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Regulatory Role of FGF-2 on Type I Collagen Expression during Endothelial Mesenchymal Transformation

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Purpose. To investigate the regulatory role of FGF-2 on type I collagen expression during endothelial mesenchymal transformation (EMT).

Methods. Corneal endothelial cells (CECs) treated with FGF-2 from the primary culture to the third passage were transformed and designated as fibroblastic CECs (fCECs). Steady state levels of both type I collagen RNAs were measured using reverse transcription–real-time PCR, and their half lives were determined in the presence of inhibitor of RNA synthesis. Limited proteolysis with pepsin was used to determine secretion of type I collagen. Protein–protein interaction was determined by coimmunoprecipitation, and subcellular localization was studied by immunofluorescence.

Results. fCECs were characterized by greatly stimulated proliferative potential, loss of contact inhibition, and multilayer fibroblastic cells. The steady state level of α1(I) collagen RNA was greatly upregulated through stabilization of the message in fCECs, whereas steady state level and half-life of the α2(I) collagen RNA were slightly increased compared with the corresponding levels in normal CECs. Of interest, fCECs predominantly secreted homotrimeric type I collagen, $[\alpha1(I)]_3$, with heterotrimeric type I collagen as a minor species. Type I collagen in fCECs was preferentially associated and colocalized with Hsp47 at Golgi apparatus as opposed to its association with protein disulfide isomerase in CECs. LY294002 (a specific PI 3-kinase inhibitor) greatly reduced the steady state levels and stability of α1(I) and α2(I) collagen RNAs and the secretion of type I collagen.

Conclusions. FGF-2 directly mediates corneal EMT through the action of PI 3-kinase, which acts on posttranscriptional regulation by affecting the stability of type I collagen RNA. (*Invest Ophthalmol Vis Sci.* 2005;46:4495–4503) DOI:10.1167/iovs.05-0818

Corneal endothelium plays a critical role in maintaining corneal hydration and corneal transparency.[1–3] The corneal endothelium rests on a self-made basement membrane (Descemet's membrane), of which type IV collagen is a major extracellular matrix (ECM) component.[4,5] Qualitative and quantitative changes in collagen expression affect the unique structure of Descemet's membrane and, subsequently, its corneal function.[6] Although the physiologic collagen phenotypes of corneal endothelial cells (CECs) are types IV and VIII collagen,[4,5,7–9] CECs also synthesize type I collagen, which is then intracellularly degraded immediately after synthesis.[10] Our investigation of the intracellular degradation of type I collagen demonstrated that the newly synthesized procollagen I in CECs is not properly folded, as evidenced by its pepsin susceptibility.[11] The improperly folded procollagen I was retained in the endoplasmic reticulum (ER), where it was associated with protein disulfide isomerase (PDI), a major molecular chaperone for ER retention of unfolded or misfolded proteins.[12–15] The ER-retained procollagen I is polyubiquitinated in the cytosol after retrograde transport from the ER and is subsequently targeted to the proteasome machinery for degradation.[16,17] Such intracellular degradation of type I collagen in CECs is essential to maintain a healthy cornea because secretion of type I collagen into Descemet's membrane would adversely affect corneal function.

When inflammation is caused by chemical, mechanical, or other injury, CECs undergo endothelial mesenchymal transformation (EMT). During this transformation, the CECs lose their characteristic contact-inhibited phenotypes and are converted to multilayer fibroblastic cells.[18–20] These morphologically altered cells simultaneously resume their proliferation ability and deposit fibrous tissue in the basement membrane environment. Thus, EMT absolutely requires a phenotypic switch of collagen gene expression: secretion of type I collagen is induced, whereas secretion of types IV and VIII collagen is stopped.[7,8,21] Such corneal fibrosis after EMT represents a significant pathophysiologic problem that causes blindness by physical blocking of light transmittance. The most common example of corneal fibrosis observed in corneal endothelium in vivo is the development of a retrocorneal fibrous membrane in Descemet's membrane.[8,20,22]

In previous studies, we reported that fibroblast growth factor-2 (FGF-2) is the direct mediator for EMT.[23–26] FGF-2 is a ubiquitous, multifunctional growth factor that regulates many cellular activities, such as cell proliferation, differentiation, angiogenesis, and wound healing.[27–32] The bioactivity of FGF-2 is mediated by high-affinity receptors with an intrinsic tyrosine kinase activity, ultimately resulting in the activation of various signal transduction cascades.[33–35] We reported that FGF-2 directly regulates cell-cycle progression as it facilitates the degradation of $p27^{Kip1}$ through the action of phosphatidylinositol (PI) 3-kinase, ultimately leading to a marked stimulation of cell proliferation.[36,37] We also showed that FGF-2 induced reorganization of the actin cytoskeleton at the cortex through the activation of PI 3-kinase and that the further inhibition of RhoA and the activation of Cdc42 cause the cells to acquire pseudopodia, thus leading to migratory phenotypes.[38] Most important, we reported that FGF-2 induces the secretion of type I collagen, the major component of retrocorneal fibrous membrane.[8,18,23] Recent studies demonstrated that the PI 3-kinase/Akt signaling pathways were directly involved in type I collagen expression in activated hepatic stellate cells and human lung fibroblasts from fibrous tissue.[39,40] In these cells, PI 3-kinase activation results in increased stabilization of α1(I) collagen RNA.

With these findings in mind, we investigated whether FGF-2 is a major facilitator of the synthesis and secretion of type I collagen through the action of PI 3-kinase, during which the growth factor initiates and completes the EMT process in CECs. We found that continuous FGF-2 stimulation of CECs

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facilitates type I collagen secretion (a combination of the homotrimeric and the heterotrimeric molecules) by stabilizing type I collagen RNAs through the action of PI 3-kinase.

Materials and Methods

Antibodies

Goat anti–type I collagen antibody that reacts with individual α1(I) and α2(I) chains was obtained from Chemicon (Temecula, CA). Mouse anti-PDI antibody and mouse anti-Hsp47 antibody were obtained from Stressgen Biotechnologies Corp. (Victoria, BC, Canada). Mouse anti-Golgi 58K protein and mouse anti–β-actin antibodies were purchased from Sigma (St Louis, MO). Mouse anti-phospho Akt (Ser473) antibody and rabbit anti-Akt antibody were obtained from Cell Signaling Technology (Beverly, MA). Fluorescein isothiocyanate (FITC)- and rhodamine-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Biotinylated secondary antibodies were purchased from Vector Laboratories Inc. (Burlingame, CA).

Cell Cultures

Rabbit primary CECs were isolated and established as previously described.[5] Briefly, the Descemet's membrane/corneal endothelium complexes of rabbit eyes purchased from Pel Freeze (Rogers, AR) were treated with 0.2% collagenase and 0.05% hyaluronidase (Worthington Biochemical, Lakewood, NJ) at 37°C for 90 minutes. Cells were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco-BRL, Grand Island, NY) supplemented with 15% fetal bovine serum (Omega, Tarzana, CA) and 50 μg/mL gentamicin (DMEM-15) in a 5% CO_2 incubator. For subculture, confluent cultures were treated with 0.05% trypsin and 5 mM EDTA for 5 minutes. Corneal stromal fibroblasts (CSFs) were isolated and maintained as previously described.[41] CSFs were used as control fibroblasts that secrete type I collagen.

To establish an in vitro model of EMT, we maintained the primary cultures (from the first day in culture) and the serially passaged CECs, up to the third passage, in DMEM-15 in the presence of FGF-2 (10 ng/mL; Chemicon) and heparin (10 μg/mL; Sigma). Third-passage CECs that were modulated to multilayer fibroblastic cells were designated fibroblastic CECs (fCECs). fCECs were continuously maintained in DMEM-15 containing FGF-2 and heparin.

Cell Proliferation Assay

Cells (4×10^3/well) were plated in 96-well tissue culture plates. After 24 hours, the medium was replaced with medium containing 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT; 50 μg/mL) and further maintained for 4 hours at 37°C. The MTT-containing medium was then discarded. Undiluted dimethyl sulfoxide (100 μL) was added to the cells, cells were incubated for 2 hours at room temperature, and the absorbency was read at 570 nm, using a 96-well plate reader (Benchmark Plus Microplate Spectrophotometer; Bio-Rad Laboratories, Inc., Hercules, CA).

Immunofluorescence Staining and Confocal Microscopy

Immunostaining procedures were performed as described previously.[11] CECs seeded on the 4-well chamber slide were maintained in culture until they reached 70% confluence. Cells were fixed and permeabilized in ice-cold methanol and acetic acid (1:1) at −20°C for 10 minutes and then blocked with 2% bovine serum albumin for 15 minutes. Cells were incubated with primary antibodies (dilution ranging from 1:50 to 1:200) at 37°C for 1 hour. After washing with phosphate-buffered saline, cells were simultaneously incubated with FITC-conjugated secondary antibody (1:100 dilution) and rhodamine-conjugated secondary antibody (1:200 dilution) at room temperature for 30 minutes. Control experiments were performed in parallel with the omission of one of the primary antibodies. Antibody labeling was examined using a laser scanning confocal microscope (LSM-510; Zeiss, Oberkochen, Germany). The 1.8-μM optical slices were taken perpendicularly to the cell membrane (apical-to-basal orientation). For fluorescein examination, a 488-nm argon laser was used in combination with a 499/505 to 530 excitation/emission filter set. For rhodamine, the 543-nm helium neon laser was used with a 543 excitation filter and a 560 emission filter. Simultaneous images of FITC and rhodamine were captured from the same optical section. Image analysis was performed using the standard system operating software provided with the microscope.

Protein Preparation and SDS-PAGE

Cells were scraped in lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 50 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM *N*-ethylmaleimide, 1 μg/mL leupeptin, and 1 μg/mL aprotinin), and the lysates were sonicated on ice. Concentration of the resultant lysates was assessed with a protein assay system (Bradford; Bio-Rad Laboratories, Inc.). Proteins were separated using SDS-PAGE, as described by Laemmli, using the discontinuous Tris-glycine buffer systems,[42] and autofluorograms were developed as described previously.[11,43]

Cross-linking Analysis

Procedures used for protein cross-linking have been previously described.[44] Cells treated with trypsin containing EDTA were combined with dithiobis (succinimidyl propionate) (DTSP), a thiol-cleavable cross-linker, to a final concentration of 2 mM and placed on ice for 30 minutes. After the reaction, DTSP was inactivated with 2 mM glycine in phosphate-buffered saline, and the cells were lysed with lysis buffer.

Immunoprecipitation and Immunoblot Analysis

Immunoprecipitation analysis was performed as described previously.[11] After cross-linking with DTSP, cell lysates were precleared with protein G-Sepharose beads and precipitated with anti–type I collagen antibody; the antigen-antibody complex was then precipitated with Sepharose beads, and the proteins bound to the Sepharose beads were eluted with Laemmli sample buffer containing dithiothreitol, boiled for 5 minutes, and subjected to SDS-PAGE. The proteins separated by SDS-PAGE were transferred to a 0.45-μm nitrocellulose membrane (Bio-Rad Laboratories, Inc.) at 0.22 ampere for 10 hours in a semidry transfer system (transfer buffer consisted of 39 mM glycine, 48 mM Tris-base, 0.37% SDS, and 20% methanol). Immunoblot analysis was performed as described previously,[11,16] using a commercial kit (ABC Vectastain; Vector Laboratories, Inc.). All washes and incubations were carried out at room temperature in TTBS (0.9% NaCl, 100 mM Tris-HCl, pH 7.5, 0.1% Tween 20). Briefly, the nitrocellulose membrane was immediately placed in the blocking buffer (5% nonfat milk in TTBS); membranes were incubated with primary antibody (1:5000 dilution for PDI; 1:1000 dilution for Akt or phospho-Akt) and with biotinylated secondary antibody (1:5000 dilution) and then incubated with ABC reagent. Membranes were treated with enhanced chemiluminescence reagent (ECL; Amersham Biosciences Corp., Piscataway, NJ) and exposed to ECL film. The relative density of the polypeptide bands detected on ECL film was estimated using Gel-doc (Bio-Rad Laboratories, Inc.).

Biosynthetic Collagen Labeling

Biosynthetic labeling of collagen was performed as previously described.[10,16] Half the cells were pretreated with LY294002 (20 μM) for 24 hours. All cells were then labeled with 200 μCi L-[2,3,4,5,-^{3}H] proline (101 Ci/mmol; Amersham Life Sciences, Arlington Heights, IL) for 24 hours in DMEM supplemented with 2% FBS, 25 μg/mL ascorbate, and 50 μg/mL β-aminopropionitrile fumarate, with or without LY294002. Proteins from the medium fraction were precipitated with ammonium sulfate, and the resultant precipitates were subjected to limited proteolysis with pepsin (100 μg/mL, pH 2.0) at 4°C for 18 hours. Pepsin was inactivated with 4 N NaOH, and proteins were separated on 6% SDS-PAGE.

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Reverse Transcription–Real-time PCR

Total RNAs were isolated using Trizol reagent (Gibco-BRL, Rockville, MD), and DNA contamination of samples was eliminated using a DNA-free kit (Ambion, Austin, TX), according to the manufacturer's instruction. First-strand cDNA was synthesized (Reverse Transcription System; Promega Corp., Madison, WI) in reaction mixtures containing 5 mM $MgCl_2$, 1× reverse transcription buffer, 1 mM each dNTP, 0.5 U RNase inhibitor, 15 U reverse transcriptase, 1.5 μg oligo$(dT)_{15}$ primer, and 1 μg preincubated RNA at 70°C for 10 minutes. Reaction mixtures were sequentially incubated at 42°C for 15 minutes, at 95°C for 5 minutes, and at 60°C for 5 minutes. Primers were designed using Primer Express software version 2.0 (Applied Biosystems, Foster City, CA) and were as follows: α1(I) collagen, sense (5′-CCTGCGTGTAC-CCCACTCA-3′) and antisense (5′-CGCCATACTCGAACTGGAATC-3′) for a 146-base pair (bp) product; α2(I) collagen, sense (5′-ATGGTG-GCAGCCAGTTTGA-3′) and antisense (5′-TATTCTTGCAGTGGTAGGT-GATG-3′) for a 126-bp product; hypoxanthine phosphoribosyl transferase (HPRT) used as a housekeeping gene for a control reaction, sense (5′-AGCTACTGTAATCAGTCAACG-3′) and antisense (5′-AGAG-GTCCTTTTCACCAGCA-3′). Real-time PCR was carried out according to the manufacturer's instructions (Light Cycler; Roche Diagnostics, Indianapolis, IN) with master mix (Fast Start DNA Master SYBR Green I; Roche Diagnostics). A 2-μL aliquot of 1:10 diluted cDNA sample from the RT reaction (2 μL H_2O as a negative control) was added to 18 μL master mix containing 100 pM each primer and 4 mM $MgCl_2$. The thermal cycle condition was 5 minutes at 95°C for preincubation, followed by 40 cycles of 3 seconds at 95°C, 15 seconds at 58°C, and 15 seconds at 72°C. Linearity of each primer was confirmed to have a correlation coefficient of >0.98 by measuring 50-fold dilutions of cDNA samples. Ct values were defined as the cycle number at which fluorescence exceeded a threshold value of 0.5. Levels were normalized to HPRT mRNA and converted to a linearized value using the formula $1.8^{(Ct_{HPRT}-Ct_{GENE\ X})}$.

Assay for Stability of RNA

To assess the stability of α1(I) and α2(I) collagen RNAs, transcription was blocked with 5,6-dichloro-1-b-D-ribofuranosyl benzimidale (DRB; 1 μM), a specific inhibitor of RNA polymerase II, or with actinomycin D (1 μM), which binds to DNA between the GC pair. Total RNAs were isolated in a time-dependent manner (0–24 hours), and the half-life of type I collagen RNAs was determined using relative RT real-time PCR.

Statistical Analysis

Results were expressed as mean ± SE, and $P < 0.02$ was considered significant.

Results

Phenotypic Characteristics of fCECs in an In Vitro EMT Model

Our unpublished data (2005) demonstrated that complete EMT requires that the cells be continuously treated with FGF-2 for at least three passages. Because we had not documented the whole process of FGF-2–mediated EMT, we investigated the EMT procedures from the beginning of FGF-2 treatment of cells to the final stage of acquiring multilayers of fibroblastic cells. For this purpose, the freshly isolated CECs from the tissue were plated and maintained in DMEM-15 containing FGF-2 (10 ng/mL) and heparin (10 μg/mL). Serially subcultured cells were continuously exposed to FGF-2 until the third passage. Figure 1A demonstrates that the characteristic polygonal primary CECs became elongated when they were maintained in DMEM-15 containing an optimal concentration of FGF-2. First-passage CECs maintained in FGF-2 throughout the culture period completely lost their polygonal morphology, became elongated, and began to lose contact inhibition (Fig. 1A). Such phenotypic alteration was further facilitated in second-passage

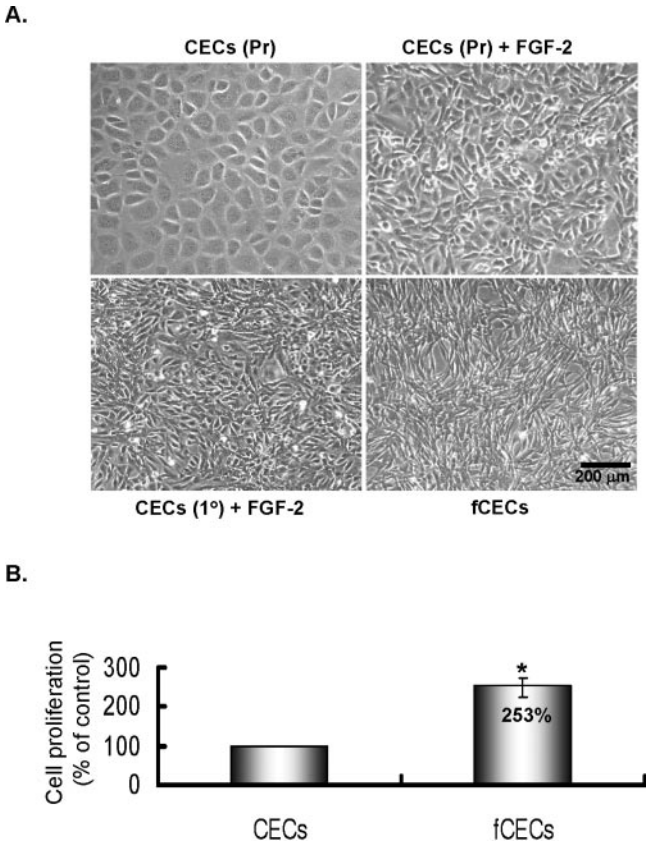

Figure 1. Phenotypic characteristics of fCECs in an in vitro EMT model. fCECs were generated as stated in "Phenotypic Characteristics of fCECs in an In Vitro EMT Model." (**A**) Phase-contrast micrographic presentation of fCECs in comparison with the primary (Pr) CECs and the first passage CECs (1°) that were maintained in DMEM-15 containing FGF-2. fCECs were also maintained in DMEM-15 containing FGF-2. Scale bar, 200 μm. (**B**) Cell proliferation was determined by MTT (A570) assay as described in "Cell Proliferation Assay" (*$P < 0.002$). Data are representative of five experiments.

CECs maintained in the presence of FGF-2 (data not shown). Third-passage CECs maintained throughout the culture period in DMEM-15 containing FGF-2 became multilayers of fibroblastic cells; therefore, we designated them fCECs (Fig. 1A). It should be noted that second-passage CECs maintained in FGF-2 showed less potential to become multilayers of fibroblastic cells than fCECs (data not shown), suggesting that continuous treatment of CECs with FGF-2 is absolutely required to complete the EMT process. In addition, cell proliferation was significantly stimulated in fCECs by 2.5-fold compared to that of CECs (Fig. 1B). On the other hand, our previous study demonstrated that even second-passage CECs, when freshly treated with FGF-2 for 24 hours, did not increase in number.[24]

Effect of FGF-2 on Type I Collagen Secretion of fCECs

Collagen phenotypes had been another marker to monitor the endothelial-to-mesenchymal transformation. Therefore, we determined the collagen phenotypes in fCECs; cells were labeled with [^{3}H] proline, and the medium fraction was subjected to limited pepsin digestion to obtain collagen molecules in triple helical conformation. Type I collagen is the major collagen species in fCECs, whereas secretion of type IV collagen is barely detectable (Fig. 2). Of interest, type I collagen in fCECs is mostly composed of $[\alpha1(I)]_3$. The ratio between the homotrimeric isotype, $[\alpha1(I)]_3$, and heterotrimeric molecule,

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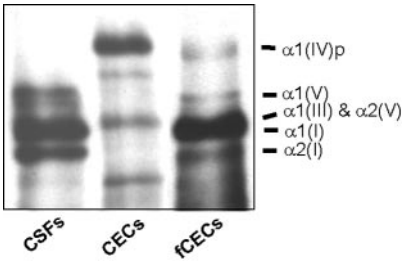

FIGURE 2. Collagen phenotypes in fCECs in comparison with those in CECs and CSFs. Cells were labeled with [^{3}H] proline, and medium fractions were subjected to limited proteolysis with pepsin. The resultant protein fractions were applied to a 6% SDS-polyacrylamide gel under reduced conditions. Data are representative of seven experiments.

$[\alpha1(I)]_2\alpha2(I)$, was 7.3:2.7, as determined by the relative density of the α1(I) and α2(I) polypeptide bands. Conversely, CECs showed a characteristic pepsin-resistant α1(IV)p band, but they lacked the pepsin-resistant fragment of α2(IV), which is frequently more susceptible to protease treatment. CECs also secreted type III collagen as a minor species. These data obtained from CECs confirm our previous findings.[7,21] CSFs, the control fibroblasts, secrete a 2:1 ratio of the α1(I) and α2(I) chains of type I collagen; they also secrete type V collagen (Fig. 2).

Effect of FGF-2 on the Expression of Type I Collagen RNAs

Secretion of the homotrimeric type I collagen, $[\alpha1(I)]_3$, led us to investigate the steady state levels of type I collagen RNAs in fCECs using relative RT real-time PCR. Steady state levels of α1(I) and α2(I) collagen RNAs were elevated in fCECs compared with the corresponding levels in CECs (Fig. 3A). The steady state level of α1(I) collagen RNA in fCECs was more than 10 times the corresponding level in CECs, whereas the steady state level of α2(I) collagen RNA in fCECs was twice the corresponding level in CECs. The high steady state level of α1(I) collagen RNA in fCECs prompted the question whether the stability of the transcript helped control the level of the transcript. Turnover rates of α1(I) and α2(I) collagen RNAs were determined by inhibiting RNA synthesis with DRB and performing relative RT real-time PCR of these mRNAs as a function of time. Figure 3B shows that fCECs contained stable α1(I) collagen RNA, the half-life of which exceeded 24 hours in contrast to the relatively short half-life of the same message in CECs (12 hours). CECs contained stable α2(I) collagen RNA, with a half-life of 24 hours—similar to our previous findings[45]—whereas the half-life of α2(I) collagen RNA in fCECs was slightly extended beyond 24 hours. These data suggest that upregulation of the steady state level of α1(I) collagen RNA may be a prerequisite for the secretion of the homotrimeric type I collagen molecules.

Effect of FGF-2 on Intracellular Trafficking of Type I Collagen

One intriguing observation of CECs is that they not only produce the physiologic collagens (types IV and VIII), they produce type I collagen, the presence of which in the basement membrane environment is biologically undesirable. Therefore, CECs remove procollagen I (the precursor of type I collagen) by intracellular degradation before it is secreted. Subsequent attempts to address the mechanism by which procollagen I is selectively degraded in CECs led us to the finding that procollagen I is not properly folded and that PDI facilitates ER reten-

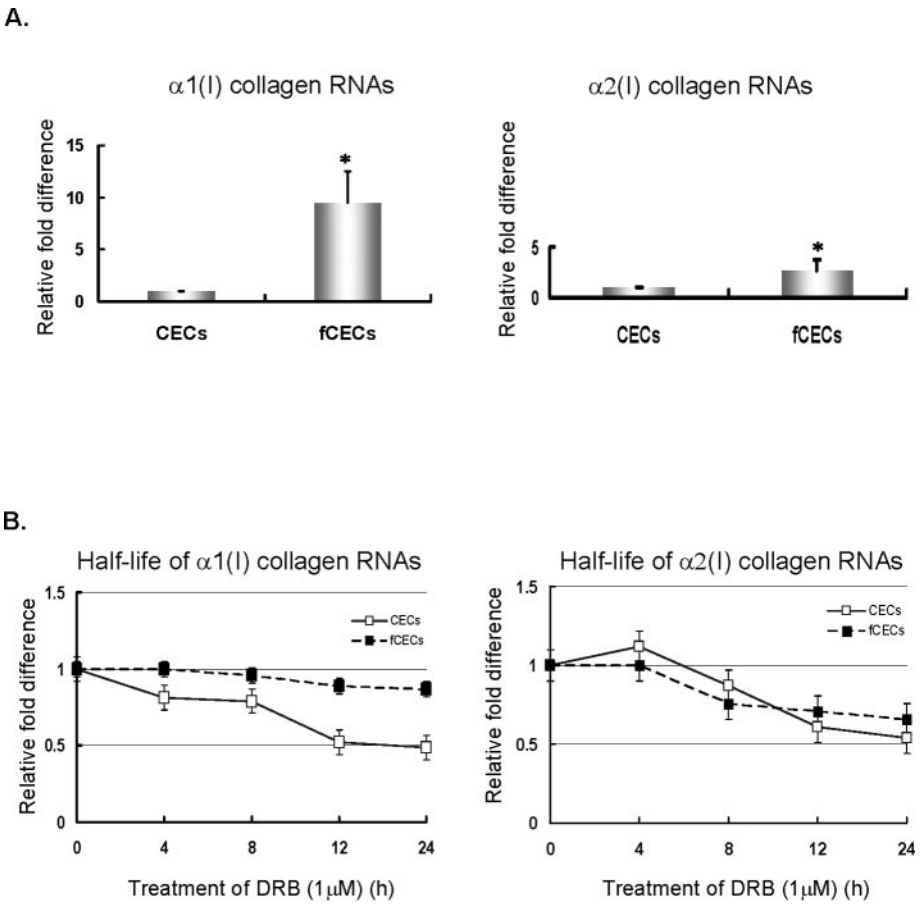

FIGURE 3. Steady state levels and half-lives of α1(I) and α2(I) collagen RNAs in fCECs and CECs. RT real-time PCR was used to determine the steady state levels and turnover rates of the two type I collagen messages. (**A**) Relative-fold difference observed in fCECs was expressed to compare with the levels in normal CECs ($^*P < 0.02$). These data represent the average of seven independent experiments. (**B**) fCECs were treated with 1 μM DRB for 4, 8, 12, and 24 hours. These data are representative of six experiments. Levels were normalized to HPRT mRNA and converted to a linearized value using the formula $1.8^{(Ct_{HPRT}-Ct_{GENE\ X})}$, as stated in "Reverse Transcription Real-time PCR." The relative-fold difference at a given time was expressed in comparison with the level before cells were treated with DRB.

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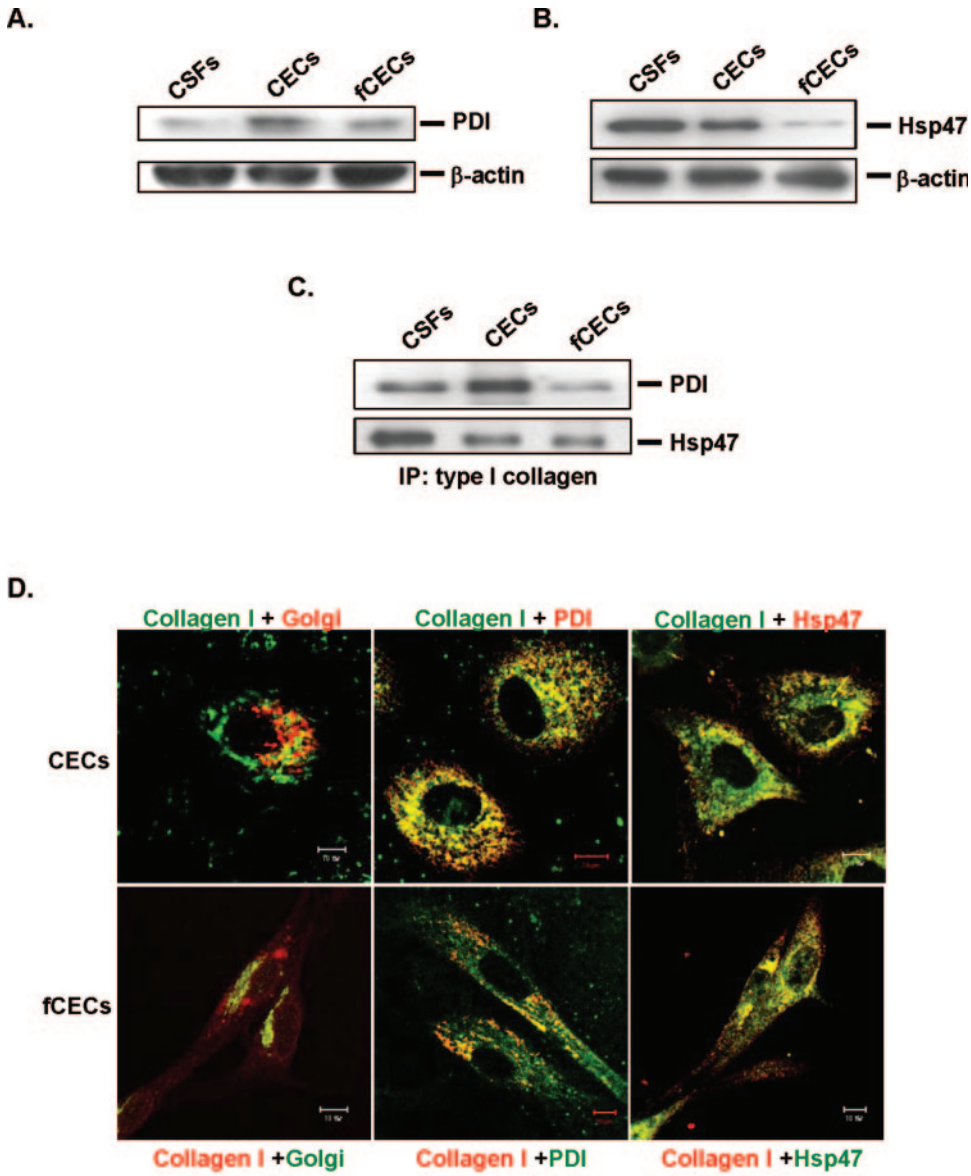

FIGURE 4. Association of type I collagen with molecular chaperones in fCECs. (**A**, **B**) Expression levels of PDI and Hsp47 were determined by immunoblot analysis in fCECs, CECs, and CSFs. β-actin was used as the control of protein concentration for immunoblotting analysis. (**C**) Cells were cross-linked with DTSP before lysis. Cell lysates were immunoprecipitated (IP) with anti–type I collagen antibody, then subjected to immunoblotting analysis with anti-PDI or anti-Hsp47 antibody. Data are representative of six experiments. (**D**) Subcellular localization of type I collagen with Golgi, Hsp47, or PDI in fCECs and CECs. Cells were fixed, permeabilized, and double-stained for anti–type I collagen (1:50 dilution) with anti-Hsp47 (1:200 dilution), anti-Golgi 58K protein (1:200 dilution), or anti-PDI (1:200 dilution) antibodies. Scale bar, 10 μm. Data are representative of six experiments.

tion of procollagen I to prevent its secretion.[16] On the other hand, Hsp47 is preferentially associated with the properly folded procollagen I that is targeted to Golgi apparatus and subsequent secretion to ECM.[46,47] Based on these findings, it is likely that fCECs use Hsp47 as a major molecular chaperone while they secrete type I collagen. We determined PDI and Hsp47 expression at the protein levels in fCECs and compared these with their expression in controls. PDI expression was slightly decreased in fCECs compared with the expression level in CECs. CSFs that secrete type I collagen produced less PDI than did CECs (Fig. 4A). To our surprise, fCECs produced far less Hsp47 than did CECs and CSFs. We, therefore, determined the interaction of procollagen I with the two molecular chaperones (PDI and Hsp47) using coimmunoprecipitation. Figure 4C shows a smaller association of procollagen I with PDI in fCECs than in CECs, whereas its association with Hsp47 in CECs and fCECs is similar. Considering the low level of Hsp47 protein in fCECs, this finding clearly suggests that in fCECs, procollagen I is associated more closely with Hsp47 than with PDI. In contrast, there is a preferential association of type I collagen with PDI in CECs. These findings were further confirmed by immunocytochemical analysis. Procollagen I in CECs was largely colocalized with PDI in the ER. However, procollagen I was not localized in Golgi apparatus, and subcellular localization of procollagen I was less coincidental with Hsp47 (Fig. 4D), confirming our previous findings.[12,16,48] Procollagen I in fCECs was preferentially colocalized with Hsp47 at the Golgi apparatus, whereas subcellular localization of procollagen I and PDI in fCECs was less coincidental than their localization in CECs (Fig. 4D).

Involvement of PI 3-Kinase in the Expression of Type I Collagen RNAs

Among FGF receptor–mediated signaling pathways, we have shown that PI 3-kinase is the major signaling molecule in the FGF-2–mediated mitogenic and morphogenic pathways in CECs.[25,36,38] Therefore, we determined whether the PI 3-kinase pathway also played a key role in FGF-2–mediated type I collagen expression and in its subsequent secretion in fCECs. The fCECs were treated with LY294002 for 24 hours, then labeled with radioactive proline. The medium was subjected to limited proteolysis with pepsin and was analyzed by SDS-PAGE. Figure 5 shows that fCECs secreted the homotrimeric molecule, $[\alpha 1(I)]_3$, as the major type I collagen, whereas CECs predominantly secreted type IV collagen; type I collagen is the

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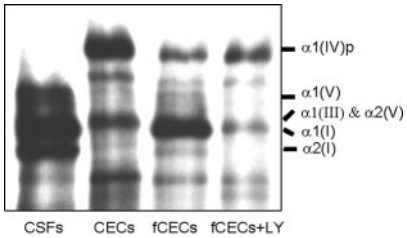


FIGURE 5. Effect of LY294002 on type I collagen secretion in fCECs. Cells were preincubated for 24 hours in the presence of LY294002 (LY; 20 μM) and labeled with [^{3}H] proline. Medium fractions were concentrated and subjected to a limited pepsin digestion. Resultant precipitates were applied to a 6% SDS-polyacrylamide gel. Data are representative of seven independent experiments.

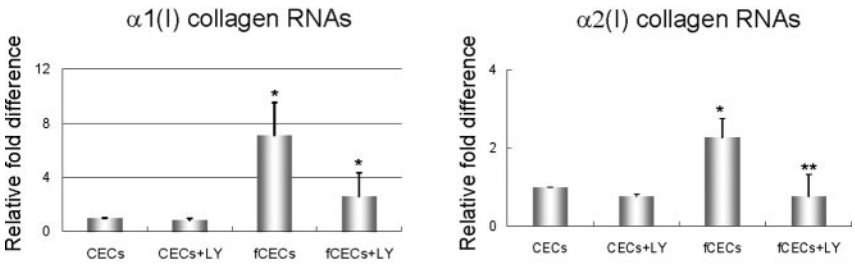
major collagen in CSFs. When fCECs were treated with LY294002, the secretion of type I collagen was greatly reduced (Fig. 5). Of interest, the secretion of type IV collagen was markedly elevated by the inhibitor, suggestive of the importance of PI 3-kinase pathways in collagen gene expression in fCECs.

We further investigated whether the PI 3-kinase pathway was involved in the expression of type I collagen RNAs in fCECs. Steady state levels of type I collagen RNAs were determined in fCECs treated with LY294002. Figure 6A shows that the inhibitor greatly reduced the steady state levels of α1(I) and α2(I) collagen RNAs in fCECs; the expression level was reduced by 63% for the α1(I) message and by 67% for the α2(I) message, from its respective levels in fCECs in the absence of the inhibitor. In CECs, expression of the two type I collagen RNAs was not influenced by the inhibitor. The reduced levels of α1(I) and α2(I) collagen RNAs in the presence of the inhibitor led us to investigate whether PI 3-kinase is involved in the turnover rate of type I collagen RNAs in fCECs. In this experiment, cells were treated with actinomycin D before they were analyzed by relative RT real-time PCR. Figure 6B demonstrated that LY294002 markedly reduced the half-lives of α1(I) and α2(I) collagen RNAs—10 hours for the α1(I) message and 8 hours for the α2(I) message—suggesting that FGF-2–mediated PI 3-kinase activation stabilizes both transcripts of type I collagen.

PI 3-kinase activation in fCECs was confirmed by immunoblot analysis using an antibody specific to the phosphorylated Akt (Ser473). Figure 7 shows that FGF-2 caused phosphorylation of Akt at Ser473 in fCECs. This observation is in agreement with our previous reports that both Ser473 and Thr308 sites of Akt were phosphorylated in CECs in response to FGF-2 stimulation.[37] Such phosphorylation of Akt at Ser473 in fCECs was completely inhibited by the addition of 20 μM LY294002 (Fig. 7). No Akt phosphorylation was observed in CECs in the absence of FGF-2 stimulation (Fig. 7).

Finally, we tested whether removing FGF-2 from fCECs could reverse the collagen phenotypes from type I to type IV; fCECs were maintained for 48 hours in the absence of FGF-2, and the medium fraction was examined for its collagen types. There were neither qualitative nor quantitative changes in collagen synthesis. fCECs maintained in the absence of FGF-2

A.



B.

	CECs	fCECs	fCECs + LY
α1(I) collagen RNA	12 h	>>24 h	10 h
α2(I) collagen RNA	24 h	>24 h	8 h

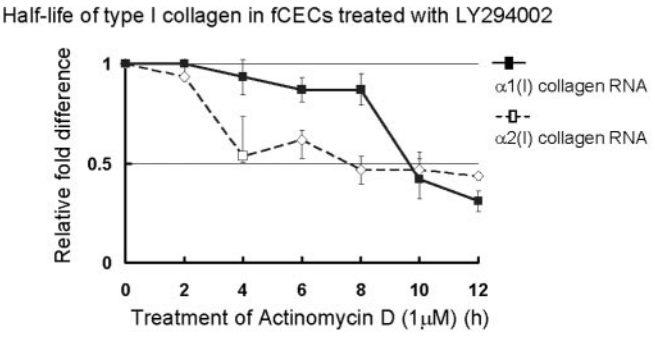


FIGURE 6. Effect of LY294002 (LY) on the steady state levels and half-lives of α1(I) and α2(I) collagen RNAs in fCECs. (**A**) Steady state levels of α1(I) and α2(I) collagen RNAs in fCECs were determined by RT real-time PCR. Data are representative of seven independent experiments (*$P <$ 0.02; **$P < 0.001$). (**B**) fCECs were pretreated with LY294002 (20 μM) for 24 hours and then treated with 1 μM actinomycin D for 2, 4, 6, 8, 10, or 12 hours. Levels of α1(I) and α2(I) collagen RNAs were determined by RT real-time PCR. Data are representative of six independent experiments. Relative-fold difference at a given time was expressed in comparison with the level before cells were treated with actinomycin D.

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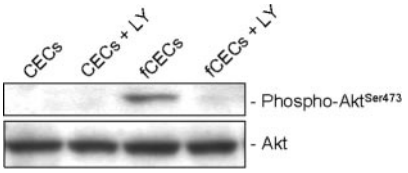

FIGURE 7. PI 3-kinase activation measured by Akt phosphorylation. Cells were incubated in the absence or presence of LY294002 (LY) for 48 hours followed by SDS-PAGE on a 10% SDS polyacrylamide gel under reduced conditions. Akt and phospho-Akt expression were determined by immunoblot analysis of the cell lysates with anti-Akt (1:500 dilution) and anti–phospho Akt^{Ser473} (1:500 dilution) antibodies. Data are representative of three experiments.

predominantly secreted the homotrimeric isotype of type I collagen, $[\alpha1(I)]_3$, into the medium (Fig. 8A). Similarly, these cells maintained relatively high steady state levels of both type I collagen RNAs (Fig. 8B). The steady state level of $\alpha1$(I) collagen RNA was four times the corresponding level in CECs, whereas the level of $\alpha2$(I) collagen RNA was slightly reduced from the corresponding level in fCECs. These data indicate that the acquired mesenchymal characteristics of fCECs are maintained in the absence of the mediator of EMT.

DISCUSSION

Corneal endothelium in vivo responds to various injuries by two distinct pathways. In the regenerative pathway, endothelial cells do not replicate but rather are replaced by migration and spreading of existing cells. In the nonregenerative pathway, transformed endothelial cells not only resume cell proliferation, they alter their cell morphology and collagen phenotypes. Thus, the endothelial-to-mesenchymal transformation observed during the nonregenerative wound healing process of corneal endothelium is involved in the irreversible modulation of some key cellular activities. We have reported that FGF-2 is the direct mediator for EMT observed in CECs.[23,24,36,38] FGF-2 greatly facilitates cell-cycle progression, leading to a marked stimulation of cell proliferation. It also reorganizes actin cytoskeleton, leading to a loss of contact inhibition and an induction of multilayers of fibroblastic cells, and it alters type IV collagen–producing cells to type I collagen–producing cells.[5,7,36,38] We had reported much of the signaling pathways of FGF-2–mediated cell-cycle progression and reorganization of actin cytoskeleton. Thus, in the present study, we investigated how type I collagen becomes the major ECM molecule during EMT.

Our unpublished data (2004) suggest that prolonged and continuous exposure of CECs to FGF-2 is necessary for acquiring mesenchymal phenotypes that secrete type I collagen. Our pilot study also showed that at least 3 passages of CECs maintained in FGF-2–supplemented medium were required. Thus, fCECs were generated from the third passage of CECs that were continuously stimulated with FGF-2. The fCECs showed characteristic phenotypes of mesenchymal cells, such as multilayers of elongated cells that retained a high proliferative potential. Using these fCECs, we determined how the secretion of type I collagen was induced during EMT.

In the present study, we found that fCECs predominantly secreted type I collagen as the homotrimeric $[\alpha1(I)]_3$ molecule and that regular type I collagen, $[\alpha1(I)]_2\alpha2(I)$, is secreted at a lower level. The homotrimeric $[\alpha1(I)]_3$ collagen molecule has been associated with rapidly growing tissues, tumors, and freshly formed scars.[49–51] Recent studies have demonstrated that *oim/oim* mice (osteogenesis imperfecta model; homozygous null for the *proα2(I)* collagen gene) synthesize exclusively the homotrimeric type I collagen isotype, $[\alpha1(I)]_3$.[52,53] In contrast to the results of these studies in which the absence of pro$\alpha2$(I) chain caused the formation of homotrimeric type I collagen, fCECs preferentially promote the assembly of the homotrimeric isotype of type I collagen. Therefore, our in vitro EMT model may represent the early stage of the wound healing process in corneal endothelium.

Attempts to elucidate how the homotrimeric type I collagen came to be present in fCECs led us to the discovery that fCECs contain high levels of $\alpha1$(I) collagen RNA. Given that $\alpha2$(I) collagen RNA is known to be stable and abundant in CECs[45] and that FGF-2 did not markedly elevate the steady state level of $\alpha2$(I) collagen RNA, this finding suggests that the steady state level of the $\alpha1$(I) collagen message may be the rate-limiting step for type I collagen synthesis during EMT. The high steady state level of $\alpha1$(I) collagen RNA is mediated by the extended half-life of the message through the action of PI 3-kinase. LY294002, a specific inhibitor of PI 3-kinase, decreased the stability of the $\alpha1$(I) collagen RNA and, subsequently, its steady state level. Similar results have been reported in human lung fibroblasts, in which PI 3-kinase activation results in an increased stabilization of $\alpha1$(I) collagen RNA and its steady state level.[40] Such posttranscriptional regulation of $\alpha1$(I) collagen RNA may be attributed to complex formation with specific stabilizing proteins at the 3′-untranslated region of $\alpha1$(I) collagen RNA.[54,55] Our data further demonstrated that LY294002 also markedly shortened the half-life of $\alpha2$(I) collagen RNA in fCECs, suggesting that PI 3-kinase is also involved in the stability of $\alpha2$(I) collagen RNA. However, this message may not be efficiently translated in fCECs, in which the heterotrimeric type I collagen, $[\alpha1(I)]_2\alpha2(I)$, is a minor collagen species.

When type I collagen synthesis is upregulated and the molecule is properly folded, fCECs must accommodate the intracellular trafficking machinery to assist the secretion of type I collagen from the ER to Golgi apparatus and, ultimately, to the plasma membrane. Our data demonstrate that procollagen I synthesized in fCECs is preferentially associated with Hsp47, which is known to bind the collagen molecule in a triple-helix conformation. Type I collagen and Hsp47 are colocalized in the Golgi apparatus in fCECs, indicating that type I

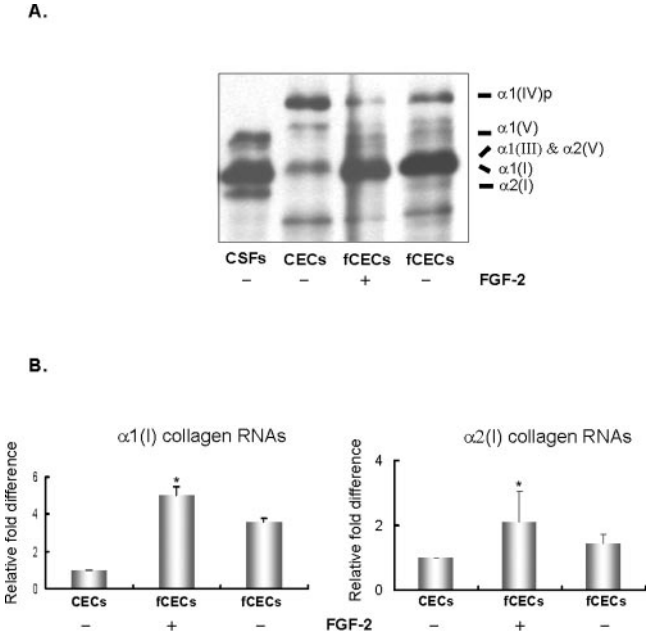

FIGURE 8. Collagen synthesis and steady state levels of type I collagen in fCECs maintained in the absence of FGF-2. fCECs were maintained in DMEM-15 for 48 hours. Collagen biosynthesis (**A**) was determined as described in Figure 2, and steady state levels of type I collagen RNAs (**B**) were measured as described in Figure 3 (*$P < 0.02$).

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collagen is correctly targeted to the secretory machinery. One intriguing observation is that fCECs produced less Hsp47 than did CECs or CSFs. Perhaps in fCECs the Hsp47 is efficiently involved in its intracellular trafficking with cargo (type I collagen) from the ER to Golgi or without cargo from the Golgi to the ER. Or perhaps the amount of Hsp47 in fCECs is sufficient to export type I collagen from the ER.

Taken together, our data demonstrate that FGF-2 induces endothelial-to-mesenchymal transformation of CECs and that FGF-2 completes the whole process of EMT as the growth factor takes part in all three major cellular activities: CECs arrested in the G1 phase of the cell cycle are adequately stimulated, the contact-inhibited CECs are modulated to the wound phenotypes (i.e., activated mesenchymal cells), and type IV collagen–producing endothelial cells are altered to type I collagen–producing fibroblastic cells. FGF-2 fulfils this mission of EMT through the action of PI 3-kinase. This particular signaling molecule can be used as a therapeutic target for the treatment of corneal fibrosis.

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