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Pathogens in Focus

Epstein-Barr virus: Inhibition of apoptosis as a mechanism of cell transformation

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Abstract

Epstein-Barr virus is a potent mitogen for human B lymphocytes and is associated with a large number of human malignancies. This large virus expresses several genes that may contribute to the transformed phenotype of infected cells and it possesses multiple strategies for the inhibition of apoptosis. The interferon-inducible protein kinase PKR is an important target for the anti-apoptotic actions of the virus and its activity is regulated by the small untranslated RNA, EBER-1. This review summarizes the mechanisms of action of EBER-1 and other Epstein-Barr virus gene products in the regulation of apoptosis, and presents a model of how EBER-1 exerts its effects on PKR activity.

Keywords: Epstein-Barr virus; Malignant transformation; Tumour virus; Apoptosis

1. Introduction

It is over 40 years since Epstein-Barr virus (EBV) was characterised as an agent associated with Burkitt's lymphoma (Young & Rickinson, 2004). Since that time this ubiquitous γ herpesvirus has been implicated in the aetiology of several additional human tumours, including post-transplant lymphomas, nasopharyngeal carcinoma, some forms of gastric carcinoma and Hodgkin's lymphoma, and possibly a number of other types of cancer (Busson, Keryer, Ooka, & Corbex, 2004; Gandhi, Tellam, & Khanna, 2004; Thompson & Kurzrock, 2004). Although there is good evidence that EBV is a contributory factor in the development of some or all of these tumours the virus is widespread in the human population and its presence clearly does not result in cancer in the majority of individuals. EBV is also the causative agent of infectious mononucleosis, but again the majority of patients who get this disease do not run a high risk of subsequently developing cancer. These facts raise some interesting questions about the relationship between the virus and its host and draw attention to the role of the immune system in protection against the carcinogenic potential of EBV.

Acquisition of EBV usually occurs in childhood and, for most people, results in a life-long asymptomatic infection in which the virus remains in a latent state. B lymphocytes are the target cells for primary infection. Following an initial burst of proliferation the majority of the infected host cells then die by apoptosis, but a small fraction enters the memory cell pool of non-dividing cells and it is this population that constitutes a reservoir of EBV-positive cells thereafter (Hammerschmidt & Sugden, 2004). Certain epithelial cell types may also become infected by EBV, but less is known about the mechanisms involved.

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2. Overview of pathogenesis

EBV has a double-stranded DNA genome of ca. 172 kilobases that is linear in the virion but becomes circularised to form a viral episome shortly after infection. The relatively large genome allows the virus to encode a large number of RNA and protein species, but only a small sub-set of these are expressed in most infected cells. As is typical of herpesviruses, EBV can either undergo a cycle of lytic infection (during which the majority of viral genes are expressed (Amon & Farrell, 2005)) or it can enter into a state of "latency" in infected cells. In the latter situation the virus does not replicate and a maximum of only six viral nuclear antigens (the EBNAs), three plasma membrane-associated proteins (latent membrane proteins or LMPs) and a small number of other viral gene products are present (Young & Murray, 2003). Prominent among the latter are two small untranslated RNA species, EBER-1 and EBER-2, which are usually found in infected cells in high abundance.

There are several aspects relevant to the pathogenesis of the various diseases with which EBV is associated (either as a causative agent or as a contributory factor). This virus is in fact one of the most potently mitogenic viruses known. When it infects resting B lymphocytes it drives a high proportion of the cells into the cell cycle, giving rise to B cell blasts that express several cellular activation antigens (Hammerschmidt & Sugden, 2004; Sugimoto, Tahara, Ide, & Furuichi, 2004). From B cells infected by EBV in tissue culture immortalised lymphoblastoid cell lines can be grown out with a high frequency. The mitogenic actions of EBV account for the extensive (albeit temporary) expansion of B cells that is characteristic of infectious mononucleosis. In addition, the rapid proliferation of virus-infected B cell blasts is believed to increase the probability of gene rearrangements, such as the translocation of the c-myc proto-oncogene to an immunoglobulin locus (Klein, 1999). Such translocations, which result in transcriptional activation of this gene, are characteristic of most forms of Burkitt's lymphoma. Inappropriate expression of the c-myc gene represents a major factor contributing to the highly malignant phenotype of this tumour.

Several recent studies have shown that, as well as promoting cell proliferation, EBV is strongly anti-apoptotic (e.g. Nanbo, Inoue, Adachi-Takasawa, & Takada, 2002; Kennedy, Komano, & Sugden, 2003). The specific viral gene products believed to be responsible for this are described in the following section. This is a very important property of the virus since it inhibits the death of proliferating B cells that may normally be very susceptible to apoptosis (especially in the absence of an appropriate, normal immunological stimulus). It also probably ensures the survival of infected cells long enough for them to enter the non-dividing memory B cell pool. These cells are intrinsically long-lived and acts as a reservoir for the virus (Thorley-Lawson, 2005). Most importantly, the ability to inhibit apoptosis makes a major contribution to the malignant transformation of infected cells.

As if these strategies were not enough to ensure the survival of the virus in its host for life, EBV is also able to evade immune surveillance very effectively when it enters into a latent state in infected cells. This is because most virus-encoded proteins are not expressed in this state (Young & Murray, 2003), and thus few antigenic peptides are generated. In some cells only one nuclear antigen (EBNA-1) is present, together with the EBER RNAs, and in infected memory B cells no viral proteins are expressed at all (Hochberg et al., 2004). The importance of immune surveillance as a protection against EBV-driven tumours in normal individuals is shown by the fact that EBV-positive lymphomas arise with a relatively high frequency in immunocompromised patients (e.g. those undergoing post-transplant immunosuppressive treatment) (Thompson & Kurzrock, 2004). The generation of peptides from the breakdown of EBNA-1 itself is inhibited by the presence of Gly-Ala repeats within the sequence of this protein (Levitskaya et al., 1995).

3. Viral virulence factors

In the case of EBV it is unlikely that there is a single "virulence factor" that accounts for the pathogenic properties of this virus. Several viral genes contribute to both the mitogenic and anti-apoptotic functions of EBV. Viral gene products for which there is firm evidence of a role in host cell immortalisation or transformation include EBNA-1 and EBNA-2, LMP-1 and LMP-2A, and the EBERs (Young & Rickinson, 2004). All of these may be expressed during latent infections, with EBNA-1 and the EBERs being almost always present. Table 1 summarises relevant features of these molecules. It is notable that all of these viral gene products have the ability to inhibit cell death, using a variety of mechanisms to achieve this end.

A novel aspect of EBV infection is the accumulation of high levels of the small virally encoded RNAs, EBER-1 and EBER-2. Several recent studies have addressed the possible mechanisms of action of these non-translated RNA species and there is accumulating evidence for the importance of EBER-1 as an important player in the strategy by which EBV transforms cells (see Sec-

Table 1
EBV gene products involved in regulation of apoptosis and cell transformation

Gene product	Description	Mechanism of action[a]
EBNA-1	Expressed in all proliferating EBV-infected cells Required for viral DNA replication **Provides survival function**	Binds to origin of replication in viral genome Regulates transcription **Destabilises p53**
EBNA-2	Regulates transcription of cellular and viral genes Essential for B cell transformation	Interacts with RBP-Jκ transcription factor **Activates transcription of anti-apoptotic genes**
LMP-1	Integral membrane protein Behaves as classical oncogene product Acts as constitutively active TNF receptor-like protein	**Activates NF-□B, MAP kinase and PI3k anti-apoptotic pathways**
LMP-2A	Integral membrane protein Not essential for B cell transformation	Mimics B cell receptor signalling **Activates MAP kinase and PI3k anti-apoptotic pathways**
EBERs	Small untranslated RNAs Very abundant in almost all EBV-infected cells	**EBER-1 binds to PKR and inhibits pro-apoptotic activity of this protein kinase** Induce expression of autocrine growth-promoting cytokines

[a] Functions believed to be important for the anti-apoptotic effects of EBV are shown in bold.

tion 4). Fig. 1 shows the structure of EBER-1, with the location of likely binding sites for a number of cellular proteins indicated. EBER-1 is highly structured, with several stem-loop regions that are created by intramolecular base-pairing (Glickman, Howe, & Steitz, 1988). The nucleotide sequence (and hence the secondary and tertiary structure) of this RNA is highly conserved among viral isolates, suggesting a critical role for the conformation of the molecule in relation to its biological function. The high abundance of EBER-1 in latently infected cells facilitates the interaction of the RNA with its ligands in vivo.

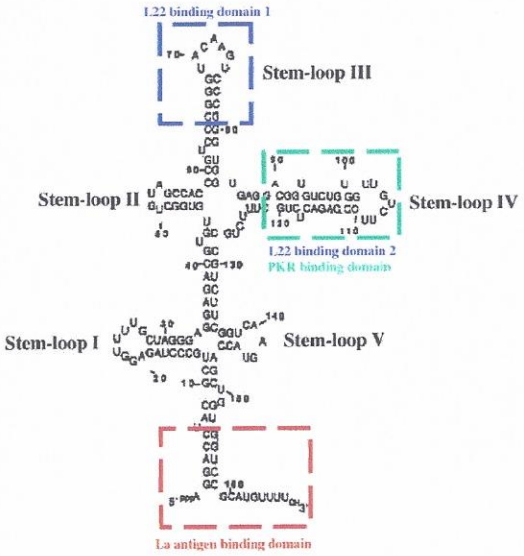

Fig. 1. Structure and protein binding sites of the small EBV-encoded RNA EBER-1. EBER-1 is a 167 nucleotide RNA that possesses a high content of secondary structure due to the formation of five clearly defined stem-loop regions (I-V). The molecule has distinct binding sites for at least three cellular proteins, indicated here by the coloured boxes. The La antigen binds to the 3′ terminal oligo (U) tract and is probably involved in transcriptional termination and/or 3′ end processing of this RNA polymerase III transcript (Gottlieb & Steitz, 1989). The dsRNA-regulated protein kinase PKR binds to stem-loop IV (Vuyisich, Spanggord, & Beal, 2002), and the ribosomal protein L22 binds to both stem-loops III and IV (Toczyski & Steitz, 1993). PKR competes partially but not completely with L22 for binding to EBER-1, suggesting that L22 associates with its two binding sites independently. On the other hand, L22 can completely out-compete PKR for binding to the RNA (Elia et al., 2004).

4. Biological function

The biological functions of the key EBV gene products that inhibit apoptosis and thereby contribute to transformation are summarised in Table 1. Consistent with their nuclear localization in infected cells, the EBNAs are DNA-binding proteins. EBNA-1 appears to serve a dual role in latent infection, on the one hand being required for viral DNA replication and maintenance of the episomal viral genome and on the other contributing to the survival of infected B cells (Kennedy et al., 2003). The DNA-binding function and transcriptional regulatory properties of EBNA-1 may not be necessary for the latter activity. Rather, a recent study suggests that EBNA-1 can inhibit apoptosis by lowering p53 levels, due to its ability to bind to the p53-regulatory protein USP7 (Saridakis et al., 2005). EBNA-2, on the other hand, transcriptionally up-regulates a number of cellular genes, such as that encoding the B cell activation antigen CD23. This activity is modulated by other EBV

gene products, viz. EBNA-LP and the EBNA-3 family. The genes thus regulated may act to inhibit apoptosis and also promote cell proliferation.

The latent membrane proteins of EBV are also strongly implicated in the transforming ability of this virus. LMP-1 acts as a classic oncogene in transformation assays, its mode of action being to stimulate several signal transduction pathways (Izumi, 2004; Morrison, Gulley, Pathmanathan, & Raab-Traub, 2004). It functions as a constitutively active homologue of the tumour necrosis factor receptor family, closely resembling CD40 (Young & Rickinson, 2004) (Table 1). An important effect of LMP-1 is the activation of the NFκB pathway, which can up-regulate the expression of several anti-apoptotic genes at the transcriptional level. The MAP kinase and phosphatidylinositol-3-kinase (PI3k)/protein kinase B (Akt) pathways are also activated by LMP-1. The Akt kinase in particular has important prosurvival actions on cells. LMP2A can mimic the presence of an active B cell receptor to provide a survival signal to the B cell (Caldwell, Wilson, Anderson, & Longnecker, 1998). Like LMP1, it can activate the PI3k/Akt pathway (Scholle, Bendt, & Raab-Traub, 2000) and it may also promote the expression of genes involved in cell cycle regulation (Portis, Dyck, & Longnecker, 2003) (Table 1).

The abundant small RNAs EBER-1 and EBER-2 do not code for any proteins but may nevertheless regulate apoptosis through their ability to bind to protein ligands (Fig. 1). One protein in particular has been suggested to have an important role in the cellular actions of EBER-1 and that is the interferon-inducible, double-stranded RNA (dsRNA)-regulated protein kinase PKR (Clemens, 1997). This enzyme controls protein synthesis at the level of polypeptide chain initiation but has also been shown to promote cell death in a number of systems (Clemens, 2004). EBER-1 inhibits the protein kinase activity of PKR in vitro because it competes with dsRNA activators for binding to the enzyme (Sharp et al., 1993) (Fig. 2). Consistent with this mode of action, expression of EBER-1 in EBV-negative cells has been shown to protect against IFN-induced apoptosis (Nanbo et al., 2002). Comparisons of EBER-positive and -negative viruses have demonstrated that the small RNA contributes significantly to the efficiency of growth transformation of B cells by EBV (Yajima, Kanda, & Takada, 2005). In addition, EBER expression has been reported to stimulate the production of autocrine growth factors such as IL-9, IL-10 and IGF-1 (Kitagawa et al., 2000; Iwakiri, Sheen, Chen, Huang, & Takada, 2005; Yang, Aozasa, Oshimi, & Takada, 2004). However it is not clear whether the

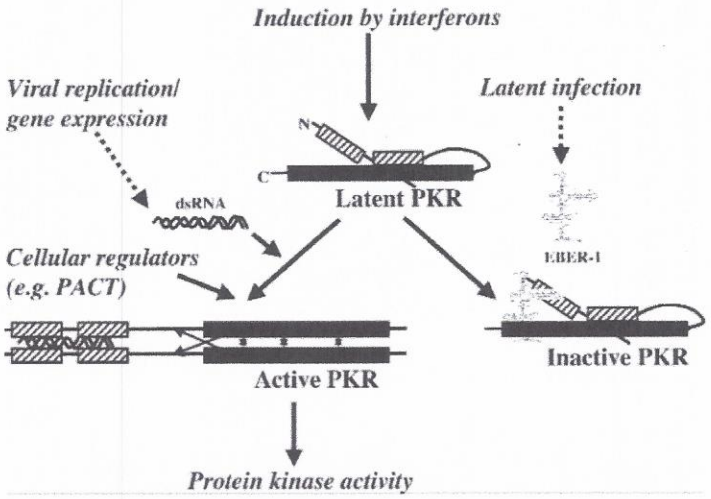

Fig. 2. A model for the regulation of activity of the protein kinase PKR by EBER-1. PKR is a protein kinase that is present at variable levels in most cell types, but can also be induced at the transcriptional level by interferons. In both cases the enzyme remains catalytically inactive until activated by the binding of viral or cellular dsRNA (or the regulatory protein PACT) to the N-terminus. Such binding relieves an auto-inhibitory interaction between the N-terminal domain and the catalytic domain in the C-terminal half of the protein. [PKR can also be activated by caspase cleavage, which separates the two domains of the kinase, thus releasing an active catalytic fragment (Saelens, Kalai, & Vandenabeele, 2001).] EBER-1 binds to part of the N-terminal regulatory domain in competition with dsRNA, but does not prevent the auto-inhibitory interaction with the catalytic domain. Thus EBER-1 abrogates activation of PKR by dsRNA without itself activating the enzyme. PKR has a number of substrates, the best characterised of which is a subunit of protein synthesis initiation factor eIF2. Phosphorylation of the latter impairs polypeptide chain initiation. PKR also possesses pro-apoptotic activity. Thus EBER-1 is able to block the inhibition of protein synthesis by dsRNA and may also inhibit the promotion of cell death mediated by one or more PKR-dependent pathways.

mechanism of induction of these proteins involves PKR or other EBER ligands such as the La antigen or ribosomal protein L22 (Elia, Vyas, Laing, & Clemens, 2004).

5. Clinical applications

Given the multiplicity of strategies by which EBV may inhibit apoptosis and transform cells it is difficult to propose a single approach by which the tumourigenic properties of the virus might be abrogated. Moreover, it is possible in some cases that, while EBV has a role to play in the establishment of malignancy, the continued presence of the virus may not always be necessary (the transcriptional activation of the c-myc oncogene as a result of gene rearrangements that may occur with increased probability in a proliferating B cell population driven by EBV infection is a case in point). Nevertheless, one can propose possible therapeutic strategies that may prove effective, individually or in combination, against certain EBV-associated tumours. Thus, down-regulation of expression of EBNA-1 or -2, the LMPs and/or the EBERs might be effective if suitable methodologies can be devised. In this regard the application of RNA interference holds promise, provided suitable siRNA vectors could be introduced into cells with adequate efficiency. [Whether RNAi would be effective against EBER expression is open to some doubt, however, given the highly structured nature of the EBERs, see Fig. 1].

If the regulation of PKR by the EBERs proves to be as critical as some recent papers would suggest, interference with the binding of these RNAs to the protein kinase might also be an effective strategy for reversing the resistance of EBV-infected cells to apoptosis. It is of interest that at least one other ligand for EBER-1, viz. the ribosomal protein L22, competes with PKR for binding to the RNA and thereby interferes with the inhibition of PKR by EBER-1 (Elia et al., 2004). Whether this fact can be exploited to diminish EBER binding to PKR in vivo remains to be investigated.

Another clever way in which the presence of EBER-1 may be exploited to work against the interests of EBV is to infect cells with a mutant adenovirus lacking the gene encoding the small viral RNA, VA-I. The latter is required for effective replication of adenovirus but can be complemented by EBER-1, probably because both RNAs act in similar ways against PKR (Bhat & Thimmappaya, 1983; Sharp et al., 1993). Thus normal EBV-negative cells and tissues, which do not express EBER-1, will remain protected against infection by the defective adenovirus but EBV-positive tumour cells should permit efficient viral infection and will be killed. The experimental efficacy of this oncolytic viral strategy against EBV-associated tumours has been recently demonstrated (Wang et al., 2005).

Finally, studies from the emerging field of microRNAs have revealed that EBV expresses several miRNA genes (Pfeffer et al., 2004). Although the functions of these small RNAs are not yet understood, some miRNAs may play roles in enhancing the ability of the virus to infect or transform its host cells. If this is the case, they may provide us with further targets for future therapeutic strategies to combat the pathogenic effects of Epstein-Barr virus infection.

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