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Mouse strain variation in SMA⁺ myofibroblast development after corneal injury

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Abstract

The purpose of present study was to investigate differences in myofibroblast development after haze-generating injury in different commonly used strains of mice. The inbred mouse strains used in this study were Balb/c, C57BL/6, C3H/HeJ and DBA/1J. All mice had uniform irregular phototherapeutic keratectomy with an excimer laser according to a previously published method to generate stromal haze. DBA/1J mice generated significantly greater density of alpha smooth muscle actin (SMA)-positive myofibroblasts in the anterior stroma compared to Balb/c ($p < 0.05$), C57BL/6 ($p < 0.05$) and C3H/HeJ ($p < 0.01$) mice. The C3H/HeJ strain had significantly lower density of SMA-positive myofibroblasts compared to other three mouse strains. These results indicate that mouse strain must be considered in designing experiments and interpreting the results of experiments in which corneal haze and myofibroblast generation is studied in mice. Further investigation of genetics underlying mouse strain variation could provide insight into the corneal wound healing and haze generation processes.

Myofibroblast generation and persistence play a crucial role in the development of stromal opacity (also referred to as haze) during the corneal wound healing response to injuries to the corneal epithelium and stroma (Mohan et al., 2003; Klingberg et al., 2013; Jester et al., 2012; Wilson SE, 2012). Myofibroblasts are fibroblastic cells that have heterogeneous origin depending on the tissue and type of injury (Barbosa et al., 2010; Hinz B., 2007; Novo et al., 2009; Saika et al., 2010; Singh et al., 2012). Myofibroblasts have a major role in the generation of disorganized extracellular matrix that contributes to opacity, extracellular matrix reorganization and wound contraction (Serini & Gabbiani, 1999). Expression of alpha-smooth muscle actin (α-SMA) is hallmark of myofibroblast cells. Over the past decade investigators of corneal wound healing have discussed potential differences in mouse strains at research meetings. The present study was undertaken to examine differences in the myofibroblast response to haze-generating irregular phototherapeutic keratectomy between several strains of commonly used mice.

Four commonly used mouse strains were included in the study: Balb/c, C57BL/6, C3H/HeJ and DBA/1J. Animals were treated in accordance with the tenets of the ARVO Statement for

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the Use of Animals in Ophthalmic and Vision Research and the Animal Control Committee at the Cleveland Clinic approved this investigation. Eight female 8 to 12 week-old mice were included for each group. Haze was generated in one eye of each animal by performing irregular phototherapeutic keratectomy (PTK) over a metallic mesh with the VISX S4 IR laser (AMO, Irvine, CA) using a previously reported technique (Mohan et al., 2008). All treated eyes received one drop of 0.3% Ciprofloxacin antibiotic twice a day until the epithelium was healed. Opacity (haze) in the cornea was photographed with a slit lamp (Topcon, Oakland, NJ) at one month after PTK (Fig.1).

Mice were euthanized at one month and the treated eye and contralateral control eye were enucleated and embedded in OCT compound (Sakura FineTek, Torrance, CA) inside a tissue mold (Fisher, Pittsburgh, PA). The tissue specimens were centered so that the block was bisected and transverse sections cut from the center of the cornea through the mouse eye. Central corneal sections (7-micrometer thick) of the eyes were cut with a cryostat (HM 505M, Micron GmbH, Walldorf, DE, Germany). Sections were placed on 25 × 75 × 1 mm microscope slides (Superfrost Plus, Fisher) and frozen at -80°C until staining was performed.

Immunofluorescence staining was performed on different tissue sections as previously described (Singh et al., 2011). Briefly, sections from the central cornea were stained for SMA (# ab5694, Abcam, San Francisco, CA) diluted 1:50 in 1% BSA for ninety minutes. Sections were then incubated with secondary antibody (A11037, Invitrogen, Carlsbad, CA) for sixty minutes before washing with phosphate buffered saline three times. Immunocytochemical controls were performed for each group by 1) omitting primary antibody or 2) substituting mouse non-specific IgG1 for the primary antibody. Cover slips were mounted with Vectashield containing 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, BC, Canada) camera and ImagePro software. All immunohistochemistry was performed at least three times and achieved consistent results in each experiment. SMA⁺ cells in each cornea were counted real time at the microscope. Counts of the density of SMA⁺ cells per unit volume tissue were performed in the central cornea by counting the number of SMA⁺ stromal cells per 400× full thickness column in the central cornea and averaging the densities for three adjacent fields (Singh et al., 2012).

Haze was generated at one month after irregular PTK in all four strains of mice but was more pronounced in the corneas of DBA/1J strain mice (Fig. 1). At one month after irregular PTK the central stroma had significantly higher SMA-positive cell density (Fig. 2) in the DBA/1J mouse strain compared to other three strains (C3H/HeJ, C57/BL6 and Balb/c). The difference between the C3H/HeJ mice strain and the Balb/c ($p=0.17$) or C57/BL6 ($p=0.078$) did not reach statistical significance.

The present study demonstrates that important differences in the corneal myofibroblast generation response to corneal injury may be noted between different strains of mice. This suggests that genetic differences between the strains have a role in regulating myofibroblast generation from precursor cells. The DBA/1J mouse strain is widely used for collagen-induced arthritis studies (Courtenay et al., 1980; Kato et al., 1996; Myers et al., 1997). Differences in wound healing responses between mouse strains have been noted in other organs (Shi et al., 1997; van den Borne et al., 2009). Further investigations into the specific genetic factors that modulate the differences between the strains of mice could provide important insights into the regulation of corneal wound healing. Investigators must consider these differences when designing studies of corneal wound healing in mice.

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Highlights Wilson

- Mice that have irregular phototherapeutic keratectomy develop stromal haze by one month after surgery
- Myofibroblast cells that develop in the cornea are primarily responsible for haze
- The myofibroblast response to injury varies with different mouse strains

Fig.1. Representative slit lamp photo of corneas from different mouse strains at one month after irregular PTK. Arrows indicate sub-epithelial stromal haze.

A- Negative Control (without primary antibody)

B- Balb/C mice

C- C57BL/6 mice

D- C3H/HeJ mice **E- DBA/1J mice**

Fig.2.

Representative images of α -smooth muscle actin (α -SMA) expressing myofibroblasts in the central cornea of different strains of mice at one month after irregular PTK. **A.** Negative control for IHC in which the primary antibody was omitted; **B.** Balb/c; **C.** C57/BL6; **D.** C3H/HeJ; **E.** DBA/1J. The left lane shows the nuclei of all cells stained blue with DAPI. The middle lane shows α -SMA+ cells stained red. The right lane is an overlay of the DAPI and α -SMA staining. The arrows indicate representative α -SMA-positive cells. Magnification is 400X. **F.** Mean $\pm$ SE density of SMA+ cells per 400 $\times$ stromal column for each mouse strain. *Indicates the mean for the DBA/1J strain was significantly different from the C57/BL6 or Balb/c strains ($p < 0.05$). # indicates the mean for the DBA/1J strain was significantly different from C3H/HeJ mouse strain ($p < 0.01$). The differences between the C57/BL6, Balb/c, and C3H/HeJ strains did not reach statistical significance. Note that quantization of α -SMA-positive cell density was performed real-time at the microscope and not from captured images.