Invasion of Herpes Simplex Virus Type 1 into Murine Epidermis: An *Ex Vivo* Infection Study

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Herpes simplex virus type 1 (HSV-1) invades its human host via the skin or mucosa. We aim to understand how HSV-1 overcomes the barrier function of the host epithelia, and for this reason, we established an *ex vivo* infection assay initially with murine skin samples. Here, we report how tissue has to be prepared to be susceptible to HSV-1 infection. Most efficient infection of the epidermis was achieved by removing the dermis. HSV-1 initially invaded the basal epidermal layer, and from there, spreading to the suprabasal layers was observed. Strikingly, in resting stage hair follicles, only the hair germ was infected, whereas the quiescent bulge stem cells (SCs) were resistant to infection. However, during the growth phase, infected cells were also detected in the activated bulge SCs. We demonstrated that cell proliferation was not a precondition for HSV-1 invasion, but SC activation was required as shown by infection of aberrantly activated bulge SCs in integrin-linked kinase (ILK)-deficient hair follicles. These results suggest that the status of the bulge SCs determines whether HSV-1 can reach its receptors, whereas the receptors on basal keratinocytes are accessible irrespective of their proliferation status.

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INTRODUCTION

Herpes simplex viruses types 1 and 2 (HSV-1, HSV-2) are ubiquitous pathogens that are well adapted to their human hosts. Upon primary infection via the skin and mucocutaneous regions, HSV can reach sensory neurons where latent infection is established. The extent of primary and recurrent mucocutaneous infections is largely a function of the host's immune status. HSV infections can be devastating in immune-compromised hosts and newborns. Normally, the skin forms an effective barrier to infection. However, patients with skin lesions are predisposed to primary, as well as recurrent HSV infections. Eczema herpeticum, a disseminated HSV-1 infection of the skin, is a complication of atopic dermatitis, suggesting the impaired epidermal barrier function and the dysregulated immune response as risk factors for higher susceptibility to HSV-1 (Wollenberg *et al.*, 2003; Bussmann *et al.*, 2008). In addition to atopic disease, skin abrasions or burns are particularly susceptible to HSV (Foley *et al.*, 1970; Shenoy *et al.*, 2015).

Although viral entry into individual cells in culture and the immune response to HSV have been studied in detail, we still have little knowledge of how the virus invades tissue and which cellular factors determine susceptibility to efficient HSV infection. Two important cellular factors are the surface receptors, herpes virus entry mediator (HVEM) and nectin-1, which interact with the viral envelope glycoprotein D (Montgomery *et al.*, 1996; Geraghty *et al.*, 1998). A further glycoprotein D receptor is 3-O-sulfated heparan sulfate (Shukla *et al.*, 1999; O'Donnell *et al.*, 2010). These receptors are essential for the fusion of the viral envelope with cellular membranes. In addition, heparan sulfate proteoglycans serve as attachment factors and thus bind the virus before it interacts with the cellular glycoprotein D receptors (Shieh *et al.*, 1992).

Mice are widely used as an animal model for studies of HSV skin, mucosal, and corneal infections, and the murine homologs of nectin-1 and HVEM support HSV-1 entry (Yoon *et al.*, 2003). Furthermore, infection studies with HSV-2 revealed that both nectin-1 and HVEM are needed for disease development in mice (Taylor *et al.*, 2007). Previously, we demonstrated that nectin-1, which is expressed on the surface of most epidermal keratinocytes, is important for HSV-1 entry into murine epidermis, whereas HVEM, which is restricted to a limited number of cells, can potentially replace nectin-1 as receptor (Petermann *et al.*, 2015). How HSV-1 invades the epidermis and reaches its receptors is still not known.

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Abbreviations: HVEM, herpes virus entry mediator; IFE, interfollicular epidermis; ICP0, infected cell protein 0; Ig, immunoglobulin; ILK, integrin-linked kinase; PFU, plaque-forming unit; p.i., post infection; SC, stem cell; TJ, tight junction; wt, wild type

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Scarification of the skin, mucosa, or cornea is generally used to infect mice efficiently, and, although this is clearly distinct from the natural invasion route, it has allowed various aspects of immune responses and viral pathogenesis to be studied. To analyze the initial infection steps in the epidermis, we established an *ex vivo* infection model using murine epidermal sheets as a tool to allow the analysis of cellular factors that determine HSV-1 susceptibility. In this paper, we characterize the *ex vivo* infection assay; we investigate how epidermal sheets have to be prepared to be susceptible to HSV-1, which cells can be infected, and whether cell proliferation contributes to viral entry. Our results indicate that basal as well as differentiated keratinocytes can be infected. The major precondition is cell viability, but infection does not require proliferation. Interestingly, quiescent stem cells (SCs) in the hair bulge were protected from HSV-1, whereas activation of SCs in the hair follicle growth phase or by genetical ablation of ILK led to infection of these cells as well.

RESULTS

Ex vivo infection of complete murine skin and epidermal sheets with HSV-1

We prepared murine tail skin to analyze susceptibility to HSV-1 by *ex vivo* infection. The skin samples were infected by submerging them in virus suspension, and infection was determined by staining the samples with an antibody against the viral protein ICP0 at various times post infection (p.i.). ICP0 is one of the first viral genes to be expressed once the viral genome is released into the nucleus (Boutell and Everett, 2013). At early times after infection, ICP0 localizes in nuclear foci, but it relocalizes to the cytoplasm later during infection (Petermann *et al.*, 2009). Thus, visualization of ICP0 expression indicates the successful entry of HSV-1 into individual cells. At 3 hours and even as late as 12 hours p.i., no ICP0 signals were detected in intact skin samples (Figure 1a), suggesting that HSV-1 cannot penetrate via either the cornified layer or the dermal layer. Also, at the edge of skin samples, no infected cells were detected. Even when the cornified layer was removed, nearly no infected cells were observed (data not shown). Only after removal of the dermis and infection of the epidermal sheets, was cytoplasmic ICP0 visible. It was present in the basal layer at 3 hours p.i. and spreading to suprabasal layers was observed as early as 6 hours p.i. (Figure 1b), demonstrating that nucleated keratinocytes can be infected. To confirm that all basal keratinocytes are susceptible to HSV-1, we visualized ICP0 in whole/mount preparations of epidermal sheets. The results indicated that all basal cells expressed cytoplasmic ICP0 at 3 hours p.i. when the epidermal sheets were infected at 100 plaque-forming unit (PFU)/cell. Infection at 10 PFU/cell resulted in fewer infected cells, and the predominantly nuclear location of ICP0 at 3 hours p.i. indicated a slower infection (Figure 1d).

Melanocytes represent the major class of non-keratinocyte cells in the epidermis (Lin and Fisher, 2007). In contrast to Langerhans cells (Sprecher and Becker, 1986), another class of non-keratinocytes in the epidermis, susceptibility of melanocytes to HSV-1 is still unknown. Our results demonstrate that the very few murine melanocytes, which were present in primary keratinocyte cultures (characterized by tyrosinase expression), were infected (Figure 1e). As nectin-1 serves as the major receptor for HSV-1 on keratinocytes (Petermann *et al.*, 2015), we also analyzed the behavior of melanocytes isolated from nectin-1-deficient epidermis. Surprisingly, melanocytes could be infected in the absence of nectin-1 (Figure 1e). These results demonstrate the susceptibility of melanocytes to HSV-1 even in the absence of nectin-1 implying the use of alternative receptor(s).

As the visualization of ICP0 expression detects only the onset of infection, we next determined production of progeny virus by plaque assays. As expected, viruses were produced upon infection of epidermal sheets (from 2×10^2 to 3×10^6 PFU/ml) (Figure 1c). In contrast, infection of intact skin with even a high virus dose (50 PFU/cell) led to a very small but a consistent increase in virus progeny (from 3×10^4 to 1×10^5 PFU/ml) (Figure 1c). These results suggest that at least some cells in the skin sample can be productively infected even though there was no detectable ICP0 expression (Figure 1a).

When isolated epidermal sheets were pre-incubated in medium for extended periods prior to infection, the number of infected cells in the basal layer decreased. In freshly prepared epidermal sheets or those pre-incubated for 3 hours, all basal keratinocytes were infected by 3 hours p.i. (Figure 2a and b). However, after pre-incubation for 16 hours, ICP0 expression could only be detected in 60% of cells, and very few infected cells were detected after 24 hours of pre-incubation (Figure 2a and b). The decreased number of infected cells correlated with an enlargement and flattening of basal keratinocytes (Figure 2a). In addition, when tight junctions (TJs) were visualized by ZO-1 staining, there was extensive relocalization throughout the epidermis following 16 hours of pre-incubation and 3 hours of infection (Figure 2c) indicative of TJ formation in the basal layer.

We further analyzed the transition of the basal keratinocytes in the incubated epidermal sheets by electron microscopy. After cultivation for 16 hours in medium, the transition into extremely flat cells in the basal layer became obvious (Figure 3a). Overall, the morphology of the epidermal sheets suggests a loss of cell polarity. In contrast, when epidermal sheets were analyzed directly after separation from the dermis, the basal layer showed no disturbance of morphology and architecture (Figure 3b). In the incubated sheets, the flat basal keratinocytes lost most of the keratin fibers, whereas suprabasal cells maintained at least some (Figure 3a). The intercellular spaces differed clearly between incubated and directly analyzed sheets. Although the wide intercellular spaces in non-incubated sheets were characterized by many contacting cell extensions and intact desmosomes, cell–cell contacts in the incubated sheets were much closer (Figure 3d and e, arrowheads). There was evidence for TJ formation in the basal layer (Figure 3d, arrowhead), which supports the ZO-1 staining of the incubated sheets (Figure 2c). In addition, incubation led to cell death in the basal as well as in the suprabasal layers, which was very rarely observed in non-incubated sheets. The decreased number of viable cells after incubation for 16 hours was confirmed by flow cytometric analysis (data not shown). Taken together, these