Steps Involved in Immortalization and Tumorigenesis in Human B-Lymphoblastoid Cell Lines Transformed by Epstein-Barr Virus

Masanobu Sugimoto,1 Hidetoshi Tahara,2 Toshinori Ide,2 and Yasuhiro Furuchi1

1GeneCare (previously AGENE) Research Institute, Kanagawa, Japan; and 2Department of Cellular and Molecular Biology, Division of Integrated Medical Science, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan

Abstract

Epstein-Barr Virus (EBV) is closely associated with the generation of various tumors, including Burkitt’s lymphoma. Human resting B cells from peripheral blood are easily transformed by EBV to actively proliferating B-lymphoblastoid cell lines (LCLs). These LCLs with normal diploid karyotypes have been believed to be “immortal”, without becoming tumorigenic. A series of recent studies, however, indicate that this initial, simple concept needs extensive reconsideration. Most LCLs from normal individuals are mortal because their telomeres shorten. Some LCLs are truly immortalized by developing strong telomerase activity and aneuploidy, accompanied by various other changes: down-regulation of p16/Rb; mutation of the p53 gene; modulation of apoptosis; and sensitivity to various chemical agents. Some post-immortal LCLs additionally develop the ability to form colonies in agarose and even become tumorigenic by developing the ability to grow in nude mice. The genetic background of LCLs markedly affects the frequency of immortalization. In summary, changes of B cells after infection by EBV are roughly divided into two steps: (a) transformation of B cells into LCLs caused by EBV proteins; and (b) immortalization and tumorigenesis of LCLs mainly regulated by the factors of host cells in cooperation with EBV proteins. The new concept as reviewed here is essential for the future study of tumorigenesis by EBV.

Introduction

Data have now accumulated indicating that Epstein-Barr virus (EBV; Ref. 1) is closely associated with the generation of Burkitt’s lymphoma, undifferentiated nasopharyngeal carcinoma, and various other tumors (2–5). Infection by EBV in vitro easily transforms resting B cells from human peripheral blood into actively proliferating lymphoblastoid cell lines (LCLs): the percentage of B cells susceptible to transformation has been estimated at between 10% and 100% (6–12). LCLs are used widely for various purposes: in etiological studies of tumorigenesis by EBV; as sources of DNA and cells to study various genetic disorders; and for studies of immunology and cellular biology. LCLs have long been believed to be immortal, having an unlimited life span with no other additional changes, although they have usually normal diploid karyotypes (6, 7, 13). A series of recent studies including ours, however, showed that this initial simple concept is incorrect (14–17). Two key technologies analyzed; and (b) immortalization and tumorigenesis of LCLs mainly regulated by the factors of host cells in cooperation with EBV proteins. The new concept as reviewed here is essential for the future study of tumorigenesis by EBV.

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Requests for reprints: Masanobu Sugimoto, GeneCare Research Institute, 200 Kajiwara, Kamakura, Kanagawa 247-0063, Japan. Phone: 81-467-46-9590; Fax: 81-467-45-2871; E-mail: sugimoto@genecare.co.jp.

Mortal and Immortal LCLs Established by Cloning on a Feeder Layer

Counter et al. (14, 15) started this kind of study. Because cloning of LCL cells by conventional culture is usually difficult, they used four LCLs (B2, B3, B4, and B5) cloned by culturing them on human fibroblast feeder layers (19). Two clones, B2 and B5, stopped proliferating (cellular crisis) at 45 and 95 population doubling levels (PDLs). The remaining two clones, B3 and B4, were immortalized by overcoming crisis. Immortalized LCLs exceeded more than 100 PDLs by keeping telomere length with activated telomerase. Counter et al. (14, 15) argued the following points: (a) proliferative crisis occurs in EBV-transformed cells and is detectable when clonal populations are analyzed; and (b) this crisis is likely masked in nonclonal populations because of the high rate of immortalization by EBV as described previously by Miller (7) and Nilsson (13).

Pre- and Post-Immortal LCLs Established by Conventional Cellular Passages

After transformation with EBV propagated in the marmoset cell line B95-8 (12), LCL cells are usually continuously cultured by passaging them in a culture bottle. We used a 25 cm² bottle, and a maximal cell density of between 5 × 10⁶ and 10⁷ in 5 ml of culture medium is attained in this condition (20). After the LCLs are established, as a total cell population, they have negative to moderate telomerase activity, raising the possibility that at least one telomerase-positive cell exists in all cell lines. If such a telomerase-positive cell is immortal without exception, all LCLs in bottles containing 5 × 10⁶–10⁷ cells should be immortalized after cellular passages by domination by immortal cells. However, according to our studies, the immortalization differs considerably. Proliferative crisis unmistakably occurs in most noncloned LCLs from normal individuals that end their lifespans after crisis below 150 PDLs. In our studies, during 150 PDLs, most noncloned LCLs kept normal diploid karyotypes, and their relatively low levels of telomerase activity fluctuated between negative and moderate ranges accompanied by shortening of the telomeres (20, 21). Part of the LCLs overcame cellular crisis and were unmistakably immortalized by developing strong telomerase activity, sometimes at very late stage PDLs, for instance at between 110 and 135 PDLs in N0003 and N0013, respectively (20–22). Thus, the mode of immortalization of noncloned LCLs was largely the same as immortalization of cloned LCLs (15), probably due to noncloned LCLs acquiring clonal characteristics after a long, continued culture by selection of a special cell population that adapts best to in vitro culture. The telomere lengths kept by immortalized LCLs were unusually short, mostly below 5 kbp, consistent with the results of genic accompanied by activated telomerase and aneuploidy. We will review here these emerging findings about the immortalization and tumorigenesis of EBV-transformed LCLs.
Counter et al. (15). Cellular immortalization was accompanied by aneuploidy without exception: All 10 immortalized LCLs thus far tested were aneuploid (21–23). Activation of telomerase and the appearance of aneuploidy were almost simultaneous, suggesting a correlation between the two events (22). Notably, all cells within an LCL shared a part of abnormal karyotypes. In Burkitt’s lymphoma cell lines, three reciprocal translocations, (t(8;14), t(2;8), or t(8;22) juxtapose an oncogene c-myc (3, 4). Abnormal karyotypes of post-immortal LCLs, for the most part, do not contain these typical translocations (22, 23). In our studies, we established two pairs of post-immortal LCLs from the same cell line, N6803/N6803-2 and T3/T3-2. Interestingly, these two pairs of post-immortal LCLs had clonal abnormal karyotypes distinct from each other even in the same pair. These results together strongly suggest that immortalized LCLs are derived from a single cell that become immortal by accidentally developing strong telomerase activity and chromosomal rearrangement. We use “post-immortal” for immortalized LCLs and “pre-immortal” for LCLs that are not yet immortalized, for the sake of convenience.

Effect of Genetic Background of LCLs on Immortalization

We established LCLs from normal individuals; patients of Werner syndrome (WS), a recessive genetic disorder causing premature ageing due to the lack of normal WRN helicase (24); and siblings from a family with hereditary type 2 diabetes mellitus (DM). During our study of the etiology of these genetic disorders using LCLs, we systematically compared the lifespans of LCLs by continuous cell culture for a long period: more than 9 years for some LCLs (16, 20, 22, 25). In a preliminary study by Salk et al. (26), the lifespans of two LCLs from WS patients were longer than those from two non-WS individuals. We investigated systematically this event by using many samples. Among 50 LCLs from normal individuals, five LCLs (10.0%; N0003, N0005, N0013, N6803, and N6803-2) were immortalized, and the remaining 45 LCLs were mortal. None of 44 LCLs (0%; P < 0.031 against normal individuals by the \( \chi^2 \) test) from WS patients was immortalized. Among 11 LCLs from the type 2 DM family, five LCLs (45.5%; T1, T3, T3-2, T5, and T6; P < 0.0040 against normal individuals; P < 0.00001 against WS patients) were immortalized. Maximal PDLs attained by pre-immortal LCLs were 150, 172, and 148 for normal individuals, WS patients, and the type 2 DM family, respectively. From these results, we judged LCLs as post-immortal if they satisfied the following criteria: (a) activation of telomerase; (b) aneuploidy; and (c) exceeding 180 PDLs. The misunderstanding that EBV-transformed cells are immortal may be due to the unusually long lifespans of these cell lines: average 86.0 PDLs for normal LCLs and 90.6 PDLs for WS LCLs (17).

A systematic, long continuous cell culture resulting in cellular crisis may not be necessary for most studies, and even if mortal LCLs appeared by chance, these LCLs were probably misjudged as dying under poor culture conditions. To investigate the influence of accidental poor culture conditions, we determined lifespans repeatedly for 32 LCLs. We found that a significantly high correlation (\( r = 0.785 \)) was between the two lifespans determined by independent experiments, indicating that the life span of each LCL is influenced by genetic factors of cells, including telomere length (25), rather than culture conditions. These results, together with the fact that post-immortal LCLs continue proliferating while mortal cells die in the same culture conditions, formally exclude the possibility that mortality of LCLs is due to accidental poor culture conditions.

Our results also suggest strongly that normal WRN helicase is essential for immortalization and that a genetic factor of the type 2 DM family increases immortalization. WRN helicase is markedly up-regulated by transformation of resting B cells by EBV and is further moderately up-regulated by immortalization of LCLs (23, 27, 28). These facts support the idea that WRN helicase is essential to immortalize EBV-transformed LCLs. Now, evidence has accumulated implicating that WRN helicase has an essential role in maintaining the telomere length. Fibroblasts from WS patients lacking the normal WRN gene have accelerated loss of telomeres (29), and LCLs from WS patients show abnormal dynamics of telomeres (20). Interaction and cooperation of WRN helicase with the telomere, telomerase, and related proteins have been suggested (30, 31).

The frequency of an immortal cell is calculated as 1 among 10^6 crisis cells for LCLs from normal individuals, 0 among 10^6 from WS patients, and 4.5 among 10^6 from the type 2 DM family, assuming that a culture bottle contains about 10^7 cells and that post-immortal cells derive from a single cell. These frequencies of LCLs from non-WS patients are within the range reported for SV40-induced immortalization of fibroblasts ranging from 1 among 10^7 to 1 among 10^9 crisis cells (32). Thus the frequency of immortalization of EBV-transformed LCLs is never unusually high compared with SV40-transformed fibroblasts. In human fibroblasts, two mortal stages, M1 and M2, have been proposed. The life span of M1 cells initially proposed by Hayflick and Moorhead (33) is prolonged by about 20 PDLs in M2 cells by transformation with the SV40 T-antigen gene (34). Although fibroblasts at the M2 stage are mostly mortal, they are rarely immortalized. Thus common points exist between the EBV-LCL and SV40-fibroblast systems; pre-immortal EBV-transformed LCLs apparently correspond to fibroblasts at the M2 stage.

Diverse Characteristics of Post-Immortal LCLs

Although all 10 post-immortal LCLs established by us have common characteristics of activated telomerase and clonal aneuploidy, they are diversified in other characteristics. Table 1 shows a list of LCLs from normal individuals at various stages of transformation, immortalization, and tumorigenesis (23): stage 0, resting B cells from peripheral blood; stage 1, pre-immortal LCLs established by infection with EBV with low telomerase activity and normal diploid karyotypes; stage 2, post-immortal LCLs by developing strong telomerase activity and aneuploidy (N0003, N0013, N6803-2); stage 3, post-immortal LCLs with an ability to form colonies in agarose (N0005); and stage 4, post-immortal LCLs with an ability to form colonies in agarose and to grow in nude mice (N6803). Other than these changes, various additional changes are accompanied by immortalization and tumorigenesis: (a) down-regulation of p16/Rb (c) modulation of apoptosis (see “Modification of Apoptosis by Cellular Senescence and Tumorigenesis”; Ref. 35); (d) up-regulation of WRN helicase (23, 28); (e) instability of chromosomes (23); and (f) change in cytotoxicity to various chemical compounds including cancer chemotherapeutic agents (36). Fig. 1 shows steps involved in immortalization and tumorigenesis of EBV-transformed LCLs from normal individuals based on combined results of our studies. Forty-five of 50 LCLs (90%) approached proliferating crisis followed by ending their lifespans within 150 PDLs, but the remaining five LCLs immortalized by overcoming crisis (25). The arrows with orange lines starting from stage 2 indicate three LCLs, N0003, N0013, and N6803-2, that continue proliferating staying at stage 2, and the orange line starting stage 3 indicates N0005 that continues proliferating staying at stage 3. Notably, the two colony-forming post-immortal LCLs, N0005 and N6803, showed marked diversification of abnormal chromosomes, indicating instability of the chromosomes and the three nontumorigenic post-immortal LCLs, N0003, N0013 and N6803-2, keep relatively stable sets of abnormal chromosomes (22, 23). Thus, the three LCLs N0003, N0013, and
Correlation with Early Studies

A large proportion of LCL cells spontaneously differentiate into smaller lymphoid cells that ultimately undergo apoptosis during conventional cell culture (35). Two distinct types of apoptosis with intermediate types exist: type 1 apoptosis in small and medium cells accompanied by relatively large internucleosomally fragmented DNA (greater than 2 kbp); and type 2 apoptosis in large lymphoblasts accompanied by smaller internucleosomally fragmented DNA (less than 2 kbp). The morphological differentiation of LCL cells into small lymphoid cells leading to type 1 apoptosis is almost completely blocked in the tumorigenic post-immortal N6803 cell line, as judged by morphological study of smeared cells stained with Giemsa and by analysis with a cell sorter (35, 37). This fact also suggests that the post-immortal LCL N6803 is an undifferentiated type. Cell clusters of crisis LCLs just before the end of their lifespans are sometimes surrounded by numerous apoptotic bodies, indicating an increase in type 1 apoptosis (35).

Correlation with Early Studies

Establishment of cell lines by the experimental addition of exogenous EBV in vitro started from 1973 when EBV from the B95-8 line (12) was available. Before the development of this technology, many lymphoblastoid cell lines established “spontaneously” presumably by endogenous EBV were reported (6). Nilsson called this type of cell line BED (B-lymphoblastoid, EBV-carrying, diploid line). Early studies using BED cells showed results consistent with those of recent studies that used EBV-transformed LCLs. However, the results were mostly preliminary and fragmented, and the evidence that the BEDs used were established by endogenous EBV is circumstantial. BED cells are unusually stable, but signs of major rearrangements and changes in chromosome aneuploidy were observed in some lines after 1–2 years of continuous culture (6, 38). Burkitt’s lymphoma cell lines form colonies in agarose, and most grow s.c. in irradiated mice or nude mice. In contrast, BED cells from healthy donors do not form colonies and are much less transplantable (39, 40). Nilsson suggested that rare colony-forming and tumorigenic BED cells are old and chromosomally altered. These results of early studies agree well with the scheme we propose in Fig. 1 and imply that the scheme can be applied to most situations with EBV-transformed LCLs.

EBV Proteins

This review shows that changes of B cells after infection by EBV are roughly divided into two steps: (a) transformation of B cells into LCLs caused by EBV proteins; and (b) immortalization and tumorigenesis of LCLs mainly regulated by the factors of host cells in cooperation with EBV proteins. Nine EBV proteins are expressed in EBV-established LCLs, five of which appear to be definitely required

<table>
<thead>
<tr>
<th>Cellular diversification</th>
<th>Cellular passages</th>
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<th>Telomere length (22, 23)</th>
<th>Karyotype (20–23)</th>
<th>Colony formation (23)</th>
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<th>Other changes</th>
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<tbody>
<tr>
<td>Resting B-cell* (stage 0)</td>
<td>Not proliferating</td>
<td>(−) (No change)</td>
<td>Normal diploid</td>
<td>(−) (−)</td>
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<tr>
<td>Pre-immortal (stage 1)</td>
<td>Limited 180 PDLs</td>
<td>+/− Shorten</td>
<td>Normal diploid</td>
<td>− −</td>
<td>N0003, N0013, N6803-2</td>
<td>Down-regulation of p16/Rb (23)</td>
<td>Mutation of the p53 gene (23)</td>
<td></td>
</tr>
<tr>
<td>Post-immortal (stage 2)</td>
<td>Unlimited 180 PDLs</td>
<td>+++ Maintained</td>
<td>Aneuploidy</td>
<td>− −</td>
<td>N0005</td>
<td>Modulation of apoptosis (35)</td>
<td>Up-regulation of WRN helicase (23, 27, 28)</td>
<td></td>
</tr>
<tr>
<td>Post-immortal (stage 3)</td>
<td>Unlimited 180 PDLs</td>
<td>+++ Maintained</td>
<td>Aneuploidy</td>
<td>+ +</td>
<td>N6803</td>
<td>Instability of chromosomes (22, 23)</td>
<td>Modulation of cytotoxicity against various chemical agents (36)</td>
<td></td>
</tr>
<tr>
<td>Tumorigenic (stage 4)</td>
<td>Unlimited 180 PDLs</td>
<td>+++ Maintained</td>
<td>Aneuploidy</td>
<td>+ +</td>
<td>N6803</td>
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* The expression of telomerase is moderate (+), somewhere between moderate and negative (+/−), negative (−), very strong (+++).
* The information of resting B cells in parentheses are assumed.

Table 1 Diversification of LCLs from normal individuals during cellular passages

Fig. 1. Steps involved in immortalization and tumorigenesis of EBV-transformed LCLs. The numbers in parentheses indicate the results of a series of our experiments starting from 50 LCLs derived from normal individuals (20, 23, 25). The orange arrows indicate LCLs that continue proliferating at present by keeping the original characteristics. See details in the text and Table 1 for the meaning of stages 0–4.

N6803-2 may stay at stage 2, whereas the N0005 might acquire tumorigenicity after additional proliferating.

Modification of Apoptosis by Cellular Senescence and Tumorigenesis

A large proportion of LCL cells spontaneously differentiate into smaller lymphoid cells that ultimately undergo apoptosis during conventional cell culture (35). Two distinct types of apoptosis with intermediate types exist: type 1 apoptosis in small and medium cells accompanied by relatively large internucleosomally fragmented DNA (greater than 2 kbp); and type 2 apoptosis in large lymphoblasts accompanied by smaller internucleosomally fragmented DNA (less than 2 kbp). The morphological differentiation of LCL cells into small lymphoid cells leading to type 1 apoptosis is almost completely blocked in the tumorigenic post-immortal N6803 cell line, as judged by morphological study of smeared cells stained with Giemsa and by analysis with a cell sorter (35, 37). This fact also suggests that the post-immortal LCL N6803 is an undifferentiated type. Cell clusters of crisis LCLs just before the end of their lifespans are sometimes surrounded by numerous apoptotic bodies, indicating an increase in type 1 apoptosis (35).
for B-cell transformation (41–45). The cooperation of these EBV proteins with cellular factors is a future problem to clarify the mechanism of immortalization and tumorgenesis of EBV-transformed LCLs.

Concluding Remarks

Studies that use EBV-transformed LCLs in experiments especially to study tumorgenesis should know whether the LCLs they use are pre- or post-immortal. At least 14 papers published in journals, including high-ranked ones, in 2003 use “immortalization”, apparently as the meaning of transformation.” Pre- and post-immortal LCLs are clearly distinguished by the following markers: Post-immortal LCLs have much higher telomerase activity and aneuploidy; and they should exceed at least 180 PDLs. EBV proteins, such as EBV nuclear antigens and latent membrane proteins, participate in transformation of resting B cells to actively proliferating cells, which is the first step in immortalization and tumorgenesis. However, various environmental factors, cellular events, and host genetic factors probably have a key role in cooperation with EBV proteins during transition of LCLs from the pre-immortal to the post-immortal stage and from the post-immortal to the tumorigenic condition.

Note Added in Proof


References

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