Eradiation of Latent Epstein-Barr Virus by Hydroxyurea Alters the Growth-Transformed Cell Phenotype

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The hallmark of infection by human herpesviruses, life-long persistence in the host, is unaffected by current antiviral therapies effective against replication of virus. In vitro studies indicated that low concentrations of the ribonucleotide reductase inhibitor, hydroxyurea, completely eliminated Epstein-Barr virus (EBV) episomes from latently infected Burkitt’s lymphoma (BL) cell subsets, providing the first example of chemotherapeutic eradication of a latent herpesvirus from any cell population. Unlike parental EBV-positive BL cells, virus-free cell progeny from one treated cell line no longer exhibited the malignant phenotype in tumorigeniuity assays. Hydroxyurea-treated primary B lymphocytes immortalized by EBV ceased to proliferate as episomes were lost. The altered growth phenotype of both BL cells and immortalized primary B cells suggests that latent EBV is an appropriate and accessible therapeutic target for treatment of some EBV-induced lymphoproliferations.

The Epstein-Barr virus (EBV) is a transforming human gamma-herpesvirus that in its latent state is associated with Burkitt’s lymphoma (BL), nasopharyngeal carcinoma, Hodgkin’s lymphoma, and lymphoproliferative disorders of immunosuppressed patients. Little consideration has been given to therapeutic strategies that target EBV in virus-associated malignancies, first, because latent herpesvirus infection is intractable to available antiviral chemotherapy [1, 2], and, second, because of the uncertainty that EBV contributes to the long-term maintenance of the malignant cell phenotype. In tumors such as BL, latency-associated viral proteins critical to successful growth transformation of primary B lymphocytes are down-regulated [3, 4].

In the latent state, the EBV genome is maintained as circular extrachromosomal DNA with multiple (but varying) copies per cell [5–8] that replicate in synchrony with cellular DNA during the cell cycle [9]. Because the ribonucleotide reductase inhibitor hydroxyurea has been shown to accelerate the loss of extrachromosomally amplified myc and drug-resistance genes from human tumor cell lines [10–12], we used the drug to treat EBV-immortalized as well as malignantly transformed lymphoid cells that reflect the heterogeneity of EBV lymphoproliferative diseases. The intent was to determine if the elimination of EBV episomes might offer a clinical strategy for antiviral intervention in EBV-associated lymphoproliferations.

Materials and Methods

Cell lines and hydroxyurea treatment. The cell lines studied were four EBV-positive BL tumor–derived lines (Akata [13], Mutu [14], Raji [15], Namalwa [16]) and four EBV-immortalized lymphoblastoid cell lines (IB4 [17], TW236, established by immortalization of umbilical cord blood lymphocytes with a throb-washing isolate; AB, obtained by transformation of adult peripheral blood lymphocytes with laboratory strain B95-8; and RR, derived from spontaneously transforming peripheral blood lymphocytes of a bone marrow transplant patient with lymphoproliferative disease). Because of spontaneous EBV DNA loss reported in long-term (>6 months) cultures of the Akata BL cells [18], newly derived EBV-positive clones established by cell sorting were used in these experiments to ensure a uniform population of EBV-infected cells. Cell cultures were seeded at 5 × 10⁵ cells/mL and continuously exposed to hydroxyurea (Sigma, St. Louis) at 50 μM. Cells were counted twice weekly, and their viability was tested by trypan blue exclusion; cells were then removed from spent medium by low-speed centrifugation and resuspended at 5 × 10⁵ cells/mL in fresh medium (RPMI 1640, 10% fetal bovine serum [FBS]) containing hydroxyurea. Aliquots of cells were frozen for determination of EBV DNA content at each time point. To assess virus reactivation, treated and untreated cell cultures were stained by indirect immunofluorescence with BZ.1 monoclonal antibody (gift of L. S. Young, University of Birmingham, Birmingham, UK) for the EBV immediate-early protein BZLF1.

Quantiﬁcation of EBV DNA. DNA was extracted from treated cells as well as untreated controls by standard chemistry (340A Nucleic Acid Extractor; Applied Biosystems, Foster City, CA). Total cellular DNA was quantified by spectrophotometry, digested
with BamHI, and electrophoresed in 0.8% agarose gels. After Southern transfer, blots were hybridized to a 32P-labeled probe (random primed DNA labeling kit; Boehringer Mannheim, Indianapolis) specific to the single-copy BamHI Z EBV DNA fragment. Blots were subsequently stripped and rehybridized to a polymerase chain reaction (PCR)–derived (primers 5'-ATCGAGAAATGT-GGCCACAG-3' and 5'-GTCTTCTTTTGGAAGACAC-3'), 32P-labeled probe specific to repetitive human DNA (base coordinates 551–800) [19], whose copy number of 400 members is highly conserved. Quantification of viral and cellular hybridization signals was done by use of a PhosphorImager (Molecular Dynamics, Sunnyvale, CA), and viral DNA determinations were expressed per amount of cellular DNA detected in each lane.

Fluorescent in situ hybridization (FISH). Cosmids cm302-21 and cMsal-B [20], containing ~40 kbp of EBV DNA each, were used to generate probes labeled by nick-translation with digoxigenin-16-UTP (Boehringer Mannheim). Probe was hybridized overnight to fixed interphase nuclei of hydroxyurea-treated or untreated cell populations as previously described [21]. Hybridized probe was detected by fluorescein-conjugated sheep antibodies to digoxigenin (Boehringer Mannheim). EBV DNA copy number was scored by counting the number of fluorescent signals in a minimum of 50 interphase nuclei.

Establishment of single-cell clones. After at least 15 population doublings, treated cell populations were cloned by cell sorting (FACStar Plus; Becton Dickinson, Braintree, MA) into 96-well plates containing irradiated foreskin fibroblast feeder layers in hydroxyurea-free medium. Individual clones were screened for the presence of EBV DNA by PCR for a reiterated region of EBV DNA, IR1, as described previously [22]. Then, growth kinetics of EBV-positive and EBV-negative clones in the presence of 50 μM hydroxyurea were determined over an 8-week period in culture.

Determination of cell growth phenotype. Growth characteristics of EBV-positive and EBV-negative clones, seeded at 1 × 10^5 cells/mL, were determined in RPMI 1640 medium containing 0.1% FBS over 1 week in culture. Anchorage-independent growth in soft agar was assessed in 0.33% agarose (SeaPlaque; FMC Bioproducts, Rockland, ME) as described [18]. Tumorigenicity of treated and untreated cell clones was assayed in 6- to 8-week-old SCID mice (St. Jude Children’s Research Hospital Animal Resources Center). Cells (2 × 10^3) from EBV-positive and EBV-negative clones suspended in 200 μL of PBS were injected subcutaneously into the left and right flank, respectively. Four mice were injected per EBV-negative clone tested. Mice were euthanized if tumors exceeded 2 cm or if mice appeared ill. All resultant tumors were screened for EBV by DNA hybridization analysis.

Results

Reduction of viral DNA from treated BL cell populations. The BL-derived cell line, Akata, has been shown to lose EBV DNA spontaneously from cell subpopulations during long-term culture [18], a phenomenon mimicking the unstable maintenance of viral episomes reported in tumors in vivo [23, 24]. To determine if hydroxyurea can accelerate episomal DNA loss from latently infected cells, we maintained newly derived EBV-positive cell clones of Akata in culture medium containing 50 μM hydroxyurea, a concentration far below achievable serum levels of the drug (500–2500 μM) observed clinically during treatment of neoplasia [25]. At the 50-μM level, cell death was not observed by trypan blue staining, and all cell lines continued to grow during continuous hydroxyurea exposure with only minimal increases in cell doubling times. By indirect immunofluorescent staining for BZLF1 protein, virus reactivation could not be demonstrated in any treated cell lines.

Southern blot analysis of total cellular DNA at serial time points during continuous treatment with hydroxyurea demonstrated a progressive reduction in EBV DNA content relative to cellular DNA (figure 1). By 15 cell population doublings, a 99.5% reduction in EBV DNA content was observed in treated cells when data were standardized by densitometry for cellular DNA loaded per lane. EBV DNA levels in untreated Akata cells did not change over the equivalent number of cell population doublings.

To determine if hydroxyurea-induced loss of extrachromosomal EBV DNA was reproducible in multiple BL lines, we treated two additional BL lines, Raji and Mutu. By Southern blot analysis, EBV DNA content was reduced by 50% in treated Raji cells, whereas in Mutu cells, a specific reduction in total EBV DNA was not observed. A further decrease in EBV DNA content was not achieved with increased drug concentrations of 100–200 μM. A fourth BL line, Namalawa, which does not contain episomes but has two copies of EBV DNA integrated into host chromosomes [16, 26, 27], predictably did not exhibit a reduction in viral DNA with treatment (data not shown).

Viral DNA content of BL cells at the single-cell level. At an individual cell level, there was complete eradication of EBV DNA from treated cell subsets in two BL lines examined, Akata and Mutu. When analyzed for EBV DNA by FISH, untreated
Akata and Mutu cells contained numerous hybridization signals per cell, each denoting a single EBV episome (as shown for Akata cells in figure 2A). By contrast, almost half of the hydroxyurea-treated Akata cells contained no signal whatsoever (figure 2B), and those that retained episomes had an overall lower copy number than did untreated cells (figure 2C). Complete episome loss was also detected in treated Mutu cell populations that had no discernable EBV DNA reduction on Southern analysis, but only in the occasional cell (not shown).

As corroboration of results obtained by FISH analysis, 57 (43%) of 131 cell clones derived from the hydroxyurea-treated Akata cell population by single-cell sorting were EBV-negative as determined by PCR analysis and by immunoblot detection of the EBV nuclear antigen 1 (EBNA1). Four (2%) of 169 cell clones established from treated Mutu BL cells were EBV-negative versus none from untreated cell populations. Cytogenetic studies of the EBV-negative clones demonstrated the identical t(8;14) translocation as found in the respective parental EBV-positive cell line.

**Growth characteristics of hydroxyurea-treated BL cells.** BL cells share growth properties intrinsic to most cancer cells: reduced serum requirements in culture, growth in soft agar, and tumorigenicity in immunodeficient mice. Because latent EBV proteins critical to cell transformation and growth proliferation in vitro (EBNA-LP, EBNA2, EBNA3a, EBNA3c, and LMP1) are not expressed in BL cells [3, 4], it has generally been assumed that virus, although important for tumor initiation, does not contribute to the long-term maintenance of the malignant cell phenotype [28]. To examine the effect of induced episomal DNA loss on BL cell growth, four cell clones each from the two tumor cell lines cured of viral episomes were compared in tumorigenicity assays to respective EBV-positive clones.

Whereas growth of EBV-positive and EBV-negative Akata clones was comparable in medium containing 10% FBS, only EBV-positive cell clones were capable of proliferation in 0.1% FBS (figure 3A); the EBV-negative counterparts ceased to grow under these conditions, and cells were no longer viable beyond 7 days. Serum dependency of EBV-negative Akata clones did not simply reflect toxic effects of prior hydroxyurea exposure, since treated cell clones that retained EBV DNA grew at the same rate in 0.1% FBS-containing medium as did untreated, EBV-positive cells (figure 3A). In soft agar growth assays, EBV-positive Akata cells formed visible colonies within 3 weeks. By contrast, no cell clone from which EBV DNA had been eliminated produced colonies (figure 3B). Notably, EBV-negative Mutu BL cell clones (unlike virus-negative Akata cells) did not show impaired growth in either liquid or semisolid growth media compared with that of their EBV-positive counterparts, perhaps reflecting additional stepwise changes in cell context that occur throughout tumor progression [29, 30].

In vivo tumorigenicity assays were done in SCID mice with virus-free cell clones derived from both treated BL lines. Four cell clones from each BL line were used, and EBV-negative cells were injected subcutaneously into 4 mice each at an inoculum of $2 \times 10^7$ cells. The respective EBV-negative parental BL clones were injected into the opposite flank as controls. At 6 weeks after injection, 10 of 15 surviving mice had developed large masses from EBV-positive Akata cells, with no evidence of tumor formation at the site of inoculation of EBV-negative Akata BL clones ($P < .001$, Fisher’s exact test, two-tailed; figure 3C). All tumors were EBV-positive by PCR assay, confirming their derivation from injected human cells. In agreement with in vitro assays, a comparable change in growth pattern was not observed with four EBV-negative clones isolated from the treated Mutu BL line. Tumors from EBV-positive and -negative Mutu clones developed in 12 of 16 versus 8 of 16 mice ($P = .27$, Fisher’s exact test, two-tailed).

**Effect of hydroxyurea on the immortalized phenotype of EBV-infected lymphoblastoid cell lines.** Because sustained growth of primary B cells infected with EBV is virus-depen-
almost identical to those of the untreated control (figure 4A), a pattern consistent with that observed with most tumor-derived lines maintained on the low concentration of drug [10, 12].

In contrast, the population doublings of a lymphoblastoid cell line (TW236) containing EBV DNA in an episomal configuration were markedly reduced in the presence of hydroxyurea (figure 4B). Moreover, the growth of a second lymphoblastoid cell line (RR), derived from spontaneously transforming peripheral blood lymphocytes of a patient with posttransplant EBV lymphoproliferative disease, could not be sustained with treatment (figure 4C). The impaired growth of hydroxyurea-treated lymphoblastoid cell lines coincided with diminished EBV episome numbers as determined by FISH analysis. The median hybridization signal count per cell in hydroxyurea-treated versus untreated TW236 cells was 6 versus 21 ($P < .001$, Wilcoxon two-sample test), a result almost identical to that achieved in a third lymphoblastoid cell line (AB; not shown). Indeed, complete absence of hybridization signals over some cells in the treated populations (figure 5) is consistent with an apparent reversal of lymphocyte immortalization accompanying drug-induced EBV DNA loss.

Discussion

Tumors arise through an accumulation of genetic changes affecting the control of cell growth [37, 38]. BL was one of the first cancers for which the multistep nature of neoplasia could be defined in terms of successive biologic and molecular events: EBV-induced B cell immortalization complemented by deregulation of c-myc through the characteristic t(8;14) translocation found in BL tumors [28]. The likely temporal sequence for these two genetic “hits” (and consequently the contribution from viral genes to the proliferation of cells carrying the translocation) has been a matter of debate [39, 40]. What our results allow, through elimination of EBV from a heterogeneous group of lymphoid cell lines from lymphoblastoid to BL, is a direct dissection of the role of virus in cell proliferation at various stages along the multistep pathway of tumor progression.

From a biologic and therapeutic standpoint, a key finding is the critical part EBV still plays in the malignant growth phenotype of Akata BL cells despite the presence of the translocated c-myc gene, confirming an earlier report of BL dependence on the presence of EBV genomes [18]. The enforced loss of episomes achieved here with hydroxyurea in short-term culture establishes a direct link between virus loss and altered growth phenotype, which as spontaneous events in long-term culture could have been merely coincidental. Our results also imply that BL dependence on EBV may not extend through all evolutionary stages of the malignancy. For example, the contradictory impact of viral DNA eradication on growth of clones derived from Akata versus Mutu BL cells most likely reflects additional karyotypic abnormalities (e.g., 11q23 rearrangement [41] [unpublished data]) present in the latter, rendering previously critical viral functions redundant. That factors in addi-
tion to the t(8;14) translocation may be needed for tumorigenesis is corroborated by clinical data that show expanded B cell clones bearing a t(8;14) rearrangement in peripheral blood lymphocytes of persons without clinical disease or a subsequent predisposition to develop lymphoma [42]. One might speculate that the subsequent infection by EBV of cells carrying that specific translocation in vivo could provide the critical second event required for malignant transformation [39].

What EBNA1, the only known viral protein expressed in Akata, contributes to tumorigenicity of BL is as yet unclear, but our finding adds to accumulating evidence for the oncogenic potential for this protein when expressed in the absence of other viral gene products [43, 44]. In dividing cells, EBNA1 is known to play a crucial role in EBV genome persistence and episomal DNA replication [45, 46]. In lymphoblastoid cell lines, on the other hand, the contribution of the nine expressed EBV latency-associated proteins to B lymphocyte immortalization is well-delineated, making the observed alteration in lymphoblastoid cell growth phenotype accompanying EBV loss almost intuitive.

Treatment of a diverse group of eight lymphoid cell lines indicated that the loss of viral episomes (most dramatic in Akata cells) reflects activity of hydroxyurea against the viral episome, not a selective antiproliferative effect against EBV-positive cells in a mixed cell population such as Akata [18]. For example, complete viral DNA loss (figure 5) in subsets of immortalized primary B lymphocytes totally dependent on EBV for growth in culture is convincing evidence for a direct antiviral effect. Two uniformly infected BL lines, Raji and Mutu, had either an overall reduction in EBV DNA content after drug exposure or, at a single-cell level, yielded EBV-negative cell clones. Finally, studies with Akata used freshly derived EBV-positive cell clones that provided a homogeneous cell population. Even if natural loss of episomes occurred in a subset of these cells, Akata cell clones with and without EBV exhibited identical growth kinetics when maintained in 50 μM hydroxyurea. Moreover, viral activation as a consequence of drug exposure was not demonstrated by immunostaining for the viral immediate-early protein BZLF1, ruling out a preferential cytolytic effect in virus-bearing cells.

An antiviral effect from hydroxyurea at nontoxic doses of drug has previously been reported with regard to human immunodeficiency virus type 1 infection both in vitro [47, 48] and in vivo [49] and is based on the drug’s ability to decrease the intracellular pool of dNTPs [50]. Hydroxyurea inhibits the cellular ribonucleotide reductase, a rate-limiting enzyme in synthesis of dNTPs required for DNA replication [34]. Propagation of episomal EBV DNA in concert with the cell cycle requires that the viral genome compete effectively for compo-

Figure 4. Hydroxyurea inhibits growth of lymphoblastoid cell lines containing EBV DNA in episomal but not integrated configuration. Primary B lymphocyte lines immortalized by EBV were cultured in presence or absence of hydroxyurea. A, IB4, which has viral genome integrated and was derived by infection of umbilical cord blood lymphocytes with laboratory EBV strain B95-8; B, TW236, cord blood lymphocyte line containing throat-washing isolate of EBV in episomal configuration; C, RR, spontaneous peripheral blood lymphocyte transformant containing episomal viral DNA, which was derived from bone marrow transplant recipient with lymphoproliferative disease.
Hydroxyurea Elimination of Latent EBV

Figure 5. Hydroxyurea-induced episomal loss from EBV-immortalized primary B lymphocytes as shown by fluorescence in situ hybridization analysis of untreated (A) and drug-treated (B) lymphoblastoid cell line TW 236. Arrows in B indicate EBV-negative interphase nuclei.

of the cellular replication machinery [2]. In the context of limiting substrate induced by low-dose hydroxyurea, competition for dNTPs may be heightened, particularly if EBV episomal replication is restricted to late S phase [51], at which time the remaining intracellular nucleotide pool will have been further depleted by ongoing cellular DNA synthesis. The variable resistance of Raji and Mutu cells to hydroxyurea-induced episome reduction most likely reflects increases in ribonucleotide reductase levels [34], reported to be markedly elevated in some DNA tumor virus–transformed cells [52]. Resistant cells provide a means for future delineation of the specific mechanism of drug activity against latent EBV.

One stage reportedly involved in hydroxyurea-induced loss of extrachromosomal DNA elements from tumor cells is their preferential entrapment into micronuclei, which are either extruded at the time of cell division or degraded by nucleases [11]. Segregation of EBV DNA into micronuclei was not observed in our FISH studies, and the physical anchorage of EBV episomes (unlike amplified double minute elements) to host cell chromosomes [53] makes that mechanism improbable with respect to the EBV DNA loss we described. Our results, suggesting selective inhibition of EBV DNA replication during latent infection, contrast with what has been reported during the productive (lytic) phase of the EBV life cycle in the presence of much higher levels of hydroxyurea. During the lytic cycle, EBV DNA replication is resistant to hydroxyurea at concentrations that simultaneously induce a dramatic reduction in synthesis of cellular DNA [54–56]. Failure of the drug to interfere with production of infectious virions may reflect the selective expression during the lytic cycle of a virally encoded ribonucleotide reductase [57], known to be resistant to inhibition by hydroxyurea [58].

The effect of hydroxyurea on maintenance of EBV episomes in tissue culture suggests two immediate applications. As an investigative tool, the drug may facilitate analysis of viral integration, a recurrent theme in tumor virus research but an issue on which little is known regarding EBV-associated tumors. Because of the technical difficulty of detecting a single integration event against a background of multicopy episomes, only a limited number of cell lines with unusually low EBV copy number have been studied and shown to have integrated EBV [59]. Clinically, the activity of hydroxyurea that we report, coupled with the drug’s well-known clinical safety profile derived from years of use in human therapy, its ability to diffuse into all tissues, including the central nervous system, and the acceptable toxicities at the low concentrations used here [34, 35], make it an attractive candidate for immediate clinical trials. On the basis of altered growth of treated cells early in the pathway to malignant transformation, we would predict greatest potential efficacy when applied to EBV-associated lymphoproliferations that mimic the immortalized lymphoblastoid cell phenotype.

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References