Differential Regulation of Key Stages in Early Corneal Wound Healing by TGF-β Isoforms and Their Inhibitors

Louise M. Carrington,[1,2] *Julie Albon,*[1,2] *Ian Anderson,*[3] *Christina Kamma,*[1,2] *and Mike Boulton*[1,2]

Purpose. Inhibition of TGF-β reduces myofibroblast differentiation and fibrosis in the cornea. Determining the actions of distinct TGF-β isoforms and their inhibitors during early corneal wound healing is an essential step in guiding therapeutic intervention.

Methods. Bovine serum-free corneal cell and wounded organ cultures were challenged with a range of concentrations of TGF-β_1, -β_2, and -β_3; IL-10; and neutralizing human monoclonal antibodies (mAbs) against TGF-β_1 (CAT-192) or -β_2, (CAT-152). Cultures were assessed for re-epithelialization, proliferation (cell counts and cresyl violet assay), morphology (histologic examination), repopulation of the area under the wound, and myofibroblast transformation (α-smooth muscle actin) between 0 and 5 days.

Results. TGF-β_1 delayed re-epithelialization, increased repopulation of the stroma, increased keratocyte proliferation and was the only isoform to promote myofibroblast differentiation. The anti-TGF-β_1 mAb, CAT-192 promoted re-epithelialization and reduced repopulation of the stroma. Exogenous TGF-β_3 had little effect on re-epithelialization but reduced repopulation of the stroma. IL-10 promoted corneal re-epithelialization at low doses but inhibited this response at high doses. Stromal repopulation was prevented by all doses of IL-10. TGF-β_2 or the anti-TGF-β_2 mAb, CAT-152 had little effect on any repair parameter.

Conclusions. The results confirm TGF-β_1 as the principal isoform in corneal wound healing and suggest that inhibition of the action of TGF-β_1 can promote corneal wound healing. Treatment with the anti-TGF-β_1 mAb CAT-192 accelerates corneal re-epithelialization but reduces cell repopulation of the stroma. The cytokines TGF-β_3 and IL-10 have opposing actions to that of TGF-β_1. (*Invest Ophthalmol Vis Sci.* 2006;47: 1886–1894) DOI:10.1167/iovs.05-0635

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TGF-β has been established as a major regulator of wound healing in most species and tissues, including the cornea.[1–3] To date, TGF-β_1 and -β_2 have been localized to both the corneal epithelium and stroma, and both are constituents of the tear fluid (Vesaluoma M, et al. *IOVS* 1996;37:ARVO Abstract 3912).[4–9] Although mRNA of the β_3 isoform has been isolated from whole rat corneas at very low levels, its tissue location is unclear, and the protein has yet to be detected in the nonpathologic cornea.[6,10]

The TGFβ receptors RI and RII are located in epithelial, stromal, and endothelial layers of the cornea.[11–13] RI and RII are present predominantly in the basal layer of corneal epithelial cells, with receptor density increasing proximal to the limbus in many species, including humans. The nonsignaling TGFβ-RIII (β-glycan receptor) has been located on both the epithelium and endothelium in vivo, but appears to be absent in keratocytes in vivo.[11]

As with many other growth factor signaling systems the levels and spatial location of each component in the TGF-β system alters dramatically after a corneal wound. All three isoforms are present in the corneal epithelium,[4,14–16] and corneal epithelial cells in culture release TGF-β_1 and -β_2.[17,18] TGF-β_2 is reported to be more strongly expressed than the other two isoforms[4] and throughout wound healing after PRK and TGF-β_1, -β_2, and -β_3 are present in the corneal epithelium.[19] In stromal cells TGF-β is upregulated,[15] but isoforms cannot be detected immunohistochemically until 2 days after PRK, when rounded cells in the ablated area express all three isoforms. TGF-β_1, -β_2, and -β_3 expression is delayed in spindle shaped fibroblasts until 10 days after PRK. Expression of all three isoforms returns to normal after 30 days. In addition, levels of TGF-β_1 in the tear film increase dramatically (Vesaluoma M, et al. *IOVS* 1996;37:ARVO Abstract 3912). Of particular interest is the finding that topical administration of 1D11, a TGF-β_1-, -β_2-, and -β_3-neutralizing antibody, to rabbit corneas after PRK and lamellar keratectomy wounds, results in a reduction in the appearance of myofibroblasts, and substantially decreases the incidence of haze in rabbits.[2,3] Taken together, this indicates a pivotal role for the TGF-β system in corneal maintenance and wound repair.

The TGF-β superfamily is a structurally related group of bioactive ubiquitous proteins with diverse and pleiotropic activities. TGF-β1 and -β2 share 80% sequence homology but can have opposite actions on biological processes such as proliferation, migration, and differentiation.[1] The role of the various isoforms of TGF-β in corneal wound healing is not fully understood, and therefore optimal treatment may rely on selective inhibition of one or more TGF-β isoforms. To manipulate the cytokine environment of the healing cornea after either trauma or elective surgery, it is necessary to understand the actions of each TGF-β isoform. It appears increasingly likely that the events occurring within the earliest stages of corneal wound healing alter prognosis.[2,3] Herein, we describe the very different actions of the three TGF-β isoforms in early corneal wound-healing events and their inhibition by human isoform-specific neutralizing antibodies. The action of the cytokine IL-10, a potential antagonist of TGF-β, was also evaluated. The results

of the study have been reported in part in abstract form (Carrington LM, et al. *IOVS* 2001;42:ARVO Abstract 5018).

METHODS

Cell Culture

Nonactivated bovine keratocytes were cultured based on established methods.[3,20] In brief, the epithelial and endothelial layers were scraped from the stroma which was incubated in 2 mg/mL collagenase (wt/vol RPMI at 37°C; Invitrogen-Gibco, Paisley, Scotland, UK) overnight at 37°C. Isolated cells were plated at 1×10^4 cells/well (24-well plate) in 1 mL of serum-free RPMI containing nonessential amino acids (Invitrogen-Gibco), glutamine, antibiotics, amphotericin B. Cultures were maintained in medium at 37°C in a standard 5% CO_2-95% air atmosphere for 48 hours, to allow attachment. Medium was replaced with fresh medium containing 0.1, 1, or 10ng/mL TGFβ_1, -β_2, or -β_3 or IL-10 (R&D, Abingdon, UK). Diluents were used as the control: 10 μL of phosphate buffered saline (PBS) containing 0.1% BSA and 0.01 M HCl (TGF-β isoform diluent) or 10 μL of PBS containing 0.1% BSA (IL-10 diluent). All treatments were applied in triplicate for up to 5 days in culture, and experiments were performed at least three times.

Organ Culture

Bovine corneas were centrally wounded with a 5-mm trephine, as previously described,[21] and the disc of epithelial-stromal tissue within the wound was excised. Sterile, serum-free DMEM containing 1% agar and 1% gelatin (BDH, Poole, UK) was used as a support, and serum-free Trowell's T8 medium (Invitrogen-Gibco) containing antibiotics, amphotericin B, and glutamine was added to the dish to a level just below the limbal region. Twice daily, 100 μL of fresh serum-free T8 medium was pipetted onto the surface of the cornea containing 1, 10, or 100 ng/mL TGFβ_1, -β_2, or -β_3 or IL-10 (R&D Systems); or neutralizing mAb against either active hTGFβ_1 (CAT-192; 0.1 nM–10 μM human IgG4; Cambridge Antibody Technology, Cambridge, UK); or hTGFβ_2 (CAT-152; 10 μM human IgG4; Cambridge Antibody Technology); or 10 μL of PBS containing 0.1% BSA (IL-10 and neutralizing antibody diluent) or 10 μL of PBS containing 0.1% BSA, and 0.01 M HCl (TGF-β isoform diluent). Cultures were maintained in serum-free T8 medium for up to 5 days ($n = 6$, per time, per treatment).

Re-epithelialization

Re-epithelialization was assessed as previously described,[20,21] by using captured macroimages of the wound area, where both the original wound cut and the leading edge of the epithelium could be seen. Re-epithelialization was expressed as the percentage of the original wound area that was re-covered.

Morphologic Assessment of Epithelium

Wounded and unwounded corneas were fixed overnight in 10% neutral buffered formalin (NBF), processed into wax. Seven-micrometer sections were stained with Harris hematoxylin and eosin.

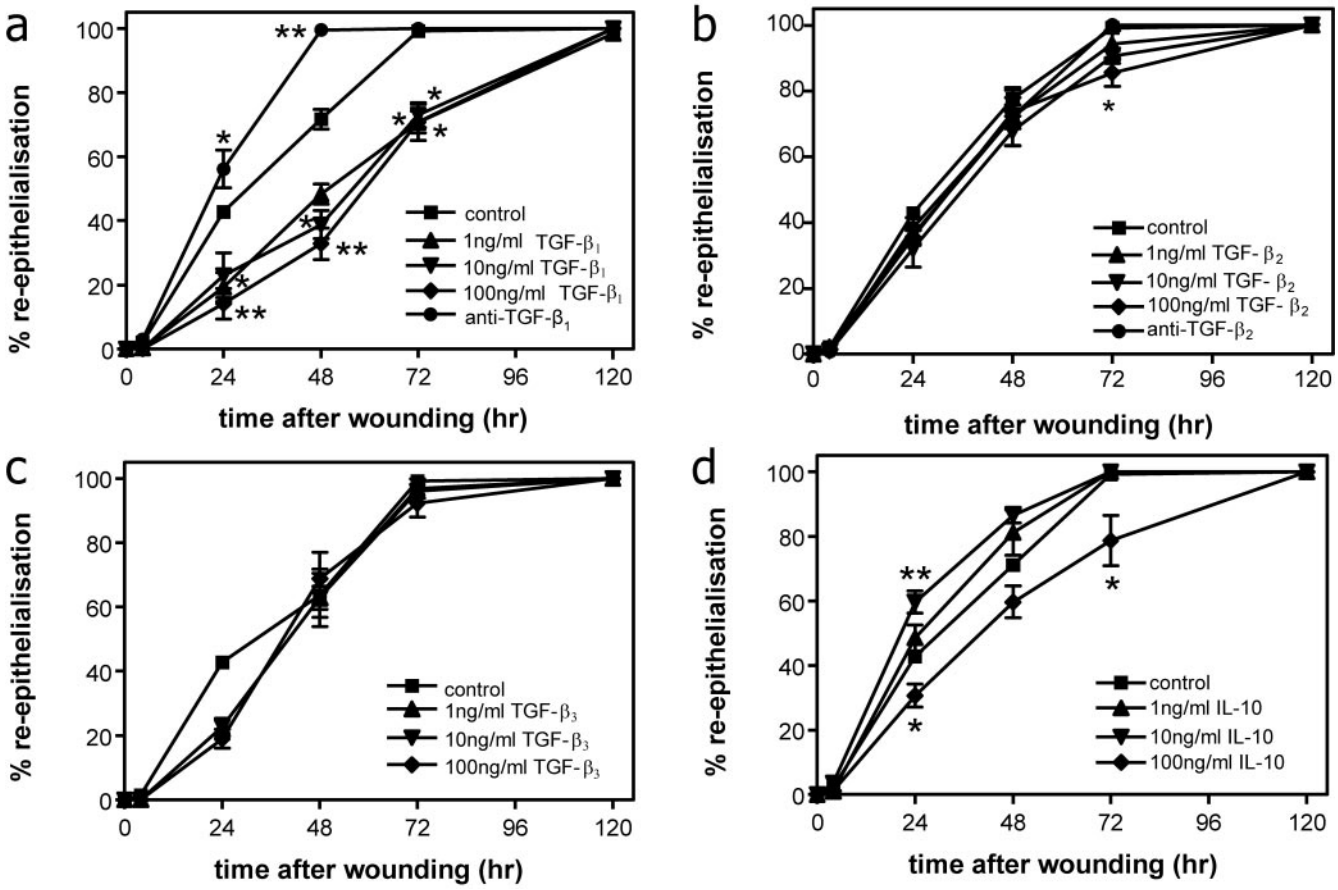

FIGURE 1. Percentage re-epithelialization of bovine corneal trephine wounds treated with 100 μL of serum-free medium twice daily, containing 1% growth factor diluent (PBS+0.1% BSA+HCL) or 1, 10, or 100 ng/mL (**a**) TGF-β_1 and 10 μM neutralizing mAb against hTGF-β_1, (**b**) TGF-β_2 and 10 μM neutralizing mAb against hTGF-β_2, (**c**) TGF-β_3, or (**d**) IL-10. Each point represents the average of at least six corneas ± SEM. Significant differences were determined by Student's *t*-test $^*P < 0.05$, $^{**}P < 0.01$.

Assessment of Proliferation in Cell Culture Using the Cresyl Violet Assay

Briefly, cells were fixed in 70% ethanol for 10 minutes at room temperature and incubated with a 0.5% solution of cresyl violet (BDH) for 1 minute at room temperature. After washing with PBS, acetic acid (33%, vol/vol ddH_2O) was added to the wells to elute the dye. The absorbance of the wells was read at 540 nm, with an ELISA reader, and wells containing 33% acetic acid were used as a blank.

Stromal Cell Density beneath the Wound

Corneas were fixed in 10% NBF overnight and processed into wax. Seven-micrometer sections were floated and incubated in 1 μg/mL bisbenzimide solution (Sigma-Aldrich, Poole, UK) for 10 minutes. Images of stained sections were captured, and the number of nuclei in the area 100 μm below the wound surface was analyzed (ImagePro Plus software; Media Cybernetics, Silver Spring, MD).

Identification of Myofibroblasts

Cultured cells were fixed at 0, 1, 2, 3, 4, and 5 days after treatment in 1% paraformaldehyde for 5 minutes. Corneal organ cultures were snap frozen in liquid nitrogen at 0, 1, 2, 3, and 5 days after treatment; embedded in optimal cutting temperature compound (OCT); and sectioned at 5-μm intervals. Cultures and sections were pretreated with 0.1% Triton-X-100 for 20 minutes, incubated with a monoclonal anti-α smooth muscle actin antibody (Sigma-Aldrich) for 2 hours followed by an Alexafluor 488-conjugated goat anti-mouse IgG (Invitrogen, Eugene, OR) for 1 hour. Counterstaining of nuclei was obtained using bisbenzimide solution incubation for 10 minutes. Cell cultures were also costained with TRITC-conjugated phalloidin (5 μg/mL in PBS; Sigma-Aldrich) for 2 hours, to identify F-actin.

Statistical Analysis

Cell counts beneath the wound were compared by using the nonparametric Mann-Whitney test. Proliferation was compared with unpaired, two-way *t*-tests (Prism 3.0; GraphPad Software, San Diego, CA) Corneal re-epithelialization was compared at each time point using either the Kruskal-Wallis test (nonparametric ANOVA) with Dunn's post test or Mann-Whitney test, as appropriate. The rate of re-epithelialization was assessed with a least-squares regression function of data between 24 and 48 hours and was expressed as the percentage of the wound area re-epithelialized in 1 hour. The calculated EC_{50} for CAT-192 in the re-epithelialization assay is indicated as the geometric mean with associated 95% confidence limits.

Results

Epithelial Wound Healing

Re-epithelialization followed a pattern similar to that previously described for this organ culture model.[20,21] Epithelial migration commenced within 12 hours after wounding, and, by 72-hours, wounds had completely re-epithelialized for corneas treated with medium alone or medium containing diluent (Fig. 1). The rate of re-epithelialization was calculated to be 1.56% ± 0.07% h^{-1}.

Corneal re-epithelialization was differentially sensitive to the three TGF-β isoforms tested. TGF-β_1 was the most potent inhibitor of re-epithelialization, inhibition was greatest at 100 ng/mL and halved the rate of re-epithelialization compared with the control (0.71% ± 0.3% h^{-1}; $P < 0.05$). The rate of re-epithelialization for corneas treated with TGF-β_2 or -β_3 was similar to that for the control ($P > 0.05$), except that TGF-β_2 at 100 ng/mL caused a small delay in re-epithelialization at 72 hours (Figs. 1b, 1c).

Administration of CAT-192 (100 μg/mL) resulted in an accelerated rate of regrowth of the corneal epithelium such that complete cover was established at 48 hours, corresponding to a re-epithelialization rate of 2.11% ± 0.1% h^{-1} ($P < 0.05$ compared with the rate observed in vehicle-treated controls, 1.56% ± 0.07% h^{-1}, Fig. 1b). In a separate experiment the concentration–response for CAT-192 was evaluated at a single time point. CAT-192 (10 ng/mL–100 μg/mL) applied to wounded corneas resulted in a concentration-related enhancement of corneal re-epithelialization measured at 40 hours, with a calculated EC_{50} of 0.51 μg/mL (0.23–1.10, 95% confidence limits; $n = 12$, Fig. 2). Neither CAT-152 or a null control IgG4 had a significant effect on the rate of re-epithelialization (Figs. 1b, 2)

IL-10 exhibited a pleiotropic effect on re-epithelialization (Fig. 1d): 1 ng/mL had no effect, 10 ng/mL increased re-epithelialization, and 100 ng/mL decreased re-epithelialization (without apparent cytotoxicity using the trypan blue assay, data not shown).

Epithelial Morphology

Epithelial morphology during re-epithelialization in control corneas and those receiving diluent was similar to that previously described for this model[20,21] and as depicted in Figure 3. Initial rounding off and retraction of the epithelium from the wound site at 4 hours was followed by migration onto the denuded stroma at 12 hours with the leading edge of the epithelium one cell deep, with no obvious stratification. Migration continued across the wound, with the leading edge of epithelium increased to 3 to 4 cells and slight hypercellularity at the original wound edge evident. By 72 hours, wound closure had occurred with the epithelium at the closure site thinner and less differentiated than the distal epithelium. After 5 days in culture the entire epithelium within the wound site

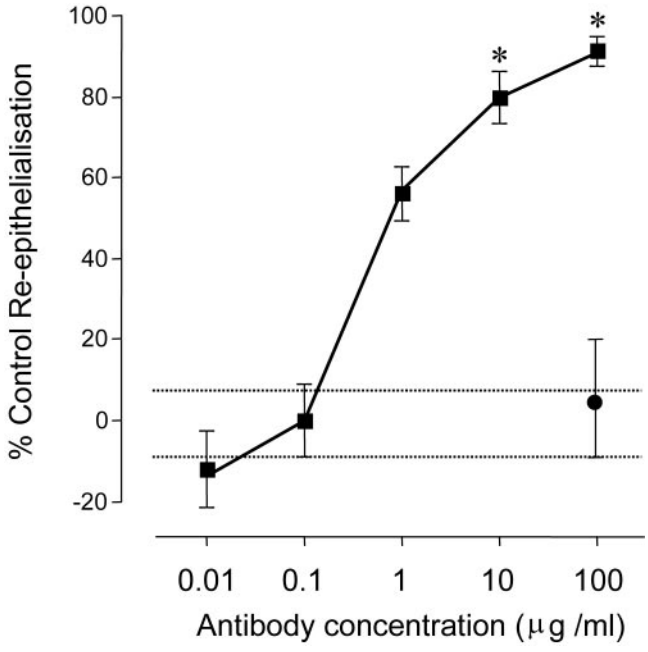

Figure 2. Dose response to CAT-192 during corneal re-epithelialization after an excisional trephine wound. The EC_{50} for CAT192 was 0.51 mg/mL (95% CI, 0.23–1.10; $n = 12$). Data are expressed as the percentage change in re-epithelialization of the vehicle-treated control group. *Dotted lines*: SEM of the vehicle control group data. A null IgG4 antibody control group (CAT-001, $n = 6$) was included. Each point represents the mean; vertical bars, SEM. The effect of the different doses of CAT-192 was compared with control treatment using the Kruskal-Wallis and Dunn tests. *$P < 0.01$.

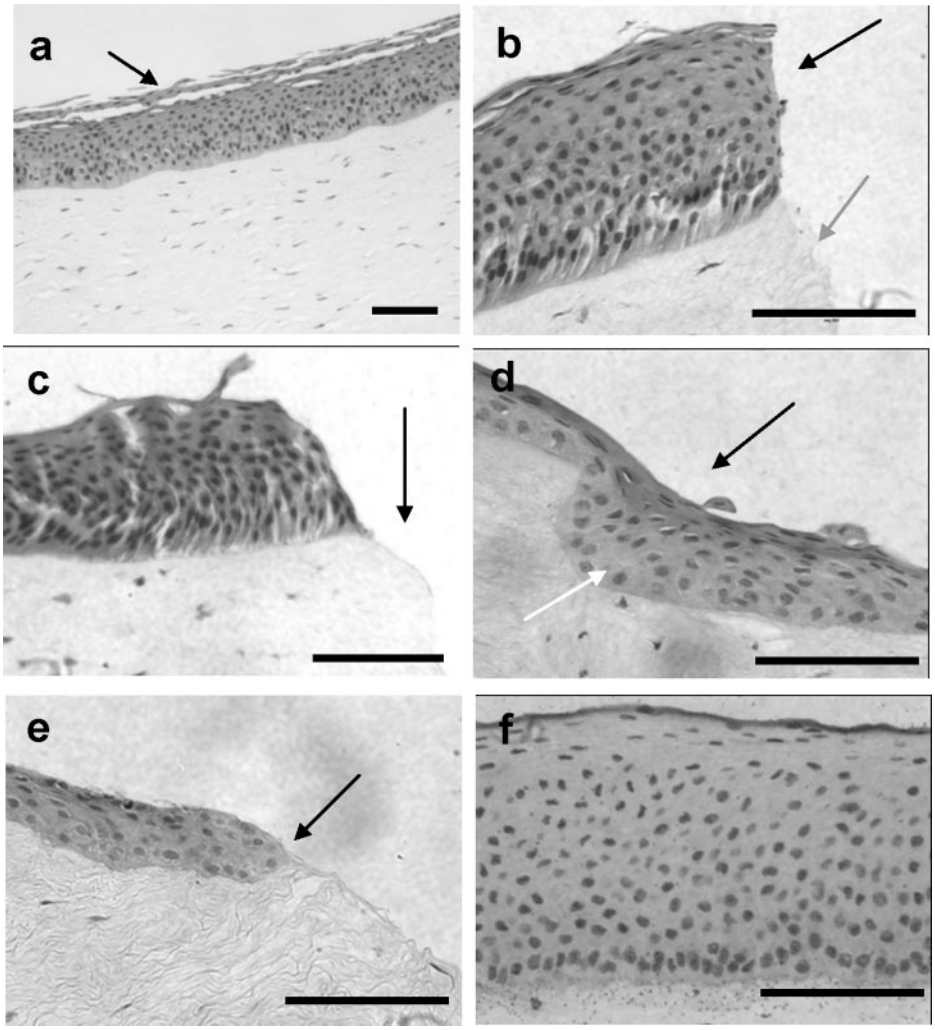

FIGURE 3. Epithelial morphology of wounded corneas at various stages of re-epithelialization. Unwounded bovine corneas (**a**) contained 8 to 10 layers of cells within the epithelium and 1 to 2 layers of desquamating, flattened superficial epithelial cells (*black arrow*). Corneas that had undergone wounding with a trephine, showed clean, sharply cut edges (**b**) through the epithelium immediately after wounding (*black arrow*), stromal cutting was less defined (*grey arrow*). Four hours after wounding (**c**), the epithelium had retracted from the cut edge of the stroma (*black arrow*). As the epithelium migrated across the denuded stroma (**d**), the epithelium over the corner of the original cut (*black arrow*) had thinned to one to three layers deep, whereas hypercellularity was often observed within the wound's edge (*white arrow*). (**e**) The leading edge of the epithelium had lost stratification and become one to two cells deep (*black arrow*). By 120 hours (**f**), stratification of the healed epithelium was evident with a slight thickening compared with unwounded corneas. Scale bar, 100 μm.

exhibited good stratification, although slight thickening of the epithelium within the wound area was common.

Morphologic differences were observed in corneas treated with TGF-β_1 (Fig. 4). This included considerable thinning of the epithelium in the wound site at all time points and was more pronounced in the corneas receiving 100 ng/mL TGF-β_1 (Fig. 4b). Stratification of the epithelium migrating into the wound was always evident, although the layers were thinner than those in control corneas. By 120 hours, re-epithelialization was complete, confirming the results of macroscopic image analysis. Corneas treated with the anti-TGF-β_1 antibody CAT-192 were similar to the control, although the epithelium appeared slightly thinner (Figs. 4c, 4d).

Corneas receiving 1 ng/mL TGF-β_2 showed a retraction of the wound edge that was not observed at higher concentrations (Fig. 4e). Thinning of the epithelium was particularly obvious at 100 ng/mL, especially at the interface between wounded and unwounded stroma. By 48 hours in culture, hypercellularity was evident at all TGF-β_2 concentrations, with sparse populations of cell nuclei in the superficial layers and loss of stratification correlated with increasing doses of TGF-β_2, and at 100 ng/mL no obvious basal cells or superficial epithelium were observed. Patches of acellularity were observed within the epithelium (Fig. 4f). By 120 hours in culture all wounds had closed, stratification of the epithelium was poor in corneas treated with higher doses of TGF-β_2. Corneas receiving a neutralizing antibody against TGF-β_2, CAT-152, showed morphologic features similar to those observed in the control corneas (Fig. 4g, 4h).

TGF-β_3, at all doses, showed considerable epithelial retraction from the wound edge at 4 hours and some cellular debris (Fig. 4i). Morphology of the epithelium was reasonably normal with some thinning toward the epithelial edge in corneas treated with higher doses. By 24 hours, migration into the wound had begun but was less advanced than in the control. Hypercellularity and extreme thinning of the epithelium was evident at the wound edge and stratification, as indicated by lack of columnar cells in Fig 4j, became less evident with increasing TGF-β_3 concentrations. By 120 hours in culture, the epithelium had an increased thickness throughout the wound, compared with the control (Fig. 4j).

IL-10 produced contrasting results, depending on dose. At 4 hours after wounding 1 ng/mL IL-10 showed neither retraction nor migration, but the epithelium was thinned to three to four cell layers, 10 ng/mL resulted in extreme thinning of the leading edge of the epithelium and some retraction, whereas 100 ng/mL prompted migration of a very thin leading edge into the wound area (Fig. 4k). At 24 and 48 hours, apart from degree of migration, epithelial morphology was similar for all three doses of IL-10. Stratification was not evident at the leading edge and hypercellularity was evident at the original wound margin. By 72 hours, differentiation of cells was evident but not confined to obvious layers, and basal cells were difficult to distinguish; 1-ng/mL-treated corneas demonstrated an

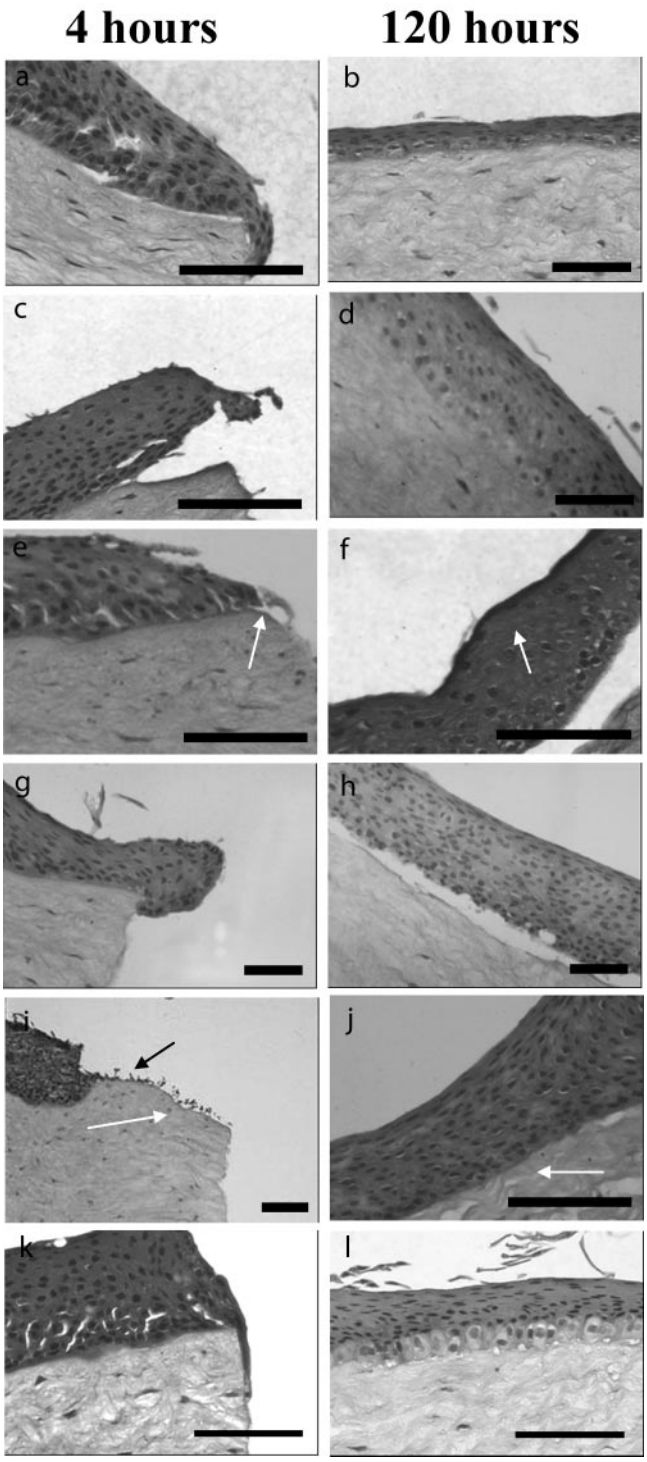

FIGURE 4. Epithelial morphology after trephine wounding 4 (**a**, **c**, **e**, **g**, **i**, **k**) and 120 (**b**, **d**, **f**, **h**, **j**, **l**) hours after treatment with 100 ng/mL TGF-β_1 (**a**, **b**) or anti-TGF-β_1 (**c**, **d**); TGF-β_2 (**e**, 10 ng/mL; **f**, 1 ng/mL; *white arrow*, an area apparently devoid of nuclei) or anti-TGF-β_2 (**g**, **h**); 100 ng/mL TGF-β_3 (**i**, **j**; *black arrow*: cellular debris and retraction of epithelium from the wound edge, *white arrow*); or IL-10 (**k**, 100 ng/mL; **l**, 10 ng/mL). Scale bar, 100 μM.

even epithelium within the wound area with some disruption of the layering, whereas both 10 and 100 ng/mL produced hypercellularity at the wound edges and a thinning of the epithelium at the site of wound closure. At 120 hours, good stratification was seen in corneas treated with all three doses (Fig. 4l).

Keratocyte Proliferation

Keratocyte Cell Culture. Bovine keratocytes in serum-free cell culture retained a stellate morphology similar to that seen in vivo, forming a monolayer with interconnecting processes. The initial seeding density of 1×10^4 cells per well, resulted in a preconfluent culture; however, after 2 days in culture the number of cells had increased to approximately 4×10^4 cells per well and the keratocytes had extended long processes toward each other, forming a network. The culture conditions allowed low-level proliferation of keratocytes throughout the 5 days of experimentation (Fig. 5). Twenty-four hours after the medium was changed, the number of keratocytes had increased to $7.3 \pm 0.1 \times 10^4$ cells and continued to increase, reaching $1.8 \pm 0.2 \times 10^5$ cells at 120 hours. Diluents had no effect on keratocyte proliferation.

TGF-β_1 had no significant effect on keratocyte numbers at either 1 or 10 ng/mL. However, at 0.1 ng/mL, TGF-β_1 more than doubled the number of keratocytes at 120 hours of culture compared with the control ($P < 0.001$; Fig. 5a). Other isoforms had no effect on keratocyte proliferation at the concentrations tested (Fig. 5b, 5c).

All three concentrations of IL-10 stimulated keratocyte proliferation in cell culture, with the cell number doubling compared with the control ($P < 0.01$l; Fig. 5d). The proliferative response appeared to be initiated earlier in cultures treated with 10 ng/mL IL-10.

Trephine-Wounded Corneas. The number of keratocytes decreased to 56% ± 2% compared with unwounded control corneas immediately after wounding, and a maximum reduction to 33% ± 1.5% was measured 4 hours after wounding. Thereafter, the number of keratocytes gradually increased in the wound area, and, by 120 hours, the number of cells beneath the wound had increased to 60% ± 7.5% of that in unwounded corneas (Fig. 6).

The TGF-β isoforms prompted very different repopulation behavior. In agreement with the results from keratocyte cell culture, the increase in cells under the wound was evident only in corneas treated with the lowest concentration of TGF-β_1 (1 ng/mL), where, by 4 and 120 hours, the repopulation of cells under the wound had increased from 33% ± 1.5% to 83% ± 4.0%, respectively, of the unwounded control (Fig. 6a). In contrast, the TGF-β_1 neutralizing mAb CAT-192 dramatically decreased keratocyte numbers at all time points, compared with the control (Fig. 6a). TGF-β3 significantly decreased the cell density under the wound at all concentrations tested (Fig. 6c). Similarly, addition of IL-10 decreased the number of cells beneath the wound (Fig. 6d). Neither TGF-β_2 nor CAT-152 had a significant effect on this response (Fig. 6b).

Myofibroblast Differentiation

Keratocytes cultures maintained in medium alone or plus diluent retained a stellate morphology and were α-smooth muscle actin negative. β-Actin was located perinuclearly and throughout the cytoplasm with no obvious stress fiber bundles (Fig. 7a). Of all the factors tested in cell culture, only high-dose TGF-β_1 prompted the differentiation of keratocytes into myofibroblasts. Prominent stress fibers were obvious when cells were stained with phalloidin, the cells lost their stellate shape and elongated into spindles (Fig. 7b). More than half of the cells staining positive for α smooth muscle actin which appeared to be organized in bundles similar to the β-actin filaments.

Only very occasional cells, if any, were α-smooth muscle actin positive in control cornea organ cultures (Figs. 7e) with

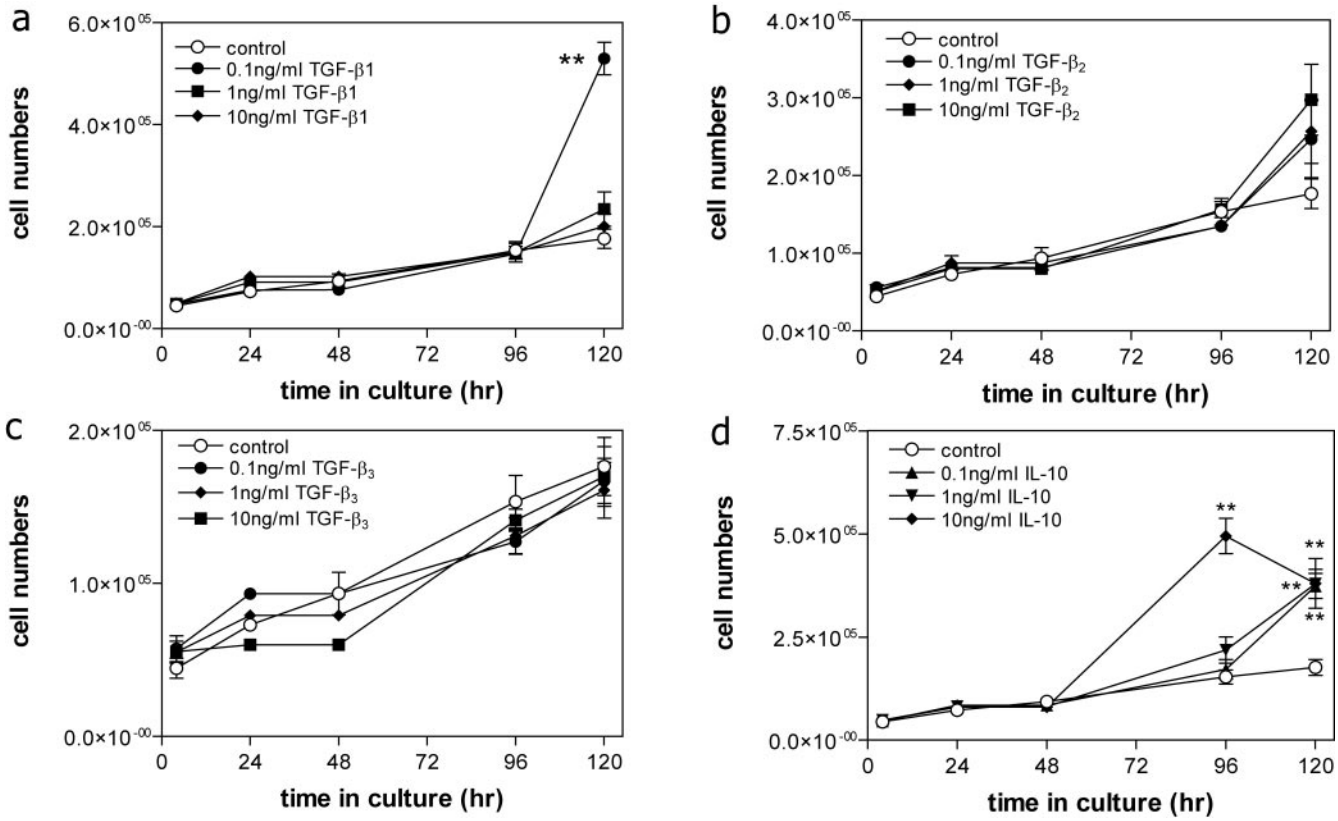

FIGURE 5. The effect of (**a**) TGF-β_1, (**b**) TGF-β_2, (**c**) TGF-β_3, and (**d**) IL-10 on the number of keratocytes in serum-free cell culture assessed using the cresyl violet assay. Each point is the average of three experiments of triplicate wells. Error bars, SEM. Data were analyzed using the Students *t*-test. $^*P < 0.05$; $^{**}P < 0.01$.

the exception of cells surrounding the lumen of blood vessels in the corneal limbus (Figs. 7c, 7d). Very occasionally, isolated cells were evident in the area under the wound after re-epithelialization had occurred. Myofibroblasts became evident in the stroma of corneas treated with 100 ng/mL of TGF-β_1 (Figs. 7f). These were located in the area directly under the wound, within the upper 150 μm of the stroma at the edge of the wound face, with isolated cells (<1% of stromal cells) appearing at 72 hours after wounding. At 120 hours, the number of α-smooth muscle actin–positive cells had significantly increased to 65.97% ± 13.57%, compared with control corneas at the same time point ($P < 0.001$). The stromal cells of corneas treated with CAT-192 (anti-TGF-β_1), TGF-β_3 or IL-10 had no obvious α-smooth muscle actin positive cells at any time point.

DISCUSSION

We present evidence that TGF-β isoforms differentially regulate several key events in early corneal wound healing. TGF-β_1 appeared to be the most active corneal isoform and was able to delay re-epithelialization, increase proliferation of keratocytes, enhance repopulation of the periwound area, and promote myofibroblast transformation. Moreover, neutralization of endogenously produced TGF-β_1 after treatment with the anti-TGF-β_1 mAb, CAT-192, mediated an effect opposite the response to exogenously added TGF-β_1. In contrast to the TGF-β_1 isoform, TGF-β_3 reduced the keratocyte repopulation of the periwound area. TGF-β_2 or neutralization of TGF-β_2 with the selective antibody CAT-152 had little effect on corneal wound healing.

The effects of TGF-β_1 reported in this study concur with the findings of other investigators in various species. First, TGF-β_1 was the only factor capable of inducing α-smooth muscle actin expression in stromal cells in cell and organ culture, a well-documented phenomenon.[3,22] Second, TGF-β_1 increased the number of cells under the wound in wounded, organ-cultured corneas, a finding in common with reports in the literature involving rabbits.[3] Third, a neutralizing antibody against the active form of TGF-β_1, inhibits the slow repopulation of stromal cells under the wound as previously shown after laser keratectomy.[23] Although not examined in this study, it is likely that these events are, at least in part, mediated via connective tissue growth factor.[24]

TGF-β_3 inhibited not only the repopulation of the stroma observed in the control but also decreased the number of cells below that seen at any time point in untreated corneas. Of note, neutralizing TGF-β_3 had no effect, either on the number of cells beneath the wound or the expression of laminin and fibronectin in the cornea,[23] and thus may act by inhibiting the action of endogenous TGF-β_1 as occurs during wound healing in the skin.[25] TGF-β_3 knockout mice demonstrate scarring in the fetal stage that does not occur after wounding in the wild-type equivalent,[26] and it appears that the ratio of TGF-β_1 to -β_3 is critical in determining the extent of fibrosis. Thus, TGF-β_3 may be a candidate for therapeutic interventions, especially because it had no detrimental effect on corneal re-epithelialization in this study.

Møller-Pedersen et al. [2] have reported that a pan neutralizing antibody (1D11) able to block all isoforms of TGFβ reduced keratocyte activation and transformation and inhibited stromal fibrosis in a rabbit model of PRK. However, in this rabbit

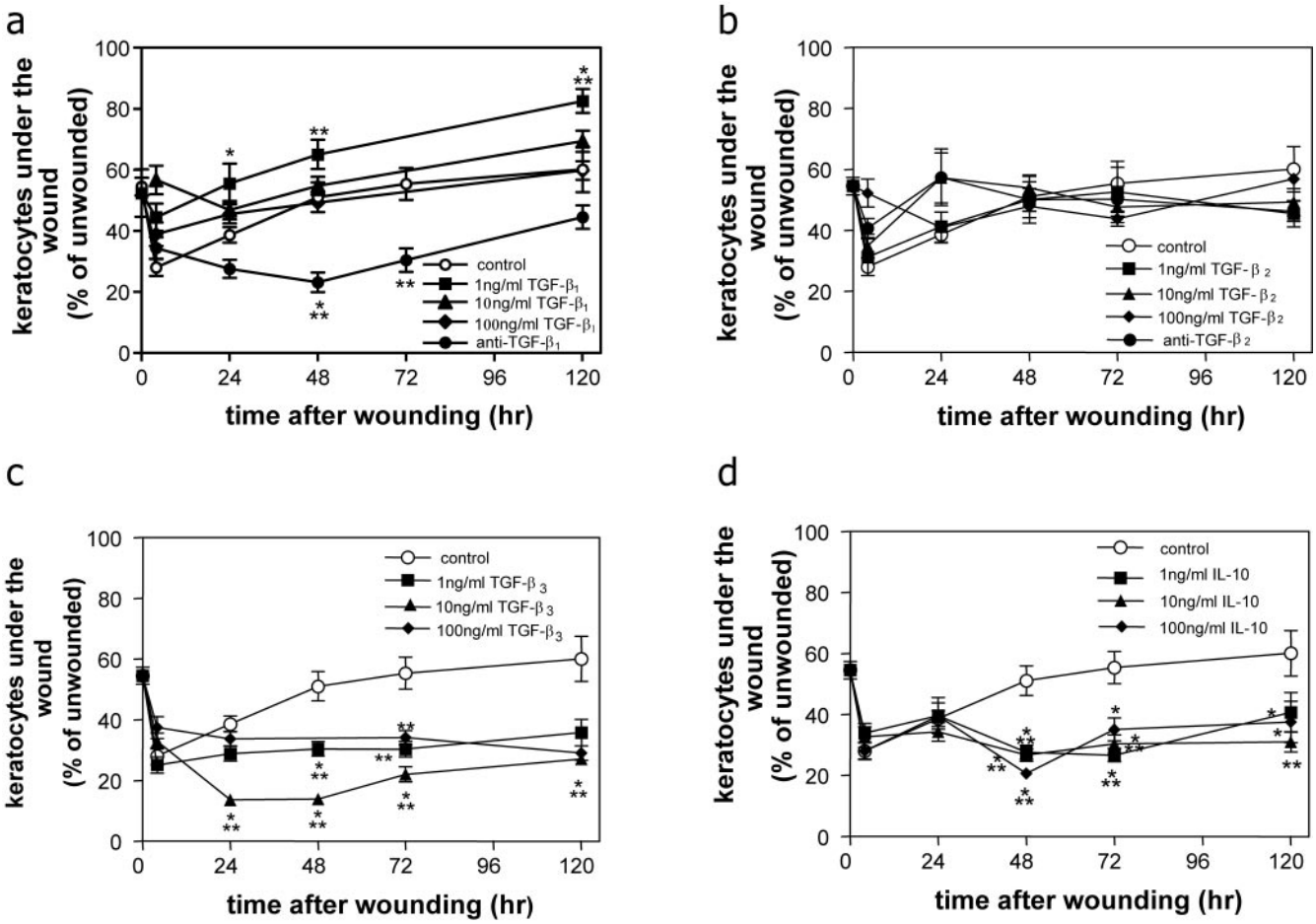

FIGURE 6. The effect of (**a**) TGF-β_1 and anti-TGF-β_1, (**b**) TGF-β_2 and anti-TGF-β_2, (**c**) TGF-β_3, and (**d**) IL-10 on the number of keratocytes beneath a trephine wound in organ-cultured corneas between 0 and 120 hours after wounding. Points represent the average number of cells under the wound as a percentage of the number at a comparable depth in that unwounded control. Each point is the average of six corneas per treatment and error bars, SEM. Data were analyzed using the Kruskal-Wallis test (*$P < 0.05$; **$P < 0.01$). Significance symbols for 4-hour time points are not shown due to lack of space.

model, the regrowth of the stroma was unaffected by pan isoform neutralization with 1D11. Our results suggest that while neutralizing TGF-β_1 may be important in preventing fibrosis, the neutralization of TGF-β_3 may well be agonistic to TGF-β_1 action. One may postulate that the best outcome (of a single agent) would be selective neutralization of TGF-β_1, and this could be achieved with the human monoclonal antibody CAT-192. This approach may reduce fibrosis, keratocyte transformation (and hence light-reflective keratocytes), as well as repopulation of the stroma. Rapid re-epithelialization would also limit additional stromal trauma. This approach would be worthy of study in a model system such as experimental PRK.

IL-10 is classically regarded as a potent anti-inflammatory cytokine and most studies into its function and effect have centered on this premise. This study is the first to report the effect of IL-10 on corneal wound healing. IL-10 was seen to have a pleiotropic effect on the epithelium, increasing re-epithelialization at 10 ng/mL, but suppressing wound coverage at 100 ng/mL.

Sources for IL-10 include TH_2 cells[27] and monocytes,[28] both of which should have no access to the cornea in nonpathologic situations. IL-10 mRNA has been isolated from the corneas of mice both before and after alkali burns[29] and in humans during corneal transplant surgery.[30] Corneal epithelial cells are a likely candidate, as the epithelia of other organs including skin produce IL-10.[31–33] Previous studies have shown that IL-10 treatment can reduce the migration of T-cells and neutrophils into HSV-1-infected mouse corneas,[34] reduce HLA-DR expression on corneal cells and infiltrating leukocytes of human herpetic stromal keratitis specimens,[35] and decrease corneal opacification in HSV-1-infected BALB/c mice.[34,36] All of these in vivo experiments were performed in the presence of a functioning immune system and were characterized by immune cell infiltration of the cornea, which could have mediated the effects of IL-10 on the corneal cells. The current study is therefore the first to show that corneal cells, in the absence of lymphocytes, can respond to IL-10 treatment and as such provides compelling evidence that the cornea contains the IL-10 receptor. Indeed, the upregulation of IL-10 Rc mRNA expression has been identified in corneas after excimer laser injury.[37]

Little is known about the mechanism by which IL-10 regulates epithelial cells and fibroblasts. IL-10 has been shown to have antagonistic effects to the actions of TGF-β,[38] although how this is achieved is unclear. IL-10 has been shown to modulate extracellular matrix components by downregulating type I collagen expression and upregulating collagenase and stomelysin mRNA in human skin fibroblasts[39]; reducing constitutive and transforming growth factor-β-stimulated, type I collagen mRNA expression in human lung fibroblast cells[40]; and downregulating the biosynthesis of fibrinogen in smooth muscle cells,[41] all of which may contribute to modulation of fibrosis.

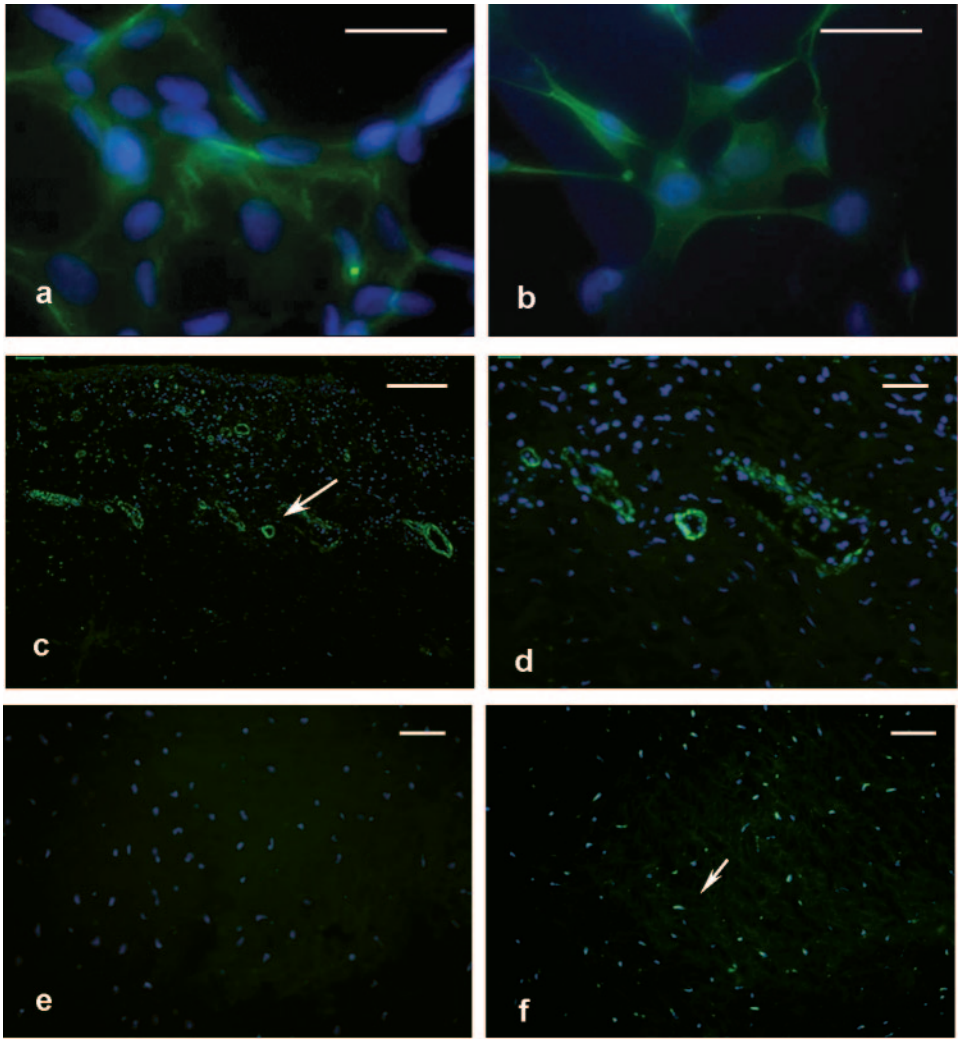

FIGURE 7. α-Smooth muscle actin staining in myofibroblasts. In serum-free cell culture, untreated keratocytes did not express α-smooth muscle actin (not shown); however, serum-treated cultures (**a**) developed α-smooth actin expression apparently within stress fibers in groups of cells after 5 days, commonly these cells were overlying the initial monolayer. In cultures treated with 10 ng/mL TGF-β_1, ~50% of cells expressed α-smooth muscle actin after 5 days (**b**). Untreated wounded bovine corneas, except for the limbal vasculature (*arrow* denotes blood vessel), which acted as an internal positive control (**c**), shown at higher magnification in (**d**), were negative for α-smooth muscle actin (**e**). Corneas treated with 100 ng/mL TGF-β_1 for 5 days contained cells within the stroma, immediately beneath the wound site, that expressed α-smooth muscle actin (**f**), indicating the presence of myofibroblasts (*arrow*). Scale bar: (**a**, **b**) 50 μm; (**c**) 100 μm; (**d**) 20 μm; (**e**) 40 μm.

Our study confirms the primary role of the β_1 isoform of TGF during corneal wound healing. Furthermore, it identifies IL-10 and TGF-β_3 as potential therapeutic regulators of corneal repair and the prevention of fibrosis. It is also likely that early application of treatment will be essential to minimize adverse healing and optimize repair. A postoperative indication such as corneal refractive surgery could be benefited by this type of biological therapy.

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