Herpes simplex virus 2 infects human endothelial ECV304 cells and induces cell apoptosis synergistically with ox-LDL

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ABSTRACT — Virus infection has been shown to accelerate atherosclerosis. Serological studies indicate a link between the Herpes simplex virus (HSV) infection and atherosclerosis, which is initiated and progression of which is promoted by such factors as oxidized low-density lipoprotein (ox-LDL)-induced endothelial dysfunction. In order to recognize the direct role of HSV-2 in endothelial dysfunction, the present study investigated the infection of HSV-2 in endothelial ECV304 cells and the induction of cell apoptosis in the presence of ox-LDL. We firstly examined the HSV-2 infection by immunohistochemical assay for viral gB protein, quantitative PCR for viral ICP4 mRNA, or via virus growth determination. Then we investigated the regulation of HSV-2 infection on the cell viability and apoptosis, in the absence or presence of ox-LDL. In addition, we analyzed the apoptosis-associated molecules in the HSV-2-infected ECV304 cells. The results demonstrated that HSV-2 infected endothelial ECV304 cells and replicated efficiently, and the virus infection significantly reduced the cell viability and significantly induced cell apoptosis; particularly, cell viability reduction and cell apoptosis induction were aggravated by the ox-LDL presence. Moreover, the western blot assay confirmed the apoptosis induction; there was a significantly high level of released cytochrome c, activated caspase 3 and lyzed Poly (ADP-ribose) polymerase (PARP) by the activated caspase 3 in the HSV-2-infected ECV304 cells, particularly the cells subject to ox-LDL. Thus, we confirmed that HSV-2 infected endothelial ECV304 cells, induced cell apoptosis, which was aggravated by ox-LDL.

Key words: Herpes simplex virus 2 (HSV-2), ECV 304 cells, Apoptosis, Ox-LDL

INTRODUCTION

Endothelial dysfunction initiates and promotes the progression of atherosclerosis, which were caused by various factors, particularly by oxidized low-density lipoprotein (ox-LDL) (Ehara et al., 2001). Circulating ox-LDL not only promotes the production of long-term pro-inflammatory cytokines by endothelial cells or macrophages (Bekkering et al., 2014; Yurdagul et al., 2014), but also impairs platelet-derived growth factor receptor activity in smooth muscle cells (Vindis et al., 2007), and promotes oxidative stress in vivo (Itabe, 2012). Moreover, ox-LDL is recognized to directly target endothelial cells and to induce apoptosis via both mitochondrial and death-receptor (Fas/FasL) apoptotic pathways (Salvayre et al., 2002; Imanishi et al., 2002; Chen et al., 2004), to promote the activation of caspases, such as caspase-9 (Chen et al., 2004). The impaired vascular vasculium then attracts monocyte adhesion to and upregulates reactive oxygen species (ROS) generation in (Li and Mehta, 2000a; Cominacini et al., 2000) vascular endothelial cells. Therefore, the endothelial cell damage promoted by ox-LDL is a key cascade in atherosclerosis development. Over-circulating ox-LDL upregulates and binds to the lectin-like ox-LDL receptor (LOX-1), type II membrane glycoprotein belonging to the C-type lectin family (Sawamura et al., 1997), in vascular endothelial cells (Li and Mehta, 2000b). Activated LOX-1 further promotes ROS, activates p38 mitogen-activated protein kinases (MAPK) (Nishimura et al., 2004; Bai et al., 2011) and even apoptosis, by the cross talk between p38 MAPK and caspase signaling pathways (Takahashi et al., 2002; Xu et al.,...
Infections have also been shown to accelerate atherosclerosis (Roivainen et al., 2000; Lalla et al., 2003; Chatzidimitriou et al., 2012; Ravnskov and McCully, 2012). Serological studies indicate a link between the infection of human cytomegalovirus (HCMV) (Nieto et al., 1996; Smieja et al., 2003) or Herpes simplex virus (HSV) (Mendy et al., 2013) and atherosclerosis. Also, Human Immunodeficiency Virus (HIV) (Guaraldi et al., 2011; Farrugia et al., 2009), Hepatitis C Virus (HCV) (Sosner et al., 2012; Vassalle, 2012), and Hepatitis B Virus (HBV) (Ghotaslou et al., 2008) have also been indicated to promote atherosclerosis. Infectious agents such as human cytomegalovirus (HCMV) (Söderberg-Naucler, 2006) cause impairment of vessel wall intact and endothelial cell (EC) dysfunction (Epstein et al., 1999; Libby et al., 2002). HSV infection has been recognized to be a risk factor for subclinical coronary atherosclerosis in HIV-infected men. Infection with multiple herpes viruses may contribute to the increased burden of atherosclerosis (Hechter et al., 2012). Different mechanisms by which HSV may contribute to atherogenesis have been described. In vascular cells, HSV infection leads to lipid accumulation (Hajjar et al., 1986), attracts leukocytes with subsequent inflammatory damage (MacGregor et al., 1980) and even activates thrombin generation and platelet adhesion (Nicholson and Hajjar, 1999). Further studies should be performed to explore novel mechanism in the pathogenesis of atherosclerosis.

In the present study, we determined HSV-2 infection in human endothelial ECV304 cells, and then investigated the infection of the virus on the ox-LDL-induced apoptosis in the cell. This study confirmed that HSV infection deteriorates the apoptosis induced by ox-LDL, which implies that HSV infection plays a direct role in ox-LDL-induced impairment of endothelial cells.

### MATERIALS AND METHODS

**Reagents, cell cultures, and virus infection**

Ox-LDL was purchased from Biomedical Technologies Inc. (Stoughton, MA, USA) and resolved in M199 medium. Human endothelial ECV304 cell line was provided by the Cell Resource Center of the Chinese Academy of Medical Sciences (Beijing, China) and was cultured in M199 medium (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% fetal bovine serum (FBS; Tianhang Bio, Hangzhou, China), 100 U/mL penicillin (Invitrogen, Carlsbad, CA, USA) and 100 μg/mL streptomycin (Invitrogen) at 37°C under 5% CO₂. M199 medium supplemented with 2% FBS was used to maintain ECV304 cells. ECV304 cells with 90% confluence were treated with 0, 15, or 60 μg/mL ox-LDL for various hours.

The HSV-2 virus stock was tittered before use, by the real-time quantitative PCR (RT-qPCR) for ICP4 gene and by the plaque forming assay (Herold et al., 1991). The serial-diluted HSV-2 (1, 0.01 or 0.1 MOI [Multiplicity of Infection]) was used to inoculate ECV304 cells for 1 hr inoculation at 37°C and was removed, then cells were washed with pre-warmed phosphate buffer solution (PBS) for three times and were substituted with M199 medium supplemented with 2% FBS. For the virus infection assay, cells were cultured for another 24 hr and were fixed for immunohistochemical assay. For the expression assay of viral ICP4 or gB, cells were inoculated for another 0, 0.5, 1, 2, 8, 12 or 24 hr. For the replication assay, after cells were inoculated for another 0, 4, 8, 12 or 24 hr, the supernatant was tittered by plaque forming assay on Vero cells. For the cell viability or apoptosis assay, cells were inoculated for another 0, 6, 12, or 24 hr, and were subject to methyl thiazolyl tetrazoliym assay (MTT assay), to annexin V-FITC (Fluorescein isothiocyanate) apoptosis assay or to western blot assay.

**Immunohistochemical assay for HSV-2 infection**

HSV-2-infected ECV304 cells were fixed with 1% paraformaldehyde, and were inoculated with mouse monoclonal antibody against HSV-2 gB (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and then were inoculated with FITC-labeled secondary goat antibody against mouse IgG (Sino Biological, Beijing, China) and were observed under fluorescence microscope.

**RNA isolation and RT-qPCR**

Total cellular RNA from 2–5 × 10⁵ ECV304 cells was prepared with Trizol and was supplemented with RNase inhibitor cocktail (Ambion, Austin, TX, USA); reverse transcription (RT) was performed with M-MLV Reverse Transcriptase (Promega, Madison, WI, USA). Quantitative analysis of mRNA expression of Infected cell protein 4 (ICP4) and β-actin was conducted with Takara One Step RT-PCR kit (Takara, Tokyo, Japan), by LightCycler 480 (Roche Diagnostics, GmbH, Mannheim, Germany). Relative quantification was determined using the Δ∆Ct method, with β-actin used as the reference gene (Livak and Schmittgen, 2001).

**Cell viability assay**

Cell viability was evaluated by MTT assay (Invitrogen). 90% confluent ECV304 cells seeded in 96-well plates post ox-LDL treatment, HSV-2 infection or...
both, were incubated with 50 μL MTT solution for 2 hr at 37°C. After incubation, the MTT solution was discarded, and 150 μL DMSO was added to dissolve the precipitate completely at room temperature. The optical density was then measured at 570 nm using a spectrophotometer (Bio-Rad, Hercules, CA, USA). The cell viability was expressed as relative viable cells (%) to control.

**Apoptosis assays**
The percentage of cells undergoing apoptosis was determined by flow cytometry using the Annexin V-FITC Apoptosis Detection Kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer’s instructions. Briefly, approximately 2 x 10^5 ECV304 cells post ox-LDL treatment, HSV-2 infection or both, were harvested and resuspended in binding buffer. The cells were mixed with annexin V-FITC and propidium iodide (PI). After incubating for 15 min in the dark, analysis was performed by a FACScan flow cytometer (Bio-Rad) to analyze cellular apoptosis. The results were expressed as the percentage of apoptotic cells from the total cells.

**Western blot assay**
Cell extracts were prepared by a standard protocol, and proteins were detected by western blot using mouse monoclonal anti-gB antibody (Santa Cruz Biotechnology), rabbit polyclone anti-β-actin antibody (Abcam, Cambridge, UK), anti-cytochrome c (Cyt c) antibody (Abcam), anti-poly (ADP-ribose) polymerase (PARP) antibody (Cell Signaling Technology Inc., Danvers, MA, USA), or anti-caspase 3 antibody (Sino Biological, Beijing, China). Goat anti-mouse IgG or goat anti-rabbit IgG (Pierce, Rockford, IL, USA) secondary antibody conjugated to horseradish peroxidase and ECL detection systems (Super Signal West Femto, Pierce) were used for target protein band detection.

**Statistical analysis**
For the analysis of relative ICP4 mRNA expression, relative cell viability, percentage of apoptotic cells, the level of Cyt c release, the level of activated caspase 3 or the level of lyzed PARP, statistical evaluations are presented as mean ± S.E. Data were analyzed using the Student’s t test.

**RESULTS**

**HSV-2 infects endothelial cell line ECV304 cells**
ECV304 has been described as a spontaneously transformed cell line derived from human umbilical vein endothelial cells (Hughes, 1996). ECV304 cells are characteristic of endothelial cells (Suda et al., 2001; Andozia et al., 2010). To investigate whether HSV-2 infected endothelial cells, we inoculated ECV304 cells with 0.1 MOI (Multiplicity of Infection) HSV-2 viruses for 12 or 24 hr, and examined the virus infection in ECV304 cells by immunofluorescent technique with polyclonal antibodies against gB, which is the main HSV-2 membrane glycoprotein. As shown in Fig. 1A, post the virus inoculation for 12 or 24 hr, more than 50% of ECV304 cells were positive in FITC, while the FITC-positive cells were less than 5% in the control ECV304 cells (HSV-2 0 hr). Next, we examined the gB expression in HSV-2-infected ECV304 cells with western blot assay; Fig. 1B indicates a high level of gB in protein level from 8 to 24 hr post inoculation (H.P.I.). To reconfirm the HSV-2 replication in ECV304 cells, we analyzed the mRNA of ICP4, which is the product of the alpha 4 gene, and regulates herpes simplex virus 1 and herpes simplex virus 2 gene expression at the transcriptional level (Yao and Courtney, 1991; Blaho and Roizman, 1991). It was also significantly expressed in the infected cells (p < 0.01 or 0.001), compared to the control group.

To further establish the infection of ECV304 cells by HSV-2, we then determined the growth curve of HSV-2 with 0.01 or 0.001 MOI in this cell line. Firstly, 0.01 MOI was adopted to infect ECV304 cells, and supernatants of infected cells were collected at 0, 4, 8, 12 or 24 H.P.I. and were tittered by the plaque forming assay. Fig. 1D indicates that from 4 H.P.I., there was detectable HSV-2 in the supernatant (0.67 ± 0.08 lg PFU/mL), and the virus titer reached a maximum of 5.74 ± 0.71 lg PFU/mL. We then repeated the experiment with 0.001 MOI; it was found that the virus titer ranged from 1.56 ± 0.22 lg PFU/mL at 8 H.P.I. to 4.64 ± 0.78 lg PFU/mL at 24 H.P.I.. Thus, we confirmed in the present study that HSV-2 infected the human endothelial cell line ECV304 and replicated efficiently.

**Ox-LDL induces apoptosis in ECV304 cells**
HSV-2 has been recognized to induce apoptosis in such types of cells as dendritic cells (DC) (Stefanidou et al., 2013), T cells (Vanden Oever and Han, 2010), and HEp-2 cells (Han et al., 2009). To further determine whether HSV-2 infection induces apoptosis in ECV304 cells, we examined the viability and apoptosis of ECV304 cells with HSV-2 infection by a MTT assay, or by a FACSflow cytometer with annexin V-FITC apoptosis detection kit. Fig. 2A shows that the viability of HSV-2-infected cells decreased significantly at both 12 and 24 hr post inoculation (p < 0.05 respectively). Although there was no obvious morphological change of the infected ECV304 cells, the virus infection with 1 MOI induced a high lev-
Fig. 1. HSV-2 infects and forms plaques in ECV304 cells. A: Immunohistochemical assay for gB protein in ECV304 cells that were infected with 0.1 MOI HSV-2 for 12 or 24 hr; the gB was detected with anti-gB polyclone antibodies and followed FITC-labeled secondary antibody. B: Western blot assay for gB expression in HSV-2-infected ECV304 cells. C: RT-qPCR analysis of ICP4 expression in mRNA level in ECV304 cells that were infected with 0.1 MOI HSV-2 for 0, 0.5, 1 or 2 hr. D: Growth curves of HSV-2 virus in ECV304 cells with 0.01 or 0.001 MOI; virus was tittered in the HSV-2-infected ECV304 cells. All quantitative results were the average of triple experiments. Statistical significance was set as ** p < 0.01, *** p < 0.001.
el of apoptosis at 12 or 24 H.P.I. (Fig. 2B; p < 0.05 or 0.01), compared to mock cells. Therefore, HSV-2 infection reduced the viability and promoted the apoptosis of ECV304 cells.

Ox-LDL deteriorates the HSV-2-induced ECV304 cell apoptosis

Ox-LDL is recognized to directly target endothelial cells and to induce apoptosis, and thus to promote the progression of atherosclerosis. To investigate the influence of ox-LDL on the HSV-2 infection-induced apoptosis in ECV304 cells, we firstly examined the viability of ECV304 cells treated with various concentrations; Fig. 3A indicates that 60 rather than 15 μg/mL ox-LDL reduced ECV304 cell viability significantly (p < 0.05 for 12 hr post treatment (H.P.T.) or p < 0.01 for 24 H.P.T.). Next, we examined the viability of cells infected with 1 MOI HSV-2 and treated with 15 μg/mL ox-LDL, with singular HSV-2 infection, singular 15 μg/mL ox-LDL treatment, or blank treatment as control. Fig. 3B shows that the combined HSV-2 infection and 15 μg/mL ox-LDL treatment induced a more significant cell viability reduction than each singular treatment (p < 0.05 respectively for 12 hr, or p < 0.01 respectively for 24 hr). Secondly, we determined the apoptosis of the cells post above-mentioned treatment. As shown in Fig. 3C, singular 15 μg/mL ox-LDL also did not promote significant cell apoptosis at both 12 and 24 H.P.I., compared to control group (whereas 60 μg/mL ox-LDL induced significant cell apoptosis, p < 0.05 at 12 H.P.T., or p < 0.01 at 24 H.P.T.); however, the lower concentration of ox-LDL significantly induced apoptosis of ECV304 cells post HSV-2 infection (p < 0.05 for 12 H.P.T., or p < 0.01 for 24 H.P.T.). Therefore, the ox-LDL treatment aggravated the HSV-2 infection-induced apoptosis of ECV304 cells.

Combined ox-LDL treatment and HSV-2 infection induce apoptotic cascade in ECV304 cell

The role of caspases in ox-LDL-induced apoptotic cascade has been identified. ox-LDL treatment promotes a significant release of cytochrome c (Cyt c) from mitochondria, which then activates caspase-9 and caspase-3 (Chen et al., 2004). To elucidate the apoptotic cascade promoted by the combined HSV-2 infection and ox-LDL treatment, we analyzed the Cyt c release, caspase 3 activation and the lysis of PARP by caspase 3 by western blot assay. As shown in Fig. 4A, the western blot analysis demonstrated that the Cyt c release was upregulated significantly in HSV-2 infected cells (p < 0.01), rather than in ox-LDL treated cells, and the released Cyt c upregulation was more significant in cells with both ox-LDL treatment and HSV-2 infection (p < 0.01 compared to the 15 μg/mL ox-LDL treatment or p < 0.05 compared to the 1 MOI HSV-2 infection). The cleaved caspase 3 (active form of caspase 3) (Fig. 3C) and lyzed PARP (Fig. 3D) were also significantly promoted by HSV-2 infection.
Moreover, the upregulation of the two above-mentioned molecules was also aggravated by the 15 μg/mL ox-LDL treatment (p < 0.05 or p < 0.01). Thus, we demonstrated aggravation of HSV-2 infection-induced apoptosis by ox-LDL in ECV304 cells.

**DISCUSSION**

Viral agents such as herpesviruses have been implicated in the inflammatory atherosclerotic process (Sorlie et al., 1994; Prasad et al., 2002). Chronic activation of inflammation by infection is hypothesized to promote atherogenesis and thrombosis (Libby et al., 2002). A seropositivity to HSV-2, but not HSV-1 is associated with premature cardiovascular diseases (Mendy et al., 2013). And, in particular, HSV-2 was implied to be a coronary atherosclerosis risk factor in HIV-infected men (Hechter et al., 2012). The mechanisms by which HSV induces atherosclerosis are multiple-step and not limited to: potential interference with vascular cells’ lipid metabolism, increases in cytokines or growth factors by vascular and inflammatory cells, and alterations of vascular endothelium by
inhibiting anticoagulant properties, inducing prothrombotic capacities and increasing the binding of inflammatory cells and platelets (Nicholson and Hajjar, 1998; Tedgui and Mallat, 2006). However, it is unknown about direct influence of HSV infection on endothelial cell itself, there is little known about the crosstalk between the HSV infection and the key atherosclerosis contributor, ox-LDL.

In the present study, we firstly confirmed the effective infection of HSV-2 in human endothelial ECV304 cells. HSV-2 infected and replicated efficiently in ECV304 cells, and HSV-2 inoculation in ECV304 cells not only promoted the expression of viral genes in mRNA and protein levels, but also produced a high level of progeny viruses. We then found that the virus infection reduced the viability, promoted the apoptosis of ECV304 cells. Furthermore, the HSV-2-induced cell viability decrease and cell apoptosis could be aggravated by the ox-LDL treatment with a sufficiently low concentration, which induced insignificant cell viability reduction and even apoptosis in ECV304 cells. 15 μg/mL ox-LDL treatment, together with 1 MOI HSV-2 infection promoted a more signif-

Fig. 4. Western blot assay of apoptosis-associated molecules in ECV304 cells post ox-LDL treatment or (and) HSV-2 infection. A: Western blot assay of Cyt c release, cleaved caspase 3 and lyzed PRAP by caspase 3 in ECV304 cells with 15 μg/mL ox-LDL treatment or (and) 1 MOI HSV-2 infection for 24 hr; ECV304 cells without any treatment was taken as blank control. B: Percentage of released Cyt c in ECV304 cells post ox-LDL treatment or (and) HSV-2 infection. C: Percentage of cleaved caspase 3 in ECV304 cells post ox-LDL treatment or (and) HSV-2 infection. D: Percentage of lyzed PRAP in ECV304 cells post ox-LDL treatment or (and) HSV-2 infection. All results were averaged for three independent experiments. * p < 0.05, **p < 0.01, ns: no significance.
icant level of cell apoptosis, than singular ox-LDL treatment or singular HSV-2 infection. There was a synergistic effect between ox-LDL treatment and HSV-2 infection in the endothelial cell impairment. The synergistic apoptosis was reconfirmed by the western blot analysis of apoptosis-associated Cyt c release, caspase 3 activation and the PARP lysis by activated caspase 3. The present study revealed a direct impairment of ECV304 cells by HSV-2 infection; this implies an important role of HSV-2 infection in atherosclerotic lesions to endothelial cells. However, we did not elucidate the mechanism underlining the synergistic effect of HSV-2 infection and ox-LDL treatment on the endothelial cell apoptosis.

The present study casts further light on the mechanism of the injury to endothelial cells exerted by HSV infection. Besides the deregulation of lipid accumulation (Hajjar et al., 1986), leukocyte attraction, which follows subsequent inflammatory damage (MacGregor et al., 1980), and even the activation of thrombin generation and platelet adhesion (Nicholson and Hajjar, 1999), HSV-2 infection leads to endothelial cell apoptosis. Furthermore, in the present study, we found the synergism of HSV-2 infection with ox-LDL in the injury to endothelial cells.

In summary, the present study indicated that HSV-2 infected endothelial ECV304 cells and promoted cell apoptosis. Moreover, the virus infection-induced cell apoptosis was aggravated by ox-LDL treatment.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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