cell death 1 was increased in LSGs from SS patients, as determined by real-time polymerase chain reaction (PCR) (10). Recently, Hjelmervik et al (13) reported that the up-regulated genes in SS salivary glands were IFN-stimulated transcription factor 3 and IFN regulatory factor (IRF-1). However, the essential genes in the generation of sialadenitis in patients with SS have not been clarified. Thus, there is a need for analysis of disease susceptibility genes in labial salivary and lacrimal glands of SS patients.

In the present study, we focused on the STAT-1 gene, one of the IFNγ-inducible genes, in LSGs from SS patients. We found that STAT-1α may be one of the susceptibility genes in the generation of SS. We also discuss the functional role of STAT-1α in SS.

MATERIALS AND METHODS

Subjects. Approval for this study was obtained from the Local Ethics Committee, and written informed consent was obtained from all patients and healthy subjects who participated. LSGs were collected from 10 healthy Japanese subjects and from 12 Japanese patients with primary SS who were receiving followup care at the Department of Internal Medicine, University of Tsukuba Hospital. All SS patients satisfied the Japanese Ministry of Health criteria for the classification of SS (14), and all had an LSG focus score of >3, as determined by the Greenspan et al method (15).

RNA extraction and complementary DNA (cDNA) synthesis. Biopsy samples were frozen in liquid nitrogen and kept at –80°C until the RNA extraction procedure. Frozen LSGs were homogenized, and total RNAs were extracted using Isogen reagent (Nippon Gene, Tokyo, Japan). The optical density of RNA was measured with a DU 640 Spectrophotometer (Beckman Coulter, Fullerton, CA), the RNA yield and quality were estimated, and total RNAs were stored at –80°C until used. We synthesized cDNA using the Revert Aid First-Strand cDNA Synthesis kit (Fermentas, Hanover, MD) with >200 ng of total RNA.

Real-time quantitative PCR. Quantitative analysis was performed using STAT-1α, STAT-1β, IFNγ-inducible 10-kd protein (IP-10), and IRF-1, with GAPDH as an endogenous control (all from Applied Biosystems, Foster City, CA). PCRs were run in an ABI Prism 7700 sequencer (Applied Biosystems). The primer and probe sequences used were as follows: for STAT-1α, 5′-GTCTCGGATAGTGGGCTC-TG-3′ (sense), 5′-TGCTGGCCTTTCTTTCATTT-3′ (antisense), and 5′-TCTCTGGCGACAGTTTCCT-3′(probe) and for STAT-1β, 5′-TTACTCCAGGCCAAAGGAAG-3′ (sense), 5′-AGGCTGGCTTGAGGTTTGTA-3′ (antisense), and 5′-TGATGGCCCTAAAGGAACTG-3′ (probe).

Protein extraction. Immediately after biopsy, tissues were minced into fragments of >1 mm^3. Cell lysates were extracted with lysis buffer (50 m*M* Tris HCl, 5 m*M* $MgCl_2$, 0.5% Nonidet P40, and 2 m*M* phenylmethylsulfonyl fluoride). Aliquots of 10 μg of total protein were prepared and stored at −80°C until used.

Western blot analysis. 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 hour and then incubated with one of the following antibodies: mouse anti–STAT-1 (1:250 dilution; BD Biosciences, San Jose, CA), mouse anti–pSTAT-1 tyrosine 701 (anti-$pTyr^{701}$) (1:1,000 dilution; BD Biosciences), rabbit anti–pSTAT-1 serine 727 (anti-$pSer^{727}$) (1:1,000 dilution; Cell Signaling Technology, Beverly, MA), mouse anti–STAT-2 (1:500 dilution; BD Biosciences), rabbit anti–pSTAT-2 (1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), or mouse anti–β-actin (1:6,000 dilution; Sigma-Aldrich, St. Louis, MO). Secondary antibody was applied for 30 minutes, using isotype-matched horseradish peroxidase (HRP)–labeled anti-mouse IgG antibody (1:2,000 dilution; Dako, Tokyo, Japan) or HRP-labeled anti-rabbit IgG antibody (1:2,000 dilution; Bio-Rad, Hercules, CA).

The dilutions were performed in 0.05% Tween 20 in phosphate buffered saline (PBS). Proteins were detected by enhanced chemiluminescence using an ECL Western blot detection kit (Amersham, Little Chalfont, UK).

Immunohistochemical analysis. Tissue samples were embedded en bloc in TissueTek OCT compound (Sakura, Torrance, CA) and frozen in liquid nitrogen. The frozen blocks were stored at –80°C until sectioned for staining. Sections (5 nm) were cut in a cryostat and mounted on silane-coated glass (Muto Glass, Tokyo, Japan). The slides were air dried at room temperature, carefully packed and sealed, and then stored at –80°C until immunohistochemical staining was performed.

Sections were thawed, dried, and then fixed with acetone for 10 minutes. Endogenous peroxidase activity was inhibited using 0.3% hydrogen peroxidase/methanol. Sections were blocked in 5% bovine serum albumin–PBS for 10 minutes and then incubated with one of the following antibodies: mouse anti-$pTyr^{701}$ (1:20 dilution), rabbit anti-$pSer^{727}$ (1:50 dilution), rabbit IRF-1 (1:50 dilution; Santa Cruz Biotechnology), goat anti–IP-10 (1:100 dilution; R&D Systems, Minneapolis, MN), or mouse anti-Fas (1:25 dilution; BD Biosciences). Isotype-matched HRP-conjugated anti-mouse IgG antibody (1:200 dilution), anti-rabbit IgG antibody (1:200 dilution), or anti-goat antibody (Dako) was added for 30 minutes. HRP activity was detected using 3,3′-diaminobenzidine (DAB; Nichirei, Tokyo, Japan) as substrate. Sections were counterstained with Mayer's hematoxylin for 10 seconds and then mounted with aqueous mounting medium. Control slides were incubated with blocking buffer containing isotype-matched antibodies instead of the primary antibody.

To quantify the staining for Tyr^{701} pSTAT-1 and Ser^{727} pSTAT-1 on ductal epithelial cells in the region examined, the number of positively stained cells in every high-power field was recorded, and the results were expressed as a percentage of the total number of ductal epithelial cells. The LSG sections were coded and were analyzed in random order by an observer who was blinded to the source of the samples.

TUNEL staining. Apoptotic cells were detected using an in situ apoptosis detection kit (Takara Bio, Shiga, Japan). Briefly, after drying at room temperature, sections were fixed with acetone for 30 minutes. Endogenous peroxidase activity

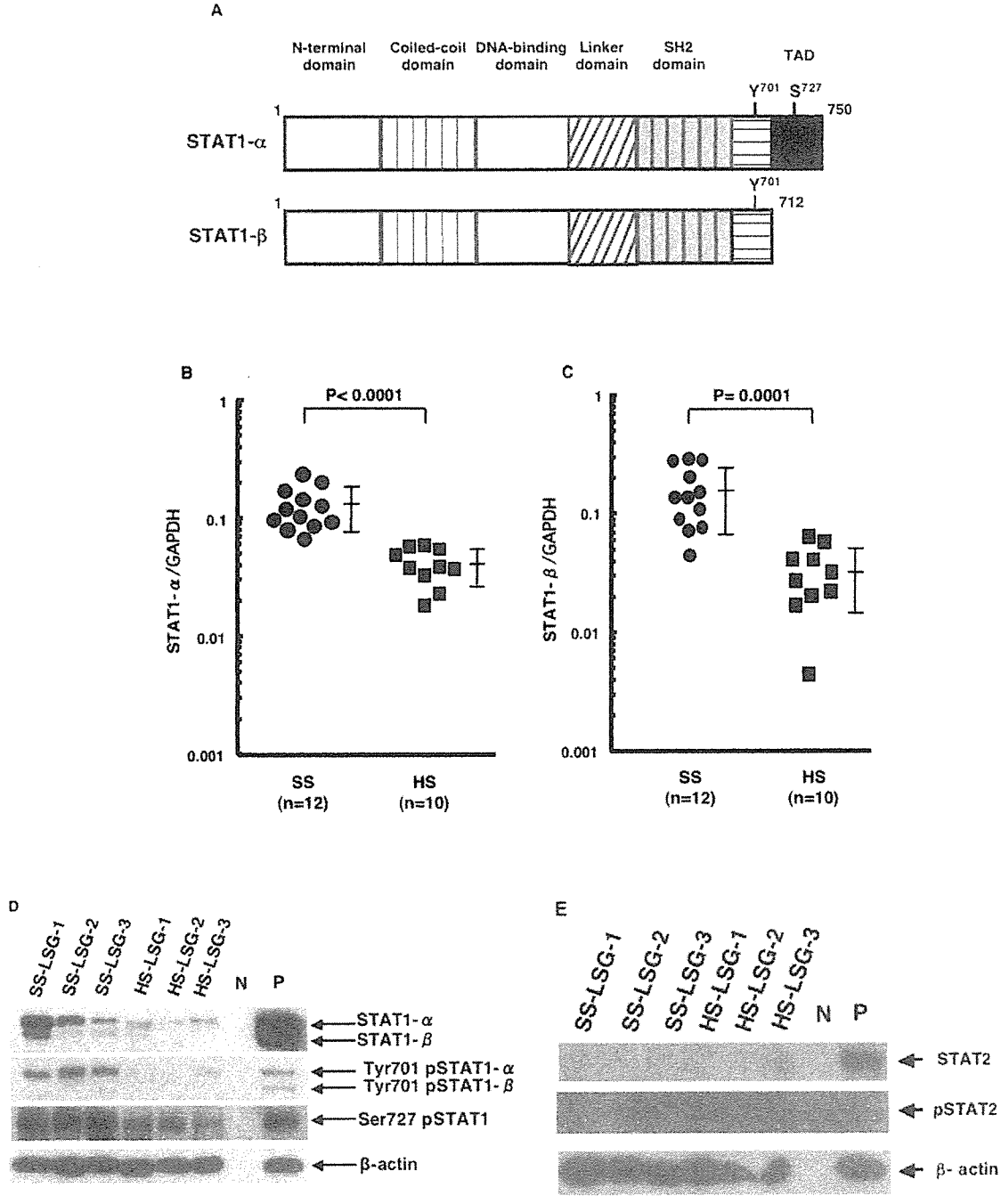

Figure 1. Expression and activation of STAT-1 in labial salivary glands (LSGs) from patients with Sjögren's syndrome (SS). **A,** Domain structure of STAT-1α and STAT-1β. Y^{701} = Tyr^{701}; S^{727} = Ser^{727}; TAD = transcription activation domain. Adapted, with permission, from ref. 50. **B** and **C,** STAT-1 mRNA in LSGs from 12 SS patients and 10 healthy control subjects (HS), as determined by real-time quantitative polymerase chain reaction. Both STAT-1α (**B**) and STAT-1β (**C**) mRNA were highly expressed in SS LSGs compared with controls. Results are expressed as the relative ratio of STAT-1α or STAT-1β to GAPDH. Each symbol represents a single subject. Bars show the mean ± SD. **D** and **E,** Western blot analysis of LSGs from 3 SS patients and 3 healthy control subjects. STAT-1α protein levels were higher in SS LSGs than in controls; however, STAT-1β protein was not clearly detected (**D**). Tyr^{701} pSTAT-1 protein was specifically detected in SS LSGs, and Ser^{727} pSTAT-1 was prominent in SS LSGs as compared with control LSGs (**D**). STAT-2 and pSTAT-2 expression was absent in all of the samples tested (**E**). Cell lysate extracted from interferon-γ–stimulated human peripheral blood mononuclear cells was used as a positive control (P), lysis buffer alone was used as a negative control (N), and β-actin was used as an internal control.

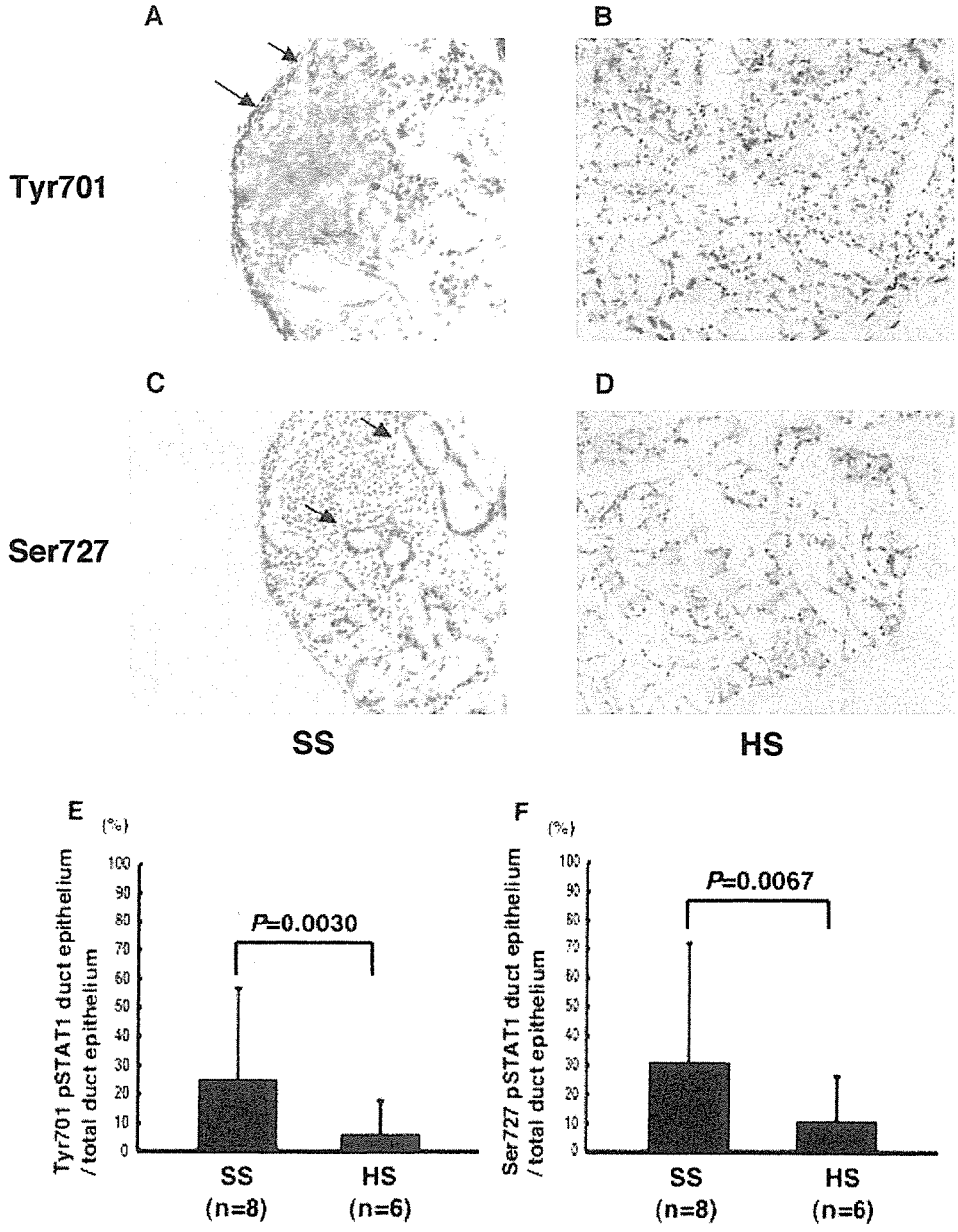

Figure 2. Differential localization of Tyr^{701} and Ser^{727} pSTAT-1 on ductal epithelium in labial salivary glands (LSGs) from patients with Sjögren's syndrome (SS). **A,** Tyr^{701} pSTAT-1 was localized in the ductal epithelium and infiltrating mononuclear cells in SS LSGs, especially in the ductal epithelium adjacent to lymphoid infiltrates **(arrows). B,** Tyr^{701} pSTAT-1 was not detected in control LSGs. **C,** Ser^{727} pSTAT-1 was localized only in the ductal epithelium in SS LSGs **(arrows). D,** Ser^{727} pSTAT-1 was not detected in control LSGs. (Original magnification × 100.) **E,** Tyr^{701} pSTAT-1–positive and **F,** Ser^{727} pSTAT-1–positive ductal epithelial cells in LSGs from SS patients and from healthy control subjects (HS). Values are the mean and SD ratio.

was blocked with 0.3% hydrogen peroxidase/methanol. Sections were treated for 5 minutes on ice with a permeabilization buffer and then incubated with a TUNEL labeling mixture for 1 hour at 37°C. An HRP-conjugated anti–fluorescein isothiocyanate antibody was added for 30 minutes at 37°C. HRP activity was detected using DAB as substrate. Mayer's hematoxylin was used for counterstaining. Selected sections were incubated with labeling solution to use as negative controls.

Statistical analysis. The Mann-Whitney U test was used for statistical analysis. *P* values less than 0.05 were considered significant.

RESULTS

High levels of STAT-1 mRNA expression in LSGs from SS patients. Since a predominant expression of Th1 cytokines has been reported in SS LSGs (4–6), we focused on 1 of the IFNγ-inducible genes, STAT-1. STAT-1α and STAT-1β mRNA were quantitatively analyzed by real-time PCR of LSGs from 12 patients with SS and 10 healthy control subjects. Both STAT-1α and STAT-1β mRNA were highly expressed in SS LSGs as compared with control LSGs (mean ± SD 0.13 ± 0.06 versus 0.04 ± 0.01 [$P < 0.0001$] for STAT-1α; 0.16 ± 0.09 versus 0.03 ± 0.02 [$P = 0.0001$] for STAT-1β) (Figures 1B and C).

Overexpression of pSTAT-1α protein in LSGs from SS patients. Western blot analysis was performed to investigate the expression of STAT-1α and STAT-1β at the protein level in LSGs from 3 patients with SS and 3 healthy control subjects. As shown in Figure 1D, the level of STAT-1α protein in the 3 SS LSG samples was higher than that in the 3 control LSG samples, whereas the expression of STAT-1β protein was low compared with that of STAT-1β mRNA. Moreover, Tyr^{701} pSTAT-1 protein was specifically detected in LSGs from the SS patients. Compared with control LSGs, Ser^{727} pSTAT-1 was prominent in SS LSGs.

No expression of STAT-2 and pSTAT-2 proteins in LSGs from SS patients. STAT-1α is the only mediator of the action of IFNγ, but it is also one of the mediators of the action of IFNα. It is known that IFNα signaling is mediated by STAT-2, STAT-1, and the IRF-9 complex (16). To examine whether IFNα relates to the phosphorylation of STAT-1α protein, the expression of STAT-2 and pSTAT-2 in LSGs from 3 SS patients and from 3 healthy control subjects was analyzed by Western blotting. As shown in Figure 1E, the expression of STAT-2 and pSTAT-2 was not detected in LSGs from SS patients or healthy controls. These findings suggest that the high expression and phosphorylation of STAT-1α proteins in LSGs from SS patients might be dependent on IFNγ rather than IFNα.

Differential localization of Tyr^{701} and Ser^{727} pSTAT-1 on the ductal epithelium in LSGs from SS patients. To localize activated STAT-1, we analyzed Tyr^{701} and Ser^{727} pSTAT-1 proteins by immunohistochemical staining of LSGs from 8 of the SS patients and 6 of the healthy control subjects. Figure 2 clearly demonstrates the localization of Tyr^{701} pSTAT-1 in the ductal epithelium and infiltrating mononuclear cells in LSGs from the SS patients, especially the ductal epithelium adjacent to lymphoid infiltrates (Figure 2A), although not in LSGs from the control subjects (Figure 2B). In contrast, Ser^{727} pSTAT-1 was observed only in the ductal epithelium of SS LSGs (Figure 2C), and there was no expression in control LSGs (Figure 2D).

Figures 4E and F show the ratio of Tyr^{701} or Ser^{727} pSTAT-1–positive ductal epithelial cells in LSGs from SS patients and healthy control subjects. Tyr^{701} pSTAT-1–positive ductal epithelium in SS LSGs (mean ± SD 25.39 ± 5.87%) was significantly increased compared with control LSGs (6.10 ± 5.82%; $P = 0.0030$) (Figure 2E). The number of Ser^{727} pSTAT-1–positive cells in SS LSGs (31.20 ± 9.62%) was also higher compared with controls (11.06 ± 4.21%; $P = 0.0067$) (Figure 2F).

High levels of STAT-1–inducible gene expression in LSGs from SS patients. To examine whether STAT-1–inducible genes are in fact induced by STAT-1, we examined by real-time PCR mRNA for IP-10, IRF-1, and Fas in LSGs from the 12 patients with SS and the 10 healthy control subjects. IP-10 mRNA was highly expressed in SS LSGs (mean ± SD 0.007 ± 0.005) compared with control LSGs (0.001 ± 0.0001; $P = 0.0002$) (Figure 3A). The expression of IRF-1 was also significantly higher in SS LSGs (0.05 ± 0.03) than in control LSGs (0.01 ± 0.0.1; $P = 0.0005$) (Figure 3B). Moreover, the mRNA level of Fas in SS LSGs (0.17 ± 0.06) was higher than in control LSGs (0.08 ± 0.04; $P = 0.0008$) (Figure 3C).

Colocalization of destructive factors with Ser^{727} pSTAT-1–positive cells in LSGs from SS patients. To characterize the function of Tyr^{701} pSTAT-1 and Ser^{727} pSTAT-1 in SS LSGs, we analyzed Tyr^{701} and Ser^{727} pSTAT-1, Fas, IRF-1, and IP-10 proteins, as well as apoptotic cells by immunohistochemical staining using sequential sections of LSGs from 4 SS patients. Figure 4 shows that Ser^{727} pSTAT-1, but not Tyr^{701} pSTAT-1, was colocalized with Fas, IP-10, IRF-1, and apoptotic cells.

DISCUSSION

Previous studies have demonstrated that the STAT family is associated with autoimmune diseases, such as the association of STAT-3 and STAT-1 with rheumatoid arthritis (17–20) and of STAT-1 with autoimmune diabetes (21,22). These observations suggest that STAT-1 functions as an effector (21,22) or regulator molecule (18,19) in autoimmune diseases. Recent studies using STAT-1–knockout or STAT-1–transgenic mice showed that STAT-1 signaling plays an important role as an effector in Th1-type T cell–mediated hepatitis

(23,24). STAT-1 is linked to abnormal glandular homeostasis in the nonobese diabetic mouse (25). Moreover, Wu et al (26) showed that IFNγ induced phosphorylation of STAT-1 in a human salivary gland cell line. These findings support the notion that STAT-1 functions as an effector molecule in Th1-type diseases, including SS (4–6).

STAT-1 is known as the mediator of IFNγ signaling: pSTAT-1 dimerizes after undergoing INFγ-induced phosphorylation, when it translocates to the nucleolus, and activates the transcription of IFN-inducible genes. Maximal activation by STAT-1 through IFNγ signaling requires both Tyr^{701} and Ser^{727} phosphorylation (27,28). STAT-1β is a naturally occurring splice variant of STAT-1α that lacks the 38 carboxyl-terminal amino acids that contain a phosphorylation site at Ser^{727}. Since there is no transactivation domain in

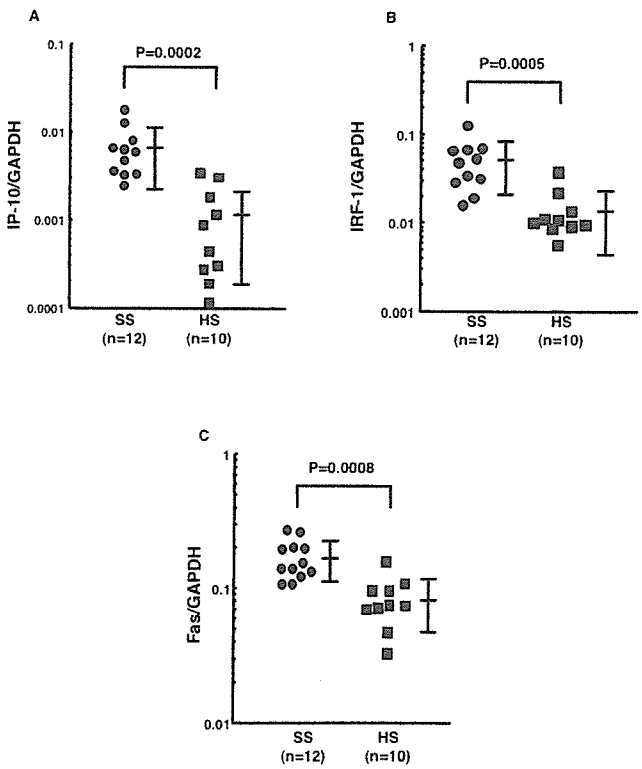

Figure 3. Expression of STAT-1–inducible genes in labial salivary glands (LSGs) from patients with Sjögren's syndrome (SS). Levels of **A,** interferon-γ (IFNγ)–inducible 10-kd protein (IP-10), **B,** IFN regulatory factor (IRF-1), and **C,** Fas mRNA in LSGs from 12 SS patients and 10 healthy control subjects (HS) were determined by real-time quantitative polymerase chain reaction. All 3 STAT-1–inducible genes were up-regulated in SS LSGs as compared with controls. Results are expressed as the relative ratio of each gene to GAPDH. Each symbol represents a single subject. Bars show the mean ± SD.

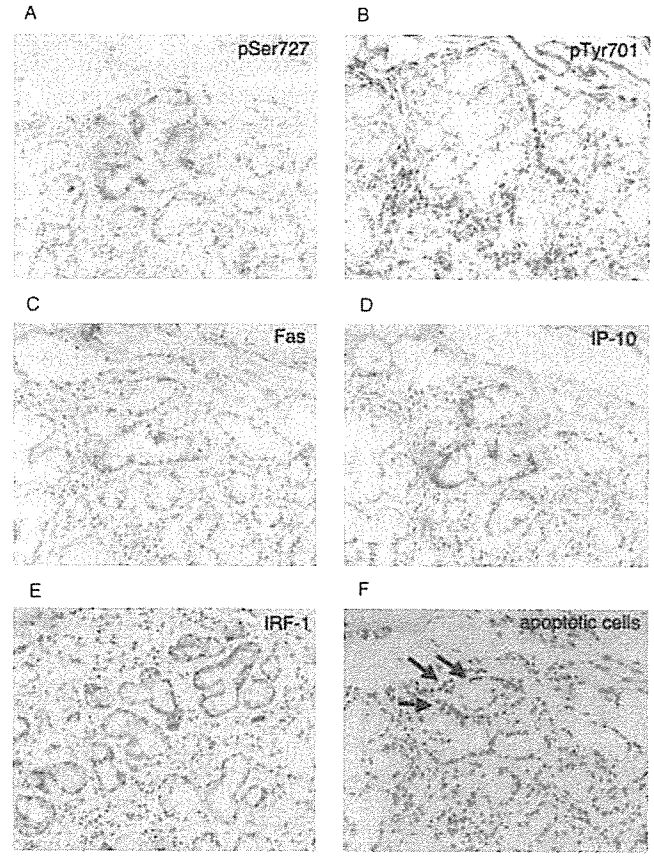

Figure 4. Colocalization of destructive factors with Ser^{727} pSTAT-1–positive cells in labial salivary glands from patients with Sjögren's syndrome. The expression of **A,** Ser^{727} pSTAT-1, **B,** Tyr^{701} pSTAT-1, **C,** Fas, **D,** interferon-γ (IFNγ)–inducible 10-kd protein (IP-10), **E,** IFN regulatory factor (IRF-1), and **F,** TUNEL-positive apoptotic cells (**arrows**) was examined by immunohistochemical analysis. Immunohistochemical staining showed that Ser^{727} pSTAT-1 was colocalized with Fas, IP-10, IRF-1, and apoptotic cells (**A, C, D, E,** and **F**). However, the localization of Tyr^{701} pSTAT-1 (**B**) was clearly different from that of the other molecules examined. (Original magnification × 200.)

STAT-1β, Tyr^{701} pSTAT-1β dimers are able to bind DNA, but are not able to activate it (29). Thus, STAT-1β is considered an antagonist of STAT-1α.

In the present study, both STAT-1α and STAT-1β mRNA were expressed in LSGs from SS patients. STAT-1α protein was highly expressed in SS LSGs, whereas the expression of STAT-1β protein was low compared with that of STAT-1β mRNA. The difference between STAT-1α and STAT-1β protein expression may be due to the instability of STAT-1β mRNA and the low efficiency of phosphorylation of STAT-1β protein. Furthermore, Tyr^{701} pSTAT-1 protein was specifically detected in SS LSGs, and Ser^{727} pSTAT-1 protein was more strongly induced in SS LSGs, suggest-

ing that the overexpression of STAT-1α protein, but not STAT-1β protein, leads to transcription of STAT-1–inducible genes in SS LSGs. In contrast, neither STAT-2 nor pSTAT-2 was detected in SS LSGs, indicating that STAT-1α may be mediated by IFNγ rather than IFNα.

Immunohistochemical analyses demonstrated that Tyr^{701} pSTAT-1 was localized in the infiltrating lymphocytes and in the adjacent ductal epithelium in SS LSGs, although Ser^{727} pSTAT-1 was identified only in the ductal epithelium of SS LSGs. The differential expression of Tyr^{701} and Ser^{727} pSTAT-1 in SS LSGs may be associated with the different functioning of STAT-1α and STAT-1β. Recently, Stephanou et al (30) showed that induction of apoptosis and Fas expression required Ser^{727} from STAT-1 but not Tyr^{701}. Therefore, STAT-1α might be essential for the induction of apoptosis of the epithelium in salivary glands. In contrast to epithelial cells, the phosphorylation of STAT-1β was dominant in mononuclear cells that infiltrated SS LSGs, resulting in resistance to apoptosis.

IP-10 is an IFNγ-induced CXC chemokine that is present in many tissues, including the heart, liver, lung, and spleen (31), and it binds to the CXCR3 chemokine receptor expressed on T cells (32,33). IP-10 plays an important role in the recruitment of T cells to sites of inflammation (34). The expression of IP-10 correlates with tissue infiltration by T cells in autoimmune diseases such as rheumatoid arthritis and multiple sclerosis (35,36). In a study of SS salivary glands, Ogawa et al (37) demonstrated that IP-10 proteins were predominantly expressed in the ductal epithelium adjacent to lymphoid infiltrates, as well as in most T cells expressing CXCR3.

IRF-1 is a downstream transcription molecule of STAT-1 in the IFN signaling pathway (38,39). IRF-1 binds to the interferon-stimulated response element and regulates IFNα/β-induced genes (40). Moreover, IRF-1 arrests the cell cycle or induces apoptosis of some cells without any stimulation by IFNγ (41,42). Kano et al (43), using IRF-1–deficient primary hepatocytes, found that IRF-1 is a critical mediator in IFNγ-induced apoptosis, suggesting that IRF-1 plays an important role in STAT-1–induced apoptosis through IFNγ signaling.

Fas, a cell surface molecule belonging to the tumor necrosis factor superfamily, is expressed in various tissues, such as the thymus, heart, liver, and spleen. Several studies have shown that IFNγ is able to stimulate various cells to express Fas (30,44), which triggers apoptosis when stimulated by its ligand (FasL) (45). In studies of SS salivary glands, Bolstad et al (10) found that Fas and FasL were expressed on ductal and acinar epithelial cells and on mononuclear cells in the inflammatory infiltrates, although FasL was most frequently detected on mononuclear cells.

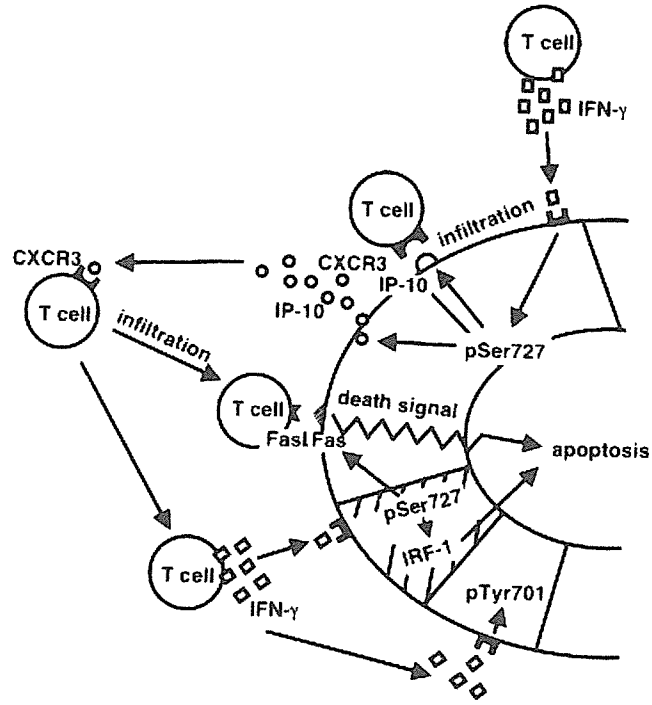

Figure 5. Possible mechanism of salivary gland destruction in Sjögren's syndrome. Ser^{727} pSTAT-1 ($pSer^{727}$) in the ductal epithelium induces interferon-γ (IFNγ)–inducible 10-kd protein (IP-10) and recruits CXCR3 (the receptor for IP-10) and FasL-positive T cells to the salivary glands. In addition, the IFNγ-induced Ser^{727} pSTAT-1 leads to the expression of Fas and IFN regulatory factor (IRF-1), resulting in apoptosis of the ductal epithelium. Tyr^{701} pSTAT-1 ($pTyr^{701}$) is also induced by IFNγ; however, $pTyr^{701}$ may not be essential for apoptosis of the ductal epithelium.

In the present study, Fas, IP-10, and IRF-1 were also expressed on the same cells as Ser^{727} pSTAT-1–positive cells. Therefore, we proposed the hypothesis that Ser^{727} pSTAT-1 induced by IFNγ leads to the expression of Fas, IRF-1, and IP-10, which results in apoptosis of the ductal epithelium and in the recruitment of T cells into the salivary glands (Figure 5). Functional analysis of Ser^{727} pSTAT-1 will be necessary to clarify the role of STAT-1 in the generation of salivary gland destruction in patients with SS.

The STAT pathway is negatively regulated at multiple steps by several groups of proteins. The suppressor of cytokine signaling (SOCS) proteins are rapidly induced by cytokines and inhibit STAT signaling through distinct mechanisms (46). In the nucleus, the activity of STATs can be negatively regulated by at least 2 molecular mechanisms: the dephosphorylation of

STATs by protein tyrosine phosphatases (47) and the suppression of STAT-mediated gene activation by members of the protein inhibitor of activated STAT family (48). These negative regulators are important for controlling the signaling strength, kinetics, and specificity of the STAT pathway. Recently, Chong et al (49) demonstrated that overexpression of SOCS-1 protects against pancreatic beta cell destruction in the NOD mouse. Their findings suggested the possibility that the overexpression of a negative regulator may be a new approach to the effective treatment of organ-specific autoimmune diseases such as SS.

In conclusion, we provided evidence for the overexpression of STAT-1 mRNA and protein in LSGs from patients with SS. In addition, we detected pSTAT-1α in the ductal epithelium of SS LSGs. These findings suggest that STAT-1 may function as a key molecule in the pathogenesis of SS.

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Altered peptide ligands regulate muscarinic acetylcholine receptor reactive T cells of patients with Sjögren's syndrome

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In the generation of Sjögren's syndrome (SS), CD4 positive αβ T cells have a crucial role. Previous studies have provided evidence about the T cell receptor (TCR) Vβ and Vα genes on these T cells, and sequence analysis of the CDR3 region indicates the presence of some conserved amino acid motifs, supporting the notion that infiltrating T cells recognise relatively few epitopes on autoantigens.[1]

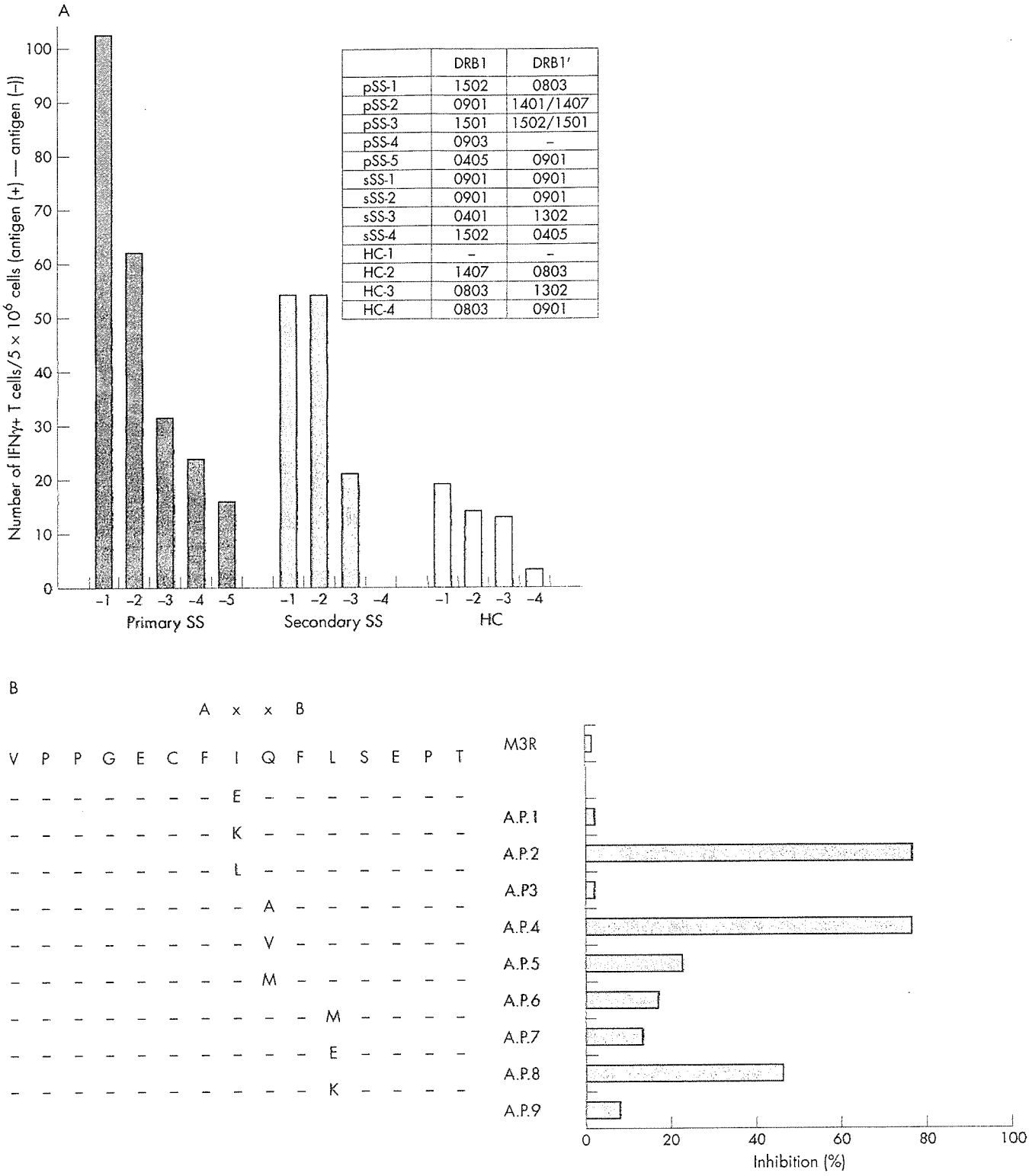
Candidate autoantigens recognised by T cells that infiltrate the labial salivary glands of patients with SS have been analysed, and Ro/SSA 52 kDa,[2] α-amylase, heat shock protein, and TCR BV6 have been identified, although Ro/SSA 52 kDa reactive T cells were not increased in peripheral blood.[3] Gordon *et al* indicated that anti-M3R autoantibodies occurred in SS and were associated with the sicca symptoms.[4] Recently, we provided evidence for the presence of autoantibodies against the second extracellular domain of muscarinic acetylcholine receptor (M3R) in a subgroup of patients with SS.[5] The M3R is an interesting molecule, because this portion has an important role in intracellular signalling,[5] although the function of anti-M3R autoantibodies remains unknown.

The mechanism through which a peptide is recognised by a TCR is flexible. If the amino acid residue of the peptide ligands for TCR is substituted by a different amino acid and can still bind to major histocompatibility complex molecules (altered peptide ligand), such an altered peptide ligand could regulate the activation of T cells. Several studies have shown that an altered peptide ligand could induce differential cytokine secretion, anergy, and antagonism of the response to the wild-type antigens.[6 7] The altered peptide ligand has the potential of being used therapeutically against T cell mediated diseases such as autoimmune diseases and allergic disorders.

As an extension to our previous study,[5] we focused in the present study on M3R reactive T cells and analysed T cell epitopes and their altered peptide ligands with the aim of regulating T cell proliferation and autoantibody production. The 25mer synthetic amino acids encoding the second extracellular domain of M3R (KRTVPPGECFIQFLSEPTITF GTAI, AA213-237) were used as the antigen for T cells, and the number of interferon (IFN) γ producing T cells was counted by flow cytometry using a magnetic activated cell sorting (MACS) secretion assay. The proportion of IFNγ-producing T cells among peripheral blood mononuclear cells (PBMCs) was high in two of five patients with primary SS (pSS) and two of four patients with secondary SS (sSS), compared with the level in four healthy control subjects (HC) (fig 1A). Three patients with SS and M3R reactive T cells (pSS-2, and sSS-1, 2) had the HLA-DR B1*0901 allele and the other patient (pSS-1) had HLA-DR B1*1502 and *0803 alleles. The 25mer amino acids contain the anchored motifs that bind to HLA-DR B1*0901. Thus, IFNγ production by T cells should be due to the recognition of antigen on the HLA molecule by the TCR on T cells.

The results shown in fig 1 were obtained as follows. Blood samples were collected from five Japanese patients with pSS and four Japanese patients with sSS followed up at the University of Tsukuba Hospital. All patients with SS satisfied both the Japanese Ministry of Health criteria for the classification of SS[8] and the revised EU-US criteria[9]. We also recruited four HC from our university. Approval for this study was granted from the local ethics committee, and written informed consent was obtained from all patients and HC who participated in this study.

Their HLA-DR allele was examined by the SSOP-PCR method, as described elsewhere. A 15mer peptide (VPPGECFIQFLSEPT) (M3R AA216–230) corresponding to the sequence of the second extracellular loop domain was also synthesised (Kurabo Industries, Osaka, Japan). PBMCs were purified with Ficoll-Paque and 5×10^6 cells were cocultured with 10 μg of M3R peptide (25mer) in 1 ml of RPMI-1640 with 10% of human AB serum (Sigma, St Louis, MO) for 12 hours at 37°C. As a positive control, 1 μg of staphylococcal enterotoxin B (Toxin Technology Inc, USA) was used. IFNγ-producing cells were identified by the MACS cytokine secretion assay (Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, the cells were incubated with 20 μg of IFNγ detection antibody (Ab; Miltenyi Biotec), 20 μg of



	DRB1	DRB1′
pSS-1	1502	0803
pSS-2	0901	1401/1407
pSS-3	1501	1502/1501
pSS-4	0903	–
pSS-5	0405	0901
sSS-1	0901	0901
sSS-2	0901	0901
sSS-3	0401	1302
sSS-4	1502	0405
HC-1	–	–
HC-2	1407	0803
HC-3	0803	1302
HC-4	0803	0901

Figure 1 (A) M3R reactive T cells. (B) Selection of altered peptide ligands.

anti-CD4-FITC Ab (Becton Dickinson, Franklin Lakes, NJ, USA), and 5 μg of anti-CD3-APC Ab (Becton Dickinson) for 10 minutes at 4°C. After double washing with a cold buffer (phosphate buffered saline/0.5% bovine serum albumin with 2 mM EDTA), the cells were incubated with 20 μg of anti-phycoerythrin microbeads (Miltenyi Biotec) for 15 minutes at 4°C. After double washing, the cells were resuspended with 500 μl buffer and then passed through an MS column (Miltenyi Biotec), which was set to mini-magnet (Miltenyi Biotec). The column was set on the Falcon tube (Becton Dickinson), bead-binding cells were eluted by 1 ml of cold buffer, and IFNγ-producing cells were analysed by FACSCalibur (Becton Dickinson).

The 15mer peptide (M3R 216–230) and its nine altered peptide ligand candidates were synthesised (Sigma) (fig 1B). The purity of each peptide was >90%. The anchor positions binding to HLA-DR B1*0901 are AA222 and AA225, which are indicated as A and B in fig 1B. PBMCs from patient pSS-2 were used in this experiment; 1×10^6 cells were cultured with 10 μg of each peptide in 1 ml of RPMI-1640 with 10% human

AB serum. IFNγ-producing T cells were identified using MACS secretion assay as described in fig 1A.

To determine the altered peptide ligands of M3R in patients with SS, we synthesised nine 15mer peptides (VPPGECFI→E/K/LQFLSEPT, VPPGECFIQ→A/V/MFLSEPT, VPPGECFIQFL→M/E/KSEPT, M3R216–230), in which the anchored motif binding to the HLA-DR B1*0901 molecule is conserved, although one amino acid to TCR was different. Altered peptide ligands were selected based on inhibition of IFNγ production by M3R reactive T cells. Figure 1B shows that M3R 223I→K and M3R 224Q→A significantly suppressed the number of IFNγ-producing T cells, suggesting that they are candidates for selection as altered peptide ligands. The inhibition of IFNγ by other cytokines may not be likely, because interleukin 4 producing T cells were not increased (data not shown).

In conclusion, we have provided evidence for the presence of M3R reactive T cells in the serum of patients with SS and shown that VPPGECFKQFLSEPT (M3R 223I→K) and VPPGECFIAFLSEPT (M3R 224Q→A) are candidate altered peptide ligands of the second extracellular domain of M3R. Our findings may provide the basis of a potentially useful antigen-specific treatment for SS using altered peptide ligands of autoantigens recognised by autoreactive T cells.

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Expression of TNF-α, tristetraprolin, T-cell intracellular antigen-1 and Hu antigen R genes in synovium of patients with rheumatoid arthritis

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Abstract. Post-transcriptional regulation through the AU-rich element (ARE) by ARE binding proteins (ARBPs) has an important role in controlling the production of cytokines, including tumor necrosis factor (TNF)-α. Therefore, expression of ARBPs may influence, or may be influenced, by the severity of rheumatoid arthritis (RA). We measured the gene expression of ARBPs, including tristetraprolin, T-cell intracellular antigen (TIA)-1 and Hu antigen R (HuR), in synovial tissues from RA and osteoarthritis patients. cDNA was constructed from synovial tissues obtained from 21 patients with RA, and those from 12 patients with osteoarthritis. Gene expression was measured using the TaqMan PCR real-time quantification method. No significant differences were observed in the expression of tristetraprolin, TIA-1 or HuR genes between RA and osteo-arthritis synovium samples. No significant relationships between expression of tristetraprolin, TIA-1 or HuR genes and TNF-α gene expression serum CRP levels in samples from RA patients were observed. A significant positive relation-ship was observed between gene expression levels of TIA-1 and HuR. While HuR stabilizes TNF-α mRNA and enhances TNF-α production, TIA-1 acts as a post-transcriptional silencer, and suppresses the production of the TNF-α protein. The clear positive relationship between the expression of these two ARBPs may imply that the expression of either gene affects the expression of the other, or the mechanisms that control the expression of these genes have some factors in common.

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Abbreviations: RA, rheumatoid arthritis; TNF, tumor necrosis factor; ARE, AU-rich element; ARBPs, ARE binding proteins; TTP, tristetraprolin; 3'UTR, 3' untranslated region; TIA, T-cell intracellular antigen; HuR, Hu antigen R; RRM, RNA recognition motif; CRP, c-reactive protein; OA, osteoarthritis; SD, standard deviation; DMARD, disease modifying anti-rheumatic drugs; PCR, polymerase chain reaction; DNA, deoxyribonucleic acid; GAPDH, glyceraldehydes-3 phosphate dehydrogenase; MAPK, mitogen-activated protein kinase; COX2, cyclooxygenase 2

Key words: AU-rich element, Hu antigen R, T-cell intracellular antigen-1, tristetraprolin, rheumatoid arthritis

Introduction

Rheumatoid arthritis (RA) is a systemic inflammatory disorder, the main lesion of inflammation being the joint synovium. In the affected synovium of patients with RA, proliferation of sinoviocytes and invasion of inflammatory cells are observed (1). Various cytokines and chemokines are produced in the proliferated synovial tissues, which further enhances inflammation and joint destruction. Although the precise mechanism that causes this vicious cycle is still unclear, it is generally accepted that the tumor necrosis factor (TNF)-α plays a central role in this inflammatory process (2,3). Thus, mechanisms that control TNF-α production may have a strong influence on the disease activity and prognosis of RA in individual patients.

The mechanisms that promote TNF-α production have been elucidated in various studies, and it has become evident that nuclear factor κB (4), and activator protein-1 (5), are key molecules that enhance TNF-α gene expression. These molecules promote the transcription of the TNF-α gene, and thus, increase the production of the TNF-α protein.

In addition to these transcriptional factors, recent evidence shows that post-transcriptional regulation is also important in

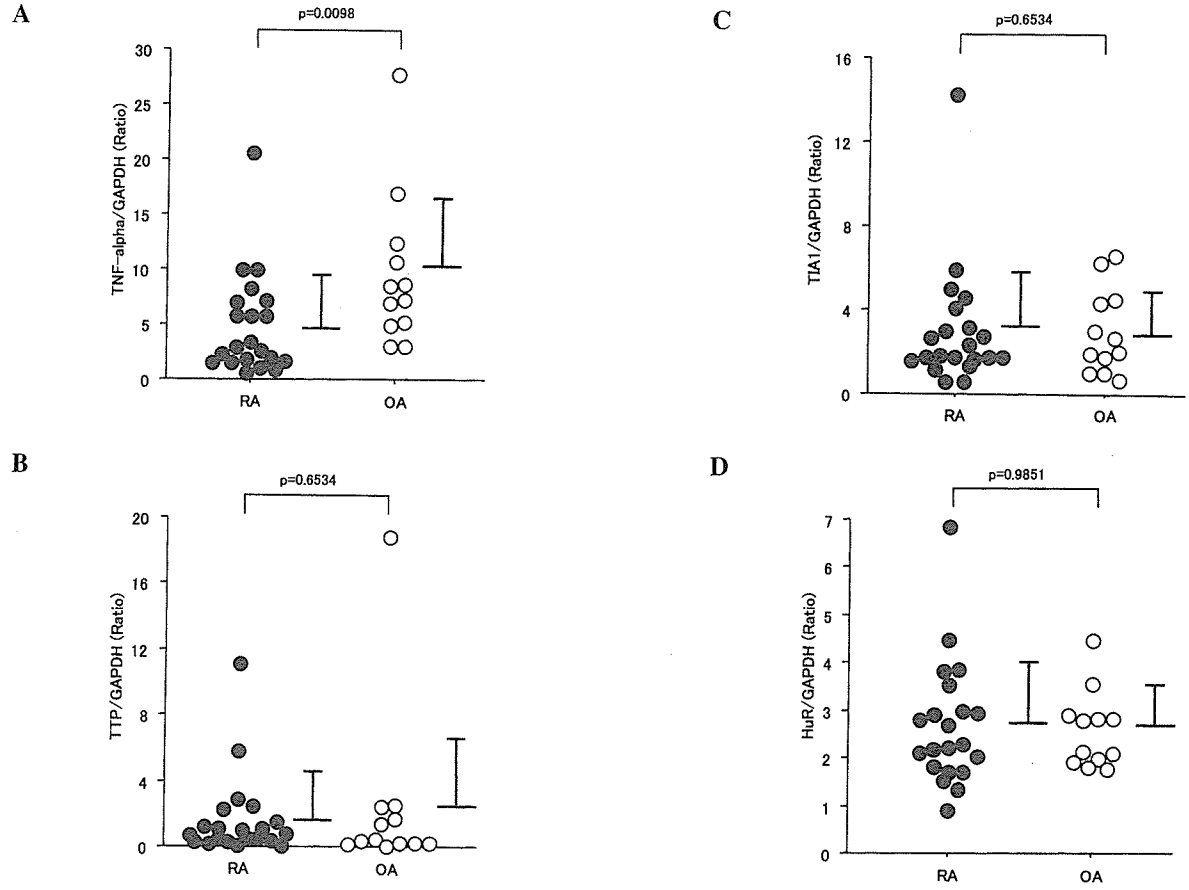

Figure 1. Expression of tumor necrosis factor (TNF)-α (A), tristetraprolin (TTP) (B), T-cell intercellular antigen (TIA)-1 (C), and Hu antigen R (HuR) (D) mRNA in synovial tissues of patients with RA and OA. p-value calculated by the Mann-Whitney U test.

the regulation of cytokine production (6,7). A group of molecules termed AU-rich element (ARE) binding proteins (ARBPs) play a pivotal role in post-transcriptional regulation of inflammatory cytokine production. Among various ARBPs, tristetraprolin (TTP) is one of the most investigated. TTP is a widely distributed phosphoprotein, possesses two zinc finger domains that form the biological active site, and is an immediate early protein that responds to various stimuli (8). It has been demon-strated that TTP induces destabilization of TNF-α mRNA by directly binding to the ARE in the 3' untranslated region (3'UTR) of TNF-α mRNA, and accelerates mRNA degradation, thereby reducing the production of TNF-α protein. It has been also demonstrated that TTP knockout mice manifest erosive arthritis, dermatitis, and body weight loss, and that these symptoms could be prevented by administration of anti-TNF-α antibodies (9).

T-cell intercellular antigen (TIA)-1 and Hu antigen R (HuR) are also ARBPs that are involved in post-transcriptional regulation of TNF-α production. TIA-1 possess three RNA recognition motif (RRM) type RNA binding domains and binds to ARE in the 3'UTR of TNF-α mRNA (10,11). Recent studies have shown that upon binding to ARE, TIA-1 works as a translational silencer, not a transcript destabilizer (12). HuR, also called HuA (13), is a member of the embryonic lethal abnormal visual protein family, and also possesses three RRMs that bind to ARE and poly A tails of various mRNAs. HuR has been shown to stabilize ARE-containing mRNAs, upon binding to such RNAs (14,15). Thus, HuR acts as an enhancer of TNF-α production. Presumably, these molecules act in concert to precisely control the production of the TNF-α protein. Therefore, we hypothesized that the severity of RA in individuals may be influenced, at least in part, by the dynamics of TTP, TIA-1 and HuR production.

In a recent study, we investigated the quantity of TNF-α and TTP mRNAs in synovium of RA patients, and reported that serum C-reactive protein (CRP) was significantly increased in patients whose synovium had a lower TTP/TNF-α gene expression ratio (16). These results suggested that post-transcriptional regulation of TNF-α by ARBPs is an important factor that affects the severity of RA. In this study, we further investigated the quantity of TNF-α, TTP, TIA-1, and HuR mRNA in synovium samples of RA and osteoarthritis (OA) obtained from operated joints. We compared RA with OA in the expression of the above four genes, and investigated the relationship of expression of these four genes. Results imply that although the expression of a single ARBP investigated in this study do not govern the severity of RA, expression of these ARBPs do affect each other and may contribute in determining the disease activity of RA.

Materials and methods

Patients and samples. Synovial tissues from 21 patients with RA, age (mean ± standard deviation, SD) 58.95±7.79 years, disease duration (mean ± SD) 17.28±10.71 years, CRP (mean

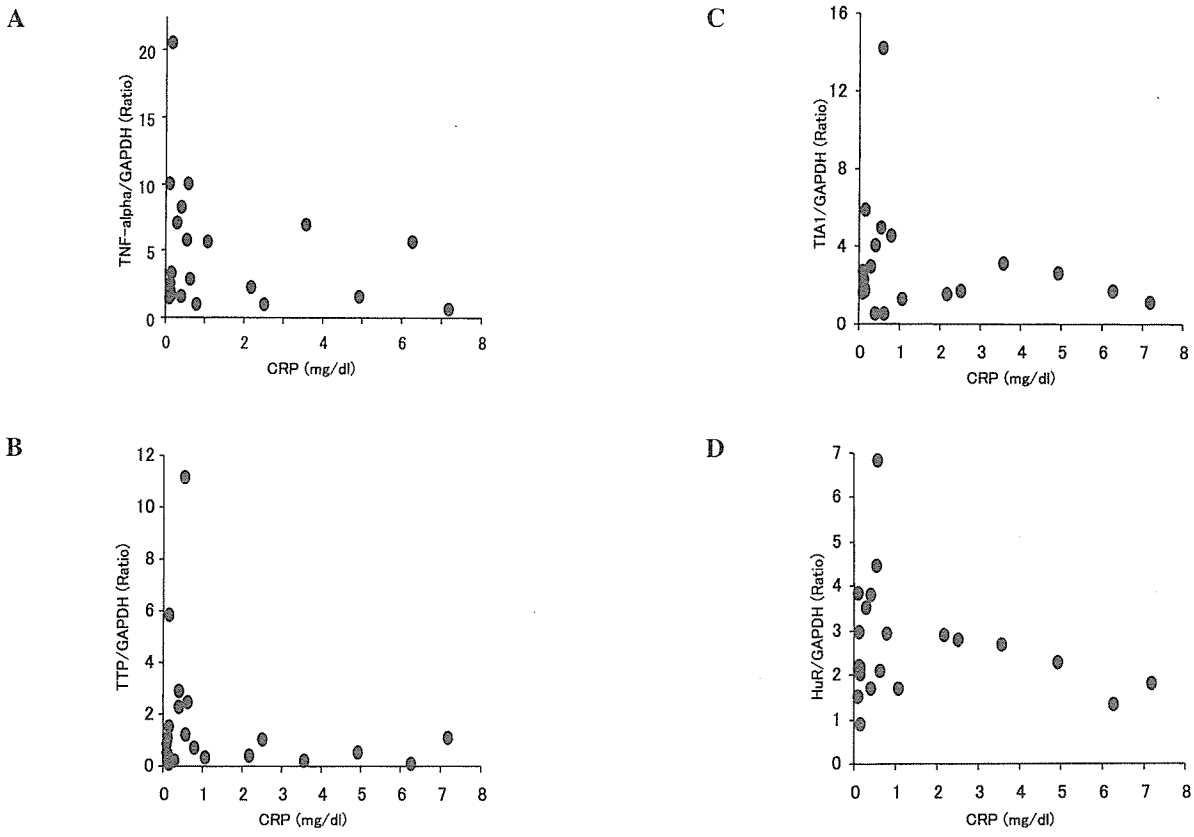

Figure 2. Relationship between C reactive protein (CRP) and gene expression. Relationship between serum CRP and expression of tumor necrosis factor (TNF)-α mRNA (A: p=0.3033, Spearman's rank correlation); tristetraprolin (TTP) (B: p=0.3213, Spearman's rank correlation); T-cell intercellular antigen (TIA)-1 (C: p=0.5088, Spearman's rank correlation); and Hu antigen R (HuR) (D: p=0.7549, Spearman's rank correlation).

± SD) 1.52±2.65 mg/dl] and 12 with OA [age (mean ± SD) 73.08±2.64 years, disease duration (mean ± SD) 17±19.80 years, CRP (mean ± SD) 0.23±0.48 mg/dl] were used. All RA patients fulfilled the American College of Rheumatology criteria for classification of RA (17). All samples were taken when the patients underwent joint replacement surgery of elbow, hand, hip or knee joints, at Tsukuba University Hospital or at Takebayashi Hospital. At the time of serum sampling, RA patients were taking 0-10 mg/day prednisolone and 0-3 disease modifying antirheumatic drugs (DMARD), including methotrexate (8 mg/week maximum), salazosulfapyridine (1000 mg/ day maximum), and 100-200 mg/day bucillamine, a DMARD commonly used in Japan. Serum CRP, rheumatoid factor and other clinical parameters were measured 0-2 days before operation. No patient showed any signs of infection at the time of serum sampling or operation. All subjects gave written informed consent and the study was approved by the local ethics committee.

Real-time PCR. Expression of TTP, TIA-1, HuR, and TNF-α genes were measured using the TaqMan PCR real-time quantification method. Total RNA was extracted from synovial tissues, and cDNA was synthesized using the RevertAid first-strand cDNA synthesis kit (Fermentas, Hanover, MD). Synthesized cDNA samples were applied to PCR and the amount of amplified products was monitored with an ABI-7300 sequence detector (Applied Biosystems Japan, Tokyo, Japan). PCR mixture (qPCR Master mix) was purchased from Eurogentec (Seraing, Belgium); magnesium concentration was 5 mM final, primer concentrations 200 nM final, and the probe concentration was 100 nM final. Thermal cycler conditions were 50°C for 2 min, 95°C for 10 min, then 45 cycles of 95°C for 15 sec and 60°C for 1 min. Standard curves for the gene of interest and glyceraldehydes-3 phosphate dehydrogenase (GAPDH) gene were generated from a standard sample in every assay. All measurements were done in triplicates. The level of gene expression was calculated from the standard curve, compensated with that of GAPDH gene, and was expressed as an expression ratio (expression of the gene of interest/expression of the GAPDH gene). The sequences of specific primers and probes are as follows: TNF-α forward: 5'TGGAGAAGGGTGACCGACTC3', TNF-α probe: 5'CGC TGAGATCAATCGGCCCGACTAT3', TNF-α reverse: 5'TCCTCACAGGGCAATGATCC3'. Primers and the probe for TTP, TIA-1, HuR, and GAPDH were purchased from Applied Biosystems.

Results

TNF-α, TTP, TIA-1 and HuR gene expression in RA or OA joint synovium. Expression of TNF-α, TTP, TIA-1, HuR genes in synovial tissues of 21 RA and 12 OA patients were measured by TaqMan real-time PCR. Expression of TNF-α gene was significantly higher in OA synovial tissues compared to RA synovial tissues (p=0.0098, Mann-Whitney U test; Fig. 1A), Expression of TTP, TIA-1 and HuR genes were not significantly different between RA and OA synovium samples (Fig. 1B-D).

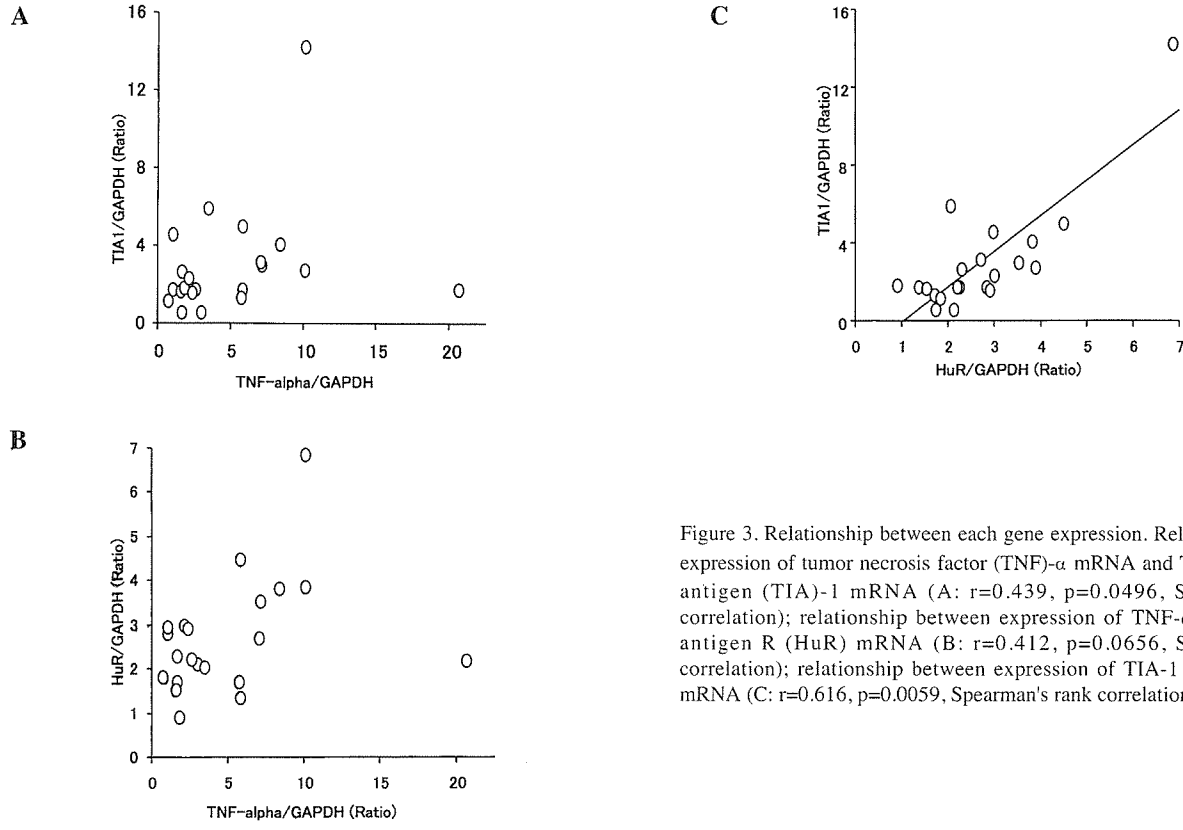

Figure 3. Relationship between each gene expression. Relationship between expression of tumor necrosis factor (TNF)-α mRNA and T cell intercellular antigen (TIA)-1 mRNA (A: r=0.439, p=0.0496, Spearman's rank correlation); relationship between expression of TNF-α mRNA and Hu antigen R (HuR) mRNA (B: r=0.412, p=0.0656, Spearman's rank correlation); relationship between expression of TIA-1 mRNA and HuR mRNA (C: r=0.616, p=0.0059, Spearman's rank correlation).

Relationship between the expression of ARBP genes in RA synovial tissues and serum CRP levels. We investigated the relation between expressions of TNF-α, TTP, TIA-1 or HuR genes in synovial tissues and serum CRP levels in RA patients. No significant correlation was observed for any of the genes studied (Fig. 2). Next, we calculated the ratio of TNF-α gene expression and TTP, TIA-1 or HuR gene expression, and compared the ratio with serum CRP. There was no significant relationship between these ratios and CRP (data not shown).

Relationship between the expression ARBP genes in RA patients. We questioned whether the expression of an ARBP gene or the TNF-α gene is significantly correlated to the expression of another. There were no significant correlation between the expression of TNF-α and TTP genes, TTP and TIA-1 genes, and TTP and HuR genes (data not shown). TIA-1 gene expression was positively correlated with TNF-α gene expression, but statistical significance was lost when adjusted for multiple comparison (r=0.439, p=0.0496 by Spearman's rank correlation; Fig. 3A). HuR gene expression also tended to be higher in patients with higher TNF-α gene expression (r=0.412, p=0.0656 by Spearman's rank correlation; Fig. 3B), but without statistical significance. Expression of TIA-1 and HuR genes were significantly correlated with each other, even after adjustment for multiple comparison (r=0.616, p=0.0059 by Spearman's rank correlation; Fig. 3C).

Discussion

The importance of TNF-α, a major inflammatory cytokine, in the pathogenic process of RA is now clear (2,3). The introduction of TNF-α antagonistic biological drugs have remarkably changed the therapeutic strategies to and the outcome of RA (18,19). However, some patients do not respond to anti-TNF-α therapies (19), and the factors that make interindividual differences in the severity of RA and responsiveness to various therapies are poorly understood. Understanding of these factors, in particular the mechanisms that control TNF-α production, may lead to development of new therapeutic strategies for refractory RA. In this study, we focused on the ARBPs that are important in the post-transcriptional regulation of TNF-α production. Differences in the expression of these molecules may affect the amount of TNF-α produced, and hence, the severity of RA in individual patients.

Rather unexpectedly, expression of the TNF-α gene was significantly higher in the synovial tissues from patients with OA than those from patients with RA. It has been shown that TNF-α plays an important role in the joint destructive process in OA, as well as in RA (20). In addition, while most OA patients were under no medication or on occasional non-steroidal anti-inflammatory drugs only, most RA patients were under more immunosuppressive therapies including DMARDs and steroids. We were unable to obtain healthy synovial tissues, which would have been preferable as controls than synovial tissues from OA patients.

In a previous study, we reported that the TTP/TNF-α gene expression ratio in the synovial tissues from RA patients is lower in patients with higher CRP (16). We considered that this finding may suggest that individual differences of TTP expression may partly account for the differences in the severity of RA. However, this finding was not reproduced in our present study, where a new set of synovial samples were used. We cannot clearly explain the reason for the discrepancy between these studies. One possible explanation is that while many of the RA samples in the previous study were from

patients who underwent synovectomy, samples in the present study were obtained from patients who underwent total joint replacement. Therefore, the disease activity was not high in many of the RA patients included in this study, compared to the RA patients included in our previous study. Theoretically, it would be ideal to measure the expression of these genes in synovial tissues from freshly diagnosed RA patients and study whether they are related to severity, drug responsiveness and prognosis of these patients. An alternative is to measure the amount of these genes in peripheral blood mononuclear cells of RA patients, which is currently underway in our laboratory.

Among the gene expression of ARBPs in the synovial tissues of RA patients, we observed a significant relationship between the expression of HuA and TIA-1. Between the gene expression of TNF-α and the ARBPs examined, tendencies towards positive relationships were observed between TNF-α and TIA-1, and TNF-α and HuR. Although HuR, TIA-1 and TTP all bind to the 3'UTR region of TNF-α mRNA, they exert different functions. HuR acts as a stabilizer (21), TTP a destabilizer (8), and TIA-1 a translational silencer (12). These proteins act in synergy to precisely control the production of TNF-α protein (22). The mechanisms that control the production of these ARBPs are not fully understood. It has been reported that the p38 mitogen-activated protein kinase (MAPK) pathway plays an important role in post-transcriptional regulation of inflammatory genes, and that the mRNAs regulated by p38 share common ARE present in their 3'UTR (23). p38 may stabilize these mRNA by inhibiting the destabilizing action of ARBPs, or by enhancing the production or function of stabilizing ARBPs. It has recently been shown that TTP mRNA is stabilized through a p38 mediated phosphorylation pathway (24). Furthermore, TTP seem to be able to destabilize its own mRNA by binding to ARE of TTP-mRNA (24). The role of p38 in the regulation of production or function of ARBPs, and how an ARBP affect the production of another are interesting but very complicated issues that await elucidation.

The results of our present study may imply that when HuR expression is enhanced by some mechanism, TIA-1 expression also increases to prevent excess production of the TNF-α cytokine. TIA-1 and HuR are also important in the regulation of cyclooxygenase 2 (COX2) production (25). Dysregulated production or RNA-binding of TIA-1 is hypothesized to be related to enhanced COX2 production. Dysregulated TIA-1, therefore, may lead to enhanced TNF-α and COX2 expression (12,25), which in turn may lead to higher disease activity in RA patients.

No significant relationship between the expression of the TTP gene and that of HuR or TIA-1 in the synovial tissues from RA patients was observed in this study. TTP seems to be one of the most important ARBPs in the regulation of TNF-α production, considering the observation that TTP knockout mice develop severe inflammatory symptoms that are attributable to TNF-α overproduction (9). In this study, we could not show a direct relationship between TTP gene expression and RA disease activity, TNF-α gene expression or expressions of TIA-1 or HuR. However, we believe it will be worthwhile to further investigate the expression of the TTP gene in RA or other inflammatory disorders. These studies may lead to finding a clue to explain the differences in the disease of inflammatory disorders in individual patients, and also may give a starting point to develop new methods to control these disorders.

In conclusion, by studying the expression of TTP, TIA-1 and HuR, the three major ARBPs that post-transcriptionally regulate the production of TNF-α, we found that in synovial tissues of RA patients, the expression of HuR and TIA-1 genes are significantly correlated to each other. Our results may give insight into to mechanisms that determine the disease activity of RA, and may promote further studies that elucidate the pathogenesis of RA.

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Gene transduction of tristetraprolin or its active domain reduces TNF-α production by Jurkat T cells

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Abstract. Tristetraprolin (TTP) is a physiological regulator of tumor necrosis factor (TNF)-α production. It destabilizes TNF-α mRNA by binding to the AU-rich element located in the 3' region of TNF-α mRNA. We wished to determine how transducing the TTP gene or its fragment gene encoding its biological active site, the tandem zinc finger (TZF) domain, affects TNF-α production, cell viability and growth of Jurkat T cells. Jurkat T cells were transduced with either the TTP or the TZF gene using retrovirus vectors. Cell growth and apoptosis was analyzed. Expression of genes before or after appropriate stimuli was measured by real-time PCR. In addition, production of the TNF-α protein was measured by enzyme immunoassay. The transduction of either gene reduced TNF-α mRNA levels under unstimulated conditions, and reduced the response to phytohemagglutinin stimulation. Production of TNF-α protein upon stimulation was also decreased in TTP/TZF-transduced cells. Transduction of either gene also affected the expression of granulocyte-macrophage colony-stimulating factor mRNA in a similar fashion, but not that of c-myc. The growth rate of TTP-transduced Jurkat T cells tended to be slower than that of TZF- or mock-transduced cells. TTP-transduced cells were more susceptible to campthothecin-induced apoptosis than others. Our results indicate that either TTP or TZF gene transduction using retrovirus vectors can reduce the production of TNF-α in Jurkat T cells although some differences were noted between TTP and TZF in cell growth and occurrence of apoptosis. These results suggest that TTP may be a potential target for new therapies against RA.

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Abbreviations: RA, rheumatoid arthritis; TNF, tumor necrosis factor; TTP, tristetraprolin; ARE, AU-rich element; 3' UTR, 3' untranslated region; GM-CSF, granulocyte-macrophage colony-stimulating factor; TZF, tandem zinc finger; DMEM, Dulbecco's modified Eagle's medium; CO_2, carbon dioxide; VSV-G, vesicular stomatitis virus G protein; EGFP, enhanced green fluorescent protein; TTP-EGFP, pGCDNsamIRESEGFP-TTP-His-FLAG; TZF-EGFP, pGCDNsamIRESEGFP-TZF-His-FLAG; PCR, polymerase chain reaction; PHA, phytohemagglutinin; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; IRES, internal ribosomal entry site; ELISA, enzyme-linked immunosorbent assay; IL-3, interleukin-3

Key words: AU-rich element, post-transcriptional regulation, retrovirus vector, rheumatoid arthritis

Introduction

Rheumatoid arthritis (RA) is an inflammatory disorder affecting mainly the joint synovium. Continuous inflammation causes joint destruction and greatly impairs the quality of life of the patients. To develop novel therapies for RA, investigations on RA are being performed to elucidate the pathogenesis of RA and the mechanisms that lead to joint erosion and destruction. Although the precise mechanism is still unclear, it is generally accepted that tumor necrosis factor (TNF)-α plays an important role in the pathogenesis of RA, through a number of studies on TNF-α knockout mice, TNF-α receptor knockout mice and TNF-α transgenic mice (1-3).

It is now well-established that the administration of anti-TNF-α antibodies to RA patients or mice with experimentally-induced arthritis results in a marked decrease in the severity of the arthritis (1,4-6). Inhibition of the functions of TNF-α by antibodies or decoy receptors is one of the most important methods currently available to control the activity of RA. Further elucidation of the mechanism that regulates TNF-α production may provide a clue for the development of new therapies of RA.

A number of steps regulate the production of proteins, including TNF-α. Generally, transcriptional regulation is recognized as the main step that controls protein synthesis, and many studies have demonstrated the importance of

transcriptional regulators including nuclear factor-κB, nuclear factor of activated T cells, and activator protein-1 (7-10). However, recently, it has become apparent that post-transcriptional regulation is also important in the regulation of protein production, especially in that of cytokines (11,12).

A number of molecules are involved in post-transcriptional regulation of TNF-α production (13,14). Among those molecules, tristetraprolin (TTP) is one of the most investigated to date (15). TTP is a widely distributed phosphoprotein encoded by an immediate-early gene, *Zfp-36* (16). It has been demonstrated that TTP knockout mice, although appearing normal at birth, eventually manifest marked medullary and extramedullary myeloid hyperplasia associated with cachexia, erosive arthritis, dermatitis, conjunctivitis, glomerular mesangial thickening, and high titers of anti-DNA and anti-nuclear antibodies (17). These symptoms could be prevented by administration of anti-TNF-α antibodies. Thus, the phenotypes observed in these mice are caused mainly by TNF-α overproduction. It was demonstrated that TTP induces destabilization of TNF-α mRNA by binding to the AU-rich element (ARE) in the 3' untranslated region (3' UTR) of TNF-α mRNA, which accelerates mRNA degradation by enhancing the removal of the polyadenylated tail from the mRNA (18), thereby reducing the production of TNF-α protein (19). Granulocyte-macrophage colony-stimulating factor (GM-CSF) mRNA also has a similar structure in its 3' UTR (20).

Past studies reported that the tandem zinc finger (TZF) region, consisting of two zinc finger motifs, is the biological active site of TTP, and can bind ARE and destabilize the mRNA of TNF-α (21,22). On the other hand, it has been reported that the introduction of the TTP gene induces cell apoptosis (23,24).

In a recent report (25), we investigated the quantity of TNF-α and TTP mRNAs in synovial tissues of RA patients. Interestingly, serum C-reactive protein was significantly increased in patients whose synovium had a lower TTP/TNF-α gene expression ratio. These results implied that individual differences in TTP production influence the activity of RA, and that inappropriate TTP production may be one of the factors that cause higher RA disease activity. We considered that TTP is a suitable potential therapeutic target of RA, and hence it is important to investigate the detailed function of TTP and the consequences of TTP-overexpression.

In this study, we transduced the TTP gene or the TZF gene, to a human T cell leukemia cell line, Jurkat T cells, using retrovirus-derived vectors. We examined the effect of these transductions on the expression of the TNF-α gene and production of TNF-α protein, as well as the effects on cell growth and induction of apoptosis. The results showed that overexpression of TTP or its active domain reduced TNF-α production by Jurkat T cells. The retrovirus-derived vectors showed high efficiencies for the transductions performed in this study, providing a valuable tool for further studies.

Materials and methods

Cell culture. 293gp is a packaging cell line derived from U293 cells. PG13 represents also a packaging cell line derived from NIH3T3 cells. These cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 1,000 mg/l glucose, L-glutamine and sodium bicarbonate (Sigma-Aldrich Japan, Tokyo, Japan) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (BioWest, Loire Valley, France), penicillin G sodium (100 U/ml) and streptomycin sulfate (100 μg/ml) (Invitrogen, Carlsbad, CA). These cells were maintained at 37°C in a humidified incubator under 10% carbon dioxide (CO_2) in air. Jurkat T cells and their transduced cells were cultured in RPMI-1640 medium with L-glutamine and sodium bicarbonate (Sigma-Aldrich Japan) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (BioWest), penicillin G sodium (100 U/ml) and streptomycin sulfate (100 μg/ml) (Invitrogen). These cells were maintained at 37°C in humidified incubator under 5% CO_2 in air.

Recombinant retrovirus vector. The structure of GCDNsap and the method to produce recombinant retroviruses packaged in the vesicular stomatitis virus-G (VSV-G) protein have been described previously (26). To construct the GCDN/OVA vector with full-length OVA cDNA and the enhanced green fluorescent protein (EGFP) gene, a *Bam*HI-*Xho*I fragment containing full-length OVA cDNA, and a *Xho*I-*Cla*I IRES/EGFP fragment obtained from GCsamI/E (27) were inserted into the GCDNsap vector. The vectors were converted to the corresponding recombinant retroviruses packaged in the VSV-G by transduction into 293gp (22). The virus titer of GCDN/OVA was 9.0×10^6 infectious units/ml on Jurkat T cells.

Orientation of the cDNA inserts and the integrity of the DNA sequences were confirmed by sequencing using the ABI PRISM Big Dye Terminator V1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) followed by comparison with the published sequences (TTP accession no. MN_003407) on the National Center for Biotechnology Information database. The pGCDNsamIRESEGFP-TTP-His-FLAG (TTP-EGFP) expression construct contains the hTTP coding region (nucleotides 60-1,040) cloned in-frame with the 3' His and FLAG tags ligated in between *Not*I and *Sal*I sites. The pGCDNsamIRESEGFP-TZF-His-FLAG (TZF-EGFP) expression construct contains the fragment, which codes the TZF (nucleotides 315-611), the biological active domain of TTP (24). The TZF fragment was ligated into the vector in-frame with the 3' His and FLAG tags between *Not*I and *Sal*I restriction enzyme sites. TTP and TZF fragments were generated by polymerase chain reaction (PCR) using primers corresponding to the 15 nucleotides at each end of the fragment and flanked by a *Not*I site at the 5' end site and a *Sal*I site at the 3' end site. The sequence is equal to the published TTP gene sequence except for a silent mutation, C→T, at position 368 (Fig. 1). The pGCDNsamIRESEGFP vector without any modification was used as the control mock vector.

Establishment of retrovirus-producing cell lines. TTP-EGFP or TZF-EGFP (at 20 μg) and VSV-G (at 10 μg) were co-transfected into 293gp cells using MBS mammalian transfection kit (Stratagene, La Jolla, CA) according to the instructions provided by the manufacturer. Each supernatant was collected, and protamine sulfate was added (final concentration: 10 μg/ml). Two ml of each supernatant with protamine sulfate was added to PG13 cells with 2 ml of

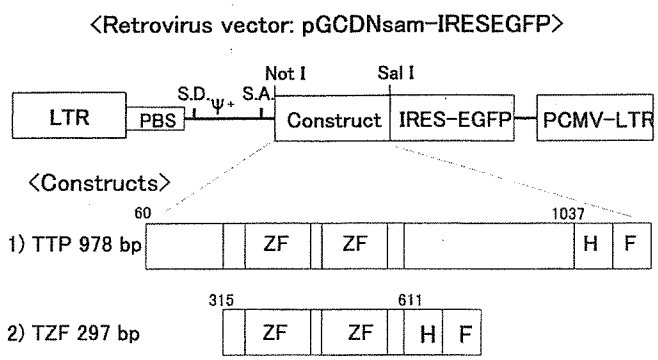

Figure 1. Schematic diagram of the retrovirus vector, pGCDNsamIRESEGFP, and the constructs of tristetraprolin (TTP) and its tandem zinc finger domain (TZF). An internal ribosomal entry site (IRES)-enhanced green fluorescent protein (EGFP) fragment was added downstream of the multicloning site of the retrovirus vector, pGCDNsamIRESEGFP. The TTP construct (total 978 bp: 60-1,037 in MN_0003407) and the TZF construct (total 297 bp: 315-611 in MN_003407) were amplified by PCR, and His6 (H) and FLAG (F) tag sequences were added. Each of these constructs was flanked by a *Not*I site at the 5' end site and *Sal*I site at the 3' end site, and was ligated into the retrovirus vector.

DMEM medium cultured in 6-well plates (Corning, New York, NY). The PG13 cells were centrifuged at 2,000 rpm for 30 min at 32°C and then incubated at 37°C in a humidified incubator under 10% CO_2. This process was repeated three times and the cells were incubated for a few days. Limiting dilution was performed for transduced cells, and the supernatant from the wells that contained cell colonies was collected and RNA copies were quantified by Northern dot blot hybridization using ^{32}P-conjugated TZF probe. The infectious efficiencies were analyzed for selected PG13 cells by detecting the inserted EGFP using a FACScalibur (Becton Dickinson, Franklin Lakes, NJ).

Transduction of the TTP or TZF gene to Jurkat T cells. To transduce Jurkat T cells with pGCDNsamIRESEGFP inserted with TTP, TZF or mock, $1.0x10^5$ cells were plated in 4 ml of the culture medium and 1 ml of each supernatant from each pGCDNsamIRESEGFP-transduced cell line, with protamine sulfate, followed by centrifugation at 2,000 rpm for 30 min. Two days after transduction, the cells were harvested and washed twice with phosphate-buffered saline, and subjected to flow cytometric analyses, using a FACScalibur analyzer. After the analysis, limiting dilution was performed using RPMI-1640 medium with hybridoma enhancing supplement (Sigma-Aldrich Japan) to obtain cell clones.

Western blotting. Cloned Jurkat T cells transduced with TTP-EGFP, TZF-EGFP or mock viruses ($1.0x10^7$ cells) were cultured and the medium was changed 1 day before harvesting. Cells were collected, centrifuged at 1,000 rpm for 5 min, resuspended in 100 μl of lysis buffer (25 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 0.5% NP40, and 2 mM phenylmethylsulfonyl fluoride) and incubated for 5 min on ice. Cell lysates were centrifuged at 15,000 rpm for 5 min. The supernatant was collected and immunoprecipitated using M2 affinity gel (Sigma, St. Louis, MO). The immunoprecipitant was separated on 4-20% gradient SDS polyacrylamide gels (Daiichikagaku-yakuhin, Tokyo), transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA) and the recombinant TTP or TZF protein expressed in the cells was detected by anti-FLAG M2 peroxidase-conjugated antibody (Sigma).

Cell proliferation assay. The cell growth of the transduced cell clones and control cells was estimated by directly counting the number of viable cells. For each clone, $1.0x10^5$ viable cells were plated in 3 ml of culture medium, and were counted every day for 5 days. Cell culture was performed in triplicate, and the experiment was repeated three times to confirm the data obtained.

Induction and detection of apoptosis. To induce apoptosis of Jurkat T cells, camptothecin (Biovision, Mountain View, CA) was added to the culture medium at a final concentration of 6 μg/ml, and the cells were incubated for 4 h. Annexin V-phycoerythrin-conjugated antibody (Becton Dickinson, San Jose, CA) was used to estimate the percentage of cells undergoing apoptosis by flow cytometry. The assays were performed according to the methods described in the product manuals.

Synthesis of cDNA for real-time PCR. Jurkat T cells transduced with TTP, TZF or mock viruses were pre-incubated in 6-well culture plates (Corning) ($1.0x10^6$ cells/well suspended in 3 ml RPMI medium). Phytohemagglutinin (PHA) (Wako, Osaka, Japan) was added at a final concentration of 5 μg/ml to the wells and the cells were harvested at the time course indicated in Figs. 4b and 5. Cells were spun down to pellets and total RNA was extracted from the cell pellets using Isogen (Nippon Gene, Tokyo), and cDNA was synthesized using RevertAid first strand cDNA synthesis kit (Fermentas, Hanover, MD), following the instructions provided by the manufacturer.

Real-time PCR. Synthesized cDNA samples were amplified with specific primers and fluorescence-labeled probes for the genes of interest, and accumulation of amplified products was monitored by an ABI 7700 sequence detector (Applied Biosystems Japan, Tokyo). qPCR Mastermix was purchased from Eurogentec (Seraing, Belgium), the magnesium concentration was 5 nM final, the primer concentration was 200 nM final, and the probe concentration was 100 nM final. Thermal cycler conditions were: 50°C for 2 min and 95°C for 10 min, then 45 cycles of 95°C for 15 sec and 60°C for 1 min. Serial dilutions of standard samples were included and standard curves for the gene of interest and the glyceraldehydes-3-phosphate dehydrogenase (GAPDH) gene were generated in every assay. All measurements were performed in triplicate. The level of gene expression was calculated from the standard curve, and expressed relative to that of GAPDH gene. The sequences of specific primers and probe are as follows: TNF-α forward, 5'-TGGAGAAGGGTGACCGACTC-3'; TNF-α probe, 5'-CGCTGAGATCAATCGGCCCGACTAT-3'; and TNF-α reverse, 5'-TCCTCACAGGGCAATGATCC-3'. The primers and probe for GM-CSF, C-MYC, and GAPDH were purchased from Applied Biosystems.

Enzyme immunoassays for measurement of TNF-α. Jurkat T cell clones transduced with TTP, TZF or mock viruses were cultured with 5 μg/ml of PHA for 12 h at a cell concentration