Herpes Simplex Virus (HSV) Modulation of Staphylococcus aureus and Candida albicans Initiation of HeLa 299 Cell-Associated Biofilm

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Abstract Although herpes simplex virus type-1 (HSV-1), and type-2 (HSV-2), Staphylococcus aureus and Candida albicans co-habit the oral and genital mucosa, their interaction is poorly understood. We determined the effect HSV has on bacterial and/or fungal adherence, the initial step in biofilm formation. HeLa229 cells were infected with HSV-1 (KOS) gL86 or HSV-2 (KOS) 333gJ at a multiplicity of infection (MOI) of 50 and 10. S. aureus (ATCC 25923) and/or C. albicans (yeast forms or germ tube forms) were co-incubated for 30 min (37 °C; 5 % CO2; 5:1 organism: HeLa cell ratio; n = 16) with virus-infected HeLa cells or uninfected HeLa cell controls. Post-incubation, the monolayers were washed (3x; PBS), lysed (RIPA), and the lysate plated onto Fungisel and/or mannitol salts agar for standard colony count. The level of HeLa-associated S. aureus was significantly decreased (P < 0.05) for both HSV-1- and HSV-2-infected cells, as compared to virus-free HeLa cell controls (38 and 59 % of control, respectively). In contrast, HSV-1 and HSV-2 significantly (P < 0.05) enhanced HeLa cell association of C. albicans yeast forms and germ tube approximately two-fold, respectively. The effect of S. aureus on germ tube and yeast form adherence to HSV-1- and HSV-2-infected cells was specific for the Candida phenotype tested. Our study suggests that HSV, while antagonist towards S. aureus adherence enhances Candida adherence. Furthermore, the combination of the three pathogens results in S. aureus adherence that is either unaffected, or partially restored depending on both the herpes viral species and the fungal phenotype present.

Introduction

Adherence to cell surfaces is an essential initial stage in microbial colonization and subsequent biofilm formation [58, 77].Shared sites of persistent colonization and chronic infection for Staphylococcus aureus, Candida albicans, and HSV are the oronasopharynx and genital tract. S. aureus and C. albicans, both commensals, are also the 2nd and 4th most common cause of bloodstream infections, respectively [54, 79]. Of the various sites of S. aureus and C. albicans co-colonization, the oronasopharynx serves as the reservoir for systemic infections [44]. Within the oronasopharynx, S. aureus, C. albicans, and HSV occupy two distinct geographic niches. In hosts with dentition, the oral mucosa is shared by HSV and C. albicans, while the anterior nasal nares are occupied by S. aureus [21]. Clinically, S. aureus is only rarely isolated from oral-pharyngeal specimens when normal tissue is present, despite in vitro findings that S. aureus adheres to buccal epithelial cells [22, 51]. Interestingly, in the presence of dentures, an abiotic surface, S. aureus forms a robust biofilm on the denture surface along with C. albicans [29, 63]. Little is known concerning genital tract co-colonization niches beyond the clinical findings that S. aureus infection is associated with genital inflammation, discharge, and dyspareunia, while C. albicans and HSV produce mucosal lesions similar to those observed in the oral cavity [25, 39,
genital tract, it is a near certainty that S. aureus and/or S. aureus this study was to determine whether HSV-1 or HSV-2 affect 53]. Using a HeLa cell model of virus infection, the focus of permanent resident of infection sites [5]. This permanent, albeit intermittent, presence of HSV virions may play a role in regulating the host microbiome. This could be accomplished via an alteration in available cell surface receptors for adherence by other members of the microbiome. [6, 8–10, 12, 16, 19, 21, 53]. Using a HeLa cell model of virus infection, the focus of this study was to determine whether HSV-1 or HSV-2 affect S. aureus and/or C. albicans germ tube and yeast form adherence, the initial step in biofilm formation.

Methods

Microbial Strains and Handling

Recombinant spread-deficient, entry proficient strains of HSV-1(KOS) gL86 and HSV-2 (KOS) 333gJ encoding a beta-galactosidase reporter activity were used [3, 37, 68, 74]. Both virus strains enter and replicate, thus have the potential to induce cell signaling, but lack the genes essential for viral cell-to-cell spread. All virus used in this study were taken from a single lot. Virus stocks were maintained at −80 °C until use. HSV entry into HeLa cells was confirmed by o-nitrophenyl-β-d-galactopyranoside (ONPG) and 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-Gal) assays, as previously described [52]. HeLa 229 cells were maintained and cultivated at 37 °C, 5 % CO2 in 1× Dulbecco’s modified Eagle’s medium (DMEM with 4.5 g/L glucose and l-glutamine, without sodium pyruvate; Mediatech) supplemented with 10 % heat-inactivated fetal bovine serum (FBS) and gentamicin (50 μg/ml). C. albicans, maintained at −80 °C until use, was initially subcultured onto Sabourad Dextrose agar. For use, C. albicans was cultured onto Fungisel medium (37 °C; 48 h; Troy Biologies). Yeast suspensions (YF) were prepared in Hanks Balanced Salts Solution (HBSS; 105 CFU/ml final concentration; 37 °C) immediately prior to use. Germ tube forms (GT) were generated by incubation in fetal bovine serum (FBS; 3 h; 37 °C; Abs600 0.3), followed by washing in HBSS (2×; 4000 x g) and re-suspension in HBSS to 105 CFU/ml final concentration.

Staphylococcus aureus ATCC 25923, maintained at −80 °C until use, was subcultured onto mannitol salts medium (37 °C; 18 h) for use. S. aureus suspensions were prepared immediately prior to use in HBSS (105 CFU/ml final concentration; 37 °C).

Polymicrobial Adherence Assay

The number of HeLa cell-associated S. aureus and C. albicans was determined as an indicator of biofilm initiation (adherence). HeLa 229 were grown overnight in 96-well (4×104 cells/well) at 37 °C, 5 % CO2 to reach 85 % final confluence. Before infection with virus, the cells were washed with 1× Opti-MEM with HEPES, sodium bicarbonate and l-glutamine (Gibco). Virus (HSV-1 (KOS) gL86 or HSV-2 (KOS) 333gJ−) was added to HeLa 299 cells at a multiplicity of infection (MOI) of 50 and 10 for 3 h at 37 °C, 5 % CO2. After viral infection, the cells were washed once each with PBS then HBSS before incubation with C. albicans YF or GT with and without S. aureus (5:1 target to cell ratio; n = 16). After incubation (30 min; 37 °C; 5 % CO2), HeLa cell monolayers were washed to remove unbound microbes (PBSx3) and lysed (RIPA, Life Technologies, 1:50 dilution; filter sterilized). The cell lysate (50 μl) was spread plated onto mannitol salts and/or Fungisel agar to select for S. aureus and C. albicans, respectively. Controls consisted of HSV-uninfected HeLa cells handled as described for virus-infected HeLa cells. For each experiment, a separate control plate to confirm the viral MOI was performed.

Imaging studies of HSV-1- and HSV-2-infected (MOI 50; 3 h; 37 °C) HeLa cell monolayers (5×104 cells/cover slip) were performed by both fluorescent microscopy and bright field microscopy. Cell monolayers were incubated (30 min; 37 °C; 5 % CO2) with S. aureus and C. albicans (5:1 target to cell). HeLa cells were then washed free of non-adherent microbes (PBSx3) and fixed (methanol). For fluorescent microscopy, monolayers were stained with FITC-conjugated Herpes Simplex Virus Type 1+2 gD antibody (Abcam), and 4',6-diamidino-2-phenylindole (DAPI; Life Technologies), then examined by epi-fluorescent microscopy. Cells that were signal positive for HSV or Candida or S. aureus (100 individual cells per microbe signal per coverslip) were secondarily scanned for the presence of additional microbe co-localization signals (1000× initial magnification; Nikon). For bright field microscopy, cell monolayers were stained with Gram’s crystal violet (Troy Biologies), then examined by light microscopy. Cells that were positive for Candida or S. aureus (100 individual cells per microbe signal per coverslip) were secondarily scanned for the presence of additional microbe co-localization signals (1000× initial magnification).
Statistical Analysis

Each adherence experiment was conducted twice in octuplicate. Each imaging study was conducted twice in triplicate. Data were evaluated by analysis of variance (ANOVA; GraphPad InStat 3.10 for Windows, GraphPad Software Inc.). Mean values were considered significantly different at \( P < 0.05 \).

Results

*Candida*, HSV-1 and HSV-2 Modulation of *S. aureus* Adherence to HeLa Cells

Since HSV-1 and HSV-2 co-localize in the oropharynx with *C. albicans*, we first determined their effect alone, and in concert, on *S. aureus* adherence to virus-infected HeLa cells as compared to *S. aureus* adherence to virus-free HeLa cells. Both HSV-1- and HSV-2-affected *S. aureus* adherence to HeLa cells (Fig. 1a, b). At the highest virus concentration (MOI 50) the level of *S. aureus* adhered to uninfected HeLa cells (24.2 CFU/well ± 3.19; \( n = 16 \)) was significantly higher (2.7-fold; \( P < 0.05 \)) than that measured for *S. aureus* adherence to HSV-1-infected HeLa cells (9.1 ± 1.1 CFU/well). Staphylococcal adherence to uninfected HeLa cells was also significantly (1.7-fold; \( P < 0.05 \)) higher than that measured for adherence to HSV-2 (MOI 50)-infected HeLa cells. The level of *S. aureus* adherence to HSV-2-infected cells at a fivefold lower MOI (MOI 10) was similar to that measured for the HSV-2 MOI 50 (1.9-fold below virus-free control).

Co-incubation of *S. aureus* with *C. albicans* germ tube (GT) forms restored the level of *S. aureus* adherence to HSV-1 and HSV-2-infected HeLa cells to that measured for uninfected HeLa cell controls. Similar to the effect of GT forms, *S. aureus* adherence to HSV-2-infected cells in the presence of *C. albicans* yeast forms (YF) resulted in staphylococcal adherence levels similar to that measured uninfected HeLa cells. In contrast, the presence of YF did not affect the HSV-1 mediated depression in *S. aureus* adherence. These results show that HSV viral entry is antagonist to *S. aureus* adherence, an antagonism that can be, in part, reversed by *C. albicans* GT and YF.

*C. albicans* Adherence to HSV-1- and HSV-2-Infected HeLa Cells in the Presence and Absence of *S. aureus*

In these assays, we determined whether *C. albicans* GT and YF adherence to HeLa cells was affected by HSV and *S. aureus* (Fig. 1c–f). Our findings showed that HSV-1 and HSV-2 significantly (\( P < 0.05 \)) enhanced *C. albicans* GT and YF adherence in a virus dose-dependent manner; maximal adherence levels of GT and YF occurred at an MOI of 50. For both candidal phenotypes, adherence was significantly higher (\( P < 0.05 \)) than that measured for adherence to virus-uninfected HeLa cell controls. *Candida* GT adherence to HSV-2-infected HeLa cells (normalized to virus-uninfected HeLa cell controls) was significantly (\( P < 0.05 \)) higher than its adherence to HSV-1-infected HeLa cells (3.6-fold vs. 1.9-fold higher, respectively). The reverse pattern was observed for YF adherence. YF significantly (\( P < 0.05 \)) and preferentially adhered to HSV-1-infected cells (2.7-fold over uninfected control) as compared to YF adherence to HSV-2-infected cells (1.9-fold over uninfected control).

The HSV-mediated enhancement of YF and GT adherence was significantly (\( P < 0.05 \)) and preferentially affected by the presence of *S. aureus*. *S. aureus* decreased GT adherence to HSV-2-infected cells to levels measured for uninfected HeLa cell control. In contrast, *S. aureus* significantly (\( P < 0.05 \)) decreased YF adherence to HSV-1-infected HeLa cells. *S. aureus* had no significant effect on YF adherence to HSV-2-infected cells or GT adherence to HSV-1-infected HeLa cells. Taken together, these results indicate that HSV promotes the association of *C. albicans* in a phenotypic-specific manner. Furthermore, these findings indicate that *S. aureus’* effect on *C. albicans* adherence was HSV-type specific with regard to fungal phenotype.

Microscopic Evaluation of *C. albicans* and *S. aureus* Adherence Pattern to HSV-1- and HSV-2-Infected HeLa Cells

We visually examined the association of *S. aureus* and *C. albicans* with HSV-1- and HSV-2-infected HeLa cells using the size, morphology, and arrangement differences between *S. aureus* and *C. albicans* to distinguish between the organisms. In the absence of HSV-1 or HSV-2, *S. aureus* and *C. albicans* (GT and YF) co-localized onto uninfected HeLa control cells (Fig. 2a). In contrast, no co-localization of staphylococci with *C. albicans* was observed on HSV-1- or HSV-2-infected HeLa cells (Fig. 2b–d). Using fluorescent microscopy with FITC-conjugated anti-HSV-gD monoclonal antibody, we further confirmed that *S. aureus* (Fig. 3b, c) did not co-localize with *C. albicans*, HSV-1 or HSV-2 (Fig. 3a, a1, 3d). However, *Candida* co-localized with HSV-1 and HSV-2 (Fig. 3a1, c). *S. aureus* and *C. albicans* were not observed microscopically to interact with one another (data not shown). Furthermore, the co-localization adherence pattern of *S. aureus* and *Candida*, in the absence of HSV, indicates a lack of physical interference with each other’s adherence.
These findings further suggest a depletion of available *S. aureus* adherence receptors subsequent to HSV cell entry.

**Discussion**

In this report, we describe an in vitro model system to study the polymicrobial interactions which occur at the initiating step in biofilm formation, i.e., adherence. The use of HeLa cells in this model system presents a unique advantage in the study of microbial interactions regarding cell surface receptors as they lack surface expression of fibronectin [4, 13, 27]. This absence of surface fibronectin more closely mimics that observed in colonization and natural infection, since the apical surface of mucosal epithelia normally lacks fibronectin [18, 33, 38, 49, 66]. This absence of fibronectin also allows for the study of alternative mechanisms by which *S. aureus* and *C. albicans*...
adhere to HSV-infected cells since both microbes adhere to fibronectin [2, 4, 13, 40, 69]. This model system for the study of virus effects on bacterial and fungal adherence was further enhanced through the use of entry and replication proficient, but non-spreading strains of HSV-1 and HSV-2 [3, 74].

HSV enters cells via endocytosis [56, 57]. Endocytosis of the virus-receptor complex results in a dynamic display of cell surface receptors available to S. aureus and C. albicans for their adherence. Interestingly there is an opposing interaction of S. aureus and C. albicans with heparan sulfates on the mammalian cell surface. S. aureus adherence to various sulfonated heparans, particularly a heparan component syndecan-1, is important in its interaction with epithelial cells [31, 36, 42, 47, 61, 73]. In contrast, the presence of cell surface heparan sulfates block C. albicans adherence to extracellular matrix proteins, e.g., laminin, and types I and IV collagen [43]. HSV cell entry causes a species specific (HSV-1 vs. HSV-2) differential depletion of heparan sulfates, and induction of heparanase secretion, which further depletes cell surface sulfonated heparans [1, 14, 20, 28, 30, 35, 41, 45, 56, 57, 70, 75]. Both these events seem critical in our observations. For instance, loss of S. aureus heparan sulfate receptors could explain the lack of its adherence to HeLa cells infected with either HSV-1 or HSV-2 (Fig. 4). Conversely, Candida preferred receptors would be unmasked by heparanase production and HSV entry depletion of surface heparan sulfates. The HSV-1 vs. HSV-2 differential depletion of sulfonated heparan receptors could explain our findings of enhanced adherence to virus-infected cells that was Candida phenotype (YF-GT) dependent (Fig. 4) [45].

Although this model begins to address the patterns measured for S. aureus and C. albicans adherence to HSV-infected cells, a secondary mechanism is likely responsible for the effects of S. aureus and C. albicans on each other’s adherence in the presence of both uninfected and HSV-infected (50 % HeLa cells infected/well, MOI 50) cells.
HSV-infected cells release interferons alpha and gamma, which in turn cause perturbation of lipid rafts altering membrane fluidity (hydrophobicity) and the cell cytoskeletal complex in non-virally infected cells [24, 34, 60, 64, 72]. Both S. aureus and C. albicans interact with hydrophobic surfaces [2, 21, 32]. The secondary extrinsic HSV-mediated effects on uninfected cells, i.e., surface hydrophobicity changes, could result in increased S. aureus and C. albicans adherence and the apparent restoration of cell binding. The lack of cell-to-cell spread of the virus in this model system will allow for determination of which virally mediated cell alteration, i.e., intrinsic, extrinsic, or a combination of changes, is responsible for the differential adherence by S. aureus and C. albicans [2, 21].

The finding herein that HSV-1 and HSV-2 renders cells refractory to S. aureus adherence, while enhancing phenotype specific C. albicans adherence, as well as predilection for differential fungal phenotype adherence mediated by HSV-1 (yeast form) vs. HSV-2 (germ tube form) begin to explain host colonization sites and may play a role directing maintenance of the candidal commensal (YF) versus pathogenic state (GT) in vivo, e.g., HSV-2 may impact the incidence of vaginal candidiasis. These positive interactions between HSV and C. albicans may also provide an environment more conducive to HSV survival. Recently it has been shown that HSV-1 becomes embedded in the fungal biofilm where it remains viable and protected from antiviral agents, as well host factors, e.g., antibodies [50]. This protection of HSV from host factors is augmented by Candida’s ability to downregulate host proinflammatory cytokine secretion, which would be detrimental to HSV replication [17, 23, 26, 76]. HSV-1 in turn has been shown to protect Candida by down-regulating antifungal immune response [15]. Both HSV and Candida would benefit from the exclusion of S. aureus from their neighborhood. S. aureus is a proinflammatory pathogen [80]. In addition to the induction of various cytokines, including interferon gamma, the chronic presence of S. aureus elicits a leukocytic infiltrate comprised neutrophils, T cells and natural killer cells [62, 78]. Such a

![Fig. 3](image-url) Lack of co-localization of S. aureus with C. albicans on HSV-1- or HSV-2-infected HeLa cells. HSV-infected HeLa cell monolayers challenge with S. aureus and C. albicans (5:1 target to cell) stained (FITC-conjugated anti-HSV gD antibody, and DAPI). Pictures are representative of findings from screening of cells that were signal positive for HSV then scanned for Candida and S. aureus (100 individual cells per microbe signal per coverslip) that were then secondarily scanned for the presence of additional microbe co-localization signals (×1000 initial magnification; Nikon). a—a4 C. albicans (a2 insert; DAPI staining) co-localize with HSV-1; b—b3 C. albicans co-localized with HSV-2 (b1 insert, C. albicans DAPI staining); mean ± SEM
proinflammatory response would be detrimental to both HSV and candidal survival.

To our knowledge this is the first report demonstrating the ability of HSV to regulate the adherence of multiple opportunistic pathogens that are part of the inter-kingdom microbiome. This study demonstrates that HSV-1 and HSV-2 modulate both fungal and bacterial adherence to cells, likely, in part, through HSV alteration of heparan sulfate cell surface display. These findings represent a paradigm shift from the current view that host factors solely control microbiome population members, to one where a life-long latent viral pathogen could co-opt the host by a specific molecular mechanism that alters biofilm initiation, thus, usurping regulatory control of microbiome membership. Further studies are needed to define the specific role the various HSV viral entry receptors play in modulation of staphylococcal and candidal adherence. By understanding the initial interactions that occur between HSV-1 and HSV-2 as permanent members of the host microbiome, and chronic colonizers, e.g., *S. aureus* and *C. albicans*, another avenue opens with regard to biofilm inhibition and eradication.

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Compliance with Ethical Standards

Conflict of interests The authors have no conflict of interests.

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