Malignant Transformation of Epstein-Barr Virus-Negative Akata Cells by Introduction of the BARF1 Gene Carried by Epstein-Barr Virus

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Epstein-Barr virus (EBV), a ubiquitous human herpesvirus that causes infectious mononucleosis, is likely to be involved in the pathogenesis of endemic Burkitt’s lymphoma (BL) and lymphoma induced in AIDS patients. Almost 100% of NPCs (nasopharyngeal carcinomas) (1) (24) and about 10% of gastric carcinomas are associated with EBV (2, 17). The EBV genome is present in more than 90% of endemic BL cases in equatorial Africa and in about 10% of sporadic BL cases (11). In vitro, EBV is able to infect resting B lymphocytes and create the immortalized lymphoblastoid cell line (LCL).

EBV-positive Akata BL cells spontaneously lost the EBV genome during long-term culture, becoming nontumorigenic, while parental EBV-positive Akata cells induced tumors in SCID mice, suggesting an important oncogenic role of EBV in these cells (8). In fresh BL biopsy tissues and early-passage BL-derived cell lines, only EBNA1, EBERs, and BARF0 are expressed and these are classified as type I latency genes. In the EBV-positive Akata BL cell line, three of the genes mentioned above plus LMP2A were expressed (7, 12). Transfection of the EBNA1 gene into EBV-negative Akata cells did not restore tumorigenicity to Akata cells. Recent data showed that transfection of EBERs, the second viral gene expressed in EBV-negative Akata cells, induced tumor formation in SCID mice (7, 11). In these cells, we observed expression of the BARF1 gene (22) but this activation was not confirmed by another laboratory (12). In our previous study, we reported that about 1 to 2% of EBV-positive Akata cells expressed a lytic antigen (22). In these cells, we observed expression of the BARF1 gene localized in the BamHI A fragment of the viral genome (23).

The BARF1 gene, encoding a 31-kDa early protein, has been shown to be able to induce malignant transformation in BALB/c3T3 cells and in the human B-cell line Loukess (15, 19, 20). Injection of BARF1-expressing BALB/c3T3 cells into newborn rats resulted in the induction of aggressive tumors, while injection of BARF1-expressing Loukess human EBV-negative B cells into the same mouse induced the formation of a small tumor that regressed after 3 weeks. In addition, this viral gene was capable of immortalizing primary epithelial cells (20) and was expressed in NPC biopsy tissues (4). Thus, the BARF1 gene has oncogenic activity (9). Expression of the BARF1 gene in the EBV-negative Loukess cell line induces expression of the proto-oncogene c-myc and B-cell activation antigens CD23 and CD21 (10). However, the known activities of BARF1 are not limited to its oncogenic ability; BARF1 has also been shown to serve as a target for antibody-dependent cytotoxicity (18). Recent reports have shown that this viral protein could play a role as a soluble receptor for human colony-stimulating factor 1 (16) and could regulate the immune response by inhibiting alpha interferon secretion from mononuclear cells (3).

We wondered if BARF1 is capable of inducing a tumoral transformation when it is expressed in EBV-negative Akata cells.

Activation of BARF1 gene expression in tumors. Since 1 to 2% of EBV-positive Akata cells expressed early proteins (22), we asked whether BARF1 is expressed in this cell line. To detect BARF1 expression, we carried out reverse transcription RT-PCR and an immunoblot assay. RT-PCR analysis was carried out with RNA extracted from EBV-positive Akata, M81, B95-8, and P3HR-1 cells. The RT-negative control came from
a PCR without reverse transcriptase (Fig. 1 right part). The presence of BARF1 transcription was clear not only in EBV-positive Akata cells but also in other EBV-positive B-cells (Fig. 1, left part). We were then interested in determining whether the BARF1 gene is expressed in tumors induced by injection of EBV-positive Akata cells in SCID mice. After 3 months of Akata cell injection, we harvested tumor biopsy tissues and examined the expression of early proteins like BARF1, ZEBRA, EA-D, and BALF2 by both the Western blot and immunofluorescence assay methods. Figure 2 shows representative immunoblots of protein extracted from tumors induced by EBV-positive Akata cells. Translational expression of BARF1 was very weak in EBV-positive Akata cells (Fig. 2A, lane 2) and absent in EBV-negative Akata cells (Fig. 2A, lane 1), as well as in another EBV-negative B-cell line, Louckes (Fig. 2A, lane 5), while its expression became more important in two EBV-positive Akata cell-derived tumors (Fig. 2A, lanes 3 and 4). Expression of EA-D, ZEBRA, and BALF2 was weakly positive in cell cultures but became significant in tumors, while expression of the EBV gp220-320 late protein was never detected in tumors or in Akata cell cultures (Fig. 2B). Expression of the early proteins was also analyzed by using an immunofluorescence assay (6) with anti-BARF1, anti-ZEBRA, anti-EA-D, and anti-BALF2 antibodies (Fig. 3). Surprisingly, a large proportion of cells from tumors effectively expressed the above-mentioned early proteins. Before injection, about 1% of EBV-positive Akata cells expressed lytic proteins (BARF1, EA-D, BALF2, and ZEBRA) while a large proportion of tumor-derived cells became positive for early antigens (more than 50% for BARF1, ZEBRA, and EA-D and 30% for BALF2). EBV-positive Akata cells do not expressed LMP1 as a type I latency protein. We also observed no reactivation of LMP1 in tumors derived from EBV-positive Akata cells. Neither RT-PCR nor immunoblot analysis showed any positive response in cell cultures or in tumors (Fig. 2B).

Expression of BARF1 and Bcl2 in BARF1-transfected, EBV-negative Akata cells. We previously showed that introduction of the BARF1 gene into the human B-cell line Louckes led to the induction of malignant transformation in newborn rats (21). We addressed the question of whether introduction of the BARF1 gene into EBV-negative Akata cells can induce malignant transformation. Four neomycin-resistant clones (pZC55A, pZC55B, pZC55C, and pZC55D) were isolated af-
ter transfection of a recombinant BARF1 plasmid into EBV-negative Akata cells by an electric field-mediated DNA transfer method. The expression of p31 BARF1 was examined by both the RT-PCR and Western blot methods. For RT-PCR analysis, we treated the extracted RNA with DNase I (Gibco) in order to eliminate any contaminant DNA present in the RNA sample. The cDNA was synthesized from RNA and amplified by Taq polymerase with primers corresponding to the entire BARF1 sequence (Fig. 1). The specificity of the amplified fragment was verified by hybridization with the radiolabeled BARF1 sequence used as probe. In this experiment, P3HR-1 cells expressing a lytic phase were used as a positive control. RT-PCR was carried out as described in the legend to Fig. 1. The size of the amplified fragment was 668 bp for the BARF1 gene. EBV-negative Akata cells transfected with the pZip vector were used as a negative control. (B) Expression of BARF1 protein in EBV-positive Akata cells and in BARF1 transfectants was analyzed by Western blotting with a Pep-1 antibody, a polyclonal rabbit antiserum prepared against a synthetic peptide corresponding to a presumed epitope, amino acids 203 to 209 (GKNDKEE), of the BARF1 protein (4). Two xenografts of NPC C17 and C18 were used as positive controls for BARF1 detection (lanes 1 and 8). A 31-kDa protein corresponding to the BARF1 protein was found in C18 and C17 at a high intensity, but BARF1 transfectants showed a weak response. (C) Activation of Bcl2 expression by the BARF1 gene. Bcl2 expression was analyzed in vector DNA-transfected Akata cells (lane 1), EBV-negative Akata cells (lane 2), EBV-positive Akata cells (lane 3), and BARF1 transfectants pZC55A, pZC55B, pZC55C, and pZC55D (lanes 4 to 7). Jurkat cells (lane 8) were used as a positive control, giving a band of 26 kDa corresponding to the Bcl2 protein. β-Actin was used as the loading control.

C18, that express a high level of BARF1 protein as positive controls. Three transfectants (pZC55A, pZC55C, and pZC55D) translated the p31 BARF1 protein, while pZC55B showed a much lower level of expression (Fig. 3B).
Akata cells transfected with vector DNA (pZip-Akata) gave a negative response. The expression of BARF1 was almost undetectable in EBV-positive Akata cells in this experiment. Immunofluorescence analysis showed that 1 to 2% of EBV-positive Akata cells spontaneously entered a lytic phase (22). In fact, when BARF1 expression was analyzed with anti-BARF1 antibody in EBV-positive Akata cells, only 0.5 to 1% of the cells became positive (Fig. 3). The small proportion of EBV-positive Akata cells expressing the lytic phase makes BARF1 expression easy to detect by the RT-PCR method but difficult to detect by the immunoblot method.

Our previous results showed that BARF1 is able to activate Bcl2 expression in rodent cells and its activation is likely associated with the oncogenesis of BARF1 (15). We therefore examined whether Bcl2 expression is upregulated by BARF1 in B cells. In this analysis, Jurkat cells were used as a positive control. As illustrated in Fig. 4C, a slight increase in Bcl2 expression was observed in three of four BARF1-transfected clones (pZC55A, pZc55C, and pZC55D) in which BARF1 expression is higher than in pZ vector-transfected cells, as well as in Akata cells. When the expression of BARF1 was low, as in the case of the pZC55B clone, the level of Bcl2 expression was also low. The Bcl2 expression in EBV-positive Akata cells was detected at a level comparable to that observed in EBV-negative Akata cells and vector DNA-transfected, EBV-negative Akata cells. Transfection of individual EBV latent genes into EBV-negative BL cells has shown that LMP1, EBNA2, and EBNA3B can up-regulate Bcl2 expression. All three of these EBV genes are not expressed, however, in EBV-positive Akata cells.

Growth in soft agar. We analyze first the growth properties of four BARF1 transfectants by examining their ability to form colonies in soft agar (SeaPlaque; FMC Bioproducts). Colony formation efficiency was determined by three independent experiments for each BARF1 transfectant. Cells (0.2 × 10^6) were cultured in complete medium containing 0.33% agarose as already described (15). High cloning efficiency was observed in three BARF1-expressing clones. The averages of these experiments were 52% for pZC55A, 71% for pZC55C, and 64% for pZC55D (Fig. 5, right side). The average obtained for pZC55B was 14%. No colony formation was observed in cells transfected by vector DNA under the same condition (Fig. 5, left side).

Tumorigenicity of BARF1-expressing Akata cells in SCID mice. Our previous observation showed that BARF1 expression in EBV-negative BL cells (Louckes) is able to induce tumors in newborn rats (21). To determine whether BARF1 expression contributes tumorigenicity to EBV-negative Akata cells, all four BARF1 transfectants (20 × 10^7 cells/mouse) were injected into SCID mice (eight SCID mice for each transfectant). In parallel, three control cell lines (EBV-positive Akata cells, EBV-negative Akata cells, and vector DNA-transfected, EBV-negative Akata cells) were also injected into six SCID mice. Two BARF1 transfectants (pZC55C and pZC55D) and EBV-positive Akata cells induced tumor formation 12 weeks after infection, while pZC55A produced tumors at 14 weeks. Tumors resulting from pZC55B developed 16 weeks after injection. Moreover, only one of eight of mice developed a tumor. This weak tumorigenicity probably comes from the low level of p31 BARF1 expression in the pZC55B clone (Fig. 4B). In contrast, EBV-negative Akata cells and vector DNA-transfected Akata cells were unable to induce any tumors in SCID mice (Table 1).

BARF1 expression confers apoptosis resistance on B cells. Bcl2 is the prototype of a family of related proteins that can be considered apoptotic death antagonists. In subsequent experiments, these various transfectants were subjected to examination for sensitivity to death by apoptosis following culture for 4 days in medium containing either 10 or 0.1% fetal calf serum (FCS). As illustrated in Fig. 6, all cell samples maintained good viability (about 90%) in standard medium containing 10% FCS after 4 days (Fig. 6, histogram, left side). When the growth
The kinetics of all cell clones were measured for 4 days in 0.1% FCS, EBV-positive Akata cells, EBV-negative Akata cells, and vector DNA-transfected cells lost viability rapidly after 3 days of culture. In contrast, three clones with high-level BARF1 expression (pZC55A, pZC55C, and pZC55D) showed significant survival (60 to 65% viability after 4 days) while pZC55B showed a decrease in viability similar to that of EBV-positive Akata cells (Fig. 6, right side). These experiments suggest that BARF1 contributes apoptosis resistance by up-regulation of Bcl2 expression. An apoptosis assay based on the visualization of DNA fragmentation was carried out. Cells were cultured for 72 h in medium containing 0.1% FCS. For analysis of apoptotic cell death, 10^6 cells were washed twice with phosphate-buffered saline (PBS) and harvested by centrifugation. The pellet was lysed in a solution containing 1% (wt/vol) sodium dodecyl sulfate, 0.5 mg of proteinase K (Boehringer, Mannheim, Germany) per ml, and 10 mM Tris-HCl (pH 8.0); incubated for 12 h at 37°C; and then incubated with RNase for a further 1 h. Protein was subsequently precipitated by addition of 5 volumes of a saturated NaCl solution. The DNA was precipitated with ethanol at −20°C. A 1.0-μg DNA sample was loaded onto a 1.5% agarose gel containing ethidium bromide for examination of DNA fragmentation as previously described (5).

Figure 7 illustrates the electrophoretic analysis of DNA extracted from cells cultured for 3 days in 0.1% serum. At this serum concentration, all cultures showed weak degradation of DNA compared with that containing 10% serum, in which no degradation of DNA was observed (Akata EBV−/10%, last lane). The presence of fragmented low-molecular-weight DNA was observed particularly in EBV-positive and -negative Akata (lane 5 and 6) and pZC55B (lane 2) cells, showing that the decline in the viability of these cells in 0.1% FCS was likely due to apoptotic death.

In EBV-positive Akata cells, four EBV genes (EBNA1, EBERs, BARF0, and LMP2A) are expressed (7, 13). Here we demonstrate that additional BARF1 gene expression was observed in EBV-positive Akata cells by RT-PCR analysis. As the BARF1 gene belongs to the early gene group, the detection of BARF1 expression in B cells probably comes from the 1 to 2% of Akata cells expressing lytic genes (22). The detection of cell death, 10^6 cells were washed twice with phosphate-buffered saline (PBS) and harvested by centrifugation. The pellet was lysed in a solution containing 1% (wt/vol) sodium dodecyl sulfate, 0.5 mg of proteinase K (Boehringer, Mannheim, Germany) per ml, and 10 mM Tris-HCl (pH 8.0); incubated for 12 h at 37°C; and then incubated with RNase for a further 1 h. Protein was subsequently precipitated by addition of 5 volumes of a saturated NaCl solution. The DNA was precipitated with ethanol at −20°C. A 1.0-μg DNA sample was loaded onto a 1.5% agarose gel containing ethidium bromide for examination of DNA fragmentation as previously described (5).

Table 1. Tumorigenicity of EBV-negative Akata cells transfected with BARF1 in SCID mice

<table>
<thead>
<tr>
<th>Cells</th>
<th>Tumorigenicity</th>
<th>Time (wk) to tumor appearance</th>
</tr>
</thead>
<tbody>
<tr>
<td>EBV− Akata</td>
<td>0/6</td>
<td></td>
</tr>
<tr>
<td>pZip EBV− Akata</td>
<td>0/6</td>
<td></td>
</tr>
<tr>
<td>EBV+ Akata</td>
<td>4/6</td>
<td>12</td>
</tr>
<tr>
<td>pZC55A</td>
<td>4/8</td>
<td>14</td>
</tr>
<tr>
<td>pZC55B</td>
<td>1/8</td>
<td>16</td>
</tr>
<tr>
<td>pZC55C</td>
<td>5/8</td>
<td>12</td>
</tr>
<tr>
<td>pZC55D</td>
<td>6/8</td>
<td>12</td>
</tr>
</tbody>
</table>

a Four-week-old SCID mice were inoculated subcutaneously with 20 × 10^6 cells of each clone. Six (for EBV-positive and -negative Akata cells and EBV-negative Akata cells transfected with the pZip vector) or eight (for BARF1 transfectants) mice were used for tumorigenicity assays.

b Number of mice that developed tumors/total number of mice.
A 1.0-μg DNA sample was loaded onto a 1.5% agarose gel solution. The DNA was precipitated with 3 volumes of ethanol at 20°C and then incubated with RNase for a further 1 h. Protein was harvested by centrifugation. The pellet was lysed in a solution containing 1% (wt/vol) sodium dodecyl sulfate, 0.5 mg of proteinase K.

BARF1 protein in EBV-positive Akata cells was, however, difficult to visualize on an immunoblot. Interestingly, a large population of EBV-positive Akata cells isolated from tumor biopsy tissues expresses the BARF1 gene. This was confirmed by immunoblotting with an intensi

![Diagram](image-url)

**FIG. 7.** Gel electrophoretic analysis of DNAs extracted from cells. EBV-negative (lane 6) and -positive (lane 5) Akata cells and BARF1 transfectants (pZC55A, pZC55B, pZC55C, and pZC55D) were cultured for 3 days in either 0.1% FCS (lanes 1 to 6) or 10% FCS (lane 7). For analysis of apoptotic cell death, 10⁶ cells were washed twice with PBS and harvested by centrifugation. The pellet was lysed in a solution containing 1% (wt/vol) sodium dodecyl sulfate, 0.5 mg of proteinase K, and 10 mM Tris-HCl (pH 8.0); incubated for 12 h at 37°C; and then incubated with RNase for a further 1 h. Protein was subsequently precipitated by addition of 5 volumes of a saturated NaCl solution. The DNA was precipitated with 3 volumes of ethanol at −20°C. A 1.0-μg DNA sample was loaded onto a 1.5% agarose gel containing ethidium bromide, electrophoresed, and photographed under UV light.

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REFERENCES


