Malignant transformation of B lymphoma cell line BJAB by Epstein–Barr virus-encoded small RNAs

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Abstract EBV-encoded small RNAs (EBERs) are non-polyadenylated and abundantly transcribed RNAs, whose functions have not yet been fully elucidated. Here, we report that the EBV-negative B lymphoma cell line, BJAB, was rendered more malignant and resistant to apoptosis by EBERs. EBER-transfected cells exhibited enhanced growth potential in SCID mice as well as in soft agar, and showed resistance to apoptotic stimuli in comparison with the vector control. EBERs inhibited the activity of the double-stranded RNA-dependent protein kinase, PKR, which is reputed to act as a tumor-suppressor. These results suggest that EBERs play an important role in the pathogenesis of EBV-associated malignancies through the inhibition of PKR.

Key words: Epstein–Barr virus; Epstein–Barr virus-encoded small RNA; Double-stranded RNA-dependent protein kinase; Apoptosis; Transformation

1. Introduction

Epstein–Barr virus (EBV) is a ubiquitous human γ herpes virus that establishes a life-long latent infection in its host. EBV is associated with malignant diseases, including Burkitt's lymphoma (BL) [1,2], nasopharyngeal carcinoma [3,4], and immunoblastic B cell lymphoma of immunosuppressed individuals. In these diseases, the different patterns of viral gene expression are classified as type 1 latency for BL, type 2 latency for nasopharyngeal carcinoma and type 3 latency for immunoblastic B cell lymphoma of immunosuppressed individuals.

In type 1 latency, viral gene expression is restricted to two proteins and two non-coding RNAs. The proteins are EBV-associated nuclear antigen 1(EBNA-1) and RK-BARF0, and the RNAs are EBV-encoded small RNA-1 and 2 (EBER-1 and EBER-2). In type 2 latency, integral membrane proteins (LMPs) are present in addition to all the genes expressed in type 1 latency. In type 3 latency, all the following viral latent genes are expressed: six EBNAs, three LMPs, RK-BARF0 and EBERs.

It has been reported that the type 1 latency program of EBV is closely related to the malignant phenotype of the BL cell line [5,6]. Moreover, infection of epithelial cells with EBV was shown to induce type 1 latency and result in malignant phenotypes [7]. These results suggest that type 1 latency genes of EBV may play important roles in the establishment of malignant phenotypes.

Of all genes expressed in type 1 latency, we focused on EBERs as the genes responsible for malignant phenotypes because they have been reported to inhibit double-stranded RNA-dependent protein kinase, PKR, which is described as an anti-oncogene product [8,9].

PKR was identified initially as an interferon-inducible enzyme that became activated during virus infection [10–12]. It is constitutively expressed in mammalian cells and is assumed to function in the control of cell proliferation. Upon activation, PKR exhibits two kinase reactions: autophosphorylation and phosphorylation of substrates such as eukaryotic initiation factor (eIF) 2α. Phosphorylation of eIF2α results in a decreased proliferation rate by inhibiting protein synthesis at the translational initiation step [13,14]. Over-expression of wild-type PKR inhibits the growth of yeast cells [15] and induces apoptosis of HeLa cells [16,17], while dominant negative mutants of PKR confer malignant phenotypes onto NIH3T3 cells [18–20]. Furthermore, recent studies have shown that PKR may be involved in stress-induced apoptosis [21–23], signal transduction and transcriptional control [24]. These results suggest that PKR may have a tumor-suppressor function.

In this report we demonstrate that an EBV-negative B lymphoma cell line, BJAB, was rendered more malignant and resistant to apoptosis by EBERs, which have an inhibitory effect on the tumor-suppressor activity of PKR.

2. Materials and methods

2.1. Cell culture

BJAB is an EBV-negative B lymphoma cell line. Cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and 2 mM L-glutamine at 37°C in 5% CO_2 humidified atmosphere.

2.2. Plasmids and transfection

EBERs and their promoters on the *Eco*RI J fragment of EBV were

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Abbreviations: EBERs, Epstein–Barr virus-encoded small RNAs; PKR, double-stranded RNA-dependent protein kinase

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amplified by the polymerase chain reaction (PCR) with primers 5′-CTCGGAGCTCCTAGGTCAGAC-3′ (5′sequence) and 5′-GCATATACAGTCAGCATATG-3′ (3′sequence). The amplified fragment was cloned into the pGEM-T vector (Promega). Both pcDNA3 vector (Invitrogen) and pGEM-T/EBERs were digested by *Aat*II and *Not*I, and the fragment of EBERs was ligated into the pcDNA3 vector. Vector plasmids with or without EBERs were introduced into BJAB cells by electroporation as follows: 5×10^6 cells were washed twice with serum-free RPMI 1640 and resuspended in 200 μl of ice-cold K-PBS containing 20 μg of linearized plasmid DNA. Electroporation was performed in the 4-mm gap cuvette with the Gene Pulser at 250 V and 975 μF. To generate clonal transfectants, the cells were transferred to 96-well tissue culture plates (5×10^3 cells/ml) at 48 h post-transfection, and cultured in 200 μl of medium containing 800 μg/ml of G418. Half of the medium was changed every 6 days until colonies emerged.

2.3. Northern blot analysis

Total RNA was extracted from BJAB cells with Isogen (Nippon Gene) according to the manufacturer's instructions. 10 μg of total RNA was loaded in each lane and separated on a 1.5% agarose gel containing 0.66 M formaldehyde. Total RNA was transferred to Hybond N+ nylon membranes (Amersham) and fixed with a UV cross linker (Spectronics Corporation). Ribosomal RNA 18S and 28S stained with ethidium bromide were visualized using the FLA 2000 system (Fuji Photo Film Co., Tokyo, Japan). Probes were amplified by PCR with primers 5′-AGGACCTACGCTGCCCTAGA-3′ and 5′-AAAACATGCGGACCACCAG-3′ for EBER 1, and 5′-AGGACAGCCGTTGCCCTA-3′ and 5′-AAAAATAGCGGACAAGCCGA-3′ for EBER 2. These probes were radiolabeled with a DNA labeling kit (Nippon Gene). Membranes were prehybridized and hybridized with labeled probes in the buffer (40% deionized formamide, 4×SSC, 10% dextran sulfate, 1×Denhardt's solution, 40 μg/ml sonicated and denatured salmon sperm DNA, 0.1% SDS, 20 mM Tris (pH 7.5)) at 42°C for 16 h. The membranes were washed twice for 15 min at room temperature, and once for 30 min at 56°C with 2×SSC containing 0.1% SDS, and exposed using a Fuji Imaging Plate. Radioactivity was detected with the BAS system (Fuji Photo Film Co., Tokyo, Japan).

2.4. Colony formation assays

For the colony formation assays, 10^3 cells were suspended in 4 ml of standard growth medium containing 0.5% soft agarose (SeaPlaque; FMC). The suspension was embedded into a base layer made of 3 ml of the same medium containing 0.6% soft agarose in 6-cm-diameter tissue culture dish. The number of colonies larger than 0.5 mm was counted after 3 weeks of incubation.

2.5. Tumorigenicity assays

Tumorigenicity of EBER-transfected and vector-transfected BJAB cells was assessed by the ability to induce tumors in SCID mice. Female SCID mice (C.B-17/lcr-scid Jcl;CLEA) were injected subcutaneously in each hind flank with 1×10^7 cells suspended in 200 μl of serum-free RPMI1640 medium. Each mouse received an injection of EBER-transfected BJAB cells in the right flank and vector-transfected BJAB cells in the left flank. SCID mice were monitored for 6 weeks to measure the tumors that developed.

2.6. Induction and detection of apoptosis

For the induction of apoptosis, cells were exposed to anti Fas monoclonal antibody (250 ng/ml) or mitomycin C (10 μg/ml). The cells were incubated for 16 h after stimulation, washed twice with phosphate-buffered saline (PBS), and suspended in 100 μl of binding buffer containing fluorescein-conjugated annexin V and propidium iodide (Trevigen). After 30 min of incubation at 20–25°C in the dark, the cells were diluted with 400 μl of binding buffer, and analyzed with FACScalibur for the detection of apoptosis.

2.7. Immunoprecipitation

For immunoprecipitation, 1×10^6 of EBER-transfected and vector-transfected BJAB cells were washed by PBS and lysed in 100 μl of Buffer 1 (20 mM Tris–HCl pH 7.6, 50 mM KCl, 400 mM NaCl, 1 mM EDTA, 5 mM 2-mercaptoethanol, 1% Triton X-100, 0.2 mM PMSF, 100 U/ml aprotinin, 20% glycerol, 100 μM Na_3VO_4, 20 mM β-glycerophosphate). Cell lysates were incubated on ice for 10 min and centrifuged at 15000 rpm for 10 min at 4°C. 0.3 μl of anti-PKR monoclonal antibody was added to supernatants and lysates were incubated on ice for 20 min. After addition of 20 μl of protein G Sepharose suspension, cell lysates were gently agitated at 4°C for 1 h, washed four times with Buffer 1, and washed once with Buffer 3 (20 mM Tris–HCl pH 7.6, 80 mM KCl, 5 mM 2-mercaptoethanol, 2 mM $MgCl_2$, 2 mM $MnCl_2$, 20% glycerol, 100 μM Na_3VO_4, 20 mM β-glycerophosphate).

2.8. Kinase assay

Immunoprecipitated PKR was incubated with [γ-^{32}P]ATP (3000 Ci/mmol, NEN Life Science Products, Inc.), in the presence or absence of poly(I)poly(C) at a concentration of 0.2 μg/ml at 30°C for 15 min, and then with 3 μg of histone H2A (Boehringer Mannheim, Germany), which is an effective substrate of PKR, at 30°C for 15 min. The phosphorylation reaction was stopped by adding 2×sample buffer (60 mM Tris pH 6.8, 2% SDS, 10% glycerol, 100 mM DTT, 0.1% BPB). SDS–PAGE was then performed and phosphorylated H2A proteins were visualized by the BAS system (Fuji Photo Film Co., Tokyo, Japan).

2.9. Western blot analysis

After the immunoprecipitation of PKR, protein G Sepharose beads bound to PKR via antibody were suspended in 20 μl of sample buffer (250 mM Tris pH 6.8, 4% SDS, 20% glycerol, 0.2% BPB), and samples were boiled with 2-mercaptoethanol at 100°C for 3 min. PKR and the other precipitated molecules detached from protein G Sepharose were separated in 10% polyacrylamide gel and transferred to a PVDF membrane (Atto, Tokyo, Japan). The membrane was blocked in PBS containing 3% milk and 0.1% Tween 20. For immunostaining, the membrane was incubated with rabbit anti-PKR polyclonal antibody (Santacruz). PKR protein was detected with the FLA 2000 system (Fuji Photo Film Co., Tokyo, Japan).

3. Results

3.1. Vector- and EBER-transfected BJAB cell clones were successfully established

To investigate the function of EBERs, we introduced an EBER-cloned vector or control vector into BJAB cells and established several clones of both transfectants (Fig. 1). The expression levels of EBERs in EBER-transfected cells were approximately 40–60% of those in Raji cells, which constitute an EBV-positive Burkitt's lymphoma cell line. Northern blot analysis revealed a higher level of EBER-1 transcript expression than EBER-2 transcript expression in both Raji cells and EBER-transfected cell clones.

3.2. EBERs enhanced the growth potential of the B lymphoma cell line, BJAB

To evaluate the effects of EBERs, we first compared the

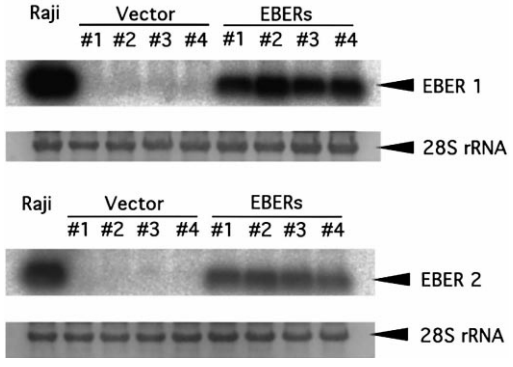

Fig. 1. Northern blot analysis of EBER-1 and EBER-2 expression in Raji and established transfectants. 10 μg of total RNA was loaded in each lane.

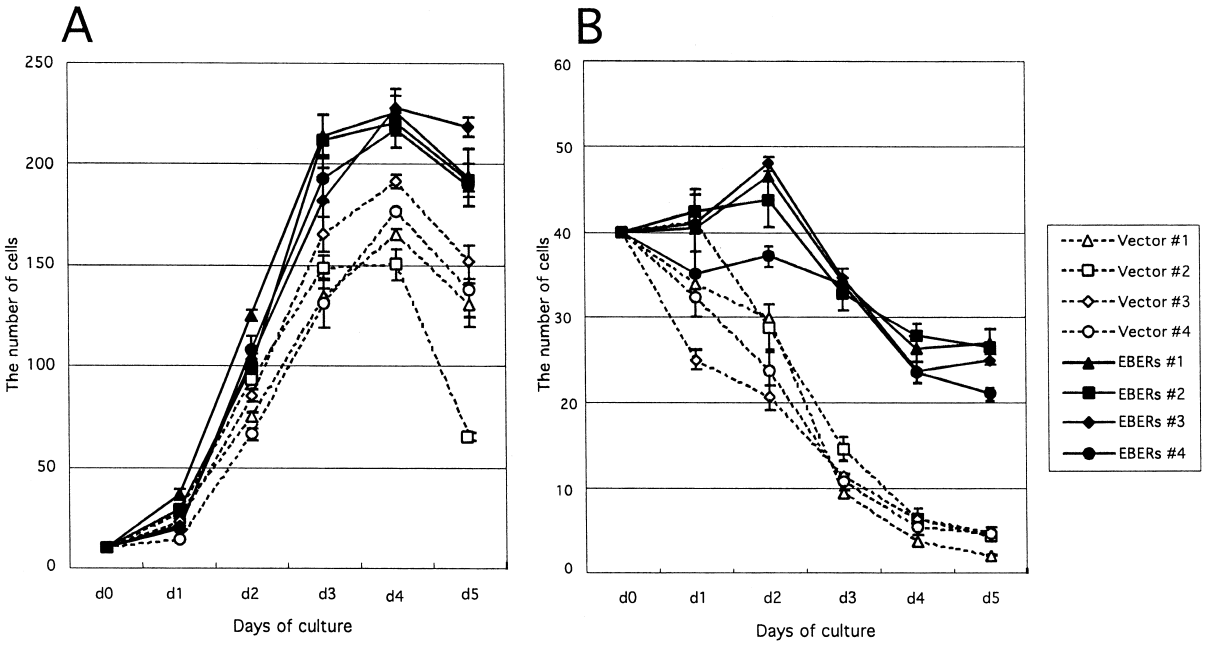

Fig. 2. Growth characteristics of EBER-transfected and vector-transfected BJAB cells. A: Growth curves of BJAB cells at a 10% serum concentration. EBER-transfected (shaded symbols) and vector-transfected (open symbols) BJAB cells were seeded at 1×10^5 cells/ml in growth medium containing 10% FCS, and cell density was monitored daily for 5 days to assess growth rate. B: Growth curves of BJAB cells at a low serum concentration. Cells were washed and seeded at 4×10^5 cells/ml in growth medium containing 0.1% FCS. Cell density was monitored daily for 5 days.

growth kinetics of EBER-transfected BJAB cells with those of vector controls at the serum concentration of 10% (Fig. 2A). In the logarithmic phase (day 1–3), EBER-transfected BJAB cells exhibited higher growth rates than the vector control in the presence of 10% serum. In the stationary phase (day 4–5), EBER-positive cells reached higher saturation densities than vector-transfected BJAB cells at the serum concentration of 10%.

We also investigated the growth kinetics of EBER- and vector-transfected BJAB cells under limited serum concentrations (Fig. 2B). EBER-transfected BJAB cells survived at low serum concentrations in comparison with vector-transfected BJAB cells, which suggests that EBERs inhibited apoptosis induced by serum deprivation.

To determine whether or not EBERs confer enhanced growth potential and tumorigenicity, we examined EBER- and vector-transfected BJAB cells for their ability to form colonies in soft agar (Fig. 3) and tumor generation in SCID mice (Table 1). Although vector-transfected BJAB cells exhibited limited ability to produce colonies in soft agar, EBER-transfected BJAB cells formed many larger colonies. In terms of tumorigenicity in SCID mice, EBER-transfected BJAB cells produced tumors at the sites of inoculation in 10 of 10 SCID mice, while vector-transfected BJAB cells did so in only 1 of 10 SCID mice (Table 1). Even in the mouse in which EBER- and vector-transfected cells formed a tumor, the size of the tumor from vector-transfected cells was much smaller than that from EBER-transfected cells.

3.3. *EBERs conferred resistance to apoptosis induced by several stimuli*

From the results indicating that EBERs contribute to survival of BJAB cells under growth-limiting conditions, we predicted that EBERs might inhibit stress-induced apoptosis. To determine whether EBERs endow resistance to apoptosis, cells were exposed to several apoptotic stimuli (anti-Fas monoclonal antibody and mitomycin C). Next, cells were subjected to staining with annexin V, which is a marker for early stage apoptosis and PI, which is a marker for late stage apoptosis (Fig. 4). Viable cells were those that were not stained by either annexin V or PI. The proportions of viable cells exposed to various apoptotic stimuli were significantly higher in EBER-transfected BJAB cells than in vector-transfected BJAB cells. These results suggest that EBERs may act as inhibitors of apoptosis induced by different stimuli.

Table 1
Tumorigenicity of BJAB cells in SCID mice

Cell line	Number of mice tumor(+)/total	Length/width/height of developed tumor (mm)
EBER-transfected BJAB	10/10	23.8/15.8/7.6[a]
Vector-transfected BJAB	1/10	10/9/3

Tumorigenicity of EBER-transfected and vector-transfected BJAB cells in SCID mice. Each mouse received an injection of vector-transfected BJAB cells (clone #1) in the left flank and EBER-transfected BJAB cells (clone #1) in the right flank. The size of formed tumors was measured 6 weeks after inoculation.
[a]Mean value.

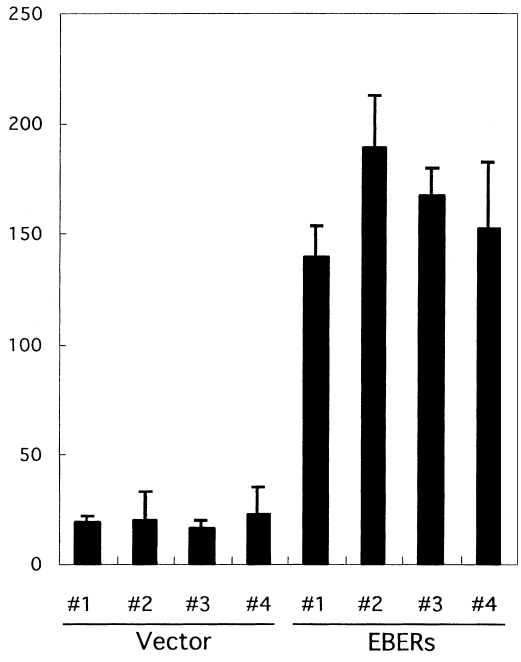

Fig. 3. Colony formation of EBER- and vector-transfected BJAB cells in soft agar. BJAB cells were plated in 0.5% soft agarose at a density of 10^3 cells/6-cm-diameter dish. The number of colonies was counted 3 weeks after plating. These results were the average of three independent experiments. The vertical bars represent standard errors of the mean.

3.4. EBERs inhibit activation of PKR, a mediator of various apoptotic signals

We carried out an in vitro kinase assay to investigate whether in BJAB cells, the stem–loop structure of EBERs can activate or inhibit the kinase activity of PKR. The results of the kinase assay are shown in Fig. 5. PKR immunoprecipitated from EBER-transfected cells exhibited lower kinase activity than that from the vector control, although the amounts of PKR protein were almost the same in both types of cells. PKR phosphorylated itself and its substrate, H2A, efficiently when incubated with polyIC. Even in the absence of polyIC, we detected autophosphorylation of PKR and phosphorylation of H2A. These results suggested that EBERs were able to inhibit the kinase activity of PKR by binding to this kinase.

4. Discussion

We have demonstrated that EBERs bestow enhanced growth potential and apoptosis resistance on BJAB cells. In the analysis of growth potential, it seemed that different growth properties were manifest under growth-limiting conditions such as confluent cultures, low-serum-concentrations and in vivo growth in SCID mice. Since EBERs comprise only non-polyadenylated small RNAs, it is interesting that EBERs can exert the malignant transformation activity that we have described.

According to a previous report, EBER-negative recombinant forms of EBV could still immortalize B lymphocytes in culture [25]. However, this immortalization of B lymphocytes was in the context of type 3 latency, which is different from the type 1 latency seen in Burkitt's lymphoma. It is probable

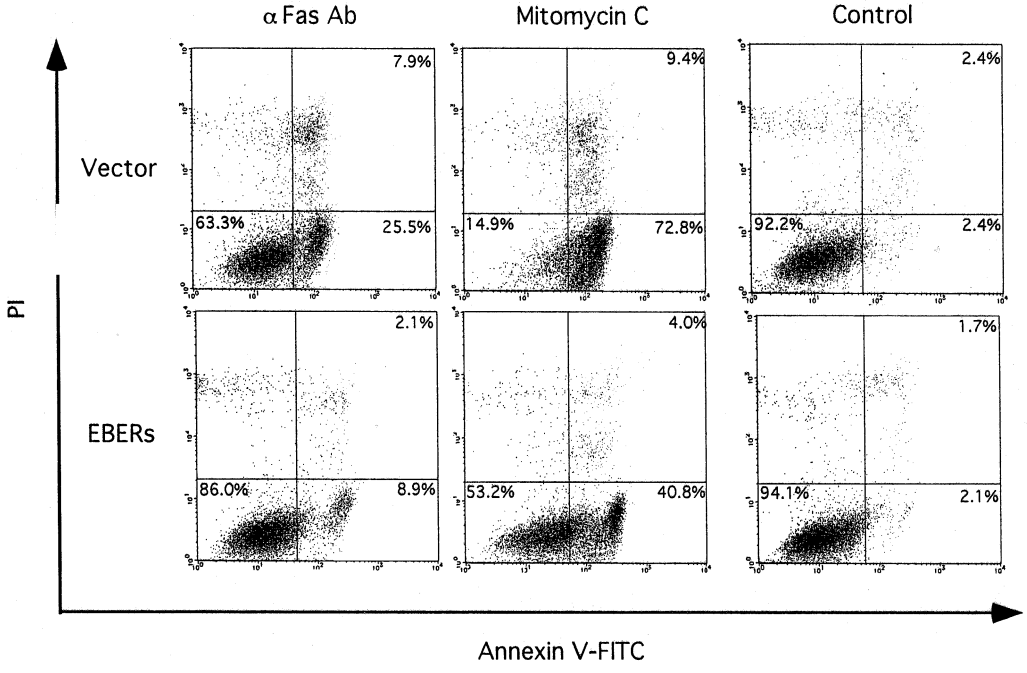

Fig. 4. Resistance to apoptosis induced by several stimuli. EBER-transfected and vector-transfected BJAB cells were exposed for 16 h to various inducers of apoptosis. They were then incubated with fluorescein isothiocyanate-conjugated annexin V and propidium iodide. Stained cells were measured with FACScalibur. Each number represents the percentage of viable cells (lower left), early apoptotic cells (lower right) and late apoptotic cells (upper right). The figures are the representative results obtained from three independent experiments with vector-transfected BJAB clone #1 and EBER-transfected BJAB clone #1. We observed essentially the same results in all the other clones.

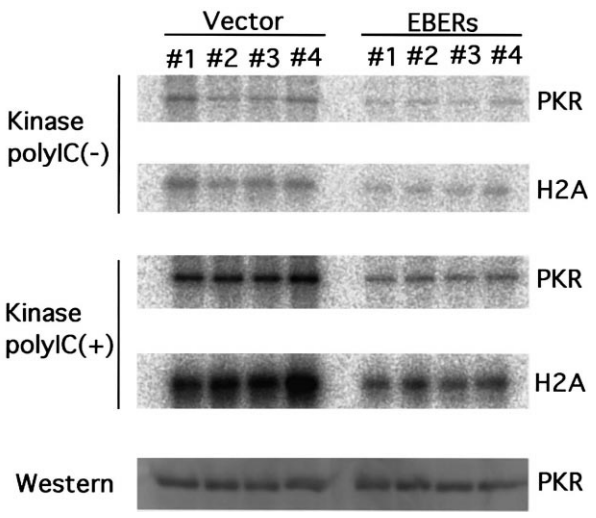

Fig. 5. In vitro kinase assay of PKR. Immunoprecipitated PKR proteins from vector-transfected (lane 1–4) and EBER-transfected (lane 5–8) BJAB cells were used for the kinase reaction in the presence or absence of polyIC. After immunoprecipitation, Western blot analysis was performed to confirm that equal amounts of PKR protein were present in these immunocomplex assays.

that EBERs play an important role in malignant transformation in the context of type 1 latency, but not in immortalization in the context of type 3 latency.

The proliferation rate of EBER-transfected BJAB cells in the medium containing 10% FCS was higher than that of vector-transfected cells at the logarithmic phase, and the saturation density was higher than that of vector controls at the stationary phase. Furthermore, EBER-transfected BJAB cells survived at a lower serum concentration in comparison with vector-transfected BJAB cells. These results strongly suggested that EBERs possess the ability to inhibit apoptosis induced by saturation and serum deprivation. EBERs may act as genes inhibiting apoptosis, whose expression is required for transformation.

In addition to saturation and serum deprivation, EBERs exhibited resistance to apoptosis induced by stress such as Fas and mitomycin C. These findings indicate that EBERs can inhibit a variety of apoptotic signals and that EBERs may target the common pathway associated with various types of apoptosis.

In general, it has been said that the transformation of cells requires expression of at least two kinds of genes: genes like c-myc promoting cell growth, and genes like bcl-2 inhibiting apoptosis. EBER-transfected BJAB cells proliferated faster than vector-transfected BJAB cells at a serum concentration of 10%, and EBER-positive cells were able to grow in soft agar and in SCID mice more efficiently than the vector controls. These data show that EBERs have not only anti-apoptotic effects, but also growth-promoting effects. According to previous reports, EBV-converted BJAB cells were able to grow in soft agar and these convertants exhibited an increased expression of c-myc [26,27]. Another report suggested that EBV enhances tumorigenicity and resistance to apoptosis through the regulation of c-myc and bcl-2 in the context of the type 1 latency program [6]. The c-myc oncogene has been shown to contribute to tumorigenesis and induction of apoptosis [28–30]. Therefore, it is possible that the anti-apoptotic activity of EBERs may play a role in protecting cells from c-myc-induced apoptosis, thereby allowing c-myc to exert its oncogenic activity.

EBERs are highly expressed transcripts, which have a stem-loop structure that is a kind of double-stranded RNA structure. So far, at least three functions have been ascribed to EBERs as follows. Firstly, EBERs bind to the PKR and interfere with its kinase activity [8,9]; Secondly, EBERs bind to the lupus antigen La protein [31]; Thirdly, EBERs bind to the ribosomal protein L22 and sequester L22 in the nucleus [32,33].

PKR has been extensively investigated as a regulator of translation, signal transduction, cell cycle and apoptosis [13,24,34]. Activation of PKR results in the phosphorylation of eIF-2α and the inhibition of protein synthesis. PKR can phosphorylate IκBα and induce the activation of nuclear factor-κB (NF-κB) [24,35,36], which leads to the expression of Fas [37,38]. PKR may cause apoptotic cell death through up-regulation of Fas [39]. In addition, a connection between PKR and p53 has been reported [40–42]. The ectopic expression of catalytically inactive mutants of PKR in NIH3T3 cells led to the formation of tumors in nude mice [18] and the resistance to apoptosis induced by various stresses such as serum-deprivation, TNF-α and Fas-induced signals [22,43]. PKR was shown to be a mediator of stress-induced apoptosis by in vitro studies using PKR knockout mouse embryo fibroblasts as well [21,23]. Moreover, the ectopic expression of two cellular inhibitors of PKR, p58 and HIV-1 TAR RNA binding protein TRBP, also resulted in the malignant transformation of cells [44,45]. Considering the important roles of PKR in cell growth and apoptosis, EBERs may induce malignant transformation of BJAB cells through the inhibition of PKR function. Our observation that the phosphorylation level of H2A by PKR is lower in EBER-transfectants as compared to vector control supports this notion.

It has been reported that in the case of murine NIH 3T3 cell lines, the stable expression of EBER-1 results in a morphologically transformed phenotype and confers the ability to form colonies in soft agar [46]. Very recently, Komano et al. reported that EBV-negative clones of the Akata cell line (which was derived from a patient with EBV-positive Japanese Burkitt's lymphoma) displayed a more malignant nature when transfected with EBERs relative to the vector control [47]. The authors suggested that EBERs may exhibit oncogenic effects through the up-regulation of bcl-2.

We have shown that EBERs have oncogenic potential and an inhibitory effect on PKR, and attempts are underway to determine the nature of these RNAs and to clarify the mechanism of EBER-induced transformation.

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