Caveolin-1 as a Novel Indicator of Wound-Healing Capacity in Aged Human Corneal Epithelium

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Excess caveolin-1 has been reported to play a role in age-dependent hyporesponsiveness to growth factors *in vitro*. Therefore, we hypothesized that caveolin-1–dependent hyporesponsiveness to growth factors in aged corneal epithelial cells might be responsible for delayed wound healing *in vivo*. To test this hypothesis, we evaluated corneal wound-healing time by vital staining using fluorescein after laser epithelial keratomileusis (LASEK). We compared wound-healing times in young, middle-aged and elderly patients. We also examined caveolin-1 levels and other aging markers, such as p53 and p21, in the corneal epithelium. Elderly patients generally had higher caveolin-1 levels in the corneal epithelia than young patients. There were, however, variations among individuals with increased caveolin-1 in some young patients and decreased levels in some elderly patients. Wound-healing time after LASEK correlated well with the corneal caveolin-1 status. Therefore, we suggest that caveolin-1 status might be responsible for delayed wound healing in elderly patients after LASEK. Caveolin-1 status might be a regulator for wound-healing capacity and a novel target for *in vivo* adjustment.

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INTRODUCTION

Aged organisms have been suggested to have the inefficient and slow wound-healing capacity (1–4). The molecular mechanisms of this age-dependent delay in wound healing have not been delineated. Endogenously produced peptide growth factors such as epidermal growth factor (EGF), transforming growth factor α (TGF-α) and platelet-derived growth factor (PDGF) play key roles in the natural wound-healing process (1,2,5). A reduced mitogenic response to growth factors in aged cells surrounding wounds may be responsible for the age-dependent, diminished capacity for wound healing (1,2). The molecular mechanism for reduced mitogenic responses in aged cells varies depending on the respective growth factor (6,7).

Caveolin is a principal structural component of caveolae membranes (8). Three mammalian caveolin genes (*caveolin-1*, *-2* and *-3*) have been identified and characterized (9). Caveolin plays an important role in growth-factor–induced cellular signal transduction by interacting with various signaling proteins, such as epidermal growth factor receptor (EGFR), G-proteins, Src-like kinases, Ha-Ras, protein kinase C, endothelial nitric-oxide synthase and integrins (10–14). We previously observed that caveolin-1 levels increase with aging (15), and caveolin-1 reduction by use of antisense oligonucleotides or small-interfering RNA recovers EGF response in senescent cells. Because EGF receptor levels on the cell surface do not change during aging, we have suggested that accumulation of excess caveolin-1 protein might be responsible for age-dependent decreases in EGF response and morphological changes *in vitro* (16,17).

The far-near ultraviolet radiation (193-nm wavelength) generated by an argon-fluoride excimer laser has been used to ablate the cornea and reshape its curvature to correct refractive errors, such as myopia, astigmatism and hyperopia (18,19). This surgery, also called laser epithelial keratomileusis (LASEK), is more commonly performed in elderly individuals who need refractive correction to improve vision and quality of life. Postoperative care for the elderly requires special concern for wound-healing time and efficiency. Because aging likely leads to delays in wound healing, we compared the status of corneal epithelia and wound-healing

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Table 1. Subject characteristics and corneal epithelial wound-healing times after LASEK.[a]

Case no.	Sex	Age, years	Wound-healing time, h		Case no.	Sex	Age, years	Wound-healing time, h		Case no.	Sex	Age, years	Wound-healing time, h	
			OD	OS				OD	OS				OD	OS
Y1	F	20	48	48	M1	F	40	48	72	E1	F	54	72	96
Y2	M	20	48	48	M2	F	40	72	72	E2	M	56	72	96
Y3	M	20	48	48	M3	F	41	72	48	E3	M	56	96	72
Y4	M	20	48	72	M4	M	41	72	72	E4	M	57	72	120
Y5	F	20	72	48	M5	F	41	72	48	E5	F	58	96	72
Y6	F	20	48	72	M6	F	42	72	72	E6	M	59	72	72
Y7	F	20	48	48	M7	F	42	72	48	E7	M	60	96	120
Y8	F	20	48	48	M8	F	42	72	48	E8	F	60	96	120
Y9	F	20	48	48	M9	F	42	72	72	E9	F	60	96	96
Y10	M	21	48	48	M10	F	43	72	72	E10	F	60	120	96
Y11	F	21	48	48	M11	F	44	72	72	E11	F	61	96	72
Y12	F	21	48	48	M12	M	45	72	72	E12	F	62	120	96
Y13	F	22	48	48	M13	M	45	72	72	E13	M	63	96	120
Y14	F	22	48	48	M14	F	45	96	72	E14	F	63	120	96
Y15	M	23	72	48	M15	F	45	72	72	E15	F	65	120	120
Y16	M	23	48	48	M16	F	47	72	96	E16	F	65	96	120
Y17	M	24	48	48	M17	M	47	48	72	E17	F	65	120	120
Y18	M	24	72	72	M18	F	48	72	96	E18	F	68	96	120
Y19	F	25	48	48	M19	M	49	96	72	E19	F	69	96	120
Y20	F	27	72	48	M20	M	49	72	96	E20	M	70	96	96
Mean ± SD		21.7 ± 2.0	52.2 ± 9.2		Mean ± SD		43.9 ± 2.9	71.4 ± 12.7		Mean ± SD		61.6 ± 4.5	99.6 ± 17.7	

[a]OD, right eye; OS, left eye.

times from young and elderly patients after LASEK. To test the possibility that caveolin-1 might be involved in regulating wound healing after LASEK, we compared expression of caveolin-1 with expression of other age-related proteins, such as p53 and $p21^{waf1/cip1}$ (p21) in the corneal epithelia from young and elderly patients.

MATERIALS AND METHODS

Human Corneal Cell Isolation and Primary Culture

Human corneal epithelial cells were isolated from tissue explants from donated human corneas and cultivated as described (20). Primary human corneal epithelial (PHCEp) cells were maintained in medium (Epilife; Cascade Biologics, Portland, OR, USA) containing 0.06 mol/L $CaCl_2$, and human corneal growth supplement (Cascade Biologics). Cells were maintained in a humidified atmosphere with 5% CO_2 at 37°C, subcultured with 0.25% trypsin-EDTA every 3 to 4 d and used for experiments.

LASEK and Clinical Observations

This study involved 60 patients (120 eyes), with myopia range –3.00 to –5.00 diopters of spherical equivalent refractive power, who had undergone LASEK for refractory correction during the period from February 2005 to January 2008. The Yong-San Hospital Institutional Review Board (IRB) at the Chung-Ang University in Korea approved the surgery (IRB# 2008-003-01). Before LASEK all patients were examined carefully by an ophthalmologist to determine the state of the cornea. Exclusion criteria included history of glaucoma or corneal disease, previous corneal or intraocular surgery and ocular inflammation. Patients were divided into three groups according to age: young group, age between 20 and 29 years; middle-aged group, between 40 and 49 years; and elderly group, older than 50 years. The mean ages were 21.7 ± 2.0 years for the young group, 43.9 ± 2.9 years for the middle-aged group, and 61.6 ± 4.3 years for the elderly group. Demographic information can be found in Table 1.

All procedures were performed by one surgeon according to the tenets of the Helsinki Declaration. Written informed consent was obtained from all patients.

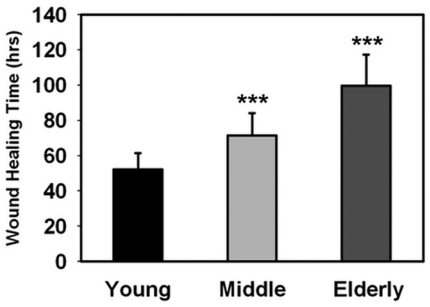

Figure 1. Comparison of corneal wound-healing times among young, middle-aged and elderly groups. Corneal wound-healing times were measured in the young (40 eyes, 20 patients), middle-aged (40 eyes, 20 patients) and elderly groups (40 eyes, 20 patients). Bar graphs show the mean wound-healing times in each age group. There was a statistically significant difference between the young and middle-aged or elderly group (***$P < 0.001$).

Before surgery, we excluded patients with corneal lesion or erosion. To minimize the influence of tear factors, we also excluded patients with a tear breakup time (TBUT) of less than 10 s (21). After application of proparacaine hydrochloride 0.5% eyedrops (Alcaine®; Alcon, Puurs, Belgium) as topical anesthesia, a standardized epithelial incision was performed by using an 8.0-mm well into which 20% alcohol was instilled and left for 15 s. The alcohol was removed, the eyes rinsed with a balanced salt solution (BSS®; Alcon) and the epithelium was removed and collected manually with a disposable surgical blade. LASEK was then performed with the MEL 80 excimer laser system™ (Carl Zeiss Meditec, Jena, Germany), at a wavelength of 193 nm, by use of a gaussian beam with a 0.7-mm spot diameter with a repetition rate of 250 Hz. The ablation depth depended on the amount of intended correction, and the ablation zone diameter was 6.5 mm equally. After ablation, balanced salt solution chilled to –4° was applied to the corneal surface for 30 s, followed by one drop of diclofenac sodium 0.1% (Voltaren®; Norvatis, Basel, Switzerland). After surgery the eyes were covered with disposable soft contact lenses (Purevision™; Bausch & Lomb, Rochester, NY, USA). Postoperatively, all patients were treated with levofloxacin 1% eyedrops (Cravit®; Santen, Osaka, Japan) four times a day until complete reepithelization was established. After LASEK, patients had a follow-up examination every 24 h until the corneal epithelium completely closed, and corneal wound-healing time was recorded. The corneal epithelium was stained with fluorescein and photographed at 3 d after LASEK with a slit-lamp digital camera (D1®; Nikon, Tokyo, Japan).

Western Blot Analysis

Tissues were harvested after LASEK and solubilized for 30 min in ice-cold lysis buffer containing 25 mmol/L HEPES, pH 8.0, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L Na_3VO_4, 1 mmol/L NaF, 1% Triton X-100 and protease inhibitors. Total protein in the lysates was determined using a BCA (bicinchoninic acid) protein assay kit (Pierce; Rockford, IL, USA), according to the manufacturer's instructions. Cell lysates containing equal amounts of protein were resolved by SDS-PAGE and transferred to nitrocellulose filters (Protran; Schleicher & Schuell, Keene, NH, USA). Blots were blocked with 5% nonfat dried milk and 0.1% Tween 20, treated with antibodies in blocking solution overnight and then washed and incubated with horseradish peroxidase–conjugated anti-IgGs (1:5000). The immune complexes were visualized by enhanced chemiluminescence.

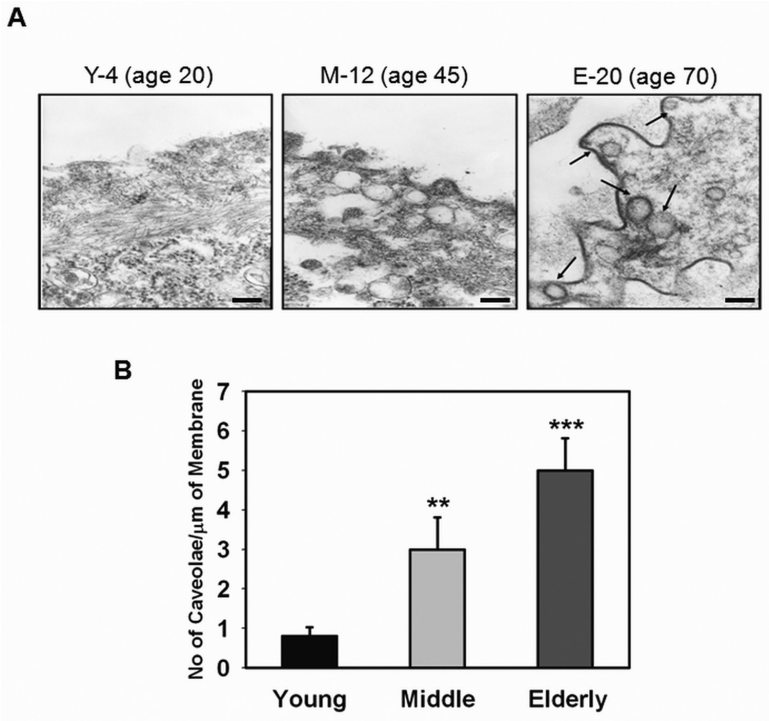

Figure 2. Caveolaelike structures in corneal epithelium from young, middle-aged and elderly patients. (A) Caveolaelike structures in corneal epithelia of one young (Y4, age 20 years), one middle-aged (M12, age 45 years), and one elderly (E20, age 70 years) patient were visualized by using electron microscopic analysis. The arrow indicates caveolae structures. Bar = 100 nm. (B) The number of caveolaelike vesicles in the corneal epithelium of the three age groups were counted and plotted as a histogram with mean ± SD. Significant differences between young and middle-aged or elderly patients are indicated with asterisks (**$P < 0.01$, and ***$P < 0.001$).

Immunofluorescence Microscopy

Corneal epithelia from young (age 23 years) and elderly (age 65 years) patients were permeabilized with 0.5% Triton X-100 and fixed with 4% paraformaldehyde. Tissues were then incubated with 1% bovine serum albumin (BSA) in 20 mmol/L sodium phosphate (pH 7.4) containing 150 mmol/L NaCl (phosphate-buffered saline [PBS]) to block nonspecific labeling. For immunofluorescence used for human tissue, the primary antipaxillin and anti–caveolin-1 polyclonal antibodies were diluted to 1:500 in 0.1% BSA in PBS. Tissues were washed and incubated simultaneously with Alexa 546-conjugated goat antirabbit secondary antibody (4 g/mL; Invitrogen, Eugene, OR, USA) and Alexa 488-conjugated phalloidin (0.67 U/mL; Invitrogen) in 0.1% BSA/PBS for 1 h. Coverslips were mounted onto slides (Immu-mount; Shandon Lipshaw, Pittsburgh, PA, USA), and photographed (400×) on a DEF 280 microscope (Leica, Wetzlar, Germany).

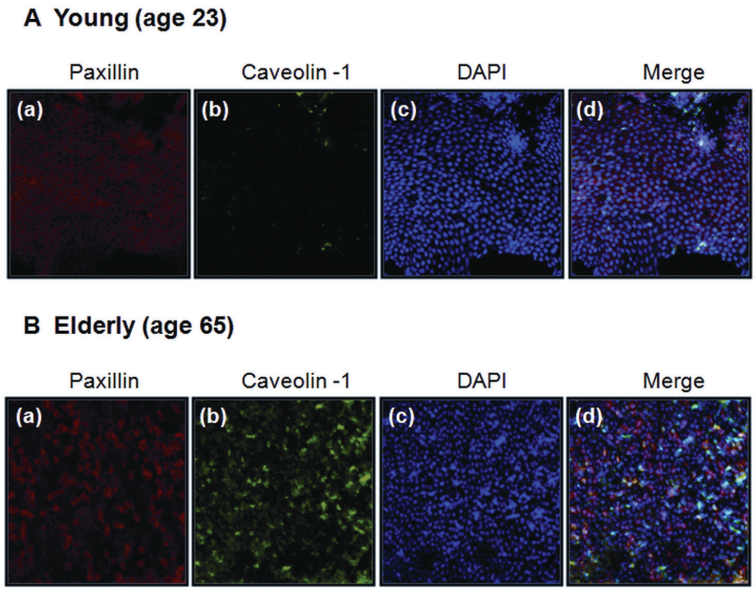

Figure 3. Immunofluorescent staining of age-related protein, paxillin and caveolin-1 expression in corneal epithelia from young and elderly patients. Young (A, age 23 years) and elderly (B, age 65 years) corneal epithelia were fixed and stained with antipaxillin and anti–caveolin-1 antibodies. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). The paxillin-, caveolin-1– and DAPI-stained images were merged to reveal any overlap. Immunofluorescence microscopy was performed as described in Materials and Methods. Each experiment was performed three times with similar results.

Electron Microscopy

Corneal epithelia from young, middle-aged and elderly patients were fixed with 3% glutaraldehyde in PBS (pH 7.4). After being washed with 0.2 mol/L sodium cacodylate buffer (pH 7.4), cell pellets were treated with 1% osmium tetroxide in cacodylate buffer for 1 h. Tissues were then dehydrated in graded ethanol through propylene oxide and embedded in epoxy resin (Polyscience, Warrington, PA, USA). Ultrathin sections were cut and stained with uranyl acetate and lead citrate. Sections were observed by use of a transmission electron microscope (H-600; Hitachi, Yokohama, Japan).

Adenoviral Caveolin-1 Vector Preparation and Transfection

Infective recombinant adenovirus was made by using the AdEasy system (22). Recombinant adenovirus expressing caveolin-1 (Adv/Cav-1) was produced by inserting wild-type caveolin-1 cDNA into a shuttle plasmid (pAdTRACK-CMV; Invitrogen, Carlsbad, CA, USA). After electroporation, homologous recombination was performed with the shuttle vector and a large adenovirus-containing plasmid (pAdEasy-1) in *Escherichia coli* BJ518. Recombinant viruses were selected with kanamycin and subjected to restriction endonuclease digestion. Infective adenovirus virions were produced after transfection of the linearized recombinant adenovirus plasmid in HEK293 cells. Virus stocks were amplified in HEK293 cells on 15-cm plates and purified by using BD Adeno-X purification kits (Clontech, Palo Alto, CA, USA). A control vector (Adv/GFP) carrying cDNA for modified green fluorescence protein EGFP was also prepared as described above.

Statistical Analysis

The Graph-Pad Prism (GraphPad, San Diego, CA, USA) was used for statistical analysis. One-way ANOVA was performed to assess differences in preoperative spherical equivalent refractive power among the young, middle-aged and elderly groups. Student *t* test was performed to assess differences in wound-healing time and relative caveolin-1 expression between young and elderly corneal epithelia. Mean ± standard deviations (SD) of experimental and control values in each group are presented. *P* values less than 0.05 were considered significant.

RESULTS

Aging Delays Corneal Wound Healing after LASEK

There was no significant difference in the preoperative refractive errors among the young, middle-aged and elderly groups ($P = 0.48$). After LASEK was performed, corneal epithelial defects were examined every 24 h until healing was complete, and individual healing times were recorded.

Wound-healing time was closely related to age (Table 1). The wound-healing time increased significantly ($P < 0.001$) with patient age, from 52.3 h for the young group (age 20–29 years) to 70.8 h for the middle-aged group (age 40–49 years) and 99.7 h for the elderly group (age >50 years) (Figure. 1).

Caveolin-1 and Paxillin Are Enriched in Caveolae from Epithelia of Elderly Patients

Electron microscopic studies of representative corneal epithelia samples from young (Y4, age 20 years), middle-aged (M12, age 45 years) and elderly (E20, age 70 years) patients revealed the presence of caveolaelike structures, which were more abundant in middle-aged and elderly patients than in young patients (Figure 2A, caveolae are indicated by arrows). Quantitative analysis of caveolae number in corneal epithelial cells showed that elderly patients have five times more caveolaelike structures than young patients ($P < 0.001$), and middle-aged patients have three times more ($P < 0.01$) (Figure 2B).

Increased caveolin-1 expression in elderly patients was also confirmed by the results of immunofluorescence microscopic analysis (Figure 3). We examined paxillin and caveolin-1 expression

and colocalization within caveolaelike structures, based on previous observations that a significant proportion of focal adhesion molecules, for example, paxillin, were recruited to the caveolae fraction in senescent human diploid fibroblast (HDF) cells (17). When young and elderly corneal tissues were coimmunostained with antipaxillin and anti–caveolin-1 antibodies, paxillin colocalized with caveolin-1 in elderly corneal epithelial cells (Figure 3B) but not in young cells (Figure 3A). These data suggest that the focal adhesion protein and caveolin-1 are upregulated in caveolae from elderly corneal tissues.

Caveolin-1 Status and Other Aging Marker Proteins in Corneal Epithelia Vary Among Individuals

We used Western blot analysis to examine the expression of caveolin-1 and other markers of aging, such as p53 and p21, in the corneal epithelia of five young (Y1–Y5) and five elderly (E5–E9) patients. Interestingly, not all elderly patients showed consistent increases in age-related proteins compared with young patients (Figure 4A). Among the five young patients, two (Y4 and Y5) showed upregulated caveolin-1 and one (Y5) showed increased p21 expression. Among the five elderly patients, two (E5 and E6) showed lower caveolin-1, p53 and p21 levels than other elderly patients. These data suggest that age-related protein expression does not correlate with chronological age but varies among individuals.

To verify these data, we then used Western blot analysis to examine caveolin-1, p53 and p21 expression in corneal epithelia from an additional five young and five elderly patients. The expression of these proteins in all 10 young (age 23 ± 3 years) and elderly patients (age 60 ± 5 years) was calculated by using densitometric analysis of each band and plotted as the relative density of β-actin (Figure 4B). As expected, caveolin-1, p53 and p21 levels were significantly (*$P < 0.05$; ***$P < 0.001$) higher in corneal epithelia from elderly (E in Figure 4B) than young patients (Y in Figure 4B).

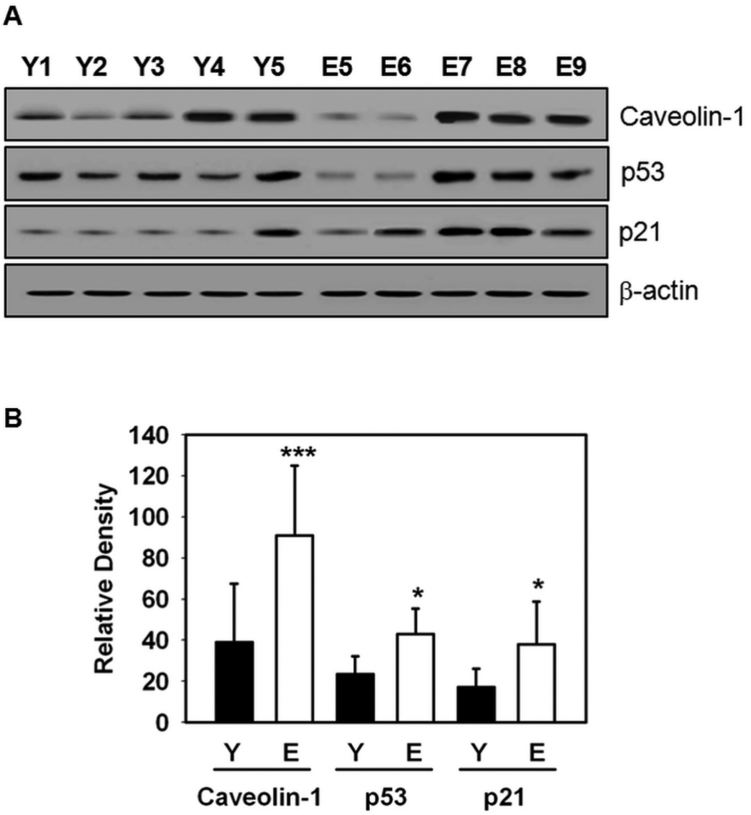

Figure 4. Individual differences in caveolin-1, p53 and p21 expression. (A) Corneal epithelia from five young (Y1–Y5: age 20 years) and five elderly patients (E5–E9; age 58–60 years) were lysed in lysis buffer, and 30 μg protein from each lysate were analyzed by Western blot by using antibodies specific against caveolin-1, p53 and p21. β-Actin was also monitored as a control. (B) Caveolin-1, p53 and p21 expression in corneal epithelium from 10 young (Y, black bars) and 10 elderly patients (E, white bars) were analyzed by Western blot. The density of each protein was measured relative to β-actin. Bars represent means ± SD. *$P < 0.05$ and ***$P < 0.001$ indicate significant differences in expression between young and elderly patients.

Wound-Healing Time Correlates Well with Caveolin-1 Status

We determined wound-healing status by examining corneal epithelialization status after fluorescein staining. Corneal epithelial defects were stained with fluorescein and then appeared green. The wound-healing time was a measure of the time to complete corneal epithelialization, indicated by the elimination of all green staining. In this study, we also compared corneal epithelialization 3 d after LASEK in two young patients (Y3 and Y4 shown in Table 1), both aged 20 years, with different caveolin-1 expression and two elderly patients (E5 and E7 shown in Table 1) with similar ages (58 and 60 years, respectively) but different caveolin-1 expression. Complete corneal epithelialization was observed 3 d after LASEK in both young patients (Y3 and Y4 in Figure 5A), but corneal defects (green-colored area) remained in some elderly patients (E5 and E7 in Figure 5A). Some degree of corneal epithelial defect was observed in 13 of 20 elderly patients (65%).

Corneal wound-healing times differed between two young patients (48 h for Y3 and 60 h for Y4) and two elderly patients (84 h for E5 and 108 h for E7). Patients with longer wound-healing times (Y4 and

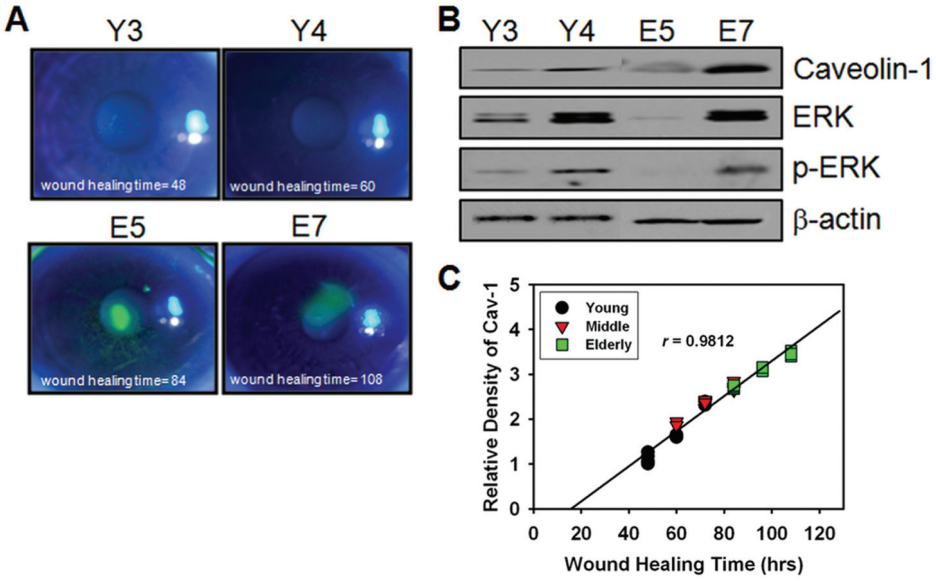

Figure 5. Correlation between corneal wound-healing time and caveolin-1 expression. (A) Photographs showing corneal wound-healing status 3 d after LASEK. Corneas from young (Y-1, age 20 years; Y-2, age 20 years) and elderly (E-5, age 58 years; E-7, age 60 years) patients were stained with fluorescein and photographed with a slit-lamp digital camera. Complete corneal epithelizations were observed in young patients. Central epithelial defects stained with fluorescein were still present in elderly patients. (B) Western blots of corneal epithelia from young (Y3 and Y4) and elderly patients (E5 and E7). Corneal epithelia were lysed in lysis buffer and 30 μg protein from each lysate was analyzed by Western blot by using antibodies against caveolin-1, p-ERK and ERK. The β-actin level was also monitored as a control. (C) The relative levels of caveolin-1 from 10 young (black circle), 10 middle-aged (red triangle) and 10 elderly (green square) patients were analyzed by using densitometry after Western blotting. The wound-healing times versus the relative caveolin-1 level for 30 patients were plotted and analyzed to get a correlation coefficient between determinations (r = 0.9812).

E7) showed upregulated caveolin-1 expression compared with the age-matched patients (Y3 and E5, respectively) (Figure 5B). At the same time, p–extracellular signal-regulated kinase (ERK) levels were upregulated in these patients with longer healing times (Figure 5B). These data indicate a strong correlation of caveloin-1 status with wound-healing time, suggesting that caveolin-1 plays a role in modulating wound healing in all age groups. Because we observed that caveolin-1 status in the corneal epithelium varies among individuals, we examined the relationship between wound-healing time after LASEK and caveolin-1 expression in a group of patients (Figure 5C). We plotted the wound-healing time versus the relative caveolin-1 level for 30 patients to get a correlation coefficient between determinations (r). The r value of 0.9812 provides strong evidence of a correlation between these two parameters.

Suppressed EGF Signaling by Caveolin-1 Overexpression in Human Corneal Epithelial Cells

Hyporesponsiveness of senescent fibroblasts to EGF stimulation was suggested to be strongly related to upregulated caveolin-1. Caveolin-1 overexpression in young HDF cells may downregulate EGF signaling (8,16,17,23). To confirm the role of caveolin-1 in corneal epithelium, we overexpressed caveolin-1 in corneal epithelium cells. We transfected corneal epithelial cells with an adenoviral vector that expressed both caveolin-1 and enhanced GFP (Adv/Cav-1) or an adenoviral vector that expressed enhanced GFP alone as a control (Adv/GFP). Fluorescence microscopic analysis revealed efficient viral vector transfection (Figure 6A). Western blot analysis showed the significant caveolin-1 induction in Adv/Cav-1–infected cells compared with Adv/GFP-infected cells (Figure 6B). When EGF signaling was compared between caveolin-1–induced and control cells, the caveolin-1–overexpressing cells showed hyporesponsiveness to EGF. Mock transfection and Adv/GFP control cells showed increased ERK phosphorylation by EGF stimulation, whereas EGF signaling was not activated in Adv/Cav-1–infected human corneal epithelial cells (Figure 6C). Therefore, our data suggest that caveolin-1 overexpression suppresses EGF mitogenic signaling in corneal epithelial cells as well as HDF cells (15).

DISCUSSION

Wound healing is a complex, tissue-dependent process. The initial action involves removing damaged tissue. Next a proliferative and migratory phase occurs, in which cells invade the wound site to initiate repair and replacement. To manipulate corneal wound healing, a greater understanding of corneal physiology at the subcellular level may be required. Changes associated with corneal wound healing following LASEK could occur in epithelium and stroma. Most corneal wounds involve damage to the epithelium, and epithelial healing time is important to the final refractive outcome.

Immediately after LASEK, growth factors, cytokines, neuropeptides and chemokines are released, initiating the wound-healing cascade and undesirable complications such as corneal haze (24). After photorefractive keratectomy, the release of varying factors such as TGF-β, EGF, PDGF, vascular endothelial growth factor, hepatocyte growth factor and interleukin-6 has been reported. These growth factors have been further characterized by a recurrent set of events in a wound-healing context: injury-induced growth factor production and release, growth factor–induced proliferative and migratory re-

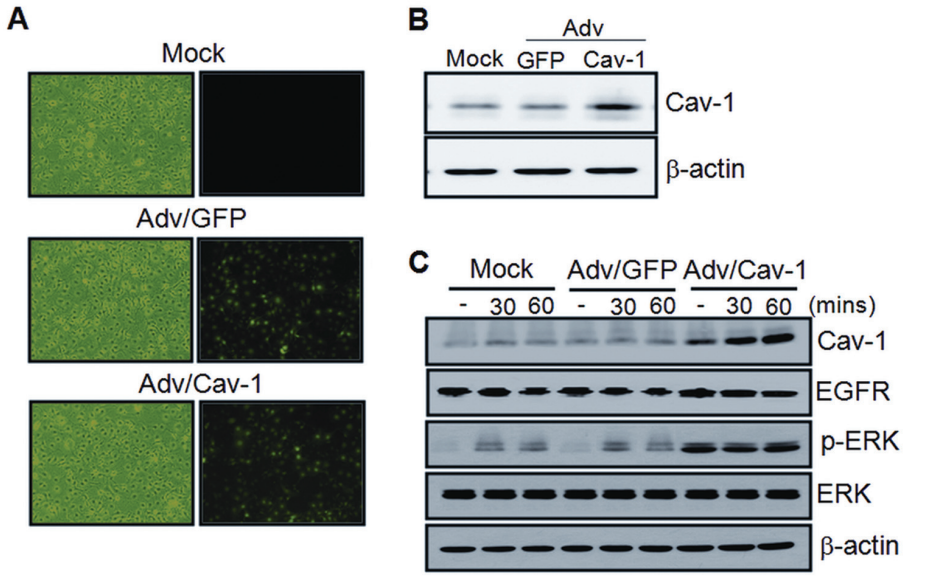

Figure 6. Adenovirus-mediated caveolin-1 upregulation in corneal epithelial cells. (A) An adenoviral vector expressing both caveolin-1 and enhanced GFP (Adv/Cav-1) and an adenoviral vector expressing enhanced GFP alone as a control (Adv/GFP) were used. The efficiency of adenoviral infection was monitored by measuring fluorescence from GFP. (B) Caveolin-1 production in Adv/Cav-1–infected human corneal epithelial cells was analyzed by Western blotting against caveolin-1 and compared with Adv/GFP-infected cells. (C) Infected cells were stimulated with 100 μg/mL EGF for the indicated time (min). Total cells were lysed in lysis buffer, and 30 μg protein from each lysate were analyzed by Western blot against caveolin-1, EGFR, p-ERK and ERK. β-Actin was also monitored as a control.

sponses and morphologic changes in target cells, additional growth factor regulation through feedback loops and a specific termination process (25). Endogenous basic fibroblast growth factor (bFGF) levels, a member of the FGF family (26), have been shown to increase at injury sites (27).

Because of growth factor involvement in wound-healing processes, there have been many efforts to improve corneal wound healing by modulating growth factors. Several researchers have shown that growth factors such as EGF, TGF-β, bFGF, hepatocyte growth factor, PDGF, interleukin-6 and keratinocyte growth factor increase epithelial healing rates *in vitro*, but their *in vivo* effects after topical application are controversial (25). Some groups have reported that topical EGF application increased the wound-healing rate *in vivo*, but others reported no benefit. Moreover, a recent study showed that eye drops containing bFGF significantly accelerated epithelial healing after LASEK by increasing corneal reepithelialization in transgenic mice (28).

It is generally accepted that corneal wound-healing time increases with age (29,30) (Table 1). We also found that mean wound-healing time after LASEK correlated well with mean patient age. Patients within similar age groups showed individual variations in wound-healing time after the LASEK procedure. By examining markers of aging, such as caveolin-1, p53 and p21 in corneal epithelia from individuals, we found that mean protein levels were higher in elderly patients, with variations among individuals. These variations might reflect the individual aging process, rather than chronological age.

In elderly patients who undergo LASEK, reduced blood supply to the lens or cornea could delay wound healing (31,32). We postulated that age-dependent inefficient responses to growth factors might also delay corneal wound healing in the elderly. We previously reported a role of caveolin-1 in the senescent phenotype and its accumulation in aged animal tissues, suggesting a suppressive role in EGF responsiveness (16,23). Therefore, we tested the role of caveolin-1 to suppress EGF signaling in corneal epithelial cells. In consequence, caveolin-1 transfection by use of an adenoviral transfection system showed hyporesponsiveness to EGF in corneal epithelial cells, as previously shown in HDF (16) and Chinese hamster ovary cells (9). Our data confirm that caveolin-1 is a mitogenic suppressive factor in the corneal epithelium. In this study, we also observed that differences in wound-healing time correlated well with caveolin-1 levels of corneal epithelial cells rather than chronological age, suggesting that caveolin-1 in human corneal epithelium might be a negative marker for intrinsic wound-healing capacity.

Caveolin is well known to regulate signaling in caveolae. Anchorage-independent transformed fibroblast growth disappeared after caveolin-1 overexpression. Suppression of caveolin-1 stimulated transformation and activated mitogen-activated protein kinase signaling (33). Overexpression of caveolin-1 also inhibited EGF-induced signaling from EGFR to the Raf/MAP kinase ERK kinase (MEK)/Erk (Raf-MEK-ERK) pathway, and the nucleus in Chinese hamster ovary cells (9). In addition, caveolin-1 overexpression in mesangial cells suppressed Raf-MEK-ERK activation and cell proliferation induced by bFGF and PDGF (34). In this study, we also found that caveolin-1 overexpression in corneal epithelial cells showed a reduction of EGF-induced ERK phosphorylation. The results of the present study suggest that caveolin-1 inhibits the signaling induced by growth factors such as EGF, bFGF and PDGF in corneas, and might be responsible for delaying wound healing in elderly patients after LASEK.

In previous reports (35,36), it has been shown that tear-film stability is mostly influenced by age and sex, and dry-eye changes are more marked in women than in men. We used TBUT to assessed tear function. Because normal eyes have a

TBUT of 15–45 s (21), we excluded cases with a TBUT of less than 10 s to minimize the influence of tear factors. Our cases had TBUT within the normal range, and no significant differences among groups were found (data not shown). However, we could not exclude the possibility that age- and sex-dependent tear factors played a role in wound healing directly or indirectly via the regulation of caveolin expression. Further studies are needed.

Taken together, these data indicate that caveolin-1 status is a useful marker of wound-healing efficiency after LASEK. Therefore, we suggest that downregulation of caveolin-1 might be a useful method to facilitate wound healing *in vivo*, especially for elderly patients undergoing LASEK. This method may also be used to facilitate wound healing in other surgeries or traumas.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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