Influence of *Staphylococcus aureus* attachment to the herpes simplex virus infected cells

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**Abstract**

The purpose of this study was to investigate the response of HeLa cells to the interaction with inactivated *Staphylococcus aureus* cells and live challenge with herpes simplex virus (HSV). The results of this study are indicating that the interaction between the HeLa cells and *S. aureus* inactivated whole cells could modulate the host cell apoptosis and cytokine production, and therefore, influence the progression of HSV infection. The pre-treatment of HeLa cells with heat inactivated bacterial whole cells protects them from the occurrence of HSV mediated cytopathic effect, while the post viral infection treatment with bacterial cells prevents the high activation of bax/bcl-2 apoptotic pathway, a process that could change the fate of the infectious process triggered by the virus, and eventually reduce its multiplication rate.

**Keywords**: *Staphylococcus aureus*, herpes simplex, HeLa cells, inflammatory cytokines, apoptosis.

**Introduction**

*Staphylococcus aureus* is a significant human pathogen that causes several diseases in humans, including skin infections, scalded-skin syndrome, toxic shock syndrome, endocarditis, septic arthritis, and osteomyelitis [1]. Although staphylococci have been typically regarded as non-invasive extracellular pathogens that damage host cells after adhering to the extracellular matrix, many studies have shown the ability of *S. aureus* to trigger an endocytic process and to be ingested by non-professional phagocytes, mouse fibroblasts [2], mouse renal cells [3], bovine mammary epithelial cells [4, 5], mouse and human osteoblast cell lines [6, 7], human osteoblasts [8], chicken osteoblasts [9, 10] as well as HeLa cells [11]. Following the internalization, *S. aureus* could modulate the apoptosis of the infected cells. The implication of soluble mediators in the modulation of bacteria–host cell interaction has been intensively studied [5, 12, 13]. On the other hand, viruses could interfere with the cellular events triggered by a bacterial infection, by disrupting the sequence of programmed cellular events, and triggering programmed cell death in the infected cells either intrinsically or by mediators of the host immune response [14]. The herpes viruses are highly disseminated in nature and highly pathogenic for humans causing infections inducing dermatologic, immunologic, or neurologic disorders [15], especially in immunocompromised individuals. Herpes viruses multiply best in metabolically active cells [16]. However, viruses have evolved some mechanisms to block endogenously or exogenously induced apoptosis if this process threatens the capacity of the cell to produce the required number and quality of infectious virus progeny [14]. Therefore, apoptosis during HSV infection represents an important virus-host interaction process, which likely influences viral pathogenesis [17]. The acute and recurrent phases of HSV infection are associated with strong cytokine responses [18]. Although wound infections produced by *S. aureus* are often complicated by other bacteria or virus co-infection, the mechanisms by which a viral infection affecting the epithelial cells can influence *S. aureus* cutaneous opportunistic infections have been poorly studied.

The purpose of this study was to investigate the consequences of the direct contact interaction between the HeLa cells and *S. aureus* inactivated whole cells upon the host cell apoptosis and cytokine production, in the presence of HSV infection.

**Materials and Methods**

The bacterial strain used in our experiments was one
S. aureus isolate from a wound secretion. In order to obtain heat inactivated bacterial bodies, the bacterial strain was grown in 10 mL of DMEM:F12 (Dulbecco’s modified Eagle’s medium:nutrient mixture F12) supplemented with 2% fetal bovine serum (FBS) for 18 hours. The obtained culture was centrifuged and the pellet was washed once with phosphate-buffered saline (PBS), resuspended in 10 mL of PBS, heat-inactivated for 20 minutes, centrifuged at 6000×g and resuspended again in 10 mL of DMEM:F12.

**Experimental models**

In the first experimental model, HeLa cells (ATCC CCL2) monolayers grown in 35 mm Petri dishes were cultured for 24 hours in the presence of heat-inactivated bacterial cells (1:1 in DMEM:F12 supplemented with 2% FBS). After 24 hours, the HeLa cells monolayer was washed with PBS and inoculated with KOS HSV-1 at a multiplicity of infection (MOI) of 0.01 for 30 minutes at 37°C. Cells were then washed and incubated in DMEM with 2% FBS at 37°C with 5% CO₂. The experiment was evaluated 24 hours after the viral infection.

In the second experimental model, HeLa cells monolayers were first infected with KOS HSV-1 at a MOI of 0.01, incubated in DMEM with 2% FBS at 37°C with 5% CO₂ for 24 hours, and then the heat-inactivated bacterial cells were added. The experiment was evaluated 24 hours after the bacterial treatment.

In order to establish a baseline for results interpretation, HeLa cells infected with HSV at a low MOI (0.01) without any bacterial treatments were used as controls.

**Immunofluorescence HSV detection**

Herpes simplex virus was detected using the Light Diagnostics™ HSV 1/2 Typing DFA Kit (Cat. No. 3291, Millipore, USA) according to the manufacturer’s protocol. Cover slips were mounted onto glass slides with 1:1 glycerol: HBSS (Hanks’ balanced salt solution) and examined with a Nikon Eclipse E600 Microscope with fluorescence module, using a FITC (Fluorescein Isothiocyanate) filter set. A bright apple-green fluorescence in the cytoplasm and/or cell membrane of the infected epithelial cells indicated the HSV presence, while the absence of fluorescent green and presence of a dull red color due to the Evans blue counterstaining indicated the lack of infection.

**Quantitative RT-PCR for apoptosis markers and cytokines assay**

Total RNA was extracted with Trizol Reagent (Invitrogen, USA) according to the manufacturer’s protocol, and thereafter 2 μg of total RNA, from each sample, was used for reverse transcription with High Capacity cDNA Reverse Transcription Kit with RNAse inhibitor (Applied Biosystems). 50 ng of cDNA was subjected to real time PCR (polymerase chain reaction) for human IL-1, IL-6, IL-8, TNF-α, Bcl2, and Bax, using pre-validated Taqman Gene Expression Assays kits (Applied Biosystems). Human β actin was used as endogenous control. Each experiment was performed three times. The ΔΔCT method was used to compare the relative expression levels.

**Results**

**The effect of heat inactivated bacterial bodies on HSV infected HeLa cells**

When HeLa cells were pretreated with heat inactivated bacterial bodies before HSV infection, the cytopathic effect was not observed, although the HSV antigen could be detected by using an immunodetection assay. By contrast, when the bacterial bodies were applied 24 hours post-viral infection, an intense cytopathic effect was observed, similar as magnitude to that observed on HeLa cells infected with HSV for 48 hours. The cytopathic effect figured as small or large syncytia, with alterations in the cell nuclei and cytoplasmic tails resulted from fusion of the cellular membranes harboring viral glycoproteins. The cytopathic effect was confirmed using the HSV antigen immunodetection (Figure 1).

**The modulation of the host cell apoptosis by the physical interaction between the HeLa cells, heat inactivated S. aureus and HSV**

Bax gene expression was significantly increased in the presence of bacterial bodies, but the attachment of bacterial bodies to the cells fails to inhibit the expression of anti-apoptotic genes, accounting for cellular activation.

The qRT-PCR assay clearly showed that the viral infection is triggering a more intensive apoptotic effect (as revealed by the up-regulation of the pro-apoptotic bax gene expression simultaneously with the drastic inhibition of the anti-apoptotic bcl-2 gene), than the bacterial bodies.

The prior contact of HeLa cells with the heat inactivated bacterial bodies followed by virus addition induced only a decrease in bax expression compared to the stimulated control, meaning that these cells are partially protected. If the virus acts first, antiapoptotic gene expression decreases, with an increase of pro-/anti-apoptotic effects ratio, a phenomenon related to the presence of extensive cytopathic effects (Figure 2).

**Analysis of some cytokines gene expression in HeLa infected cells**

In our experiment, the HSV1 virus increases the expression of tumor necrosis factor alpha (TNF-α), independently to the presence of bacterial bodies. In the presence of concomitant viral infection and bacterial bodies, expression of IL-1 and IL-8 gradually increases from 24 hours to 48 hours accounting for a more pronounced pro-inflammatory effect. IL-6 expression is inhibited by virus, but increased by the bacterial bodies. Post-treatment of HeLa cells with bacterial bodies after the viral infection also increases the IL-6 mRNA level. The addition of bacterial bodies before the viral infection is generally inducing a strong increase in the expression of TNF-α, IL-6 and IL-8 (Figure 3).

**Discussion**

Epithelial cells are responsible for prevention of invasive infections through the mechanical barrier and also, the initiation of local immune responses. The defense reactions of mammalian cells are triggered or modulated following the interaction between the bacterial components
and the epithelial cells receptors. This may activate various intracellular signaling pathways implicated in the generation of antimicrobial compounds [19] or in the production of pro-inflammatory cytokines [20].

Our aim was to study the effects of the direct contact interaction between the HeLa cells and S. aureus inactivated whole cells upon the host cell apoptosis and cytokine production, when a simultaneous viral infection with HSV is occurring.

Both viral and bacterial pathogens have a machinery to modulate the host cells signaling pathways upon infection. One of the major components of microbial pathogenesis is to modulate the apoptosis of the infected host cells at critical time points. The level of apoptosis in HeLa cells infected with HSV is very low, probably due to the manipulation of the host cell for viral multiplication, needing metabolically active cells. However, when bacterial bodies are added at 24 hours after infection, the anti-apoptotic genes expression is decreased, demonstrating that the administration of bacterial cell wall associated antigens following a previous viral infection, could change the fate of the infectious process triggered by the virus, and eventually reduce its multiplication rate.

One of the major pathways, by which the pathogens activate the host cell, is the modulation of the cytokine profile. Different inflammatory mediators are expressed at different times after pathogen exposure. IL-1 and TNF-α, the most important pro-inflammatory cytokines are locally produced by many cell types and are responsible for early responses triggering an inflammatory cascade [21]. The pro-inflammatory cytokine, IL-6, is one of the key mediators of the “acute-phase response” in inflammation [22]. IL-8 is acting both as a chemo-attractant and activator of polymorphonuclear leukocytes and is produced by intestinal and vaginal epithelial cells consecutively to bacterial aggression, demonstrating that epithelial cells act as a link between the bacterial antigens and the specific and nonspecific host immune cells, found in the mucosa-associated lymphoid tissue [23]. The synthesis of cytokines at the site of infection is chronologically coordinated and is decisive for an effective inflammatory response.

Figure 1 – HSV antigen immunodetection: (A) HeLa cells treated 24 hours with heated bacterial bodies followed by HSV infection for another 24 hours; (B) HeLa cells infected with HSV for 24 hours, followed by treatment with heated bacterial bodies for another 24 hours.

Figure 2 – Graphic representation of quantitative expression of bax and bcl-2 genes in HeLa cells. MV24/48: Cell control for viral infection at 24/48 hours; MBF24/48: Control of heated bacterial cells fractions at 24/48 hours; V0B24BF: Viral infection before adding bacterial fraction; B0V24BF: Bacterial infection before HSV infection.

Figure 3 – Graphic representation of quantitative expression of IL-1b, IL-6, IL-8 and TNF genes in HeLa cells. HELA cell control; MV24/48: Cell control for viral infection at 24/48 hours; MBF24/48: Control of heated bacterial cells fractions at 24/48 hours; V0B24BF: Viral infection before adding bacterial fraction; B0V24BF: Bacterial infection before HSV infection.
response in order to return to the normal state [24, 25]. The chronic inflammation and/or infection are produced by disturbances in the well-balanced order and extent of the inflammatory response [25].

The efficient clearance of the viral infection is dependent on the occurrence of a pro-inflammatory reaction and of a type 1 immune response, characterized by the activation of immune cells (i.e., macrophages, NK and cytotoxic T-cells). However, the epithelium represents the first line of host defense. After the viral infection, the epithelial cells produce pro-inflammatory cytokines, which attract and activate phagocytes at site of infection. HSV could suppress the pro-inflammatory cytokines expression by decreasing the mRNA stability, and therefore interfering with the antiviral host response [26]. The epithelial cells produce pro-inflammatory cytokines, interfering with the antiviral host response [26]. The treatment of HeLa cells with bacterial bodies 24 hours before the viral infection is increasing the expression level of TNF-α, IL-6 and IL-8 pro-inflammatory cytokines genes. The bacterial antigens are also increasing the level of IL-1 in the treated cells, accounting for the occurrence of a more pronounced pro-inflammatory effect, possibly favoring in vitro a more rapid and efficient elimination of the viral infection.

Previous studies have shown that the level of IL-6 in eukaryotic cells infected by S. aureus is influenced by any change occurred in the adherence phase of this microbial cell to the eukaryotic cell (i.e., the lack of different microbial adhesions) [27, 28]. On the other hand, Strindhall et al. (2004) showed that clinical isolates of S. aureus express a different capacity to stimulate the cytokine production by the human endothelial cells, which was not directly correlated with the adhesion capacity, indicating the implication of other bacterial components in the eukaryotic cells activation [28].

Taken together, the results of this study could suggest that both viruses and bacterial antigens may interfere with the key processes in host cell by modulating the levels of pro- and anti-inflammatory cytokines and promoting apoptosis.

Conclusions

The results of this study are demonstrating that the direct contact interaction between the HeLa cells and S. aureus inactivated whole cells could modulate the host cell apoptosis and cytokine production, and therefore, influence the progress of HSV infection. The pre-treatment with heat inactivated bacterial bodies protects HeLa cells from the occurrence of HSV mediated cytopathic effect, while the post-viral infection treatment with bacterial antigens prevents the high activation of bax/bcl-2 apoptotic pathway, a process that could change the fate of the infectious process triggered by the virus, and eventually reduce its multiplication rate. In our study, the infection of HeLa cells with HSV in the presence of inactivated bacterial bodies is triggering the synthesis of the pro-inflammatory cytokines TNF-α, IL-6 and IL-8, whose levels are generally increasing in time, suggesting that bacterial antigens could contribute to the decrease of viral multiplication rate.

Conflict of interests

The authors declare that they have no conflict of interests.

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