Detection of Epstein–Barr virus-encoded small RNA 1 and latent membrane protein 1 in synovial lining cells from rheumatoid arthritis patients

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Abstract

Several investigators have demonstrated an association between Epstein–Barr virus (EBV) and the pathogenesis of rheumatoid arthritis (RA). However, there is no direct evidence that this virus exists in the synovial cells of patients with RA. We attempted to detect EBV in synovial cells from RA patients. Specimens of synovial tissues from 34 patients with RA and from 20 patients with osteoarthritis (OA), and from one patient with psoriatic arthritis as controls, were examined for evidence of the EBV by in situ hybridization. The specimens were also tested by immunoperoxidase staining for expression of the CD21 molecule (EBV receptor), EBV nuclear antigen (EBNA)-2 and latent membrane protein (LMP)-1. EBV-encoded small RNA-1 (EBER) was demonstrated in synovial lining cells from eight (23.5%) out of 34 RA patients but in none of 20 OA patients (P < 0.05) nor in the one psoriatic arthritis patient. Interestingly, EBER localized in synovial lining cells that were located at the apex of villus proliferating lesions. Furthermore, LMP-1 was also detected in synovial lining cells at the top of villus lesions. Nevertheless, CD19 and CD21 molecules, and EBNA-2 were not demonstrated in such lesions. The incidence of EBV-positive in synovial lining cells with severely infiltrated lymphocytes tended to be higher than that in moderately infiltrated ones. This is the first evidence that EBV exists in chronically inflamed synovial lining cells of human joints in 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 be associated with infectious mononucleosis, African Burkitt’s lymphoma and nasopharyngeal carcinoma (1). Additionally, several investigators have explored whether EBV could be pathologic in rheumatoid arthritis (RA) (2–6). Shimizu et al. have also found that sera from patients with RA preferentially reacted with EBV nuclear antigen (EBNA)-3 (7). However, whether or not EBV is directly associated with the pathogenesis of RA remains unclear. To examine the hypothesis that EBV may play an important role in the progressive proliferation of synovial cells in patients with RA, we undertook detection of EBV by in situ hybridization for the presence of EBV-encoded small RNA (EBER) in synovial lining cells and immunostaining for the expression of CD21 molecules or of latent membrane protein (LMP)-1 and EBNA-2.

Methods

All RA patients fulfilled the criteria of the American College of Rheumatology (ACR; formerly, the American Rheumatism

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Association) (8). Thirty-three out of 34 patients with RA and 20 patients with osteoarthritis (OA) who underwent total joint replacement of knee or hip joints were identified from 1990 through 1994. One of the synovial samples obtained from an RA patient was operated on for biopsy of synovium. This specimen demonstrated inflamed synovitis of the right wrist joint which was judged to be compatible with RA by a pathologist. For this study, we obtained informed consent from each patient or normal individual and followed the Helsinki Declaration (Hong Kong Amendment, September 1989). X-rays of the patient’s joints revealed stage III or IV as defined by Steinberger’s RA stage classification (9). All OA patients fulfilled the diagnosis criteria of the ACR (10,11). One psoriatic arthritis patient showed typical psoriatic skin rash and erosive osteolytic lesion in the knee, and this patient underwent total joint replacement of the knee. We examined this psoriatic synovial tissue for control as non-rheumatoid inflammatory synovitis. Although we wanted to examine more psoriatic synovial tissues, we could not obtain them except for this sample. Under light microscopy, synovial tissue from the patients with RA exhibited marked proliferation of synovial cells resulting in a villus appearance of the membrane, fibrosis, hyalinization, granuloma and/or prominent lymphocytic infiltration associated with lymph follicle formation. We classified materials according to the degree of infiltration by lymphocytes in order to analyze the pathological characterization of EBV-positive samples as follows: type 1, fibrinoid degeneration and non-specific granulation; type 2, lymphocyte and plasma cell infiltration and non-specific granulation; type 3, mixed type (type 1 and type 2); type 4, scar. Almost all samples of OA showed fibrosis, but three samples showed proliferative synovial cells. One sample of psoriatic arthritis showed proliferative synovial cells.

In situ hybridization of synovium for EBER-1
Formalin-fixed, paraffin-embedded sections of the synovial tissues were mounted on glass slides coated with 3-aminopropytriethoxysilane (Probe-on; Fischer Scientific, Pittsburgh, PA), deparaffinized, digested with pepsin and dehydrated. The EBER probe, a probe of 40 bp (5’-AGCAGAGTCTGGG-AAGACAACCACAGACACCGTCCTCACC) that recognizes a region of the EBV genome transcribed in latently infected cells, was applied (12). The EBER was detected with a kit from Iatoron Laboratories (Chiba, Japan) by the method given in the manufacturer’s manual. The criteria for positivity was a positive nuclear mass staining of synovial cells for EBER-1 at the synovial lining cells under low magnification (×40) but not positive staining with a sense probe. We always performed control staining with a sense probe to exclude non-specific staining.

Enzyme immunostaining of synovium for LMP-1
Synovial tissue sections were deparaffinized and immunostained with mouse monoclonal anti-CD21 antibodies (Dako, Glostrup, Denmark) to the human EBV receptor and with anti-EBNA-2 antibody (PE2) and LMP-1 antibody (CS1-4 and S12) to detect these molecules. We employed a LSAB kit system (Dako) following the method given in the manufacturer’s manual. Deparaffinized sections were treated with microwave radiation before staining with PE2, CS1-4 and S12 antibodies.
Table 1. Detection of EBER in synovial lining cells by *in situ* hybridization

<table>
<thead>
<tr>
<th>EBER</th>
<th>RA type 1</th>
<th>RA type 2</th>
<th>RA type 3</th>
<th>RA type 4</th>
<th>OA</th>
<th>Psoriatic arthritis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Negative</td>
<td>8</td>
<td>8</td>
<td>7</td>
<td>3</td>
<td>20</td>
<td>1</td>
</tr>
</tbody>
</table>

*Type 1, fibrinoid degeneration and non-specific granulation; type 2, lymphocyte and plasma cell infiltration and non-specific granulation; type 3, mixed type (type 1 and type 2); and type 4, scar.*

The $\chi^2$ test was used for statistical comparison. Statistical calculation was performed with a statistical software package (STATVIEW II; Abacus Concepts, Berkeley, CA).

**Results**

To identify the type of synovial cells which was latently infected, we employed *in situ* hybridization to detect EBER. Eight synovial tissue specimens from 34 patients with RA had nuclear and perinuclear staining for EBER at the apex of areas of villus appearance in sites of progressive proliferation of synovial cells (Fig. 1A and B). In contrast, none of the specimens from 20 patients with OA and from one patient with psoriatic arthritis showed the specific staining (RA versus OA: $P < 0.05$, Table 1). A sense probe was employed as a control and did not give a positive staining (Fig. 1C). The incidence of EBER-positive cases in a group of RA patients with prominent infiltration of lymphocytes in the synovium tended to be higher than that in cases without such infiltration (Table 1). One of the EBER-positive synovium samples obtained from RA patients was operated on for biopsy of the synovium. This specimen demonstrated inflamed synovitis of the right wrist joint which was judged to be compatible with RA by a pathologist. This patient was a 31-year-old female and she had been suffering from a soft tissue mass in her right wrist distal of the styloid process. An orthopedician operated on the lesion for the purposes of differential diagnosis. After 27 months since the pathological diagnosis had been made, she fulfilled the ACR criteria for RA. This result indicated that the EBV was associated with the early stage of RA. To confirm the sensitivity and specificity of our *in situ* hybridization system, we investigated human EBV-positive post-pyelothorax lymphoma, Raji cells lymphoma which had developed in SCID mice and Raji cells in culture. Under these conditions, EBER was detected only in tumor cells with our *in situ* hybridization system. In particular, in Raji cells in culture, the positivity of EBER exceeded 97%. It did not seem possible that EBER probes would react with a cellular RNA in the absence of EBV infection, since we had never detected positive signals in negative control cells of EBV. Synovial cells from the patients with RA and OA were negative for the EBV receptor (CD21) on immunostaining. None of the synovial cells, especially at the apex of areas of villus appearance in the synovium, reacted with the anti-par-B-cell antibody (anti-CD19) and anti-EBNA-2 antibody (PE2). Lymph follicles of synovial tissue from the patients with RA reacted strongly with anti-CD19. Synovial lining cells in the synovial tissue from four out of the eight EBER-positive specimens were specifically stained by the two different mAb to LMP-1 (CS1-4 and S12). The location of the LMP-1-positive synovial cells was the same as that of the EBER-positive ones (Fig. 2A and B). None of the EBER-negative cases was positive for LMP-1.

**Discussion**

Data have been accumulating to suggest that patients with RA have antibodies which react with an antigen in the nucleus of EBV-transformed B cells called RA-associated nuclear antigen (RANA) (2). Also, the number of EBV-infected peripheral lymphocytes in RA patients tends to be more than in normal individuals and RA patients display an impairment in their ability to generate EBV-specific cytotoxic T lymphocytes (4). Some investigators have explained the association of the
EBV with RA on the basis of the hypothesis of molecular mimicry, i.e. RANA was identical to EBNA-1 (6), antibody to EBNA-1 reacted with a 62 kDa in the synovium of patients with RA (5), and a homology in amino acid sequence existed between gp110, which was a component of EBV capsid antigen, and HLA-DR4 (13). It has been reported, however, that EBV could not be detected in the synovium of RA patients by immunofluorescence and Southern blot assay (14). Newkirk et al. demonstrated that their PCR detected EBV DNA in RA blood (15). We examined synovial tissue by the in situ EBER EBV-encoded small RNA-1 of nuclear antigen reactive with antibody in rheumatoid arthritis. Arthritis & Rheum. 31:315.

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Abbreviations

EBR  EBV-encoded small RNA-1
EBNA  EBV nuclear antigen
EBV  Epstein–Barr virus
LMP  latent membrane protein
OA  osteoarthritis
RA  rheumatoid arthritis
RANA  RA-associated nuclear antigen

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Epstein–Barr virus association with RA synovitis

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