American Journal of Pathology, Vol. 152, No. 1, January 1998

Epstein-Barr Virus LMP1 Modulates the Malignant Potential of Gastric Carcinoma Cells Involving Apoptosis

Lai-Fa Sheu,* Ann Chen,* Yu-Hui Wei,* Kuo-Chieh Ho,† Jye-Young Cheng,‡ Ching-Liang Meng,§ and Wei-Hwa Lee*

From the Departments of Pathology, Surgery,‡ and Dentistry,§ Tri-Service General Hospital, National Defense Medical Center, and the Department of Botany,† National Taiwan University, Taipei, Taiwan, Republic of China*

About 10% of gastric carcinomas including lymphoepithelioma-like carcinoma and adenocarcinoma are associated with Epstein-Barr virus (EBV) infection. In EBV-associated gastric carcinomas, the tumor cells express Epstein-Barr nuclear antigen 1 (EBNA-1) but not EBNA-2, -3A, -3B, or -3C, leader protein, or latent membrane proteins (LMPs) because of gene methylation. Only a few exceptional cases have LMP1 expression in tumor cells as demonstrated by immunohistochemical studies. To elucidate the biological effects of LMP1 and the significance of its restricted expression in EBV-associated gastric carcinomas, the LMP1 gene was transferred into EBV-negative gastric carcinoma cell lines (SCM1 and TMC1) and into EBV-negative nasopharyngeal carcinoma (NPC) cells (HONE-1) as a control. The biological effects of LMP1 in gastric carcinoma cells were monitored *in vitro* and *in vivo*. These results showed that the consequence of LMP1 expression is a growth enhancement in NPC cells, but it is a growth suppression in gastric carcinoma cells. The LMP1-expressing gastric carcinoma cells had a reduced growth rate, colony-forming efficiency, mean colony size, and tumorigenicity and a lower malignant cytological grade. The reduced growth rate, colony-forming efficiency, and mean colony size were partially reversible *in vitro* with treatment with LMP1 antisense oligonucleotide. In addition, enhanced apoptosis was found in the LMP1-expressing gastric carcinoma cells. This suggests that LMP1 may negatively modulate the malignant potential of gastric carcinoma cells via an enhancement of apoptosis. We concluded that the restriction of LMP1 expression in EBV-associated gastric carcinomas may lead to a growth advantage for tumor cells by avoiding LMP1 apoptotic effects and immunologically mediated elimination. *(Am J Pathol 1998, 152:63–74)*

Epstein-Barr virus (EBV), a human herpesvirus, can cause infectious mononucleosis and is closely associated with endemic Burkitt's lymphoma, nasopharyngeal carcinoma (NPC), lymphoproliferative diseases in immunocompromised patients,[1] nasal T-cell lymphomas,[2,3] and some Hodgkin's diseases.[4] EBV was first shown to be associated with gastric carcinomas by Burke et al in 1990.[5] This association was characterized by the presence of the EBV genome in gastric carcinoma with marked stromal lymphocytic infiltration, a type histologically similar to nasopharyngeal lymphoepithelioma. EBV involvement has also been demonstrated in gastric adenocarcinoma and adjacent dysplastic epithelia.[6] The evidence of carcinogenesis of EBV has been shown, including an almost uniform involvement of EBV in tumor cells with monoclonal proliferation and an elevation of EBV-specific antibodies in patients.[7,8] Approximately 10% of gastric carcinomas are associated with EBV infection.[9] In EBV-associated gastric carcinomas, the tumor cells exclusively express Epstein-Barr nuclear antigen 1 (EBNA-1) but not EBNA-2, -3A, -3B, or -3C, leader protein, or latent membrane proteins (LMPs) because of gene methylation.[7,10] Only a few exceptional cases express LMP1 in tumor cells as demonstrated by immunohistochemical studies.[9,11]

LMP1 is an integral membrane protein that consists of a 24-amino-acid amino-terminal cytoplasmic domain, six membrane-spanning hydrophobic domains separated by short reverse turns, and a 200-amino-acid carboxyl-terminal cytoplasmic domain.[12,13] LMP1 aggregates in patches on the plasma membrane[14] and can engage the tumor necrosis factor receptor (TNFR) family-associated proteins for the TNFR family.[15,16] The interaction of LMP1 with these TNFR family-associated proteins may prove to be important in the biological effects of LMP1. LMP1 has properties of a classical oncogene. LMP1 can transform rodent fibroblast cell lines as assayed by tumorigenicity in nude mice and foci formation.[17,18] In Burkitt's lym-

Supported by grants from the National Science Council (NSC 85-2331-B-016-040), Tri-Service General Hospital (TSGH-C85-44 and TSGH-C86-46), and National Defense Medical Center (NDMC-85), Taipei, Taiwan, Republic of China.

Accepted for publication October 17, 1997.

Address reprint requests to Dr. Lai-Fa Sheu, Department of Pathology, Tri-Service General Hospital, 8, Sec. 3, Ting-Chow Road, Taipei, Taiwan, Republic of China.

phoma cells, LMP1 can induce villous projections, growth in a tight clump, NF-κB activity, and the expression of activation markers (CD23 and CD40), adhesion molecules (ICAM1, LFA1, and LFA3), and *bcl-2* proto-oncogene[19–22] and can inhibit p53-triggered apoptosis.[23] EBV recombinant genetic analysis indicates that LMP1 is essential for primary B-lymphocyte transformation.[24] In squamous epithelial cells, the consequences of LMP1 expression are inhibition of cellular differentiation,[25] morphological transformation,[26] and induction of epidermal growth factor receptors.[27] In transgenic mice, LMP1 is able to alter keratin expression and induce hyperplasia of the skin.[28] However, LMP1 is cytotoxic in a variety of cell lines and induces apoptosis in squamous epithelial cells when expressed at a high level.[29,30] Collectively, it seems that the biological effects of LMP1 are complicated.

Although the relationship between EBV and gastric carcinoma has been demonstrated, the biological effects of EBV infection and the significance of restricted LMP1 expression in EBV-associated gastric carcinomas are still unknown. To our knowledge, there has been no EBV-associated gastric carcinoma cell line or EBV-immortalized gastric epithelial cell line established. Establishing an EBV-infected gastric carcinoma *in vitro* may be complicated because of the absence of the EBV receptor (CD21) in gastric epithelium and gastric carcinoma cells.[31] The evaluation of biological effects of EBV infection in gastric carcinoma cells could not be performed in our study. However, the expression of LMP1 gene in EBV-negative gastric carcinoma cells by gene transfer is one of the more convenient methods for the initial evaluation of the biological effects of EBV infection and the significance of restricted LMP1 expression in EBV-associated gastric carcinomas. In this study, the LMP1 gene was introduced into gastric carcinoma cells, and its biological effects were monitored *in vitro* and *in vivo*. We showed that LMP1 is able to negatively modulate the malignant potential of gastric carcinoma cells possibly via a process involving the enhancement of apoptosis.

Materials and Methods

Cells and Animals

Two gastric carcinoma cell lines (SCM1 and TMC1) were used for this study. The SCM1 cell line was originally derived from the gastric specimen of a patient with poorly differentiated tubular adenocarcinoma of the stomach. The TMC1 cell line was derived from metastatic tumor cells in the lymph node of a patient with poorly differentiated adenocarcinoma of the stomach. Both cell lines were EBV negative, and they were cultured in RPMI 1640 medium containing 10% fetal calf serum (FCS) and incubated at 37°C in an incubator with 5% CO_2 and water saturation. The EBV-negative NPC cell line (HONE-1)[32] was used as a comparative study. Pathogen-free nude (BALB/c nu/nu) mice were used for tumorigenicity assays.

Construction of LMP1 Expression Vector

The LMP1 gene was excised from the *Eco*RI D fragment of the B95.8 strain of EBV by *Mlu*I digestion. This LMP1 gene is a wild type without the specific mutations in exon 3 found in the NPC strain of EBV.[33,34] After being end-blunted by T4 DNA polymerase, the LMP1 gene was cloned into the pMAMneo expression vector (Clontech, Palo Alto, CA). The orientation of the LMP1 gene relative to the MMTV promoter was ascertained by restriction mapping. The reconstructed plasmid (pMAM-LMP1) containing the LMP1 gene and the control plasmid (pMAM-neo) were used for cell transfection to establish the LMP1-expressing cells and vector-transfected control cells, respectively.

DNA Transfection

DNA transfection was performed by the lipofection method according to the manufacturer's suggestion (GIBCO BRL, Gaithersburg, MD). In brief, adherent cells (2×10^5 cells) in a 35-mm tissue culture dish were transfected with 1 ml of a DNA-liposome complex. The DNA-liposome complex was prepared by mixing solution A (1 μg of plasmid DNA diluted into 100 μl of Opti-MEM I) and solution B (6 μl of lipofectamine diluted into 100 μl of Opti-MEMI). After incubation of the DNA-liposome complex at room temperature for 15 to 45 minutes, 0.8 ml of Opti-MEM I medium was added and mixed gently; then the diluted complex solution was overlaid onto rinsed cells. The cells were incubated at 37°C in a CO_2 incubator. After 8 hours, 1 ml of growth medium containing 20% FCS was added without removing the transfection mixture. The medium was replaced with fresh growth medium 24 hours after the start of transfection. At 72 hours after transfection, cells were subcultured at a 1:10 dilution in growth medium containing G418 (400 μg/ml). G418-resistant colonies were selected. Colonies containing more than 20 cells were counted after 14 days.

Detection of LMP1 Expression by Immunohistochemical Stain

Detection of LMP1 expression in transfected cells and their induced tumors in nude mice were performed by *in situ* immunostaining. The cells were grown on coverslips to approximately 70% confluence, washed twice with ice-cold phosphate-buffered saline (PBS), and then fixed in methanol/acetone (1/1, v/v) at −20°C for 30 minutes. The fixed cultured cells were rinsed in Tris-buffered saline (TBS; 145 mmol/L NaCl and 20 mmol/L Tris, pH 7.6), incubated with 2% H_2O_2 in methanol for 10 minutes to destroy endogenous peroxidase activity, blocked with normal serum from rabbits (1:5 dilution) for 5 minutes, and then incubated with mouse monoclonal antibody S12[35] against LMP1 for 2 hours. After three washes with TBS, the cells were exposed to rabbit anti-mouse IgG antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA) for 60 minutes and washed with TBS, and the positive staining was devel-

oped in aminoethylcarbazole chromogen solution (Dako, Carpinteria, CA). The cells were lightly counterstained with hematoxylin and mounted for examination. The formalin-fixed and paraffin-embedded tissue sections of the tumor masses derived from the tumorigenicity assay in nude mice were dewaxed, pretreated by wet autoclaving for antigen retrieval,[36] rinsed in TBS, and stained by the same procedures as the culture cells except that the secondary antibody was replaced by rabbit anti-mouse IgG antibody conjugated with alkaline phosphatase (Santa Cruz Biotechnology), the positive staining was developed in naphthol phosphate-new fuchsin solution (Dako), and the light hematoxylin counterstain was omitted.

Western Immunoblotting

Proteins were extracted from the transfected cells, and tumor masses were derived from the tumorigenicity assay in nude mice and the metastatic NPC in neck lymph node. After washing with PBS, the cells and tumors were lysed in RIPA buffer (150 mmol/L NaCl, 50 mmol/L Tris, pH 7.5, 5 mmol/L EDTA, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and 1 mmol/L phenylmethylsulfonyl fluoride), homogenized by sonication, and centrifuged at 15,000 × *g* for 10 minutes at 4°C. The extracted proteins in the supernatant were quantified by the Lowry assay and then stored in aliquots at −70°C before use. For SDS-polyacrylamide gel electrophoresis, extracted protein mixed with an equal volume of sample buffer (0.02% bromophenol blue, 4% SDS, 4% 2-mercaptoethanol, 20% glycerol, and 50 mmol/L Tris) was boiled for 8 minutes and separated on an 8.5% polyacrylamide gel. The separated proteins were electrotransferred to nitrocellulose membrane (Amersham, Arlington Heights, IL), probed with mouse anti-LMP1 protein monoclonal antibody S12, detected with horseradish-peroxidase-conjugated rabbit anti-mouse IgG antibody (Serotech), and developed in enhanced chemiluminescence detection reagents (Amersham). For the LMP1-positive control and quantitative analysis, the B95.8 cells were included in this assay. The detection of *bcl-2* expression in LMP1-expressing gastric carcinoma cells and vector-transfected cells was performed according to previously described methods.[37]

Growth Rate Assessment

Cells were plated at a density of 1×10^4 cells/ml on 24-well plates in RPMI 1640 medium containing 10% FCS, and this growth medium was refreshed every other day. Each day, triplicate cultures were trypsinized individually, and the number of viable cells from each culture was determined by the trypan blue exclusion method.

Colony-Forming Assay

The vector-transfected and LMP1-expressing gastric carcinoma cells were trypsinized, and viable cells were counted. On a 100-mm culture dish, 1000 cells were seeded in RPMI 1640 medium containing 10% FCS, and this growth medium was refreshed every 3 days. After 2 weeks, the culture cells were washed with PBS, fixed in a 10% buffered formalin, and stained with a 1% solution of crystal violet to determine the colony number. This assay was carried out in triplicate in three independent experiments. For the measurement of colony-forming efficiency, the number of colonies per dish, counted under low magnification (×5), was divided by the number of viable cells seeded and expressed as a percentage. The mean colony size was measured by counting the number of cells per colony at ×200 magnification in 60 randomly selected colonies.

Tumorigenicity Assay in Nude Mice

LMP1-expressing gastric carcinoma cells and vector-transfected cells (2×10^6) were simultaneously transplanted subcutaneously at different locations on the back of each nude mouse. After transplantation, tumor development was monitored daily. After 30 days, the tumor masses were dissected, weighed, and cut into three pieces. One piece was fixed in neutralized 10% formalin for histopathological examination, and the others were frozen in liquid nitrogen and preserved at −70°C for other relevant studies.

Morphological Study

The morphological characteristics of cultured LMP1-expressing cells and vector-transfected cells were examined in a subconfluent condition. The cellular morphology of each tumor mass derived from the tumorigenicity assay in nude mice was evaluated on serial tissue sections using hematoxylin and eosin (H&E) staining.

Detection of Apoptotic DNA Fragmentation

The DNA fragmentation assay was performed as previously described.[38] In brief, the LMP1-expressing gastric carcinoma cells and vector-transfected cells were cultured to approximately 70% confluence in RPMI 1640 medium containing 10% FCS. The floating cells were collected by aspiration of the medium, and the adherent cells were collected by trypsinization. These cells were washed twice in PBS containing 1 mmol/L EDTA and 0.1 mg/ml proteinase K, pelleted and lysed in 1 mmol/L EDTA containing 0.6% SDS. Sodium chloride was added to 1 mol/L, and the solution was mixed by gentle inversion and incubated at 4°C overnight. After centrifugation at 15,000 × *g* for 60 minutes at 4°C, low molecular weight DNA was ethanol precipitated from the supernatant. DNA fragmentation was analyzed by subjecting 30 μg of DNA to electrophoresis on a 1.5% agarose gel using Tris-acetate/EDTA buffer, staining with ethidium bromide, and photography under ultraviolet illumination.

Antisense Treatment

To specify the biological effects of LMP1, the cells were treated with LMP1 antisense oligonucleotide for growth

Table 1. Comparison of the LMP1 Transfection in Gastric and Nasopharyngeal Carcinoma Cells

	SCM1		TMC1		HONE1	
	Number of colonies	Size of colonies	Number of colonies	Size of colonies	Number of colonies	Size of colonies
pMAMneo						
Experiment 1	63	176	51	132	70	187
Experiment 2	71	182	53	124	75	199
Mean	67	179	52	128	73	193
pMAM-LMP1						
Experiment 1	58	150	40	98	102	242
Experiment 2	50	142	35	107	90	213
Mean	54	146	38	103	96	228

The number of colonies and the size of colonies are expressed as the mean of triplicate data of each experiment.

rate assessment and the colony-forming assay. LMP1 antisense oligonucleotide (5′ AG GTC GTG TTC CAT CCT CAG GGC 3′), complementary to a 23-bp sequence overlapping the start codon of the LMP1 mRMA, was synthesized using an Applied Biosystem's 380B DNA synthesizer (Applied Biosystems, Foster City, CA) with a phosphorothioate substitution on each base. Purity was assayed by polyacrylamide gel electrophoresis after two cycles of ethanol precipitation. Sterile aliquots of 1 mmol/L stock solution were stored at −20°C and thawed on ice immediately before use in each experiment. The LMP1-expressing gastric carcinoma cells and vector-transfected cells were cultured in growth medium containing 3 μmol/L LMP1 antisense oligonucleotides. The following procedures were the same as previously described for growth rate assessment and the colony-forming assay. Treatment of the control oligonucleotide, which consisted of the same nucleotides in randomized sequence (5′ CT AGC AGT GCG AGG CGT TCT CTC 3′), was performed in the control study.

Results

Comparison of LMP1 Transfection Effects in Gastric Carcinoma and NPC Cells

Transfections, under the same conditions, were performed in gastric carcinoma cells (SCM1 and TMC1) and NPC cells (HONE-1) to evaluate the differences of LMP1 transfection effects in gastric carcinoma and NPC cells. Fourteen days after transfection, the G418-resistant colonies containing more than 20 cells were counted. The mean colony size was measured by counting the number of cells per colony at ×200 magnification in 60 randomly selected colonies. This assay was carried out in triplicate in two independent experiments. These results are summarized in Table 1. Using this approach in gastric carcinoma cells (SCM1 and TMC1), we found consistently that pMAM-LMP1 transfection of cells that express LMP1 resulted in the recovery of fewer drug-resistant cell colonies and a smaller mean colony size than when the control plasmid (pMAMneo) was introduced alone. However, the NPC cells (HONE-1) transfected with pMAM-LMP1 resulted in the recovery of more drug-resistant cell colonies and a larger mean colony size than when the pMAMneo was introduced alone. To determine the expression level of LMP1 in transfected gastric carcinoma and NPC cells, Western analysis was performed on the pooled drug-resistant cells. B95.8 cells and a specimen of metastatic NPC with EBV association in neck lymph node (the residual lymphoid tissue was removed under a dissecting microscope) were also included as controls. As shown in Figure 1, the LMP1 expression levels of LMP1-transfected gastric carcinoma and NPC cells are nearly equal, but they are obviously less than in metastatic NPC cells and B95.8 cells. According to these results, the consequence of the LMP1 expression in NPC cells is a growth enhancement, but it is a growth suppression in gastric carcinoma cells.

Establishment of LMP1-Expressing Gastric Carcinoma Cells

To further characterize the biological effects of LMP1 in gastric carcinoma cells, two monoclonal drug-resistant cell lines (SCM-L14 and -L22) with persistent LMP1 expression were randomly selected from pMAM-LMP1-

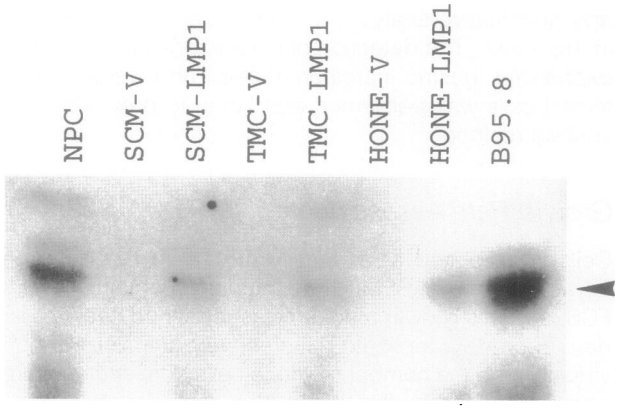

Figure 1. Western blot assay for LMP1 expression in pMAM-LMP1-transfected gastric carcinoma and NPC cells. Equal amounts of extracted protein (800 μg) were loaded in each lane except that 50 μg of extracted protein of B95.8 cells and 150 μg of extracted protein of metastatic NPC were loaded for quantitative analysis. NPC, metastatic NPC; SCM-V, vector-transfected SCM1 cells; SCM-LMP1, pMAM-LMP1-transfected SCM1 cells; TMC-V, vector-transfected TMC1 cells; TMC-LMP1, pMAM-LMP1-transfected TMC1 cells; HONE-V, vector-transfected HONE-1 cells; HONE-LMP1, pMAM-LMP1-transfected HONE-1 cells. The **arrow** indicates the positive band of LMP1, approximately 65 kd.

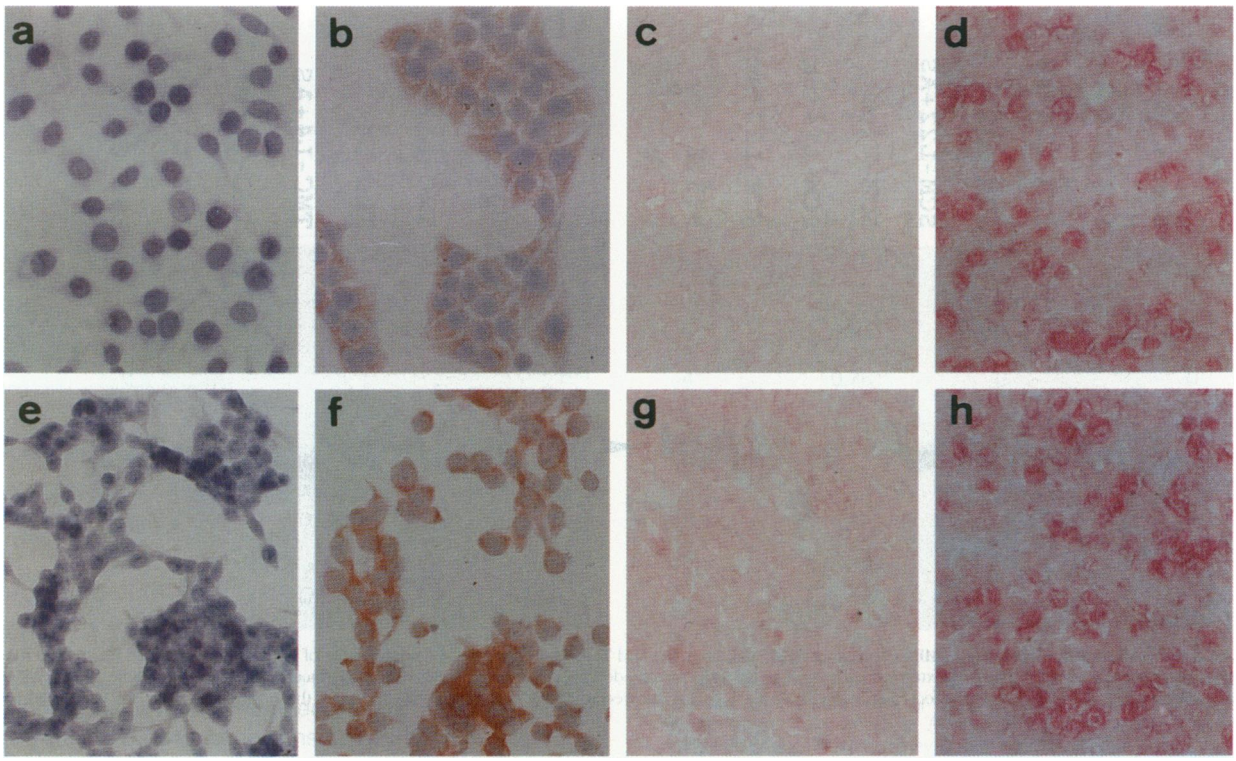

Figure 2. Immunostaining for LMP1 expression in LMP1-expressing gastric carcinoma cells. **a:** Vector-transfected SCM1 cells. **b:** LMP1-expressing SCM1 cells (SCM-L22). **c:** Induced tumor derived from vector-transfected SCM1 cells. **d:** Induced tumor derived from LMP1-expressing SCM1 cells (SCM-L22). **e:** Vector-transfected TMC1 cells. **f:** LMP1-expressing TMC1 cells (TMC-L1). **g:** Induced tumor derived from vector-transfected TMC1 cells. **h:** Induced tumor derived from LMP1-expressing TMC1 cells (TMC-L1). Anti-LMP1; magnification, ×200.

transfected SCM1 cells and two (TMC-L1 and -L4) from pMAM-LMP1-transfected TMC1 cells. LMP1 expression in these selected LMP1-expressing gastric carcinoma cell lines and in the tumors induced in nude mice was demonstrated by *in situ* immunostaining and Western blot analysis. In culture, LMP1 expression was restricted to the cytoplasm. In the tumors induced in nude mice, LMP1 expression was demonstrated in the nuclei and cytoplasm (Figure 2). These selected LMP1-expressing gastric carcinoma cell lines all expressed LMP1 at approximately 65 kd as demonstrated by Western blot analysis (Figure 3). LMP1 expression was undetectable in vector-transfected SCM1 and TMC1 cells. Thus, these selected LMP1-expressing gastric carcinoma cell lines were confirmed to constantly express LMP1 *in vitro* and *in vivo*. In addition, the LMP1 expression levels in these selected LMP1-expressing gastric carcinoma cell lines were lower than in B95.8 cells.

Reduced Growth Rate of LMP1-Expressing Gastric Carcinoma Cells in Vitro

The growth rates of LMP1-expressing gastric carcinoma cells and vector-transfected cells were evaluated by daily counting of viable cells. As shown in Figures 4 and 5, the LMP1-expressing SCM1 (SCM-L14 and -22) and TMC1 (TMC-L1 and -L4) cells showed an obviously slower growth rate than that of vector-transfected SCM1 and TMC1 cells. These findings suggest that the LMP1-expressing gastric carcinoma cells have a reduced growth rate *in vitro*.

Reduced Colony-Forming Efficiency and Colony Size of LMP1-Expressing Gastric Carcinoma Cells in Vitro

The results of the colony-forming assay are summarized in Table 2. The colony-forming efficiency of LMP1-expressing SCM1 cells was less than that of vector-transfected SCM1 cells. The mean colony size of LMP1-expressing SCM1 cells was smaller than that of vector-transfected SCM1 cells. These results indicate that LMP1-expressing gastric carcinoma cells had a reduced colony-forming efficiency and mean colony size *in vitro*. Because some of the LMP1-expressing and vector-transfected TMC1 cells grew in suspension, the colony-forming assay could not be tested in LMP1-expressing TMC1 cells.

Reduced Tumorigenicity and Growth Rate of LMP1-Expressing Gastric Carcinoma Cells in Vivo

A subcutaneous tumorigenicity assay in nude mice was performed to test the difference in tumorigenicity of LMP1-expressing gastric carcinoma cells and vector-

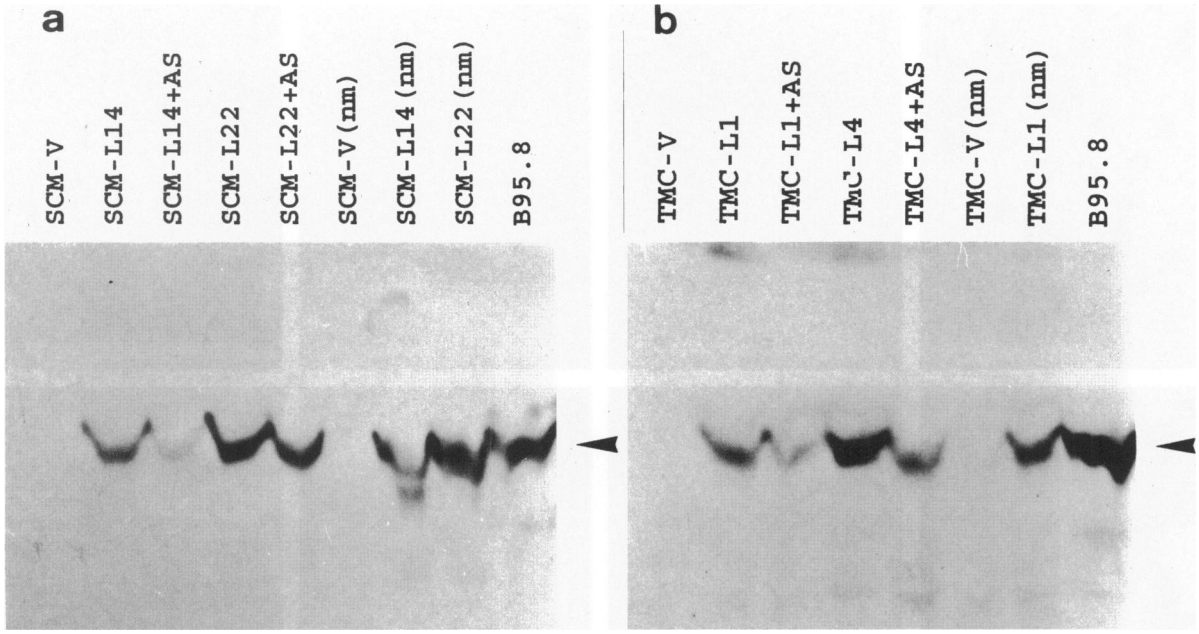

Figure 3. LMP1 expression in selected LMP1-expressing SCM1 cell lines (**a**) and TMC1 cell lines (**b**). Equal amounts of extracted protein (250 μg) were loaded in each lane except that only 50 μg of extracted protein of B95.8 cells was loaded for quantitative analysis. nm, induced tumor mass in nude mouse; AS, with antisense treatment; SCM-V, vector-transfected SCM1 cells; TMC-V, vector-transfected TMC1 cells. The **arrow** indicates the positive band of LMP1, approximately 65 kd.

transfected cells. As shown in Table 3, the tumorigenesis frequency of SCM-L14 was 71% and of SCM-L22 cells was 62%, which was significantly lower than that of vector-transfected SCM1 cells (100%; $P < 0.05$). The growth rates of SCM-L14 and -L22 cells, assessed by the weight of the induced tumor masses, were significantly slower than that of vector-transfected SCM1 cells ($P < 0.05$).

Similar results were obtained from LMP1-expressing TMC1 cells (Table 4). The tumorigenesis frequency of TMC-L1 cells was 13% and of TMC-L4 cells was 0%, which was significantly lower than that of vector-transfected TMC1 cells (100%; $P < 0.05$). The growth rate of LMP1-expressing TMC1 cells was also significantly slower than that of vector control TMC1 cells in the animal

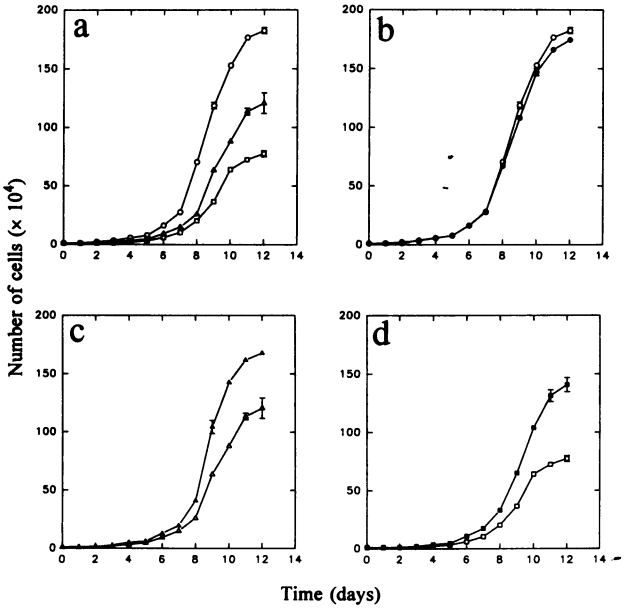

Figure 4. Growth curves of LMP1-expressing SCM1 cells and vector-transfected cells with or without LMP1 antisense treatment. **a:** Growth curves of SCM-L14 (△), SCM-L22 (□), and vector-transfected cells (○). **b:** Growth curves of vector-transfected cells (○) and with LMP1 antisense treatment (●). **c:** Growth curves of SCM-L14 cells (△) and with LMP1 antisense treatment (▲). **d:** Growth curves of SCM-L22 cells (□) and with LMP1 antisense treatment (■).

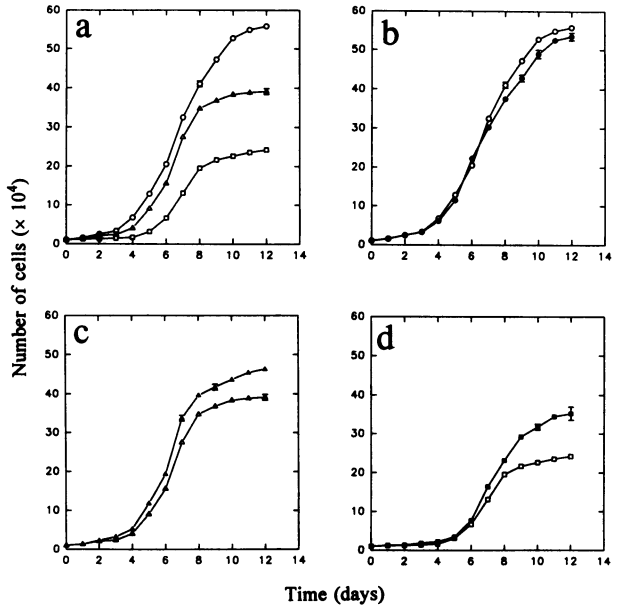

Figure 5. Growth curves of LMP1-expressing TMC1 cells and vector-transfected cells with or without LMP1 antisense treatment. **a:** Growth curves of TMC-L1 (△), TMC-L4 (□), and vector-transfected cells (○). **b:** Growth curves of vector-transfected cells (○) and with LMP1 antisense treatment (●). **c:** Growth curves of TMC-L1 cells (△) and with LMP1 antisense treatment (▲). **d:** Growth curves of TMC-L4 cells (□) and with LMP1 antisense treatment (■).

Table 2. Colony-Forming Efficiency (CFE) of LMP1-Expressing SCM1 Cells

Cell lines	CFE (%)		Mean colony size (cells/colony)	
	AS (−)	AS (+)	AS (−)	AS (+)
SCM-V	34	32	235	224
SCM-L14	12	21	163	194
SCM-L22	8	17	138	186

CFE, colony-forming efficiency; AS (−), without LMP1 antisense treatment; AS (+), with LMP1 antisense treatment. CFE = (colonies formed/cells inoculated) × 100.

test ($P < 0.05$). These findings suggest that LMP1-expressing gastric carcinoma cells have a reduced tumorigenicity and growth rate *in vivo*.

Morphological Alterations of LMP1-Expressing Gastric Carcinoma Cells

Morphological alterations of the LMP1-expressing gastric carcinoma cells were examined *in vitro* and *in vivo*. In subconfluent culture conditions, the vector-transfected SCM1 cells predominantly displayed more spindle shapes and grew in a less organized pattern (Figure 6a). However, the LMP1-expressing SCM1 cells showed polygonal or ovoid shapes and grew in a cohesive and well organized pattern (Figure 6b). The vector-transfected SCM1 cell tumors induced in nude mice showed a higher malignant cytological grade, characterized by increased cellular polymorphism, conspicuous eosinophilic nucleoli, frequent mitosis, and relatively scanty cytoplasm (Figure 6c). In contrast, the LMP1-expressing SCM1 cells displayed a lower malignant cytological grade, characterized by monotonous tumor cells with polygonal or ovoid nuclei, inconspicuous nucleoli, occasional mitosis, and moderate cytoplasm (Figure 6d). No identifiable differences between the mucin production of vector-transfected or LMP1-expressing SCM1 cells were found by serial tissue section examination. Similarly, a lower grade of malignant cytological features was found in LMP1-expressing TMC1 cells. The cultured vector-transfected TMC1 cells had a tendency to grow clumped together, and some of them formed clusters (Figure 6e). In contrast, the LMP1-expressing TMC1 cells mostly grew in a homogeneous monolayer attached pattern (Figure 6f). The vector-transfected TMC1 cell tumors induced in nude mice showed a higher malignant cytological grade, characterized by cellular polymorphism with giant cell formation, prominent eosinophilic nucleoli, frequent mitosis, and scanty cytoplasm (Figure 6g). However, the LMP1-expressing TMC1 cell tumors displayed a lower malignant cytological grade, characterized by monotonous tumor cells with polygonal or ovoid nuclei, inconspicuous nucleoli, occasional mitosis, and increased production of mucin accumulated in the intercellular spaces (Figure 6h). Similar morphological alterations were also observed in the pooled LMP1-expressing SCM1 and TMC1 cells (data not shown). These results suggest that the LMP1-expressing gastric carcinoma cells displayed a lower malignant cytological grade than that of vector-transfected cells.

Table 3. Tumorigenesis of LMP1-Expressing SCM1 Cells (SCM-L14 and -L22) and Vector-Transfected Cells (SCM-V)

Mouse	Tumor weight (g)		
	SCM-V-derived tumors	SCM-L14-derived tumors	SCM-L22-derived tumors
1	0.144	0.028	0.013
2	0.063	0	0
3	0.124	0.042	0.033
4	0.250	0.100	0.030
5	0.190	0	0.005
6	0.287	0.113	0.032
7	0.246	0.103	0.066
8	0.272	0.085	0.037
9	0.246	0.278	0.180
10	0.260	0	0
11	0.182	0.069	0.030
12	0.140	0.145	0.005
13	0.057	0	0
14	0.233	0.055	0
15	0.116	0.048	0
16	0.333	0.078	0.063
17	0.398	0	0
18	0.128	0	0
19	1.165	0.214	0
20	1.681	0.432	0.227
21	1.889	0.788	0.292

Tumorigenesis was 100% for SCM-V-derived tumors, 71% for SCM-L14-derived tumors, and 62% for SCM-L22-derived tumors. Differences were considered significant at $P < 0.05$ by Fisher's exact test for tumorigenicity and Mann-Whitney *U* test for growth rate (assessed by weight).

Table 4. Tumorigenesis of LMP1-Expressing TMC1 Cells (TMC-L1 and -L4) and Vector-Transfected Cells (TMC-V)

Mouse	Tumor weight (g)		
	TMC-V-derived tumors	TMC-L1-derived tumors	TMC-L4-derived tumors
1	0.804	0	0
2	0.653	0	0
3	0.596	0	0
4	0.761	0	0
5	0.556	0	0
6	0.314	0	0
7	0.643	0.190	0
8	0.285	0.025	0
9	0.507	0	0
10	0.869	0	0
11	0.690	0	0
12	0.707	0	0
13	0.655	0	0
14	0.341	0	0
15	0.716	0	0

Tumorigenesis was 100% for TMC-V-derived tumors, 13% for TMC-L1-derived tumors, and 0% for TMC-L4-derived tumors. Differences were considered significant at $P < 0.05$ by Fisher's exact test for tumorigenicity and Mann-Whitney *U* test for growth rate (assessed by weight).

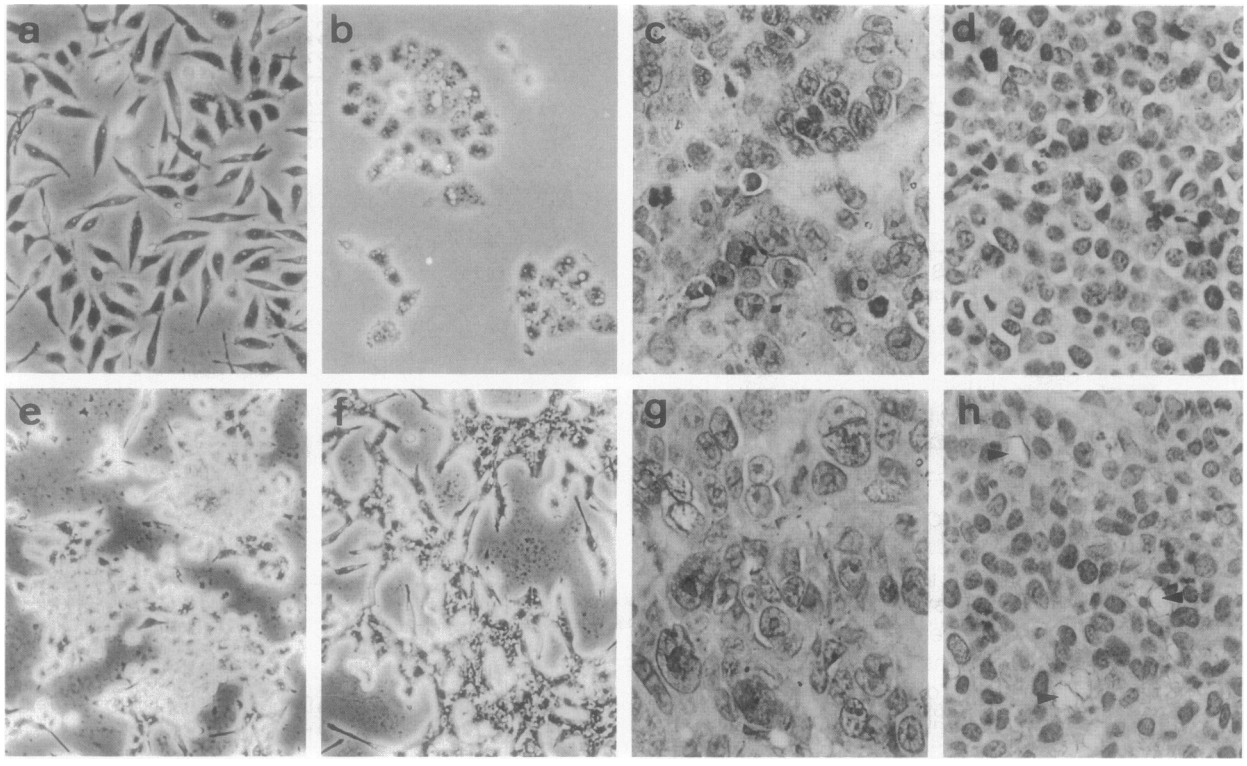

Figure 6. Morphological alterations of LMP1-expressing gastric carcinoma cells. **a:** Vector-transfected SCM1 cells. **b:** LMP1-expressing SCM1 cells (SCM-L22). **c:** Tumor induced by vector-transfected SCM1 cells. **d:** Tumor induced by LMP1-expressing SCM1 cells (SCM-L22). **e:** Vector-transfected TMC1 cells. **f:** LMP1-expressing TMC1 cells (TMC-L1). **g:** Tumor induced by vector-transfected TMC1 cells. **h:** Tumor induced by LMP1-expressing TMC1 cells (TMC-L1). The **arrows** indicate mucin accumulated in the intercellular space. H&E stain (**c**, **d**, **g**, and **h**); magnification, × 200 (**a**, **b**, **d**, **e**, **f**, and **h**) and × 300 (**c** and **g**).

Enhanced Apoptosis of LMP1-Expressing Gastric Carcinoma Cells

Under routine conditions, more floating cells appeared in the culture of LMP1-expressing gastric carcinoma cells than in the culture of vector-transfected cells. To characterize this phenomenon, the low molecular weight DNA isolated from the total cell population, including attaching and floating cells, of the LMP1-expressing gastric carcinoma cells and vector-transfected cells were analyzed by agarose gel electrophoresis. DNA fragmentation in the ladder pattern was noted in LMP1-expressing SCM1 cells (SCM-L14 and -L22) and TMC1 cells (TMC-L1 and -L4), but it was not clearly identified in vector-transfected cells (Figure 7). In serum starvation conditions, DNA fragmentation in the ladder pattern was also found in vector-transfected SCM1 and TMC1 cells (data not shown). These results suggest that apoptosis was enhanced in LMP1-expressing gastric carcinoma cells.

Lack of Induced bcl-*2 Expression in LMP1-Expressing Gastric Carcinoma Cells*

The expression of *bcl-2* in LMP1-expressing gastric carcinoma cells and vector-transfected cells was assayed by Western blot. No LMP1-inducible *bcl-2* expression in LMP1-expressing gastric cells or constitutive *bcl-2* expression in vector-transfected cells was detectable. The expression of *bcl-2* was detected only in MCF-7 cells as a positive control (data not shown). These results suggest that LMP1 cannot induce *bcl-2* expression in gastric carcinoma cells, which are compatible with the similar findings in primary B cells, rodent fibroblasts, and immortalized squamous epithelial cells.[30,39]

Specificity of the Biological Effects of LMP1 by LMP1 Antisense Treatment

After antisense treatment for 3 days, the LMP1 expression level of LMP1-expressing SCM1 cells (SCM-L14 and

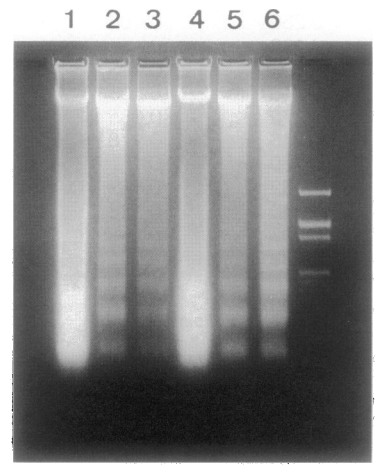

Figure 7. DNA fragmentation of LMP1-expressing gastric carcinoma cells. **Lane 1**, vector-transfected SCM1 cells; **lane 2**, SCM-L14 cells; **lane 3**, SCM-L22 cells; **lane 4**, vector-transfected TMC cells; **lane 5**, TMC-L1 cells; **lane 6**, TMC-L4 cells. The molecular marker, pCEP4 plasmid (Invitrogen) was digested by *Hin*fI.

-L22) and TMC1 cells (TMC-L1 and -L4) was assayed by Western blotting, which showed obvious inhibition of LMP1 expression (Figure 3). After antisense treatment, the vector-transfected SCM1 and TMC1 cells showed no significant changes in growth rate, but the LMP1-expressing SCM1 and TMC1 cells showed an obviously increased growth rate when compared with the relative LMP1-expressing gastric carcinoma cells without antisense treatment (Figures 4 and 5). In the colony-forming assay with antisense treatment, the vector-transfected SCM1 cells showed a slightly reduced colony-forming efficiency and mean colony size, but the LMP1-expressing SCM1 cells (SCM-L14 and -L22) showed an increased colony-forming efficiency and mean colony size (Table 2). The cellular morphology and colony shape of LMP1-expressing and vector-transfected SCM1 cells showed no obvious change after antisense treatment. The growth rate, colony-forming efficiency, and mean colony size of LMP1-expressing gastric carcinoma cells was partially reversed by LMP1 antisense treatment. For treatment with the control oligonucleotide, there were no significant findings except a negligible toxic effect (data not shown). Therefore, it is conceivable that the reduction of growth rate, colony-forming efficiency, mean colony size, and tumorigenicity and the morphological alterations of LMP1-expressing gastric cells resulted from the biological effects of LMP1.

Discussion

In this study, we explored the biological effects of the LMP1 gene (from the B95.8 strain of EBV without the specific 30-bp deletion in exon 3 found in the NPC strain of EBV[33,34]) in gastric carcinoma by transfecting it in EBV-negative gastric carcinoma cells. The comparison of LMP1 transfection effects between gastric carcinoma and NPC cells were performed, and these results suggest that the consequence of LMP1 expression in NPC cells is a growth enhancement, but it is a growth suppression in gastric carcinoma cells. Multiple monoclonal LMP1-expressing gastric carcinoma cell lines were randomly selected for the additional characterization of the biological effects of LMP1 in gastric carcinoma cells. The LMP1-expressing gastric carcinoma cells had a reduced growth rate, colony-forming efficiency, mean colony size, and tumorigenicity and a lower malignant cytological grade when compared with vector-transfected cells. When LMP1 expression was blocked with LMP1 antisense *in vitro*, the reduced growth rate, colony-forming efficiency, and mean colony size of LMP1-expressing gastric carcinoma cells was partially reversed. In addition, there was no LMP1-inducible *bcl-2* expression to protect the gastric carcinoma cells from apoptosis induced by LMP1. Our findings showed that LMP1 can negatively modulate the malignant potential of gastric carcinoma cells possibly via the enhancement of apoptosis. LMP1 is a unique viral oncoprotein on account of its oncogenic potential in rodent fibroblast, B-lymphocytes, and epithelial cells,[40] but it also exhibits cytotoxicity in a variety of cell lines[29] and induces apoptosis in squamous epithelial cells when expressed at high levels.[30] Collectively, these findings suggest that LMP1 has paradoxical biological effects: an oncogenic potential associated with cytotoxicity. However, our findings also suggest that gastric carcinoma cells are more sensitive to the toxic effects of LMP1 than NPC cells.

Oncogenes associated with toxicity have also been demonstrated in v-*abl*,[41] v-*src*,[42] v-*rel*,[43] and c-*myc*.[44] For example, c-*myc* is essential for cell proliferation, but it also can induce cellular apoptosis. The activation of apoptosis by c-*myc* is dependent on regions that are also essential for transformation.[44] Similarly, mutant analysis has shown that the same domains of LMP1 are responsible for both the transforming function and the toxic effects.[29] The co-expression of c-*myc* and *bcl-2* is commonly present in human follicular lymphoma.[45] In addition, the apoptotic effects of c-*myc* can be blocked by the ectopic expression of *bcl-2*.[46] Similarly, the coexistence of activation of oncogenes and inactivation of the *p53* gene has been commonly observed in human cancers.[47–49] It was proposed that development of tumors would involve the deregulation of cellular proliferation and suppression of apoptosis.[46] In EBV-infected normal B cells, the EBV gene products that initiate and maintain B cell growth and immortalization *in vitro* include six nuclear antigens, EBNAs 1, 2, 3a, 3b, 3c, and LP, and two latent membrane proteins, LMPs 1 and 2.[50] The associated overexpression of *bcl-2* also was found.[51] The *in vitro* studies have suggested that LMP1 is essential for primary B cell transformation,[24] which can induce *bcl-2* expression to protect B cells from apoptosis[20] and cooperate with EBNA2 for the induction of B cell activation.[52] On the other hand, there were no similar effects demonstrated in the EBV-infected normal T cells. In EBV-infected normal human thymocytes ($CD21^+$), the EBV genome is in linear form, and its gene expression includes EBNA1 from the Fp promoter, EBNA2, ZEBRA, RAZ, and gp350/220 but no LMPs or EBERs, which has some of the same specific characteristics of lytic replication.[53] However, outgrowth of immortalized T-cell clones from EBV-infected thymocytes have not been observed, which suggests that, in contrast with B cells, EBV may be incapable of immortalizing T cells *in vitro* due to the lack of suppression of apoptosis.[54]

The EBV-encoded LMP1 gene can induce the expression of ICAM1, LFA1 and LFA3,[19,25] which are the essential mediators for conjugate formation between T lymphocyte and target cells.[55] In addition, cytotoxic T lymphocyte epitopes have also been identified in the LMP1 gene.[56] Collectively, it was predicted that the expression of LMP1 may itself be influenced by biological effects in host cells and the patient's immunosurveillance function. In EBV-associated neoplasms, LMP1 expression is frequently observed in NPC,[57] Hodgkin's diseases,[4,58] nasal T-cell lymphomas,[2,3] AIDS-related lymphomas, and post-transplant lymphomas,[59–62] but it is rarely present in endemic Burkitt's lymphoma[63–65] or gastric carcinomas.[7,9,11] A characteristic genotype of LMP1 with a 30-bp deletion and several single-base mutations at the carboxyl-terminal end of exon 3 were initially identified in NPC.[33,34] This mutant LMP1 gene may also be demon-

strated in EBV-associated Hodgkin's diseases,[66] Burkitt's lymphoma,[67] B-immunoblastic lymphoma, and peripheral T-cell lymphomas.[68–70] It has been shown to induce a more aggressive malignant transformation of epithelial cells[71] and has nonimmunogenicity in a murine carcinoma model that may escape immunologically mediated elimination.[72] In NPC, the elevated expression of adhesion molecules LFA3 and ICAM1 in tumor cells may be induced by LMP1,[73] but the NPC cells' ability to escape from immunosurveillance may be due to the defective activity of natural killer cells in NPC patients[74,75] and/or the nonimmunogenicity of the specific mutant LMP1 gene.[72] In addition, the LMP1 apoptotic effects may be overcome by the overexpression of *bcl-2* in NPC.[36,76] Thus, the consequence of LMP1 expression in NPC is an oncogenic potential. LMP1 is also frequently expressed in Hodgkin's disease,[58,77,78] nasal T-cell lymphomas,[2,3] AIDS-related lymphomas, and post-transplant lymphomas,[59–62,79,80] in which LMP1 apoptotic effects may be inhibited by the expression of *bcl-2*,[81] EBV-encoded BHRF-1 protein (a viral homologue of *bcl-2*),[3,82,83] or other unknown factors. In addition, the defective immunofunction of these patients and/or nonimmunogenicity of the specific mutant LMP1 may also provide a growth advantage for tumor cells. In EBV-associated Burkitt's lymphoma, the LMP1 gene may be a mutant variant,[67] but LMP1 expression was mainly restricted by the extensive methylation of EBV DNA.[84] This restriction may be attributed to the absence of *bcl-2* expression[65] to inhibit the LMP1 apoptotic effects. Consequently, this effect may result in down-regulation of LFA1, LFA3, and ICAM1 expression and the ability to escape from immunosurveillance.[85] In gastric carcinoma, EBV infection is independent of *bcl-2* expression.[86] Our study also showed that there was no LMP1-inducible *bcl-2* expression in LMP1-expressing gastric carcinoma cells. The EBV-specific T-cell-mediated immunity is normally retained in patients with EBV-associated gastric carcinoma.[7] It is conceivable that the restricted LMP1 expression due to gene methylation in EBV-associated gastric carcinomas[7] may provide a growth advantage for tumor cells by avoiding the LMP1 apoptotic effect and/or escaping from the patient's immunosurveillance function.

In conclusion, this study demonstrated that gastric carcinoma cells were more sensitive to the toxic effects of LMP1 than NPC cells. LMP1 was capable of negatively modulating the malignant potential of gastric carcinoma cells, including the reduction of growth rate, colony-forming efficiency, tumorigenicity, and malignant cytological grade of tumor cells possibly via the enhancement of apoptosis. Thus, restriction of LMP1 expression in EBV-associated gastric carcinomas may account for the advantage of tumor development and progression and the ability to escape from the immunologically mediated elimination.

Acknowledgments

We thank Professor Yu-Sun Chang (Department of Microbiology and Immunology, Chang-Gung College of Medicine and Technology, Taoyuan, Taiwan, ROC) for kindly providing the S12 antibody, Jin-Yan Ding and Chi-Fang Liu for their excellent technical assistance, and Dr. Fu-Gong Lin for his statistical analyses.

References

1. Miller G: Epstein-Barr virus: biology, pathogenesis, and medical aspects. Virology. Edited by Fields BN, Knipe DM. New York, Raven Press, 1990, pp 1921–1958
2. Harabuchi Y, Imai S, Wakashima J, Hirao M, Kataura A, Osato T, Kon S: Nasal T-cell lymphoma causally associated with Epstein-Barr virus: clinicopathologic, phenotypic, and genotypic studies. Cancer 1996, 77:2137–2149
3. Chiang AKS, Tao Q, Srivastava G, Ho FCS: Nasal NK- and T-cell lymphomas share the same type of Epstein-Barr virus latency as nasopharyngeal carcinoma and Hodgkin's disease. Int J Cancer 1996, 68:285–290
4. Herbst H, Niedobitek G, Kneba M, Hummel M, Finn T, Anagnostopoulos I, Bergholz M, Krieger G, Stein H: High incidence of Epstein-Barr virus genomes in Hodgkin's disease. Am J Pathol 1990, 137: 13–18
5. Burke AP, Yen TS, Shekitka KM, Sobin LH: Lymphoepithelial carcinoma of the stomach with Epstein-Barr virus demonstrated by polymerase chain reaction. Mod Pathol 1990, 3:377–380
6. Shibata D, Weiss LM: Epstein-Barr virus-associated gastric adenocarcinoma. Am J Pathol 1992, 140:769–774
7. Imai S, Koizumi S, Sugiura M, Tokunaga M, Uemura Y, Yamamoto N, Tanaka S, Sato E, Osato T: Gastric carcinoma: monoclonal epithelial malignant cells expressing Epstein-Barr virus latent infection protein. Proc Natl Acad Sci USA 1994, 91:9131–9135
8. Levine PH, Stemmermann G, Lennette ET, Hildesheim A, Shibata D, Nomura A: Elevated antibody titers to Epstein-Barr virus prior to the diagnosis of Epstein-Barr virus-associated gastric adenocarcinoma. Int J Cancer 1995, 60:642–644
9. Harn HJ, Chang JY, Wang MW, Ho LI, Lee HS, Chiang JH, Lee WH: Epstein-Barr virus-associated gastric adenocarcinoma in Taiwan. Hum Pathol 1995, 26:267–271
10. Sugiura M, Imai S, Tokunaga M, Koizumi S, Uchizawa M, Okamoto K, Osato T: Transcriptional analysis of Epstein-Barr virus gene expression in EBV-positive gastric carcinoma: unique viral latency in the tumor cells. Br J Cancer 1996, 74:625–631
11. Kida Y, Miyauchi K, Takano Y: Gastric adenocarcinoma with differentiation to sarcomatous components associated with monoclonal Epstein-Barr virus infection and LMP1 expression. Virchows Arch A Pathol Anat 1993, 423:383–387
12. Fennewald S, van Santen V, Kieff E: The nucleotide sequence of a messenger RNA transcribed in latent growth transforming virus infection indicates that it may encode a membrane protein. J Virol 1984, 51:411–419
13. Hennessy K, Fennewald S, Hummel M, Cole T, Kieff E: A membrane protein encoded by Epstein-Barr virus in latent growth-transforming infection. Proc Natl Acad Sci USA 1984, 81:7201–7211
14. Liebowitz D, Wang D, Kieff E: Orientation and patching of the latent infection membrane protein encoded by Epstein-Barr virus. J Virol 1986, 58:233–237
15. Rothe M, Wong SC, Henzel WJ, Goeddel DV: A novel family of putative signal transducers associated with the cytoplasmic domain of the 75 kD tumor necrosis receptor. Cell 1994, 78:681–692
16. Mosialos G, Birkenbach M, Yalamanchili R, VanArsdale T, Ware C, Kieff E: The Epstein-Barr virus transforming protein LMP1 engages signalling protein for the tumor necrosis factor receptor family. Cell 1995, 80:389–399
17. Wang D, Leibowitz D, Kieff E: An EBV membrane protein expressed in immortalized lymphocyte transforms established rodent cells. Cell 1985, 43:831–840
18. Baichwal VJ, Sugden B: Transformation of Balb 3T3 cells by the BNLF-1 gene of Epstein-Barr virus. Oncogene 1988, 2:461–467
19. Wang D, Liebowitz D, Wang F, Gregory C, Rickinson A, Larson R, Springer T, Kieff E: Epstein-Barr virus latent infection membrane protein alters the human B-lymphocyte phenotype: deletion of the amino terminus abolishes activity. J Virol 1988, 62:4173–4184

20. Henderson S, Rowe M, Gregory C, Croom-Carter D, Wang F, Longnecker R, Kieff E, Rickinson A: Induction of bcl-2 expression by Epstein-Barr virus latent membrane protein 1 protects infected B cells from programmed cell death. Cell 1991, 65:1107–1114
21. Hammarskjold ML, Simurda MC: Epstein-Barr virus latent membrane protein transactivates the human immunodeficiency virus type 1 long terminal repeat through induction of NF-κB activity. J Virol 1992, 66:6496–6501
22. Laherty CD, Hu HM, Opipari AW, Wang F, Dixit VM: The Epstein-Barr virus LMP1 gene induces A20 zinc finger protein expression by activating nuclear factor κB. J Biol Chem 1992, 267:24157–24160
23. Okan I, Wang Y, Chen F, Hu LF, Imreh S, Klein G, Wiman KG: The EBV-encoded LMP1 protein inhibits p53-triggered apoptosis but not growth arrest. Oncogene 1995, 11:1027–1031
24. Kaye KM, Izumi KM, Kieff E: Epstein-Barr virus latent membrane protein 1 is essential for B-lymphocyte growth transformation: Proc Natl Acad Sci USA 1993, 90:9150–9154
25. Dawson CW, Rickinson AB, Young LS: Epstein-Barr virus latent membrane protein inhibits human epithelial cell differentiation. Nature 1990, 344:777–780
26. Fahraeus R, Rymo L, Rhim JS, Klein G: Morphological transformation of human keratinocyte expressing the LMP gene of Epstein-Barr virus. Nature 1990, 345:447–449
27. Miller WE, Shelton Earp H, Raab-Traub N: The Epstein-Barr virus latent membrane protein 1 induces expression of the epidermal growth factor receptor. J Virol 1995, 69:4390–4398
28. Wilson JB, Weinberg W, Johnson R, Yuspa S, Levine AJ: Expression of the BNLF-1 oncogene of Epstein-Barr virus in the skin of transgenic mice induces hyperplasia and aberrant expression of keratin 6. Cell 1990, 61:1315–1327
29. Hammerschmidt W, Sugden B, Baichwal VR: The transforming domain alone of the latent membrane protein of Epstein-Barr virus is toxic to cells when expressed at high level. J Virol 1989, 63:2469–2475
30. Lu JJY, Chen JY, Hsu TY, Yu WCY, Su IJ, Yang CS: Induction of apoptosis in epithelial cell by Epstein-Barr virus latent membrane protein 1. J Gen Virol 1996, 77:1883–1892
31. Oda K, Tamaru J, Takenouchi T, Mikata A, Nunomura M, Saitoh N, Sarashina H, Nakajima N: Association of Epstein-Barr virus with gastric carcinoma with lymphoid stroma. Am J Pathol 1993, 143:1063–1071
32. Glaser R, Zhang HY, Yao K, Zhu HC, Wang FX, Li GY, Wen DS, Li YP: Two epithelial tumor cell lines (HNE-1 and HONE-1) latently infected with Epstein-Barr virus that were derived from nasopharyngeal carcinomas. Proc Natl Acad Sci USA 1989, 86:9524–9528
33. Hu LF, Zabarowsky ER, Chen F, Cao SL, Ernberg I, Klein G, Winberg G: Isolation and sequencing of the Epstein-Barr virus BNLF-1 gene (LMP1) from a Chinese nasopharyngeal carcinoma. J Gen Virol 1991, 72:2399–2409
34. Chen ML, Tsai CN, Liang CL, Shu CH, Huang CR, Sulitzeanu D, Liu ST, Chang YS: Cloning and characterization of the latent membrane protein (LMP) of a specific Epstein-Barr virus variant derived from the nasopharyngeal carcinoma in the Taiwanese population. Oncogene 1992, 7:2131–2140
35. Mann KP, Staunton D, Thorley-Lawson DA: Epstein-Barr virus encoded protein found in plasma membrane of transformed cells. J Virol 1985, 55:710–720
36. Sheu LF, Chen A, Meng CL, Ho KC, Lin FG, Lee WH: Analysis of bcl-2 expression in normal, inflamed, dysplastic nasopharyngeal epithelia, and nasopharyngeal carcinoma: association with p53 expression. Hum Pathol 1997, 28:556–562
37. Haldar S, Negrini M, Monne M, Sabvioni S, Croce CM: Down-regulation of bcl-2 by p53 in breast cancer cells. Cancer Res 1994, 54: 2095–2097
38. Wagner AJ, Small MB, Hay N: Myc-mediated apoptosis is blocked by ectopic expression of bcl-2. Mol Cell Biol 1993, 13:2432–2440
39. Martin JM, Veis D, Korsmeyer SJ, Sugden B: Latent membrane protein of Epstein-Barr virus induces cellular phenotypes independently of expression of Bcl-2. J Virol 1993, 67:5269–5278
40. Knecht H, Brousset P, Bachmann E, Sandvej K, Odermatt BF: Latent membrane protein 1: a key oncogene in EBV-related carcinogenesis? Acta Haematol 1993, 90:167–171
41. Ziegler SF, Whitlock CA, Goff SP, Gifford A, Witte ON: Lethal effect of the Abelson murine leukemia virus transforming product. Cell 1981, 27:477–486
42. Tarpley WG, Temin HM: The location of v-src in a retrovirus determines whether the virus is toxic or transforming. Mol Cell Biol 1984, 4:2653–2660
43. Schwartz RC, Witte ON: A recombinant murine retrovirus expressing v-rel is cytopathic. Virology 1988, 165:182–190
44. Evan GL, Wyllie AH, Gilbert CS, Littlewood TD, Land H, Brooks M, Waters CM, Penn LZ, Hancock DC: Induction of apoptosis in fibroblast by c-Myc protein. Cell 1992, 69:119–128
45. Tsujimoto Y, Cossman J, Jaffe E, Croce M: Involvement of the bcl-2 gene in human follicular lymphoma. Science 1985, 228:1441–1443
46. Wagner AJ, Small MB, Hay N: Myc-mediated apoptosis is blocked by ectopic expression of bcl-2. Mol Cell Biol 1993, 13:2432–2440
47. Cooper GM: Role of oncogenes and tumor suppressor genes in the pathogenesis of neoplasms. Oncogenes. Edited by Cooper GM. Boston, Jones and Bartlett Publishers, 1995, pp 162–180
48. Hollstein M, Sidransky D, Vogelstein B, Harris CC: p53 mutations in human cancer. Science 1991, 253:49–53
49. Vogelstein B, Kinzler KW: The multistep nature of cancer. Trends Genet 1993, 9:138–141
50. Kieff E, Liebowitz D: Epstein-Barr virus and its replication. Virology. Edited by Fields BN, Knipe DM. New York, Raven Press, 1990, pp 1889–1920
51. Tsujimoto Y: Overexpression of the human bcl-2 gene product results in growth enhancement of Epstein-Barr virus-immortalized B cells. Proc Natl Acad Sci USA 1989, 86:1958–1962
52. Wang F, Gregory C, Sample C, Rowe M, Liebowitz D, Murray R, Rickinson A, Kieff E: Epstein-Barr virus latent membrane protein (LMP1) and nuclear proteins 2 and 3C are effectors of phenotypic changes in B lymphocytes: EBNA-2 and LMP1 cooperatively induce CD23. J Virol 1990, 64:2309–2318
53. Kelleher CA, Kaufman Paterson RL, Dreyfus DH, Streib JE, Xu JW, Takase K, Jones JF, Gelfand EW: Epstein-Barr virus replicative gene transcription during de novo infection of human thymocytes: simultaneous early expression of BZLF-1 and its repressor RAZ. Virology 1995, 208:685–695
54. Kaufman Paterson RL, Kelleher CA, Streib JE, Amankonah TD, Xu JW, Jones JF, Gelfand EW: Activation of human thymocyte after infection by EBV. J Immunol 1995, 154:1440–1449
55. Springer TA: Adhesion receptors of the immune system. Nature 1990, 346:425–434
56. Khanna R, Burrows SR, Kurilla MG, Jacob CA, Misko IS, Scully TB, Kieff E, Moss DJ: Localization of Epstein-Barr virus cytotoxic T cell epitopes using recombinant vaccinia: implications for vaccine development. J Exp Med 1992, 176:169–176
57. Fahraeus R, Fu HL, Ernberg I, Finke J, Rowe M, Klein G, Falk K, Nilsson E, Yadav M, Busson P, Tursz T, Kallin B: Expression of Epstein-Barr virus-encoded proteins in nasopharyngeal carcinoma. Int J Cancer 1988, 42:329–338
58. Pallesen G, Hamilton-Dutoit SJ, Rowe M, Young LS: Expression of Epstein-Barr virus latent gene products in tumor cells of Hodgkin's disease. Lancet 1991, 1:320–322
59. Hamilton-Dutoit SJ, Rea D, Raphael M, Sandvej K, Delecluse HJ, Gisselbrecht C, Marelle L, van Krieken JHJM, Pallesen G: Epstein-Barr virus-latent gene expression and tumor cell phenotype in acquired immunodeficiency syndrome-related non-Hodgkin's lymphoma: correlation of lymphoma phenotype with three distinct patterns of viral latency. Am J Pathol 1993, 143:1072–1085
60. Carbone A, Gloghini A, Volpe R, Boiocchi M, Tirelli U, Italian T: High frequency of Epstein-Barr virus latent membrane protein-1 expression in acquired immunodeficiency syndrome-related Ki-1 (CD30)-positive anaplastic large-cell lymphomas. Am J Clin Pathol 1994, 101:768–772
61. Delecluse HJ, Kremmer E, Rouault JP, Cour C, Bornkamm G, Berger F: The expression of Epstein-Barr virus latent proteins is related to the pathological features of post-transplant lymphoproliferative disorders. Am J Pathol 1995, 146:1113–1120
62. Chetty R, Biddolph S, Kaklamanis L, Cary N, Stewart S, Giatromanolaki A, Gatter K: bcl-2 protein is strongly expressed in post-transplant lymphoproliferative disorders. J Pathol 1996, 180:254–258
63. Rowe DT, Rowe M, Evan GI, Wallace LE, Farrell PJ, Rickinson AB: Restricted expression of EBV latent genes and T-lymphocyte de-

tected membrane antigen in Burkitt's lymphoma cells. EMBO J 1986, 5:2599–2607
64. Rowe M, Rowe DT, Gregory CD, Young LS, Farrell PJ, Rupani H, Rickinson AB: Differences in B cell growth phenotype reflect novel patterns of Epstein-Barr virus latent gene expression in Burkitt's lymphoma cells. EMBO J 1987, 6:2743–2751
65. Niedobitek G, Agathanggelou A, Rowe M, Jones EL, Jones DB, Turyaguma P, Oryema J, Wirght DH, Young LS: Heterogeneous expression of Epstein-Barr virus latent proteins in endemic Burkitt's lymphoma. Blood 1995, 86:659–665
66. Knecht H, Bachmann E, Brousset P, Sandvej K, Nadal D, Bachmann F, Odermatt BF, Delsol G, Pallesen G: Deletions within the LMP1 oncogene of Epstein-Barr virus are clustered in Hodgkin's disease and identical to those observed in nasopharyngeal carcinoma. Blood 1993, 82:2937–2942
67. Chen WG, Chen YY, Bacchi MM, Bacchi CE, Alvarenga M, Weiss LM: Genotyping of Epstein-Barr virus in Brazilian Burkitt's lymphoma and reactive lymphoid tissue: type A with a high prevalence of deletions within the latent membrane protein gene. Am J Pathol 1996, 148: 17–23
68. Sandvej K, Peh SC, Andresen BS, Pallesen G: Identification of potential hot spots in the carboxy-terminal part of the Epstein-Barr virus (EBV) BNLF-1 gene in both malignant and benign EBV-associated diseases: high frequency of a 30-bp deletion in Malaysian and Danish peripheral T-cell lymphomas. Blood 1994, 84:4053–4060
69. Knecht H, Bachmann E, Brousset P, Rothenberger S, Einsele H, Lestou VS, Delsol G, Bachmann F, Ambros PF, Odermatt BF: Mutational hot spots within the carboxy terminal region of the LMP1 oncogene of Epstein-Barr virus are frequent in lymphoproliferative disorders. Oncogene 1995, 10:523–528
70. Chang YS, Su IJ, Chung PJ, Shu CH, Ng CK, Wu SJ, Liu ST: Detection of an Epstein-Barr virus variant in T-cell lymphoma tissues identical to the distinct strain observed in nasopharyngeal carcinoma in the Taiwanese population. Int J Cancer 1995, 62:673–677
71. Hu LF, Chen F, Zheng X, Ernberg I, Cao SL, Christensson B, Klein G, Winberg G: Clonability and tumorigenicity of human epithelial cells expressing the EBV encoded membrane protein LMP1. Oncogene 1993, 8:1575–1583
72. Trivedi P, Hu LF, Chen Fu, Christensson B, Masucci MG, Klein G, Winberg G: Epstein-Barr virus (EBV)-encoded membrane protein LMP1 from a nasopharyngeal carcinoma is non-immunogenic in a murine model system, in contrast to a B cell-derived homologue. Eur J Cancer 1994, 30A:84–88
73. Busson P, Zhang Q, Guillon JM, Gregory CD, Young LS, Clausse B, Lipinski M, Rickinson AB, Tursz T: Elevated expression of ICAM1 (CD54) and minimal expression of LFA3 (CD58) in Epstein-Barr-virus-positive nasopharyngeal carcinoma cells. Int J Cancer 1992, 50:863–867
74. Lynn TC, Wang JH, Yang CS, Tu SM: Natural killer cell activity in patients with nasopharyngeal carcinoma. Chin J Microbiol Immunol 1986, 19:177–182
75. Lakhdar M, Oueslati R, Ellouze R, Thameur H, Cammoun M, Khedhiri N, Kastally R: High interferon titer and defective NK-cell activity in the circulation of nasopharyngeal carcinoma patients. Int J Cancer 1989, 43:543–548
76. Lu QL, Elia G, Lucas S, Thomas A: Bcl-2 proto-oncogene expression in Epstein-Barr-virus-associated nasopharyngeal carcinoma. Int J Cancer 1993, 53:29–35
77. Herbst H, Dallenbach F, Hummel M, Niedobitek G, Pileri S, Muller-Lantzsch N, Stein H: Epstein-Barr virus latent membrane protein expression in Hodgkin and Reed-Sternberg cells. Proc Natl Acad Sci USA 1991, 88:4766–4770
78. Jiwa NM, Oudejans JJ, Bai MC, van der Brule AJC, Horstman A, Vos W, van der Valk P, Kluin PHM, Walboomers JMM, Meijer CJLM: Expression of bcl-2 protein and transcription of the Epstein-Barr virus bcl-2 homologue BHRF-1 in Hodgkin's disease: implications for different pathogenic mechanisms. Histopathology 1995, 26:547–553
79. Schlaifer D, Brousset P, Attal M, Massip P, Payen C, Marchou B, Huguet F, Muller C, Laurent G, Pris J, Delsol G: bcl-2 proto-oncogene and Epstein-Barr virus latent membrane protein-1 expression in AIDS-related lymphoma. Histopathology 1994, 25:77–82
80. Camilleri-Broet S, Davi F, Feuillard J, Bourgeois C, Seilhean D, Hauw JJ, Raphael M: High expression of latent membrane protein 1 of Epstein-Barr virus and bcl-2 oncoprotein in acquired immunodeficiency syndrome-related primary brain lymphomas. Blood 1995, 86: 432–435
81. Hockenbery D, Nunez G, Milliman C, Schreiber RD, Korsmeyer SJ: Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. Nature 1990, 348:334–348
82. Henderson S, Huen D, Rowe M, Dawson C, Johnson G, Rickinson A: Epstein-Barr virus-coded BHRF-1 protein, a viral homologue of bcl-2, protects human B-cells from programmed cell death. Proc Natl Acad Sci USA 1993, 90:8479–8483
83. Oudejans JJ, van den Brule AJC, Jiwa NM, de Bruin PC, Ossenkoppele GJ, van der Valk P, Walboomers JMM, Meijer CJLM: BHRF1, the Epstein-Barr virus (EBV) homologue of the BCL-2 proto-oncogene, is transcribed in EBV-associated B-cell lymphomas and in reactive lymphocyte. Blood 1995, 86:1893–1902
84. Ernberg I, Falk K, Minarovits J, Busson P, Tursz T, Masucci MG, Klein G: The role of methylation in the phenotype-dependent modulation of Epstein-Barr nuclear antigen 2 and latent membrane protein genes in cells latently infected with Epstein-Barr virus. J Gen Virol 1989, 70: 2989–3002
85. Gregory CD, Murray RJ, Edwards CF, Rickinson AB: Down-regulation of cell adhesion molecules LFA-3 and ICAM-1 in Epstein-Barr virus-positive Burkitt's lymphoma underlies tumor cell escape from virus-specific T cell surveillance. J Exp Med 1988, 167:1811–1824
86. Gulley ML, Pulitzer DR, Eagan PA, Schneider BG: Epstein-Barr virus infection is an early event in gastric carcinogenesis and is independent of bcl-2 expression and p53 accumulation. Hum Pathol 1996, 27:20–27