博 士 学 位 論 文

培養ヒト角膜線維芽細胞の貪食能にプラス
ミノーゲンが与える影響

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Doctoral Dissertation

Stimulation of Phagocytic Activity in Cultured Human Corneal Fibroblasts by Plasminogen

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論文題目

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Stimulation of Phagocytic Activity in Cultured Human Corneal Fibroblasts by Plasminogen

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PURPOSE. Plasminogen has been detected in the corneal stroma after tissue injury and interacts with corneal fibroblasts during wound healing. We examined the effect of plasminogen on phagocytic activity of corneal fibroblasts.

METHODS. Cultured human corneal fibroblasts were exposed to plasminogen and then incubated with fluorescent microparticles before measurement of phagocytic activity by confocal microscopy or flow cytometry. The binding of corneal fibroblasts to immobilized plasminogen was quantitated with a real-time biomolecular interaction assay. The production of urokinase-type plasminogen activator (uPA), matrix metalloproteinases (MMPs), and IL-1β by corneal fibroblasts was measured by fibrin zymography, by immunoblot analysis or gelatin zymography, or with an enzyme-linked immunosorbent assay, respectively.

RESULTS. Plasminogen increased phagocytic activity of corneal fibroblasts in a concentration- and time-dependent manner, with the maximal effect apparent at 30 μg/mL and 24 hours. Corneal fibroblasts bound to immobilized plasminogen in a manner dependent on time and cell number, and the stimulatory effect of plasminogen on phagocytic activity was blocked in the presence of epsilon-aminocaproic acid, an inhibitor of plasminogen binding to cell surface receptors. Plasminogen-induced phagocytic activity was not associated with changes in the production of uPA, MMPs, or IL-1β by corneal fibroblasts.

CONCLUSIONS. Plasminogen induced phagocytic activity in corneal fibroblasts in a manner dependent on its binding to the cell surface. This effect was not associated with increased production of proteases or IL-1β. Thus, plasminogen may promote the clearance of foreign particles or damaged tissue components by corneal fibroblasts early after tissue injury.

Keywords: phagocytosis, corneal fibroblasts, plasminogen

Keratocytes are the principal resident cells of the corneal stroma. They are embedded among collagen lamellae and form a three-dimensional network through the establishment of intercellular gap junctions.[1,2] Under normal conditions, keratocytes are quiescent and contribute to the maintenance of corneal shape and transparency by mediating the slow but steady turnover of collagen, proteoglycans, and other extracellular matrix (ECM) proteins.[3–5] Given the absence of a vascular system in the cornea, the wound healing process in this tissue differs from that in other tissues, such as the skin.[6,7] The cornea faces the external environment and, thus, is vulnerable to various types of insult. In response to corneal injury, stromal keratocytes surrounding the injury site become activated and participate in the repair process.[8,9]

Phagocytosis is a primitive host defense system for the removal of damaged tissue components or causative pathogenic agents,[10–12] with polymorphonuclear leukocytes (PMNs) and macrophages being key mediators of this process. In addition to these "professional" phagocytic cells, however, various other cell types possess phagocytic activity, although phagocytosis is not their primary biological function. Keratocytes are an example of such "nonprofessional" phagocytic cells. Activated keratocytes (corneal fibroblasts) undergo cell division and an increase in cell size, and they manifest increased phagocytic activity.[13–16] Previously, we showed that keratocytes mediate the uptake and long-term storage of Indian ink particles or latex beads in vitro and in vivo.[17,18] We also showed that the activity of lysosomal enzymes in corneal fibroblasts is increased when the cells are cultured with latex beads.[19,20] These findings suggest that one function of keratocytes during the early phase of stromal wound healing may be the removal of components or causative agents of damaged tissue by phagocytosis before arrival of blood-derived cells, such as PMNs and macrophages. Such a function may be especially important in the case of corneal infection, in which pathogen elimination early during the infection process is important to prevent stromal melting, a potentially serious pathologic condition of the cornea characterized by excessive degradation of stromal collagen.[21–23]

Plasminogen is the zymogen of the fibrinolytic enzyme plasmin, which is responsible for the degradation of fibrin clots in the body.[24] Plasminogen is converted to plasmin by plasminogen activators, such as urokinase-type plasminogen
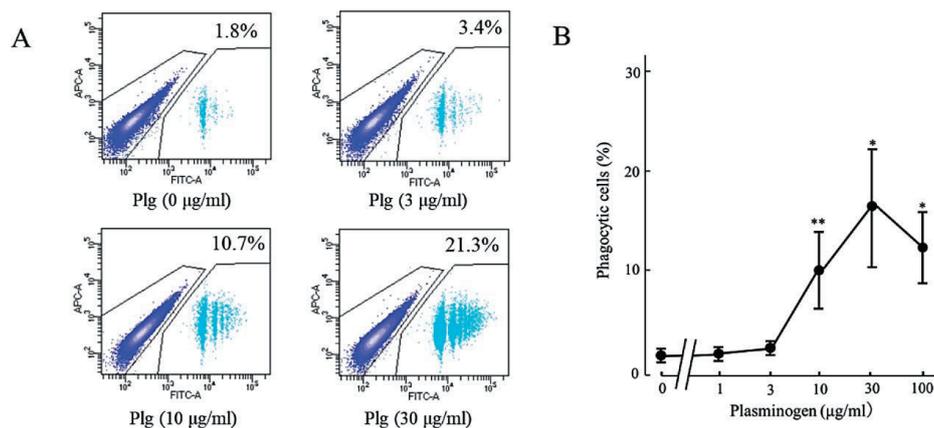
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Figure 1. Concentration-dependent effect of plasminogen on phagocytosis in human corneal fibroblasts. Cells were cultured with the indicated concentrations of plasminogen (Plg) for 24 hours and then exposed to fluorescent microparticles in serum-free medium for an additional 24 hours. Phagocytic activity then was measured by flow cytometry. Representative flow cytometric profiles of APC versus FITC fluorescence are shown in **A**, with the *light* and *dark blue dots* representing cells that had taken up the microspheres and those that had not, respectively. Quantitative analysis of cells that had taken up the microspheres is shown in **B**, with the data representing mean ± SEM values from three independent experiments. *$P < 0.05$, **$P < 0.01$ versus the value for cells incubated without plasminogen (Dunnett's multiple comparison test).

activator (uPA).[24] The uPA-plasminogen system has a central role in the pathogenesis of stromal melting and corneal ulceration. Thus, corneal fibroblasts secrete pro-matrix metalloproteinases (pro-MMPs) that are activated by plasmin and then degrade ECM components during tissue remodeling.[25,26] Plasminogen is synthesized predominantly by the liver and circulates in the blood. Under normal conditions, corneal fibroblasts also synthesize plasminogen at a low level, with plasminogen being present at a low concentration ($\sim$1 µg/mL) in the cornea.[27] However, the concentration of plasminogen in human tear fluid was shown to increase to 30 to 40 µg/mL during corneal wound healing.[28] The level of plasmin activity in tear fluid also is increased during inflammation, ulceration, or infection of the cornea.[29] These findings suggest that plasminogen has a key role in the early phase of corneal wound healing.

To investigate how plasminogen might contribute to the initial phase of corneal stromal wound healing, in addition to its role as an activator of pro-MMPs at later stages, we examined its effect on the phagocytic activity of cultured human corneal fibroblasts.

METHODS

Cell Isolation and Culture

Human corneal fibroblasts were isolated from the corneoscleral rim of corneas obtained for corneal transplantation surgery from The Eye-Bank for Sight Restoration (New York, NY, USA). The tissue was used in accordance with the tenets of the Declaration of Helsinki. The endothelial layer of the cornea was removed mechanically, and the remaining tissue was immersed in dispase (2 mg/mL in MEM; Sigma-Aldrich Corp., St. Louis, MO, USA) for 1 hour at 37°C. After removal of the epithelial sheet, the remaining stromal tissue was incubated with collagenase A of *Clostridium histolyticum* (2 mg/mL in MEM; Sigma-Aldrich Corp.) for 5 hours at 37°C to yield a single-cell suspension of corneal fibroblasts. The isolated cells were maintained under 5% CO_2 in air at 37°C in MEM supplemented with 10% fetal bovine serum (FBS; Gibco-BRL, Grand Island, NY, USA). The medium was changed twice a week, and the cells were subcultured by 1:2 splitting every week. They were used for experiments after four to seven passages.

Assay of Plasminogen Binding to Corneal Fibroblasts

The binding of plasminogen to corneal fibroblasts was measured with a real-time cellular-molecular interaction assay system, the IAsys resonant mirror biosensor (Affinity Sensors, Cambridge, UK), as described previously.[30] In brief, an IAsys carboxylate cuvette was equilibrated with PBS, carboxylate on the cuvette surface was activated by exposure to 200 mM *N*-hydroxysuccinide and 50 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) for 7 minutes, the activation solution was removed and washed out with PBS, and human plasminogen (10 µg/mL; Hyphen BioMed, Paris, France) in 10 mM sodium acetate (pH 5.0) was added for 5 minutes to allow its immobilization on the cuvette surface. After the removal of the plasminogen solution and washing of the cuvette with PBS, the unbound surface of the cuvette was blocked by exposure to 1 M ethanolamine. The immobilization of plasminogen on the cuvette surface was confirmed by detection of an increase in arc seconds. The binding of corneal fibroblasts (2.5×10^4 to 2×10^5 cells/mL) to immobilized plasminogen then was measured.

Preparation of Plasminogen-Linked Microparticles

Fluorescent polystyrene particles with a diameter of 3.0 µm and a carboxylate coating (Fluoresbrite yellow green [YG] carboxylate microspheres) were obtained from Polysciences (Warrington, PA, USA). The particles have an excitation peak at 441 nm and an emission peak at 486 nm (green fluorescence). The coupling of plasminogen to the microbeads was performed with reagents and buffers supplied by Polysciences. A suspension of the microspheres (4.7×10^8 particles in 0.28 mL) was centrifuged at 1000g for 5 minutes, and the particle pellet was suspended in 200 µL of coupling buffer and centrifuged again. The particles then were suspended in 85 µL of coupling buffer and allowed to react with EDAC (2 mg in 10 µL of coupling buffer) for 15 minutes at room temperature with occasional agitation. Plasminogen (150 µg in 150 µL coupling buffer) then was incubated with the activated particles for 1 hour at room temperature, the mixture was
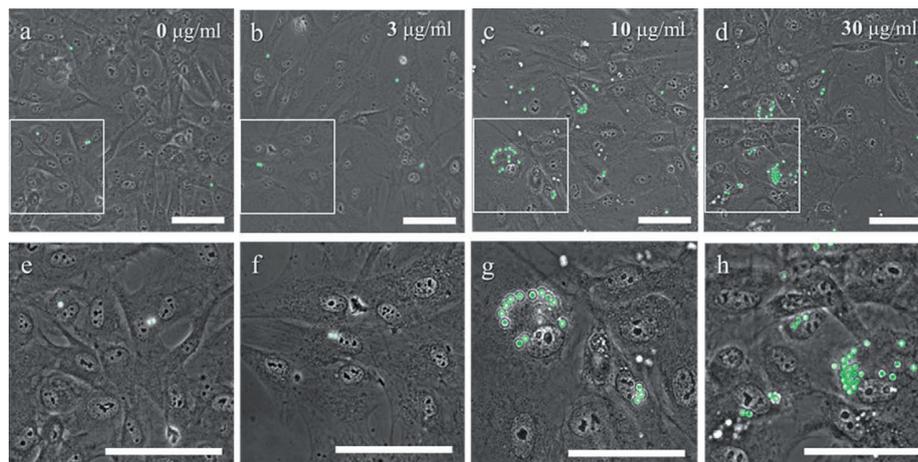
FIGURE 2. Direct visualization of microparticle ingestion by human corneal fibroblasts with a laser-scanning confocal microscope. Cells were incubated with the indicated concentrations of plasminogen for 24 hours and then exposed to fluorescent microparticles in serum-free medium for an additional 24 hours before observation by laser-scanning confocal microscopy. The *boxed regions* in **A–D** are shown at higher magnification in **E–H**, respectively. *Scale bars*: 50 μm.

centrifuged at 1000*g* for 10 minutes, and the amount of protein in the supernatant was measured to provide an estimate of unreacted plasminogen with the use of a Qubit protein assay kit (Invitrogen, Eugene, OR, USA). The protein-linked micro-beads were washed twice with 200 μL wash/storage buffer, suspended in 200 μL wash/storage buffer, and maintained at 4°C until use. The amount of plasminogen cross-linked to the microbeads was estimated to be 110 μg per 4.7×10^8 particles.

Quantification of Phagocytosis by Flow Cytometry

Corneal fibroblasts (1×10^6 cells in a 35 mm² plate) were cultured in MEM supplemented with 10% FBS for 24 hours, washed twice with serum-free MEM, and exposed to MEM containing test agents, including human plasminogen (Hyphen BioMed) and the lysine analog epsilon-aminocaproic acid (EACA; Kyowa Hakko Kogyo, Tokyo, Japan), for 24 hours before replacement of the medium with 1 mL serum-free MEM containing Fluoresbrite YG carboxylate microspheres (Poly-sciences; 1×10^8 particles/mL) and incubation of the cells for

an additional 24 hours at 37°C. The cells then were washed five times with ice-cold PBS to remove free microparticles before isolation by exposure to 0.25% trypsin and centrifugation. The proportion of cells that had taken up the microspheres then was determined by flow cytometry based on forward and side scatter characteristics with a FACSCanto II instrument (BD Biosciences, Franklin Lakes, NJ, USA), which is equipped with 488-nm blue and 633-nm red lasers. BD FACSDiva CS&T Research Beads (BD Biosciences) were used for optimization of the instrument setup and checking of the stability of the cytometer. Samples containing 30,000 cells were examined at a medium flow rate (60 μL/minute), and the fluorescence intensity of FITC and allophycocyanin (APC) was measured with the use of 530/30 and 630/20 bandpass filters, respectively. Aggregation was excluded based on forward and side scatter characteristics. The data were analyzed with FACSDiva software (BD Biosciences). Phagocytic activity was expressed as the percentage of cells that had taken up the fluorescent microparticles.

Direct Visualization of Phagocytic Activity by Laser-Scanning Confocal Microscopy

Corneal fibroblasts exposed to test agents and fluorescent microspheres as described above were washed, collected, and replated at a density of 5000 cells per well in eight-well glass chamber slides (BD Biosciences). Direct observation of the ingested beads was performed with an LSM 510 META laser-scanning confocal microscope (Zeiss, Gottingen, Germany).

Sample Preparation for Assay of Cathepsin B Activity, Fibrin, or Gelatin Zymography, and Immunoblot Analysis

Corneal fibroblasts were incubated in the absence or presence of plasminogen (10 μg/mL) for 24 hours and then exposed to fluorescent microparticles (1×10^8 particles/mL) in serum-free MEM for an additional 24 hours at 37°C. The cells were washed five times with ice-cold PBS and then lysed in a solution containing 10 mM sodium phosphate buffer (pH 7.2), 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, and 0.2% NaN₃. The cell lysates were centrifuged at 17,000*g* for 5 minutes at 4°C, and the resulting supernatants (10 μg of
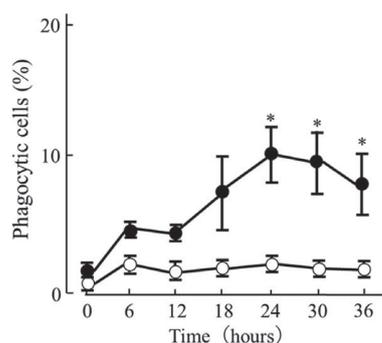

FIGURE 3. Time-dependent effect of plasminogen on phagocytosis in human corneal fibroblasts. Cells were incubated in the absence (*open circles*) or presence (*closed circles*) of plasminogen (10 μg/mL) for the indicated times and then exposed to fluorescent microparticles in serum-free medium for an additional 24 hours. Phagocytic activity then was measured by flow cytometry. Data are means ± SEM from three independent experiments. *$P < 0.05$ versus the corresponding value for cells cultured without plasminogen (Dunnett's multiple comparison test).
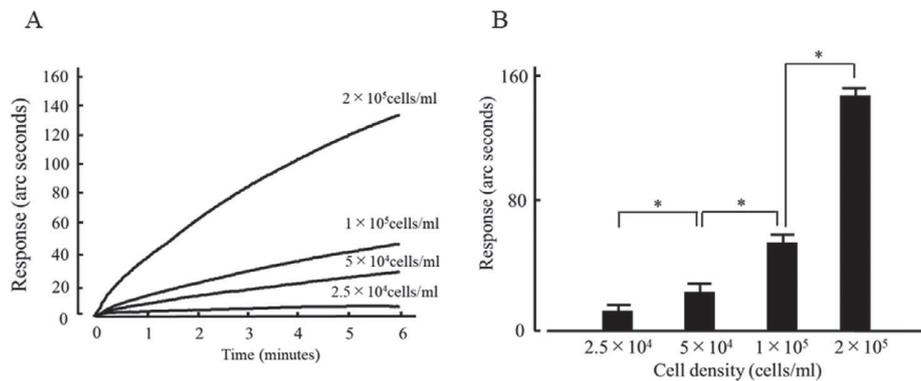
FIGURE 4. Binding of human corneal fibroblasts to immobilized plasminogen. Cells at the indicated densities were incubated with immobilized plasminogen, and cell binding to the plasminogen was analyzed with a real-time biomolecular interaction assay system. Representative assay traces (**A**) and quantitation of binding at 6 minutes (**B**) are shown. The data in **B** are means ± SEM of triplicates from a representative experiment. *$P <$ 0.05 (Dunnett's multiple comparison test).

protein) then were assayed for cathepsin B activity or subjected to fibrin zymography, gelatin zymography, or immunoblot analysis.

Assay of Cathepsin B Activity

Intracellular cathepsin B activity was determined with the use of a Cathepsin B Activity Assay Kit (Abcam, Cambridge, UK). Fluorescence was measured with a microplate reader (AR-VOmx2 multilabel counter; Perkin Elmer, Waltham, MA, USA) at excitation and emission wavelengths of 400 and 505 nm, respectively.

Fibrin and Gelatin Zymography

The expression of uPA was determined as described previously.[30] In brief, cell lysates and molecular markers were subjected to nondenaturing electrophoresis on a 10% polyacrylamide gel containing bovine fibrinogen (0.55 mg/mL) and thrombin (0.056 NIH U/mL; both from Sigma-Aldrich, Corp.). The gel then was washed with 2.5% Triton X-100 for 60 minutes, incubated for 36 hours at 37°C in a reaction buffer containing

0.5 M glycine-NaOH (pH 8.4), stained with Coomassie Blue R-250 for 1 hour, and then incubated in destaining solution (30% methanol, 10% acetic acid). The activities of MMP-2, MMP-3, and MMP-9 were detected by gelatin zymography with the use of a Gelatin Zymo Electrophoresis Kit (Life Laboratory Company, Yamagata, Japan). The intensity of bands was measured with a LAS-1000 system (Fuji Film, Tokyo, Japan).

Immunoblot Analysis

Immunoblot analysis was performed as described previously.[31] In brief, cell lysates were 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 then was incubated for 1 hour at room temperature with 5% dried skim milk in PBS containing 0.1% Tween 20 before exposure overnight at 4°C to antibodies specific for MMP-1 (R&D Systems, Minneapolis, MN, USA). Immune complexes then were detected with horseradish peroxidase–conjugated secondary antibodies and chemiluminescence reagents (GE Healthcare Bio-Sciences, Little Chalfont, UK).
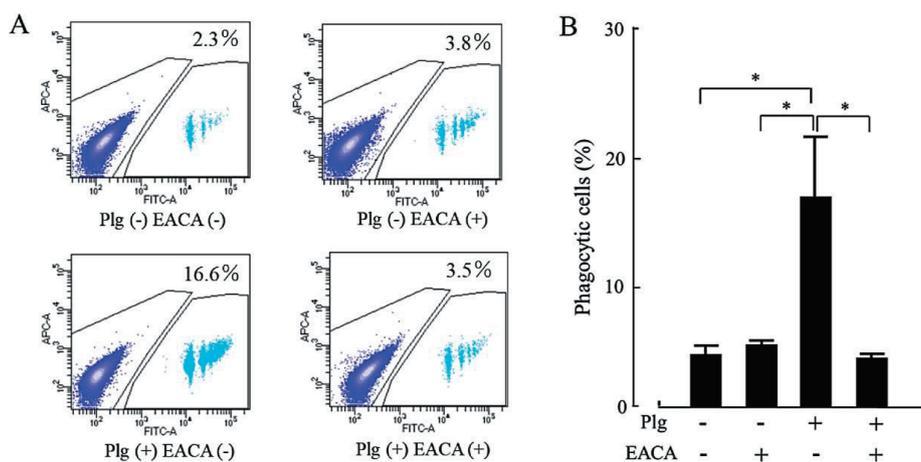

FIGURE 5. Inhibition by EACA of plasminogen-induced phagocytosis in human corneal fibroblasts. Cells were incubated with or without plasminogen (Plg, 10 μg/mL) and EACA (0.1 M) for 24 hours and then were exposed to fluorescent microparticles in serum-free medium for 24 hours. Phagocytic activity then was measured by flow cytometry. Representative flow cytometric profiles of APC versus FITC fluorescence are shown in **A**, with the *light* and *dark blue dots* representing cells that had and had not taken up the microspheres, respectively. Quantitative analysis of cells that had taken up the microspheres is shown in **B**, with the data representing mean ± SEM values from three independent experiments. *$P <$ 0.05 (Dunnett's multiple comparison test).
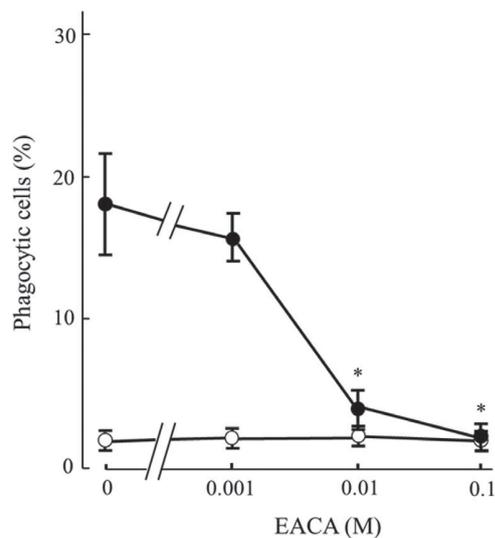
Figure 6. Concentration dependence of the inhibitory effect of EACA on plasminogen-induced phagocytic activity in human corneal fibroblasts. Cells were incubated for 24 hours in the absence (*open circles*) or presence (*closed circles*) of plasminogen (10 μg/mL) and with the indicated concentrations of EACA, and then were exposed to fluorescent microparticles in serum-free medium for 24 hours, after which phagocytic activity was determined by flow cytometry. Data are means ± SEM from three separate experiments. *$P < 0.05$ versus the corresponding value for cells incubated without EACA (Dunnett's multiple comparison test).

RT and Real-Time PCR Analysis

Reverse transcription (RT) and real-time polymerase chain reaction (PCR) analysis was performed as described previously.[32] In brief, total RNA was isolated from corneal fibroblasts with the use of an RNeasy Kit (Qiagen, Valencia, CA, USA) and then was subjected to RT with random primers and a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). The resulting cDNA was subjected to real-time PCR analysis with SYBR Premix Ex Taq (Takara Bio, Shiga, Japan) and an ABI 7900HT Sequence Detection System (Applied Biosystems). The PCR conditions included an initial incubation at 94°C for 2 minutes followed by 40 cycles of 94°C for 30 seconds, 50°C for 30 seconds, and 72°C for 30 seconds. The PCR primers (forward and reverse, respectively) were 5′-CCAAATGGGCTTGAAGCTG-3′ and 5′- GGTATCCGTGTAGCA CATTCTGTC-3′ for pro–MMP-1, and 5′-ATCTGCCTGCCCTC GATGTATAA-3′ and 5′-TTTCAGCTGCTCCGGATAGAGATAG-3′ for uPA. The amounts of pro–MMP-1 and uPA mRNAs were calculated with the ΔΔCt (cycle threshold) method and were normalized by that of β-actin mRNA.

Assay of IL-1β Secretion

The concentration of IL-1β in culture supernatants was measured with an ELISA kit (Quantikine, R&D Systems).

Statistical Analysis

Quantitative data are presented as means ± SEM and were analyzed with Dunnett's multiple comparison test. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of Plasminogen on Phagocytic Activity of Corneal Fibroblasts

We first examined the effect of prior exposure to plasminogen on the phagocytic activity of human corneal fibroblasts. To quantitate the phagocytic activity of the cells, we measured uptake of fluorescent microspheres by flow cytometry. Thus, cells that had phagocytosed the fluorescent microparticles were separated from those that had not based on fluorescence intensity (Fig. 1A). The phagocytic activity of the cells was negligible in the absence of plasminogen exposure and was increased by such exposure in a concentration-dependent manner, with the maximal effect being apparent at a concentration of 30 μg/mL (Fig. 1B). Laser-scanning confocal microscopy also revealed the presence of ingested fluorescent microparticles in cells that had been exposed to plasminogen at 10 or 30 μg/mL for 24 hours, with few cells that had been incubated in the presence of plasminogen at 0 or 3 μg/mL containing such particles (Fig. 2). The stimulatory effect of plasminogen (10 μg/mL) on phagocytic activity of corneal fibroblasts also increased in a time-dependent manner, with the maximal effect apparent for cells that had been treated with plasminogen for 24 hours (Fig. 3). Thus, these results showed that plasminogen increased the uptake of fluorescent micro-beads by corneal fibroblasts in a concentration- and time-dependent manner.

Plasminogen Binding to Corneal Fibroblasts and Its Necessity for Promotion of Phagocytic Activity

To investigate the mechanism by which plasminogen stimulates the phagocytic activity of corneal fibroblasts, we examined whether plasminogen binds to the cells and whether such binding is required for this stimulatory effect. The binding of plasminogen to cells was measured with a resonant mirror biosensor. The assay revealed that corneal fibroblasts bound to immobilized plasminogen in a time- and cell number–dependent manner (Fig. 4).

EACA is a lysine analog that inhibits plasminogen binding to fibrin or cell surface receptors by competing with their lysine-
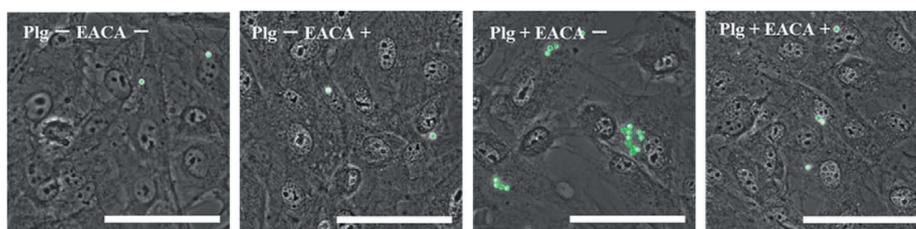
Figure 7. Direct visualization of the effect of EACA on plasminogen-induced microparticle ingestion by human corneal fibroblasts with a laser-scanning confocal microscope. Cells were incubated with or without plasminogen (Plg, 10 μg/mL) and EACA (0.1 M) for 24 hours and then were exposed to fluorescent microparticles in serum-free medium for 24 hours before examination by laser-scanning confocal microscopy. *Scale bars*: 50 μm.
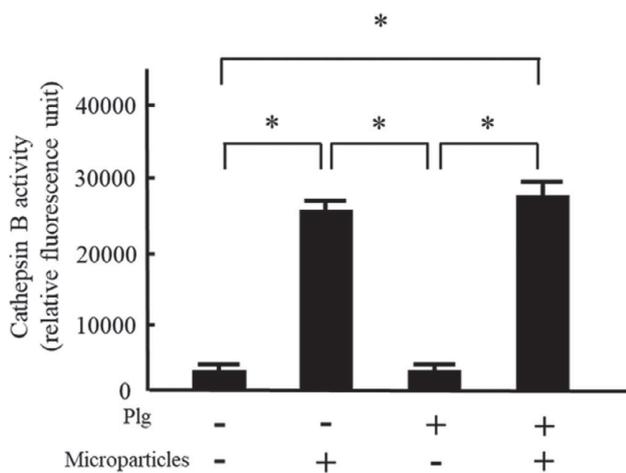
Figure 8. Effects of plasminogen and microparticle ingestion on cathepsin B activity in human corneal fibroblasts. Cells were incubated with or without plasminogen (Plg, 10 μg/mL) for 24 hours, exposed (or not) to fluorescent microparticles in serum-free medium for 24 hours, and then assayed for intracellular cathepsin B activity with a fluorescence-based assay. Data are means ± SEM from three independent experiments. *P < 0.05 (Dunnett's multiple comparison test).

containing binding sites.[33] Therefore, we next examined the effect of EACA on the plasminogen-induced phagocytic activity of corneal fibroblasts by flow cytometry and laser-scanning confocal microscopy. Flow cytometry revealed that EACA blocked the stimulatory effect of plasminogen on phagocytic activity (Fig. 5), and that this action of EACA was concentration-dependent (Fig. 6). Laser-scanning confocal microscopy also showed that the plasminogen-induced increase in the number of ingested fluorescent microparticles in the cells was greatly attenuated in the presence of EACA (Fig. 7). Together, these data suggested that the binding of plasminogen to corneal fibroblasts is necessary for the stimulatory effect of plasminogen on phagocytic activity in these cells.

Cathepsin B Activity in Corneal Fibroblasts During Phagocytosis

Exposure of corneal fibroblasts to plasminogen alone did not affect the intracellular activity of cathepsin B, whereas incubation of the cells with microparticles per se resulted in a significant increase in cathepsin B activity regardless of whether the cells had been exposed to plasminogen (Fig. 8). Thus, these results suggested that contact of the cells with the microparticles or their low level of phagocytosis under basal conditions is sufficient to induce upregulation of lysosomal enzyme activity in a manner independent of plasminogen.

Phagocytosis of Plasminogen-Coated Microparticles by Corneal Fibroblasts

To examine the possible opsonic action of plasminogen, we measured the phagocytic activity of corneal fibroblasts with plasminogen-coated microbeads. Such microparticles were not taken up by corneal fibroblasts to a greater extent than were uncoated microparticles (Fig. 9), suggesting that the stimulatory effect of plasminogen on phagocytic activity was not due to opsonization.

Production of MMPs, uPA, and IL-1β by Corneal Fibroblasts During Phagocytosis

Finally, we explored whether the plasminogen-induced engulfment of microparticles by corneal fibroblasts affects the production of uPA, MMPs (MMP-1, -2, -3, or -9), or IL-1β by these cells. Immunoblot analysis of cells that had been incubated in the absence or presence of plasminogen (10 μg/mL) for 24 hours, and then with or without microparticles for 24 hours revealed that the abundance of MMP-1 was not affected by plasminogen or microparticles (Figs. 10A, 10B). RT- and real-time PCR analysis also showed that the amount of MMP-1 mRNA in corneal fibroblasts was not affected by incubation of the cells with plasminogen or microparticles (Fig. 10C). Similarly, gelatin zymography revealed that the activity of MMP-2 was not affected by exposure of the cells to plasminogen or microparticles (Fig. 11). Neither MMP-9 nor
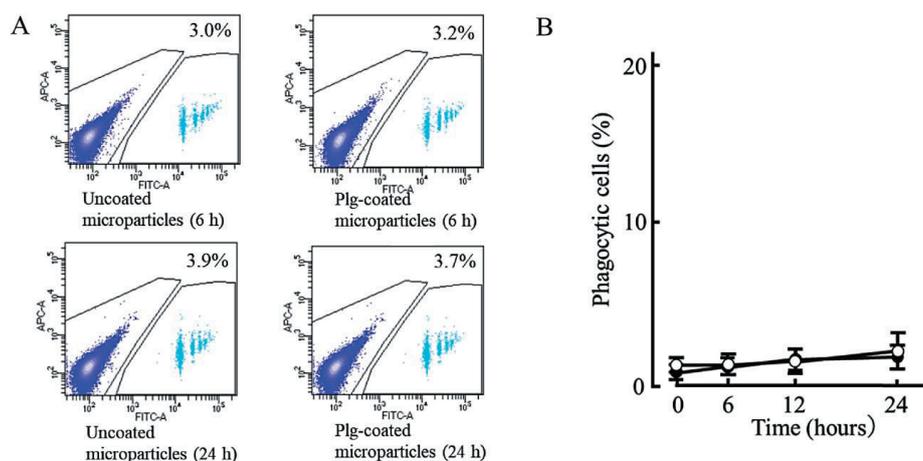

Figure 9. Phagocytosis of plasminogen-coated microspheres by human corneal fibroblasts. Cells were incubated with plasminogen (Plg)–coated fluorescent microparticles or uncoated fluorescent microparticles in serum-free medium for the indicated times, after which phagocytic activity was measured by flow cytometry. Representative flow cytometric profiles of APC versus FITC fluorescence are shown in **A**, with the *light* and *dark blue dots* representing cells that had and had not taken up the microspheres, respectively. Quantitative analysis of cells that had taken up the uncoated (*open circles*) or plasminogen-coated (*closed circles*) microspheres is shown in **B**, with the data representing mean ± SEM values from three independent experiments.
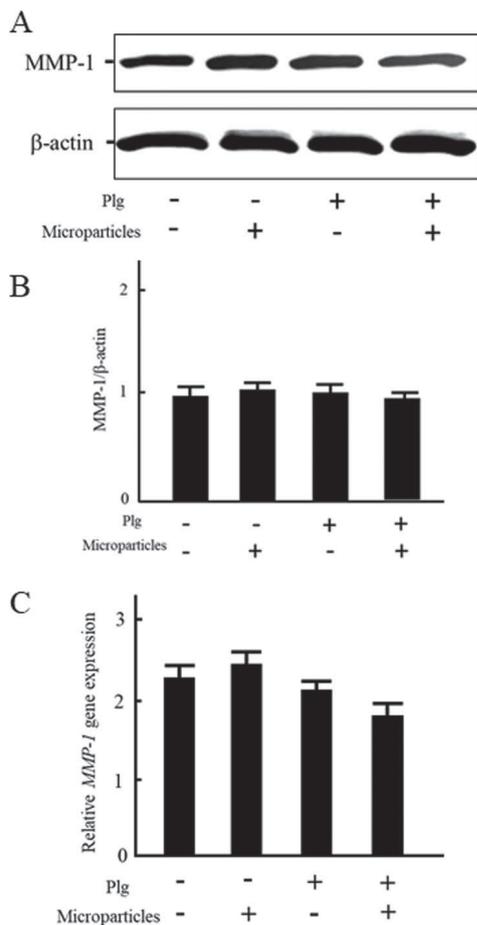
FIGURE 10. MMP-1 expression by human corneal fibroblasts undergoing plasminogen-induced phagocytosis. (**A**) Cells were incubated with or without plasminogen (Plg, 10 µg/mL) for 24 hours and then in the absence or presence of fluorescent microspheres in serum-free medium for 24 hours. Cell lysates then were prepared and subjected to immunoblot analysis with antibodies to MMP-1 and to β-actin (loading control). (**B**) Densitometric quantitation of the MMP-1/β-actin band intensity ratio for blots similar to that in **A**. Data are means ± SEM from three independent experiments. No statistically significant difference was apparent among the four groups. (**C**) Total RNA isolated from cells treated as in **A** was subjected to RT and real-time PCR analysis of MMP-1 mRNA. Data were normalized by β-actin mRNA abundance and are means ± SEM from three separate experiments. No statistically significant difference was apparent among the four groups.

MMP-3 was detected in corneal fibroblasts under any of the conditions tested (Fig. 11). Fibrin zymographic analysis of cell lysates also showed that the amount of uPA was not affected by exposure of the cells to plasminogen or microparticles (Figs. 12A, 12B), whereas RT- and real-time PCR analysis showed that the amount of uPA mRNA was similarly unaffected (Fig. 12C). Together, these results indicated that expression of the proteolytic enzymes MMP-1, MMP-2, MMP-3, MMP-9, and uPA in corneal fibroblasts was not affected by phagocytosis regardless of the absence or presence of plasminogen.

Analysis with an ELISA of the culture supernatants of corneal fibroblasts that had been incubated in the absence or presence of microparticles for 6, 12, or 24 hours after prior incubation with or without plasminogen (10 µg/mL) for 24 hours also revealed that neither plasminogen nor the microparticles had an effect on the concentration of IL-1β, which was <10 pg/mL under all tested conditions (data not shown).
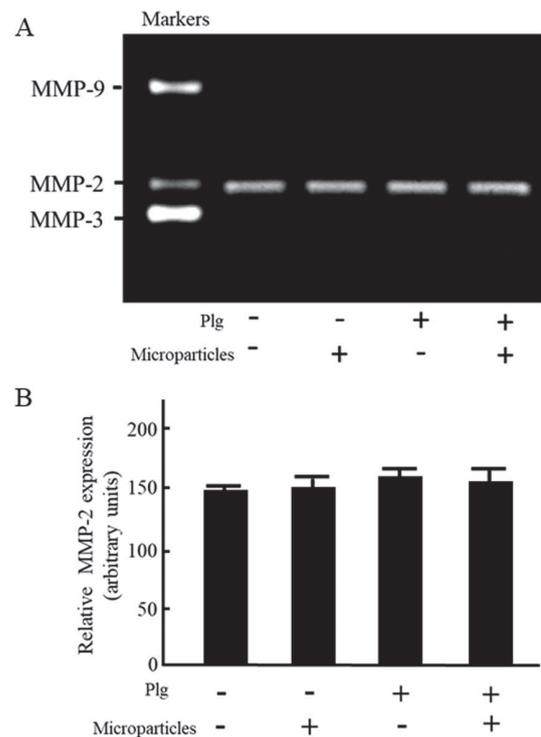

FIGURE 11. Expression of MMP-2, MMP-3, and MMP-9 by human corneal fibroblasts undergoing plasminogen-induced phagocytosis. Cells were incubated with or without plasminogen (Plg, 10 µg/mL) for 24 hours and then in the absence or presence of fluorescent microspheres in serum-free medium for 24 hours. Cell lysates then were prepared and subjected to gelatin zymographic analysis. Representative results (**A**) and quantitative data (means ± SEM) for MMP-2 expression from three independent experiments (**B**) are shown. No statistically significant difference was apparent among the four groups.

DISCUSSION

We have shown that plasminogen increases the phagocytic activity of human corneal fibroblasts in culture. Our findings suggested that plasminogen contributes to the clearance of foreign particles or damaged tissue components in the corneal stroma by stimulating the phagocytic activity of resident corneal fibroblasts.

At least two possible mechanisms exist by which plasminogen might induce phagocytic activity in corneal fibroblasts: through its binding to receptors on the cell surface, or through its binding to the targeted particles and function as an opsonin. Our findings that plasminogen binds to corneal fibroblasts, that such binding is required for stimulation of phagocytic activity, and that plasminogen-coated microparticles were not phagocytosed by the cells to a greater extent than were uncoated particles suggested that plasminogen does not act as an opsonin, but rather stimulates phagocytosis by direct binding to corneal fibroblasts.

Phagocytic cells are classified as professional or nonprofessional, with the former including PMNs, dendritic cells, monocytes, and tissue-resident macrophages, and the latter including other cell types, such as epithelial cells and fibroblasts.[34,35] Professional phagocytes express receptors on their surface that detect material for removal and are specialized for this purpose.[11] On the other hand, nonprofessional phagocytic cells are not specialized for this purpose, but perform other key biological functions.[11] Thus, corneal
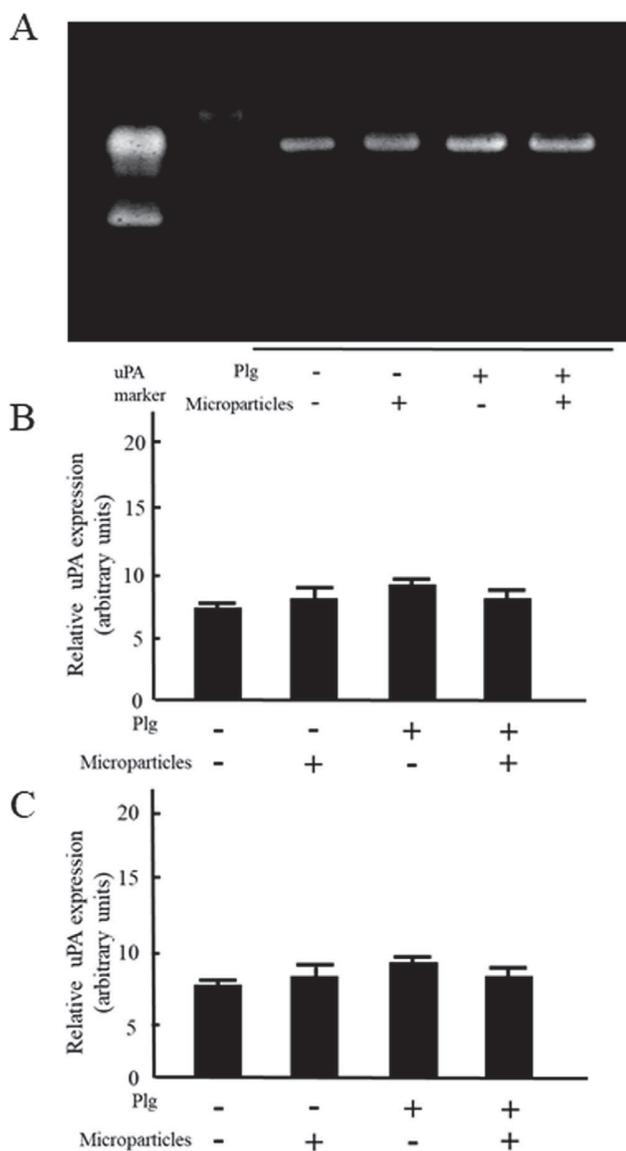
FIGURE 12. Expression of uPA by human corneal fibroblasts undergoing plasminogen-induced phagocytosis. (A) Cells were incubated with or without plasminogen (Plg, 10 µg/mL) for 24 hours and then in the absence or presence of fluorescent microspheres in serum-free medium for 24 hours. Cell lysates then were prepared and subjected to fibrin zymography for the detection of uPA. (B) Quantitation of uPA expression for experiments as in A. Data are means ± SEM from three independent experiments. No statistically significant difference was apparent among the four groups. (C) Total RNA isolated from cells treated as in A was subjected to RT- and real-time PCR analysis of uPA mRNA. Data were normalized by the amount of β-actin mRNA and are means ± SEM from three separate experiments. No statistically significant difference was apparent among the four groups.

fibroblasts possess phagocytic activity, but they also synthesize and degrade ECM components to maintain corneal homeostasis.

The phagocytic activity of professional phagocytes, such as macrophages[36] and dendritic cells[37] is stimulated by plasmin. Active plasmin, but not the inactive enzyme, thus increased the uptake of microparticles by these cells. However, the addition of EACA, an inhibitor of plasminogen or plasmin binding to cells, did not attenuate the stimulatory effect of plasminogen or

plasmin on phagocytosis.[37] On the other hand, we have now shown that prior exposure to plasminogen increased the phagocytic activity of corneal fibroblasts and that this effect was inhibited by EACA. Thus, the binding of plasminogen to corneal fibroblasts appears to be required for the stimulatory effect of plasminogen on the phagocytic activity of these cells. Together, these observations suggested that the activation of phagocytosis may occur via different mechanisms in professional versus nonprofessional phagocytes. Further studies in which the ingestion activities of the two types of phagocyte are directly compared will be required to confirm and explain such differences in their regulation.

In the event of corneal injury or infection, it is important that damaged tissue components or pathogens are removed promptly. Given that the cornea is an avascular tissue, the arrival of professional phagocytic cells at the site of injury or infection is delayed compared to other tissues, with the role of resident nonprofessional cells in this process, thus, being initially more important. Cultured corneal fibroblasts were shown previously to have phagocytic activity,[19,20] and the plasminogen concentration is thought to be increased at sites of corneal injury.[28,29] We showed that plasminogen increases the phagocytic activity of cultured corneal fibroblasts. Together, these various observations suggested that plasminogen-induced phagocytosis by corneal fibroblasts may have an important role in the early stage of the corneal response to injury or infection, until the arrival of professional phagocytic cells.

Later stages of the corneal response to injury or infection can be associated with the excessive degradation of stromal collagen. Our previous studies provided support for the notion that corneal fibroblasts have a key role in such collagen degradation through the synthesis and secretion of the collagenolytic enzyme MMP-1.[31,32] Infiltrating immune cells also have been shown to positively modulate this function of the stromal resident cells.[38] In the case of bacterial infection of the cornea, factors released from bacteria also promote collagen degradation by keratocytes.[32,39]

We found that exposure of corneal fibroblasts to plasminogen did not affect the intracellular activity of cathepsin B, whereas the presence of microparticles per se increased cathepsin B activity in a manner independent of prior exposure of the cells to plasminogen. These results suggested that lysosomal enzyme activity is regulated by contact of the cells with microparticles or by phagocytosis in an all-or-none fashion, rather than being dependent on the extent of phagocytosis. Further studies will be needed to clarify the mechanism of such regulation.

The stimulation of microparticle phagocytosis in corneal fibroblasts by plasminogen was not associated with any change in the expression of uPA or MMPs (MMP-1, -2, -3, and -9). The uptake of latex beads by corneal fibroblasts in primary culture was shown previously to result in the release of collagenase activity,[40] which is now known to be attributable to MMPs. However, our results now indicated that phagocytosis in corneal fibroblasts does not result in the initiation of a proteolytic cascade involving uPA, plasminogen/plasmin, and MMPs. We measured the amounts of uPA and MMPs at 24 hours after the addition of microparticles, whereas the previous study measured collagenase activity at 3 days,[41] with this difference in incubation period possibly contributing to the difference in findings.

We also found that plasminogen-induced phagocytosis was not associated with an increase in the release of IL-1β by corneal fibroblasts. This finding was consistent with our previous observations that keratocytes phagocytose India ink particles or latex beads and store them for a long period of time in vivo without any apparent inflammation.[17,18] The removal of

foreign bodies and damaged tissue components by corneal fibroblasts without induction of IL-1β expression may serve to avoid excessive inflammation in the avascular tissue.

The concentration of plasminogen in the cornea has been found to be approximately 1 μg/mL under normal conditions, and inflammatory cytokines induce a 2- to 3-fold increase in plasminogen synthesis in the cornea.[27] The plasminogen concentration in human tear fluid was increased to 30 to 40 μg/mL after keratectomy.[25] We found that the stimulatory effect of plasminogen on the phagocytic activity of corneal fibroblasts was apparent at approximately 10 to 100 μg/mL, concentrations that are likely to be achieved under pathologic conditions. Thus, plasminogen may contribute to the removal of tissue debris in the cornea after keratectomy. Therefore, the appearance of plasminogen in the corneal stroma during the early phase of stromal damage or inflammation may serve to stimulate the phagocytic activity of resident corneal fibroblasts to remove foreign material or tissue debris before the arrival of professional phagocytes, such as PMNs and monocytes, from blood vessels at the limbus. In addition, plasminogen is thought to contribute to the pathophysiology of conditions that are associated with the excessive degradation of stromal collagen, such as corneal ulceration, by activating proteolytic enzymes, such as MMPs, secreted from corneal fibroblasts stimulated by IL-1 or bacterial pathogens.[31,32,41]

In summary, we showed that plasminogen promotes phagocytic activity of corneal fibroblasts. Although plasminogen has been shown previously to contribute to ECM degradation by corneal fibroblasts under inflammatory conditions,[31,32,39] plasminogen-induced phagocytosis of microparticles did not affect the expression of uPA or MMPs by corneal fibroblasts. Plasminogen likely has a regulatory role in the clearance of infectious agents or damaged tissue components by corneal fibroblasts during the early phase of inflammation or wound healing before the arrival of professional phagocytes at corneal stromal lesions.

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