Decreased expression of signaling lymphocytic-activation molecule-associated protein (SAP) transcripts in T cells from patients with rheumatoid arthritis

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Abstract

The function of Epstein–Barr virus (EBV)-specific cytotoxic T cells is disturbed in rheumatoid arthritis (RA) patients but the mechanism for this disturbance has remained unknown. In a recent study searching for the causative gene of X-linked lymphoproliferative syndrome, the gene possibly linked to EBV-specific cytotoxic T cells or NK cell-mediated cytotoxic activity to EBV-infected cells was discovered, and its product is now referred to as signaling lymphocytic-activation molecule-associated protein (SAP) or Src homology 2 domain-containing protein (SH2D1A). In the present study, we attempted to investigate the involvement of the SAP gene in RA using a quantitative real-time PCR; the expression level of SAP transcripts in peripheral leukocytes or T cells was examined for patients with RA. The expression level of SAP transcripts in peripheral leukocytes of 21 RA patients was significantly lower than that of 13 normal individuals (P = 0.0007), four patients with palindromic RA, 11 with inactive systemic lupus erythematosus (SLE) or 17 with chronic renal diseases. The decreased expression of SAP transcripts in RA patients was also observed in peripheral CD2+ T cells compared with normal individuals. There was no mutation in the coding region of SAP cDNAs derived from peripheral leukocytes of five RA patients. The decreased expression of SAP transcripts in peripheral leukocytes or T cells of RA patients might lead to the failure of the immune system to eliminate the EBV-infected synovial lining cells in joints of RA patients. Our findings have suggested that decreased expression of the SAP gene might be involved in the onset or progress of RA.

Introduction

Epstein–Barr virus (EBV), a human herpes virus, is capable of remaining latent in host lymphocytes subsequent to primary infection. This virus is known to cause infectious mononucleosis, polyclonal B cell lymphoma, African Burkitt’s lymphoma and nasopharyngeal carcinoma (1). It has been proposed that latent infection with EBV might contribute to the pathogenesis of rheumatoid arthritis (RA) by inducing polyclonal B cell activation (2–7). However, whether or not EBV is directly associated with the pathogenesis of RA remains unclear. Most recently several investigators including us have reported the expression of EBV DNA, RNA or associated proteins in synovial tissue specimens or synovial cells from patients with RA using PCR, in situ hybridization or immunohistochemical procedures (8–12). One of the reasons for the findings described above might be the dysfunction of the EBV-specific cytotoxic T cells in RA patients (13,14).

Recently, in an attempt to locate the causative gene of X-linked lymphoproliferative syndrome (XLP), different groups
have independently identified in XLP families mutations in a novel gene (15–17) and its product is now referred to as signaling lymphocytic-activation molecule (SLAM)-associated protein (SAP) or Src homology 2 domain-containing protein (SH2D1A). The four tyrosine-based motifs (TXXXYV/I) in the cytoplasmic domain of SLAM, also known as CDw150 (18), may be involved in the recruitment of Src homology 2 (SH2)-domain-containing protein tyrosine phosphatase-2 (SHP-2) as well as the association between SLAM and SAP (15). Namely, SAP competes with SHP-2 for binding to phosphorylated SLAM (15). Similar to the SALM/SAP pathway, it has been proposed that SAP functions as a regulator of the signal transduction pathway initiated by 2B4, which is primarily expressed on NK cells and a subset of CD8+ T lymphocytes (19–23). 2B4 is a surface molecule involved in the activation of NK cells mediated by cytotoxicity. The abnormal function of 2B4 molecules appeared to be directly involved in the inability of NK cells to lyse EBV-infected cells (24,25). The defective protein of SAP plays an important role in the pathogenesis of the inherited immunodeficiency XLP.

In the present study, we examined whether aberrant expression or mutation of the SAP gene was observed in RA patients or not.

Methods

Patients

The patients with RA, systemic lupus erythematosus (SLE) and palindromic RA in the present study fulfilled the criteria of the American College of Rheumatology (formerly the American Rheumatism Association) or characteristics described by Hench et al. (26–28). The patients with SLE who were employed as disease controls in this study were in the inactive stage and taking <15 mg prednisolone daily. All chronic renal disease patients had had their diagnosis confirmed by renal biopsy. All of the patients were seen in our outpatient clinic at the Nihon University School of Medicine. For this study, we obtained informed consent from each patient and the normal individuals following the guidelines of the Helsinki Declaration (Hong Kong Amendment, September 1989). Some of the RA patients were taking one disease-modifying antirheumatic drug, including low-dose prednisolone (<7.5 mg) or non-steroidal anti-inflammatory drugs, and a few patients were taking methotrexate (<7.5 mg/week). The mean age of the patients with RA was 57.4 years, the mean C-reactive protein was 2.82 mg/dl, the mean erythrocyte sedimentation rate was 45.6 mm/h, the mean rheumatoid arthritis hemagglutinin test was 215-fold, the mean white blood cell count was 8050/µl and the mean number of peripheral lymphocytes was 1677/µl.

Cell preparation

A 14-ml sample of human blood was collected from each patient into tubes containing EDTA. Peripheral leukocytes were prepared from the buffy coat after centrifugation at 1500 r.p.m. for 15 min. CD2+ T cells were obtained using anti-CD2 antibody-coated magnetic beads (Dynabeads; Dynal, Oslo, Norway). The RNA was immediately extracted by the following method and stored at −70°C.

Quantitative real-time PCR using Taq-Man probe

Total RNA was prepared from the peripheral leukocytes or T cells by a standard procedure using RNAzol B (Tel-Test, Friendswood, TX). Single-strand DNA was synthesized from 2 µg of total RNA using oligo(dT) primer and the Superscript pre-amplification system (Life Technologies, Grand Island, NY) according to the manufacturer’s recommendation. After the reaction, 399 µl of distilled water was added to 21 µl of reaction mixture to give a total volume of 420 µl. The expression level of SAP transcripts was measured by the real-time Taq-Man RT-PCR technique (29). The PCR was performed in a total volume of 50 µl containing 200 nM each of the SAP cDNA-specific primers (5′-AGAGTCCACCCGCGCATAGTGA-3′, and 5′-TACCTTGTGACTCTAGCAG-3′), 100 nM Taq-Man probe (5′-CGTGTACTGCCATTGTGCTGTATCCAGG-3′; PE Applied Biosystems, Foster City, CA) and 1×Universal Master Mix (PE Applied Biosystems). An automatic thermocycler (ABI Prism 7700 sequence detection system; PE Applied Biosystems) was employed for 50 cycles at 95°C for 30 s and 62°C for 90 s, after preheating at 50°C for 120 s and 95°C for 10 min. The standard curve was obtained using serial 1:10 dilutions of standard plasmid DNA with Escherichia coli transfer RNA solution (1 µg/ml). The quantity of SAP transcripts in each sample was determined by extrapolation to the x-axis of the standard curve in Fig. 1. The expression level of GAPDH transcripts was also determined and used to normalize the expression level of SAP transcripts by the following equation: the expression level of SAP transcripts/ the expression level of GAPDH transcripts.

Primers and the Taq-Man probe for GAPDH were purchased from PE Applied Biosystems.

Isolation of SAP cDNAs from RA patients

The SAP cDNA was amplified from single-stranded cDNAs of leukocytes from five RA patients by PCR. The PCR was performed in a total volume of 20 µl containing 500 nM each of the primers (5′-AAGAGTCCACCCGCGCATAGTG-3′, and 5′-TACCTTGTGACTCTAGCAG-3′), 0.2 mM dNTP, 5% DMSO, 1 U Taq DNA polymeraseEX Taq (Takara, Tokyo, Japan), 1×PCR buffer (Takara) and 5 µl of single-stranded cDNA. An automatic thermal cycler (ABI Prism 9700) was employed for 30 cycles at 94°C for 30 s, 60°C for 1 min and 72°C for 2 min, after pretreatment at 94°C for 3 min and on ice for 5 min.

Determination of SAP cDNA sequence

Nucleotide sequence of the SAP cDNAs obtained from leukocytes of five RA patients was determined using direct sequence method. Sequence was performed using an ABI Prism 377 DNA sequencer (PE Applied Biosystems) and reaction kits (BigDye terminator cycle sequencing ready reaction kit; PE Applied Biosystems). The nucleotide sequences were compared with that of the wild-type SAP cDNA deposited in the GenBank/EMBL (AF073019).

Statistical analysis

The Student’s t-test and F-test were used for statistical comparison. The statistical calculations were performed with a statistical software package (Statview II; Abacus Concepts, Berkeley, CA).
Decreased SAP mRNA expression in patients with RA

Fig. 1. A linear relationship between threshold cycles and the amount of SAP cDNA was observed ranging from 0.1 fg/µl and 1 ng/µl. The standard curve was obtained using serial 1:10 dilutions of standard plasmid DNA with E. coli transfer RNA solution (1 µg/ml). The quantity of SAP transcripts in each sample was determined by extrapolation to the x-axis of the standard curve. The expression level of GAPDH transcripts was also determined by the same procedure. In all experiments, the correlation coefficient was between 0.996 and 0.998.

Results

Determination of the linear range of the amplification

To determine the linear relationship between the threshold and the long starting copy number, samples containing defined amounts of standard cDNA were amplified. As shown in Fig. 1, a linear relationship was detected for SAP cDNA, at least ranging between 0.1 fg/µl and 1 ng/µl. A linear relationship was also detected for GAPDH cDNA, at least ranging from 0.1 fg/µl to 1 ng/µl. In all experiments, the correlation coefficient was between 0.996 and 0.998.

Decreased expression of SAP transcripts in peripheral leukocytes in RA patients.

Figure 2 shows the expression level of SAP transcripts normalized with that of GAPDH transcripts. The expression level of SAP transcripts in the peripheral leukocytes of RA patients was lower than that of normal individuals (RA, 0.0058 ± 0.052, n = 21; normal, 0.024 ± 0.021, n = 13, P < 0.01 Student’s t-test). Also, there was a predisposition that the expression level of palindromic RA patients was lower than that of normal individuals (palindromic RA; 0.0108 ± 0.006, n = 4). As shown in Table 1, we also examined the expression level of SAP transcripts in other diseases and found no significant difference between these patients and normal individuals [SLE, 0.0195 ± 0.020, n = 11; chronic nephritis, 0.0253 ± 0.021, n = 17; including minimal change nephrotic syndrome, 0.0129 ± 0.010, n = 5; chronic glomerulonephritis, 0.0142 ± 0.008, n = 3; membranous nephropathy, 0.0397 ± 0.026, n = 6; and membranoproliferative glomerulonephritis, 0.0282 ± 0.019, n = 3].
Decreased SAP mRNA expression in patients with RA

The expression level of SAP transcripts in peripheral CD2⁺ T cells of RA patients is decreased. The expression level of SAP transcripts normalized with that of GAPDH transcripts in peripheral CD2⁺ T cells is indicated. The expression level of SAP transcripts in peripheral CD2⁺ T cells of five RA patients (0.0182 ± 0.011) was significantly lower than that of five normal individuals (0.0779 ± 0.01) (P < 0.005, RA patients versus normal, Student’s t-test). Values are mean ± SD.

Table 1. SAP mRNA expression in peripheral leukocytes in other patients

<table>
<thead>
<tr>
<th>Condition</th>
<th>SAP/GAPDH [mean ± SD (range)]</th>
<th>n</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLE</td>
<td>0.0195 ± 0.020 (0.0021–0.0714)</td>
<td>11</td>
<td>NS</td>
</tr>
<tr>
<td>Chronic nephritis</td>
<td>0.0253 ± 0.021 (0.0071–0.0857)</td>
<td>17</td>
<td>NS</td>
</tr>
<tr>
<td>RA</td>
<td>0.0056 ± 0.052 (0.00003–0.0162)</td>
<td>21</td>
<td>0.0007</td>
</tr>
<tr>
<td>Normal</td>
<td>0.0239 ± 0.021 (0.0015–0.0806)</td>
<td>13</td>
<td></td>
</tr>
</tbody>
</table>

*aSample versus normal (Student’s t-test)

Decreased expression of SAP transcripts in peripheral CD2⁺ T cells of RA patients

The SAP gene is known to be expressed mainly in T cells. Therefore, to specify the type of peripheral leukocytes exhibiting decreased expression of SAP transcripts, we examined SAP gene expression in CD2⁺ T cells (Fig. 3). The expression level of SAP transcripts in peripheral CD2⁺ T cells of five RA patients was significantly lower than that of normal individuals (RA, 0.0182 ± 0.011; normal, 0.0779 ± 0.010, P < 0.005 Student’s t-test).

SAP cDNAs from RA patients showed no mutation

The SAP cDNAs were obtained from five RA patients. The expression level of SAP transcripts in peripheral leukocytes of RA-S (the number is the patient’s serial no.), RA7, RA10, RA11 and RA23 was 0.0162, 0.0160, 0.0028, 0.0050 and 0.0015 respectively. Determination of nucleotide sequence of the SAP cDNAs did not detect any mutations in their coding region compared with wild-type SAP cDNA. No deletions which were reported in XLP patients (15–17) were found.

Discussion

Patients with RA have antibodies that react with an antigen in the nucleus of EBV-transformed B cells called RA-associated nuclear antigen (RANA) (2). In addition, the number of EBV-infected peripheral lymphocytes in RA patients tends to be more than in normal individuals and RA patients exhibit an impairment in their ability to generate EBV-specific cytotoxic T lymphocytes (13,14). Some investigators have explained the association between EBV and RA in relation to the molecular mimicry hypothesis, i.e. RANA is identical to EBNA-1 (6), the antibody to EBNA-1 reacts with a 62 kDa protein in the synovium of RA patients (5), and a homology in amino acid sequences exists between gp110, which is a component of the EBV capsid antigen, and HLA-DR4 (30). It has been reported, however, that EBV cannot be detected in the synovium of RA patients by immunofluorescence and Southern blot assay (31). Although we demonstrated that EBV DNA is amplified by PCR in almost all synovial and peripheral mononuclear cell samples from osteoarthritis (OA), EBV DNA amplification in OA synovial tissues is thought to be due to contamination with B lymphocytes, since EBV DNA is amplified by peripheral B lymphocytes from normal individuals. Recently, Edinger et al. have demonstrated that EBV DNA can be detected in the synovium in patients with RA and OA by PCR as we have previously reported (9). Saal et al. have also demonstrated by PCR and RT-PCR that EBV DNA and EBV-encoded small RNA (EBER)-1 transcripts can be detected in the synovium of RA patients, and that the EBV genome is more frequently present in the synovium of RA patients (29 of 84) than in that of non-RA patient controls (eight of 81) (10). As we had thought that PCR study alone could not elucidate the role of EBV in rheumatoid synovitis, we also examined the synovial tissue by the in situ hybridization method. The localization of EBER-1 and latent membrane protein (LMP)-1 was in proliferative sites at the apex of areas of villus formation in synovial cells. Most recent papers also demonstrated that EBER-1 or EBV DNA was detected in the synovial lining or synovial cells (11,12). Other investigators have reported that EBER-1 was not detectable in the synovial membranes of patients with RA (32,33). There is a discrepancy between their results and ours; the reasons for this discrepancy, however, are unclear.

It has been demonstrated that LMP-1 is involved in the malignant transformation of fibroblast cells (34). LMP-1 can also induce bcl-2 expression that inhibits the apoptosis of B lymphocytes (35). The expression of LMP-1 on synovial cells may cause an aberrant proliferation of synovial cells. One may question why LMP-1 was not expressed in all the EBER-1⁺ cases. We do not have clear answers to this question. However, it is well known that in nasopharyngeal carcinoma, LMP-1 is expressed in only 35–65% of EBV nucleic acid-positive cases (36,37). One may also wish to know why EBV signals were present in some areas but not in others, and why synovial cells in RA exhibited a latent type 2 EBV infection which has been described only for malignant tumors and not for non-neoplastic cells. One possibility may be that the EBER-1⁻ and LMP-1⁻ lesions sustain lytic infections of EBV. Takeda et al. demonstrated BZLF1 and gp350/220 antigens in RA synovial lining cells, which were the proteins detected during the lytic phase (11). However, BHLF-1 RNAs, which also were the most abundant transcripts detected during the lytic phase, was not be detected by in situ hybridization (32). The synovium in RA undergoes marked proliferation, leading to the destruction of bone at the joints similar to that which...
occurs with malignant tumors. In our previous study, EBER-1 and LMP-1 were mainly expressed at the sites of proliferation of synovial lining cells. Because the synovium in RA is neoplastic in nature, it may not be extraneous that synovial cells in RA exhibit latent type 2 EBV infection.

We attempted to find the gene causing a disturbance in the function of EBV cytotoxic T cells in RA patients, paying special attention to the SAP encoded by the XLP disease gene (15). SLAM is a cell-surface antigen to which SAP binds, and a new T cell signal-transduction pathway is initiated by the co-receptor molecule SLAM. It was found that the recruitment of SHP-2 to phosphorylated SLAM is blocked by the binding of SAP to the sequence surrounding the most membrane-proximal tyrosine residue. As SHP-2 can act as a negative regulator of signal transduction (38), the binding of SAP could have a positive effect on co-stimulation by SLAM–SLAM interactions on the interface between T and B cells. As EBV-transformed B cells express SLAM at a high level, the failure of the immune system to eliminate EBV-infected cells like XLP in RA patients appears to be caused by defective EBV-specific T cell and cytotoxic T cell responses (15–17). The mechanisms key to sustaining the elimination of EBV-infected cells might be the same as those for the elimination in patients with XLP as mentioned by Sayos et al. (15). In contrast to XLP, at least in five patients with RA, there is no major genetic mutation in the coding region of the SAP gene as mentioned in ‘result’. These patients were female except for RA10. The disease activities of RA11 and RA23 were more severe, and their titers of rheumatoid factor and the concentration of soluble CD4 were higher than others. The onsets of disease for RA7 and RA10 were < 1 year. RA5 did not have particular characteristics as RA. These observations indicated the various stage or characteristics for RA did not affect the expression level of SAP transcripts and also SAP cDNA sequence.

Mechanisms of the decrease of SAP transcripts are still unknown. Genomic polymorphism at promoter or enhancer regions might exist in RA patients or some cytokines might inhibit the expression of SAP mRNA. As SAP primarily exists in T cells, the disturbance of SAP mRNA expression should affect SLAM-induced signal-transduction events in T cells. RA patients have impaired IFN-γ production by Tn cells in peripheral blood, indicative of a Tn,2-like phenotype (39). SLAM during T cell activation induces IFN-γ production and redirects the Tn,2 phenotype to a Tn,1/Tn,0 phenotype, and an inadequate response of the Tn subset in RA patients could result from impaired function of the SLAM/SAP pathway. In the another study, the expression levels of SLAM were significantly up-regulated in the synovial fluid and synovial tissue T cells from patients with RA compared with peripheral blood T cells from the same patients. Furthermore, anti-SLAM mAb increased the production of IL-10, IFN-γ and tumor necrosis factor-α by in vitro-activated synovial fluid mononuclear cells, supporting the hypothesis that signaling through SLAM may play a role in the regulation of synovial inflammation in patients with RA (40). Given the fact that SLAM has recently been shown to be a high-affinity self-ligand, synovial T cells may stimulate their own cytokine production through homophilic SLAM–SLAM interactions. As the frequency of IFN-γ-producing CD4+ and CD8+ cells is significantly increased in synovial fluid when compared with peripheral blood (41), the SAP regulation in RA synovium may be different in peripheral blood.

Sayos et al. have reported that SAP exist in EBV+ Burkitt’s lymphoma line Raji B cells (15). To eliminate the possibility that B cells resembling Raji cells might exist in RA and that SAP mRNA expression is disturbed as a result, we separated the CD2 T cells. As the regulation of the SAP–SLAM pathway might be variable in age (42) and whether it was receiving severe immunosuppressive treatment, we employed age-matched chronic nephritis and inactive SLE as control in this study. This hypothesis is supported by evidence indicating that primary EBV infection remains silent in most small children, and usually causes mononucleosis in adolescents and young adults (42), and EBV+ lymphoma appears on patients with immunosuppressants after organ transplantations. SAP exists in not only T cells, but also B cells and NK cells, and increased expression of SAP transcripts in CD8+ T cells compared with CD4+ T cell subsets. As the total numbers of peripheral leukocytes and lymphocytes were remarkably low in the active state of SLE, active SLE was not a precise disease control.

Most recently, several investigators have demonstrated that in patients with XLP, altered 2B4 function by the lack of functional SAP or SH2D1A proteins may contribute to the inefficient control of EBV+ B cells by NK cells (24,25,43). CD48 is the ligand for 2B4 (44) and the expression on EBV-transformed B cells is at least 10-fold greater than that on EBV− B cells (45). The up-regulation of CD48 on EBV-transformed B cells may act as a signal to specifically activate NK cells via 2B4 and induce lysis of transformed cells. Impaired 2B4/SAP pathway may contribute to fail to eliminate EBV-infected cells by NK cells in RA patients.

Based on the lower level of binding of SAP to the SAP docking site on SLAM or 2B4, we conclude that dysfunctional SLAM/SAP or 2B4/SAP-induced signal-transduction pathways may be responsible for the ineffective T cell or NK cell response in sustaining the elimination of EBV-infected cells in patients with RA.

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Abbreviations

EBER: Epstein–Barr virus-encoded small RNA
EBV: Epstein–Barr virus
LMP: latent membrane protein
OA: osteoarthritis
RA: rheumatoid arthritis
RANA: RA-associated nuclear antigen
SAP: SLAM-associated protein
SHP-2: Src homology 2-domain-containing protein tyrosine

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**References**


