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Proof for EBV's Sustaining Role in Burkitt's Lymphomas

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Abstract

We have found that not all Epstein-Barr viral (EBV) plasmids are duplicated each cell cycle. This inefficiency is intrinsic to EBV's mechanism of DNA synthesis in latently infected cells and necessarily leads to a loss of EBV plasmids from proliferating cells. If EBV provides its host cells advantages that allow those cells that retain EBV to outgrow those that lose it, then such proliferating populations will be EBV-positive. EBV-associated human tumors are EBV-positive. Thus the presence of EBV plasmids in most cells of a tumor demonstrates that EBV sustains these tumors *in vivo*. The virus can provide multiple selective advantages to tumor cells, including promoting cell proliferation and inhibiting cell death. In the case of Burkitt's lymphomas (BL), most current evidence indicates that the tumor requires the virus minimally to block apoptosis.

Keywords

Burkitt's lymphoma; Epstein-Barr virus; tumor virus; tumor survival

Introduction

Multiple viruses cause cancers in their natural hosts; that is, infections by them are risk factors for the development of these cancers. For example, Rous Sarcoma virus induces sarcomas in 100% of susceptible strains of chickens [1]; infection with Hepatitis B Virus gives a 100-fold increase in the likelihood of people developing primary hepatocellular carcinoma [2]. The causal contributions of viruses to cancers can be assessed prospectively by determining the frequency of developing the viral-associated cancers in infected and uninfected populations. The roles tumor viruses play in sustaining tumors is more difficult to determine, but of great therapeutic importance. So long as tumor viruses help sustain their associated tumors, targeting these viruses either to eliminate the viral genomes or to inhibit their gene products in tumor cells should be therapeutically beneficial.

One approach to testing maintenance functions for tumors has been to engineer the conditional expression of proto-oncogenes or oncogenes in various cells of transgenic mice and measure both the formation and potential reversal of tumors as a function of expression of the transforming genes. These studies indicate that tumor dependence on the continued expression of the proto-oncogenes or oncogenes is not certain and varies with the type of tumor and the duration of the initial expression [3-5]. These observations, though intriguing in themselves, are equivocal about any role tumor viruses might play in maintaining tumors. A second approach to addressing whether tumor viruses maintain their associated tumors is to analyze transformed cells. Early studies demonstrated that cells transformed *in vitro* by mutant viruses

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having temperature sensitive transforming genes were temperature sensitive for their transformed phenotypes [6,7]. This approach clearly is not applicable to the study of viral tumors derived from human patients. These tumors have now been studied by inhibiting viral genes expressed in cells derived from tumors. For both cervical carcinomas carrying human papillomaviruses (HPV) and Burkitt's lymphomas carrying Epstein-Barr virus (EBV), inhibiting viral genes in cell culture induces cell death in the tumor derived cells [8-10]. These findings indicate that both HPV and EBV likely sustain their associated tumors *in vivo*.

We have found independent evidence that EBV sustains all of the cancers in which its plasmid genome is present in the bulk of tumor cells. In this review we shall detail this evidence and evidence for the selective advantages that EBV provides the tumors it sustains.

Studies of the replication of EBV's plasmid genome: the proof

We found proof for EBV's sustaining its associated tumors serendipitously through our studies of the replication of EBV's plasmid genome. EBV's DNA genome is generally found as an extrachromosomal element, a plasmid, in latently infected cells, those cells not supporting a productive infection. Two viral genetic elements mediate its replication; all else is provided by the cell. *OriP*, EBV's origin of plasmid replication, consists of two clusters of binding sites in its DNA for the viral protein EBNA1 [11,12]. One cluster of four sites, termed DS, is a licensed origin of DNA synthesis [13-15]. The second cluster consisting of twenty sites, termed FR, affects maintenance of plasmids in proliferating cells [16-18]. EBNA1 on binding DS recruits the cellular origin recognition complex, ORC, required for the initiation of DNA synthesis [14,15]. EBNA1 on binding FR likely tethers EBV's plasmid to AT-rich sites in human chromosomes via EBNA1's AT-hook domains [19] to foster the maintenance of EBV DNA in proliferating cells.

We wanted to examine the synthesis of EBV's plasmids and their partitioning to daughter cells to measure the efficiency of these events and to learn if they were coupled mechanistically. To do so we used a method developed by Andrew Murray and his colleagues to visualize segments of DNA in live yeast cells [20]. A polymer of binding sites for the lac repressor was introduced in an EBV-derived plasmid carrying *OriP* and a selectable marker. This plasmid was introduced into cells, clones of cells carrying the plasmids selected, and the cells were transduced with retroviruses expressing the lac repressor fused to red fluorescent protein (RFP). The lac repressor binds its DNA binding site specifically with high affinity but can be induced to dissociate from DNA on exposure to its inducer, IPTG. Cells were carried in IPTG until we wanted to visualize the EBV-derived plasmids, at which time the IPTG and the selective drug were washed away and the cells monitored with a fluorescent microscope. The results of these observations are diagrammed in Figure 1 [21]. They indicate that 84% of EBV-derived plasmids are synthesized each S-phase; all of these duplicated plasmids remain co-localized as single signals within the resolution of the fluorescent microscope through to anaphase when they separate as do sister chromatids. 88% of these co-localized pairs partition symmetrically; one to one daughter cell, the other to the other. The remaining 12% of these pairs partition asymmetrically; two to one daughter cell, none to the other. These rates of partitioning for the duplicated plasmids differ from the 50% that would be determined by a random process, indicating that EBV has evolved a non-random mechanism for its partitioning. The 16% of plasmids that fail to be duplicated in each S-phase are distributed randomly to daughter cells yielding a loss of 8% of plasmid DNAs in the population of daughter cells. This loss has also been confirmed with intact EBV plasmids in endothelial and B-lymphoid cells [21]. It underlies the evidence for EBV's sustaining its associated tumors. Every population of proliferating EBV-positive cells loses 8% of the viral genomes each cell cycle; after 8 cycles, only 50% of the viral DNA will remain; after 50 cycles, only 1% will remain in the population. However, if those cells that retain EBV are provided selective advantages by EBV such that they survive

or proliferate more efficiently than do their sister cells that have lost EBV, then a population of proliferating cells will remain EBV-positive.

Two sets of observations when coupled show that EBV-positive tumors do retain EBV plasmids. First, biopsies from EBV-positive tumors have been examined with immunocytochemistry and *in situ* hybridization to detect viral antigens and RNAs in single cells. These studies indicate that the majority of cells identified as being malignant express viral antigens or viral RNAs and thus are infected [22-25]. Second, the state of EBV DNA has been characterized in biopsies and cell lines derived from them. These analyses indicate that the viral DNA in these populations of cells exists predominantly as closed circular, extrachromosomal molecules [26-28]. Thus most cells in most EBV-positive tumors retain EBV DNAs as plasmid molecules, indicating the virus must provide these tumors one or more selective advantages.

What are the selective advantages that EBV provides tumors?

The notion that tumor viruses can provide host cells a selective advantage is antithetical to the traditional roles of host and parasite. Viruses are obligate cellular parasites that depend on a host cell to reproduce. The host-virus relationship favors the virus: the virus produces progeny, while the cell is likely to die by lysis, by programmed cell death, or by an alerted immune response. But in the atypical case of a tumor virus and a host tumor cell, the roles appear to be reversed. The tumor cell depends on the virus for properties that sustain it. The host-virus relationship favors the host: the tumor cell is sustained by viral gene(s), while the virus is usually incapable of producing progeny. For EBV-associated malignancies, functional studies have revealed two broad selective advantages the virus can afford tumor cells. EBV is capable of both promoting cell proliferation and inhibiting cell death. Here we shall outline evidence for the contributions EBV can make to transformed cells and consider those it likely makes to Burkitt's lymphomas.

EBV fosters the proliferation of B cells

EBV can drive resting, primary B cells to proliferate (a process referred to as transformation). It has become clear that one viral gene central to EBV's ability to transform cells is the latent membrane protein 1 (LMP1). Not only does the deletion of LMP1 reduce EBV's transformation efficiency 100-fold [29,30], but the withdrawal of LMP1's signaling causes transformed cells to cease proliferation [31]. LMP1 is thought to foster proliferation by mimicking activated CD40. Like CD40, LMP1 can influence multiple signaling pathways including NF- κ B, JAK-STAT, and AP1, however it does so in a ligand-independent fashion [32-35]. Interestingly, the loss of LMP1 does not entirely preclude transformation. LMP1-null virus can still transform cells if feeder cells are provided, but these infected cells, unlike their wild-type counterparts, do not grow in SCID mice [29].

That B cells infected with a LMP1-null variant of EBV can proliferate under certain conditions parallels EBV-positive Burkitt's lymphomas, which also proliferate in the absence of LMP1. Primary tumors lack detectible LMP1 expression [36]. In addition, the robust signaling typically downstream of LMP1 is also lacking. For example, transcriptional profiling of BL biopsies has revealed a consistent downregulation of NF- κ B target genes when compared to other mature, aggressive B-cell lymphoma biopsies [37,38]. Likewise, EBV-positive cell lines derived from Burkitt's lymphomas do not display the constitutive activation of either JAK/STAT or AP-1 pathways present in EBV-positive cell lines that do express LMP1, such as those derived from post-transplant lymphoproliferative disorder (PTLD) or Hodgkin's lymphomas. [39-41]. One explanation for the absence of LMP1 in Burkitt's lymphomas is that cells expressing LMP1 are killed by the immune response; those that don't express it survive. This explanation is not wholly satisfying, though, for at least two reasons. Other viral proteins

(EBNA3A, 3B, 3C, LP, and LMP2A) [36,42,43] are expressed in subsets of Burkitt's lymphomas and can elicit cytotoxic T cell responses as can LMP1 [44,45]. Furthermore, if LMP1 were necessary to foster proliferation of cells progressing toward Burkitt's lymphoma, then the loss of its expression could only be compensated for by changes in the host cell that provided similar proliferative stimuli. This compensation is found, for example, in the constitutive activation of JAK/STAT or AP-1 pathways in both EBV-positive and EBV-negative Hodgkin's lymphoma cell lines [39,41]. In contrast, none of the pathways central to LMP1-mediated proliferation are efficiently activated in Burkitt's lymphoma. Thus, it seems reasonable that LMP1 is not expressed in BL both because it is immunogenic, and because it does not provide an optimal proliferative stimulus for the evolving tumor.

Given that LMP1 is not responsible for the proliferative stimulus in Burkitt's lymphomas, what is? A favorite candidate for this role is the c-myc oncogene, which is almost always translocated in Burkitt's lymphoma to one of the three immunoglobulin loci [46]. For example, transgenic mice that mimic the juxtaposition of the IgH E μ enhancer and the c-myc locus succumb to B-cell lymphomas at an early age. [47]. In addition, in a lymphoblastoid cell line, high levels of c-myc expression can compensate for the loss of EBNA2 function (and the concomitant loss of LMP1 which is transcriptionally regulated by EBNA2) to support proliferation [48]. While these data are consistent with the hypothesis that the translocated c-myc drives proliferation in BL, they are limited by being derived from experimental systems which oblige cells to evolve myc-dependent proliferation. Burkitt's lymphoma evolves *in vivo* under no such obligations. C-myc has not been rigorously shown to sustain the proliferation of Burkitt's cells; it may be required, for example, to initiate a proliferate state independent of LMP1 but not to sustain it. This latter possibility is consistent with multiple studies using mouse models in which tumors induced by c-myc can evolve to be independent of continued c-myc expression [4,5,49]. It is, therefore, possible that EBV provides proliferative stimuli to Burkitt's lymphomas with genes other than LMP1, that cellular oncogenes including c-myc provide these stimuli, or both viral and cellular oncogenes cooperate to foster proliferation.

EBV provides multiple blocks to apoptosis in B cells

EBV not only drives the proliferation of primary B cells, it also prevents them from dying by apoptosis [50,51]. Throughout B cell development and function, apoptosis removes defective B cells and maintains the homeostasis of surviving B cells [reviewed in 52]. B-lymphocytes are controlled by multiple regulators of apoptosis; they are "primed" for apoptotic cell death. EBV, having evolved a lifecycle adapted to B cells, survives amidst the apoptotic triggers used to regulate these cells by disarming them.

Burkitt's lymphomas also may be prone to apoptosis, reflecting their cell of origin. There is evidence for this notion *in vivo*: BL biopsies are marked with frequent apoptotic tumor cells. In fact, a defining histopathological pattern of Burkitt's lymphoma is a "starry sky" appearance, resulting from the infiltration of the tumor by phagocytic macrophages capable of engulfing apoptotic tumor cells [53]. The apoptotic signal affecting the tumor cells could originate from either internal or external sources over the course of tumor evolution. Misregulated c-myc sensitizes cells to apoptosis [reviewed in 54], and other less characterized oncogenic lesions may do so too. As the tumor develops, a hostile microenvironment may also stimulate apoptotic cell death. Burkitt's lymphomas grow extremely rapidly [55], possibly making nutrients and oxygen limiting for cell growth and survival. Lastly, once clinical treatment for the tumor has begun, the chemotherapeutic drugs commonly used are themselves potent inducers of apoptosis [37].

Burkitt's lymphomas, evolving under the selective pressure to block apoptosis from any of these effectors – oncogene misregulation, a hostile microenvironment, and even

chemotherapeutic drugs – can use EBV to escape this pressure. In fact, most viral genes expressed latently in B cells have been reported under some conditions to block apoptosis. EBNA3A and 3C (present in a subset of BLs) downregulate the expression of the pro-apoptotic tumor suppressor Bim coordinately, and can confer resistance to cytotoxic drugs including nocodazole, cisplatin, and roscovitine [56]. The ability to inhibit Bim expression may be particularly important in Burkitt's lymphoma, because deficiencies in Bim accelerate myc-induced tumor formation in mice [57]. A truncated form of EBNA-LP found in some BL lines has been shown to protect cell against apoptosis induced by verotoxin 1 or staurosporine [58]. EBNA1, found in all EBV-positive Burkitt's lymphoma, has been reported to diminish p53-induced apoptosis, but this activity has not been determined in the context of a B cell [10,59]. LMP2A, detected in some BLs *in vivo*, can modulate apoptosis induced in B cells by the B cell receptor [36,60]. The viral RNAs have also been demonstrated to possess anti-apoptotic activity and are ubiquitously expressed in Burkitt's lymphoma. The EBERs, short, non-coding and non-polyadenylated RNAs, can block cell death induced by hypoxia or interferon alpha in BL cells [61,62]. Recently, one of the many viral miRNAs, BART5, has been shown to inhibit the expression of the proapoptotic protein PUMA in epithelial cells, though it remains to be determined whether this activity occurs in B cells, too [63].

While it is clear that multiple viral genes could block apoptosis under various experimental conditions, it is not known which ones do so in Burkitt's lymphoma *in vivo*. These blocks not only may differ among tumors but also may change over the course of tumor evolution. For example, one subset of EBV-positive Burkitt's lymphomas (referred to as WP-restricted BL) that arise with a truncated viral genome, express a broader array of genes than do most EBV-positive Burkitt's lymphomas and exhibit greater resistance to multiple apoptotic stimuli [42, 43,64]. One related early-passage tumor displays a substantial heterogeneity of viral gene expression between cells and a correlative substantial variation in apoptotic resistance [64]. This heterogeneity, not seen in other similarly studied Burkitt's lymphomas, is consistent with this tumor, at presentation, progressing in the midst of changing selective pressures by acquiring an alternate set of anti-apoptotic viral genes. That multiple viral genes can block apoptosis and that different Burkitt's lymphomas express different combinations of these anti-apoptotic genes indicates that EBV contributes survival functions to these tumors in multiple ways.

Counterexamples?

A few Burkitt's lymphomas lose EBV genomes when propagated in cell culture [65]. The rate at which these cells evolve to lose EBV is hastened when certain viral genes are expressed independently in them [66]. An extreme example of this evolution is provided by nasopharyngeal carcinomas. These tumors are generally EBV-positive when biopsied but when placed in cell culture generally evolve to lose their EBV plasmids [67]. Cells that retain EBV *in vivo* and lose it on propagation in cell culture could be interpreted to counter the notion that EBV affords the tumor cells selective advantages. We interpret these examples, however, to indicate that Burkitt's lymphoma cells can evolve to become independent of EBV's growth and survival genes. This evolution requires genetic or epigenetic changes in the tumor cell so that it differs from other Burkitt's lymphoma cell lines that continue their dependence on EBV in culture. In this interpretation, the loss of dependence on EBV is not surprising; it parallels the loss of dependence on cellular oncogenes observed in many other types of tumors. We propose that EBV provides nasopharyngeal carcinomas selective advantages that favor tumor cells *in vivo* but not in cell culture. One such advantage afforded them by EBV may be the inhibition of MHC class I genes that has been described [68].

Proof and consequences

An intrinsic property of the synthesis of EBV's plasmid genome is that not all viral DNAs are synthesized each cell cycle. A consequence of this inefficiency is that proliferating cells can retain EBV only if the virus provides those cells that do retain it the ability to outgrow those that lose it. Burkitt's lymphomas generally retain EBV plasmids and thus we can conclude that these tumors make use of the tumor virus to sustain themselves. What are these uses? Burkitt's lymphomas may use viral functions to promote their proliferation, but they appear minimally to use viral genes to insure their survival by blocking apoptosis. A consequence of these tumors depending on EBV for its selective advantages is that forcing the loss of the virus from the tumors, or inhibiting the functions of its survival genes in the tumors, would provide a therapeutic benefit to patients with Burkitt's lymphoma.

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Abbreviations

EBV	Epstein-Barr virus
BL	Burkitt's lymphoma
EBNA	Epstein-Barr Nuclear Antigen
LMP	Latent Membrane Protein
DS	Dyad Symmetry
FR	Family of Repeats
IPTG	isopropyl- β -D-thiogalactoside

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Figure 1. A model of the synthesis and partitioning of EBV plasmids

Shown is one cell in the G₂ phase of the cell cycle with EBV plasmids identified with letters. Four plasmids were duplicated in the prior S phase to yield co-localized pairs of plasmids (AA¹, BB¹, CC¹, and DD¹); one was not (E). Studies with EBV plasmids visualized in live cells have shown that 16% of the plasmids fail to be duplicated each S-phase [21]. These studies have also shown that during mitosis (M-phase) 88% of the co-localized plasmids are partitioned symmetrically. This partitioning is represented here as plasmids A and A¹, B and B¹, and C and C¹ each being distributed to separate daughter cells in G₁ phase. The 12% of the co-localized plasmids that do not partition symmetrically, represented by D and D¹, and the 16% of the plasmids that are not duplicated in S phase, represented by E, partition randomly. In the

example depicted here, all three plasmids (D, D¹, A) have been distributed to the same daughter cell. These defects in synthesis and partitioning lead to daughter cells having increased numbers of plasmids as shown for the upper cell in G₁ and decreased numbers of plasmids as shown for the lower cell in G₁ relative to the parental cell in G₂. The defect in plasmid synthesis leads to the loss of plasmids from a population of proliferating cells. The parental cell prior to S-phase had five plasmids; the two daughter cells have only nine plasmids or four and one-half plasmids per cell.