# 博 士 学 位 論 文

## 角膜実質細胞によるコラーゲンゲル収縮能に コラーゲン受容体 Endo180 が与える影響

近 畿 大 学 大 学 院
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**西　田　功　一**

Doctoral Dissertation

# Requirement for the collagen receptor Endo180 in collagen gel contraction mediated by corneal fibroblasts

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論文題目

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# Requirement for the collagen receptor Endo180 in collagen gel contraction mediated by corneal fibroblasts

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ABSTRACT

The interaction of keratocytes with extracellular matrix components plays an important role in the maintenance of corneal transparency and shape as well as in the healing of corneal wounds. In particular, the interaction of these cells with collagen and cell-mediated collagen contraction contribute to wound closure. Endo180 is a receptor for collagen that mediates its cellular internalization. We have now examined the role of Endo180 in collagen contraction mediated by corneal fibroblasts (activated keratocytes). Antibodies to Endo180 inhibited the contractile activity of mouse corneal fibroblasts embedded in a three-dimensional collagen gel and cultured in the presence of serum, with this effect being both concentration and time dependent and essentially complete at an antibody concentration of 0.2 μg/ml. Whereas corneal fibroblasts cultured in a collagen gel manifested a flattened morphology with prominent stress fibers under control conditions, they showed a spindlelike shape with few stress fibers in the presence of antibodies to Endo180. Antibodies to Endo180 had no effect on the expression of α–smooth muscle actin or the extent of collagen degradation in collagen gel cultures of corneal fibroblasts. Immunohistofluorescence analysis did not detect the expression of Endo180 in the unwounded mouse cornea. However, Endo180 expression was detected in keratocytes migrating into the wound area at 3 days after a corneal incisional injury. Together, our results suggest that Endo180 is required for the contraction of collagen matrix mediated by corneal fibroblasts and that its expression in these cells may contribute to the healing of corneal stromal wounds.

Keywords : Endo180; collagen gel contraction; corneal stroma; wound healing; corneal fibroblast

## 1. Introduction

The cornea plays an important role in vision as a refractory component of the eye [1]. The transparency and dome-shaped structure of the cornea are both important for this role and are dependent on the arrangement of corneal cells—including epithelial cells, stromal keratocytes, and endothelial cells—and the extracellular matrix (ECM) as well as on interactions between the cells and ECM components. In particular, the molecular structure and alignment of collagen fibers (mostly composed of type I collagen) and the interactions of these fibers with keratocytes and other ECM proteins such as proteoglycans are key to the maintenance of both corneal transparency and shape [2, 3]. In contrast to the skin, in which wound healing is associated with the formation of fibrotic scars, the healing of corneal wounds must take place without fibrotic changes and scarring if the transparency and shape of the tissue are to be maintained.

The interaction between cells including activated keratocytes (corneal fibroblasts) and collagen has been studied in a three-dimensional (3D) culture system in which the cells are embedded in a collagen matrix [4, 5, 6]. Interaction of collagen molecules in the matrix with the cells results in gel contraction mediated by the cells. This contraction is influenced by multiple factors including cytokines [7], cell differentiation [8], cell adhesion molecules [9], and the secretion of proteolytic enzymes [10－14].

Endo180, also known as urokinase-type plasminogen activator (uPA) receptor–associated protein (uPARAP), is related to the macrophage mannose receptor [15], is expresssed predominantly on mesenchymal cells [16, 17], and plays a pivotal role in collagen internalization by cells [18, 19]. It binds to collagen and enhances the adhesion of cells to this ECM protein [20, 21]. Furthermore, Endo180 promotes the migration of fibroblasts on collagen [20], and it interacts with the complex formed by pro-uPA and the uPA receptor [22].

The mechanism of collagen gel contraction mediated by fibroblasts in 3D culture involves repetitive engagement and disengagement of the cells with the collagen matrix [4]. The principal cellular receptors for collagen are thought to be integrins [23, 24], with integrin $\alpha 2\beta 1$ being thought to be largely responsible for the reorganization and contraction of collagen mediated by cells [9, 25, 26]. Given that Endo180 also functions as a receptor for collagen, however, we have now examined the possible role of Endo180 in collagen contraction mediated by mouse corneal fibroblasts in 3D culture.

## 2. Materials and methods

### *2. 1. Corneal wounding and immunohistofluorescence analysis*

Adult male C57BL/6 mice were anesthetized by intraperitoneal injection of sodium pentobarbital (Kyoritsu Seiyaku, Tokyo, Japan) at 50 mg/kg and topical administration of 0.4% oxybuprocaine eye drops (Santen Pharmaceutical, Osaka, Japan). The right central cornea was then subjected to a nonpenetrating incision injury with a microsurgical blade (Straight; Mani, Utsunomiya, Japan). The animals were killed 1 or 3 days after the injury, and the eyes were enucleated, fixed in Super Fix (70% methanol and 8% formaldehyde in buffer) (Kurabo, Osaka, Japan), embedded in paraffin, sectioned at a thickness of 4 μm, and processed for immunohistofluorescence analysis. The sections were incubated in Tris-NaCl blocking (TNB) buffer [0.1 M Tris-HCl (pH 7.5), 0.15 M NaCl, 0.5% Blocking Reagent (Perkin Elmer, Waltham, MA)] for 1 h at room temperature before exposure overnight at 4℃ to rabbit polyclonal antibodies to Endo180 (ab70132; Abcam, Cambridge, UK) at a dilution of 1:500 in TNB buffer. They were then washed three times with Tris-NaCl-Tween (TNT) wash buffer [0.1 M Tris-HCl (pH 7.5), 0.15 M NaCl, 0.05% Tween-20] and incubated for 30 min at room temperature with horseradish peroxidase–conjugated goat antibodies to rabbit immunoglobulin G (Nichirei Biosciences, Tokyo, Japan). Immune complexes were detected with the use of the fluorescence-based tyramide signal amplification (TSA) system (Perkin Elmer), and the sections were mounted with the use of Vectashield containing 4′, 6-diamidino-2-phenylindole (DAPI) (Vector, Burlingame, CA) for observation with a laser confocal microscope (C2; Nikon, Melville, NY). In vivo experiments were approved by the Animal Care and Use Committee of Kindai University Faculty of Medicine and were performed in compliance with the ARVO (The Association for Research in Vision and Ophthalmology) Statement for the Use of Animals in Ophthalmic and Vision Research.

### *2. 2. Cell isolation and culture*

Primary cultures of corneal fibroblasts were established from C57BL/6 mice as previously described [27]. In brief, eyes of mice at postnatal day 1 were enucleated and the cornea was removed with the use of sterile surgical forceps under a stereo dissection microscope. The explants were placed in 35-mm culture dishes in minimum essential medium (MEM) (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) (Gibco-BRL, Grand Island, NY) in order to allow the outgrowth of corneal fibroblasts. The isolated cells were then grown to confluence in 35-mm culture dishes and were harvested for experiments after four to seven passages.

### *2. 3. Three-dimensional culture of corneal fibroblasts in a collagen gel matrix*

Three-dimensional culture of mouse corneal fibroblasts in a collagen gel matrix was performed as previously described [6]. In brief, type I collagen (Nitta Gelatin, Osaka, Japan), 10 × MEM, FBS, and reconstitution buffer [0.05 M NaOH, 0.26 M $Na_2CO_3$, 0.2 M HEPES (pH 7.3)] were mixed, and corneal

fibroblasts were added to the mixture to yield a final cell density of $1 \times 10^5$ cells/ml and final collagen concentration of 2 mg/ml. Portions (300 μl) of the cell suspension were transferred to the wells of a 24-well tissue culture plate that had been coated with 0.1% bovine serum albumin (Sigma-Aldrich), and the plate was then incubated at 37℃ for 1 h to allow gel formation. MEM (300 μl) containing 10% FBS with or without rabbit polyclonal antibodies to Endo180 (ab70132, Abcam) or control rabbit serum (ab7487, Abcam) was then added on top of each gel. Alternatively, for examination of the effects of transforming growth factor–β (TGF-β) on the expression of Endo180 and α–smooth muscle actin (α-SMA) in corneal fibroblasts, MEM (300 μl) with or without recombinant mouse TGF-β1 (R&D Systems, Minneapolis, MN) was added on top of each gel.

*2. 4. Assay of collagen gel contraction*

Collagen gel cultures of corneal fibroblasts were incubated at 37℃ for up to 5 days, during which time the gels detached spontaneously from the plastic plates and floated in the medium. The cell-mediated contraction of the gels was measured by determining the gel diameter.

*2. 5. Fluorescence staining of F-actin*

Cells cultured in collagen gels for 24 h were fixed for 10 min at room temperature with 3.7% formaldehyde in phosphate-buffered saline (PBS), washed with PBS, permeabilized for 5 min with 0.1% Triton X-100 in PBS, and incubated at room temperature first for 30 min with PBS containing 1% bovine serum albumin and then for 1 h with Alexa Fluor 488–conjugated phalloidin (Thermo Fisher Scientific, Waltham, MA) at a dilution of 1:200 in PBS to stain F-actin and with propidium iodide (Thermo Fisher Scientific) at a dilution of 1:200 to stain nuclei. The cells were then examined with a laser-scanning confocal microscope (Axiovert200M; Carl Zeiss, Tokyo, Japan).

*2. 6. Assay of collagen degradation*

The amount of degraded collagen in 3D culture supernatants was assessed by measurement of the amount of hydroxyproline generated from collagen fragments as previously described [28]. In brief, after removal of nondegraded collagen by ultrafiltration, the culture supernatants were subjected to hydrolysis for 24 h at 110℃ with 6 M HCl and the amount of hydroxyproline in the hydrolysates was then measured by spectrophotometry.

*2. 7. Immunoblot analysis*

Cells embedded in collagen gels were pulverized in 200 μl of extraction buffer [10 mM sodium phosphate buffer (pH 7.2), 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 0.2% $NaN_3$], and the cell lysates were then centrifuged at 17,000 × *g* for 5 min at 4℃. The resulting supernatants (10 μg of

protein) were then subjected to SDS-polyacrylamide gel electrophoresis on an 8% to 16% gradient gel, the separated proteins were transferred to a polyvinylidene difluoride membrane, and the membrane was then exposed for 1 h at room temperature to 5% dried skim milk in PBS containing 0.1% Tween-20 (PBST) before incubation overnight at 4℃ with rabbit polyclonal antibodies to α-SMA (ab5694, Abcam) at a dilution of 1:500 and rabbit polyclonal antibodies to Endo180 (ab70132, Abcam) at a dilution of 1:500 in PBST. The membrane was washed with PBST before incubation for 1 h at room temperature with horseradish peroxidase–conjugated goat secondary antibodies (ab205718, Abcam) at a dilution of 1:5000 in PBST and detection of immune complexes with chemiluminescence reagents (GE Healthcare Bio-Sciences, Little Chalfont, UK). Band intensities were measured with the use of Image J software (NIH, Bethesda, MD) and were normalized by those for β-actin probed as an internal control.

*2. 8. RT and real-time PCR analysis*

Total RNA was isolated from corneal fibroblasts cultured in collagen gels with the use of an RNeasy Kit (Qiagen, Valencia, CA) and was then subjected to reverse transcription (RT) with random primers and a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). The resulting cDNA was subjected to real-time polymerase chain reaction (PCR) analysis in a 96-well plate with the use of SYBR Premix Ex Taq (Takara Bio, Shiga, Japan) and an ABI 7900HT Sequence Detection System (Applied Biosystems). The PCR conditions included incubation at 94℃ for 2 min followed by 40 cycles of 94℃ for 30 s, 50℃ for 30 s, and 72℃ for 30 s. The PCR primers (forward and reverse, respectively) were 5′-ATGGCAACTGGAGGCAATATGAG-3′ and 5′-GGCTGCAGGTCAGCAGGTTTA-3′ for Endo180 and 5′-AAGAGCATCCGACACTGCTGAC-3′ and 5′-AGCACAGCCTGAATAGCCACATAC-3′ for α-SMA. The amounts of the mRNAs were calculated with the ΔΔCt (cycle threshold) method, and that of Endo180 mRNA was normalized by that of β-actin mRNA.

*2. 9. Statistical analysis*

Quantitative data are presented as means ± SEM and were compared with Student's unpaired *t*-test or Dunnett's multiple comparison test. A P value of $<0.05$ was considered statistically significant.

## 3. Results

*3. 1. Expression of Endo180 during mouse corneal wound healing*

We first examined whether keratocytes express Endo180 after an incisional injury to the mouse cornea. Endo180 expression was not detected in any cellular components of the unwounded cornea by immunohistofluorescence analysis (Fig. 1a). One day after incision, the remaining epithelial cells had migrated into the V-shaped injury site and filled the gap in the stroma by forming an epithelial plug. No keratocytes were detected within a distance of ～50 μm from the incision. Again, no cells expressing Endo180 were

apparent at this time in any layer of the cornea (Fig. 1b). At 3 days after the incision, keratocytes had migrated into the acellular zone near the injury site and these migratory cells were found to express Endo180 (Fig. 1c). These results thus indicated that keratocytes up-regulate the expression of Endo180 during corneal wound healing.

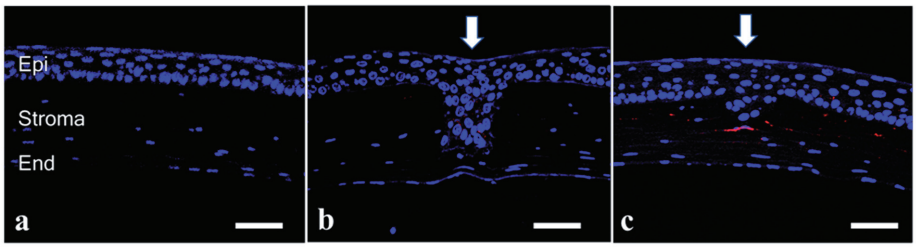


Fig. 1. Endo180 expression in the wounded mouse cornea.
Sections of the mouse cornea were subjected to immunohistofluorescence staining of Endo180 (red fluorescence) and to staining of nuclei with DAPI (blue fluorescence). Representative images of the unwounded cornea (a) and of the cornea at 1 day (b) or 3 days (c) after infliction of a nonpenetrating incisional wound are shown. Arrows indicate the wounded area. Scale bars, 50 μm. Epi, epithelium; End, endothelium.

*3. 2. Effect of antibodies to Endo180 on collagen gel contraction mediated by corneal fibroblasts*

To investigate the possible role of Endo180 in collagen gel contraction mediated by corneal fibroblasts, we examined the effect of antibodies to this protein on gel diameter. Incubation of the collagen gel cultures for 3 days in the presence of various concentrations of antibodies to Endo180 revealed that they inhibited collagen gel contraction in a concentration-dependent manner, with this effect being significant at a concentration of 0.02 μg/ml and essentially complete at 0.2 μg/ml (Fig. 2). Similarly, incubation of the 3D cultures for various times in the presence of antibodies to Endo180 at 2 μg/ml showed that the antibodies inhibited collagen contraction in a time-dependent manner (Fig. 3). Whereas gels exposed to control rabbit serum had begun to contract at 2 days and achieved their minimal diameter at 4 days, the diameter of those incubated in the presence of antibodies to Endo180 remained largely unchanged for up to 5 days. These results thus suggested that Endo180 is necessary for collagen gel contraction mediated by corneal fibroblasts.

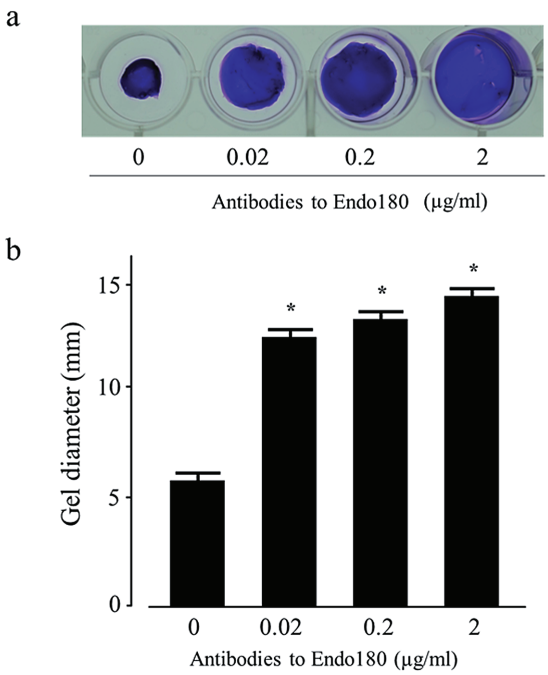


Fig. 2. Concentration-dependent inhibitory effect of antibodies to Endo180 on collagen gel contraction mediated by corneal fibroblasts.
a. Representative images of collagen gel cultures of corneal fibroblasts that had been incubated for 3 days in the presence of the indicated concentrations of antibodies to Endo180.
b. The diameter of collagen gels treated as in a was determined as the mean + SEM of results from four independent experiments, each performed in triplicate with a different cell preparation. *$P < 0.01$ versus the absence of antibodies to Endo180 (Dunnett's test).

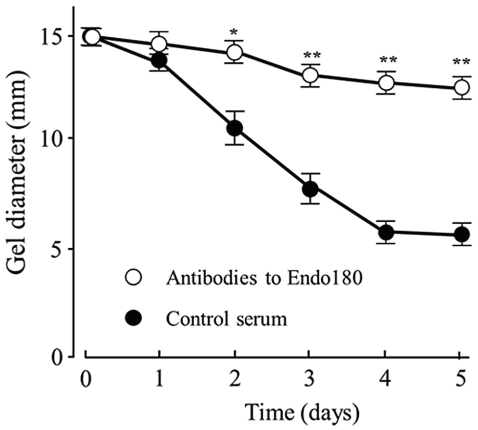


Fig. 3. Time course of the inhibitory effect of antibodies to Endo180 on collagen gel contraction mediated by corneal fibroblasts.
Cells in collagen gels were incubated for the indicated times in the presence of antibodies to Endo180 (2 μg/ml) or control rabbit serum (2 μg/ml), after which the extent of collagen gel contraction was determined by measurement of gel diameter. Data are means ± SEM from four independent experiments, each performed in triplicate with a different cell preparation. *$P < 0.05$, **$P < 0.01$ versus the corresponding value for control serum (Student's unpaired *t*-test).

*3. 3. Effect of antibodies to Endo180 on the morphology of corneal fibroblasts embedded in a collagen gel*

We examined the effect of antibodies to Endo180 on the morphology of corneal fibroblasts in collagen gel cultures by staining of the cells for F-actin with phalloidin. Cells cultured for 24 h in the presence of control rabbit serum manifested a flattened morphology with prominent stress fibers (Fig. 4a and c). In contrast, those cultured with antibodies to Endo180 (2 μg/ml) showed a spindlelike morphology with many fewer stress fibers (Fig. 4b and d), suggesting that the interaction of Endo180 with the collagen matrix is a key determinant of cell morphology.

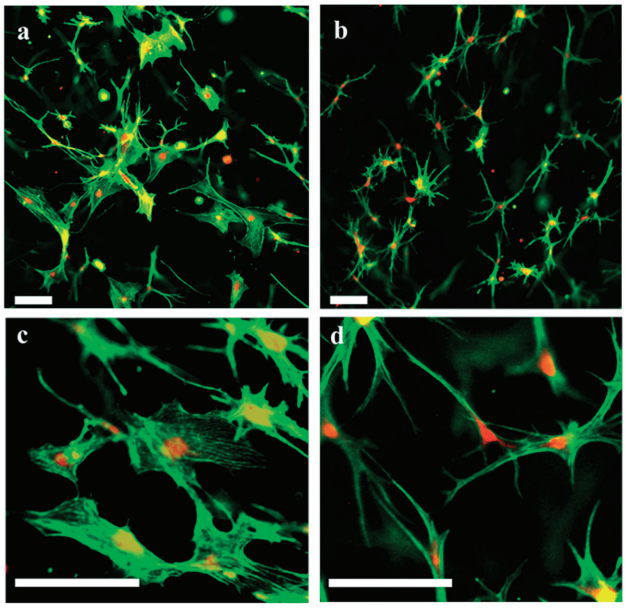


Fig. 4. Effect of antibodies to Endo180 on the morphology of corneal fibroblasts in collagen gel cultures.
Cells were cultured for 24 h in collagen gels in the presence of control rabbit serum (2 μg/ml) (a, c) or antibodies to Endo180 (2 μg/ml) (b, d). They were then stained with fluorescently labeled phalloidin to detect F-actin (green fluorescence) and with propidium iodide to detect nuclei (red fluorescence) before examination with a fluorescence microscope. Scale bars, 50 μm.

*3. 4. Effect of antibodies to Endo180 on collagen degradation by corneal fibroblasts*

To examine whether collagen degradation might contribute to the Endo180-dependent collagen gel contraction mediated by corneal fibroblasts, we measured the amount of degraded collagen after incubation of the 3D cultures for 3 days in the presence of antibodies to Endo180 (2 μg/ml) or control serum (2 μg/ml). Only a low level of collagen degradation was apparent under both conditions, and there was no significant difference between the two (Fig. 5) These results thus suggested that Endo180 does not affect collagen degradation by corneal fibroblasts.

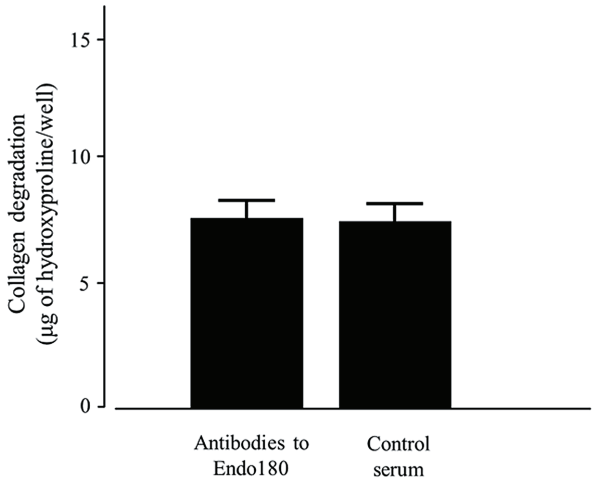


Fig. 5. Effect of antibodies to Endo180 on collagen degradation by corneal fibroblasts.
Corneal fibroblasts embedded in collagen gels were incubated for 3 days in the presence of antibodies to Endo180 (2 μg/ml) or control rabbit serum (2 μg/ml), after which the amount of degraded collagen was determined. Data are expressed as micrograms of hydroxyproline per well and are means + SEM from four separate experiments, each performed in triplicate with a different cell preparation. There was no significant difference between the two groups ($P > 0.05$, Student's unpaired *t*-test).

*3. 5. Effects of TGF-β on Endo180 and α-SMA expression in corneal fibroblasts*

Given that TGF-β also induces collagen gel contraction mediated by corneal fibroblasts [8, 29], we investigated the possible effect of this growth factor on the expression of Endo180 in collagen gel cultures of these cells. Immunoblot analysis revealed that exposure of corneal fibroblasts to TGF-β1 did not increase the abundance of Endo180 but did induce a significant increase in that of α-SMA (Fig. 6a and b). Consistent with these results, RT and real-time PCR analysis showed that, whereas TGF-β1 increased the amount of α-SMA mRNA in the cells, it had no effect on that of Endo180 mRNA (Fig. 6c). These findings thus indicated that, whereas TGF-β up-regulates the expression of α-SMA in corneal fibroblasts, it does not regulate that of Endo180.

*3. 6. Effect of antibodies to Endo180 on α-SMA expression in corneal fibroblasts cultured in a collagen gel*

Finally, we examined whether Endo180 might contribute to the regulation of α-SMA expression in 3D cultures of corneal fibroblasts. Immunoblot analysis of cell lysates revealed that exposure of the cells to antibodies to Endo180 (2 μg/ml) for 3 days had no effect on the abundance of α-SMA compared with that in cells exposed to control rabbit serum (Fig. 7).

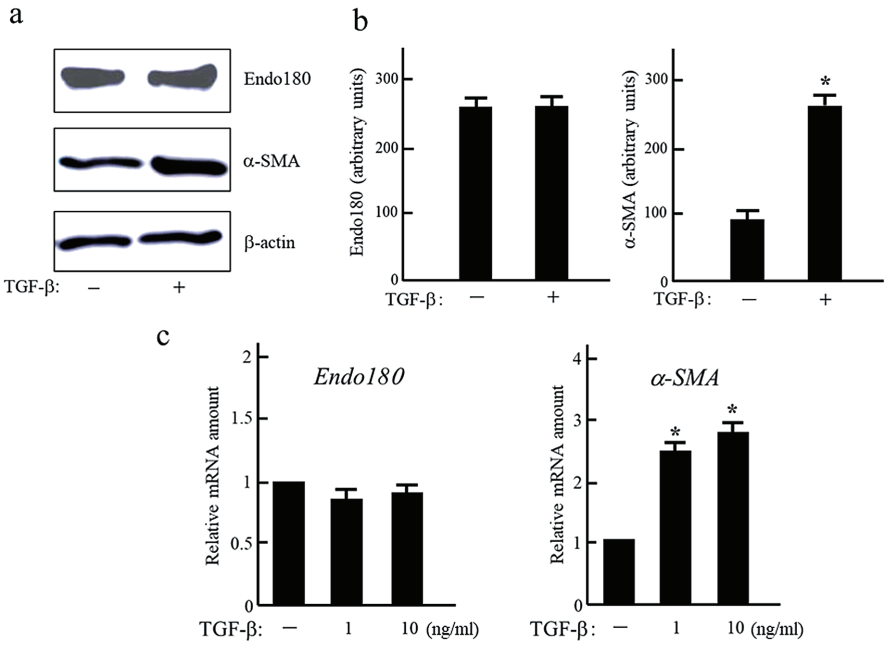


Fig. 6. Effects of TGF-$\beta$ on Endo180 and $\alpha$-SMA expression in corneal fibroblasts cultured in collagen gels.
a, b. Cells in collagen gels were cultured for 48 h in the absence or presence of TGF-$\beta$1 (10 ng/ml), after which cell lysates were subjected to immunoblot analysis with antibodies to Endo180, to $\alpha$-SMA, and to $\beta$-actin (loading control). Representative results (a) and quantitative data (means + SEM) for the amounts of Endo180 and $\alpha$-SMA normalized by that of $\beta$-actin from three independent experiments, each performed in triplicate with a different cell preparation (b), are shown.
c. Cells in collagen gels were cultured for 24 h in the absence or presence of TGF-$\beta$1 (1 or 10 ng/ml), after which total RNA was isolated from the cells and subjected to RT and real-time PCR analysis of Endo180 and $\alpha$-SMA mRNAs. Data are means + SEM from three separate experiments, each performed in triplicate with a different cell preparation. $^*P < 0.05$ versus the value for cells incubated without TGF-$\beta$1 (Dunnett's test)

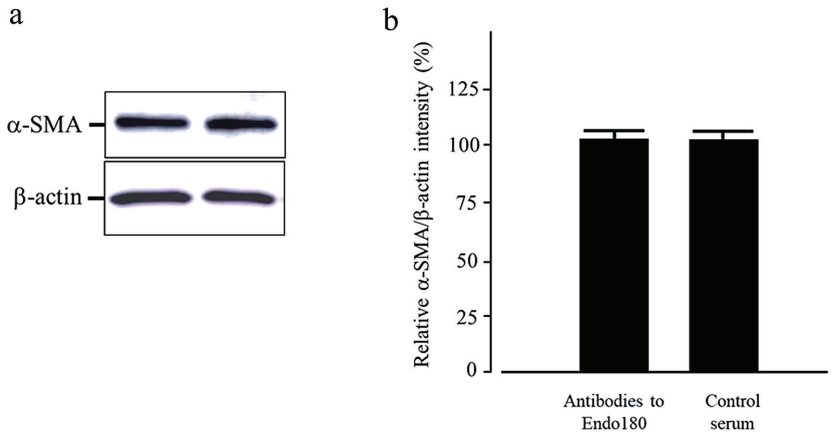


Fig. 7. Effect of antibodies to Endo180 on the expression of $\alpha$-SMA in corneal fibroblasts cultured in a collagen gel.
Cells in collagen gels were cultured for 3 days in the presence of antibodies to Endo180 (2 μg/ml) or control rabbit serum (2 μg/ml), after which cell lysates were subjected to immunoblot analysis with antibodies to $\alpha$-SMA and to $\beta$-actin (loading control). Representative results (a) and quantitative data (means + SEM) for the amount of $\alpha$-SMA normalized by that of $\beta$-actin from three independent experiments, each performed in triplicate with a different cell preparation (b), are shown.

4. Discussion

We have here shown that Endo180 participates in collagen gel contraction mediated by corneal fibroblasts. We thus found that antibodies to Endo180 inhibited such gel contraction in both a concentration- and time-dependent manner, with the extent of inhibition essentially being complete at an antibody concentration of 0.2 μg/ml.

Corneal incision injury was previously shown to result in keratocyte death at and around the injury site within 24 h, with activated keratocytes subsequently gathering under the regenerating epithelium [30]. In the present study, we found that Endo180 was not detectable in the unwounded cornea or at 1 day after an incisional injury. However, at 3 days after incision, the expression of Endo180 was apparent in keratocytes migrating into the wound area, suggesting that Endo180 and its interaction with collagen may play a role in the healing process. Given that Endo180 was not detected in the unwounded cornea, it is unlikely to play a role in the maintenance of corneal shape and transparency in the steady state.

Most studies of Endo180 to date have focused on its role in the internalization of collagen [20, 31, 32]. We have now examined its role in the generation of contractile force by corneal fibroblasts cultured in a 3D collagen matrix. We found that, whereas cells cultured in such a matrix under control conditions manifest a flattened morphology with prominent stress fibers as a result of their interaction with collagen, those cultured in the presence of antibodies to Endo180 showed a spindlelike morphology with few stress fibers, indicating that Endo180 plays an important role in the interaction of corneal fibroblasts with collagen fibers in this model system.

Integrins are thought to serve as the principal collagen receptors in epithelial and mesenchymal cells, and the integrin $\beta 1$ chain has been shown to play a key role in collagen gel contraction mediated by fibroblasts [33－35]. Integrins serve to connect the ECM to the actin cytoskeleton [36], and integrin-dependent collagen gel contraction depends simply on the interaction of integrins with collagen fibrils. However, our present results now show that another collagen receptor, Endo180, directly participates in collagen gel contraction mediated by corneal fibroblasts.

The transformation of fibroblasts into myofibroblasts has been shown to play an important role in collagen contraction mediated by these cells [37－39]. TGF-$\beta$ was thus previously found to promote collagen gel contraction mediated by corneal fibroblasts, and this effect was correlated with an increase in the expression of the myofibroblast marker $\alpha$-SMA [8, 29]. We also found that TGF-$\beta$ increased both the expression of $\alpha$-SMA in corneal fibroblasts (Fig. 6) and gel contraction mediated by these cells (data not shown). However, we did not detect an effect of TGF-$\beta$ on Endo180 expression in corneal fibroblasts. Previous studies have shown that Endo180 is up-regulated by TGF-$\beta$ in other cell types [40, 41], whereas another study found that Endo180 expression and collagen internalization were both down-regulated by TGF-$\beta$ [42]. These observations suggest that the regulation of Endo180 expression by TGF-$\beta$ may be dependent on cell type. We found that antibodies to Endo180 had no effect on the abundance of $\alpha$-SMA in our 3D cultures of corneal

fibroblasts, suggesting that the stimulatory effect of Endo180 on the contractility of corneal fibroblasts is not mediated by an increase in α-SMA expression and promotion of myofibroblast differentiation. Fibroblasts cultured on a rigid surface were shown to up-regulate the expression of α-SMA, whereas those on a flexible collagen-coated surface up-regulated expression of integrin α2β1, suggesting that surface rigidity influences the differential expression of these proteins [43].

In summary, our results suggest that Endo180 is required for collagen gel contraction mediated by corneal fibroblasts in vitro. We also detected the expression of Endo180 in migrating keratocytes during corneal wound healing. Further studies are needed to elucidate the role of this protein in the interaction of keratocytes with the collagen matrix and wound healing in vivo.

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