LABORATORY INVESTIGATION

Infection of endotheliotropic human cytomegalovirus of trabecular meshwork cells

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Abstract

Purpose Human cytomegalovirus (HCMV) infections can cause endotheliitis which is associated with an elevation in the intraocular pressure (IOP). However, the mechanism of the IOP elevation has not been established. The purpose of this study was to determine whether HCMV strains which are capable of infecting corneal endothelial cells can also replicate and induce anti-viral responses, and can reorganize the actin cytoskeleton in trabecular meshwork cells.

Study design Experimental study design.

Methods Cultured primary human trabecular meshwork cells (HTMCs) were infected with the Towne or TB40/E strains of HCMV. TB40/E is trophic for vascular endothelial and corneal endothelial cells. Real-time PCR, western blot, and fluorescent immunostaining have been used to determine whether HCMV-infected HTMCs will support the expression of viral mRNA and protein, allow viral replication, and elicit anti-viral host responses. We also determined whether lytic replication was present after HCMV infection.

Results HCMV infection led to the expression of viral mRNA and proteins of IE1, glycoprotein B(gB), and pp65. TB40/E infection induced interferon- β , a sign of host anti-viral immune response and monocyte chemotactic protein-1 (MCP-1) as IOP-related chemokine. Together with the induction of the regulators of actin cytoskeleton, myosin phosphatase Rho interacting protein (MPRIIP) and MCP-1, TB40/E induced a high level of expression of viral proteins, including IE1, gB, and pp65 as well as actin stress fiber formation, and achieved pathogenically high viral titers.

Conclusions Human trabecular meshwork cells support the replication of endotheliotropic TB40/E strain of HCMV which indicates that this strain may have high virulence for trabecular meshwork.

Keywords Cytomegalovirus · Corneal endothelial cells · Trabecular meshwork · Secondary glaucoma · Endotheliitis

Introduction

Human cytomegalovirus (HCMV) infection can lead to endotheliitis which is associated with an elevation in the intraocular pressure (IOP) and endothelial cell loss [1]. Its chronic and relapsing nature resembles the clinical characteristics of Fuchs iridocyclitis and Posner-Schlossman syndrome. These diseases are characterized by chronic and relapsing elevations in the IOP, and HCMV infection has been recognized as one important causative pathogen leading to these disorders.

In HCMV endotheliitis, the IOP elevation and endothelial inflammation frequently recur during the long course of the disease process. The IOP elevation can last for months or years, and the eye can become refractory to IOP lowering medications. Hence, patients often require filtering surgery. The findings strongly suggest that the HCMV may actively

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proliferate in the trabecular meshwork and corneal endothelial cells.

The IOP elevation during the long disease course of HCMV endotheliitis will damage the trabecular meshwork cells which can then lead to a severe reduction in aqueous outflow. However, there is no definitive proof that surgically-obtained trabecular meshwork tissue was permissive for HCMV infection. Because filtering surgery is generally performed after years of IOP elevation, the trabecular meshwork cells were already damaged. This may explain the absence of HCMV proteins in the trabecular meshwork specimens.

When HCMV infects permissive cells, latent infection can be established. During the latency phase, detection of viral protein antigens is very difficult unless the specimen is collected during the reactivation phase. We have shown that corneal endothelial cells can be infected by the Towne strain, the TB40/E strain, and a clinically-isolated strain of HCMV [2, 3]. However, the efficiency of infection measured by TCID₅₀ is markedly different depending on the strain, and vascular endotheliotropic TB40/E was also tropic for corneal endothelium. We hypothesized that endotheliotropic HCMV strain will infect trabecular meshwork cells more readily than non-tropic strains of HCMV.

The purpose of this study was to determine whether endotheliotropic strain of HCMV can infect trabecular meshwork cells and reorganize the actin stress fibers. In addition, we examined whether HCMV infection will induce strong immune responses which may explain the increased outflow resistance of the trabecular meshwork in eyes with HCMV infection.

Materials and methods

All protocols and methods adhered to the tenets of the Declaration of Helsinki, and the study was approved by the Ethics Committee of Tottori University.

Cells and virus

Human trabecular meshwork cells (HTMC) were isolated from trabecular tissues stripped from cadaver eyes donated for research purposes (ScienCell). The HTMCs were propagated to confluence on 6- or 96-well plates in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 15% fetal bovine serum.

A standard laboratory strain, Towne, and an endotheliotropic HCMV strain, TB40/E, were used. The Towne strain is a representative laboratory strain together with AD169, and is one of most widely used HCMV strains. The Towne strain has been used for vaccine development [4].

The TB40/E strain was created by transfecting human foreskin fibroblast cells (HFF) transfected by a TB40-BAC4 clone and propagated by standard procedures as described [2]. The viral titers were designated by the 50% tissue culture infection dose (TCID₅₀) method using human foreskin fibroblast cells infected with serially diluted supernatants.

Real-time RT-PCR

Total RNA was isolated from HTMCs at the indicated time points after infection by the two HCMV strains and reverse transcribed using the QuantiTect Reverse Transcription Kit (Qiagen). The cDNAs were amplified and quantified by the LightCycler (Roche) using the QuantiTect SYBR Green PCR kit. The sequences of the real-time PCR primer pairs were:

IE-1:

forward 5'-CCTCCAAGGTGCCACGGCCCGA-3',
reverse 5'-CATCCTCCCATCATATTA-3'.

UL83 (pp65):

forward 5'-GTCAGCGTTCGTGTTTCCCA-3',
reverse 5'-GGGACACAACACCGTAAAGC-3'.

Glycoprotein B (gB):

forward 5'-GGGACACAACACCGTAAAGC-3',
reverse 5'-ATGATGCCCTCRTCCARGTC-3'.

Interferon- β (IFN- β):

forward 5'-CATTACCTGAAGGCCAAGGA -3',
reverse 5'-CAATTGTCCAGTCCCAGAGG-3'

Myosin phosphatase Rho interacting protein (MPRIP):

forward 5'-CTCTCCACACACGAGCTGAC-3',
reverse 5'-TCTTCTGGTGCGTTTCTTCC-3'

Monocyte chemotactic protein-1 (MCP-1):

forward 5'-AGGTGACTGGGGCATTGAT-3',
reverse 5'-GCCTCCAGCATGAAAGTCTC-3'

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH):

forward 5'-AGCCACATCGCTCAGACAC-3',
reverse 5'-GCCCAATACGACCAATCC-3'.

To measure the degree of viral DNA replication, HTMCs were infected with either the TB40/E or the Towne strain, and viral DNA was extracted from the cells at the indicated time points with the QiaAmp DNA mini kit (Qiagen). The extracted DNA was amplified with the LightCycler (Roche) and normalized to known dilutions of synthesized pp65 DNA fragments.

Fig. 1 Induction of the mRNA of the genes of human cytomegalovirus (HCMV) in human trabecular meshwork cells (HTMC) after HCMV infection. HTMCs were infected with the Towne strain or the TB40/E strain of HCMV, and the induction of the mRNA of IE1 (a), pp65 (b), and glycoprotein B (gB) (c) was determined by RT-real-time PCR. Viral genes, IE1, pp65, and gB, were sequentially induced after infection by the Towne strain or the TB40/E strain of HCMV. TB40/E induced significantly higher amounts of IE1, pp65, and gB. * $P < 0.05$, ** $P < 0.05$; $n = 8$

Fig. 2 Induction kinetics of viral gene proteins in HTMCs after HCMV infection. HTMCs were infected with the Towne strain or the TB40/E strain at MOI of 1 or 5, and the extracted viral proteins were assayed by western blot analyses. The proteins of IE1, pp65,

and UL44 were sequentially expressed after the HCMV infection. Infection by the TB40/E strain led to stronger inductions of pp65 and UL44

Fig. 3 Induction of interferon response in HTMCs after infection by the TB40/E strain of HCMV. The extracted mRNA at 6 h post infection was evaluated for interferon using real-time RT-PCR for interferon-β. TB40/E infection induced significantly higher levels of interferon-β than Towne. **P* = 0.016, ***P* = 0.0001; *n* = 5

Western blot

HTMCs were infected with the TB40/E strain or the Towne

Fig. 4 Induction of myosin phosphatase Rho interacting protein (MPRIP) in HTMCs after infection by the TB40/E strain of HCMV. HTMCs were infected with the TB40/E strain or the Towne strain. The level of mRNA induction of myosin phosphatase Rho interacting protein (MPRIP) was evaluated using RT-PCR. TB40/E infection induced significantly higher levels of MPRIP at 12 h PI than Towne. **P* = 0.0001; *n* = 5

strain at a selected multiplicity of infection (MOI). Cell lysates were harvested at 1, 2, and 3 days post infection

Fig. 5 Induction of MCP-1 in HTMCs after infection by HCMV. HTMCs were infected with the TB40/E strain or the Towne strain. The level of mRNA induction of MCP-1 was evaluated using RT-PCR. TB40/E infection prolonged the induction of higher levels of MCP-1 at 24 h, while induction of MCP-1 by Towne was observed at 6 h PI and was transient. * $P = 0.04$, ** $P < 0.001$; $n = 5$

(PI), and 10 μ g of protein/lane were electrophoresed. The transferred membranes were stained with antibodies for HCMV IE1 (NEA-9221, Perkin Elmer), pp65 (CA003, EastCoast Bio), or UL44 (CH167, Santa Cruz) [5]. The proteins were made visible with horseradish peroxidase-conjugated secondary antibody and chemiluminescence substrate as described [2].

Immunocytochemistry

HTMCs were infected with HCMV at MOI of 5 and stained for HCMV proteins with mouse anti-HCMV monoclonal antibody against IE-1 (MAB8129, Millipore) or pp65 (ab31624, Abcam), or glycoprotein B (gB; ab6499, Abcam). IE1, pp65, and gB were made visible by incubation with Alexa 488 conjugated-secondary antibody and stained for actin cytoskeleton using phalloidin. Images were photographed with a confocal microscope (LSM710, Zeiss).

Electron microscopy (EM)

HTMCs were infected with either the TB40/E strain or the Towne strain of HCMV and fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer. After post fixation with 1% OsO₄ in 0.1 M sodium cacodylate buffer, cells were stained with 1% uranyl acetate. Cells were dehydrated, embedded in Epon 812, and sectioned with a microtome. The sections were stained with 4% uranyl

acetate and 0.4% lead citrate and were examined by transmission electron microscopy (TEM).

Statistical analyses

Data are presented as the mean $\pm$ standard error of the mean (SEMs). Statistical analyses were performed by *t* test, Mann Whitney U test, or ANOVA with post hoc test.

Results

When HTMCs were infected with the Towne strain of HCMV at an MOI of 5, an induction of the viral immediate early protein, IE1, was detected at 6 h, and there was a gradual increase to 24 h post infection (Fig. 1a right). The transcriptions of the delayed early proteins, gB, and leaky late protein, pp65, were also increased at 24 h PI (Fig. 1b, c, right).

When HTMCs were infected with the TB40/E strain, IE1 was induced at 6 h. The induction kinetics of gB and pp65 were similar to those following the standard laboratory Towne strain infection, however the induction levels were significantly higher (Fig. 1).

HCMV-infected HTMCs were examined for IE1, pp65, and DNA polymerase processivity subunit UL44 by western blot analysis (Fig. 2). Consistent with the gene transcription outcomes, the IE1 protein was promptly induced at 1-day PI with both strains. Notably, TB40/E induced the expression of pp65 as early as 1-day PI at low MOI.

Fig. 6 Induction of viral gene proteins and remodeling of actin cytoskeleton after HCMV infection. HTMCs were infected with the TB40/E strain (a) or the Towne strain (b) of HCMV at MOI of 5 and immunostained for IE1, pp65, and glycoprotein B (Green). TB40/E infection induced significant remodeling of the actin cytoskeleton (c, pp65, green). Actin was stained using phalloidin (red). Nucleus was labelled by DAPI (blue). *Viral assembly compartment

In contrast, Towne infection required 3 days at high MOI for induction. With TB40/E infection, the UL44 induction

was also earlier at 2 days PI compared to 3 days PI with the Towne strain.

We next determined whether HCMV infection will induce the antiviral responses of the host cells, regulation of cytoskeleton, and MCP-1 as an IOP-related cytokine [14]. When HTMCs were infected with the TB40/E strain, there was a significant induction of interferon- β as early as 6 h PI (Fig. 3). In contrast, infection by the Towne strain did not induce interferon- β .

We then examined whether HCMV infection will induce the regulatory factors for actin cytoskeleton (Fig. 4). After infection by the TB40/E strain of HCMV, the induction of the myosin phosphatase Rho interacting protein (MPRIIP) was significantly increased in the HTMCs. Again, Towne infection did not induce MPRIIP appreciably. Both strains induced MCP-1 in response to HCMV infection, however, a prolonged induction was observed after TB40/E infection at 24 h PI (Fig. 5).

The location of the viral protein expression and actin stress fiber formation after HCMV infection was determined by immunostaining (Fig. 6). Intense IE1 staining of the nuclei of HTMCs was detected at 2 day PI with nuclear F-actin [6]. The expression of IE1 moved to the assembly compartment in the cytoplasm at 3 days PI. (Fig. 6a, b; upper panel) The expression of pp65 was also detected in the nuclei at 2 days PI (Fig 6c.), and localized to the viral assembly compartments in the cytoplasm at 3 days PI. (Fig. 6a, b; lower panel) The expression of gB was delayed and observed at 3 days PI. (Fig. 6a, b; middle panel).

When HTMCs were infected by TB40/E, an owl's eye appearance was observed in the nucleus with IE staining earlier than after Towne infection (Fig. 6a; white arrow), and it was detected as early as 1 day PI. (Fig. 6a; upper left) The level of expression increased in the cytoplasm at 3 days PI.

The expressions of pp65 and gB were observed in the nuclei at 2 days PI, and they were also localized in the viral assembly compartment, at the same time distinct stress fibers of actin were formed.

TB40/E infection induced significant remodeling of the actin cytoskeleton (Fig. 6c) Thus, the TB40/E infection was more robust with an earlier expression of viral proteins and actin stress fiber formation.

The replication of viral DNA in HTMCs was assessed after infection with the TB40/E strain or the Towne strain (Fig. 7). Both strains induced significant increases of viral genome at 2 days PI at MOI of 5, and the increase continued, and both strains supported an efficient replication at MOI 1. Interestingly, TB40/E infection induced 5 times more viral replication than the Towne strain at day 3 PI.

HCMV infected HTMC were examined by TEM. Infection by either the TB40/E strain or the Towne strain led

Fig. 6 (continued)

Fig. 7 Viral genome replication for HCMV in human trabecular meshwork cells (HTMCs). HTMCs were infected with the HCMV and examined for viral genomic copy equivalent using real-time PCR. Both the TB40/E strain and Towne strain induced significant increases of the viral genome. TB40/E infection induced 5 times more viral replication than Towne at day 3 PI. $n = 6$

to many viral particles and electron-dense particles (dense bodies) in the cytoplasm of the HTMCs (Fig. 8).

We compared the increase in the levels of the TB40/E and Towne strains in HTMC using titration by TCID₅₀. HTMCs were infected with TB40/E or Towne at MOI of 1 and 5, and titrated (Fig. 9). TB40/E led to a continuous increase until day 11 PI and reached 10⁷ TCID₅₀. In contrast, the increase of the Towne strain reached a plateau at day 5 PI, and the maximum viral titer remained 10 times lower than that of TB40/E.

Discussion

Earlier studies show that infection of the anterior segment of the eye by HCMV can cause endotheliitis or iritis. Both diseases are characterized by an elevation of the IOP which is not observed in HCMV retinitis. Thus, anterior segment infections by HCMV may belong to a unified spectrum of a disease. Under these conditions, IOP elevation can be caused by an increase in the outflow resistance in which the trabecular meshwork cells play a major role.

Fig. 8 Production of infectious virions after HCMV infection of human trabecular meshwork cells (HTMCs). HTMCs were infected with the TB40/E strain or the Towne strain of HCMV at MOI of 0.1 and examined by electron microscopy. Viral particles (arrow) and dense bodies (arrowhead) can be seen in the cytoplasm. Bars indicate 500 nm (TB40/E) and 200 nm (Towne)

Steroid treatment is known to induce steroid-induced glaucoma and impair the outflow function of the trabecular meshwork. When the trabecular meshwork cells were exposed to dexamethasone *in vitro*, there was a remodeling of the actin fibers and an induction of contraction [7]. Thus, stress fiber formation and actin remodeling were suggested as possible mechanisms of secondary glaucoma. Our results show that HCMV can replicate in HTMCs and reorganize the actin cytoskeleton of the cells [2]. This may explain why there was an elevation in the IOP associated with HCMV infection.

In eyes with open angle glaucoma, IL-4, IL-6, IL-8, IL-16, CCL2(MCP-1), TGF- β 1, CCL13, CCL15, CCL22,

CCL24, CXCL13, CXCL16, and TNF- α were found to be significantly elevated in the aqueous humor [8–13]. Of these, the elevation of MCP-1 is consistent, and MCP-1 is a representative cytokine whose level is significantly correlated with the elevation in the IOP [14]. MCP-1 is well known to attract mononuclear cells and also induce collagen synthesis by TGF- β 1 induction [15]. It also induces the production of matrix metalloproteinase-1 and the tissue inhibitor of metalloproteinase-1 (TIMP-1). All of these changes may explain the induction of fibrotic changes in the outflow system leading to IOP elevation.

Very recently, it was reported that the AD169 strain of HCMV can infect HTMCs [16]. AD169 infection induced TGF β 1 but its induction required at least 3 days, and the induction levels remained within 10–20% of that of mock infection. Moreover, AD169 infection did not induce any mRNA of cytoskeleton-related molecules including fibronectin, α -SMA, and collagen type 1A. This suggests less fibrogenic ability of AD169 which is in marked contrast to dexamethasone treatment to HTMCs, which induced fibronectin and α -SMA.

We have shown that HCMV can proliferate in primary corneal endothelial cells, and TB40/E is tropic for corneal endothelium as well as vascular endothelium [2]. This led us to hypothesize that endotheliotropic strains can cause more relevant disease characteristics. The results showed that the endothelial cell tropic TB40/E proliferates very efficiently in trabecular meshwork cells with marked actin cytoskeleton stress fiber formation. This implies that the endothelial type of HCMV infection is the cause of the higher virulence.

In HCMV endotheliitis, the copy numbers of the DNA of HCMV in the aqueous humor determine the frequency of recurrences, degree of IOP elevation, and endothelial cell loss [17, 18]. This indicates that the copy number of the viral genome determines the course of the disease and refractoriness. The adaptive Towne strain also proliferated in HTMCs, however, for HTMCs and corneal endothelial infections the TCID₅₀ remained 10 times lower than the TB40/E. The TB40/E strain proliferated well in corneal endothelial cells and required 4 days to reach a plateau with high tissue culture infective dose (TCID) titers. Thus, the trabecular meshwork cells supported both the endotheliotropic and adaptive strains, yet in HTMCs the TB40/E strain proliferated more efficiently and had a higher virulence.

Contraction and reorganization of the actin stress fibers presumably play important roles in IOP regulation through the conventional outflow channels. Rho kinase inhibitors reorganize or disrupt F-actin fibers through Rho GTPase proteins; they were recently made available for use on patients to reduce the IOP by targeting the outflow through Schlemm's canal. HCMV diverts the actin fiber transport system of the host to organize nuclear egress, viral assembly, and egress from the cytoplasm. Thus, HCMV infection

Fig. 9 Efficient growth of HCMV in HTMCs. HTMCs were infected with TB40/E or Towne at MOI of 1 and 5 and were titrated for tissue culture infectious dose (TCID₅₀). Both strains had efficient growth in HTMCs. The maximum viral titer was significantly higher for the TB40/E strain. n = 4

disrupts or reorganizes the actin fiber cytoskeleton. For example, US-28 protein of HCMV activates the RhoA-mediated motility and the ROCK pathway [19, 20]. In the intracellular viral assembly complex of the host, RhoB is diverted to reorganize or disrupt actin stress fibers leading to efficient viral production [21].

Adaptive HCMV strains, including the Towne and AD169 strains, proliferate well in fibroblasts, however, macrophages, dendritic cells, vascular endothelial cells, and epithelial cells do not facilitate efficient proliferation. These strains are deficient by as many as 20 genes [22], including pUL128, pUL130, and ppUL131A, which mediate viral entry into endothelial cells or epithelial cell lineage [23, 24]. However, whether these factors and other viral genes determine the pathogenicity of HCMV has not been determined.

We observed the induction of IE1 transcript and gene products in the HTMCs. The induction of IE1 determines the latency or reactivation of HCMV in the infected cells. A strong induction of IE1 by the TB40/E strain indicates a facilitated activation of viral gene induction (Figs. 1, 2). However, IE1 expression is not a reliable marker of viral infection because it can be induced without viral replication. IE1 is induced even in non-permissive animal cells because of its potent promotor activity.

We showed a strong induction of the pp65 protein which strongly inhibits antiviral interferon responses, and it can serve as the major target of the host acquired immune

system. The HCMV proteins, including pp65, provoke strong acquired immunity of CD8⁺ T cells through MHC class I recognition pathway. Because of the high prevalence of HCMV infection in elderly subjects [25], up to 30% of CD8⁺ T cells in their bodies are responsive to HCMV antigens [26]. This substantial expansion of memory inflated CD8⁺ T cells is often facilitated by antigen presentation in non-hematopoietic cells including endothelial cells [3, 27]. This immunostimulatory effect of infected cells is characterized by potent interferon responses which we showed for TB40/E infection.

gB mediates the adhesion of HCMV to the host heparan sulfate proteoglycan. Thus, neutralization of gB by antibody generation is an important strategy by the host that prevents cell-to-cell spread or cell fusion after infection. Collectively, the strong expression of pp65 and gB by the trabecular meshwork cells and the reactivity of the host acquired immunity may determine the clinical characteristics of the disease.

There are some limitations to our study. Our analysis was based on an in vitro infection model of cultured trabecular meshwork cells. This may not reflect the in vivo role of trabecular meshwork including the conventional outflow of aqueous humor. In addition, the inflammatory cells recruited by CMV-induced inflammation in vivo, may cause remodeling of trabecular meshwork. However, our finding that the endotheliotropic strain of HCMV is

pathogenic in both the corneal endothelium and trabecular meshwork is important information leading to a better understanding of the disease etiology.

In conclusion, HCMV can infect trabecular meshwork cells and promote the reorganization of the actin cytoskeleton. This then presumably leads to an IOP elevation in the host. Understanding the viral and host aspects of HCMV infection may provide important clues to develop efficacious treatment of refractory anterior segment infection by HCMV.

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