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HSV-1 Enters Melanocytes and Induces their Apoptosis

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Abstract

Herpes simplex virus type 1 (HSV-1) enters into the host and makes diseases. HSV-1 can infect many types of cells but epidermis is a target in which keratinocytes are the main attacked cells. Melanocytes are important components of the epidermis and have a close relationship with keratinocytes. Many studies are on "the effects of HSV-1 on keratinocytes"; less is known about the effects of HSV-1 on melanocytes. In this study, we treated murine Melan-A cell line with HSV-1 *in vitro* and draw a conclusion that HSV-1 can infect melanocytes and induce melanocytes apoptosis for the first time.

Keywords: Melanocytes; Apoptosis; Herpes simplex virus type 1; Keratinocytes

Abbreviations: HSV: Herpes Simplex Virus; rEGFP-HSV-1: recombinant Enhanced Green Fluorescent Protein-tagged Herpes Simplex Virus-1; MOI: Multiplicity of Infection; HBV: Hepatitis B Virus; FBS: Fetal Bovine Serum; OD: Optical Density; ANOVA: Analysis of Variance

Introduction

Vitiligo is a depigmenting disorder that does not cause much physical impairment, but patients have disturbances in mental health and quality of life. About 1% of world's people have vitiligo. Depigmenting disorder is due to the destruction of melanocytes in which apoptosis is the most important reason.

Over the past few decades, some factors destroying melanocytes in the apoptotic process have been determined, such as melanocyte autoantibodies cytotoxic CD8+ T cells [1], serum antibodies to melanocyte-specific antigens [2] and 4-tertiary butyl phenol [3]. In recent years, studies have focused on the effects of the virus on vitiligo.

Soylu et al. [4] and Zhu et al. [5] suggest that hepatitis B virus (HBV) is related to vitiligo; Shukla [6] and Grimes et al. [7] discovered Cytomegalovirus DNA in skin biopsy specimens of patients with vitiligo, suggesting that vitiligo may be triggered by Cytomegalovirus infection; Erf et al. [8] suggested a strong causative link between turkey herpes virus infection and Smyth line chicken vitiligo which is an animal model to study human vitiligo; Adiloglu et al. [9] detected higher rate of serum anti-human herpes virus 6 IgG in vitiligo patients than in the controls, suggesting a tentative role of HSV 6 infection in vitiligo.

Among all the virus mentioned related to vitiligo, only HSV mainly attacks the skin, coincidentally, vitiligo is a skin disorder, therefore, the pathogenic role of HSV is really susceptible.

HSV, an important human pathogen, belongs to herpes virus family. Latent infection and periodical relapse are the characteristics of the virus. The virus is divided into 2 types: HSV-1 and HSV-2. Skin is the main target which HSV attacks and keratinocytes are an entry site for HSV.

Petermann et al. [10] suggested that HSV-1 enter into keratinocytes depending on the presence of nectin-1 and herpes virus entry mediator (HVEM) on keratinocytes surface. The modes of HSV entry are both by direct fusion with the plasma membrane and via endocytic vesicles. HSV establishes a primary infection in the epithelium, which is followed by latent infection of neurons, maintaining the ability to reactive [11].

Under some stimulus, such as pressure, poor immunity and depression, HSV-1 can be activated and infect epithelial tissue along the nerve. HSV may suppress the apoptotic pathway and thus block apoptosis of sensory neurons [12]. However, one of the most important host cell defenses against HSV infection is programmed cell death, an attempt of self-sacrifice to prevent the spread of infection [13]. Indeed, HSV-1 is cytopathic to keratinocytes [14], also induces apoptosis of ocular cells [15] and immature dendritic cells [16].

On the basis of the foregoing, we hypothesized that HSV can infect melanocytes and induces melanocytes apoptosis. To test this hypothesis we treated murine Melan-A cell line with HSV-1and measured the proliferation and apoptosis of the cells.

Materials and Methods

Cell cultures

Murine melanocytes Melan-A cell line was provided by Yuxiao Hong first affiliated hospital of China Medical University, China. Cells were grown in Roswell Park Memorial Institute medium modified (RPMI; Hyclone, USA) supplemented with 10% Fetal Bovine Serum (FBS), penicillin (100 U ml^{-1}), streptomycin (100 μg ml^{-1}) and cholera toxin (CT, Sigma-Aldrich, 200ppm L^{-1}) in a humidified 10% CO_2 atmosphere at 37°C. The cell densities were maintained at between 0.25×10^6 cell mL^{-1} and 0.5×10^6 cell mL^{-1}. Cell viability and density were monitored using a hemocytometer and trypan blue staining, and viability was maintained at >90%.

Virus and infections

rEGFP-HSV-1, a recombinant virus of HSV-1gene and enhanced green fluorescent protein gene, was purchased from Beijing Five Plus Molecular Medicine Institute. The titer was 1×10^7 TU ml^{-1}.

Observing virus infection using confocal microscope

Add one sterile cover slip into each well of the prepared six-well plate. The logarithmic phase of Melan-A cells was added at the concentration of 5×10^4 ml^{-1}, 2 ml $well^{-1}$. Add rEGFP-HSV-1 at the MOI of 3 after cultured for 24 h when the cells attached on the coverslip. The cells were continued to culture for another 24 h before taking the coverslip out. Put the coverslip under the confocal microscope and observe the position of green fluorescence protein.

Infection efficiency assay

Melan-A cells were cultured in 6-wells plates at the concentration of 5×10^4 ml^{-1}, 2 ml $well^{-1}$. Add rEGFP-HSV-1 at the MOI of 3, 7, 15, 30 after 24 h when the cells adherent. Continued to culture the cells for another 24 h. Then the cells were harvested. After washing with cold 1x PBS twice, cells were resuspended in cold 1x PBS. We detected infection efficiency by measuring the density of green fluorescent protein in the cells using a flow cytometer (CALIBUR, BD and the United States).

Proliferation assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay

Melan-A cells were added into a 96-well plate at the concentration of 1.5×10^4 ml^{-1}, 100 µl $well^{-1}$. Add rEGFP-HSV-1 at the MOI of 1, 2, 3, 4, 5, 6, 7 after cultured for 24 h when the cells adherent. The cells were continued to culture and measured the OD value using MTT assay every 8 h for 48 h [17].

MTT assay

20 µl of MTT solution (5 mg ml^{-1}) was added to each well and the cells were cultured for another 4 h. Culture medium was then removed and 150 µl of DMSO was added to each well. After shaking the culture plates for 5 min, the optical density (OD) was measured using a spectrophotometer (ND-1000, Thermo Fisher, United States) at a wavelength of 570 nm. The cell viability rate (%) was calculated as (OD treated/OD control) × 100%.

Observing cytomorphology of infected cells

Melan-A cells were cultured in a culture dish (60 mm) at the concentration of 5×10^4 ml^{-1}, 5 ml per culture dish. Add rEGFP-HSV-1 at the MOI of 1, 2 and 3 after cultured for 24 h when the cells attached.

The cells were continued to culture for 48 h and counted up the percentage of normal cells, shrinkage cells, and death cells respectively, using inverted microscope. Selected 3 different areas to take photos and calculated the mean value ± SEM.

Apoptosis assay

Melan-A cells were cultured in 6-well plates at 1×10^5 cells $well^{-1}$, 2 ml $well^{-1}$. Add rEGFP-HSV-1 at the MOI of 1, 2 and 3 after cultured for 24 h when the cells attached. The cells were continued to culture for 48 h to measure the percentage of the apoptotic cells.

After collecting the cells and washing 3 times using cold 1x PBS, added 200 µl 1x binding buffer (50 mmol L^{-1} HEPES, 700 mmol L^{-1} NaCl, 12.5 mmol L^{-1} $CaCl_2$, pH 7.4) to resuspend the cells.

Added 5 µl Annexin-V-APC, mixing up before incubated at room temperature for 15 min in dark. Added 5 µl Propidium Iodide Staining Solution (PI) and 300 µl 1x binding buffer, incubated for another 5 min. Analyzed the apoptotic cells using flow cytometry.

Statistical analysis

All assays were repeated 3 times. Data are presented as mean ± SEM and were tested for normality and equal variance. One-way analysis of variance (ANOVA) was applied by using SPSS 20.0 software (SPSS Inc., United States). P-values less than 0.05 were considered statistically significant. The tests were one-sided; there are no adjustments for multiple comparisons. The data met the assumptions of the tests.

Results and Discussion

HSV-1 infects Melan-A cells

To determine whether HSV-1 can infect Melan-A cells, we treated Melan–A cells with HSV-1 at the MOI of 3. As indicated in Figure 1A, the green fluorescent protein was detected in the cells after co-culturing for 24 h, suggesting that HSV-1 entered the cells and replicated. For detecting the infection efficiency, we treated Melan-A cells with different MO of HSV-1 and measured using flow cytometry. The result showed that the infection efficiency was in a titer-dependent manner (Figures 1B and 1C).

HSV-1 inhibits the proliferation of Melan-A cells

Higher MOI of HSV-1 contributed to higher infection efficiency. In accordance with these, higher MOI greatly reduced the proliferation of Melan-A cells. In this section, we observed that HSV-1 inhibited the proliferation of Melan-A cells compared to the control in a titer-dependent manner (Figure 2), while the inhibition of growth was significant after 8 h.

HSV-1 induces apoptosis of Melan-A cells

In this section, we observed the morphology of Melan-A cells infected with HSV-1 under an inverted microscope and analyzed apoptotic cells by flow cytometry. Normal Melan-A cells got confluent after being cultured for 24 h, with obvious dendrites, clear cell nuclear and cytoplasm as in Figure 3A(a).

Cells infected by HSV-1, deformed in shape and dendrites disappearing as in Figure 3A(b), even death Figures 3A(c) and 3A(d).The high titer of HSV-1, the more cells death. Figures 3A(b)-3A(d) are pictures of cytomorphology of Melan-A cells treated by HSV-1 at the MOI of 1, 2, 3. Figure 3A(a) is control. We observed cytomorphology of infected cells under inverted microscope and calculated the percentage of normal, shrinkage, and death cells (Figure 3B). The percentage of apoptotic cells increased with the increasing titer of HSV-1. (Figures 3C and 3D), suggesting that HSV-1 can induce Melan-A cells apoptosis by titer-depended manner.

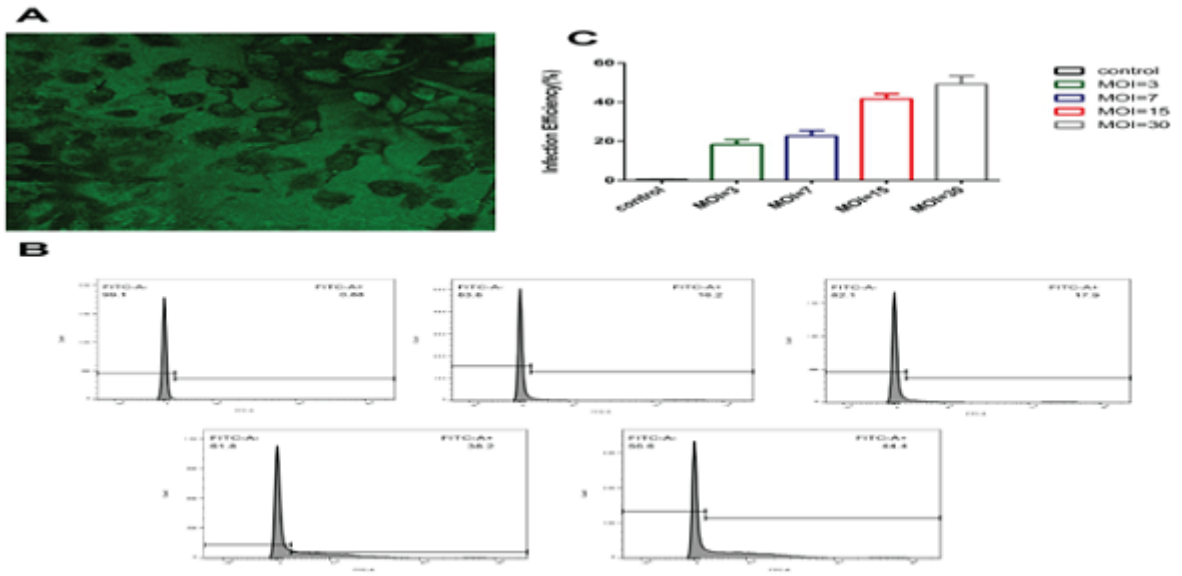

Figure 1: The virus entered the cells and the infection efficiency was increased with increasing titer. (A): Put one sterile coverslip into each well of prepared six-well plate. Melan-A cells were added at the concentration of 5×10^4 ml^{-1}, 2 ml $well^{-1}$. Added rEGFP-HSV-1 at the MOI of 3 after cultured for 24 h later when the cells attached on the coverslip. The cells were continued to culture for another 24 h before taking the coverslip out. Put the coverslip under the confocal microscope and observed the position of green fluorescence. The site of the arrow (↓) is a fluorescent protein. (B) and (C): Melan-A cells were cultured with rEGFP-HSV-1 at the MOI of 3, 7, 15, 30. Melan-A cells were cultured in 6-wells plates at the concentration of $5x10^4$/ml. Add rEGFP-HSV-1 at the MOI of 3, 7, 15, 30 after 24 h later when the cells attached. Continue to culture the cells for another 24 h. Then detected the efficiency of virus infection utilizing green fluorescence. (B, a) is control, (B, b), (B, c), (B, d), (B, e) are tested samples: MOI=3, 7, 15, 30, respectively. All the experiments were repeated 3 times and one-way analysis of variance (ANOVA) was applied by using SPSS 20.0 software. $P<0.05$ was considered statistically significant.

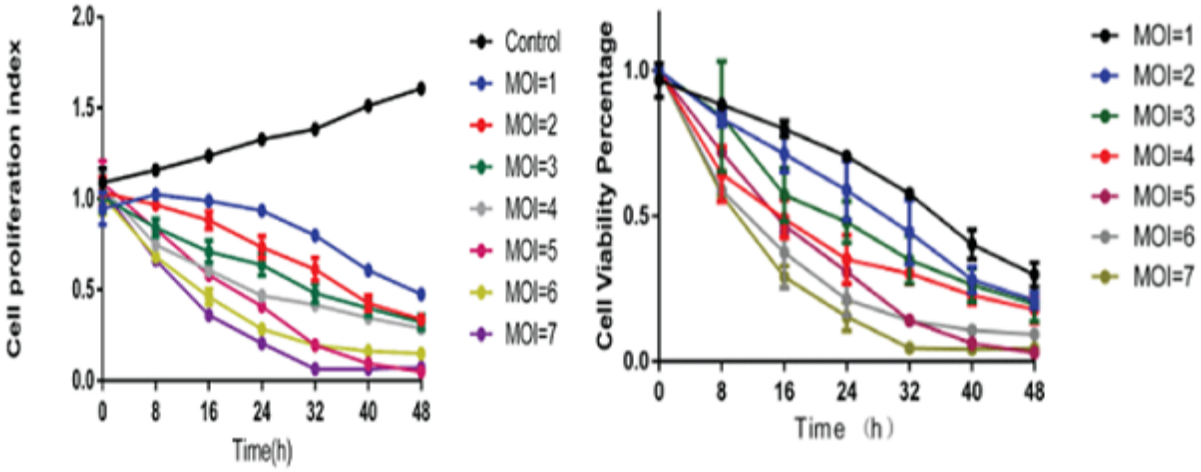

Figure 2: HSV-1 inhibits Melan-A cells proliferation. Melan-A cells were added into 96-well plate at the concentration of 1.5×10^4/ml, 100 μl/well. Add rEGFP-HSV-1 at the MOI of 1, 2, 3, 4, 5, 6, 7 after cultured for 24 h when the cells attached. The cells were continued to culture and measured the OD value using MTT assay every 8 h for 48 h; wavelength of 570 nm was used. Figure 2a is cell proliferation; 2b is cell viability percentage figure. All the experiments were repeated 3 times, one-way analysis of variance (ANOVA) was applied by using SPSS 20.0 software. $P<0.05$ was considered statistically significant.

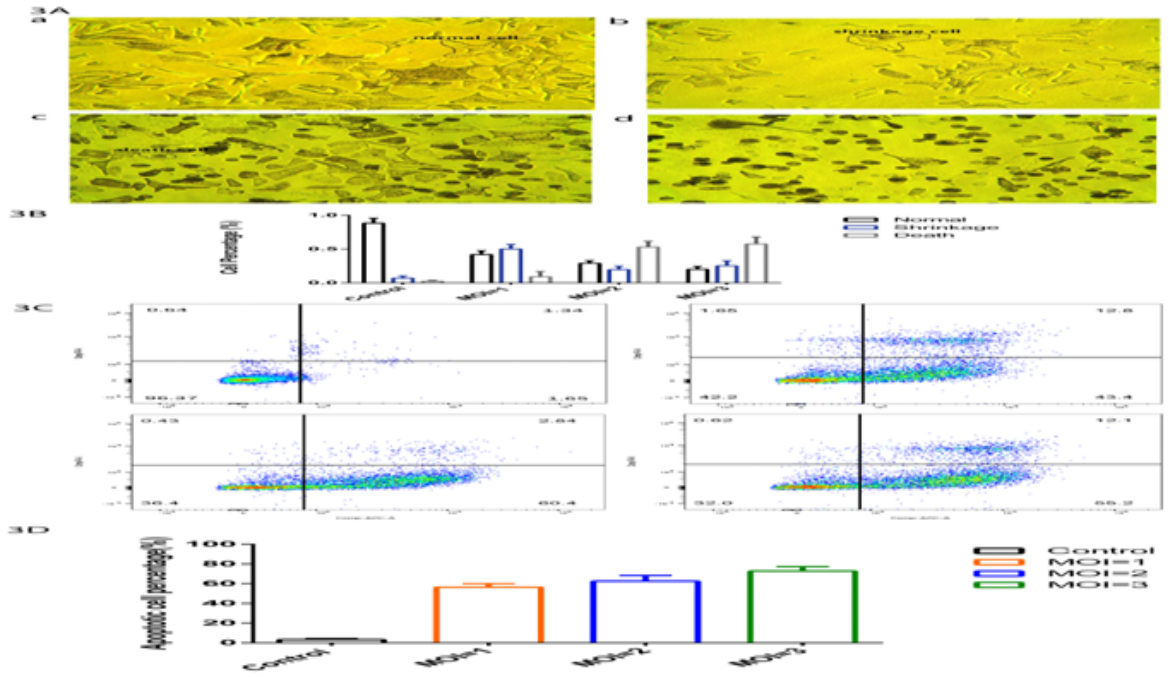

Figure 3: HSV-1 induces Melan-A cells apoptosis. (A), (B): Melan-A cells were cultured in culture dish (60 mm) at the concentration of 5 × 10^4/ml, 5 ml per culture dish. Add rEGFP-HSV-1 at the MOI of 1, 2, 3 after cultured for 24 h later when the cells attached. The cells were continued to culture for 48 h and counted up the percentage of normal cells, shrinkaged cells and death cells using microscope. Selected 3 different areas to take photos and calculated the mean value ± SEM. (A, a) normal cells; (A, b): infected cells (MOI=1); (A, c): infected cells (MOI=2); (A,d): infected cells (MOI=3). (C), (D): Melan-A cells were cultured in culture dish (60 mm) at the concentration of 5 × 10^4/ml, 5 ml per culture dish. Add rEGFP-HSV-1 at the MOI of 1, 2, 3 after cultured for 24 h later when the cells attached. The cells were continued to culture for 48 h to detect the percentage of the apoptotic cells. Collected the cells and washed 3 times using cold 1× PBS, added 200 μl 1x binding buffer to resuspend the cells, added 5 μl Annexin-V-APC, mixing up before incubated for 15 min at room temperature in dark. Added 5 μl Propidium Iodide Staining Solution (PI) and 300 μl 1x binding buffer, incubated for another 5 min, detected the apoptotic cells using flow cytometry. All the experiments were repeated 3 times and one-way analysis of variance (ANOVA) was applied by using SPSS 20.0 software. $P<0.05$ was considered statistically significant.

Discussion

HSV-1 is a common human pathogen mainly attacking epidermal cells and neurons. HSV-1 has 2 kinds of infection mechanisms: one is a lytic infection and the other is latent infection [18]. The lytic infection has been detected when infecting epidermal cells such as keratinocytes [14] and melanoma [19]. But the virus can't replicate without host cell materials, so it evolves a mechanism which inhibits cells apoptosis by expressing latent-associated transcription, and it is called latent infection which is common when infecting neurons [20]. However, virus infection is detrimental to cells, so many infected cells clear virus by apoptosis. Even in latent infection, host cells also express pro-apoptotic regulators Bcl2l11 [21]. The relationship between herpes virus and cell apoptosis has been studied before. Besides HSV, it shows that bovine herpesvirus type 1 (one kind of herpes virus) [22] could also induce cells apoptosis.

Melanocytes and keratinocytes are the main components of human epidermal cells; melanocytes connect with keratinocytes through synapse and work together. Although HSV-1 infects epidermis mainly via keratinocytes and many researches are on the topic of "HSV-1 and keratinocytes", less is known about the effects of HSV-1 on melanocytes. In this study, we infected murine Melan-A cell line with HSV-1 *in vitro*, observing the cytomorphology and comparing the proliferation and apoptosis with controls. In section 1, we first observed that HSV-1 can enter Melan-A cells (Figure 1A) and infection efficiency was dose-dependent. Interestingly, the increasing rate of infection efficiency was decreased as HSV-1 concentration increasing (Figure 1C), suggesting that HSV-1 enters Melan-A cells via specific receptor as the same method as keratinocytes [10]. But if the specific receptor is nectin-1, HVEM or some others? It needs further study. In section 2, we detected the proliferation of Melan-A cells treated by HSV-1. The proliferation of treated cells inhibited compared to the controls (Figure 2). We can conclude that the cells whether treated with HSV-1 from Figure 2A. In order to find out whether the cells death is due to apoptosis or cell necrosis, we carried out the third experiment. In this section, we infected Melan-A cells with HSV-1 at the MOI of 1, 2, 3 and detected apoptosis at 48 h. It showed that the death of Melan-A cells was due to apoptosis and the apoptosis rate was dose-dependent. But which apoptosis signaling pathway was triggered by HSV-1 needs further study. Our results coincide with the conclusion that HSV-1 induces cells apoptosis and filled the gap of limited knowledge on the types of cells which can be infected by HSV-1. Our findings suggest that HSV-1 can enter melanocytes and induce

melanocytes apoptosis, so HSV-1 infection might be a trigger of vitiligo.

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Authors' Contribution

Xing-Hua Gao conceived and designed the experiments; Jing Lan performed the experiments and analyzed the data; Yuxiao Hong, Ruiqun Qi and Song Zheng contributed materials tools; Xing-Hua Gao and Jing Lan wrote the paper. All authors read and approved the final manuscript.

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