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Comparison of anti-inflammatory and anti-angiogenic effects of JAK inhibitors in IL-6 and TNFα-stimulated fibroblast-like synoviocytes derived from patients with RA

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Rheumatoid arthritis (RA) involves synovial tissue proliferation, inflammation, and angiogenesis, and contributes to joint destruction. Angiogenesis is a key therapeutic target for the treatment of RA, and Janus kinase (JAK) inhibitors have emerged as a promising therapy. In this study, we compared the inhibitory effects of five JAK inhibitors, including tofacitinib (TOF), baricitinib, peficitinib, upadacitinib, and filgotinib, on interleukin (IL)-6-induced inflammation in RA synovial tissues. All five inhibitors effectively suppressed IL-6-induced inflammatory and angiogenic factors, including vascular endothelial growth factor, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1, by inhibiting the phosphorylation of signal transducer and activator of transcription (STAT)1 and STAT3. Overall, the results suggest that while all five JAK inhibitors are effective in reducing IL-6-induced inflammatory and angiogenic factors, their efficacy may differ owing to specific molecular mechanisms and pharmacological properties.

Keywords Rheumatoid arthritis, JAK inhibitors, STAT, IL-6, TNF-alpha

Rheumatoid arthritis (RA) is characterized by synovial tissue proliferation, inflammation, vasodilation, and angiogenesis, which results in joint destruction[1–3]. This has a negative impact on activities of daily living and quality of life[4].

Angiogenesis is an important therapeutic target in the treatment of RA[5]. Angiogenic mediators such as growth factors, pro-inflammatory cytokines, chemokines, extracellular matrix, and cell adhesion molecules play important roles in RA, including tumor necrosis factor alpha (TNFα), interleukin-6 (IL-6), intercellular adhesion molecule-1 (ICAM1), matrix metallopeptidase-1 (MMP1), vascular endothelial growth factor (VEGF), vascular cell adhesion molecule-1 (VCAM1), and monocyte chemoattractant protein-1 (MCP1)[6]. TNFα and IL-6 play important roles in synovial inflammation and angiogenesis[7], and VEGF acts specifically on vascular endothelial cells and strongly promotes angiogenesis[8]. VEGF, ICAM1, and VCAM1 expression is increased in patients with RA and has been implicated in disease activity[9,10]. MCP1 and MMP1 contribute to RA progression by promoting inflammatory cell infiltration[11,12].

Inflammatory cytokine activation of the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway is a key event in the etiology and progression of RA, and JAK inhibitors have recently received attention as a new oral therapy for RA[13]. Compared with conventional therapies, JAK inhibitors, such as tofacitinib (TOF), baricitinib (BAR), peficitinib (PEF), upadacitinib (UPA), and filgotinib (FIL), significantly reduce pain and inflammatory responses, such as those induced by C-reactive protein[14–18]. The JAK family

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includes four members, JAK-1, JAK-2, JAK-3, and tyrosine kinase 2, which bind to various cytokine receptors and transduce cellular signals[19]. Inflammatory cytokines such as IL-6 induce the phosphorylation of STAT1 and STAT3 via JAK; phosphorylation of STAT1 and STAT3 increases the expression of inflammatory cytokines and exacerbate arthritis[20,21]. JAK inhibitors inhibit the nuclear transcription induced by activated STAT dimers and selectively inhibit pain processing by directly regulating the effects of multiple cytokines[22,23]. However, the mechanism of the JAK/STAT pathway is complex, and there are various combinations of JAK and STAT, and research is ongoing to elucidate its detailed mechanism[24,25]. Five JAK inhibitors have been approved in Japan. TOF inhibits JAK1 and JAK3[26], whereas BAR selectively inhibits JAK1 and JAK2[27]. PEF is a pan-JAK inhibitor that, like other JAK inhibitors, has been shown to be safe[18]. In addition, UPA and FIL are JAK inhibitors with relatively high JAK1 selectivity[14,16]. Thus, the five JAK inhibitors differ in their selectivity and may have different inhibitory effects on JAK/STAT signaling; however, there are no reports on the differences in the inhibitory effects in synovial tissue. This study aimed to compare the inhibitory effects of TOF, BAR, PEF, UPA, and FIL on IL-6-induced inflammation-related factors.

Materials and methods

Preparation of human RA synovium

Preparation of human RA synovium was performed as previously described[28]. Synovial tissues were obtained from six patients with RA undergoing total knee joint replacement surgery. Demographic characteristics of RA patients who provided synovial tissue were summarized in Table 1. All the patients met the criteria outlined in the 1987 Revised Guidelines of the American College of Rheumatology for RA[29]. All samples were acquired in accordance with the ethical principles outlined in the World Medical Association Declaration of Helsinki for Medical Research Involving Human Subjects. The study protocol was approved by the Ethics Committee of Kobe University Graduate School of Medicine (B230035) and all participants provided written informed consent. The medication history, including the use of JAK inhibitors and biological agents, was carefully examined.

Histological analysis

Synovial tissues were snap-frozen, soaked in hexane on dry ice, and embedded in mounting medium. Samples were sectioned at 8 µm using a cryostat (Leica, Wetzlar, Germany). The sections were fixed with 4% paraformaldehyde for 15 min, washed, and stained. The sections were placed on silane-coated glass slides (Dako Japan, Tokyo, Japan) and stained with hematoxylin (Muto Pure Chemicals, Tokyo, Japan) and eosin (Fujifilm, Tokyo, Japan). Images of the sections were obtained using an all-in-one fluorescence microscope (BZ-X700; Keyence, Osaka, Japan) and the histopathological parameters of each sample were evaluated by two separate examiners (YS and KI) in a blinded fashion ($n=6$). The observed lumen formation was quantified according to the Rooney's score subscale (proliferating blood vessels)[30,31]. A comparison was made between the two groups: a group using JAK inhibitors (JAKi group) and a group not using JAK inhibitors (non-JAKi group).

Immunohistochemistry

Immunohistochemical analyses were conducted in two groups (JAKi and non-JAKi) ($n=6$). Immunohistochemistry was performed using fluorescence-labeled primary antibodies against α-smooth muscle actin (αSMA) (1:200; Sigma-Aldrich, St. Louis, MO, US) and VEGF (1:200; Bioss, Boston, MA, US). Images of the sections in vivo were obtained using an all-in-one fluorescence microscope (BZ-X700; Keyence), and positive areas were automatically calculated using a hybrid cell count application (BZ-H4C, Keyence) in the BZ-X Analyzer software (BZ-H4A, Keyence).

Preparation of fibroblast-like synoviocytes derived from patients with RA (RA-FLS) cultures

Preparation of RA-FLS was performed as previously described[28]. Primary synoviocytes were derived from the synovial tissues of patients with RA and cultured. The tissue specimens underwent a process involving mincing and subsequent incubation with trypsin (0.5 mg/mL; Sigma-Aldrich, St. Louis, MO, US) for 15 min at 37 °C. Subsequently, the synovium was treated with Dulbecco's modified Eagle's medium (DMEM; Gibco/

	Patient identifier	Sex	Age (years)	Age at onset of RA (years)	Disease duration	RF	CCP	CRP	ESR	DAS28 ESR	TJC(28)	SJC (28)	VAS general health	Current disease modifying therapy
JAKi group ($n=3$)	1	Female	27	23	4	pos	pos	2.04	52	6.64	10	9	90	Upadacitinib, Methotorexate, Prednisolone
	2	Male	59	55	4	neg	pos	0.24	14	2.91	1	1	16	Peficitinib, Methotrexate, Prednisolone
	3	Male	80	77	3	pos	NA	3.29	112	6.26	5	4	82	Peficitinib, Prednisolone
Non-JAKi group ($n=3$)	4	Female	70	44	26	pos	pos	3.40	87	5.62	2	2	93	Methotrexate, Prednisolone
	5	Female	82	66	16	neg	pos	0.90	26	4.84	3	2	85	Methotrexate, Sulfasalazine, Prednisolone
	6	Female	85	74	11	pos	NA	1.52	107	6.63	6	6	93	Glimumab, Prednisolone, Tacrolimus

Table 1. Demographic characteristics of RA patients who provided synovial tissue. RF, rheumatoid factor; CCP, cyclic citrullinated peptide; CRP, C reactive protein; ESR, erythrocyte sedimentation rate; DAS28, disease activity score 28; TJC, tender joint count; SJC, swollen joint count; VAS, visual analog score; NA, not available.

Life Technologies, Grand Island, NY, USA) containing 0.2% collagenase (Sigma-Aldrich, St. Louis, MO, US) at 37 °C for 15 h. Dissociated cells were cultured overnight in DMEM supplemented with 10% fetal bovine serum (Lonza, Walkersville, MD, US) and 100 U/mL penicillin–streptomycin. All experiments were performed using cells from passages 3 to 5.

RA-FLS stimulation assay with JAK inhibitors and conventional disease modifying anti-rheumatic drugs (DMARDs)

RA-FLS (1×10^5 cells/well) were seeded in 6-well culture dishes and pre-cultured in DMEM at 37 °C for 24 h. The JAK inhibitors TOF, BAR, PEF, UPA, FIL (Cayman Chemical, Michigan, USA) or Bucillamine (BUC) (Tront Research Chemicals, Toronto, Canada), as a representative of conventional DMARDs, dissolved in dimethyl sulfoxide (DMSO) (Nacalai tesque, Kyoto, Japan) were added to the medium, followed by stimulation with TNFα (10 ng/mL) (R&D Systems) or IL-6 (100 ng/mL) and soluble IL-6 receptor (sIL-6R) (100 ng/mL) (R&D Systems, Minneapolis, MN, US) 2 h after the addition of the JAK inhibitors or BUC. The concentration of each JAK inhibitor and BUC was determined based on estimated blood concentrations in the clinical dosage when stimulating with TNFα: TOF (0.3 μM), BAR (0.3 μM), PEF (1.0 μM), UPA (0.3 μM), FIL (2.0 μM), and BUC (2.0 μM). Lower and higher doses of JAK inhibitors than those used clinically were also studied when stimulating with IL-6 and sIL-6R: TOF (0.1, 0.3, 1.0 μM), BAR (0.1, 0.3, 1.0 μM), PEF (0.1, 1.0, 5.0 μM), UPA (0.1, 0.3, 1.0 μM), or FIL (0.1, 2.0, 10.0 μM). DMSO was administered as a control instead of a stimulant.

Quantitative reverse transcriptase–polymerase chain reaction (RT–PCR)

After stimulation for 24 h, RNA were extracted from the cells using a QIA shredder and RNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol as previously described[32]. Briefly, 1 μg of total RNA was reverse-transcribed to first-strand cDNA using 1.25 μM oligo-dT primer in 40 μL PCR buffer II containing 2.5 mM $MgC1_2$, 0.5 mM dNTP mix, 0.5 U of RNase inhibitor, and 1.25 U of murine leukemia virus reverse transcriptase (PerkinElmer/Applied Biosystems, Foster City, CA, USA), at 42 °C for 1 h. The relative mRNA expression levels of inflammatory (ICAM1 and MMP1) and angiogenic (VEGF, VCAM1, and MCP1) factors were analyzed using SYBR Green RT–PCR on an ABI Prism 7500 sequence detection system (Applied Biosystems, Foster City, CA, USA). Relative gene expression was normalized to that of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using the comparative cycle threshold (Ct) method. The difference between the mean Ct values of the gene of interest and those of the housekeeping gene is denoted as ΔCt, whereas the difference between the ΔCt and the Ct value of the calibrator sample is denoted as ΔΔCt. The $\log_2$ (ΔΔCt) value gives the relative level of gene expression. The primer sequences used to detect human ICAM1, MMP1, VEGF, VCAM1, MCP1 and GAPDH are listed in Table 2.

Western blot analysis

After stimulation with IL-6 and sIL-6R for 24 h, Western blot analysis was performed as previously described[32]. Briefly, the cells were washed with Tris-buffered saline with Tween-20 (TBST) and lysed in a buffer containing 25-mM Tris, 1% Nonidet P-40, 150 mM NaCl, 1.5 mM ethylene glycol tetraacetic acid, and protease/phosphatase inhibitor mix (Roche Diagnostics, Basel, Switzerland). The lysates were centrifuged at 15,000×g for 10 min at 4 °C to remove cellular debris. Next, the cellular debris-free lysates were collected and mixed with 4x electrophoresis sample buffer; 15 μL of cell lysates (1.0×10^7 cells/mL) were electrophoresed on a 7.5–15% sodium dodecyl sulphate-polyacrylamide gradient gel (Biocraft, Tokyo, Japan) and electrically transferred onto a polyvinylidene difluoride blotting membrane (GE Healthcare Life Sciences, Little Chalfont, UK). The membrane was blocked with 5% skimmed milk in TBST at 25 °C for 30 min, incubated with antibodies against anti-STAT1, anti-phosphorylated STAT1 (pSTAT1), anti-STAT3, and anti-pSTAT3 (Cell Signaling Technology, Danvers, MA, US) at 4 °C for 12 h, and further incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody at 25 °C for 1 h. The proteins were subsequently visualized using ECL Plus reagent (GE Healthcare Life Sciences, Little Chalfont, UK) on a chemiluminescence analyzer (LAS-3000 mini; Fujifilm, Tokyo, Japan). The expression of the beta-actin protein was detected using a mouse anti-β-actin polyclonal antibody (Sigma-Aldrich, St. Louis, MO, US) as the primary antibody. Protein expression was determined semi-quantitatively using National Institutes of Health ImageJ software and digitally captured images. Four different samples were analyzed in this experiment.

Statistical analysis

Statistical analyses were conducted using the EZR software (Saitama Medical Center, Jichi Medical University, Saitama, Japan)[33]. The results are expressed as the mean ± standard deviation. Statistical comparisons between

Gene	Forward primer sequence (5′–3′)	Reverse primer sequence (5′–3′)
ICAM1	AACCAGAGCCAGGAGACACTG	GCGCCGGAAAGCTGTAGATG
MMP1	CATGCCATTGAGAAAGCCTTCC	AGAGTTGTCCCGATGATCTCC
VEGF	TTCATGGATGTCTATCAGCG	GCTCATCTCTCCTATGTGCT
VCAM1	GGCGCCTATACCATCCGAAA	TATGACCCCTTCATGTTGGC
MCP1	TTCCCCTAGCTTTCCCCAGA	TCCCAGGGGTAGAACTGTGG
GAPDH	ATGGGGAAGGTGAAGGTCG	GGGGTCATTGATGGCAACAATA

Table 2. Primer sequences.

groups were performed using the Kruskal-Wallis test. Post-hoc analysis was performed using the Steel Dwass test. A probability value < 0.05 was considered statistically significant.

Results

JAK inhibitors inhibited angiogenesis and VEGF expression in RA synovial tissues

Hematoxylin and eosin (HE) staining showed that the JAKi group exhibited marked degeneration in the sub-lining and deep lining layers, with decreased lymphocyte infiltration and angiogenesis compared to the non-JAKi group (Fig. 1a). Rooney's subscale (proliferating blood vessels) was significantly lower in the JAKi group than in the non-JAKi group (2.3 ± 1.2 and 7.3 ± 1.2, respectively, $p < 0.05$) (Fig. 1b). Fluorescent and HE staining of αSMA showed inhibition of angiogenesis in the JAKi group (Fig. 2a). Positive areas and signaling intensities of αSMA were significantly lower in the JAKi group than in the non-JAKi group (115138.0 ± 47226.7 and 355301.0 ± 59326.8 μm^2, respectively, $p < 0.001$) (Fig. 2b). Additionally, fluorescent staining showed that VEGF expression was suppressed and positive areas and signaling intensities were significantly lower in the JAKi group than in the non-JAKi group (29299.8 ± 19048.6 and 521010.0 ± 107511.9 μm^2, respectively, $p < 0.001$) (Fig. 2a, b).

JAK inhibitors suppressed the IL-6 + sIL-6R-induced mRNA expression of ICAM1, VCAM1, VEGF, MCP1, and MMP1

The expression of inflammatory and angiogenic factors in response to each JAK inhibitor and BUC is summarized in Table 3. Administration of any JAK inhibitor at estimated blood concentrations suppressed all the inflammatory and angiogenic factors when stimulated with IL-6 and sIL-6R, but no inflammatory and angiogenic factors when stimulated with TNFα. Furthermore, the expression levels of all inflammatory and angiogenic factors decreased with increasing doses of IL-6 and sIL-6R (Fig. 3). No significant difference was observed in the mRNA expression of anti-inflammatory or anti-angiogenic factors between each JAK inhibitors and BUC when stimulated with TNFα. At estimated blood concentrations, administration of TOF did not suppress all the inflammatory and angiogenic factors compared to BUC when stimulated with IL-6 and sIL-6R. In contrast, BAR significantly reduced the mRNA expression of ICAM1 and VEGF. Similarly, PEF, UPA and FIL significantly reduced the mRNA expression of ICAM1, VEGF, VCAM1 and MCP1 (Supplemental Fig. 1).

JAK inhibitors suppressed the IL-6 + sIL-6R-induced expression of pSTAT1 and pSTAT3

All JAK inhibitors significantly suppressed pSTAT1 and pSTAT3 levels compared to those in the control (Fig. 4).

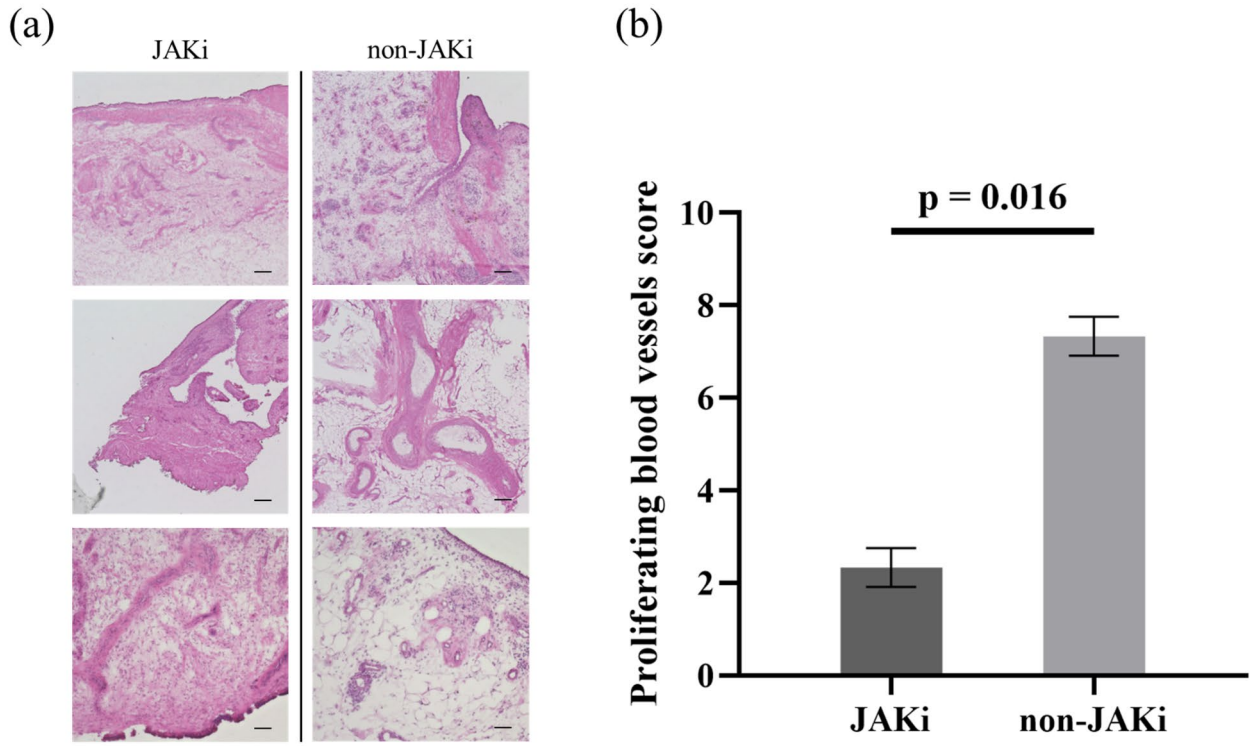

Fig. 1. (**a**) Histological analysis of the synovium. Hematoxylin and eosin staining of a typical specimen after JAKi or non-JAKi treatment. (**b**) Comparison of Rooney's proliferating blood vessel scores of the two groups. $n = 3$ in each group. Scale bars, 200 μm (**a**).

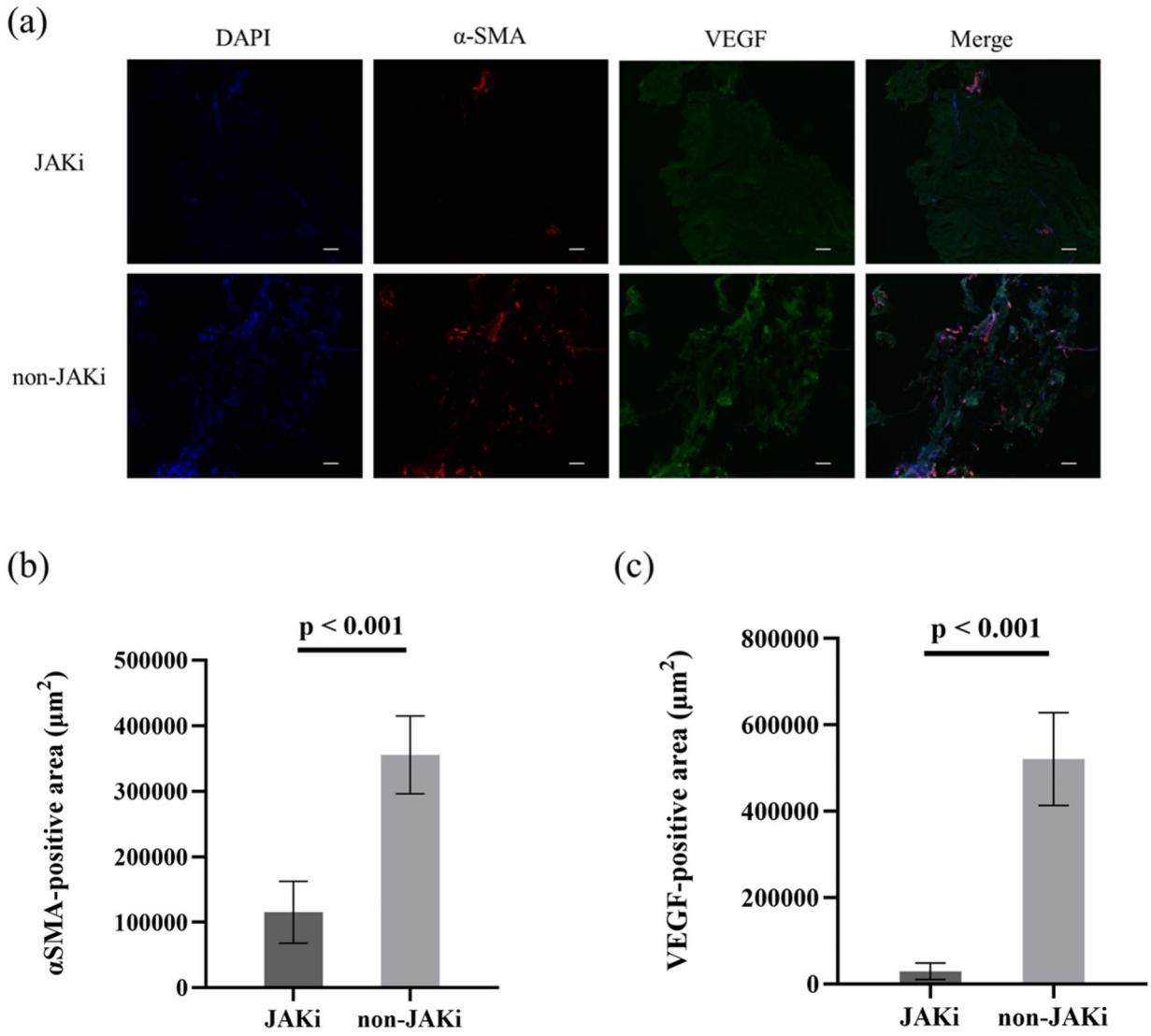

Fig. 2. (**a**) Representative of αSMA (red) and VEGF (green) in the synovium tissue. (**b**, **c**) Quantitative assessment of expression of αSMA and VEGF between the groups. A significant suppression in the expression of αSMA and VEGF was observed in the JAKi group than in the non-JAKi group. Scale bars, 200 μm (**a**). αSMA, α-smooth muscle actin; VEGF, vascular endothelial growth factor.

Discussions

In the present study, we confirmed that JAK inhibitors inhibit angiogenesis and VEGF expression in RA synovial tissue as well as suppress IL-6-induced inflammatory and angiogenic factors in vitro.

A comparison of the anti-inflammatory effects of the five JAK inhibitors (TOF, BAR, PEF, UPA, and FIL) revealed that all of these drugs were more effective than the placebo in treating RA[34–37]. However, the specific differences in efficacy were not statistically significant[34]. These JAK inhibitors vary in their selectivity for different JAK isoforms, with TOF exhibiting the most extensive cytokine suppression via the JAK/STAT pathway[38]. Further studies are required to compare the anti-inflammatory effects of these JAK inhibitors.

In the present study with TNFα stimulation, no significant differences were observed in the suppression of inflammation-related factors between each drug. This may be due to the fact that JAK inhibitors inhibit the JAK/STAT pathway but not inflammation via other pathways induced by TNFα such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and activator protein 1[39]. In contrast to the TNFα stimulation, all five JAK inhibitors suppressed IL-6-induced inflammatory and angiogenic factors, and their inhibitory effect increased in a dose-dependent manner. All five JAK inhibitors suppressed pSTAT1 and pSTAT3 expression. This indicated that at the estimated blood concentrations, all five drugs effectively inhibited IL-6-induced inflammatory and angiogenic factors by inhibiting the phosphorylation of STAT1 and STAT3.

We also compared the anti-inflammatory and anti-angiogenic effects of JAK inhibitors with BUC as a representative of conventional DMARDs. In this study, there was no significant difference in the mRNA expression

		JAK inhibitor dose	Tofacitinib	Baricitinib	Peficitinib	Upadacitinib	Filgotinib	Bucillamine
TNFα	ICAM1	–	0.82 ± 0.17	0.78 ± 0.21	0.81 ± 0.12	0.87 ± 0.10	0.86 ± 0.08	0.80 ± 0.19
	MMP1	–	0.91 ± 0.10	0.85 ± 0.14	0.84 ± 0.11	0.87 ± 0.12	0.83 ± 0.12	0.82 ± 0.17
	VEGF	–	1.01 ± 0.19	0.93 ± 0.20	0.92 ± 0.18	0.93 ± 0.14	0.86 ± 0.14	0.89 ± 0.16
	VCAM1	–	0.97 ± 0.15	0.96 ± 0.10	1.04 ± 0.10	1.08 ± 0.07	0.97 ± 0.12	0.99 ± 0.10
	MCP1	–	0.89 ± 0.20	0.86 ± 0.23	0.98 ± 0.21	0.91 ± 0.17	0.80 ± 0.16	0.86 ± 0.21
IL6 and sIL6-R	ICAM1	Low	0.80 ± 0.24	0.70 ± 0.21	0.64 ± 0.15	0.55 ± 0.20	0.95 ± 0.07	–
		Medium	0.72 ± 0.12	0.58 ± 0.21	0.44 ± 0.16	0.41 ± 0.15	0.47 ± 0.13	0.93 ± 0.29
		High	0.48 ± 0.17	0.32 ± 0.13	0.25 ± 0.11	0.29 ± 0.13	0.36 ± 0.09	–
	MMP1	Low	0.98 ± 0.11	0.95 ± 0.20	0.95 ± 0.16	0.88 ± 0.30	0.95 ± 0.09	–
		Medium	0.82 ± 0.15	0.75 ± 0.14	0.67 ± 0.14	0.68 ± 0.22	0.72 ± 0.23	0.86 ± 0.15
		High	0.71 ± 0.15	0.65 ± 0.13	0.57 ± 0.14	0.57 ± 0.17	0.50 ± 0.24	–
	VEGF	Low	0.98 ± 0.21	0.83 ± 0.07	0.99 ± 0.19	0.81 ± 0.28	0.94 ± 0.04	–
		Medium	0.83 ± 0.08	0.66 ± 0.14	0.58 ± 0.10	0.61 ± 0.15	0.66 ± 0.16	0.90 ± 0.11
		High	0.65 ± 0.15	0.54 ± 0.14	0.44 ± 0.08	0.48 ± 0.06	0.43 ± 0.13	–
	VCAM1	Low	1.02 ± 0.27	0.91 ± 0.21	0.83 ± 0.24	0.67 ± 0.17	0.90 ± 0.05	–
		Medium	0.86 ± 0.16	0.77 ± 0.15	0.60 ± 0.16	0.58 ± 0.18	0.60 ± 0.25	0.91 ± 0.12
		High	0.72 ± 0.14	0.72 ± 0.20	0.36 ± 0.06	0.52 ± 0.15	0.27 ± 0.19	–
	MCP1	Low	0.91 ± 0.44	0.75 ± 0.28	0.83 ± 0.39	0.67 ± 0.15	0.78 ± 0.09	–
		Medium	0.63 ± 0.23	0.53 ± 0.27	0.41 ± 0.18	0.40 ± 0.13	0.48 ± 0.13	0.83 ± 0.19
		High	0.49 ± 0.24	0.24 ± 0.13	0.17 ± 0.05	0.21 ± 0.05	0.24 ± 0.10	–

Table 3. Relative expression of each target induced with TNFα or IL-6 and sIL-6R. Values are expressed as the mean ± standard deviation. The concentrations of each JAK inhibitors and bucillamine are as follows; Tofacitinib: 0.1, 0.3, 1.0, Baricitinib: 0.1, 0.3, 1.0, Peficitinib: 0.1, 1.0, 5.0, Upadacitinib: 0.1, 0.3, 1.0, Filgotinib: 0.1, 2.0, 10.0, Bucillamine 2.0 (μM). The concentration medium was determined by reference to estimated blood concentrations in clinical practice.

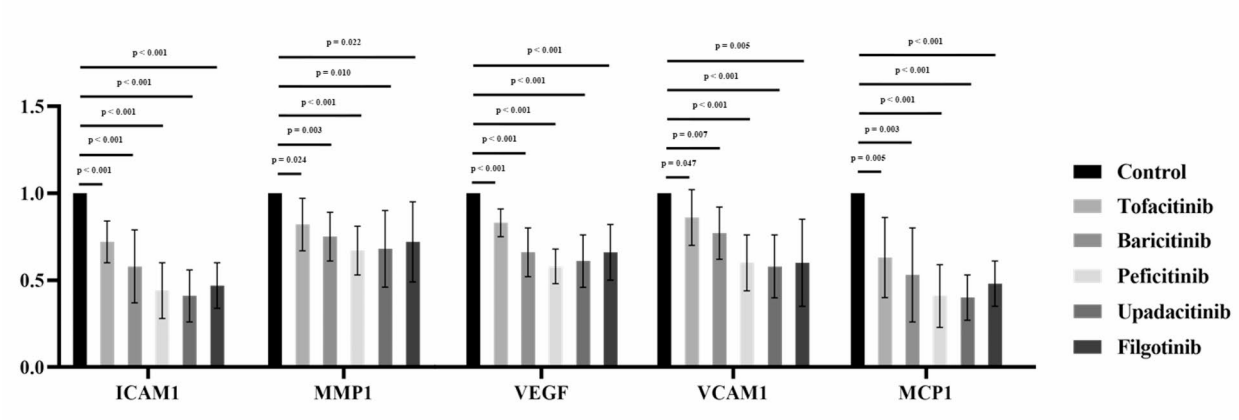

Fig. 3. Expression of the inflammatory and angiogenic factors in estimated blood concentrations in the clinical dosage of each JAK inhibitor. Administration of any JAK inhibitor at estimated blood concentrations suppressed all the inflammatory and angiogenic factors when stimulated with IL-6 and sIL-6R. JAK, Janus kinase; IL-6, interleukin-6; sIL-6R, soluble IL-6 receptor; TOF, tofacitinib; BAR, baricitinib; PEF, peficitinib; UPA, upadacitinib; FIL, filgotinib; ICAM1, intercellular adhesion molecule-1; MMP1, matrix metallopeptidase-1;VEGF, vascular endothelial growth factor; VCAM1, vascular cell adhesion molecule-1; MCP1, monocyte chemoattractant protein-1.

of anti-inflammatory or anti-angiogenic factors between JAK inhibitors and BUC when stimulated with TNFα. Whereas, the inhibitory effects of JAK inhibitors, with the exception of TOF, suppressed the mRNA expression of almost all categories of inflammatory and angiogenic factors significantly more than BUC when stimulated with IL-6 and sIL-6R. Conventional DMARDs such as BUC exert their anti-inflammatory effects mainly by suppressing the production of a wide range of cytokines via the NF-κB pathway, including IL-6 and TNFα[40]. JAK inhibitors, on the other hand, exert their anti-inflammatory effects mainly by suppressing IL-6 via the JAK/STAT pathway and therefore have a different mechanism of action compared to conventional DMARDs. Although there is no previous literature comparing the direct anti-inflammatory effects of JAK inhibitors and conventional

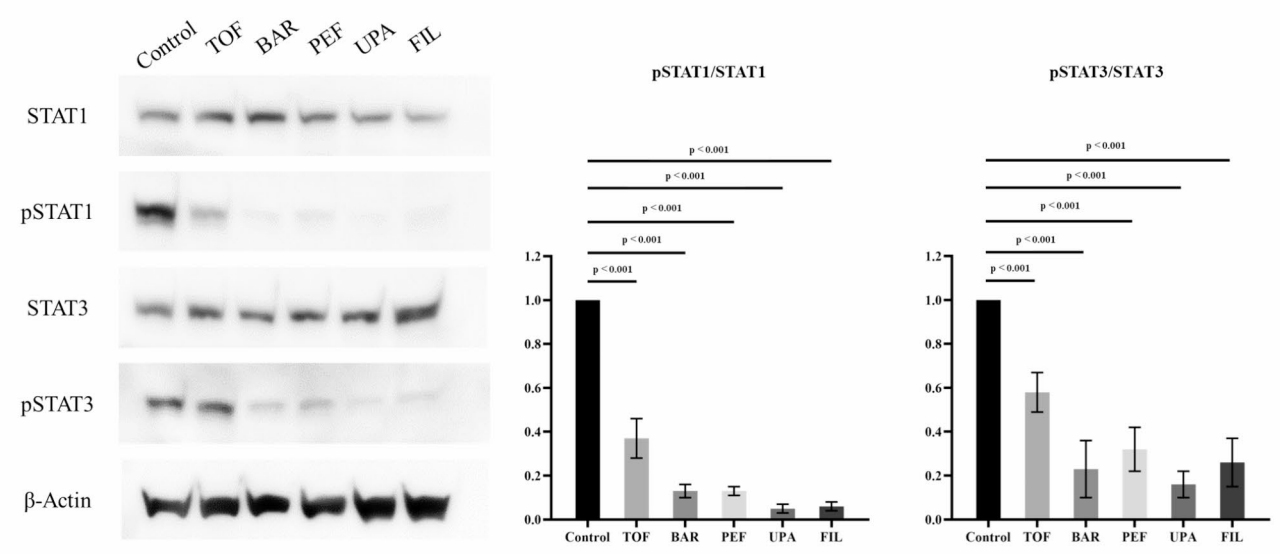

Fig. 4. (**a**) Western blotting was performed to analyze STAT1, pSTAT1, STAT3, pSTAT3, and β-actin. (**b**, **c**) Comparison of the ratios of pSTAT1 to STAT1 and pSTAT3 to STAT1. Protein expression was determined by semi-quantitative analysis of digitally captured images. All JAK inhibitors significantly suppressed pSTAT1 and pSTAT3 levels compared to those in the control. Compared to TOF, BAR, PEF, UPA, and FIL suppressed pSTAT1 and pSTAT3 levels. STAT, signal transducer and activator of transcription; JAK, Janus kinase; TOF, tofacitinib; BAR, baricitinib; PEF, peficitinib; UPA, upadacitinib; FIL, filgotinib.

DMARDs in human synovial tissue, there are reports of better remission rates with JAK inhibitors compared to conventional DMARDs in clinical practice[41]. Although not significantly different in all categories, these results suggest that JAK inhibitors could have a better anti-inflammatory effect than conventional DMARDs.

The present study had some limitations. First, a small population of human synovial tissues was evaluated, and it was difficult to completely standardize aspects such as drug history and years of illness, which may have affected the results and should be considered in future studies. Second, there are insufficient reports on the long-term effects of JAK inhibitors, particularly their side effects. Further data collection and extensive investigations are necessary to draw comprehensive clinical conclusions.

In conclusion, our results suggest that all five JAK inhibitors were effective in reducing IL-6-induced inflammatory and angiogenic factors. This may be due to the specific molecular mechanisms and pharmacological profiles of each drug contribute to the nuanced differences in their efficacy. Further studies focusing on the detailed pharmacodynamics and long-term clinical outcomes of these inhibitors are warranted to fully elucidate their comparative benefits in the treatment of RA.

Data availability

The datasets generated and/or analyzed during the current study are not publicly available but are available from the corresponding author upon reasonable request.

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Y.S.: Data curation, formal analysis, validation, visualization, writing—original draft.K.I.: Formal analysis, investigation, methodology, software, writing—review & editing.S.H.: Conceptualization, project administration, resources, writing—review & editing.K.W., K.A., T.K., M.T., Y.K., N.N.: Data curation.T. Maeda, K.T.: Formal analysis.T. Matsumoto, R.K.: Supervision.T. Matsubara: Supervision, writing—review & editing.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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