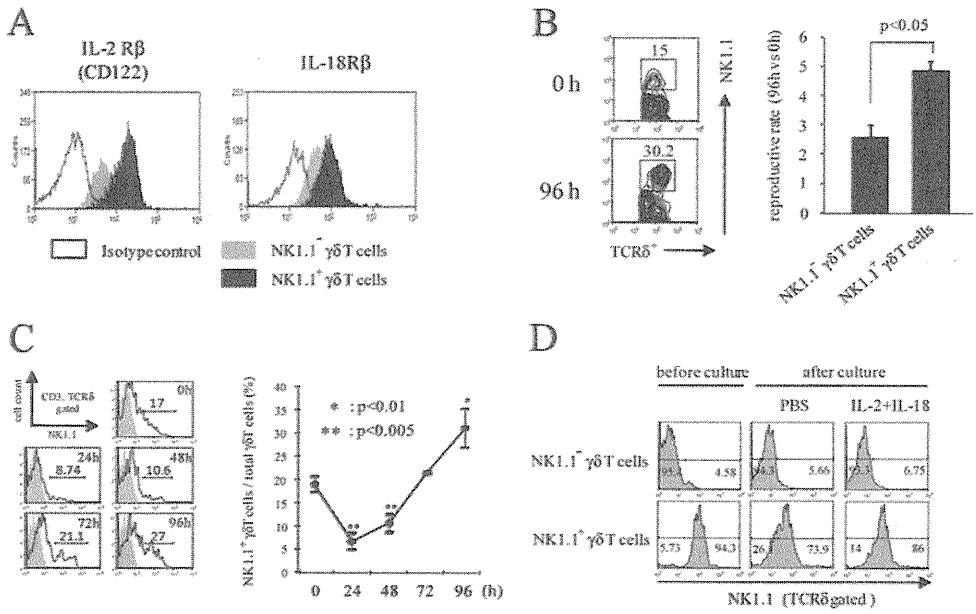

Figure 5. High expression levels of IL-2Rβ and IL-18Rβ and increased proliferation of NK1.1$^+$ γδT cells, after stimulation with IL-18 plus IL-2. (*A*) The expression levels of IL-2 receptor β (IL-2Rβ) and IL-18Rβ on NK1.1$^+$ and NK1.1$^-$ γδT cells were analyzed by flow cytometry. Splenocytes from B6 mice were enriched by a TCRγ/δ$^+$ isolation kit, as described in MATERIALS AND METHODS. Cells were stained with anti-CD3, anti-TCRδ, anti-NK1.1, anti-IL-2Rβ, and anti-IL-18Rβ mAbs, and isotype-matched immunoglobulin. (*B*) Splenocytes from B6 mice were cocultured with IL-18/IL-2 for 96 hours. (*Left*) Representative flow cytometry demonstrates NK1.1$^+$ γδT cells of splenocytes after culturing with IL-18/IL-2 for 96 hours. After culturing, cells were stained with anti-CD3ε, anti-TCRδ, and anti-NK1.1 mAbs for flow cytometry. (*Right*) Reproduction rate of NK1.1$^+$ and NK1.1$^-$ γδT cells under 0-hour and 96-hour culture conditions. Data represent mean ± SEM; $P < 0.05$. (*C*) Splenocytes from C57BL/6 mice were cocultured with IL-18/IL-2 for 0, 24, 48, 72, and 96 hours. (*Left*) The expression of NK1.1 in γδT cells in each group of cultured cells was determined by flow cytometry. Representative flow cytometry demonstrates the expression of NK1.1 in cultured γδT cells with IL-18/IL-2 for 0, 24, 48, 72, and 96 hours. (*Right*) Proportion of NK1.1$^+$ γδT cells among total γδT cells at 0, 24, 48, 72, and 96 hours of culture conditions. Data represent mean ± SEM; $P < 0.05$. Each group of cultured cells was stained with anti-CD3ε, anti-TCRδ, and anti-NK1.1 mAbs. Data are representative of at least two independent experiments. *$P < 0.01$. **$P < 0.005$. (*D*) Splenocytes from C57BL/6 mice were cocultured with IL-18/IL-2 for 0, 24, 48, 72, and 96 hours. Culture cells were analyzed by flow cytometry. Data are representative of at least two independent experiments. (*E*) Sorted NK1.1$^-$ γδT cells and NK1.1$^+$ γδT cells were cocultured with PBS or IL-18/IL-2 for 96 hours, as described in MATERIALS AND METHODS. At 96 hours after coculturing with PBS or IL-18/IL-2, the expression of NK1.1 in γδT cells was analyzed by flow cytometry. Data are representative of at least two independent experiments.

Vγ4$^+$ γδT cells are detected in the blood, lung, liver, spleen, and lymph nodes, whereas TCR Vγ5$^+$ γδT cells are present in the skin, TCR Vγ6$^+$ γδT cells are present in the lung, tongue, and uterus, and TCR Vγ7$^+$ γδT cell are present in IELs. Interestingly, TCR Vγ1$^+$ and TCR Vγ7$^+$ γδT cells produce predominantly Th1 cytokines, although TCR Vγ4$^+$ γδT cells secrete Th1 cytokines (29). Our results showed that NK1.1$^+$ γδT cells as well as NK1.1$^-$ γδT cells infiltrating the lungs of mice with IL-18/IL-2–induced ILD contained all TCR Vγ (Vγ 1, 2, 4, 5, 6, and 7) and TCR Vδ (Vδ 1–8) repertoires, suggesting polyclonal

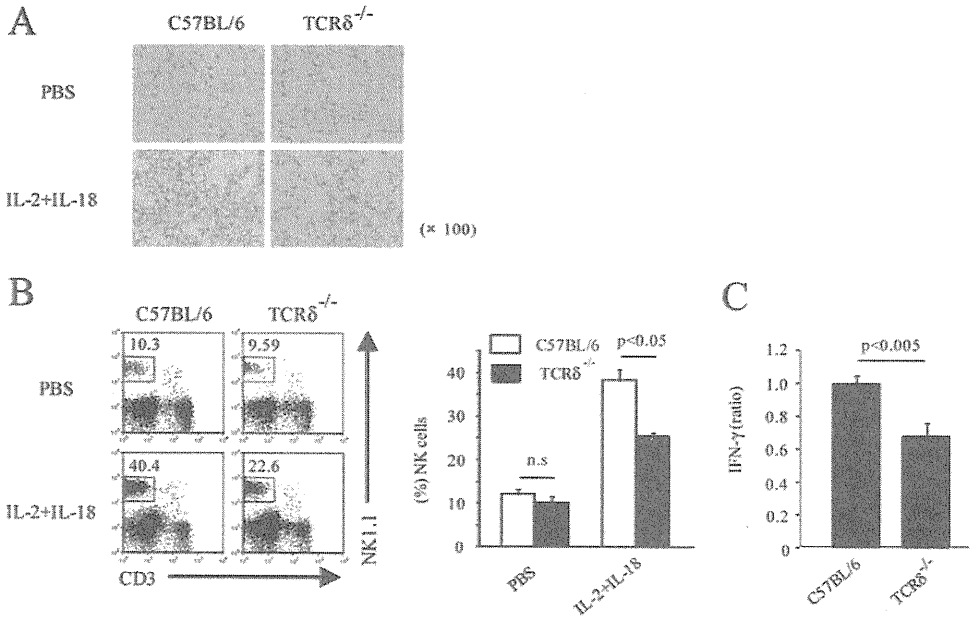

Figure 6. Amelioration of IL-18 plus IL-2–induced ILD, and reduced number of pulmonary NK cells and production of IFN-γ, in TCRδ$^{-/-}$ mice. (*A*) Lung tissues were harvested from B6 and TCRδ$^{-/-}$ mice at 6 hours after treatment with IL-18/IL-2 for 4 days. Lung tissues were stained with hematoxylin and eosin. Original magnification, ×100. (*B*) Lung tissues were harvested from B6 and TCRδ$^{-/-}$ mice at 6 hours after treatment with IL-18/IL-2 for 4 days. Pulmonary lymphocytes were isolated as described in MATERIALS AND METHODS. Pulmonary lymphocytes were analyzed by flow cytometry. Data are representative of at least three independent experiments, and graph shows the pooled data of three experiments. Data represent mean ± SEM; $P < 0.05$. (C) Lung tissues were harvested from B6 and TCRδ$^{-/-}$ mice at 6 hours after injection with IL-18/IL-2 for 4 days. IFN-γ in lung supernatants was measured by ELISA. The ratios of IFN-γ production in the lungs of B6 and TCRδ$^{-/-}$ mice were calculated relative to those of B6 mice. IFN-γ production in the lungs of B6 mice was assumed to be 1.0. Data are representative of at least three independent experiments, and graph shows the pooled data of three experiments. Data are mean ± SEM. $P < 0.05$.

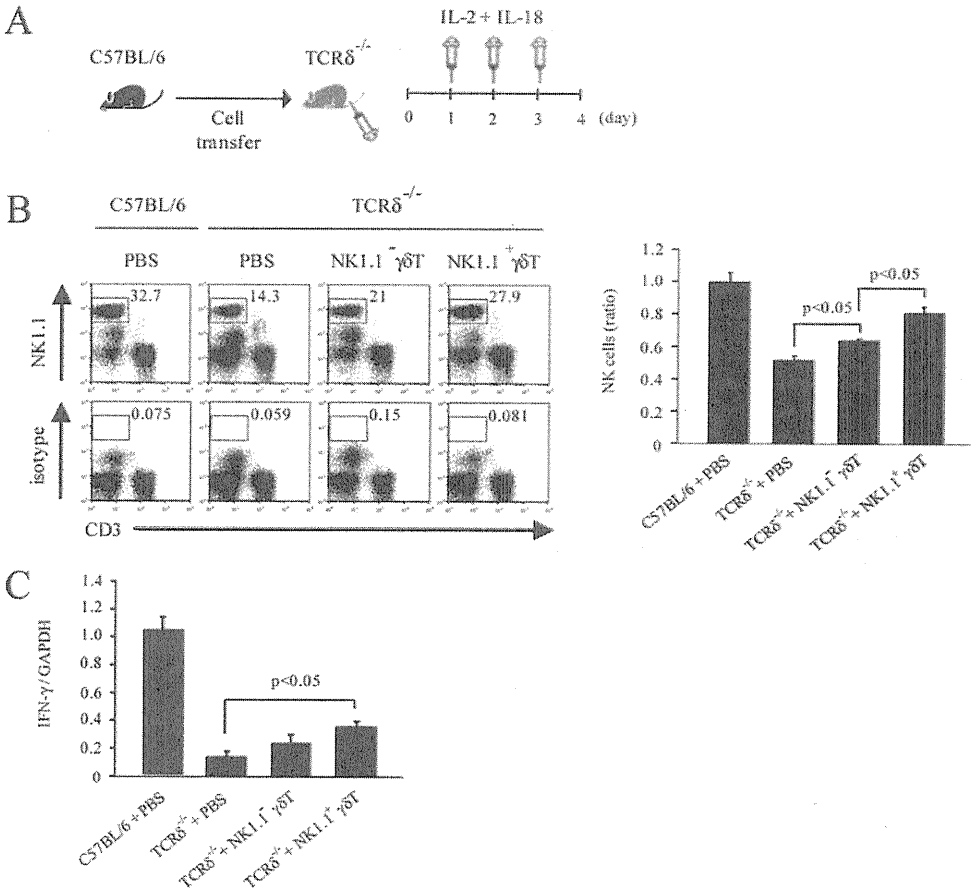

Figure 7. NK1.1$^+$ γδT cells accelerate the severity of IL-18 plus IL-2–induced ILD. (*A*) Naive B6 mice were harvested, and NK1.1$^-$ γδT and NK1.1$^+$ γδT cells were purified from splenocytes, as described in MATERIALS AND METHODS. These cells were transferred into TCRδ$^{-/-}$ mice (2×10^5/mouse). At 24 hours after the transfer, TCRδ$^{-/-}$ recipient mice were treated with IL-18/IL-2 for 3 days. At 24 hours after the last injection, lung tissues were analyzed. (*B*) Three B6 mice received transfers with PBS, and three TCRδ$^{-/-}$ mice received transfers with PBS, NK1.1$^+$, or NK1.1$^-$ γδT cells. At 24 hours after cell transfer, these mice were treated with IL-18/IL-2 for 3 days. Pulmonary lymphocytes were isolated as described in MATERIALS AND METHODS. Pulmonary lymphocytes were analyzed by flow cytometry. Data are shown as a ratio of NK cells compared with those in control mice. The value of control mice is indicated by the mean of three independent experiments as 1.0. Other values represent mean ± SEM of three independent mice, using the average value of the control mouse. The values as mean ± SEM for B6 mice + PBS were 1 ± 0.061; for TCRδ$^{-/-}$ mice + PBS, 0.514 ± 0.030; for TCRδ$^{-/-}$ mice + NK1.1$^-$ γδT cells, 0.636 ± 0.015; and for TCRδ$^{-/-}$ mice + NK1.1$^+$ γδT cells, 0.809 ± 0.043; $P < 0.05$. (C) Three B6 mice received transfers with PBS, and three TCRδ$^{-/-}$ mice received transfers with PBS, NK1.1$^+$, or NK1.1$^-$ γδT cells. At 24 hours after cell transfer, these mice were treated with IL-18/IL-2 for 3 days. Lung mRNA was extracted, and the expression of IFN-γ mRNA was analyzed by RT-PCR. Data are shown as a ratio of IFN-γ–producing cells, compared with those in control mice. The value of control mice was shown by the mean of three independent experiments as 1.0. Other values represent the mean ± SEM of three independent mice, using the average of the control mouse. Values as mean ± SEM for B6 mice + PBS were 1 ± 0.094; for TCRδ$^{-/-}$ mice + PBS, 0.132 ± 0.041; for TCRδ$^{-/-}$ mice + NK1.1$^-$ γδT cells, 0.219 ± 0.059; and for TCRδ$^{-/-}$ mice + NK1.1$^-$ γδT cells, 0.327 ± 0.048; $P < 0.05$.

γδT cell expansion in the lung. These results indicate that NK1.1$^+$ γδT cells may recognize a wide variety of antigens, and thus are unlikely invariant NK T cells. However, whether NK1.1$^+$ γδT cells recognize any antigens in IL-18/IL-2–induced ILD remains unknown.

Crowe and colleagues (29) reported that the expression of NK1.1 in TCR αβ NK T cells was down-regulated by stimulation with TCR within 24 hours, and it returned to the naive level within 6 days *in vivo*. In our study, we examined the expression level of the NK1.1 molecule in NK1.1$^+$ γδT cells after stimulation with IL-18/IL-2. The expression of NK1.1 in γδT cells was also down-regulated within 24 hours and 48 hours, but it increased to the 1.5 times level at 96 hours, similar to TCR αβ NK T cells. Moreover, NK1.1$^-$ γδT cells did not express the NK1.1 molecule, even after culturing with IL-18/IL-2 for 96 hours, indicating that NK1.1$^+$ γδT cells may have different cell populations than NK1.1$^+$ γδT cells. Further examination will be necessary to clarify whether NK1.1$^+$ γδT cells develop from immature NK1.1$^-$ γδT cells or NK1.1$^+$ γδT cells.

NK cells and IFN-γ are believed to play important roles in the pathogenesis of IL-18/IL-2–induced ILD mice. Label and colleagues (30) and Gardner and colleagues (31) emphasized the role of γδT cells in the regulation of NK cell functions such as proliferation and cytokine expression. We showed that the infiltration of NK cells in the lung after injection with IL-18/IL-2 was significantly lower in TCRδ$^{-/-}$ mice than in B6 mice. Lung tissue staining clearly showed that cell infiltration was inhibited in TCRδ$^{-/-}$ mice compared with B6 mice. Although the rate of pulmonary NK cells was low, the histological result showed a clear difference between TCRδ$^{-/-}$ and B6 mice. The histological findings may be attributable to effects not only by NK cells but also by γδT cells. The results demonstrated that γδT cells participated in the proliferation of NK cells after injection with IL-18/IL-2. Furthermore, the production of IFN-γ in lung tissue from TCRδ$^{-/-}$ mice treated with IL-18/IL-2 was significantly lower compared with that in B6 mice. In addition, we observed evidence that the number of pulmonary NK cells was significantly increased and the expression of IFN-γ mRNA tended to be higher via the adoptive cell transfer of NK1.1$^+$ γδT cells into TCRδ$^{-/-}$ mice compared with NK1.1$^-$ γδT cells. Therefore, these findings in TCRδ$^{-/-}$ mice support the notion that NK1.1$^+$ γδT cells may enhance the accumulation of NK cells in the lung in IL-18/IL-2–induced ILD.

The CD161 molecule in humans is homologous with murine NK1.1. Previous studies reported the presence of CD161$^+$ γδT cells in peripheral blood mononuclear cells in patients with HIV (32) and multiple sclerosis (33). However, no reports on CD161$^+$ γδT cells in patients with interstitial pneumonitis are available. Thus, further experiments on human γδT cells in lungs from patients with ILD are necessary for a better understanding of the pathogenesis of ILD.

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ORIGINAL ARTICLE

Importance of serine727 phosphorylated STAT1 in IFNγ-induced signaling and apoptosis of human salivary gland cells

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Abstract

Aim: It is reported that in salivary glands of Sjögren's syndrome (SS), interferon gamma (IFNγ) and IFNγ-inducible genes containing signal transducers and activators of transcription 1 (STAT1) are upregulated and play a crucial role in the pathogenesis of SS. The aim of this study is to clarify which phosphorylation of STAT1, serine727 (Ser^{727}) or tyrosine701 (Tyr^{701}) of STAT1, is important for IFNγ signaling and IFNγ-induced apoptosis in salivary gland cells.

Methods: We established STAT1 Tyr^{701} variant (tyrosine to phenylalanine; Y701F) and STAT1 Ser^{727} variant (serine to alanine; S727A), which were transfected into human salivary gland (HSG) cells. HSG cells transfected with these mutant-STAT1 were analyzed on the expression of IFNγ-inducible genes and apoptosis after stimulation with IFNγ.

Results: In Y701F mutant-STAT1 transfected HSG cells (Ser^{727}-dominant HSG cells), IFNγ-inducible genes such as IP10, IRF1, and Fas expression were increased after stimulation with IFNγ. In Ser^{727}-dominant HSG cells, the induction of apoptosis after stimulation with IFNγ was also increased compared with S727A mutant-STAT1 transfected HSG cells (Tyr^{701}-dominant HSG cells).

Conclusion: Phosphorylation of Ser^{727} in STAT1 might be more important in IFNγ signaling and the induction of apoptosis in HSG cells than phosphorylation of Tyr^{701}. Accordingly, we propose that phosphorylation of Ser^{727} in STAT1 could be a potentially suitable new therapeutic target for SS patients to prevent the destruction of salivary glands.

Key words: apoptosis, human salivary gland cells, Interferon gamma, signal transducers and activators of transcription 1, Sjögren's syndrome.

INTRODUCTION

Sjögren's syndrome (SS) is a chronic autoimmune disease characterized pathologically by focal lymphocytic infiltration of exocrine glands, especially lachrymal and salivary glands, and clinically by dry eyes and dry mouth.[1] Inflammatory cytokines, especially Interferon

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gamma (IFNγ), are considered to play an important role in the destruction of the exocrine glands, based on the detection of IFNγ messenger RNA (mRNA) expression in the salivary glands of SS patients.[2] Increased concentrations of IFNγ contribute to the upregulation of human leukocyte antigen (HLA) class II and co-stimulatory molecules on epithelial cells and antigen-presenting cells. IFNγ also upregulates Fas and caspase-8 expression and therefore fosters apoptosis.[3] Moreover, we also reported the upregulation of several IFNγ-inducible genes containing signal transducers and activators of transcription 1 (STAT1) in the salivary glands of SS patients.[4] Thus, the IFNγ signaling seems to play a crucial role in the pathogenesis of SS.

STAT1 is known as the mediator of IFNγ signaling. Maximal activation by STAT1 of the IFNγ signaling requires phosphorylation of both tyrosine701 (Tyr^{701}) and serine727 (Ser^{727}).[5,6] We reported previously the induction of STAT1-α phosphorylation and the different localization of Tyr^{701}-phosphorylated STAT1-α and Ser^{727}-phosphorylated STAT1-α in the labial salivary glands of patients with SS.[7]

Stephanou *et al.*[8] reported that induction of apoptosis and Fas expression by ischemia/reperfusion in cardiac myocytes required Ser^{727} of STAT1 but not Tyr^{701}, suggesting that Ser^{727}-phosphorylated STAT1-α is essential for the induction of apoptosis. However, it is not clear which phosphorylation of STAT1 induces apoptosis by IFNγ in salivary gland cells. To clarify this question, we established a STAT1 Tyr^{701} variant (tyrosine to phenylalanine; Y701F) and STAT1 Ser^{727} variant (serine to alanine; S727A), which were transfected into human salivary gland (HSG) cells. HSG cells transfected with these mutant-STAT1 were analyzed on the expression of IFNγ-inducible genes and apoptosis after stimulation with IFNγ.

MATERIALS AND METHODS

Production of mutant-STAT1 and transformation of mutant-STAT1 into HSG cells

We made the Tyr^{701} mutant (tyrosine to phenylalanine; Y701F) and Ser^{727} mutant (serine to alanine; S727A) STAT1 DNA fragments (Fig. 1a). These two mutant-STAT1 DNA fragments were inserted into myc-His vectors by double digestion with BamH I and Nod I (Fig. 1b). 1.0×10^5/mL of HSG cells were pre-cultured overnight, and then culture medium was changed to medium without sera. Mutant-STAT1 Y701F and S727A were transformed into HSG cells using FuGENE6 (Roche Applied Science, Mannheim, Germany). HSG cells transfected with mutant-STAT1 were selected by zeosin. HSG cells with Y701F mutant-STAT1 were Ser^{727}-dominant HSG cells, and HSG cells with S727A mutant-STAT1 were Tyr^{701}-dominant HSG cells.

IFNγ-inducible gene expression in mutant-STAT1-transfected HSG cells after stimulation with IFNγ

Human salivary gland cells transfected with Y701F, S727A mutant-STAT1 and empty vector were stimulated with IFNγ (2000 U/mL) for 24 h. These HSG cells were trypsinized and total RNA and cell lysate were extracted from HSG cells. Complementary DNA (cDNA) was synthesized by cDNA synthesis kit (Fermentas International, Burlington, ON, Canada). Reverse transcription–polymerase chain reaction (RT-PCR) and quantitative PCR were performed with cDNA using the human IFNγ-inducible protein 10 (IP10), interferon regulatory factor 1 (IRF1), Fas and CD40 specific primers. Human-glyceralaldehyde-3-phosphate dehydrogenase (GAPDH) was amplified to assess the cDNA yield and to analyze as the internal control.

IFNγ-inducible gene expression was examined at protein levels by Western blot analysis using the cell lysate of HSG cells. Total proteins were fractionated on sodium dodecyl sulfate-polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked in 100% Block-Ace (Dainippon, Osaka, Japan) for 1 h and then incubated with rabbit anti-Fas antibody (1 : 500 dilution; Cell Signaling Technologies, Beverly, MA, USA) or mouse anti-β-actin antibody (2 μg/mL; BioVision, Mountain View, CA, USA) at 4°C overnight. Secondary antibodies, anti-rabbit IgG horseradish peroxidase (HRP) linked antibody (1 : 2000 dilution; Cell Signaling Technologies) or anti-mouse IgG HRP linked antibody (1 : 2000 dilution; Dako, Tokyo, Japan), were applied at room temperature for 1 h, and then proteins were detected by enhanced chemiluminescence using an ECL Western blot detection kit (Amersham, Little Chalfont, UK).

Apoptosis of mutant-STAT1-transfected HSG cells after stimulation with IFNγ

Human salivary gland cells transfected with Y701F, S727A mutant-STAT1 and empty vector were cultured with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and peni-

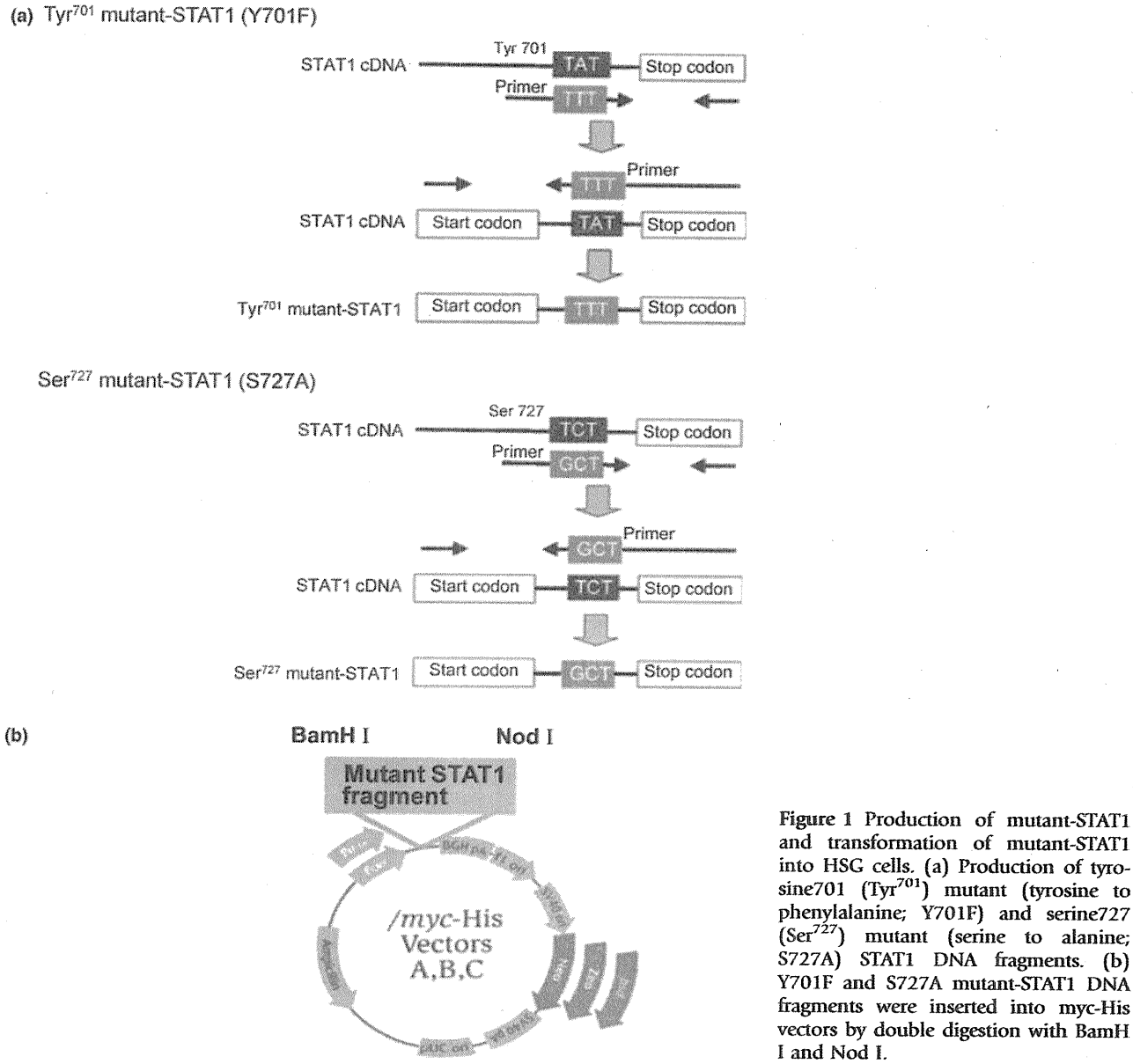

Figure 1 Production of mutant-STAT1 and transformation of mutant-STAT1 into HSG cells. (a) Production of tyrosine701 (Tyr^{701}) mutant (tyrosine to phenylalanine; Y701F) and serine727 (Ser^{727}) mutant (serine to alanine; S727A) STAT1 DNA fragments. (b) Y701F and S727A mutant-STAT1 DNA fragments were inserted into myc-His vectors by double digestion with BamH I and Nod I.

cillin/streptomycin. After stimulation with IFNγ (2000 U/mL) for 24 h, cells were trypsinized and harvested. Harvested HSG cells were washed in phosphate-buffered saline (PBS) and resuspended in binding buffer containing annexin-V-fluorescein isothiocyanate to monitor apoptosis-associated plasma membrane alteration for 20 min at room temperature. The samples were analyzed with a FACS Calibur flow cytometer (BD-Biosciences, Mountain View, CA, USA), and data were analyzed with FlowJo software (Tree Star, Ashland, OR, USA).

RESULTS

IFNγ-inducible gene expression was increased in Ser^{727}-dominant HSG cells after stimulation with IFNγ

Figure 2 shows the expression of IFNγ-inducible gene mRNA in mutant-STAT1 and empty vector-transfected HSG cells after stimulation with IFNγ. In Y701F mutant-STAT1-transfected HSG cells (Ser^{727}-dominant HSG cells), IP10 and IRF1 mRNA expression were increased after stimulation with IFNγ, whereas Fas

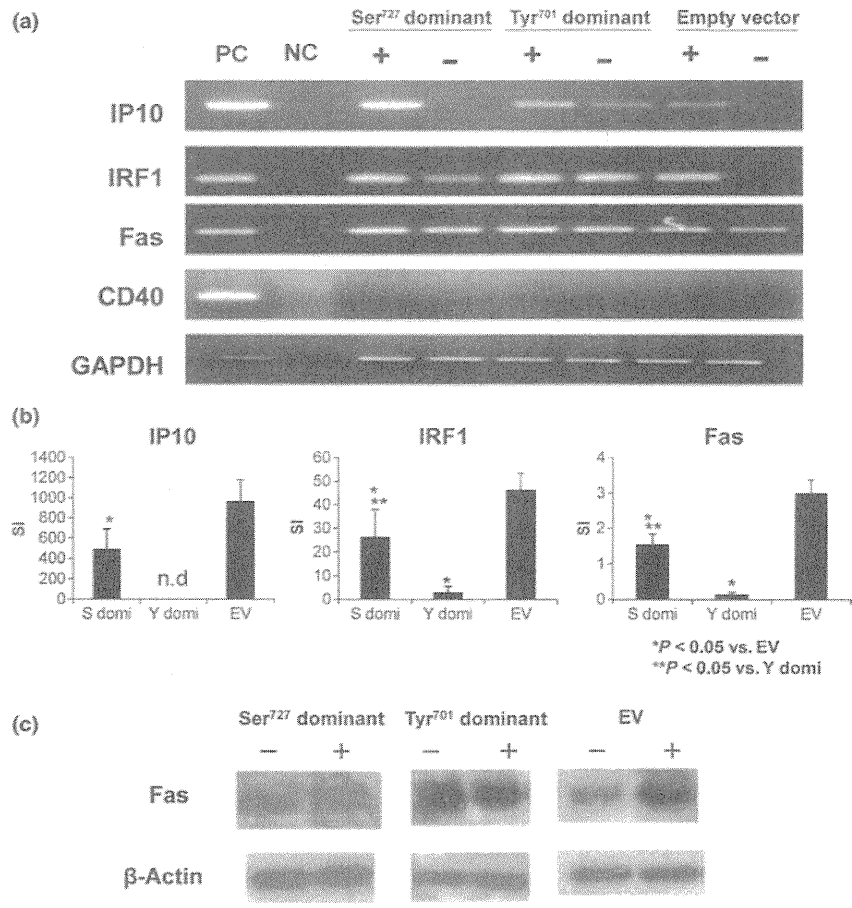

Figure 2 IFNγ-inducible gene expression in mutant-STAT1-transfected human salivary gland (HSG) cells after stimulation with IFNγ. (a) HSG cells transfected with Y701F, S727A mutant-STAT1, and empty vector were stimulated with IFNγ (2000 U/mL) for 24 h. mRNA expression of IFNγ-inducible genes (IP10, IRF1 and Fas) were analyzed by reverse transcription – polymerase chain reaction (RT-PCR). The expression of IP10, IRF1 and Fas were increased in Ser^{727}-dominant HSG cells after stimulation with IFNγ. (b) The results of quantitative PCR are shown as stimulation indexes (S.I), which were calculated by expression levels after stimulation with IFNγ/expression levels before stimulation. In Ser^{727}-dominant HSG cells, IRF1 and Fas mRNA expression levels were significantly increased after stimulation with IFNγ compared with Tyr^{701}-dominant HSG cells ($P < 0.05$, Mann–Whitney *U*-test). (c) Western blot analysis demonstrated that Fas protein expression in Ser^{727}-dominant HSG cells was increased after stimulation with IFNγ, although in Tyr^{701}-dominant HSG cells this was not increased. IP10, IFNγ-inducible protein 10; IRF1, interferon regulatory factor 1; GAPDH, human-glyceralaldehyde-3-phosphate dehydrogenase; PC, positive control; NC, negative control; +, after stimulation with IFNγ; −, before stimulation with IFNγ; EV, empty vector; S domi, Ser^{727}-dominant HSG cells; Y domi, Tyr^{701}-dominant HSG cells; n.d, not determined.

mRNA expression was increased slightly in RT-PCR analysis (Fig. 2a). On the other hand, in S727A mutant-STAT1-transfected HSG cells (Tyr^{701}-dominant HSG cells), only IP10 mRNA expression was increased slightly, whereas IRF1 and Fas mRNA expression were not increased after stimulation with IFNγ in RT-PCR analysis (Fig. 2a). The expression of CD40 mRNA was not detected in any type of HSG cell, neither before nor after stimulation with IFNγ. The results of quantitative PCR on IP10, IRF1 and Fas mRNA expression are shown as stimulation indexes (S.I.), which were calculated by expression levels after stimulation with IFNγ/expression levels before stimulation. In Ser^{727}-dominant HSG cells, IRF1 and Fas mRNA expression levels were significantly increased after stimulation with IFNγ compared with Tyr^{701}-dominant HSG cells ($P < 0.05$, Mann–Whitney *U*-test). S.I of IP10 in Tyr^{701}-dominant HSG cells was not determined, because the expression of IP10 before stimulation was not detected by quantitative PCR analysis (Fig. 2b).

Western blot analysis showed that in Ser^{727}-dominant HSG cells, Fas protein expression was also increased after stimulation with IFNγ, whereas in Tyr^{701}-dominant HSG cells, Fas protein expression was not increased (Fig. 2c).

Induction of apoptosis after stimulation with IFNγ was increased in Ser^{727}-dominant HSG cells

Figure 3 shows the induction of apoptosis after stimulation with IFNγ detected by annexin-V in mutant-STAT1 and empty vector-transfected HSG cells. The population of apoptotic cells before stimulation with IFNγ was 26.8%, 24.5% and 11.9%, and after stimulation with IFNγ, the population of apoptotic cells was changed to 34.9%, 22.8% and 12.9%, in Ser^{727}-dominant HSG cells, Tyr^{701}-dominant HSG cells, and empty vector transfected-HSG cells, respectively. Stimulation index (S.I.) with IFNγ was 1.30, 0.93 and 1.08 in Ser^{727}-dominant HSG cells, Tyr^{701}-dominant HSG cells, and empty vector transfected-HSG cells, respectively. The induction of apoptosis after stimulation with IFNγ was increased in Ser^{727}-dominant HSG cells compared with Tyr^{701}-dominant HSG cells.

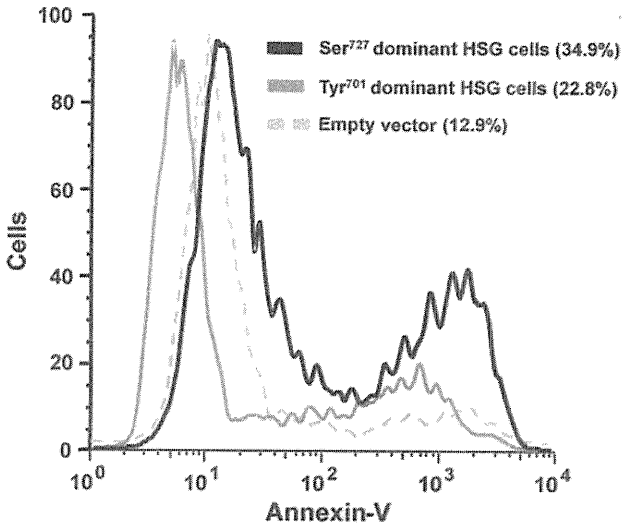

Figure 3 Apoptosis of mutant-STAT1-transfected human salivary gland (HSG) cells after stimulation with IFNγ. HSG cells transfected with Y701F, S727A mutant-STAT1, and empty vector were stimulated with IFNγ (2000 U/mL) for 24 h and then apoptotic cells were detected by annexin-V. The histogram of FLOWJO software (Tree Star, Ashland, OR, USA) showing the population of apoptotic cells after stimulation with IFNγ was 34.9%, 22.8% and 12.9%, in Ser^{727}-dominant HSG cells, Tyr^{701}-dominant HSG cells, and empty vector transfected-HSG cells, respectively. The induction of apoptosis after stimulation with IFNγ was increased in Ser^{727}-dominant HSG cells compared with Tyr^{701}-dominant HSG cells.

DISCUSSION

In Y701F mutant-STAT1-transfected HSG cells (Ser^{727}-dominant HSG cells), the expression of IFNγ-inducible genes such as IP10, IRF1 and Fas, were increased after stimulation with IFNγ. Moreover, in Ser^{727}-dominant HSG cells, the induction of apoptosis after stimulation with IFNγ was also increased compared with S727A mutant-STAT1-transfected HSG cells (Tyr^{701}-dominant HSG cells). These findings indicated that phosphorylation of Ser^{727} in STAT1 might be more important in IFNγ signaling and induction of apoptosis in HSG cells than phosphorylation of Tyr^{701}.

Previously, we reported that Tyr^{701}-phosphorylated STAT1 was localized in infiltrating lymphocytes and the adjacent ductal epithelium, while Ser^{727}-phosphorylated STAT1 was localized only in the ductal epithelium of labial salivary glands from SS patients (SS LSGs).[7] We also revealed that IP10, IRF1 and Fas genes were highly expressed in SS LSGs and colocalized with Ser^{727}-phosphorylated STAT1 but not with Tyr^{701}-phosphorylated STAT1.[7] We proposed that STAT1, especially Ser^{727}-phosphorylated STAT1, might function as a key molecule in the pathogenesis of SS, including the destruction of salivary glands.[7] Interestingly, these previous findings accord with the results in the present study.

In the present study, we showed the correlation of Ser^{727}-phosphorylated STAT1 with IFNγ signaling and the induction of apoptosis in HSG cells *in vitro*. In the pathogenesis of SS, IFNγ might induce phosphorylation of STAT1, especially Ser^{727}, in HSG cells, which cause IFNγ signaling and apoptosis. Thus, Ser^{727}-phosphorylated STAT1 might have essential roles in the destruction of salivary glands in patients with SS. On the other hand, Tyr^{701}-phosphorylated STAT1 might suppress IFNγ signaling and apoptosis. We previously showed that Tyr^{701}-phosphorylated STAT1 was localized in infiltrating lymphocytes in SS LSGs.[7] Thus, IFNγ might induce phosphorylation of Tyr^{701} of STAT1 in infiltrating lymphocytes, resulting in resistance to apoptosis.

In conclusion, phosphorylation of Ser^{727} in STAT1 might be important in IFNγ signaling and the induction of apoptosis in HSG cells. Accordingly, we propose that phosphorylation of Ser^{727} in STAT1 could be a potentially suitable new therapeutic target for SS patients to prevent the destruction of salivary glands.

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Magnetic resonance imaging of wrist and finger joints distinguishes secondary Sjögren's syndrome with rheumatoid arthritis from primary Sjögren's syndrome with articular manifestations

Sirs,

It is sometimes difficult for clinicians to differentiate the arthritic condition of rheumatoid arthritis (RA) from that of Sjögren's syndrome (1). We have recently reported the importance of anti-cyclic citrullinated peptide (CCP) antibodies for this differentiation (2). In addition, magnetic resonance imaging (MRI) is also very useful for the recognition of arthritis especially in early arthritis patients who do not show abnormalities by x-ray (3-5). We recruited 29 patients of SS who fulfilled the diagnostic criteria of SS according to the American-European Consensus Group (6) with articular manifestations from the Unit of Translational Medicine, Department of Immunology and Rheumatology, Nagasaki University Graduate School of Biomedical Sciences. Informed consent was obtained and the protocol was approved by the Institutional Review Board of Nagasaki University. All patients complained of morning stiffness and tender or swollen joints at more than one site on the wrist and finger joints. No patients had radiographic erosion on hands and feet plain radiographs at the first MRI study. All patients had been previously diagnosed as primary SS by physicians, but 9 of the 29 SS patients fulfilled the 1987 criteria of the American College of Rheumatology (ACR) for RA (7) after the diagnosis of primary SS, designated as secondary SS with RA. Baseline characteristics (age, duration of SS to the entry, serum IgG, prevalence of anti-SS-A/SS-B antibodies) of 29 patients, with or without fulfillment of RA, were not significantly different (data not shown). Sixteen of 29 SS patients underwent the second MRI. Indications for taking the second MRI were determined by each physician. The mean interval between the first MRI and second MRI was 10.9 months.

Plain MRIs of both wrists and finger joints were acquired using a 1.5T system (Sigma; General Electric Medial Systems, Milwaukee, WI) with an extremity coil. Coronal T1-weighted spin-echo and short-time inversion recovery (STIR) images were acquired as previously described (3, 8). The images were evaluated for bone oedema, bone erosion, and synovitis at 15 sites on each hand, including the distal radioulnar joint, the radiocarpal joint, the midcarpal joint, the first carpometacarpal joint, the second through fifth carpometacarpal joints (together), the first through fifth metacarpophalangeal joints, and the first through fifth proximal interphalengeal joints separately, as we recently reported (3, 8).

Table I. Autoantibodies and MRI finding in primary SS with articular manifestations and secondary SS with RA.

	First MRI and autoantibodies at baseline		
	Primary SS (n=20)	Secondary SS with RA (n=9)	*p*-value
Symmetric synovitis	11 (55%)	9 (100%)	<0.05
Bone oedema	0 (0%)	2 (22%)	NS
Bone erosion	1 (5%)	2 (22%)	NS
Tenosynovitis	8 (40%)	6 (67%)	NS
Anti-CCP antibodies	1 (5%)	5 (56%)	<0.01
IgM-RF	8 (40%)	5 (56%)	NS
	Second MRI		
	Primary SS (n=11)	Secondary SS with RA (n=5)	*p*-value
Symmetric synovitis	8 (73%)	5 (100%)	NS
Bone oedema	0 (0%)	5 (100%)	<0.0005
Bone erosion	1 (9%)	5 (100%)	<0.005
Tenosynovitis	4 (36%)	5 (100%)	<0.05

IgM-RF; latex-enhanced immuno-electrometric assay (Dade Behring, Marburg, Germany; cut-off value, 14IU/ml) and anti-CCP; DIASTAT Anti-CCP (Axis-Shield, Dundee, UK; cut-off value, 4.5 U/ml), respectively. P-value was calculated by chi-square test and Fisher's exact probability test.

Results are summarised in Table I. Frequency of bone erosion, tenosynovitis and prevalence of IgM-RF were not statistically different in the presence or absence of RA. Bone edema was found only in secondary SS with RA patients (0% *vs.* 22%). The frequency of symmetrical synovitis and the prevalence of anti-CCP antibodies were significantly higher in secondary SS with RA. Prevalence of anti-CCP antibodies in secondary SS with RA patients was low as compared with patients of early-stage RA in our other cohorts (3, 8). It might come from small number of patients in the present study. None of the patients showed Jaccoud's arthropathy and few other extraglandular manifestations, except articular manifestations, were recorded. Interestingly, MRI-proven bone oedema was found in all patients with secondary SS with RA while no patients with primary SS showed bone edema even on the second MRI. In addition to bone oedema, other findings of symmetrical synovitis, bone erosion and tenosynovitis were more frequently found in secondary SS with RA patients as compared with patients of primary SS with articular manifestations at second MRI.

This study marks the first observational finding that MRIs of wrist and finger joints are clinically effective at differentiating the condition of arthritis in SS patients. Additional examinations, including musculoskeletal ultrasonography especially power Doppler ultrasonography (9, 10) with longer follow-up periods, are required to strengthen our results.

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ORIGINAL ARTICLE

Contribution of an adenine to guanine single nucleotide polymorphism of the matrix metalloproteinase-13 (*MMP-13*) −77 promoter region to the production of anticyclic citrullinated peptide antibodies in patients with HLA-DRB1*shared epitope-negative rheumatoid arthritis

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Abstract We examined whether matrix metalloproteinase-13 (MMP-13) contributes to disease susceptibility or severity of rheumatoid arthritis (RA). Eighty-seven patients with RA whose disease duration was <2 years and 71 healthy controls were enrolled in the study. Adenine (A) to guanine (G) single nucleotide polymorphism (SNP) of the −77 *MMP-13* promoter region in RA and healthy controls was determined by polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) technique. Human leukocyte antigen (HLA)-DRB1 genotyping was also performed using the same populations. Anticyclic citrullinated peptide (anti-CCP) antibodies from RA patients at entry were studied, and their relationships were examined. The genotype and allele frequency of SNP of *MMP-13* at −77 did not differ between RA patients and healthy controls. We focused on the RA patients who were negative for HLA-DRB1*shared epitope (SE) alleles and found that the seropositivity of anti-CCP antibodies with a titer >25 U/ml was high in the A/A genotype compared with the G/G genotype. The same characteristic was also found in HLA-DRB1*0405 allele-negative patients. Our data suggest that SNP of the −77 *MMP-13* promoter region acts as a surrogate marker of anti-CCP antibody production in HLA-DRB1*SE allele-negative RA patients, which may reflect RA severity.

Keywords MMP-13 · Rheumatoid arthritis · Polymorphism · Anti-CCP antibodies

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Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by joint destruction. Although the etiology of the disease remains unknown, recent evidence suggests that both genetic and environmental factors contribute to RA susceptibility and severity [1]. The most prominent genetic component in RA heritability is the human leukocyte antigen (HLA) locus on chromosome 6, which accounts for one third of RA genetic susceptibility [2]. In addition, several non-HLA genes are also considered to contribute to RA development [3].

Joint destruction in RA occurs by the degradation of type I, II, and III collagen, which constitutes cartilage and bone. Matrix metalloproteinases (MMPs) play an important role in this process. One such metalloproteinase is MMP-13, which degrades type II collagen of the interstitial collagens. MMP-13 expression is recognized in the synovial lining layer, vascular endothelial cells, fibroblast-like synoviocytes, monocytes, osteoblasts, and cartilage cells in rheumatoid synovial tissues. The intensity of MMP-13 messenger RNA (mRNA) expression in rheumatoid synovial tissues correlates with a severe clinical course of RA [4]. Serum MMP-13 concentrations in early-stage RA

patients are high, but decrease in response to anti-rheumatic treatment [5]. These data indicate that MMP-13 is one of the most important MMPs involved in the development of RA.

In a study of allele-specific effects on the regulation of MMP-13 expression in relation to the −77 A>G polymorphism, it was found that the *MMP-13* −77 A allele has higher promoter activity than the *MMP-13* −77 G allele [6]. A previous study also found that the functional disability assessed by the Steinbrocker Index in patients with RA is high in the *MMP-13* −77 A/A genotype compared with the −77 G/G genotype [7]. Based on these previous findings, we investigated whether the *MMP-13* −77 A>G polymorphism is associated with anticyclic citrullinated (anti-CCP) peptide antibody production in early-stage RA from the Japanese population.

Patients and methods

Study population

Eight-seven patients with RA diagnosed according to the 1987 revised criteria of the American College of Rheumatology for classification of the disease [8] and who had visited our early arthritis clinic as previously reported [9, 10] were enrolled in the study. The Early Arthritis Clinic opened in 2001 as part of the Unit of Translational Medicine, Department of Immunology and Rheumatology, Graduate School of Biomedical Sciences, Nagasaki University. Patients were referred from an area in the western part of Japan, Nagasaki Prefecture, which has approximately 450,000 inhabitants. The control samples were 60 unrelated healthy Japanese individuals. Each individual provided a signed consent form to participate in the study, which was approved by the Institutional Review Board of Nagasaki University. Baseline clinical manifestations and variables included gender; age; localization of arthritis; duration of morning stiffness; number of tender joints; number of swollen joints; C-reactive protein level (CRP) measured by latex turbidimetric immunosorbent assay (Daiichi Pure Chemicals, Fukuoka, Japan); immunoglobulin M (IgM)-rheumatoid factor (RF) positivity measured by latex-enhanced immunonephelometric assay, cutoff value 14 IU/ml (Dade Behring, Marburg, Germany); positive status for anti-CCP antibodies measured by enzyme-linked immunosorbent assay (ELISA), cutoff value 4.5 U/ml (DIASTAT Anti-CCP; Axis-Shield, Dundee, UK); and MMP-3 measured ELISA, cutoff value 59.7 ng/ml for women and 121.0 ng/ml for men (Daiichi, Japan). We summarize some of this information in Table 1.

Table 1 Patient characteristics

Characteristics	Total cohort (n = 87)
Sex, no. female/male	70/17
Age, mean ± SD (years)	53.26 ± 14.14
Mean disease duration, months ± SD	5.66 ± 5.91
No. (%) seropositive for anti-CCP antibodies[a]	54 (62)
No. (%) seropositive for anti-CCP antibodies with a titer >25 U/ml	39 (45)
No. (%) positive for HLA-DRB1*SE alleles	47 (54)
No. (%) positive for HLA-DRB1*0405 allele	38 (44)

SD standard deviation, *SE* shared epitope, *anti-CCP* anti-CCP anticyclic citrullinated peptide

[a] Anti-CCP antibodies cutoff value 4.5 U/ml

DNA isolation and genotyping

Genomic DNA was extracted from peripheral blood samples by a standard procedure. The *MMP-13* −77 A>G polymorphism was examined according to the published method [6]. In brief, a 445-bp DNA fragment, including the polymorphic site, was amplified by polymerase chain reaction (PCR) using a set of oligonucleotide primers: sense 5′-GATACGTTCTTACAGAAGGC-3′; antisense 5′-GACAAATCATCTTCATCACC-3′. The PCR products were digested with 1 U of *Bsr*I (New England Biolabs Inc., Beverly, MA, USA), which claves the G allele, generating two fragments 197 and 248 bp in size. The digests were analyzed on 3% agarose gels. HLA-DRB1 genotyping was also performed, as we previously described [11].

Statistical analysis

Distributions of the *MMP-13* −77 A>G polymorphism in RA patients and healthy controls were determined using Fisher's exact test. The Chi-square test was used for comparison. A p value <0.05 was considered statistically significant.

Results

Baseline characteristics and distribution of MMP-13 −77 A>G polymorphism between early-stage RA patients and healthy controls

Baseline characteristics of the 87 patients are given in Table 1. As the mean disease duration from the onset of articular symptoms to entry was 5.66 ± 5.91 months, our population is considered to be early-stage RA patients. Fifty-four of 87 patients (62%) were seropositive toward

Table 2 Genotype and allele frequency of the −77 polymorphism of the matrix metalloproteinase-13 (*MMP-13*) gene in patients and controls

	Patients [*n* (%)]	Controls [*n* (%)]	*P* value	Odds ratio	95% CI
Genotype					
A/A	23 (26)	18 (30)	0.64	0.84	0.40–1.74
A/G	39 (45)	27 (45)	0.98	0.99	0.51–1.92
G/G	25 (29)	15 (25)	0.62	1.21	0.57–2.55
Presence of allele					
A	62 (71)	45 (75)	0.62	0.83	0.39–1.74
G	64 (74)	42 (70)	0.64	1.19	0.58–2.47
Allele frequency					
A	85 (49)	63 (53)	0.54	0.86	0.54–1.38
G	89 (51)	57 (48)	0.54	1.16	0.73–1.84

CI confidential interval

anti-CCP antibodies. The carriership of HLA-DRB1*shared epitope (SE) alleles and the HLA-DRB1*0405 allele was 54 and 44%, respectively, which is similar to previous reports from Japanese populations [11, 12]. We compared genotype, presence of the allele, and allele frequency of *MMP-13* −77 A>G polymorphism between RA patients and healthy controls. As shown in Table 2, there was no statistically significant difference between the two populations.

Percentage of RA patients with anti-CCP antibodies >25 U/ml is high in the *MMP-13* −77 A/A genotype compared with the −77 G/G genotype among the HLA-DRB1*SE allele-negative population in patients with RA

We next tried to examine the relationship between the *MMP-13* −77 A>G polymorphism and RA severity. Syversen et al. [13] recently revealed that titers of anti-CCP antibodies in early-stage RA patients at baseline reflect the further radiographic bone destruction. They set the anti-CCP antibody titers as 25 U/ml [13]. Thus, we examined the distribution of anti-CCP antibodies based on the cutoff value of 25 U/ml, but we found no statistically significant difference among *MMP-13* −77 A/A, −77 A/G, and −77 G/G polymorphisms (data not shown). To exclude the influence of HLA-DRB1*SE alleles on the *MMP-13* −77 A>G polymorphism, we carried out the same analysis in HLA-DRB1*SE allele-negative RA patients and found that the percentage of patients with anti-CCP antibody titers >25 U/ml was statistically high in the *MMP-13* −77 A/A genotype compared with the *MMP-13* −77 G/G genotype (Fig. 1). A similar result was also obtained in HLA-DRB1*0405 allele-negative RA patients [patients with anti-CCP antibody titer >25 U/ml was 7 of 12 in *MMP-13* −77 A/A genotype (58%) and 3 of 16 in *MMP-13* −77 G/G genotype (19%); $p = 0.039$, −77 A/A genotype vs. −77 G/G genotype].

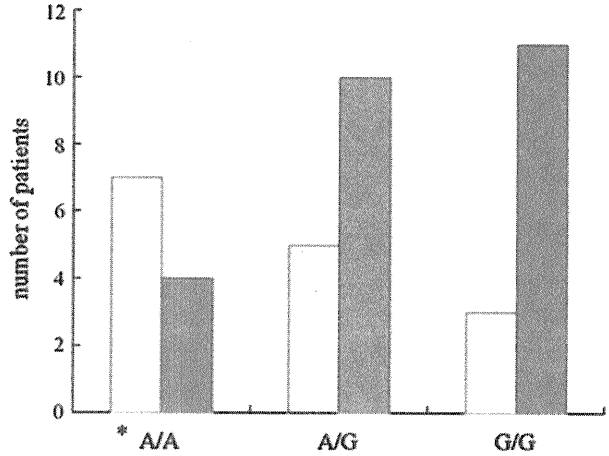

Fig. 1 Association of the −77 polymorphism of the matrix metalloproteinase-13 (*MMP-13*) gene with levels of anticyclic citrullinated peptide (anti-CCP) antibodies in the human leukocyte antigen (HLA)-DRB1*shared epitope (SE) allele-negative population in patients with rheumatoid arthritis (RA). *Open bars* show the number of RA patients with anti-CCP antibody titer >25 U/ml, whereas *gray bars* indicate those with anti-CCP antibody titer <25 U/ml. The percentage of RA patients with anti-CCP antibody titer >25 U/ml was statistically significantly high in the *MMP-13* −77 A/A genotype compared with the *MMP-13* −77 G/G genotype. Data were calculated by chi-square test, as described in "Patients and methods"

Discussion

RA is a multifactorial disorder with an estimated heritability of 60% [14]. As the mean disease duration from onset to entry of our 87 RA patients was <6 months, the study population were considered to be early-stage RA patients. Early-stage patients reflect the disease process well compared with established patients with RA. The *MMP-13* −77 A>G polymorphism did not differ between RA patients and healthy controls, suggesting that *MMP-13* is not an RA-susceptible gene.

We next investigated whether *MMP-13* determines RA severity. We focused on the relationship between anti-CCP antibodies with the *MMP-13* −77 A>G polymorphism in patients with RA. Anti-CCP may be directly involved in RA pathogenesis. Locally produced anti-CCP generates immune complexes and may contribute to initiating and sustaining synovial inflammation by triggering monocyte and granulocyte activation and cytokine production [15, 16]. Therefore, patients with high levels of anti-CCP might have severe progression compared with patients with low levels. Actually, the presence of anti-CCP antibodies

in RA is considered to be a prognostic factor toward further radiographic progression [13]. Patients with anti-CCP antibody levels >25 U/ml are especially more likely to develop radiographic progression [13]. Susceptibility to and severity of the disease has been associated with variations in *HLA* genes. Both radiographic progression and anti-CCP antibodies have proved to correlate positively with the presence of HLA-DRB1*SE alleles. Probably due to the influence of HLA-DRB1*SE alleles, we found no association between the *MMP-13* −77 A>G polymorphism and anti-CCP antibodies in our entire population. However, the distribution of anti-CCP antibody-positive patients, defined as having antibody levels >25 U/ml, differs between −77 A/A and −77 G/G genotypes of the *MMP-13* polymorphism in HLA-DRB1*SE allele-negative patients with RA. Considering that MMP-13 expression in the rheumatoid synovial tissues of patients with the *MMP-13* −77 A/A genotypes might be high, the production of anti-CCP antibodies as well as joint destruction might become obvious in these patients. Accordingly, our data appear to support the previous finding [7].

In summary, our data indicate an association between the *MMP-13* −77 A>G polymorphism and production of anti-CCP antibodies in patients with HLA-DRB1*SE allele-negative early-stage RA. Recently, strong combined gene–environment effects in anti-CCP antibody-positive RA have been identified, such as in the interactions of HLA-DRB1*SE alleles with smoking and drinking coffee [17]. As we did not include environmental factors in this report, the more precise consideration of environmental factors with the *MMP-13* −77 A>G polymorphism, HLA-DRB1*SE alleles, and anti-CCP antibodies might lead to new insights into the gene–environment effects of RA.

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Conflict of interest All authors declared no conflict of interest.

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Brief Communication

Immune Complexome Analysis of Serum and Its Application in Screening for Immune Complex Antigens in Rheumatoid Arthritis

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BACKGROUND: Analysis of circulating immune complexes (CICs) produced during an immune response may be useful in elucidating some aspects of this process. Identification of antigens incorporated into CICs provides information that may be helpful in developing diagnostic and treatment strategies for autoimmune diseases, infection, cancer, and transplantation therapy, and such information might be more relevant than information on free antigens. Because CICs may contain many antigens, comprehensive identification and profiling of such antigens is more effective than immunoblotting detection.

METHODS: We developed a novel proteomic strategy (immune complexome analysis) in which immune complexes (ICs) are separated from serum, digested directly with trypsin, and then subjected to nano-liquid chromatography–tandem mass spectrometry for identifying and profiling antigens in CICs. We applied this strategy to the analysis of CICs in 21 rheumatoid arthritis (RA) patients. Serum samples from 13 healthy donors and 8 osteoarthritis patients were used as controls.

RESULTS: CICs containing thrombospondin-1 (TSP-1) and platelet factor 4 (PF4) were found in the serum of 81% and 52% of RA patients, respectively, and in none of the controls.

CONCLUSIONS: The ICs in the serum of a majority of the RA patients contained TSP-1 or PF4, and these ICs may have potential as alternative biomarkers. Our technique for immune complexome analysis uses routine clinical samples, simple protocols, and widely available equipment. This method may be generally applicable to the study of the relationship between CICs and certain diseases associated with the immune response in animals and humans.

Immune complexes (ICs)[8] are products of reactions that involve noncovalent interactions between foreign antigens or autoantigen and antibody molecules. Circulating ICs (CICs) are possibly pathogenic unless they are removed by phagocytosis. Importantly, the identification of antigens in CICs might be different from that of free antigens because CICs are the direct and real-time products of an immune response. For a long time, CICs were thought to represent a common pathway for the pathogenesis of a large variety of diseases (e.g., immunologic diseases); however, the relationship is still unclear. Therefore, information on antigens in CICs is useful to reveal whether CICs play an important role in a particular disease. In the future, such information might provide new insights into pathophysiology and could form the basis for novel diagnostic and treatment strategies for autoimmune diseases, infection, cancer, and transplantation therapy. CICs present in the human body are likely to contain many different antigens that could reflect underlying disease and/or differences between individuals. Therefore, comprehensive identification and profiling of such antigens might be more effective than immunoblotting detection of individual antigens. Microarrays are widely used to detect multiple antigens *(1)*; however, the analytical comprehensiveness of this technique is fundamentally limited because preparation and selection of antigens or antibodies is required and because only molecules represented on microarrays can be identified. CICs and ICs in synovial fluid are likely to contribute to the pathogenesis of rheumatoid arthritis (RA) through the activation of the complement cascade, direct destruction of cartilage, and production of tumor necrosis factor α in synovial tissues *(2, 3)*. Considering that very different autoantibodies have been shown to be associated with RA *(1)*, comprehensive profiling of antigens incorporated in ICs may be effec-

[8] Nonstandard abbreviations: IC, immune complex; CIC, circulating immune complex; RA, rheumatoid arthritis; anti-CCP, anti–citrulline-containing protein/peptide; RF, rheumatoid factor; TSP-1, thrombospondin-1; PF4, platelet factor 4.

tive in improving our understanding; however, such studies have been very limited to date *(3–5)*.

In this report, we propose a novel proteomic strategy (immune complexome analysis) that entails separation of ICs from serum, direct tryptic digestion, and nano-liquid chromatography–tandem mass spectrometry for the identification and profiling of antigens in CICs. We have applied this strategy to RA as a model disease.

Serum samples were collected from RA patients (n = 21; 31–84 years; 18 females) at Sasebo Chuo Hospital who fulfilled the American College of Rheumatology criteria. The mean (SD) values for disease activity scores for C-reactive protein and erythrocyte sedimentation rate in the RA patients were 3.05 (1.12) and 3.69 (1.02), respectively. Nine of 10 RA patients were positive for anti–citrulline-containing protein/peptide (anti-CCP) antibody, but the results for the other 11 patients were not available. Sera from healthy donors (n = 13; 21–32 years; 9 females) and osteoarthritis patients (n = 8; 45–80 years; 4 females) were collected at Nagasaki University Hospital and used as controls. Whole blood was collected into tubes containing coagulation accelerator. After removing the clot by centrifugation at 1300*g* for 10 min at 4 °C, we stored the resulting supernatant (serum) at −80 °C. All experiments were performed in accordance with the Helsinki Declaration, with approval from the institutional ethics committees.

CICs were purified by magnetic beads with immobilized protein G (PureProteome™ Protein G Magnetic Bead System; Millipore). Beads (40 μL) were incubated with 10 μL of serum diluted with 90 μL PBS (9.0 mmol/L Na_2HPO_4, 2.9 mmol/L NaH_2PO_4, 137 mmol/L NaCl) for 30 min with gentle mixing. The beads with bound ICs were recovered with a magnet and washed 3 times with 500 μL PBS. The beads were resuspended in 100 μL of 10 mmol/L dithiothreitol and incubated at 56 °C for 45 min. We then added 100 μL of 55 mmol/L iodoacetamide and incubated the mixture at room temperature for 30 min in the dark. Subsequently, we added trypsin (Promega) to a final concentration of 0.5 g/L and incubated the mixture overnight at 37 °C. We then added 100 mL/L trifluoroacetic acid to stop the digestion and recovered the supernatant containing the peptide digests of antigens and antibodies. Finally, the volume of this mixture was vacuum-reduced to approximately 80 μL. The peptide mixture (1 μL) was subjected to nano-liquid chromatography–electrospray ionization–tandem mass spectrometry (LCQ Fleet; Thermo Fisher Scientific). The sample was loaded onto a nano precolumn [300 μm (i.d.) × 5.0 mm, LC-18; Chemicals Evaluation and Research Institute]. Peptides were separated on a nano-HPLC column [75 μm (i.d.) × 15 cm, Acclaim PepMap 100 C18, 3 μm; Dionex] with gradient elution and ion spray into the mass spectrometer at a spray voltage of 1.2–2.0 kV. Tandem mass spectrometry data were searched against a human subdatabase of the public nonredundant protein database of the International Protein Index (version 3.67; European Bioinformatics Institute). The filter criteria (single-, double-, and triple-charged peptides with a correlation factor and protein probability) were adjusted by maintaining the empirically determined protein false-discovery rate at zero. More details of protein identification by nano-liquid chromatography–tandem mass spectrometry can be found in the Data Supplement that accompanies the online version of this brief communication at http://www.clinchem.org/content/vol57/issue6.

The proteins identified by immune complexome analysis of RA patients and controls (healthy donors and osteoarthritis patients) are summarized in Table 1. Peptides derived from IgG and keratin were ignored. Peptide assignments determined as likely to be correct on the basis of a false-discovery rate of zero were confirmed by manual verification of fragmentation spectra (Fig. 1). Known human proteins (n = 34), such as apolipoproteins, complement proteins, coagulation proteins, and adhesion proteins, were identified in more than one independent sample of RA patients. The detection of several complements (C1, C3, C4) indicated that both the extraction of ICs from serum and peptide mapping by our method were successful.

IgG and C3 have been found together on the cartilage surface *(6)*, and RA-associated autoantibodies are enriched in synovial fluid *(7)* relative to serum. It is unclear, however, whether such enrichment is caused by deposition or local synthesis. Considering that CICs containing some of the antigens (clusterin, apolipoprotein E, and vitronectin) identified in this study have also been found in arthritic joints of RA and osteoarthritis patients *(4)*, our results suggest that CICs possibly accumulate in lesion sites.

The best-known diagnostic marker for RA is rheumatoid factor (RF), which is bound to the Fc domain of IgG. RF has a poor specificity (80.8%) for RA *(8)*, however, and is occasionally found in healthy persons (4%) *(9)*. Anti-CCP antibody has a greater specificity (98%) for RA than RF; however, its sensitivity (68%) is lower than RF (78.6%) *(3)*, and the relevant antigens of anti-CCP antibody remain obscure *(10)*. Of the antigens we have identified, thrombospondin-1 (TSP-1) incorporated into CICs was 100% specific for RA (95% CI, 84%–100%) and appeared more sensitive (81%; 95% CI, 58%–94%) than RF and anti-CCP antibodies (Table 1, Fig. 1). TSP-1 has been reported to be present in synovial tissues of RA patients *(11)*, and plasma TSP-1 concentrations have been shown to be increased in RA patients *(12)*. No information is available, however,

Table 1. Summary of proteins in ICs extracted from serum.

Protein	Accession[a]	RA (n = 21), frequency/peptide hit[b]	OA[c] (n = 8), frequency/peptide hit[b]	Healthy donors (n = 13), frequency/peptide hit[b]
Apolipoprotein				
Apolipoprotein A-I	IPI00021841.1	16/1–4	8/1–2	11/1–2
Apolipoprotein A-II	IPI00021854.1	2/1	0	1/1
Apolipoprotein B-100	IPI00022229.1	20/2–6	6/1–9	7/1–4
Apolipoprotein C-III	IPI00021857.1	11/1–2	7/1–2	1/1
Apolipoprotein E	IPI00021842.1	12/1–3	3/1–3	2/1
Isoform 1 of clusterin	IPI00291262.3	17/1–2	6/1–3	9/1–2
Complement				
Complement C1r subcomponent	IPI00296165.6	19/1–7	8/1–3	12/2–5
Complement C1s subcomponent	IPI00017696.1	12/1–4	2/1	6/1–2
Complement component 1, q subcomponent, B chain precursor	IPI00477992.1	11/1–3	4/1–2	3/2
Complement C1q subcomponent subunit A	IPI00022392.1	7/1	2/1	5/1–2
Complement C1q subcomponent subunit C	IPI00022394.2	12/1–2	5/1–2	3/1
Complement component 3	IPI00739237.1	16/2–6	3/2–4	12/2–7
Complement C4-A	IPI00032258.4	21/3–9	7/1–6	11/2–3
Complement component 4B preproprotein	IPI00418163.3	7/1–2	3/1	6/1–2
C4b-binding protein, α chain	IPI00021727.1	21/3–8	8/5–13	13/5–9
Isoform 1 of C4b-binding protein, β chain	IPI00025862.2	3/1	0	3/1
Complement factor H	IPI00029739.5	12/1–3	2/1–2	2/1
Coagulation proteins				
Plasminogen	IPI00019580.1	20/2–8	8/1–7	12/4–5
Prothrombin (fragment)	IPI00019568.1	19/1–8	8/2–7	12/2–3
PF4	IPI00022446.1	10/1[d]	0	0
PF4 variant	IPI00022295.1	3/1[d]	0	0
Adhesion proteins				
Isoform 1 of fibronection	IPI00022418.1	21/9–18	8/4–23	11/3–12
Vitronection	IPI00298971.1	21/1–4	8/1–4	11/1–3
Isoform B of fibulin-1	IPI00218803.3	15/1–5	4/1–2	5/1
Others				
TSP-1	IPI00296099.6	17/1–5	0	0
Histidine-rich glycoprotein	IPI00022371.1	19/3–5	8/3–7	13/1–6
Isoform HMW of kininogen-1	IPI00032328.2	18/2–6	7/1–6	12/2–4
Putative uncharacterized protein albumin	IPI00022434.4	18/1–12	8/2–5	7/4–10
Galectin-3-binding protein	IPI00023673.1	12/1–2	3/1–2	7/1–3
Vitamin K–dependent protein S	IPI00294004.1	8/1	3/1–2	1/1
Rheumatoid factor RF-ET9 (fragment)	IPI00384404.4	2/1	0	0
Rheumatoid factor D5 light chain (fragment)	IPI00816799.1	5/1	0	1/1
VH3 protein (fragment)	IPI00383732.1	4/1	0	3/1
Anti–folate-binding protein (fragment)	IPI00915820.1	2/1	1/1	1/1

[a] The accession is simply a series of digits that are assigned consecutively to each sequence record processed by International Protein Index.
[b] Peptide hit, number of peptides identified by liquid chromatography–tandem mass spectrometry analysis.
[c] OA, osteoarthritis.
[d] Eleven of 21 RA patients were positive for PF4 and/or its variant (2 RA patients were positive for both).

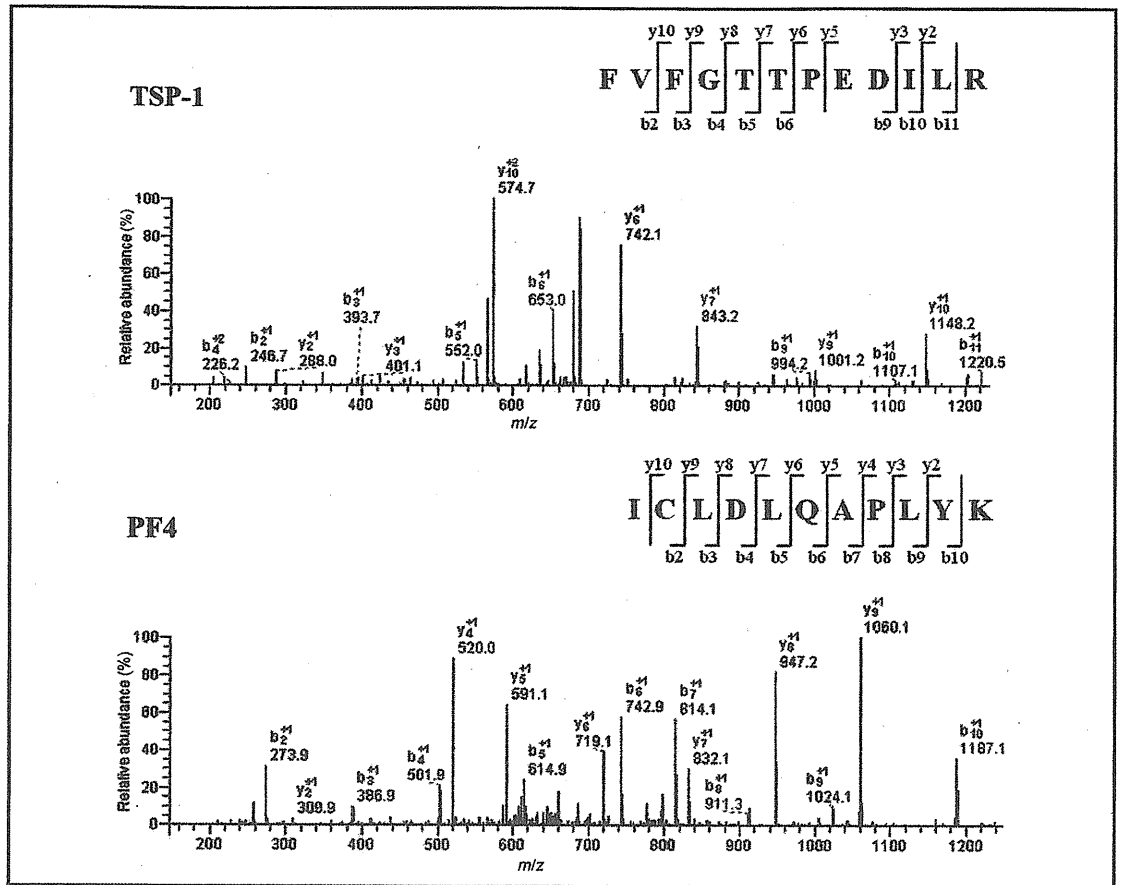

Fig. 1. Fragmentation spectra of TSP-1 and PF4 in CIC obtained by nano-liquid chromatography–tandem mass spectrometry.

Data on the masses of each tryptic peptide and its fragmentation ions were compared with those predicted for all tryptic peptides from all proteins in the International Protein Index.

about whether TSP-1 is present in ICs. This report is the first to show that ICs in the sera of RA patients contain TSP-1, and the IC may be a promising marker for RA. Recent studies have suggested that the TSP-1/transforming growth factor/connective tissue growth factor pathway plays an important role in angiogenesis and erosive arthritis lesions *(13, 14)*.

Although platelet factor 4 (PF4) was less sensitive (52.4%; 95% CI, 30%–75%) than TSP-1, our method also specifically detected PF4 or a variant incorporated into CICs in RA patients for the first time (Table 1, Fig. 1). Recently, Xiao et al. demonstrated that the ICs following the binding of anti-PF4 antibodies to PF4 were present and that they stimulated human neutrophil activation and cell adhesion *(15)*. This adhesion mechanism enables leukocytes to migrate from the blood and affect inflamed synovium. Furthermore, PF4 is known to be an angiostatic chemokine *(16)*, and the production of anti-PF4 antibodies can accelerate angiogenesis.

To investigate potential nonspecific binding of the identified proteins to the protein G beads, we spiked healthy donor sera with TSP-1 (recombinant) and PF4 (natural) at concentrations 10-fold higher than their typical concentrations in blood. Neither TSP-1 nor PF4 was recovered with the protein G beads, suggesting that their detection in the immune complexome analysis was not due to nonspecific binding and that detection of these proteins in CICs was biologically relevant.

Anti-CCP antibodies are now widely recognized as a specific marker of RA *(10)*, so we also searched the mass spectra for peptides in which citrulline had been substituted for arginine; however, the detection fre-

quency of citrullinated antigens incorporated into CICs was much lower in this study than that of non-citrullinated antigens. Similarly, other studies that used mass spectrometry reported that citrullinated peptides did not yield a better calculated fit with the fragmentation products than arginine-containing peptides in the joints of RA patients *(4)* or that citrullinated antigens were not found in the serum of RA patients *(3)*. On the other hand, some citrullinated antigens have been found in synovial fluid *(3)* or plasma *(5)* via an immunodetection method with the corresponding antibodies. The discrepancy may be attributed to the difference in methods, but it is also possible that not only citrullinated antigens but also noncitrullinated ones are involved in the pathogenesis of RA.

In conclusion, this report is the first to reveal that ICs containing TSP-1 or PF4 are present specifically in the serum of RA patients, and these ICs may have potential as alternative biomarkers for the diagnosis of RA. This method for immune complexome analysis may be generally applicable to the study of the relationship between CICs and immune response–related disease treatments in animals and humans.

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