Lysophosphatidic Acid Augments the Gene Expression and Production of Matrix Metalloproteinases-1 and -3 in Human Synovial Fibroblasts in Vitro

Koji Mizuno,* Michika Komiya, Katsuki Okuyama, Keisuke Imada, and Takashi Sato*

Department of Biochemistry, School of Pharmacy, Tokyo University of Pharmacy and Life Sciences; 1432–1 Horinouchi, Hachioji, Tokyo 192–0392, Japan.

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Rheumatoid arthritis (RA) is an inflammatory disease with joint dysfunction following cartilage degradation. The level of lysophosphatidic acid (LPA) has been reported to be augmented in human synovial fluid from patients with RA. However, it remains to be elucidated whether LPA participates in cartilage destruction. In the present study, we have demonstrated that the production of promatrix metalloproteinases (proMMPs)-1 and -3 was augmented along with an increase of extracellular signal-regulated kinase (ERK)1/2 phosphorylation through LPA receptor 1 (LPAR1) in human synovial fibroblasts. These results suggest that LPA transcriptionally increases MMP production by the activation of an LPAR1/ERK1/2 signal pathway in human synovial fibroblasts. Thus, LPA is likely to be a pathological candidate for cartilage degradation in RA.

Key words rheumatoid arthritis; cartilage destruction; lysophosphatidic acid; matrix metalloproteinase; extracellular signal-regulated kinase (ERK)1/2; human synovial fibroblast

INTRODUCTION

Rheumatoid arthritis (RA) is a joint disease characterized by synovial inflammation leading to protease-dependent cartilage destruction. Synovial fibroblasts, which are a dominant cell type of arthritic synovium, have been reported to be activated in response to inflammatory cytokines, and then invade into cartilage tissue to form pannus. Thus, synovial fibroblasts are likely to participate in the initiation and progression of injured joint destruction. On the other hand, matrix metalloproteinases (MMPs) have been reported to play important roles in joint destruction in RA. Since different sets of MMPs have been expressed in synovial fibroblasts in RA, both MMPs-1 and -3 have been involved in the aggravation of joint destruction due to the degradation of type I collagen and non-collagenous components.

A bioactive lipid, lysophosphatidic acid (LPA), is produced by the autotaxin-catalyzed hydrolysis of lysophosphatidylcholine. Since six types of LPA receptors (LPAR1–6) have been identified, LPA exhibits biological activities through different LPAR in various tissues and cell species. Recent studies have demonstrated the involvement of LPA in the pathogenesis of RA. However, it remains unclear how LPA modulates the expression of MMPs in synovial fibroblasts. The aim of this study is to clarify the regulatory mechanisms of LPA-induced MMP expression in normal human synovial fibroblasts.

MATERIALS AND METHODS

Cell Culture and Treatment Normal human synovial fibroblasts (NHSFs) (5.0 × 10^5 cells/well) (Cell Systems Bio-technologie Vertrieb GmbH, St. Katharinen, Germany) in 12-well culture plates (AGC Techno Glass, Shizuoka, Japan) were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (Sigma-Aldrich Japan, Tokyo, Japan) as previously reported. After the confluence, the cells were washed three times with a phosphate buffered saline, and then treated for 10 min (for extracellular signal-regulated kinase (ERK)1/2 phosphorylation) or 24 h (for real-time PCR and Western blot analysis of MMPs and LPAR1–6) with or without LPA (1-oleoyl lysophosphatidic acid sodium salt: Cayman, Ann Arbor, MI, U.S.A.) (1–100 µM) under a serum-free condition. In addition, an LPAR1/LPAR3 inhibitor, Ki16425 (Cayman) and an ERK inhibitor, U0126 (LC Laboratories, Woburn, MA, U.S.A.) were administrated at 30 min prior to the LPA treatment.

Real Time PCR Total RNA was isolated from cells using ISOGEN II (Nippon Gene, Toyama, Japan), and then the aliquot of RNA (500 ng) was subjected to reverse transcriptase reaction for the synthesis of cDNA using a PrimeScript RT reagent (TaKaRa Bio, Shiga, Japan) according to the manufacturer’s instructions. Aliquots of an equivalent of 2.5 ng of total RNA of the transcript were subjected to real-time PCR for MMPs-1 and -3, LPAR1–6, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs using SYBR Premix Ex Taq II (TaKaRa Bio) and their specific primers (Table S1).

Western Blot Analysis As most MMPs are extracellularly secreted as proform (proMMP), the harvested culture medium was analyzed for MMP production as described previously. The medium (1 mL) and aliquots (50 µg protein) of cell lysate were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 12.5% (w/v) acrylamide/Tris–glycine gel for proMMPs-1 and -3 and unphosphorylated ERK1/2 (ERK) and phosphorylated ERK1/2 (Thr202/Tyr204) (p-ERK), respectively. The separated protein was electrotransferred onto polyvinylidene fluoride membranes. The membranes were reacted with goat anti-proMMP-1 (Sigma-Aldrich, St. Louis, MO, U.S.A.) (1:2000

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dilution), sheep anti-proMMP-3 antibodies (graciously provided by Prof. H. Nagase, the Kennedy Institute of Rheumatology, University of Oxford, Oxford, U.K.) (1:1000 dilution), and rabbit antibodies against p-ERK (1:1000 dilution) and ERK (1:1000 dilution) (Cell Signaling Technology, Beverly, MA, U.S.A.), which were then complexed with horseradish peroxidase-conjugated rabbit anti-goat immunoglobulin G (IgG), donkey anti-sheep IgG, or goat anti-rabbit IgG. Next, the immunoreacted proMMP-1, proMMP-3, unphosphorylated ERK, and p-ERK were visualized with enhanced chemiluminescence-Western-blotting detection reagents (GE Healthcare Bio-Sciences, Tokyo, Japan) according to the manufacturer's instructions. The relative amounts of the proteins were quantified by densitometric scanning using Image J (ver. 1.43u, National Institutes of Health, Bethesda, MD, U.S.A.).

**Statistical Analysis** Statistical analysis of the data was performed using one-way ANOVA, followed by Fisher’s multiple comparisons to evaluate differences between the means. p-Values <0.05 were considered statistically significant.

**RESULTS**

Transcriptional Augmentation of proMMP-1 and -3 Production by LPA in Human Synovial Fibroblasts We first examined the effect of LPA on the mRNA expression of MMP-1 and MMP-3 in NHSFs. As shown in Figs. 1A and B, both mRNA expressions were augmented by LPA in a dose-dependent manner. In addition, Figs. 1C and D showed that LPA dose-dependently increased the production of proMMPs-1 and -3. Furthermore, a similar observation was detectable in NHSFs which were maintained for 1 h under serum-free conditions prior to LPA stimulation (Fig. S1), indicating that LPA transcriptionally facilitated the production of proMMPs-1 and -3. Moreover, the LPA-augmented production of proMMPs-1 and -3 was detectable for at least 72 h (Fig. S2). Given the results that LPA (30 µM) significantly increased the production and gene expression of proMMPs-1 and -3 (Fig. 1), we have performed further experiments using LPA at 30 µM.

Involvement of LPAR₁/LPAR₃ in the Regulation of proMMP-1 and -3 Production by LPA in NHSFs Next, we examined which types of LPAR were associated with the LPA-augmented production of proMMPs-1 and -3 in NHSFs. As shown in Fig. 2A, LPAR₁ mRNA was found to be predominantly detectable, whereas LPAR₂ and LPAR₅ mRNA were slightly expressed in NHSFs. In addition, the LPA-augmented proMMP-1 production and gene expression in NHSFs were suppressed by an LPAR₁/LPAR₃ inhibitor, Ki16425, in

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(A) MMP-1

(B) MMP-3

(C) proMMP-1

(D) proMMP-3

Fig. 1. Transcriptional Augmentation of proMMP-1 and -3 Production by LPA in NHSFs

A, B: Quantitative real-time PCR for MMPs-1 and -3. C, D: Western blot analysis for proMMPs-1 and -3. Data are represented as means ± standard error of the mean (S.E.M.) for three independent experiments. *, **, and ***, significantly different from untreated cells (p<0.05, 0.01, and 0.001, respectively).
a dose-dependent manner (Figs. 2B, D). Similar results were observed in the production and mRNA expression of MMP-3 in the LPA- and/or Ki16425-treated NHSFs (Figs. 2C, E), indicating the involvement of LPAR1/LPAR3 signaling in the LPA-mediated transcriptional augmentation of MMP production in NHSFs.

**LPA-Augmented proMMP-1 and -3 Production Results from the Activation of the LPAR1/LPAR3-ERK1/2 Pathway in NHSFs** A previous report has shown that LPAR1 activation leads to ERK phosphorylation, which in turn facilitates gene transcription, cell cycle progression, and proliferation in various cell species. Therefore, we hypothesize that LPA might promote proMMP production through the activation of an LPAR1/LPAR3-ERK signal pathway. In this regard, we examined whether LPA increased the phosphorylation of ERK1/2 via LPAR1/LPAR3 in NHSFs. As shown in Fig. 3A, LPA was found to augment ERK1/2 phosphorylation (5.9 fold vs. untreated cells). In addition, the level of phosphorylated ERK was decreased by adding Ki16425 (55% inhibition against the LPA treatment), indicating that the ERK signal pathway existed at the downstream of LPAR1/LPAR3 in NHSFs. In addition, an ERK inhibitor, U0126, was found to decrease the LPA-augmented proMMP-1 and -3 production and mRNA expression (Figs. 3B–E). Thus, these results suggest that LPA activated the ERK1/2 signal pathway through LPAR1/LPAR3, which in turn transcriptionally accelerated the production of proMMPs-1 and -3 in NHSFs.

**DISCUSSION**

Matrix metalloproteinases have been reported to play important roles in cartilage degradation in RA. In addition, it has been reported that the expression of MMPs in RA is regulated by various inflammatory factors such as cytokines and chemokines. Furthermore, LPA has been reported to be associated with the pathogenesis of RA due to its increase in
synovial fluids and the aggravation of inflammation. In this study, we demonstrated that LPA transcriptionally augmented the production of proMMPs-1 and -3 in NHSFs, suggesting that LPA is a pathological candidate for cartilage destruction in RA. Moreover, our finding that the proMMP inducible activity of LPA was maintained for at least 72h allows us to speculate that LPA stimulation is persistent rather than transient in NHSFs.

Lin et al. have reported that LPA increases the expression of interleukin 1β and interleukin 8 via LPAR1 and/or LPAR3 in human umbilical endothelial cells. In addition, Miyabe et al. have reported that LPA induces the production of inflammatory cytokines and cell migration via LPAR1 in human synovial fibroblasts. In the present study, we demonstrated that NHSFs predominantly express LPAR1, and while the expression of LPAR2 and LPAR5 was little, that of LPAR2, LPAR4, and LPAR5 was barely detectable. Furthermore, we found that the LPA-augmented proMMP-1 and -3 production was suppressed by a LPAR2/LPAR5 inhibitor, Ki16425, in NHSFs. Therefore, these LPA-mediated inflammatory reactions in RA are likely to be caused by the activation of an LPAR signal pathway in synovial fibroblasts.

It has been reported that LPAR1 activation leads to ERK phosphorylation, which in turn facilitates gene transcription, cell cycle progression, and proliferation in various cell species. Since mitogen-activated protein kinases such as c-Jun N-terminal kinase (JNK), ERK1/2, and p38 have been reported to play important roles in the regulation of MMP expression in a cell-type specific manner, it remains unclear whether ERK signaling might exist at the downstream of LPAR in synovial fibroblasts from RA patients. In this study, we demonstrated that LPA increases the phosphorylation of ERK1/2 in NHSFs. In addition, the LPA-augmented proMMP-1 and -3 production was found to be suppressed by U0126. Thus, these results at least partially suggest a pathological mechanism of joint destruction in RA, in which LPA...
facilitates MMP expression by activating an ERK1/2 signal pathway through LPAR1 in synovium.

In conclusion, we have demonstrated for the first time that LPA transcriptionally increases MMP production by the activation of an LPAR1/ERK1/2 signal pathway in human synovial fibroblasts. Thus, we have provided novel evidence that LPA is a pathological candidate for cartilage degradation in RA. Moreover, the inhibition of LPAR signaling may become a therapeutic strategy to prevent joint destruction in RA.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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