Cooperative interaction between Bcl-2 and Epstein–Barr virus latent membrane protein 1 in the growth transformation of human epithelial cells

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The Epstein–Barr virus latent membrane protein 1 (LMP-1) is essential for virus-induced B cell immortalization and can protect B lymphoma cell lines from apoptosis signals *in vitro* via induction of cellular Bcl-2 expression. However, we have reported that high-level expression of LMP-1 in epithelial cells (RHEK-1 cells) itself induces apoptosis. This apoptotic event occurs in the absence of detectable Bcl-2 expression in the LMP-1-transfected epithelial cells. In this study, we transfected the *bcl-2* gene into the LMP-1-containing cells and examined the effect of Bcl-2 upon LMP-1-mediated apoptosis, and upon the growth phenotype of the transfected cells. The results show that ectopic expression of Bcl-2 specifically blocks apop-

totic death induced by LMP-1. This was observed from cell culture viability and from gel electrophoresis and flow cytometric assays of the degree of DNA fragmentation in cultured cells. Furthermore, co-expression of LMP-1 and Bcl-2 in RHEK-1 cells enabled the cells to grow under low-serum conditions and to form colonies in semi-soft agar medium. These results suggest, therefore, that these two proteins play important complementary roles in the process of EBV-associated epithelial cell transformation. It appears significant, therefore, that LMP-1 and Bcl-2 are frequently co-expressed in the malignant cells of an EBV-positive epithelial tumour, nasopharyngeal carcinoma.

Introduction

Epstein–Barr virus (EBV), a human herpesvirus which infects B cells and epithelial cells *in vivo*, has cell growth-transforming potential and is associated with Burkitt's lymphoma (BL; Epstein *et al.*, 1964), nasopharyngeal carcinoma (NPC; Henle *et al.*, 1970; Old *et al.*, 1966) and immunoblastic B-cell lymphoma of the immunosuppressed (Young *et al.*, 1989). EBV infection of resting B cells *in vitro* leads to the outgrowth of immortalized lymphoblastoid cell lines in which six nuclear antigens (EBNAs), three latent membrane proteins (LMPs), and two small nonpolyadenylated RNAs (EBERs) are expressed (Kieff, 1996). From genetic analysis with virus recombinants, the viral genes encoding EBNA2, EBNA3A, EBNA3C and LMP-1 are essential for the immortalization process (Cohen *et al.*, 1991; Kaye *et al.*, 1993; Tomkinson *et al.*,

1993). In addition, the EBNA-1 gene is required for maintenance of the EBV genome. Of these, it is interesting that only EBNA-1 and LMP-1 are expressed in the epithelial tumour, NPC; EBNA-1 is always present whilst the expression of LMP-1 is detectable in 30–65% of NPC biopsies (Fahraeus *et al.*, 1988; Young *et al.*, 1988).

LMP-1 is a 62 kDa integral membrane protein (Mann *et al.*, 1985) consisting of six membrane-spanning domains flanked by a short N-terminal sequence and a large C-terminal domain, both situated on the cytosolic face of the membrane (Liebowitz *et al.*, 1986). The LMP-1 protein is detectable by immunofluorescence as punctate patches in the membrane and in fractionation experiments is associated with the detergent-insoluble fraction of the cytoskeleton (Liebowitz *et al.*, 1987). Several lines of evidence suggest a key role for LMP-1 as an effector of virus-induced cell transformation (Wang *et al.*, 1988). Thus transfection of the *LMP-1* gene into immortalized, but non-tumorigenic, rodent fibroblasts induces a fully transformed phenotype which enables the cell to grow as tumour *in*

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vivo (Baichwal & Sugden, 1988; Wang *et al.*, 1985). Using a similar approach, expression of LMP-1 in certain human epithelial cell lines results in morphological transformation of the cells, inhibition of normal cell differentiation and effects upon cell growth (Dawson *et al.*, 1990; Fahraeus *et al.*, 1990). Furthermore, it has been shown that mice expressing an LMP-1 transgene in skin epithelium have a phenotype suggestive of disturbed epithelial cell differentiation (Wilson *et al.*, 1990).

Very recently, we have re-examined the effect of LMP-1 in human epithelial cells by expressing the protein at higher levels than achieved in the earlier studies; in these circumstances, we found that LMP-1 could induce apoptosis (Lu *et al.*, 1996). Similar transfection experiments have also been carried out in B lymphoma cell lines and in that situation it was found that LMP-1 expression leads to the induction of Bcl-2 (Gregory *et al.*, 1991; Henderson *et al.*, 1991). Bcl-2 is a 26 kDa, putative membrane-associated protein (Chen-Levy & Cleary, 1990) that functions as a cell death suppressor; hence its induction by LMP-1 in B cells can protect those cells from programmed cell death. In epithelial cells, however, neither transient nor stable transfection with LMP-1 has any detectable effect in Bcl-2 status. (Rowe *et al.*, 1994; Lu *et al.*, 1996).

Although Bcl-2 expression is best studied in cells of the haematopoietic lineage, there is evidence that it can also alter epithelial survival. For instance, using a *bcl-2*-transfected human mammary gland epithelial cell line, MTSV-1-7, it was found that Bcl-2 expression conferred a survival advantage, especially after the cells have reached confluence (Lu *et al.*, 1995). As a result, the transfectants 'piled up' on a plastic culture dish and formed structures similar to those observed in normal mammary gland tissue. We were particularly interested in the observation that Bcl-2 is very often expressed along with LMP-1 in EBV-positive NPC tumour cells (Lu *et al.*, 1993a). This led us to examine the effect which ectopic Bcl-2 expression might have on the growth/survival phenotype of our LMP-1-transfected epithelial cells.

Methods

■ **Cells.** RHEK-1, a non-malignant immortalized human epithelial cell line, and the LMP-1-expressing RHEK-1 cell transfectant (LMP-135) have been described previously (Lu *et al.*, 1996). To generate Bcl-2-expressing cell lines, plasmid pCAJ-SV2-bcl-2-neo (Tsujimoto, 1989) was transfected into LMP135 cells by electroporation at 960 μ F and 0.15 kV. Mock-transfected control cell lines were generated by transfecting pCAJ-SV2-neo gene into LMP135 and RHEK-1 cells. Selection began at 48 h post-transfection by diluting a cell suspension 1:10 in Dulbecco's modified Eagle's medium (DMEM) containing 800 μ g/ml of G418. The surviving cells were collected and expanded for further analysis. The cell cultures were maintained in 5% CO₂ at 37 °C in DMEM supplemented with 10% foetal calf serum (FCS), 100 U/ml of penicillin G and 100 μ g/ml of streptomycin sulphate.

■ **Immunoblots.** Equivalent amounts of proteins were solubilized in sample buffer (1% Triton X-100, 5 mM EDTA in TBS pH 7.6 plus protease inhibitors) with 2-mercaptoethanol and separated by SDS-PAGE. The gel was blotted and the relevant protein was detected with

anti-LMP-1 monoclonal antibody CS.1-4 (Dako) or anti-Bcl-2 monoclonal antibody (clone 124, Dako), followed by washing three times with washing buffer (10 mM TBS, 0.2% Tween 20). The immune complexes were detected by addition of a 1:10 000 dilution of streptavidin-horse-radish peroxidase-conjugated goat anti-mouse IgG for 1 h followed by addition of ECL substrate (Amersham) according to the manufacturer's protocol. Anti-c-Myc MAb (9E10), anti-c-Jun MAb (KM-1), anti-Ras MAb (F235), anti-p53 MAb (PAb 1801), anti-Rb MAb (IF8), anti-c-Fos MAb (4-10G), anti-TGF- β 1 (V), anti-c-Fgr (N-47) and anti-ICAM-1 (C-19), (produced by Santa Cruz) were also used for testing expression of the corresponding antigens.

■ **Indirect immunofluorescence assay.** Cells were trypsinized, washed three times with PBS and air-dried before fixing in methanol (for LMP-1) or 1:1 methanol-acetone (for Bcl-2) at -20 °C for 10 min. The slides were dried at room temperature and stained either with LMP-1-specific MAb CS.1-4 or Bcl-2-specific MAb clone 124 (Dako) at a 1:50 dilution at 37 °C for 1 h, followed by washing three times. The slides were incubated with a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse Ig (Sigma) at 1:500 dilution for another 1 h at 37 °C. After washing four times with PBS, the slides were counterstained with 0.01% Evans' blue for 5 min, washed three times with double-distilled water and air-dried. The slides were examined by fluorescence microscopy.

■ **DNA content, fragmentation and cell-cycle analysis by flow cytometry.** Analysis of the DNA content by flow cytometry was performed as described previously (Mann *et al.*, 1987). Briefly, subconfluent proliferating cells and cells grown in serum-starved medium for 48 h were trypsinized, washed twice with PBS, fixed in 50% ethanol in PBS at -20 °C for 30 min and centrifuged at 250 g at 4 °C for 10 min. The cells were washed twice with PBS, digested with RNase A at a concentration of 1000 U/ml for 30 min and stained with 100 μ g/ml of propidium iodide. Cell-cycle profiles and distributions were determined by flow cytometry analysis using the Lysis II program on a FACScan flow cytometer (Becton-Dickinson).

■ **In vitro growth assays.** Replicate cultures from each of the samples in the exponential growth phase were seeded in fresh DMEM medium, supplemented with 0.1, 1 or 10% FCS at a density of 5×10^5 cells/ml, and counted daily. For the serum-starvation assay, cells were cultured in DMEM medium containing 10% FCS 24 h prior to deprivation of the serum. Subsequently, the numbers of viable and non-viable cells from each culture were determined by the Trypan blue exclusion method.

■ **DNA fragmentation analysis by agarose gel electrophoresis.** This was done as described by Lu *et al.* (1996). Briefly, 2×10^6 cells from each cell line were cultured for 24 h, and then the medium was replaced with serum-free DMEM for 48 h. Both floating and adherent cells were collected, washed twice in Ca²⁺/Mg²⁺-free PBS containing 1 mM EDTA, pelleted and lysed in 1 mM EDTA containing 0.6% SDS. NaCl was added to 1 M, the solution mixed and then incubated at 4 °C overnight. Low molecular mass DNA was ethanol-precipitated from the supernatant and analysed for fragmentation in 1.5% agarose gels in Tris-acetate-EDTA buffer, stained with ethidium bromide and observed under UV illumination.

■ **Anchorage-independent growth assay.** The transformation assay was performed as described previously by Baichwal & Sugden (1988). Cells were diluted to 10^5 cells/ml in DMEM. The cell suspension (100 μ l) was mixed with 2 ml gel of 0.33% agarose-DMEM and then layered over a 2 ml gel of 0.5% agarose in a 60 mm diameter dish. The agarose was set by placing the plates at 4 °C for 20 min and then cultured at 37 °C. Seven to twelve days after plating, 2 ml 0.33% agarose-DMEM was added to the cells. The number of cells that grew to form colonies was determined with a microscope.

Results

Co-expression of Bcl-2 and LMP-1 in RHEK-1 cells

To investigate the effects of Bcl-2 expression in this experimental system, we introduced the human Bcl-2 expression plasmid pCΔj-SV-2-bcl-2-neo (Tsujimoto, 1989) into

the parental RHEK-1 cell line and into the previously derived LMP-1-positive RHEK-1 cell clone LMP135. As an additional control, these same cell lines were transfected with the empty vector control pCΔj-SV-2-neo. In each case, drug-resistant colonies were established by selection in G418-containing medium. In all, we selected for further study eleven clones

Fig. 1. (A) Western blot analyses of LMP-1 and Bcl-2 expression in representative *bcl-2*-transfected LMP-1/RHEK-1 (Bcl-2/LMP) cells, mock-transfected LMP-1/RHEK-1 (vec/LMP) cells, parental LMP-1/RHEK-1 (LMP135) cells, *bcl-2*-transfected RHEK-1 (Bcl-2/RHEK) and mock-transfected RHEK-1 (vec/RHEK) control cells. Expression of Bcl-2 and LMP-1 was detected by using the Bcl-2-specific MAb clone 124 and LMP-1-specific MAb CS.1-4. (B) Indirect immunofluorescence staining of Bcl-2 and LMP-1 in representative cells. LMP-1 staining in Bcl-2/LMP (ai), vec/LMP (a ii), Bcl-2/RHEK (a iii) and Bcl-2 staining in Bcl-2/LMP (b i), vec/LMP (b ii), Bcl-2/RHEK (b iii). The cells were counterstained with 0.01 % Evans' blue.

Fig. 2. Morphology of the Bcl-2- and LMP-1-co-expressing cells in culture. (a) Bcl-2/LMP, (b) vec/LMP, (c) Bcl-2/RHEK and (d) vec/RHEK clones.

carrying the Bcl-2 plasmid on the LMP135 cell background (Bcl-2/LMP), three clones carrying the Bcl-2 plasmid on the RHEK-1 cell background (Bcl-2/RHEK), four clones carrying the empty vector on the LMP135 cell background (vec/LMP) and four clones carrying the empty vector on the RHEK-1 cell background (vec/RHEK).

Each of the transfectants was characterized with respect to LMP-1 and Bcl-2 expression by immunoblotting cell extracts with the anti-LMP-1 and anti-Bcl-2 specific MABs. Representative results from immunoblotting are shown in Fig. 1(A). Expression of LMP-1 was retained in all transfectants of the LMP135 cell line, and was detected as three bands with molecular masses of 64, 62 and 54 kDa, as reported previously (Lu *et al.*, 1996). Expression of Bcl-2 as a 26 kDa species was seen in all transfectants receiving the Bcl-2 expressing plasmid. Note in particular the three clones in which both LMP-1 and Bcl-2 were co-expressed. Expression of LMP-1 and Bcl-2 proteins was also demonstrated at the single cell level (Fig. 1B) by indirect immunofluorescence using the same antibodies. This confirmed the maintenance of high levels of characteristic LMP-1 staining in all transfectants on the LMP135 background (Fig. 1B, *ai* and *aii*). Bcl-2 transfectants on the LMP135 and on the RHEK cell backgrounds showed strong Bcl-2 staining (Fig. 1B, *bi* and *biii*), whereas control transfectants remained Bcl-2-negative (Fig. 1B, *bii*).

Under microscopic examination, the eleven selected Bcl-2/LMP clones were morphologically similar to each other and

distinct in growth characteristics from control transfectant of the LMP135 line and from both Bcl-2 and control transfectants of the RHEK parent line. The differences are illustrated in Fig. 2. In Bcl-2/LMP clones represented by Fig. 2(a), the cells appear larger and the pattern of growth less ordered than in the various control transfectants (Fig. 2b, c, d). In particular, the introduction of Bcl-2 on the RHEK parental cell background had no obvious effect on the morphology of the cells, in contrast to the effects of Bcl-2 when introduced on the LMP135 cell background.

LMP-1-induced apoptotic cell death is blocked by ectopic Bcl-2 co-expression in epithelial cells

In our earlier work, overexpression of LMP-1 in RHEK-1 epithelial cells was shown to induce apoptosis (Lu *et al.*, 1996), and this effect was more apparent under serum-deficient culture conditions. To evaluate the effect of Bcl-2 on the above phenomenon, three representative Bcl-2/LMP clones were compared with a control transfectant of LMP135, with LMP135 itself as well as a Bcl-2 transfectant of RHEK-1 cells. Standard numbers of all six clones were seeded in medium containing 10, 1, 0.1 or 0% FCS, and viable cell counts were made for adherent and for floating cell populations at various time-points following the initiation of the culture. In addition, morphological changes were observed by light microscopy and DNA fragmentation, a sign of apoptosis, was assayed by agarose gel electrophoresis and by flow cytometry.

All three Bcl-2/LMP clones consistently grew to a higher cell density in growth medium containing 10% FCS than any of the control cell populations (Fig. 3A, upper panel), this was because the Bcl-2/LMP cells did not appear to display any of the density-dependent inhibition of growth shown by the other clones. In addition, the Bcl-2/LMP clones were able to proliferate and maintain cell numbers at higher levels in 1% FCS, 0.1% FCS (data not shown) and even in serum-free medium (Fig. 3A, lower panel). On the other hand, the number of LMP135 and vec/LMP cells decreased rapidly during serum starvation, whereas the Bcl-2/RHEK cells were less susceptible but still did not show an increase in cell number (Fig. 3A, lower panel).

Differences between the different clones in this experiment were also apparent if one assayed the percentage of dead cells appearing in the culture (Fig. 3B). Both the LMP135 parental cells and the control vec/LMP transfectant showed significant numbers (up to 10%) of dead cells accumulating in the cultures over the first 48 h, even in 10% FCS, whereas this was not seen in the Bcl-2/LMP clones nor in the Bcl-2/RHEK transfectant (Fig. 3B, upper panel). These differences were much more dramatically shown when the experiment was done in serum-free medium. Levels of death in the LMP135 and vec/LMP transfectant cells then reached 60% within 48 h, whereas the Bcl-2/LMP1 and Bcl-2/RHEK cells were largely protected from cell death (Fig. 3B, lower panel). Note therefore that

Fig. 3. (A) Growth curves of Bcl-2/LMP clones 1, 2, 3, LMP135, vec/LMP and Bcl-2/RHEK cells. Cells (5×10^5) of each line were cultured either in DMEM containing 10% FCS (upper panel) or in serum-free medium (lower panel) and were counted at different times. Data were obtained for each time-point in triplicate. (B) Cell viability in 10% FCS culture medium (upper panel) or in serum-free medium (lower panel). Cells were collected at various time-points after plating and cultured either in 10% FCS medium or in serum-free medium. Cell viability was determined by the trypan blue exclusion method. The number of dead cells as a percentage of the inoculated cells is shown. Values are the means of three independent determinations $\pm$ SD.

Bcl-2 was able to affect the susceptibility of RHEK cells to serum starvation but this was not accompanied by enhancement of proliferative potential; the latter was only apparent in the Bcl-2/LMP1 co-expressing clones.

We also used agarose gel electrophoresis to analyse low molecular mass DNA isolated from the dying cells that became detached from the plate under these various conditions. Fig. 4

demonstrates the pattern of DNA fragmentation seen in cells detaching from the LMP135 and vec/LMP serum-starved cultures, consistent with their death occurring by an apoptotic mechanism (Fig. 4, vec/LMP and LMP135). However, in the Bcl-2/LMP clones (Fig. 4, Bcl-2/LMP clones 1, 2 and 3), this level of DNA fragmentation was not observed. Less dramatic differences were observed between the RHEK clones, because

Fig. 4. Agarose gel electrophoresis analysis of DNA fragmentation in cells undergoing apoptosis. Low molecular mass DNA was taken from Bcl-2- and LMP-1-co-expressing cells Bcl-2/LMP clones 1 to 3, vec/LMP, LMP135, Bcl-2/RHEK, vec/RHEK and RHEK-1. The cells were cultured in serum-starved medium for 48 h, harvested and the low molecular mass DNAs were analysed by 2% agarose gel electrophoresis and visualized by ethidium bromide staining and UV fluorescence. ϕ X174 DNA (digested with *Hae*III) served as molecular mass markers.

the parental and control cells (lacking LMP-1) showed much lower levels of apoptosis than did LMP135 cells (Fig. 4, Bcl-2/RHEK, vec/RHEK and RHEK-1).

Effects of interaction between Bcl-2 and LMP-1 on the distribution and progression of the cell cycle

To further evaluate the consequence of Bcl-2 expression on LMP-1-expressing epithelial cells, we examined the distribution of the cells throughout the cell cycle by flow cytometry. As shown in Fig. 5(a) and Table 1, under 10% FCS conditions there were no significant differences in cell-cycle distribution and progression in proliferating cells between Bcl-2/LMP clones and the parental LMP135 clone. However, under serum-

starvation conditions the high peak A_0 on the DNA histogram, which shows the cells with fragmented DNA, was observed in LMP135 and vec/LMP cells but not in the Bcl-2/LMP clones (Fig. 5b, Table 1). These results obtained from an analysis of those cells still adherent are in agreement with the data obtained from the study of fragmented DNA in the floating-cell population observed by agarose gel electrophoresis (Fig. 4), in which fragmented DNAs were observed in LMP135 and vec/LMP control cells but not in Bcl-2/LMP clones 1, 2, 3, Bcl-2/RHEK, vec/RHEK and RHEK-1 cells. Thus, co-expression of Bcl-2 and LMP-1 reduced the LMP-1-mediated induction of DNA fragmentation observed, especially during serum deprivation. These results thus provided evidence that Bcl-2 expression could block apoptosis induced by high-level expression of LMP-1 in human epithelial cells.

Transformed morphology and anchorage-independent growth of Bcl-2 and LMP-1 co-expressed human epithelial cells

As described above, co-expression of Bcl-2 and LMP-1 in RHEK-1 cells resulted in altered cellular morphology and unlimited cell growth even in low serum. To further evaluate the transformed phenotype of the Bcl-2/LMP clones, an anchorage-independent growth assay was performed. As shown in Fig. 6, all three representative Bcl-2/LMP clones analysed (Fig. 6a, b, c) had the ability to grow and form colonies in 0.33% semi-soft agar (mean colony forming efficiency 47%) whereas the vec/LMP (Fig. 6d) and LMP135 (Fig. 6e) control cells did not. Furthermore, RHEK parental cells transfected with Bcl-2 also did not acquire colony-forming ability in soft-agar medium (Fig. 6f). Taken together, these results indicate that Bcl-2 may play a dual role in the growth transformation of LMP-1-transfected epithelial cells, on the one hand by inhibiting LMP-1-induced apoptosis and on the other by interacting with LMP-1 to promote cellular growth.

Expression of oncogenes and tumour suppressor genes in the Bcl-2/LMP clones

We next asked whether the unique growth phenotype of the Bcl-2/LMP clones was associated with identifiable changes in the expression of certain other cellular oncogenes or tumour suppressor genes in the cells. Results of immunoblots are shown in Fig. 7. There were no significant differences in the expression of c-Fos, c-Jun, p53 and Ras in Bcl-2/LMP clones 1, 2, and 3 compared to the various control cell populations. Expression of Rb, TGF- β 1, c-Fgr and ICAM-1 proteins was not detected (data not shown). However, when compared to RHEK-1 cells and transfectants on the RHEK background, c-Myc protein was detected at significantly higher levels in the Bcl-2/LMP clones as well as in the original LMP135 cells and its control transfectants. The up-regulated expression of c-Myc in high-level LMP-1-expressing cells (LMP135) and vec/LMP in Fig. 7 was also detected in two other independently

Fig. 5. DNA histograms of cells cultured in 10% FCS (a) or serum-starved for 48 h (b). Ethanol-fixed (i) Bcl-2/LMP (ii) vec/LMP, (iii) LMP135 and (iv) Bcl-2/RHEK cells were stained with propidium iodide and analysed for DNA content (fluorescence). A total of 1×10^4 cells was analysed for each histogram. Peaks representing fragmented DNA (A_0) and the G_0/G_1 , S and G_2/M phases of the cell cycle are indicated.

Table 1. Cell cycle analysis of proliferating and serum-starved cells

Cell line	Serum*	Percentage of cells (mean $\pm$ SE) [†] in:			
		A_0	G_0/G_1	S	G_2/M
Bcl-2/LMP clone 1	+	2.0 ± 0.3	59.9 ± 0.6	22.1 ± 0.4	15.8 ± 0.4
Bcl-2/LMP clone 2	+	2.0 ± 0.4	59.0 ± 0.8	27.0 ± 0.5	12.1 ± 0.3
Bcl-2/LMP clone 3	+	1.7 ± 0.4	56.4 ± 1.0	28.8 ± 0.5	13.6 ± 0.4
vec/LMP	+	5.2 ± 0.5	58.2 ± 1.2	23.4 ± 0.4	15.2 ± 0.5
LMP135	+	6.2 ± 0.6	58.1 ± 1.0	22.6 ± 0.4	14.0 ± 0.2
RHEK-1	+	3.5 ± 0.2	61.9 ± 0.4	15.5 ± 0.5	19.6 ± 0.4
Bcl-2/LMP clone 1	—	3.2 ± 0.1	53.9 ± 0.5	22.2 ± 0.3	21.7 ± 0.4
Bcl-2/LMP clone 2	—	2.7 ± 0.4	55.3 ± 0.4	23.3 ± 0.3	19.8 ± 0.4
Bcl-2/LMP clone 3	—	2.5 ± 0.6	55.1 ± 0.8	22.3 ± 0.6	20.2 ± 0.5
vec/LMP	—	31.1 ± 0.6	35.1 ± 1.2	21.8 ± 0.3	12.5 ± 0.4
LMP135	—	38.0 ± 0.6	31.1 ± 1.0	15.6 ± 0.5	14.4 ± 0.3
RHEK-1	—	3.1 ± 0.3	61.4 ± 0.5	17.1 ± 0.4	19.0 ± 0.2

[†] Results of duplicate experiments involving 10^4 cells.

* Cells were maintained in 10% FCS (+) or serum-starved (—) for 48 h.

established LMP-1-positive RHEK clones, LMP118 and LMP124 (Lu *et al.*, 1996; data not shown), indicating that it correlated consistently with LMP-1 transfection, as seen previously (Evan *et al.*, 1992).

Discussion

We previously reported that constitutive over-expression of EBV LMP-1 in human epithelial cells induced apoptosis (Lu *et al.*, 1996). In the present report, we demonstrate that this effect of LMP-1 is counteracted by co-expression of Bcl-2, protection from apoptosis being evident both from the degree of DNA fragmentation as monitored by agarose gel electro-

phoresis (Fig. 4) and from DNA content analysis (Fig. 5). The induction of apoptosis by LMP-1 and its protection by Bcl-2 were observable in cells cultured in 10% FCS–DMEM and were even clearer under serum-starved conditions; in serum-depleted cultures protection by Bcl-2 persisted for at least 8 days, by which time all LMP-1-positive Bcl-2-negative clones had died. These effects of Bcl-2 were reproducible in several independent clones established in an LMP-1-positive background. Moreover, Bcl-2 and LMP-1 coexpression induced a marked morphological change in the cells and significantly enhanced their proliferative potential under *in vitro* conditions; thus the Bcl-2/LMP clones showed no density-dependent inhibition of growth in 10% FCS, grew well in low-serum-

Fig. 6. Anchorage-independent growth assay for Bcl-2 and LMP-1 co-expressed and control cell lines. Transfected cells that co-express Bcl-2 and LMP-1 could grow on semi-soft agar medium: (a) Bcl-2/LMP clone 1, (b) Bcl-2/LMP clone 2, (c) Bcl-2/LMP clone 3; control cells could not grow in semi-soft agar: (d) vec/LMP, (e) LMP135, (f) Bcl-2/RHEK.

Fig. 7. Western blot analyses of c-Myc, c-Fos, c-Jun, p53, Ras and actin expression in *bcl-2*-transfected, mock-transfected LMP135 cells and RHEK-1 cells. Expression of c-Myc, c-Fos, c-Jun, p53, Ras and actin was detected by the ECL method as described in Methods.

containing medium and formed colonies in semi-soft agar. These data indicate that Bcl-2 and LMP-1 interact cooperatively to alter epithelial cell-growth potential.

We chose to examine the effects of Bcl-2 on LMP-1-induced apoptosis not only because of this protein's ability to

inhibit apoptosis in a variety of hematopoietic cell lines and tissues, but also in view of the fact that LMP-1 and Bcl-2 are often co-expressed in NPC tissues (Lu *et al.*, 1993a). The protective effect of Bcl-2 that we have observed does not appear to be a result of altering cell cycle progression since there was no change in cell cycle profile attributable to expression of Bcl-2; other studies have also reported that Bcl-2 expression has no effect on cell cycle progression in pro-B cells (Hockenberry *et al.*, 1990) and does not block the establishment of quiescence in serum-starved NIH 3T3 cells (Reed *et al.*, 1991). Although many hematopoietic cell lines and at least one fibroblast cell line can be protected from apoptosis by Bcl-2 expression, this phenomenon may not be universal, since Bcl-2 overexpression was not sufficient to make an IL-2-dependent T-cell line or an IL-6-dependent myeloma cell line interleukin independent (Hockenberry *et al.*, 1990). Therefore, the dual effect of Bcl-2 both on the susceptibility of LMP-1-positive epithelial cells to apoptosis and on the morphology/growth patterns of the cells *in vitro* is very significant.

The mechanism through which Bcl-2 promotes tumorigenesis is unique (Reed *et al.*, 1987), since it involves an enhancement of cell survival rather than any direct effect on cell proliferation/cell cycle progression. In this study, Bcl-2 itself neither affected cell cycle progression nor enhanced cell proliferation in the control Bcl-2/RHEK cells. Reed *et al.* (1990b) showed that Bcl-2 expression has been found to lower the growth factor requirements of NIH 3T3 fibroblasts for

DNA synthesis, though the level of DNA synthesis obtained was less than that in cells treated with growth factors. Moreover, B lymphocytes from *bcl-2* transgenic mice exhibit an extended life-span without enhanced proliferation (McDonnell *et al.*, 1989). Similar results have been obtained in other studies in various types of epithelial cells, in which Bcl-2 could protect from apoptosis but did not enhance cell proliferation (Q. L. Lu *et al.*, 1993*b*; P. J. Lu *et al.*, 1995).

It is notable that in the human B cell system, LMP-1 has been reported to induce Bcl-2 expression and inhibit apoptosis (Gregory *et al.*, 1991; Henderson *et al.*, 1991; Rowe *et al.*, 1994). In contrast, LMP-1 does not induce Bcl-2 expression in fibroblasts (Martin *et al.*, 1993) or in epithelial cells (Rowe *et al.*, 1994; Lu *et al.*, 1986). Nevertheless, immunohistochemical expression of Bcl-2 has been detected in 80% of NPC, especially in the undifferentiated nasopharyngeal carcinoma which is closely associated with EBV infection, and 67% (6/9) of the LMP-1-positive NPC tissues examined in a previous study were Bcl-2 positive (Lu *et al.*, 1993*a*). This suggests that the pathogenesis of NPC might involve as one step EBV infection leading to LMP-1 expression in epithelial target cells, and as another step upregulation of cellular Bcl-2 via some virus-independent mechanism.

The most striking finding in the present study is that Bcl-2 expression not only efficiently inhibits LMP-1-induced apoptosis but also functionally cooperates with LMP-1 in the transformation process of epithelial cells. The *bcl-2* gene can cooperate with cellular and viral oncogenes leading to cellular immortalization and/or transformation in B cells and in fibroblasts (Strasser *et al.*, 1990; McDonnell & Korsmeyer, 1991; Martin *et al.*, 1993; Vaux *et al.*, 1988). In this context, it has been postulated that Bcl-2 expression acts by prolonging the life-span of cells which may otherwise be programmed to die. In the context of epithelial cells, it is important to note that no increase in the incidence of epithelial malignancies has been reported in the *bcl-2* transgenic mice (McDonnell *et al.*, 1989; McDonnell & Korsmeyer, 1991), and *in vitro* studies showed that overexpression of Bcl-2 by gene transfer was unable to immortalize epithelial cells (Nataraj *et al.*, 1994), or to malignantly transform SV40-immortalized epithelial cells (Lu *et al.*, 1995). The effect of Bcl-2 in our system may allow EBV-positive epithelial cells to tolerate high level LMP-1 expression and therefore render them capable of responding fully to LMP-1 signals; this allows expression of the complete LMP-1 phenotype.

Very significantly, among the oncogenes and tumour suppressor genes detected in this study, c-Myc protein was found to be over-expressed in all the original LMP-1-expressing clones; these high levels of c-Myc were not affected by over-expression of Bcl-2. The *c-myc* gene is expressed at elevated levels in a variety of human tumours, indicating that its deregulation may be an important component in multi-stage carcinogenesis (Field & Spandidos, 1990; Hunter, 1991; Spencer & Groudine, 1991). Experimental

evidence also suggests that Myc expression can promote cell proliferation, block differentiation and sensitize cells to apoptosis (Evan *et al.*, 1993; Luscher & Eisenman, 1990). However, it is not clear if the diverse activities of *c-myc* are mediated via a common set of effector molecules which trigger distinct effects dependent on cellular context or if different effectors mediate the different responses. In our study, it is possible that LMP-1-induced apoptosis might be occurring as a result of the over-expression of the *c-myc* oncogene (Askew *et al.*, 1991; Evan *et al.*, 1992; Shi *et al.*, 1992). We found that over-expression of Bcl-2 in these cells prevented apoptosis and prolonged the survival time of the cells without altering c-Myc levels, in agreement with a previous report that Bcl-2 abrogates c-Myc-induced apoptosis without affecting the c-Myc mitogenic function (Abdallali *et al.*, 1992). If LMP-1-induced apoptosis is a result of up-regulated c-Myc protein expression, the ability of Bcl-2 expression to protect LMP-1-positive cells suggests that this form of apoptosis shares a common mechanism with apoptosis induced in hematopoietic lines and fibroblasts as described previously (Askew *et al.*, 1991; Evan *et al.*, 1992; Shi *et al.*, 1992). It has been reported that Bcl-2 has no effect on cell cycle progression (Hockenberry *et al.*, 1990) and in our system Bcl-2 did not affect cell proliferation (Fig. 3A, upper panel), cell cycle progression (Fig. 5, iv), or cause cell transformation of the parental LMP-1-negative RHEK-1 cells (Fig. 6f). Very importantly, however, Bcl-2 did bring about growth transformation when expressed on an LMP-1-positive cell background. This cooperation between Bcl-2 and LMP-1 resulting in growth transformation of epithelial cells might be similar to that seen between Bcl-2 and cellular oncogenes such as *c-myc* in certain circumstances in lymphoid cells (Vaux *et al.*, 1988; Nunez *et al.*, 1989; Reed *et al.*, 1990*a*). However, the exact mechanism of this synergy between Bcl-2 and LMP-1 is not yet clear and is still worthy of further investigation, particularly in view of its potential relevance to the multi-step pathogenesis of NPC.

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