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Epstein-Barr Virus Promotes Epithelial Cell Growth in the Absence of EBNA2 and LMP1 Expression

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We attempted to infect primary gastric epithelia (PGE) with recombinant Epstein-Barr virus (EBV) carrying a selectable marker that made it possible to select EBV-infected cells. Cells dually positive for EBV-determined nuclear antigen (EBNA) and cytokeratin were detected in 3 of 21 primary cultures after 3 days of EBV inoculation. From one culture, EBV-infected cell clones were repeatedly obtained at a frequency of 3 to 5 cell clones per 10^6 cells. EBV-infected clones had enhanced population doubling and grew to attain a highly increased saturation density, together with acquisition of marked anchorage independence. The infected clones retained the ultrastructural morphology characteristic of gastric mucosal epithelium and have been growing stably for more than 18 months (corresponding to at least 300 generations) so far, in clear contrast to the parental PGE cells, which ceased growth after 60 generations. The p53 gene of the parental PGE cells was found to be overexpressed, perhaps thereby conferring the basal potential for long-term survival in vitro. Moreover, EBV infection accelerated, to a significant extent, the growth rate and agar clonability of NU-GC-3 cells, an established EBV-negative but EBV-susceptible human gastric carcinoma cell line. Both EBV-converted PGE and NU-GC-3 clones, like EBV-positive gastric carcinoma biopsy specimens, expressed a restricted set of EBV latent infection genes characterized by the absence of EBNA2 and latent membrane protein 1 (LMP1) expression. These results indicate that EBV infection causes a transformed phenotype on PGE in the setting of possible unregulated cell cycling and renders even established gastric carcinoma cells more malignant via a limited spectrum of viral latent-gene expression. This study may reflect an in vivo scenario illustrating multiphasic involvement of EBV in carcinogenesis of gastric or other epithelial cancers.

Epstein-Barr virus (EBV) is a ubiquitous virus which infects the majority of the human population and is the causative agent of infectious mononucleosis (27). Recently, increasing evidence has linked EBV infection to various epithelioid malignancies as well as lymphoid ones. The very strong association between EBV and nasopharyngeal carcinoma (NPC) is already well known (27). Moreover, the viral genome is detected in rare carcinomas with intense lymphoid stroma (termed lymphoepithelioma-like carcinoma) arising in the salivary glands (29), thymus (6), and stomach (33). In addition, an increasing number of studies have suggested a causal relationship between EBV and primary gastric carcinoma of the more common adenocarcinoma type (11, 33). About 5 to 15% patients with gastric carcinoma in all parts of the world have EBV DNA in 100% of carcinoma cells (11, 13, 18, 30, 34). Analysis of the terminal sequence of EBV plasmid DNA in gastric carcinoma cells indicated that tumor cells arose from a single EBV-infected cell, thus suggesting that EBV infection had occurred in the very early stage of tumor development (13). Gastric carcinoma cells express a limited number of EBV genomes, similar to those in Burkitt's lymphoma, which are EBV-determined nuclear antigen 1 (EBNA1), two small nonpolyadenylated RNAs known as EBER1 and EBER2, the transcripts from the *Bam*HI-A region (BARF0), and latent membrane protein 2A (LMP2A) (13, 37). This is different from the pattern in NPC, in which LMP1 is also expressed in carcinoma cells in about half of the patients (27, 47). Concerning the effects of EBV products on epithelial cells, LMP1 has some pleiotropic biological activities but other gene products do not. LMP1 induces epidermal hyperplasia in transgenic mice (41), alters keratin gene expression in human keratinocytes (7), inhibits cell differentiation in some immortalized epithelial cell lines (5), induces expression of the epidermal growth factor receptor (21), and blocks p53-mediated apoptosis through activation of the A20 gene (8). Thus far, there has been no evidence that EBV provides a continuing contribution to the growth phenotype of EBV-positive gastric carcinoma, which is negative for LMP1 expression.

Although the interaction between EBV and lymphoid cells has been studied extensively, the remarkable resistance of epithelial cells to EBV infection in vitro has hampered studies of the role of EBV in epithelial malignancies. Recently, we generated EBV recombinants with a selectable marker, which makes it possible to select EBV-infected cells even when the efficiency of infection is low or the EBV-uninfected population in culture is able to proliferate (34, 45). Using the recombinant virus, we found that various carcinoma cell lines can be infected with EBV (14, 44) and that their virus convertants consistently express a limited number of EBV latent genes, as EBV-positive gastric carcinoma cells do (14), thus indicating that the system could be a model for EBV oncogenesis. The present study focused mainly on the effect of EBV infection on primary gastric epithelial cells by applying our infection system and demonstrated that EBV promotes epithelial cell growth in the absence of EBNA2 and LMP1 expression.

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MATERIALS AND METHODS

Culture of primary gastric epithelia. Primary gastric epithelia (PGE) were prepared from endoscopically collected or surgically resected gastric specimens from noncancerous patients. The tissues were transferred to cold Hanks' balanced salt solution, minced with blades, and treated at 37°C for 30 min with a mixture of collagenase type I (100 U/ml; GIBCO BRL, Rockville, Md.), hyaluronidase type IV-S (0.05%; Sigma, St. Louis, Mo.) and dispase (2 U/ml; GIBCO BRL) in Hanks' balanced salt solution. The suspension was pipetted several times to completely disperse PGE during incubation and then passed through a mesh. The filtrate was washed twice with DM201 culture medium (Wako Pure Chemical Industries, Osaka, Japan) containing 10% fetal calf serum (FCS) (GIBCO BRL), penicillin (100 U/ml), streptomycin (100 μg/ml), and gentamicin (100 μg/ml). The cells were resuspended in the same medium and seeded into six-well culture plates. Cells adhered to the wells within the following 2 days and slowly grew as a monolayer with small islands. The adherent cells in these cultures were of epithelial origin morphologically and were positive for cytokeratins by immunofluorescence staining.

EBV infection and transfection of PGE cells. Recombinant EBV of the Akata strain (rEBV) carrying the neomycin resistance gene (Neo^r) was used as a source of virus for infection (35, 43). Implanted PGE cells were exposed to rEBV through cell-free infection as previously described (14, 44). Two days after virus inoculation, the cells were reseeded into 24-well culture plates at 5×10^4 cells/ml/well in a medium containing G418 (200 μg/ml; GIBCO BRL) for selection. The Neo^r gene (pcDNA3 vector; Invitrogen, Carlsbad, Calif.) was transfected by using the Lipofectamine Plus reagent (GIBCO BRL), and then the cells were subjected to G418 selection as described above.

Immunofluorescence assay. Expression of EBNA was examined on acetone-methanol-fixed cells by anticomplement immunofluorescence with reference human serum (titer, 1,280). Expression of EBNA2 and LMP1 was tested on acetone-methanol-fixed cells by streptavidin-biotin immunofluorescence with mouse monoclonal antibodies (MAbs) PE2 (46) (a gift of E. Kieff, Harvard Medical School, Boston, Mass.) and CS1-4 (Dako, Glastrup, Denmark), respectively. EBV lytic infection was assessed on acetone-fixed cells by indirect immunofluorescence with MAb C1 (39) (a gift of D. A. Thorley-Lawson, Tufts University, Boston, Mass.), specific to the viral envelope antigen, gp350, and MAb R3 (25) (a gift of G. Pearson, Georgetown University, Washington, D.C.), reactive to the viral early protein encoded by BMRF1. Cytokeratins were stained with a mixture of MAbs AE1 and AE3 (Dako) by indirect immunofluorescence.

To examine the expression of the EBV receptor, CD21, cells were prepared by treatment with 2 mM EDTA–phosphate-buffered saline (pH 7.2) at 37°C for 10 to 15 min, washed with cooled culture medium, and reacted with MAbs OKB7 (Ortho Diagnostics, Raritan, N.J.) and HB-5 (Becton Dickson, San Jose, Calif.) at 4°C for 30 min. The second reaction involved the use of a fluorescein isothiocyanate-labeled $F(ab')_2$ fragment of rabbit antibody to mouse immunoglobulin G (Dako) followed by flow cytometric analysis.

Southern blot analysis. Purified cellular DNA (5 μg) was digested with *Bam*HI, size fractionated by electrophoresis in a 0.7% agarose gel, and transferred to a nylon membrane (Hybond N+; Amersham International plc, Little Chalfont, United Kingdom). Probe DNA was labeled with [α-^{32}P]dCTP (3,000 Ci/ml) by random priming. To detect the EBV genome, the *Bam*HI-K probe was used. The 1.9-kb *Xho*I-a subfragment from *Bam*HI-Net and the *Eco*RI-I and *Bam*HI-C fragments were also used as probes to investigate EBV integration into the cellular DNA, which has occasionally been reported to occur at the termini or within the *Bam*HI-C region of the genome (12, 19, 26). Hybridization was performed at 42°C overnight in 50% formamide–5× Denhardt's solution–5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.5% sodium dodecyl sulfate (SDS) containing salmon testis DNA (100 μg/ml; Sigma). The blot was washed twice in 2× SSC–0.1% SDS for 10 min at room temperature and once in 0.1× SSC–0.1% SDS for 10 min at 65°C and then exposed to X-ray film at −80°C for 15 h.

Morphological characterization. Phase-contrast micrographs of cultured cells were obtained with an inverted microscope. For ultrastructural studies, cells were grown in culture chamber slides (Becton Dickinson), fixed in 2.5% glutaraldehyde, and postfixed in 1% osmium tetroxide. Fixed cells were stained with 1% uranyl acetate, and the slides were examined with an electron microscope.

Immunoblotting. Cells were lysed in SDS-polyacrylamide gel electrophoresis loading buffer, sonicated, and boiled for 5 min. A volume of lysate equal to 10^5 cells was separated in 10% polyacrylamide gels and transferred to a nitrocellulose membrane. After overnight blocking with 5% nonfat dry milk in Tris-buffered saline (TBS-M [pH 7.6]), the membrane was incubated for 2 h at room temperature with human sera optimally diluted (1:50 to 1:200) in TBS-M to detect EBNAs, washed three times with TBS-M containing 0.1% Tween 20 (TBS-TM), and then reacted for 30 min with horseradish peroxidase-conjugated sheep antibodies to human immunoglobulin G (diluted 1:2,000 in TBS-M [Amersham]). Expression of EBNA2 and LMP1 was examined by using MAbs PE2 and CS14, and antibody reaction and washing were done in TBS and TBS-T solutions, respectively. After the second antibody reaction, the filters were washed five times with TBS-T, immersed in the enhanced chemiluminescence solutions (Amersham) as specified by the manufacturer, and subjected to autoradiography. We also examined cytokeratin and p53 expression by immunoblotting with a mixture of MAbs AE1 and AE3 (Dako) and rabbit polyclonal antibody CM1 (Novocastra Laboratories, Newcastle, United Kingdom), respectively. Cell lysates from B lymphoblastoid cell lines (LCL) immortalized by the Akata or B95-8 strain of EBV were used as EBV-positive controls.

***In situ* hybridization.** In situ hybridization was performed to investigate EBV-encoded small RNA 1 (EBER1) expression (40). To do so, 10^4 cells detached by trypsin treatment were dispensed into wells of an eight-chamber slide glass (Nunc-InterMed, Tokyo, Japan) and incubated until the cultures reached 60 to 80% confluence. Then the slides were air dried and fixed with freshly prepared 4% paraformaldehyde–0.1 M phosphate buffer (pH 7.4) overnight at 4°C. After being briefly washed with 0.1 M phosphate buffer, the cells were treated with proteinase K (10 to 30 μg/ml) for 20 min at 37°C, rinsed again in 0.1 M phosphate buffer, and dehydrated in a 70 to 100% ethanol series. Details of the hybridization procedures and probe sequence are described elsewhere (14).

RT-PCR. Reverse transcription-PCR (RT-PCR) analysis was carried out to investigate the expression of LMP2A, LMP2B, and BARF0 and the utilization of EBNA promoters (promoters in *Bam*HI-C, *Bam*HI-W, and *Bam*HI-Q; Cp, Wp, and Qp, respectively). Total cellular RNA was isolated by guanidium isothiocyanate-phenol extraction with TRIzol reagent (GIBCO BRL) as specified by the manufacturer. Extracted RNA was heated for 5 min at 90°C and rapidly cooled on ice. cDNA synthesis was performed for 60 min at 37°C with Moloney murine leukemia virus RTase (GIBCO BRL), using 100 pmol of random hexamer (Takara, Otsu, Japan) followed by 10 min of heating at 94°C to inactivate RTase. The cDNA samples were then subjected to 30 cycles of PCR in a thermal cycler. Each cycle consisted of denaturation for 30 s at 94°C, annealing for 30 s at 45 to 55°C, and extension for 1 min at 72°C. The reaction mixture contained buffers and reagents as described previously (37), with 20 pmol of each primer and cDNA (equivalent to 5×10^4 cells/tube) in a volume of 50 μl. A 5-μl samples of the PCR products was electrophoresed on a 2% agarose gel and blotted onto nylon membranes, and specific amplified DNA was detected with a ^{32}P-end-labeled oligoprobe. Details of primer and probe sequences are described in our previous reports (14, 37). RNAs from LCL and Akata cells (38) were used as positive controls. The quality of the RNA was checked by parallel amplification of β-actin mRNA.

Analysis of cell growth and tumorigenicity in mice. The population-doubling time and saturation (maximal) cell density were determined by seeding the same number of viable cells into wells of 12-well culture plates (2 ml of medium) or 6-well culture plates (5 ml of medium) and counting the number of cells in each well every day for 10 days. Growth curves in liquid culture were obtained at standard and low serum concentrations. Anchorage-independent growth ability was assessed by plating cells in six-well culture plates at variable cell numbers (from 0.25×10^4 to 5×10^4 viable cells) suspended in DM201 medium–0.4% agarose (SeaPlaque; Takara) containing 20% FCS as a triplicate culture for each cell number. They were incubated at 37°C under 5% CO_2 for 4 weeks. Tumorigenicity was tested by inoculating 10^7 cells subcutaneously into athymic nude mice and SCID mice (female, BALB/c background), which were subsequently observed for progressive tumor formation for 12 weeks.

DNA sequencing. Exons 5 to 8 of the p53 gene were PCR amplified with fluorescently labeled dideoxy chain-terminating nucleotides (Thermo Sequenase Dye terminator cycle-sequencing premix kit; Amersham) basically as specified by the supplier. Primers for PCR and direct sequencing were based on published sequence (32). PCR products were purified and analyzed on an automated DNA sequencer (model 373; Applied Biosystems Inc., Foster City, Calif.).

RESULTS

EBV infection of primary gastric epithelial cells. We could obtain a sufficient number of cells for infection experiments from 21 independent PGE cultures. Successful EBV infection was discerned in three of these cultures by coexpression of EBNA and cytokeratins (up to 0.2%), and the following selection culture produced several G418-resistant clones from a single PGE culture (referred to as PGE-5). In the other primary cultures, cells became extinct by 10 days after addition of G418 to the medium or ceased to grow within 1 month even in the absence of G418. The drug-resistant PGE-5 cells have been growing stably for more than 18 months (corresponding to at least 300 generations) so far, with weekly subcultivation by diluting them 1:10. All of the G418-resistant PGE-5 clones showed virtually 100% positivity for EBNA by immunofluorescence assay (Fig. 1A and 1B). EBV infection of the resistant clones was confirmed by Southern hybridization with an EBV *Bam*HI-K probe (Fig. 1C). In each EBV-carrying clone, hybridization of *Bam*HI-digested DNA with the *Xho*I-a probe detected a single terminal fragment band, and rehybridization of the same blots with the *Eco*RI-I probe also detected a single band identical in size to that detected by the *Xho*I-a probe

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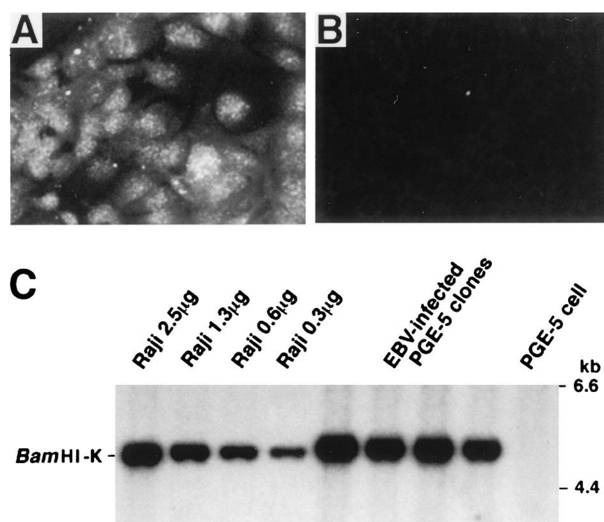

FIG. 1. Detection of EBV in G418-resistant PGE-5 clones. (A) Immunofluorescent staining of EBNA in a G418-resistant PGE-5 clone with EBV-seropositive human serum. Magnification, ×400. (B) Immunofluorescent staining in the same PGE-5 clone with EBV-seronegative human serum as a control. Magnification, ×400. (C) Southern blot analysis of G418-resistant PGE-5 clones. All DNA samples were digested with *Bam*HI, and the blot was probed with a *Bam*HI-K fragment of EBV DNA. Serially diluted Raji cell DNAs served as positive controls. Each lane of PGE-5 clones and parental PGE-5 cells contained 5 μg of DNA. All G418-resistant PGE-5 clones were estimated to carry more than 25 copies of the EBV genome per cell.

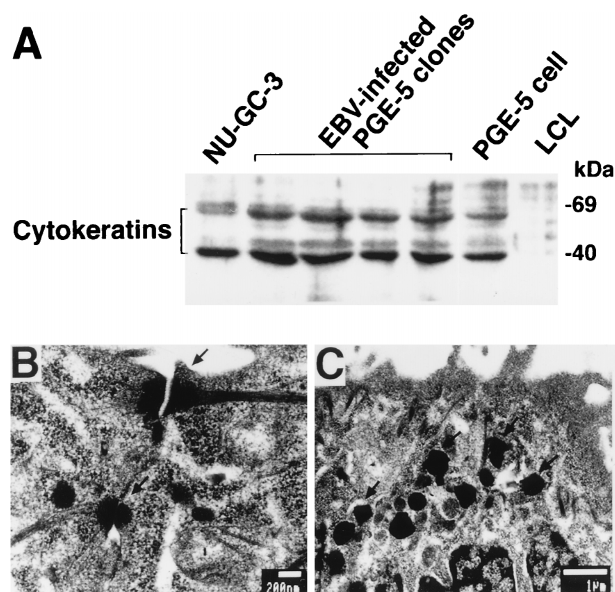

FIG. 2. Demonstration of the epithelial nature of PGE-5 cells. (A) Immunoblot analysis for cytokeratin expression in EBV-infected and -uninfected PGE-5 cells. A mixture of MAbs AE1 and AE3 detected cytokeratins as multisized specific bands between 40 and 66 kDa. A gastric carcinoma cell line, NU-GC-3, served as a positive control, and LCL served as a negative control. (B and C) Transmission electron micrographs of EBV-infected PGE-5 cells show the junctional complex (arrows) (B) and mucus granules (arrows) (C). Bars in panels B and C denote 200 nm and 1 μm, respectively. Magnifications, ×8,000 (B) and ×20,000 (C).

(data not shown). The *Bam*HI-C probe identified the standard *Bam*HI-C fragment in all clones tested. These results signified that EBV is maintained in the G418-resistant PGE-5 clones as an episomal form, not integrated into the cellular DNA. Unexpectedly, parental PGE-5 cells could be propagated, although slowly, for a certain period; they became extinct after 30 passages (the life span was approximately 60 generations). EBV-infected, G418-resistant cell clones were reproducibly obtained from the PGE-5 culture of passages 5 through 8 at the frequency of 3 to 5 cell clones per 10^6 initial cells.

Immunofluorescence analysis showed cytokeratin expression in 100% of the cells. Western blot analysis also confirmed cytokeratin expression (Fig. 2A). Electron microscopic observation demonstrated that PGE-5 cells had junctional complexes (tight junctions and desmosomes) and many electron-dense mucus granules (Fig. 2B and C). These findings provided conclusive evidence for the gastric mucosal epithelium origin of PGE-5 cells. Parental PGE-5 cells of passages 5 through 8 previously stored at −152°C were thawed and transfected with a Neo^r gene, thereby providing a total of six Neo^r-carrying clones (neo-PGE-5), which served as a control for the following analyses.

We could not detect the expression of CD21 in PGE-5 cells by flow-cytometric analysis with anti-CD21 MAbs (OKB7 and HB-5) or by sensitive RT-PCR (data not shown). Moreover, addition of MAb OKB7, which recognizes and blocks the EBV-binding site on the CD21 molecule, to the culture did not reduce the incidence of emergence of EBV-infected PGE clones. These results were compatible with our previous data indicating that direct EBV infection of epithelial cells could be mediated by a novel epithelium-specific binding receptor for EBV (14, 44).

EBV gene expression in PGE-5 clones. PGE-5 clones infected with rEBV were examined for the expression of EBV genes. All EBV-infected clones were positive for EBNA1 but negative for the other EBNAs and LMP1 in immunoblot (Fig. 3A) and immunofluorescence assays (data not shown). In situ hybridization studies showed that EBER1 expression was observed specifically in the nuclei of all cells of each EBV-infected PGE-5 clone (Fig. 3B). RT-PCR analysis revealed that infected clones used exclusively Qp, not Cp or Wp, for EBNA1 transcription (Fig. 3C), confirming that a restricted program of latency is maintained in these cells (22, 31). The expression of LMP1, LMP2A, LMP2B, and BARF0 was further examined by RT-PCR, which showed that EBV-infected PGE-5 clones expressed LMP2A and BARF0 but not LMP1 or LMP2B (Fig. 3C). EBV replicative antigen-expressing cells were rarely seen (<0.1%) in virus-infected clones. Taken together, these results indicated that the viral latency in EBV-infected PGE-5 clones was similar to that typically observed in EBV-associated gastric carcinoma cells (13, 37) and LMP1-negative NPC cells (47).

Morphological and growth characteristics of EBV-infected PGE cells. Morphologically, individual cells of neo-PGE-5 clones as well as EBV-uninfected parent PGE-5 had a so-called cobblestone shape, as a population, they showed a pavement-like growth appearance (Fig. 4A). EBV-infected PGE-5 cells, on the other hand, had bipolar spindle-shaped morphology at the single-cell level. They were highly condensed, with a seemingly parallel orientation to each other, near to the saturation phase (Fig. 4B). Furthermore, when their growth kinetics were compared, EBV conversion clearly gave rise to enhanced population doubling with higher maximum confluence (Fig. 4B and C). The doubling time and maximal cell density of each cell group cultured in 2 ml of 10% FCS-containing medium in 12-well plates were estimated to be 85 h and 4.5×10^5 cells/well for neo-PGE-5 clones and 50 h and 9.2×10^5 cells/well for EBV-infected PGE-5 clones. These values for neo-PGE-5 and EBV-infected PGE-5 clones were calculated as an average of the results from four clones. A reduction of the

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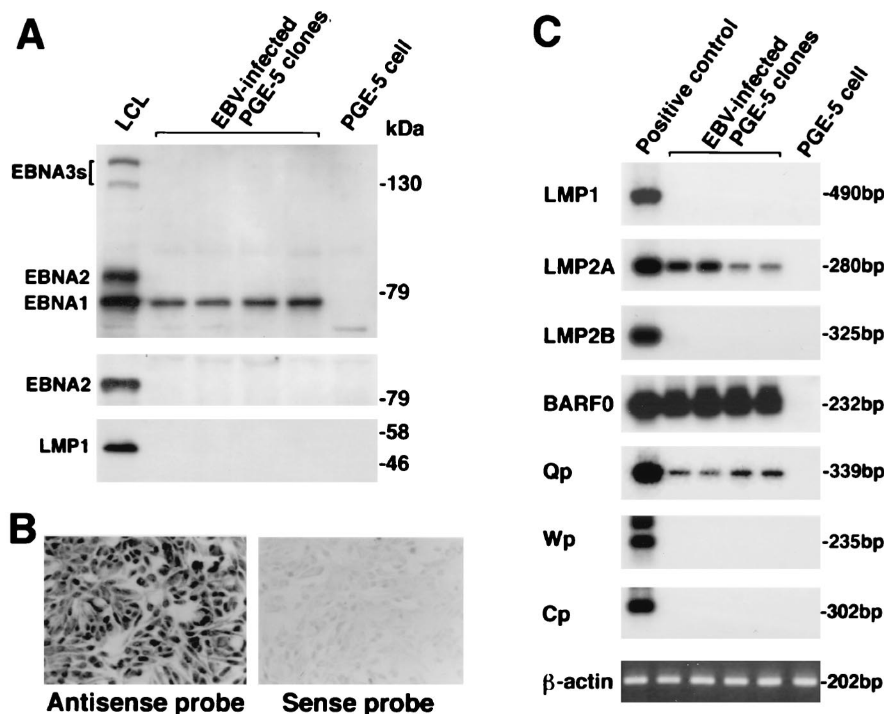

FIG. 3. Analysis of EBV latent-gene expression in EBV-infected PGE-5 cell clones. (A) Immunoblotting for detection of EBNAs and LMP1. The blots were probed with pooled human sera for EBNA1, EBNA2, EBNA3A, EBNA3B, and EBNA3C (top blot), MAb PE2 for EBNA2 (middle blot), and MAb CS1-4 for LMP1 (bottom blot). Protein samples extracted from 10^5 cells were loaded per slot. (B) In situ hybridization for EBER1. An EBV-infected PGE-5 clone was hybridized with the antisense oligoprobe (left) and with the sense oligoprobe (right). Intense nuclear signals are evident only with the antisense probe. (C) RT-PCR analysis of EBV latent-gene expression and EBNA promoter usage in EBV-infected clones. Akata cells were used as a positive control for detection of Qp-initiated EBNA mRNA, and LCL was used as a positive control for detection of LMP1, LMP2A, LMP2B, and BARF0 mRNAs and Cp- or Wp-initiated EBNA mRNAs. Parental PGE-5 cells served as a negative control.

concentration of FCS in the medium to 0.1% still allowed EBV-infected clones to grow with a slightly prolonged doubling time of 70 h and a lower saturation density of 7.2×10^5 cells/well, whereas Neo^r-transfected clones could not proliferate (Fig. 4C). In agreement with the results of fluid culture, a striking difference in clonability in semisolid agar was also found between neo-PGE-5 and EBV-infected PGE-5 clones (Table 1). In the soft-agarose assay, neo-PGE-5 clones formed only small colonies (each comprising less than 20 cells) during the first 2 weeks; however, most of them stopped growing thereafter and finally died by 3 weeks after seeding (Fig. 5A). In sharp contrast, EBV-infected clones continuously grew and finally formed large colonies by 3 weeks after seeding (Fig. 5B). Two of three EBV-infected clones examined transiently produced subcutaneous tumors in SCID mice (10 to 15 mm in diameter) around 6 weeks postinoculation, although the tumors regressed after 10 weeks. PGE-5 cells and neo-PGE-5 clones did not form tumors in SCID mice. None of them was tumorigenic in nude mice, irrespective of EBV infection.

Overexpression of the p53 gene in EBV-infected and -uninfected PGE-5 cells. To investigate the possibility that a cellular gene confers intrinsic growth potential on parental PGE-5 cells, which is represented by their prolonged survival in vitro, we analyzed the expression of a tumor suppressor gene, p53. Immunoblot analysis clearly showed that p53 accumulated not only in all EBV-infected clones but also in parental PGE-5 cells (Fig. 6). On the other hand, p53 was not detected in other two PGE cultures. Subsequent nucleotide sequencing of exons 5 to 8 of the p53 gene by the PCR-based method failed to identify mutations or deletions in PGE-5 cells but detected a single nucleotide mutation in intron 5.

Growth alteration of a gastric carcinoma cell line by EBV infection. We further investigated whether EBV endows an already established carcinoma cell line with more malignant phenotypes. Of the various human epithelial cell lines that we previously succeeded in infecting with EBV in vitro, an EBV-negative gastric carcinoma cell line, NU-GC-3 (1), was used for this purpose. EBV-converted NU-GC-3 clones displayed, as already reported (14), the same program of viral gene expression as EBV-associated gastric carcinoma cells and EBV-infected PGE-5 cells in the present study (Fig. 7A). In medium containing 10% FCS, the growth rate and saturation density of EBV-infected NU-GC-3 clones (population doubling time, 24 h; maximal cell density, 5.0×10^6 cells/well [Fig. 7B]) distinctly differed from those of Neo^r-transfected NU-GC-3 clones (population doubling time, 38 h; maximal cell density, 2.9×10^6 cells/well [Fig. 7B]). In addition, the anchorage-independent growth ability of NU-GC-3 was significantly enhanced by EBV infection (Table 2). Repeated tests showed that such EBV-dependent growth differences in NU-GC-3 were retained for at least 6 months after isolation of those clones.

DISCUSSION

We demonstrated here that EBV is capable of infecting and immortalizing primary epithelial cells, although only in one culture. Moreover, we found that EBV could convert already

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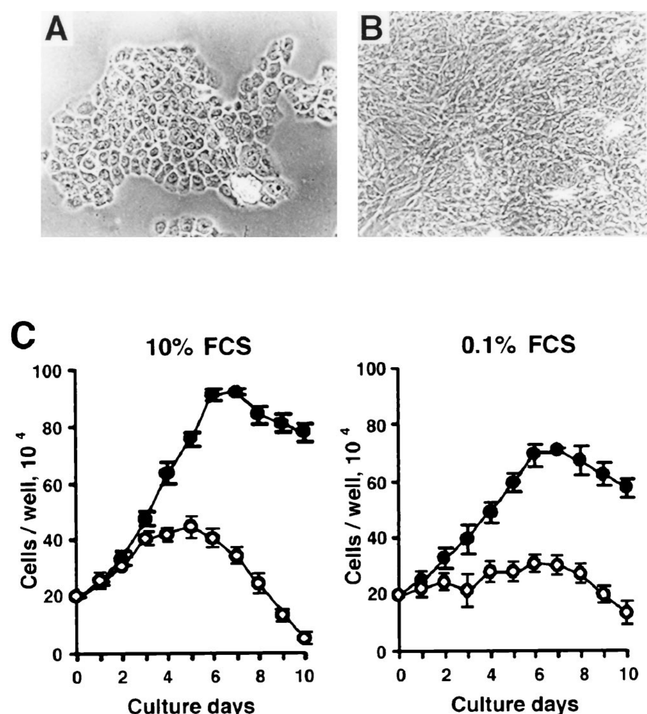

FIG. 4. Growth characteristics of Neo^r-transfected and EBV-infected PGE-5 clones. (A) Neo^r-transfected PGE-5 clone. Magnification, ×100. (B) EBV-infected PGE-5 clone. Magnification, ×100. Both clones were detached by trypsinization, seeded into separate wells of 12-well plates under the same culture conditions, and photographed at near the plateau phase (5 days after passage). Differences between the two cell types are easily recognizable (see the text for details). (C) Growth kinetics of EBV-infected (solid circles) and Neo^r-transfected (open circles) PGE-5 cells at normal and low FCS concentrations. Individual data are plotted as a circle with a vertical bar, which represents the mean ± standard error calculated from the results of four clones.

established carcinoma cells into ones with more malignant phenotypes. In such EBV-induced events, the patterns of EBV expression in both primary-culture cells and carcinoma cells were identical to those of EBV-associated gastric carcinoma (13) and LMP1-negative NPC (47), i.e., so-called type I latency characterized by the absence of EBNA2 (due to Qp usage) and LMP1 expression (28). Although the effects of EBV on inducing epithelial cell growth have been suggested to be caused by LMP1 (5, 7, 8, 21), we could not detect LMP1 expression in these cells even by a very sensitive RT-PCR assay. Our results

TABLE 1. Growth of PGE-5 cells in soft agarose

Cell	No. of colonies per 10^4 cells[a]
Neo^r-transfected PGE-5	
Clone 1	6 ± 4
Clone 2	9 ± 3
Clone 3	6 ± 3
Clone 4	0
rEBV-infected PGE-5	
Clone 1	295 ± 29
Clone 2	281 ± 14
Clone 3	210 ± 7
Clone 4	255 ± 14

[a] The results are expressed as means ± standard errors of results from three wells. Colonies consisting of >50 viable cells were counted.

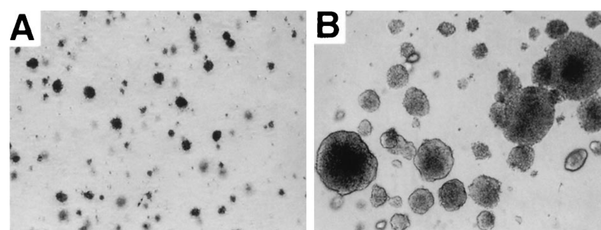

FIG. 5. Growth in soft agarose of EBV-infected PGE-5 clones and controls. (A) Neo^r-transfected PGE-5 clone as a control. Magnification, ×40. (B) EBV-infected PGE-5 clone. Magnification, ×40. A total of 10^4 cells at the logarithmic phase were seeded per well in six-well plates. The number of viable colonies was counted and photographed 4 weeks after seeding. Almost all colonies in the control culture consisted of dead cells, whereas the virus-infected clone formed many large colonies.

indicated that EBV genes other than LMP1 exerts the effects on epithelial cell growth.

We have recently provided evidence that EBV is necessary for the malignant phenotype of the Burkitt's lymphoma-derived Akata cell line by comparing EBV-positive and -negative Akata cell clones (36) and by reinfecting EBV-negative cell clones (15). Both EBV-positive and EBV-reinfected Akata cells represent type I latency. The present results strongly suggest that the persistence of EBV with type I latency contributes to the malignant growth phenotype not only of lymphoid neoplasias but also of epithelioid neoplasias. Several studies have shown the potential roles of EBNA1 and EBERs in oncogenic or growth-promoting effects (3, 16, 42). Although LMP2A has not yet been demonstrated to act as a direct effector of cell growth, it may associate with the maintenance of EBV latency through phosphorylation by *src* family tyrosine kinases and a mitogen-activated protein kinase (20, 24).

Among 21 primary epithelial cultures examined, EBNA was detected in a small fraction of cells in 3 cultures only. As we reported recently (14), cocultivation of primary-culture cells with EBV-producing Akata cells revealed a 10-fold-higher efficiency of EBV infection than that with cell-free virus preparations, even though gastric epithelium seems to be relatively resistant to EBV infection. It is not known whether there is a specific cell type susceptible to EBV or whether the state of cell differentiation influences the susceptibility to EBV.

Moreover, our results suggest that EBV alone is not sufficient to immortalize primary epithelial cells, because we could obtain immortalized cells from only one culture even though EBV infection was detected in three cultures. PGE-5 cells seemed to have already acquired intrinsic growth potential, since they could be propagated for up to 30 passages without EBV infection, in contrast to other primary-culture cells, which became extinct within 1 month. A point mutation in intron 5 similar to that found in PGE-5 cells was also found in a sample from a patient with gastric epithelial dysplasia (32). Although

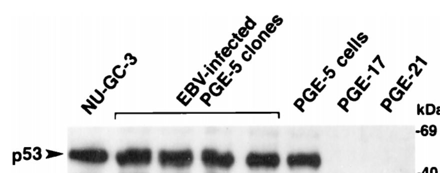

FIG. 6. Immunoblot analysis of p53 expression in EBV-infected and -uninfected PGE-5 cells. The gastric carcinoma cell line NU-GC-3 was used as a positive control for p53 overexpression. PGE-17 and PGE-21 denote other PGE lysates.

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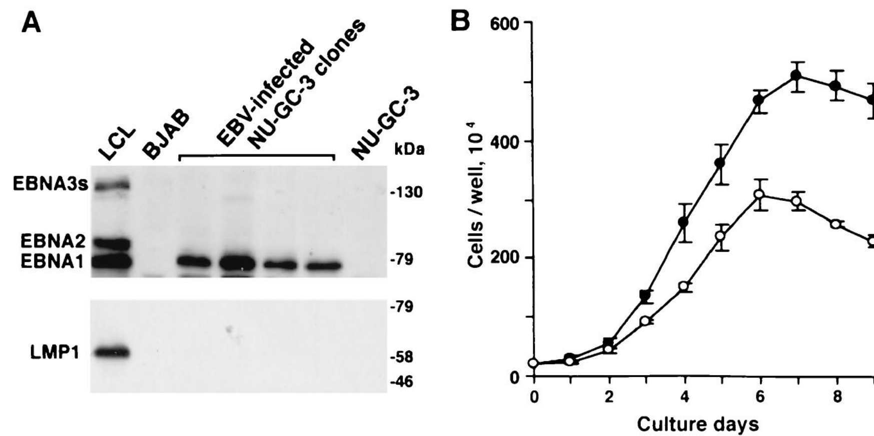

FIG. 7. (A) Detection of EBNAs (top blot) and LMP1 (bottom blot) in EBV-converted NU-GC-3 cells by immunoblotting. (B) EBV induced-growth alteration of NU-GC-3 cells. Growth curves of EBV-infected (solid circles) and Neo^r-transfected NU-GC-3 (open circles) clones in medium containing 10% FCS are shown. The assay was carried out in six-well culture plates. Data are expressed as described in the legend to Fig. 4C.

the relationship between intron mutation and p53 accumulation has not been clarified, loss of functions of the p53 tumor suppressor gene may confer basic growth potential to PGE-5 cells, and addition of EBV function leads to immortalization. A recent study documented frequent p53 accumulation in potentially premalignant lesions of the nasopharyngeal epithelium as well as in fully malignant cells, suggesting that p53 overexpression may precede EBV infection in an early stage of NPC development (10). Accumulation of p53 protein was reported to occur in about 60 to 70% of patients with gastric carcinoma and precancerous lesions (adenoma and dysplasia) (4, 32), and, more interestingly, in about 40% of those with the precancerous intestinal metaplasia of the gastric mucosa (23, 32). A relatively small-scale survey showed that the p53 gene is overexpressed in more than half of the patients with EBV-positive gastric carcinoma tested (9). Thus, p53 alterations may be an additional event required for the genesis of EBV-associated epithelial malignancies, by way of the auxiliary functions for cell cycling (2, 17) or inhibitory effects of apoptotic epithelial cell death (45).

To our knowledge, our research has demonstrated for the first time that EBV infection showing type I latency is functionally associated with epithelial growth promotion. Further studies are necessary to determine which genes expressed in type I latency play this role. In this regard, since the two kinds of cells used in this study, PGE-5 and NU-GC-3, were sensitive to the EBV-induced growth promotion, they will serve as a useful indicator to identify the viral gene(s) responsible for such activities.

TABLE 2. Growth of NU-GC-3 cells in soft agarose

Cell	No. of colonies per 2,000 cells[a]
Neo^r-transfected NU-GC-3	
Clone 1	42 ± 17
Clone 2	39 ± 17
Clone 3	38 ± 22
Clone 4	37 ± 14
rEBV-infected NU-GC-3	
Clone 1	679 ± 34
Clone 2	423 ± 26
Clone 3	317 ± 20
Clone 4	208 ± 16

[a] The results are expressed as means ± standard errors of results from three wells. Colonies consisting of >50 viable cells were counted.

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