burlingame, CA) to allow visualization of all nuclei in the tissue sections. Irrelevant isotype-matched primary antibody, secondary antibody alone and tissue sections from naïve eyes were used for negative controls for each immunocytochemistry experiment. The sections were viewed and photographed with a Nikon Eclipse E800 microscope equipped with a digital camera (SpotCam RT KE, Diagnostic Instruments Inc., Sterling Heights, MI).

2.5. Quantification of SMA-positive cells

Six corneas for each time point were used to quantify SMA-positive cells in the tissues. The SMA-positive cells in six randomly selected, non-overlapping, full-thickness central corneal columns extending from the anterior stromal surface to the posterior stromal surface were counted following a method reported previously (Mohan et al, 2003). The diameter of each column was a 400X microscope field.

2.6. Statistical analysis

Statistical analysis was performed with StatView software 4.5 (Abacus Concepts, Berkeley, CA). Variance was expressed as the standard error of the mean (SEM). Statistical comparisons between the groups were performed using analysis of variance (AVOVA) with the Bonferonni-Dunn adjustment for repeated measures.

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3. Results

3.1. Biomicroscopic evaluation of haze

The PTK-treated corneas of both the groups collected at the 4-week time point showed different levels of clinical haze. The mouse corneas that had irregular PTK after epithelial scrape (group 4) showed significantly higher levels of haze than the corneas that underwent regular PTK after epithelial scrape (group 3). The biomicroscopic grading of the clinical corneal haze in the animals 4 weeks after irregular PTK with epithelial scrape (group 4) showed prominent haze compared to the corneas of regular PTK with epithelial scrape (group 3) that showed less haze (Figure 1). No corneal haze was observed during biomicroscopic studies in epithelial scrape only (group 1) or naïve (group 2) corneas. Also, none of the mouse corneas examined at the 3 weeks or earlier time points after PTK had corneal haze (data not shown).

3.2. Immunofluorescence detection of myofibroblasts

The formation of haze in mouse corneas was also confirmed by the immunocytochemical detection of myofibroblasts (Figure 2) and quantification of SMA-positive cells in tissue sections (Figure 3). All tissue sections of control and PTK treated mouse eyes collected after 1, 2 or 3 weeks following PTK showed 4 or less SMA-positive cells/400x column in the stroma (data not shown). In contrast, mouse corneas collected 4 weeks after regular or irregular PTK, with epithelial removal, (groups 3 and 4), demonstrated higher myofibroblast cell densities (p value 0.001 or <0.0001). The highest numbers of SMA-positive cells (34 ± 7.9) were detected in the corneas having irregular PTK after epithelial scrape (group 4). The corneas having regular PTK with epithelial scrape (group 3) also showed significantly higher SMA-positive cells (22 ± 6.6) than the control, but less than the mouse corneas that had irregular PTK. The most remarkable feature of the PTK technique was that it selectively induced haze in the anterior stroma, similar to the . Careful evaluation of corneal sections from the different groups showed that 70% or more of the SMA-positive cells were present in the anterior stroma beneath the epithelium and the remaining 10–30% SMA-positive cells in the central or posterior stroma. A few of the corneas also showed 1 to 4 SMA-positive cells in the epithelium and/or endothelium but these cells were excluded from the counting.

4. Discussion

Corneal haze is a common complication that occurs in patients after excimer laser photorefractive keratectomy (Seiler and McDonnell, 1995; Lipshitz et al., 1997; Sakimoto et al., 2006). Clinically significant corneal haze has been observed in the eyes after excimer laser PRK (Lipshitz et al., 1997; Hersh et al., 1997; Shah et al, 1998; Siganos et al., 1999; Kuo et al., 2004) and the frequency and intensity of the complication is related to the level of attempted correction. The incidence of corneal haze following PRK in humans (Kuo et al., 2004) or rabbits (Mohan et al., 2003) increases with increasing attempted correction and increasing volume of stromal tissue removal (Moller-Pedersen et al., 1998). Myofibroblasts, and the disordered extracellular matrix materials these cells produce, are the primary causes of corneal haze following PRK (Jester et al., 1999b; Stramer et al., 2003, Mohan et al., 2003; Netto et al., 2006).

The roles of laser-induced stromal surface irregularity, cytokines and growth factors released from corneal epithelium and tears, influx of bone marrow-derived cells, extracellular matrix, stromal remodeling, and corneal crystallins has been studied in haze development following PRK in vivo using various animal models (Mohan et al., 2003; Wilson et al., 2003; Jester et al., 1999a; Jester et al., 2002; Jester et al., 1995; Jester et al, 1999b; Funderburgh et al., 2001; Javier et al., 2006). Recently, the structural and functional integrity of the regenerating

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epithelial basement membrane has been shown to play a critical role in determining whether a particular cornea develops haze (Netto et al., 2006).

One of the major limiting factors restricting further investigation of molecular mechanisms and the function of select genes controlling corneal wound healing and basement membrane repair is the lack of a suitable model to provide a broad range of genetic testing. The mouse is the preferred host for completing such studies and for developing new strategies for treating various disorders and diseases. However, the mouse model is not currently suitable for studying corneal myofibroblast biology because an appropriate technique for inducing haze is not available. Thus, haze does not typically develop in the mouse cornea following epithelial scrape, photorefractive keratectomy or other mechanical injuries.

The excimer laser (193nm) commonly generates stromal irregularity and haze in human and rabbit corneas, especially when used for higher corrections (Netto et al., 2006; Sakimoto et al., 2006). The mouse cornea is unsuitable for performing photorefractive keratectomy (PRK) with excimer laser because its thickness (~100–130 micron) is approximately 3–4 times thinner than rabbit cornea (400–450 microns) or human (~500–700 microns) cornea. It was, therefore, postulated that phototherapeutic keratectomy could be used to generate stromal irregularity and corneal haze in the mouse eye. Our previous studies showed that stromal surface irregularity induced with a fine mesh and the excimer laser significantly increased formation of haze in rabbit corneas associated with structural and functional defects in the epithelial basement membrane (Netto et al., 2006a; Netto et al., 2006b). This knowledge led us to hypothesize that surface irregularity produced by phototherapeutic keratectomy could induce opacity in the mouse cornea to provide a useful mouse model for studying myofibroblast biology. This study confirms that stromal surface irregularity promotes haze formation in the mouse—presumably by triggering structural and functional defects in the epithelial basement membrane.

The biomicroscopic and immunocytochemical analyses performed in this study showed that haze can be induced in the anterior stroma of mouse cornea, especially with a PTK technique that generates stromal surface irregularity. Appearance of significant haze and detection of SMA-positive cells in the PTK-treated corneas (group 3 and 4) was noted at the 4 week time point and not at earlier tested time points (1, 2 or 3 weeks). This is an interesting observation, as our earlier studies performed in rabbit eyes showed the appearance of SMA-positive cells and haze around 2–3 weeks after photo refractive keratectomy (PRK), with a peak around 4 weeks after PRK (Netto et al., 2006a; Netto et al., 2006b; Mohan et al., 2003). The delayed appearance of SMA-positive cells in the mouse corneas after excimer laser surgery could be due to the difference in surgical technique or species wound healing differences. Additional study is needed to determine what factors are most important. We speculate that differences in the timing of myofibroblast appearance in the wounded corneas of different species are due to differences in marker protein expression, such as alpha smooth muscle actin, in the stroma of different species, based on the study performed by Jester and his coworkers (Jester et al. 2005) that showed human, mouse, rabbit, chicken and pig corneal keratocytes have diverse protein expression patterns.

The data from this study provides optimal PTK excimer laser conditions for producing haze preferentially in the anterior stroma of the mouse cornea. Irregular PTK performed after epithelial scraping was found to be the most effective method for generating myofibroblasts in the mouse cornea, and thus for studying corneal biology—particularly corneal haze formation associated with myofibroblast generation *in vivo*. The availability of mutant and genetically altered strains of mice will substantially enhance the capacity for investigating the roles of selected genes in this myofibroblast biology and haze development.

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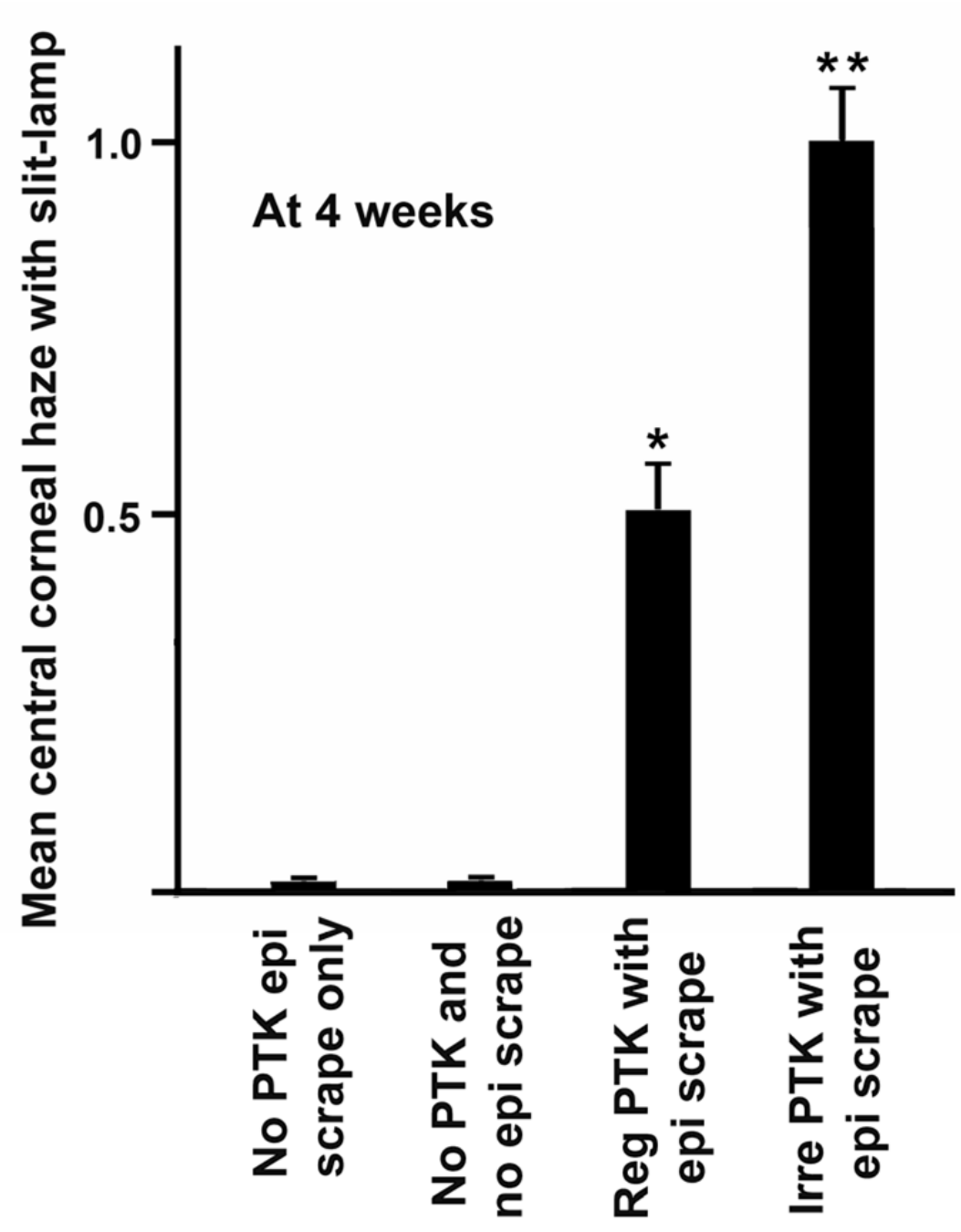

Figure 1.
Biomicroscopic quantification of haze in the central corneas of the mouse eyes of different groups at 4 weeks after PTK/epithelial scrape. None of the corneas showed haze at time points earlier than 4 weeks.

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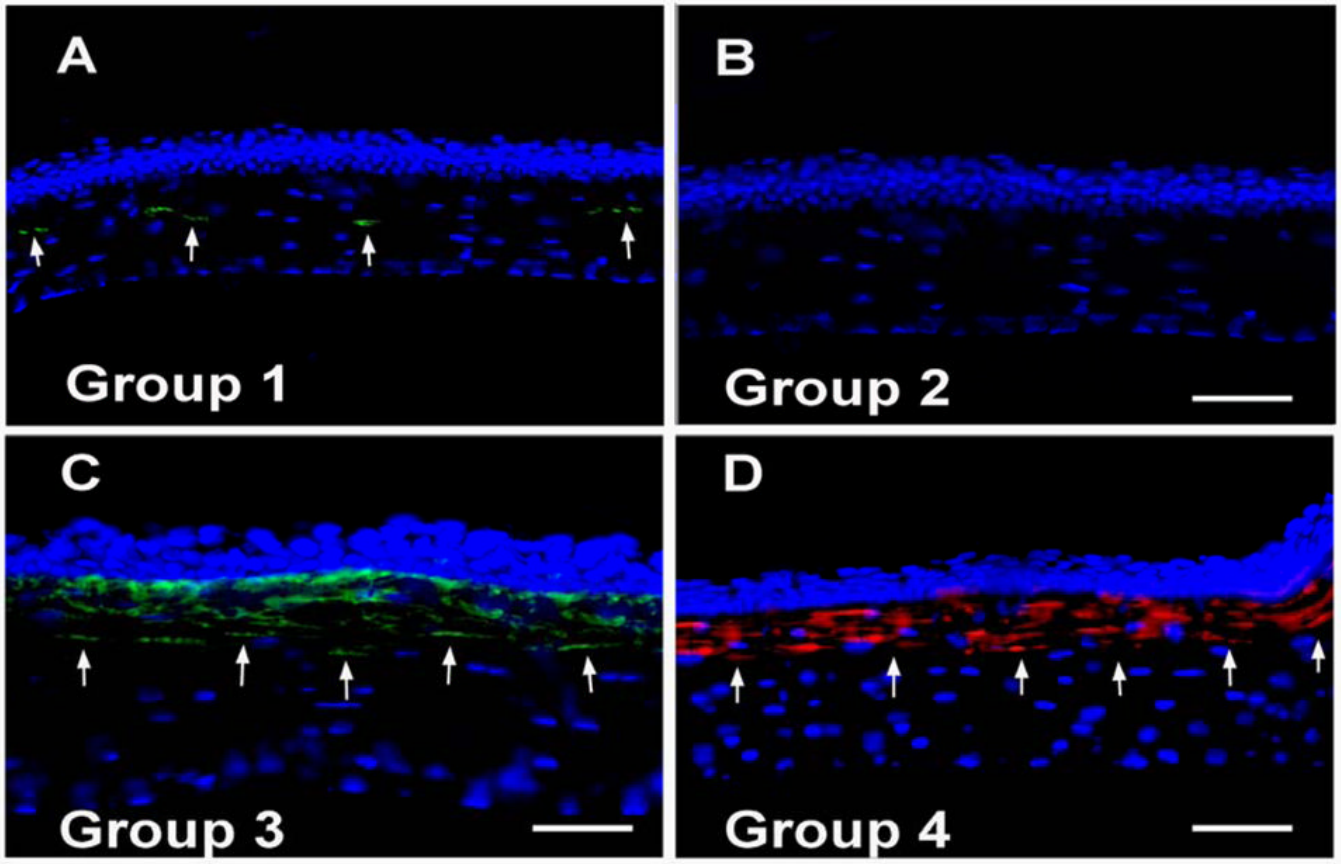

Figure 2.

Alpha-smooth muscle actin staining in the mouse corneas of groups 1 to 4 collected 4 weeks after PTK, epithelial scrape or no treatment. Cell nuclei were stained blue with DAPI and SMA-positive cells were stained green or red. Panels A to D show representative images of the SMA-positive cells detected in the corneas of groups 1 to 4, respectively, collected 4 weeks after treatment. In some experiments, secondary antibody with green fluorescence was used, and in others, secondary antibody with red fluorescence was used. The bar is 50 μm. Magnification 200×.

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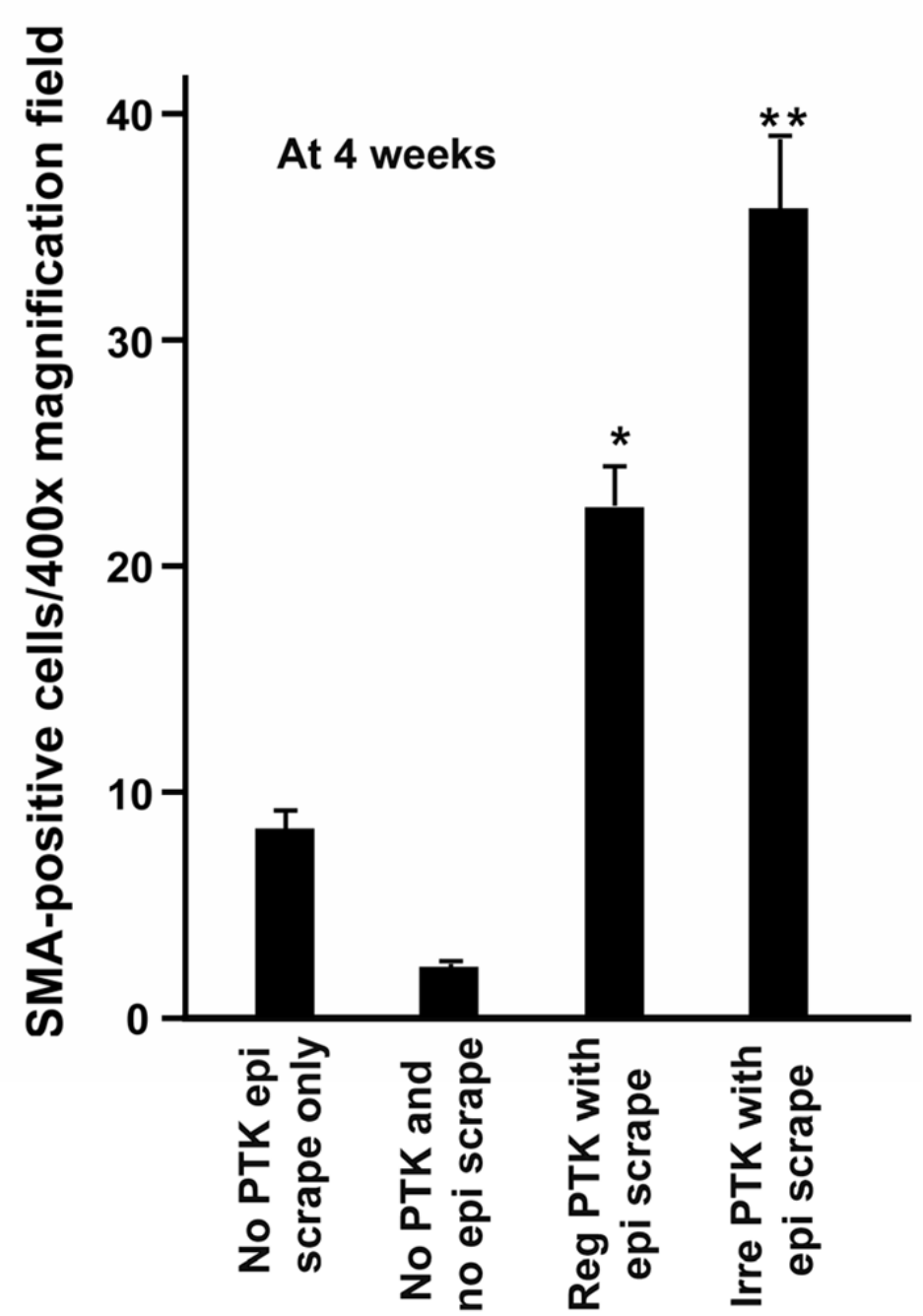

Figure 3.

Quantification of SMA-positive cells/400x column in the mouse corneas of groups 1 to 4 collected 4 weeks after PTK, epithelial scrape or no treatment. ** represents the mouse corneas that received irregular PTK after epithelial scrape (group 4), and showed significantly higher SMA-positive cells ($p < 0.0001$) in the anterior stroma compared to the control corneas (group 1 and 2). * denotes mouse corneas that received regular PTK with epithelial scrape (group 3) that showed statistically significant (p 0.001) higher number of SMA-postive cells in the stroma compared to control tissues (group 1 and 2). The error bars represent the SEM.