Abstract

Objectives: Atherosclerosis is the leading cause of death in the United States, and human cytomegalovirus (HCMV) infection may play a role in the development of this disease. Diminished expression and/or activity of endothelial nitric oxide synthase (eNOS) are an early event in atherogenesis. In the current study, we investigated the effects of HCMV infection on eNOS activation in human aortic endothelial cells (HAECs).

Methods and results: We found that HCMV inhibited eNOS phosphorylation/activation in HAECs. The signaling upstream of eNOS involving Akt and PDK1 were also suppressed by the HCMV infection. Moreover, HCMV infection increased the expression of PTEN (phosphatase and tensin homolog deleted on chromosome 10). Silencing PTEN expression with specific siRNA reversed the inhibitory effects on eNOS activation in HCMV-infected cells indicating the involvement of PTEN in mediating HCMV’s inhibitory effects. Next we observed that the activation of p38 MAPK stress signaling pathway mediates HCMV’s effects on PTEN up-regulation and eNOS inactivation.

Conclusions: In summary, our findings suggest that inhibition of eNOS leading to endothelial dysfunction may be a basis of the proatherogenic effects of HCMV. Importantly, upregulation of PTEN and activation of stress signal p38 MAPK are involved in HCMV’s inhibitory effects on eNOS activation.

Keywords: Cytomegalovirus; eNOS; Akt; PTEN; p38 MAPK

1. Introduction

There is increasing evidence that chronic infection with certain organisms is associated with atherosclerosis [1]. Human cytomegalovirus (HCMV), a member of the Herpesviridae family, is a widespread opportunistic pathogen that infects 50% to 90% of adult populations. In addition to monocytes, endothelial cells presents a site of latency for this virus [2–4]. Recent studies have indicated...
that HCMV infection could be one of the causal factors for atherosclerosis. Mouse CMV (MCMV) infection has been shown to promote atherogenesis [5,6]. In humans, HCMV DNA and antigens have been shown in atherosclerotic lesions [7,8]. HCMV re-activation may be involved in the pathogenesis of solid organ allograft rejection, and graft atherosclerosis [9,10]. Existing HCMV infection appears to be a strong independent risk factor for restenosis after coronary atherectomy [11–13]. Although still far from consistent, population studies have generally demonstrated an association between HCMV seropositivity and the increased risk of coronary atherosclerosis [2,3,14,15]. While disturbed coagulation and inflammation could be involved in HCMV-induced vasculogenesis, the mechanisms for HCMV-induced vascular diseases are not well understood. Understanding the mechanisms can lead to preventive and therapeutic measures to eliminate this potentially curative causal factor for vascular diseases.

Endothelium-derived NO, as an endogenous vasodilator, prevents vascular inflammation and thrombus formation by inhibiting platelet and leukocyte adherence. Reduced eNOS expression and activity is a common feature in various cardiovascular diseases including HCMV-seropositive state [16]. It is, therefore, logical to hypothesize that HCMV infection may promote atherogenesis by repressing eNOS activation, resulting in endothelial dysfunction. In this study, we examined this hypothesis, and explored related signaling pathways. We found that HCMV suppresses Akt and eNOS activation, up-regulates PTEN, and activates stress signaling p38 MAPK pathway. All these signals could be related to the inhibitory effect of HCMV infection in eNOS activity.

2. Materials and methods

2.1. p38 MAPK siRNA and antibodies

PTEN siRNA was obtained from Cell Signaling (Beverly, MA). p38 MAPK siRNA and JNK siRNA were purchased from Ambion (Austin, Texas). For Western blot analysis and immunofluorescence staining, monoclonal and polyclonal antibodies were obtained from Cell Signaling (Beverly, MA) and anti-IE (HCMV immediate early gene) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2. Cell culture

Primary human aortic endothelial cells (HAECs) were obtained from Cell Applications (San Diego, CA) and grown in EGM medium from Cambrex (East Rutherford, NJ) containing basal media, 2% fetal bovine serum, growth factors, cytokines, and supplements. Cells cultured up to five passages were used in experiments. HAECs were obtained in compliance with World Medical Association Declaration of Helsinki in the Ethical Principles for Medical Research Involving Human Subjects.

2.3. Infection of HAECs with HCMV

The VHL/E (a generous gift from Dr. Waldman) strain of HCMV was used in the study [17]. Subconfluent HAEC monolayers were infected with HCMV at multiplicity of infection (MOI) 1 as described previously [18]. Supernatant and cell fractions were harvested at various postinfection (p.i.) times.

2.4. siRNAs and endothelial cell transfection

Silencing of PTEN and p38 MAPK gene expression in primary aortic endothelial cells was achieved using the siRNA technique. Transfection of HAECs was carried out using LipofectAMINE™ 2000 (Invitrogen, Carlsbad, California) according to the manufacturer’s instruction.

2.5. Western blot analysis

HAECs were collected from mock- and HCMV-infected cells and washed with ice-cold PBS. Cells were lysed in protein lysis buffer [20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 10 μg/ml of each protease inhibitors (aprotinin, leupeptin and pepstatin), and 1 mM phenylmethylsulfonyl fluoride] for 1 h on ice. Protein concentration was measured by the Bradford method (Bio-Rad). Fifteen μg of protein per lane was separated by 10% or 12% SDS-polyacrylamide gels and transferred to PVDF membranes. The membrane was blocked in 5% nonfat powdered milk in TBST (50 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween 20). The membrane was incubated with the primary antibody in 2% powdered milk in TBST, washed extensively with TBST, and then incubated with secondary anti-rabbit or anti-mouse horseradish peroxidase-labeled antibody. Bands were visualized with ECL (Amersham Biosciences, Piscataway, NJ) according to the manufacturer’s instruction.

2.6. Real-time quantitative RT-PCR

Total RNA from treated cells was extracted with Trizol (Invitrogen) according to the manufacturer’s protocol. The mRNAs were reverse-transcribed into cDNAs with an iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer’s instruction. Real-time PCR was performed by using iCycler iQ real-time PCR detection system (Bio-Rad). Primers were designed through Beacon Designer 2.0 software. The primers for human PTEN were, forward: 5'-CAAGATGTATGTGGAAACTATTTCCAATG-3' and reverse: 5'-CCTTCTAAGCTGGCAGACCAACAA-3'. The mRNA levels were acquired from the value of threshold
cycle (Ct) of the real-time PCR and normalized against the house-keeping gene β-actin.

2.7. Immunofluorescence staining

For immunofluorescence assays, cells were grown on glass coverslips, and infected. After infection, cells were washed with PBS, fixed with 4% paraformaldehyde for 10 min, and permeabilized with 0.2% Triton X-100 for 5 min. The coverslips were blocked with 1% BSA, incubated with the primary antibody, washed extensively with PBS, and then incubated with secondary anti-rabbit or anti-mouse FITC or Texas Red-labeled antibody. One percent BSA in PBS was used for blocking nonspecific binding sites and for dilution of primary and secondary antibodies. The DNA dye 4′,6′-Diamidino-2-phenylindole dihydrochloride (DAPI) was added at a concentration of 0.1 μg/ml and incubated for 15 min to counterstain double-stranded DNA in nuclei. The slides were examined with a Leica DMLS epifluorescent microscope equipped with a Leica DC 100 digital camera and the data was analyzed with Image-Pro Plus V4.5 software (Media Cybernetics, Inc).

3. Results

3.1. HCMV inhibits eNOS activation

We infected HAECs with VHL/E, a clinical isolate whose natural endothelial cytopathogenicity has been preserved by propagation in endothelial cells. The eNOS activation was then monitored in HCMV-infected cells by measuring eNOS phosphorylation at ser 1177, which has been shown to be involved in the activation of eNOS. As shown in Fig. 1A, while the eNOS protein expression remained unchanged, phosphorylated eNOS was dramatically decreased in the infected cells. The inhibition started as early as day 2 post-infection and was almost undetectable by day 4 post-infection.

Insulin, which stimulates eNOS phosphorylation by activating PI3K pathway, plays an important role in regulating vascular function [19]. Therefore, we examined whether HCMV infection impairs the insulin-stimulated eNOS activation. HAECs, after infection with HCMV for 5 days, were stimulated with insulin for 30 min. As shown in Fig. 1B, insulin stimulated eNOS phosphorylation in the uninfected cells, but failed to stimulate eNOS phosphorylation in the infected cells.

3.2. HCMV inhibits Akt pathway

Since Akt activation enhances eNOS expression/activity [20], we investigated whether HCMV infection inhibits eNOS activation by suppressing Akt. Akt is activated by phosphorylation at Ser-473 and Ser-308. We detected the phosphorylation of Akt in HCMV-infected cells. As shown in Fig. 2A, Akt phosphorylation at Ser-473 was inhibited by HCMV infection in a time-dependent manner. However, the total Akt level was not affected suggesting that HCMV infection may block signaling upstream of Akt. Phosphoinositide-dependent kinases PDKs (PDK1 and PDK2) are the Akt upstream kinases, which directly phosphorylate Akt on Thr-308 and Ser-473, respectively [21,22]. Accordingly, we measured the phosphorylation of PDK1 in HCMV-infected cells using anti-phospho-(Ser-241) PDK1 antibody. As shown in Fig. 2B, PDK1 phosphorylation at Ser-241 was inhibited by HCMV infection.

3.3. Up-regulation of the PTEN in HCMV-infected cells

Next we investigated the upstream molecules responsible for the inhibition of eNOS activation. PTEN antagonizes PI3K/Akt signaling by dephosphorylating PIP_3 [23,24]. We, therefore, examined whether PTEN is involved in the effects of HCMV. We first measured PTEN activation in HCMV-infected cells. Since PTEN can be activated by dephosphorylation at the C-terminal (Ser 380) [25–28], we determined the PTEN phosphorylation at the Ser380/Thr382/Thr383. As shown in Fig. 3A, HCMV infection decreased PTEN phosphorylation at Ser380/Thr382/Thr383 indicating the activation of PTEN by HCMV infection. We also showed that the PTEN protein was increased by HCMV infection (Fig. 3A) and the increased expression was specifically seen by immunofluorescence stains in the HCMV-infected cells (Fig. 3B). Additionally, PTEN mRNA levels in the infected cells were also significantly increased (Fig. 3C) indicating that HCMV infection can induce PTEN expression at mRNA level. Taken together, these data indicate that HCMV infection can specifically induce PTEN activation and expression.

3.4. The involvement of the PTEN in HCMV-induced inhibition of eNOS phosphorylation and Akt activation

To examine whether the PTEN up-regulation was involved in HCMV-induced inhibition of the Akt pathway and eNOS activation, we blocked PTEN expression using PTEN-specific siRNA. As shown in Fig. 4, HCMV infection induced PTEN expression and reduced the phosphorylation of Akt and eNOS (lane 2). PTEN siRNA specifically inhibited the HCMV-induced PTEN upregulation in a concentration-dependent manner (lane 3–5). As PTEN expression was decreased, the inhibition of phosphorylation of Akt and eNOS was reversed, suggesting that PTEN up-regulation is directly involved in HCMV-induced inhibition of Akt signaling and eNOS activation.

3.5. HCMV infection leads to activation of p38 MAPK pathway

To identify intracellular stress signaling pathways that may mediate HCMV-induced inhibition of Akt signaling
and eNOS activation, we examined the kinetics of p38 MAPK activation. As shown in Fig. 5A, exposure of HAECs to HCMV stimulated p38 MAPK activity, whereas JNK pathway, another MAPK kinase, remained unchanged (data not shown). Next, we determined whether HCMV infection could activate MKK3 and MKK6, two cellular kinases that are known to phosphorylate p38 MAPK [29,30]. MKK3 and MKK6 phosphorylation was activated at late stage of the HCMV infection (Fig. 5B) suggesting that increased MKK3/6 activity may account for p38 MAPK activation at late stage of infection. The phosphorylation of MLK3, an upstream kinase that can directly phosphorylate M KK3/MKK6, was also increased in HCMV-infected cells (Fig. 5B).

3.6. Role of p38 MAPK pathway in HCMV-induced eNOS inhibition

To examine whether activation of p38 MAPK was involved in HCMV-induced up-regulation of the PTEN
Fig. 2. HCMV infection inhibited Akt pathway. (A) Reduced Akt phosphorylation in HCMV infected cells. HAECs were infected with HCMV for different time. Cell lysate was prepared. Phosphorylated Akt was detected by Western blotting using anti-phospho (Ser-473) Akt antibody. Membranes were stripped and reprobed for the total Akt using anti-Akt antibody. Representative blots of three separate experiments are shown. (B) Reduced PDK1 phosphorylation in HCMV infected cells. HAECs were infected with HCMV for different time. PDK1 phosphorylation was detected from the infected HAECs using anti-phospho (Ser-241) PDK1 antibody. Total PDK1 was measured using anti-PDK1 antibody. Representative blots are shown.

Fig. 3. HCMV infection stimulated PTEN expression. (A) Increased PTEN activation and expression by HCMV infection. HAECs were infected with HCMV and the cell lysate was prepared at different postinfection time. PTEN phosphorylation was determined by anti-phospho (Ser380/Thr382/Thr383) PTEN antibody; and its expression was measured using anti-PTEN antibody. β-actin was measured as loading control. Representative blots from five independent experiments are shown. (B) Increased PTEN expression in HCMV-infected cells. HCMV infected HAECs (day 6) were stained with anti-IE antibody-FITC (green) and anti-PTEN antibody-Texas Red (red). Merged image shows co-localization. (C) HCMV infection increased PTEN mRNA. Total RNA from mock and infected cells was extracted and the mRNAs were reverse-transcribed into cDNAs. PTEN mRNA levels were quantified by real-time PCR and normalized to β-actin mRNA. Results are expressed as percentage of the control.
and inhibition of the insulin signaling, we suppressed p38 MAPK expression using p38 MAPK-specific siRNA. As shown in Fig. 6, p38 MAPK expression was specifically suppressed by p38 MAPK siRNA indicating that the siRNA is efficient. Along with the reduction in p38 MAPK expression, PTEN expression also fell, suggesting that p38 MAPK pathway is involved in HCMV-induced PTEN expression. Furthermore, phosphorylation of Akt and eNOS in HCMV-infected cells was completely reversed by the p38 MAPK siRNA treatment.

4. Discussion

In the present study, we have shown that HCMV infection inhibits basal and insulin-stimulated eNOS phos-

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Fig. 4. Involvement of PTEN in HCMV-induced Akt and eNOS inactivation. HAECs were infected with HCMV for 6 days before transfected with different amounts of PTEN specific siRNA (25–150 nM). Twenty-four hours after transfection, total protein lysates from mock-infected and infected cells were prepared and blotted with antibodies against PTEN, phosphorylated Akt and phosphorylated eNOS. β-actin was measured as loading control. Representative blots of five separate experiments are shown.

Fig. 5. HCMV infection activated p38 MAPK pathway. (A) Increase of phosphorylated p38 MAPK by HCMV infection. HAECs were infected with HCMV for different times and cell lysate was prepared. Phosphorylated p38 MAPK were detected by Western blotting using anti-phospho (Thr-180/Tyr-182) p38 MAPK antibody. The membrane was stripped and re-blotted with anti-p38 MAPK antibody. β-actin was measured as loading control. Representative blots of five separate experiments are shown. (B) Activation of p38 MAPK in HCMV-infected cells. HCMV-infected HAECs (day 6) were stained with anti-IE antibody-FITC (green), anti-phospho (Thr-180/Tyr-182) p38 MAPK antibody-Texas Red (red) and DAPI (blue). Merged image shows co-localization. (C) HCMV infection activated p38 MAPK pathway. The phosphorylation of MLK3 and MKK6 in mock and HCMV infected cells was detected by anti-phospho (Thr-277/Ser-281) MLK3 antibody and anti-phospho (Ser-189/207) MKK3/MKK6 antibody, respectively. β-actin was measured as loading control. Representative blots of five separate experiments are shown.
phorylation and activation, which is likely to be the results of the Akt inactivation through the up-regulation of PTEN by the HCMV infection. Stress signal p38 MAPK appears to be involved in the PTEN-triggered Akt and eNOS inactivation in HCMV-infected cells. The significant eNOS inhibition starts at 2 days post infection and persists to as long as the experimental periods (8 days post infection). The eNOS inactivation is accompanied by the simultaneous Akt inactivation and p38 activation, which show a time-dependent change in intensity and lags behind the changes in eNOS. The proposed pathway by which HCMV infection inhibits eNOS activation is shown in Fig. 7.

HCMV, a widespread opportunistic pathogen, causes acute, latent, and chronic infections. Although the primary infection may be asymptomatic in immunocompetent individuals, the virus can cause a wide variety of severe diseases in immunocompromised hosts. Involvement of HCMV infection has been discovered in atherosclerosis [14,16,31,32], thrombosis [33,34], allograft rejection [35,36] and restenosis [37,38]. However, the precise mechanisms of atherogenesis are not clear. Endothelium-derived NO is an endogenous anti-atherogenic factor. Deregulation of eNOS and subsequent decrease in NO production are prominent features of various vascular diseases and early events in atherogenesis.

Although it has been shown that HCMV-seropositive individuals have impaired NO-dependent vasodilation [16], little data is available about whether HCMV infection interferes with eNOS activation. One recent study shows that HCMV infection in human heart transplant recipients is associated with high levels of endogenous inhibitor of NOS, asymmetric dimethylarginine (ADMA). They found that HCMV infection impairs dimethylarginine dimethylaminohydrolase (DDAH) activity that results in increased ADMA [39]. Our results indicate that HCMV infection may also be able to reduce NO production through inhibition of eNOS phosphorylation/activation. As far as we are aware, our study is the first to show the inhibition of eNOS activation by HCMV infection. Since NO is a key molecule that plays
critical role in regulating vascular function, we propose that the reduction in eNOS activation as a result of HCMV infection is a plausible link in accelerated atherogenesis in HCMV-infected patients.

In this study, we also addressed the signal transduction pathways for HCMV-induced inhibition of eNOS. As is well-known, eNOS activity is regulated by post-translational modifications through several signaling pathways, including the ERK MAPK pathway [40], the AMP-activated protein kinase (AMPK) pathway [41] and the Akt pathway. Insulin induces eNOS activation through the IRS/P13K/PDK/Akt cascade, which plays a key role in insulin signaling in target tissues. Our results show that HCMV infection prevents eNOS activation by inhibiting Akt pathway, although inhibition of other pathways by HCMV infection cannot be ruled out. We have noted the report that HCMV infection induces Akt phosphorylation in fibroblasts [42]. While this discrepancy could be a cell specific phenomenon, it could also result from differences in the experimental conditions. It is also possible that HCMV infection induces Akt phosphorylation in the early stages of infection, and prolonged HCMV infection decreases Akt phosphorylation. This dual effect was observed in the occurrence of apoptosis in endothelial cells infected with HCMV. In early stages of infection, HCMV was shown to induce anti-apoptotic factors such as ERK MAPK and PI3K-signaling pathways that promotes cell survival [43,44]; however, in the later stages of infection, HCMV promoted cell death [18]. Akt kinase, an established anti-apoptotic factor, may be altered by HCMV infection to fit the needs of cell regulation.

We further investigated the mechanisms for HCMV-induced inhibition of the Akt signaling and eNOS activation. Our results show that HCMV infection can induce both PTEN activation and expression. Importantly, silencing PTEN by specific siRNA reversed the HCMV’s inhibitory effects on Akt and eNOS activation, suggesting that the up-regulated PTEN could be the mediator of these effects. PTEN is a member of serine/threonine/tyrosine phosphatase subfamily of protein phosphatases. It dephosphorylates PtdIns (3,4,5)P3 into PtdIns(4,5)P2 and thus antagonizes PI3K-dependent signaling pathways and Akt activation [45]. PTEN has been shown to play critical role in embryonic development, cell growth, apoptosis, differentiation and migration [46]. PTEN is also widely expressed in cardiomyocytes, fibroblasts, endothelial cells and vascular smooth muscle cells [47], where it may modulate cell survival/apoptosis, metabolism and function. Our finding suggests that PTEN may be an important negative regulator of the eNOS activation in vessel wall and be involved in the development of endothelial dysfunction.

In searching for the mechanisms and pathways for HCMV-induced PTEN upregulation and eNOS inhibition, we found that HCMV infection activated stress signal p38 MAPK. To determine if HCMV infection also increased activity of other MAPKs, we examined the activity of JNK and found no evidence of significant increases for JNK throughout the time course of the HCMV infection. This observation indicates that the p38 MAPK activation is a specific event. Inhibition of p38 MAPK fully reversed HCMV-induced upregulation of PTEN and inhibition of Akt and eNOS phosphorylation indicating the involvement of p38 MAPK pathway in mediating HCMV’s effects. It is known that p38 MAPK pathway plays an essential role in regulating many cellular processes including inflammation, cell differentiation, cell growth and death. Activation of p38 MAPK by extracellular stimuli such as bacterial pathogens and cytokines triggers downstream responsive genes. Several investigators have described the induction of the p38 MAPK activation following HCMV infection [48,49].

Our observation provides important evidence for the cross talk between stress signaling and Akt pathway, which leads to eNOS inactivation in the HCMV-infected cells. Our findings also indicate a novel mechanism that this cross talk may be mediated by the PTEN upregulation.

In summary, we have demonstrated that HCMV infection exerts an inhibitory effect on eNOS activation and Akt signaling in endothelial cells. HCMV infection inhibits Akt pathway by activating stress-response signal p38 MAPK pathway, and up-regulating PTEN. HCMV-induced eNOS inactivation may play an important role in endothelial dysfunction and ensuing vascular diseases in those with chronic or reactivated latent HCMV infection. The elucidation of the molecular mechanisms leading to the inactivation of Akt pathway and eNOS inhibition through PTEN and stress signaling regulation will offer potential targets to attenuate the HCMV-induced cytopathic effects, hence, vascular diseases.

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References


