Suppression of the Malignant Phenotype in Somatic Cell Hybrids between Burkitt’s Lymphoma Cells and Epstein-Barr Virus-immortalized Lymphoblastoid Cells despite Deregulated c-myc Expression

Jürgen Wolf, Michael Pawlita, Jörn Bullerdiek, and Harald zur Hausen


ABSTRACT

To approach the question whether the absence of specific cellular gene functions may be involved in Burkitt’s lymphoma pathogenesis, somatic cell hybrids were established between a malignant Epstein-Barr virus (EBV) positive Burkitt’s lymphoma cell line (BL 60) and a nonmalignant EBV-immortalized lymphoblastoid cell line (IARC 277) derived from the same individual. The hybrids revealed a near tetraploid karyotype including one copy of the 8q* chromosome resulting from the Burkitt’s lymphoma-specific translocation t(8;22) in addition to three apparently normal copies of chromosome 8. Although the hybrid cells exhibited the deregulated c-myc expression pattern of the parental Burkitt’s lymphoma cell line with highly abundant transcripts originating from the 8q* chromosome, their growth characteristics in tissue culture as well as in nude mice were identical to that of the parental nonmalignant lymphoblastoid cell line. These data indicate that, at least in the system described here, the malignant phenotype of Burkitt’s lymphoma cells can be suppressed by introduction of an additional set of apparently normal chromosomes from the same individual and that EBV infection and c-myc deregulation may not be sufficient for maintenance of the malignant phenotype.

INTRODUCTION

Suppression of the tumorigenic phenotype in somatic cell hybrids between malignant and nonmalignant cells has strongly supported the view that absence of specific cellular gene functions might represent a critical condition for malignant transformation (1–3).

Until now this mechanism has been implicated mainly in the development of tumors of epithelial and mesenchymal origin, whereas malignancies of the hematopoietic and lymphatic system may be considered as models for the dominance of activated oncogenes. A prototype for these latter malignancies is endemic BL, a malignant B-cell lymphoma, occurring in Africa and New Guinea (4). Seroepidemiological studies (5) as well as the detection of the viral DNA in the tumor (6) have provided strong evidence for an etiological role of EBV in the development of endemic BL.

In addition specific chromosomal translocations are consistently found in BL which involve the c-myc gene on chromosome 8 and immunoglobulin gene loci on the chromosomes 14, 22, or 2 (7–9). The c-myc allele involved in these translocations is either truncated or shows somatic mutations in, or around, the first exon (10–12). These structural alterations as well as the proximity of the c-myc gene to immunoglobulin enhancer elements caused by the translocation have led to the hypothesis that a deregulated c-myc gene expression contributes to the malignant phenotype of BL (13, 14).

However, despite extensive studies of EBV infection and c-myc expression in BL (12, 15–19), the precise mechanisms by which these two postulated oncogenic functions act in the process of malignant transformation are not well understood. It remained an open question whether further, as yet unidentified, mechanisms contribute to the development of BL.

Using the technique of somatic cell hybridization we approached the question whether the absence of specific cellular gene functions may be involved in BL pathogenesis. Cells of a highly tumorigenic EBV-positive BL cell line carrying a variant t(8;22) translocation were fused with nontumorigenic EBV-immortalized B-lymphoblastoid cells originating from the same individual. All hybrid clones investigated were nontumorigenic in nude mice despite presence of EBV and continued deregulation of the c-myc gene.

MATERIALS AND METHODS

Cells. The Burkitt’s lymphoma cell line BL 60 and the lymphoblastoid cell line IARC 277 were kindly provided by G. M. Lenoir (20). BL 60 originates from a 4-year-old North African female and was obtained from a tissue sample taken at diagnosis. BL 60 was shown to be EBV positive and to carry a chromosomal translocation t(8;22). IARC 277 was established by spontaneous outgrowth of peripheral blood lymphocytes of the same patient (20). All cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics. The concentration of viable cells was determined in 0.25% trypan blue in a Neubauer chamber.

Electrotransfection. The electrotransfection procedure was performed as described by Potter et al. (21). Briefly, 1 × 10⁶ cells and 10 μg linearized DNA of the plasmid pSV2neo (22) in a total volume of 200 μl ice cold PBS were placed in an electroporation chamber with an electrode distance of 4 mm. A high voltage pulse of 2200 V (capacitance 960 nF) was applied with an electropulsing device (constructed by W. Ansorge, European Molecular Biology Laboratory, Heidelberg, West Germany). After 5 min on ice, 20 ml growth medium were added and cells were incubated for 24 h. Then, 2 × 10⁶ cells/ml medium were transferred to 96-well microtiter plates (150 μl/well). Within 5 days more than 99% of the cells died. Three weeks after seeding 6-thioguanine (Sigma) in 96-well microtiter plates (150 μl/well). Within 5 days more than 99% of the cells died. Three weeks after seeding 6-thioguanine-resistant colonies were obtained with a frequency of 4.2 × 10⁻⁶. About 10% of these colonies proved to be stably HGPRT⁺ when seeded in hypoxanthine-aminopterine-thymidine selective medium.

Cell Fusion and Hybrid Selection. Cells (1 × 10⁶) of each fusion partner were attached to 60-mm plastic Petri dishes treated with 100 μg/ml concanavalin A (Serva) as described elsewhere (23). The plates were then washed twice with PBS and covered with 2 ml of prewarmed (37°C) 50% (wt/vol) polyethylene glycol 1500 (Boehringer) for 90 s, followed by washing with PBS and subsequent cultivation in RPMI medium as described above. Forty-eight hours later, the cells, now detached from the plastic dishes and in suspension, were transferred to 24-well tissue culture plates (1 ml/well). Costar) and the medium was changed to selective medium containing 1200 μg/ml G418, 1 × 10⁻⁶ M
SYNOPSIS OF MALIGNANT PHENOTYPE IN SOMATIC CELL HYBRIDS

RESULTS

Establishment of Somatic Cell Hybrids between the Burkitt's Lymphoma Cell Line BL 60 and the Lymphoblastoid Cell Line IARC 277. The EBV-positive Burkitt's lymphoma cell line BL 60 carries a variant t(8;22) translocation and is highly tumorigenic in nude mice (29). The BL 60 cell line and the nontumorigenic EBV-immortalized lymphoblastoid cell line IARC 277 were derived from the same individual (20) but harbor different EBV genomes (30). To establish somatic cell hybrids between these cell lines two selectable markers had to be introduced. To avoid a possible selection for tumorigenic variants, no marker was introduced into the nonmalignant lymphoblastoid fusion partner IARC 277. Rather, BL 60 cells were rendered neomycin resistant by transfection of the plasmid pSV2neo (22). Subsequently, spontaneous HGPRT" mutants were selected in 6-thioguanine-containing medium. A "universal fuser" subline of BL 60, termed BL60-P7 (neomycin-resistant HGPRT"), was fused with the IARC 277 cell line. Seven hybrid clones were analyzed in detail.

Karyotype and c-myc RFLP Analysis. The hybrid clones were cytogenetically characterized by near tetraploid karyotypes (modal counts, 82–93) in comparison to the near diploid parental cell lines BL60-P7 and IARC 277 (modal counts, 45–47). In each hybrid clone one copy of the Burkitt's lymphoma-derived (8;22)(8pter→8q24::22q11→22qter) translocation chromosome (chromosome 8q) was identified in all metaphases as well as the 22q" translocation chromosome and three apparently normal copies of chromosome 8. In addition the presence of 4 marker chromosomes, three originating from the parental BL cell line and one originating from the parental LCL, showed the BL/LCL hybrid nature of the clones (Fig. 1). The presence of the BL60-P7-derived chromosome 8q" in the hybrid cells was confirmed by demonstration of the PuVI RFLP in c-myc exon 1 (19). In the variant t(8;22) translocation in BL 60 the breakpoint on chromosome 8q" lies 3' of the c-myc gene, leaving the latter nonrearranged. One PuVI restriction site, however, is abolished in c-myc exon 1 on chromosome 8q" so that in addition to the 0.86-kilobase germ line fragment a new fragment can be detected in Southern blot analysis of PuVI-digested BL 60 DNA probed with c-myc exon 1. Six of the 7 hybrids showed the predicted 1.8-kilobase fragment with

![Fig. 1. Cytogenetic analysis of a representative hybrid clone. The G-banded metaphase of hybrid clone 3 demonstrates a near tetraploid chromosome complement. The 8q-translocation chromosome (large arrow) and the 22q chromosome (small arrow) resulting from the reciprocal translocation are marked. The presence of additional marker chromosomes I: del(4)(p13 or 14→qter), II: 3pter-3q26 or 27::?, IV: t(19;?) (pter or qter) of the parental BL60-P7 cell line, and II: t(18;?) (18pter-18q11::?) of the parental IARC 277 cell line further confirms the BL/LCL hybrid nature of the cells. The cytogenetic nomenclature is according to ISCN (45).](cancerres.aacrjournals.org)
approximately one-third of the intensity of the germ line band (Fig. 2). Hybrid clone 4 contained an aberrant band, probably due to additional loss of a PvuII site.

The presence of EBV DNA sequences was demonstrated in the parental and hybrid cells by Southern blot analysis of BamHI-digested cellular DNA probed with the BamHI-W fragment of EBV (data not shown).

Growth Characteristics in Vitro. In suspension culture all hybrid clones demonstrated the typical phenotype of EBV-immortalized LCL by forming large, macroscopically visible clumps (31), indistinguishable from those observed in the parental nonmalignant lymphoblastoid cells. In contrast the parental Burkitt’s lymphoma cells grew as individual cells without clumping (data not shown).

Comparison of the growth kinetics of the parental cells and the hybrids again showed no difference between the parental lymphoblastoid cells and the hybrids. When seeded at a concentration of $1 \times 10^5$ cells/ml in fresh medium, the parental Burkitt’s lymphoma cells proliferated to a final concentration of about $3 \times 10^6$ cells/ml within 1 week. Under the same conditions the parental lymphoblastoid cells as well as the hybrid clones reached a final concentration of only about $7 \times 10^5$ cells/ml before growth arrest occurred (Fig. 3).

The parental cell lines BL 60 and IARC 277 showed no difference for serum requirement. Also both cell lines were not clonable in soft agar. Therefore these parameters were not analyzed in the hybrid clones.

Tumorigenicity in Nude Mice. The parental cell lines (BL60-P7, IARC 277) and the hybrid clones were tested for their tumorigenic potential in nude mice by inoculation of $1 \times 10^7$ cells into each flank of preirradiated nude mice (29). No difference was seen between the in vivo growth pattern of the hybrid clones and the parental lymphoblastoid cells. Initial growth of well-circumscribed spherical masses occurred after a latency period of 2 weeks for all cell lines. While the grafts of the parental Burkitt’s lymphoma cell line formed large progressively growing tumors reaching a size of 4–5 cm diameter without any sign of regression within 5 weeks after injection, the grafts of the parental lymphoblastoid cell line as well as of the hybrid clones reached a size of 0.5–1.0 cm diameter. Then the growth of these grafts stopped and they started to regress, partly with necroses (Fig. 4; Table 1). Two animals that carried hybrid grafts (clones 2 and 5) with extended necroses died in a cachectic state 7 weeks after injection. For all other hybrid grafts as well as for the grafts of the parental lymphoblastoid...
could be observed. Cell line complete regression within 9 weeks after injection (Lanes 1-7) were separated on an agarose gel (ethidium bromide staining, top), cell lines BL60-P7 (Lane B) and IARC 277 (Lane L) and the hybrid clones 1-7.

**Table 1 Initial take number and growth behavior of parental cells and hybrid clones in preirradiated nude mice**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Initial take no.* 2 weeks postinjection</th>
<th>Graft size 5 weeks postinjection (diameter in cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BL 60-P7</td>
<td>19/20</td>
<td>19</td>
</tr>
<tr>
<td>IARC 277</td>
<td>8/10</td>
<td>8</td>
</tr>
<tr>
<td>Hybrid</td>
<td><strong>1</strong></td>
<td><strong>1</strong></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>7</td>
</tr>
</tbody>
</table>

* Number of injection sites with initially growing graft/total number of injection sites.

**Fig. 5.** Northern blot analysis of c-myc expression in parental cell lines and hybrid clones. Equal amounts of total cellular RNA (5 µg/lane) of the parental cell lines BL60-P7 (Lane B) and IARC 277 (Lane L) and the hybrid clones 1-7 (Lanes 1-7) were separated on an agarose gel (ethidium bromide staining, top), transferred to Nylon filters, and probed with c-myc exon 3 (bottom); kb, kilobase.

**c-myc Transcription.** c-myc transcripts in the BL cell line BL 60 originate from the translocation chromosome 8q* and are more abundant compared to EBV-immortalized lymphoblastoid cells, probably due to loss of a transcriptional elongation block near the 3' end of exon 1 (19). We wanted to analyze whether this deregulated c-myc expression pattern of the parental Burkitt's lymphoma is changed in the nonmalignant hybrid clones. Therefore, we first determined the steady state level of c-myc RNA in the parental cell lines and the hybrid clones by Northern blot analysis of total cellular RNA probed with c-myc exon 3. A 222-base pair fragment of total cellular RNA in all hybrids was found to be in the range of that present in BL60-P7, which is significantly higher than in IARC 277 (Fig. 5).

By Northern blot analysis we could not distinguish whether the abundant c-myc transcripts present in the hybrid cells originated from the 3 copies of germ line chromosome 8 or from the BL60-P7-derived 8q* chromosome, because the t(8;22) translocation does not affect the size of the c-myc transcripts. We took advantage of the PvuII RFLP in c-myc exon 1 to identify the origin of the transcripts. A 222-base pair c-myc exon 1 cDNA fragment extending from 181 base pairs upstream to 41 base pairs downstream of the germ line PvuII site, which is lost on the chromosome 8q*, was generated first by reverse transcription and subsequently by enzymatic amplification (polymerase chain reaction), using two synthetic 20mer oligonucleotides (Fig. 6a). Following PvuII digestion cDNA derived from transcripts of the c-myc gene on chromosome 8q* remained undigested (i.e., 222 base pairs long), whereas cDNA derived from germ line c-myc transcripts was digested into two fragments of 181 and 41 base pairs. As shown by ethidium bromide staining of the PvuII-digested amplified cDNA fragments (Fig. 6b) as well as after hybridization with an internal diagnostic oligonucleotide (Fig. 6c), only transcripts from chromosome 8q* could be found with this highly sensitive method in the hybrid clones 1-7. After long-term exposure we could detect traces of germ line-derived c-myc transcripts with an intensity of less than 1% of the 8q*-derived transcripts. No c-myc cDNA fragments were obtained in control experiments in which reverse transcriptase was omitted.

**c-myc transcription was also analyzed in vivo.** From three regressing, partly necrotic hybrid grafts (clones 2, 5, and 7), we could isolate intact RNA to determine steady state level and chromosomal origin of c-myc transcripts as described above. c-myc transcripts in these regressing hybrid grafts were found to be highly abundant and derived almost exclusively from the BL60-P7 8q* chromosome as observed in tissue culture (data not shown).

These results indicate that despite their lymphoblastoid cell-like growth pattern in vitro and in vivo the hybrid clones show the deregulated c-myc expression pattern of the parental Burkitt's lymphoma cell line.

**Fig. 6.** Analysis of the origin of c-myc transcripts in parental cell lines and hybrid clones. a, Schematic representation of the c-myc locus and the positions and orientations of the synthetic oligonucleotides used as primer for reverse transcription and enzymatic amplification (o1, 20mer, coding strand), as second PCR primer (o2, 20mer, noncoding strand), and the diagnostic oligonucleotide (o3, 20mer, noncoding strand) used for hybridization. The cDNA fragments that are obtained from c-myc transcripts originating from the germ line chromosome 8 and the 8q* chromosome, respectively, after reverse transcription using o1 as primer and PCR primed by o1 and o2 followed by PvuII digestion are shown schematically. b, Separation of the amplified cDNA fragments of the parental cell lines BL60-P7 (Lanes B), IARC 277 (Lanes L), and the hybrid clones 1-7 (Lanes 1-7) with (+) and without (-) PvuII digestion in 8% polyacrylamide gels. A linearized 2959-base pair (bp) plasmid DNA fragment which can be cut by PvuII into 2514- and 445-base pair fragments was added after amplification and co-digested to monitor completeness of PvuII digestion. In Lane L+, 10 times more amplification products were loaded onto the gel to visualize the small 41-base pair fragment. In c, the gel shown in b was electroblotted and hybridized with end-labeled oligonucleotide o3.
DISCUSSION

Using the technique of somatic cell fusion, we have approached the question whether the absence of specific cellular functions might represent an essential condition in the pathogenesis of endemic BL. Hybrids between a highly tumorigenic BL cell line and a nontumorigenic LCL derived from the same individual were shown to be nontumorigenic despite the presence of EBV and the continued deregulation of the c-myc gene.

Suppression of the malignant phenotype has already been observed for a wide spectrum of murine and human tumor cells fused with normal fibroblasts or keratinocytes (32–36). Whether this tumor suppression effect is also a characteristic of lymphoma/lymphocyte hybrids, however, has still been unclear. For instance, murine T-lymphoma/lymphocyte hybrids retained the tumorigenic phenotype (37, 38). In addition, the murine plasmacytoma/lymphocyte hybrids commonly used for the production of monoclonal antibodies show progressive growth in vivo (3). It thus remained an open question whether these results reflected a dominance of phenotype of the malignant lymphoma or whether they were due to loss of the chromosomes which are thought to be necessary for tumor suppression. In the seven BL/LCL hybrid clones presented here, nontumorigenicity correlates well with a stable, near tetraploid karyotype.

In previous studies with BL/LCL hybrids which were established to analyze the regulation of the translocated c-myc gene (39, 40), no conclusions concerning a putative tumor suppression mechanism could be drawn, because the parental LCL, despite its lymphoblastoid phenotype in tissue culture, was highly tumorigenic in nude mice after introduction of selection markers (39).

Our result that the EBV-positive BL/LCL hybrids are not tumorigenic despite deregulated c-myc expression may have implications for the understanding of endemic BL. A three-step development of endemic BL has been suggested by Klein (41). According to this model, primary EBV infection is the first event and leads to B-lymphocyte immortalization. Subsequent T-cell suppression and polyclonal B-cell proliferation stimulated by parasite infection, e.g., holoendemic malaria, cause an increase in the number of dividing B-cells which increases the risk of genetic alterations. The chromosomal translocation is the third step and finally leads to the deregulation of the c-myc gene and the development of a malignant cell clone. Alternatively, Lenoir and Bornkamm (42) have suggested that the malaria-induced triggering of the B-cell system and the chromosomal translocation precede EBV infection. Despite the different temporal sequence in these two scenarios, the two critical events remain the same: EBV infection and c-myc deregulation.

Experiments in which the two postulated critical factors were introduced into B-lymphocytes appeared to support these models. EBV-induced immortalization and subsequent transfection of a constitutively expressed c-myc gene were reported to render human B-lymphocytes tumorigenic, suggesting that EBV infection and c-myc deregulation might be sufficient for malignant transformation in this cell type (43).

The results reported here, in some contrast, indicate that, at least in somatic BL/LCL hybrids, EBV and c-myc deregulation may not be sufficient for maintenance of the tumorigenic phenotype.

At present we cannot exclude that specific features of the experimental system of somatic cell hybrids contribute to the suppression of the malignant phenotype. For instance, gene dosage effects in the near tetraploid hybrids could result in an increased production of a factor inhibiting the product of the activated c-myc gene.

The discrepancy between the tumorigenicity of the c-myc transfectants in the study mentioned above and our nontumorigenic BL/LCL hybrids might also be due to the use of different cell lines and experimental conditions in the tumorigenicity assays. The c-myc transfectants were reported to induce tumors within 5 weeks after inoculation into untreated nude mice, in contrast to the control LCLs which did not obtain c-myc expression plasmids. No further information was provided on size and long-term growth pattern of the transfectants. In the present study, 5 weeks after inoculation into preirradiated nude mice, most of the LCL and hybrid grafts appeared as small tumors but regressed completely in the following weeks. Similar results, albeit with an overall reduced take rate, were also obtained with a small number of nonirradiated recipient nude mice (data not shown). The in vivo growth pattern observed for the parental cell lines in the present study is in accordance with the results of Gurdsevitch et al. (29) who tested a large number of BL cell lines and LCLs for their tumorigenic potential in nude mice.

Our view that EBV infection and c-myc deregulation may not be sufficient for tumorigenicity of B-lymphocytes is supported by recent results of Hotchin et al. (44). These authors also introduced a constitutively expressed c-myc gene into an EBV-immortalized lymphoblastoid cell line and reported that the transfectants had altered growth properties in vitro but were not tumorigenic in vivo.

Taken together, our results suggest that the parental LCL contributes one or more specific functions which prevent tumor formation of the hybrids and which are absent in the parental BL cell line.

We are currently investigating the EBV latent gene expression in the hybrid cells described here. These experiments may reveal whether a modification of the viral gene expression is involved in tumor suppression. However, other mechanisms have also to be considered, e.g., regulation of cell proliferation, tumor angiogenesis, modification of the differentiation state, and expression of specific cell surface antigens.

ACKNOWLEDGMENTS

We thank Gilbert Lenoir for providing the cell lines, Georg Bornkamm for the c-myc and EBV plasmids, Britta Klevenz for excellent technical assistance in tumorigenicity assays, and Magnus von Knebel-Doeberitz, Regine Heilbronn, Michael Boshart, and Valerie Bosch for helpful discussion and critical reading of the manuscript.

REFERENCES

SUPPRESSION OF MALIGNANT PHENOTYPE IN SOMATIC CELL HYBRIDS


Suppression of the Malignant Phenotype in Somatic Cell Hybrids between Burkitt’s Lymphoma Cells and Epstein-Barr Virus-immortalized Lymphoblastoid Cells despite Deregulated c- myc Expression

Jürgen Wolf, Michael Pawlita, Jörn Bullerdiek, et al.


Updated version  Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/50/10/3095

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.