Inhibition by Epigallocatechin Gallate of IL-1–Induced Urokinase-Type Plasminogen Activator Expression and Collagen Degradation by Corneal Fibroblasts

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PURPOSE. The proinflammatory cytokine interleukin (IL)-1 is implicated in corneal ulceration and promotes collagen degradation by corneal fibroblasts cultured in a three-dimensional (3D) collagen gel. Epigallocatechin-3-gallate (EGCG), the principal polyphenol in extracts of green tea, has various beneficial health effects, some of which appear to be mediated through direct or indirect inhibition of protease activity. We therefore examined the effect of EGCG on IL-1β–induced collagen degradation by corneal fibroblasts embedded in a collagen gel.

METHODS. Human corneal fibroblasts were cultured in a type 1 collagen gel. Collagen degradation was assessed by measurement of hydroxyproline in acid hydrolysates of culture supernatants. The expression of urokinase-type plasminogen activator (uPA) was examined by real-time and RTPCR analysis and by fibrin zymography, and that of the collagenase matrix metalloproteinase 1 (MMP1) was detected by immunoblot analysis.

RESULTS. EGCG inhibited IL-1β–induced, plasminogen-dependent collagen degradation by corneal fibroblasts in a concentration-dependent manner. It also attenuated the IL-1β–induced expression of uPA at both mRNA and protein levels. EGCG inhibited the IL-1β–induced conversion of exogenous plasminogen to plasmin as well as the plasminogen-dependent activation of pro-MMP1 in the 3D cultures without a substantial effect on pro-MMP1 abundance.

CONCLUSIONS. EGCG inhibits IL-1β–induced collagen degradation by corneal fibroblasts, with this effect likely being mediated by suppression of the upregulation of uPA, the uPA-mediated conversion of plasminogen to plasmin, and the plasmin-mediated activation of pro-MMP1. EGCG thus warrants further investigation as a potential treatment for corneal ulcer.

Keywords: corneal fibroblasts, collagen degradation, EGCG, uPA, wound healing

Excessive collagen degradation in the corneal stroma can lead to the development of corneal ulcer.1 The causes of this serious pathological condition are varied, but the participation of the proinflammatory cytokine interleukin (IL)-1 has been well documented.2,3 Keratocytes, the principal resident cells of the corneal stroma, are connected via gap junctions to form a three-dimensional (3D) network structure and are quiescent in the healthy cornea.4,5 These cells mediate the slow turnover of extracellular matrix components, such as collagen and proteoglycans in the stroma, which they achieve via the synthesis and degradation of these molecules.6,7 However, under inflammatory conditions, such as those associated with corneal infection or injury, keratocytes become activated and undergo a change in phenotype. They thus transform into fibroblasts, migrate to the site of inflammation, and increase their degradation of extracellular collagen fibrils. This degradation of collagen is mediated by a proteolytic cascade that includes urokinase-type plasminogen activator (uPA), plasmin generated from plasminogen, and matrix metalloproteinases (MMPs).8,9 The upregulation of MMP production by activated corneal fibroblasts thus plays a key role in degradation of the extracellular matrix associated with corneal ulceration.10,11

We previously showed that, in the presence of plasminogen, IL-1α and IL-1β enhanced collagen degradation by corneal fibroblasts in a 3D model culture system in which corneal fibroblasts are maintained in a collagen gel matrix.12 In addition, we recently found that collagen enhances IL-1β–induced uPA expression in corneal fibroblasts, with the pathway for IL-1β–induced collagen degradation by these cells being strictly dependent on uPA production and mediated by the conversion of plasminogen to plasmin by uPA and the activation of MMPs by plasmin.13
(-)-Epigallocatechin-3-gallate (EGCG), the principal polyphenol in extracts of green tea, has been shown to possess various beneficial effects on cancer,14–16 allergy,17 hypertension,18 atherosclerosis,19 diabetes,20 as well as bacterial and viral infection.21 EGCG has thus been found to suppress tumor growth and angiogenesis by inhibiting MMP2 and -9 expression in tumor cells.22–24 Topical administration of EGCG was also shown to inhibit corneal neovascularization in rabbits through suppression of the expression of VEGF and cyclooxygenase-2,25 to attenuate inflammatory responses of the ocular surface in a mouse model of dry eye,26 and to limit the development of corneal opacity caused by alkali-burn injury to the cornea in mice.27 EGCG binds to uPA and interferes with its ability to recognize its substrates, thereby inhibiting its enzymatic activity.28 Furthermore, EGCG attenuates the activity of the uPA gene promoter and thereby downregulates the amount of uPA mRNA.29

On the basis of these various observations, we have now examined whether EGCG might inhibit collagen degradation by corneal fibroblasts in the corneal stroma. In particular, we investigated the effects of EGCG on IL-1β-induced collagen degradation as well as on uPA and MMP1 expression by corneal fibroblasts in our 3D culture system.

**METHODS**

**Cell Isolation and Maintenance**

Corneal fibroblasts were isolated from the corneoscleral rim of human 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. After removal of the epithelial sheet and endothelial layer, the stromal tissue was treated for 5 hours at 37°C with collagenase A of Clostridium histolyticum (2 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) in MEM to obtain a single-cell suspension of corneal fibroblasts. The isolated cells were maintained under 5% CO2 in air at 37°C in MEM supplemented with 10% fetal bovine serum (Gibco-BRL, Grand Island, NY, USA). The cells were used for experiments after four to seven passages.

**3D Culture of Corneal Fibroblasts**

Culture of corneal fibroblasts in a collagen gel was performed as previously described.13 In brief, type I collagen (Nitta Gelatin, Osaka, Japan), 10X MEM, reconstitution buffer (0.05 M NaOH, 0.26 M NaHCO3, and 0.2 M HEPES [pH 7.3]), and corneal fibroblast suspension were mixed on ice in a volume ratio of 7:2:1:1. The final density of corneal fibroblasts was 3 × 104 cells/ml, and the final collagen concentration was 2 mg/ml. The mixture (300 µl per well) was transferred to the wells of a 24-well tissue culture plate, which was then incubated at 37°C for 1 hour to allow gel formation. MEM (300 µl) containing various agents, including recombinant human IL-1β (5 ng/ml; R&D Systems, Minneapolis, MN, USA), human plasminogen (50 µg/ml; Hyphen BioMed, Paris, France), and EGCG (3–300 µM; Cayman Chemical, Ann Arbor, MI, USA) was then added on top of each collagen gel. The resulting cultures were incubated for 30 minutes or 24 hours before analysis.

**Cell Viability Assay**

Corneal fibroblasts (1 × 10⁵ cells) in 180 µl of culture medium containing various concentrations of EGCG (0–300 µM) were seeded in 96-well plates and cultured for 72 hours. After the addition of 20 µl of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-
diphenyltetrazolium bromide] (Sigma-Aldrich) to each well, the cells were cultured for an additional 4 hours. Formazan was dissolved by the addition of 100 µl of dimethyl sulfoxide to each well, and absorbance was measured at 562 nm with a VERSAmax plate reader (Japan Molecular Devices, Tokyo, Japan). The assay was performed with six wells per condition.

**Fibrin Zymography**

The production of uPA by corneal fibroblasts cultured in a collagen gel was examined by fibrin zymography as described previously.30 In brief, cell lysates and molecular mass markers were subjected to electrophoresis on a 10% polyacrylamide gel containing bovine fibrinogen (0.55 mg/mL; Sigma-Aldrich) and thrombin (0.056 NIH U/mL; Sigma-Aldrich). The gel was washed with 2.5% Triton X-100 for 1 hour, incubated for 36 hours at 37°C in a reaction buffer containing 0.5 M glycine-HCl (pH 8.4), stained for 1 hour with Coomassie Brilliant Blue R-250, and then destained with a solution comprising 30% methanol and 10% acetic acid. The intensity of bands corresponding to uPA was measured with the use of a LAS-1000 system (Fuji Film, Tokyo, Japan) calibrated with human standard uPA (0.1 IU).

**Immunoblot Analysis**

Immunoblot analysis was performed as previously described.13 In brief, cell lysates were subjected to SDS-PAGE on an 8% to 16% gradient gel, the separated proteins were transferred to a polyvinylidene difluoride membrane, and the membrane was then 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 MMP1 (R&D Systems), for the phosphorylated p65 subunit of nuclear factor-kappaB (NF-kB; Cell Signaling, Danvers, MA, USA), for phosphorylated IκB-α (Cell Signalling), or for β-actin (Cell Signalling). Immune complexes were detected with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence reagents (GE Healthcare Bio-Sciences, Little Chalfont, UK). Band intensities were measured with the use of ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA) and were normalized by that for β-actin.

**RT and Real-Time PCR Analysis**

Real-time and RT-PCR analysis were performed as described previously.31 In brief, total RNA was isolated from corneal fibroblasts cultured in a collagen gel with the use of a RNeasy Kit (Qiagen, Valencia, CA, USA). The RNA was then subjected to RT with random primers and a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), and the resulting cDNA was subjected to real-time 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°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′-ATCTGCCTGGCCCTCAGTG TATAA-3′ and 5′-TCTGAGCTGGCCAGATAAGTAG-5′ for uPA and 5′-ATCTGCCTGGCCCTCAGTG TATAA-3′ and 5′-TCTGAGCTGGCCAGATAAGTAG-5′ for pro-MMP1. The amounts of uPA and pro-MMP1 mRNAs were calculated with the ΔΔCt (cycle threshold) method and were normalized by that of β-actin mRNA.
Assay of Collagen Degradation

Measurement of degraded collagen in 3D culture supernatants was performed as previously described. In brief, supernatants from collagen gel incubations were subjected to ultrafiltration with a Vivaspin 500 Centrifugal Ultra Filter (100,000 MWCO; GE Healthcare Bio-Sciences) to remove native collagen fibrils with a molecular size of more than 100 kDa. The filtrate was then subjected to hydrolysis with 6 M HCl for 24 hours at 110°C, and the amount of hydroxyproline in the hydrolysates was measured by spectrophotometry.

Chromogenic Assay of Plasmin Activity

Plasmin activity was measured with an assay based on the chromogenic substrate H-D-valyl-L-leucyl-L-lysine-p-nitroaniline (S-2251; Chromogenix, Milan, Italy). Culture supernatants (100 μL) were incubated at 37°C with 100 μL of 0.5 mM S-2251, and the release of p-nitroaniline was measured with the use of a Thermomax microplate reader (Molecular Devices, Sunnyvale, CA USA). Plasmin activity was estimated as S-2251–degrading activity.

Statistical Analysis

Quantitative data are presented as means ± SEM and were analyzed with Dunnett’s multiple comparison test or Tukey’s multiple comparison test. A P value < 0.01 was considered statistically significant.

RESULTS

Inhibitory Effect of EGCG on IL-1β–Induced Collagen Degradation by Corneal Fibroblasts

We first examined the effect of EGCG on IL-1β–induced collagen degradation by corneal fibroblasts in the absence or presence of plasminogen. In the absence of plasminogen, only a low level of collagen degradation was apparent even in the presence of IL-1β. However, in the presence of plasminogen, IL-1β induced marked collagen degradation, and this effect was inhibited by EGCG in a concentration-dependent manner (Fig. 1). The inhibitory effect of EGCG was significant at 30, 100, and 300 μM. Collagen gels containing corneal fibroblasts and exposed to IL-1β and plasminogen manifested a visible reduction in size and became pultaceous as a result of collagen degradation. Such changes were less apparent in the presence of EGCG at 30 μM and were not observed at 100 or 300 μM.

Effect of EGCG on Cell Viability

To investigate whether the inhibitory effect of EGCG on collagen degradation by corneal fibroblasts might be due to cytotoxicity, we examined the viability of corneal fibroblasts cultured in a collagen gel with various concentrations of EGCG with the use of the MTT assay. EGCG did not affect cell viability at concentrations up to 30 μM (Fig. 2). At concentrations of 100 and 300 μM, however, EGCG induced a significant loss of viability. On the basis of these results, we focused on the effects of EGCG at a concentration of 30 μM in subsequent experiments.

Effect of EGCG on IL-1β–Induced uPA Production by Corneal Fibroblasts

To investigate the mechanism by which EGCG inhibits IL-1β–induced collagen degradation by corneal fibroblasts, we first examined the possible effect of EGCG on cell-associated uPA abundance in 3D cultures in the presence of IL-1β. Fibrin zymography of cell lysates revealed that EGCG inhibited the expression of uPA in a concentration-dependent manner, with this effect being significant at 30, 100, and 300 μM (Figs. 3A, 3B), similar to the concentration dependence for the inhibitory effect of EGCG on IL-1β–induced collagen degradation. Furthermore, real-time and RT-PCR analysis showed that the
IL-1β-induced increase in the amount of uPA mRNA was significantly inhibited by EGCG at concentrations of 10, 30, 100, and 300 μM (Fig. 3C), indicating that the IL-1β-induced expression of uPA was attenuated by EGCG at both the mRNA and protein levels.

**Effects of EGCG on Pro-MMP1 Production and Activation in Corneal Fibroblast Cultures**

We next examined the effects of EGCG on the production and activation of pro-MMP1 in 3D cultures of corneal fibroblasts. Immunoblot analysis of cell lysates revealed that exposure of corneal fibroblasts in collagen gels to IL-1β increased the abundance of pro-MMP1 and that this effect was inhibited slightly by EGCG at 30 μM (Fig. 4A, B). The presence of plasminogen in the cultures resulted in the conversion of pro-MMP1 produced in the presence of IL-1β to active MMP1, and this effect of plasminogen was inhibited by EGCG. Real-time and RT-PCR analysis also showed that the IL-1β-induced increase in the amount of pro-MMP1 mRNA was not affected by EGCG at concentrations of 10 to 100 μM, although it was prevented by EGCG at 300 μM (Fig. 4C), likely as a result of the loss of cell viability induced by EGCG at this concentration (Fig. 2). Together, these results thus suggested that EGCG has only a small inhibitory effect on the IL-1β-induced production of pro-MMP1 by corneal fibroblasts but that it markedly attenuates the plasminogen-dependent activation of pro-MMP1 in 3D cultures.

**FIGURE 2.** Effect of EGCG on the viability of corneal fibroblasts. Corneal fibroblasts were incubated for 72 hours in the presence of EGCG at the indicated concentrations, after which cell viability was evaluated with the MTT assay. Data are expressed as a percentage of the value for cells not exposed to EGCG and are means ± SEM from three separate experiments. *P < 0.01 versus the value for cells cultured without EGCG (Dunnett’s multiple comparison test).

**FIGURE 3.** Concentration-dependent inhibition by EGCG of IL-1β-induced uPA expression in corneal fibroblasts. (A, B) Cells were cultured for 24 hours in a collagen matrix and in the presence of IL-1β (5 ng/mL) and the indicated concentrations of EGCG, after which the amount of uPA in cell lysates was examined by fibrin zymography. A marker for uPA was included in the analysis. Representative results (A) and quantitative data (means ± SEM) from three independent experiments (B) are shown. *P < 0.01 versus the value for cells cultured without EGCG (Dunnett’s multiple comparison test). (C) Cells were cultured for 24 hours in collagen gels and in the absence or presence of IL-1β (5 ng/mL) and with the indicated concentrations of EGCG, after which total RNA was isolated from the cells and subjected to real-time and RT-PCR analysis of uPA mRNA. Data were normalized by the amount of β-actin mRNA and are means ± SEM from three separate experiments. *P < 0.01 versus the value for cells incubated with IL-1β alone (Dunnett’s multiple comparison test).
Effect of EGCG on Plasmin Activity in 3D Cultures of Corneal Fibroblasts

We investigated whether EGCG might affect plasmin activity in culture supernatants of corneal fibroblasts incubated in the presence of IL-1β and plasminogen. The level of plasmin activity in the culture supernatants was reduced by EGCG in a concentration-dependent manner, with this effect being significant at concentrations of 30 and 100 μM (Fig. 5).

Effect of EGCG on IL-1β–Induced NF-κB Signaling in Corneal Fibroblasts

NF-κB is a transcription factor that regulates the expression of various inflammation-related genes. We investigated whether EGCG might affect the IL-1β–induced activation of the NF-κB signaling pathway in corneal fibroblasts cultured in a collagen gel. IL-1β increased the phosphorylation of both the p65 subunit of NF-κB and the endogenous NF-κB inhibitor IκB-α (Fig. 6). These effects of IL-1β were not influenced by plasminogen, but they were prevented in the presence of EGCG (30 μM), indicating that EGCG directly suppresses the IL-1β–induced activation of NF-κB signaling.

DISCUSSION

We have here shown that EGCG attenuates IL-1β–induced collagen degradation by corneal fibroblasts. This effect was associated with suppression of uPA expression at both the mRNA and protein levels as well as with inhibition of both the conversion of plasminogen to plasmin and the activation of pro-MMP1. These results suggest that the inhibitory effect of EGCG on collagen degradation is mediated by prevention of the IL-1β–induced upregulation of uPA in corneal fibroblasts and consequently of the uPA-mediated activation of plasminogen, the plasmin-dependent activation of pro-MMP1, and the MMP1-mediated degradation of collagen. Furthermore, EGCG inhibited the IL-1β–induced activation of the NF-κB signaling pathway, which may account for its attenuation of uPA expression.

MMPs are largely responsible for the degradation of collagen in the corneal stroma.7,34,35 MMP1 is released by a variety of cell types including fibroblasts and is capable of cleaving fibrillar collagen.9,36 The synthesis and release of MMP1 by corneal fibroblasts contribute to the process of corneal destruction.37 MMPs are produced by cells as inactive proenzymes, and the activation of pro-MMP1 is thought to be...
mediated by plasmin.\textsuperscript{34} Whereas plasmin is present at only a low level in the healthy cornea, its abundance is increased in corneal disorders.\textsuperscript{38,39} Plasmin is generated from plasminogen by uPA, which has been shown to play a role in severe allergic conjunctivitis,\textsuperscript{40} leukocyte infiltration into the cornea,\textsuperscript{41} and collagen degradation by corneal fibroblasts.\textsuperscript{13} The conversion of plasminogen to plasmin by uPA and the activation of pro-MMPs by plasmin constitute the primary pathway for collagen degradation by corneal fibroblasts.\textsuperscript{13,42} We have now shown that EGCG suppresses the activation of pro-MMP1 produced by corneal fibroblasts in response to IL-1\textbeta stimulation, and that this effect appears to be mediated by inhibition of the upregulation of uPA without a marked effect on the expression of pro-MMP1.

EGCG has previously been shown to inhibit collagen production or collagen degradation by various cell types. It thus suppressed both collagen production and collagenase activity in cultures of hepatic stellate cells\textsuperscript{43} as well as inhibited the degradation of type II collagen by cartilage-like cell sheets.\textsuperscript{44} EGCG also downregulated the release of MMP\textgamma, -8, and -9 in 3D cocultures of macrophages and gingival fibroblasts.\textsuperscript{45} We found that EGCG suppressed collagenolytic activity but did not substantially affect the production of MMP1 in 3D cultures of corneal fibroblasts.

The transcription factor NF-JB plays an important role in regulation of the expression of a large number of genes in response to cell stimulation with proinflammatory cytokines. Whereas NF-JB forms a complex with its inhibitor I\textbeta in the cytoplasm of resting cells, cell stimulation induces the dissociation of NF-JB from I\textbeta and the translocation of NF-JB subunits from the cytoplasm to the nucleus. Such nuclear translocation of NF-JB has been demonstrated in corneal fibroblasts.\textsuperscript{46} We have also previously shown that IL-1 induced the phosphorylation and degradation of I\textbeta and the activation of NF-JB in corneal fibroblasts.\textsuperscript{47} Expression of the uPA gene has been shown to be regulated by NF-JB, with the gene promoter region having been found to contain binding sites for NF-JB.\textsuperscript{48} We have now shown that EGCG inhibited both the activation of NF-JB and the upregulation of uPA mRNA in corneal fibroblasts stimulated with IL-1\textbeta,\textsuperscript{48} consistent with the notion that the inhibitory effect of EGCG on uPA expression is mediated by suppression of NF-JB activation.

Many compounds, including the chelator EDTA, a thiol-containing peptide, and cysteine, have been investigated for their potential to treat corneal ulcer.\textsuperscript{49,50} In addition, MMP

**FIGURE 5.** Concentration-dependent inhibition by EGCG of the conversion of plasminogen to plasmin in 3D cultures of corneal fibroblasts. Collagen gels containing corneal fibroblasts were incubated for 24 hours in the presence of IL-1\textbeta (5 ng/mL), plasminogen (50 \mu g/mL), and the indicated concentrations of EGCG, after which culture supernatants were assayed for plasmin activity with the use of a chromogenic assay. Data are means \pm SEM from three separate experiments. *P < 0.01 versus the value for cells incubated in the absence of EGCG (Dunnett’s multiple comparison test).

**FIGURE 6.** Inhibition by EGCG of IL-1\textbeta–induced activation of NF-JB signaling in corneal fibroblasts. Cells were cultured in a collagen gel in the absence or presence of plasminogen (50 \mu g/mL, plg), IL-1\textbeta (5 ng/mL), and EGCG (50 \mu M) for 30 minutes, after which cell lysates were subjected to immunoblot analysis with antibodies to phosphorylated (p-) forms of I\textbeta or the p65 subunit of NF-JB. Representative results (A) and quantitative data for the p-I\textbeta/\beta-actin ratio or the p-p65/\beta-actin ratio (means \pm SEM) from three independent experiments (B) are shown. *P < 0.01 versus the value for cells incubated without addition (Dunnett’s multiple comparison test).
inhibition has been shown to suppress corneal ulceration in animal models or to attenuate collagen degradation by corneal fibroblasts.\(^ {51-53}\) However, none of these agents has been shown to be effective for the treatment of corneal ulcer in human patients. Our results now indicate that EGCG inhibits collagen degradation by corneal fibroblasts, not by substantially attenuating pro-MMP1 production, but by suppressing the upregulation of uPA in these cells. This distinct mechanism of action for the inhibition of collagen degradation by EGCG may prove advantageous for the treatment of corneal ulcer.

In the clinical setting, not only resident keratocytes but also infiltrated inflammatory cells contribute to the excessive degradation of stromal collagen associated with corneal ulcer. The effects of EGCG on the functional interactions between keratocytes and inflammatory cells also warrant investigation. EGCG has previously been shown to have multiple effects on inflammatory signaling, corneal alkali-burn injury, and corneal neovascularization.\(^ {25-27}\) Our present results now implicate EGCG as a candidate therapeutic agent for the treatment of corneal ulcer. In summary, we have shown that EGCG inhibits IL-1\(\beta\)-induced, plasminogen-dependent collagen degradation by corneal fibroblasts. EGCG did not substantially attenuate pro-MMP1 production by these cells but rather suppressed the upregulation of uPA, the conversion of plasminogen to plasmin, and the activation of pro-MMP1 in response to stimulation with IL-1\(\beta\) (Fig. 7). This inhibitory effect on uPA expression was associated with attenuation of NF-\(\kappa\)B activation. Given that NF-\(\kappa\)B is also thought to upregulate expression of the pro-MMP1,\(^ {54,55}\) it remains unclear why EGCG inhibited expression of the uPA gene but not that of the pro-MMP1 gene. Regardless, our results suggest that EGCG warrants further investigation for its potential as a novel therapeutic agent for corneal ulcer.

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Inhibitory Effect of EGCG on Collagen Degradation


