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Collagenolytic Activity of Keratocytes Cultured in a Collagen Matrix

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Abstract: To study the mechanism of collagen degradation by keratocytes, we developed the new in vitro model in which keratocytes were cultured three-dimensionally in a collagen matrix. Subcultured rabbit keratocytes were embedded in a type I collagen matrix and cultured in serum-free medium. Collagenolytic activity of the cells was determined by measuring the amount of hydroxyproline released into the medium from degraded collagen. Activities of collagenase in the medium were also measured, using collagen labeled with fluorescein isothiocyanate as a substrate. The presence of plasminogen was required for collagen degradation by keratocytes. In the presence of plasminogen, the amount of collagen degradation depended on both the cultivation period and the number of cells. The addition of interleukin-1 (IL-1) stimulated the collagen degradation in a dose-dependent manner. This stimulatory effect of IL-1 was completely inhibited by the addition of IL-1 receptor antagonist (IL-1ra). Collagenase activity in the medium was stimulated by the addition of IL-1, and IL-1ra antagonized this stimulatory effect. These findings indicate that our present model may be useful for investigating the mechanism of collagen degradation by keratocytes. **Jpn J Ophthalmol 1998;42:79–84**

Key Words: Collagen, collagenase, interleukin-1, interleukin-1 receptor antagonist, keratocytes.

Introduction

Collagen metabolism (ie, the synthesis and degradation of collagen) is an essential process in the maintenance of the normal integrity and function of the cornea. Excess degradation of extracellular collagen often leads to a disastrous pathological condition, such as ulceration. Besides the inflammatory cells (leukocytes and macrophages), keratocytes are thought to contribute to collagen degradation in the ulcerating cornea.[1]

The mechanism of collagen degradation in the ulcerating cornea has been extensively investigated but is not yet fully understood. Matrix metalloproteases (MMPs) including collagenase are thought to play a central role in the breakdown of the extracellular matrices of the corneal stroma.[1] It has been reported that keratocytes synthesize MMPs both in vitro and in vivo.[2–9] Keratocytes have also been found to produce tissue inhibitors for metalloproteases.[10] The MMPs produced by the keratocytes were in latent form requiring some activation. Although in vitro activation of purified collagenase has been studied in detail, the physiologically relevant pathways of procollagenase activation have remained a subject for speculation. The plasminnogen/plasmin system has been proposed as one of the physiological activators of MMPs.[2,11,12] These findings suggest there is a complicated mechanism in collagen degradation by keratocytes in vivo.

Recently, many effects of growth factors and cytokines on inflammatory processes have been demonstrated. Interleukin-1 (IL-1), one of the inflammatory cytokines, was reported to enhance the breakdown of extracellular matrix by various types of cells,[5,13-16]

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and binding sites for IL-1 were detected on keratocytes from the normal cornea and keratoconus.[17] Furthermore, the injection of IL-1 into the corneal stroma induced corneal neovascularization.[18] These findings suggest that IL-1 plays a role in corneal inflammation.

The cellular functions of keratocytes used to be investigated using cells cultured on a plastic plate. Like other connective tissues, however, keratocytes in vivo are surrounded by extracellular matrix protein (collagen and proteoglycans).[1] Recent studies revealed the importance of extracellular matrices for the regulation of cellular functions.[19] We previously reported that extracellular collagen influenced various characteristics of keratocytes, such as cell shape and mitotic activity.[20] Gap junctions were observed between keratocytes cultured both in a collagen matrix and in vivo.[12] Furthermore, we found that production of procollagenase by keratocytes was stimulated by the presence of extracellular collagen.[22] In the present study, to understand the mechanism that regulates collagen degradation by keratocytes, we investigated the collagenolytic properties of keratocytes cultured three-dimensionally in a collagen matrix.

Materials and Methods

Albino male rabbits were purchased from Hokusetsu Sangyo (Settsu, Osaka). Care and treatment of animals adhered to the Guiding Principles in the Care and Use of Animals (DHEW publication, NIH 85-23). Eagle's minimum essential medium (MEM) was purchased from the Research Foundation for Microbial Diseases of Osaka University (Suita, Osaka). Dried MEM powder was purchased from Nissui Pharmaceutical (Tokyo). Porcine type I collagen solution (3 mg/mL in HCl, pH 3.0) was obtained from Nitta Gelatin (Yao, Osaka). Insulin-like growth factor-1 (IGF) and human epidermal growth factor (EGF) were purchased for Earth Pharmaceutical (Akoh, Hyogo); basic fibroblast growth factor (bFGF) was from Takara Shuzo (Kyoto); interleukin-6 was from Boehringer Mannheim (Mannheim, Germany); bovine plasminogen was from JCR Pharmaceutical (Ashiya, Hyogo, Japan); human recombinant IL-1α, IL-1β, and IL-1 receptor antagonist (IL-1ra) were supplied by Otsuka Pharmaceutical (Osaka); purified collagenase from human fibrosarcoma cells was supplied by Kanebo (Osaka).

Cell Culture in Collagen Gel

Rabbit keratocytes were cultured and passed in MEM containing 10% fetal calf serum as described previously.[20] In all experiments, we used cultured keratocytes within six passages. Cultivation of keratocytes in a collagen matrix was performed as previously described.[20] Type I collagen solution was mixed with a tenfold concentration of the MEM and neutralized by 0.2N NaOH. Suspensions of cultured keratocytes were added, and the collagen solution was plated in a 48-multiwell plate (300 μL/well) and incubated at 37°C for 1 hour to form a gel. The MEM (300 μL) was placed over the gel, and the cells were cultivated for another 24 hours. The number of cells and length of cultivation time were varied in certain experiments. In some experiments, human plasminogen (100 μg/mL) was added to the medium, and in the later experiments in this series, EGF, IL-1α, IL-1β, IGF, bFGF, or IL-6 was added (each at 10 ng/mL). The IL-1ra effects were tested at 1 μg/mL.

Collagen Degradation by Keratocytes

Collagenolytic activity of the keratocytes was estimated by measuring the amount of hydroxyproline (HYP) generated by acid hydrolysis from collagen fragments (molecular weight: < 100 kDa). After cultivation, the media were collected. Nondegraded collagen in the medium was removed by ultrafiltration using Ultrafree® (C3HK, cutoff molecular weight, 100 kDa, Japan Millipore, Tokyo. The samples were then hydrolyzed in 6N HCl at 110°C for 24 hours, and the amount of HYP in hydrolysates was measured according to the method described by Bergman and Loxely.[23] In the preliminary study, we confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis that undegraded collagen was completely removed from the medium by the ultrafiltration.

Collagenase Activity

In some experiments, collagenase activity in the culture medium was also measured. An aliquot of the collected medium was mixed with soybean trypsin inhibitor (final concentration = 450 μg/mL; from Sigma, St. Louis, MO, USA) and fluorescein isothiocyanate (FITC)-labeled collagen solution (from Collagen Research Laboratory, Tokyo). The samples were incubated at 35°C for 16 hours. Undegraded collagen was then precipitated by the addition of ethanol. After centrifugation at 30 000 $\times$ g for 15 minutes, the fluorescence intensity of FITC in the supernatant was measured fluorophotometrically. The FITC-labeled purified collagenase from human fibrosarcoma cells was used as a control. One unit of collagenase activity was defined as 1 μg of collagen degraded per minute at 35°C.

Statistics

Data are expressed as mean ± SEM of triplicated measurements. Statistical significance was determined by Student's *t*-test.

Results

First, we investigated the collagenolytic activity of keratocytes cultured in a collagen matrix. When keratocytes (10×10^3 cells) were cultured in collagen gel with unsupplemented medium, the amount of HYP in the culture medium increased slightly in proportion to the cultivation period (Figure 1). At 48 hours, the amount of HYP in the medium was about 6 μg/mL. When plasminogen (100 μg/mL) was added to the medium, however, the amount of HYP increased significantly over the amount in cultures without plasminogen, in proportion to the cultivation period. At 48 hours, the amount of HYP in the medium was about 60 μg/mL and had reached a plateau.

Different numbers of keratocytes were cultured in the presence or absence of plasminogen (100 μg/mL) for 24 hours (Figure 2). In the absence of plasminogen, the amount of HYP in the medium was about 10 μg/mL, regardless of the number of cells cultured. In the presence of plasminogen, the amount of HYP increased in proportion to the number of the cells. When more than 30×10^3 cells were embedded in a collagen matrix, the amount of HYP was significantly greater than that in the control cultures without cells ($P < 0.005$). These findings demonstrated that keratocytes cultured in a collagen matrix degrade extracellular collagen but require plasminogen for this activity.

To determine the effects of growth factors and cytokines on collagen degradation by keratocytes, we added EGF, IL-1α, IL-1β, IL-6, IGF, or FGF to medium containing plasminogen. As shown in Figure 3, after 24 hours of cultivation only IL-1α and IL-1β

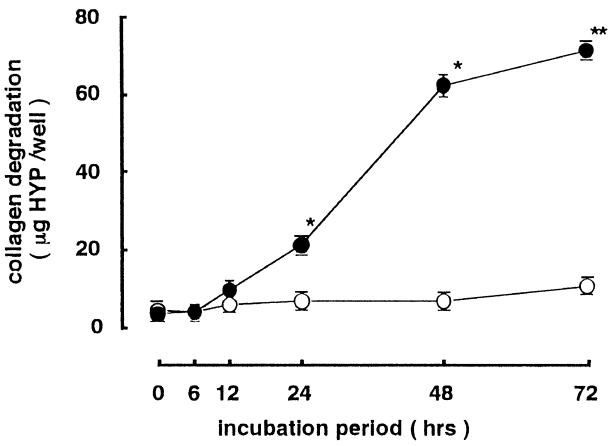

Figure 1. Effect of incubation period on collagen degradation by keratocytes. Keratocytes were cultured in collagen matrix for various periods either in the presence (closed circles) or absence (open circles) of plasminogen (100 μg/mL). Each point shows mean ± SEM in triplicated assay. $*P < 0.005$, $**P < 0.001$—compared with unsupplemented medium.

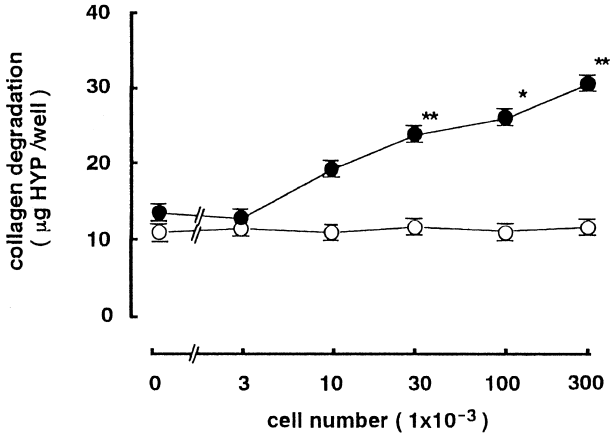

Figure 2. Effect of cell number on collagen degradation. Different numbers of keratocytes were cultured in collagen matrix for 24 hours either in the presence (closed circles) or absence (open circles) of plasminogen (100 μg/mL). Each point shows mean ± SEM in triplicated assay. $*P < 0.005$, $**P < 0.001$—different from cultures without cells.

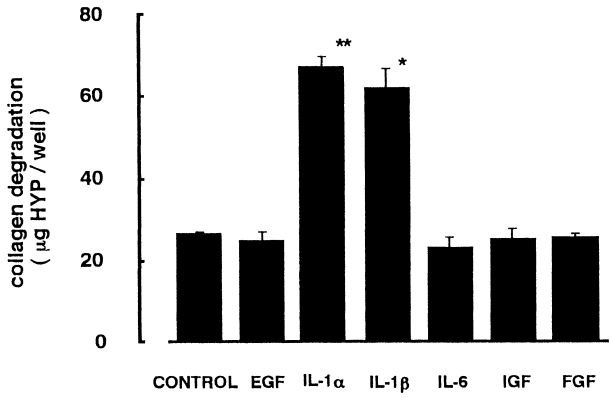

Figure 3. Effects of cytokines and growth factors on collagen degradation. In the presence of 100 μg/mL of plasminogen, keratocytes (10×10^3) were cultured for 24 hours in collagen matrix covered with Eagle's minimum essential medium (MEM; control) or with MEM plus epidermal growth factor (EGF; 10 ng/mL), IL-1α (10 ng/mL), IL-1β (10 ng/mL), insulin-like growth factor (IGF; 10 ng/mL), fibroblast growth factor (FGF; 10 ng/mL), or IL-6 (10 ng/mL). Each point indicates mean ± SEM in triplicated assay. $*P < 0.005$, $**P < 0.001$—compared with control.

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significantly increased the amount of HYP in the medium. The addition of other growth factors and IL-6 had no effect.

Next, keratocytes were cultured in a collagen matrix in the absence or presence of plasminogen and various concentrations of IL-1α for 24 hours (Figure 4). In the presence of plasminogen, IL-1α increased the amount of HYP in the medium in a dose-dependent manner. At an IL-1α concentration of 1 ng/mL or higher, the amount of HYP in the medium was significantly higher than that in the control culture without IL-1α ($P < 0.05$). In the absence of plasminogen, however, IL-1α did not affect the amount of HYP in the medium, indicating that IL-1α stimulated collagen degradation by keratocytes provided that plasminogen was present. The effect of IL-1β on collagen degradation by keratocytes was identical (data not shown).

To examine whether the effects of IL-1 on keratocytes were mediated through IL-1 receptors, we studied the effect of IL-1ra (Figure 5). The IL-1ra alone did not affect the amount of HYP in the medium at any concentrations of IL-1ra tested. In the presence of IL-1α (10 ng/mL), however, the amount of HYP decreased in proportion to the concentration of IL-1ra added. At IL-1ra concentrations of 1 μg/mL or higher, the amount of HYP in the medium in the presence of IL-1α was almost the same as that in the absence of IL-1α. These findings demonstrate that the stimulatory effect of IL-1 on collagen degradation by keratocytes in mediated by IL-1 receptors on the cell surface.

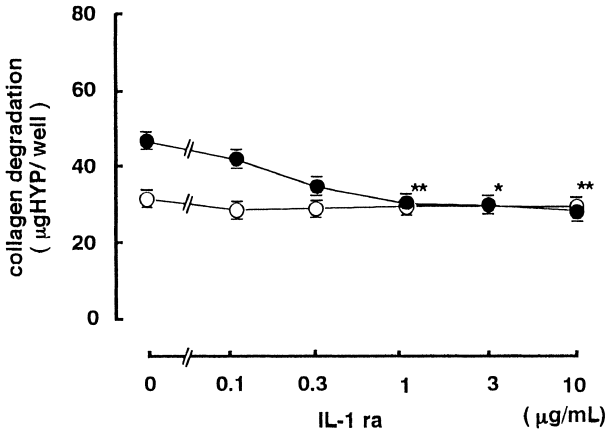

Figure 5. Effects of IL-1ra on collagen degradation. Keratocytes were cultured for 24 hours in collagen matrix in the presence of plasminogen (100 μg/mL) and various concentrations of IL-1ra with IL-1 (closed circles, 10 ng/mL) or without IL-1 (open circles). Each point shows mean ± SEM in triplicated assay. $*P < 0.01$, $**P < 0.005$—different from cultures with IL-1 and plasminogen.

No collagenase activity was detected in the medium in the absence of plasminogen (data not shown). In the presence of plasminogen alone, collagenase activity was about 0.05 U/mL (Figure 6). The addition of IL-1α at 10 ng/mL brought the enzyme activity in the medium to 0.69 U/mL, a level significantly higher than in control cultures ($P < 0.001$). On the other hand, the enzyme activity was significantly decreased by the addition of IL-1ra (1 μg/mL)

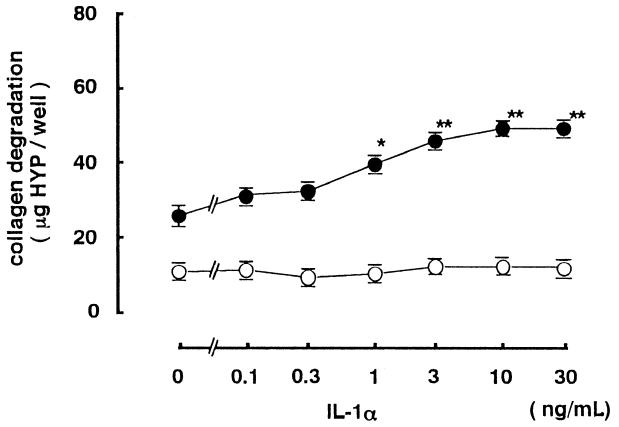

Figure 4. Effects of IL-1α on collagen degradation. Keratocytes (10×10^3) were cultured in collagen matrix with (closed circles) or without (open circles) plasminogen (100 μg/mL) for 24 hours. Each point shows mean ± SEM in triplicated assay. $*P < 0.05$, $**P < 0.005$—compared with medium without IL-1α.

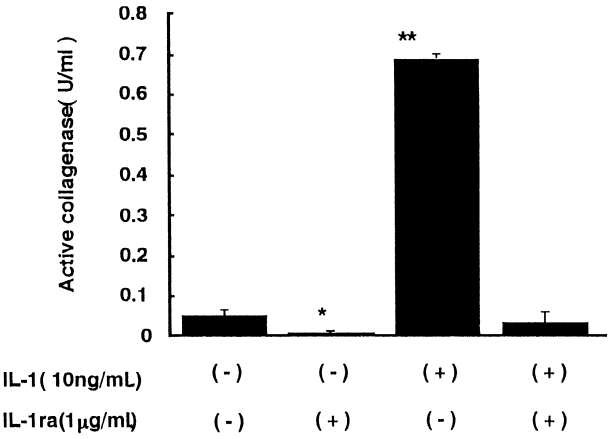

Figure 6. Effects of IL-1 and IL-1ra on collagenase activity. Keratocytes (10×10^3) were cultured in collagen matrix with plasminogen (100 μg/mL) for 24 hours. IL-1α (10 ng/mL), IL-1ra (1 μg/mL), or both were added to medium. Each point shows mean ± SEM in triplicated assay. $*P < 0.05$, $**P < 0.001$.

($P < 0.05$). Furthermore, in cultures with both IL-1 and IL-1ra, the enzyme activity was almost the same as that in control cultures. These findings show that IL-1 and IL-1ra modulate collagenase synthesis by keratocytes.

Discussion and Conclusions

The present study demonstrates that keratocytes, when cultured in a collagen matrix, degrade extracellular collagen. The IL-1 stimulated collagen degradation by keratocytes, and this stimulatory action of IL-1 was inhibited by IL-1ra. Furthermore, collagen degradation by keratocytes, with or without IL-1, required the presence of plasminogen. Collagenase activity was detected in the culture medium and depended on the presence of plasminogen.

Excessive collagen degradation in corneal stroma is one of the clinical features of corneal ulcer. Matrix metalloproteinases including collagenase (MMP-1) is thought to play a central role in the collagen degradation of corneal ulcer. The synthesis of MMPs by cells is thought to be regulated by various cytokines and growth factors. Among them, the roles of IL-1 in inflammation, which have been investigated extensively, include the stimulation of production of MMPs, prostaglandins, and the plasminogen activator by fibroblasts in the connective tissue.[5,14,24,25] It was recently reported that IL-1 stimulated IL-8 synthesis by keratocytes[26] and that IL-1 had an angiogenetic effect in the cornea.[18] Fini et al reported that IL-1 stimulated the production of MMPs by keratocytes.[3,5,14] We also reported that procollagenase synthesis by keratocytes in a collagen gel was stimulated by IL-1.[22] Our present results confirmed that IL-1 initiates inflammation and accelerates the breakdown of collagen by keratocytes.

We found that IL-1ra inhibited the stimulatory effect of IL-1 on both collagen degradation and collagenase synthesis by keratocytes. The IL-1ra, found in and purified from the monocyte-conditioned medium,[27] is the third member of the IL-1 gene family, and its sequence shows similarity to those of IL-1α and IL-1β.[28] The IL-1ra binds to IL-1 receptors of the cell surface without transducing a signal to the nucleus and is therefore thought to be a physiological inhibitor of IL-1. The IL-1ra has been reported to block IL-1-induced changes both in vitro and in vivo.[29] Therefore, IL-1ra may be effective for the reduction of collagen degradation in ulcerating cornea.

In the present study, collagenolytic activity of keratocytes and its stimulation by IL-1 were dependent on the presence of plasminogen. Matrix metalloproteinases including collagenase secreted from the cells are in an inactive form and require some activation mechanism. The plasminogen activator/plasmin system is reportedly one of the physiological activators of metalloproteases.[2,11,12] Plasmin activity has been detected in tears from inflamed eyes.[30,31] aprotinin, a serine protease inhibitor, reportedly demonstrates therapeutic efficacy against ulcerative corneal diseases.[31] Furthermore, keratocytes synthesize a urokinase-type plasminogen activator.[32,33] These findings indicate that the plasminogen in our model may act as the activator of collagenase. They also confirm the contribution of a plasminogen activator/plasmin system to corneal ulceration, which was demonstrated by Berman et al.[2,11,12] Our findings indicate that the present experimental model in which keratocytes were cultured in a collagen matrix with plasminogen is adequate to study the process of collagen degradation by keratocytes in vivo.

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