Human Keratocytes Cultured on Amniotic Membrane
Stroma Preserve Morphology and Express Keratocan

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PURPOSE. To develop a new method of expanding human corneal keratocytes in serum while maintaining their characteristic morphology and keratocan expression.

METHODS. Human keratocytes were isolated from central corneal buttons by digestion in 1 mg/mL of collagenase A in DMEM and seeded on plastic or the stromal matrix of human amniotic membrane (AM) in DMEM with different concentrations of FBS. On confluence, cells on AM were continuously subcultured for six passages on AM or plastic. In parallel, cells cultured on plastic at passages 3 and 11 were reseeded on AM. Cellular morphology and cell–cell networks were assessed by phase-contrast microscopy and a cell viability assay, respectively. Expression of keratocan was determined by RT-PCR and Western blot analysis.

RESULTS. Trophined stroma yielded 91,600 ± 26,300 cells (ranging from 67,000 to 128,000 cells per corneal button). Twenty-four hours after seeding, cells appeared dendritic on AM, even in 10% FBS but fibroblastic on plastic. Such a difference in morphology correlated with expression of keratocan assessed by RT-PCR and Western blot, which was high and continued at least to passage 6 on AM, even in 10% FBS, but was rapidly lost each time when cells on AM were passaged on plastic. Fibroblasts continuously cultured on plastic to passages 3 and 11 did not reverse their morphology or synthesize keratocan when reseeded on plastic in 1% FBS or on AM.

CONCLUSIONS. Human keratocytes maintain their characteristic morphology and keratocan expression when subcultured on AM stromal matrix even in the presence of high serum concentrations. This method can be used to engineer a new corneal stroma. (Invest Ophthalmol Vis Sci. 2005;44:5136–5141)
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The extracellular matrix of the corneal stroma contains a dense network of collagen fibrils and proteoglycans arranged in an astonishing order to allow transparency for clear vision. Keratocytes, which are the cells in the corneal stromal matrix, are dendritic in shape,1 form extensive cellular networks,2–5 and synthesize collagens I, V, VI, and XII6–5 and keratan sulfate-containing proteoglycans such as lumican, keratocan, and imucin.6–8 Among all keratan sulfate-containing proteoglycans, keratocan is uniquely synthesized by mouse keratocytes in vivo,9 whereas lumican and imucin are widely distributed.10–13

To investigate how keratocytes maintain corneal stromal transparency, it is important to expand their number by subculturing. Unfortunately, all such attempts fail to maintain the normal phenotype of keratocytes. When cultured on a plastic substrate in a serum-containing medium, bovine14 and rabbit15 keratocytes rapidly lose their dendritic morphology14,15 and acquire a fibroblastic morphology.14 At the same time, they start expressing integrin α5β116 and α-smooth muscle actin,17,18 a marker for myofibroblasts,19 especially when seeded at a low density.18 In addition, such subculturing condition reduces the ratio of keratan sulfate-containing proteoglycans to dermatan sulfate-containing proteoglycans.14,20,21

Without knowing which factor(s) in the serum is detrimental to the maintenance of the kerocyte’s phenotype, a serum-free medium has been adopted to culture bovine keratocytes so as to maintain the dendritic morphology and a normal ratio of keratan sulfate-containing proteoglycans to dermatan sulfate-containing proteoglycans.14 Under such a serum-free culturing condition, these keratocytes secrete lumican, keratocan, and imucin.22,23 Nevertheless, this serum-free culturing method precludes ex vivo expansion and subculturing.14,24

Herein, we report our success in developing a new culture system to achieve ex vivo expansion of human corneal keratocytes while maintaining their characteristic dendritic morphology and continuous expression of keratocan, even in the presence of high concentrations of serum, by growing them on the stromal matrix of the human amniotic membrane (AM). The significance of this finding is further discussed.

MATERIALS AND METHODS

The tissue culture plastic plates (six-well) and 30 mm culture dishes were purchased from BD Biosciences (Lincoln Park, NJ); culture plate inserts used for fastening AM from Millipore (Bedford, MA); amphotericin B, Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), gentamicin, Hank’s balanced salt solutions (HBSS), HEPES buffer, phosphate buffered saline (PBS), 0.05% trypsin/0.53 mM EDTA, and RNA extraction reagent (Trizol) from Invitrogen-Gibco (Grand Island, NY); 4% to 15% gradient SDS-polyacrylamide gel and horseradish secondary anti-rabbit antibody from Bio-Rad (Hercules, CA); collagenase A from Roche Diagnostics (Indianapolis, IN); aminobenzamidine, EDTA tetrasodium salt, guanidine, hydrochloric acid, isopropanol, chloroform, endo-β-galactosidase, sodium acetate, and urea from Sigma-Aldrich (St. Louis, MO). A cell viability assay (Live/Dead Assay) was obtained from Molecular Probes (Eugene, OR).

Isolation of Human Keratocytes

Human corneas stored in humid chambers for less than 4 days were obtained from the Florida Lions Eye Bank (Miami, FL). An 8-mm Barron’s trephine was used to remove a central corneal button. After the corneal epithelium was scrapped off with a cell scraper and
Descemet’s membrane was peeled off, the remaining corneal stroma was cut into 0.5 × 0.5-mm pieces. These stromal pieces (~12 per cornea) were then incubated at 37°C for 45 minutes in DMEM containing 1 mg/mL collagenase A in a plastic dish. After incubation, collagenase A was removed by pipetting, and the digested stromal pieces were incubated in a second aliquot of collagenase A for another 45 minutes or until the tissue became smeared onto the bottom of the dish. The digested tissue was then centrifuged at 800g for 5 minutes and resuspended in 1.5 mL of DMEM containing 20 mM HEPES, 50 μg/mL gentamicin, and 1.25 μg/mL amphotericin B per cornea. This keratocyte-containing cell suspension was then seeded on plastic dishes or the stromal side of the AM.

**Primary Culture of Keratocytes on Plastic or AM**

Human AM preserved according to the method described by Lee and Tsenge was kindly provided by Bio-Tissue (Miami, FL). After thawing, human AM was incubated in HBSS containing 0.1% EDTA for 30 minutes at 37°C, and the amniotic epithelium was then denuded with an epithelial scraper (Amino; Innova, Toronto, Ontario, Canada). Epithelia denuded AM with the stromal side facing up was tightened to a small plastic insert of 32-mm diameter, using a rubber band in a manner similar to that described elsewhere. The keratocyte cell suspension prepared from one corneal button was seeded on each 3-mm insert or a 35-mm plastic dish. Cells were cultured in a medium containing DMEM supplemented with 10% FBS and the medium was changed every 2 to 3 days. In a separate experiment, cultures grown on AM containing DMEM containing 10% FBS were continuously subcultured at 1:3 split on plastic. Cells on plastic at passages 3 and 11 were seeded on plastic in DMEM containing 1%, 5%, or 10% FBS or on AM stromal matrix in DMEM containing 10% FBS to see whether there was any reversibility in morphology and keratocan expression.

**Subculture of Keratocytes on Plastic and AM**

When the primary culture on AM reached 70% to 80% confluence, cells were dissociated into single cells by incubation in HBSS containing 0.05% trypsin and 0.53 mM EDTA at 37°C for 20 minutes, followed by vigorous pipetting. After centrifuging at 800g for 5 minutes, cells were resuspended in DMEM containing 10% FBS, subdivided into two equal parts, with one being seeded onto AM stroma and the other on a plastic dish. They were cultured in DMEM containing 10% FBS. The AM culture was subcultured to either AM or plastic in the same manner as described above for a total of six passages. In parallel, cells grown on plastic in DMEM containing 10% FBS were continuously subcultured at 1:3 split on plastic. Cells on plastic at passages 3 and 11 were seeded on plastic in DMEM containing 1%, 5%, or 10% FBS or on AM stromal matrix in DMEM containing 10% FBS to see whether there was any reversibility in morphology and keratocan expression.

**Morphologic Analysis Using a Cell Viability Assay**

At each passage on AM or plastic, cell morphology was documented by phase-contrast microscopy and in some instances analyzed by cytoplasmic staining in a cell viability assay, according to a method described by Poole et al. and the manufacturer (Live/Dead Assay; Molecular Probes) as a means to enhance the three-dimensional visibility of the keratocan and cell-cell interaction and morphology. The assay is based on the principle that live cells produce an intense green fluorescence in the cytoplasm when cell-permeant calcein becomes fluorescent by the action of an intracellular esterase.

Briefly, after the removal of the culture medium, cells were washed twice with HBSS and incubated for 40 minutes with 0.5 mL green fluorescent stain consisting of 2 mM calcein-AM, and 4 mM ethidium homodimer in PBS. After cells were washed with PBS, they were examined by an epifluorescence microscope (Te-2000u Eclipse; Nikon, Tokyo, Japan).

**Reverse Transcription–Polymerase Chain Reaction**

Total RNA was extracted (TRIzol; Invitrogen-Gibco) reagent from two 8-mm central corneal buttons that had been minced with a blade and sonicated at 6000 rpm (Tissue Tearor sonicator; Biospec Products, Inc., Racine, WI) as a positive control. Total RNA was similarly extracted from cells cultured on plastic or AM. Total RNA equivalent to 1 × 10⁶ cultured cells or one corneal button was subjected to RT-PCR based on a protocol recommended by Promega (Madison, WI). The final concentration of RT reaction was 10 mM Tris-HCl (pH 9.0 at 25°C), 5 mM MgCl₂, 50 mM KCl, 0.1% Triton X-100, 1 mM each dNTP, 1 μ/L recombinant RNase in ribonucleases inhibitor, 15 U avian myeloblastosis virus (AMV) reverse transcriptase, 0.5 μg Oligo(dT)₁₅ primer and total RNA in a total volume of 20 μL. The reaction was kept at 42°C for 60 minutes. One tenth of the RT product was used for subsequent PCR with the final concentration of PCR reaction being 10 mM Tris-HCl (pH 8.3 at 25°C), 50 mM KCl, 1.5 mM Mg(OAc)₂, 1.25 U Taq DNA polymerase in a total volume of 50 μL, with primers shown in Table 1. The PCR mixture was first denatured at 94°C for 5 minutes then amplified for 30 cycles (94°C, 1 minute; 60°C, 1 minute; 72°C, 1 minute) using a programmable thermal controller (PTC-100; MJ Research, Inc., Watertown, MA). After amplification, 15 μL of each PCR product and 3 μL of 6× loading buffer were mixed and electrophoresed on a 1.5% agarose gel in 0.5× Tris-boric acid-EDTA (TBE) containing 0.5 μg/mL ethidium bromide. Gels were photographed and scanned.

**Western Blot Analysis**

Total cellular protein was isolated from cells cultured on AM and plastic at P2 and P4 with extraction reagent (TRIzol; Invitrogen-Gibco) for total RNA (see total RNA isolation). After complete removal of the aqueous phase that contain total RNA and precipitation of DNA with ethanol, the protein in the phenol:ethanol supernatant was precipitated with isopropl alcohol. The protein pellet was then extracted in 4 M guanidine-HCl containing 10 mM sodium acetate, 10 mM sodium EDTA, 5 mM aminobenzamidine, and 0.1 M e-amino-n-caproic acid at

**Table 1. RT-PCR Primer Sequences**

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<th>cDNA Location</th>
<th>Size of PCR Product (bp)</th>
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Products, Inc.) for use as a positive control. Proteins extraction was blade and sonicated at 6000 rpm (Tissue Tearor sonicator; Biospec Elmer, Boston, MA). Two normal human corneas were minced with a volume of 2 ml 6 M urea. The protein concentration was measured by spectrophotometer at optical density of 280 nm.

RESULTS

Morphologic Differences in Primary Cultures

Cell suspension obtained after collagenase digestion yielded 91,600 ± 26,500 cells (ranging from 67,000 to 128,000 cells per corneal button). Within 24 hours after seeding, cells attached to plastic and AM matrix and exhibited a distinctly different morphology. On AM stroma, cells were dendritic or stellate and formed a connecting network when grown in the presence of 1% or 10% FBS for 1 week (Figs. 1A, 1B, respectively). Cells on AM matrix projected their dendritic processes in a three-dimensional pattern. In contrast, cells on plastic up to passage 10 (not shown) grew rapidly on plastic with 10% FBS. All micrographs were taken at the same magnification. Bar, 25 μm.

Four cultures were incubated in distilled water and the water-insoluble fraction was dissolved in 0.1 M Tris-acetate solution (pH 6.0) containing 6 M urea. The protein concentration was measured by spectrophotometry at optical density of 280 nm. One-hundred-microgram protein aliquots were incubated with endo-β-galactosidase (0.1 U/ml, Sigma-Aldrich) at 37°C overnight. Equal volume of 2× SDS sample buffer was added into samples, boiled for 5 minutes, electrophoresed on an SDS-PAGE gradient (4%-15%) gel, and transferred to a nitrocellulose membrane. These membranes were preincubated with blocking buffer and probed with an affinity-purified polyclonal antibody raised against a synthetic peptide (RSERQVYEVHDSDDWTIH)27 corresponding to 18 N-terminal amino acids of the predicted human keratocan protein (a gift from Albert de la Chapelle, Ohio State University, Columbus, OH). Immunoreactivity was visualized with an enhanced chemiluminescence reagent (Perkin Elmer, Boston, MA). Two normal human corneas were minced with a blade and sonicated at 6000 rpm (Tissue Tearor sonicator; Biospec Products, Inc.) for use as a positive control. Proteins extraction was performed by using the procedure described for expanded cells but omitting the extraction reagent (TRIzol; Invitrogen-Gibco).

To display better the difference in cellular morphology of these two culture systems, we used the cytoplasmic stain in a cell viability assay (Live/Dead Assay; Molecular Probes) to demarcate the entire cytoplasm. Indeed, most of the cells grown on AM stroma in 10% FBS had a triangular-shaped cell body, and their cytoplasm was stretched into many thin dendritic processes (Fig. 2A). These processes formed extensive intercellular networks in a three-dimensional pattern (Fig. 2B). In contrast, cells grown on plastic in 10% FBS maintained spindle-shaped cytoplasm with no intercellular contact (Figs. 2C, 2D).

Morphologic Differences in Continuous Passages

Cells continued to maintain a dendritic morphology and formed widespread networks when continuously passaged from the primary culture, so long as they were grown on AM stromal matrix. As shown in Figure 3, such a dendritic morphology was maintained up to passages 2 and 4 (Figs. 3A, 3C, respectively). Similarly, labeling with green fluorescent stain showed that extensive intercellular networks were maintained (Fig. 3B, 3D, respectively). In contrast, cells immediately adopted a spindle shape within 24 hours when subcultured from the primary AM culture to a plastic dish (Fig. 3E), with a marked reduction in the formation of intercellular networks (Fig. 3F). Such a dramatic change in cell morphology from dendritic to spindle shaped was consistently observed each time cells in an AM culture were subsequently cultured on a plastic dish for the total of six passages tested so far (not shown).

Cells that had continuously been cultured on plastic up to passage 3 were seeded to AM stromal matrix to test whether the morphology could be reversed. Phase-contrast microscopy confirmed the fibroblastic morphology of the passage-4 cells cultured on plastic in DMEM containing 10% FBS (Fig. 4A). The passage-4 cells on AM stroma remained spindle shaped and did not revert to a dendritic morphology (Fig. 4B). Furthermore, cell–cell networks were absent in the former but maintained in the latter (not shown). Even if they were cultured on plastic with 1% FBS, their spindle shape was not changed (not shown). The same result was obtained when we used cells continuously cultured on plastic up to passage 10 (not shown).

Keratocan Expression

Reverse Transcription–Polymerase Chain Reaction.

Total RNA was extracted from cells seeded on plastic and AM, and RT-PCR was used to determine the expression of keratocan transcript with a size of 1059 bp. Keratocan was not expressed in cells continuously cultured on plastic at passage 3 when seeded in DMEM containing different serum concentrations (1%, 5%, or 10% FBS) or on AM. Keratocan was expressed in the normal control corneal stroma (K; Fig. 4). In primary cultures, cells grown on plastic barely expressed keratocan transcript in 1% FBS, but rapidly lost keratocan expression in 5% or 10% FBS (Fig. 5). In contrast, cells expressed abundant amounts of keratocan transcript in 1%, 5%, and 10% FBS, with the highest noted in 5% FBS (Fig. 5).

To determine whether such a difference in keratocan expression correlates with the morphologic changes noted, we continued to subculture the primary cells on AM for a total of six passages. For each passage, cells cultured on AM were equally divided and subcultured on either AM or plastic. All cells were grown in DMEM containing 10% FBS. As shown in Figure 6, using glyceraldehyde-3-phosphate dehydrogenase (GAPDH; with a size of 573 bp) as a loading control, we noted that cells subcultured on plastic showed reduced expression of FBS, cells on plastic reached confluence in 6 days, and cells on AM did so in 14 to 17 days.
keratocan transcript at passages 1 and 2 but did not express keratocan transcript thereafter up to passage 5. In contrast, cells subcultured on AM expressed abundant amounts of keratocan transcript at passages 1 and 2, and continued to do so up to passage 5. This dramatic difference was maintained at passage 6, the last passage tested up to the time of the present report (not shown). Furthermore, keratocan expression was lost when cells were subcultured from passages 1 to 2 on plastic (P1→P2).

Unlike the aforementioned expression pattern of keratocan, transcripts of lumican (1015 bp) and collagen III-a1 (568 bp) were uniformly expressed up to passage 5 by cells grown on AM and plastic (Fig. 6). As a control, the normal cornea stroma (K) expressed keratocan and lumican, but not collagen III-a1 (Fig. 6). The finding that collagen III-a1 is not expressed by normal corneal stroma, but is expressed in wounded cornea, has been reported.28

Cells continuously cultured on plastic with 10% FBS up to passage 3 did not express any keratocan transcript when subcultured on plastic, even in 1% FBS, or seeded back on AM (Fig. 4). The same result was obtained for cells continuously cultured on plastic for up to passage 11 (not shown).

**Western Blot Analysis.** To correlate transcript expression with protein expression, we performed Western blot analysis.

**Figure 2.** Cytoplasmic staining using a cell viability assay. In primary AM cultures, cells formed an extensive cell network (A) with some showing extensive dendritic processes projected in three dimensions (B). In contrast, cells in primary plastic cultures did not form a network (C) and appeared spindle shaped without dendritic processes (D). (A, C) Same magnification; (B, D) higher magnification. Bar, 25 μm.

**Figure 3.** Morphologic differences between cells cultured on plastic or AM, after subculturing. Phase-contrast microscopy shows cells cultured on AM with a dendritic morphology at passages 2 (A) and 4 (C). Cells also formed extensive cell–cell networks (B, D, respectively). In contrast, cells cultured on D at passage 1 immediately adopted a fibroblastic morphology when they were subcultured on plastic (E), without forming cell–cell networks (F). (D, E, F) Cell viability staining. All micrographs were taken at the same magnification. Bar, 25 μm.

**Figure 4.** Changes in morphology and keratocan expression. Cells that have continuously been grown on plastic for three passages were seeded on plastic and AM. The fibroblastic morphology noted on plastic (A) remained spindle shaped and cells on AM (B) did not revert to a dendritic morphology. Micrographs were taken at the same magnification. Bar, 25 μm. Expression of keratocan transcript (1059 bp) by RT-PCR was found in the normal corneal stroma (K), but was not detected in cells on plastic with 1%, 5%, or 10% FBS or on AM with 10% FBS. The expression of GAPDH (573 bp) served as a loading control.

**Figure 5.** RT-PCR analysis of expression of keratocan transcript in primary cultures. Total RNA was extracted from primary plastic and AM cultures. With GAPDH (573 bp) as a loading control, expression of keratocan transcript (1059 bp) was barely detected on plastic with 1% FBS, but was absent in 5% or 10% FBS. In contrast, keratocan transcript was readily detected in cells on AM in 1%, 5%, and 10% FBS (with the highest noted in 5% FBS) and in normal corneal stroma (K).
Proteins extracted by guanidine HCl from cells grown on AM and plastic at passages 2 and 4 clearly expressed a positive band of 50 kDa, which was consistent with keratan$^7$ expressed by normal corneal stroma as a positive control (Fig. 7). In contrast, this protein band was not detected in proteins extracted from cells cultured on plastic at passages 2 and 4 (Fig. 7).

**DISCUSSION**

The results of the present study provide strong experimental evidence that dendritic morphology and keratocan expression by cultured human keratocytes can be maintained on AM stromal matrix during their continuous expansion in the presence of high concentrations of serum for at least six passages. This accomplishment represents a significant advance in the field of keratocyte biology, because all previous attempts based on conventional plastic cultures have failed. Earlier studies have used the dendritic morphology and formation of extensive intercellular networks as the hallmark of keratocytes in bovine culture systems.\(^ {14}\) Such a characteristic dendritic morphology can be achieved on plastic culture only in a serum-free medium, but is rapidly lost in a serum-containing medium.\(^ {14,15}\)

Because human keratocytes on AM stromal matrix continue to maintain such a characteristic morphology even in the presence of serum, this new culture system can allow ex vivo expansion of keratocytes for further manipulations and studies without losing its phenotype. As a result, we believe that this new culture system can be used as the first step toward engineering the human corneal stroma.

In our study, the dendritic morphology correlated well with the expression of keratocan transcript and protein. Among all keratan sulfate-containing proteoglycans, keratocan is uniquely expressed by keratocytes.\(^ {9,22}\) Unlike lumican and collagen III-a1, which were uniformly expressed by cells on both plastic and AM, keratocan was expressed only by cells on AM. This finding further supports the notion that keratocan expression is a specific hallmark for keratocytes. This new culture system based on AM stromal matrix will help us to investigate how the keratocan gene is expressed and to determine whether expression of keratocan influences the corneal stromal transparency.

It is worth reiterating that the phenotype of keratocytes with respect to dendritic morphology and keratocan expression is easily lost on plastic when serum is added, but can be maintained on AM, even in the presence of high serum. Such a contrast in serum modulation provides a clue from which one might probe the mechanism by which the keratocyte phenotype is maintained. We have reported that TGF-β1 signaling is activated on plastic but is suppressed when human corneal fibroblasts are cultured on AM stromal matrix.\(^ {28}\) Furthermore, addition of exogenous TGF-β1 in serum-free DMEM with insulin, transferrin, and selenium activates further TGF-β signaling, leading to activation of downstream expression of α-smooth muscle actin in human corneal fibroblasts on plastic cultures, but not in those on AM stroma matrix.\(^ {29}\) We thus speculate that an important mechanism that causes keratocytes to lose their phenotype on plastic cultures, especially in the presence of serum, is the activation of TGF-β1 signaling. In contrast, TGF-β1 signaling is not activated because of the suppressive action of AM stromal matrix. This putative mechanism is not only pathologically important to prevent myofibroblast differentiation as reported,\(^ {29,31}\) (Choi TH, et al. IOVS 1999;40:ARVO Abstract S328), but is also physiologically relevant in maintaining the keratocyte phenotype. Future investigation into the molecular mechanism whereby TGF-β signaling is suppressed by AM stromal matrix will not only unveil how AM transplantation prevents scar formation in ocular surface reconstruction (for reviews see Refs. 29,32,33), but also how the normal phenotype of fibroblasts of different tissues can be maintained in vitro using this new culture system.

**References**

can cDNA and genomic DNA and the characterization of its ex-
22598.
10. Ujita M, Shinomura T, Kimata K. Molecular cloning of the mouse
11. Ying S, Siraishi A, Kao CW-C, et al. The cloning of mouse kerato-
can cDNA and genomic DNA and the characterization of its ex-
lagen fibril assembly: skin fragility and corneal opacity in the
lumican-null mice: defects in collagen fibril structure and packing
in the posterior stroma. Invest Ophtalmol Vis Sci. 2000;41:3565–
3573.
synthesis by bovine keratocytes and corneal fibroblasts: mainte-
15. Jester JV, Barry-Lane PA, Cavanagh HD, Petroll WM. Induction of
α-smooth muscle actin expression and myofibroblast transforma-
16. Masur SK, Cheung JKH, Antohi S. Identification of integrins in
cultured corneal fibroblasts and in isolated keratocytes. Invest
Ophtalmol Vis Sci. 1993;34:2690–2698.
17. Desmoulieère A, Geinoz A, Gabbiani F, Gabbiani G. Transforming
growth factor β1 induces α-smooth muscle actin expression in
granulation tissue myofibroblasts and in quiescent and growing
18. Masur SK, Dewal HS, Dinh TT, et al. Myofibroblasts differentiate
19. Gabbiani G, Chapronnier C, Hüttner I. Cytoplasmic filaments and
gap junctions in epithelial cells and myofibroblasts during wound
20. Dahl IM, Johnsen W, Anseth A, Prydz H. The synthesis of glyco-
aminoglycans by corneal stroma cells in culture. Exp Cell Res.
21. Dahl IM, Coster L. Proteoglycan biosynthesis in cultures of corneas
and corneal stroma cells from adult rabbits. Exp Eye Res. 1978;27:
175–190.
22. Berryhill BL, Beales MP, Hassell JR. Production of prostaglandin D
synthase as a keratan sulfate proteoglycan by cultured bovine
keratocyte phenotype to bovine keratocytes made fibroblastic by
24. Jester JV, Petroll WM, Cavanagh HD. Corneal stromal wound
healing in refractive surgery: the role of myofibroblasts. Prog Retin
25. Lee SH, Tseng SCG. Amniotic membrane transplantation for per-
sistent epithelial defects with ulceration. Am J Ophtalmol. 1997;
123:303–312.
26. Grueterich M, Espana E, Tseng SC. Connexin 43 expression and
proliferation of human limbal epithelium on intact and denuded
27. Pellegrata NS, Dieguez-Lucena JL, Joensuu T, et al. Mutations in
25:91–95.
28. White J, Werkmeister JA, Ramshaw JA, Birk DE. Organization of
fibrillar collagen in the human and bovine cornea: collagen types
29. Tseng SCG, Li D-Q, Ma X. Suppression of transforming growth
factor isoforms, TGF-β receptor II, and myofibroblast differentia-
tion in cultured human corneal and limbal fibroblasts by amniotic
both normal conjunctival fibroblasts and pterygial body fibroblasts
31. Choi TH, Tseng SCG. In vitro and in vitro demonstration of
epithelial cell-induced myofibroblast differentiation of keratocytes
and an inhibitory effect by amniotic membrane Cornea. 2001;20:
197–204.
32. Solomon A, Rosenblatt M, Monroy DC, et al. Suppression of inter-
leukin-1a and interleukin-1b in the human corneal epithelial cells
2001;85:444–449.
33. Tseng SCG, Tsubota K. Amniotic membrane transplantation for
ocular surface reconstruction. In: Holland EJ, Mannis MJ, eds.