JOURNAL OF VIROLOGY, Dec. 1996, p. 9003–9007 Vol. 70, No. 12
0022-538X/96/$04.00+0

Differential Tumorigenicity between Epstein-Barr Virus Genome-Positive and Genome-Negative Cell Lines with t(11;14)(q13;q32) Derived from Mantle Cell Lymphoma

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Received 1 July 1996/Accepted 10 September 1996

Epstein-Barr virus (EBV) genome has been detected in several human lymphoproliferative diseases, but the oncogenic function of EBV is not fully understood. We previously established EBV-positive (SP-50B) and EBV-negative (SP-53) cell lines with the t(11;14)(q13;q32) chromosome abnormality from a single patient with mantle cell lymphoma. Monoclonal EBV DNA in a circular episomal form was demonstrated in the SP-50B cells by Southern blot hybridization with the EBV-terminal fragment probe. SP-50B cells were positive for not only EBV-encoded nuclear antigen-1 (EBNA1) but also latent membrane protein-1 and EBNA2. None of the EBV-encoded proteins was expressed in SP-53 cells. The isogenic EBV-infected and EBV-free cell lines of neoplastic clones made it possible to examine a tumorigenic role of EBV. Only EBV-positive SP-50B cells possessed malignant phenotypes, such as growth ability in low serum, colony formation in soft agarose, and tumorigenicity in nude mice. On the other hand, a lymphoblastoid B-cell line established by infecting the patient's normal B lymphocytes in vitro with exogenous EBV had no tumorigenicity. These results suggested that EBV infection, if it occurred in neoplastic lymphoma cells, could play a role in acquisition of malignant phenotypes.

Epstein-Barr virus (EBV) is a ubiquitous human herpesvirus which infects B lymphocytes and certain epithelial cells and is implicated in several human malignancies, including Burkitt's lymphoma and nasopharyngeal carcinoma (18). Although EBV infection was also detected in Hodgkin's lymphoma (34, 35) and non-Hodgkin's lymphoma, especially in immunosuppressed individuals (9, 17, 39), the oncogenic function of EBV in these lymphoproliferative diseases is not well delineated.

Structural and functional alterations of oncogene loci induced by chromosomal translocations are consistently found in many hematopoietic malignancies and are implicated in tumorigenesis. Burkitt's lymphomas (BLs), for example, are characterized by specific reciprocal chromosomal translocations that involve the c-*myc* oncogene locus on chromosome 8q24 and various immunoglobulin gene loci on chromosome 14, 2, or 22, resulting in activation of the c-*myc* gene. While the deregulation of the c-*myc* gene is thought to play a pathogenetic role in BL, in the case of EBV-positive BL, EBV infection is also considered as an additional pathogenetic element. Recently, Shimizu et al. isolated EBV-positive and EBV-negative clones in vitro from a t(8;14)(q24;q32)-carrying BL cell line which was originally 100% positive for EBV and demonstrated that only the EBV-positive clone exhibits tumorigenicity (28). On the other hand, EBV-infected human B lymphoblastoid cells acquired tumorigenicity when transfected with constitutively expressed c-*myc* gene (16). These results suggest that both EBV infection and c-*myc* activation are necessary for acquisition of tumorigenicity in endemic BL. Thus, while several studies to explore tumorigenesis of BL in conjunction with EBV infection and c-*myc* gene activation have been reported, the tumorigenic role of EBV in other non-Hodgkin's lymphomas has not been well investigated, possibly because of the lack of ideal model systems.

Mantle cell lymphoma (also called intermediate lymphocytic lymphoma) is a form of intermediate-grade non-Hodgkin's lymphoma that originates from mature B cells in the primary follicle and mantle zone of peripheral lymphoid organs (3, 12, 31). The chromosome translocation t(11;14)(q13;q32) is characteristic of mantle cell lymphoma and occurs in up to 70% of the cases (4, 23, 24, 37). As a result of t(11;14)(q13;q32) chromosome translocation, the *bcl-1* locus on chromosome 11q13 is juxtaposed to an immunoglobulin heavy chain gene on chromosome 14. The subsequent deregulation of the cyclin D1/*PRAD1* oncogene, a member of the cyclin G1 gene family, is considered to contribute to tumorigenicity (11, 20, 24).

We previously established EBV-positive (SP-50B) and EBV-negative (SP-53) cell lines from a single patient with mantle cell lymphoma (7). The paired availability of the EBV-infected and EBV-free cell lines of neoplastic clones with the same origin is valuable in the investigation of the tumorigenic role of EBV.

Both the SP-50B and SP-53 cells had the same phenotypic markers as original lymphoma cells in respect of surface and cytoplasmic immunoglobulins and other cell markers, such as CD5, CD10, and CD19 (7). They possessed the t(11;14)(q13;q32) chromosome translocation, indicating that these cell lines were derived from the original lymphoma cells. Molecular analyses revealed a rearrangement of the cyclin D1/*PRAD1* gene in both cell lines (26). Thus, SP-50B and SP-53 cells had similar characteristics except for EBV infection. We had also attempted to infect the EBV-negative SP-53 cells in vitro with exogenous EBV by incubating the cells in the supernatant of the B95-8 cell line, but it was unsuccessful (7). A third cell line derived from the same patient, SP-52EB, was established by infecting the peripheral blood cells with the B95-8 virus and had no chromosome abnormality (7). SP-52EB is considered to be a lymphoblastoid cell line (LCL) originated from normal B lymphocytes. These cell lines had been preserved in liquid nitrogen, and each cell line within the 30th passage was used in this study. EBV infection in these cell lines was further confirmed by examining for the presence of EBV-encoded small RNA (EBER) by the in situ hybridization method. SP-50B and

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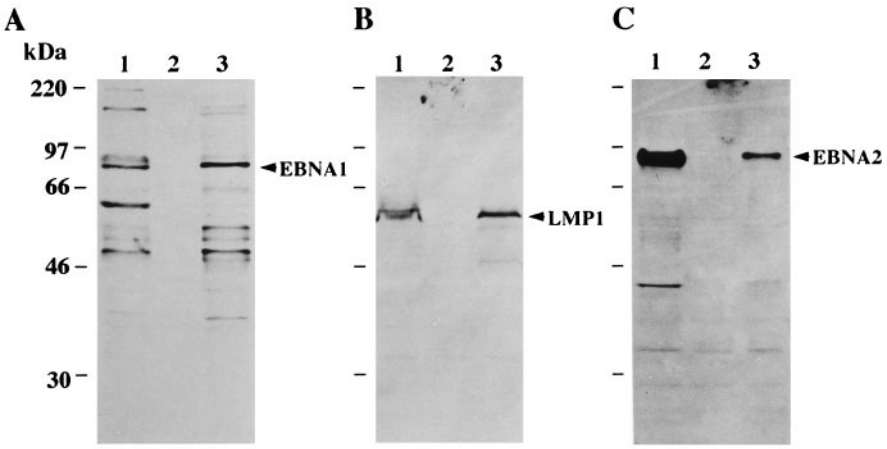

FIG. 1. Immunoblot analysis of EBV-encoded proteins. Proteins (corresponding to 3×10^5 cells) were subjected to sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis and transferred onto a nitrocellulose filter as described previously (6). The proteins were reacted with a polyclonal antibody specific to EBNA1 (A), with anti-LMP1 monoclonal antibody CS1-4 (25) (B), or with anti-EBNA2 monoclonal antibody PE2 (38) (C); this was followed by incubation with peroxidase-labeled protein A or anti-mouse antibody. The blots were developed by the enhanced chemiluminescence method. EBNA1, LMP1, and EBNA2 bands are indicated by arrows. Positions of mass markers are indicated on the left. Lanes 1, SP-50B; lanes 2, SP-53; lanes 3, SP-52EB.

SP-52EB cells were strongly positive for EBER, as expected, whereas none of the SP-53 cells expressed EBER, confirming that SP-53 is an EBV-free cell line (data not shown). Immunoblot analysis demonstrated that both SP-50B and SP-52EB cells were positive for not only EBNA1 but also LMP1 and EBNA2 (Fig. 1). This pattern of viral gene expression was similar to that observed in some cases of EBV-associated B-cell lymphoproliferative diseases and in EBV-infected LCL, where a full set of latent EBV genes is expressed (8, 38). We next tested clonality of EBV in the SP-50B and SP-52EB cells by Southern blot hybridization analysis using the EBV-terminal fragment probe (22). A major single band was demonstrated at 12 kb in DNA from SP-50B cells, indicating that the viral DNA was monoclonal in a circular episomal form with fused termini, whereas EBV DNA from SP-52EB cells showed multiple bands between 14 and 8 kb, representing polyclonal EBV infection (Fig. 2).

The growth ability of each cell line was tested in RPMI 1640 medium containing only 0.1% fetal calf serum (FCS) (GIBCO BRL, Gaithersburg, Md.) at the initial cell concentration of 4×10^5/ml. Representative results from two independent experiments are shown in Fig. 3. SP-50B cells showed continuous growth and reached the maximum cell density at around 10^6 cells per ml at 3 days. Thereafter, the number of viable cells decreased, but some cells still survived over a period of 7 days in 0.1% FCS. In contrast, SP-53 cells showed no increase in cell number over the first 2 days and then gradually lost viability. Almost all cells died after 7-day culture. The growth curve of SP-52EB cells was similar to that of SP-53 cells.

The growth properties of these three cell lines were also studied by examining their ability to form colonies in soft agarose (Fig. 4A). SP-50B cells formed visible colonies 7 to 14 days after plating, while SP-53 and SP-52EB cells did not form colonies under the same conditions. A group of more than 20 viable cells was scored as a clone 14 days following seeding. The cloning efficiency of the SP-50B cells from the average of three experiments was 27.2%.

We next tested the ability of the cell lines to form tumors in vivo when injected subcutaneously into 4- to 5-week-old athymic nude mice. SP-50B cells gave rise to tumors with a high take incidence at all inoculum doses tested (Table 1). The palpable subcutaneous nodules (0.5-cm diameter) could usually be detected within 2 weeks after inoculation of 0.5×10^7 to 1×10^7 SP-50B cells and progressed to apparent tumors with no sign of regression or necrosis (Fig. 4B). In comparison with SP-50B cells, SP-53 cells exhibited a considerably lower tumorigenicity. Even with 5×10^7 cells, the SP-53 cells failed to yield any detectable tumors. The minimum inoculum dose of the SP-53 cells capable of producing a tumor was 10^8 cells in this system, and the tumor appeared in only one of four mice inoculated with this dose. Likewise, SP-52EB had no tumorigenicity. When 0.5×10^8 to 1×10^8 SP-52EB cells were inoculated, small nodules were occasionally observed within 2 weeks, but this was followed by complete regression within another 2 weeks. The nude mouse tumor cells were positive for EBNA1, as well as EBNA2 and LMP1, and monoclonal EBV

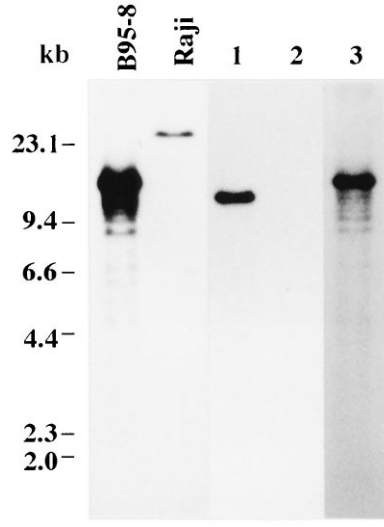

FIG. 2. Clonality analysis of EBV genomes. Total cellular DNAs were digested with *Bam*HI, subjected to electrophoresis through a 0.75% agarose gel, transferred onto a nylon membrane filter, and hybridized to the ^{32}P-labeled *Xho*I fragments that represent unique sequences at the right end of the EBV genome. Raji has a monoclonal EBV DNA in an episomal form. B95-8 shows polyclonal EBV infection with linear DNA. Positions of size markers are shown on the left. Lane 1, SP-50B; lane 2, SP-53; lane 3, SP-52EB.

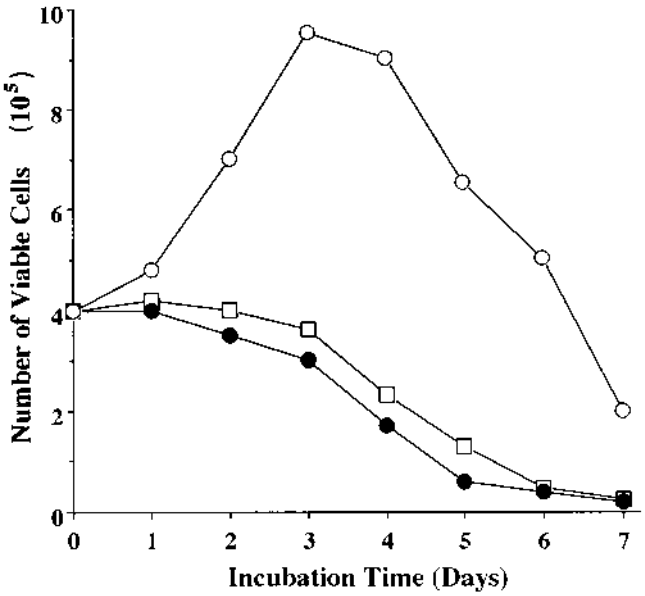

FIG. 3. Growth curves of SP-50B (open circles), SP-53 (solid circles), and SP-52EB cells (open squares) in 0.1% FCS after resuspension at a density of 4×10^5 cells per ml. Cell proliferation was monitored daily for 7 days. The number of viable cells was counted by the trypan blue-dye exclusion method.

DNA was demonstrated (data not shown). Chromosome analysis showed that the tumor cells had a human karyotype containing the t(11;14)(q13;q32) chromosome abnormality (data not shown). These results confirmed that the tumors were derived from in vivo growth of the injected SP-50B cells.

In the present study, we have performed a comparative study of tumorigenicity between SP-50B and SP-53 lymphoma cell lines having similar characteristics except for EBV infection. The results are summarized in Table 2. SP-50B cells exhibited a far higher tumorigenicity than SP-53 cells, as shown by growth ability in low serum, clonogenicity in soft agarose, and tumorigenicity in nude mice. Neither of the SP-53 and SP-52EB lines grew in soft agarose nor did they produce progressively growing tumors in nude mice. The molecular events in the acquisition of the malignant phenotypes of EBV-infected lymphoma cells are poorly understood. The immortalizing function of EBV infection itself seems not to be associated with tumorigenic capabilities, as suggested by the evidence that the EBV-infected SP-52EB LCL showed no tumorigenicity. Other pathogenetic events, in addition to EBV infection, are likely to be involved in the higher tumorigenesis of SP-50B cells.

Deregulated cellular oncogenes induced by abnormal chromosomal rearrangements are known to correlate with tumor progression and tumorigenesis. The most well-characterized oncogene in conjunction with EBV is c-*myc*, associated with t(8;14)(q24;q32) in BL. Several studies indicated that the c-*myc* activation and EBV infection cooperatively play a tumorigenic role in BL. The expression of an activated c-*myc* gene caused the tumorigenic conversion of normal EBV-infected LCL (5, 16), and conversion of an EBV-negative BL line (BJAB) to a permanently EBV-carrying subline led to acquisition of tumorigenic capabilities by increasing the level of c-*myc* expression (14, 15, 36). Furthermore, additional evidence comes from a recent study that the presence of the EBV genome was indispensable for the sustenance of malignant phenotypes of BL, as concluded by a comparative study with EBV-positive and

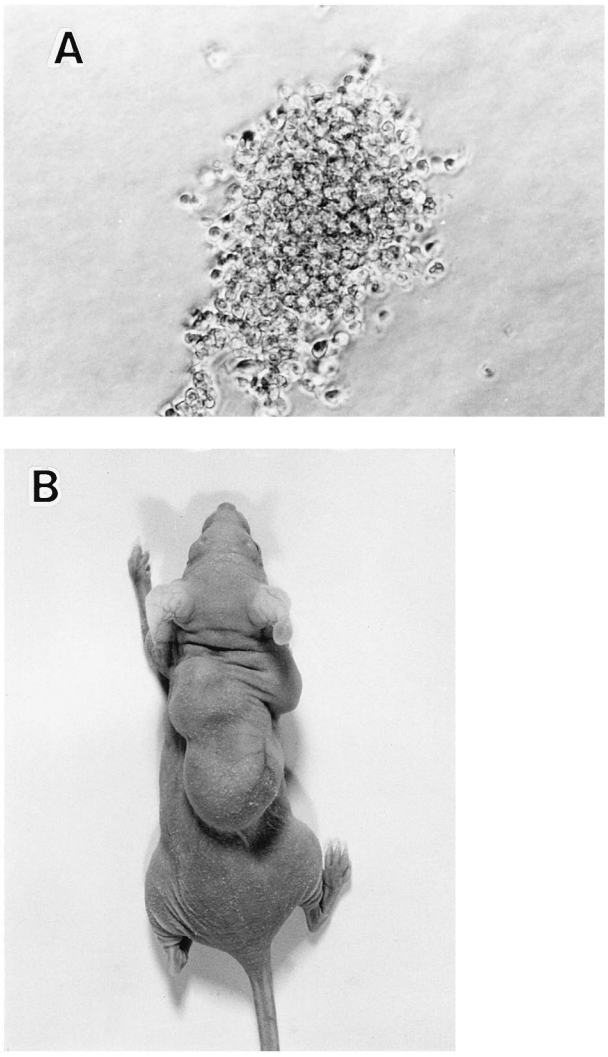

FIG. 4. (A) Growth of SP-50B cells in soft agarose. One thousand cells suspended in medium containing 0.3% agarose (Sea Plaque; FMC BioProduct, Rockland, Maine) were layered onto an underlayer formed by 0.5% agarose in medium. A colony formation was photographed under an inverted microscope 14 days following seeding. (B) A nude mouse bearing a tumor initiated by an injection with SP-50B cells. The nude mouse was photographed 8 weeks after inoculation with 5×10^6 cells.

EBV-negative BL clones (28). The expression of c-*myc* gene was examined in tumorigenic SP-50B and nontumorigenic SP-53 lines by Northern (RNA) blot analysis. The c-*myc* gene was expressed at similar levels in both cell lines, indicating that

TABLE 1. Tumorigenicity assay in nude mice[a]

No. of cells inoculated	No. of mice with tumors/no. of mice inoculated		
	SP-50B	SP-53	SP-52EB
5×10^6	3/4	ND[b]	ND
1×10^7	3/3	0/3	0/3
5×10^7	3/3	0/3	0/3
1×10^8	3/3	1/4	0/3

[a] Four-to-five-week-old nude mice were inoculated subcutaneously with various numbers of cells. Data are the number of mice with tumors 8 weeks after inoculation.

[b] ND, not done.

TABLE 2. Characteristics of SP-50B, SP-53, and SP-52EB cells

Item(s) tested	SP-50B	SP-53	SP-52EB
Chromosome	t(11;14)	t(11;14)	Normal
EBNA1 and EBER	+	−	+
LMP1 and EBNA2	+	−	+
EBV clonality	Monoclonal		Polyclonal
Growth in low serum	+	−	−
Colony formation in soft agarose	+	−	−
Tumorigenicity in nude mice	+	±[a]	−

[a] Tumor formation was observed only when a high dose of cells (10^8) was inoculated.

the difference in tumorigenicity between SP-50B and SP-53 lines was not due to activation of the c-*myc* gene (data not shown).

The t(11;14)(q13;q32) chromosomal translocation in mantle cell lymphoma is considered to be structurally similar to the t(8;14)(q24;q32) in BL. The deregulated expression of the cyclin D1 oncogene is thought to be a critical step in tumorigenesis in mantle cell lymphoma (11, 20, 24). Having demonstrated that SP-53 cells had little tumorigenesis, it is unlikely that the presence of the t(11;14)(q13;q32) alone is sufficient for the potentiation of malignancy. It is hypothesized, therefore, that the EBV gene product(s) might further regulate the function of the already overexpressed cyclin D1 oncogene directly or indirectly, and that this might be related to the stronger tumorigenicity of the SP-50B cells. Rearrangement of the cyclin D1 gene in both SP-50B and SP-53 cells was already confirmed, while no rearrangement was observed in SP-52EB cells (26). Further comparative studies including cyclin D1 protein analysis will be needed to better understand the mechanisms responsible for the difference of tumorigenicity of these cell lines.

SP-50B cells expressed LMP1 and EBNA2. These EBV-encoded proteins are essential for the growth transformation and immortalization of B lymphocytes. LMP1 upregulates *bcl-2* oncogene, which renders B lymphocytes resistant to apoptotic death (10). Recently, Mosialos et al. demonstrated that LMP1 interacts with tumor necrosis factor receptor-associated factor leading to activation of NF-κB, which is likely to be relevant to the growth transformation function of LMP1 (19). EBNA2 induces transactivation of the cellular oncogene c-*fgr* and, in combination with LMP1, transactivates *bcl-2* (1, 13, 33). Both LMP1 and EBNA2 are considered to be associated with tumorigenicity, since transfection of transcriptionally active gene constructs into rodent fibroblasts resulted in acquisition of malignant phenotypes, including growth ability in low serum, colony formation in soft agarose, and tumor formation in nude mice (27, 32). But, the tumorigenic role of LMP1 and EBNA2 in human lymphoma cells is still unclear. Recently, it was reported that expression of cyclin D2, another member of the cyclin G1 gene family, was induced by LMP1 and EBNA2 (2, 29, 30). The findings imply that expression of the cyclin D2 following EBV infection subverts the cell cycle control and thereby leads to uncontrolled cell proliferation. On the other hand, cyclin D1 is usually undetectable in EBV-infected BL lines and LCLs (21). In this context, it would be interesting to examine whether or how EBV infection in t(11;14)(q13;q32)-carrying lymphoma cells affects the constitutively expressed cyclin D1 oncogene.

In summary, by utilizing the isogenic EBV-positive and -negative lymphoma cell lines, which are, to our knowledge, the first cell lines available from mantle cell lymphoma, we have successfully demonstrated the putative role of EBV in acquisition of malignant phenotypes of other non-Hodgkin's lymphoma besides BL.

We thank S. Imai for the gift of EBV *Xho*I probe. We are also grateful to M. Isobe for the analysis of c-*myc* expression.

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