Induction of apoptosis in epithelial cells by Epstein–Barr virus latent membrane protein 1

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Epstein–Barr virus (EBV) induces human B cell transformation and is closely associated with nasopharyngeal carcinoma. The expression of an EBV latent membrane protein, LMP-1, protects B cells from apoptosis by up-regulating the expression of a cellular oncogene, bcl-2. LMP-1 also transforms rodent fibroblasts and affects the differentiation, morphology and growth of human and rodent epithelial cells. In this report, we describe a novel finding that high level expression of the LMP-1 gene in a human epithelial cell line (RHEK-1) induces apoptosis, characterized by chromosomal DNA fragmentation in the transfected cells. In particular, such an effect was more apparent under serum starvation. We also found that in the transfected RHEK-1 cells, LMP-1 expression neither affected bcl-2 expression nor led the cells to grow in semi-solid soft agar medium. These results indicate that LMP-1 may participate in the development of EBV-associated epithelial malignancy via a mechanism different from that seen in B cell or fibroblast transformation.

Introduction

Epstein–Barr virus (EBV) is the causative agent of infectious mononucleosis (Henle & Henle, 1979) and is also strongly linked to two malignancies of B cell origin, the immunoblastic lymphoma seen in immunosuppressed patients (Young et al., 1989) and endemic Burkitt’s lymphoma (BL; Epstein et al., 1964). In addition, EBV is closely associated with an epithelial malignancy, nasopharyngeal carcinoma (NPC; Henle et al., 1970; Old et al., 1966). A small subset of virus genes is expressed in latently infected (immortalized) B cells, including six nuclear antigens, EBNA (Epstein–Barr virus nuclear antigens) 1, 2, 3a, 3b, 3c and LP (leader protein), three latent membrane proteins, LMPs 1 and 2a/2b, and two small RNAs (EBERs, Epstein–Barr virus-encoded RNAs; Kieff & Leibowitz, 1990). Several of these virus proteins have been shown to be required for activation and immortalization of infected B cells to lymphoblastoid cell lines (LCLs) in vitro and are expressed in immunoblastic types of B cell lymphoma. In addition to EBNA-1, which is required for maintenance of the EBV episomal genome, the only virus-encoded protein yet found in EBV-associated, non-B cell malignancies is LMP-1, and this is detectable in 30–65 % of NPC (Fahraeus et al., 1988; Young et al., 1988).

LMP-1, encoded by the BNLF-1 open reading frame, is a 62 kDa integral membrane protein (Hennessy et al., 1984; Mann et al., 1985) containing six transmembrane segments. Both the amino-terminal (approximately 25 amino acids) and the carboxy-terminal (around 200 amino acids) domains of this 386 amino acid protein are cytoplasmic and very little of the protein is exposed on the outside of the cells (Liebowitz et al., 1987). LMP-1 is likely to have an important role in the initiation and/or maintenance of the immortalized state in B cells (Mann et al., 1985). Introduction of the LMP-1 gene can also alter the phenotype of EBV-negative sporadic BL cell lines (Wang et al., 1988), reproducing some aspects of the in vitro-transformed LCL phenotype. These include the appearance of increased villous projections, growth in tight clumps and increased expression of activation and adhesion molecules and of the bcl-2 proto-oncogene (Wang et al., 1988; Henderson et al., 1991). This indicates that LMP-1 is an important effector of changes in cell growth. Work in mouse systems indicated that the

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effects of LMP-1 were not restricted to B cells. Firstly, LMP-1, in the absence of any other virus gene products, functions as a transforming gene in rodent cells in vitro, inducing anchorage-independent and tumorigenic growth of both Rat-1 (Wang et al., 1985) and BALB/3T3 (Baichwal & Sugden, 1988) rodent fibroblasts. Secondly, transgenic mice that express LMP-1 in skin epithelial cells develop a dermatological disease, indicating a disturbance of the differentiation process (Wilson et al., 1990). Subsequently it was also shown that LMP-1 can affect the differentiation, morphology and growth of human epithelial cell lines (Dawson et al., 1990; Fahraeus et al., 1990; Niedobitek et al., 1992; Zheng et al., 1994).

An interesting feature of such gene transfection experiments was that over-expression of LMP-1 appeared to be toxic to all cell lines tested (Hammerschmidt et al., 1989), including six human B lymphoid lines, BALB/3T3 fibroblasts, 143/EBNA-1 cells (human osteosarcoma cell line expressing EBV EBNA-1 gene) and Hep-2 cells (human carcinoma cell line). The nature of such cytotoxicity is unknown, the apparently toxic effects of LMP-1 being inferred from the low yield of stable transfectants from cultures in which transient transfection gave high LMP-1 expression. We therefore hypothesized that under some circumstances, expression of the LMP-1 gene may induce apoptosis. Apoptosis is an active form of physiological cell death. Unlike necrosis (Duvall & Wyllie, 1986; Kerr et al., 1972), apoptosis is characterized by chromatin condensation and nuclear DNA fragmentation of the cells in intranucleosomal regions (Arends et al., 1990). Recently, EBV infection has been found to be associated with increased apoptosis in BL cells (AW-Ramos; Hideaki & Glenda, 1993). In addition, Kawanishi (1993) demonstrated that nucleosome laddering characteristic of apoptosis was induced in Raji cells by infection with the P3HR-1 strain of EBV. However, the exact mechanism for such a virus-induced apoptotic phenomenon is not clear. The purpose of the present study is therefore to analyse the effects of high level LMP-1 expression on a human epithelial cell line (RHEK-1) and to clarify the nature of the cytotoxicity mediated by LMP-1.

Methods

**Cells.** RHEK-1 (a kind gift from Dr J S. Rhim, National Cancer Institute, Bethesda, Md., USA) is a non-malignant cell line originally established from normal human foreskin keratinocytes infected with a hybrid virus, adenovirus 12-simian virus 40 (Ad12–SV40; Rhim et al., 1985). Although this cell line is immortal in culture, the cells retain their flat epithelial morphology and a number of markers specific to epithelial cells. Also, they do not form colonies in soft agar and are non-tumorigenic in athymic nude mice.

To generate LMP-1-expressing cell lines, the entire LMP-1 coding region (Baer et al., 1984; Bankier et al., 1983) was cloned into a RHB'11 expression vector, which was derived from the N2 vector system (Armentano et al., 1987) by deleting the neo gene and replacing it with a DNA segment containing multiple cloning sites. The LMP-1-containing construct is referred to as LMP-1/RHB'11 and the expression of the LMP-1 gene is driven by the murine leukaemia virus long terminal repeat (LTR) promoter. The RHEK-1 cells were cotransfected with LMP-1/RHB'11 plasmid and a neomycin resistance marker by electroporation at 960 μF and 0.15 kV. A mock-transfected control cell line was generated by cotransfection of RHB'11 vector and the neo gene into RHEK-1 parental 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 μg/ml of G418. The concentration of G418 was subsequently reduced to 400 μg/ml after 1 week of selection. 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 % fetal calf serum (FCS), 100 U/ml of penicillin G and 100 μg/ml of streptomycin sulphate.

**Immunoblotting.** Cells were trypsinized and washed three times with PBS. The cell pellets were resuspended in 1 ml of lysis buffer (1% Triton X-100, 5 mM-EDTA in TBS pH 7.6 plus protease inhibitors), dispersed by thorough sonication, solubilized with 2% SDS, boiled for 5 min and analysed by 10% PAGE. After electrophoresis, the proteins were transferred to a nitrocellulose membrane and the membrane was treated with 4% non-fat milk, 10 mM-TBS and 0.2% Tween-20. The blots were incubated with a 1:100 dilution of the LMP-1-specific MAb CS-1-4 (Rove et al., 1987; Dako) for 1 h at 37 °C and washed three times with washing buffer (10 mM-TBS, 0.2% Tween-20). The immune complexes were detected by the addition of a 1:10000 dilution of streptavidin–horseradish peroxidase-conjugated goat anti-mouse IgG for 1 h and the blots were then washed three times with washing buffer. The ECL substrate (Amersham) was used according to the manufacturer’s protocol.

**Indirect immunofluorescence assay.** Cells were trypsinized, washed three times with PBS and air-dried before fixation in methanol at −20 °C for 10 min. The slides were dried at room temperature and stained with LMP-1-specific MAb CS-1-4 in a 1:100 dilution at 37 °C for 1 h, followed by washing three times with PBS. 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 (Sigma) for 5 min. washed three times with double-distilled water and air-dried. After mounting, the slides were examined under a fluorescence microscope and photographed with ASA-400 Fuji film.

**Determination of cell viability and growth curves.** Replicate cultures from each of the cell lines were plated at 10⁶ cells/well on 6-well plates in DMEM containing 10% FCS. For the serum starvation assay, cells were cultured on the same medium 24 h prior to the deprivation of serum. Subsequently, on each day of analysis, triplicate cultures were trypsinized individually and the numbers of viable and non-viable cells from each culture were determined by the Trypan blue exclusion method.

**Fluorescence intensity, fragmented DNA and cell-cycle analysis by flow cytometry.** Immunofluorescence quantification of LMP-1 and its correlation with DNA content by flow cytometry were performed as described previously (Mann et al., 1987). Briefly, subconfluent proliferating cells and cells starved of serum for 48 h were trypsinized and washed twice in PBS. The cells were 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 then incubated with PBS containing 2% FCS and 0.1% sodium azide at 4 °C for 10 min and washed twice with PBS. Cold PBS (200 μl) containing mouse MAB CS-1-4 at a 1:50 dilution was added, incubated at 4°C for 30 min and the cells were washed three times with cold PBS. FITC-conjugated goat anti-mouse Ig antibodies (200 μl; 1:100 dilution) were added and the cell suspension was then incubated at 4°C for 30 min, followed by washing four times with cold PBS. Some cells could then be used directly for flow cytometric analysis of LMP-1.
Cell-cycle profiles and distributions were determined by flow cytometry (Becton-Dickinson). Previously (Wagner medium was replaced with serum-free DMEM for 48 h. From both types FCS and cultured either for 2 days or for 24 h, after which time the staining. Other cells were digested with RNase A at a concentration of 1000 U/ml for 30 min and stained with 100 μg/ml of propidium iodide. Cell-cycle profiles and distributions were determined by flow cytometry analysis using the Lysis II program on a FACSScan Flow Cytometer (Becton-Dickinson).

DNA fragmentation analysis by agarose gel electrophoresis. DNA fragmentation assay was performed as described previously (Wagner et al., 1993). A total of 2 x 10⁶ cells from each cell line were plated on 100 mm diameter plates in DMEM containing 10% FCS and cultured either for 2 days or for 24 h, after which time the medium was replaced with serum-free DMEM for 48 h. From both types of culture the floating cells were collected by aspiration of the medium and the adherent cells were collected by trypsinization. The cells were washed twice in Ca²⁺/Mg²⁺-free PBS containing 1 mM-EDTA, pelleted and the adherent cells were collected by trypsinization. The cells were of culture the floating cells were collected by aspiration of the medium and lysed in 1 mM-EDTA containing 0.6% SDS. Sodium chloride was added to 1 M and the solution was mixed by gentle inversion and incubated at 4 °C overnight. After centrifugation for 20 min in a microcentrifuge at 4 °C, low molecular mass DNA was ethanol-precipitated from the supernatant. DNA was analysed for fragmentation by subjecting samples to electrophoresis in 1.5% agarose gels in Tris-acetate/EDTA buffer, stained with ethidium bromide and photographed under UV illumination.

Anchorage-independent growth assay. The transformation assay was done as previously described by Baichwal & Sugden (1988). Briefly, cells were diluted to 10⁵ cells per ml in DMEM. The cell suspension (100 μl) was mixed with 2 ml of 0.35% 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 up to 20 rain and then cultured at 37 °C. Seven to 12 days after plating, 2 ml of 0.35% agarose DMEM was added to the cells. The number of cells that grew to form colonies was determined with a microscope.

Results

Expression of transfected LMP-1 in human epithelial RHEK-1 cells

To determine the effects of LMP-1 on the biological characteristics of human epithelial cells, the LMP-1 gene was cloned into an expression vector and transfected into RHEK-1 cells. After subcloning and screening for LMP-1 in the transfected cells with an indirect immunofluorescence test, 29 LMP-1-positive clones and three mock transfectant clones (RR-1, RR-2 and RR-3) were obtained. The expression and levels of LMP-1 protein were assessed in six random clones and three mock transfectants by Western blot analysis. Fig. 1 (a) shows representative results from three LMP-1-positive clones and one mock transfectant. Significantly high levels of LMP-1 expression were identified in clones LMP118 (lane 3) and LMP135 (lane 5), but a relatively lower level in clone LMP124 (lane 4). There was no LMP-1 expression in mock-transfected clones (RR-3 representative data, lane e) or in the parental RHEK-1 cells (lane 7). Note that two different forms of LMP-1 protein were detected in the positive control B cell line, B95.8 (lane 2), the latent (67 kDa) and lytic phase (54 kDa) forms, as previously reported (Hudson et al., 1985; Rowe et al., 1992). Interestingly, the major band of LMP-1 protein detected in all three epithelial clones was of 62 kDa; other bands were seen at around 64 kDa, which may be another form of LMP-1 (Rowe et al., 1987), and at 54 kDa, similar in size to the lytic cycle-associated form of LMP-1 seen in B95.8 cells (lane 2). Two bands of molecular mass 62 kDa and 52–54 kDa were also observed by Peng et al. (1985) and the latter was also detected in NPC biopsies by others (Fahraeus et al., 1988; Young et al., 1989). From Fig. 1 (a), when 12 μg (lane 2) of total cellular protein from B95.8 cells were used, densitometric analysis showed that the total amounts of LMP-1 protein detected in LMP118 (lane 3), LMP124 (lane 4) and LMP135 (lane 5) were about 1.0, 0.45 and 0.8 times that detected in B95.8 (lane 2). Therefore, allowing for the differences in cell loading, LMP-1 expression in LMP118, LMP124 and LMP135 cells was about 60, 27 and 48 times that in B95.8 cells. The same results were obtained (data not shown) with another LMP-1-specific MAb, S12 (Mann et al., 1985). To confirm the unusually high levels of epithelial cell expression of LMP-1, 0.2, 0.4, 1, 2, 5, 10 and 20 μg of total protein prepared from B95.8 cells and 0.2, 0.5, 2, 5, 10, 20 and 40 μg of total protein prepared from a B95.8-transformed human LCL were loaded alongside 0.2 μg protein from LMP135 and the strength of LMP-1 signal analysed by densitometry. The results are shown in Fig. 1 (b) and (c); the amount of LMP-1 protein detected in LMP135 cells was equal to that in 10 μg total cellular protein from B95.8 cells and in 5 μg total cellular protein from the LCL.

The expression of LMP-1 in the cells was further confirmed by both indirect immunofluorescence and flow cytometry analyses using LMP-1-specific MAb CS.1-4 (Fig. 2a, b). Immunofluorescence staining of clones LMP118 and LMP135 showed a similar strong signal (LMP135, Fig. 2a, i), whereas the fluorescence intensity of clone LMP124 was lower (Fig. 2a, ii). This could also be demonstrated when levels of LMP-1 in LMP135 and LMP124 were compared by flow cytometry analysis (Fig. 2b). LMP118 cells and other LMP-1-positive clones gave results similar to those seen for LMP135 in this analysis (data not shown). Immunofluorescence staining of B95.8 and B95.8-transformed human LCL cells were also analysed in this experiment for comparison, as shown in Fig. 2 (a, v and iv).

The morphologies of clones LMP135 (Fig. 3; representative also of LMP118 and other LMP-1-positive clones) and LMP124 (Fig. 3c) were much more spindle-like when compared to the parental RHEK-1 cells (Fig. 3a) and the mock-transfectant control clone RR-3 (Fig. 3b; representative of RR-1 and RR-2). These observed changes in morphology were consistent with those described previously (Fahraeus et al., 1990). In addition, the growth rates of clones LMP118, LMP124 and LMP135 were significantly slower than those of RR-2 and RR-3 mock-transfected control lines and RHEK-1 parental cells (Fig. 4). From these growth curves, the doubling times of LMP118, LMP124, LMP135, RR-2, RR-3 and RHEK-1 cell lines were calculated as 43.5, 39, 42.5, 29, 28 and 34 h, respectively (Fig. 4). In addition, all the clones and control cells...
used in this study, as well as RHEK-1 cells, could not be grown in semi-solid soft agar medium (data not shown).

**Increased cell death caused by LMP-1 expression**

Interestingly, under normal culture conditions there were always more floating cells and dead cells in LMP118, LMP124, and LMP135 as well as other LMP-1-positive clones than in the parental cell line or the mock-transfected clones. To quantify this, we placed equivalent numbers of cells in DMEM containing 10% FCS or in medium lacking serum and analysed the cells at various time points. The results are shown in Fig. 5. Small but significant differences in the numbers of non-viable
Fig. 3. Morphology of the cells in culture. (a) RHEK-1 cells; (b) RR-3 cells (mock transfectant); (c) LMP124; and (d) LMP135. Bar marker represents 50 μm.

Fig. 4. The growth curves of LMP118, LMP124, LMP135, RR-2, RR-3 and RHEK-1 cells. 5 x 10^5 of each cell line were cultured in DMEM containing 10% FCS and were counted at different times. Data obtained from each time point were assayed in triplicate. The doubling time (g) was determined by the equation g = (ln 2t)/(ln N) – (ln N_0), where t is the time interval t_1-t_2 during which the cell number N_0 increases to the cell number N in the exponential phase of growth.

Fig. 5. Cell viability in 10% FCS culture medium or in serum-free medium. Cells were collected at various times after plating and cultured in 10% FCS medium (a) or in serum-free medium (b). Cell viability was determined by Trypan blue exclusion. The number of dead cells as a percentage of the inoculated cells is shown. Values are the means of three independent determinations ±SD.

increase in cell loss when assayed under serum-free conditions; there was very significant cell detachment and cell death which increased with prolonged starvation (Fig. 5 b). This effect was much more marked in cells expressing high levels of LMP-1 (LMP118 and LMP135) than in those expressing low levels (LMP124).

Effect of expression of LMP-1 on susceptibility to apoptosis and on cell-cycle progression

It is known that cells undergoing apoptosis exhibit a characteristic DNA fragmentation pattern consisting of degradation of DNA into discrete oligonucleosomal fragments, and this is detectable by flow cytometric analysis of the DNA content of individual cells. Therefore, we performed cell-cycle analyses of LMP-1-transfected cell lines using flow cytometry.
Fig. 6. DNA histograms of cells cultured in 10% FCS (o) or serum-starved for 48 h (b). Ethanol-fixed LMP118 (i), LMP135 (ii), LMP124 (iii), RR-2 (iv), RR-3 (v) and RHEK-1 (vi) cells were stained with propidium iodide and analysed for DNA content (fluorescence). A total of 1 x 10⁴ cells were analysed for each histogram. Peaks representing fragmented DNA (A₀) and the G₀/G₁, S and G₂/M phases of the cell cycle are indicated.

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

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Serum*</th>
<th>A₀ (mean ± se)</th>
<th>G₀/G₁ (mean ± se)</th>
<th>S (mean ± se)</th>
<th>G₂/M (mean ± se)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RHEK-1</td>
<td>+</td>
<td>1.3 ± 0.3</td>
<td>56.0 ± 0.4</td>
<td>22.9 ± 0.4</td>
<td>22.4 ± 2.7</td>
</tr>
<tr>
<td>RR-2</td>
<td>+</td>
<td>1.1 ± 0.1</td>
<td>58.3 ± 0.6</td>
<td>18.7 ± 1.3</td>
<td>25.1 ± 2.5</td>
</tr>
<tr>
<td>RR-3</td>
<td>+</td>
<td>1.0 ± 0.2</td>
<td>56.3 ± 0.6</td>
<td>16.9 ± 0.2</td>
<td>28.9 ± 2.4</td>
</tr>
<tr>
<td>LMP124</td>
<td>+</td>
<td>4.5 ± 0.6</td>
<td>58.6 ± 0.8</td>
<td>20.8 ± 0.8</td>
<td>13.3 ± 0.1</td>
</tr>
<tr>
<td>LMP135</td>
<td>+</td>
<td>3.7 ± 1.3</td>
<td>57.5 ± 0.8</td>
<td>25.6 ± 0.6</td>
<td>13.3 ± 0.1</td>
</tr>
<tr>
<td>LMP118</td>
<td>+</td>
<td>8.0 ± 0.6</td>
<td>57.0 ± 0.7</td>
<td>23.7 ± 0.3</td>
<td>14.5 ± 0.5</td>
</tr>
<tr>
<td>RR-1</td>
<td>-</td>
<td>3.3 ± 0.1</td>
<td>63.6 ± 0.3</td>
<td>15.6 ± 0.6</td>
<td>20.5 ± 0.4</td>
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<tr>
<td>RR-2</td>
<td>-</td>
<td>1.4 ± 0.1</td>
<td>48.3 ± 0.3</td>
<td>15.5 ± 0.3</td>
<td>36.7 ± 0.2</td>
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<tr>
<td>RR-3</td>
<td>-</td>
<td>1.6 ± 0.1</td>
<td>48.1 ± 0.3</td>
<td>15.0 ± 0.2</td>
<td>36.4 ± 0.2</td>
</tr>
<tr>
<td>LMP124</td>
<td>-</td>
<td>8.8 ± 0.1</td>
<td>56.6 ± 0.4</td>
<td>18.7 ± 0.6</td>
<td>18.0 ± 0.3</td>
</tr>
<tr>
<td>LMP135</td>
<td>-</td>
<td>26.6 ± 7.6</td>
<td>42.8 ± 5.3</td>
<td>16.4 ± 0.4</td>
<td>17.3 ± 0.4</td>
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<tr>
<td>LMP118</td>
<td>-</td>
<td>37.4 ± 3.1</td>
<td>33.4 ± 0.4</td>
<td>14.8 ± 0.3</td>
<td>16.6 ± 1.3</td>
</tr>
</tbody>
</table>

* Cells were maintained in 10% FCS (+) or serum-starved (-) for 48 h.
+ Results of duplicate experiments involving 10⁴ cells.

following DNA staining with propidium iodide. Fig. 6 shows the distributions obtained from LMP118, LMP135, LMP124, RR-2, RR-3 and RHEK-1 cells when analysed either in 10% FCS (Fig. 6a, i-vi) or after 48 h in serum-free medium (Fig. 6b, i-vi). There was an additional peak (A₀) on the DNA histogram in the LMP-1-transfected cells when cultured either in serum-containing medium (Fig. 6a, i-iii) or, more particularly, after 48 h serum starvation (Fig. 6b, i-iii). This peak represents cells with < 2c DNA content, specifically cells with fragmented DNA (Afanas'ev et al., 1986; Telford et al., 1991). Under serum-free conditions, such cells constituted 37.4% (± 3.1%) of the LMP118 line, 26.6% (± 7.6%) of the LMP135 line and 8.8% (± 0.1%) of LMP124 line (a LMP-1-positive cell line expressing LMP-1 at a lower level; Table 1). Note that these values are from analysis of the adherent population only and therefore do not include the many LMP118, LMP135 and LMP124 cells which had already detached from the monolayer. Many fewer cells were found with a < 2c DNA content (3.3% ± 0.1%; Table 1) in the serum-starved RHEK-1 cell line (Fig. 6b, vi) and almost none were detected in the control transfectant (Fig. 6b, iv, v). We also analysed the other clones expressing LMP-1 at high levels and obtained similar results to those seen in LMP135 and LMP118 clones (data not shown).

Note that when the percentages of cells in G₀/G₁, S and G₂/M were examined (Table 1), it seemed that the expression of LMP-1 had no significant effect on the cell-cycle distribution of proliferating cells when compared to that seen in the parental RHEK-1 cells. However, cell-cycle analysis of the mock-transfectant RR-2 and RR-3 clones showed increased numbers of these cells in the G₂/M phase (Fig. 6b, iv, v; Table 1), suggesting that these clones were more mitotic under both culture conditions. In fact, we routinely observed that RR-2, RR-3 (see also Fig. 4) and RR-1 mock-transfectant clones tended to show more rapid growth than RHEK-1 cells.

We further analysed low molecular mass DNA isolated from the various clones either cultured in the proliferating state or in serum starvation for 48 h. This assay was conducted on DNA from the total cell population, including both attached and detached cells. When the samples were analysed by gel electrophoresis (Fig. 7a), by far the most prominent DNA fragmentation was observed in LMP-1-expressing cell lines LMP118 and LMP135 cultured in DMEM containing 10% serum; no DNA fragmentation was found in parental RHEK-1 or mock-transfectant RR-3 clones. In addition, the extent of DNA fragmentation correlated with the number of floating
dead cells in each case. Note that DNA fragmentation was also observed in the adherent cell population as judged by gel electrophoresis of the low molecular mass DNA of adherent transfected cells following serum starvation (data not shown). These observations were in agreement with the data shown in Fig. 6, in which apoptotic cells in the adherent cell population were detected by flow cytometry of DNA content. In addition, by acridine orange-staining of nucleic acid, the LMP-1-positive clones cultured in 10% FCS or serum-free medium showed cells with chromatin condensation and cellular shrinkage, indicative of the process of apoptosis (data not shown).

**Test for bcl-2 expression in LMP-1-positive epithelial cells**

The expression of Bcl-2 protein in LMP118, LMP124, LMP135, RR-3, RHEK-1 and Raji cells was assayed by Western blot (Fig. 7b). The Bcl-2 protein was only detected in bcl-2-transfected RHEK-1 (lane 1) and Raji cells.

**Discussion**

We observed that constitutive expression of the LMP-1 gene in human epithelial cells induced apoptosis. This effect was demonstrated both by the morphology of the cells in culture and by the degree of DNA fragmentation as monitored by flow cytometry and agarose gel electrophoresis. Induction of apoptosis by LMP-1 was most dramatically observed after serum starvation, suggesting that LMP-1 is able to drive epithelial cells into apoptosis when environmental growth factors are removed and the cells would normally enter a quiescent state. Our findings also suggest that the cytotoxicity observed following over-expression of the LMP-1 gene in several cell lines (Hammerschmidt et al., 1989) is mediated, at least in part, by apoptosis. However, the molecular pathway linking LMP-1, apoptosis and the fragmentation of chromosomal DNA in epithelial cells remains to be elucidated.

Hu et al. (1993) transfected the LMP-1 gene derived from B95.8 cells into RHEK-1 cells and showed that only one of 12 transfected clones caused tumors in SCID mice. The LMP-1 level of the clone was only 0.4 times that of the B95.8 cells used as a positive control. By contrast, in our study the levels of LMP-1 in clones LMP118, LMP124 and LMP135 were about 60, 27 and 48 times that of the B95.8 cells, respectively. It has been observed that LMP-1 is toxic to cells when expressed at high levels but transforms rodent cells when expressed at lower levels (Hammerschmidt et al., 1989). We infer that the novel features of LMP-1 action detailed here but...
not observed by Hu et al. (1993) may be explained by the differences in levels of expression obtained in the two studies. In addition, it has also been reported that the B95.8 LMP-1 gene expressed under the control of a strong promoter (retrovirus LTR), gave rise to bands of 62 kDa and 52–54 kDa (Wang et al., 1985), whereas Hu et al. (1993) observed only the 63 kDa band using the SV40 promoter.

Since LMP-1 has also been reported to function as an oncogene to transform rodent cells, it is interesting to see that LMP-1 can induce apoptosis when expressed at a high level. It is not known how LMP-1 is capable of regulating two processes with distinctly opposite outcomes. However, several studies have demonstrated such paradoxical functions of oncogenes in cells; for example, c-Myc is essential for cell replication and is also able to induce apoptosis in fibroblasts (Evan et al., 1992). The activation of apoptosis by Myc has been shown to be dependent on regions that were also shown to be essential for transformation (Evan et al., 1992). Similarly, in analyses of several LMP-1 mutants, it has been shown that the transforming and toxic domains of LMP-1 coincide (Hammerschmidt et al., 1989), suggesting that the same region of the protein may be responsible for both effects.

It has been proposed that for tumours to develop, one needs not just positive growth deregulation but also activation of a pathway that can block apoptosis (Wagner et al., 1993). It is of interest to note that over-expression of Myc occurs in many neoplasias which are evidently capable of escaping from the programmed cell death pathway. In at least one instance, that of malignant lymphomas derived from the t(14;18) translocation, the induction of apoptosis by over-expression of Myc may be blocked by the high levels of Bcl-2 expressed (McDonnell et al., 1989; Tsujimoto et al., 1985). A similar situation exists in BL, where Myc is over-expressed (Cory, 1986; Spencer & Groudine, 1991) and where tumour cells are very often EBV-positive. Interestingly, the EBV gene BHRF1 encodes a protein with significant similarity to Bcl-2 (Cleary et al., 1986) and this may therefore protect cells from apoptosis following translocation of a c-myc allele.

In our study, LMP-1 induced apoptosis of epithelial cells but did not modulate the expression of the proto-oncogene bcl-2 as determined by Western blot. The latter result is consistent with other investigators’ observations (Rowe et al., 1994). This situation is therefore different to that seen in B cell transfection where LMP-1 up-regulated bcl-2 expression and protected B cells from apoptosis (Gregory et al., 1991; Henderson et al., 1991). The different effects of LMP-1 expression in B cells (protection from apoptosis) and epithelial cells (sensitization to apoptosis) may be due in part to the different levels of expression obtained in the two systems and also to the fact that LMP-1 does not induce Bcl-2 expression in epithelial cells.

It has been proposed that apoptosis may be an important defence mechanism at the cellular level. Many mammalian cells undergo apoptosis when exposed to viruses (reviewed in White et al., 1991; White, 1993). Premature death of infected cells may limit virus propagation to surrounding uninfected cells by minimizing virus production. Because of this, viruses encode gene products which inhibit apoptosis to defeat this host suicide defence mechanism. For example, expression of adenovirus E1A protein serves not only to activate resting cells but also sensitizes the cells to apoptosis; the latter is normally prevented by expression of the E1B 19K protein and in permissive cells thereby maximizes adenovirus production (White et al., 1991). High level expression of the EBV LMP-1 protein may have similar effects to adenovirus E1A protein. In B cells, sensitization to apoptosis is counteracted by LMP-1 induction of Bcl-2. In epithelial cells, other EBV proteins may counteract or modulate the effects of LMP-1 expression, especially BHRF-1 in the context of the lytic cycle. During the pathogenesis of EBV-carrying tumours of epithelial origin, other cellular changes may be necessary to counteract the apoptotic effect of LMP-1 expression; for example, Bcl-2 expression is always increased in NPC cells (Lu et al., 1993), probably via a LMP-1-independent mechanism.

Taken together, our results demonstrate that over-expression of LMP-1 in a human epithelial cell line induces apoptosis. In particular, this effect was more apparent under conditions of serum starvation. In addition, we also found that expression of transfected LMP-1 in the cells had no effect on the expression of cellular proto-oncogene, bcl-2. These results suggest, therefore, that the involvement of LMP-1 in the development of EBV-associated epithelial tumours is complex and not necessarily analogous to its action in B cell systems.

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**References**


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