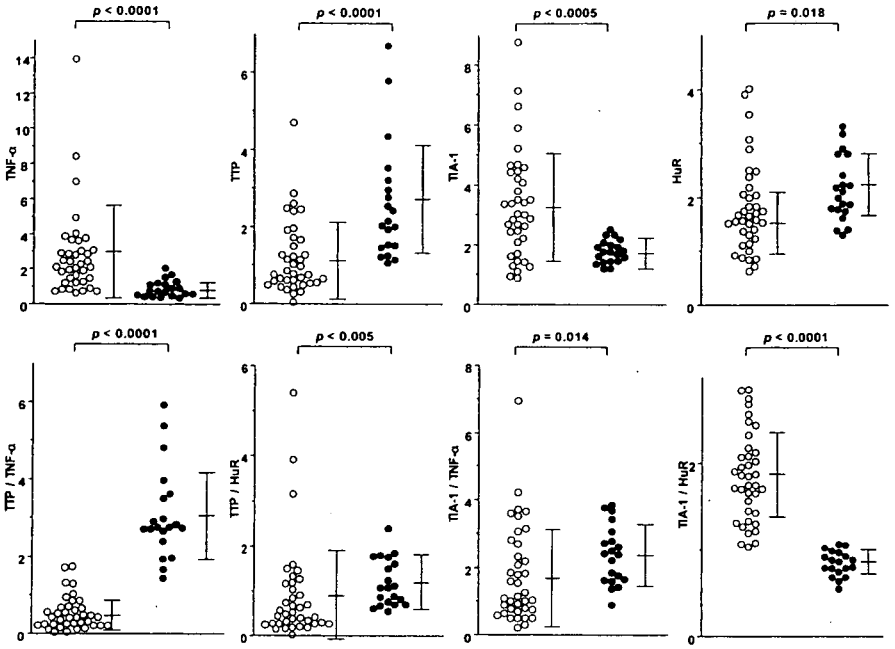

Figure 1. Expression levels of the tumor necrosis factor α (TNFα) gene and 3 adenine/uridine-rich element binding protein (tristetraprolin [TTP], T cell intracellular antigen 1 [TIA-1], and Hu antigen R [HuR]) genes in peripheral blood mononuclear cells from 38 patients with active rheumatoid arthritis at week 0 (open circles) and from 20 healthy control subjects (solid circles). Bars show the mean ± SD. *P* values were calculated by Mann-Whitney U test.

and 2.84 ± 1.99 at week 2; for TTP, 1.20 ± 0.95 at week 0 and 1.17 ± 1.32 at week 2; for TIA-1, 3.34 ± 1.79 at week 0 and 3.88 ± 1.79 at week 2; for HuR, 1.79 ± 0.83 at week 0 and 2.06 ± 0.91 at week 2) (Figure 2). However, the TTP:HuR gene expression ratio decreased 2 weeks after initiation of infliximab therapy (0.90 ± 1.09 at week 0 and 0.71 ± 0.88 at week 2; $P = 0.015$), while no significant changes were noted in the TTP:TNFα, TIA-1:TNFα, and TIA-1:HuR ratios (for TTP:TNFα, 0.55 ± 0.43 at week 0 and 0.50 ± 0.39 at week 2; for TIA-1:TNFα, 1.80 ± 1.42 at week 0 and 1.94 ± 1.19 at week 2; for TIA-1:HuR, 1.85 ± 0.52 at week 0 and 1.88 ± 0.34 at week 2) (Figure 2).

At week 54, the TNFα gene expression level and the TIA-1:HuR gene expression ratio increased from those observed at week 0 (for TNFα, 2.80 ± 2.48 at week 0 and 5.05 ± 3.89 at week 54 [$P = 0.015$]; for TIA-1:HuR, 1.85 ± 0.52 at week 0 and 2.35 ± 0.71 at week 54 [$P = 0.010$]). TTP gene expression increased from that at week 2 (1.17 ± 1.32 at week 2 and 1.61 ± 0.94 at week 54; $P = 0.0065$). In contrast, the TIA-1:TNFα gene expression ratio decreased, from 1.94 ± 1.19 at week 2 to 1.22 ± 0.81 at week 54 ($P = 0.026$). Fluctuations in TIA-1 gene expression and the TTP:TNFα and TTP:HuR gene expression ratios differed greatly among individual patients (Figure 2).

Relationship between TNFα and ABP gene expression levels in patients with RA. We next examined the correlation between the gene expression levels in PBMC samples from healthy control subjects and those in samples from patients with RA at week 0, week 2, and week 54. We anticipated that although posttranscriptional regulation of TNFα production would be adequately executed in healthy individuals, some disturbance might be present in patients with active RA. These disturbances may be partially responsible for the higher disease activity in these patients to whom infliximab is prescribed. In particular, we were interested in investigating whether the correlation between TNFα and the ABPs that have been shown to suppress TNFα production (TTP and TIA-1) would be altered. In addition, we were interested in determining whether a disturbance in posttranscriptional regulation of TNFα production, if it does exist, would be affected by infliximab therapy.

In the control samples, gene expression of TNFα correlated with the expression levels of all genes examined (for TTP and TNFα, r = 0.64 and $P = 0.0017$; for

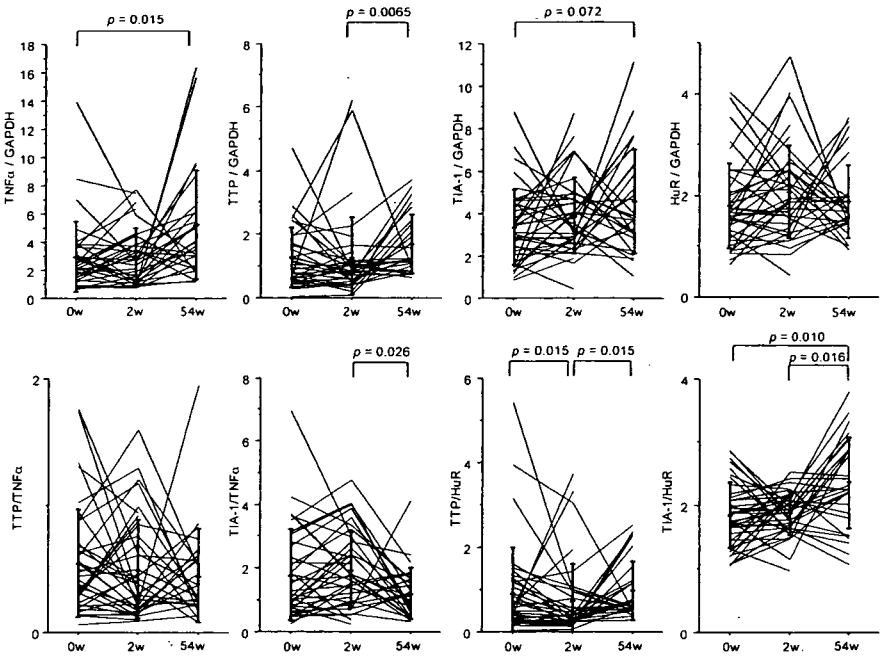

Figure 2. TNFα and adenine/uridine-rich element binding protein gene expression levels in peripheral blood mononuclear cells from patients with rheumatoid arthritis, before (0w) and 2 weeks and 54 weeks after administration of the first dose of infliximab. Bars show the mean ± SD. *P* values were calculated by Wilcoxon's rank sum test. See Figure 1 for definitions.

TTP and HuR, r = 0.64 and $P = 0.0017$; for TIA-1 and TNFα, r = 0.62 and $P = 0.0030$; for TIA-1 and HuR, r = 0.73 and $P = 0.0001$; for TNFα and HuR, r = 0.60 and $P = 0.0041$; for TTP and TIA-1, r = 0.59 and $P = 0.0049$) (Figure 3A). In samples obtained from patients with RA at week 0, the correlations between TTP and TNFα and between TIA-1 and HuR were significant (for TTP and TNFα, r = 0.40 and $P = 0.016$; for TIA-1 and HuR, r = 0.87 and $P < 0.0001$) (Figure 3B), while the correlation between TTP and HuR was not significant. Similar correlations were noted at week 2 and at week 54 (for TTP and TNFα, r = 0.34 and $P = 0.039$ at week 2 and r = 0.39 and $P = 0.042$ at week 54; for TIA-1 and HuR, r = 0.94 and $P < 0.0001$ at week 2 and r = 0.84 and $P < 0.0001$ at week 54) (Figures 3C and D).

Interestingly, the significant relationships for gene expression between TIA-1 and TNFα, TNFα and HuR, and TTP and TIA-1 were not observed in samples obtained from patients with RA at week 0. However, significant relationships between TIA-1 and TNFα and between TNFα and HuR gene expression were observed in week 2 and week 54 samples (for TIA-1 and TNFα, r = 0.18 and $P = 0.27$ at week 0, r = 0.38 and $P = 0.022$ at week 2, and r = 0.51 and $P = 0.009$ at week 54; for TNFα and HuR, r = 0.10 and $P = 0.54$ at week 0, r = 0.45 and $P = 0.007$ at week 2, and r = 0.72 and $P = 0.0002$ at week 54 (Figures 3B, C, and D). These observations suggest that regulatory mechanisms that control the expression of these molecules are disturbed in patients with active RA and are somewhat restored after the initiation of anti-TNFα therapy.

Relationship between TNFα and ABP gene expression and efficacy of infliximab therapy. Our working hypothesis was that differences in the regulation of ABP production might lead to differences in the severity of RA and the efficacy of TNFα-blocking agents. Thus, we anticipated that we might observe some differences in the expression of these molecules between patients whose disease responded to infliximab and infliximab nonresponders.

At the time of this study, 27 patients with RA had received at least 9 courses of infliximab therapy (week 54). At week 54, 18 patients (66.7%) had achieved at least an ACR20 response; 14 patients (51.9%) had achieved an ACR50 response, and 8 patients (29.6%) had achieved an ACR70 response. The 14 patients who achieved an ACR50 response at week 54 were included in the responder group, while the 9 patients who did not

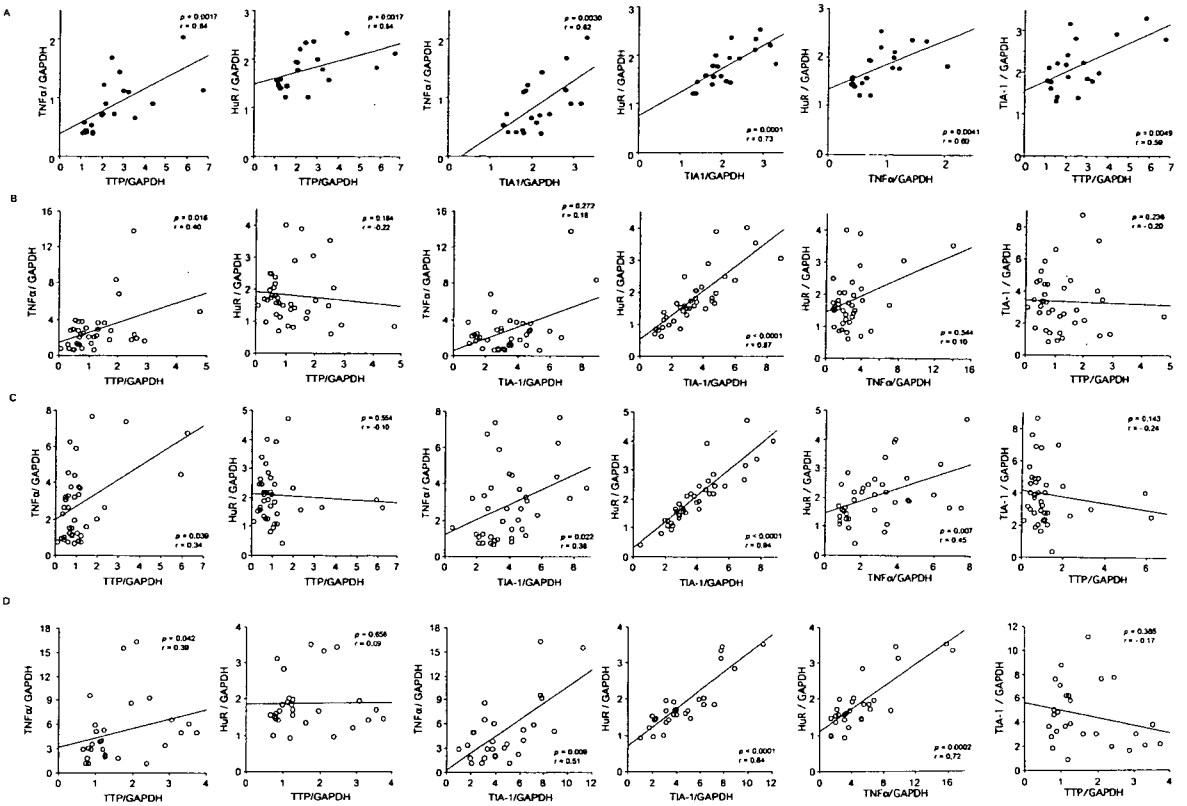

Figure 3. Relationships between expression levels of tumor necrosis factor α (TNFα) and adenine/uridine-rich element binding protein genes in peripheral blood mononuclear cells obtained from patients with rheumatoid arthritis (RA; open circles [n = 38]) and from healthy control subjects (solid circles [n = 20]). **A,** Healthy control subjects. **B,** Patients with RA, before initiation of infliximab therapy. **C,** Patients with RA, 2 weeks after administration of the first dose of infliximab. **D,** Patients with RA, 54 weeks after initiation of infliximab therapy. *P* values were calculated using Pearson's correlation coefficient. See Figure 1 for other definitions.

achieve at least an ACR20 response at week 54 were included in the nonresponder group. No significant differences in the expression of TNFα and ABP genes were observed between these 2 groups (for TNFα, 3.27 ± 3.53 in responders and 2.18 ± 1.25 in nonresponders; for TTP, 1.20 ± 1.31 in responders and 1.09 ± 0.50 in nonresponders; for TIA-1, 3.55 ± 1.64 in responders and 2.86 ± 1.01 in responders; for HuR, 1.72 ± 0.68 in responders and 1.86 ± 0.92 in nonresponders) (Figure 4). The TIA-1:HuR gene expression ratio at week 0 tended to be higher in the responder group than in the nonresponder group (2.051 ± 0.471 and 1.626 ± 0.365, respectively [P = 0.059 by Mann-Whitney U test]). No statistically significant differences were observed between responders and nonresponders for other clinical parameters measured at week 0 or week 2, including serum CRP levels, the erythrocyte sedimentation rate, and other ACR-defined improvement parameters, and the matrix metalloproteinase 3 level (data not shown).

DISCUSSION

In this study, we investigated the role of ABPs in the pathogenesis of RA, as well as any association between gene expression and the efficacy of anti-TNFα therapy, by monitoring PBMC samples obtained before and after infliximab therapy. A similar study using synovial tissue would have been preferable but is quite impractical. We anticipated that the gene expression levels of TNFα and the ABPs in PBMCs would reflect the inflammation status of patients with RA, and we focused on TTP, TIA-1, and HuR, which are clinically important ABPs. Results of previous studies suggested that TTP and TIA-1 are antiinflammatory factors, while HuR is considered an inflammation-accelerating factor

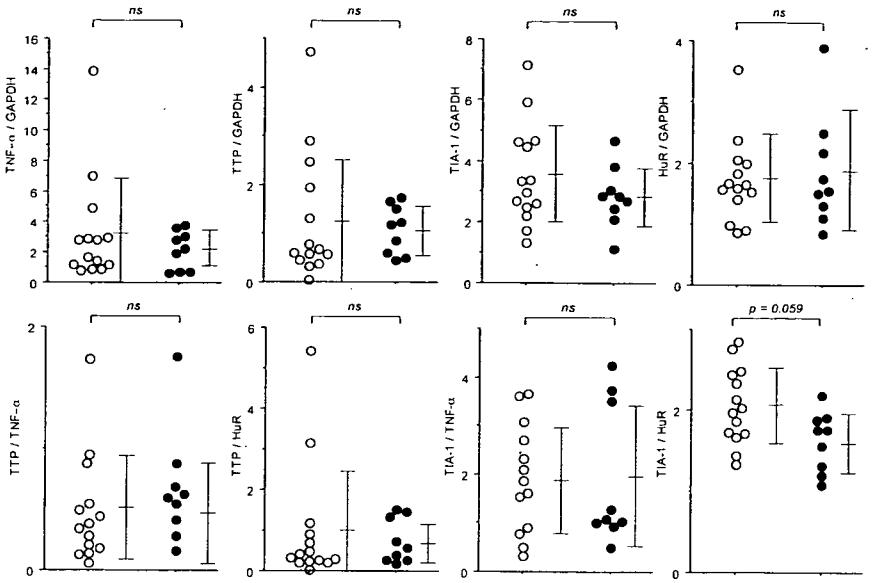

Figure 4. Relationships between expression levels of TNFα and adenine/uridine-rich element binding protein genes in peripheral blood mononuclear cells, and efficacy of infliximab therapy. Open circles represent the 14 rheumatoid arthritis patients who achieved 50% improvement according to the American College of Rheumatology response criteria (ACR50) at week 54. Solid circles represent the 9 patients who had not achieved at least an ACR20 response at week 54. Bars show the mean ± SD. *P* values were calculated by Mann-Whitney U test. NS = not significant (see Figure 1 for other definitions).

(28,33,34,36,39,41,42). We speculated that the balance or imbalance in the production of these factors may induce differences in TNFα production, and hence, RA activity.

The aim of anti-TNFα therapy is to neutralize TNFα in the circulation and suppress the harmful effects of this cytokine in vivo. In doing so, the physiologic mechanisms that control TNFα gene transcription may be attenuated in the short term, resulting in increased TNFα production. If this is the case, differences in the posttranscriptional regulation of TNFα production may affect the disease activity or efficacy of TNFα-blocking agents in individual patients with RA. Therefore, examining the gene expression of TNFα and ABP may provide not only a better understanding of the pathogenesis of RA but also a clue to the factors that affect and allow us to predict the efficacy of TNFα-blocking drugs.

Our results showed that prior to the start of infliximab therapy, the TNFα gene was overexpressed and the TTP gene was underexpressed in patients with RA compared with healthy control subjects. Furthermore, the TTP:TNFα and TTP:HuR ratios were significantly lower in patients with RA than in healthy control subjects. Because TTP is a destabilizer of TNFα mRNA, and various stimuli including TNFα itself promote TTP production (33), our results imply that the negative feedback mechanism of TTP production is not sufficient to counter the excessive TNFα production that occurs during active RA. TIA-1 gene expression and the TIA-1:HuR expression ratio were higher in patients with RA than in control subjects, while the TIA-1:TNFα ratio was lower. Because TIA-1 is a translational silencer of TNFα, it is conceivable that TIA-1 is produced as another negative feedback mechanism against TNFα overproduction to compensate for the TTP decrement in patients with active RA, although such compensation is still not sufficient to mitigate the symptoms of RA activity. Interestingly, although the scatter in expression of the TIA-1 gene was small among healthy control subjects, it was quite large among patients with RA. This may reflect the interindividual differences in the regulation of TIA-1 expression, which become evident when a person acquires an inflammatory disorder mediated by TNFα.

Considering the crucial role of excessive TNFα production in the RA inflammatory process, abnormal regulation at the posttranscriptional level may be one of the factors that promote this excessive TNFα production, and thus, more severe arthritis. We previously reported that TTP gene expression is significantly higher in RA synovial tissue compared with that in OA synovial

tissue (40). One of the explanations for this discrepancy is that in the previous study, RA synovial samples were obtained from patients who had undergone surgery, and disease activity greatly varied among these patients. In contrast, all of the PBMC samples used in the present study were obtained from patients with active arthritis and were obtained just prior to initiation of anti-TNFα therapy. Another interpretation is that TTP production may be higher at sites of active inflammation (e.g., synovial tissue in patients with RA) than in PBMCs.

To investigate the effect of infliximab on the production of TNFα posttranscriptional regulatory factors, we compared the gene expression levels of TNFα and the 3 ABPs before and 2 weeks and 54 weeks after administration of the first dose of infliximab. There were no significant changes in gene expression, but the TTP:HuR ratio significantly decreased after infliximab therapy. This change may have resulted from the decrease in TTP gene expression and the increase in HuR gene expression in response to infliximab-induced TNFα removal. The results hinted at this situation but were not statistically significant. In addition, the TIA-1:TNFα ratio tended to increase at week 2. This may also have resulted from a subtle reduction in TNFα gene expression and an increase in TIA-1 gene expression. When gene expression at week 0 and week 54 were compared, TNFα gene expression and the TIA-1:HuR ratio were significantly higher in week 54 samples than in week 0 samples. Inhibition of the function of the TNFα protein by infliximab may have led to a relative increment in expression of the TNFα gene and the TIA-1 gene.

Interestingly, parameters investigated in this study tended to fluctuate toward the reverse direction when changes between week 0 and week 2 and those between week 2 and week 54 were compared. In addition, there seemed to be a large interindividual difference in the fluctuation of these parameters, especially TTP, TIA-1, HuR, the TIA-1:TNFα ratio, and the TIA-1:HuR ratio. These results imply that the impact of infliximab on the posttranscriptional regulation of TNFα production varies among individual patients with RA. These variations may affect the long-term efficacy of anti-TNFα drugs. Recently, it has been discussed whether infliximab therapy could be postponed in some patients without causing a flare in disease activity (45). Gene expression levels of TNFα and ABPs that are involved in the posttranscriptional regulation of TNFα production are possible candidates for parameters that could be used to predict whether infliximab may be withdrawn without a severe flare in disease activity.

A significant positive relationship was observed between the gene expression of TNFα and that of TTP, in PBMC samples from both patients with RA and healthy control subjects. This result is consistent with results of a previous study showing induction of TTP protein biosynthesis by TNFα production in macrophages as a part of a negative feedback mechanism (33). The correlation between TNFα and TTP was most prominent in healthy subjects, which may imply that the balance of TNFα and TTP gene expression is inappropriately regulated in at least some patients with RA. The positive relationship of TNFα and TIA-1, in both patients with RA and healthy control subjects, implies that a negative feedback mechanism between TNFα and TIA-1 may also exist. In addition, a strong positive relationship was observed between the gene expression of TIA-1 and that of HuR in both patients with RA and healthy control subjects, implying that either a common mechanism controlling the expression of TIA-1 and HuR is present or that one of these proteins controls the expression of the other. Interestingly, we also observed a strong positive relationship between the expression of TIA-1 and that of HuR genes in synovial tissue obtained from patients with RA after surgery (46).

Recently, it was shown in HuR-transgenic mice that HuR and TIA-1 act in concert to suppress TNFα production, suggesting that HuR may be an inflammation suppressor in vivo (26), contrary to previous studies in which HuR was reported to be a proinflammatory factor (39,41,42). Thus, it seems that ABPs do not function independently of each other, and the precise roles of these ABPs in vivo are still to be elucidated. In contrast to what is observed in healthy control subjects, positive correlations between TTP and HuR gene expression and between TTP and TIA-1 were not present in RA samples obtained before and 2 weeks after initiation of infliximab therapy. It is possible that an imbalance in gene expression is one of the causes of excessive TNFα production, which in turn leads to higher disease activity in patients with RA.

Currently, it is not clear why the efficacy of TNFα-blocking agents differs greatly among patients with RA. We wanted to find some clues that may help answer this question. The TIA-1:HuR gene expression ratio tended to be higher in the responder group, but the difference between responders and nonresponders was not statistically significant. It may be possible that patients with lower gene expression of HuR and higher expression of TIA-1 have decreased TNFα mRNA stability and translation, and hence, lower TNFα production. In these patients, infliximab might be more effective in neutralizing circulating TNFα than in patients

with a lower TIA-1:HuR expression ratio. Measurement of such gene expression in patients prior to infliximab therapy might be a useful predictor of the potential efficacy of infliximab. However, we were unable to draw a definitive conclusion in this study, and additional studies with a larger number of samples should be performed to confirm our observations and speculations. In a recent study, it was shown that failure to suppress serum CRP at week 2 of therapy identified the majority of patients who were nonresponders by week 12 (47). However, in our series of patients, we did not find a significant relationship between a reduction in the CRP level at week 2 and the efficacy of infliximab at week 54.

Our study had several limitations. Although we included all of our patients who were receiving infliximab therapy, the cohort number was not large enough for strong statistical analyses. Protein analyses would also be useful to accurately determine the ABP levels actually present in the cells. Other ABPs that were not studied here may also have important roles in the pathogenesis of RA. Furthermore, the ABPs studied here can also affect the mRNA of other inflammatory molecules such as cyclooxygenase 2 (48–50); these interactions may have impacted our findings and therefore should be considered in any interpretation of the data.

In conclusion, by analyzing the gene expression levels of TNFα and ABP in PBMCs, we observed a relationship between the expression of TTP, TIA-1, and HuR that might have an impact on TNFα gene expression and thereby protein production. Our results also implied that the TIA-1:HuR gene expression ratio before infliximab therapy may predict the efficacy of treatment. Further studies are necessary to enhance our understanding of RA pathogenesis and to identify possible targets of therapy as well as parameters that predict the efficacy of pharmaceutical agents.

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AUTHOR CONTRIBUTIONS

Dr. Sumida had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Sugihara, Tsutsumi, E. Suzuki, Sumida.

Acquisition of data. Sugihara, Tsutsumi, E. Suzuki, T. Suzuki, Ogishima, Hayashi, Chino, Ishii, Mamura, Goto, Matsumoto, Ito, Sumida.

Analysis and interpretation of data. Sugihara, Tsutsumi, Wakamatsu, Sumida.

Manuscript preparation. Sugihara, Tsutsumi, Sumida.

Statistical analysis. Sugihara, Tsutsumi, Sumida.

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parental genetic testing identified an identical nucleotide missense substitution in the mother, but not in the father.

Improved clinical symptoms after inhibition of interleukin 1 (IL 1) activity by anakinra was reported in all of the cryopyrin-associated autoinflammatory syndromes, although treatment failure has also been reported.[1–4] Therapy with IL 1 antagonism was begun in our patient at 9 years and a clinical response was reported within days. On taking Anakinra (~0.90 mg/kg/day) his energy level improved, his rash appeared less frequently and his arthritis and conjunctivitis were resolved. His erythrocyte sedimentation rate, white cell count and platelet count all improved (table 1). The patient's family temporarily reduced the dose of anankinra to 0.30 mg/kg/day, and he again experienced clinical and laboratory evidence of disease flare, indicating a dose-dependent response (table 1).

In all, 60 different dominantly inherited missense mutations within exon three of CIAS1 have been associated with the three heterogeneous cryopyrin-associated autoinflammatory syndromes.[5 6] Previous patients described with characteristics of both familial cold-induced autoinflammatory syndrome and Muckle–Wells syndrome have had other unique cryopyrin mutations that were distinct from the new mutation described here.[1 7] It is interesting to note that two other mutations that would cause different amino acid substitutions at this site (Thr436Iie; Thr436Asn) were reported in patients with chronic infantile neurological cutaneous articular syndrome. [6] These findings would suggest that specific amino acid substitutions at this site are solely responsible for the clinical phenotype. However, phenotypic heterogeneity, including incomplete penetrance among family members, has been described among individuals carrying identical mutations.[7 9 10] Thus there is probably an effect of additional modifier genes or environmental factors in the phenotypic expression of the cryopyrin-associated autoinflammatory syndromes.[6]

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DNA microarray analysis of labial salivary glands of patients with Sjögren's syndrome

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Sjögren's syndrome (SS) is a chronic autoimmune disease characterised by dry eyes, dry mouth and focal lymphocytic infiltration in lacrimal and salivary glands. The infiltrating lymphocytes are mainly CD4 α/β T cells,[1] especially T helper 1 (Th1) type T cells, because they produce both interferon (IFN)γ and interleukin 2.[2 3] To understand the pathogenesis of SS, several molecules in labial salivary glands (LSGs) have been screened by microarray analysis in human SS. Hjelmervik *et al*[4] and Gottenberg *et al*[5] reported that the upregulated genes in SS salivary glands were IFN-inducible genes, such as IFN-stimulated transcription factor 3, IFN-regulatory factor 1 and B cell-activation factor of the TNF family. However, there is little or no information on the essential genes involved in the generation of sialoadenitis in patients with SS. We screened abnormally expressed genes in LSGs of patients with SS using cDNA microarray technology to elucidate the SS susceptibility genes.

The cDNA array was performed using total RNA from LSGs of three patients with primary SS and from three healthy subjects (control) with a DNA chip including 775 genes (JGS, Tokyo, Japan). The DNA chip contained immunoglobulins, human leucocyte antigens (HLAs), complements, T cell receptors (TCRs), IFN-inducible proteins, cytokines, transcriptional factors and other autoimmune disease-related genes. Abnormally

Abbreviations: HLA, human leucocyte antigen; SS, Sjögren's syndrome; IFN, interferon; LSG, labial salivary gland Th1, T helper cell; TCR, T cell receptor

Table 1 Genes expressed in labial salivary glands of patients with Sjögren's syndrome (SS)

Gene name	SS/HS
Upregulated genes	
Immunoglobulin (Ig)	
Ig heavy constant γ3	8.40*
Igγ chain, Ig rearranged γ chain, V-J-C region	2.99*
Ig κ variable 1D8	3.25*
Rearranged Ig mRNA for μ heavy chain enhancer and constant region	2.23*
Human leucocyte antigen (HLA)	
Human MHC HLA class I cell surface antigen (HLA-B 5102) mRNA	6.56*
HLA-F	3.31*
HLA-DRβ1	6.22*
HLA-DP light chain	3.01*
RING6 (HLA class II α chain-like product)	2.41*
MHC class II DQ	2.35*
MHC class II DR	7.28*
Complement	
Complement C3	3.96*
T cell receptor (TCR)	
TCRβ chain (T cell receptor β chain)	2.11*
Interferon (IFN)-inducible genes	
IP-30 (IFNγ-inducible protein)	2.17*
I-5111 (IFN-inducible gene)	2.25*
IP-10 (IFNγ-inducible chemokine)	2.31*
IFNα/β-inducible p44	2.1*
IFN-α-inducible protein 27	2.19*
Staf50 mRNA	2.04*
Thymosin β	
Thymosin β4	2.03*
Thymosin β10	2.12*
Transcription factor	
STAT1α	2.88*
STAT1β	2.07*
I κ B	2.02*
Downregulated genes	
LIM protein (cystein-rich protein 3)	0.44*

MHC, major histocompatibility complex.
The average of patients with SS healthy subjects based on cDNA array fluorescent data healthy subjects (n = 3, each). The Mann–Whitney U test was used to compare the control and SS groups.
*p<0.05

expressed genes in SS LSG represented those with expression level over twofold or below 0.5-fold of that of LSGs of the controls. The Mann–Whitney U test was used for statistical analysis. p Values <0.05 were considered significant.

A total of 24 upregulated genes were identified in LSGs (table 1). These included immunoglobulins, HLAs, complements, TCRs, IFN-inducible proteins, thymosin β and transcriptional factors. We also identified one single downregulated gene, LIM protein. Among the abnormally expressed genes in SS LSG, expression level 19 was significantly higher than in the controls. These included thymosin β4 and thymosin β10, the regulators of apoptosis activity and inflammation-related genes such as HLA-DR and TCRβ. Upregulation of HLA, complements, TCRs and immunoglobulins should be due to the infiltration of T cells and B cells in LSGs from patients with SS. Interestingly, many IFNγ-inducible genes, such as IP10, STAT1α and STAT1β, were highly expressed in SS, and our previous study reported that STAT1 could function as a key molecule in the pathogenesis of SS.[6] These findings suggest that Th1 cells and Th1 type cytokines function as destructive factors in the generation of sialoadenitis in LSGs of patients with SS. Thymosin β has anti-apoptotic activity,[7] suggesting that it may function as a protective factor against salivary gland destruction in patients with SS.

Recent studies reported the presence of germinal centre-like structures in the salivary glands of patients with SS, and that the germinal centre formed by infiltrating mononuclear cells (B cells, T cells and others) produces autoantibodies and exhibits apoptosis.[8 9] In this study, the significant upregulation of 10 genes—for example, IgHCγ, HLA class I, HLA class II, C3 and TCRβ—might be due to mononuclear infiltrates and might be responsible for the formation of a germinal centre, in LSGs of patients with SS.

In conclusion, cDNA microarray analysis demonstrated upregulation of IFNγ-related genes in LSGs of patients with SS, indicating that Th1 cells might play a crucial role in the generation of sialoadenitis in those patients.

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Overexpression of Phosphorylated STAT-1α in the Labial Salivary Glands of Patients With Sjögren's Syndrome

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Objective. **To clarify the molecular mechanisms of Sjögren's syndrome (SS), we analyzed the functional role of the STAT-1 gene, one of the interferon-γ (IFNγ)–inducible genes, in labial salivary glands (LSGs) from SS patients.**

Methods. **The expression of STAT-1 messenger RNA (mRNA) was examined by real-time polymerase chain reaction (PCR) analysis, and the phosphorylation of STAT-1 protein (Tyr^{701} and Ser^{727} pSTAT-1) was investigated by Western blot and immunohistochemical analyses. The expression of IFNγ-inducible 10-kd protein (IP-10), IFN regulatory factor 1 (IRF-1), and Fas was also examined by real-time PCR and immunohistochemical analyses.**

Results. **STAT-1α and STAT-1β mRNA were highly expressed in LSGs from SS patients. The level of STAT-1α protein in SS LSGs was higher than that in 3 control LSGs, whereas STAT-1β protein was not clearly detected by Western blot analysis. Moreover, Tyr^{701} and Ser^{727} pSTAT-1α proteins were specifically detected in SS LSGs. Immunohistochemical analysis showed localization of Tyr^{701} pSTAT-1 in infiltrating lymphocytes and the adjacent ductal epithelium from SS patients. Ser^{727} pSTAT-1 was localized only in the ductal epithelium of SS LSGs. The STAT-1–inducible genes IP-10 and IRF-1 and the Fas genes were highly expressed in SS LSGs and were colocalized with Ser^{727} pSTAT-1–positive, but not Tyr^{701} pSTAT-1–positive, cells.**

Conclusion. **We found evidence of the up-regulation of STAT-1α mRNA and protein in LSGs from SS patients, as well as the presence of pSTAT-1α in ductal epithelium from SS patients. Our findings suggest that STAT-1α, especially Ser^{727} pSTAT-1, may function as a key molecule in the pathogenesis of SS.**

Sjögren's syndrome (SS) is a chronic autoimmune disease characterized by focal infiltration of lymphocytes into lacrimal and salivary glands, which leads to dry eyes and dry mouth. In SS, several autoantibodies are produced, and they are classified as non–organ-specific antibodies, such as anti-SSA and anti-SSB (1), and organ-specific antibodies, such as anti–type 3 muscarinic acetylcholine receptor antibody (2). SS in the absence of other autoimmune diseases is classified as primary, and SS in the presence of rheumatoid arthritis, systemic lupus erythematosus, or other connective tissue diseases is classified as secondary.

The infiltrating lymphocytes are mainly CD4+ α/β T cells (3), especially Th1-type T cells, because they produce both interferon-γ (IFNγ) and interleukin-2 (4–6). B cells, macrophages, and natural killer cells are found less frequently than T cells (7). These cells lead to salivary gland destruction via the production of inflammatory cytokines and the interaction between Fas and FasL (8–10). However, the mechanisms of the onset and progression of SS are poorly understood.

To clarify the pathogenesis of SS, several molecules in labial salivary glands (LSGs) have been investigated in animal models of SS as well as in humans with SS. In the MRL/*lpr* mouse, the expression of sialadenitis-associated genes was analyzed by microarray, and 13 genes were found to be up-regulated (11). Highly expressed genes in the conjunctival epithelium in patients with SS were examined using the introduced amplified fragment length polymorphism method (12). Moreover, the expression of Fas, FasL, CTLA-4, and programmed

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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

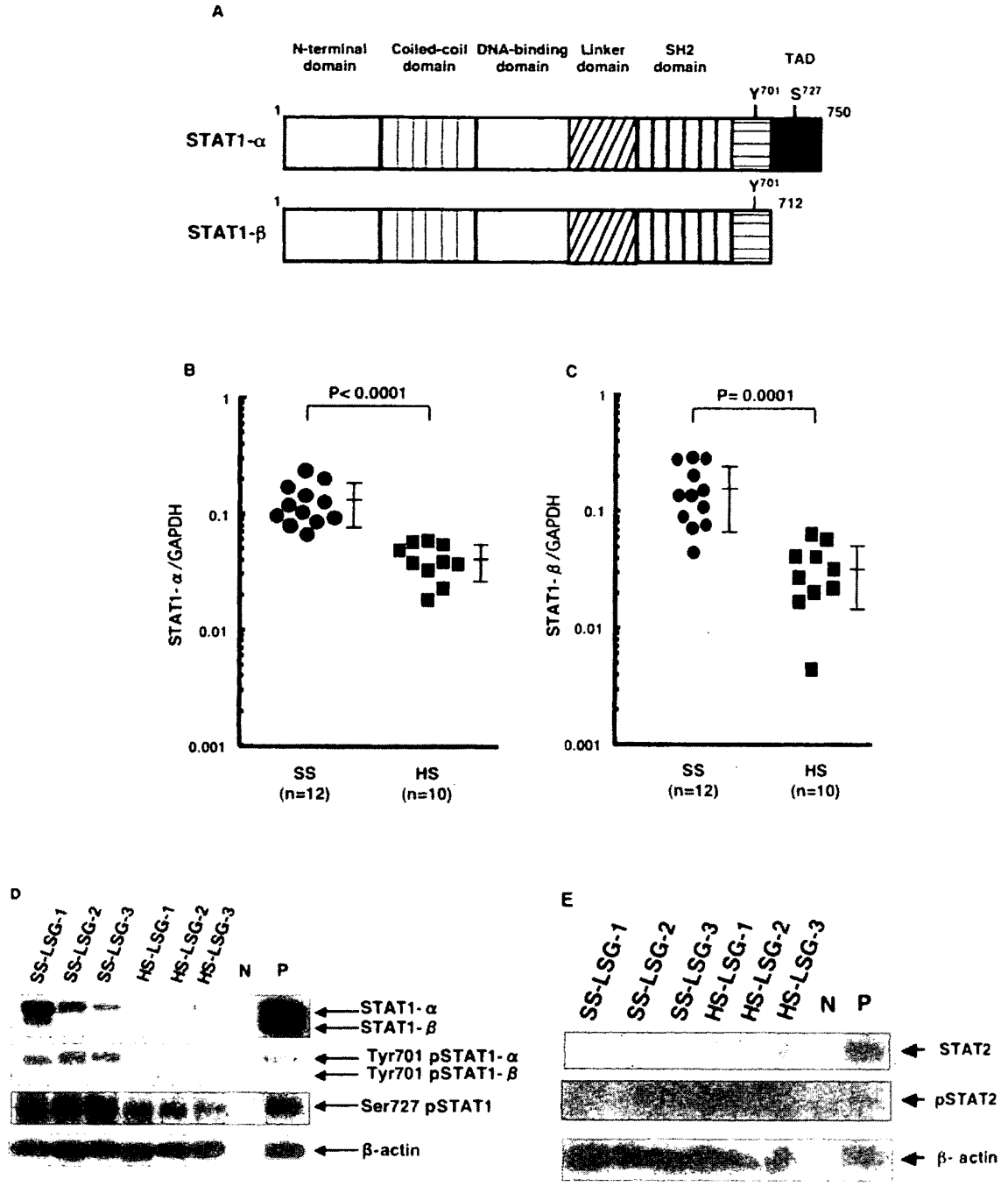

Figure 1. Expression and activation of STAT-1 in labial salivary glands (LSGs) from patients with Sjögren's syndrome (SS). **A,** Domain structure of STAT-1α and STAT-1β. Y^{701} = Tyr^{701}; S^{727} = Ser^{727}; TAD = transcription activation domain. Adapted, with permission, from ref. 50. **B** and **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.

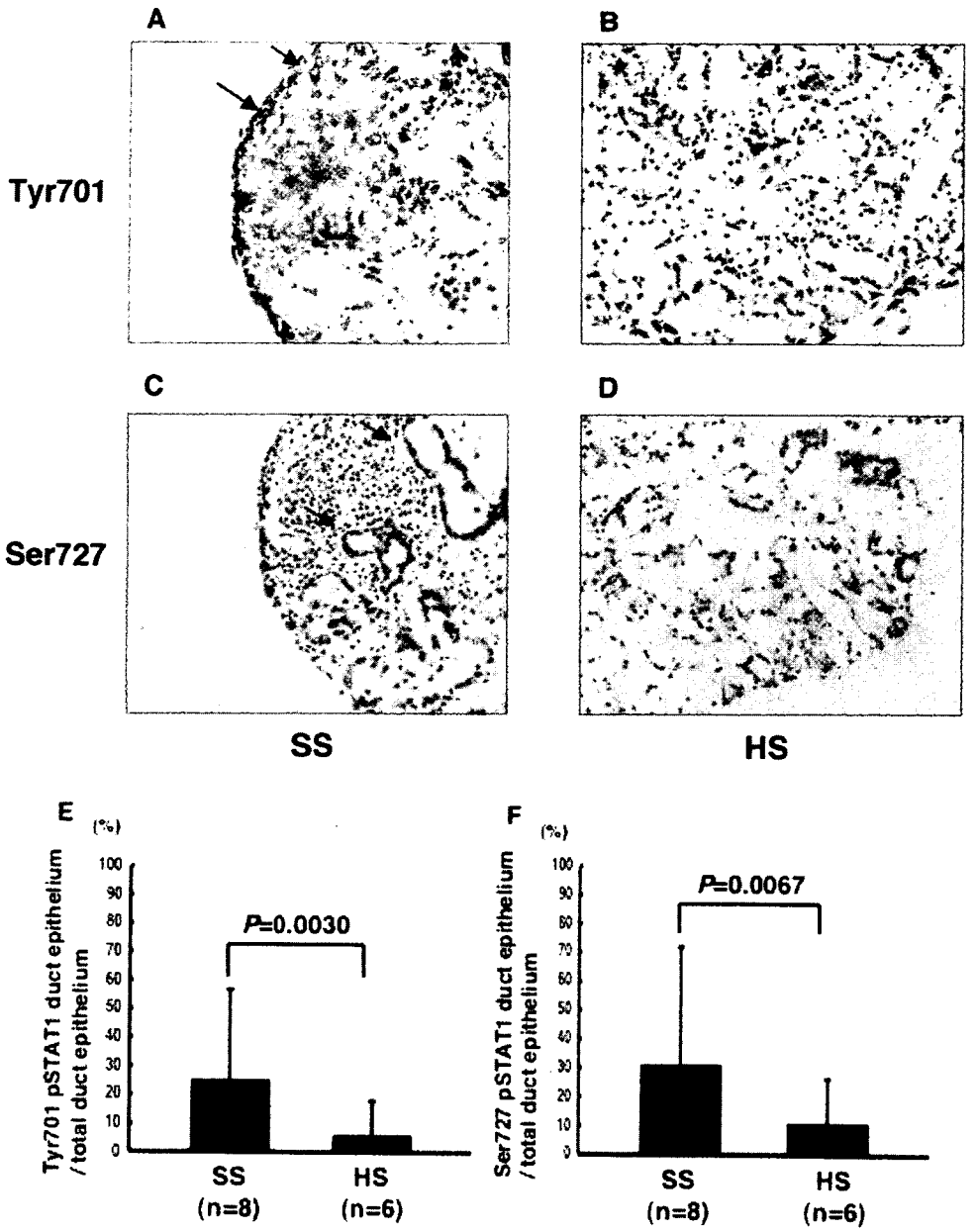

Figure 2. Differential localization of Tyr^{701} and Ser^{727} pSTAT-1 on ductal epithelium in labial salivary glands (LSGs) from patients with Sjögren's syndrome (SS). **A,** Tyr^{701} pSTAT-1 was localized in the ductal epithelium and infiltrating mononuclear cells in SS LSGs, especially in the ductal 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 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.

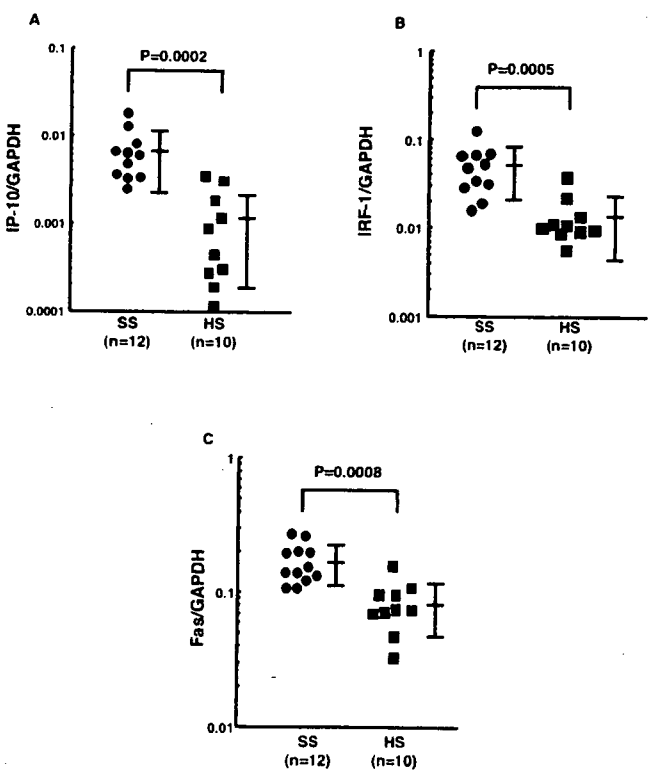

Figure 3. Expression of STAT-1–inducible genes in labial salivary glands (LSGs) from patients with Sjögren's syndrome (SS). Levels of **A**, interferon-γ (IFNγ)–inducible 10-kd protein (IP-10), **B**, IFN regulatory factor (IRF-1), and **C**, Fas mRNA in LSGs from 12 SS patients and 10 healthy control 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.

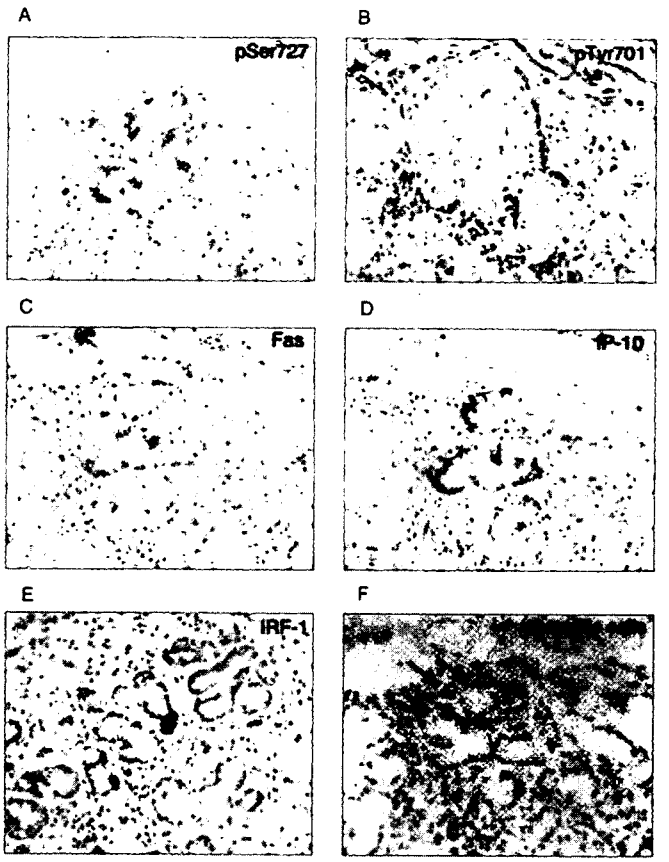

Figure 4. Colocalization of destructive factors with Ser^{727} pSTAT-1–positive cells in labial salivary glands from patients with Sjögren's syndrome. The expression of **A**, Ser^{727} pSTAT-1, **B**, Tyr^{701} pSTAT-1, **C**, Fas, **D**, interferon-γ (IFNγ)–inducible 10-kd protein (IP-10), **E**, IFN 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.)

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.

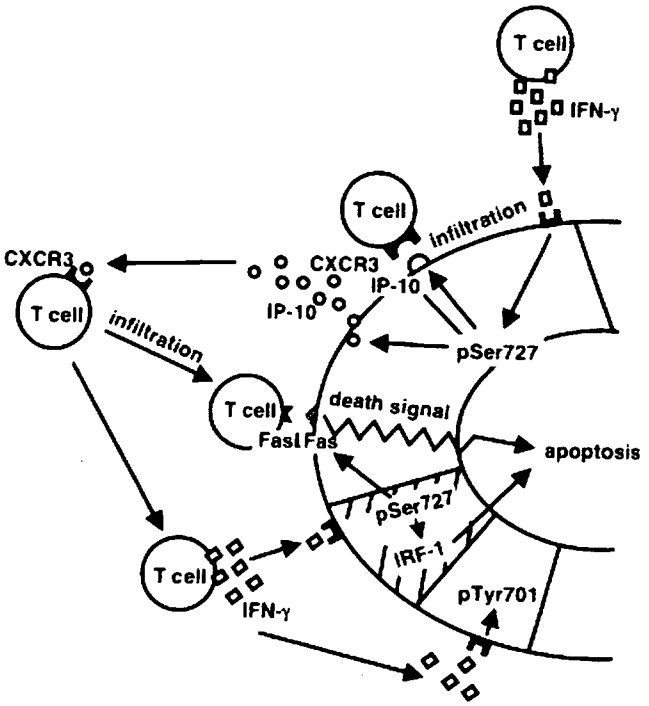

Figure 5. Possible mechanism of salivary gland destruction in Sjögren's syndrome. Ser^{727} pSTAT-1 ($pSer^{727}$) in the ductal epithelium induces interferon-γ (IFNγ)–inducible 10-kd protein (IP-10) and recruits CXCR3 (the receptor for IP-10) and FasL-positive T cells to the salivary glands. In addition, 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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ORIGINAL ARTICLE

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Altered peptide ligands control type II collagen-reactive T cells from rheumatoid arthritis patients

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Abstract We previously reported that peripheral blood mononuclear cells from HLA-DRB1*0101 Japanese patients with rheumatoid arthritis (RA) were highly reactive to 256–271 peptide of type II collagen (CII). In this report, we tried to regulate the CII reactivity of T cells from RA patients with HLA-DRB1*0101 by altered peptide ligand (APL), which is a single amino acid substitution of the T-cell epitope on CII 256–271 peptide. Antagonistic activity of 21 APLs was assessed using three different T-cell lines. Results showed that 262 (G→A) APL of CII 256–271 exhibited antagonistic activity in all T-cell lines and it was suggested that the application of CII APL might be a new therapeutic strategy in the regulation of RA.

Key words Altered peptide ligand (APL) · Antagonist · Rheumatoid arthritis (RA) · T cells · Type II collagen

Introduction

T-cell activation depends on the ability of the T-cell receptor (TCR) to recognize 8–20 amino acid peptides that are bound to major histocompatibility complex (MHC) molecules. The way TCR recognizes peptide is flexible. If the amino acid residue of peptide ligands for TCR is substituted for a different amino acid and can still bind to MHC molecules (altered peptide ligands; APLs), these APLs could regulate the activation of T cells. Several studies have shown that APL had a potential to induce differential cytokine secretion, anergy, and antagonism of the response to the wild-type antigens.[1–3] Therefore, it is possible to use APL as a therapeutic agent against T-cell-mediated diseases such as autoimmune diseases and allergic disorders.

Rheumatoid arthritis (RA) is generally considered to be a T-cell-mediated autoimmune disease. Type II collagen (CII), a molecule abundant in articular cartilage, is an attractive candidate as a target antigen (autoantigen) responsible for pathogenicity of RA. We previously reported that peripheral blood mononuclear cells (PBMCs) from RA patients with HLA-DRB1*0101 haplotype, which is one of the major alleles in Japanese RA patients, reacted to CII 256–271 peptide and this CII fragment was suggested to be a major T-cell epitope in RA patients with this haplotype.[4] In the present study, we established three different CII256–271-specific T-cell lines (E01, H01, H07) from two HLA-DRB1*0101-positive RA patients and tried to regulate CII reactive T cells by inducing TCR antagonism using 21 different APLs.

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Patients and methods

Patients

Two RA patients, who met the 1987 classification criteria of the American College of Rheumatology,[5] were selected after confirmation that their PBMCs included CII 256–271 peptide-reactive T cells. Their HLA-DRB1 haplotypes were DRB1*0101/*0405 and DRB1*0101/*0901. They agreed to participate in the present study and written informed consent was obtained from these subjects before collection of blood samples. The study design was approved by the Ethical Committee of the University of Tsukuba.

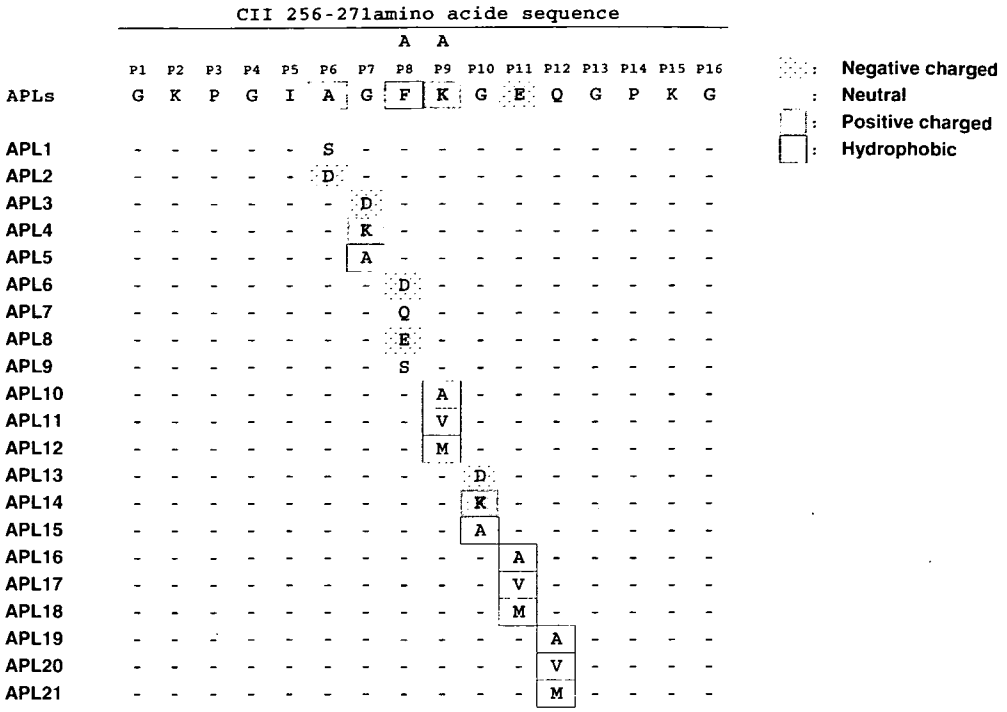

Fig. 1. Design for altered peptide ligands (*APL*). Two anchor positions on CII 256–271 peptide. CII 263 (F) and CII 264 (K), were reported and a single amino acid around these residues was substituted. Twenty-one analog peptides were designed. *A* indicates an anchor residue, which was a binding site to HLA molecule. The *dash* indicates the identical amino acid residue to that of CII 256–271 peptide

Peptides

It was reported that CII 263 (F) and CII 264 (K) were the dominant residues at the binding to DR1 molecule,[6] and therefore, the TCR contact site was considered to be around these residues. The amino acid sequence of APLs was designed so that a single amino acid of the TCR contact site, between CII 261 and CII 267, was changed to an amino acid that had a different charge and was of similar molecular size. Twenty-one APLs were set in this examination, and they were synthesized by Qiagen (Tokyo, Japan) including CII 256–271 wild-type peptide. Purity of each peptide was higher than 90%. The sequences of the peptides are shown in Fig. 1.

Generation of antigen-specific T-cell lines

Peripheral blood mononuclear cells from two RA patients were isolated using Ficoll-Paque (Pharmacia Biotechnology, Piscataway, NJ, USA). 2×10^6 PBMCs were suspended in RPMI-1640 containing 2 mM L-glutamine (Gibco BRL, Grand Island, NY, USA), 100 IU/ml penicillin/streptomycin (Gibco BRL), and 10% autologous serum, and cultured at 37°C in 5% CO_2 with 20 μg/ml of CII 256–271 peptide and interleukin (IL)-2 (30 U/ml, Immunace 35; Shionogi, Osaka, Japan). Cells were restimulated with the CII peptide and limiting dilution was carried out at 10, 50, or 100 cells/well in the presence of 2×10^4 feeder cells, which was the autologous B-cell line infected Epstein–Barr virus and irradiated (100 Gy), and pulsed with CII peptide. Cells were restimulated at 7-day intervals, and 6 lines (E01–E06) and 10 lines (H01–H10) were established from each patient. These T-cell lines were examined for antigen specificity by proliferative response to several concentration of CII 256–271 peptide. Restriction of the DRB1 molecule was confirmed using anti-DP, DR, and DQ antibody and L-cell transfectant expressing HLA-DR 1.[3] From the results, 1 line (E01) and 2 lines (H01 and H07) were established from each individual as DRB1-restricted T-cell lines.

Measurement of TCR antagonism by APLs

Whether APL could function as an antagonist for TCR was determined as previously reported.[1] Briefly, feeder cells were pulsed with a suboptimal concentration (5 μM) of CII 256–271 peptide for 2 h at 37°C under 5% CO_2, washed twice, and irradiated. Feeder cells prepulsed with the CII peptide were incubated with each APL (100 μM) for 12 h, and thereafter, antigen-specific T cells (1×10^5) were added. After incubation for 72 h, cell proliferative response was estimated. Antagonistic activity of APL was expressed as percentage of inhibition of the CII 256–271 peptide response. It was judged as positive when the percentage of inhibition was more than 80%.

Evaluation of cell proliferative response

Cell proliferative response was measured using a bromodeoxyuridine enzyme-linked immunosorbent assay (ELISA) system (Cell Proliferation ELISA kit; Roche Diagnostics, Mannheim, Germany).