Role of vasodilator-stimulated phosphoprotein in human cytomegalovirus-induced hyperpermeability of human endothelial cells

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Abstract. Atherosclerosis (AS) is a common chronic vascular disease and epidemiological evidence demonstrates that infection is closely associated with the occurrence of AS, including infection by human cytomegalovirus (HCMV) and Chlamydophila pneumoniae. The aim of the present study was to investigate the effect of HCMV AD169 infection on the barrier function of human umbilical vein endothelial cells (HUVECs) and to understand the role of vasodilator-stimulated phosphoprotein (VASP) during this process. In cultured HUVEC-CRL-1730 cells, knockdown of VASP expression with small interfering (si)RNA-VASP resulted in impaired cellular barrier function. Furthermore, knockdown of Ras-related C3 botulinum toxin substrate 1 (Rac1) using siRNA-Rac1 could induce downregulation of VASP expression in HUVEC-CRL-1730 cells. Additionally, following the infection of the cells by HCMV, cellular morphological alterations could be observed under an inverted microscope, the mRNA and protein levels of Rac1 and VASP were transiently reduced, and what appeared to be a time-dependent impairment of the barrier function was observed. Finally, transfection of siRNA-VASP or siRNA-Rac1 into HCMV-infected HUVEC-CRL-1730 cells resulted in increased impairment of the cellular barrier function. Taken together, these data demonstrated that HCMV infection could induce impairment of the barrier function in monolayer HUVEC-CRL-1730 cells via interference with Rac1/VASP expression.

Introduction

Atherosclerosis (AS) is a common chronic vascular disease that may lead to cardiovascular and cerebrovascular disease, affecting the health of the individual (1). A number of epidemiological studies have revealed that AS occurrence is closely associated with infection, including infection with human cytomegalovirus (HCMV) or Chlamydophila pneumoniae (2,3). Endothelial cells (ECs) expressing the 36 kDa annexin II receptor on their surface are potential hosts of HCMV (4). Bentz et al. (5) revealed that HCMV infection of human microvascular ECs could result in a decrease in the cellular barrier function and an increase in permeability. This effect was associated with cytoskeletal rearrangements, including actin stress fiber depolymerization, and decreased expression of tight junction proteins, containing occludin and vascular endothelial-cadherin (6). Vascular endothelial impairment and barrier function dysfunction are important factors in the initiation of AS lesions; they facilitate the movement of monocytes accompanied by peroxidized lipids across the vascular endothelium, which are then deposited in the intima where monocytes absorb lipids, resulting in the formation of foam cells, which accumulate into atherosclerotic plaques (7). Therefore, elucidating the mechanism by which HCMV infection leads to the reduction of EC barrier function and promotes increased permeability through the rearrangement of the cytoskeleton may improve understanding of the process of AS formation.

Ena/vasodilator-stimulated phosphoprotein (VASP) homology (EVH) proteins are actin-associated proteins involved in a range of dynamic processes that are dependent on cytoskeletal remodeling and cellular polarity, including axon guidance and formation, lamellipodial and filopodial dynamics, platelet activation and cell migration (8). Additionally, as a main member of the EVH family, VASP was also revealed to serve a crucial role in establishing and maintaining the barrier functions of endothelial and epithelial...
cells, which are closely associated with tight junction protein 
ZO-1 (ZO-1) at tight junctions (9,10). ZO-1, which is located 
near the tightly connected EC envelope, has a molecular 
weight of 225 kDa (11,12) and contains an SH3 domain (10). 
VASP consists of three functional regions: EVH1, EVH2 and 
proline-rich regions (PRR), of which PRR can bind to an 
SH3 domain. In a previous study utilizing human umbilical 
vein endothelial cells (HUVECs), VASP was phosphorylated 
by protein kinase A and distributed to the cell-cell junction, 
while the binding between phosphorylated VASP and ZO-1 
was significantly enhanced; also, the polymerization of tight 
junctions was increased and EC permeability was significantly 
reduced (9). These data demonstrated that VASP and ZO-1 
could jointly regulate EC barrier function. However, further 
studies are required.

The Rho family of GTPases contains >20 members, of 
which transforming protein RhoA (RhoA), Ras-related C3 
obutulin toxin substrate 1 (Rac1) and cell division control 
protein 42 homolog (Cdc42) are the main players involved in 
the regulation of cell-cell connections and potential actomyosin 
networks (13,14). RhoA- and Rac1-mediated signaling path-
ways can respectively disrupt or maintain cell barrier function 
by coordinating actomyosin contractions and barrier alterations 
in various cell types (15,16). Furthermore, Rac1 and Cdc42 
activities are required to maintain barrier integrity (17,18) 
by mediating the formation of actin filaments that associate 
with proteins from junctional complexes, including ZO-1 and 
α-catenin at the cell periphery (19). In addition, Rac1 regulates 
the alterations of endothelial permeability by mediating skeletal 
protein remodeling (20). It has been demonstrated that VASP 
is a downstream effector of Rac1 in osteosarcoma cells (21). 
Therefore, Rac1-mediated VASP activation may be involved in 
maintaining the barrier function of ECs.

In the present study, HCMV-induced EC barrier dysfunc-
tion was used to investigate the role of Rac1-mediated VASP 
activation in regulating vascular permeability, which may 
contribute to elucidating the molecular mechanism underlying 
the development of AS following HCMV infection.

Materials and methods

Plasmids, small interfering (si)RNAs and antibodies. 
To generate green fluorescent protein (GFP)-VASP overexp-
ression plasmids, the VASP cDNA sequence was cloned into 
the pEGFP-C1 (304 mg/ml; Clontech Laboratories, Inc., 
Mountainview, CA, USA) multicloning site between the 
EcoRI and BamHI restriction sites. To generate flag tag protein 
(Flag)-Rac1 overexpression plasmids, the Rac1 DNA sequence 
was cloned into the pflag-CMV (285 mg/ml; Clontech 
Laboratories, Inc.) multicloning site between the EcoRI 
and BamHI restriction sites. The 21-nucleotide RNA oligos 
corresponding to human Rac1 (Genbank accession number, 
006908) were synthesized by Shanghai GenePharma Co., Ltd. 
(Shanghai, China) as follows: siRNA1 targeted nucleotides 
439-459 (sense, 5'-GGAGATTGTGTGCTGTAAAAA-3' and 
anti-sense, 5'-UUUACAGCACCAUCCUC-3'), siRNA2 
targeted nucleotides 199-217 (sense, 5'-CUAUGUGUUCUG 
ACAAUUA-3' and anti-sense, 5'-AAUUGUCAACAGACA 
GUAG-3') and siRNA3 targeted nucleotides 328-346 (sense, 
5'-CAUCCUAGGGGAACUAAA-3' and anti-sense, 5'-UUU 
AGUCCCUACUAGGAUG3'), with dTdT overhangs at each 3' 
terminus. The following RNA oligos containing 21 nucleotides 
corresponding to human VASP (Genbank accession number, 
003370) were also synthesized by Shanghai GenePharma Co., 
Ltd.: siRNA1 targeted nucleotides 942-960 (sense, 5'-CAU 
CCUGCAAGAGAATTU and anti-sense, 5'-UUCAU 
CUUOOGCAAGGUUGTG-3'), siRNA2 targeted nucleotides 
1,002-1,020 (sense, 5'-CGCCAGUCUAGUUAATT and 
anti-sense, 5'-UAUCACUUGAGUCGUUGCGT-3') and 
siRNA3 targeted nucleotides 1,024-1,042 (sense, 5'-GG 
ACUACCAGGGAGAAATT and anti-sense, 5'-UUU 
CACCCCCUGUAGUUCCGA-3'). A negative control siRNA 
(5'-AGUUCAGCAGCAGAGUUCdTdT-3') was also used in 
the experiment (Shanghai GenePharma Co., Ltd.). The 
anti-Rac1 (cat. no. 610651; dilution 1:1,000; BD Biosciences, 
Franklin Lakes, NJ, USA), anti-VASP (cat. no. 0010-10; dilu-
tion, 1:1,000; ImmunoGlobe GmbH, Himmelstadt, Germany), 
anti-GAPDH (cat. no. SC-69778; dilution, 1:5,000) and 
antibeta-tubulin (cat. no. SC-73242; dilution, 1:5,000) (both Santa 
Cruz Biotechnology, Inc., Dallas, TX, USA) antibodies were 
used in the study.

Cell culture and transfection. Two endothelial cell lines, 
the primary HUVECs and HUVEC-CRL1730, were obtained from 
the Key State Laboratory of Virology of Wuhan University 
(Wuhan, China). The cells were cultured in Dulbecco's modified 
Eagle's medium (DMEM, Hyclone; GE Healthcare, Logan, UT, 
USA) supplemented with 10% fetal bovine serum (FBS; Gibco; 
Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/ml 
penicillin G and 100 U/ml streptomycin sulfate at 37°C in 
a humidified incubator supplemented with 5% CO₂. Prior to trans-
fection, the HUVEC-CRL1530 cells were seeded at a density 
of 2x10⁵ cells/well in 6-well plates and grown overnight in 
DMEM (without FBS or antibiotics) until they reached 60-70% 
confluence. Transfection of siRNAs or plasmids was carried 
out using Lipofectamine® 2000 reagent (Invitrogen; Thermo 
Fisher Scientific, Inc.). Lipofectamine and siRNAs or plasmids 
were each diluted in DMEM. Diluted Lipofectamine was then 
mixed with diluted siRNAs or plasmids, and this mixture was 
icubated for 20 min at room temperature for complex forma-
tion. Following the addition of DMEM (2 ml/well) to the cells, 
the Lipofectamine/siRNA or Lipofectamine/plasmid mixture 
was added to each well, resulting in a final concentration of 
100 pmol siRNAs or 4 µg plasmids. At 48 h after transfection, 
the cells were collected for western blotting or incubated with 
TRIZol (Invitrogen; Thermo Fisher Scientific, Inc.) for reverse 
transcription-quantitative (RT-q) PCR analysis.

HCMV titration assay and observation of cell morphology. The 
HCMV AD169 He was obtained from the Perinatal Laboratory of 
Tongji Medical College, Huazhong University of Science 
and Technology (Wuhan, China). The HUVEC-CRL1730 cells 
were seeded at 5x10⁵ cells/well in 96-well plates and cultured 
in DMEM (supplemented with 2% FBS and antibiotics) at 
37°C in 5% CO₂. To determine the CCID₅₀/ml, serial 10-fold 
dilutions followed by serial 8-fold dilutions of the HCMV 
viral sample were made in DMEM. The following day the 
cells were inoculated a 96-well plate with the same dilution of virus, 
6 replicate wells were set up for each dilution each well 
was inoculated with 0.1 ml diluted virus solution in a 37°C, 5%
CO₂ incubator. Following HCMV infection, the cells become larger and rounder and specific ‘hawk-eye’ changes appeared, which were observed under a light microscope (Olympus IX 73 DP80, Olympus Corporation, Tokyo, Japan). Following inoculation, depending on the speed of the HUVEC-CRL1530 cell lesions, observations were continued for several days until the cytopathies did not progress. Cytopathic effect (CPE) is a specific lesion that occurs when cells are infected with a virus. The virus dilution corresponding to 50% CPE is the titer of the virus. The dilution of half of the lesions was taken as the 50% infection unit; CCID50 (where CCID50 represents the 50% cell culture infectious dose in HUVEC-CRL1530 cells).

**Endothelial monolayer cell permeability assay. In vitro cell permeability analysis was performed with fluorescein isothiocyanate (FITC)-dextran (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) as follows.** Following treatment with a time gradient HCMV (10⁻⁵ dosage) infection at 0, 4, 8, 12 and 24 h or plasmid and siRNA transfection, the HUVEC-CRL-1730 cells were seeded into the upper chambers of a Costar Transwell 24-well plate at a density of 1×10⁵ cells/cm² and initially plated with 1% gelatin (membrane diameter, 6.5 µm; pore size, 0.4 µm). Following adherence, the cells were cultured in serum-free DMEM for 24 h. The medium in the upper layer was subsequently replaced with medium containing 100 µg/ml FITC-dextran (100 µl) and the lower chamber was filled with normal medium. After incubation for 45 min, the fluorescence intensity of the sample was measured in a black 96-well plate with 100 µl sample from the upper and lower chambers. An excitation wavelength of 490 nm and an emission wavelength of 520 nm were used to measure the fluorescence in each well using a microplate reader (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The experiments were repeated three times.

**Isolation of RNA and RT-qPCR analysis.** The HUVEC-CRL-1730 cells were seeded into the 6-well plate with HCMV infection and/or plasmid and siRNA transfection and the mRNA expression was measured at 0, 4, 8, 12, 16, 24 and 48 h. Total RNA was extracted from cells using TRIzol reagent, and 2 µg total RNA was used for first-strand cDNA synthesis with a RevertAid™ First-Strand cDNA Synthesis kit (Fermentas; Thermo Fisher Scientific, Inc.) according to the manufacturer’s protocol. The semi-quantitative PCR was performed using the CFX96 Real Time PCR Detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) in the presence of SYBR Green (Thermo Fisher Scientific, Inc.). All qPCRs were run in triplicate and repeated at least three times. Relative mRNA expression was calculated using the 2⁻ΔΔCq method (22) using GAPDH as the internal reference. The primers used were as follows: VASP, sense 5'-GGAAAGTCAAGCAGGAGGAGGAGGAG-3'; Rac1, sense 5'-AGACCGAGCTGTTGAGTAAAAT-3' and antisense 5'-CAGGACTCACAAGGAGG-3'; GAPDH, sense 5'-AGGTCACACTGACAGTT-3' and antisense 5'-GCCTCAAGATCATCAGCAAT-3'. Rac1 reaction parameters were 94°C for 40 sec, 54°C for 30 sec and 72°C for 30 sec for 40 cycles. VASP reaction parameters were 94°C for 40 sec, 60°C for 30 sec and 72°C for 30 sec for 40 cycles.

**Western blotting.** The HUVEC-CRL-1730 cells were seeded into the 6-well plate treatment with HCMV infection and/or plasmid and siRNA transfection at the protein expression was measured at 0, 4, 8, 12, 16, 24 and 48 h. Following that, the cells were washed with PBS and lysed in 200 µl modified RIPA buffer (Thermo Fisher Scientific, Inc.). Protein concentration was measured with a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Inc.). Equal amounts of total protein (10 µg/lane) were loaded, electrophoresed on 10% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with Tris-buffered saline containing 5% nonfat milk for 2 h at room temperature, then probed with antibodies against VASP (1:1,000 dilution), Rac1 (1:1,000 dilution) and GAPDH at 4°C overnight. The membranes were then incubated with horseradish peroxidase-linked goat anti-rabbit IgG (cat. no. 16473-1-AP; 1:1,000 dilution) and anti-mouse IgG (cat. no. 10285-1-AP; 1:1,000 dilution) (both ProteinTech Group, Inc., Chicago, IL, USA) secondary antibodies for 2 h at room temperature. The bound antibodies were detected by Pierce™ ECL Western Blotting substrate (Thermo Fisher Scientific, Inc.). Quantification of band densities was performed using ImageJ software (version 1.5.0.26; National Institutes of Health, Bethesda, MD, USA). The experiments were repeated three times.

**Statistical analysis.** Data are presented as the mean ± standard error of the mean. Statistical significance between groups was tested using one-way analysis of variance followed by a Bonferroni's post-hoc test. P<0.05 was considered to indicate a statistically significant difference.

**Results**

HCMV infection induces increased EC permeability. The normal primary HUVECs appeared closely linked and flat with typical spindle shapes (Fig. 1A). After 6 passages, the morphology of primary HUVECs had changed when viewed under an inverted microscope, with an increased number of irregular protrusions decreased density and more cellular debris in the cytoplasm (Fig. 1B). After 7 days of HCMV infection, the morphology of the primary HUVEC cells was altered, with evident nuclear enlargement and cell swelling, and gradual rounding of the cells (Fig. 1C). Typical dense basophilic inclusion bodies surrounded by a halo in the nucleus and ‘owl eye’-like alterations of the nuclei were observed. The normal HUVEC-CRL-1730 cells appeared closely linked and flat with typical spindle shapes (Fig. 1D). After 10 days of HCMV infection, the HUVEC-CRL-1730 cell boundaries became blurred and numerous cells merged to form giant cells, and a number of the cells died (Fig. 1E).
Next, the effect of HCMV infection on the permeability of the two EC cell lines was studied. First, the 50% cell culture infective dose (CCID$_{50}$) of HCMV was determined to be $10^{-5}$ by a virus titration assay. The monolayer cells were infected with CCID$_{50}$ HCMV AD169 for 4, 8, 12 and 24 h. A Transwell assay was subsequently used to analyze the intercellular permeability of FITC-labeled dextran. As revealed in Fig. 1F and G, the permeability of HUVEC-CRL-1730 cells was significantly increased after HCMV infection compared with the negative control group ($P<0.05$), while no significant difference was identified in primary HUVECs. These results indicated that HCMV infection increased the permeability of monolayer HUVEC-CRL-1730 cells, leading to a decline in cell barrier function.

VASP is involved in maintaining EC permeability. VASP is involved in maintaining EC permeability in HUVEC-CRL-1730 cells via binding to ZO-1 at the cell junction (9). In order to verify whether the expression level of VASP affects the permeability of ECs, a loss-of-function assay was utilized by transfecting HUVEC-CRL-1730 cells with siRNA-VASP or the overexpression plasmid pEGFP-VASP. siRNA-VASP significantly inhibited VASP gene transcription in the cells, which resulted in the protein expression level being decreased to 85% ($P<0.05$; Fig. 2A and B) and the mRNA expression level of VASP being reduced to 79% ($P<0.05$; Fig. 2C) compared with the siRNA-NC group. On the contrary, compared with the pEGFP-C1 group, transfection with pEGFP-VASP upregulated VASP protein and mRNA expression by 72 and 68%, respectively (both $P<0.05$; Fig. 2B and C).

In addition, a Transwell assay was used to detect permeability 24 h after transfection. VASP knockdown revealed a 40±2% increase in endothelial permeability (both $P<0.05$; Fig. 2D) compared with the control or siRNA-NC groups, whereas the overexpression plasmid resulted in no significant difference in endothelial permeability.

HCMV infection reduces the expression of Rac1 and VASP in HUVEC-CRL-1730 cells. The Rac1/VASP signaling pathway is involved in the regulation of endothelial permeability (23) and HCMV infection increased the permeability of the monolayer HUVEC-CRL-1730 cells. To investigate the potential signaling pathway involved in HCMV infection-induced hyperpermeability in ECs, the expression of Rac1 and VASP at the mRNA and protein levels were examined in HCMV-pretreated cells over a range of times. The results demonstrated that HCMV infection significantly inhibited Rac1 and VASP mRNA expression starting at 8 and
12 h, respectively, compared to the 0 h group (all P<0.05; Fig. 3A and B); maximum inhibition was achieved at 12 h.

HCMV infection also significantly inhibited Rac1 and VASP protein expression starting at 8 and 12 h, respectively, compared with the 0 h group (all P<0.05; Fig. 3A). Maximum Rac1 and VASP protein inhibition was achieved at 24 and 48 h, respectively. Therefore, the data demonstrated that Rac1 and VASP expression levels can be affected by HCMV infection, which also suggested that VASP expression was positively associated with Rac1. These data suggest that HCMV infection-induced increases in EC permeability may be achieved by altering the expression levels of VASP and/or Rac1.

**HCMV infection induces hyperpermeability through the Rac1/VASP signaling pathway in HUVEC-CRL-1730 cells.** HCMV infection inhibited the expression of Rac1 and VASP, and, therefore, to investigate the association between Rac1 and VASP, silencing or over-expression of Rac1 was carried out using siRNA-Rac1 or a pflag-Rac1 plasmid, respectively. At the mRNA and protein level, siRNA-Rac1 oligonucleotides demonstrated a significant inhibitory effect on Rac1 and VASP expression, and the pflag-Rac1 plasmid significantly increased Rac1 and VASP expression compared with the control group and their respective negative controls (all P<0.05; Fig. 4A-C). These data indicated that VASP may act as a downstream effector of Rac1 in HUVEC-CRL-1730 cells.

Following transfection with siRNA-Rac1 or the pflag-Rac1 plasmid in HUVEC-CRL-1730 cells, a Transwell assay was used to detect endothelial permeability at 24 h (Fig. 4D). Rac1 knockdown revealed significant increase in endothelial permeability compared with the control and siRNA-NC groups (P<0.05), which is a 45±2% increase compared with the control group, whereas the Rac1 overexpression plasmid resulted in no significant difference in endothelial permeability. Furthermore, the potential role of the Rac1/VASP signaling pathway in the regulation of HCMV infection-induced hyperpermeability in the cells was analyzed.

After HCMV infection for 24 h, the cells were transfected with siRNA-Rac1, the pflag-Rac1 overexpression plasmid, siRNA-VASP, the pEGFP-VASP overexpression plasmid or their respective controls using Lipofectamine 2000. After 48 h, HUVEC-CRL-1730 cell permeability was assessed using a Transwell assay. The endothelial permeability of Rac1 knockdown cells significantly increased compared with the control and siRNA-NC groups (both P<0.05), which was an increase of 58±3% compared to the control cells (Fig. 4E). The endothelial permeability of the Rac1 overexpressing cells revealed no significant alteration. As demonstrated in Fig. 4F, the endothelial permeability of VASP knockdown cells was...
also significantly increased compared with the control and siRNA-NC groups (both P<0.05), an increase of 50±3% compared to the control cells. The endothelial permeability of the VASP overexpressing cells revealed no significant alteration. These data suggested that HCMV infection induces hyperpermeability via the Rac1/VASP signaling pathway.

Discussion

AS is a common chronic vascular pathological process and the most common cause of cardiovascular and cerebrovascular diseases (24). The pathogenesis of AS is complex; the high etiological risk factors associated with the development of AS include genetic polymorphisms and susceptibility, long-term exposure to various chemicals, smoking, hypertension and hyperlipidemia (25). Furthermore, numerous epidemiological studies and clinical retrospective analyses have demonstrated that viral or bacterial infections, including HCMV (26) and Chlamyphila pneumonia (27), may affect the formation of AS.

EC injury is the first step in AS development and is frequently caused by specific chemicals, high fat diets, genetic factors, trauma and pathogen infection (3). Yi et al (28) found HCMV DNA, antigens, and immediate-early (IE) genes and proteins in the internal carotid arteries of patients suffering ischemic stroke. Blum et al (29) found a high-titer of immunoglobulin G anti-CMV antibodies in the sera of patients with high-risk atherosclerotic cardiovascular disease. Additionally, a clinical cohort study revealed that CMV infection is an independent risk factor for AS and coronary heart disease (30). These studies suggest that the viral infection is closely associated with the development and progression of AS.

ECs are potential hosts of HCMV, and the entry of HCMV into cells is mediated by cellular annexin II (4), a 36 kDa HCMV-binding protein that exists on the cellular membrane. In the present study, in order to demonstrate that endothelial damage can be caused by viral invasion, the effect of HCMV infection on the morphology of ECs was observed. Under normal physiological conditions, HUVECs were closely connected, flat, long and spindle-shaped, and exhibited the typical ‘paving stone’ morphology. After the cells were infected with HCMV for 7-10 days, the nuclei had increased in size and some of the cells had become rounded. In addition, the boundaries between cells became blurred and numerous cells merged to form giant cells, while a number of cells died. The data from the current study are consistent with that of a previous report, which demonstrated that, following HCMV infection, the cells from the two HUVEC cell lines became rounded and intercellular fissure formation occurred (27). This
alteration promoted the attraction of a variety of inflammatory cells and factors through ECs, which began to accumulate peroxidized lipids from the blood vessels, and gradually evolved into foam cells, which are involved in atherosclerotic plaque formation (31). These phenomena suggest that the reduction of barrier function, which is closely associated with the morphological alterations of ECs, is crucial to the development of AS.

In the present study, an increase in Rac1 expression was demonstrated to induce hyperpermeability, which disrupted...
EC barrier function. Baumer et al. (32) demonstrated that Rac1 activation likely contributed to the barrier-stabilizing effects of cyclic adenosine monophosphate in the microvascular endothelium. These effects may in part, be mediated by the Epac/Rap1 signaling pathway (33). Cytoskeletal rearrangements are the structural basis for the modification of EC morphology and associated barrier function (34). VASP, an actin-associated protein, belongs to a family of Ena/VASP proteins, which localize to the intercellular junction, focal adhesion terminals of stress fibers and highly dynamic areas of change at the cellular membrane (35). VASP has been revealed to serve an important role in the maintenance of endothelial barrier function (36,37), and paracellular permeability (38). Luyer et al. (39) demonstrated that the expression of the tight junction protein, ZO-1, was abolished and levels of phosphorylated VASP increased under low intestinal blood flow conditions in a rat model hemorrhagic shock and, at the same time, the permeability of the intestinal mucosa and bacteria translocation also increased. Bogatcheva et al. (40) reported that the bacterial endotoxin lipopolysaccharide secreted by human pulmonary microvascular ECs can cause the redistribution of VASP- and ZO-1-associated proteins. In VASP-depleted pulmonary microvascular ECs, the cell barrier function was reduced, and the mechanism involved may be associated with the cell-cell tight junction damage caused by the disorder of the ZO-1-F-actin arrangement (41).

In the current study, after HCMV infection, endothelial permeability and the expression of Rac1 and VASP were analyzed at the mRNA and protein levels in two HUVEC cell lines. The mRNA level of Rac1 and VASP reach the minimum level at 12 h. The reason for the increase after 12 h may be associated with HCMV infection-induced activation of cytokine-associated signaling pathways. For example, following HCMV infection, the cellular factor, nuclear factor-κB p105 subunit, was activated and its expression increased, which was important for the efficient transcription of IE genes (42); also, hypoxia-inducible factor (HIF)-1α transcription was clearly stimulated (43). The aforementioned results also demonstrated that HCMV-induced hyperpermeability was accomplished by decreasing Rac1 and VASP expression in HUVEC-CRL-1730 cells.

In addition, a further decrease of VASP expression using siRNA-VASP in HCMV-infected cells aggravated the permeability increase. These results indicated that HCMV-induced endothelium hyperpermeability may be mediated by Rac1 and VASP-associated cell-cell junction remodeling. In addition, as VASP is a downstream effector of Rac1 in osteosarcoma cells, interference of Rac1 in the cells led to a significant reduction in VASP expression (21). Additionally, consistent with previous reports, the EC data from the current study also revealed that interfering with the expression of Rac1 using siRNA-Rac1 could inhibit the expression of VASP. Therefore, HCMV-induced endothelial cytoskeleton remodeling may be controlled by the Rac1/VASP signaling pathway. In conclusion, the current study demonstrated that HCMV infection could induce impairment of the barrier function in monolayer HUVEC-CRL-1730 cells via downregulation of Rac1-mediated VASP expression. The present study identified that the presence of pathogens, which cause a breakdown in the barrier function of endothelial cells had a high correlation with the occurrence and development of atherosclerosis. These results indicate that an effective strategy for the prevention of atherosclerosis would be to strengthen the protection of endothelial cells.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YT, LW and YH designed the study and wrote the manuscript. XX and YM analyzed the data. LZ, JZ and LX performed the cell experiments.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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