Tumorigenicity of EBNA2-transfected cells

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Abstract The Epstein-Barr virus nuclear antigen 2 (EBNA2) gene is thought to be important for transformation by Epstein-Barr virus (EBV), but the mechanism of this transformation is little understood. Here, to examine the transforming ability of EBNA2, we transfected a rat fibroblast cell line F2408 with a recombinant EBNA2 expression plasmid and examined cell morphology, colony formation in soft agar, and tumorigenicity in nude mice. The morphology of transfected clones was similar to those of untransfected cells, but two of seven clones grew in soft agar, and four clones of seven clones reproducibly formed tumors in nude mice. These four clones showed EBNA2 expression, but non-tumorigenic clones did not. These results indicate that the expression of EBNA2 is correlated with tumorigenicity.

Key words: EBNA2; Tumorigenicity; Transfection

1. Introduction

Epstein-Barr virus (EBV) is well known to be an etiological agent in endemic Burkitt lymphoma, nasopharyngeal carcinoma (NPC) and lymphomas in immunodeficiency [1]. Recently, Hodgkin's disease [2], Ki-1-positive anaplastic large cell lymphoma [3,4] and a part of gastric carcinoma [5] have also been reported to be closely related to EBV. However, the mechanism of tumorigenesis by EBV is not well understood. The transforming genes of EBV are suspected to be in the region of EBV associated nuclear antigens (EBNAs) and latent membrane protein (LMP)1 [6]. LMP1 can transform established rodent cells. Expression of LMP1 resulted in altered cell morphology and growth in low serum and in soft agar. Further, Rat-1 cells expressing LMP1 were tumorigenic in nude mice [7]. LMP1 was also expressed in 40% of nasopharyngeal carcinoma [8].

EBNA2, encoded by the IR1 and the U2 regions of EBV genome, seems to be important for B cell immortalization, because P3HR-1 virus, the genome of which lacks the EBNA2 coding region cannot immortalize lymphocytes [9]. There are also reports that EBNA2 is essential for B-lymphocyte growth transformation [10,11]. These findings suggest the important role of EBNA2 in transformation. Transactivation of CD21 [12], CD23 [13], c-fgr [14] and LMP1 [15,16] has been demonstrated and thought to be a mechanism of lymphocyte immortalization by EBNA2. But the process and mechanism of tumorigenesis by EBNA2 is now unclear.

Here, we examined the transforming phenotype of EBNA2 transfected rat fibroblast cell line F2408 and found a correlation between the tumorigenicity and the expression of EBNA2.

2. Materials and methods

2.1 Transfection

Recombinant pZ-E2 (pZip NEO SV(X)1/EBNA2 DNA) [17], obtained from E. Kieff (Harvard University), contains the EBNA2 open reading frame driven by the murine leukemia virus long terminal repeat. Fisher rat F2408 fibroblasts were transfected with recombinant pZ-E2 plasmid by the calcium phosphate transfection method After selection in medium supplemented with the neomycin analog G418, we obtained seven G418-resistant F2408 clones (F2408-E2).

2.2. Southern blot analysis

DNAs were extracted by proteinase K, phenol/chloroform method, from cultured cells. Ten micrograms of DNA from each specimen were digested with the restriction enzymes *Hin*dIII and *Bam*HI, subjected to electrophoresis on a 0.8% agarose gel, transferred to a nylon membrane (Biodyne, Pall) by blotting and hybridized with labeled E2 DNA.

2.3. Northern blot analysis

RNAs were purified from cultured cells with guanidium thiocyanate followed by centrifugation in cecium chloride solution [18]. Poly (A^+) RNAs were selected with oligo-dT latex (Oligotex, Roche), electrophoresed through 1.4% agarose gels containing formaldehyde, transferred to nylon membranes (Biodyne, Pall), and hybridized with the labeled E2 DNA as a probe.

2 4 Indirect immunofluorescence staining

Specimens of frozen tissue or cultured cells on chamber slides (Nunc) were fixed with equal quantities of acetone and methanol for 5 min at 4°C. Monoclonal anti-EBNA2 antibody PE2 (Dako) were diluted 20 times with PBS and reacted for 90 min at 37°C. After washing twice with PBS, biotinylated goat anti-human IgG or anti-mouse IgG (BRL) was diluted 50 times with PBS and reacted for 60 min at 37°C After washing twice with PBS, 200 times-diluted streptavidin–fluorescein isothiocyanate conjugate (BRL) was reacted for 45 min at room temperature. After washing three times with PBS, the slides were sealed with glycerin containing 0 22 M 1,4-diazobicyclo-octane (DABCO) (Wako, Osaka).

3. Results

3.1. Transformed phenotypes of EBNA2-transfected cells

We transfected pZ-E2 into rat fibroblast F2408, selected seven clones and examined various transformed phenotypes of these clones. These clones contained the EBNA2 gene detected by Soutern blotting analysis (data not shown). The morphology and the saturation densities of seven clones were similar to those of untransfected cells or cells transfected with the plasmid without E2. Two of these clones grew in 0.33% soft agar (Table 1).

Subcutaneous inoculation of E2 transfected clones at 2×10^6 cells per mouse into nude mice induced tumors in four of the

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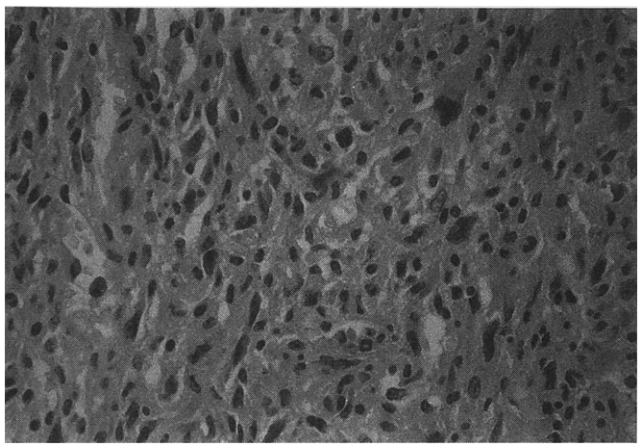

Fig. 1. Histological appearance of a tumor induced in a nude mouse 6 weeks after injection of 2×10^6 F2408-E2 cells. H-E staining. × 400.

seven clones after 2–3 weeks, whereas cells transfected with vector plasmid without E2 did not induce tumors (Table 1). These tumors were undifferentiated sarcomas with many mitotic cells (Fig. 1) and few collagen fibers stained with the Azan–Mallory staining. Southern blot analysis of these tumor tissue showed retention of E2 DNA (Fig. 2).

These results indicated that EBNA2 conferred tumorigenicity and anchorage independency on the established cell line.

Table 1
Transforming activity of EBNA-2

Clones	Saturation density* ($\times 10^6$ cells/10 cm plate)	Colony formation in soft agar+	Growth in low serum** ($\times 10^5$ cells/plate)	Tumorigenicity in nude mice ++
EBNA2				
Cl.14	0.99	0	0.50	0/3
Cl.23	0.83	0	0.87	0/3
Cl.28	ND§	0	0.65	0/3
Cl.9	1.10	0	3.0	6/6
Cl 16	0.98	0	4.0	6/6
Cl.18	ND	123.5	3.5	3/3
Cl.24	ND	27.0	3.5	3/3
pZipNeo				
Cl 2	0.76	0	0.56	0/3
Cl.4	ND	ND	0.25	0/3
Cl.6	1.06	0	0.88	0/3
F2408	0.89	0	0.75	0/3

*F2408 cells containing the indicated clones were plated onto 10 cm plates. The number of cells in two plates was counted daily, and the maximum cell number (average for two plates) is shown as the saturation density.

+2×10^4 cells were seeded into 6 cm plastic plate with 0.33% noble agar in DMEM supplemented with 5% FCS on top of 0 5% base agar. The number of colonies larger than 0.125 mm was counted after culture for 3 weeks. Average for two experiments is shown.

**2×10^5 cells were seeded in 10 cm plastic plate with 10 ml medium containing 0.1% FCS. Medium was changed each 3 or 4 days. The number of cells were counted after 10 days. Average for three experiments is shown.

++2×10^6 G418-resistant cells were injected into nude mice. (The number of mice with tumour)/(the number of mice inoculated) is shown.

§ND. not done.

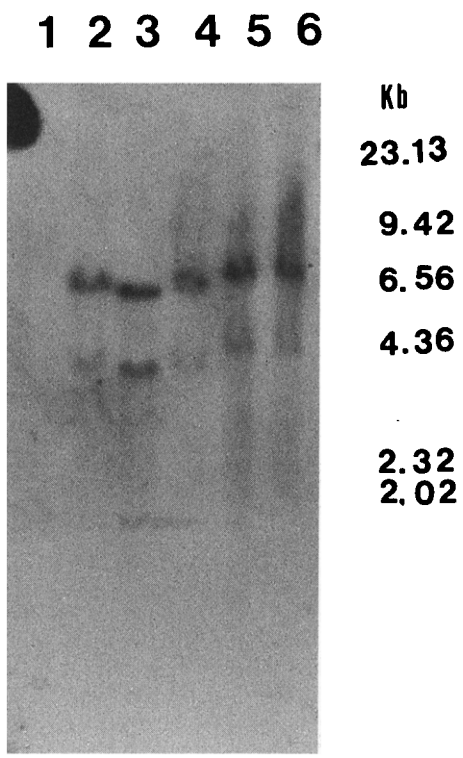

Fig. 2. Southern blot analysis of tumors induced by F2408-E2 Cl.9. Lane 1, F2408-pZip; lane 2, F2408-E2 Cl.9; lane 3 through 6, tumors 1, 2, 3, 4 induced by F2408-E2 Cl.9, respectively. Each lane contained 30 μg of DNA treated with 150 units of the restriction enzyme *Hm*dIII.

Next we examined cell growth of these clones in media with low serum. Tumorigenic clones grew slightly in low serum, but the non-tumorigenic or control clones did not grow under these conditions (Table 1). Therefore, EBNA2 provided the cells with a growth capacity on low serum conditions as well as tumorigenicity.

3.2. Expression of EBNA2

To explain the difference between tumorigenic and non-tumorigenic clones, we studied the expression of ENBA2 on these clones. Poly(A^+) RNAs selected from total RNAs of F2408-E2 clones and control clones were analysed by Northern blot hydbridization. We detected a distinct hybridization band in

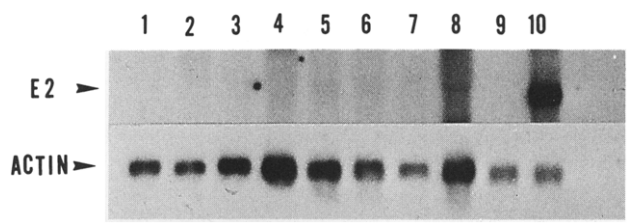

Fig. 3. EBNA2 RNA expression in transfected F2408-E2. Northern blot was prepared by electrophoresis of polyadenylated RNA from 200 μg each total RNA in 1 4% agarose-2.2 M formaldehyde gel, followed by transfer to a nylon membrane filter. The blot was hybridized successively with 3×10^6 cmp/ml labelled E2 DNA or actin sequence. Lane 1, pZip-transfected F2408 Cl.2; lane 2, F2408-E2 Cl.14; lane 3, F2408- E2 Cl.23, lane 4, F2408-E2 Cl.28; lane 5, F2408-E2 Cl.9; lane 6, F2408-E2 Cl.16; lane 7, F2408-E2 Cl.18; lane 8, F2408-E2 Cl.24; lane 9, P3HR1 cells which delete EBNA2 gene; lane 10, Raji cells as positive control for EBNA2.

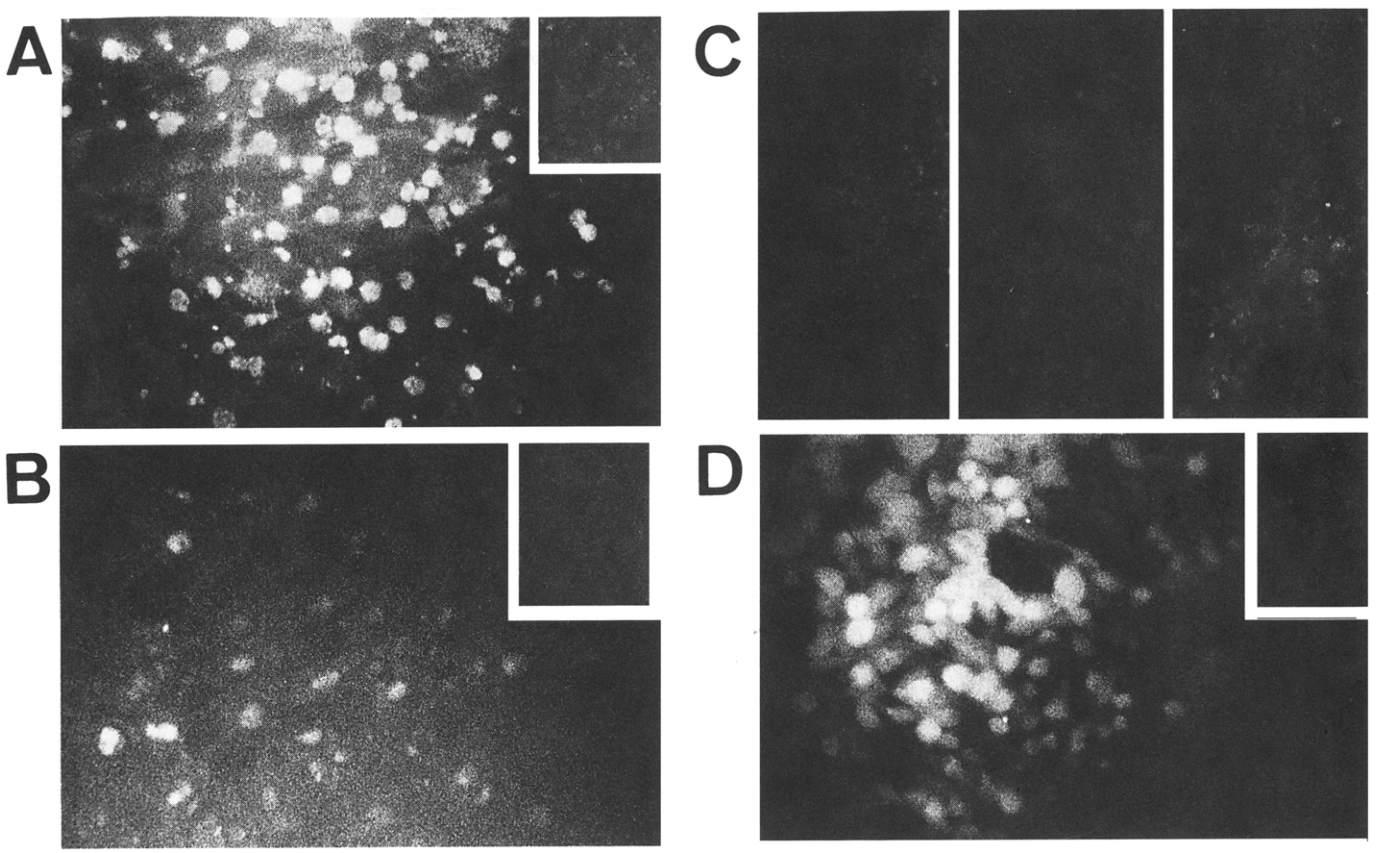

Fig. 4. Immunofluorescence of E2-expressing cells. Cells were stained with monoclonal antibody PE2. The nuclei of the cells were stained. A, F2408-E2 Cl.24 cells, × 100, B, F2408-E2 Cl.9 cells, × 100; C, F2408- E2 Cl.23 cells (left), F2408-E2 Cl.28 cells (middle), and F2408-pZip Cl.4 cells (right), × 50; D, recultured cells of F2408-E2 Cl.9, × 100. Control stainings with PBS were shown in parentheses, × 50.

one clone 24, but not in other clones (Fig. 3). Because the expression of EBNA2 may be too low to be detected by Northern blotting, we tried another method. Immunofluorescence staining using monoclonal EBNA2 antibody showed distinct fluorescence in clone 24 and weak fluorescence in other tumorigenic clones, whereas no fluorescence was detected in non-tumorigenic clones and clones transfected with the vector only (Fig. 4A, B, C). Nuclei of recultured cells from induced tumor also showed fluorescence when stained with this antibody (Fig. 4D).

All these results suggest that the EBNA2 gene of EBV causes tumorigenicity.

4. Discussion

We showed tumorigenicity in nude mice and growth ability in low serum of EBNA2-expressing cells.

Although we detected EBNA2 expression in only one clone by Northern blotting, we could show all the tumorigenic clones to express EBNA2 by immunofluorescence staining. This fact suggests that weak EBNA2 expression may be sufficient to induce tumors and that this may be related to unsuccessful detection of EBNA2 expression in various human tumors.

We also showed that some EBNA2-transfected clones were anchorage-independent, and tumorigenicity and decreased serum requirement did not correlate with anchorage independency. Higher expression of EBNA2 may be necessary to induce anchorage independency than to induce tumorigenicity. Alternatively, dissociation of tumorigenicity and decreased serum requirement from anchorage independency has been reported in cells transfected with the E6 gene of human papillomavirus type 16 [19].

Dambaugh et al. reported that Rat-1 cells expressing EBNA2 grew in medium with 0.5% fetal calf serum but remained contact inhibited, anchorage-dependent and non-tumorigenic in mice [17]. This report differs from our data on many points but the efficiency of tumorigenicity or anchorage independency may be dependent on the type of cells transfected.

We examined the state of c-*myc* in tumorigenic and non-tumorigenic clones, because rearrangement and overexpression of the c-*myc* gene has been reported with EBV-induced Burkitt lymphoma [20,21]. But we found no difference in the restriction enzyme digestion pattern of the c-*myc* gene between tumorigenic and non-tumorigenic clones by Southern blot analyses (data not shown). This experiment showed that the difference between tumorigenic and non-tumorigenic clones was not due to activation of the c-*myc* gene.

These results indicate that the EBNA2 gene possesses transforming activity.

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