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1．研究テーマ
角膜潰瘍の病態解明と新しい治療法の開発

2．本年度の研究業績

(1) 学会・研究会等における発表 有 ・ (無) (学会名・演題)

(2) 学会誌等に発表した論文 (有) ・ 無 (雑誌名・論文名)

Qin Li, Ken Fukuda, Ying Lu, Yoshikuni Nakamura, Tai-ichiro Chikama, Naoki Kumagai, and Teruo Nishida. Enhancement by neutrophils of collagen degradation by corneal fibroblasts. *J LEUKOCYTE BIOL* (in press)

-日中医学協会助成事業-

角膜線維芽細胞のコラーゲン分解に対する triptolide の影響

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Abstract

Purpose: To investigate the role of triptolide on collagen degradation by corneal fibroblasts.

Methods: Three-dimensional gels of type I collagen containing rabbit corneal fibroblasts were incubated with medium containing interleukin 1β (IL-1β) in the presence of triptolide, and the extent of collagen degradation was assessed by measurement of released hydroxyproline. The release of matrix metalloproteinases (MMPs) from cultured corneal fibroblasts was measured by gelatin zymography and immunoblot analysis, and the abundance of MMPs mRNA in these cells was determined by reverse transcription combined with real-time polymerase chain reaction analysis.

Results: Triptolide inhibited IL-1β stimulated collagen degradation by rabbit corneal fibroblasts in a dose and time dependent fashion. The expression of MMP-1, 3 and 9 was inhibited by triptolide at both protein and mRNA level.

Conclusions: Triptolide inhibited collagen degradation by corneal fibroblasts through the suppression of MMPs expression by fibroblasts, suggesting that triptolide might be an effective medicine for the treatment of corneal ulceration in the future.

Key Word corneal fibroblasts, corneal ulceration, collagen degradation, triptolide, matrix metalloproteinases

Introduction

The cornea is an avascular and transparent tissue. In order to assure its transparency, collagen fibrils are more densely and neatly packed in the cornea than any other tissue in the body. Corneal fibroblasts, which are major resident cells in the corneal stroma, play an important role in collagen metabolism, because corneal fibroblasts both synthesize and

degrade collagen fibrils through the secretion of the matrix-degrading enzymes such as matrix metalloproteinases (MMPs). We also previously established the assay system to measure collagenolytic activity of corneal fibroblasts using this three dimensional collagen gel culture and reported interleukin 1 and elastase derived from *pseudomonas aeruginosa* stimulate collagen degradation by corneal fibroblasts with different mechanisms.[1,2]

Triptolide, a diterpene triepoxide, is an extract from the Chinese herb *Tripterygium wilfordii* hook f (TWHF) that has been used in traditional Chinese medicine for the treatment of rheumatoid arthritis.[3] Triptolide shows anti-inflammatory activity in immune cells such as T cells, B cells, and monocytes.[4,5] It also shows potent immunosuppressive property to prolong heart, kidney and bone marrow allograft survival in animal model.[6,7] In recent study, triptolide also reportedly acts on tissue resident cells such as epithelial cells, fibroblasts, chondrocytes, or synoviocyte. In addition, it inhibited the expression of MMPs in chondrocytes and synoviocyte.[8,9] Therefore, it is possible that triptolide has inhibitory effect on collagen degradation by corneal fibroblasts through the down regulation of MMPs in corneal fibroblasts.

The collagen degradation assay established by us, in which corneal fibroblasts are embedded in collagen gels, exerts total collagenolytic activity in vitro. It provides a model system with which to investigate the mechanism of corneal stromal ulceration. With this model system, we have now investigated the effects of triptolide on IL-1 induced collagen degradation, as well as the expressions of MMPs by corneal fibroblasts.

Methods

1 Cell culture; 2 Three-dimensional culture; 3 Measurement of collagenolytic activity; 4 Gelatin Zymography; 5 Immunoblot analysis; 6 Quantitative RT-PCR analysis of MMP-1, 2, 3 and 9 mRNA.

Results

Effect of triptolide on collagen degradation by rabbit corneal fibroblasts

Corneal fibroblasts (1×10^5) were cultured for 48 hours in collagen gels with various concentrations of triptolide (0.03 μM to 3.0 μM) in the absence or presence of IL-1β (0.1 ng/ml). In the absence of IL-1β, triptolide had no significant effect on

collagen degradation at any concentration examined. However, triptolide inhibited IL-1 induced collagen degradation in a dose dependent manner, being statistically significant at a concentration of 0.3 μM and 3 μM compared to those cultured in the absence of IL-1β (Fig. 1).

Effect of triptolide on expression of MMPs by corneal fibroblasts

We then investigated if triptolide down-regulates MMP expression by corneal fibroblasts by Western blotting and gelatin zymography. Immunoblot analysis with antibodies to rabbit MMP-1 of the culture supernatant revealed a small amount of 57-kDa immunoreactive bands corresponding to proMMP-1 and a typical doublet of 49- and 45-kDa immunoreactive bands corresponding to active MMP-1 (Fig. 2). Addition of 0.1 ng/ml IL-1β stimulated the density of bands corresponding to proMMP-1 and active MMP-1. Triptolide decreased the intensity of both proMMP-1 and active MMP-1 in dose dependent manner. Active MMP-1 at 45 kDa band could not be observed in those treated with 3 μM triptolide. Immunoblot analysis with antibodies to MMP-3 revealed that corneal fibroblasts produced a small mount of 57-kDa immunoreactive protein corresponding to proMMP-3 and 45-kDa immunoreactive protein corresponding to active MMP-3. [10] Addition of 0.1 ng/ml IL-1 stimulated the density of bands corresponding to proMMP-3 and active MMP-3. The addition of triptolide decreased the intensity of bands corresponding to both proMMP-3 and active MMP-3 in dose dependent manner.

Gelatin zymography of the culture supernatant revealed three major bands of 89, 65 and 57 kDa (Fig. 3), corresponding to intermediate MMP-9, proMMP-2 and active MMP-2, respectively. [11] When incubations were performed in the additional presence of IL-1β, proMMP-2 was not altered and active MMP-2 was significantly increased; the intermediate MMP-9 disappeared, gelatinolytic bands at 92 and 77 kDa, corresponding to proMMP-9 and active MMP-9 were observed. Triptolide did not affect the bands of proMMP-2 and active MMP-2; however the intensity of both proMMP-9 and active MMP-9 was slightly decreased in dose dependent manner. These observation demonstrated that IL-1β stimulated the amount of MMP-1, 2, 3, 9. Triptolide inhibited the overexpression of MMP-1, 3, 9 induced by IL-1.

Inhibitory effect of triptolide on mRNA expression of MMPs by corneal fibroblasts

We then investigated the effect of triptolide on the abundance of MMP mRNAs in corneal fibroblasts by reverse transcription combined with real-time polymerase chain reaction analysis. When IL-1β was added, the abundance of MMP-1, 2, 3 and 9 mRNA was increased. The addition of 3 μM triptolide decreased the abundance of IL-1 induced MMPs mRNA (Fig. 4).

Discussion

Our study clearly demonstrated that triptolide inhibited IL-1-induced collagen degradation by corneal fibroblasts in vitro. Triptolide is not a MMP inhibitor to bind the active sites of MMPs. It did not inhibit the activation of proMMPs to active MMPs. It inhibited the synthesis and secretion of MMPs at both protein and mRNA level.

In the current study, with our model by rabbit corneal fibroblasts in three dimensional culture system, we showed that triptolide inhibited IL-1 induced collagen degradation by corneal fibroblasts. Our work has characterized MMPs as novel targets of its inhibitive effect on collagen degradation. Synthesis of MMPs is the primary level of regulation determining collagenolytic activity in cornea. Cultured corneal fibroblasts reportedly produce MMP-1, -2, -3 and –9. [12] MMP-1 is the main collagenolytic enzyme to degrade collagen type I. MMP-3 although could not degrade collagen type I, it participates the activations of other MMPs. [13] [14] [15] The two gelatinases, MMP-2 and MMP-9 have the capacity to further degrade type I collagens after MMP-1 cleavage and subsequent denaturation of the three collagen chains. [16] Triptolide inhibited the synthesis and secretion of proMMPs by corneal fibroblasts at both protein and mRNA level. The activation of proMMPs to active MMPs was not altered by triptolide.

Corneal ulceration, the disintegration of corneal stroma resulting from excessive degradation of collagens, is a devastating disorder that can cause blindness. Here we show for the first time that triptolide can effectively suppress IL-1-induced collagen degradation by corneal fibroblasts in vitro. The inhibition of MMPs at both mRNA and protein in a similar fashion suggests that MMPs are targets of triptolide, and their observed inhibition may be one of the mechanisms for its possible effects in the patients with corneal ulceration. Therefore, besides its known immunosuppressive and

anti-inflammatory activity, triptolide may also be an effective anti-ulcer agent for corneal ulceration that deserves additional research.

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Fig 1

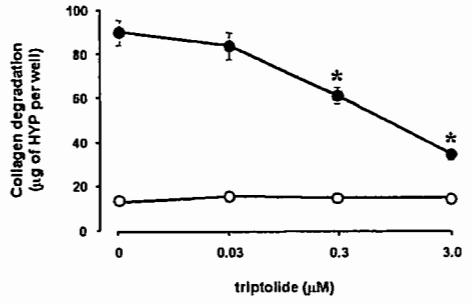

Fig 2

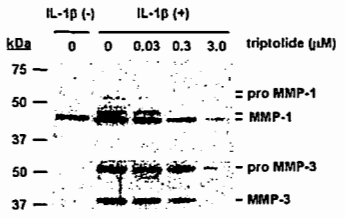

Fig 3

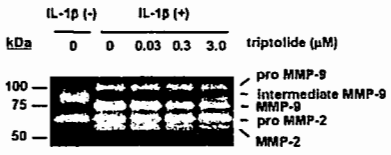

Fig 4