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Role of bcl-2 in Epstein-Barr Virus-Induced Malignant Conversion of Burkitt's Lymphoma Cell Line Akata

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We have demonstrated that Epstein-Barr virus (EBV) confers enhanced growth capability in soft agarose, tumorigenesis in the SCID mouse, and resistance to apoptosis in the Burkitt's lymphoma cell line Akata. Subsequently, we have shown that EBV-encoded small RNAs (EBERs) are responsible for these phenotypes. We constantly observed the upregulation of bcl-2 oncoprotein expression upon EBV infection and expression of EBERs. To test whether these phenotypes were due to the upregulation of bcl-2 expression, we introduced bcl-2 into EBV-negative Akata cells at various levels encompassing the range at which EBV-positive cells expressed it. As cells expressed bcl-2 at higher levels, they became more capable of growing in soft agarose and became resistant to apoptosis. However, clones expressing bcl-2 at a higher level than EBV-positive Akata cells were negative in the tumorigenesis assay in the SCID mouse. On the other hand, introduction of bax into EBV-positive Akata cells reduced the resistance to apoptosis; however, it failed to reduce the growth capability in soft agarose. These data indicate that EBV targets not only bcl-2, but also an unknown pathway(s) to enhance the oncogenic potential of Akata cells.

Previously we established a system to test whether any cellular phenotypes of latency I Burkitt's lymphoma (BL) cells were due to Epstein-Barr virus (EBV), by using a cell line of BL origin, Akata, which has several unique characteristics among BL cell lines (24–27). We have demonstrated that EBV contributes to growth capability in soft agarose, tumorigenesis in immunodeficient mice, and resistance to apoptosis in Akata cells (12, 24). We also reported that EBV-determined nuclear antigen 1 (EBNA1) was not responsible for these phenotypes (12). Similar results were reported by two independent groups (4, 23). We further clarified that EBV-encoded RNAs (EBER-1 and -2) are responsible for these phenotypes (11).

The question that remained to be answered was the mechanism by which EBV contributes to these phenotypes. We constantly observed the upregulation of bcl-2 oncoprotein expression upon EBV infection or expression of EBERs in EBV-negative Akata cell clones (11, 12). A similar finding was also described by Ruf et al. (23). Distinct from other oncogenes, bcl-2 fosters cell survival rather than promoting cell proliferation. Since it is well known for its antiapoptotic function (20), it was assumed that the resistance to apoptosis was due to upregulation of bcl-2 protein. BL cells are predisposed to c-*myc*-induced apoptosis, since BL cells possess immunoglobulin (Ig)/c-*myc* translocation, which results in constitutive activation of the c-*myc* gene (9). Therefore, we hypothesized that upregulation of bcl-2 expression by EBV infection would protect cells from c-myc-induced apoptosis and allow c-myc to exert its oncogenic functions. To test this idea, we employed two approaches: (i) introduction of *bcl-2* into EBV-negative Akata cells to test whether any phenotypes were restored and (ii) introduction of *bax* into EBV-positive Akata cells to antagonize the function of bcl-2 to determine whether any phenotypes were reduced.

Effect of bcl-2 expression on oncogenic potential and resistance to apoptosis in Akata cells. First, we introduced bcl-2 expression vector pBcl-2 into EBV-negative Akata cells. This pcDNA3-based vector carried human *bcl-2*α under control of a human cytomegalovirus promoter. We successfully isolated clones that expressed low to very high levels of bcl-2 protein (Fig. 1A). The expression of bcl-2 protein was detected by Western blot analysis with antihuman bcl-2 monoclonal antibody bcl-2/100 (Pharmingen). Neomycin resistance gene (*neo*)-transfected cell clones and EBV-reinfected cell clones were also isolated for use as negative and positive controls, respectively. The average relative signal intensity representing the amount of bcl-2 protein expressed was quantified by densitometric analysis and dot plotted in Fig. 1A. In this experiment, the level of bcl-2 expression detected by Western blot analysis appeared to be within the semiquantitative range. The average level of relative bcl-2 expression of EBV-reinfected cell clones was between those of clones with low and medium levels of bcl-2 expression. The growth rates among these cell clones were almost the same under serum-rich and low-serum conditions, except for clones with high and extra-high levels of bcl-2 expression under low-serum conditions. Using these cell clones, we carried out a soft agarose cloning assay, apoptosis assay, and tumorigenesis assay in SCID mice.

For the soft agar colony assay, 10^4 cells were embedded in 0.4% SeaPlaque agarose containing RPMI 1640 and 12% fetal bovine serum as described previously (11). After 2 to 3 weeks of incubation, colonies that contained more than 100 live cells were counted. The mean values of the number of colonies that

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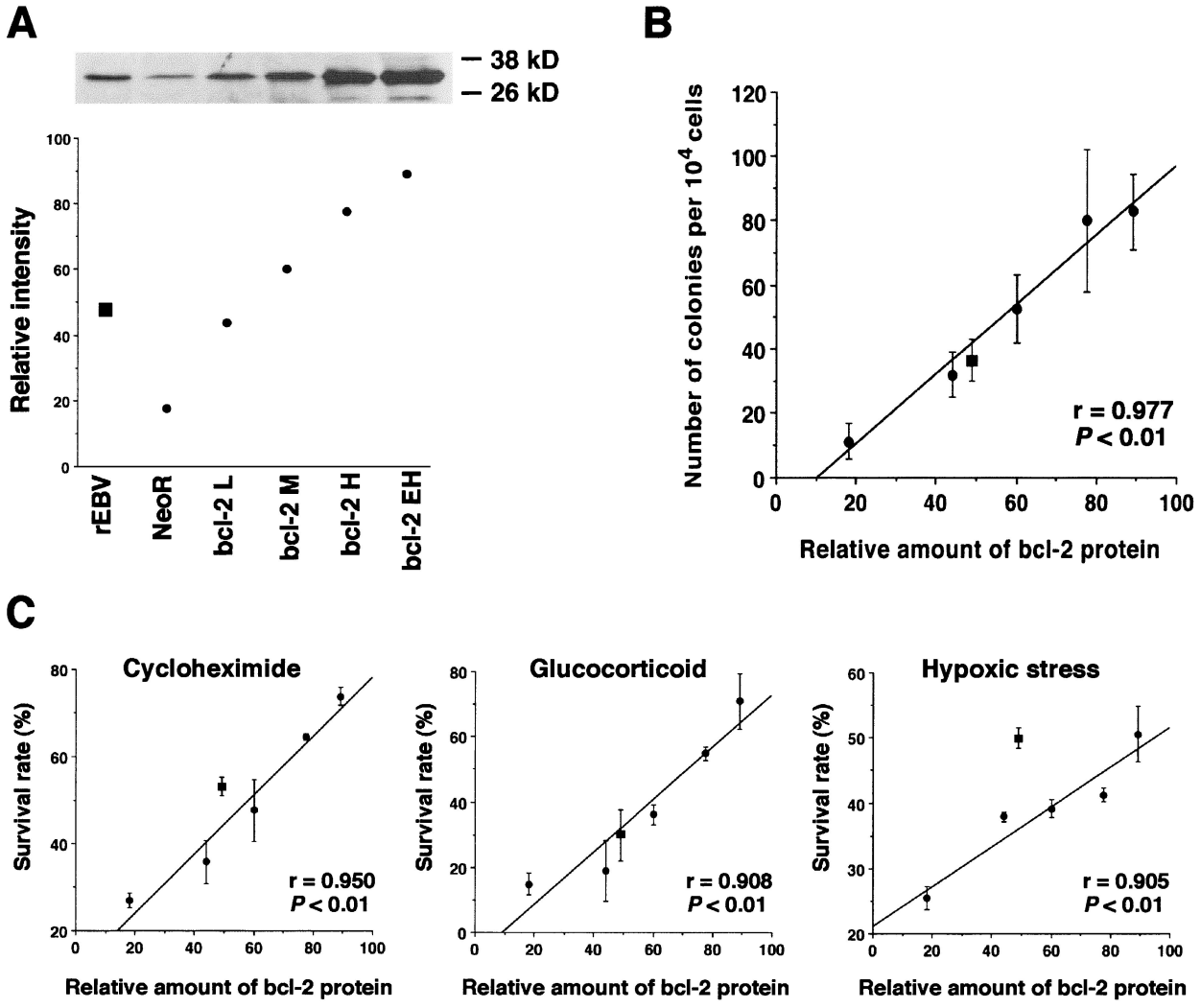

FIG. 1. Effects of bcl-2 expression on clonability in soft agar and resistance to apoptosis in Akata cells. (A) Western blot analysis detecting bcl-2 protein of *bcl-2*-transfected cells, which expressed low (L), medium (M), high (H), and extra high (EH) levels. The data presented here are representative of four clones tested. The average relative signal intensity representing the amount of bcl-2 protein expressed in these cells was quantified by densitometric analysis and dot plotted. (B) Clonability in soft agarose. The mean values of the number of colonies that emerged in soft agar were plotted against the relative amounts of bcl-2 protein. Each dot represents the average number of colonies that emerged per 10^4 cells. (C) Resistance to apoptosis. The mean values of the survival rates against apoptotic stimuli were plotted against the relative amounts of bcl-2 protein. ●, *bcl-2*-transfected cells; ■, EBV-reinfected cells. Horizontal bars represent the mean values of each group. The bars show the mean values ± standard deviation of four clones.

emerged in soft agarose were plotted against the relative amounts of bcl-2 protein. As a result, the number of colonies in soft agar was found to be in direct proportion to the relative amount of bcl-2 protein (Fig. 1B).

For the apoptosis assay, cells in the log phase were exposed to cycloheximide (20 μg/ml; Wako, Osaka, Japan), glucocorticoid (1 μM; Pharmacia and Upjohn), and a 100% CO_2-saturated humidified atmosphere (hypoxic stress) as described previously (11). Viability of cells was quantified by a colorimetric assay (Cell Titer 96; Promega). The percent survival rate (%SR) was calculated by the formula %SR = {[(A_{570} of the sample) − (A_{570} of the blank)]/[(A_{570} of the control) − (A_{570} of the blank)]} × 100. The mean values of %SRs against all apoptotic stimuli for each clone were plotted against the relative amounts of bcl-2 protein (Fig. 1C). As a result, %SRs were also found to be in direct proportion to the relative amount of bcl-2 protein. It was noted that cells became resistant to hypoxic stress with a minimal increase of bcl-2 expression. This is consistent with the previous finding that upon hypoxic stress, the greatest difference of susceptibility to apoptotic cell death was seen between EBV-positive and -negative clones (11).

The tumorigenic potential of clones expressing higher levels of bcl-2 than EBV-infected Akata cells was tested. A total of 1.5×10^7 cells were inoculated into the thigh subcutis of 4-week-old male SCID mice as described previously (11). Those clones failed to develop tumor masses in the SCID mice (Table 1). Interestingly, the malignant phenotype of bcl-2-expressing Akata cell clones scored differently in the soft agarose colony assay and tumorigenesis assay in the SCID mouse. Historically, these results have been thought to reflect the

TABLE 1. Tumorigenicity of *bcl-2*-transfected Akata cell clones in SCID mice[a]

Cell clone	No. of mice with tumors/ no. tested
EBV	
Positive	3/3
EBV Negative	0/3
EBV Reinfected	8/9 (2/3, 3/3, 3/3)
EBER transfected	7/15 (1/3, 2/3, 2/3, 1/3, 1/3)
neo^r transfected	0/15 (0/3, 0/3, 0/3, 0/3, 0/3)
bcl-2 transfected	0/15 (0/3, 0/3, 0/3, 0/3, 0/3)

[a] Five cell clones transfected with *neo*^{r}, EBER, or *bcl-2* plasmid and three EBV-reinfected clones derived from an EBV-negative Akata cell clone (1.5×10^7 cells each) were individually inoculated into 4-week-old male SCID mice (Fox Chase C.B-17/Icr-scid Jcl; Clea, Tokyo, Japan). Mice were sacrificed 8 weeks after inoculation, and the developed tumors were measured. The tumors ranged from 0.8 to 4.5 cm in diameter.

"tumor cell" phenotype; however, our data suggested that this was not the case.

Effect of bax expression on oncogenic potential and resistance to apoptosis in Akata cells. Second, we attempted to antagonize the function of bcl-2 by using bax, a homologue of bcl-2. bax binds to bcl-2 and inhibits its antiapoptotic function (21). We speculated that if the malignant phenotype and resistance to apoptosis depend on bcl-2 protein, expression of bax in EBV-positive Akata cells should lead to a loss of these phenotypes. We transfected bax expression plasmid pBax into both EBV-negative [EBV(−)] and -positive [EBV(+)] Akata cells. The expression vector for bax (pBax) was constructed by inserting the *bax*-α cDNA downstream of the SRα promoter, which drives transcription of a bicistronic mRNA for *bax* and *neo*^{r} mediated by an encephalomyocarditis virus internal ribosomal entry site sequence. We isolated G418-resistant cells that were designated EBV(−)/*neo*^{r}, EBV(−)/*bax*, EBV(+)/*neo*^{r}, and EBV(+)/*bax*. Expression of bcl-2 and bax protein in these cells was tested by Western blot analysis with antihuman bcl-2 monoclonal antibody bcl-2/100 and a rabbit anti-human bax polyclonal antibody (Pharmingen) (Fig. 2A). A small amount of bax protein was detected in EBV(−)/*neo*^{R} and EBV(+)/*neo*^{r} cells; in contrast, EBV(−)/*bax* and EBV(+)/*bax* cells expressed approximately 2.1- and 2.5-fold more bax protein than EBV(−)/*neo*^{r} and EBV(+)/*neo*^{r}, respectively. The levels of bcl-2 protein expression in these cells were almost the same, except for EBV(−)/*bax* cells. They expressed 1.9-fold more bcl-2 protein than the others. Since expression of bax might oversensitize EBV(−) cells to apoptosis, cells expressing bcl-2 protein at a higher level seemed to be selected in the cloning process for EBV(−)/*bax* cells. A slightly reduced growth rate was seen in bax-transfected cells.

Cells were subjected to a soft agarose colony assay and apoptosis assay (Fig. 2B and C). Both EBV(−)/*neo*^{r} and EBV(−)/*bax* cells hardly formed colonies in soft agarose. The number of colonies seen for EBV(+)/*neo*^{r} cells was significantly higher than that for EBV(−)/*neo*^{R} cells, which is consistent with previous findings (11, 12). The number of colonies of EBV(+)/*bax* cells was not significantly less than that of EBV(+)/*neo*^{R} cells. In the apoptosis assay, EBV(+)/*neo*^{R} cells were more resistant to apoptosis than EBV(−)/*neo*^{R} cells in response to all stimuli. A slight reduction of %SRs was seen in EBV(−)/*bax* cells compared with EBV(−)/*neo*^{R} cells. In contrast, a significant reduction of %SRs was found in EBV(+)/*bax* cells compared with EBV(+)/*neo*^{R} cells. There is a report that bax protein functions in both bcl-2-dependent and -independent fashions (10, 29). Therefore, it remains a possibility that the phenotype seen in EBV(+)/*bax* cells might be partly due to the bcl-2-independent function of bax protein.

Using the transfectants derived from an EBV-negative Akata cell clone expressing various levels of bcl-2 proteins encompassing the range of EBV-reinfected Akata cell clones, we demonstrated that: (i) bcl-2 expression conferred resistance to apoptosis, (ii) bcl-2 expression contributed to the growth capability in soft agarose, (iii) the effects of bcl-2 expression in these assays were dose dependent, and (iv) bcl-2 expression was insufficient to support tumorigenesis in the SCID mouse. In the bax study, we demonstrated that the bax expression reduced the resistance to apoptosis, whereas the effect on the

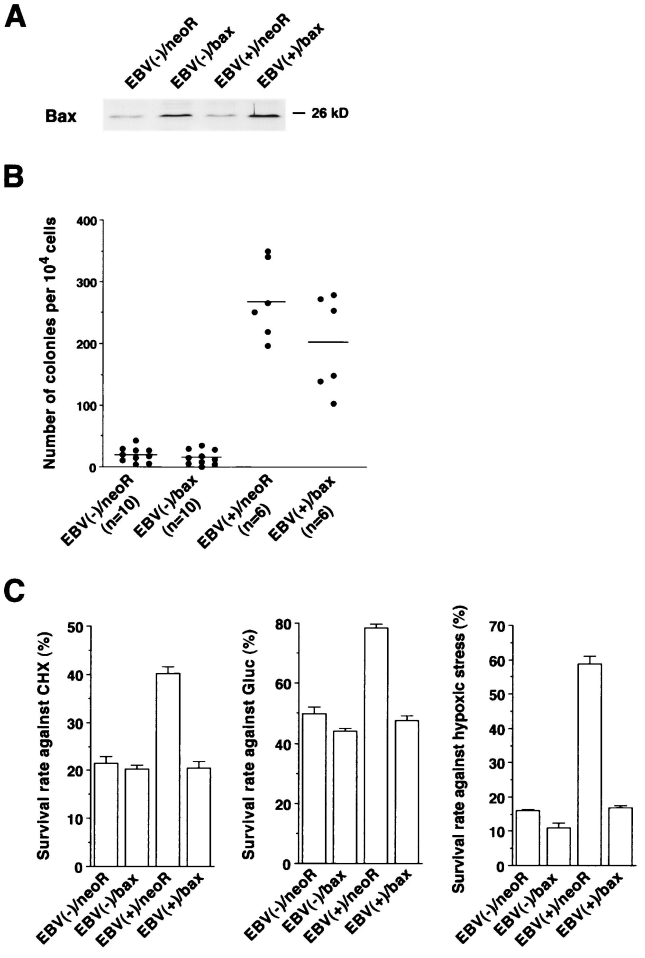

FIG. 2. Effects of bax protein expression on clonability in soft agar and resistance to apoptosis of Akata cells. (A) Expression of bcl-2 and bax proteins in transfected cells. (B) Clonability in soft agarose. Each dot represents the average number of colonies that emerged per 10^4 cells. Horizontal bars represent the mean values of each group. (C) Resistance to apoptosis against apoptotic stimuli. The bars show the mean values ± standard deviation of three independent experiments. By *t* test analysis, the differences between mean values from EBV(+)/*bax* and EBV(+)/*neo*^{R} cells were significant at $P < 0.001$ against all apoptotic inducers.

growth capability in soft agarose was modest. Those data strongly support the idea that EBV targets not only bcl-2, but also an unknown cellular factor(s) to confer the malignant phenotype and resistance to apoptosis seen in the EBV-positive Akata cells.

The tumorigenic potential of bcl-2 has been clearly demonstrated in rodent systems by transfection of the bcl-2 expression plasmid into NIH 3T3 cells in vitro (22), and in a *bcl-2* transgenic mouse study in which follicular lymphoproliferations progressed in the long term to high-grade malignant lymphoma (15, 16). Furthermore, it is widely accepted that *bcl-2* synergizes with the c-*myc* oncogene in tumor progression. This was suggested by clinical investigations indicating that activation of both c-*myc* and *bcl-2* may have conferred an aggressive clinical outcome in lymphoma cases (3, 8, 19). This idea was also demonstrated in a transgenic mouse study, in which *bcl-2*/c-*myc* double transgenic mice displayed accelerated lymphomagenesis (6, 14). In mammalian cells, deregulated expression of c-*myc* has been shown to contribute not only to tumorigenesis (13), but also to induce apoptosis in various cell lines, including BL cell lines (1, 5, 17). The mechanism of *bcl-2*/c-*myc* synergy seems to be that bcl-2 protects cells from c-myc-induced apoptosis (2, 28). Like Akata cells (26), all of the BL cells possess a chromosomal translocation involving the c-*myc* locus, which is believed to result in constitutive activation of the c-*myc* gene (9). Therefore, BL cells were thought to be predisposed to c-myc-induced apoptosis. Our data imply that EBV infection upregulates expression of bcl-2 protein to protect cells from c-myc-induced apoptosis and to allow c-myc to exert its oncogenic functions. However, other unknown pathways remain to be verified to explain the mechanism by which EBV contributes to the genesis of BL.

The role of bcl-2 in the development of BL has been largely unknown. Although attempts to detect bcl-2 protein expression in tumor biopsy samples failed (7, 18), several lines of evidence supported the hypothesis that BL cell lines with type I latency expressed bcl-2 protein at a low level (18, 23). Since (i) the level of bcl-2 expression in type I BL cell lines is relatively low compared to that in type III BL cell lines and EBV-immortalized lymphoblastoid cell lines (18) and (ii) there is no ideal tissue culture system available to demonstrate the role of bcl-2 in the type I BL cell lines, the significance of bcl-2 expression in the development of BL remains to be validated.

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