Abstract. Rheumatoid arthritis (RA) is an autoimmune disease that causes chronic inflammation in synovial tissues. Hyperplasia of synovial tissues leads to the formation of pannus that invades the joint cartilage and bone, resulting in joint destruction. Fas ligand (FasL), which is a member of the tumor necrosis factor superfamily, contributes to the pathogenesis of autoimmune diseases, including RA. The current study attempted to identify genes whose expressions in rheumatoid fibroblast-like synoviocytes (RA-FLS) were regulated by FasL, using cDNA microarray. A total of four individual lines of primary cultured RA-FLS were incubated either with recombinant human FasL protein or PBS as an unstimulated control for 12 h. Gene expression was detected using a microarray assay. The results revealed the expression profiles of genes in RA-FLS regulated by Fas and investigated the functions of the genes that were regulated. Among the genes in this profile, the mRNA expression changes of the following genes were indicated to be of note using RT-qPCR: Dual specificity phosphatase 6, epiregulin, interleukin 11, angiopoietin-like 7, protein inhibitor of activated STAT 2 and growth differentiation factor 5. These genes may affect the pathogenesis of RA by affecting apoptosis, proliferation, cytokine production, cytokine-induced inflammation, intracellular signaling, angiogenesis, bone destruction and chondrogenesis. To the best of our knowledge, the current study is the first study to reveal the expression profile of genes in RA-FLS regulated by FasL. The data demonstrated that FasL may regulate the expression of a number of key molecules in RA-FLS, thus affecting RA pathogenesis. Further studies of the genes detected may improve the understanding of RA pathogenesis and provide novel treatment targets for RA.

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease that causes chronic inflammation in synovial tissues. Hyperplasia of synovial tissues leads to the formation of pannus, which invades joint cartilage and bone, resulting in joint destruction. Previous reports have indicated that a number of features of transformed long-lived cells are observed in hyperplastic synovial tissues of patients with RA, such as oncogene expression, resistance to apoptosis, and the presence of somatic mutations (1-3). Several explanations for resistance to apoptosis of rheumatoid fibroblast-like synoviocytes (RA-FLS) have been proposed, including deregulation of the Bcl-2 family of proteins critical to the intrinsic apoptosis pathway, deregulation of NF-κB signaling, p53 mutations, and low expression of PUMA; these are all found in RA synovium and FLS, which provides an explanation for the lack of p53-induced FLS apoptosis (4). In addition, it has been reported that hyperproliferation of RA synovial cells involves the abnormal function of death receptors such as Fas and death receptor 3 (5,6).

Fas ligand (FasL)/TNFSF6, a member of the tumor necrosis factor (TNF) superfamily, is expressed by various cell types in arthritic synovium, including T cells, synovial fibroblasts, and macrophages (7), and can promote apoptosis in activated primary B cells, T cells, dendritic cells, and synovial fibroblasts through Fas (8,9). Inhibition of the Fas/FasL pathway contributes to synovial hyperplasia of RA (10-12). Apoptosis through the Fas/FasL pathway in RA synovial cells is inhibited by pro-inflammatory cytokines present within the synovium (8). Meanwhile, the Fas/FasL system may have a pro-inflammatory effect in RA (13,14). Audo et al demonstrated that membrane-bound FasL induces apoptosis as well as proliferation, whereas soluble FasL stimulates only proliferation (13). Moreover, soluble FasL activates
several signaling pathways in RA-FLS, such as extracellular signal-regulated kinase (ERK)-1/2, phosphatidylinositol 3-kinase, caspase 8, and c-jun N-terminal kinase (13). However, the mechanisms and cell targets for these effects are still poorly understood.

Decoy receptor 3 (DcR3)/TR6/M68/TNFRSF6b, a member of the TNF receptor superfamily, binds to 3 ligands belonging to the TNF superfamily: Fasl, LIGHT, and TL1A (15). Overexpression of DcR3 may benefit tumors by helping them avoid the cytotoxic and regulatory effects of Fasl (16,17), LIGHT (18), and TL1A (19). In our previous studies, we demonstrated that DcR3 overexpressed in RA-FLS and stimulated by TNFα protects cells from Fas-induced apoptosis (20). We previously also reported that DcR3 could play a role as a ligand by binding to membrane-bound TL1A in the pathogenesis of RA (21-24).

Furthermore, the expression profiles of genes regulated by DcR3 and TL1A in RA-FLS have been revealed by the use of cDNA microarrays in our previous studies (25,26), suggesting that signaling through DcR3 and its ligands is involved in the pathogenesis of RA. However, the contribution of Fasl, another ligand of DcR3, to the pathogenesis of RA remains to be fully elucidated.

In the current study, we searched for genes whose expressions in RA-FLS were regulated by Fasl using a cDNA microarray. The gene expression profiles revealed a series of genes that may play a significant role in the pathogenesis of RA via the Fasl-Fas signaling pathway. Further study is needed to reveal the difference of the gene expression profiles among the ligands, which might result in better understanding the role of the Fasl/TL1A/DcR3 signaling system in the pathogenesis of RA.

Materials and methods

Isolation and culture of synovial fibroblasts. RA-FLS were obtained from ten patients (samples 1-10) with RA who fulfilled the 1987 criteria of the American College of Rheumatology (formerly, the American Rheumatism Association) (27) during total knee replacement surgery from September 2014 to April 2019. Patients included one male and nine females aged 70.4±8.5 years old. Their C-reactive protein levels and erythrocyte sedimentation rates were 1.4±2.6 mg/dl and 25.6±14.0 mm/h, respectively. As for the drug therapy for RA, five patients were administered oral methotrexate (MTX) (average MTX dose, 8.8±4.6 mg/week), two were administered tacrolimus (1.5±0.5 g/day), two were administered salazosulfapyridine (1.0±0.0 g/day), and two were administered bucillamine (150.0±50.0 mg/day). Prednisolone (PSL) was used to treat three patients (average PSL dose, 4.7±0.6 mg/day). None of the patients had been treated with biological disease-modifying anti-rheumatic drugs (bioDMARDs) or Janus kinase inhibitors.

Synovial samples were collected from the patients, all of whom provided informed written consent to participate in this study in accordance with the World Medical Association Declaration of Helsinki Ethical Principles for Medical Research Involving Human Subjects. The protocol, including consent procedures, was approved by the Kobe University Graduate School of Health Sciences Ethics Committee (approval no. 308). Tissue specimens were minced and digested in Dulbecco's modified Eagle's medium (DMEM; Merck KGaA) containing 0.2% collagenase (Merck KGaA) for 2 h at 37°C with 5% CO₂. Dissociated cells were cultured in DMEM supplemented with 10% fetal bovine serum (Merck KGaA) and 100 U/ml of penicillin/streptomycin (Meiji Seika Pharma Co., Ltd.). Following incubation overnight and the removal of non-adherent cells, adherent cells were further incubated in fresh medium. All experiments were conducted using cells from passages 3 to 4 (20).

Gene expression profiling. Four individual cell lines (samples 1-4) of primary cultured RA-FLS (2x10⁶ cells/well) were incubated with 1,000 ng/ml of recombinant human Fasl protein (R&D Systems) or were left untreated with OPTI-MEM medium (Thermo Fisher Scientific, Inc.) as control for 12 h at 37°C with 5% CO₂. The concentration of Fasl was determined by a preliminary experiment based on the previous reports using Fasl (28,29). After incubation, RNA was extracted with QIAshredder (Qiagen GmbH) and an RNeasy Mini Kit (Qiagen GmbH) according to the manufacturer's protocol. Extraction of total RNA was performed for each sample separately.

Gene expressions were detected by a microarray assay (Human Genome U133 Plus 2.0, GeneChip® 3' Expression Array; Thermo Fisher Scientific, Inc.). The labeling of RNA probes, hybridization, and washing were carried out according to the manufacturer's protocol.

RT-qPCR analysis for mRNA expression of genes regulated by FasL. RA-FLS (samples 5-10) were cultured in six-well plates at a density of 2x10⁶ cells/well with 1,000 ng/ml of FasL or serum-free medium only as a control. RNA was extracted using the QIAshredder and RNeasy mini kits according to the manufacturer's protocols. Oligo (dT)-primed first-strand complementary DNA (cDNA) was synthesized (2 µg total RNA) using a High Capacity cDNA Transcription kit (Applied Biosystems; Thermo Fisher Scientific). Relative expression levels of mRNA encoding DUSP6, EREG, IL-11, ANGPTL7, PIA2, and GDF5 were compared using TaqMan® real-time PCR on a StepOne™ real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). Pre-designed primers and probes for DUSP6 (Hs04329643_ s1), EREG (Hs00914313_m1), IL-11 (Hs01055413_g1), ANGPTL7 (Hs00221727_m1), PIA2 (Hs00915227_m1), GDF5 (Hs00167060_m1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Hs99999905_m1) were obtained from Applied Biosystems (Thermo Fisher Scientific, Inc.). Comparative analysis of each of these genes in individual patients was performed using StepOne™ 2.1 software (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. All amplifications were conducted in duplicate. The mRNA expression levels of each gene were calculated using the comparative threshold cycle (ΔΔCq) method as previously described (30).

Statistical analysis. Values are expressed as the mean ± standard deviation unless otherwise indicated. As for the data analysis of the microarray assay, Avadis 3.3 Prophetic software (Strand Life Sciences) was used for statistical analysis (31).
Differentially expressed genes were extracted by a paired t-test, with P values <0.05 considered to indicate statistical significance and fold-change >2.0, and ordered into hierarchical clusters using the Euclidean algorithm as the distance measure and the complete algorithm as the linkage method.

The data analysis of the RT-qPCR assay was as follows. The Wilcoxon signed ranked test was used to evaluate the differences between mRNA expression levels of genes in the control group and FasL-stimulated group. Statistical analyses were conducted using Statcel (version 3; OMS Publishing, Inc.). P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Microarray analysis (gene expression profiling of RA-FLS stimulated by FasL).** The microarray analysis used in the current study (Human Genome U133 Plus 2.0, GeneChip® 3’ Expression Array) was able to detect the expression of 27,420 genes.

The microarray analysis revealed that FasL upregulates or downregulates the expressions of various genes in RA-FLS. We used the NCBI's UniGene database (https://www.ncbi.nlm.nih.gov/UniGene/clust.cgi?ORG=Hs&CID=55682) to identify the genes. Among the 1039 genes differentially upregulated by FasL, 806 were annotated in the database. Twenty of the 806 genes upregulated by FasL are shown in Table I. Gene annotations of 1190 among the 1518 genes differentially downregulated by FasL were also in the database. Twenty of the 1190 downregulated genes by FasL are shown in Table II. Hierarchical clustering analysis was performed for genes for which expression changes were detected in at least 2 of the 4 samples, which was 247 genes. The results of hierarchical clustering analysis for these 247 genes are illustrated in Fig. 1.

**Functional annotation.** The 246 genes regulated by FasL were classified into categories registered in the David Bioinformatics Database (https://david.ncifcrf.gov/) according to their biological functions. The most significant 10 functional categories were as follows: Transcriptional activator activity, positive regulation of metabolic process, positive regulation of cellular metabolic process, positive regulation of nitrogen compound metabolic process, regulation of phosphorylation, positive regulation of biological process, regulation of phosphate metabolic process, regulation of MAPK cascade, regulation of multicellular organismal process (Table III).

**mRNA expression detected by RT-qPCR.** Based on the microarray assay, we confirmed the mRNA expressions of genes by real-time PCR. Fig. 2 shows the mRNA expression levels of the 3 most upregulated genes. DUSP6 was upregulated 21 times by FasL compared to the control, EREG was upregulated 24 times by FasL compared to the control, and...
IL-11 was upregulated 9 times by FasL compared to the control. Fig. 3 shows the mRNA expression levels of the 3 most downregulated genes. ANGPTL7 was downregulated 0.15 times by FasL compared to the control, PIAS2 was downregulated 0.58 times by FasL compared to the control, and GDF5 was downregulated 0.11 times by FasL compared to the control.

**Discussion**

Genome-wide gene expression cDNA microarrays provide a powerful technique to investigate the pathophysiology of a variety of diseases, including tumors (32-34), immune-mediated diseases (35,36), and inflammatory diseases (37-39). Using microarray assays, we previously revealed the expression profiles of genes in RA-FLS regulated by DcR3 (25) and TL1A (26). Subsequently, based on the profile regulated by DcR3, we investigated the significance of IL-12B p40 (22), tryptophan hydroxylase 1 (24), and centrosomal protein 70 kDa (23) as regulated by DcR3 in RA-FLS in detail. The profile regulated by TL1A included the following noteworthy genes: Spectrin repeat-containing nuclear envelope 1, Fc receptor-like 2, PYD (pyrin domain)-containing 1, cell division cycle 45 homolog, signal transducer and activator of transcription 5B, and interferon regulatory factor 4 (26).

To the best of our knowledge, this is the first study to reveal the expression profiles of genes in RA-FLS regulated by FasL. Among the genes in this profile, the following genes were of note: Dual specificity phosphatase 6 (DUSP6), epiregulin (EREG), interleukin 11 (IL-11), angiopoietin-like 7 (ANGPTL7), protein inhibitor of activated STAT 2 (PIAS2), and growth differentiation factor 5 (GDF5); these genes were all highly regulated by FasL.

DUSP6 regulates CD4+ T-cell activation and differentiation by inhibiting T-cell receptor dependent ERK 1/2 activation (40). It has been reported that DUSP6 promotes endothelial inflammation through the inducible expression of TNF-α-induced intercellular adhesion molecule-1 via nuclear factor-κB, which is independent of ERK signaling (41).

Epiregulin is a growth regulator that belongs to the epidermal growth factor (EGF) family and mediates the dose-dependent increase in proliferation of primary mouse keratinocytes (42). EREG is increased in patients with RA and is associated with the development of IL-6 amplifier activation (43). EREG triggers the temporal regulation of growth factors such as amphiregulin, betacellulin, transforming growth factor (TGF)-α, fibroblast growth factor 2, placental growth factor 2, and tenasin C, contributing to the early phase of inflammation; each growth factor reciprocally regulates EREG in affected tissue during the late phase of inflammatory disease development (44). Secretion of vascular endothelial growth factor-A and EREG from RA-FLS was inhibited upon treatment with the aryl hydrocarbon receptor.
antagonist GNF351, resulting in attenuation of RA-FLS cell migration, along with cytokine-induced RA-FLS cell proliferation (45).

IL-11 signaling appears to be initiated by the binding of IL-11 to IL-11 receptor α chain (IL-11Rα), which then binds gpl30, the signaling unit of the IL-6 cytokine family (46). IL-11 attenuates the inflammatory response through downregulation of proinflammatory cytokine release and nitric oxide production (47,48). IL-11 contributes to RA angiogenesis directly and indirectly. IL-11 promotes endothelial cell migration and tube

Figure 1. Heat map showing the result of hierarchical clustering. The heat map illustrates the expression values mapped to a color gradient from low (blue) to high expression (red). The horizontal dendrogram illustrates the similarity of functions between neighboring genes. The vertical dendrogram shows similarities in gene expression between neighboring samples.
formation mediated through IL-11Rα ligation. Vascular endothelial growth factor and IL-8 produced from IL-11-treated RA-FLS contribute to the indirect effect of IL-11 on angiogenesis (49). In addition, IL-11 plays a key role in osteoclast formation via the gp130/Jak signaling pathway (50).

ANGPTL7 is a member of angiopoietin family that exerts pro-angiogenic activities on endothelial cells. ANGPTL7 expression has been identified in some cancer cells induced by hypoxia (51). ANGPTL7 induces proinflammatory responses in macrophages, including the induction of immune gene expression, the promotion of proinflammatory cytokine secretion, enhanced phagocytosis, and antagonized anti-inflammatory signaling through the P38 MAPK signaling pathway (52). Down-regulation of a positive regulator of inflammation might have a negative effect for inflammation in patients with RA.

PIAS proteins inhibit activated STAT and play important roles in regulating many important cellular events, such as cell survival, migration, and signal transduction in many cell types (53,54). PIAS proteins also modulate the activity of several transcription factors and act as E3 ubiquitin ligases in the sumoylation pathway (54-57). In a similar fashion, down-regulation of a negative regulator of inflammation might have a positive effect for inflammation in patients with RA. Lao et al reported that PIAS3 regulates migration and invasion through the Rac1/PAK1/JNK pathway in RA-FLSs (53).

GDF5 is a member of the TGF-β superfamiley and is most closely related to the bone morphogenetic protein subfamily. GDF5 increases glycosaminoglycan synthesis (58) and cartilage and bone formation (59). GDF5 is present in the synovium membrane and cartilage of patients with RA and is actively involved in the regulation of cartilage maintenance and repair (60). GDF5 is associated with joint destruction in patients with osteoarthritis (61) and RA (62). GDF5 in RA-FLS was
suppressed by IL-1β and had a strong chondrogenic-promoting effect on TGF-β3-induced chondrocyte differentiation in RA-FLS (60).

In the present study, at the first brush, we exhaustively investigated and revealed the gene expression profiles regulated by FasL in RA-FLS by the microarray assay. Secondly, we confirmed the universality of the gene expression pattern by a different method, RT-qPCR assay, using the different samples of RA-FLS from those used for the microarray assay. In order to obtain the pathological homogeneity among the samples as much as possible, the patients who underwent similar clinical features were recruited; who had been treated only with conventional DMARDs, not with biological DMARDs or targeted synthetic DMARDs, and who had their knee joint destructed severely resulting total knee replacement surgery. Therefore, we considered that there were no differences among the 10 samples. The samples 1-4 used for the microarray and 5-10 for the RT-qPCR assay were randomly selected.

The expression profiles of genes regulated by TL1A were elucidated by use of a microarray assay in our previous report (26). TL1A and FasL are bound and inhibited by the common decoy receptor, DcR3. Therefore, clarifying the relationship between the expression profiles of genes regulated by FasL and those regulated by TL1A might help us to better understand the role of the FasL/TL1A/DcR3 signaling system in the pathogenesis of RA. Further study is needed to reveal the relationship between these gene expression profiles.

The limitations of the current study include its small sample size and that it presents gene expression data only. The results of the current study revealed a series of genes whose expression is regulated by FasL in RA-FLS with microarray analysis, and the mRNA expression of some genes of note was confirmed by RT-qPCR assay. However, in addition to the expression analysis of each gene, how the genes regulated by FasL in RA-FLS are involved in the pathogenesis of RA also requires further investigation. In the current study, we aimed to analyze exhaustively the gene expression pattern regulated by FasL in RA-FLS. Therefore, the assay for expression of proteins coded by each gene should also be performed in the further studies.

In conclusion, the current study is the first, to the best of our knowledge, to report the expression profile of genes in RA-FLS regulated by FasL. These data demonstrate that FasL may regulate the gene expression of various key molecules in RA-FLS, thus affecting the pathogenesis of RA, including apoptosis, proliferation, cytokine production, cytokine-induced inflammation, intracellular signaling, angiogenesis, bone destruction, and chondrogenesis. FasL may have pleiotropic actions not only protectively but also detrimentally for RA. Further investigation of the genes detected in this profile may provide a deeper understanding of the pathogenesis of RA and new targets for its treatment.

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Availability of data and materials

The datasets generated and analyzed during the current study are available in the NCBI’s Gene Expression Omnibus (GEO) repository, GEO series accession no. GSE153378 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE153378).

Authors’ contributions

KF conceived and designed the current study, was involved in data collection and analysis, confirmed the authenticity of all the raw data, wrote and gave final approval of the manuscript. YM conceived and designed the current study, was involved in data collection and analysis, confirmed the authenticity of all the raw data and gave final approval of the manuscript. TosM and TomM collected the data and gave final approval of the manuscript. SH conceived and designed the current study, was involved in data collection and analysis and gave final approval of the manuscript. RK conceived and designed the current study and gave final approval of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Kobe University Graduate School of Health Sciences Ethics Committee (approval no. 308). All the participants provided written informed consent to participate in the present study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Rheumatoid arthritis is a disease characterized by the immune system attack on joints. Various studies have been conducted to understand its etiology and potential therapeutic targets. One study by Fukuda K, Miura Y, Maeda T, Hayashi S and Kuroda R, using cDNA microarray analysis, discovered that tumor necrosis factor-like ligand 1A (TL1A) is a TNF-like ligand regulated by tumor necrosis factor receptors. This finding was published in Biomed Rep 1: 1‑5, 2019.

The study by Fukuda K, Miura Y, Maeda T, Hayashi S and Kuroda R focused on the role of TL1A in rheumatoid arthritis synovial fibroblasts. They found that TL1A regulates the expression of various genes involved in the disease, such as genes associated with proliferation and migration of fibroblast-like synoviocytes.

In another study, the roles of other receptors were also explored. For instance, the decoy receptor 3 (DcR3) was shown to regulate the expression of tryptophan hydroxylase 1 in rheumatoid synovial fibroblasts, as reported by Maeda T, Miura Y, Fukuda K, Hayashi S and Kurosaka M in Mol Med Rep 13: 3647‑3652, 2016.

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