

Concentration-dependent effects of transforming growth factor β 1 on corneal wound healing

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Purpose: There is an unmet challenge to promote wound healing in non-healing wounds such as in the post-LASIK (laser-assisted in situ keratomileusis) cornea. Using human corneal fibroblasts (HCFs) in cell culture, we investigated the concentration dependence of the growth factor transforming growth factor β 1 (TGF β 1) on wound closure. Although high concentrations of TGF β 1 leads to scarring, we asked whether low concentrations of TGF β 1 could promote wound healing without generating a large fibrotic response.

Methods: HCFs were cultured in supplemented serum-free media (SSFM). Cell migration was assessed by scratch-wounding. SMAD 2/3 and p38 mitogen-activated protein kinase (p38MAPK) localization and α -smooth muscle actin (α -SMA) organization were evaluated by immunocytochemistry. Active TGF β was quantified using a luciferase bio-assay.

Results: We found that neutralizing antibody to TGF β 1 reduced cell migration by 73%, compared to immunoglobulin G (IgG) control, establishing that endogenous TGF β 1 (determined to be 0.01 ng/ml) is necessary to promote cell migration. To evaluate the concentration-dependent effects of TGF β 1 on wound closure, HCF migration was quantified to determine the impact of increasing concentrations of TGF β 1 (0.01–1.0 ng/ml). Compared to control (cells in SSFM), the higher concentrations (0.1 and 1.0 ng/ml TGF β 1) significantly decreased cell migration (63%–86%), induced myofibroblast differentiation (83%–88%), increased SMAD 2/3 localization into the nucleus (72%–79%) and inhibited the activation of p38MAPK (51%–63%). In contrast, addition of the lower concentration of TGF β 1 (0.01 ng/ml TGF β 1) promoted a cell migration rate that was similar to endogenous TGF β , reduced SMAD 2/3 nuclear localization, and stimulated p38MAPK activation. A TGF β 1 blocking antibody and the p38MAPK inhibitor, SB202192, was used to demonstrate that p38MAPK activation is necessary for TGF β 1-induced cell migration.

Conclusions: Together, our data demonstrate that low concentrations of TGF β 1 promote p38MAPK activation that is a key to HCF migration, suggesting that a low concentration of TGF β may be useful in treating non-healing corneal wounds.

The identification of signaling pathways that promote fibroblast migration into a corneal wound to promote healing without a fibrotic response is an essential area for study. In a normal wound healing response, resident keratocytes are activated to become fibroblasts and myofibroblasts. Activated resident corneal fibroblasts and bone marrow derived fibrocytes migrate into the wound site [1]. The fibroblast-secreted proteases remodel damaged extracellular matrix (ECM) and secrete new ECM that acts as “glue” sealing the wound [2,3].

After laser-assisted in situ keratomileusis (LASIK), the central flap region is not repopulated with stromal cells and the cornea remains unhealed [4,5]. This results in a dramatic decrease in corneal tensile strength [6,7]. Weakening of the cornea after LASIK has been linked to corneal ectasia whereby the post-LASIK cornea exhibits collagen fibril thinning and decreased interfibril distance [8]. Furthermore, because the central cornea remains acellular, there is an increased risk for corneal edema [4,5]. Although these defects

occur in a small percentage of LASIK patients, they are potentially severe complications that can lead to loss of vision and may become a greater public health issue with the aging of the population who have LASIK corneas.

To advance our understanding of the role of transforming growth factor β (TGF β) in wound healing, we have investigated the concentration dependence of TGF β to wound closure in vitro. A dual role in wound healing has been proposed for TGF β : It promotes fibroblast cell proliferation and cell migration necessary to repopulate wounded tissue, however it also generates adherent myofibroblasts, which aid in wound closure by contracting wounded tissue but their persistence in a healing wound leads to scarring. Thus, anti-TGF β antibodies that neutralize TGF β , significantly reduce myofibroblast differentiation and scarring [9], however, they also inhibit cell repopulation [10,11]. These data suggest that TGF β promotes wound healing and that TGF β 's divergent actions may be concentration dependent.

In corneal stromal epithelial and endothelial cells, activation of the p38 mitogen-activated protein kinase (p38MAPK) pathway after wounding is key to increased cell migration that is necessary for wound closure [11-13]. In an effort to identify conditions that promote regenerative healing in the corneal stroma, we investigated the relationship

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between TGF β 1 concentration and human corneal fibroblast (HCF) cell migration, wound closure, activation of p38MAPK and SMAD 2/3 pathways in vitro. After evaluating a range of concentrations, we determined that addition of 0.01 ng/ml TGF β most closely resembled the activity of endogenous TGF β for promoting cell migration, wound closure, and p38MAPK activation without generating a large fibrotic response.

METHODS

Antibodies and reagents: Transformed mink lung epithelial cells (TMLC) containing the plasminogen activators inhibitor-1 (PAI-1) promoter fused to the luciferase gene were a generous gift of Dr. Daniel Rifkin, New York University, New York, NY. SMAD 2/3 antibody was from Santa Cruz Biotechnology (sc-133098; Santa Cruz, CA), α -smooth muscle actin (α -SMA) antibody was from Sigma (clone 1A4; St. Louis, MO). P38MAPK antibody (ab31828) and Phospho-p38MAPK antibody (ab32557) and TGF β 1 antibody (ab10517) was from Abcam (Cambridge, MA). Secondary Alexa-488 was from Jackson ImmunoResearch (West Grove, PA). Immunoglobulin G (IgG) Antibody was from Jackson ImmunoResearch. TGF β RI inhibitor, SB431542 and p38MAPK inhibitor, SB202190, was from Tocris Bioscience (Ellisville, MO). Bovine collagen (PureCol) was from Advanced Biomatrix (San Diego, CA). TGF β 1 was from R&D Systems (Minneapolis, MN).

Preparation of human corneal cells: HCF were derived from the stroma of human corneas that were not suitable for transplantation (obtained from National Disease Research Interchange (NDRI), Pittsburgh, PA). Stromal keratocytes were isolated as previously described [14]. To produce fibroblasts, freshly isolated corneal stromal keratocytes were cultured in DMEM-F12 with antibiotic antimycotic (ABAM) and gentamicin (all from Sigma) plus 10% fetal bovine serum (FBS, Atlanta Biological, Lawrenceville, GA). All experiments were done on 10 μ g/ml bovine collagen I (Advanced Biomatrix) in supplemented serum-free media (SSFM: DMEM/F-12, 1X RPMI-1640 Vitamin Mix, 1 \times ITS Liquid media supplement (5 μ g/ml each of insulin, transferrin, and 0.05 μ g/ml sodium selenite), 1 μ g/ml glutathione; (all from Sigma), 1 mM sodium pyruvate, 0.1 mM MEM non-essential amino acids; (all from Gibco-BRL) with ABAM (antibiotic antimycotic, Sigma) and gentamicin (Invitrogen, Carlsbad, CA).

Migration assay: HCFs were seeded at confluency (1 \times 10⁵ cells/well) on 10 μ g/ml collagen in SSFM. The next day cultures were scraped wounded with a 200 μ l pipette tip, medium was replaced. After an incubation at 37 °C for 24 h, migration was assessed with **T-Scratch** software developed by Koumoutsakos group (CSE laboratory), at ETH, Zürich, Switzerland. Briefly, images taken at 24 h for each treatment are imported into this application. This software determines a percent wound closure (the space remaining in the scratch

wound) compared to the control (time zero after wounding). For inhibition studies we added 2.5 μ g/ml TGF β 1 antibody, 2.5 μ g/ml matched IgG control, 10 μ M SB202192, or 10 μ M SB431542.

Immunocytochemistry: Cells were fixed with 3% p-formaldehyde (Fisher Scientific, Fair Lawn, NJ) in PBS for 15 min at RT and permeabilized with 0.1% Triton X-100 for 1 min at RT. After blocking non-specific binding with 3% normal mouse serum (Jackson ImmunoResearch), cells were incubated with anti- α -SMA antibody or anti-SMAD 2/3 antibody or anti-p38MAPK antibody followed by Alexa-488 secondary. After washing, coverslips were mounted on slides for viewing with a Zeiss Axioskop microscope and images were captured using a Zeiss Axioscope with a SPOT-2 CCD camera (Diagnostic Instruments, Sterling Heights, MI) and processed by Adobe Photoshop (Adobe, San Jose, CA) software. Photoshop images were exported into the **MetaMorph** image analysis software package (version 6.3r3; Molecular Devices, Sunnyvale, CA) to determine relative cell area.

TGF β activity assay: This bioassay detects active TGF β . HCFs were co-cultured with TMLC containing the PAI-1 promoter fused to the luciferase gene. Extracellular TGF β binds to its receptors and signals intracellularly to activate the PAI-1 promoter. HCFs and TMLC were plated together, each at 1 \times 10⁵ cells per well in 24 well dishes in DMEM, 10% FBS, 1 mM L-glutamine with antibiotics. After 24 h the media were replaced with (0.1% BSA in DMEM) and further incubated for 24 h. Luciferase activity was measured using the Bright-Glo detection system (Promega, Madison, WI) and luminescence was determined using a Synergy 2 multi-mode Microplate Reader (Biotek, Winooski, VT). Addition of known amounts of recombinant human TGF β 1 (R&D Systems) to TMLC cells was used to generate the standard curve.

BrdU staining (data not shown): Proliferation assays were attempted using two methods. First, HCFs were seeded at low density on collagen in 100 mm plate in either SSFM alone or with increasing concentrations of TGF β (0.01, 0.1, or 1.0 ng/ml TGF β). After 24 h 10 μ M BrdU (Cell Signaling Technology, Beverly, MA) was added for 4 h before fixation with methanol at -20 °C for 10 min. DNA was then denatured in 2 M HCL for 1 h and integrated BrdU was detected by anti-BrdU monoclonal antibody (Cell Signaling Technology) followed by FITC-conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch) for 30 min and counter stained by propidium iodide. Slides were evaluated using Zeiss Axioscope with a SPOT-2 CCD camera (Diagnostic Instruments). In the second method, HCFs were seeded at confluency on collagen in a 100 mm plate in SSFM. After 24 h, cells were scratch wounded (using a grid to ensure an equal number of scratches per plate) in the presence of either SSFM or SSFM with increasing TGF β concentrations (as above)

with 10 μ M BrdU. After 4 h, cells were fixed and stained for BrdU as above. Differences between conditions were not observed using either technique.

Activation of p38MAPK by western blot: HCFs were seeded at confluence 2×10^6 on collagen in SSFM. The next day cells were scratch wounded using a grid to produce consistent wounding per plate. Media was exchanged and reagents were added. After 4 h, cells were lysed in RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton) with protease inhibitors (Roche Applied Scientific, Indianapolis, IN) and the phosphatase inhibitors (HALT; Thermo Scientific, Rockford, IL). Lysates were western blotted for p38MAPK and phospho-p38MAPK. Ratios of P-p38MAPK/p38MAPK are graphed.

Statistical analysis: Standard error between experiments was calculated. All experiments were repeated at least 3 times. P-values were calculated using the student's *t*-test. *p-value <0.05, **p-value <0.01, ***p-value <0.001.

RESULTS

Neutralizing TGF β activity inhibits cell migration: Endogenous TGF β is increased in cells at the wound edge of wounded corneal fibroblasts in vitro [15]. To confirm that endogenous TGF β is necessary for HCF migration, after scratch-wounding of confluent cells, we blocked TGF β by adding neutralizing TGF β 1 antibody or matched IgG (control). Neutralizing antibody inhibits total TGF β 1 activity since it binds latency-associated peptide (LAP)-TGF β preventing the generation of new TGF β 1 as well as binding active TGF β [16]. A caveat to this experiment is that LAP may contribute to cell migration (unrelated to activation of TGF β) [17], however, this has not been demonstrated in HCFs.

HCF migration was visualized by comparing time zero after wounding (Figure 1A) to 24 h post-wounding (Figure 1B-D). To eliminate the contribution of serum components to cell migration, all studies were performed on HCFs grown under supplemented-serum free conditions (SSFM, see methods) on collagen. These data show that under SSFM conditions, at 24 h, HCFs have almost completely repopulated the wound area (86%; Figure 1B) whereas, the anti-TGF β 1 antibody inhibited cell migration by 73% (Figure 1C) compared to the IgG control (Figure 1D). Percent migration into the wound compared to time zero was quantified using **T-Scratch** software (see methods; Figure 1E). Because TGF β neutralizing antibody inhibited cell migration, we sought to determine the levels of active TGF β in HCFs when plated in SSFM using the sensitive TMLC bioassay (see methods). TMLCs contain the PAI-1 (plasminogen activator inhibitor-1) promoter linked to the luciferase gene and TGF β activates PAI-1 [18]. We have co-cultured TMLC cells with HCFs to quantify TGF β activity in HCF in unwounded cells [19]. Using this assay we determined that HCFs under SSFM conditions have 0.01 ng/ml active TGF β (either

secreted or localized to their cell surface; Figure 1F). This assay does not distinguish between TGF β isoforms however, TGF β 1 neutralizing antibody reduced active TGF β to undetectable amounts (data not shown), suggesting that this is a main isoform.

Increasing TGF β 1 concentration correlates with decreasing cell migration: HCFs were scratch-wounded under SSFM conditions (-) and migration rates were compared to HCFs treated with exogenously added TGF β 1: 0.01 ng/ml, 0.1 ng/ml, and 1 ng/ml (Figure 2A-E). Percent cell migration was determined using **T-Scratch** software (Figure 2F) and summarized in Table 1. All TGF β 1 concentrations tested decreased cell migration rates compared to the endogenous TGF β 1 in SSFM, however differences between SSFM and addition of 0.01 ng/ml were slight and were not statistically significant. The effect of TGF β 1 on cell proliferation was also tested using BrdU staining. However, after 24 h in culture, no difference in cell proliferation was found (data not shown).

Increasing TGF β 1 concentration generates fibrotic markers: Since TGF β is known to promote fibrotic markers, we next investigated the impact of TGF β concentration on myofibroblast differentiation as characterized by α -SMA stress fiber organization and increased cell area. HCFs were treated with increasing concentrations of TGF β 1 and after 72 h, cells were fixed and immunostained for α -SMA (Figure 3A-D). As expected, the number of cells with α -SMA stress fibers increased with TGF β 1 concentration (Figure 3E and Table 1). Furthermore, using **Metamorph** analysis software, we found that increasing TGF β 1 concentrations resulted in a corresponding increase in their area compared to cells grown without adding TGF β 1 (Figure 3F). Of note is that 1.0 ng/ml and 0.1 ng/ml promote similar phenotypic changes in α -SMA organization and increased cell area suggesting that 0.1 ng/ml is a threshold concentration for promoting a fibrotic response. Addition of 0.01 ng/ml TGF β 1 also generated myofibroblasts after 3 days in culture but approximately 60% fewer myofibroblasts were visualized after treatment with 0.01 ng/ml compared to the two higher concentrations. As predicted, HCFs treated with TGF β neutralizing antibody had no α -SMA stress fibers (data not shown).

TGF β 1 concentration affects p38MAPK and SMAD 2/3 activation: Activation of p38MAPK promotes cell migration and regenerative wound healing in epithelial and endothelial corneal cells [11-13]. In contrast, activation of SMAD 2/3 is correlated with fibrotic wound healing [20]. Immunocytochemical detection of nuclear versus cytoplasmic localization of p38MAPK and SMAD 2/3 is an effective method to detect their activation only at the leading edge since their activated forms are localized to the nucleus. Thus, to determine the effect of TGF β 1 on p38MAPK and SMAD 2/3 activation, HCFs were seeded at confluence and scratch wounded in the presence of either SSFM alone, or increasing concentrations of TGF β 1. Nuclear localization of p38MAPK

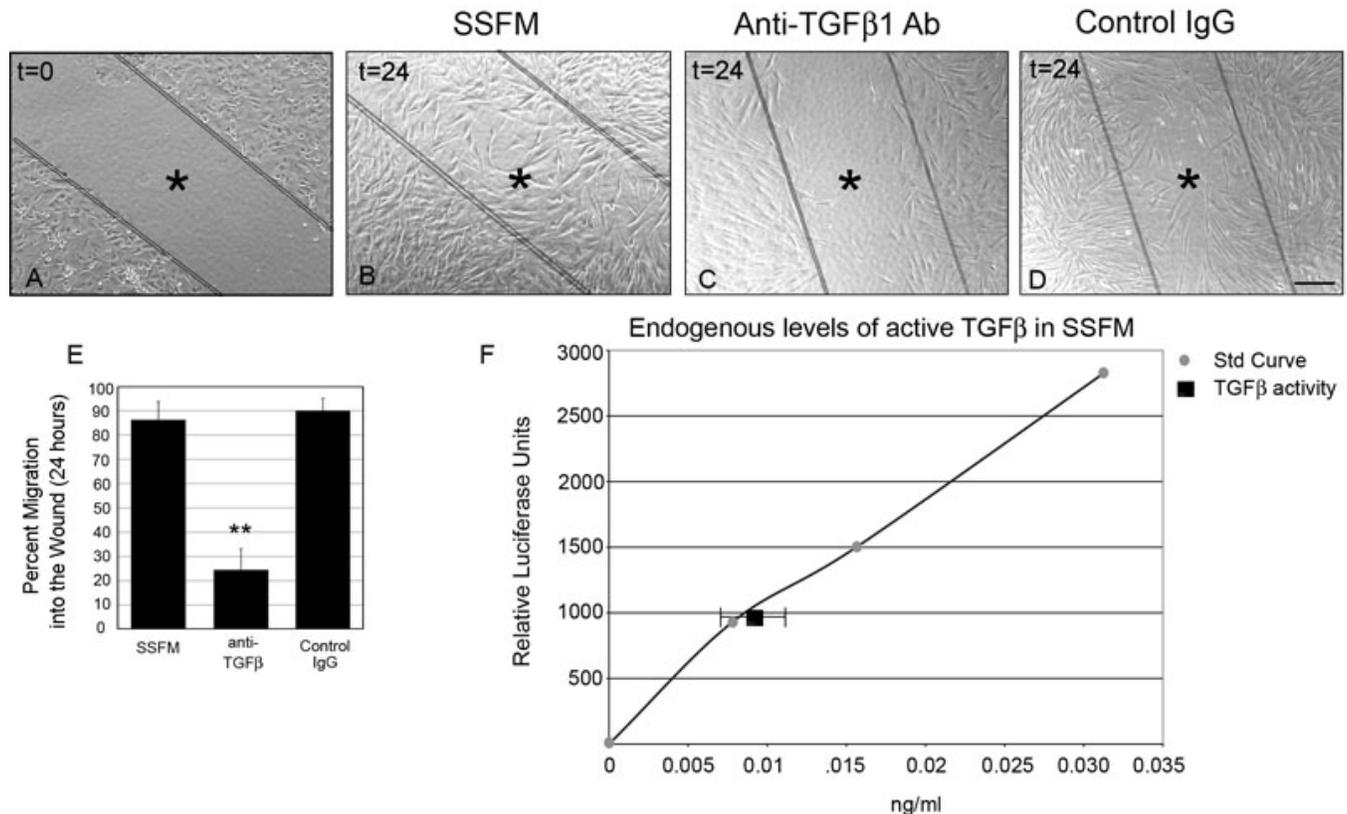


Figure 1. Endogenously secreted TGFβ1 promotes wound healing. HCFs were seeded on collagen in SSFM at 1×10^5 cells/ml in a 24 well dish. The next day cells were scratch-wounded and imaged (A) time zero, or incubated for 24 h with either (B) SSFM (C) 2 μg/ml anti-TGFβ1 antibody or (D) 2 μg/ml matched IgG control. Bar=200 μm, *inside lines denoting wounded area. E: Using T-Scratch software, percent cell migration into the wound margin at 24 h compared to time zero was calculated. Each condition was compared to SSFM, **p-value <0.01. F: To determine endogenous levels of TGFβ, HCFs were co-cultured with TMLC, which contain the PAI-1 promoter fused to the luciferase gene. This assay demonstrated that HCFs have 0.01 ng/ml active TGFβ. Experiments were repeated at least three times with similar results.

and SMAD 2/3 in leading edge cells was analyzed at several time points, from 1 to 8 h after wounding. At 4 h, changes in nuclear localization of p38MAPK and SMAD 2/3, in migrating cells was easily quantified. In both cases, activation is visualized by translocation to the nucleus. In SSFM (Figure 4A) and 0.01 ng/ml TGFβ1 (Figure 4B), p38MAPK was activated as indicated by its translocation to the nucleus in the leading edge cells (arrows). This is in contrast to 0.1 ng/ml TGFβ1 (Figure 4C) and 1.0 ng/ml TGFβ1 (Figure 4D) in which p38MAPK was excluded from nuclei (arrows). Data from multiple images of only leading edge cells were quantified for p38MAPK nuclear exclusion (Figure 4 I). Importantly, these data show that addition of 0.01 ng/ml TGFβ1 closely resembles that of the endogenous levels of TGFβ for the activation of p38MAPK suggesting that it is a key to promoting cell migration. In contrast the two higher concentrations inhibited p38MAPK activation. These data are supported by western blots for phospho-p38MAPK and p38MAPK after scratch wounding (Panel A, in Appendix 1).

We next analyzed SMAD 2/3 activation. As predicted, SMAD 2/3 nuclear localization increased with TGFβ1

concentration (Figure 4E-H, arrows). However a low level of SMAD 2/3 activation is compatible with cell migration since the leading edge cells have detectable SMAD 2/3 in the nucleus compared to the nuclear exclusion in the cells behind the leading edge (Figure 4E-G). Quantification of leading edge cells that have discrete SMAD 2/3 localization to the nucleus is shown in Figure 4J. Data from Figure 1, Figure 2, Figure 3, and Figure 4 are summarized in Table 1. Next we sought to determine if activation of p38MAPK and SMAD 2/3 is necessary for cell migration.

P38MAPK nuclear localization is necessary for cell migration: To assess the importance of p38MAPK activation and SMAD 2/3 to cell migration, HCFs were seeded at confluence and scratch wounded in the presence of specific inhibitors to p38MAPK and SMAD 2/3. Four h after wounding, cells were fixed and immunostained for p38MAPK and SMAD 2/3. Other cultures of scratch wounded cells were incubated with inhibitors for 18 h and imaged the next day for wound closure.

The p38MAPK inhibitor (SB202190) prevented translocation of p38MAPK to the nucleus (Figure 5A) and

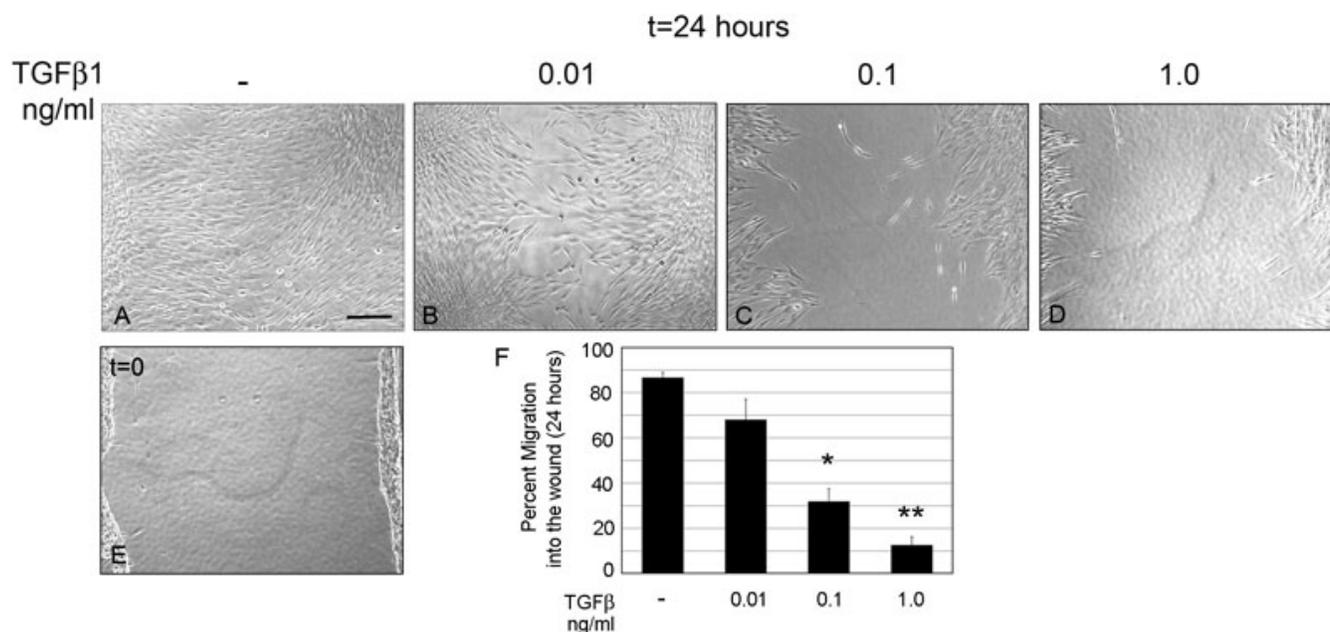


Figure 2. Increasing TGF β 1 concentrations reduces HCF migration. HCFs were seeded on collagen in SSFM at 1×10^5 cells/ml in a 24 well dish. The next day cells were scratch-wounded and incubated for 24 h in (A) SSFM, (B) 0.01 ng/ml TGF β 1, (C) 0.1 ng/ml TGF β 1, (D) 1.0 ng/ml TGF β 1, or (E) imaged at time zero. Bar=200 μ m. F: Using T-Scratch software, percent cell migration into the wound margin at 24 h compared to time zero was calculated. Each condition was compared to SSFM, *p-value <0.05, **p-value <0.01. Experiments were repeated at least three times with similar results.

TABLE 1. SUMMARY OF WOUND HEALING DATA AFTER TGF β 1 TREATMENT.

Data summary	TGF β 1			
	-	0.01 ng/ml	0.1 ng/ml	1.0 ng/ml
% Cell migration into the wound	86.6 \pm 2.3	68.0 \pm 9.0	31.9 \pm 5.6	12.6 \pm 3.6
% Cells with α -SMA stress fibers	2.2 \pm 1.4	36.1 \pm 4.4	90.2 \pm 8.0	85.3 \pm 11.3
Cell area (pixel intensity 1000 \times)	7.4 \pm 0.9	10.5 \pm 1.4	17.1 \pm 2.2	16.9 \pm 1.2
% Leading-edge cells with p38MAPK nuclear exclusion	11.9 \pm 6.7	18.7 \pm 9.9	62.7 \pm 13.3	74.8 \pm 6.4
% Leading-edge cells with SMAD 2/3 in nuclei	3.0 \pm 3.0	8.4 \pm 8.0	75.2 \pm 18.7	82.3 \pm 11.6

HCFs were untreated (-), or treated with 0.01 ng/ml TGF β 1, 0.1 ng/ml TGF β 1, or 1.0 ng/ml TGF β 1. This table summarizes the data from Figure 2 (% cell migration into the wound), Figure 3 (% cells with α -SMA stress fibers and cell area), Figure 4 (% of cells with p38MAPK detected outside the nuclei in leading edge cells and % of cells with SMAD 2/3 concentrated in the nuclei in leading edge cells).

also inhibited cell migration after scratch wounding (Figure 5K), demonstrating that preventing activation of p38MAPK inhibits cell migration. Since phosphorylation of SMAD 2/3 by p38MAPK is necessary for full activation, SMAD 2/3 nuclear translocation was also prevented [21,22] (Figure 5F). DMSO control cultures shown in Figure 5B,G,L, were similar to cells in SSFM alone (Figure 4A,E). To determine if blocking all TGF β 1 signaling could prevent TGF β -mediated activation of p38MAPK, neutralizing antibody to TGF β 1 was added. We found that TGF β 1 antibody prevented activation of p38MAPK (Figure 5C) and SMAD 2/3 (Figure 5H), as well as cell migration (Figure 5M). As expected, cells that were treated with control IgG (Figure 5D,I,N) demonstrated nuclear immunostaining and wound closure rates similar to that seen in cells in SSFM alone (Figure 4A,E and Figure 1D). These data are supported by western blots for phospho-

p38MAPK and p38MAPK after scratch wounding (Panel B, in Appendix 1).

Next, we assessed the importance of SMAD 2/3 activation to wound closure. The SB431542 inhibitor at 10 μ M prevents activin receptor-like kinase (ALK 4,5,7) and TGF β RI signaling (SMAD 2/3 activation) but does not inhibit p38MAPK activation [23]. In cells treated with 10 μ M SB431542, p38MAPK was still localized to the nucleus in the leading edge cells (Figure 5E), but SMAD 2/3 was excluded from the nucleus (Figure 5J), and cells migrated at rates similar to controls (DMSO; Figure 5O). Since SMAD 2/3 is excluded from the nucleus and cells still migrate, supports the hypothesis that a low level of SMAD 2/3 activation is not necessary for cell migration. These data are quantified in bar graphs below the images in Figure 5. Left to right: Exclusion of p38MAPK from the nucleus in leading edge cells,

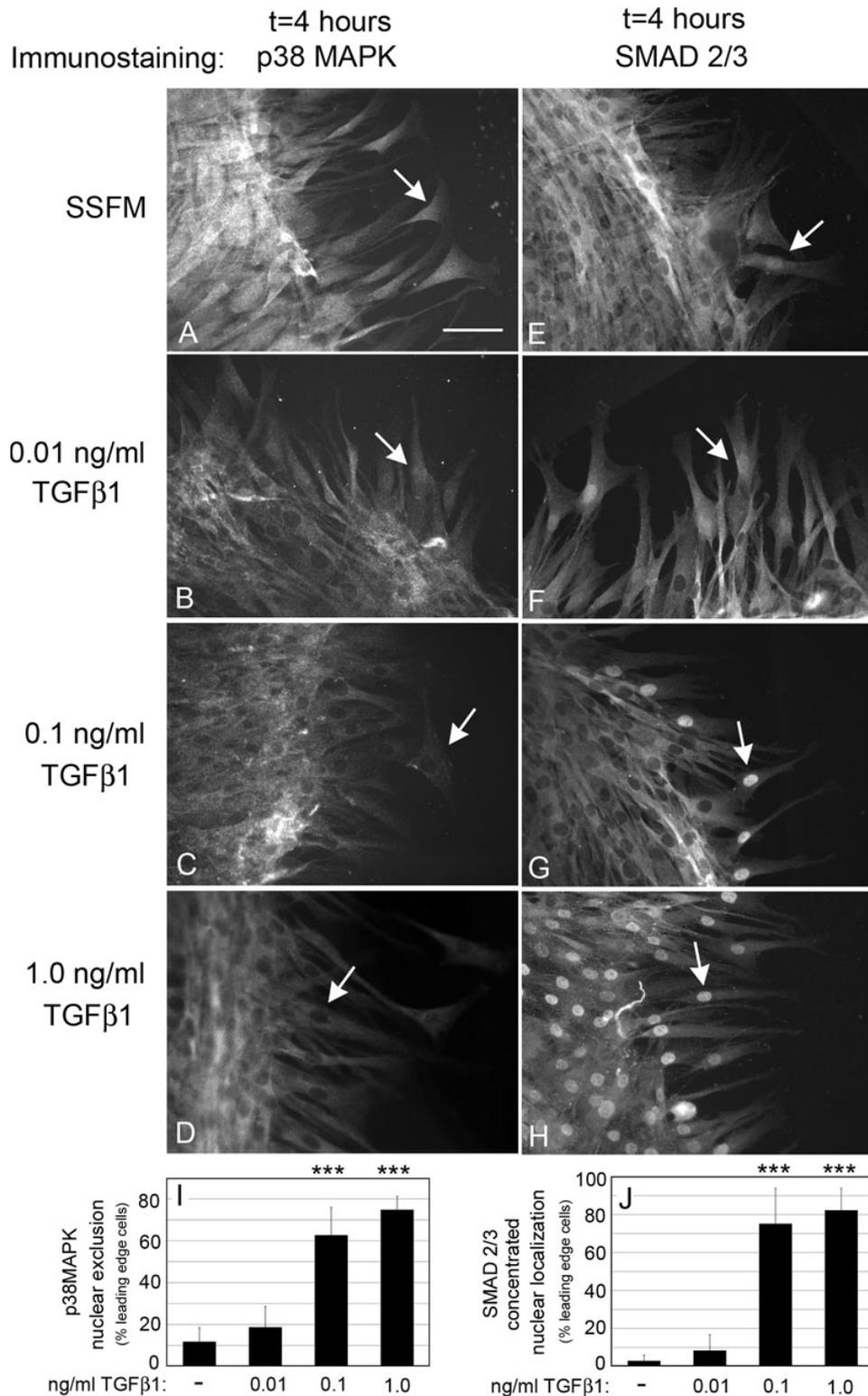


Figure 4. Increasing TGFβ1 concentrations results in a loss of nuclear p38MAPK. HCFs were seeded on collagen in SSFM at 1×10^5 cells/ml in a 24 well dish. The next day cells were scratch-wounded and incubated in (A, E) SSFM, (B, F) 0.01 ng/ml TGFβ1, (C, G) 0.1 ng/ml TGFβ1, or (D, H) 1.0 ng/ml TGFβ. After 4 h cells were fixed and immunostained for p38MAPK (A-D) or SMAD 2/3 (E-H). Bar=50 μm. Arrows point to nuclei in which we detected either the nuclear localization or nuclear exclusion of p38MAPK and SMAD 2/3. The percent of cells in which p38MAPK was excluded from nuclei of the leading edge cells is shown in (I). The percent of cells with SMAD 2/3 concentrated in nuclei in the leading edge cells is shown in (J). Image J software was used for quantification. Each condition was compared to SSFM. ***p-value <0.001. Experiments were repeated at least three times with similar results.

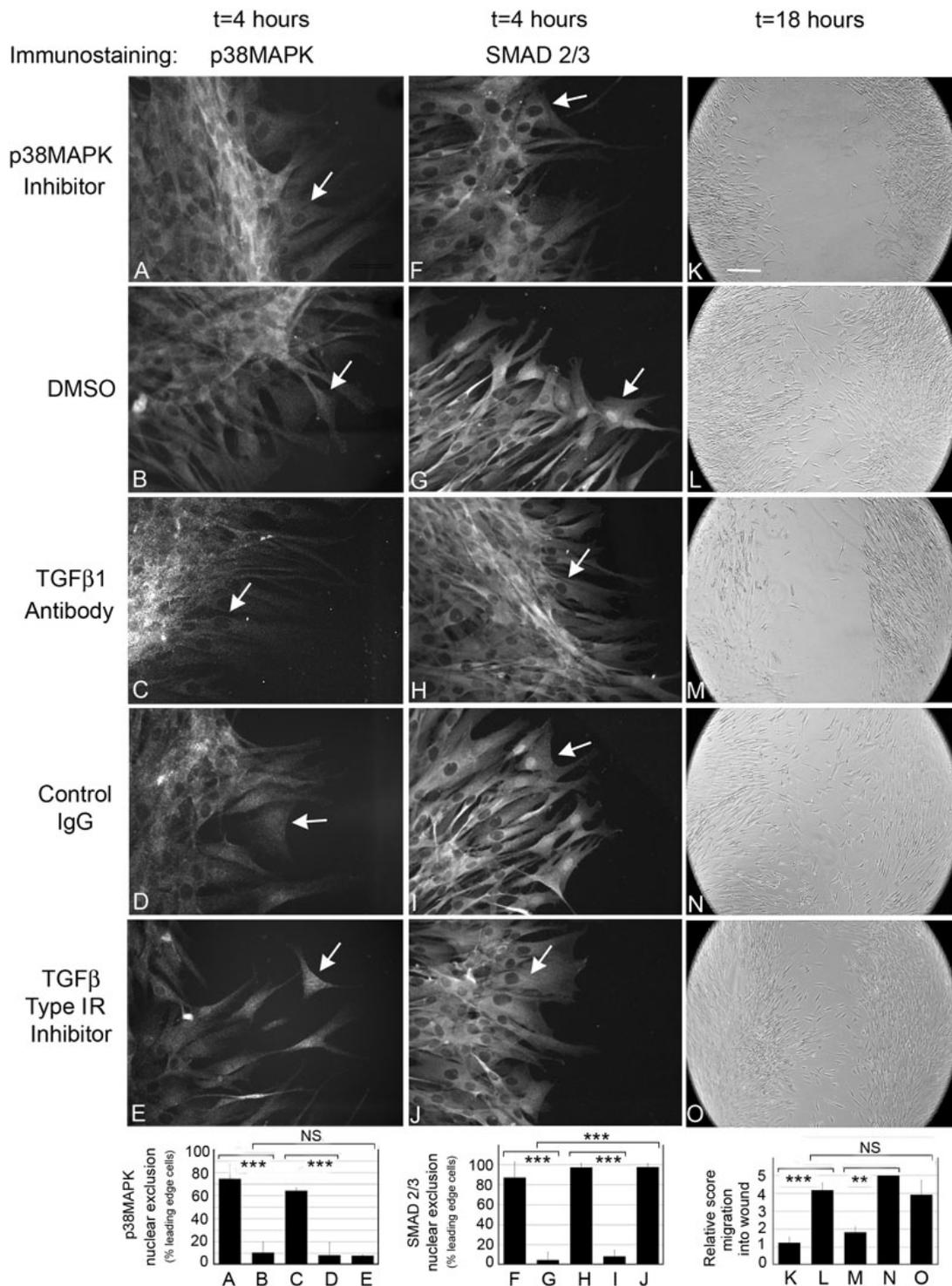


Figure 5. Nuclear p38MAPK localization is necessary for HCF migration. HCFs were seeded on collagen in SSFM at 1×10^5 cells/ml in a 24 well dish. The next day cells were scratch-wounded and incubated in (A, F, K) p38MAPK inhibitor, 10 μ M SB202190, (B, G, L) DMSO, (C, H, M) TGF β 1 antibody, (D, I, N) Control IgG, or (E, J, O) TGF β RI (ALK5) inhibitor 10 μ M SB431542. After 4 h cells were fixed and immunostained for p38MAPK (A-E) or SMAD 2/3 (F-J). Arrows denote the nuclei of leading edge cells in which p38MAPK and SMAD 2/3 were either localized or excluded (Bar=50 μ m) or after 18 h cells were imaged (K-O). Bar=200 μ m. DMSO is the control for addition of SB202190 or SB431542. Quantification of all data are shown in the bar graphs below the images. Left to right: Exclusion of p38MAPK from the nucleus in leading edge cells, exclusion of SMAD 2/3 from the nucleus in leading edge cells, cell migration into the wound. Nuclear localization was counted using Image J software. Two non-biased people scored cell migration, 0 (less migration) to 5 (most migration). **p-value <0.01, ***p-value <0.001. NS=not significant. Experiments were repeated at least three times with similar results.

exclusion of SMAD 2/3 from the nucleus in leading edge cells, cell migration into the wound.

Taken together, the data presented in Figure 4, Figure 5, and Appendix 1 demonstrate that TGF β 1-mediated activation of p38MAPK is necessary for cell migration and wound closure in vitro. Furthermore, that addition of a low concentration of TGF β 1 such as 0.01 TGF β 1 (additive to endogenous TGF β) promotes p38MAPK activation.

DISCUSSION

Non-healing after LASIK: Although LASIK has restored clear vision to millions of people, the post-LASIK cornea remains acellular and unhealed and thus there is a need to promote cell repopulation into the unhealed cornea [4,5]. It is possible that the lack of cell repopulation after LASIK is because LASIK remodeling of the stroma alters the ECM in a way that may inhibit cell migration from the non-wounded peripheral cornea into the wounded central cornea. It is also possible that, since the LASIK cut intersects the epithelium only at the edge of the flap, pro-migratory cytokines originating in the cut epithelium may not reach the flap bed. Our in vitro study shows that endogenous TGF β promotes cell migration. However, the fact that post-LASIK wounds do not heal, suggests that endogenous TGF β is not impacting wound closure post-LASIK. Thus, we investigated if adding a low concentration of TGF β 1 (equal to the levels of endogenous TGF β) may be useful in promoting cell migration. We demonstrate that TGF β 1 at a low concentration (0.01 ng/ml) is similar to endogenous TGF β for promoting cell migration, suggesting that in the absence of cell migration (and perhaps endogenous TGF β), addition of this low concentration of TGF β could substitute for endogenous TGF β . A recent study by Mi et al. [24] aimed at increasing flap adhesion after a "LASIK-like cut" tested TGF β 1 as well as other cytokines for their ability to promote corneal wound healing. After a LASIK cut of bovine corneas, the corneas were excised placed in organ culture and treated for 4 weeks with a cytokine: TNF α , IL-1, Fas ligand, or TGF β 1. Addition of these cytokines resulted in greater flap adherence but the corneas did not remain transparent. Based on our data, the lowest concentration of TGF β 1 that Mi et al. [24] tested, 0.1 ng/ml, would generate a significant myofibroblast response. At this concentration, we report that 90% of all cells are myofibroblasts. Consistent with our findings, the authors predicted that a lower concentration could be useful in increasing flap adhesion without generating corneal haze.

The impact of TGF β 1 concentration on p38MAPK and SMAD 2/3 signaling: TGF β binds to the TGF β Receptors I and II, serine/threonine kinases that initiate downstream signaling pathways. Our data support the finding that TGF β (ligand) concentration differentially affects p38MAPK and SMAD 2/3, signaling pathways downstream of TGF β 1. Previous studies have demonstrated that pathway activation also depends on receptor expression, receptor kinase activity, and

expression of receptor binding partners [25,26]. We investigated the importance of TGF β concentration to p38MAPK activation and its effect on human corneal fibroblast wound healing in vitro because of reports demonstrating that p38MAPK activation is necessary for cell migration in other cell types [11-13,27]. Consistent with previous studies, we found p38MAPK activation in actively migrating cells. Our findings however add new insights into the TGF β 1-mediated regulation of p38MAPK since a concentration dependence of TGF β on p38MAPK was revealed: Whereas low concentrations of TGF β 1, endogenous and (0.01 ng/ml), induced p38MAPK activation, 0.1 ng/ml and 1.0 ng/ml TGF β prevented activation of p38MAPK. The biphasic data for low and high concentrations of TGF β 1 suggests that TGF β receptor occupancy initiates different signals that regulate p38MAPK activation. Furthermore, these data support our hypothesis that addition of 0.01 ng/ml TGF β 1 could be used to stimulate cell migration in vivo.

SMAD 2/3 activation by TGF β was also investigated. SMAD's translocation to the nucleus is often used as a marker for the presence of active TGF β and thus served as an important control for visualizing and verifying the effect of increasing concentrations of active TGF β 1. We found that as expected for a classic dose dependent result, increasing concentrations of TGF β 1 led to greater nuclear concentration of SMAD 2/3, which correlated with reduced migration by cells at the wound edge. However, a low level of SMAD 2/3 translocation to the nucleus was visualized even under the most migratory conditions (SSFM and 0.01 ng/ml TGF β 1), suggesting that SMAD 2/3 activation may be necessary for cell migration. However, the finding that cells migrated after treatment with the SMAD 2/3 inhibitor, SB431542, suggested that this is not the case. In contrast, others have shown that TGF β -mediated inhibition of cell proliferation is dependent on SMAD 2/3 activation [21]. Thus perhaps, the minimal SMAD 2/3 translocation to the nucleus prevents cell proliferation as the cells are actively migrating.

Conclusion—Together our data suggest that addition of a low concentration of TGF β may be useful for promoting human corneal fibroblast migration into a non-healing wound, without generating a large fibrotic response. These in vitro data lay the groundwork for future in vivo studies that will assess the effects of low levels of TGF β on flap adhesion and corneal clarity.

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Appendix 1. p38MAPK activation demonstrated by western blot.

HCFs were seeded at confluence in SSFM on collagen in 100 mm plates. The next day cells were scratch-wounded (grids were used to generate consistent scratching) and reagents were added. After 4 h, cells were lysed with RIPA buffer with protease and phosphatase inhibitors. Lysates were western blotted with Phospho-p38MAPK antibody or p38MAPK antibody. Relative Pixel Intensity (RPI) was calculated for each band. **A:** No TGF β (-) or plus TGF β , 0.01,

0.1, 1.0 ng/ml). SSFM is 100%. Each condition was compared to SSFM, *p value <0.05. **B:** Lane 1, p38MAPK Inhibitor (SB202190); Lane 2, DMSO; Lane 3, TGF β 1 antibody; Lane 4, Control IgG; Lane 5, TGF β RI inhibitor (SB431542). Control IgG is 100%. p value<0.05. Experiments were repeated 3 times with similar results. To access the data, click or select the words “[Appendix 1.](#)” This will initiate the download of a compressed (pdf) archive that contains the file.