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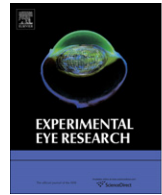

Corneal myofibroblast generation from bone marrow-derived cells

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ABSTRACT

The purpose of this study was to determine whether bone marrow-derived cells can differentiate into myofibroblasts, as defined by alpha-smooth muscle actin (SMA) expression, that arise in the corneal stroma after irregular phototherapeutic keratectomy and whose presence within the cornea is associated with corneal stromal haze. C57BL/6J-GFP chimeric mice were generated through bone marrow transplantation from donor mice that expressed enhanced green fluorescent protein (GFP) in a high proportion of their bone marrow-derived cells. Twenty-four GFP chimeric mice underwent haze-generating corneal epithelial scrape followed by irregular phototherapeutic keratectomy (PTK) with an excimer laser in one eye. Mice were euthanized at 2 weeks or 4 weeks after PTK and the treated and control contralateral eyes were removed and cryo-preserved for sectioning for immunocytochemistry. Double immunocytochemistry for GFP and myofibroblast marker alpha-smooth muscle actin (SMA) were performed and the number of SMA+GFP+, SMA+GFP−, SMA−GFP+ and SMA−GFP− cells, as well as the number of DAPI+ cell nuclei, per 400× field of stroma was determined in the central, mid-peripheral and peri-limbal cornea. In this mouse model, there were no SMA+ cells and only a few GFP+ cells detected in unwounded control corneas. No SMA+ cells were detected in the stroma at two weeks after irregular PTK, even though there were numerous GFP+ cells present. At 4 weeks after irregular PTK, all corneas developed mild to moderately severe corneal haze. In each of the three regions of the corneas examined, there were on average more than 9× more SMA+GFP+ than SMA+GFP− myofibroblasts. This difference was significant ($p < 0.01$). There were significantly more ($p < 0.01$) SMA−GFP+ cells, which likely include inflammatory cells, than SMA+GFP+ or SMA+GFP− cells, although SMA−GFP− cells represent the largest population of cells in the corneas. In this mouse model, the majority of myofibroblasts developed from bone marrow-derived cells. It is possible that all myofibroblasts in these animals developed from bone marrow-derived cells since mouse chimeras produced using this method had only 60–95% of bone marrow-derived cells that were GFP+ and it is not possible to achieve 100% chimerization. This model, therefore, cannot exclude the possibility of myofibroblasts also developed from keratocytes and/or corneal fibroblasts.

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1. Introduction

Corneal myofibroblast generation is associated with injury to the cornea (Jester et al., 1999b; Mohan et al., 2003). These cells are integral to wound contraction and regression that occur following incisional procedures such as radial keratotomy (Garana et al., 1992) and the development of "haze" or stromal opacity following excimer laser surface ablation procedures such as photorefractive keratectomy (PRK) or phototherapeutic keratectomy (Mohan et al., 2003; Netto et al., 2006).

Many studies have demonstrated that myofibroblasts can develop from corneal fibroblasts *in vitro* when the cells are exposed to transforming growth factor beta (Masur et al., 1996; Jester et al., 1999a, 2002). These studies have naturally underlain the widely held dogma that myofibroblasts in the cornea are solely derived from keratocytes and their progeny cells. Recently, however, studies in many other tissues, including lung, skin and liver, have demonstrated that myofibroblasts can be derived from bone marrow-derived cells (Direkze et al., 2003; Hashimoto et al., 2004; Ogawa et al., 2006). In the present study, chimeric mice expressing

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enhanced green fluorescent protein (EGFP) in bone marrow-derived cells are used to conclusively demonstrate that corneal myofibroblasts can originate from bone marrow-derived precursor cells.

2. Materials and methods

2.1. Animals and surgery

All animals were treated in accordance with the tenets of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The Animal Control Committee at the Cleveland Clinic approved the animal studies included in this work. Anesthesia was obtained with an intraperitoneal injection of 130 μg ketamine and 8.8 μg xylazine per gram of body weight and 1 drop of 1% proparacaine HCl (Alcon, Ft. Worth, TX, USA) applied topically to the eye when phototherapeutic keratectomy was performed. Euthanasia was performed with an inhaled overdose of 5% isofluorane gas.

The EGFP chimera mice were generated in our laboratory using an adaptation of the method of Van Parijs et al. (1999). In brief, C57BL/6J (Catalog 000664, The Jackson Laboratory, Bar Harbor, Maine) recipient mice received a total of 800 rads of whole body radiation in two doses (400 rads and 400 rads) 4 h apart using a 137Cs Mark 1 irradiator (J.L. Sheperd & Associates, Glendale, CA). Recipient mice received gentamycin 5 mg/kg IP 2 days prior and 7 days after irradiation. The tibia and femur of C57BL/6-TgN(ACT-bEGFP)1Osb mice (Catalog 003291, The Jackson Laboratory) were isolated after euthanasia. This EGFP-expressing transgenic mouse line expresses an enhanced green fluorescent protein cDNA under the control of a chicken beta-actin promoter and cytomegalovirus enhancer in all cells except erythrocytes (Okabe et al., 1997). The end of the bones were cut with Wescott scissors and the bone marrow was flushed from the bones with a 10cc syringe and a 30 gauge needle using 1–3 ml of DMEM medium (Fisher Scientific, Pittsburgh, PA) containing 10% fetal bovine serum (FBS, Fisher Scientific) under sterile conditions. The bone marrow cells were collected into a 6 cm sterile tissue culture dish (Fisher Scientific) containing 4 ml of the DMEM medium with FBS. The cells were washed twice with 2 ml of sterile phosphate buffered saline (0.137 M sodium chloride, 0.0027 M potassium chloride, and 0.0119 M phosphate buffer) in a 50 ml test tube and pelleted at 500 rpm for 5 min in a centrifuge at 4 °C. Red blood cells were lysed by incubating cell pellet in 5 ml of modified Gormori's Tris Azo Coupling (TAC) Buffer for 10 min at room temperature. The reaction mixture was centrifuged and resulting pellet was suspended in 2 ml of DMEM medium with FBS. After cell counting, 1–3 million cells (in 200 μl) was injected intravenously into the tail vein of the irradiated C57BL/6J recipient mice. This procedure reconstitutes the thymus, spleen, bone marrow and peripheral blood of the recipient mice with EGFP-positive bone marrow-derived cells.

Four weeks after injection, 100 μl of blood was drawn from recipient mice by cutting the tip of the anesthetized tail and the blood was processed for fluorescence-activated cell sorting (FACS) using the Easy-Lyse Whole Blood Erythrocyte Lysing Kit (Leinco Technologies, St. Louis, MO). In brief, the blood was collected in tubes containing 0.8 ml of 0.5 M EDTA to prevent coagulation and 2 ml of 1× Easy lysis buffer was added. The mixture was incubated for 10 min at room temperature. After centrifugation at 500 RPM for 2 min, the cell pellet was washed twice with 1× FACS washing buffer and cells were fixed in 0.5 ml of 1× FACS fixative. The flow cytometry was performed with a Becton–Dickinson (Franklin Lakes, New Jersey) FACScan. Twenty-four chimeric mice showing greater than 60% (range 61–90%) EGFP+ bone marrow-derived cells were subsequently used in this study.

Haze-generating irregular phototherapeutic keratectomy (PTK) was performed on the chimeric mice in one eye selected at random with a VISX S4IR excimer laser (Abbott Laboratories, Irvine, CA) as previously reported (Mohan et al., 2008). Briefly, the corneal epithelium was scrapped with a #64 Beaver blade (Becton–Dickinson) and PTK was performed by firing 45 pulses of laser (ablation depth approximately 10 μm) with a beam diameter of 2 mm on the central cornea, sparing the limbus. The irregularity was generated by positioning a fine mesh screen in the path of laser for the final 50% of the pulses (Mohan et al., 2008).

2.2. Cornea tissue preparation and immunocytochemistry

At 2 weeks (3 eyes) or 4 weeks (21 eyes) after PTK, haze formation was gauged with slit lamp biomicroscope and the animals were euthanized. The experimental and contralateral control eyes were removed with 0.12 forceps and Westcott scissors, embedded in liquid OCT compound (Sakura FineTek, Torrance, CA) within a 15 mm × 15 mm × 5 mm mold (Fisher Scientific, Pittsburgh, PA) and snap frozen using previously reported methods (Mohan et al., 2003). The frozen tissue blocks were maintained at −85 °C. Tissue sections (7 μm) were cut with a cryostat (HM 505M, Micron GmbH, Walldorf, Germany) and maintained frozen at −85 °C until staining was performed.

Double-immunofluorescence staining was performed on experimental and control tissue sections to study the co-expression of EGFP and alpha-smooth muscle actin (α-SMA) in corneas. The polyclonal rabbit anti-green fluorescent protein antibody (Cat. #AB3080, Millipore, Eugene, OR) was diluted 1:50 in PBS with 1% bovine serum albumin (BSA, Promega, Madison, WI) and placed on the slides for 90 min at room temperature. Sections were washed in PBS and the secondary antibody, goat anti-rabbit IgG (H + L) Green, Alexa Fluor 488 (Cat. #A11034, Invitrogen, Carlsbad, CA) was applied at a concentration of 1:100 in PBS for 60 min at room temperature. The sections were subsequently incubated for 60 min with normal rabbit serum (Cat. # 011-000-120, Jackson ImmunoResearch, West Grove, PN) diluted 1:5 with PBS. After washing again with PBS, a fourth incubation was performed with excess un-conjugated, donkey anti-rabbit IgG (H + L) (Cat. #711-007-003, Jackson ImmunoResearch, West Grove, PN) diluted 1:200 in PBS for 60 min at room temperature. Alpha-smooth muscle actin (α-SMA) was detected by incubating tissue sections with a rabbit polyclonal antibody (Cat. # ab5694, Abcam, Cambridge, MA) diluted 1:50 in PBS with 1% BSA for 90 min at room temperature. Sections were washed with PBS and then incubated with Alexa Fluor 594 (Cat. # A11037, Invitrogen, Carlsbad, CA) secondary antibody, goat anti-rabbit IgG (H + L) (Red) diluted 1:500 in PBS for 60 min at room temperature. Immunocytochemistry controls were performed by omitting one or both primary antibodies.

Coverslips 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.

2.3. Quantification of cells

SMA+ and GFP+ cells were counted real time under the microscope. Counts were made on sections from ten different corneas that were removed at 4 weeks after PTK. In each case, counts of SMA+/GFP+, SMA+/GFP−, SMA−/GFP+ and SMA−/GFP− cells in randomly selected, full thickness 400× microscopic fields from the 1) central, 2) mid-peripheral and 3) periphery peri-limbal cornea were performed, as previously described (Mohan et al., 2003).

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