Involvement of S100A4 in Stromal Fibroblasts of the Regenerating Cornea

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Purpose. S100A4 is a member of the S100 family of calcium-binding proteins. Members of the S100 family have been implicated in a variety of cellular events, including growth, signaling, differentiation, and motility. It has been suggested that S100A4 modulates cell shape and motility by interacting with components of the cytoskeleton. In the present study, the distribution patterns of S100A4 were investigated in normal and regenerating mouse corneas.

Methods. Rabbit cDNA libraries were prepared from cultures of corneal fibroblasts. S100A4 was identified as the most abundant message present. Expression of S100A4 in the cornea was determined using Northern blot analysis, in situ hybridization, and immunohistochemistry. Distribution patterns of S100A4 in primary corneal fibroblast cultures treated with either FGF-2/heparin or TGFβ1 were analyzed by immunofluorescence.

Results. S100A4 mRNA was rarely detected in keratocytes or epithelial cells of the normal rabbit cornea. Likewise, S100A4 antigen was not found in normal mouse corneas. However, after removal of the corneal epithelium, fibroblasts are activated and had readily detectable S100A4 expression 6 days after wounding. In the in vitro equivalent of activated keratocytes, cultured rabbit corneal fibroblasts, S100A4 was restricted to the cytoplasm. In contrast, in cultures treated with TGFβ1, which induces a myofibroblast phenotype, more than 90% of the cells showed a nuclear localization of S100A4.

Conclusions. The findings show that S100A4 is expressed in the keratocyte phenotypes that appear in stromal tissue of corneas recovering from damage, the fibroblasts, and myofibroblasts. Its expression and distinct subcellular redistribution patterns suggest that S100A4 may be involved in the interconversions that occur between keratocytes, fibroblasts, and myofibroblasts during corneal wound healing. (*Invest Ophthalmol Vis Sci.* 2003;44:4255–4262) DOI:10.1167/iovs.03-0578

The S100 proteins are a large subfamily of Ca^{2+}-binding proteins characterized by the presence of two EF-hand structural motifs (for reviews, see Refs. 1–3). They are called S100 because of their biochemical property of remaining soluble after precipitation with 100% ammonium sulfate.[4] S100 genes are clustered on the long arm of human chromosome 1, region 21, in a locus called the epidermal differentiation complex.[5,6] However, the S100 proteins are found in a wide range of cell types and have been shown to regulate a variety of cellular processes, such as cell proliferation and differentiation, extracellular signal transduction, intercellular adhesion, and motility (for reviews, see Refs. 7,8).

One interesting member of the S100 family is S100A4, also known as p9Ka,[9] calvasculin,[10] fibroblast-specific protein 1 (Fsp1),[11] and metastasin 1 (mts1).[12] The best known role ascribed to S100A4 is the ability to cause cell shape changes, particularly in cells induced to elongate.[13] For example, S100A4 mRNA has been demonstrated to be upregulated in carcinogen- or oncogene-transformed fibroblasts and when rat pheochromocytoma cells are induced to elongate with nerve growth factor.[14,15] Consistent with a role in cell shape change is the primarily cytoskeletal localization of S100A4 in cultured cells.[16–18] This cytoskeletal S100A4 staining pattern was virtually identical with that of the phalloidin-visualized actin cytoskeleton, leading to the suggestion that S100A4 may directly interact with F-actin.[16–18] S100A4 has also been associated with stress fibers, tropomyosin, and nonmuscle myosin.[10,19] Not surprisingly, S100A4 has also been implicated in cellular motility and invasiveness.[15] It has been demonstrated that S100A4 levels correlate with the motility of cells grown on various substrates.[20] Most recently, it has been shown that S100A4 is capable of enhancing endothelial cell motility in vitro and stimulating corneal neovascularization in vivo, suggesting that S100A4 protein plays a role in angiogenesis.[21] Enhanced motility contributes to invasiveness, and thus it is not surprising that S100A4 is involved in metastasis. Elevated levels of S100A4 were found in epithelial cell lines with high metastatic potential, whereas related benign cell lines showed low or undetectable levels.[12] Ectopic expression of S100A4 in a benign rat mammary cell line increased its potential to metastasize and colonize the lungs and lymph nodes.[16]

The ability to change shape and to become motile are primary requirements for those epithelial and mesenchymal cells that are involved in wound repair.[22] After a wound occurs, epithelial cells surrounding the injury site proliferate and migrate to resurface the denuded tissue.[23] Likewise, within the stroma, the appearance of fibroblasts and myofibroblasts in the region of the injury are characteristic of the wound response.[24] Wound healing has been extensively studied in the cornea, where it has been shown that within the stroma, the normally quiescent keratocyte is converted into the motile and highly secretory fibroblast and later into the contractile myofibroblast.[25] Both fibroblasts and myofibroblasts play critical roles in the healing process. Fibroblasts move about the wounded area, where they secrete collagenases, proteases, and extracellular matrix components, all of which are used in reconstructing the damaged stroma.[26] Myofibroblasts appear at the wound site at a later time, and, because of their contractile nature, promote wound closure.[27]

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Mechanisms underlying the interconversions among the corneal keratocytes, fibroblasts, and myofibroblasts have been explored in cultured keratocytes.[28–32] For example, keratocytes grown in the absence of serum have a phenotype identical with that seen in vivo, whereas the addition of serum results in activation and a resultant fibroblast phenotype.[29,30] The addition of TGFβ to keratocytes or corneal fibroblasts induces the formation of myofibroblasts.[31,32]

During our investigations on the characterization of the stroma that underlies the limbal and corneal epithelia, we found that S100A4 was one of the most abundantly expressed genes in fibroblast cultures prepared from each of these regions. Given the association of S100A4 in the regulation of cytoskeletal changes and cell motility, we investigated whether this S100 protein was involved in corneal wound repair. We report that corneal keratocytes normally express low levels of S100A4 in vivo, but that after wounding, S100A4 was readily observed in the stromal cells as well as in the cells lining the new blood vessels that had formed. Primary cultures of keratocytes showed little S100A4, whereas activated fibroblasts and myofibroblasts revealed a prominent expression of S100A4. Most interesting was the observation that S100A4 exhibited a growth factor–dependent translocation from the cytoplasm to the nucleus when fibroblasts were experimentally converted to myofibroblasts.

Materials and Methods

All experimental procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the University of Pennsylvania Animal Care and Ethics Committee.

Isolation of Rabbit Keratocytes for mRNA

We isolated keratocytes from the limbal and corneal compartments, as previously described.[33] Briefly, the central cornea button covered a 5-mm radius, which was at least 5 mm away from the limbal zone and therefore was free from any limbal or conjunctival cell types. The limbal sample was defined as a 2-mm wide zone in which the K3 keratin was expressed suprabasally. These activated keratocytes are typically referred to as fibroblasts and were generated by their outgrowth from cultured small pieces (<1 mm^3) of explanted limbal or corneal stroma. The stromal pieces were placed in 1 mL Chang's medium (Irvine Scientific, Santa Ana, CA) with 100 U/mL penicillin and 100 μg/mL streptomycin in a 35-mm Petri plate, and left undisturbed in a 37°C and 5% CO_2 incubator for 4 days. Under these conditions, fibroblasts migrated from most of the stromal pieces. After that, the culture medium was changed every 3 days. Ten to 12 days later, the cells were suspended by trypsinization (0.125% trypsin and 0.01% EDTA in phosphate-buffered saline [PBS]), and the dissociated single cells were then plated in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. Subconfluent cultures were fed every 3 days by removing old medium and adding the fresh 37°C medium.

SSH Library Screening

Cultured corneal fibroblasts from the central and limbal regions of the ocular surface were expanded to generate sufficient cell mass for mRNA preparations. Fibroblast-specific cDNA libraries were made using subtractive suppression hybridization (SSH; BD Biosciences-Clontech, Palo Alto, CA). Messages common to corneal and limbal fibroblasts were subtracted from the libraries by cross-hybridization of the two cDNAs, and highly expressed messages were normalized. The cDNA libraries made specific for limbal and corneal fibroblasts were cloned into pCR2.1 (Invitrogen, Carlsbad, CA) and were plated at low density. Colonies were chosen at random and used to make plasmid DNA. The DNA was spotted on a nylon membrane (Hybond; Amersham, Arlington Heights, IL), which were hybridized with probes generated from the two tissue-specific cDNAs, according to Clontech's procedures.

Probes

The rabbit S100A4 cDNA was excised from pCR 2.1 as a 350-bp *Eco*RI fragment. The cDNA fragment was gel purified with commercial reagents (Qiagen, Valencia, CA). S100A4 cDNA was labeled with [α-^{32}P]dCTP (3000 Ci · mmol^{-1}; NEN, Boston, MA) using random primer reagents supplied by Roche Diagnostics (Indianapolis, IN). Probes were separated from unincorporated nucleotides by size-exclusion chromatography through G-50 minicolumns (Amersham Pharmacia Biotech, Piscataway, NJ) and used for Northern blot detection. Linearized pCR 2.1 plasmids carrying the rabbit S100A4 sequences were used to generate labeled sense and antisense riboprobes with [α-^{35}S] UTP (800 Ci · mmol^{-1}; NEN) and T7 and SP6 RNA polymerase (Promega, Madison, WI). The labeled riboprobes were purified by size-exclusion chromatography through G-50 columns. Riboprobes were precipitated and resuspended in 21 μL of diethyl pyrocarbonate (DEPC)-treated H_2O, quantified by scintillation counting, and used for in situ hybridization.

Northern Blot Analysis

Messenger RNA isolated from cultured limbal and corneal fibroblasts was converted to cDNA by PCR synthesis (SMART system; BD Biosciences-Clontech). Equal quantities of both cDNAs were electrophoresed on a 1% agarose gel, transferred to nylon membranes (Hybond; Amersham), and UV cross-linked. Hybridization was performed at 72°C with ^{32}P-labeled S100A4 cDNA, according to previously described procedures. The blot was washed twice in 2× SSC and 0.5% SDS at 55°C for 20 minutes. High-stringency washing was performed in 0.2× SSC and 0.5% SDS at 68°C for 1 hour. Blots were either exposed to a phosphorescence imager (Amersham) or to autoradiograph film (Xomatic; Eastman, Kodak, Rochester, NY). Signal intensity was quantified with the software accompanying the phosphorescence imager (ImageQuant; Amersham).

In Situ Hybridization

Five-micrometer sections were made from paraffin-embedded rabbit corneal tissue. The sections were deparaffinized in xylene and rehydrated through a graded series of ethanol-PBS solutions. Rehydrated sections were fixed with 4% paraformaldehyde in PBS for 10 minutes in a cold room. The slides were washed twice for 5 minutes with 0.5× SSC (20× SSC: 175.3 g NaCl, 88.2 g Na citrate in 1 L DEPC H_2O) at room temperature. Slides were then incubated with proteinase K (1 μg/mL) in RNase buffer (10 mL of 1 M Tris [pH 8.0], 100 mL of 5 M NaCl, made up to 1 L with DEPC H_2O) for 10 minutes. Slides were again washed twice for 10 minutes with 0.5× SSC. Sections were prehybridized in rHB2 buffer (50% formamide, 0.3 M NaCl, 20 mM Tris [pH 8.0], 5 mM EDTA, 1× Denhardt's solution, 10% dextran sulfate, 10 mM dithiothreitol) at 48°C for 2 hours. For each slide, 2 μL of radiolabeled probe (300,000 counts/μL) was combined with 1 μL of tRNA (50 mg/mL), denatured by boiling for 3 minutes, and mixed with 17 μL of rHB2 solution. Denatured probe was added to the prehybridization solution on the slides and gently mixed. Sections were coverslipped, and hybridization was performed overnight at 55°C. The next day, the slides were washed twice for 10 minutes with 2× SS and 2 mM EDTA at room temperature, incubated in RNase solution containing 20 μg/mL RNase A for 30 minutes at room temperature, and washed twice for 10 minutes with 2× SSC/EDTA. High-stringency washing was performed in 0.1× SSC, EDTA, and β-mercaptoethanol (3 mL 20× SSC, 1.2 mL 0.5 M EDTA, 525 μL mercaptoethanol, made up to 600 mL with DEPC H_2O) for 2 hours at 65°C. Sections were dehydrated for 2 minutes each in 50% and 90% ethanol containing 0.3 M ammonium acetate and air dried overnight. Slides were exposed to emulsion (catalog. no. 02757-50; Ilford Scientific Products, Basildon, UK) for 2 weeks, developed, and counterstained with hematoxylin and eosin.

Immunohistochemistry

Polyclonal affinity-purified rabbit anti-mouse S100A4 antibody was kindly provided by Eugene M. Lukanidin (Department of Molecular Cancer Biology, Danish Cancer Center, Copenhagen, Denmark). The specificity of this antibody to mouse S100A4 has been described.[34] Five-micrometer sections were deparaffinized, rehydrated, and rinsed in Tris-buffered saline (TBS). Slides were treated for 1 minute in 10 mM citrate (pH 6.0) and 1 mM EDTA in a pressure cooker to retrieve the antigen. To exhaust endogenous peroxidase, sections were incubated in 1% H_2O_2 for 30 minutes. Blocking was performed with 10% NGS in TBS for 1 hour at room temperature. The blocking solution was removed, and the sections were incubated with the primary antibody diluted 1:250 in TBS containing 10% NGS for 1 hour at room temperature. Slides were washed in TBS and incubated with the secondary antibody, biotinylated goat anti-rabbit IgG (Vectastain; Vector Laboratories) diluted 1:200 in 10% NGS and TBS for 1 hour at room temperature, washed in TBS, and incubated with the avidin-biotin complex (ABC; Vectastain; Vector Laboratories), according to the manufacturer's recommendations. Detection was performed with DAB (Sigma-Aldrich, St. Louis, MO). Sections were counterstained with hematoxylin.

Removal of the Corneal Epithelium by Topical Application of Phorbol Ester

During studies of the mitogenic effects of phorbol myristate (TPA) on corneal epithelium, we noted that, at high concentrations, TPA acts as an irritant and effects a complete removal of the corneal epithelium. Therefore, we used this strategy to wound the corneas and achieve a fibroblastic response. Briefly, we anesthetized SENCAR mice (7 weeks old) with γ-hydroxybutyric acid (intraperitoneal injection of 100 μL of 10% solution in PBS). We administered a single topical application of 50 μL of 1.0% TPA in petrolatum to the surface of both eyes. Control mice were administered petrolatum only. One, 6, and 12 days after treatment, mice (three per group) were killed, and eyes were surgically removed and processed for histology and immunocytochemistry, as described earlier.

Keratocyte, Fibroblast, and Myofibroblast Cultures for Immunocytochemistry

To perform immunocytochemistry on keratocytes (quiescent corneal fibroblasts) rabbit corneas (Pel-Freeze, Rogers, AR) were treated with collagenase according to the method of Masur et al.[35] These freshly isolated keratocytes were plated at 10^5 cells/mL on type I collagen (10 μg/mL) in serum-free medium: DMEM-F12 supplemented with L-glutamine, glutathionine, RPMI vitamin mix, sodium pyruvate (Invitrogen-Gibco), antibiotic-anti-mycotic mix, gentamicin (Sigma-Aldrich), and insulin-transferring selenium supplement (Invitrogen-Gibco).[29] It has been demonstrated that the keratocyte phenotype can be maintained under these conditions. Cultured keratocytes were studied between 24 hours and 1 week of isolation.

To generate corneal fibroblasts, keratocytes were released by collagenase treatment of corneas and the cells plated in DMEM/F12 (Invitrogen-Gibco) and 10% FBS. For evaluation of pure cultures of fibroblasts and myofibroblasts, the primary cultured corneal fibroblasts were resuspended, plated on fibronectin, and grown in serum-free cultures to which specific growth factors were added. Specifically, cultures were trypsinized, and cells resuspended in DMEM/F12+soybean trypsin inhibitor (1 mg/mL) and then plated on fibronectin-coated glass coverslips (10 μg/mL; Sigma-Aldrich) at a cell density of 5×10^5 cells/mL. Cells were allowed to attach for 24 hours in serum-free medium and then growth factors were added according to previous studies.[28,31] Fibroblast cultures were generated by growth in DMEM/F12, 10% FBS, FGF-2 (20 ng/mL; Invitrogen-Gibco), heparin (5 μg/mL; Invitrogen-Gibco), and myofibroblast cultures by growth in DMEM/F12, 1%FBS, and TGFβ1 (0.25 ng/mL; R&D Systems, Minneapolis, MN). At various times after addition of the growth factor—4 hours, 24 hours, and 3 days—cells were fixed in 3% *p*-formaldehyde in PBS (pH 7.4; Sigma-Aldrich) for 15 minutes at room temperature and subsequently immunodetected with rabbit anti-mouse S100A4 antibody (1:200 dilution) for 60 minutes. Anti-S100A4 was visualized with Alexa 488 goat anti-rabbit antibody-conjugated to FITC (1:800 dilution; Molecular Probes, Eugene, OR). All coverslips also underwent immunodetection with mouse monoclonal anti-α-smooth muscle actin-Cy3 (1:40 dilution; Sigma-Aldrich) to identify myofibroblasts. The cells were viewed with a microscope (Axioskop; Carl Zeiss Meditec, Thornwood, NY) and the images recorded on computer (Photoshop; Adobe Systems, Mountain View, CA). Each experimental condition was repeated at least three times.

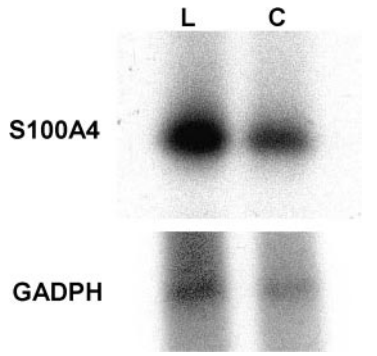

Figure 1. Northern blot analysis of S100A4 mRNAs from rabbit limbal and corneal fibroblasts. *Top*: mRNAs from rabbit limbal and corneal fibroblasts were hybridized with a cDNA fragment of S100A4. An intense signal was observed in both corneal and limbal fibroblasts. *Bottom*: the probe on the same blot was stripped off and rehybridized with a GAPDH cDNA probe as control.

Results and Discussion

Expression of S100A4 in Limbal and Corneal Keratocytes

To identify genes preferentially expressed in limbal and corneal fibroblasts, we prepared cultured rabbit limbal and corneal fibroblast cDNA libraries using subtractive suppression hybridization. Several preferential limbal and corneal genes were identified from these libraries, including apolipoprotein D, and RNase4; however, of all the messages analyzed by Northern blot, S100A4 was the most abundant gene in both limbal and corneal keratocytes (Fig. 1). Elevated levels of S100A4 are known to be associated with growth and morphologic changes that occur in cultured cells, particularly those stimulated by the presence of serum.[36] Because the starting mRNA populations used in this study were obtained from cultured limbal and corneal fibroblasts grown in the presence of serum, the high levels of S100A4 in these cells reflect the growth environment. Furthermore, cells in culture often display an activated phenotype (e.g., high proliferation rate), and elevated levels of S100A4 have been shown to correlate with activated cells.[15,36,37]

To determine the distribution of S100A4 within the normal cornea, we used in situ hybridization to detect mRNA and immunocytochemistry to detect antigen. In situ hybridization showed little if any S100A4 mRNA in the limbal (Fig. 2A) and corneal (Fig. 2B) keratocytes of normal rabbit cornea. It has been reported that the specialized mesenchymal cells that constitute the dermal papilla of the mouse hair follicle, as well as the epithelial cells within the bulge region of the follicle, express S100A4.[38] Therefore, as a positive control for our corneal in situ experiments, we examined rabbit hair follicles, and detected a strong signal for S100A4 mRNA in these two

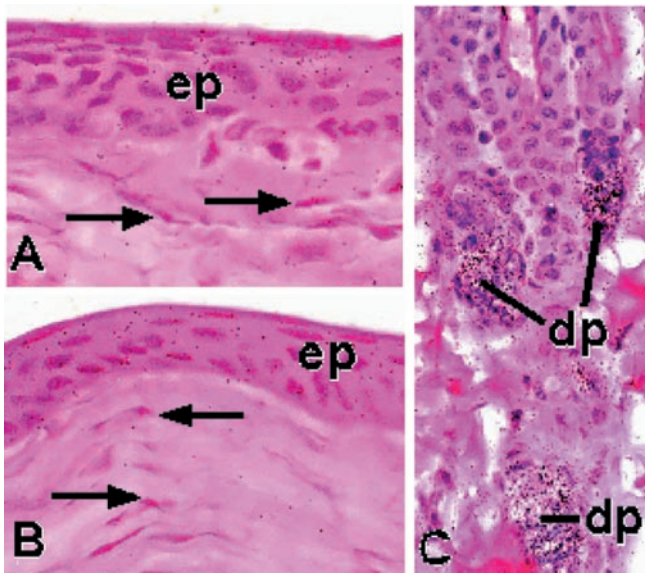

FIGURE 2. S100A4 mRNA is not detected in keratocytes of limbal (**A**) and corneal (**B**) stroma, but is observed in the specialized mesenchymal cells of the hair follicle (**C**). Rabbit limbus (**A**), cornea (**B**), and skin (**C**) were processed for in situ hybridization with ^{35}S-labeled antisense (**A**–**C**) and sense (data not shown) probes to S100A4. Little if any signal was noted in the limbal and corneal keratocytes (*arrows*) and epithelium (ep). In contrast, a strong signal was observed in the specialized mesenchymal cells that constituted the dermal papilla (dp) of the hair follicle (**C**).

follicular zones (Fig. 2C). We also detected S100A4 mRNA in occasional superficial cells of the corneal and limbal epithelium (Figs. 2A, 2B), but the signal was extremely weak.

Consistent with the in situ hybridization data, immunocytochemical staining of mouse corneas with an anti-S100A4 polyclonal antibody revealed that S100A4 antigen was hardly detectable in the normal limbal and corneal keratocytes (Fig. 4A). The low expression patterns of S100A4 mRNA and antigen in the normal corneal keratocytes most likely reflect the quiescent nature of these cells. Likewise, the low expression S100A4 message and protein in the normal corneal and limbal epithelia is indicative that this S100 protein is not involved in proliferation and/or differentiation of these tissues. Similar low expression of S100A4 has been observed in the epidermal keratinocytes and dermal fibroblasts from normal human and mouse skin.[38,39] As a positive control for our immunocytochemical studies, we stained the skin of the backs of mice and detected S100A4-positive cells in the dermal papilla of the hair follicle (Fig. 4B). This is consistent with previous reports that demonstrate S100A4 staining in a specialized population of mesenchymal cells known as the dermal papilla.[38] Although they resemble the fibroblast phenotypically, dermal papilla cells have been demonstrated to be biochemically distinct.[40–42] Furthermore, wheras the fibroblast population is normally static, the dermal papilla cells migrate at specific times during the hair growth cycle.[43] Therefore, S100A4 expression in the dermal papilla cells may be related to the migratory role that has been postulated for this protein.[15]

Expression of S100A4 in Keratocytes after Wounding

Because corneal wounding activates the quiescent keratocytes within the corneal stroma in vivo,[44] we used a corneal wound-healing model to obtain activated fibroblasts. Twenty-four hours after a single topical application of 1% TPA in petrolatum to the surface of the cornea, a complete loss of the corneal epithelium was noted (Fig. 3A). At this time, the stroma contained an occasional necrotic body and was characterized by a marked absence of keratocytes (Fig. 3A). We have used topical application of TPA in petrolatum at lower concentrations (0.01%–0.5%) to stimulate cell proliferation in mouse cornea, limbus, epidermis, and hair follicle.[45–47] After a single application of 0.5% TPA, no evidence of epithelial damage or necrosis was noted[47]; however, a 1% TPA concentration (a twofold

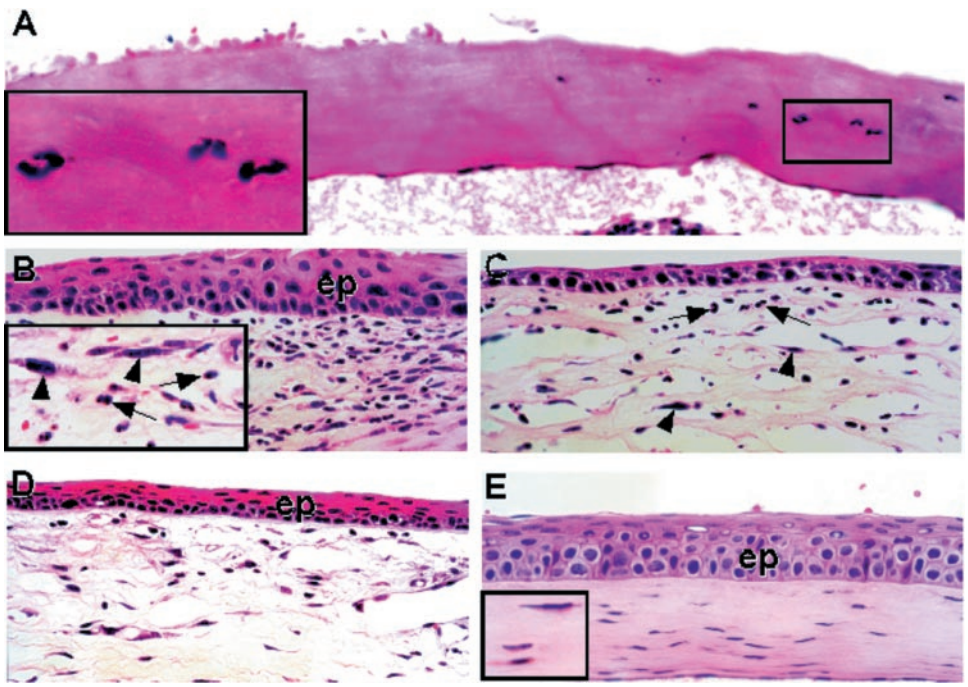

FIGURE 3. Wound repair of the cornea and limbus after topical application of TPA. (**A**) Paraffin-embedded section of a mouse cornea 24 hours after topical application of 1% TPA in petrolatum. Note the complete loss of the epithelium and the absence of keratocytes within the stroma. *Boxed area*: remnants of necrotic keratocytes at higher magnification. (**B**) Paraffin-embedded section of the peripheral cornea 6 days after treatment, as described in (**A**). Note the hyperplastic epithelium (ep) and marked increase in stromal cells. *Boxed area*: fibroblasts (*arrowhead*) and infiltrating neutrophils (*arrows*) at higher magnification. (**C**) Paraffin-embedded section of the central cornea 6 days after treatment, as described in (**A**). At this time, the stroma consisted of fibroblasts (*arrowheads*) and inflammatory cells (*arrows*). (**D**) Paraffin-embedded section of the peripheral cornea 12 days after treatment, as described in (**A**). Note the diminution of inflammatory cells within the stroma and the thinner epithelium (ep). (**E**) Paraffin-embedded section of a mouse cornea treated topically with petrolatum for 6 days. Note the well-organized corneal epithelium (ep) and the avascular stroma consisting of solely of keratocytes. *Boxed area*: thin, flattened keratocytes at higher magnification.

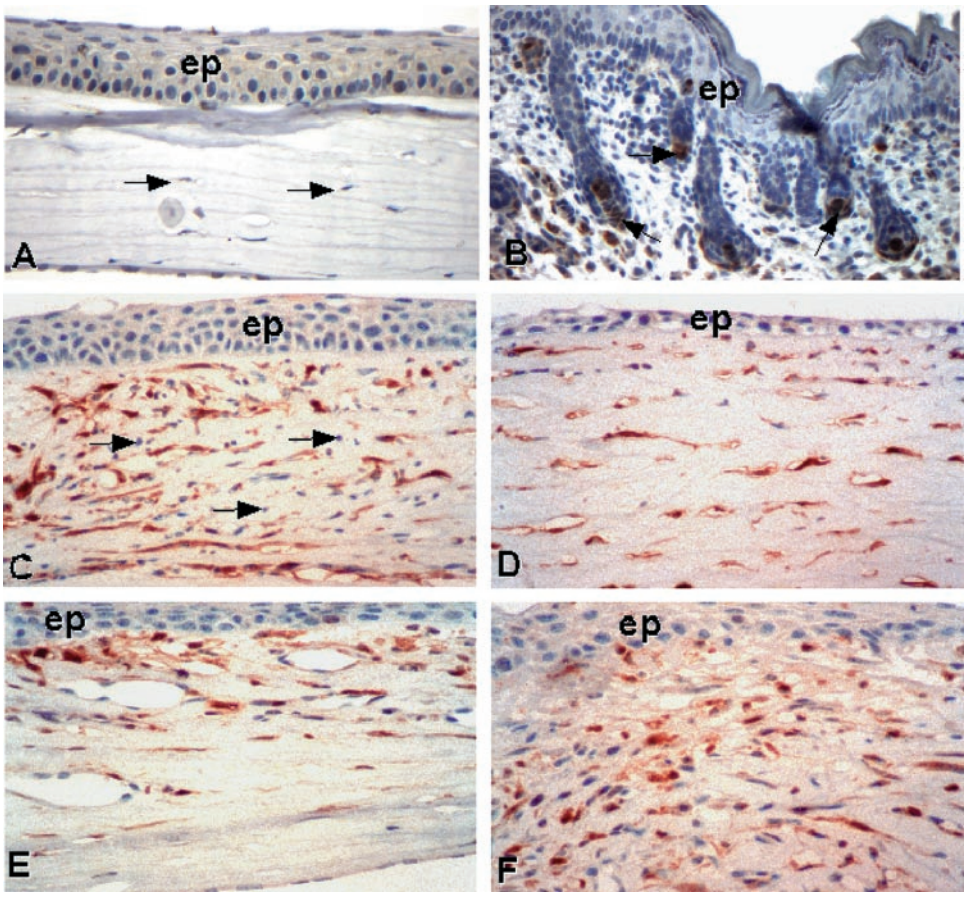

FIGURE 4. S100A4 is expressed in mouse fibroblasts during corneal wound healing. Immunohistochemical staining of mouse cornea 6 days after treatment with petrolatum (**A**), showing little if any S100A4 expression in either the corneal epithelium (ep) or stromal keratocytes (*arrows*). Immunohistochemical staining of mouse back skin (**B**; positive control) showing S100A4 expression primarily restricted to the specialized mesenchymal cells (*arrows*) of the hair follicle. Immunohistochemical staining of mouse peripheral (**C**, **E**) and central (**D**, **F**) corneas 6 (**C**, **D**) and 12 (**E**, **F**) days after topical treatment with 1% TPA in petrolatum. Immunohistochemical staining was performed with a polyclonal rabbit anti-mouse S100A4 IgG, biotinylated secondary IgG, the ABC, and the substrate DAB. Note that all corneal stromal fibroblasts expressed S100A4 antigen at 6 and 12 days after treatment, whereas the inflammatory infiltrate (**C**, *arrows*) and the epithelium (ep) showed no specific staining for S100A4 antigen.

increase) appeared to be toxic, indicating that TPA has a very narrow range between stimulation and toxicity.

Six days after TPA treatment, the corneal surface was completely covered by a new epithelium (Fig. 3B). The peripheral corneal epithelium consisted of 1 to 2 layers of cuboidal cells and 3 to 4 layers of rounded, nucleated wing cells. The epithelium lacked the highly ordered, stratified appearance of the petrolatum-treated control (Fig. 3E). The central corneal epithelium consisted of 1 to 2 layers of cuboidal cells, separated by wide intercellular spaces (Fig. 3C). Little if any S100A4 reactivity was observed in either the peripheral or corneal epithelium at this time (data not shown). The stroma underlying the newly formed epithelium contained many large fibroblasts and the occasional vessel (Fig. 3B). At this time, greater numbers of fibroblasts were observed in the peripheral corneal stroma than in the central corneal stroma (Figs. 3B, 3C). An inflammatory infiltrate consisting primarily of neutrophils and leukocytes was also present throughout the stroma (Figs. 3B, 3C). Six days after wounding, a dramatic increase in S100A4 staining was detected in the fibroblasts in both the central (Fig. 4D) and peripheral (Fig. 4C) corneal stroma. S100A4 staining was not observed in the inflammatory cells that persisted in the peripheral corneal stroma (Fig. 4C).

Twelve days after TPA treatment, the peripheral corneal epithelium was thinner than at the 6-day time point and consisted of a single layer of cuboidal basal cells, several layers of flattened wing cells, and enucleated superficial cells (Fig. 3D). The central corneal epithelium was more stratified, containing two to three layers of cuboidal cells and several layers of flattened nucleated superficial cells (data not shown). S100A4 reactivity was not detectable in the peripheral and central corneal epithelium (Figs. 4E, 4F). Fibroblasts were the major cell type present in the stroma, and the inflammatory infiltrate was reduced, with very few neutrophils or inflammatory cells present. Expression of S100A4 was still noted in the fibroblasts 12 days after TPA treatment (Figs. 4E, 4F). The density of fibroblasts expressing S100A4 was reduced in the peripheral cornea (Fig. 4E), whereas the central corneal stroma still contained numerous S100A4-expressing cells (Fig. 4F).

As we and others have shown, wounding of the cornea results in the disappearance of keratocytes followed by a subsequent repopulation with increased numbers of activated keratocytes or fibroblasts.[48] The demonstration that S100A4 is abundantly expressed in fibroblasts after a corneal wound indicates that S100A4 protein can serve as a marker of activated keratocytes or fibroblasts. Hepatocyte growth factor (HGF) and keratinocyte growth factor (KGF) mRNAs have also been reported to be upregulated in mouse corneal keratocytes after a corneal epithelial scrape wound.[44] Similar to S100A4, both HGF and KGF mRNAs were still expressed 7 days after wounding. Our finding of S100A4 expression in fibroblasts 12 days after wounding indicates that fibroblasts continue to show an activated phenotype well after reepithelialization has been completed. One of the requirements for proper corneal wound repair is the migration of activated fibroblasts into the wounded stroma.[25,26] As mentioned previously, S100A4 has been implicated in processes of cell motility and invasiveness,[15] and we suggest that S100A4 may play a role in fibroblast migration within the corneal stroma.

Changes in the Subcellular Distribution of S100A4 Caused by the Conversion of Corneal Fibroblasts into Myofibroblasts

It has been shown that corneal keratocytes when placed in serum-containing culture assume a fibroblast phenotype and

that these fibroblasts can be experimentally converted to myofibroblasts.[28,31,32] Because fibroblasts and myofibroblasts are the primary cell types involved in corneal stromal wound repair, we used this experimental system to compliment and extend our in vivo perturbation experiments, by assessing the in vitro S100A4 distribution patterns in keratocytes, fibroblasts, and myofibroblasts. When freshly isolated keratocytes were plated on collagen and grown in the absence of serum for 3 days, only diffuse, minimal expression of S100A4 antigen was noted in these cells (Fig. 5A).

For the study of growth factor–induced differentiation of fibroblasts and myofibroblasts, growth factors were added to primary corneal fibroblast cultures. Addition of FGF and heparin to the culture medium (serum included) resulted in a culture in which most of the cells are fibroblasts (Fig. 6A). S100A4 was primarily detected in the cytoplasm of these fibroblasts. As verification that this staining pattern was specific for S100A4, a second rabbit polyclonal antibody to S100A4 (Dako, Carpinteria, CA) yielded identical cytoplasmic staining. Western blot analysis of rabbit corneal fibroblast lysates using this antibody detected a single band of approximately 12 kDa, which corresponds to S100A4. After 1 (Fig. 6B) and 4 (Fig. 6C) hours' exposure of the keratocytes to FGF and heparin, S100A4 was concentrated at the tips of the fibroblast projections, regions known to be engaged in actin assembly. Such an early location of S100A4 to the cell periphery after the addition of FGF and heparin is consistent with the involvement of this protein in cell shape changes.[13]

Addition of TGFβ to the culture medium for 3 to 4 days converted fibroblasts into myofibroblasts (Fig. 6D) identified by the presence of α-smooth muscle actin in stress fibers (Fig. 6E).[49–51] In these differentiated myofibroblasts, S100A4 staining was primarily restricted to the nucleus (Fig. 6D). The translocation of S100A4 into the nucleus occurred between 4 and 24 hours after the addition of TGF-β (Figs. 6F, 6G). At 4 hours, these cells were fibroblastic, and S100A4 was primarily cytoplasmic, consistent with their phenotype, whereas by 24 hours, S100A4 was primarily detected in the nucleus, consistent with myofibroblast differentiation.

In typical cultures derived from cornea stroma and grown in medium with serum, the cells are predominantly fibroblasts with approximately 12% to 20% myofibroblasts.[28] In these mixed cultures, a heterogeneous pattern of S100A4 localization was detected. The myofibroblasts had nuclear S100A4, whereas most of the cells were fibroblasts and had cytoplasmic S100A4.

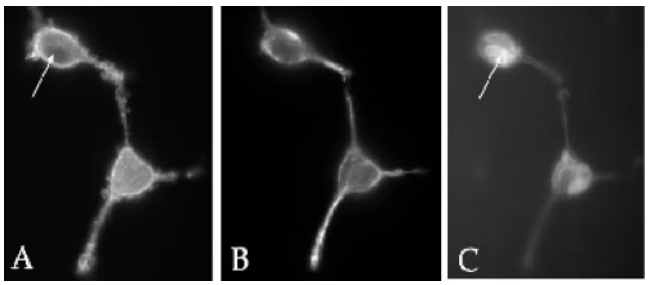

FIGURE 5. Rabbit corneal keratocytes express minimal S100A4 in culture. Freshly isolated keratocytes, plated on collagen and grown in serum-free medium have a neuronal appearance. Immunodetected S100A4 (**A**), vimentin (**B**), and Hoechst nuclear dye (**C**) were visualized in the same cells. After 3 days in culture, expression of S100A4 was limited to the cell periphery (**A**) and was minimal compared with that in fibroblasts and myofibroblasts (Fig. 6). Immunodetection of the fibroblast intermediate filament protein, vimentin (**B**) confirmed that the cells were keratocytes (fibroblast lineage) rather than epithelial or endothelial in origin. (**A**, **C**) Absence of S100A4 in the keratocyte nucleus.

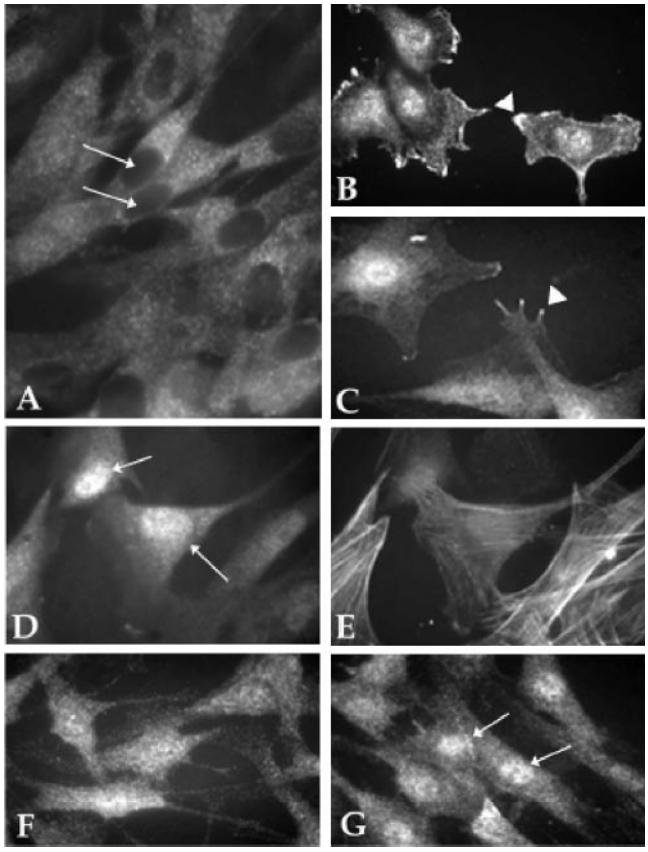

FIGURE 6. Cultured rabbit corneal fibroblast and myofibroblasts express S100A4. (**A**, **C**) Immunofluorescence micrographs of subconfluent rabbit corneal fibroblasts exposed to FGF and heparin in 10% FBS. (**A**, *arrows*) Nuclei without immunodetectable S100A4. After 1 (**B**) or 4 (**C**) hours of FGF and heparin treatment, S100A4 localized in the footlike processes at the cell periphery (*arrowheads*). (**D–G**) In contrast, S100A4 was highly expressed in the nuclei (*arrows*) of myofibroblasts differentiated after 3 days of TGF-β treatment. (**D**, **E**) Micrographs of the same myofibroblasts immunodetected for S100A4 (**D**) and for α-smooth muscle actin (**E**). Four hours after the initial exposure to TGF-β, S100A4 was in the nuclei and cytoplasm (**F**), whereas at 24 hours, antibody to S100A4 detected an increasingly nuclear pattern (**G**, *arrows*). Magnification, $\times$100.

This suggests that within the wound-activated cornea there is a stimulus-dependent translocation of S100A4 from the cytoplasm to the nucleus as cells differentiate from fibroblasts to myofibroblasts. Because S100A4 does not contain a nuclear localization signal, it most likely enters the nucleus in association with another molecule. In vitro studies have shown that S100A4 is capable of interacting with many non-S100 protein targets.[10,19,52–54] Most recently, it has been reported that the tumor-suppressor protein p53 interacts with S100A4.[55,56] The C-terminal portion of p53 is a multifunctional domain that is responsible for nuclear translocation, and S100A4 has been shown to interact with a region of the p53 molecule that contains two of the three proposed nuclear localization regions of p53.[56] Thus, it is possible that S100A4 enters the nucleus combined with p53. Transfection of p53 expression constructs into S100A4-inducible cell lines significantly increases the level of p53-dependent apoptosis on S100A4 induction.[56] This has led to the suggestion that S100A4 and p53 cooperate in the promotion of apoptosis. It is recognized that myofibroblasts are involved in the formation of granulation tissue, fibrosis, and, ultimately, scarring.[51] Normal healing is accompanied by loss of α-smooth muscle actin expression, either by reversion of myofibroblasts to fibroblasts or myofibroblast apoptosis.[25,57]

At present, it is not clear how myofibroblasts are removed; however, one possibility is that after wound contraction is completed, S100A4 in conjunction with p53 induces myofibroblast apoptosis.

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