p16INK4A -independence of Epstein–Barr virus-induced cell proliferation and virus latency

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Epstein–Barr virus (EBV) has the ability to promote cell cycle progression following the initial infection of primary resting B-lymphocytes and to cause cell cycle arrest at the onset of the viral replicative cycle. Various mechanisms have been proposed for the proliferative effects, including the up-regulation of cyclin D2 by the viral EBNA-2 and EBNA-LP proteins, direct binding of EBNA3C to the retinoblastoma protein (pRb), and down-regulation of the p16INK4A tumour suppressor by the viral LMP1 product. To try to gain insight into the relative importance of these mechanisms, the ability of EBV to immortalize lymphocytes from an individual who is genetically deficient for p16INK4A was examined. From detailed analyses of the resultant lymphoblastoid cell lines it is concluded that p16INK4A status has little bearing on EBV’s ability to manipulate the cell cycle machinery and a model to accommodate the previously proposed routes taken by EBV to bypass the restriction point is presented.

DNA viruses use a variety of strategies to facilitate the replication of their genomes but a common theme is to encode viral proteins that alter host cell proliferation. Among the well-characterized examples are proteins such as simian virus 40 T-antigen, human papillomavirus (HPV) E6 and adenovirus E1A, which override the G1 phase control point at least in part through a direct interaction with the retinoblastoma gene product (pRb) (Classon & Harlow, 2002; Jansen-Durr, 1996). The presence of pRb inevitably results in the activation of the p53 pathway via E2F-mediated induction of p14ARF. The same viruses therefore specify additional proteins to disarm the p53-dependent defences (Classon & Harlow, 2002; Jansen-Durr, 1996). An unusual locus specifies two quite different proteins by translating a shared second exon in different reading frames resulting in the translation of both p14ARF and a second product p16INK4A (Chin et al., 1998; Serrano et al., 1993). p16INK4A is an inhibitor of the cyclin D-dependent kinases, Cdk4 and Cdk6, which normally initiate the phosphorylation and functional inactivation of pRb (Chin et al., 1998; Palermo & Peters, 1996; Ruas & Peters, 1998). Thus, both products encoded by this locus have the ability to cause cell cycle arrest; in the case of p16INK4A by preventing the phosphorylation of pRb and in the case of p14ARF by blocking the Mdm2-mediated destruction of p53, thereby inducing expression of the p21CIP1 Cdk inhibitor (Bringold & Serrano, 2000; Chin et al., 1998). Although its functional properties suggest that p16INK4A could play a role in the G1 cell cycle control point, upstream of pRb, it is a relatively stable protein whose levels do not fluctuate significantly during the cell cycle. Indeed, the most dramatic changes in p16INK4A abundance occur when primary cells undergo a senescence-like arrest in response to stress or as a consequence of telomere erosion (Alcorta et al., 1996; Hara et al., 1996; Loughran et al., 1996; palmero et al., 1997; Reznikoff et al., 1996).

The human gammaherpesvirus Epstein–Barr virus (EBV) has the ability to promote cell cycle progression following the initial infection of primary resting B-lymphocytes and to cause cell cycle arrest at the onset of the viral replicative cycle (Flemington, 2001; Sinclair, 2003; Sinclair et al., 1998). It is therefore clear that EBV must impinge on the cell cycle machinery, it is important to stress that it has no direct equivalent of proteins such as T-antigen or HPV E6 and E7 (Sinclair et al., 1998), although it has been shown that one viral gene product, EBNA3C, can interact directly with pRb and confer resistance to p16INK4A overexpression in rodent fibroblasts (Parker et al., 1996). The relevance of this interaction in virus-infected cells remains unproven, however. EBV infection of primary B-lymphocytes has been shown to cause a dramatic up-regulation of cyclin D2 (Kempkes et al., 1995; Sinclair et al., 1994), which is attributable to the combined actions of two viral genes EBNA-2 and EBNA-LP (Sinclair et al., 1994). A likely outcome will be the activation of Cdk4 and Cdk6 leading to hyperphosphorylation of pRb, as is observed in these cells (Cannell et al., 1996). Finally, there is compelling evidence that the
viral LMP1 protein can down-regulate the expression of p16\textsuperscript{INK4A} in primary human fibroblasts (Yang et al., 2000). This occurs in part through effects on the localization of critical transcription factors (Ohtani et al., 2003). However, at present it remains uncertain whether any of these three routes are sufficient to explain the ability of EBV to manipulate the host cell cycle in infected cells.

To try to gain further insight into these possibilities, we instigated a study on B-lymphocytes from an individual who is effectively p16\textsuperscript{INK4A} deficient. The individual is homozygous for a 19 bp germline deletion in the \textit{INK4A/ARF} locus (Gruis et al., 1995). As described in detail elsewhere and illustrated in Fig. 1, the associated frame-shift results in the production of two chimaeric proteins: p14/p16, in which the amino-terminal 88 residues of p14\textsuperscript{ARF} are fused to the last 76 residues of p16\textsuperscript{INK4A}, and p16/X, comprising the first 74 residues of p16\textsuperscript{INK4A} followed by 64 amino acids specified by the +1 reading frame (Brookes et al., 2002). Detailed \textit{in vitro} and \textit{in vivo} analyses indicate that neither protein shows any residual INK4A-associated activity whereas the p14/p16 product retains all the known functions of p14\textsuperscript{ARF} (Brookes et al., 2002).

Primary B-lymphocytes were isolated from peripheral blood after fractionation of mononuclear cells using a density gradient and positive selection with anti-CD19 coated paramagnetic beads [as described in (Sinclair & Farrell, 1995a, b; Sinclair et al., 1994)]. Subsequently, the cells were infected with the B95-8 strain of EBV (Baer et al.,...
Cells from the p16<sup>INK4A</sup>-deficient patient were studied in parallel with cells from normal adult blood donors. Activation of the infected cells appeared to have occurred by 7 days post-infection and outgrowth was clear for both sets of cells by 14-days post-infection. Thus it appears that immortal cell lines grew out with similar rates suggesting that the initial stages of immortalization are not affected by p16<sup>INK4A</sup> status; however, it is not possible to discount subtle changes in the initial stages of immortalization. In all subsequent experiments we compared two lymphocytic cell lines (LCLs) from normal donors (designated N1 and N2 cell lines) and two LCLs from the p16<sup>INK4A</sup>-deficient donor (designated L1 and L2). The N cells expressed p16<sup>INK4A</sup> protein as anticipated, detectable with monoclonal antibodies that recognize epitopes in either the amino-terminal (Koh <i>et al</i>, 1995) or carboxy-terminal halves of the protein (Lukas <i>et al</i>, 1995; Parry <i>et al</i>, 1995) (Fig. 1B). Moreover, in normal LCLs p16<sup>INK4A</sup> forms a complex with Cdk6 that can be co-precipitated, as predicted for a normally functional INK4 protein (data not shown). L1 and L2 cells only express the p14/p16 fusion protein, detected using the DCS50 monoclonal antibody that recognizes the carboxy terminus of p16<sup>INK4A</sup>. This protein is incapable of associating with Cdk4 and Cdk6 (Brookes <i>et al</i>, 2002). As described elsewhere, the p16/X fusion protein, which would also be encoded by the L1 and L2 cells, appears to be inherently unstable and difficult to visualize (Brookes <i>et al</i>, 2002). No gross changes to the phosphorylation of pRB were observed, as exemplified by staining with antibodies that recognize total pRb or pRb specifically phosphorylated on serine-780 or serine-807 plus serine-811 (Fig. 1B).

The phenotypes of the four LCLs were then compared using FACS analysis (Fig. 2). All displayed the characteristic phenotype for LCLs (Rickinson, 2002); CD20<sup>+</sup> (with a mean fluorescence intensity of 70.7 ± 1.3 for N cells and 50.5 ± 0.2 for L cells), with high expression of the B-lymphocyte activation marker CD23 (with a mean fluorescence intensity of 129.5 ± 14.3 for N cells and 112.1 ± 43.7 for L cells), coupled with low expression of the germinal centre marker CD10 (with a mean fluorescence intensity of 4.0 ± 0.4 for N cells and 4.8 ± 0.3 for L cells) and detectable expression of CD54 (with a mean fluorescence intensity of 6.7 ± 1.1 for N cells and 9.1 ± 1.1 for L cells). Expression of the EBV genes EBNA-2, EBNA-LP and LMP1 was also equivalent in the four cell lines (data not shown).

**Fig. 2.** Characterization of the p16<sup>INK4A</sup>-positive and -negative LCLs. (A) Surface expression of the indicated markers was analysed in duplicate by FACS using fluorescently labelled antibodies from Becton Dickinson. The percentage of cells with positive staining is indicated on the y-axis with the standard deviation shown as the error bar. The filled bar represents N1, the stippled bar N2, the open bar L1 and the hatched bar L2. (B) Each cell line was diluted to 3 × 10<sup>5</sup> cells ml<sup>−1</sup> and the cell density was recorded over a 10 day period in duplicate; the standard deviation is shown as the error bar. (C) Cells were fixed with 80 % ethanol, stained with propidium iodide as described previously (Cannell <i>et al</i>, 1998) and their DNA content analysed by FACS in duplicate.
The establishment of the LCLs allowed us to undertake an analysis of their cell cycle characteristics. All four cell lines proliferated in an equivalent manner in normal growth conditions (10% FCS), reaching a similar saturation density (Fig. 2B) and FACS analysis of propidium iodide-stained cells revealed an equivalent cell cycle profile (Fig. 2C). Thus, the proliferative capability and cell cycle profile of the LCLs was unaffected by the loss of p16INK4A.

Since apoptosis is often preceded by cell cycle arrest (Andoh, 2000; King & Cidlowski, 1998; Meikrantz & Schlegel, 1995; Wang et al., 2000), we questioned whether p16INK4A status resulted in differences in the potential of the LCLs to survive in response to exposure to the pro-apoptotic agent anti-FAS (Fig. 3). Early stages in apoptosis were detected by measuring caspase-3 activity (Fig. 3A) (Frost & Sinclair, 2000) and also by assaying for the cleavage

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**Fig. 3.** The propensity of the N and L LCLs to undergo apoptosis was assessed. (A) FAS was ligated on L and N cells in duplicate using an anti-FAS antibody. Extracts were prepared at the indicated times and their ability to cleave the caspase-3 substrate DEVD-AMC was determined as described previously (Frost & Sinclair, 2000). (B) Total protein extracts were prepared, fractionated on SDS-PAGE gels and the status of PARP determined by Western blot analysis as described previously (Frost & Sinclair, 2000). The dashed arrow represents the p85 cleavage product of PARP and the solid line the full-length protein. (C) The number of viable cells was determined in triplicate after anti-FAS addition and is plotted on the y-axis with the standard deviation shown as the error bar.
and subsequent degradation of PARP (Fig. 3B) (Frost & Sinclair, 2000); both were altered with similar kinetics in the L and N cells. Furthermore, cell survival (Fig. 3C) and DNA fragmentation (data not shown) were assayed to measure late stages of apoptosis. No differences were found in the response of the L and N cells to anti-FAS, or to two other pro-apoptotic agents, staurosporin or etoposide (data not shown). This clearly demonstrates that p16INK4A status has no effect on the propensity of LCLs to undergo apoptosis.

We then investigated whether the balance between latency and virus replication was altered in the L and N cells. The expression of an early marker of viral lytic cycle, Zta, was assessed by both Western blot analysis (Fig. 4A) and FACS analysis (Fig. 4B). The expression of Zta was equivalent in both L and N cells (mean fluorescence intensity of 3.7 for N cells and 3.0 for L cells), with a similarly small proportion of positive cells. This suggests that p16 status does not influence the ability of cells to enter the early stages of the viral lytic cycle.

Our investigation of the expression and function of p16INK4A in EBV-immortalized LCLs revealed that p16INK4A protein is present in LCLs and furthermore that it is capable of forming inhibitory complexes. From this we can conclude that the ability of LMP1 to down-regulate the expression of p16INK4A, observed in rodent fibroblasts, either does not occur in EBV-transformed LCLs or it is insufficient to fully negate the expression of p16INK4A in these cells. It is therefore relevant to question whether p16INK4A plays a role in mediating the cell cycle effects driven by EBV in LCLs. Our investigations revealed that the proliferation of cells in normal conditions, the propensity to undergo apoptosis and the spontaneous activation of the viral lytic cycle were unaffected by the p16INK4A status in LCLs.

In summary, the signal transduction events that mediate immortalization and then drive cell cycle progression in LCLs are not influenced by the expression of functional p16INK4A in LCLs. Furthermore, other events associated with cell cycle arrest, such as apoptosis and the onset of the viral lytic cycle, are similarly unaffected by the expression of p16INK4A in LCLs. Our current working model to account for this encompasses previously proposed mechanisms to regulate the function of the restriction point by EBV. (i) Infection with EBV could result in transcription of p16INK4A being down-regulated by LMP1 and thus less protein being expressed. Importantly, LMP1 does not switch-off expression of p16INK4A, since it is still detectable in LCLs. (ii) In addition, expression of cyclin D2, Cdk4 and Cdk6 are up-regulated by EBV and so could alter the stoichiometry between cyclin-bound and p16INK4A-bound forms of Cdk4 and Cdk6 within the cells. (iii) In addition, the function of pRb could be directly modulated by association with EBNA3C. This trio of routes available to EBV to modulate the function of the restriction point could be required allow EBV to regulate the proliferation of infected cells from diverse lineages.

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