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Original article

The growth factor midkine may play a pathophysiological role in rheumatoid arthritis

Emiko Shindo[1], Toshihiro Nanki[1], Natsuko Kusunoki[1], Kotaro Shikano[1], Mai Kawazoe[1], Hiroshi Sato[1], Kaichi Kaneko[1], Sei Muraoka[1], Makoto Kaburaki[1], Yoshikiyo Akasaka[2], Hideaki Shimada[3], Tomoko Hasunuma[1], and Shinichi Kawai[1]

[1]Division of Rheumatology, Department of Internal Medicine, Toho University School of Medicine, 6-11-1, Omori-nishi, Ota-ku, Tokyo, 143-8541 Japan.

[2]Division of Chronic Inflammatory Diseases, Advanced Medical Research Center, Toho University Graduate School of Medicine, 6-11-1, Omori-nishi, Ota-ku, Tokyo, 143-8541 Japan.

[3]Division of General and Gastroenterological Surgery, Department of Surgery, Toho University School of Medicine, 6-11-1, Omori-nishi, Ota-ku, Tokyo, 143-8541 Japan.

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Correspondence to:

Toshihiro Nanki, MD, PhD

Division of Rheumatology, Department of Internal Medicine,

Toho University School of Medicine.

6-11-1, Omori-nishi, Ota-ku, Tokyo, 143-8541 Japan.

Tel: +81-3-3762-4151, FAX: +81-3-3298-0045

E-mail: toshihiro.nanki@med.toho-u.ac.jp

Abstract

Objectives. Midkine (MK) is involved in cell proliferation, differentiation, migration, and survival. In this study, we measured serum MK levels in rheumatoid arthritis (RA) and investigated the correlation of serum MK with RA disease activity. Expression and effect of MK in RA synovial tissue were also examined.

Methods. Serum MK and production of inflammatory mediators by rheumatoid synovial fibroblasts (RSFs) were measured by enzyme-linked immunosorbent assay. MK expression in synovial tissue was examined by immunohistochemistry. MK receptor expression was analyzed by RT-PCR and Western blotting.

Results. RA patients had a significantly higher serum MK level than healthy controls. In RA patients, the MK level was correlated with DAS28-ESR, disability index of the Health Assessment Questionnaire, and rheumatoid factor level. The serum MK level tended to be decreased by anti-TNF therapy. MK was expressed by synovial lining cells in RA synovial tissues and it enhanced the production of IL-6, IL-8 and CCL2 by RSFs. RSFs expressed LDL receptor-related protein 1, candidate receptor for MK.

Conclusions. The serum MK level could be a marker of disease activity in RA and an indicator of a poor prognosis. MK may have a role in the pathogenesis of RA via induction of inflammatory mediators.

Introduction

Midkine (MK) is a heparin-binding growth factor that was originally identified as the retinoic acid-response gene product [1-3]. It has been reported that MK has a role in cell proliferation, differentiation, migration, and survival [4-6]. In addition, the pathological role of MK in malignant tumors has been widely analyzed [7-9]. Several types of cancer cells express MK [1, 10], and cancer patients with high serum levels of MK are reported to have a poor prognosis [11]. Furthermore, MK enhances tumor cell growth and invasion [12]. Moreover, MK might modulate inflammatory responses [13, 14], since it has been reported to induce the migration of inflammatory cells [15-17] and suppresses the expansion of regulatory T cell by blocking the development of tolerogenic dendritic cells [18]. Thus, MK might have a role in inflammatory diseases as well as cancer.

Rheumatoid arthritis (RA) is characterized by chronic inflammation of multiple joints. The synovial tissue of RA patients features proliferation of synoviocytes, accumulation of inflammatory cells (including lymphocytes and macrophages), production of inflammatory mediators, and angiogenesis [19]. Several biologic agents are effective for RA, including monoclonal antibodies against tumor necrosis factor-α (TNF-α) and interleukin (IL)-6 receptor. However, the risk of severe infection is increased by these biologic agents [20, 21] and some patients do not respond to them. Therefore, novel therapies that are more effective for RA and safer than conventional treatment are still needed.

It has been reported that MK is expressed in RA synovial tissue [15, 22] and that MK levels are elevated in the serum [22] and synovial fluid [15] of RA patients. These reports suggest that MK may play a role in the pathogenesis of RA. In this study, we analyzed the relation between the serum MK level and disease activity in RA, and we also investigated the stimulatory effect of MK on cultured synovial fibroblasts obtained from RA patients.

Methods

Samples

Peripheral blood samples were obtained from 146 RA patients and 85 healthy subjects. All of the RA patients fulfilled the classification criteria of the American College of Rheumatology/European League Against Rheumatism [23]. Among the 146 RA patients, 121 (82.8%) were women. The mean age ± standard deviation (SD) was 60.9 ± 14.0 years, the disease duration was 125.7 ± 115.2 months, and the C-reactive protein (CRP) level was 0.57 ± 0.13 mg/dl. In addition, the disease activity score (DAS)28-erythrocyte sedimentation rate (ESR) [24] was 2.76 ± 1.46 and the disability index of the Health Assessment Questionnaire (HAQ) [25] was 0.58 ± 0.87. Of the 146 RA patients, 118 (80.8%) were positive for rheumatoid factor (RF) and 113 (77.4%) were positive for anti-cyclic citrullinated protein (CCP) antibodies. Sixty-eight patients were taking prednisolone and 101 patients were using methotrexate. Other medications were salazosulfapyridine in 2 patients, tacrolimus in 6, cyclosporin in 1, infliximab in 11, etanercept in 28, adalimumab in 7, abatacept in 3, golimumab in 3, and tocilizumab in 13. Blood samples from microscopic polyangiitis (n=3), adult-onset Still's disease (n=2), systemic lupus erythematosus (n=3), and polymyositis (n=2) were also obtained. Among the healthy subjects, 76 (89.4%) were women and their age was 63.2 ± 12.1 years. The ages and gender ratio were not significantly different between RA patients and healthy controls. Blood samples from healthy controls were obtained at Kitasato University Kitasato Institute Hospital. The subjects were healthy on the basis of their medical histories. They did not have a present or past history of organopathy, such as heart, lung, liver or kidney disease. They were normal based on physical examination and clinical laboratory tests. Synovial tissue samples were obtained from 3 patients with RA and 3 patients with osteoarthritis (OA) who underwent total knee or hip replacement. The experimental protocol was approved in advance by the Ethics Committees of Toho Medical Center Omori Hospital

(approval number: 24-207, 26-22), Toho University School of Medicine (2703024007), and Kitasato University Kitasato Institute Hospital (13028).

Measurement of MK

Serum was obtained from peripheral blood samples of the RA, polyangiitis, adult-onset Still's disease, systemic lupus erythematosus, polymyositis and healthy controls and was frozen at -80°C until analysis. Concentrations of MK and RF were determined with enzyme-linked immunosorbent assay (ELISA) kits (Cellmid, Sydney, Australia), according to the manufacturer's instruction. To examine whether RF interfered with the MK assay, serum samples from 3 RA patients were incubated with Immunoglobulin Inhibiting Reagent® (Bioreclamation IVT, Baltimore, MD, USA) for 18 h at 4°C before determination of the MK level.

Sharp score

To evaluate radiographic changes of the hands, the Sharp score was calculated [26]. Erosions were evaluated at 16 sites of each hand and wrist and joint space narrowing was assessed at 15 sites on each side. The score for erosions at each site ranged from 0 to 5, giving a total score between 0 and 160, while the score for joint space narrowing at each site ranged from 0 to 4, give a total score between 0 and 120. The score was independently evaluated by ES and KS, and the mean score was calculated.

Immunohistochemistry

Synovial tissues were fixed with freshly prepared 4% (v/v) paraformaldehyde in Tris buffered saline (TBS). Sections (3 μm) were immersed in ethanol containing 3% (v/v) H_2O_2 for 5 min to block endogenous peroxidase activity. Then the sections were incubated with EzBlock

Chemi® (Atto, Tokyo, Japan) for 10 min to block nonspecific binding. Sections were subsequently incubated for 2 h with polyclonal goat anti-human MK antibody (2 μg/ml; R&D Systems, Minneapolis, MN, USA) or normal goat serum as a control. Next, the sections were washed four times with TBS and expression was detected using a Vectastain® Elite ABC kit (Vector Laboratories, Burlingame, CA, USA). Finally, the sections were washed in TBS and counterstained with hematoxylin.

Cell culture

Rheumatoid synovial fibroblasts (RSFs) were prepared from synovial tissue samples as described previously [27]. In brief, synovial tissue was digested for 2 h with 0.2% (w/v) bacterial collagenase (Wako Pure Chemical Industries, Osaka, Japan) and then suspended in Roswell Park Memorial Institute (RPMI) 1640 medium with 10% (v/v) fetal bovine serum (FBS), 100 units/ml penicillin, and 100 g/ml streptomycin. The cells were incubated for several days at 37°C under 5% CO_2, after which nonadherent cells were removed. Fibroblast-like adherent cells from the third or fourth passages were used as RSFs. These cells were cultured overnight in 96-well plates (2×10^4 cells/well), and then were incubated with MK (0, 5, or 50 ng/ml; Wako Pure Chemical Industries) for 48 h at 37°C in RPMI 1640 medium containing 10% FBS. Levels of IL-6, IL-8 and chemokine (C-C motif) ligand 2 (CCL2) and chemokine (C-X3-C motif) ligand 1 (CX3CL1) in the culture supernatant were assessed by using ELISA kits (Life Technologies, Waltham, MA, USA, for IL-6 and CCL2; R&D Systems for IL-8 and CX3CL1), according to the manufacturer's instructions. To measure prostaglandin E_2 (PGE_2) level, RSFs were plated in 96-well plates (2×10^4 cells/well) and cultured for 48 h at 37□C with MK (0, 5, or 50 ng/ml) in medium containing 1% FBS. Three μM arachidonic acid (Cayman Chemical, Ann Arbor, MI, USA) was added to each well for last 30 min. Then, concentration of PGE_2 in the medium was measured by an

ELISA kit according to the recommendations of the manufacturer (Cayman Chemical).

Reverse transcription-polymerase chain reaction (RT-PCR)

RSFs were seeded in culture medium containing 10% FBS, and total RNA was extracted with an RNeasy Mini kit according to the recommendations of the manufacturer (QIAGEN GmbH, Hilden, Germany). Reverse transcription was performed with a SuperScript first-strand synthesis system for RT-PCR according to the recommendations of the manufacturer (Invitrogen, Carlsbad, CA, USA), with 1 μg of total RNA from the cells as a template. Equal amounts of each reverse-transcribed product were amplified by PCR with HotStar *Taq* polymerase (QIAGEN GmbH). The primer sequences and numbers of basepairs were as follows: for anaplastic lymphoma kinase (ALK) (814 bp), sense 5'-AGA TCC TCC TGA TGC CCA CT and antisense 5'-AGG ATC TTG TCC TCT CCG CT; for protein tyrosine phosphatase, receptor type Z1 (PTPRZ1) (196 bp), sense 5'-TGC TTC CAG ACT GCA CAC TT and antisense 5'-CCT GGT AAA ACT CTT TCA GTG TCT C; for Notch2 (817 bp), sense 5'-TGG CAC ATG TGT TGA TGG GA and antisense 5'-GTT GGA GAG GCA CTC GTT GA; for LDL receptor-related protein 1 (LRP1) (419 bp), sense 5'-CTG GCG AAC AAA CAC ACT GG and antisense 5'-GCA CGT CAG ACC AGT ACA CA; and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (598 bp), sense 5'-CCA CCC ATG GCA AAT TCC ATG GCA and antisense 5'-TCT AGA CGG CAG GTC AGG TCC ACC. After initial denaturation at 95°C for 15 min, PCR involved amplification for 28 cycles of 30 sec at 94°C, 30 sec at 56°C, and 30 sec at 72°C, followed by elongation for 5 min at 72°C. The amplified complementary DNA fragments were resolved by electrophoresis on a 2% agarose gel, and were detected under ultraviolet light using LAS-3000 (Fujifilm, Tokyo, Japan) after staining the gel with ethidium bromide.

Western blot analysis

RSFs were lysed in M-PER® mammalian protein extraction reagent (Thermo Fisher Scientific, Waltham, MA, USA) with Halt™ protease inhibitor cocktail (Thermo Fisher Scientific) according to the recommendations of the manufacturer. The protein content of the lysates was determined with Qubit® 3.0 Fluorometer using Qubit® protein assay (Thermo Fisher Scientific). Then cell lysates were adjusted to 30 μg of protein and were applied to Bolt® Bis-Tris Plus gel (4–12%) (Thermo Fisher Scientific) for eleectrophoresis in MOPS buffer. Next, the proteins were electroblotted onto Immobilon-P polyvinylidene difluoride membranes with iBlot® 2 Dry Blotting System (Thermo Fisher Scientific). The membranes were incubated with primary antibodies, monoclonal rabbit anti-human Notch2 antibody (Cell Signaling Technology, Danvers, MA, USA), monoclonal rabbit anti-human LRP1 antibody (Abcam plc, Cambridge, UK), or monoclonal mouse anti-human GAPDH antibody (Santa Cruz Biotechnology, Dallas, TX, USA), with a dilution of 1:200 (Notch2), 1:3000 (LRP1) and 1:100 (GAPDH), and the secondary antibody (HRP-conjugated goat anti-rabbit antibody or HRP-conjugated goat anti-mouse antibody) was added (at a dilution of 1:2000) and incubation was performed for 2.5 h using iBind Flex Western System (Thermo Fisher Scientific). Protein bands were detected with enhanced Novex® ECL Chemiluminescent Substrate Reagent Kit (Invitrogen) using LAS-3000 (Fujifilm).

Statistical analysis

Results are expressed as the mean ± SD and/or median with interquartile range. Statistical analysis was performed with Prism software (ver. 6; GraphPad Software, La Jolla, CA, USA). The significance of between-group differences of the serum MK concentration was determined by the Mann-Whitney U-test. Simple linear regression analysis was used to assess correlations between the serum MK level and the DAS28-ESR, HAQ score, Sharp score, or

RF level. To compare IL-6, IL-8, CCL2 and PGE_2 production by RSFs, analysis of variance was applied with Bonferroni's correction. In all analyses, $p<0.05$ was considered to indicate statistical significance.

Results

Serum MK level

As shown in Figure 1, the serum level of MK was significantly higher in the RA patients than in the healthy controls [RA: 787.7 ± 68.8 (mean ± SD), 540.8 (391.7-781.1) median (interquartile range); healthy controls: 373.6 ± 15.1, 334.4 (284.8-426.1) pg/ml, p<0.0001]. We also measured serum MK levels of patients with microscopic polyangiitis, adult-onset Still's disease, systemic lupus erythematosus, and polymyositis. Although the MK concentrations were vary in the patients; microscopic polyangiitis (n=3; 240.5, 458.1, 378.3 pg/ml), adult-onset Still's disease (n=2; 76.7, 232.5), systemic lupus erythematosus (n=3; 146.1, 339.8, 686.6), and polymyositis (n=2; 47.0, 31.0), they were not significantly increased compared to healthy controls. To determine whether the serum MK level could be a useful biomarker of RA disease activity, we examined the correlation between serum MK and DAS28-ESR. In the RA patients, we found that the serum MK level was significantly correlated with DAS28-ESR (r=0.223, p=0.0194) (Figure 2A). Since HAQ is widely used for measuring functional disability in daily life, the correlation between the HAQ score and serum MK was examined, and a significant correlation of serum MK with the HAQ score was identified (r=0.329, p=0.0143) (Figure 2B). The Sharp score for radiographic bone destruction of the finger and wrist joints was also analyzed for a correlation with serum MK. There was little correlation between serum MK level and Sharp score; the p value was less than 0.05, but correlation coefficient was less than 0.1 (r=0.097, p=0.0013) (Figure 2C). Furthermore, serum MK was strongly correlated with the RF titer (r=0.615, p<0.00001) (Figure 2D).

Since RF often interferes with the assay other serum proteins, we evaluated the influence of RF on the ELISA for MK. First RF in serum was blocked with Immunoglobulin Inhibiting Reagent® and then we measured MK using the ELISA. After blocking RF, the

measured concentration of MK was not altered, while the concentration of RF was significantly decreased (data not shown), suggesting that RF did not affect the assay for MK. The anti-CCP antibody titer was not correlated with the serum MK level (data not shown).

Four of the RA patients started treatment with infliximab, an anti-TNF-α monoclonal antibody, and the concentration of MK was measured several times. The clinical courses, including serum MK levels, are shown in Figure 3. In all the patients, CRP and ESR were lowered after infliximab treatment, and dosage of prednisolone could be decreased. Serum MK level tended to be lowered although it was sometimes fluctuated.

MK expression in RA synovium

Expression of MK in the synovial tissues harvested from RA and OA patients was examined by immunohistochemistry. There was strong expression of MK in the synovial lining cells of RA synovial tissue (Figure 4), while MK expression was minimal in OA synovial tissue.

Stimulatory effect of MK on RSFs

Finally, we investigated the *in vitro* effects of MK on RSFs. After RSFs were incubated with MK for 48 h, the concentrations of IL-6, IL-8, CCL2, CX3CL1 and PGE_2, which are thought to be involved in the pathogenesis of RA [28-30], in the culture supernatant were determined with ELISA kits. It was found that stimulation with MK significantly increased the production of IL-6 and IL-8 by RSFs in a dose-dependent manner (Figure 5A and B). Production of CCL2 by RSFs was also enhanced after treatment with MK (Figure 5C). Production of PGE_2 was not significantly altered by the MK stimulation (Figure 5D). Expression of CX3CL1 was not detected by this method.

We also examined the expression of candidate MK receptors, ALK, PTPRZ1, Notch2 and LRP1 [31], in RSFs. Notch2 and LRP1 mRNAs were expressed, but expression

of ALK and PTPRZ1 was not detected by RT-PCR (Figure 6A). The protein expression of LRP1, but not Notch2, was detected in RSFs by Western blotting (Figure 6B).

Discussion

In this study, we showed that the serum MK level is elevated in RA patients and is correlated with clinical indexes of RA. In addition, MK expression was obvious in RA synovial tissue, and stimulation of RSFs with MK enhanced the production of IL-6, IL-8 and CCL2.

Previous study has shown that the serum MK level is elevated in RA patients and is correlated with the RF titer [22]. In this study, we found that the serum MK level was correlated with DAS28-ESR and that MK tended to be decreased after treatment with an anti-TNF-α antibody. These results indicate that the serum MK level could be a useful marker of RA disease activity. Moreover, serum MK was correlated with HAQ, indicating that MK also could be an indicator for a poor prognosis of RA. In fact, MK was also correlated with the RF titer, which is a well-known poor prognostic factor [32]. Therefore, it is possible that the serum MK level could be a useful disease activity marker as well as a prognostic factor. In this study, we retrospectively evaluated clinical courses, including serum MK, ESR and CRP levels, in 4 patients treated with anti-TNF-α antibody. We further need to prospectively analyze the changes of serum MK level and the disease activities in detail by the treatment in large number of patients to identify the characteristics of MK as a marker for activity of RA.

We found that MK was expressed in the RA synovial lining, which is consistent with a previous report [15, 22], while there was minimal MK expression in OA synovium. The MK level in synovial fluid was also reported to be higher in RA compared with OA [22]. In the present study, we found that stimulation with MK enhanced the production of IL-6, IL-8 and CCL2 by cultured RSFs. IL-8 is a chemokine (C-X-C motif) ligand 8 (CXCL8), which induces blood vessel formation and angiogenesis and also exhibits a chemoattractant activity for neutrophils and dendritic cells [28, 33, 34]. CCL2 is thought to induce migration of monocytes into inflamed RA synovial tissue [29]. Thus, our findings suggest that MK might play a role in inflammatory cell migration via enhancement of chemokine production by

RSFs. Moreover, it was reported that MK induces neutrophil migration [15, 17], so MK could both directly and indirectly promote inflammatory cell accumulation in RA synovial tissue and synovial fluid. IL-6 has a wide range of effects on immune cells and contributes to the pathogenesis of autoimmune diseases [35], and it has been reported that inhibition of IL-6 signaling by anti-IL-6 or anti-IL-6 receptor monoclonal antibodies is effective for RA [36, 37]. Our findings indicated that MK may contribute to the production of cytokines such as IL-6, which regulate immune cell activation.

As candidate receptors for MK, ALK, PTPRZ1, Notch2, and LRP1 are reported [31]. We found that LPR1 was expressed in mRNA and protein levels in RSFs. LPR1 might be a responsible receptor for MK stimulation in RSFs.

It has been reported that MK enhances endothelial cell proliferation and vascular growth [12] and also upregulates the differentiation of osteoclasts *in vitro* [22]. Therefore, MK may contribute to angiogenesis and bone destruction in the synovial tissues in RA, as well as stimulating RSFs. In a murine model of antibody-induced arthritis, it was found that MK expression was upregulated in the affected joints after arthritis developed [22]. In addition, development of antibody-induced arthritis was significantly inhibited in MK-deficient mice compared with wild-type mice [22]. Thus, MK could be a potential therapeutic target in patients with RA.

In conclusion, the serum MK level could be a marker of RA disease activity and an indicator of a poor prognosis. It also seems possible that MK plays a role in the pathogenesis of RA via induction of IL-6, IL-8 and CCL2.

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Competing interests

Hideaki Shimada received research grant from Cellmid Ltd. Other authors declare that they have no competing interests.

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Figure legends

Figure1. Serum MK level. The serum MK concentration was determined by ELISA in 146 RA patients and 85 healthy controls.

Figure 2. Correlation of the serum MK level with the DAS28-ESR, HAQ, Sharp score, and RF in the patients with RA. Correlations between MK and the DAS28-ESR (A), HAQ score (B), Sharp score (C), and RF (D) were determined in the 146 patients. Each point represents an individual patient. $P<0.05$ was considered to indicate statistical significance.

Figure 3. Clinical courses by anti-TNF-α treatment. Clinical courses of each individual, including the treatment, serum levels of MK, ESR and CRP in 4 patients who received infliximab, are shown (A to D). PSL: prednisolone, MTX: methotrexate, IFX: infliximab.

Figure 4. Expression of MK in synovial tissue. MK expression in synovial tissue harvested from RA patients (n=3) and OA patients (n=3) was analyzed by immunohistochemistry. Sections were counterstained with hematoxylin and representative photomicrographs are shown. Bar =100 μm. MK was strongly expressed by synovial lining cells in RA synovial tissue, whereas MK expression was minimal in OA synovial tissue.

Figure 5. Stimulatory effect of MK on RSFs. After RSFs were incubated with MK for 48 h, the concentrations of IL-6 (A), IL-8 (B), CCL2 (C) and PGE_2 (D) in the culture supernatant were measured by ELISA. Data are the mean ± SD for one of three independent experiments analyzed in triplicate. $^*p<0.05$, $^{**}p<0.005$ vs. MK (0 ng/ml).

Figure 6. Expression of candidate receptors for MK in RSFs. Expression of mRNA of ALK,

PTPRZ1, Notch2, and LRP1 was analyzed by RT-PCR in RSFs from 3 RA patients (A). Expression of Notch2 and LRP1 in 3 RSFs was examined by Western blotting (B). M: molecular weight marker.

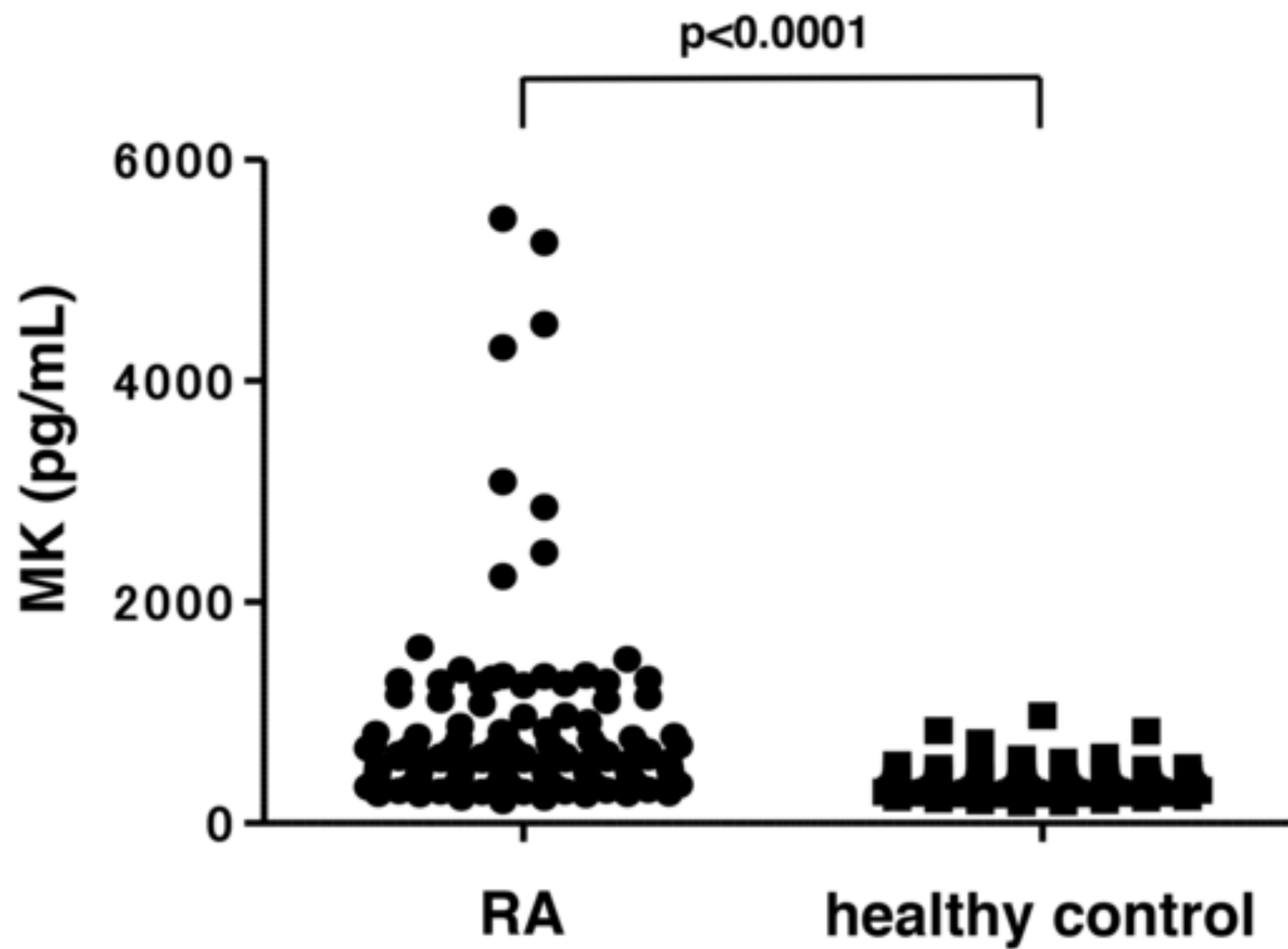

Figure 1

Figure2

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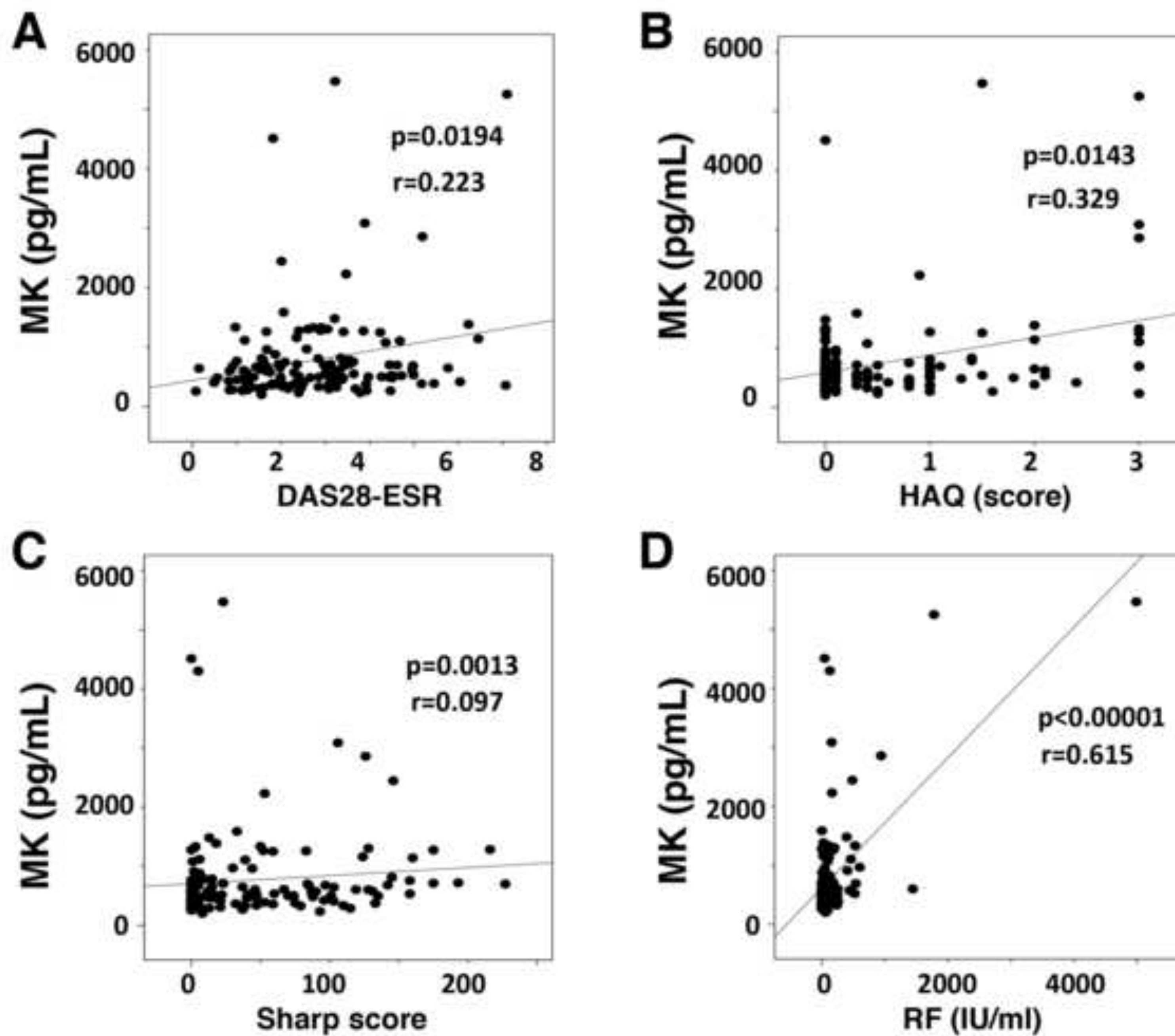

Figure 2

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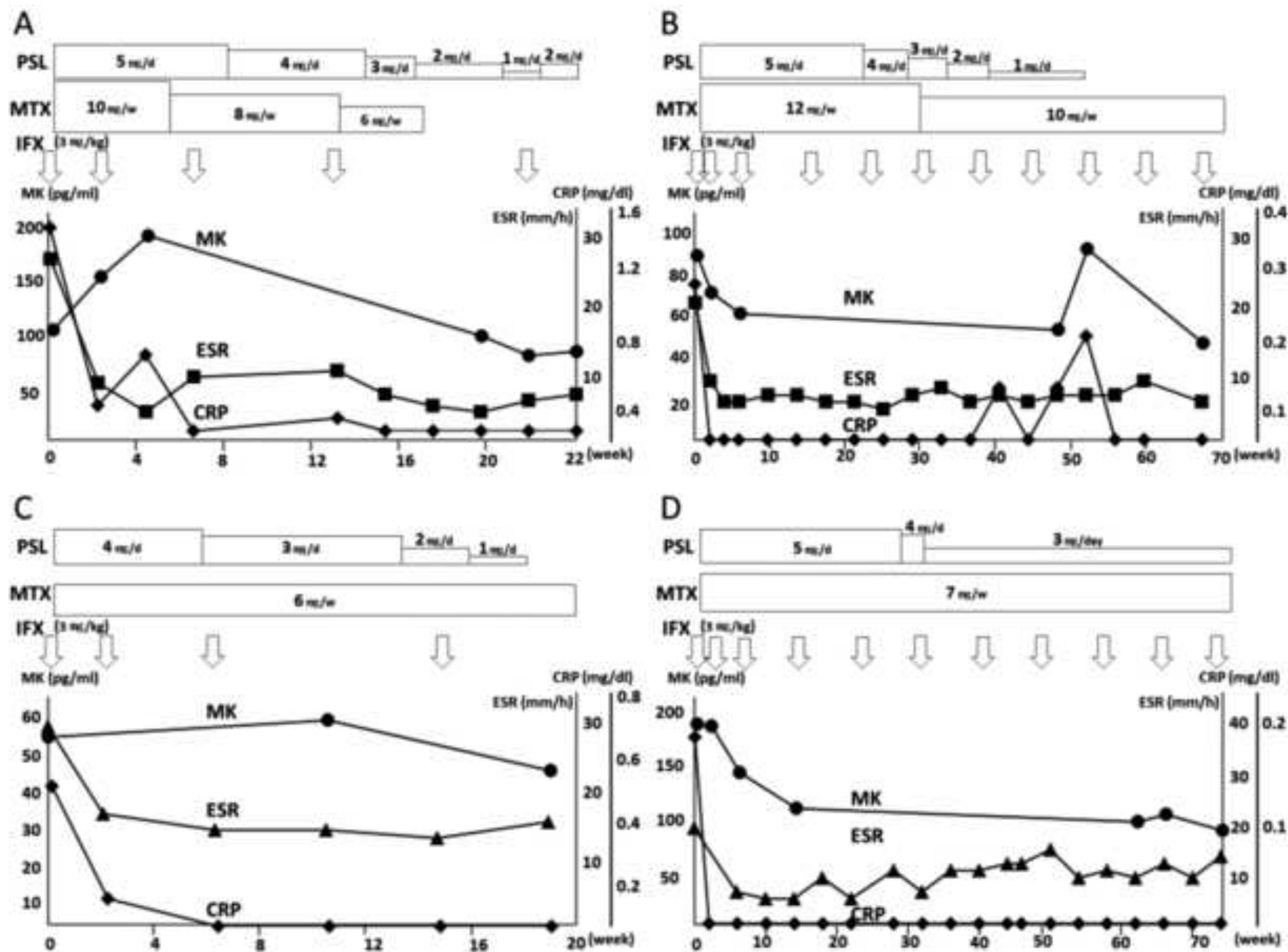

Figure 3

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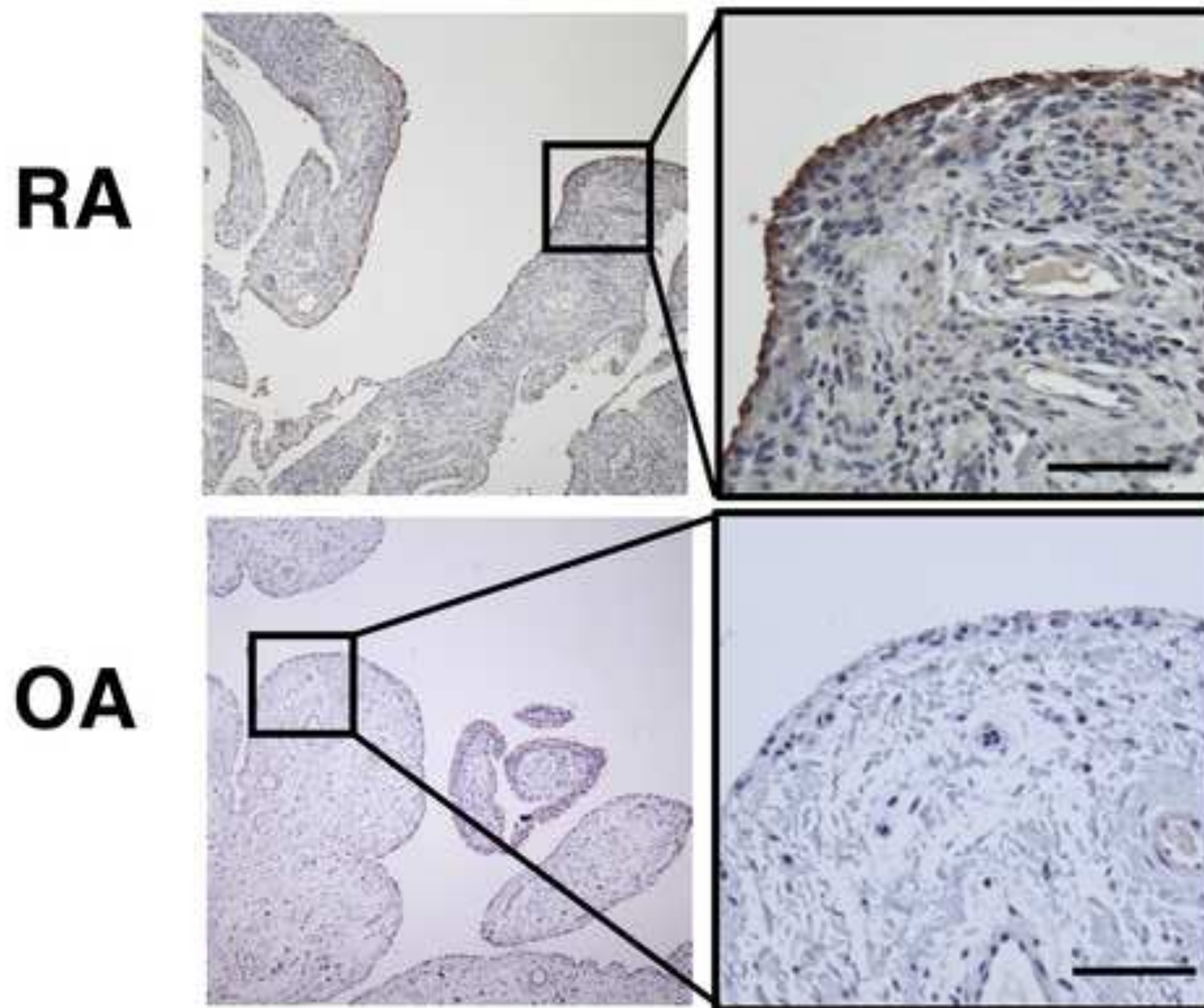

Figure 4

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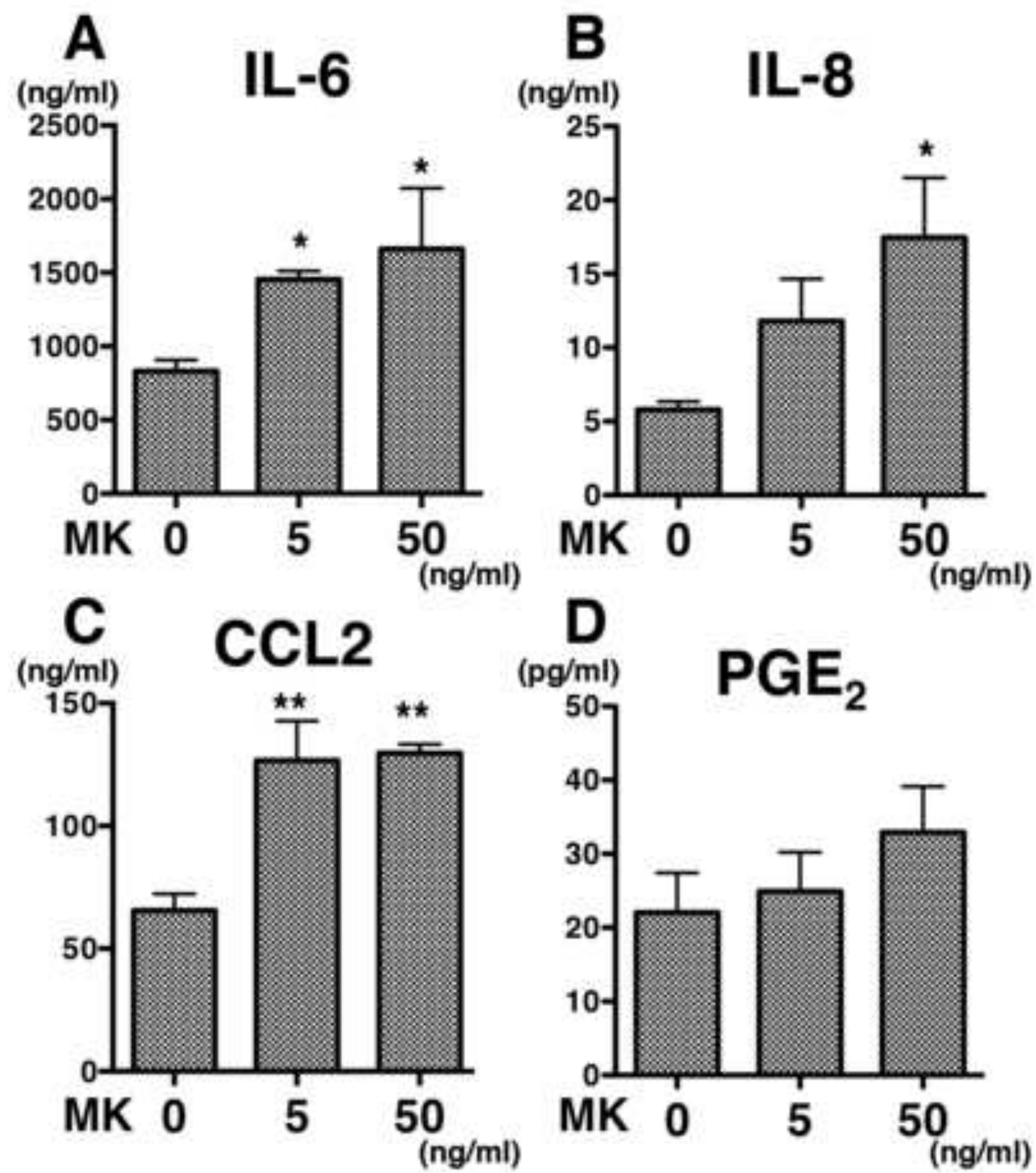

Figure 5

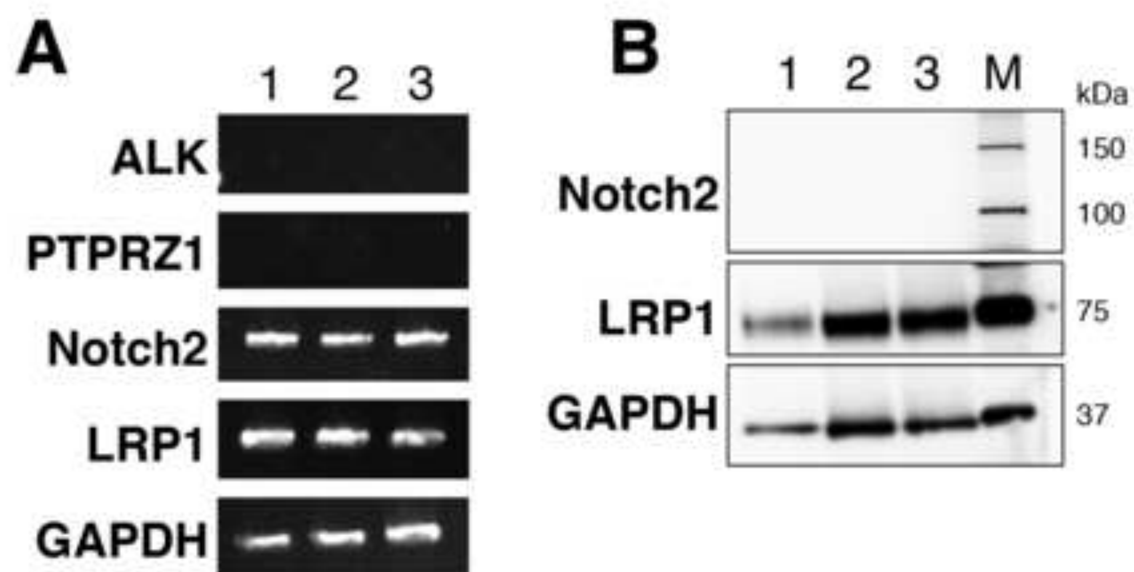

Figure 6