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Review

Epstein-Barr virus infection and persistence in nasopharyngeal epithelial cells

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Abstract

Epstein-Barr virus (EBV) infection is closely associated with undifferentiated nasopharyngeal carcinoma (NPC), strongly implicating a role for EBV in NPC pathogenesis; conversely, EBV infection is rarely detected in normal nasopharyngeal epithelial tissues. In general, EBV does not show a strong tropism for infecting human epithelial cells, and EBV infection in oropharyngeal epithelial cells is believed to be lytic in nature. To establish life-long infection in humans, EBV has evolved efficient strategies to infect B cells and hijack their cellular machinery for latent infection. Lytic EBV infection in oropharyngeal epithelial cells, though an infrequent event, is believed to be a major source of infectious EBV particles for salivary transmission. The biological events associated with nasopharyngeal epithelial cells are only beginning to be understood with the advancement of EBV infection methods and the availability of nasopharyngeal epithelial cell models for EBV infection studies. EBV infection in human epithelial cells is a highly inefficient process compared to that in B cells, which express the complement receptor type 2 (CR2) to mediate EBV infection. Although receptor(s) on the epithelial cell surface for EBV infection remain(s) to be identified, EBV infection in epithelial cells could be achieved via the interaction of glycoproteins on the viral envelope with surface integrins on epithelial cells, which might trigger membrane fusion to internalize EBV in cells. Normal nasopharyngeal epithelial cells are not permissive for latent EBV infection, and EBV infection in normal nasopharyngeal epithelial cells usually results in growth arrest. However, genetic alterations in premalignant nasopharyngeal epithelial cells, including p16 deletion and cyclin D1 overexpression, could override the growth inhibitory effect of EBV infection to support stable and latent EBV infection in nasopharyngeal epithelial cells. The EBV episome in NPC is clonal in nature, suggesting that NPC develops from a single EBV-infected nasopharyngeal epithelial cell, and the establishment of persistent and latent EBV infection in premalignant nasopharyngeal epithelium may represent an early and critical event for NPC development.

Key words Epstein-Barr virus, nasopharyngeal carcinoma, integrins, epithelial cells, latency

Epstein-Barr virus (EBV) infection is closely associated with nasopharyngeal carcinoma (NPC), though its role in the pathogenesis of NPC is not well-defined. EBV infection is readily achieved in human B cells, resulting in their transformation into proliferative lymphoblastoid cell lines. Latent and life-long EBV infection is eventually established in memory B cells. EBV infection in human epithelial cells is highly inefficient; when it does occur, it is mainly lytic in nature, and lytic infection in oropharyngeal epithelial cells has been postulated to be the source of infectious EBV for transmission. Although latent infection of EBV in normal nasopharyngeal epithelial cells is uncommon, genetic alterations such as p16 inactivation or activation of the cyclin D1/CDK4 pathway in premalignant nasopharyngeal epithelial cells may be supportive for the latency establishment of EBV infection. Furthermore, the establishment of persistent EBV infection in premalignant nasopharyngeal epithelial cells has been postulated to be an early and crucial event in the development of NPC. In this review, we discuss the essential events involved in EBV infection and the establishment of stable infection in premalignant nasopharyngeal cell models.

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Close Association Between EBV Infection and Undifferentiated NPC

EBV was first discovered by Epstein, Achong, and Barr in 1964 as a herpesvirus in tumor cell lines derived from Burkitt's lymphoma[1]. Subsequent studies demonstrated that EBV is a B-lymphotrophic herpesvirus and has the ability to transform cultured human B cells into proliferative lymphoblastoid cell lines[2]. EBV infection is the major cause of infectious mononucleosis, which is a self-limiting proliferative disease of EBV-infected B cells. The transforming property of EBV in B cells and the dependence of Burkitt's lymphoma on EBV persistence have consigned EBV as the first human tumor virus[3]. EBV infection is also associated with other human malignancies, including NPC, Hodgkin's disease, and gastric carcinoma, as well as lymphoproliferative disorders in immune-compromised patients[4]. The undifferentiated histological type of NPC, is 100% associated with EBV infection, regardless of the ethnic origin of the patient in diverse geographic regions[5,6]. In contrast to the transforming property of the virus observed in B cells, EBV infection and the expression of viral genes do not readily transform primary nasopharyngeal epithelial cells into proliferative or immortalized cell lines[4,7,8]. Although its role in the pathogenesis of NPC remains to be elucidated, the malignant transformation of EBV-infected nasopharyngeal epithelial cells is believed to be a multi-step process over a long period of time and involve an intricate interplay between EBV genes and host genetic alterations in premalignant nasopharyngeal epithelial cells[8-10]. Here, we review the recent findings related to the mechanisms involved in the entry of EBV and the establishment of stable latent infection of EBV in nasopharyngeal epithelial cells, which has long been postulated as an early event in NPC pathogenesis.

EBV Entry Into Nasopharyngeal Epithelial Cells

Despite that EBV is commonly present in undifferentiated NPC cells, infection in human epithelial cells cultured *in vitro* is difficult[8], which is in stark contrast to the ease of infecting B cells *in vitro*[2]. EBV readily infects B cells through the initial binding of the viral envelope protein gp350 with the complement receptor type 2 (CR2) on the surface of B cells[11]. The subsequent interaction of viral envelope proteins gp42, gLgH, and gB with the human leukocyte antigen (HLA) class II protein on the B cell surface triggers the fusion of the viral envelope with the host cell membrane to facilitate EBV entry[12-14]. Conversely, the entry mechanism into nasopharyngeal epithelial cells is much less clearly defined[8]. In general, human epithelial cells do not express the CR2 commonly present on the surface of B cells, which may explain the low infection efficiency of EBV in nasopharyngeal epithelial cells. The ectopic expression of CR2 in SV40-immortalized keratinocytes (SVKs) and well-differentiated squamous carcinoma cells (SCC12F) facilitates EBV infection in these cells and interestingly leads to the establishment of stably EBV-infected clones of cells expressing latent viral genes[15,16]. These early studies indicated that latent EBV infection can be established in permissive clones of CR2-expressing epithelial cells. Nonetheless, a physiologically relevant entry mechanism of EBV into epithelial cells has not yet been identified.

A polymeric IgA against the EBV antigen(s) may mediate EBV entry into pharyngeal epithelial cells via an endocytic route[17-19]. The IgA-bound EBV may enter the pharyngeal epithelial cells via secretory component (SC)-mediated endocytosis. Elevated levels of IgA against EBV-specific antigens are found in the mucosal secretions from NPC patients[18,19]; indeed, this EBV-IgA-SC- mediated endocytosis may represent a physiologic route of EBV entry into nasopharyngeal epithelial cells *in vivo*.

A breakthrough in EBV infection in human epithelial cells was achieved by directly co-culturing epithelial cells with EBV-producing Akata cells (an EBV-infected lymphoma cell line). Cell-cell contact is required in the co-culture method to mediate the entry of EBV into epithelial cells and greatly improves the infection efficiency by approximately 1,000 folds compared to a cell-free infection method using the viral supernatant harvested from EBV-producing cell lines[20]. However, CR2 is not involved in the co-culture infection method, as most infected epithelial cell lines are negative in CR2 expression, and other receptors have been proposed to participate in the EBV infection in human epithelial cells[21]. These receptors most likely have much lower affinity with EBV than CR2, and intimate cell-cell contact is required to enhance the binding of the virus released from EBV-producing B cells to the epithelial cell surface.

EBV infection in epithelial cells could also be achieved by loading EBV particles onto the surface of B cells, which are then "transferred" to epithelial cells for infection[22]. The loading of EBV onto the surface of B cells was mediated through the interaction of EBV gp350 with the CR2 of B cells[22], and a redistribution of CR2 focusing at the synapses of B cells and epithelial cells was observed. Again, the epithelial cells infected by this method were negative for CR2 expression[22]. Later studies revealed that integrins on the epithelial cell surface are involved in mediating EBV infection in human epithelial cells[23-26]. Fusion of the viral envelope and epithelial cell membrane was found to be triggered by the interaction of gLgH with integrin αvβ5, αvβ6, or αvβ8[24,25]. In other studies, the routes of EBV entry into epithelial cells were also examined using *in vitro* polarized oropharyngeal epithelial cells[23,26]. Cell-free EBV virions were able to directly infect oropharyngeal epithelial cells at the basolateral surface through an interaction of EBV *BMRF2* with integrin α5β1 located on the membrane of oropharyngeal epithelial cells[26]. EBV could also infect epithelial cells at the apical membrane through the cell-cell contact route. Moreover, EBV was found to also spread directly across lateral membrane to infect adjacent epithelial cells[26]. A later study showed that the transfer of EBV infection from B cells to epithelial cells could also be achieved at the basolateral surface due to the polarization of the EBV-binding molecules and adhesion molecules in epithelial cells[23]. In this process, CD11b on B cells interacts with heparan sulfate moieties of CD44v3 and lymphocyte endothelial epithelial-cell adhesion molecule (LEEP-CAM) on epithelial cells to facilitate the adhesion of EBV-loaded B cells to the basolateral surface of epithelial cells[23]. The entry of EBV also involves Arg-Gly-Asp (RGD)-binding or Lys-Gly-Asp (KGD)-binding integrins, as the addition of both RGD peptides and KGD peptides

could inhibit infection by up to 40%[23]. All these studies showed the importance of integrins on the surface of human epithelial cells in mediating EBV infection.

EBV can infect both epithelial cells and B cells. Viral gp42 functions as the switch of tropism between epithelial cells and B cells. EBV produced from B cells have a higher tropism for infecting epithelial cells than B cells and *vice versa*[13]. The viral envelope protein gp42 facilitates the EBV infection in B cells but impedes EBV entry into epithelial cells. The interaction between HLA class II molecules on B cells and viral gp42 facilitates the entry of EBV to B cells, however, it also leads to the targeting of gp42 to the endosome for degradation, thereby reducing its levels in EBV virions produced from B cells[13,14]. Hence, EBV virions generated from B cells will have low expression of gp42, which facilitates its infection in epithelial cells. Such EBV particles generated from B cells bind to epithelial cells via the interaction of complex gLgH to the integrins αvβ6 and αvβ8 on the epithelial cell surface, with viral gB functioning as a co-receptor[25,27]. This dual tropism of EBV allows the virus to shuttle between epithelial cells and B cells during its life cycle of infection and plays an important role in establishing life-long EBV infection in humans.

A recent report has shown that different strains of EBV may vary in their efficiency of infecting epithelial cells and B cells[28]. In this study, the M81 EBV strain isolated from NPC tissues of a Chinese patient was shown to be genetically related to EBV strains isolated from Chinese NPC tissues and genetically distant from the B95.8 EBV strain isolated from infectious mononucleosis (B-cell origin). Interestingly, the M81 EBV strain could infect epithelial cells at a much higher efficiency than the B95.8 EBV strain[28]. Although the factors governing the tropism of various strains have not yet been clearly defined, the levels and polymorphisms of gB (gp110), another glycoprotein essential for mediating viral infection in both epithelial and B cells, appear to be involved[28]. This finding raises the possibility that a specific strain of EBV may be involved in the pathogenesis of NPC, which is endemic among southern Chinese. Furthermore, M81 EBV more readily undergoes spontaneous lytic reactivation after infecting both epithelial and B cells. EBV infection in NPC is predominantly latent in nature, whereas lytic replication has been shown to play an important role in the generation of lymphoma in immune-suppressed mice reconstituted with human hematopoietic cells[29]. The relative importance of lytic and latent EBV contributing to human tumorigenesis remains to be further elucidated. The isolation of more EBV strains from NPC patients and healthy control subjects and the characterization of their genotypes, biological properties, and transforming potential will clarify the role of EBV strain variations in NPC pathogenesis.

We have recently reported that EBV could establish latent infection in telomerase-immortalized nasopharyngeal epithelial cells harboring a *p16* deletion or overexpressing cyclin D1/Bmi-1[30-32]. These immortalized nasopharyngeal epithelial cells could be infected with EBV by either cell-cell co-culture or cell-free virus infection with the Akata EBV strain[30-32]. The expression of latent EBV genes, including EBV-encoded small RNAs (*EBERs*), EBV nuclear antigen 1 (*EBNA1*), latent membrane protein 1 (*LMP1*), and *LMP2*, could be detected in infected nasopharyngeal epithelial cells representative of type II latent EBV infection, which is also a characteristic of EBV gene expression in infected NPC[30-32]. In addition, the expression of EBV-encoded microRNAs (miRNAs) was also detected in EBV-infected NPC (Tsao *et al*., unpublished observations). Interestingly, transforming growth factor (TGF)-β1, a cytokine commonly present in inflammatory tissues, strongly enhanced the efficiency of EBV infection in nasopharyngeal epithelial cells[32], and the enhancement of EBV infection was not only limited to immortalized nasopharyngeal epithelial cells but was also observed in primary nasopharyngeal epithelial cells prior to immortalization of nasopharyngeal epithelial cells. However, the mechanisms underlying TGF-β1-mediated EBV infection are unclear. Our preliminary findings indicated that the TGF-β1-induced relocation of integrins and the alteration of actin dynamics may be involved (Tsao *et al*., unpublished observations). These observations may suggest a role of pro-inflammatory cytokines in nasopharyngeal mucosa to facilitate EBV infection in premalignant nasopharyngeal epithelial cells *in vivo*.

Establishment of Latent Infection and Persistence of EBV Episomes in Infected Nasopharyngeal Epithelial Cells

EBV infection in B cells initiates a growth and proliferation program, but this does not occur in epithelial cells[33]. Distinct viral promoters are turned on during the establishment of latent EBV infection in B cells and epithelial cells, and differential latent EBV genes are transcribed (type III latency program in B cells and type II latency program in epithelial cells). The switching of viral promoter usage occurs during the immortalization of B cells after primary infection. The Wp promoter is activated at the initial phase of the primary infection in B cells, followed by activation of the Cp promoter to transcribe various *EBNAs*[34-36]. In the type III latency characteristic of EBV infection in B cells, the transcription and alternative splicing of long transcripts are initiated from either the Wp or Cp promoter, resulting in the expression of 6 nuclear antigens (EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, and EBNALP). Other latent viral genes, including *EBER*, *LMP1*, and *LMP2*, as well as EBV miRNAs are also expressed, and the expression of these viral genes contributes to the transformation of B cells into proliferative lymphoblastoid cell lines[34,35]. In contrast, neither the Wp nor Cp promoter is activated during EBV infection in epithelial cells[37]. *EBNA1* expressed in EBV-infected epithelial cells is transcribed exclusively from the Qp promoter. *EBERs*, *LMP1*, and *LMP2* genes and EBV miRNAs are also detected in EBV-infected epithelial cells. This is referred to as type II latency. However, the expression of *EBNA2* and *EBNA3C*, which are well documented as cell cycle promoters in EBV-infected B cells (type III latency), is not detected in EBV-infected epithelial cells (type II latency). This differential expression of EBV genes in latently infected nasopharyngeal epithelial cells may prevent the transformation of EBV-infected epithelial cells into proliferative clones. The cellular factors regulating these distinct profiles of latent viral

gene expression are not well understood but are believed to involve both transcriptional and epigenetic regulation.

Our recent report shows that telomerase-immortalized nasopharyngeal epithelial cell lines could be infected by EBV at variable infection rates[32]. EBV-infected nasopharyngeal epithelial cells are rapidly lost in culture, which could be attributed to both the rapid loss of EBV episomes in infected nasopharyngeal epithelial cells and growth inhibition after EBV infection[31], which may explain the infrequent rate of EBV infection detected in normal nasopharyngeal epithelial cells. EBV infection in pharyngeal epithelium is believed to be predominantly lytic in nature and is postulated to be the major source of infectious virus shed into saliva for transmission. Additional events are likely to be involved in converting NPC cells to become permissive for latent EBV infection. The detection of EBV infection in preinvasive nasopharyngeal lesions and the clonal origin of EBV in NPC clearly suggest that premalignant and cancerous nasopharyngeal epithelial cells could support latent EBV infection[4,38]. However, specific genetic alterations in premalignant nasopharyngeal epithelial cells and NPC cells may dampen the cellular response to growth arrest and senescence stimuli upon viral infection to support the establishment of latent EBV infection[4,8,9,39]. We have recently reported that stable EBV infection could be established in immortalized nasopharyngeal epithelial cells after they had acquired specific genetic alterations, including the overexpression of cyclin D1 and/or Bmi-1 as well as the inactivation of p16[30,40]. cyclin D1 overexpression is common in NPC and co-exists with EBV infection in dysplastic nasopharyngeal epithelial cells. The p16 gene is frequently deleted or inactivated by methylation in premalignant nasopharyngeal epithelial cells prior to EBV infection[9,41]. Bmi-1 overexpression is also detected in 38.7% of NPC cases[42]. Interestingly, the overexpression of Bmi-1 or cyclin D1, as well as the silencing of p16, also enhances the efficiency of the immortalization of primary nasopharyngeal epithelial cells, indicating that these genetic events are involved in the early pathogenesis of NPC[8,30,31].

Cell differentiation has a crucial role in regulating the lytic reactivation of EBV in infected B cells and epithelial cells. IgG binding to receptors on EBV-infected B cells triggers the B-cell receptor (BCR) pathway to activate the differentiation of B cells into plasma cells and the lytic reactivation of EBV. Lytic EBV infection could be detected in the upper differentiating layers of the EBV-infected epidermis of oral hairy leukoplakia (OHL) in immune-compromised human immunodeficiency virus (HIV) carriers[43]. The undifferentiated property of NPC in endemic areas may be a pre-requisite for the establishment of latent EBV infection. EBV infection is not detected in squamous carcinoma arising from other regions of the head and neck. These non-NPC head and neck cancers have a more differentiated histological property compared with EBV-associated NPC. Genetic alterations that promote cell cycle progression, such as cyclin D1 overexpression, perturb cellular differentiation and may support latent EBV infection in premalignant nasopharyngeal epithelial cells[44,45]. We have also observed that the overexpression of cyclin D1 suppresses the responses of immortalized nasopharyngeal epithelial cells to serum-induced differentiation[31]. The lytic promoter (Zp), which is crucial for the initiation of EBV lytic reactivation, contains a differentiation responsive element[46,47]. The suppressive effect of cyclin D1 on differentiation may suppress the lytic reactivation of EBV in infected cells. EBV lytic genes, including *BZLF1*, *BRLF1*, *BMRF1*, and *BGLF4*, were found to be generally down-regulated in nasopharyngeal epithelial cells overexpressing cyclin D1[31]. Similarly, the overexpression of *ΔNp63*, a gene highly expressed in basal undifferentiated cells, in EBV-infected gastric epithelial cells suppresses EBV lytic gene expression[48,49]. Furthermore, the knockdown of *ΔNp63* in EBV-infected telomerase-immortalized normal oral keratinocytes induces EBV reactivation[48,49]. The epigenetic regulation of the EBV genome also plays an important role in regulating lytic gene expression[49,50]. The lytic cycle reactivation of EBV usually requires the methylation of specific viral promoter elements[51]. BZLF1 binds to methylated DNA in a subset of lytic promoters, resulting in gene transcription to facilitate the progression of the lytic cycle and the generation of infectious viral particles[52,53].

In NPC, EBV-infected cells express EBV miRNAs and EBV genes characteristic of type II latency, including *EBNA1*, *LMP1*, *LMP2A/B*, and *EBERs*[5,7,9]. Among these latently expressed genes, *EBNA1* is essential for the persistence of the EBV genome in all EBV-associated cancers, including NPC[54,55], and controls the replication and mitotic segregation of EBV episomes, which replicate only once per cell cycle and are maintained at approximately 20–100 copies in infected NPC cells. The mitotic partitioning of EBV episomes requires the interaction of EBNA1 with the family of repeats (FR) element at the origin of replication, *oriP*[56-58]. The FR element consists of 20 tandem copies of a 30-bp sequence, each of which contains the 18-bp palindromic EBNA1-binding site, followed by a 12-bp AT-rich sequence[59,60]. EBNA1 binds as a dimer to each recognition site throughout the cell cycle. The tethering of EBV episomes to the condensed cellular chromosomes in mitosis ensures the proper segregation of the episomes[61]. A recent study reported that EBV segregation may be suboptimal in NPC cells[62], which is likely a reason accounting for the loss of EBV episomes that is commonly observed in NPC-derived cell lines propagated in culture. Although the selective advantage of EBV infection in NPC is not apparent in EBV-infected NPC cells cultured *in vitro*, the expression of EBV-encoded genes may provide anti-apoptotic and other growth advantages to EBV-infected NPC cells *in vivo*[4,7,8]. Interestingly, EBV episomes could be stably maintained in NPC xenografts passaged in immune-suppressed animals over a long period of time, confirming the selective growth advantage of EBV-infected NPC cells propagated *in vivo*. The characterization of these selective growth advantages will contribute to the understanding of the role of EBV infection in NPC pathogenesis.

The regulation of lytic and latent EBV infection plays a crucial role in the persistence of EBV episomes in infected NPC cells. Key events regulating the lytic and latent switch in EBV have been extensively reviewed[49,51]. The establishment of stable EBV requires the suppression of BZLF1 and BRLF1 levels in infected epithelial cells. The promoter for *BZLF1* is regulated by cellular transcription factors including Yin Yang (YY)1, E protein (E)2-2, myocyte enhance factor (MEF)-2D, and the zinc finger E-box-binding homebox (ZEB)

s. ZEB1 and ZEB2 are strong repressors of the Zp promoter[63-65], and high levels of ZEB1 and ZEB2 are observed in cell lines harboring latent EBV infection[63,65]. Interestingly, the host cell miRNA-200b and miRNA-429 were able to suppress the expression of ZEBs and activate lytic EBV infection in gastric carcinoma cells[66,67]. The regulation of the promoter for *BRLF1* is less well defined. Interestingly, BRLF1 may play a more important role in the activation of lytic infection in differentiating epithelial cells[48]. The role of host cell factors in regulating the latent and lytic switch of EBV in infected epithelial cells remains to be further elucidated with the identification of an NPC-specific EBV strain, improved epithelial cell infection methods and the establishment of appropriate epithelial cell models for investigation.

Conclusions and Future Directions

Recent success in the development of efficient methods to infect epithelial cells and the establishment of nasopharyngeal epithelial cell models has allowed systematic investigations of EBV entry and persistence in nasopharyngeal epithelial cells. Nonetheless, the detailed mechanistic pathways leading to EBV infection and persistence in nasopharyngeal epithelial cells are yet to be unveiled. The identification of host factors involved in the regulation of the latent and lytic switch in nasopharyngeal epithelial cells will contribute to knowledge on the benefit of persistent EBV infection in NPC. The recent cloning and characterization of an NPC-specific EBV strain is a major step forward in studying the biology of EBV infection in nasopharyngeal epithelial cells and its role in NPC development.

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