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Kaohsiung Journal of Medical Sciences (2013) 29, 606–610

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ORIGINAL ARTICLE

EBV-encoded small RNA1 and nonresolving inflammation in rheumatoid arthritis

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Received 30 July 2012; accepted 1 October 2012
Available online 13 August 2013

KEYWORDS
EBER1;
Inflammation;
Rheumatoid arthritis

Abstract Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by perpetuated inflammation in multiple joints. To date, there is no cure for RA, and the causal factor for non-resolving inflammation in RA remains unclear. In this study, we initially observed expression of Epstein–Barr virus-encoded small RNA1 (EBER1) in the synovial tissue of all five patients who showed nonresolving RA inflammation. By contrast, EBER1 was detected in the synovial tissue of only one out of seven patients with advanced osteoarthritis (OA; $p < 0.01$, Fisher's exact test). To confirm this finding, we conducted a second study on synovial tissue samples taken from 23 patients with nonresolving RA inflammation and 13 patients with OA. All synovial samples from patients with nonresolving inflammation of RA showed positive expression of EBER1 (23/23, 100%), whereas none of the synovial samples from patients with OA showed expression of EBER1 (0/13, 0%; $p < 0.001$, by Fisher's exact test). *In vitro*, transfection of RA synovial fibroblasts with EBER1 induced the production of interleukin-6. Taken together, these data strongly suggest that nonresolving RA inflammation is strongly related to the presence of EBER1, which might be, at least partially, responsible for synovial fibroblast interleukin-6 production.
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http://dx.doi.org/10.1016/j.kjms.2013.04.002

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by perpetuated inflammation in multiple joints, which usually causes deformity and disability in cases without adequate treatment. At present, only 30—50% of RA patients can achieve complete remission with the use of various kinds of disease-modifying antirheumatic drugs [1]. In 2010, Nathan and Ding [2] suggested that nonresolving inflammation is a major driver of many chronic inflammatory diseases, and that RA is a typical example. They emphasized that to cure, and not to palliate RA, it may be necessary to synergize current anti-inflammatory therapy with other therapies that target the causal factors of the disease. However, the causal factor of nonresolving inflammation in RA remains unclear.

We proposed the very straightforward concept that some persistent stimulation derived from microbial organisms, such as bacteria or viruses, might play a role in refractory RA (RA with nonresolving inflammation). In this study, we investigated the expression of Epstein—Barr virus (EBV)-encoded small RNA1 (EBER1) in refractory RA [3].

Materials and methods

Patient details and sample collection

This study was approved by the institutional review board (No. 98-4059B) at Kaohsiung Chang Gung Memorial Hospital, Kaohsiung, Taiwan. All RA patients were diagnosed according to the American Rheumatism Association 1987 revised criteria for the classification of RA [4] and followed up at Kaohsiung Chang Gung Memorial Hospital. Although the general condition of the patients showed some improvement with disease-modifying antirheumatic drugs, all patients had suffered from at least one large joint disability due to persistent joint inflammation despite medical treatment. Therefore, joint replacement was performed. All patients gave written informed consent when they were discharged from the ward, and they were followed up at the outpatient department.

In the first study, in 2010, synovial tissue samples were collected from five patients with nonresolving RA inflammation (also termed refractory RA), who were receiving joint replacement because of severe joint destruction, and seven patients with osteoarthritis (OA) who were receiving total knee arthroplasty. The clinical characteristics and

Table 1 Clinical characteristics of five patients.

Characteristic	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
Gender	Female	Female	Female	Male	Female
Age (y)	54	38	56	79	77
RA disease duration (y)	>10	6	>10	6	>10
RF (IU/mL)	130	332	303	3340	832
Anti-CCP (U/mL)	90.9	—	—	—	—
Medication					
Leflunomide	+	+			
Methylprednisolone	+				
Hydroxychloroquine	+				
Methotrexate		+		+	
Rituximab		+			
Associated condition	Severe left knee swelling and disability	Recurrent knee joint effusion	Refractory right wrist swelling	Refractory knee disability and swelling	Refractory knee swelling and disability
	Depression		SLE in remission	Hypertension	Chronic subdural hemorrhage
	Osteoporosis			Osteoporosis	Peptic ulcer
	Acoustic neuroma				Glaucoma
Operation	Left total knee arthroplasty in January 2010	Right knee arthroscopic synovectomy in August 2009	Arthrodesis of the right radiocarpal joint and hemiresection arthroplasty of the distal radioulnar joint in September 2008	Left-side total knee arthroplasty in May 2009	Right-side total knee arthroplasty in October 2009

Anti-CCP = anti-cyclic citrullinated peptide antibody; RA = rheumatoid arthritis; RF = rheumatoid factor; SLE = systemic lupus erythematosus.

laboratory findings are shown in Table 1. In the second study, synovial tissue samples were collected from 23 patients with nonresolving RA inflammation and 13 patients with OA, all of whom were undergoing total knee arthroplasty.

Histopathology examination

All synovial tissue samples were fixed in 10% buffered formalin and embedded in paraffin, and 5-μm sections were stained with hematoxylin and eosin as previously described [5].

Detection of EBER1 by RNA *in situ* hybridization

The paraffin-embedded tissue blocks were sectioned in 4-μm slices. EBER1 was detected by RNA *in situ* hybridization, as previously described by Fan and Gulley [6]. To avoid experimental bias, synovial tissue samples from patients with refractory RA and OA were processed simultaneously.

In vitro synthesis of EBER1 and measurement of IL-6 production

Human RA synovial fibroblasts were purchased from Cell Applications Inc. (San Diego, CA, USA). The pGEMT/EBER1 plasmid was kindly provided by Professor Yu-Sun Chang from the Graduate Institute of Basic Medical Sciences, Chang Gung University, Tao-Yuan, Taiwan. EBER1 was prepared by *in vitro* transcription as previously described [6]. Synovial fibroblasts were seeded in 24-well plates at a density of 5×10^4/well. After 24 hours, cells were transfected with 10 μg/mL of purified EBER1 or 10 μg/mL of poly(I-C) (Sigma-Aldrich, St. Louis, MO, USA) using Lipofectamine 2000 (Invitrogen, Grand Island, NY, USA). At 24 hours, 48 hours, and 72 hours, supernatants were collected for measurement of interleukin-6 (IL-6) production. Levels of IL-6 in cell culture supernatants were quantified using Quantikine human IL-6 kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol.

Results

In the initial study, the synovial tissue from all five patients with nonresolving inflammation of RA exhibited strong inflammation, including hypertrophy of the synovial lining with plasma cell and lymphocyte infiltration, as well as neovascularization. Using RNA *in situ* hybridization, we found a strong expression of EBER1 in the synovial specimens from all five patients with refractory RA. EBER1 expression was observed in the synovial lining cells, plasma cells, endothelial cells, or infiltration lymphocytes (Table 2 and Fig. 1). However, of the synovial tissue samples obtained from patients with OA, only one patient's sample was positive for EBER1 (1/7, 14%). There was a statistically significant difference in EBER1 expression between these two groups ($p < 0.01$, Fisher's exact test).

We expanded the study and assessed EBER1 expression in synovial samples taken from 23 patients with refractory RA and 13 patients with OA. Expression of EBER1 was observed in all samples from patients with refractory RA (23/23, 100%), with EBER1 expression predominantly located in the synovial lining cells, plasma cells, and endothelial cells. However, expression of EBER1 was not detected in any of the synovial specimens from patients with OA (0/13, 0%; Table 3). There was a highly statistically significant difference in EBER1 expression between these two groups ($p < 0.001$, Fisher's exact test).

Table 2 Expression of Epstein–Barr virus-encoded small RNA1 (EBER1) in synovial lining cells, plasma cells, endothelial cells, and lymphocytes in five consecutive patients with refractory rheumatoid arthritis (RA) receiving surgical intervention.

Age (y)	Gender	Synovial lining cell	Plasma cell	Endothelial cell	Lymphocytes
54	Female	+	+	+	+
38	Female	+	+	+	Equivocal
56	Female	+	+	+	Equivocal
79	Male	+	+	Equivocal	−
77	Female	+	+	+	−

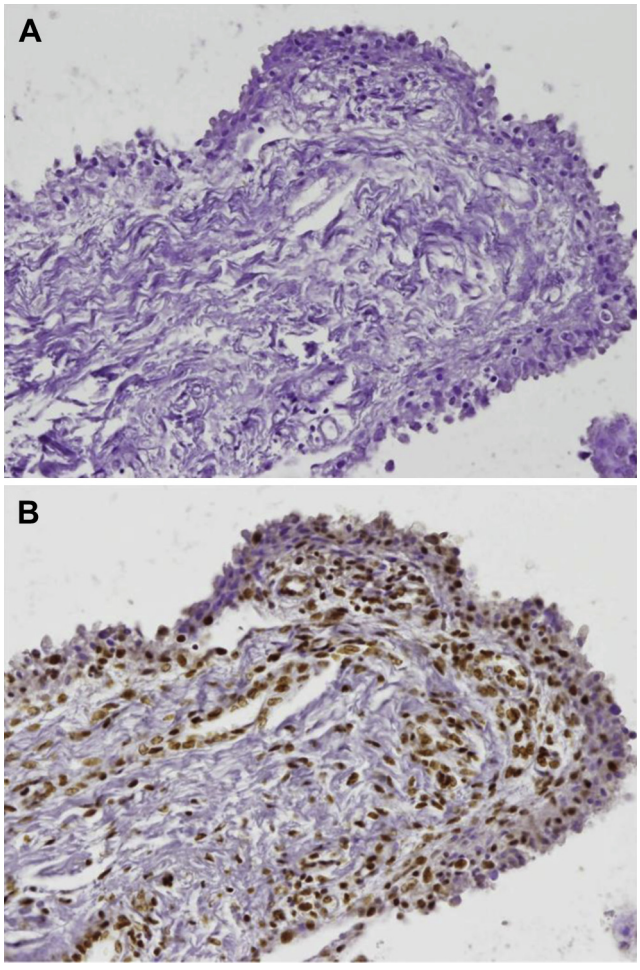

Figure 1. Detection of Epstein–Barr virus-encoded small RNA1 (EBER1) by RNA *in situ* hybridization in a patient with refractory rheumatoid arthritis (RA) compared to a patient with osteoarthritis (OA). (A) Negative control using a sense probe for EBER1 (200×) in RA. (B) EBER1 detected by RNA *in situ* hybridization (200×) in a patient with RA.

Table 3 Expression of Epstein–Barr virus-encoded small RNA1 (EBER1) in synovial tissues of the second study.

EBER1 expression[a]	RA ($n = 23$)	OA ($n = 13$)	p
Synovial lining cells,	23 (100)	0 (0)	<0.001
Plasma cells	23 (100)	0 (0)	<0.001
Endothelial cells	23 (100)	0 (0)	<0.001
Infiltration lymphocytes	15 (65.2)	0 (0)	<0.001

Data are presented as *n* (%).
[a] There was a significant difference in EBER1 expression between rheumatoid arthritis (RA) group and osteoarthritis (OA) group ($p < 0.0001$, Fisher's exact test).

Furthermore, to investigate the effect of EBER1 on human RA synovial fibroblasts, we tested whether *in vitro* synthesized EBER1 could induce the production of IL-6. As shown in Fig. 2, EBER1-induced IL-6 production by human RA synovial fibroblasts was detected at 24 hours and reached a peak at 72 hours, which indicated EBER1-mediated activation of human RA synovial fibroblasts.

Discussion

EBV has been speculated to be related to RA since 1976, when Aslpaugh and Tan [7] discovered that patients with seropositive RA have a high frequency of antibodies to a nuclear antigen present only in EBV-transformed lymphocytes. In 1978, Slaughter et al. [8] reported that lymphocytes taken from RA patients spontaneously transformed into cell lines more often and more quickly than lymphocytes taken from normal controls. Furthermore, infection of RA lymphocytes with EBV induced the production of more IgM rheumatoid factor [8]. However, in early studies, EBV-DNA was not detected within the synovial tissues of RA patients. In 1997, Takei et al. [9], using a highly sensitive technique, were the first to report that EBER1 was observed in the synovial lining cells in 23.5% (8/34) of chronic RA patients. In 2000, Takeda et al. [10] detected EBER1 by RNA *in situ* hybridization in only five of 32 patients with RA. However, in contrast to our study, in which the patients were all positive for rheumatoid factor and refractory arthritis, the patients in Takeda's study were heterogeneous for RA and were not limited to patients with refractory RA. Here, we report the expression of EBER1 in the synovial tissue of all the patients in our study with refractory RA, showing that the presence of EBER1 in synovial tissue is strongly related to nonresolving inflammation in RA.

However, it is unclear whether EBER1 is the cause of persistent inflammation in RA. EBER1 is a poly(A)-, noncoding RNA that is expressed abundantly in all forms of cells latently infected with EBV. Its biologic function has not been made clear until 2009, when Iwakiri et al. [11] discovered that EBER1 induces cell signaling through activation of Toll-like receptor 3 and induces type I interferon

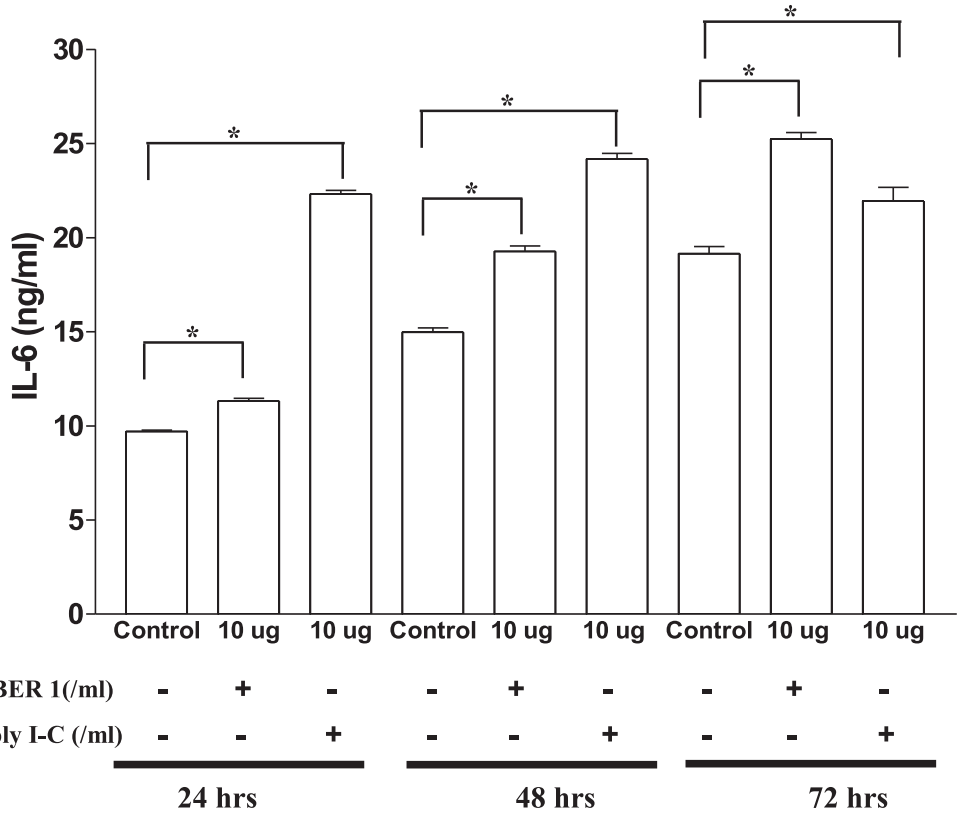

Figure 2. Production of interleukin (IL)-6 protein by human rheumatoid arthritis (RA) synovial fibroblasts treated with Epstein–Barr virus-encoded small RNA1 (EBER1) and poly(I-C). Values are expressed as the mean and standard error of the mean. *$p < 0.05$.

and proinflammatory cytokine production in lymphoblastoid cell lines. Moreover, human monocyte-derived dendritic cells treated with EBER1 showed mature phenotype and antigen presentation capacity [11]. Toll-like receptor 3 is also expressed in synovial fibroblasts [12], endothelial cells [13], and plasma cells [14], which suggests that EBER1 may also be involved in the activation of these cells. Our current observation of the positive staining of EBER1 in synovial lining cells, endothelial cells, and plasma cells seems compatible with this hypothesis. Moreover, we report the novel observation that EBER1 is capable of activating primary human RA synovial fibroblasts, resulting in the induction of IL-6 production (Fig. 2).

Further evidence to support our hypothesis that EBER1 is associated with refractory RA is provided by a study from Sweden published in 2010 [15]. Anti-CD20 treatment was given to RA patients who had been nonresponsive to anti-TNF-α treatment. Prior to treatment, EBV was identified in 15 (43%) out of the 35 patients. Bone marrow and blood samples obtained from these 15 EBV-positive patients 3 months after anti-CD20 treatment were EBV-negative. Moreover, a significantly better anti-CD20 therapy effect and lower relapse rate were observed in the EBV-positive patients as opposed to non-EBV patients [15]. This finding suggests that EBV infection in refractory RA patients plays an important role in the nonresolving RA inflammation.

In this study, we observed that poly(IC) induced higher IL-6 production than EBER1 from human RA synovial fibroblasts, suggesting that non-EBV-derived RNA could also stimulate synovial fibroblasts to produce IL-6. Indeed, Brentano et al. [16] reported that RNA release from necrotic synovial fluid cell from patients with RA activated RA synovial fibroblasts to release IL-6 *in vitro* [16]. However, it is unknown whether RNA can activate synovial fibroblasts *in vivo*. In the future, studies to determine whether RNAs other than EBER1 are present in the synovial fluid and tissues of RA patients are worth pursuing. Furthermore, it would be interesting to assess and compare the expression of EBER1 among RA and non-RA inflammatory arthritis, and determine whether EBER1 is specific for RA.

In brief, although the initial etiology of RA is still unclear, our study suggests that nonresolving inflammation in RA is strongly associated with the presence of EBER1 in synovial tissue.

Acknowledgments

The authors gratefully acknowledge the patients and their families for participating in these studies. They thank Professor Yu-Sun Chang at the Graduate Institute of Basic Medical Sciences, Chang Gung University, Tao-Yuan, Taiwan, for providing pGEMT/EBER1 plasmid. We would like to thank Chang Gung Medical Foundation, Kaohsiung Chang Gung Memorial Hospital Tissue Bank (CLRPG8B0031) for technical support. This study was supported by National Science Council, Taiwan, ROC (Grant 99-2314-B-182-011) and Chang Gung Medical Foundation, Kaohsiung Chang Gung Memorial Hospital, Kaohsiung, Taiwan (Grant BMRP743).

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