Histol Histopathol (1998) 13: 461-467

Histology and Histopathology

Invited Review

Interactions between Epstein-Barr virus and the cell cycle control machinery

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Summary. Epstein-Barr virus (EBV) persists in the majority of the world's human population. In the majority of cases the infection is asymptomatic, but EBV is associated with a number of human diseases, such as infectious mononucleosis, Burkitt's lymphoma, nasopharyngeal carcinoma, Hodgkin's disease, gastric carcinomas and other lymphomas and lymphoproliferative diseases. In this review the evidence linking EBV with these diseases is reviewed together with recent advances in understanding the interactions between EBV and the cell cycle control machinery.

Key words: Epstein-Barr Virus, Cell cycle, Cancer, Lymphoma, Leukaemia, Carcinoma

Epstein-Barr virus life cycle

Epstein-Barr virus (EBV) was the first human tumour virus to be identified (Epstein et al., 1964) and its potential role as a causative agent of disease has been the subject of intense investigation over the last 30 years. EBV is a member of the gamma Herpes virus family. The virus displays a limited host range, infecting humans and new world monkeys and a restricted tissue tropism, favouring lymphocytes and epithelial cells (reviewed in (Rickinson and Keiff, 1996)). For the majority of the adult population, exposure to the virus occurs at a young age; 90% of the adult population are sero-positive (Henle and Henle, 1979). If primary infection is delayed until teenage or early adult years, it frequently elicits a strong immune response resulting in infectious mononucleosis (reviewed in (Steven, 1996)). The majority of infected people remain asymptomatic although there is much evidence to suggest that the virus persists for life (see below). Unfortunately, for a small proportion of infected people, EBV is thought to contribute to the development of one of a number of EBV related diseases. EBV is classically associated with Burkitt's lymphoma in equatorial Africa (Epstein et al., 1964), nasopharyngeal carcinoma in China (Henle and Henle, 1976; Ho et al., 1976), and infectious mononucleosis through out the western world (Evans et al., 1968; PHLSLaboratories, 1971), the range of diseases has recently expanded to include post-transplant lymphoproliferative disease (reviewed in (Swinnen, 1996)), Hodgkin's Disease (Anagnostopoulos et al., 1989; Weiss et al., 1987, 1989), gastric carcinomas (Niedobitek and Herbst, 1994) and other lymphomas (evidence reviewed in (Su, 1996)).

Current models suggest that EBV is transmitted orally and once it is in the mouth, it can infect B-lymphocytes with high efficiency (Rickinson and Keiff, 1996). EBV enters these cells through interactions between one of its coat glycoproteins, gp340/220 and a cell surface receptor, CD21 (reviewed in (Nemerow et al., 1994)). The complex is internalised, the virus uncoats, circularises its DNA and starts expressing viral genes within a few hours. The virus encodes its own origin of replication (OriP) and origin binding protein (EBNA-1) thus ensuring that (i) the viral genome is maintained for the lifetime of the cell and (ii) that it will be duplicated and passed to both daughter cells if an infected cell proliferates (Rickinson and Keiff, 1996).

Much evidence suggests that EBV can reside in a latent form within asymptomatic hosts; the genome is maintained but few viral genes are expressed (see below). An accumulation of evidence suggests that naive B-lymphocytes are the site of EBV latency (as discussed in (Thorley-Lawson et al., 1996)); between one and sixty per million of these cells contain EBV DNA and exhibit a restricted pattern of viral gene expression (Miyashita et al., 1995). EBV can also be found in epithelial cells in vivo (Sixby et al., 1984; Greenspan et al., 1985); this is a site for lytic replication and virus shedding into the oropharynx (Yao et al., 1985) which may amplify the viral load within an individual and so play a role in the transmission of EBV to new hosts.

The two central issues relating to EBV and human health concern the life-long persistence of the virus within the infected host and the association of EBV with human diseases. In this review, the evidence linking EBV with cancer and the recent progress towards linking

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Table 1. Association of EBV with human diseases.

DISEASE		UNDIFFERENTIATED NASOPHARYNGEAL CARCINOMA	UNDIFFERENTIATED GASTRIC CARCINOMA	BURKITT'S LYMPHOMA		HODGKIN'S DISEASE
				Endemic	Sporadic	
EBV in disease	Frecuency of positivity in disease	100%	90%	nearly 100%	15 to 25%	<50% in Europe and North America 70% in Mexico
EBV in cells	Abnormal cell type	Epithelial cells	Epithelial cells	B lymphocytes		Reed-Sternberg cells
	Frequency of EBV genome in these cells	Nearly 100%	100%	100%		Nearly 100%
Genome	Clonality	yes	yes	yes		yes
Gene expression	Latency type	II	I	I		II

EBV with the disruption of cell cycle control machinery will be discussed.

Phenotype of Epstein-Barr virus infected cells.

Since EBV is prevalent within the population, yet EBV associated diseases are relatively rare, certain criteria must be met before associating EBV with a specific disease. In early studies, the importance of EBV in disease was assessed serologically (Henle and Henle, 1970, 1979a,b; Henle et al., 1968). Indeed, rising titres of serum antibodies against EBV lytic proteins are good prognostic markers for early stage Nasopharyngeal Carcinoma (NPC) (as reviewed in (Yip et al., 1996)). With the onset of new technologies, sensitive polymerase chain reaction (PCR) and in situ hybridisation (ISH) assays have been employed to identify the viral genome in biopsies. The presence of EBV in DNA isolated from biopsy material can be assessed by the amplification of regions of the viral genome by PCR, this type of analysis is also used to identify specific strains of EBV (as reviewed in (Gratama and Ernberg, 1995)). ISH is frequently used to detect two abundant viral products, the small non-poly adenylated RNAs (EBER-1 and EBER-2) (Chao et al., 1996). This allowed the presence of EBV to be detected in situ, distinguishing EBV-positive tumour cells from their neighbouring EBV-negative normal cells and any latent EBV carrying normal B-lymphocytes. With this type of analysis, the association of EBV with tumour cells has been definitively related to a number of pathological conditions (Table 1) (Herbst, 1996); for all of the diseases listed the EBV-positive cases contain a clonal population of EBV-transformed cells which implies that in these cases EBV provides one of the necessary steps for cellular transformation in vivo.

NPC can be divided into several sub-types which differ in their association with EBV. The most common sub-type, undifferentiated NPC, is strongly associated with EBV. In contrast, squamous cell and non-keratinizing NPC display variable associations with EBV, so the relationship between EBV and these subtypes of NPC is still under investigation (Niedobitek et al., 1996). This disease is highly prevalent amongst the southern Chinese (Ho, 1972). Recently, a link between bcl-2 expression and survival was identified in EBV associated NPC (Vera Sempere et al., 1997).

Gastric carcinoma is also divided into several sub-types, which show distinct associations with EBV. The undifferentiated subtype is highly related to EBV while the differentiated type and the more common poorly differentiated type are less often EBV-positive. Un-differentiated carcinoma, representing 10% of gastric carcinoma, is a very frequent malignancy in China, (as discussed in (Osato and Imai, 1996)).

Burkitt's lymphoma (BL) is divided into sporadic or endemic sub-types depending on the geographical area, although no histopathological differences are found between them. Endemic BL, typically represented in western Africa, has a strong association with EBV (almost 100%). The rarer sporadic cases in the rest of the world are less frequently associated with EBV (15-20%) (Rickinson and Keiff, 1996) (Table 1).

Hodgkin's disease (HD) has a similar mixed association with EBV; 60% of cases in China, 70% in Mexico and 97% in Peru are EBV-positive while in Europe and North America the frequency of cases associated with EBV is below 50% (Herbst, 1996).

From these brief descriptions of EBV-associated diseases, it is clear that there are distinct molecular mechanisms that can lead to the development of each disease. Furthermore, EBV is involved in the transformation of a wide range of cell types from lymphocytes to epithelial cells.

The initial approach taken towards elucidating the mechanism(s) that EBV uses to transform cells is to study which EBV genes are expressed in each disease. Since the virus has the potential to encode 90 or more genes (Farrell, 1993) and novel reagents for viral genes are continually being developed, this line of enquiry is necessarily ongoing. To date, three patterns of gene expression have been described in tumour material (Fig. 1) (extensively reviewed in (Gratama and Ernberg, 1995; Rickinson and Keiff, 1996)). Since none of these are associated with lytic virus replication and they are characterised by the expression of distinct sub-sets of

viral genes, they have been termed latency I, II and III.

All three of these forms of latency contain the EBV genome, express the two small non-polyadenylated RNA transcripts EBER1 and EBER2 and the viral protein EBNA-1 (EBV nuclear antigen). In latency I, viral gene expression is limited to these products. In latency II, the membrane proteins LMP-1 (latent membrane protein 1), LMP-2A and LMP-2B proteins are also expressed. In addition to these, the expression of EBNA-LP, -2, -3A, -3B, and 3C are included in latency III.

Correlation between latency type and disease have been studied using a variety of detection techniques, including ISH, RT-PCR and immunohistochemistry; the general pattern is described in Table 1, although variations in the patterns have been observed in some cases. This suggests that different sub-sets of viral genes are involved in the development of gastric carcinoma and Burkitt's lymphoma compared with NPC and HD (Table 1). However, this analysis might mask a transient requirement for other viral genes at an early stage.

These distinct patterns of viral gene expression within tumour types leads one to question whether any of them contribute to the transformed phenotype. Recently, much progress has been made towards understanding the ability of EBV to transform primary B-lymphocytes into immortal cell lines (LCLs) using viral mutants (reviewed in (Henderson et al., 1994; Farrell, 1995; Rickinson and Keiff, 1996)). Initially the contributions of individual viral genes to the process were assessed using recombinant viruses. This revealed that many EBV genes contribute to initiating and maintaining the immortal phenotype of LCLs; the loss of EBNA-2, -3A, -3C, -LP or LMP-1 prevents immortalisation by mutant viruses. In contrast, the loss of EBNA-LP, EBNA-3B, LMP-2A or LMP-2B does not prevent immortalisation but both EBNA-LP (Hammerschmidt and Sugden, 1989; Mannick et al., 1991) and LMP-2 (Brielmeier et al., 1996) contribute to the efficiency of the event. It is suggested that EBNA-1 is required for B-lymphocyte immortalisation, but this has not been formally tested at present.

More recently, attempts have been made to identify the minimal region of the viral genome required to immortalise primary B-lymphocytes by constructing virus particles containing large deletions in their viral DNA. Two groups have demonstrated that more than half of the genome can be deleted without losing the ability to generate LCLs. Kempkes et al. constructed a recombinant virus with only 41% of the viral genome (71Kb out of 172Kb) (Kempkes et al., 1995) and Robertson et al. showed that this could be further reduced to 64Kb (Robertson and Kieff, 1995; Robertson et al., 1994). Indeed, the virus particle itself has been shown to be inessential for immortalisation, electroporation with 71Kb of viral DNA in the context of an *E. coli* plasmid is sufficient to immortalise primary B-lymphocytes (Kempkes et al., 1995).

Altered cell cycle control by Epstein-Barr virus

The most convincing experimental evidence to suggest that EBV reprograms cell cycle control as part of its transformation strategy comes from its ability to immortalise B-lymphocytes in vitro; infection of B-lymphocytes from healthy donors drives the cells from a resting or quiescent state into continual proliferation resulting in the outgrowth of continually cycling immortal lymphoblastoid cell lines (LCLs). In recent years this in vitro assay has been used to identify

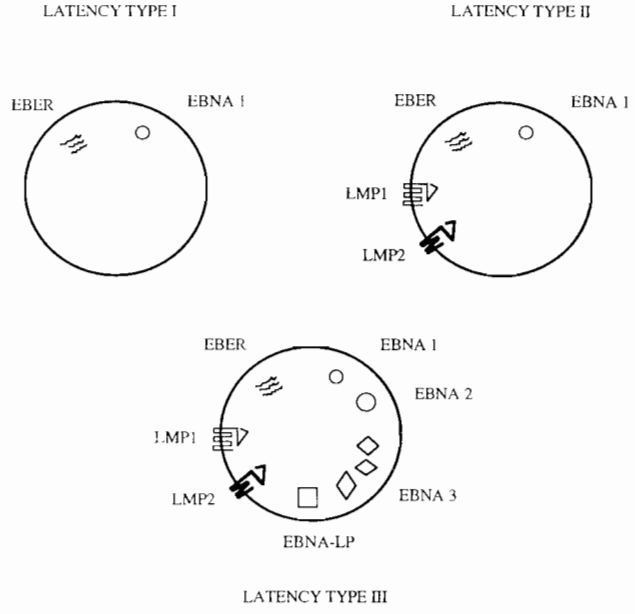

Fig. 1. The viral genes expressed during latency I, II, and III.

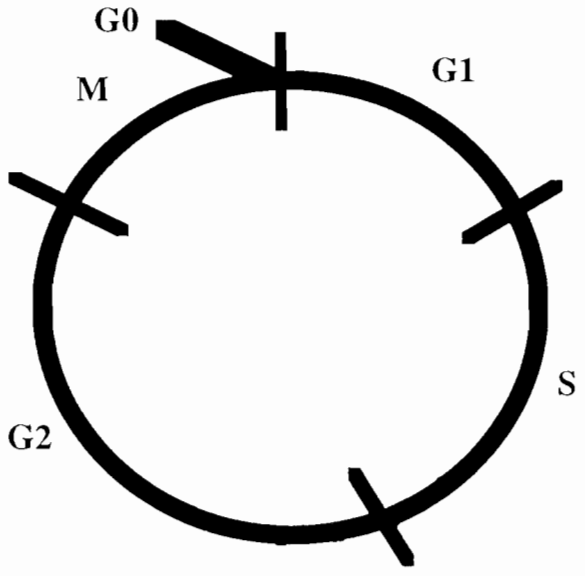

Fig. 2. The 5 phases of the mammalian cell cycle are shown,

changes to cell cycle control machinery driven by infection with EBV.

Eukaryotic cells are replicated in an ordered manner, with defined events occurring in a specific sequence, the stages being defined by the events that are undertaken in each phase; S phase involves the duplication of DNA and M phase defines mitosis or cell division (Fig. 2). Before S phase commences there is a growth phase, G1, and between S phase and mitosis there is a second growth phase G2. A further phase describes cells that are not currently replicating termed G0.

Progression around the cell cycle is regulated in response to many intracellular and extracellular signals, these are integrated into simple stop or go messages by a complex network of signal transduction pathways that channel information towards specific regulatory events. Current models suggest that the cyclin-dependent protein kinases act as integration points of much of this signalling. The kinases consist of a regulatory cyclin subunit plus a cyclin-dependent protein kinase (cdk). Cyclins D1, D2 and D3 complex with either cdk4 or cdk6 in the early G1 phase of the cell cycle, they are believed to be involved in regulating the activity of the restriction point that controls the transition through the late G1 phase of the cell cycle. The cyclin E/cdk2 complex is thought to act at the G1/S boundary, cyclin A/cdk2 regulates the transition between S and G2, the cyclin B/cdk2 complex is implicated in the initiation of mitosis (M) (Motokura and Arnold, 1993; Sherr, 1993; Draetta, 1994; Bartek et al., 1996). To date, investigations between EBV and the cell cycle control machinery have focused on events that feed into the restriction point and these will be discussed below, disruption of other areas of cell cycle control by EBV may still await discovery.

The restriction point is frequently disrupted in human cancer (Bartek et al., 1996; Taya, 1997) and, as such, signal transduction pathways that lead to it have been the subject of intense investigation. Indeed, the restriction point is mediated by the retinoblastoma protein (pRb) which was originally identified by virtue of its role as a tumour suppressor gene. pRb is a member of the 'pocket-protein' gene family which also include p130 and p107. It interacts with and inactivates a number of transcription factors including members of the E2F family that directly regulate the expression of several genes required for S phase. The activity of pRb is regulated in response to signalling by the cyclin D/cdk 4, cyclin D/cdk 6 and cyclin E/cdk 2 complexes which allow the release of active E2F. The activity of the cyclin/cdk complexes can be further regulated by phosphorylation and by the action of a series of cdk inhibitors (cdkIs) notably p15, p16, p21 and p27. In addition to these factors, the restriction point is also regulated by the presence of damaged DNA via the action of p53, which is an important tumour suppressor gene.

The effects of EBV on the expression or activity of any components of the restriction point are of interest to understanding how EBV is able to re-program proliferation controls of infected cells. The remainder of this review will be dedicated to recent advances in this area.

A number of studies have been undertaken to question whether EBV uses a similar mechanism to transform cells as the "small" DNA tumour viruses such as SV40, human papilloma virus and adenovirus. These viruses encode oncogenes that directly interact with pRb and p53 and functionally inactivate them (Ludlow, 1993). It therefore seems pertinent that any interaction between EBV proteins and these tumour suppressors should be investigated. EBNA-LP has been proposed as a candidate for this function. It has been found to colocalise with pRb in the LCL cell line IB4 (Jiang et al., 1991) and EBNA-LP can also interact with both pRb and p53 in vitro association assays (Szekely et al., 1993). It has been suggested that this may occur via interactions with the heat shock proteins (Mannick et al., 1995; Kitay and Rowe, 1996). However, a study aimed at directly questioning whether EBNA-LP has any effect on the function of pRb or p53, comes to a negative conclusion (Inman and Farrell, 1995). Another EBV gene that has been suggested as a candidate to regulate the function of pRb is EBNA 3c (Parker et al., 1996). This is able to interact with pRb in in vitro association assays and appears to influence signal transduction to the restriction point in certain cell types when it is expressed at high levels in rat embryo fibroblasts (Parker et al., 1996). So, although EBNA-LP and EBNA-3c are candidate proteins that can interact with p53 and/or pRb in vitro, no functional changes to the activity of p53 and pRb have been detected to date in vivo and so the potential contributions from these interactions to the disruption of cell cycle control by EBV remain to be determined. Although the relevance of EBNA-LP to cell cycle control has not been established as yet, it is interesting to note that it is itself a potential target for cell cycle regulation; EBNA-LP is differentially phosphorylated on serine residues at distinct stages of the cell cycle, being maximal at G2/M (Kitay and Rowe, 1996).

A different approach has been taken to address whether pRb and p53 remain active in EBV-immortalised LCLs. Somewhat surprisingly, the expression of pRb and p53 are found to increase after EBV infection of primary B-lymphocytes, suggesting a potential role for both tumour suppressor genes at an early stage of cell cycle activation (Allday et al., 1995; Szekely et al., 1995; Cannell et al., 1996) Transfection experiments show that p53 can be independently induced by either LMP-1 or EBNA-2, but induction is more efficient when resting B-lymphocytes are transfected with both EBV genes (Chen and Cooper, 1996), it has been suggested that this upregulation may occur through the activation of the transcription factor NF-kB. Szekely showed that the levels of p53 in EBV-infected B-lymphocytes can decrease with time in culture, however, the level of expression in established LCLs remains higher than that seen in primary cells

(Allday et al., 1995; Szekely et al., 1995). The function of p53 in the DNA damage response pathway in LCLs is not clearly disrupted by EBV; the cells accumulate p53 and undergo rapid apoptosis in response to cis-platin (Allday et al., 1995) and gamma irradiation (Lalle et al., 1995). However, when p53 is overexpressed in Burkitt's lymphoma cell lines, the G1/S cell cycle arrest is maintained but apoptosis is inhibited, suggesting that EBV may be able to modulate the activity of p53 (Okan et al., 1995). Although pRb levels increase following infection with EBV, its activity is also modulated by hyperphosphorylation (Cannell et al., 1996). This suggests that the cyclin-dependent kinase complexes that phosphorylate pRb are activated by EBV. Consistent with this is the observation that cyclin D2 expression is induced in LCLs and following EBV infection of primary B-lymphocytes (Palmero et al., 1993; Sinclair et al., 1994; Hollyoake et al., 1995; Kempkes et al., 1995; Cannell et al., 1996). Furthermore, using transfection it has been shown that the expression of only two viral genes, EBNA-2 and EBNA-LP, is necessary to activate the upregulation of cyclin D2, suggesting that these two proteins alone can drive quiescent primary B cells from G0 to the G1 stage of the cell cycle (Sinclair et al., 1994). Interestingly, in some cell lines, the expression of LMP-1 can also modulate signal transduction to pRb, via the induction of cyclin D2 expression (Arvanitakis et al., 1995). However, in other B-cell lines, LMP-1 induces a G2/M cell cycle arrest (Floettmann et al., 1996). It has also been shown that LMP-1 can modulate p53 mediated apoptosis in epithelial cell lines possibly via the action of A20, a TNF and CD40 responsive gene (Fries et al., 1996). Other cyclins and cdks are also upregulated after EBV infection of primary B lymphocytes including cyclin E, the cyclin D-dependent kinases cdk4 and cdk6 and the cyclin E-dependent kinase cdk2 (Sinclair et al., 1994; Hollyoake et al., 1995; Kempkes et al., 1995; Cannell et al., 1996). In addition, two cyclin-dependent kinase inhibitors, p16 and p27 are clearly downregulated in LCLs (Cannell et al., 1996). These studies suggest that EBV does not use a simple mechanism to inactivate p53 and pRb, in contrast to the small DNA tumour viruses it appears to modulate the activity of p53 and pRb via a combination of subtle interactions with the signal transduction pathways that intersect at p53 and pRb. Further investigations are required to determine both the relevance of these effects and the molecular mechanisms by which they are achieved.

EBV also reprograms the cell cycle control machinery at the onset of the viral lytic cycle. The viral protein BZLF1, which acts as a "master switch" for inducing the lytic cycle appears to have three functions: (1) BZLF1 transactivates the promoters for several lytic cycle genes (reviewed in (Sinclair and Farrell, 1992)). (2) BZLF1 represses activity of the major latency promoter Cp (Kenney et al., 1989; Sinclair et al., 1992). (3) BZLF1 interacts with p53 directly (Zhang et al., 1994) and causes increases in the levels of the cdkIs p27 and p21, consistent with the observed a G0/G1 cell cycle arrest (Cayrol and Flemington, 1996a,b).

Thus, EBV interacts with the cell cycle control machinery in a complex way both during the immortalisation of B-lymphocytes and at the onset of the viral lytic cycle.

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