ARTICLE ADDENDUM

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Integrins as triggers of Epstein-Barr virus fusion and epithelial cell infection

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Epstein-Barr virus is a ubiquitous orally-transmitted human herpesvirus that is carried by most of the adult population. It establishes latent infections in B lymphocytes, reactivates periodically from latency and can be amplified in epithelial cells where it is thought more commonly to undergo lytic replication. Entry into either cell involves fusion of the virus envelope with a cell membrane. Fusion with a B cell requires four envelope glycoproteins, gB and a ternary complex of gHgLgp42. Fusion is triggered by an interaction between gp42 and HLA class II. Fusion with an epithelial cell requires three envelope glycoproteins, gB and a binary complex of gHgL. The presence of gp42 blocks infection and blocks the interaction of gHgL with a specific receptor on the epithelial cell surface. We recently demonstrated that both integrins αvβ6 and αvβ8 can serve as specific receptors for gHgL and that on binding to gHgL, even in a soluble form, can provide the trigger for direct virus fusion with the epithelial cell plasma membrane. It reveals yet another way in which an integrin can be used by a pathogen to invade a cell.

Epstein-Barr virus (EBV) is an orally transmitted human gammaherpesvirus that is carried by more than ninety percent of the adult population. Most individuals are infected asymptomatically in childhood, but a delayed primary infection may manifest as acute, self-limiting infectious mononucleosis. Long term carriage is usually benign, but can be associated with development of either B cell or epithelial malignancies, most notably nasopharyngeal carcinoma (NPC), reflecting the primary tropism of the virus for these two cell types and its ability to establish latency in each.[1] In the persistent state in the healthy carrier the virus is thought to traffic almost continuously between B cells and epithelial cells. The reservoir of latent virus is maintained in the long lived B memory compartment, within which terminal differentiation into a plasma cell can lead to reactivation into productive lytic replication.[2] Released virus may then be amplified in epithelial cells,[3,4] where lytic replication is thought to be more common. Virus may be shed in saliva for transmission to a new host or infect new B cells for replenishment of the latent reservoir and maintenance of what could be termed a "cycle of persistence."

As an enveloped virus, EBV enters cells by fusion of its envelope with a cell membrane. There are several paradigms for virus: cell fusion, most of which ultimately invoke the action of a single virus "fusion protein" and all of which require some mechanism by which a fusion-inducing conformational change in the fusion protein can be triggered.[5] The model for the herpesviruses is, however, complicated by the fact that, although the structure of the conserved glycoprotein gB closely resembles that of the vesicular stomatitis G protein, which is a class III fusion protein,[6] it is unable to mediate fusion in the absence of a second conserved heterodimer of glycoproteins gH and gL. It is currently unclear whether it is gB alone, activated by an interaction with gHgL or a complex of gB and gHgL that is

the ultimate fusogen,[7] though the ability of Herpes simplex virus gHgL to mediate hemifusion in the absence of gB[8] perhaps suggests that all three proteins are directly involved.

The trigger that activates the core fusion machinery, gB and gHgL, differs among the herpesviruses. For EBV the trigger also differs between a B cell and an epithelial cell, and these differences have important implications for pathogenesis and spread.[9] Fusion with a B cell occurs within an endosome at low pH, but a pH-induced conformational change is not relevant to the trigger.[10] A third glycoprotein, gp42, which binds to gH[11] in some, but not all of the gHgL complexes in virus,[12] interacts with HLA class II and it is instead this interaction which provides the trigger.[13] One current hypothesis is that binding of HLA class II to gp42, which does induce a slight conformational change in gp42, alters the arrangement of relevant glycoproteins in the virus envelope.[14]

Fusion with an epithelial cell takes place at the cell surface[10] and, consistent with the lack of constitutive expression of HLA class II, involves only gB and gHgL. Just as a binary complex of gHgL is incapable of mediating entry of a B cell, a ternary complex of gHgLgp42 is incapable of mediating entry of an epithelial cell.[12] Addition of saturating amounts of a soluble form of gp42, which can bind to gHgL in trans and rescue the ability of a gp42 null virus to enter a B cell, blocks the ability of virus to enter an epithelial cell.[12] As a result of the different requirements for fusion with the different cell types the relative amounts of binary and ternary complexes in the virion can influence its tropism. In an HLA class II-positive B cell some ternary complexes are lost as gp42 interacts and traffics with HLA class II. The glycoprotein and its partners are degraded, presumably in the protease-rich peptide loading compartment. The virus emerging from a B cell thus carries less gp42 and is slightly better at infecting an epithelial cell. In contrast, no gp42 is lost to this pathway in an HLA class II-negative epithelial cell and the gp42-rich virus emerging from an epithelial cell can be as much as two logs more infectious for a B cell than virus made in a B cell.[9] This switch in tropism presumably favors the movement between epithelial cells and B cells during the cycle of persistence.

Until very recently, however, the model for virus fusion and entry into an epithelial cell lacked an identifiable trigger. The assumption was, since the presence of gp42 was essential to B-cell infection but blocked epithelial infection, that the trigger required a direct interaction between gHgL and an unknown epithelial cell surface molecule that is not available on a B cell. Screening of cDNA expression libraries turned up no candidates. We knew, however, that a soluble form of gHgL (gHtgL) made in baculovirus could bind directly to an epithelial cell and not a B cell, that soluble gp42 could block this binding[15] and that a monoclonal antibody to gHgL, which neutralized epithelial but not B-cell infection, did the same. We therefore took a biochemical approach to identify the molecule to which gHgL bound, with the expectation that it might be not only a specific gHgL receptor but also the missing trigger of fusion.

Scatchard analysis indicated that gHtgL bound saturably to epithelial cells with a K_D of approximately 10^{-9} M, although the number of binding sites varied depending on the particular epithelial cell line that was examined. The ectodomain of gH includes a KGD motif that could potentially serve as an integrin ligand, so we tested the possibility that the binding partner of gHgL was an integrin. Integrins can exist in a closed or an open conformation and the difference in the affinity of the two conformations can be as much as 9,000-fold.[16] Manganese locks integrins in an open conformation and in its presence gHtgL now bound with a K_D of approximately 10^{-12} M. In addition, a peptide corresponding to sequences in gH that included the KGD motif, but not a scrambled peptide, could both block binding of gH and infection of epithelial cells.

To begin to narrow down what integrin(s) might be acting as a gHgL receptor we examined the effects of inclusion of the matrix proteins fibronectin and vitronectin, which are the natural ligands of many integrins expressed on epithelial cells. Vitronectin and, to a slightly lesser extent, fibronectin competitively reduced gHtgL binding and reduced infection of epithelial cells. Vitronectin also blocked fusion with epithelial cells in a cell based fusion assay. No corresponding effects were seen on the interaction of virus with B cells. Of the integrins that were expressed on the epithelial cell lines we were using, those that bound fibronectin and vitronectin included $\alpha5\beta1$, $\alpha v\beta3$, $\alpha v\beta5$, $\alpha v\beta6$, $\alpha v\beta8$ and, presumably, $\alpha v\beta1$, although this latter pairing was not specifically examined. Some of the B-cell lines that failed to bind gHtgL also expressed $\alpha5\beta1$, which ruled it out as the receptor. A hamster cell line expressing only the human integrin $\alpha v\beta3$ also failed to bind gHtgL. However, reduction of expression of αv by transfection of a cognate siRNA both reduced gHtgL binding and infection of an epithelial cell. The KGD motif in gH is followed by a leucine residue at the plus-4 position which is characteristic of ligands that bind integrins $\alpha v\beta6$[17] and $\alpha v\beta8$.[18] We therefore examined the effects of soluble forms of integrins $\alpha v\beta6$ and $\alpha v\beta8$. Both integrins, but not a soluble form of $\alpha v\beta3$, blocked gHtgL binding, could be reciprocally precipitated with gHtgL and blocked epithelial cell infection. Even more exciting, however, they were also able to trigger epithelial cell fusion in a cell based fusion assay and trigger fusion of hamster cells, lacking any human integrins, that had been transfected with gB and gHgL.

There are many viruses that use integrins to facilitate entry into target cells, including adenoviruses, hantaviruses, picornaviruses, reoviruses and herpesviruses such as Kaposi Sarcoma Herpesvirus and human cytomegalovirus.[19] In most cases it is thought that it is the ability of integrins to elicit those signaling responses that lead to cytoskeletal reorganization and virus uptake that is of paramount importance. EBV so far appears to be unique in its additional adaptation to use integrins as triggers of fusion in the absence of endocytosis. Fusion with an epithelial cell and entry of virus into the cytoplasm is completely unaffected by chlorpromazine or sodium azide.[10] By analogy with the effects of HLA class II binding on gp42 it seems possible that integrin binding might produce at least a subtle conformational change in gHgL that perhaps could effect an interaction between gHgL and gB. Bimolecular complementation assays have revealed an interaction between Herpes

simplex virus gHgL and gB, following initiation of fusion, that is essential to the event.[7]

Whether or not integrin signaling is, as well, important to subsequent downstream events such as intracellular trafficking remains to be fully evaluated. However, in this respect it is of interest that while inhibitors of actin dynamics facilitate entry of EBV into an epithelial cell, perhaps because of disruption of cortical actin, they also block its subsequent transport to the nucleus (Valencia and Hutt-Fletcher, unpublished observations). This suggests a possible role for integrin-induced actin remodeling in infection and it is noteworthy that another EBV glycoprotein, the BMRF2 protein, interacts with integrins α3, α5 and β1 and is important to infection of, though not fusion with polarized epithelial cells.[20] The potential and relative roles of gHgL and the BMRF2 protein in integrin signaling and actin remodeling need further exploration.

It remains possible that integrins in addition to αvβ6 and αvβ8 (or, formally, also molecules other than integrins) can bind gHgL and trigger fusion. The effects of integrin-blocking antibodies have not been conclusive, but suggest that while αvβ1 does not bind gHgL, αvβ5 probably does. Work is ongoing to evaluate this further. The use of αvβ6 as a trigger of fusion may, however, be of particular relevance to the role of EBV in development of NPC.

Undifferentiated forms of NPC uniformly carry EBV and the tumors are common cancers in Southern China and Southeast Asia. Despite some recent declines, the age-standardized incidence rate for men in Hong Kong, for example, remains as high as 20.2 per 100,000.[21] On a worldwide basis NPC probably represents the biggest tumor burden associated with EBV. The fact that the tumors carry EBV episomes with identical numbers of terminal repeats has provided strong support for the contention that virus infection and expression of latency proteins and transcripts provides the critical, initiating, oncogenic insult.[22] Infection of a cell is followed by circularization of the virus genome as a result of random recombination of variably reiterated terminal repeat sequences. The number of terminal repeats remaining in the retained episome is thus particular to each infecting event. Infection of a preexisting tumor would produce cells carrying episomes with variable and not identical numbers of repeats. However, there is also evidence for evolution towards a predominant episomal form as one cell gains a growth advantage over its peers[23] and some studies have found the genome in only a small portion of tumor cells in early NPC and progressively increased numbers of cells at more advanced stages.[24] The possibility that the timing of infection with EBV is not always coincident with tumor initiation has important implications for the role that the dynamics of virus amplification might play in tipping the balance towards frank neoplasia. High antibody titers to EBV lytic cycle proteins, presumably reflective of productive replication, are prognostic for development of NPC in ethnic populations at high risk.[25] Molecular alterations, including chromosome 3p and 9p loss, have been detected in normal epithelia, dysplastic epithelia and NPC in similar groups.[26] EBV infection has been detected in all high grade dysplastic lesions and NPC, but not in low grade lesions or nasopharyngeal tissue of normal adults.[26] These latter findings further raise the important question as to whether preexisting changes might increase susceptibility to infection and infection then drive more aggressive cell growth.

Integrin αvβ6 is expressed at low levels on normal epithelium, but its expression is rapidly upregulated during tissue remodeling, including that accompanying wound healing, inflammation and carcinogenesis.[27] In addition, αvβ6, of all the integrins, has the highest affinity for the TGFβ1 latency associated peptide (LAP).[28] Binding of LAP to αvβ6 causes local activation of endogenous TGFβ1,[29] and TGFβ1 induces EBV lytic reactivation in B cells.[30] We have also found that dysplastic cells are more likely to express the complement receptor CR2/CD21 (Jiang and Hutt-Fletcher, unpublished), which allows for efficient attachment of EBV and increases infection rates significantly.[15] Contact between dysplastic epithelial cells expressing αvβ6, and latently infected B cells in the oropharynx may then be followed by a perfect storm of events, induction of EBV replication in one cell and vulnerability to infection in the other, compounding the potential for EBV to affect tumor progression in a cell that has already undergone molecular alterations and may be more prone to support virus latency.

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