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Research Paper

Preliminary study on Herpes simplex virus type 1 infection of human oral epithelial cell *in vitro*

Jie Zhao, Weibin Sun*, Juan Wang

Institute of Stomatology, Nanjing Medical University, Nanjing 210029, Jiangsu Province, China

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Abstract

Objective: To explore the functions and mechanisms of herpes simplex virus type 1(HSV-1) while infecting human oral epithelial cells in vitro(being similar to the infection in vivo). **Methods**:An abundance of HSV-1 strains amplified in Vero cells were used to infect human oral epithelial cells. The culture supernatant was collected to infect Vero cells again. Morphology of HSV-1 was identified by inverted microscope and transmission electron microscope. Nucleic acid of the virus was detected by PCR. **Results**:The infected human oral epithelial cells didn't display an obvious cytopathic effect(CPE) under inverted microscope(while Vero cells which were infected by the culture supernatant showed typical(CPE). The virus particles were not observed in the cytoplasm nor in nucleus of human oral epithelial cells, however under transmission electron microscope in the cytoplasm of Vero cells, the nucleic acid of HSV-1 could be detected in infected human oral epithelial cells, by PCR. **Conclusion**:HSV-1 can successfully infect human oral epithelial cells. This model may provide a useful approach for studying the pathogenesis of herpes virus-associated periodontal disease.

Key words: herpes simplex virus type 1; human oral epithelial cells; transmission electron microscope; polymerase chain reaction

INTRODUCTION

Periodontal disease, one of the most common human oral diseases in human race, is a kind of chronic infectious diseases which is caused by the invasion of microorganism and resulting in the destruction of periodontal tissue[1]. During the past 100 years, the majority of researchers considered that bacterial plaque was the most important etiological factor of periodontal disease. Since the mid 1990s, many studies have indicated that herpes simplex virus(HSV) is often detected in periodontitis sites, especially herpes simplex virus type 1(HSV-1)[2-5]. These results indicated that HSV-1 may play a significant role in the development of periodontal disease. Some researchers presumed that the damage to the epithelium barrier of the host may be one of the most important pathogenesis of HSV-1-associated periodontal disease. However, the mechanism has rarely been researched owing to a lack of a model where HSV can infect human oral epithelial cells in vitro directly. In this study, we abundantly amplified HSV-1 strains in Vero cells which were used to infect human oral epithelial cells, and established a model that HSV-1 could infect human oral epithelial cells in vitro. The present study provides a basis of experimental technique for the further study of the pathogenesis of herpesvirus-associated periodontal disease.

MATERIALS AND METHODS

Materials

RPMI 1640, DMEM, calf serum(Gibco, USA); PCR kit(Promega, USA);DNA Marker Ⅲ(TIANGEN biotech CO, China); agarose(Oxoid, British); protease K(Sigma, USA).

Culture of Vero cells, abundance amplification of HSV-1 and determination of the titer

Vero cells were cultured in Dulbecco's Modified Eagle's medium supplemented with 10% fetal bovine serum and 100 U/ml penicillin-streptomycin-

*Corresponding author
E-mail address: wbsun@njmu.edu.cn

gentamycin, and subcultured every 3 to 4 days by 0.25% trypsin supplemented with 0.02% EDTA. HSV-1 strains were abundantly amplified in Vero cells and viral titer was detected by plaque forming experiment[6]. The titer of HSV-1 was standardized to 10^6 PFU/ml.

HSV-1 infects KB cells

KB cells were cultured in RPMI1640 supplemented and subcultured every 2 days by 0.25% trypsin. The protocol that HSV-1 infected KB cells was as below: KB cells were subcultured in a 6-well plate at 37℃ for the night. After cells confluensed to 70%-80% on the second day, a sufficient quantity of HSV-1 was incubated on KB monolayer according to the titer of HSV-1 and the quantity of HSV-1 needed for per cell. The 6-well plate was placed in CO_2 incubator for 2 h so as to make the virus well-distributed, and shaken every 15-30 min. The unabsorbed viruses were removed after washing by PBS twice. Then 1.5 ml maintenance medium was added to the cultures per well. Maintenance medium was supplemented with 2% fetal bovine serum and other components as the growth promoting medium. We replaced the medium every one or two days, keeping the volume of medium 1.5 ml per well all the time.

This experiment was divided into four groups: Three groups were according to the titer of HSV-1,:0.5PFU/cell,1.0 PFU/cell, 3.0 PFU/cell. At the same time, the culture supernatant of normal Vero cells instead of HSV-1 was collected as the blank control.

Transfer infection of HSV-1 to Vero cells

After HSV-1 infecting KB cells for 48 h, the culture supernatant of infected groups and the blank control group were collected. All supernatant were stored at -70℃. Vero cells were subcultured in a 24-well plate in an atmosphere of a CO_2 incubator for the night. After cells confluensed to 70%-80% on the second day, the collected culture supernatant of different titers of HSV-1 (0.5 PFU/cell, 1.0 P FU/cell, 3.0 PFU/cell) infected KB cells were incubated on Vero monolayer. 8 wells of Vero cells were chosen to infect for every titer of HSV-1. The plate was placed in a CO_2 incubator after sloshing gently. After 2 h virus adsorption, the culture supernatant was removed and cells were washed by PBS twice. Lastly, a 0.5 ml maintenance medium was added to the cultures per well and it was incubated continually in a CO_2 incubator until the end of the experiment. Meanwhile, we collected the culture supernatant of KB cells of blank control as in the above control.

Determination of CPE Ratio

We observed the morphology of cells through inverted microscope for HSV-1 transfer infected Vero cells for 48 h, and scored CPE ratio of infected Vero cells in the three infected groups. The defining standards are shown below:if there was no CPE recorded-, if CPE ratio < 25% recorded +, if CPE ratio between 25% and 50% recorded ++, if CPE ratio >50% recorded +++.

Morphological observation of infected cells

Inverted microscope observation: The morphology of the cells was inspected and observed for microscopically detectable alterations through inverted microscope for HSV-1 infected KB cells for 0 h, 24 h and 48 h respectively and HSV-1 transfer infected Vero cells for 0 h, 24 h, 48 h respectively. TEM observation: Infected KB cells and Vero cells 48 h were digested, centrifuged, and rinsed twice by PBS, fixed by 2.5% glutaraldehyde at 4℃, dehydrated through a graded ethanol series and embedded in epoxy resins. Ultrathin slices were double stained with uranyl acetate and lead citrate, and then observed through TEM(JEM1010).

Detection of HSV-1 DNA using PCR

After HSV-1 infecting KB cells for 48 h, cells were rinsed twice with precooling PBS and collected into Apendoff's tubes. Protease K was added to the cell clumps, mixed sufficiently and incubated at 55℃ for the night. Then cells were denaturized at 94℃ for 5 minutes and centrifuged in 12 000 r/min for 5 min. Now, the supernatant fluid was the genome DNA of KB cells. The amplification by polymerase chain reaction(PCR) used the genome DNA of KB cells as PCR template and special sequences of HSV-1 as primers. The primer sequences of HSV-1 for PCR amplification were designed according to the glycoprotein D gene order of HSV-1 recorded in GenBank. The primers were as follows:upstream primer 5′-TGGGGTCCGCAGCAAATATGCCTTG-3′,downstream primer 5′-GGTGCGCTGGTTCTCGGGGATGAAG-3′. The size of amplification fragment was 709 bp. Primers were synthesized by Shanghai Biocolor Bioscience & Technology CO. At the same time, negative control and positive controls were established: The negative control was the culture supernatant of normal Vero cell and positive control was HSV-1. The amplification protocol comprised of an initial period of denaturation at 94℃ for 5 min 35 cycles of denaturation at 94℃ for 1 min, annealing at 65℃ for 1 min, extension at 72℃ for 1 min, and a final period of extension at 72℃ for 8 min. The PCR products were detected through 1% agarose gel electrophoresis, identified by ultraviolet transilluminator, and photographs were taken by gel imaging system.

Statistical analysis

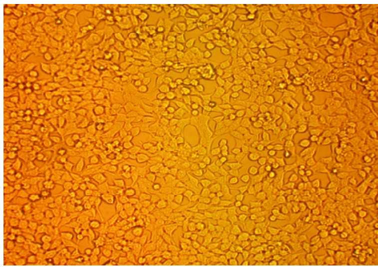
Statistical analysis was performed using SPSS13.0 software. Statistical tests were considered significantly

when their type I error was less than 0.05. The Kruskal-Wallis test and Median test were used.

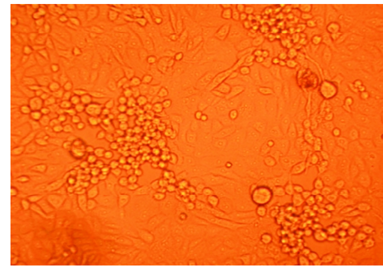
RESULTS

Inverted microscope observation

Cell morphology was observed by inverted light microscope for HSV-1 infected KB cells and HSV-1 transfer infected Vero cells for 0 h, 24 h and 48 h respectively. The infected KB cells in three different titer groups didn't display obvious cytopathic effect (CPE) under inverted microscope at either 24 h or 48 h. The infected KB cells were cerioid in as good condition as were the uninfected ones. Meanwhile, Vero cells infected by the culture supernatant of infected KB cells showed typical CPE. CPE could be detected at 24 h i.p, including vacuolisation in the cytoplasm, rounding and distention of the cells and aggregation clustering. Confluent cells and giant cells could be also observed. The amount of CPE was increased at 48h i.p(Fig 1). As control, Vero cells which cultured with the culture supernatant of KB cell in blank control didn't display obvious cytopathic effect(CPE) at neither 24 h nor 48 h.



HSV-1 infected KB cells for 48 h. The infected KB cells didn't display obvious cytopathic effect(CPE) under inverted microscope.



The culture supernatant infected Vero cells for 48 h. Typical CPE could be detected in Vero cells which infected by the culture supernatant of infected KB cells. Confluent cells and giant cells could be also observed.

Fig 1 Morphologic observation of infected cells under inverted microscope(× 100).

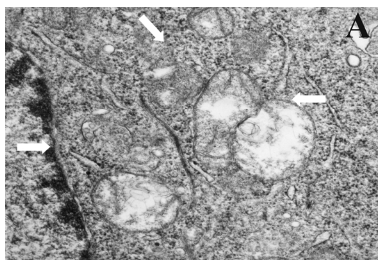
Ultrastructure observation through TEM

The cellular membrane and perinuclear membrane of the HSV-1-infected KB cells had intact double-units membrane structure. The cytoplasm was densely packed with numerous organelles. The golgi apparatus, endoplasmic reticulum, and ribosomes were copious. The nuclei contained chromatin material distributed in a diffuse pattern. Some mitochondria were markedly swollen with fragmentation or even vanishing of the cristae and showed vacuolar and myelonic degeneration. There were no mature virus particles in endochylema or nucleus (Fig 2A).

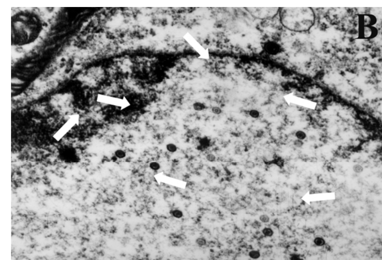
In contrast to the normal Vero cells, the infected cells became rounded and shrunk. Many organelles were swollen: mitochondria and endoplasmic reticulum we found ballooning dilatation, the size and the quantity of cytolysosome increased. The nuclear chromatin of the HSV-1-infected Vero cells was often enriched and clumped at the periphery of the nuclear membrane. Some other changes also appeared in some cells such as: conjugation of cells, the appearance of multinucleated giant cells and vacuoles in endochylema, numerous immature virus particles were prominent within the nucleus (Fig 2B).

Determination of CPE Ratio

All the data figures of CPE Ratio (of the transfer infected Vero cells) in three experimental groups were collected and two non-parameter tests were used for statistical analysis. Results of Kruskal-Wallis test were χ^2=12.958, P = 0.002 < 0.05;results of Median test were χ^2=12.686, P = 0.002 < 0.05. Results of these two non-parameter



The appearance of HSV-1-infected KB cells. Pupiform and vacuolate degeneration of mitochondria could be defected in infected KB cells.



The appearance of transfer infected Vero cells. Numerous immature virus particles were detected in the nucleus of transfer infected Vero cells.

Fig 2 Below refers Virus particles that were noted on the surface of infected cells(TEM, × 20 000)

tests revealed significant difference of CPE ratio of infected Vero cells in three different groups, that is, the CPE ratio of infected Vero cells was in relation to the titer of the virus when HSV-1 infected KB cells(Tab 1, Fig 3).

Tab 1 Statistical analysis of CPE Ratio of infected Vero cells in the three groups

Titer	-(%)	+(%)	++(%)	+++(%)	*n*	Mean rank
0.5 PFU/cell	1(12.5)	3(37.5)	4(40.0)	0(0.0)	8	6.44
1.0 PFU/cell	0(0.0)	1(12.5)	4(50.0)	3(37.5)	8	12.75
3.0 PFU/cell	0(0.0)	0(0.0)	1(12.5)	7(87.5)	8	18.31

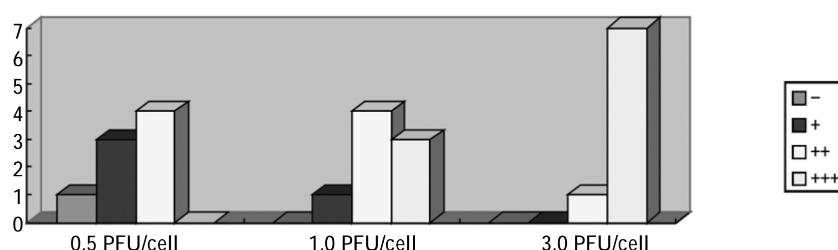
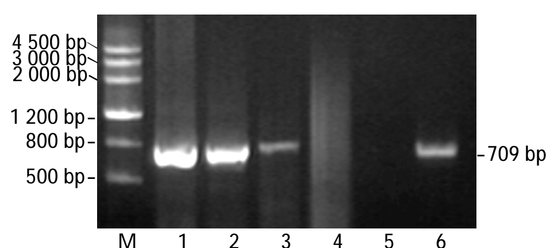


Fig 3 CPE Ratio of infected Vero cells in the three groups

Detection of HSV-1 DNA using PCR

The genome DNA of KB cells in three experimental groups infected by three different titers(0.5 PFU/cell, 1.0 PFU/cell,3.0 PFU/cell) of HSV-1 were amplified by PCR, and the PCR products were detected through 1% agarose gel electrophoresis. The PCR products of the glycoprotein D gene order of HSV-1 were visualized at the expected location(709 bp) on agarose gels after comparing with the DNA marker. The length of the PCR products was the same as predicted previously. Statistical analysis indicated that the expression of HSV-1 gene fragments could be detected in all of the three experimental groups and in the positive control. These results testified that specific gene fragments of HSV-1 were included in human oral epithelial cells which were infected by HSV-1 in vitro. On the contrary, there was no HSV-1 gene fragment in uninfected KB cells, as no signal was detected in KB cells of blank control group (Fig 4).



M:DNA Marker; 1:KB cell infected with 3.0 PFU of HSV-1 virus/cell; 2:KB cell infected with 1.0 PFU of HSV-1 virus/cell; 3:KB cell infected with 0.5 PFU of HSV-1 virus/cell; 4:Normal KB cell; 5:Negative control; 6:Positive control.

Fig 4 Detection of HSV-1 DNA content in infected human oral epithelial cells and controls

DISCUSSION

Herpes virus is a family of enveloped double-stranded DNA viruses, with genome lengths of 120-250 kbp. Human herpes viruses are classified into three groups based upon their biological characters: α-herpes viruses [as herpes simplex virus(HSV)], β-herpes viruses(as human cytomegalovirus) and γ-herpes viruses(as Epstein-Barr virus). Herpes simplex viruses are divided into two types: herpes simplex virus type 1(HSV-1) and herpes simplex virus type 2 (HSV-2)[7-8]. Between the two types, HSV-1 has a closer relationship with oral infection. Human-being is the only natural host of HSV-1. Since the mid 1990s, the relationship between herpes viruses and periodontal disease has been studied, due to of the fast development of molecular biological technique. Parra et al[2]. investigated the presence of HSV in gingival crevicular fluid samples from 30 patients with advanced periodontitis and 26 subjects with gingivitis using the polymerase chain reaction technique. The result showed that HSVs were detected in 20% of the periodontitis patients, but none of the gingivitis subjects showed a positive HSV identification. Contreras et al. determined the presence of HSV in biopsies of periodontitis lesions from 20 adults using the nested polymerase chain reaction method[3]. Kamma et al[4] examined the occurrence of HSV in subgingival plaque samples collected from 16 early-onset periodontitis patients during the maintenance phase of therapy. HSV was detected in 34.5% of active periodontitis sites and in 9.4% of stable sites[4]. Contreras et al[5]. used type-specific polymerase chain reaction to detect the presence of HSV-1 and HSV-2 in periodontal pocket samples from 26 patients who had previously been revealed to

have periodontal HSV. HSV-1 was detected in all 26 periodontal pocket specimens and HSV-2 was not detected[5]. These results indicated that HSV-1 maybe plays a significant role in the development of periodontal disease.

Though many studies demonstrated certain associations have been established with the infection of herpes virus and the induction and development of periodontal disease, the etiopathogenesis of periodontal disease doesn's seem to be understood well at present. Slots reported herpes viruses may cause periodontal pathosis as a consequence of virally induced impairment of the periodontal immune defense, resulting in heightened virulence of resident bacterial pathogens[9].

It is known that epithelium barrier is one of the most important defense mechanisms of periodontal tissue. Some researchers presumed that damage to the epithelium barrier of the host maybe imply one of the most important pathogenesis of HSV-1-associated periodontal disease. However, the mechanism has rarely been researched for lack of the model that HSV infects human oral epithelial cells in vitro directly. So establishing the model that HSV-1 infect human oral epithelial cell in vitro is not only helpful to the study of the pathogenesis of herpes virus-associated periodontal disease, but also a guide to choose effective medicine during medical therapy clinically. KB cells have been widely used as an in vitro experimental model of the epithelium, including a model of bacterial infection of the epithelium[6,10-11].

The process of virus infecting cells is divided into four stages: adhesion, entry, uncoating and activation and regulation of genome. After that, different viruses have distinct results: Many viruses were secreted out of the host cells as intact virus after virus subunits assembly. Some viruses produced progeny defective virus through the interaction with cells, leading to persistent infection clinically. There were still some viruses that were able to transfer their foreign genes into chromosome of host cells and steadily express them.

After the DNA virus infected the host cells successfully, host cells were divided into two groups according to their property: permissive cells and non-permissive cells. If the virus could replicate after infecting host cells and cause host cells death, this cell is called permissive cell and this kind of infection is a lytic infection. A permissive cell is usually the natural host of the virus. If replication can't occur completely, this cell is called a non-permissive cell and this kind of infection is know as a transforming infection. In this case, host cells can survive. This study found that the HSV-1-infected KB cells didn't display obvious cytopathic effect (CPE) under inverted microscope. Under TEM, the organelles in the cytoplasm of infected KB cells we didn' t found any obvious abnormity, besides that some mitochondria were markedly swollen, showing vacuolar and myelonic degeneration. There were no mature virus particles in either endochylema or nucleus. These results manifested that KB cells are the non-permissive cell of HSV-1. HSV-1 infects KB cells leading to transforming infection or virus incubated in the cells causing latent infection. Meanwhile, Vero cells infected by the culture supernatant of infected KB cells showed typical CPE through inverted microscope. Under TEM, numerous virus particles were noted in the infected Vero cells. Also, the PCR results showed that the glycoprotein D gene order of HSV-1 can be detected in KB cells which were infected by HSV-1 in vitro. These results presented that the latent viruses were activated and produced infectious viral particles. All of these indicated that HSV-1 could successfully infect human oral epithelial cells in vitro and express its gene in the cells. HSV-1 could transfer its DNA genes or fragment of its genome into chromosome of KB cells and steadily express them in progeny virus after cell division.

To make a difference to bacteria infecting cells, adhesion and invasion of a virus into host cells must have specific virus receptors to media. The Virus receptor, combined with a certain virus specifically and media viral entry, is a component of cytoplasmic membrane of host cells. Virus receptors can be a protein, carbohydrate or a lipoid. The first step of virus infecting cells is binding of the virus to certain receptors on the surface of the cytomembrane of target cells. Different viruses match different receptors on different target cells. Some receptors exist on the surface of many cells, but some others merely exist on the surface of one kind or a few kinds of cells specifically. Some viruses can match many kinds of receptors, but some others share one kind of receptor with other virus. HSV-1, when infested in the target cells, glycoprotein C(gC) of HSV-1 first combined to glycosaminoglycan heparan sulfate which expresses on the surface of the susceptible cells through an electrostatic binding mode. In this step viruses are gathered onto the surface of the target cells. Then, glycoprotein B(gB) and glycoprotein D(gD) bond to their receptors respectively, thus promoting the adsorption of viruses and cells more steadily[12-15]. The virus receptors on the surface of the cell are multicopy. The quantity of virus receptors is important for bonding and invading of virus to target cells. Every sensitive cell contains 10^4-10^5 receptor molecule on its cytoplasmic membrane. If the quantity of virus receptor on the target cell is less than certain amount, this cell couldn't been infected by this kind of virus. The result of this study showed that KB cell could be infected by HSV-1, which indicated there were HSV-1 receptors existing on the surface of

KB cells. HSV-1 infecting KB cells caused a transforming infection or latent infection, perhaps because the quantity of HSV-1 receptors on KB cells were not enough and viral particles were unable to bind and invade cells completely

Oral mucosa, as a protective barrier, plays an important role in the process of microbes infection because it is the interface of oral exterior and interior environments. This protective barrier consists of a mechanical protective barrier and chemical protective barrier. Gingival epithelium adheres to every tooth with dentogingival junction, sealing the interface of soft tissue and tooth, like a mechanical protective barrier. Meanwhile, epithelial cell also effects as the chemical protective barrier through producing and secreting many antimicrobial peptides, chemotatic factors and cytokines. These molecules could induce the production of leukocytes, stimulating the host defense to respond, so as to maintain the balance between health and disease[16-17]. These antimicrobial peptides are parts of the innate immune system, a complex set of responses that keep microbial invaders in check and maintain the microbial ecology of the healthy mucosa[18]. According to the study results of AIDS, human herpes virus 8(HHV8-which is related to AIDS closely) except for a decreased the level of defense in oral epithelium cells and damage to the chemical protective barrier of oral mucosa after the infecting of oral epithelium cells, resulting in serious AIDS. This study confirmed that HSV-1 could infect human oral epithelial cell. However, it will be a long time as further needs research is necessary to explain the precise mechanism of how HSV-1 induced or aggravated periodontal disease through impairing periodontal affects immune defense after invading into oral epithelium cells.

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