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류마티스관절염에서 피루브산염키나아제 M2 발현 증가의 의미에 대한 연구

Increased pyruvate kinase M2 expression and its implication in rheumatoid arthritis

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Abstract

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Background and Objective

Pyruvate kinase M2 (PKM2), a key glycolytic enzyme, is an emerging molecule involved in cancer biology and inflammatory process. The aim of this study was to investigate the expression of PKM2 in the synovial tissue, synovial fluid (SF), and plasma of patients with rheumatoid arthritis (RA) and to determine the clinical relevance of PKM2 expression levels. Also, in vitro study tried to evaluate the effects of PKM2 on biological activities of RA-fibroblast like synoviocytes (FLSs) and osteoclastogenesis.

Methods

The expression of PKM2 in the synovial tissues was assessed by immunohistochemistry and double immunofluorescence. SF (RA, n=25; osteoarthritis, n=5) and plasma (RA, n=139; control, n=47) PKM2 levels were measured by enzyme-linked immunosorbent assay (ELISA). The association of plasma PKM2 was analyzed with clinical variables including disease activity and radiographic progression or circulating levels of proinflammatory cytokines (tumor necrosis factor (TNF)-α, interleukin (IL)-6, and vascular endothelial growth factor (VEGF)). It was investigated whether RA-FLSs and macrophages could release PKM2 into the extracellular space upon inflammatory stimulation. The effect of recombinant PKM2 was also examined on osteoclast differentiation. Furthermore, the impact of PKM2 knockdown on migration and proliferation of RA-FLSs was tested using small-interfering RNA (siRNA) technology.

Results

In the synovial tissues of RA patients, PKM2 was upregulated and was mainly expressed in RA-FLSs. PKM2 levels in SF were significantly higher in patients with RA than in those with osteoarthritis ($p < 0.001$) and were significantly correlated with SF inflammatory cell counts including the number of SF mononuclear cells ($p < 0.01$). Additionally, plasma PKM2 levels were significantly elevated in RA patients ($p < 0.001$) and the levels were significantly correlated with clinical inflammatory indices including the Disease Activity Score 28-joint ($p < 0.001$) and serum levels of IL-6 and VEGF (both $p < 0.05$). Also, in early RA (disease duration $\leq$12 months, odds ratio (OR)=1.007 for plasma PKM2 concentrations) and elderly-onset RA (age at onset $\geq$60 years, OR=4.583 for RA patients with high PKM2 levels) subgroups, plasma PKM2

levels were an independent predictor for subsequent radiographic progression. It was observed in vitro that PKM2 was released from macrophages stimulated with lipopolysaccharide, TNF-α, or IL-6, but not from RA-FLSs. RANKL-induced osteoclast differentiation was dose-dependently augmented by recombinant PKM2 ($p < 0.01$ by the trend test). Moreover, PKM2 knockdown significantly decreased TNF-α induced migration and proliferation of RA-FLSs (both $p < 0.01$).

Conclusion

PKM2 was upregulated in RA synovial tissues and plasma PKM2 levels were associated with inflammatory burden and the progression of joint damage in RA patients. Intracellular PKM2 could regulate TNF-α induced migration and proliferation of RA-FLSs. Additionally, extracellular PKM2 released from activated macrophage could promote osteoclastogenesis in the inflamed joint tissue. These findings suggest that PKM2 is a novel player in the pathogenesis of RA and that PMK2 could be a potential target for RA in controlling inflammation and preventing joint damage.

Keywords: Pyruvate Kinase M2, Rheumatoid Arthritis, Inflammation, Synoviocytes, Osteoclast differentiation, joint damage

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Contents

List of tables and figures

1. Introduction

Rheumatoid arthritis (RA) is a chronic systemic inflammatory autoimmune disease, which features chronic arthritis in the diarthrodial joints leading to serious joint destruction [1]. In a recent systematic review by Cross *et al*., the estimated global prevalence of RA was 0.24% and RA was ranked as the 42nd highest contributor to disability among the 291 conditions studied [2]. They summarized that the burden of RA could be continuously increasing because of the world population growth and increasing aging. However, despite many advances in RA research, we have unmet clinical needs for new prognostic biomarkers and optimal therapeutic agents yet [3].

The synovium is the main target of RA-related inflammation and the RA synovial tissue has known to be under chronic hypoxic condition because of synoviocyte proliferation, immune cell infiltration, and upregulated cell metabolism [4-6]. It was reported that partial oxygen pressure in synovium is inversely correlated with the intensity of synovitis or the number of T cells and macrophages in RA synovial tissue [6]. Additionally, the levels of inflammatory cytokines such as tumor necrosis factor (TNF)-α, vascular endothelial growth factor (VEGF), and interleukin (IL)-6 are significantly upregulated in hypoxic conditions [6-8]. Moreover, the expression of matrix metalloproteinases (MMPs) and the migration activity of fibroblast-like synoviocytes (FLSs) are increased in hypoxic conditions [7-8].

Hypoxic microenvironment and the higher expression of inflammatory cytokines in the RA synovial tissue could change glucose metabolism from oxidative phosphorylation to glycolysis [9-10]. Lactate generated from glycolysis has been

reportedly associated with synoviocyte proliferation, angiogenesis, and pannus formation [10]. Glycolysis inhibitors could suppress the activation of immune cells, the production of anti-glucose-6-phosphate isomerase (GPI) antibodies, and the severity of arthritis in K/BxN murine arthritis model [10-11]. Upregulated glycolysis and downregulated oxidative phosphorylation are observed during the Warburg effect in cancer cells [12]. Cancer cells need rapid production of energy and metabolic precursors for continuous cell division and proliferation. Also, cancer cells have to adapt to the hypoxic environment secondary to increased oxygen requirement. Although biological functions of the Warburg effect remain to be elucidated, cancer cells and other proliferating or developing cells increase glucose uptake rate and produce lactate through enhanced glycolysis, even in the presence of oxygen and fully functioning mitochondria [13].

In the Warburg effect, a key regulator is pyruvate kinase (PK) that engages in final phase of glycolysis that converts phosphoenolpyruvate (PEP) to pyruvate [12]. PK has 4 isoforms (L, R, M1, and M2) [14]. PKL and PKR are expressed in liver and erythrocytes, respectively. PKM1 and PKM2 are encoded by PKM gene and their expression is controlled by tissue-specific promoters and alternative splicing. PKM1 is mainly expressed in skeletal muscle, heart, and brain but PKM2 is found in proliferating cells and tumor tissues. On the stimulation with activators, tetrameric PKM2 rapidly converts PEP into pyruvate for oxidative phosphorylation. On the other hand, dimeric PKM2 is an inactive form with a low kinase activity supporting to the dominance of glycolysis over oxidative phosphorylation [15-17].

PKM2 has been reported to be upregulated in many cancers and to promote the proliferation of various cancer cells [15, 18-19]. Other than engaging in glycolysis, in cancer cells, dimeric PKM2 could also translocate in the nucleus and act as a protein kinase which regulates gene expression associated with inflammation, metabolism, and cell proliferation [20-23]. Moreover, PKM2 is secreted from cancer cells into the extracellular space. It was observed that extracellular PKM2 promotes the proliferation and migration of cancer cells through epidermal growth factor (EGF) and Wnt pathways [24-25].

Recently evidences are increasing that PKM2 could play a role in the pathogenesis of inflammatory diseases and that glycolysis can be a therapeutic target in inflammatory as well as malignant diseases. PKM2 deficient CD4+ T cells reduced homocysteine-induced interferon (IFN)-γ production and PKM2 translocation to the nucleus in IL-23 stimulated T cells [26-27]. In acute infection models, lipopolysaccharide (LPS) induced the translocation of PKM2 in the lung tissue and PKM2 modulated inflammasome activation in myeloid cells [28-29]. Recently, it was reported that the expression of PKM2 was upregulated in RA synovium, especially in patients with synovial low partial oxygen pressure [31-32], in the muscle tissues from dermatomyositis [33], and in the intestinal epithelial cells of Crohn's disease [34]. Moreover, it was published that the plasma levels of PKM2 were higher in several rheumatic diseases including RA, systemic lupus erythematosus (SLE), and spondyloarthritis than in healthy donors [35] and that serum levels of PKM2 were higher in patients with inflammatory bowel disease (IBD) [36]. Extracellular PKM2 was also found to be released from activated neutrophils and neutralizing antibody against PKM2 could inhibit angiogenesis [30].

However, a literature search revealed that there has been no study to investigate the clinical implications and the biological functions of PKM2 upregulation in RA.

The aims of this study were (1) to determine the expression levels of PKM2 in synovial tissue and fluid as well as in the circulation of RA patients, (2) to examine the association of PKM2 levels with clinical variables including RA disease activity and radiographic joint damage, (3) to investigate the role of PKM2 in biological activity of RA-FLSs or receptor activator of nuclear factor-κB ligand (RANKL) induced osteoclastogenesis.

2. Materials and Methods

2.1. Study population

For plasma samples, 139 patients with RA were consecutively enrolled from Rheumatology clinic at Seoul National University Bundang Hospital (SNUBH) between Aug 2009 and Aug 2012. When first evaluated at SNUBH, the patients were recruited if they agreed for blood sampling. Additionally, synovial fluid (SF) samples from another set of 25 RA patients and 5 osteoarthritis (OA) patients were collected between Jan 2010 and Dec 2017 when they had a flare-up in the knee arthritis. Synovial tissues were obtained from patients undergoing joint-replacement surgery or synovectomy (3 elbow RA, 2 knee RA, and 3 knee OA). All RA patients were diagnosed according to the 1987 American Rheumatism Association criteria [37]. Plasma samples from 47 sex- and age-matched healthy donors (at a 3:1 ratio), including 10 samples from the SNUBH Biobank, served as controls.

This study was approved by the Seoul National University Bundang Hospital Institutional Review Board (IRB No. B-0905/075-013) and was performed according to the recommendations of the Declaration of Helsinki. Informed written consent was obtained by all participants.

2.2. Clinical and radiographic assessment

Clinical data were collected at the time of blood sampling; age, disease duration, smoking status, and body mass index, medication use including antirheumatic drugs, complete blood cell counts, erythrocyte sedimentation rate (ESR), serum C-reactive protein (CRP) level, and the presence of rheumatoid factor (RF) or anti-cyclic citrullinated peptide (anti-CCP) antibodies. RA disease activity was assessed according to the 28-joint count Disease Activity Score (DAS28) [38]. Patients with active RA were defined as those with DAS28 >3.2, and patients with remission were defined as those with DAS28 <2.6 [39]. Early RA was defined as disease duration of 12 months or less and elderly-onset RA was as symptom onset $\geq$ 60 years.

Radiographs of the hands and feet were taken at baseline and repeated after a mean (±SD) 26.8 ±16.6 months in 126 (90.6%) patients with RA. Radiographic damage was assessed using the modified Sharp/Van der Heijde score (SHS) [40]. Erosive disease was defined as SHS erosion score ≥1 at the baseline [41]. Δ SHS ≥ 1 unit/year was defined as radiographic progression, and erosive or narrowing disease progression was defined as a ΔSHS ≥1 unit/year in the corresponding subscore according to the previous literatures [42-43].

2.3. Cell lines and reagents

RAW264.7, a murine macrophage cell line, and THP-1, a human monocytic leukemia cell line, were bought from American Type Culture Collection (ATCC). Recombinant human TNF-α and soluble RANKL were purchased from Peprotech (Rocky Hill, NJ, USA). Recombinant PKM2 was from Biovision (Milpitas, CA, USA). Collagenase type

II was from Worthington (Columbus, Ohio, USA). Anti-PKM2 antibody was bought from Cell Signaling Technology (Danvers, MA, USA). Anti-β-actin antibody was from Enogene Biotech (NY, USA) and anti-vimentin antibody from Leica biosystems (Nussloch, Germany). Anti-CD68, anti-CD3, and Texas Red-X conjugated secondary antibodies were obtained from Thermo Scientific (Waltham, Massachusetts, USA) and anti-CD20 antibody was from Dako (Glostrup, Denmark).

Enzyme-linked immunosorbent assay (ELISA) kits detecting pro-MMP-1, total MMP-3, or pro-MMP-13 were purchased from R&D Systems (Minneapolis, MN, USA). PKM2 ELISA kits were obtained from Schebo Biotech (Netanyastrasse, Giessen, Germany) and Elabscience (Huston, TX, USA). Dulbecco's modification of Eagle's medium (DMEM) and minimum essential medium Eagle-alpha modification (α-MEM) were purchased from Welgene (Daegu, Korea). Fetal bovine serum (FBS) was from Atlas Biologicals (Fort Collins, CO, USA) and penicillin-streptomycin was from Gibco (Carlsbad, CA, USA) Tartrate resistant acid phosphatase (TRAP) staining kit was from Takara (Shiga, Japan).

2.4. Preparation of plasma, serum, SF, and synovial tissue samples

Peripheral venous blood was collected using serum tubes pre-added clotting activator and vacutainers containing ethylenediaminetetraacetic acid (EDTA; Becton-Dickinson, Franklin Lakes, NJ, USA). Plasma and serum were isolated by centrifuge at 2095 g for 10 min at 4℃ to remove red cells and clot materials.

Additionally, aspirated SF samples from the knee joints were centrifuged at 2095 g for 15 min at 4℃ and the supernatant was used in this study. All EDTA plasma, serum, and SF samples were aliquoted and stored at -80℃ until analysis. The postsurgical synovial tissue was fixed with 4% buffered paraformaldehyde and paraffin-embedded and stored until immunohistochemical staining was performed.

2.5. Immunohistochemistry (IHC) for PKM2 in synovial tissue

Paraffin-embedded synovial tissue was cut by 4-μm thickness and then deparaffinized with xylene and rehydrated with ethanol. Following procedures were performed using Lab Vision™ UltraVision™ LP Detection System (Thermo Scientific, Waltham, Massachusetts, USA). Slides were dipped into citrate buffer (pH 6.0) for 15 min for antigen retrieval and then incubated in hydrogen peroxide for 10 min for blocking of endogenous peroxidase. Serial sections were applied with anti-PKM2 antibody at 1:1600 for 3 h at room temperature. The slides were incubated in diaminobenzidine (DAB), and counterstained with hematoxylin. Slides were washed with phosphate-buffered saline (PBS) 3 times for 5 min at each step. To semi-quantitatively analyze, the number of PKM2-immunostained cells were manually counted among synovial lining and sublining stromal cells. At least 3 high-power fields without the area of lymphoid follicles or aggregates were randomly selected and a minimum of 1,000 cells per each slide were analyzed.

2.6. Double immunofluorescence (IF)

Paraffin-embedded synovial tissue was cut by 4-μm thickness and then deparaffinized with xylene and rehydrated with ethanol. Following procedures were performed using Lab Vision™ UltraVision™ LP Detection System. Slides were dipped into citrate buffer (pH 6.0) for 15 min for antigen retrieval and then incubated in hydrogen peroxide for 1 h for blocking of endogenous peroxidase. After blocking, slides were incubated with anti-vimentin antibody (1:100), anti-CD68 antibody (1:100), anti-CD20 antibody (1:400), or anti-CD3 antibody (1:50) overnight at 4℃. Then, the slides were incubated with goat anti-mouse IgG antibody conjugated with Texas Red-X for 1 h at room temperature in the dark. At the next, all slides were incubated with anti-PKM2 antibody (1:100) at overnight at 4℃ and then with goat anti-rabbit IgG antibody conjugated with Alexa Fluor®-488 for 1 h at room temperature in the dark. Subsequently, 4′,6-diamidino-2-phenylindole (DAPI) was used for nuclear staining. The slides were washed with PBS 3 times for 5 min at each step. Quantitative analysis of PKM2 colocalization with each cell-specific marker was performed using the Colocalization Threshold plugin of Image J software (NIH, Bethesda, Maryland, USA) [44, 45].

2.7. Measurement of PKM2, proinflammatory cytokines, and MMPs

PKM2 in plasma and SF samples was analyzed by using commercial ELISA kits from Schebo Biotech, according to a modified manufacturer's instruction. Briefly, unknown samples (diluted at 1:200 for plasma from controls, at 1:500 for plasma from RA patients, and at 1:5000 for SF samples) were added to 96-well plates precoated with

anti-PKM2 antibody. After overnight incubation at 4℃ and repeated washings, the plates were incubated with a biotin-conjugated anti-PKM2 monoclonal antibody for 30 min at room temperature. PKM2 in the samples was detected with a streptavidin-coupled horseradish peroxidase reaction, and the plates were read at 450 nm using a spectrophotometer (Molecular Devices, San Jose, California, USA). The levels of PKM2 released from stimulated RA-FLSs and THP-1 cells were also analyzed by the ELISA kits. The serum levels of TNF-α, IL-6, and VEGF were analyzed with a Luminex 100 system (Luminex, Austin, TX, USA) using a magnetic bead-based immunoassay (R&D systems). In serum-free media collected from stimulated RA-FLSs (2×10^5 cells/well in 6-well plates), MMP-1, MMP-3, and MMP-13 levels were measured using the ELISA kits, according to manufacturer's instructions. All measurements were performed in duplicate.

2.8. Isolation and culture of RA-FLSs

Synovial tissues were finely chopped and then incubated in a solution of 0.3% collagenase type II in PBS with 1% penicillin-streptomycin for 2 h at 37℃. After harshly pipetting, digested tissue sample was centrifuged and resuspended with DMEM containing 10% FBS and 1% penicillin-streptomycin. And then, isolated cells were cultured at 37℃ in a humidified CO2 incubator to adhere onto the culture dish and medium was changed every 3 days. Adherent cells were maintained and passaged and those between 3 and 5 passage number were used for RA-FLSs experiments.

2.9. Culture and differentiation of THP-1 cell line

THP-1 cell line was cultured in RPMI-1640 containing 10% FBS and was stimulated with phorbol 12-myristate 13-acetate (PMA) for 24 h for the differentiation into macrophage-like cells. After the incubation with PMA, suspended THP-1 cells turned to adherent cells. These macrophages were incubated in RPMI-1640 containing 10% FBS without PMA for another 24 h for cell recovery and then used for the experiments.

2.10. PKM2 knockdown by small interfering RNA (siRNA)

PKM2 siRNA (Santa Cruz, Texas, USA) and control siRNA (Thermo Scientific) were transfected by electroporation using Gene pulser 4-mm microcuvette (Bio-Rad, CA, USA). Electroporation was performed with 200 nM of siRNA at a single 20 ms-pulse of 200 V/cm. Transfected RA-FLSs were seeded in 6-well plates at the density of 2×10^5 cells/well. After 2 days, PKM2 knockdown was confirmed by immunoblot. Briefly, cell lysates were obtained using RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS). Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. Anti-PKM2 and anti-β-actin antibodies were used as primary antibodies.

2.11. Cell migration

RA-FLSs were seeded in 6-well plates at the density of 2×10^5 cells/well and, after 1 day, scraped using a sterile cell scraper (3-mm width). RA-FLSs were stimulated with TNF-α (10 ng/mL) in complete media and then migrated cells in the scratch area were counted under the microscope at 24 h, 48 h, and 96 h.

2.12. Cell proliferation

RA-FLSs were seeded in 48-well plates at the density of 3×10^3 cells/well and incubated in DMEM with 10% FBS. To measure proliferation, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed at 0, 24, 48, and 96 h. After adding 0.5 mg/mL MTT solution to each well, the plates were incubated for 2 h at 37℃. And then dimethyl sulfoxide (DMSO) was added to dissolve formazan crystal and absorbance was analyzed at 570 nm.

2.13. Osteoclast differentiation

RAW264.7 cells were cultured in DMEM containing 10% FBS and 1% penicillin-streptomycin at 37℃ in a humidified CO2 incubator. After seeding in the 48-well plates at the density of 1×10^3 cells/well, RAW264.7 cells were cultured in α-MEM containing 10% FBS and 1% penicillin-streptomycin with various osteoclastogenic

stimuli (25-50 ng/mL of soluble RANKL, 25-50 ng/mL of LPS, or a combination of 50 ng/mL of TNF-α and 50 ng/mL of IL-6). Under the condition of recombinant human PKM2 0-20 ng/mL, TRAP staining was done after 5 days using a commercial TRAP staining kit according to the manufacturer's instructions. To evaluate osteoclast differentiation, TRAP-positive multinucleated cells were counted under a light microscope.

2.14. Statistical analysis

Data are presented as mean±standard deviation or median [interquartile ranges]. Continuous variables were compared using Student's t-test, Mann-Whitney U test, or Kruskal-Wallis test. Multiple comparison was corrected using the Bonferroni method. Chi-square test or Fisher's exact test was performed for the comparison of categorical variables. Bivariate correlations were analyzed by Spearman's correlation coefficients. Nonparametric Jonckheere trend test was used to test for a linear trend across the groups. Binary logistic regression analysis was performed to find independent variables associated with radiographic progression. Multivariate models were created using the variables with a statistical significance of $p < 0.1$ from the univariate analyses. P or corrected p (p_c) values < 0.05 were considered statistically significant. All data were analyzed using SPSS Statistics for Windows version 25 (IBM Corp., Armonk, NY, USA).

3. Results

3.1. The expression of PKM2 in RA and OA synovial tissues

When the expression of PKM2 was immunohistochemically analyzed in the synovial tissue, PKM2 was detected in all synovial tissue samples. PKM2-positive cells were distributed in both the lining and sublining layers (Fig. 1A). PKM2 was also immunostained in mononuclear inflammatory cells and vascular endothelial cells. The intensity of PKM2 was much stronger and the fraction of PKM2-positive cells was significantly higher in RA synovial tissues than in OA synovial tissues ($p < 0.0001$, Fig. 1B).

3.2. Cellular expression of PKM2 in RA synovial tissues

To explore which cells upregulated PKM2 expression, double IF staining was performed in RA synovial tissues. Anti-vimentin antibody was used to detect FLSs and anti-CD68, anti-CD20, and anti-CD3 antibody were used to label macrophages, B cells, and T cells, respectively. PKM2 was expressed in all four types of cells. The colocalization analysis showed a significantly higher degree of colocalization between PKM2 and vimentin in the synovial tissues. The finding suggests that PKM2 is upregulated mainly in RA-FLSs, compared to in infiltrated or resident immune cells in RA synovial tissues.

3.3. Extracellular PKM2 levels in SF samples

Because PKM2 was upregulated in RA synovial tissues and SF is produced by the synovial membrane, extracellular PKM2 could be expected to be detected in SF samples. Table 1 summarizes the characteristics of SF samples analyzed. The cell number of SF inflammatory cells including white blood cells, polymorphonuclear neutrophils (PMN), lymphocytes, and macrophages/monocytes were significantly higher in RA than in OA. Also, PKM2 levels were significantly increased in RA ($p < 0.01$, Fig. 3A) and were positively correlated with the numbers of SF inflammatory cells (all $p < 0.05$, Fig. 3B-F).

3.4. Plasma PKM2 levels and their clinical implications in RA patients

Since PKM2 was observed to be upregulated at inflammation sites in RA patients and SF PKM2 levels were associated with the intensity of inflammation – inflammatory cell counts, it was further studied whether PKM2 levels were elevated in the circulation of RA patients. Additionally, the association of PKM2 was investigated with the circulating proinflammatory mediators such as TNF-α, IL-6, and VEGF or clinical variables including radiographic joint damage and progression. Table 2 presents baseline clinical features of study participants (139 RA patients and 47 controls) who provided plasma samples.

When compared to controls, plasma PKM2 and serum TNF-α, IL-6, and VEGF levels were significantly increased in total subjects with RA and patients with active RA (all p

<0.001, Fig. 4). Also, circulating IL-6, TNF-α, and PKM2 levels were significantly higher even in inactive RA subgroup than in controls. Circulating PKM2 levels were significantly correlated with IL-6 and VEGF expression (both $p < 0.0001$, Fig. 5), but not with TNF- α.

In univariate analyses to investigate the association between plasma PKM2 levels and clinical variables of RA patients, PKM2 was positively correlated with age ($p < 0.05$), swollen and tender joint counts (both <0.001), ESR ($p < 0.0001$), and CRP ($p < 0.0001$, Table 3). Eventually, plasma PKM2 levels were significantly related with RA disease activity index, DAS28-ESR or DAS28-CRP scores ($p < 0.0001$, Fig. 6). Additionally, plasma PKM2 levels were significantly correlated with the counts of peripheral white blood cells, PMNs, and monocytes, as were SF PKM2 levels (Table 3, Fig. 6). In addition, patients with erosive disease or elderly-onset had higher PKM2 levels than those without (both $p < 0.05$, Fig. 7A-B). Moreover, patients taking methotrexate (MTX), an antirheumatic and antimetabolite drug for RA treatment, showed a significantly lower levels of PKM2 than those not taking ($p < 0.01$, Fig. 7C). However, PKM2 levels were not associated with baseline SHS scores.

When RA patients were classified into high and low PKM2 subgroups according to the 90^{th} percentile of the controls, high PKM2 subgroup (n=91) had significantly higher inflammatory indices including acute phase reactants and white blood cell counts and showed a significantly higher prevalence of active patients (Table 4). Interestingly, high PKM2 subgroup was more likely to have male patients ($p < 0.001$) and patients with a history of smoking ($p < 0.05$, Table 4). But, between high and low PKM2 subgroups, the proportion of radiographic progressors as well as baseline SHS scores was comparable.

RA patients were stratified into radiographic progressors (n=46) and non-progressors (n=80) according to the predefined definition, i.e., Δ SHS ≥1 unit/year. Radiographic progressors were significantly older and had significantly more patients at age ≥60 years (both $p < 0.01$, Table 5) than non-progressors. Also, they had a longer disease duration ($p < 0.05$) and had significantly higher SHS scores (i.e., more radiographic damage) at baseline ($p < 0.001$). But the proportion of early RA or elderly-onset RA was comparable between progressors and non-progressors. Additionally, DAS28 scores and the levels of acute phase reactants were not different between the two subgroups. Moreover, serum concentrations of proinflammatory cytokines IL-6 and TNF-α and plasma PKM2 levels were comparable between progressors and non-progressors, while VEGF levels were significantly increased in the radiographic progressors ($p < 0.05$, Table 5 and Fig. 8A)

However, after RA patients were subgrouped on the basis of the disease duration, Early RA patients with radiographic progression had a significantly higher level of PKM2, but not VEGF levels, at baseline ($p < 0.05$, Fig. 8B). PKM2 levels were also significantly elevated in early RA patients with erosive or joint narrowing disease progression ($p < 0.05$, Fig. 8C-D). In addition, RA patients at age ≥60 years with high PKM2 levels were more likely to have radiographic progression than those with low PKM2 levels (20/32 (62.5%) versus 4/15 (25.7%), $p=0.03$ by Fisher's exact test).

Because plasma PKM2 levels were significantly associated with radiographic progression in patients with early RA and at age ≥60 years, multivariable analyses were performed to test plasma PKM2 level as an independent predictor of radiographic progression in these subgroups. In multivariable analysis, plasma PKM2 level or high

PKM2 remained as a significant independent variable associated with radiographic progression in both subgroups (Table 6).

3.5. PKM2 release from activated RA-FLSs and macrophages

PKM2 was immunostained mainly in RA-FLSs in synovial tissues but extracellular PKM2 levels were significantly correlated with the number of neutrophils or monocytes/macrophages in the blood or SF. Therefore, the cellular source of extracellular PKM2 was explored at next steps. Because Zhang *et al.* recently reported that activated neutrophils could release PKM2 [30], PKM2 levels in the conditioned media were investigated after RA-FLSs and macrophages derived from THP-1 cells in the present study.

RA-FLSs and macrophages were stimulated with 100 ng/mL of LPS, 10 ng/mL of TNF-α, or 50 ng/mL of IL-6 for 24 h. PKM2 levels were not increased in the culture media from stimulated RA-FLSs (Fig. 9A). However, in stimulated macrophages, PKM2 levels were significantly elevated ($p < 0.05$, Fig 9B) when compared to the non-stimulated condition.

3.6. Osteoclast differentiation enhanced by recombinant PKM2 and osteoclastogenic stimuli.

Since plasma PKM2 levels were independently associated with radiographic progression and macrophages secreted PKM2 when stimulated with proinflammatory

mediators, the effect of extracellular PKM2 on osteoclastogenesis was examined. To determine the concentration of recombinant PKM2 to be used, a pilot study was done to analyze SF PKM2 levels with primary antibodies to detect monomeric, dimeric, and tetrameric PKM2 (the ELISA kits from Elabscience). The measured PKM2 levels were 17.3 to 24.8 ng/mL in SF samples from 5 RA patients.

When RAW264.7 cells were treated with recombinant PKM2 (0-20 ng/mL), the number of TRAP (+) multinucleated cells was not significantly increased (Fig. 10A). But, in the conditions combined with suboptimal dose of RANKL, extracellular PKM2 significantly enhanced osteoclast differentiation (all $p < 0.0001$, Fig. 10B) in a dose dependent manner ($p < 0.0001$ by the trend test). Although the number of osteoclasts did not significantly increase when stimulated by PKM2 with LPS or TNF-α/IL-6, in vitro osteoclastogenesis tended to be promoted with increasing concentrations of recombinant PKM2 ($p=0.006$ for PKM2 and LPS and $p=0.046$ for PKM2 and TNF-α/IL-6 by the trend test, Fig. 10C-D).

3.7. The effect of PKM2 knockdown on migration, proliferation, and MMPs production of RA-FLSs

RA-FLSs were the main cells that upregulated PKM2 expression in RA synovial tissues, but they did not actively release PKM2. To investigate the effect of intracellular PKM2 on the biological activity of RA-FLSs, PKM2 knockdown was performed with its specific siRNA introduction. Immunoblot analysis confirmed efficient knockdown of PKM2 in RA-FLSs (Fig. 11A).

The migration of PKM2-knockdowned RA-FLSs was significantly suppressed 48 and 96 h after the stimulation with 10 ng/mL of TNF-α (both $p < 0.001$, Fig. 11B). Additionally, the proliferation of PKM2-knockdowned RA-FLSs was significantly inhibited during 24 to 96 h under the treatment with TNF-α or not (all $p \leq 0.01$, Fig. 11 C). However, after PKM2 knockdown, the production of matrix-degrading enzymes (MMP-1, MMP-3, and MMP-13) was not significantly changed (Fig. 11D).

Table 1. Characteristics of synovial fluid samples

Variables	RA (n=25)	OA (n=5)	p value
Female	23 (92)	5 (100)	n.s
Age (years)	57.9 [42.0~56.0]	59.0 [56.0~62.5]	0.7266
SF White blood cells (mm^3)	14766 [4250.0~12850.0]	232 [170.0~290.0]	0.0005
SF PMNs (mm^3)	10669.9 [1758.0~9708.0]	1.4 [0.0~3.0]	0.0005
SF Macrophages/Monocytes (mm^3)	2803.4[942.5~3985.5]	163.2[76.5~255.0]	0.0005
SF Lymphocytes (mm^3)	1506.9[498.5~1939.5]	66.2[28.5~96.0]	0.0014
SF Mononuclear cells (mm^3)	4310.3 [2140.0~5872.0]	229.4[166.0~287.5]	0.0005

All SF samples were collected from the knee joints. Values are expressed as numbers (percentages) or median [25~75 percentiles]; RA, rheumatoid arthritis; OA, osteoarthritis; PMN, Polymorphonuclear neutrophil. n.s, not significant.

Table 2. Baseline characteristics of study population who provided blood samples

Variables	RA (n=139)	Control (n=47)
Age (years)	55.0 [44.0~63.5]	54.0 [46.0~62.0]
Female	120 (86.3%)	40 (85.1%)
Smoker	21 (15.1%)	
Disease duration (months)	32.0 [6.0~90.5]	
WBC (mm^3)	7,080 [5,900~8,600]	5,380 [4,740~6,385]
ESR (mm/h)	18 [7.5~34.5]	7 [3.0~16.0]
CRP (mg/dL)	0.6 [0.1~1.5]	0.0 [0.0~0.2]
DAS28-ESR	4.1 [2.9~5.2]	
Active disease	94 (67.6%)	
Inactive disease	45 (32.4%)	
DAS28-CRP	3.7 [2.7~4.8]	
Total SHS (0-448)	4.0 [0.0~23.0]	
Erosion subscore	2.0 [0.0~12.0]	
Joint space narrowing subscore	2.0 [0.0~10.0]	
Erosive disease	85 (61.2%)	
Seropotitivity	130 (93.5%)	
RF positivity	116 (83.5%)	
Anti-CCP positivity	119 (87.5%)	
Current steroid user	66 (47.5%)	
Current MTX user	76 (54.7%)	
Current DMARD user	94 (67.6%)	
Comorbidities		
Osteoporosis	25 (18.5%)	
HTN	34 (24.5%)	
Diabetes	10 (7.19%)	
Dyslipidemia	9 (6.47%)	

HBV	5 (3.62%)

Values are expressed as numbers (percentages) or median [25~75 percentiles]; WBC, white blood cells; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; DAS28, disease activity score in 28 joints; SHS, Sharp van der Heijde score; RF, rheumatoid factor; anti-CCP, anti-cyclic citrullinated peptide antibody; MTX, methotrexate; DMARD, disease-modifying antirheumatic drug; HTN, hypertension; HBV, hepatitis B virus.

Table 3. Bivariate correlation between PKM2, proinflammatory cytokines and clinical variables

Variables	IL-6	TNF-α	VEGF	PKM2
Age	n.s	n.s	n.s	0.192 ($p=0.024$)
Disease duration	-0.228 ($p=0.007$)	n.s	-0.247 ($p=0.003$)	n.s
Swollen joint count	0.333 ($p=6.08\times10^{-5}$)	n.s	0.213 ($p=0.012$)	0.347 ($p=2.82\times10^{-5}$)
Tender joint count	0.369 ($p=7.60\times10^{-6}$)	n.s	0.229 ($p=0.007$)	0.297 ($p=3.81\times10^{-4}$)
DAS28-ESR	0.467 ($p=6.79\times10^{-9}$)	n.s	0.333 ($p=6.03\times10^{-5}$)	0.472 ($p=4.33\times10^{-9}$)
DAS28-CRP	0.447 ($p=3.45\times10^{-8}$)	n.s	0.269 ($p=0.001$)	0.401 ($p=9.85\times10^{-7}$)
WBC	0.367 ($p=8.58\times10^{-6}$)	n.s	0.236 ($p=0.005$)	0.361 ($p=1.25\times10^{-5}$)
Neutrophils	0.285 ($p=0.001$)	-0.258 ($p=0.002$)	0.244 ($p=0.004$)	0.334 ($p=5.97\times10^{-5}$)
Lymphocytes	n.s	-0.236 ($p=0.005$)	n.s	n.s
Monocytes	0.228 ($p=0.007$)	-0.189 ($p=0.025$)	n.s	0.188 ($p=0.027$)
Hemoglobin	-0.195 ($p=0.021$)	n.s	-0.171 ($p=0.045$)	-0.321 ($p=1.19\times10^{-4}$)
Platelet	0.275 ($p=0.001$)	n.s	0.277 ($p=0.001$)	0.301 ($p=3.10\times10^{-4}$)
ESR	0.472 ($p=4.61\times10^{-9}$)	n.s	0.366 ($p=9.48\times10^{-6}$)	0.591 ($p=1.82\times10^{-14}$)

CRP	0.497 ($p=5.09x10^{-10}$)	n.s	0.261 (p=0.002)	0.474 ($p=3.71x10^{-9}$)
SHS joint narrowing score	n.s	n.s	-0.177 (p=0.038)	n.s

Data are presented as bivariate correlation coefficient Spearman r and p value. n.s, not significant. DAS28, disease activity score in 28 joints; WBC, white blood cells; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; SHS, Sharp van der Heijde score.

Table 4. Clinical features of rheumatoid arthritis (RA) patients with high versus low plasma PKM2

Variables	Low PKM2 (n=48)	High PKM2 (n=91)	p value
Age (years)	50 [44~61]	58 [44~64]	0.09
Male	0 (0.0%)	18 (19.8%)	3.32×10^{-4}
Disease duration (months)	42.0 [6.0~119.2]	30.0 [5.7~86.8]	0.258
Early RA	16 (33.3%)	38 (41.8%)	0.333
Elderly-onset RA	5 (10.4%)	26 (28.6%)	0.014
Smoking	3 (6.2%)	18 (19.8%)	0.045
Swollen joint count	0.5 [0.0~3.8]	3.0 [1.0~7.0]	0.001
Tender joint count	0.0 [0.0~3.0]	3.0 [1.0~7.0]	0.001
PGVAS	72 [41~94]	70 [48~90]	0.88
ESR (mm/h)	12 [4~17]	24 [14~43]	4.93×10^{-7}
CRP (mg/dL)	0.16 [0.01~0.67]	1.01 [0.21~2.24]	3.95×10^{-5}
DAS28-ESR	3.05 [2.04~4.62]	4.43 [3.62~5.79]	8.86×10^{-6}
DAS28-CRP	2.99 [2.27~4.12]	3.97 [3.05~5.14]	1.03×10^{-4}
Active RA	21 (43.8%)	73 (80.2%)	1.25×10^{-5}
WBC (/mm^3)	6,220 [5,255~7,753]	7,450 [6,230~9,040]	0.002
Neutrophils (/mm^3)	3,959 [3,219~5,717]	5167 [4,177~6,430]	0.003
Monocytes (/mm^3)	450 [362~623]	563 [413~689]	0.043
Hb (g/L)	12.9 [12.1~13.9]	12.5 [11.6~13.6]	0.035
Platelet ($x10^3/mm^3$)	262.5 [224~304]	273 [224~347]	0.191
IL-6 (pg/mL)	3.7 [1.9~8.3]	10.4 [3.6~37.4]	1.19×10^{-4}
TNF (pg/mL)	1.9 [0.09~2.9]	1.9 [0~3.9]	0.84
VEGF (pg/mL)	79.9 [49.5~139.2]	126.9 [79.4~191.1]	2.71×10^{-4}
Baseline SHS scores	3.5 [0.0~32.8]	5.0 [0.0~18.3]	0.897
Erosive RA	25 (52.1%)	60 (65.9%)	0.111

Annual change in SHS scores	0.0 [0.0~1.6]	0.0 [0.0~1.7]	0.862
Radiographic Progressor	14/46 (30.4%)	32/80 (40.0%)	0.283
Methotrexate (MTX)	32 (66.7%)	44 (48.4%)	0.039
Leflunomide	10 (20.8%)	15 (16.5%)	0.526
Combination therapy with MTX	25 (52.1%)	31 (34.1%)	0.039

Values are expressed as numbers (percentages) or median [25~75 percentiles]. PGVAS, Patient global visual analogue scale; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; DAS28, disease activity score in 28 joints; WBC, White blood cells; Hb, hemoglobin; SHS, Sharp van der Heijde score.

Table 5. Clinical features of rheumatoid arthritis (RA) patients with radiographic progression versus those without progression

Variables	Non-progressor (n=80)	Progressors (n=46)	p value
Age	51.5 [43~61]	60.5 [46.8~67]	0.007
Age > 60	23 (28.7%)	24 (52.2%)	0.009
Menopause	31/70 (44.3%)	29/41 (70.7%)	0.007
Disease duration	24 [6.0~83.5]	56.1 [8.8~131.0]	0.043
Early RA	34 (42.5%)	13 (26.3%)	0.112
Elderly onset RA	14 (17.5%)	14 (30.4%)	0.093
Swollen joint count	2.0 [0.0~5.8]	3.0 [0.0~6.0]	0.861
Tender joint count	2.0 [0.0~5.0]	2.0 [0.0~7.3]	0.905
PGVAS	70 [41~90]	70 [50~90]	0.811
ESR (mm/h)	17.5 [8.3~34.3]	16.5 [6.8~29.5]	0.723
CRP (mg/dL)	0.50 [0.12~1.40]	0.67 [0.11~2.18]	0.586
DAS28-ESR	4.10 [2.80~5.22]	4.10 [2.86~4.83]	0.982
DAS28-CRP	3.66 [2.62~4.79]	3.79 [2.79~4.66]	0.7
Active RA	53 (66.2%)	32 (69.6%)	0.702
WBC (/mm^3)	6,945 [5,750~8,553]	7,580 [6,200~9,040]	0.157
Neutrophils (/mm^3)	4,446 [3,460~5,604]	5,505 [3,996~6,513]	0.024
Monocytes (/mm^3)	465.5 [379.5~640.5]	600.5 [403.3~729.3]	0.1
Hb (g/dL)	12.8 [11.9~13.6]	12.6 [11.6~13.7]	0.229
Platelet (x10^3/mm^3)	280 [229~337]	261 [226~345]	0.74
IL-6 (pg/mL)	6.8 [3.2~18.3]	6.1 [2.1~50.4]	0.804
TNF (pg/mL)	2.5 [0.5~3.9]	1.8 [0.0~3.9]	0.304
VEGF (pg/mL)	96.7 [60.6~150.9]	136.0 [83.4~189.2]	0.019

PKM2 (U/mL)	86.5 [47.7~213.3]	124.1 [56.9~255.8]	0.184
Baseline SHS scores	2.0 [0.0~17.8]	10.0 [3.5~33.0]	4.40 x10-4
Erosive RA	43 (53.8%)	35 (75.1%)	0.013
Annual change in SHS scores	0.0 [0.0~0.0]	2.2 [1.4~3.5]	2.10 x10-23
PD dose (mg/day)	0.0 [0.0~0.0]	2.2 [1.4~3.5]	0.037
HTN	13 (16.2%)	16 (34.8%)	0.017
Osteoporosis	10 (12.5%)	13 (28.3%)	0.027

Values are expressed as numbers (percentages) or median [25~75 percentiles]. PGVAS, Patient global visual analogue scale; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; DAS28, disease activity score in 28 joints; WBC, White blood cells; Hb, hemoglobin; SHS, Sharp van der Heijde score; PD, prednisolone.

Table 6. Multivariable logistic regression analysis of radiographic progression

	B	SE	Exp(B)	p value
Patients with disease duration ≤12 months (Early RA)*				
PKM2	0.007	0.003	1.007 [1.001~1.012]	0.012
Patients with age ≥60 years (Elderly-onset RA)†				
High PKM2	1.522	0.689	4.583 [1.189~17.675]	0.027

* adjusted with age, age at onset, absolute neutrophil counts, VEGF, and menopause;
† adjusted with age, high TNF-α, high VEGF, hydroxychloroquine use, methotrexate use, and erosive disease at baseline.

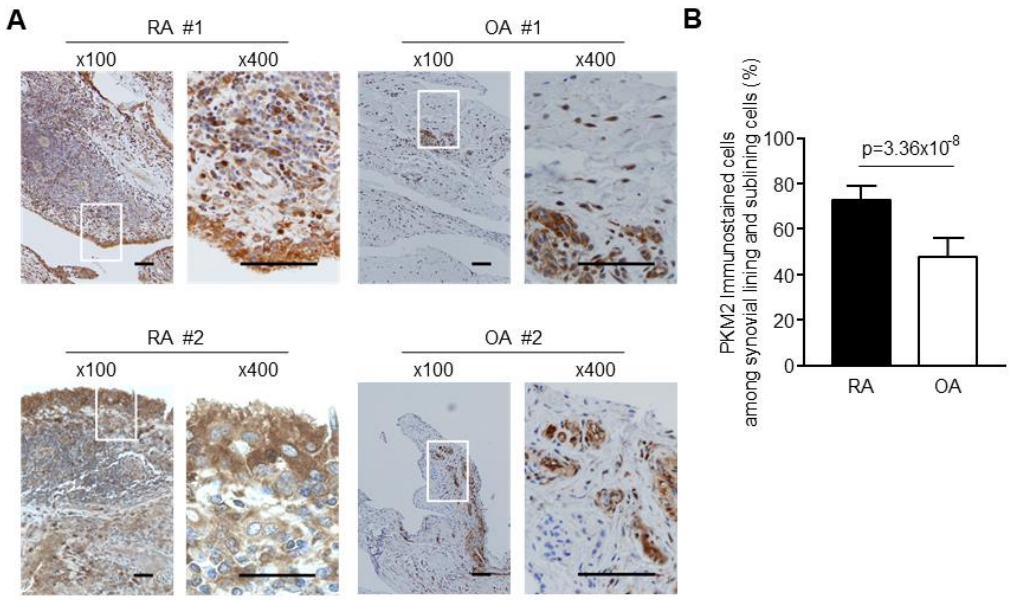

Figure 1. Immunohistochemical staining for PKM2 in synovial tissues. (A) Representative light-microscopy photographs showing that PKM2 expression was increased in synovial tissues from two rheumatoid arthritis (RA) patients. PKM2-positive cells were detected in the lining layer, sublining layer, and lymphoid follicular area of RA synovium. Vascular endothelial cells were also PKM2-positive. White boxes in the left panel (original magnification, 100×) indicate the magnified regions shown in the right panel (original magnification, 400×). (B) Semi-quantitative analysis of the expression of PKM2 in synovial tissues of 3 elbow RA, 2 knee RA, and 3 knee OA. When comparing the number of PKM2-positive cells, the fraction of PKM2-positive cells was significantly higher among synovial lining and stromal cells of RA synovial membrane. P values were calculated by student's t-test. Scale bars=200 μm.

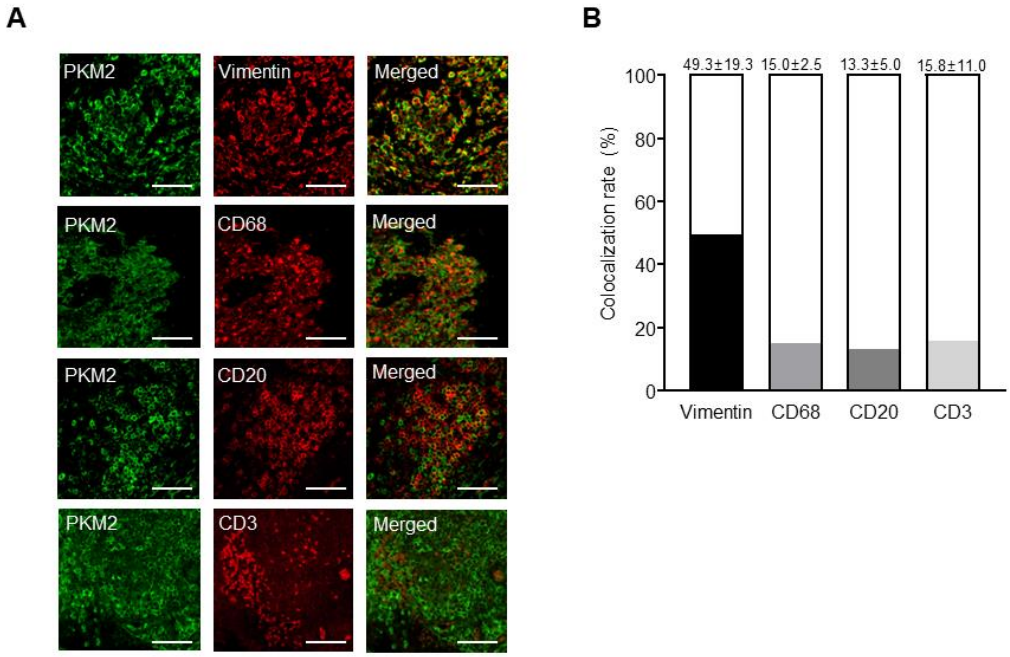

Figure 2. Double immunofluorescence staining for PKM2 and cell-specific markers. (A) Representative confocal images showing PKM2 (green) expression in vimentin-positive (synovial fibroblasts), CD68-positive (macrophages), CD20-positive (B cells) and CD3-positive (T cells) cells (original magnification, 400×). (B) Colocalization analysis of each cell-specific marker in PKM2-positive cells (n=4). Colocalization rates were estimated using R-total values of colocalization threshold of Image J. P values were calculated by student's t-test. Scale bars=100 μm.

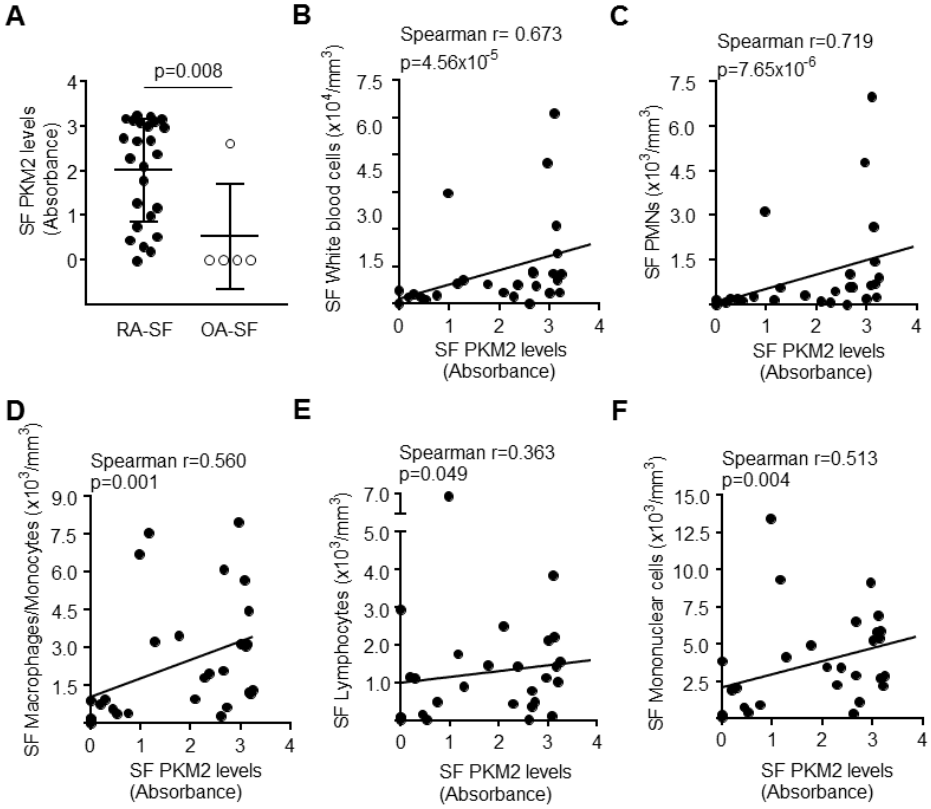

Figure 3. PKM2 levels in synovial fluid (SF) samples and its correlation with the counts of inflammatory cells. (A) SF PKM2 levels were significantly upregulated in rheumatoid arthritis (RA) patients, compared to osteoarthritis (OA) patients. SF PKM2 levels were significantly positively related with the counts of SF inflammatory cells; total white blood cells (B), polymorphonuclear neutrophils (PMN, C), macrophages/monocytes (D), lymphocytes (E), and mononuclear cells (F). P values and correlation coefficients were calculated by Spearman correlation analysis.

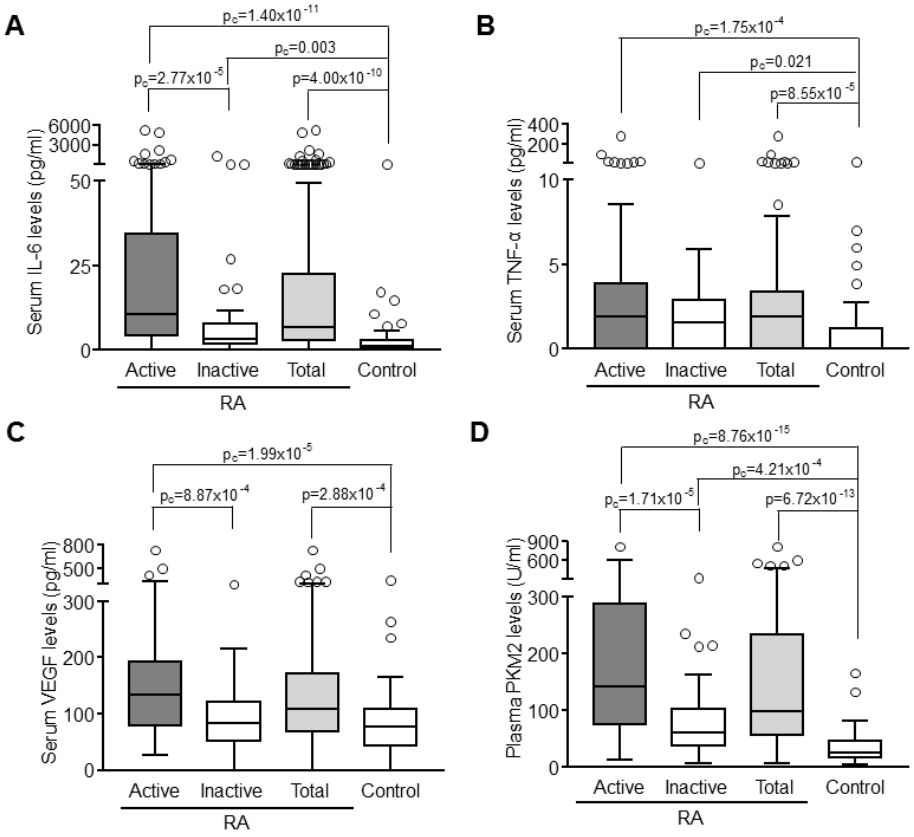

Figure 4. Circulating concentration of PKM2, IL-6, TNF-α, and VEGF in rheumatoid arthritis patients and controls. RA patients were subdivided into active and inactive subgroups according to their DAS28 scores. Circulating IL-6 levels (A), circulating TNF-α levels (B), circulating VEGF levels (C), and circulating PKM2 levels (D) were significantly increased in active RA patients, compared to inactive RA patients and control. Corrected Pc values were calculated by Mann-Whitney U test with the Bonferroni correction.

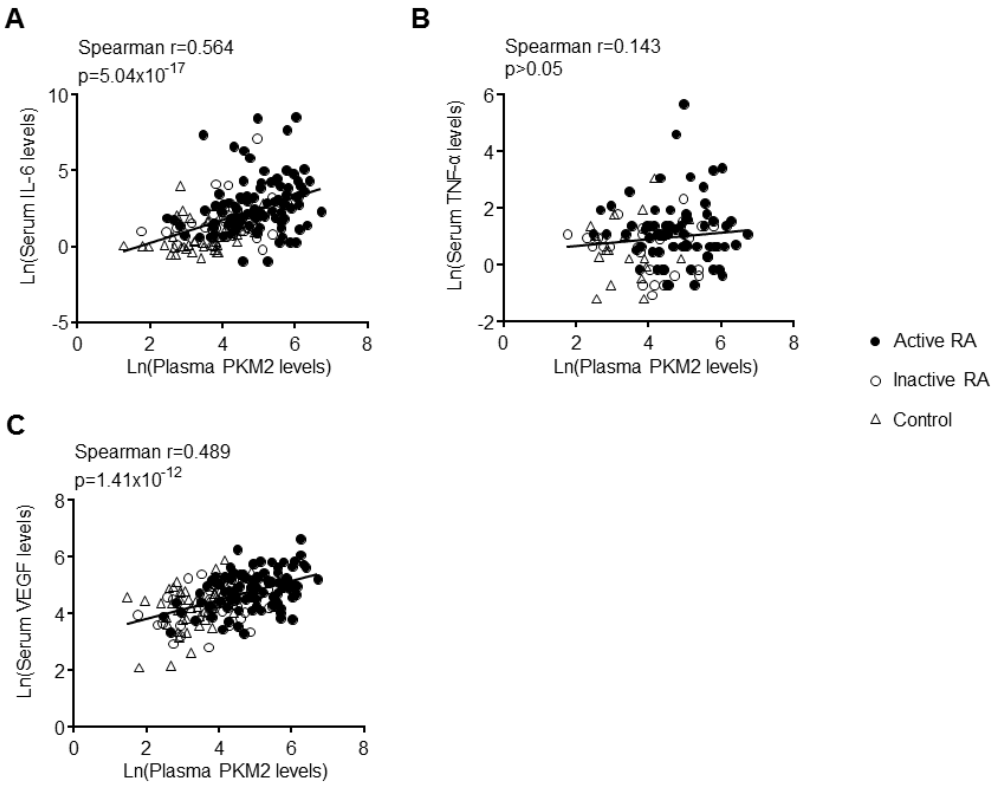

Figure 5. Correlations of plasma PKM2 levels with serum IL-6, TNF-α, and VEGF levels. Their levels were presented as log values in the graphs, but the statistical analysis was performed using original values. Plasma PKM2 levels were significantly positively correlated to serum IL-6 (A) and VEGF levels (C), but not correlated to serum TNF-α levels (p>0.05) (B). P values and correlation coefficients were calculated by Spearman correlation analysis.

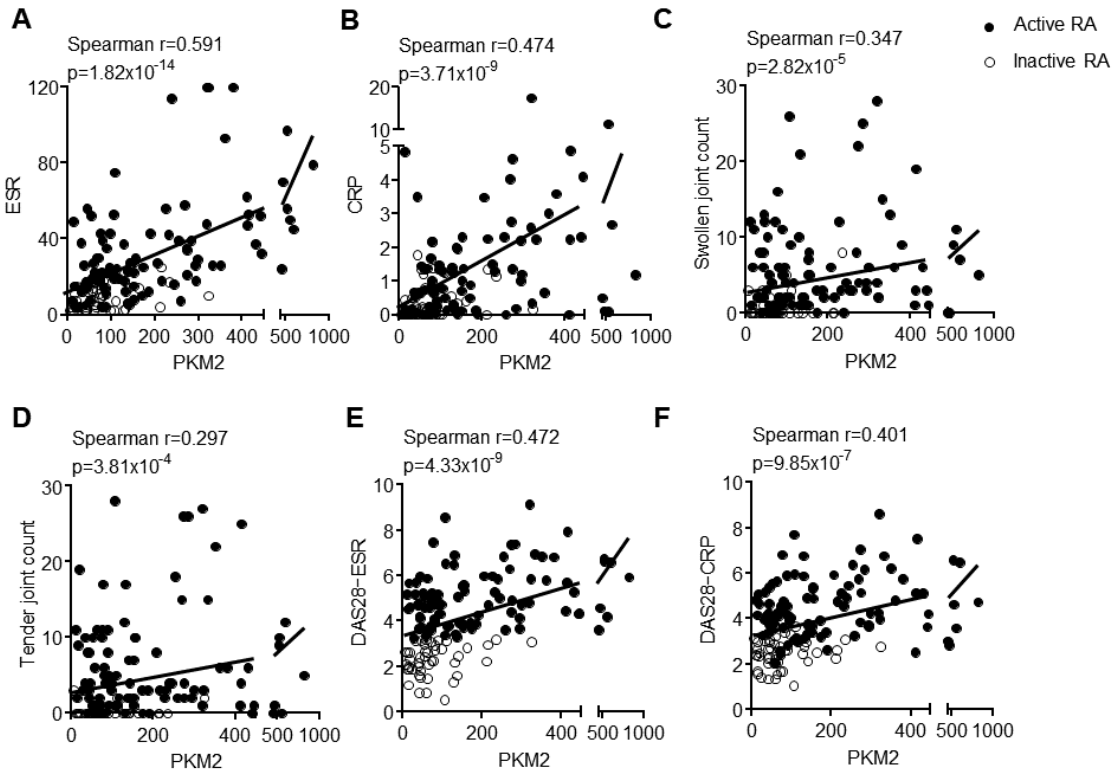

Figure 6. Bivariate correlations between plasma PKM2 levels and disease activity indices in RA patients. Plasma PKM2 levels were significantly positively related with ESR (A), CRP (B), swollen joint count (C), tender joint count (D), DAS28-ESR (E), DAS28-CRP (F). All p values <0.0001. P values and correlation coefficients were calculated by Spearman correlation analysis.

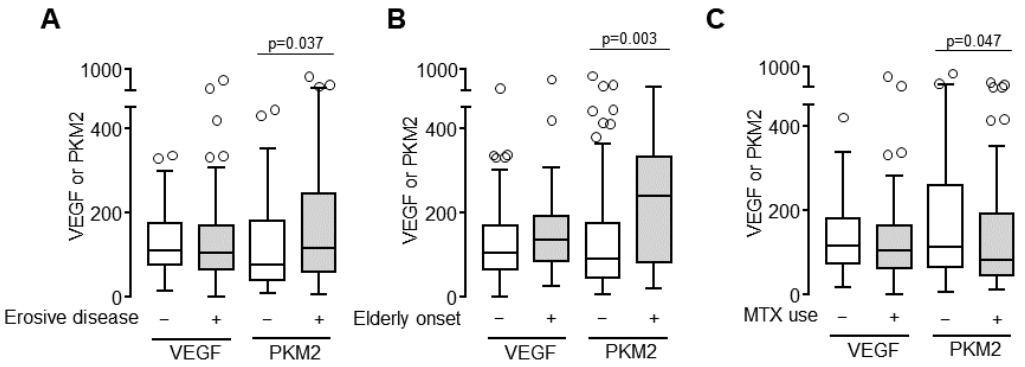

Figure 7. Comparison of plasma PKM2 levels in rheumatoid arthritis (RA) subgroups according to erosive disease, methotrexate (MTX) usage, or age at disease onset. Plasma PKM2 levels were significantly upregulated in RA patients with erosive disease (A) or elderly onset (B) and RA patients who took MTX (C), but serum VEGF levels were comparable. Elderly onset was defined as onset age ≥60 years. P values were calculated by Mann-Whitney U test.

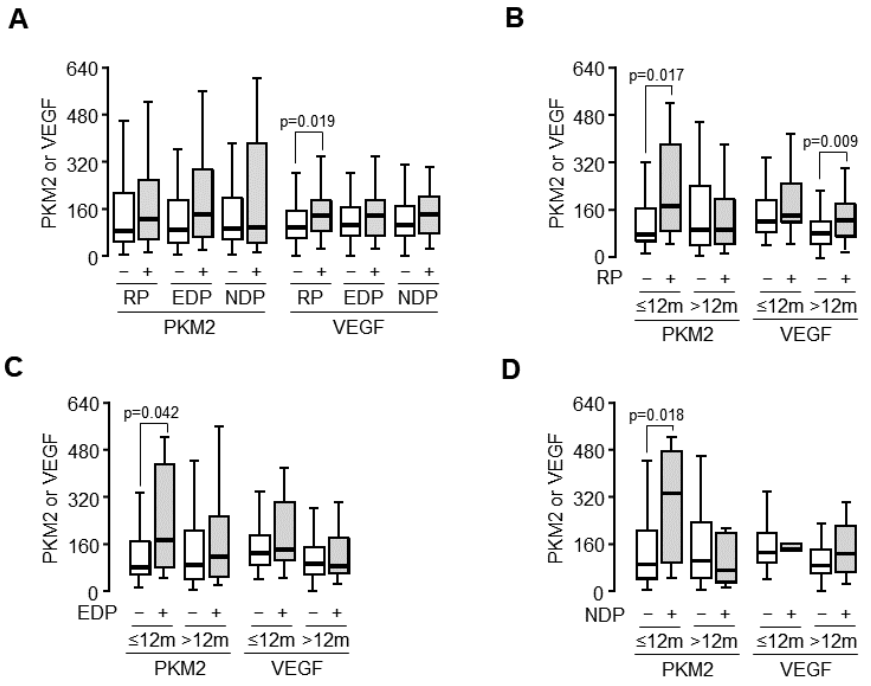

Figure 8. Comparison of plasma PKM2 levels in radiographic progressors and non-progressors in RA patients. (A) Plasma PKM2 levels were not significantly upregulated in RA patients with radiographic progression (RP), erosive disease progression (EDP), narrowing disease progression (NDP), compared to RA patients without them. When patients were divided into subgroups according to the disease durations, plasma PKM2 levels were upregulated in early RA patients with RP (B), EDP (C), NDP (D), but serum VEGF levels were not. P values were calculated by Mann-Whitney U test.

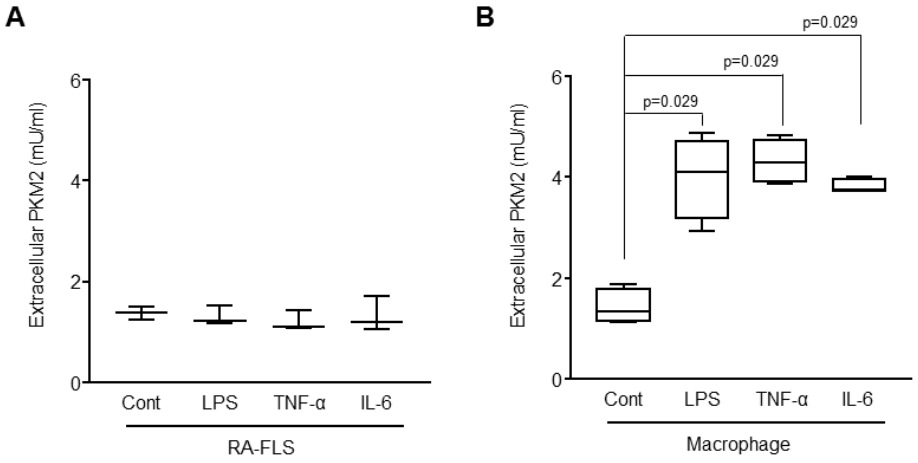

Figure 9. Extracellular PKM2 release from activated RA-FLSs and macrophages. (A) When PKM2 levels were measured in conditioned media, extracellular PKM2 levels were not increased when RA-FLSs were stimulated with LPS, TNF-α, or IL-6 (n=3). (B) However, 24 h after treated with these inflammatory mediators, activated macrophages (n=4) actively released PKM2 in the culture supernatant. P values were calculated by Mann-Whitney U test.

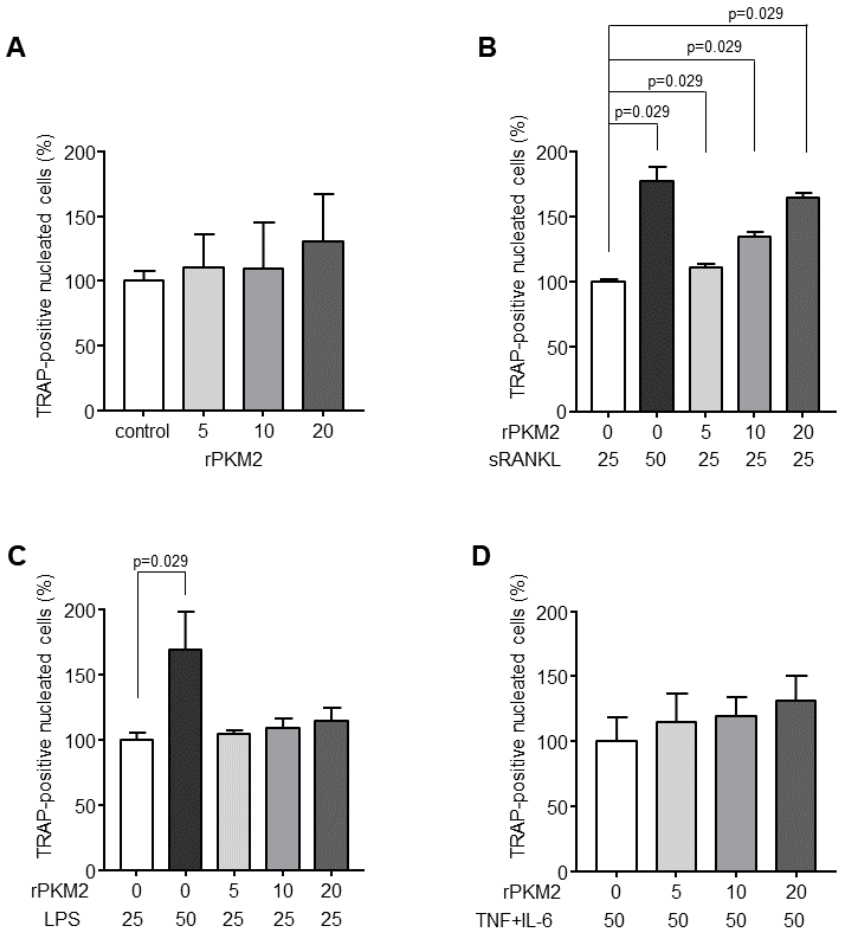

Figure 10. Osteoclast differentiation enhanced by recombinant PKM2 and osteoclastogenic stimuli. (A) When stimulated with PKM2 alone, the number of differentiated osteoclasts was not significantly changed. (B) In the existence of suboptimal RANKL dose (25 ng/mL), PKM2 dose-dependently significantly enhanced osteoclastogenesis ($p < 0.001$ by the trend test). (C) When combined with suboptimal LPS dose (25 ng/mL), also PKM2 dose-dependently increased the numbers of osteoclasts ($p < 0.01$ by the trend test). (D) Although the treatment of PKM2 plus TNF-α/IL-6 didn't show an significant increment in the number of osteoclasts, in vitro osteoclastogenesis tended to be higher with increasing concentrations of PKM2 ($p < 0.05$ by the trend test). P values were calculated by Mann-Whitney U test.

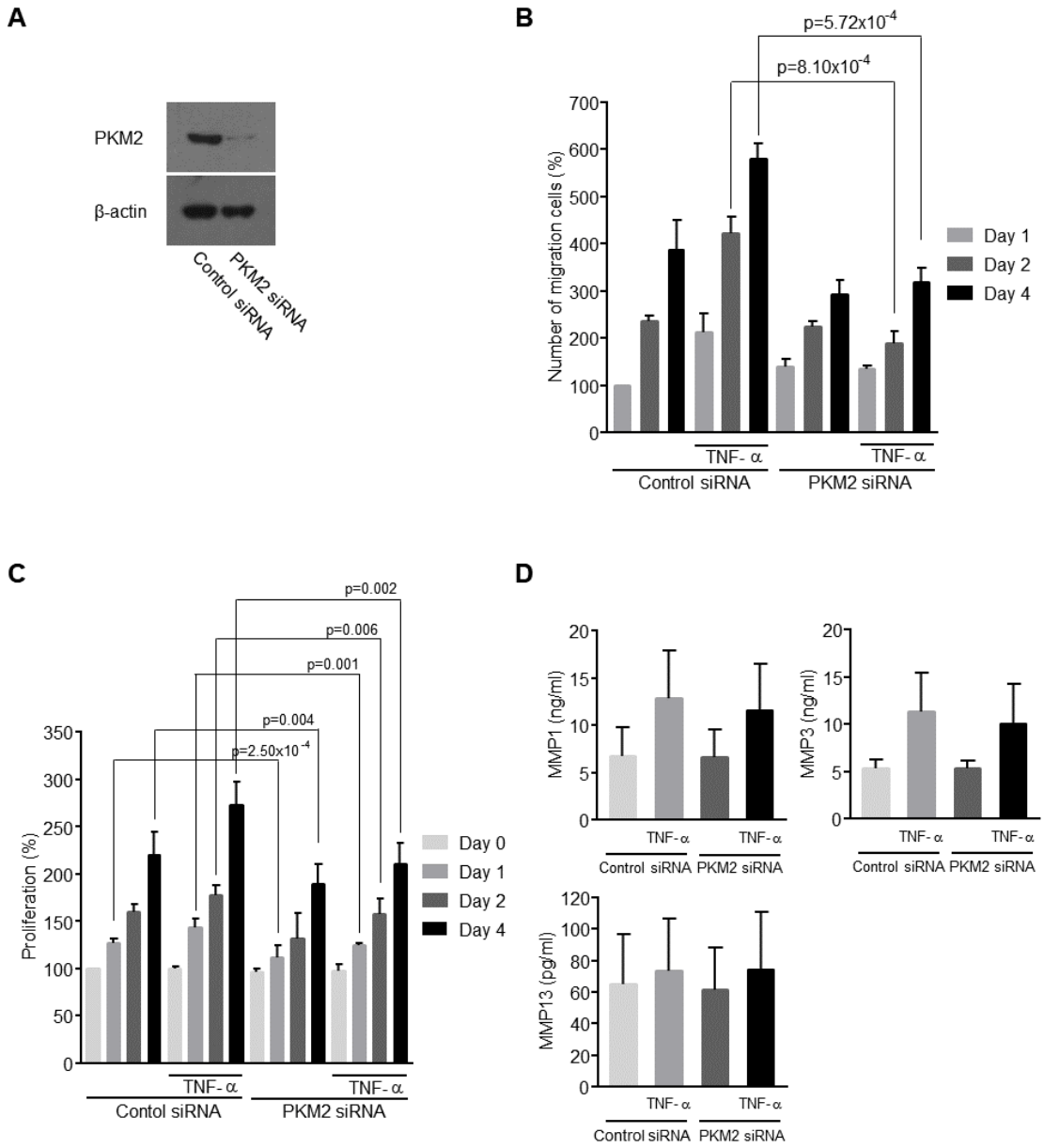

Figure 11. The effect of PKM2 knockdown on migration, proliferation, and MMP production of RA-FLSs. (A) Immunoblot analysis showed siRNA treatment successfully downregulated PKM2 expression in RA-FLSs. (B) After the PKM2 knockdown, TNF-α induced 2-dimensional migration of RA-FLSs was significantly decreased. (C) With or without stimulation with TNF-α, PKM2-knockdowned RA-FLSs showed a significant suppression of cellular proliferation. (D) However, MMP-1, MMP-

3, and MMP-13 production were not significantly affected by siRNA knockdown of PKM2. P values were calculated by student's t-test.

4. Discussion

This thesis provided new findings about PKM2 with regard to RA. It was identified that PKM2 expression was upregulated in RA synovial tissues, especially in mainly RA-FLSs, and was increased in the SF and peripheral circulation in patients with RA. PKM2 levels in the SF and plasma were significantly correlated with a variety of inflammatory markers or disease activity indices. Additionally, plasma PKM2 levels were associated with radiographic progression in early RA patients. The clinical observations are considered to be linked to the in vitro results from the experimental parts of this thesis. Extracellular PKM2 could be released from macrophages, when activated by proinflammatory cytokines or a toll-like receptor (TLR) 4 ligand, and could enhance osteoclastogenesis even under the suboptimal concentrations of classical osteoclastogenic molecules including RANKL. Besides, intracellular PKM2 was critically involved in regulating cellular proliferation and migration of RA-FLSs as a mediator of joint destruction.

Although the RA treatment strategy including biologic agents has been greatly improved in the past two decades, most cohorts reported that work disability remains high at 23 to 37% about 10 years after the onset of RA [53-56]. Even in an early RA cohort, 10% lost their employment due to RA after 3 year [57]. In this context, work disability is a significant and substantial burden to the society, the families, and RA patients. Work disability results from the complex interaction of a variety of factors including physical or mental status, social conditions, and personal factors. However,

most studies showed that RA patients with work disability have more joint counts involved, more severe radiographic damage, poorer functional status including the health assessment questionnaire (HAQ)-disability [58]. A systematic review described that joint damage progresses constantly over the first 20 years of RA and the correlation coefficients between joint damage and disability are 0.31 to 0.75 [59].

Bone and cartilage damage or joint destruction is the hallmark of RA and can largely result from the cumulative inflammatory burden [60]. This outcome of RA is dependent on the complex cellular interactions of activated immune cells, stromal cells, and osteoclasts. Histopathologically, RA synovium is characterized by dramatic synovial hyperplasia, infiltration into the synovium by lymphocytes, activated macrophages, and other cell types, and intense angiogenesis. The lining layer of RA synovium is also converted into a pannus structure - a hyperplastic synovial lining containing a higher number of activated FLSs and macrophages that invade into cartilage matrix and subchondral bone. Such over-proliferation of synoviocytes and homing and infiltration of immune cells increase oxygen consumption and result in a hypoxic microenvironment. Tissue hypoxia activates hypoxia inducible factor (HIF)-1α and increases the transcription of many HIF-responsive gene such as VEGF and PKM2 [61]. Therefore, hypoxia in the RA joint could reprogram cellular bioenergetic processes to promote glycolysis rather than oxidative phosphorylation. It has been recently revealed that both activated innate and adaptive immune cells are in a shift from the quiescent state to a Warburg phenotype to support their proliferation, production of cytokines, and other important functions such as phagocytosis [62].

In the recent study of Biniecka *et al*. [32], hypoxic synovial tissue had a significantly higher expression of key glycolysis enzymes, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and PKM2, and lower content of adenosine triphosphate (ATP) [32]. Their findings are in line with the IHC results of the present study, showing the number of PKM2-immunostained cells were significantly higher in RA synovium than in OA synovium. Though these proportions might not be representative of the levels in active RA synovitis because the joint-replacement surgery had been done in clinically inactive joints, the proportion of PKM2-positive cells was much higher in FLSs than in immune cells. In RA joints, FLSs transform into cancer-like cells which have a highly proliferative ability with suppression of apoptosis. In fact, it has been reported that PKM2 could suppress apoptosis and promote proliferation and migration of various cancer cells [63, 64]. This study also showed that PKM2 knockdown significantly decreased migration and proliferation of RA-FLSs. RA-FLSs are involved in cartilage destruction through their aggressive proliferation and migration. Recently, several researchers reported that inhibitors of 6-phosphofructo-2-kinase (PFK-2) or hexokinase-2 (HK-2), glycolytic enzymes located upstream of PKM2, modulated the invasiveness of FLSs and attenuated experimental arthritis [65, 66]. Given the previous studies, in conjunction with the current results, PKM2 may become another therapeutic target for RA.

RA-FLSs produce matrix-degrading enzymes, such as MMPs, which also contribute to cartilage destruction in RA patients. When RA-FLSs were stimulated with a TLR2-ligand Pam3CSK4, RA-FLSs significantly induced the expression of MMP-1, MMP-3, MMP-2 and MMP-9 [67]. In addition, TLR2-activation was reported to induce PKM2

nuclear translocation and increase glycolysis in RA-FLSs [31]. Moreover, hypoxia-induced MMP-3 expression is regulated by HIF-1α and PKM2 can promote transactivation of HIF-1 target genes through a direct interaction with HIF-1α [68, 69]. Based on the above findings, it was expected that PKM2 knockdown could reduce TNF-α induced expression of MMPs in RA-FLSs. However, a significant change in the production of MMP-1, MMP-3, and MMP-13 was not observed with PKM2 knockdown in the present study. It may be explained by the recent findings that there are functional differences between RA-FLSs subsets and that highly proliferating and invasive cells have a lower expression of MMPs while low-proliferating cells have their higher expression [70].

Previous studies revealed that PKM2 could be released into the extracellular space from colorectal cancer cells, breast cancer cells, or murine neutrophils although its secretory mechanism remains unknown [24,25,30]. Extracellular PKM2 released from cancer cells could augment the migration through PI3K/Akt and Wnt/β-catenin signaling pathways and enhance the proliferation through EGF receptor activation [24,25]. Zhang *et al*. found that topical application of recombinant PKM2 promoted angiogenesis in cutaneous wound healing assay in a murine model [30]. But, the biological actions of extracellular PKM2 have not been studied in non-malignant human cells. Since about 50% of RA-FLSs expressed PKM2 in RA synovial tissue, it had been expected that transformed RA-FLSs could actively secrete PKM2 as cancer cells. However, even when they were stimulated with several proinflammatory mediators, the levels of extracellular PKM2 were not elevated in conditioned media of RA-FLSs. The

number of macrophages in RA synovium was reportedly to correlate with clinical symptoms and the degree of joint damage [71]. Additionally, Palsson-McDermott *et al*. revealed that LPS increased PKM2 expression in macrophages and PKM2 activator inhibited LPS-induced HIF-1α and IL-1β [72]. Therefore, it was examined whether macrophages could release PKM2 when activated with inflammatory stimuli. And, for the first time, the present study found that two major cytokines in RA pathogenesis, TNF-α and IL-6, and LPS significantly promoted the secretion of extracellular PKM2 from THP-1 differentiated macrophages.

In RA, there are 3 different forms of bone damage: (1) bone erosions at the joint margins by the inflamed synovium, (2) periarticular osteopenia adjacent to the inflamed joints, and (3) systemic bone loss leading to osteoporosis [73]. All forms of bone loss are mediated by the imbalance between osteoclastogenesis and osteoblastogenesis. Osteoclastogenesis is dependent on macrophage colony stimulating factor (M-CSF) as a survival factor and RANKL as a main driver for the differentiation from the mononuclear cells of monocyte/macrophage lineage. RANKL is known to be produced by activated T or B cells and RA-FLSs in RA synovial tissue [73]. Notably, Danks *et al*. showed that RANKL expression on FLSs is predominantly responsible for the osteoclastogenesis during inflammatory arthritis than T cells using conditional RANKL knockout mice [74]. Especially, in RA-FLSs, RANKL expression is inducible directly by IL-6 and IL-1β and indirectly by TNF-α and IL-17 [75]. Therefore, the reduction in these inflammatory cytokines can decrease the secretion of RANKL from RA-FLSs and lead to the suppression of RANKL-dependent osteoclast differentiation. In in vitro

experiments using RAW264.7 as osteoclast precursors, recombinant PKM2 significantly and dose-dependently enhanced osteoclast formation under the suboptimal levels of RANKL. This finding suggests that extracellular PKM2 released from activated macrophages or neutrophils could augment RANKL-dependent osteoclastogenesis in RA joints. In this regard, it is interesting that plasma levels of PKM2 were significantly elevated in even clinically remitted patient with RA than controls in the current study. It has been described that radiographic progression could develop in about 20% of RA patients with clinical remission due to subclinical synovial inflammation [76-78]. Considering the synergistic effect between PKM2 and low-level RANKL, PKM2 might explain the underlying mechanisms of progressive joint damage in RA patients with clinical remission or low disease activity.

Based on the result demonstrating that PKM2 was secreted from activated macrophages, it is not surprising that PKM2 levels of SF or plasma were markedly elevated in RA patients. Since Eigenbrodt *et al*. developed an ELISA assay to measure serum PKM2 in 1997 [79], circulating or fecal PKM2 has been reported as a potential diagnostic biomarker of cancer, especially gastrointestinal malignancy [80]. But, like other tumor markers, plasma PKM2 levels were elevated in 37% of healthy individuals and 67% of patients with an acute inflammation when used a cutoff level of 15 U/mL [81]. Moreover, Oremek *et al*. published that plasma PKM2 levels were increased in 82% of RA patients, 71% of SLE patients, and 82% of spondyloarthritis patient [35]. With the exception of Almousa *et al*.'s study on IBD [36], no study has investigated the clinical significance of elevated extracellular PKM2 in non-malignant diseases until now. The current study was the first to show that PKM2 levels of SF or plasma were

significantly correlated with inflammatory cell counts such as neutrophils and monocyte/macrophages. Such relationship may be consistent with that these cells are the sources of extracellular PKM2. Additionally, plasma PKM2 levels were significantly associated with elevated inflammatory and disease activity indices in RA patients. These results suggest that plasma PKM2 levels could reflect the inflammatory burden and might be regarded as a novel potential marker of disease activity in RA.

In this study concerning plasma PKM2 levels in RA patients, it was notable that its levels were significantly associated with radiographic progression of early RA patients both on univariate and multivariate analyses. These clinical observations were in line with the experimental findings, which showed that PKM2 could regulate the proliferation and migration of RA-FLSs and could promote RANKL-dependent osteoclast differentiation. VEGF is also released from activated RA-FLSs and macrophages and the expression of both VEGF and PKM2 is regulated by HIF-1α [82].

Furthermore, serum VEGF levels were reported to be elevated in RA and associated with the development of radiographic damage after 1 year [83]. Moreover, extracellular PKM2 may be involved in angiogenesis [30]. Therefore, it was quite understandable that circulating PKM2 and VEGF levels were highly correlated with each other in RA patients. But VEGF levels were elevated in established RA with radiographic progression but PKM2 levels were upregulated in early RA with radiographic progression. This finding is not readily explainable with data in the present study and this phenomenon requires further study to replicate the observation and to interpret correctly.

There are some limitations to this study. First, the effect of PKM2 on joint damage in vivo was not tested even though glycolysis inhibitors significantly suppressed experimental murine arthritis [65, 66]. In contrary to the oncogenic effects of PKM2 in vitro studies, a conditional PKM2 knockout mouse model showed an acceleration of BRCA1-loss-driven breast cancer. Second, the number of RA patients, who provided blood sample, was not enough to generalize. Because of the heterogeneous nature of RA, a larger study is warranted to confirm our results observed in early RA. Nevertheless, this study revealed that plasma PKM2 levels were an independent variable associated with radiographic progression in early RA. Third, it is not known whether cytoplasmic or nuclear PKM2 is involved in the effects of PKM2 knockdown on migration and proliferation in RA-FLS. Fourth, the present study did not study whether activated lymphocytes could be another cellular source of extracellular PKM2.

In conclusion, PKM2 was upregulated in RA synovial tissues and plasma PKM2 levels were associated with inflammatory burden and the progression of radiographic damage. PKM2 was also involved in migration and proliferation of RA-FLSs as well as osteoclastogenesis. These findings suggest that PKM2 is a novel mediator in the pathogenesis of RA and that PMK2 could be a potential target for RA in controlling inflammation and preventing joint damage.

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국문 초록

류마티스 관절염에서 피루브산염키나아제 M2 발현 증가의 의미에 대한 연구

배경 및 목적

악성종양발생과 관련된 당분해경로 효소인 피루브산염키나아제 M2 (Pyruvate kinase M2, PKM2)는 염증반응에 역할하는 물질로 최근 주목을 받고 있다. 본 연구는 류마티스관절염 환자의 활막조직, 활막액 및 혈액에서 PKM2의 발현을 조사하고 PKM2 발현의 임상적 의미를 연구하고자 하였다. 또한 체외 실험을 통하여 류마티스관절염 섬유아세포양 활막세포의 활성 및 파골세포 분화에 미치는 PKM2의 영향을 파악하고자 하였다.

방법

류마티스관절염 및 골관절염 환자의 활막조직에서 PKM2 발현에 대한 면역조직화학과 면역형광법을 조사하였으며, 활막액 검체 (류마티스관절염 환자, n=25; 골관절염 환자, n=5) 및 혈액 검체 (류마티스관절염 환자, n=139; 건강대조군, n=47)에서 효소면역측정법으로 PKM2 농도를 측정하였다. 혈장 PKM2 농도와 류마티스관절염 질병활성도, 방사선학적 관절손상 진행, 염증유발 시토카인 (종양괴사인자, 인터루킨-6, 혈관내피성장인자) 등의 연관성을 조사하였다. 염증유발성 자극으로 활성화된 대식세포 및 류마티스관절염 섬

유아세포양 활막세포에서 PKM2를 세포외로 분비하는 지 관찰하였으며 재조합 PKM2를 이용하여 파골세포분화에 미치는 영향을 연구하였다. 또한, small-interfering RNA 기법으로 PKM2 유전자를 억제한 후 류마티스관절염 섬유아세포양 활막세포의 세포증식과 세포이동의 변화를 관찰하였다.

결과

류마티스관절염 환자군의 활막조직과 활막세포에서 PKM2 의 발현이 유의하게 높았다. 활막액 PKM2 농도도 골관절염 환자군에 비해 유의하게 높았으며 ($p < 0.001$), 활막액내 단핵구를 포함한 염증세포의 수와 유의한 양의 상관관계가 있었다 ($p < 0.01$). 부가적으로, 혈장 PKM2 농도는 류마티스관절염 환자에서 유의하게 증가되어 있었으며 ($p < 0.001$), Disease Activity Score 28-joint 등 염증 정도를 반영하는 여러 임상 지표 ($p < 0.001$) 와 혈액내 인터루킨-6 혹은 혈관내피성장인자 농도 (두 경우 $p < 0.05$) 와 유의한 양의 상관관계가 있었다. 또한, 조기 류마티스관절염 (이환기간 12 개월 이하, 혈장 PKM2 농도의 교차비=1.007) 및 고령에서 발생한 류마티스관절염 (증상발생 60 세 이상, 높은 농도의 혈장 PKM2 의 교차비=4.583) 환자군에서 혈장 PKM2 농도는 관절 손상 진행의 독립적인 예측변인이었다. 지질다당질, 종양괴사인자 및 인터루킨-6 로 활성화된 대식세포에서 PKM2 를 세포외로 분비함이 관찰되었고, 재조합 PKM2 존재하에 농도의존적으로 염증 유도 파골세포분화가 항진되었다 (경향분석에 따른 $p < 0.01$). PKM2 유전자 발현이 억제된 류마티스관절염 섬유아세포양 활막세포는 종양괴사인자 유도 세포증식과 이동이 억제되었다 (두 경우 $p < 0.01$).

결론

PKM2 발현이 류마티스관절염 환자에서 증가함을 확인하였으며 PKM2 발현 증가는 류마티스관절염 염증 및 질병활성도 정도와 비례하며 방사선학적 관

절손상 증가와 관련이 있었다. 세포내 PKM2는 류마티스관절염 섬유아세포양 활막세포의 세포증식과 이동을 조절하고 활성 대식세포에서 분비된 세포외 PKM2는 염증이 있는 관절조직에서 파골세포 분화를 항진시킬 수 있을 것으로 판단된다. 이러한 결과는 류마티스관절염 병태생리에서 PKM2의 새로운 역할을 보여주었으며 관절손상 진행을 예측하는 생체 지표로서의 혈액내 PKM2와 염증을 조절하고 관절 손상을 억제할 수 있는 치료 목표 물질로서의 PKM2를 활용할 수 있을 가능성을 제시한다.

주요어: 피루브산염키나아제, 류마티스관절염, 류마티스관절염 섬유아세포양 활막세포, 활막, 활막액, 관절 손상

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