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Stromal fibroblast–bone marrow-derived cell interactions: Implications for myofibroblast development in the cornea

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Abstract

The purpose of this study was to test the hypothesis that mouse corneal stromal fibroblast and bone marrow-derived cell interactions augment corneal myofibroblast generation and, if so, to study whether such interactions are mediated by paracrine or juxtacrine mechanisms. Mouse bone marrow-derived cells and mouse corneal stromal fibroblasts were obtained from both mice with green fluorescent protein (GFP) expressed in all cells and normal GFP– BL6 control mice. To study the interactions of the different cell types, GFP+ cells of one type were co-cultured with GFP– cells of the other type in Primaria plates (to monitor juxtacrine signaling) or Transwell System plates (to monitor paracrine effects mediated by soluble mediators). Both cell types were cultured at a cell density of 1×10^5 cells/ml. The percentage of alpha smooth muscle actin+ myofibroblasts was significantly higher (ANOVA, $p < 0.001$) when bone marrow-derived cells and mouse corneal stromal fibroblasts were co-cultured compared to when bone marrow-derived cells and mouse corneal stromal fibroblasts were cultured alone (control). The *in vitro* studies using GFP+ corneal fibroblasts or GFP+ bone marrow-derived cells demonstrated conclusively that both cells types could transform into myofibroblasts. However, the percentage of alpha smooth muscle actin+ myofibroblasts generated from either cell type precursor was higher when both cells were co-cultured together (juxtacrine) as compared to when bone marrow-derived cells and mouse corneal stromal fibroblasts were co-culture in different compartments of Transwell System (paracrine). Thus, more alpha smooth muscle actin+ GFP+ myofibroblasts were generated from GFP+ corneal stromal fibroblasts when GFP– bone marrow-derived cells were present and more alpha smooth muscle actin+ GFP+ myofibroblasts were generated from GFP+ bone marrow-derived cells when GFP– corneal stromal fibroblasts were present. Polyclonal anti-human latency associated peptide (LAP) (transforming growth factor- β 1) neutralizing antibody (a-LAP) and/or transforming growth factor- β type I receptor kinase inhibitor (LY-364947) inhibited the generation of alpha smooth muscle actin+ myofibroblasts from either precursor cell in Transwell System co-culture experiments. These data suggest that TGF β is a paracrine modulator that regulates the generation of myofibroblasts from either corneal fibroblasts or bone marrow-derived cell precursors.

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1. Introduction

Persistent corneal stromal opacity or scarring, also referred to as haze, is associated with myofibroblast generation and the deposition of abnormal extracellular matrix material in the stroma (Jester, et al., 1999a; Mohan, et al., 2003; Netto, et al., 2006). Under normal conditions, keratocytes are relatively quiescent and their primary function is to maintain collagen and other extracellular matrix components in the stroma (West-Mays and Dwivedi, 2006). During the wound healing process, a complex stromal response is initiated that can lead to the formation of alpha smooth muscle actin expressing myofibroblasts and the deposition of large quantities of abnormal extracellular matrix components, including collagen types that are not found in the normal uninjured cornea (Guarino, et al., 2009; Jester, et al., 1999a; Mohan, et al., 2003; Wynn, et al., 2007, 2008).

Myofibroblasts are fibroblastic cells that may be derived from a variety of precursor cells, including fibroblasts, epithelial cells and bone marrow-derived cells (Novo, et al., 2009; Barbosa, et al., 2010; Saika, et al., 2010). Corneal myofibroblasts can be derived from bone marrow-derived cells in vivo in mice (Barbosa, et al., 2010), and other studies have demonstrated that bone marrow-derived precursors give rise to myofibroblasts in lung, liver, heart and skin tissues (Direkze, et al., 2003; Fathke, et al., 2004; Hashimoto, et al., 2004; Ishii, et al., 2005; Mori, et al., 2005). Studies from Bucala, et al (1994) and Abe, et al (2001) have demonstrated unique circulating fibroblast-like cells termed “fibrocytes” derived from bone marrow stem cells that migrate to injury sites.

Transforming growth factor- β (TGF β) produced by corneal epithelial cells, corneal stromal fibroblasts, and possibly other cell types, is an important regulator of the wound healing response in the cornea, including the generation of myofibroblasts (Masur, et al., 1996; Jester, et al., 1999b; Saika, et al., 2006; Wilson, et al., 1994; Zhang and Phan, 1999; Tandon, et al., 2010; Imanishi, et al., 2000). Once generated, myofibroblasts themselves secrete TGF β and can thereby sustain their own activation through autocrine mechanisms (Thannickal, et al., 2004; Flanders, 2004; Kaur, et al., 2009).

Our working hypothesis is that corneal myofibroblasts can be generated from both bone marrow-derived precursors and corneal-derived precursors, and that the dominant precursor in a particular cornea is determined by the type of injury, genetic factors, and perhaps other unknown factors such as paracrine or juxtacrine interactions between the different cells present during the corneal stromal wound healing response. In this study, we examined the cellular interactions that may underlie the differentiation of either bone marrow-derived cells or corneal stromal fibroblasts into myofibroblasts.

2. Method and material

2.1. Animals

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. Eight to twelve week old C57BL/6 or C57BL/6-Tg(UBC-GFP)30 Scha/J female mice were obtained from The Jackson Laboratory (Bar Harbor, Maine). The transgenic mice express enhanced green fluorescent protein (GFP) under the direction of the human ubiquitin C promoter. Mice homozygous for the transgene are viable, fertile, normal in size and do not display any gross physical or behavioral abnormalities. These mice express GFP in all tissues examined except red blood cells.

2.2. Isolation of adherent bone marrow-derived cells

Primary cultures were established using previously described methods (Penn et al., 1993; Fei et al., 1990) with slight modification. Briefly, both upper femurs of the mice were removed and maintained in chilled PBS. All soft tissues were removed and the femur was placed in a sterile petri-plate with MEM-F [Eagle's minimum essential medium (MEM) with Earle's salts + 15% heat-inactivated FBS, henceforth designated MEM-F]. Femurs were flushed with MEM-F medium using 26 gauge needles and the suspension was passed through 18–22 G needles multiple times to prepare a single-cell suspension. The cells were counted and cultured at a concentration of 1×10^7 cells per ml in 35 or 60 mm BD culture dishes (BD Biosciences, San Jose, CA) in MEM-F media for five to seven days. The supernatant containing the non-adherent cells was poured off and the adherent cells were washed with PBS twice and then trypsinized with 0.5% trypsin-EDTA for experiments.

2.3. Isolation of mouse corneal stromal fibroblasts

Corneas were removed from mouse eyes using a 2 mm trephine and the Descemet's-endothelium complex stripped away with 0.12 mm forceps. The remaining epithelium and stroma were incubated in 5 mg/ml of Dispase II (Roche Diagnostic, Mannheim, Germany) at 4°C overnight. The loose epithelial sheets were removed and the corneal stromal disc was cut into small segments with a scalpel under the dissecting microscope. The stromal fragments were cultured in 25 cm² culture flask (BD Biosciences, San Jose, CA) with sufficient MEM-F medium to cover the explants without floating them off the bottom. The culture medium was changed every 48 hours until primary corneal fibroblast cells grew to near-confluence. Primary cells were trypsinized and either stored in liquid nitrogen or immediately processed for further experiments. One to three passage corneal fibroblasts were used for all experiments (Kaur et al., 2009).

2.3. Cytokines and inhibitors

Polyclonal goat anti-human latency associated peptide (LAP) (TGF- β 1) neutralizing antibody (Catalog Number: AB-246-NA) was obtained from R&D Systems, Inc. (Minneapolis, MN) and has been shown to bind mouse LAP (Tsang et al., 1995). Transforming growth factor- β type I receptor kinase inhibitor (LY-364947) was obtained from Sigma (St Louis, MO). LY-364947 is a selective, ATP-competitive inhibitor of transforming growth factor- β type I receptor kinase. Preliminary dose response experiments were performed with a-LAP and LY-364947 using concentrations from 1 to 10 ng/ml. The 5.0 ng/ml concentration of both TGF β inhibitors had similar potency to the 10 ng/ml concentration. We used the 5.0 ng/ml concentration as the standard concentration for the subsequent experiments presented in this manuscript.

2.4. Co-culture condition of bone marrow derived cells with mice corneal stromal fibroblasts

Bone marrow-derived cells (1×10^5 cells per ml) were plated in 12-well flat-bottom plates (Corning Costar Co. NY, USA) with cover slips (Cat no: 12-545-100, Fisher, Pittsburgh, PA) or Primaria slides (BD Biocoat-354559, Bedford, MA) in MEM-F medium for three days. Mouse corneal stromal fibroblasts (1×10^5 cells per ml) isolated from normal or GFP C57/BL/6 mice were loaded directly onto the mouse bone marrow-derived cell cultures on Primaria slides or separated from mouse bone marrow-derived cells using a Transwell System with 0.4 μ m diameter pore size (Cat no: 3460, Corning Costar Co., NY, USA). The co-culture systems were maintained for 72 hours in MEM-F medium. Some of the co-cultures were treated with 2.0 ng/ml recombinant human transforming growth factor (TGF) β 1, 5.0 ng/ml anti-LAP (TGF- β 1) antibody (A-LAP) or 5.0 ng/ml transforming growth

factor- β type I receptor kinase inhibitor (LY-364947). All experiments were conducted in triplicate at least three separate times using pooled cells from several different animals.

2.5. Immunocytochemistry protocol

Immunofluorescence staining was performed per the Invitrogen (Carlsbad, CA) protocol with slight modification. Briefly, media was removed from the cells grown in Transwell system or Primaria slides. Cells were rinsed twice with phosphate buffered saline (0.1M PBS, pH 7.4). Cells were then fixed in the dark with 4% paraformaldehyde in PBS for 30 minutes at room temperature with gentle agitation. Cells were then washed twice with PBS for one minute with gentle agitation. Cells were permeabilized with permeabilization solution (0.25% Triton® X-100 (Sigma, St. Louis, MO) in PBS) in the dark for five minutes at room temperature with gentle agitation. Cells were subsequently washed twice in PBS for one minute with gentle agitation. Blocking solution [5% BSA (Sigma, St. Louis, MO) in PBS, pH 7.4] was added and the cells were incubated for one hour at room temperature with gentle agitation. Cells were washed twice in PBS for one minute each with gentle agitation. Cells were stained for alpha smooth muscle actin (ab5694, chicken antibody, Abcam, San Francisco, CA) diluted 1:50 in 1% BSA and/or GFP antibody (ab13970, goat antibody, Abcam) diluted 1: 200 in 1% BSA for 90 minutes and then incubated with respective secondary antibodies [A11037; Alexa Fluor 594 goat anti—rabbit IgG, Invitrogen (1:200 dilution) or A11039; Alexa Fluor 488 goat anti—chicken IgG, Invitrogen (1:500 dilution)] for 60 minutes. Immunocytochemistry controls were performed by omitting primary antibodies. Coverslips were mounted with Vectashield containing DAPI (Vector Laboratories Inc., Burlingame, CA) to allow visualization of all nuclei in the cells. All immunocytochemistry experiments were performed at least three times to insure results were consistent. The slides were viewed and photographed with a Leica DM5000 microscope equipped with Q-Imaging Retiga 4000RV (Surrey, BC, Canada) camera and ImageProsoftware.

2.6. Quantification of cells

For each slide or coverslip, the total number of cell nuclei and the number of alpha smooth muscle actin+ and/or GFP+ cells were counted in randomly selected, noncontiguous 400X microscopic fields until 100 cells were counted. The results were expressed as the percentage of alpha smooth muscle actin+ and/or GFP+ cells / total DAPI positive cells counted real time at the microscope.

2.7. Statistical analysis

Statistical comparisons between the different experimental conditions were performed using analysis of variance (ANOVA). $p < 0.01$ was considered statistically significant due to the large number of tests performed. Results are expressed as the mean $\pm$ standard error.

3. Results

3.1. Direct co-culture experiments

When GFP+ adherent bone marrow-derived cells were cultured in the same well with GFP– corneal stromal fibroblast for 72 hours on Primaria slides, approximately 80–90% of corneal, as well as bone marrow-derived cells, become alpha smooth muscle actin+. The percentage of alpha smooth muscle actin+ GFP+ bone marrow-derived cells was significantly higher (ANOVA, $p < 0.001$) when co-cultured with GFP– corneal stromal fibroblasts compared to when GFP+ adherent bone marrow-derived cells were cultured alone (control) on Primaria slides (Fig. 1 and Fig. 2).

There was approximately a 5% to 10% decrease in the formation of alpha smooth muscle actin+ GFP+ bone marrow-derived cells when 5.0 ng/ml monoclonal anti-LAP (TGF- β 1) antibody and/or 5.0 ng/ml transforming growth factor- β type I receptor kinase inhibitor (LY) was added in the co-culture media, as compared to when GFP+ bone marrow-derived cells and GFP- corneal stromal fibroblast were co-cultured in the same culture without TGF β blockers in MEM-F media, but the results did not reach statistical significance ($p > 0.01$) (Fig. 2).

When GFP+ corneal stromal fibroblast were cultured together in the same well with GFP- bone marrow-derived cells for 72 hours on Primaria slides, approximately eighty to ninety percent of corneal, as well as bone marrow-derived cells, become alpha smooth muscle actin +. The percentage of alpha smooth muscle actin+ GFP+ corneal stromal fibroblast was significantly higher (ANOVA, $p < 0.001$) when co-cultured in same well with GFP- bone marrow-derived cells compared to when GFP+ corneal stromal fibroblast cells were cultured alone in Primaria slides (control) (Fig. 3 and Fig. 4).

There was decrease in the formation of alpha smooth muscle actin + GFP+ corneal stromal fibroblast when 5.0 ng/ml anti-LAP (TGF- β 1) antibody and/or 5.0 ng/ml transforming growth factor- β type I receptor kinase inhibitor (LY) was added in the co-culture media, as compared to when bone marrow-derived cells and corneal stromal fibroblast are co-cultured in the same culture without TGF β inhibitors in MEM-F media but results did not reach statistical significance ($p > 0.01$) (Fig. 4).

3.2. Transwell System co-culture experiments

GFP+ SMA+ corneal myofibroblast differentiation increased significantly ($p < 0.001$) when GFP+ corneal stromal fibroblast (top chamber) were co-cultured with adherent GFP- bone marrow-derived cells (bottom chamber) of a Transwell System compared to GFP+ corneal stromal fibroblast cells cultured alone in the top chamber (control) (Fig. 5).

When 5.0 ng/ml monoclonal anti-LAP (TGF- β 1) antibody and/or 5.0 ng/ml transforming growth factor- β type I receptor kinase inhibitor (LY) were present in the co-culture media, the percentage alpha smooth muscle actin+ corneal myofibroblasts decreased significantly (ANOVA, $p < 0.01$), as compared to when cells were co-cultured in the Transwell System with adherent GFP- bone marrow-derived cells in normal MEM-F media without inhibitors (Fig. 5).

However, GFP+ SMA+ corneal myofibroblast differentiation was at least 50% lower ($p < 0.01$) when GFP+ corneal stromal fibroblast were co-cultured with adherent GFP- bone marrow-derived cells in Transwell Systems compared to when both the cells were cultured together in Primaria slides to monitor juxtacrine effects in all experiments. GFP+ alpha smooth muscle actin+ myofibroblasts differentiated from

GFP+ bone marrow-derived cells (top chamber) at significantly increased numbers ($p < 0.001$) when co-cultured with GFP- corneal stromal fibroblast cells in the bottom chamber of a Transwell System, compared to adherent GFP+ bone marrow-derived cells cultured alone in the top well of a Transwell System (control) (Fig. 6). When 5.0 ng/ml anti-LAP (TGF- β 1) antibody or 5.0 ng/ml transforming growth factor- β type I receptor kinase inhibitor (LY) was added to the co-culture medium, the percentage alpha smooth muscle actin+ GFP+ bone marrow-derived cells decreased significantly ($p < 0.01$), as compared to when the cells were co-cultured in normal MEM-F media (Fig. 6).

Nevertheless, the GFP+ alpha smooth muscle actin+ bone marrow-derived myofibroblast differentiation was more than 50% lower ($p < 0.01$) when GFP- corneal stromal fibroblast

were co-cultured with adherent GFP+ bone marrow-derived cells in Transwell Systems compared to when both the cells were cultured together in Primaria slides to monitor juxtacrine effects.

4. Discussion

Several studies have demonstrated that myofibroblasts that develop in the cornea after injury can be derived from bone marrow-derived cells (Barbosa, et al., 2010; Saika, et al., 2010). In another study in rabbits, PRM-151 (an inhibitor of differentiation of circulating monocytes into fibrocytes and profibrotic macrophages) inhibited myofibroblast generation in the cornea after photorefractive keratectomy when administered by sub-conjunctival injection (Santhiago, et al., 2011). The specific pathophysiological roles of myofibroblasts generated from bone marrow-derived cell precursors or corneal fibroblast precursors—individually or in combination—remains to be elucidated in the corneas of mice, rabbits, or humans in vivo.

Transforming growth factor beta (TGF β) has been shown to be a key growth factor regulating myofibroblast development and persistence in both corneal and non-corneal tissues (Masur, et al., 1996; Jester, et al., 1999b; Flanders, 2004; Tandon, et al., 2010). The hypotheses examined in this study were: (i) Do interactions between corneal fibroblasts and bone marrow-derived cells promote the development of myofibroblasts? (ii) Can either cell type serve as the myofibroblast precursor during these cell interactions? and (iii) Is TGF β signaling involved in possible interactions between corneal fibroblasts and bone marrow-derived cells that give rise to myofibroblasts?

When GFP+ bone marrow-derived cells and GFP- corneal stromal fibroblasts were cultured in same well in vitro, significantly more (>80%) of the bone marrow-derived cells became GFP+ alpha smooth muscle actin+ myofibroblasts (Fig. 1 and 2), compared to when GFP+ bone marrow-derived cells were cultured alone (approximately 30%). Similarly, when GFP+ corneal stromal fibroblasts and GFP- bone marrow-derived cells were cultured in the same well in vitro, significantly more (>80%) of the corneal fibroblasts became GFP+ alpha smooth muscle actin+ myofibroblasts (Fig. 3 and 4), compared to when GFP+ corneal stromal fibroblasts were cultured alone (less than 10%). These results conclusively demonstrate that corneal myofibroblasts can develop from either corneal fibroblasts or bone marrow-derived cells, and that interactions between the two cell types augment myofibroblast development from either precursor cell type.

Co-culture in the same well experiments cannot distinguish between juxtacrine or paracrine interactions between the cell types regulating augmented myofibroblast development. Therefore, Transwell System culture experiments were performed to determine whether paracrine interactions between corneal fibroblasts and bone marrow-derived cells can modulate development of myofibroblasts. Seventy to 80% of GFP+ corneal stromal fibroblast cells become alpha smooth muscle actin+ myofibroblasts when they were cultured in a Transwell System with bone marrow-derived cells in the other chamber (Fig. 5). Similarly, 60 to 70% of GFP+ bone marrow-derived cells become alpha smooth muscle actin+ myofibroblasts when they are cultured in a Transwell System with stromal fibroblasts in the opposite well (Fig. 6). These experiments suggest that the interaction between corneal fibroblasts and bone marrow-derived cells can be regulated by paracrine interactions between the cell types mediated by soluble factors. These Transwell System experiments do not exclude the possibility that juxtacrine interactions are also important in interactions between corneal fibroblasts and bone marrow-derived cells in situ.

TGF β Type 1 receptor kinase has been shown to reverse the effect of TGF β via effects on SMAD signaling (Zhou et al., 2011). TGF β 1 LAP complexes with, and inactivates, all TGF β isoforms of humans and mice (Miller et al., 1992). When the TGF β inhibitors TGF β

type 1 receptor kinase inhibitor (LY) and/or anti-LAP (TGF β 1) antibody (a-LAP) were included in the medium in Transwell System experiments with either GFP+ corneal stromal fibroblasts (Fig. 5) or GFP+ bone marrow-derived cells (Fig. 6) as the monitored precursor cells, the percentage alpha smooth muscle actin+ myofibroblasts decreased significantly, suggesting that TGF β is an important factor in modulating paracrine interactions between corneal fibroblasts and bone marrow-derived cells that promote myofibroblast differentiation of either cell type. These experiments do not rule out a role for other cytokines, such as platelet-derived growth factor (PDGF) A (Bostrom et al., 1996), PDGF-BB (Kaur et al., 2009; Tang et al., 1996), interleukin (IL)-6 (Gallucci et al., 2006), or others (Saika et al., 2004; Yin et al., 2005, 2008). Inclusion of the TGF β inhibitors also tended to decrease myofibroblast generation in same-well co-culture experiments, but the results did not reach statistical significance. If a larger number of cultures would have been included in these experiments, these results could have become statistically significant. We hypothesize that the TGF β that regulates precursor cell differentiation into myofibroblasts in situ originates primarily from the corneal epithelium and is modulated by intact epithelial basement membrane, whether the precursor cell that gives rise to the myofibroblast is a keratocyte-derived cell or a bone marrow-derived cell (Netto, et al., 2006). Animal experiments are in progress to test this hypothesis.

These experiments conclusively demonstrate that corneal fibroblasts and bone marrow-derived cells can serve as precursors for development of myofibroblasts and suggest that interactions between the two cell types may be important in myofibroblast generation in the intact cornea. Presently, it is not known what conditions or injury types will trigger one of these cell types to be the predominant precursor for myofibroblast development in a particular cornea. For example, there could be differences between injury-related responses and infection-related responses. Importantly, it is also not presently known whether myofibroblasts that differentiate from corneal fibroblasts have identical functions to myofibroblasts that differentiate from bone marrow-derived cells, or alternatively whether these myofibroblasts have different functions depending on the cell of origin. These alternative functions might include excretion of different matrix materials or differing capacity for tissue contraction. These potential differences in generation and function can be studied using the marker methods used in the present experiments and are currently under investigation.

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- Bone marrow-derived cells can give rise to myofibroblasts in vitro.
- Corneal stromal fibroblasts can give rise to myofibroblasts in vitro.
- One cell type augments myofibroblast generation in the other cell type.
- TGF β regulates the cell interactions that augment myofibroblast development.

Fig. 1.

Representative alpha smooth muscle actin (SMA) immunocytochemistry images of GFP+ bone marrow-derived cells (BMC) when cultured in the same well with normal GFP- mouse corneal stromal fibroblasts (MSF) under varying conditions on Primaria slides for 72 hours. The nuclei of all cells were stained blue with DAPI in each panel. The left lane shows green GFP staining. The middle lane shows red alpha smooth muscle actin staining. The right lane is an overlay of the DAPI, alpha smooth muscle actin and GFP staining. **A.** GFP+ bone marrow-derived cells cultured along with mouse corneal stromal fibroblasts (controls had primary antibody omitted). Note that the light background staining for GFP in a few cells could be autofluorescence of the GFP protein in the cells. **B.** GFP+ bone marrow-derived cells cultured without mouse corneal stromal fibroblasts; **C.** GFP+ bone marrow-derived cells cultured along with mouse corneal stromal fibroblast in same well. Note the heavily GFP+ cells that are also alpha smooth muscle actin+, indicating bone marrow-derived cells that have differentiated into myofibroblasts.

Fig. 2.

Graphical results for the percentage of alpha smooth muscle actin+ cells using a repeated measure ANOVA (mean $\pm$ SE), when GFP+ adherent bone marrow-derived cells were cultured in the same well with GFP- corneal stromal fibroblasts for 72 hours on Primaria slides. *Indicates the result is significant compared to the corresponding controls at $p < 0.01$. The percentages of alpha smooth muscle actin+ GFP+ bone marrow-derived cells (BMC) and/or alpha smooth muscle actin+ mouse corneal stromal fibroblast (MSF) were significantly higher ($p < 0.001$) compared to their respective controls, when culture together in the same culture dish. Note that quantitation was performed real time at the microscope and not from captured images.

Fig. 3.

Representative alpha smooth muscle actin immunocytochemistry images of GFP+ mouse corneal stromal fibroblasts (MSF) when cultured in the same well with GFP- bone marrow-derived cells (BMC) under varying conditions on Primaria slides for 72 hours. The nuclei of all cells were stained blue with DAPI. The left lane shows green staining for GFP. The middle lane shows red alpha smooth muscle actin staining. The right lane is an overlay of the DAPI, alpha smooth muscle actin and GFP staining. **A.** GFP+ mouse corneal stromal fibroblasts cultured alone; **B.** GFP- bone marrow-derived cells cultured alone; **C.** GFP+ mouse corneal stromal fibroblasts cultured along with GFP- bone marrow-derived cells in the same well.

Fig. 4.

Graphical results of the percentage alpha smooth muscle actin+ cells when GFP- adherent bone marrow-derived cells were cultured in the same well with GFP+ corneal stromal fibroblasts for 72 hours on Primaria slides and compared to controls. Data were analyzed using a repeated measure ANOVA (mean $\pm$ SE). *Indicates the result is significant compared to the corresponding controls at $p < 0.01$. The percentage alpha smooth muscle actin+ GFP+ mouse corneal stromal fibroblasts and/or SMA+ bone marrow-derived cells were significantly higher ($p < 0.001$) compared to their respective controls, when cultured together in the same culture dish. Note that quantification was performed real time at the microscope and not from captured images.

Fig. 5.

Graphical results showing the quantification of the percentage alpha smooth muscle actin+ GFP+ mouse corneal stromal fibroblasts. Data were analyzed with a repeated measure ANOVA (mean $\pm$ SE). **Indicates the result was significant compared to the corresponding controls at $p < 0.01$. The percentage of alpha smooth muscle actin+ mouse stromal fibroblast-derived myofibroblasts increased significantly ($p < 0.001$) when they were co-cultured with GFP– bone marrow-derived cells, as compared to when mouse stromal fibroblasts were culture alone (control). When 5.0 ng/ml polyclonal anti-LAP (TGF- β 1) neutralizing antibody (a-LAP) or 5.0 ng/ml transforming growth factor- β type I receptor kinase inhibitor (LY), or both, were present in the co-culture media, the percentage alpha smooth muscle actin+ myofibroblasts decreased significantly (ANOVA, $p < 0.01$), as compared to when cells were co-culture in the Transwell system with adherent GFP– bone marrow-derived cells in normal MEM-F media * Indicates the result is significant compared to when cells were co-culture without any TGF β blockers. No additive effect of both inhibitors was noted. Note that quantitation was performed real time at the microscope and not from captured images.

Fig. 6.

Graphical results showing the quantification of the percentage of alpha smooth muscle actin + GFP+ bone marrow-derived cells. Data were analyzed using a repeated measure ANOVA (mean $\pm$ SE). **Indicates the result is significant compared to the corresponding control without mouse corneal stromal fibroblasts in the bottom well at $p < 0.01$. The percentage alpha smooth muscle actin+ adherent bone marrow-derived cells increased significantly ($p < 0.001$) when they were co-cultured with corneal stromal fibroblasts with a membrane (0.4 μ m sized pores) between them in a Transwell System, as compared to bone marrow-derived cells culture alone (control). When 5.0 ng/ml polyclonal anti-LAP (TGF- β 1) neutralizing antibody (a-LAP) or 5.0 ng/ml transforming growth factor- β type I receptor kinase inhibitor (LY), or both, were added in the co-culture media, the percentage alpha smooth muscle actin + GFP+ bone marrow-derived cells decreased significantly ($p < 0.01$) as compared to when the cells were co-culture in normal MEM-F media *Indicates the result is significant compared to when cells were co-culture without TGF β blockers. No additive effect of including both blockers was noted. Note that quantitation was performed real time at the microscope and not from captured images.