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Ocular cytomegalovirus latency exacerbates the development of choroidal neovascularization

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Abstract

Age-related macular degeneration (AMD) is a complex, multifactorial, progressive disease which represents a leading cause of irreversible visual impairment and blindness in older individuals. Human cytomegalovirus (HCMV), which infects 50–80% of humans, is usually acquired during early life and persists in a latent state for the life of the individual. In view of its previously described pro-angiogenic properties, we hypothesized that cytomegalovirus might be a novel risk factor for progression to an advanced form, neovascular AMD, which is characterized by choroidal neovascularization (CNV). The purpose of this study was to investigate if latent ocular murine cytomegalovirus (MCMV) infection exacerbated the development of CNV in vascular endothelial growth factor (VEGF)-overexpressing VEGF-A^{hyper} mice. Here we show that neonatal infection with MCMV resulted in dissemination of virus to various organs throughout the body including the eye, where it localized principally to the choroid in both VEGF-overexpressing-VEGF-A^{hyper} and wild-type(WT) 129 mice. By 6 months post-infection, no replicating virus was detected in eyes and extraocular tissues, although virus DNA was still present in all eyes and extraocular tissues of both VEGF-A^{hyper} and WT mice. Expression of MCMV immediate early (IE) 1 mRNA was detected only in latently infected eyes of VEGF-A^{hyper} mice, but not in eyes of

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Author contributions statement

JX and XL performed experimental work and statistical analysis. XZ contributed to plaque assay and RT-PCR. BM contributed to EM work. ZD contributed to data analysis. YL contributed to data interpretation of ddPCR. DGE contributed to the analysis of CNV detected by fluorescein angiography and OCT. MZ conceived the study and supervised the work. MZ, JX, and BM wrote the paper. All the authors read and approved the final manuscript.

No conflicts of interest were declared.

Data availability statement

The data that support the findings of this study are available from the corresponding author MZ, upon reasonable request.

WT mice. Significantly increased CNV was observed in eyes of MCMV-infected VEGF A^{hyper} mice compared to eyes of uninfected VEGF-A^{hyper} mice, while no CNV lesions were observed in eyes of either infected or uninfected WT mice. Protein levels of several inflammatory/angiogenic factors, particularly VEGF and IL-6, were significantly higher in eyes of MCMV-infected VEGF-A^{hyper} mice, compared to uninfected controls. Initial studies of ocular tissue from human cadavers revealed that HCMV DNA was present in four choroid/retinal pigment epithelium samples from 24 cadavers. Taken together, our data suggest that ocular HCMV latency could be a significant risk factor for the development of AMD. © 2020 Pathological Society of Great Britain and Ireland. Published by John Wiley & Sons, Ltd.

Keywords

cytomegalovirus; age-related macular degeneration; choroidal neovascularization; inflammation; VEGF

Introduction

Age-related macular degeneration (AMD) is the leading cause of irreversible visual impairment and blindness in the elderly population [1]. The advanced stage, neovascular (or ‘wet’) AMD, is characterized by the invasion of new blood vessels from the choriocapillaris into the sub-retinal pigment epithelium (RPE) and/or subretinal spaces (choroidal neovascularization, CNV), which may ultimately result in blindness and accounts for the majority of cases of vision loss in AMD [2–4]. While the precise events which contribute to the development of AMD remain uncertain, a number of studies, using various animal models, have implicated several possible immunological/inflammatory mechanisms [5–7]. In particular, the laser-induced CNV mouse model outlines the angiogenic aspect of neovascular AMD [8,9] and is one of the most widely studied. Although this model of acute injury is reproducible and cost-effective [10], its clinical relevance is limited by the somewhat artificial nature of the CNV induced via laser-stimulated photocoagulation [9].

Changes in levels of pro- and anti-angiogenic growth factors derived from the RPE, particularly vascular endothelial growth factor (VEGF) [11–13], have been considered as a primary, underlying cause of CNV pathogenesis. As a result, VEGF-A-overexpressing mice (VEGF-A^{hyper}), initially developed by Nagy and coworkers [14], have been used as a model of neovascular AMD. These mice exhibit an approximately two- to three-fold increase in VEGF-A protein levels compared with wild-type mice, and develop CNV later in life [15–17]. Since it has previously been reported that ocular neovascularization may occur during the development of cytomegalovirus (CMV) retinitis, a disease with acute symptoms that is found predominantly in immunosuppressed individuals [18–20], we wondered whether neovascular AMD might also have a viral component, especially since it exhibits a significantly higher association with anti-human CMV (HCMV) IgG titers compared with either dry AMD or non-AMD controls [21].

HCMV infects the majority of the human population [22] and is typically acquired in early life when the immune system is immature [23]. The incidence of congenital infection ranges from 0.5% to 2.4% of all live births [24–28], and more than 12% of 1-year-old US infants

were observed to be infected with HCMV [23]. Following primary infection, HCMV persists for the life of the host through cycles of latency and reactivation [29]. Latency can occur in multiple sites/cell types including vascular endothelial and hematopoietic cells [30], while HCMV can be reactivated by a variety of factors including immunosuppression, inflammation [31–34], and cell differentiation [35–37]. Although it is usually kept in check by a healthy immune system and thought to be mostly asymptomatic, increasing evidence suggests that HCMV infection is associated with some long-term diseases including atherosclerosis [38–40], vascular restenosis [41,42], and other vascular pathologies such as hypertension [43,44].

Since CMVs are species-specific, murine cytomegalovirus (MCMV) infection of mice has been widely used to model human diseases in which HCMV is thought to be involved. Although MCMV DNA could not be detected within eye tissues several months after systemic MCMV infection of adult C57 mice, Cousins *et al* [45] showed that systemic MCMV infection was associated with more severe laser-induced CNV, possibly due to activation of macrophages. Our recent studies [46] have produced the novel finding that acquisition of systemic MCMV infection by BALB/c mice during early life is associated with MCMV persistence at several sites including the choroid/RPE. Therefore, the purpose of this study was to investigate whether ocular MCMV could exacerbate the development of CNV following neonatal infection of VEGF-overexpressing VEGF-A^{hyper} and wild-type 129 mice.

Materials and methods

Ethics statement

The human eyes used were de-identified samples purchased at the Georgia Eye Bank Inc and were not from living individuals. The IRB Augusta University (AU) Committee determined that this project did not meet the definition of human subject research under the purview of the IRB according to federal regulations. The breeding and treatment of animals in this study were reviewed and approved by the Institutional Animal Care and Use Committee at Augusta University (Protocol #2009–0252), and also adhered to the ARVO (the Association for Research in Vision and Ophthalmology) Statement and Guidance for the Use of Animals in Ophthalmic and Vision Research.

Virus

The MCMV strain K181 was prepared and used as described in supplementary material, Supplementary materials and methods.

Mice

The VEGF-A hypermorphic mouse (VEGF-A^{hyper}) was purchased from the Jackson Laboratory (Bar Harbor, ME, USA; stock No 027314) and maintained on the 129S1/SvImJ background. Information about this mouse is described in supplementary material, Supplementary materials and methods.

Experimental design – animal model

A hundred plaque-forming units (pfu) of MCMV or culture medium as control was injected intraperitoneally (i.p.) into VEGF-A^{hyper} or wild-type 129 mice at < 3 days after birth. At several subsequent time points, spectral-domain optical coherence tomography (SDOCT) and fluorescein angiography were performed on four groups of mice to determine if pathology indicative of CNV was present, as well as to verify the overall integrity of retinal structure as described in supplementary material, Supplementary materials and methods. Virus- and mock-infected VEGF-A^{hyper} and wild-type 129 mice were sacrificed at 2 weeks or 6 months post-infection (p.i.) and the tissues were harvested for further examination as described in supplementary material, Supplementary materials and methods.

Experimental design – human eyes

Twenty-four pairs of fresh eyes from 24 human cadavers (death-to-preservation times < 12 h) were obtained from Georgia Eye Bank, Inc (Atlanta, GA, USA). Donor information, including age, gender, primary cause of death, and medical history, is listed in supplementary material, Table S1. Immediately following reception of eye tissues, the anterior segment (cornea, ciliary body, and iris), posterior eye cup (choroid/RPE), and retina (neural retina) were carefully dissected and stored at –80 °C. Tissues were subsequently pulverized in a mortar under liquid nitrogen and tissue powders stored at –80 °C for DNA extraction and droplet digital PCR (ddPCR) assay for the presence of HCMV DNA.

Statistical analysis

Means ± standard error of the mean (SEM) were calculated for all data. Unpaired nonparametric Mann–Whitney tests were used to calculate the statistical significance of observed differences between groups in all experiments using the GraphPad Prism software (version 7; GraphPad Software, San Diego, CA, USA). *P* values less than 0.05 were considered statistically significant.

Results

MCMV disseminates to the eye of both VEGF-A^{hyper} and wild-type 129 mice following systemic infection of neonatal mice

100 pfu of MCMV was injected i.p into VEGF-A^{hyper} or wild-type 129 mice at < 3 days after birth. All mice survived viral infection and appeared healthy, with no significant difference in body weight among virus-infected and uninfected VEGF-A^{hyper} and wild-type 129 mice at all time points analyzed (Figure 1A).

At day 14 post-infection (p.i.), mice were sacrificed and eyes and extraocular tissues were collected for analyses. Replicating virus was recovered from the majority of eyes of both VEGF-A^{hyper} and wild-type 129 mice (Figure 1B). Likewise, MCMV DNA was detected in all eyes from both groups of MCMV-infected mice at day 14 p.i. (Figure 1C). In ocular tissues, MCMV infection was observed mainly in the choroid (Figure 1D) and sclera, and was present in all eyes of both VEGF-A^{hyper} (5/5) and WT mice (5/5). Although a few MCMV EA-positive cells were observed in the ciliary body and iris, no MCMV EA staining was located in the neural retina. The average number of EA-positive cells in the choroid (in

nine sections) was 19.2 ± 1.6 per eye for VEGF-A^{hyper} mice and 21.2 ± 1.3 per eye for WT mice, while in the sclera, there were 45.6 ± 2.1 per eye for VEGF-A^{hyper} and 40.2 ± 1.8 per eye for WT mice. Not surprisingly, MCMV was also recovered from systemic tissues/organs including salivary glands, lungs, and kidneys in both groups of MCMV-infected mice (6/6 in each group, Figure 1B). H&E staining showed no remarkable pathological changes in the eyes of VEGF-A^{hyper} or wild-type 129 mice at 14 days p.i. (Figure 1E).

MCMV persists in ocular tissue of both VEGF-A^{hyper} and wild-type 129 mice, but expression of the *MCMV IE1* gene occurs only in VEGF-A^{hyper} mice

At 6 months p.i., no replication-competent MCMV was recovered from eyes or systemic tissues/organs including lungs (0/6), kidneys (0/6), and salivary glands (0/6) of either VEGF-A^{hyper} or wild-type 129 mice. Nevertheless, as reported in our previous investigation of BALB/c mice [46], which are more susceptible to MCMV infection compared with other mouse strains [47,48], MCMV DNA was detected in all eyes of both VEGF-A^{hyper} (9/9) and WT mice (8/8) infected with virus at < 3 days after birth (Figure 1F), whereas MCMV DNA was undetectable in peripheral blood leukocytes of all latently infected mice (0/4, results not shown). Although latent ocular infection of BALB/c mice was associated with expression of MCMV immediate early (IE) genes [46], *MCMV IE1* and *IE3* mRNA was undetectable in all eyes of latently infected wild-type 129 mice (Figure 1G). In contrast, *MCMV IE1* mRNA was detected in 6/13 eyes of VEGF-A^{hyper} mice at 6 months post-neonatal infection (Figure 1G), while no *MCMV IE3* or late gene *gB* transcripts were detected in eyes of either VEGF-A^{hyper} mice or wild-type 129 mice at this time (results not shown).

MCMV-infected VEGF-A^{hyper} mice exhibit increased choroidal neovascularization compared with uninfected VEGF-A^{hyper} mice

Multiple techniques were used to evaluate CNV lesions in infected and uninfected VEGF-A^{hyper} and WT mice at 6 months p.i.

SD-OCT is a noninvasive imaging technique providing high-resolution, cross-sectional images of the retinal microstructure *in vivo* [49,50] and is widely used to monitor retinal abnormalities including CNV lesions [51,52]. Similar to previous observations on VEGF-A^{hyper} C57 mice or white mice [17], cataracts were present in eyes of most VEGF-A^{hyper} mice at 6 months of age. Although we did not perform SD-OCT examinations in some infected (3/18) and uninfected (1/22) VEGF-A^{hyper} eyes due to the presence of severe cataracts, we did perform SD-OCT examinations in the majority of mice using an Envisu R2210 system. This experiment demonstrated that CNV lesions were observed in significantly more eyes (13/15) from MCMV-latently-infected VEGF-A^{hyper} mice compared with uninfected VEGF-A^{hyper} mice (9/21) (chi-square, $p < 0.01$). In addition to an increased frequency of mice exhibiting CNV, MCMV-infected VEGF-A^{hyper} mice (Figure 2A, lower right) also exhibited significantly more lesions per eye (Figure 2B, left) and a larger average lesion volume (Figure 2B, right), compared with uninfected VEGF-A^{hyper} mice (Figure 2A, upper right). CNV lesions were not observed in any eyes of infected (0/8) or uninfected (0/8) wild-type 129 mice (Figure 2A, lower left and upper left).

Due to the presence of cataracts, fluorescein angiography was successful in only half of infected (10/22) and uninfected (12/24) VEGF-A^{hyper} eyes, although the results were similar to the SD-OCT data. As shown in Figure 2C, an increased frequency of CNV was observed in MCMV-infected VEGF-A^{hyper} mice (6/10 positive), compared with uninfected VEGF-A^{hyper} mice (3/12 positive) (chi-square, $p < 0.01$).

To more precisely localize CNV lesions, frozen eye sections were stained with hematoxylin and eosin (H&E) (Figure 2D) as well as with anti-CD31 antibody (Figure 2E), a widely used marker of endothelial differentiation, although not entirely specific. The majority of CNV lesions in both MCMV-infected and uninfected VEGF-A^{hyper} mice were located above the RPE layer, either within the subretinal space as indicated in Figure 2D (upper right corner) or growing into the inner retina as indicated by Figure 2D,E (both in lower right corner). Consistent with previous studies by other investigators, which have shown that progressive, age-dependent photoreceptor degeneration occurs in VEGF-A^{hyper} mice [15–17], we observed a significantly thinner outer nuclear layer (ONL) in 6-month-old VEGF-A^{hyper} mice, compared with wild-type 129 mice in non-CNV areas (Figure 3A,B). In contrast, latent MCMV infection appeared to produce no remarkable effects on photoreceptor degeneration in VEGF-A^{hyper} mice as there was no significant difference in the thickness of the ONL between MCMV-infected and uninfected VEGF-A^{hyper} mice (Figure 3A,B).

Electron microscopy

To determine if other pathological changes typical of AMD, such as deposits at basal or apical aspects of the RPE, are associated with VEGF high expression and/or MCMV infection, eyes were collected from MCMV-infected VEGF-A^{hyper} and WT mice at 6 months p.i., as well as from age-matched, uninfected controls, and processed for examination by electron microscopy. Although a thinner ONL was observed in infected and uninfected VEGF-A^{hyper} mice in non-CNV areas, as shown in Figure 3D,F, no remarkable pathological changes such as deposits or cell death were observed in the RPE layer or inner retina. However, in areas where CNV developed, the morphology of nearby photoreceptors was disturbed, with shortening and loss of outer segments (Figure 3G, indicated by arrowheads). Furthermore, we observed that pigmented RPE-like cells appeared to proliferate and/or migrate along new vessels into the inner retina, as indicated by arrows in Figure 3H.

MCMV infection of VEGF-A^{hyper} mice increases ocular VEGF-A levels

Protein levels of VEGF-A in plasma and eyes of VEGF-A^{hyper} and WT mice were assayed by ELISA. Significantly higher levels of VEGF were present in plasma (Figure 4A) and eyes (Figure 4B) of VEGF-A^{hyper} mice, compared with WT mice, irrespective of the presence of virus. However, significantly more VEGF was detected in eyes of MCMV-infected VEGF-A^{hyper} mice compared with eyes of uninfected VEGF-A^{hyper} mice at 6 months p.i. (Figure 4B), whereas similar amounts of VEGF were detected in eyes of infected and uninfected WT mice. These results indicate that the presence of latent ocular MCMV further increased VEGF protein levels in eyes of VEGF-A^{hyper} mice.

The previous studies of Cousins et al [45] showed that systemic MCMV infection was associated with more severe laser-induced CNV in the C57 mouse strain. One possible mechanism might be related to macrophage activation [45]. Therefore, in order to localize VEGF production in the eye and also to determine if MCMV infection was related to recruitment of macrophages to CNV lesions, we performed immunofluorescence staining for both VEGF and the macrophage marker F4/80. As shown in Figure 4C, only a few VEGF-positive cells were observed in the choroid and retinal vessels from MCMV-infected and uninfected WT mice. In contrast, many VEGF-positive cells were found in the choroid, RPE layer, and inner retina of both MCMV-infected and uninfected VEGF-A^{hyper} mice. Not surprisingly, VEGF staining was particularly strong in areas with CNV lesions. F4/80-positive cells were rarely observed in WT eyes whether MCMV-infected or uninfected, whereas some F4/80-positive macrophages were observed in CNV lesions in both MCMV-infected (Figure 4C, lower right) and uninfected (Figure 4C, upper right) VEGF-A^{hyper} mice. Although some F4/80-positive macrophages were also VEGF-positive in both infected (Figure 4C, lower right) and uninfected (Figure 4C, upper right) VEGF-A^{hyper} mice (indicated by yellow arrows), the majority of VEGF-positive cells were not macrophages. Furthermore, as shown in Figure 4D, similar numbers of macrophages were observed in CNV lesions of infected VEGF-A^{hyper}, and uninfected VEGF-A^{hyper} mice.

Latent ocular MCMV infection is associated with upregulation of inflammatory/angiogenetic factors in VEGF-A^{hyper} mice

HCMV has been shown to induce angiogenesis via the production of many inflammatory/angiogenic factors [53] including VEGF [45,54,55], transforming growth factor beta (TGF- β) [56], and the cytokines/chemokines IL-6 [57,58] and C-C motif chemokine ligand 5 (CCL5) [59], while our previous studies have indicated that the receptor-interacting protein kinase 3 (RIP3) enhances innate immune responses against ocular MCMV infection [60,61]. In addition, previous studies have showed that GFAP-positive glial cells are activated following ocular MCMV infection [62], and activated glial cells might promote CNV [16]. To determine if latent ocular MCMV infection or VEGF high expression was associated with increased expression of inflammatory/angiogenic factors, the protein levels of these factors were measured by western blot and ELISA. Protein levels of ocular RIP3 (Figure 5A,B) and GFAP (Figure 5A,F) were altered by both MCMV infection and VEGF overexpression since they were significantly higher in MCMV-infected WT eyes, as well as in both infected and uninfected VEGF-A^{hyper} eyes, compared with uninfected WT eyes at 6 months p.i., although significantly more RIP3 protein was detected in infected VEGF-A^{hyper} eyes compared with MCMV-infected WT eyes or uninfected VEGF-A^{hyper} eyes. Protein levels of ocular TGF- β 1 (Figure 5A,C) and CCL5 (Figure 5G) spiked during initial systemic MCMV infection but there was no association with VEGF overexpression since the ocular protein levels of both factors were similarly elevated in eyes of both MCMV-infected WT and MCMV-infected VEGF-A^{hyper} mice, compared with uninfected VEGF-A^{hyper} eyes. In contrast, IL-6 protein levels were significantly elevated in eyes of latently infected VEGF-A^{hyper} mice only, but not in eyes of mice belonging to the other three experimental groups (Figure 5H).

HCMV DNA is present in human ocular tissue

Since systemic MCMV infection of mice early in life resulted in MCMV latency in the choroid/RPE, we hypothesized that HCMV might spread to and become latent in the choroid/RPE of human eyes. To test this hypothesis, the anterior segment (cornea, ciliary body, and iris), posterior eye cup (choroid/RPE), and retina (neural retina) were dissected from 48 fresh eyes of 24 human cadavers obtained from Georgia Eye Bank, Inc. DNA was extracted and analyzed for the presence of HCMV DNA by ddPCR, a technology emulsifying an oil-based PCR reaction into thousands of droplets, each of which then acts as a PCR micro-reaction and increases the chances of rare target sequences being detected [63], as evidenced by its use in the detection of various pathogens [64–66] including latent HCMV infections [67,68]. As shown in supplementary material, Table S2, HCMV DNA was detected in four posterior eye cups (choroid/RPE) of four individual cadavers. In all four cases, infection was unocular and no HCMV DNA was detected in contralateral eye tissue from the same cadaver. In addition, very low HCMV DNA copy numbers were detected in the retina of a fifth cadaver (1 of 48 retina samples), while no HCMV DNA could be detected in all 48 anterior segment samples. This result confirms our hypothesis that the human choroid/ RPE is a relatively common site for HCMV latency.

Discussion

The data presented in this article indicate that latent ocular MCMV infection exacerbates choroidal neovascularization in VEGF-A-overexpressing mice, suggesting that closer study of the role of cytomegaloviruses in AMD may be warranted. Systemic MCMV infection spreads to the choroid and becomes latent in both VEGF-overexpressing VEGF-A^{hyper} and wild-type 129 mice but *MCMV IE1* gene expression is activated only in the eyes of latently infected VEGF-A^{hyper} mice. Since *MCMV IE* genes are first activated in the absence of *de novo* synthesis of viral proteins during virus reactivation [35], overexpression of VEGF-A could activate IE gene expression via the following pathways:

1. Cell proliferation and differentiation. Cytomegalovirus can be reactivated by several forms of stimulation including cell differentiation [35–37]. CNV requires proliferation/differentiation of choroidal endothelia and pericytes [69,70], in addition to differentiation of blood hematopoietic stem cells and monocytes to macrophages [69]. Our previous studies have shown that choroidal endothelia, pericytes, and RPE cells may contain latent virus following systemic neonatal MCMV-infection of BALB/c mice [46]. Macrophages originating from CD34⁺ progenitor cell-derived monocytes may also contain latent MCMV [30,34], although MCMV DNA was not detected in peripheral blood leukocytes in this study. Therefore, proliferation and differentiation of latently infected ocular cells or even macrophages – during the development of CNV in VEGF-A^{hyper} mice – could activate *MCMV IE* genes.
2. Immunosuppression. Cytomegaloviruses can be reactivated from latency by immunosuppression [31–34]. In addition to its crucial role in promoting the growth of new blood vessels, VEGF also exhibits some immunosuppressive properties. For instance, VEGF can suppress the function of T cells and modulate

anti-tumor immunity via the recruitment of regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), and inhibition of the differentiation and activation of dendritic cells [71]. In addition, VEGF might also modulate anti-virus immunity [72]. Therefore, overexpression of VEGF-A in the choroid/RPE layer of VEGF-A^{hyper} mice could create an immunosuppressed environment to benefit MCMV latency and facilitating reactivation, resulting in *IE* gene expression. In addition, signaling events associated with inflammation are related to HCMV reactivation [30]. Recent studies suggest that the key inflammatory mediator IL-6 has multiple effects on HCMV reactivation ranging from increased *IE* gene expression to more efficient reactivation of infectious progeny [36,73,74]. Reactivation of latent virus from dendritic cells of healthy individuals can be significantly abrogated using neutralizing antibodies specific for IL-6 [74,75]. Therefore, increased production of IL-6 during ocular MCMV latency of VEGF-A^{hyper} mice might also contribute to the activation of MCMV gene expression.

Expression of *MCMV IE1*, but not *IE3* or the *gB* late gene, was detected in eyes of MCMV-infected VEGF-A^{hyper} mice at 6 months p.i. *IE1* is a protein considered to be a key activator of the virus productive cycle and expression of the *IE1* gene has been used as a marker of reactivation, since it is presumed that the classic cascade of gene expression and progeny virus production will follow. However, the latent state is more dynamic than originally appreciated, and major immediate-early enhancer (MIE) expression alone does not guarantee full reactivation and production of progeny virus [35]. Ectopic expression of the *IE1* proteins might not be sufficient to drive viral genome synthesis or infectious progeny production in infected cells [76], although it does influence the cellular environment [35]. Previous studies have suggested that HCMV *IE1* alone can trigger a pro-inflammatory host transcriptional response via a STAT1-dependent mechanism [77,78]. In contrast, another study reported that the presence of MCMV *IE1* potently inhibits the pro-inflammatory cytokine response including TNF α and IFN β [79]. Therefore, expression of *IE1* in the eye could modulate the production of inflammatory/angiogenic factors in MCMV latently infected VEGF-A^{hyper} mice, thereby facilitating the development of CNV. In this study, approximately one half of the eyes (7/13) of infected mice did not express the *IE1* gene at 6 months p.i. Since there is evidence which suggests that CMV latency is a highly dynamic condition, in which *IE* genes are expressed intermittently [80], latently infected eyes which did not express *IE1* at 6 months p.i. would still express *IE1* at other times during the course of the disease. Nevertheless, there is still the possibility that other viral genes or non-coding RNAs are expressed and contribute to disease. Previous results indicate that HCMV IL-10 [81] can bind to the human IL-10 receptor and induce the production of VEGF and TGF- β [82].

Several inflammatory/angiogenic factors including CCL5, TGF- β 1, RIP3, and IL-6 were significantly upregulated in eyes of both latently infected VEGF-A^{hyper} and wild-type 129 mice, compared with uninfected control eyes from age-matched VEGF-A^{hyper} or WT mice. Interestingly, levels of several cytokines, particularly IL-6, a principal cytokine in infection, cancer, inflammation, and angiogenesis [83], were significantly higher in eyes of latently infected VEGF-A^{hyper} mice compared with latently infected WT mice. IL-6 can be produced

by almost all stromal cells and immune cells as a result of various types of stimulation [83]. It is a potent pleio-tropic cytokine and has context-dependent pro- and anti-inflammatory properties [84] while mediating a plethora of physiological functions [85–89]. IL-6 also plays a significant role in ocular angiogenesis. Previous studies from Izumi-Nagai *et al* [90] have demonstrated that IL-6 plays a critical role in CNV generation associated with AMD via activation of signal transducer and activator of transcription-3 (STAT3). Likewise, a study by Rojas *et al* [91] showed that IL-6 expression was essential for angiotensin II-induced increases in retinal VEGF expression, leukostasis, and vascular remodeling. Furthermore, IL-6 contributes to HCMV-induced angiogenesis [57,58] and was significantly induced during HCMV infection of endothelial cells. Virus-free supernatants from infected endothelial cells induced angiogenesis and neovessel formation, which was significantly reduced following neutralization of IL-6 [58]. Therefore, increased production of IL-6 during ocular MCMV infection of VEGF-A^{hyper} mice might exacerbate the development of CNV directly or indirectly via activation of STAT3 and subsequent production of VEGF.

In summary, the results presented herein demonstrate that acquiring systemic MCMV infection neonatally leads to ocular latency in 129 mice; prior overexpression of VEGF-A in MCMV latently infected VEGF-A^{hyper} mice induces activation of ocular virus gene expression and production of inflammatory/angiogenic factors including IL-6 and VEGF, and therefore enhances the development of CNV. Since a significant percentage of the human population acquires HCMV during early life and HCMV DNA was detected in the choroid/RPE of approximately 17% of the human cadavers analyzed, we would predict that ocular HCMV latency could be a significant risk factor for the development of AMD, although further studies are needed to determine which cells/tissue types within the RPE/choroid contain latent virus and virus gene expression in human AMD eyes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. MCMV disseminates to and becomes latent in the eye following systemic infection of neonatal VEGF-A^{hyper} or wild-type 129 mice. (A) Postnatal changes in body weight in male (left) and female (right) neonatally-infected VEGF-A^{hyper} or WT mice, compared with uninfected control male and female mice of the same age ($n = 20-30$). (B) Titer of MCMV (upper) and MCMV positivity rate (lower) at day 14 p.i. in eyes, salivary glands, lungs, and kidneys determined by plaque assay ($n = 5-8$; $*p < 0.05$). (C) PCR assay for MCMV DNA in eyes of VEGF-A^{hyper} and WT mice at day 14 p.i. All mice tested positive. (D) Merged photomicrographs of ocular staining for RPE65 (red), MCMV EA (green), and DAPI (blue)

from an infected WT mouse (left) and an infected VEGF-A^{hyper} mouse (right) at day 14 p.i. The majority of MCMV EA-positive cells are located in the choroid (scale bars: 50 μ m). (E) Photomicrographs of ocular H&E staining of an infected WT mouse; an infected VEGF-A^{hyper} mouse at day 14 p.i.; an age-matched, uninfected control WT mouse; and an age-matched uninfected VEGF-A^{hyper} mouse (scale bars: 100 μ m). (F) PCR assay for MCMV DNA in eyes of VEGF-A^{hyper} and WT mice at 6 months p.i. All eyes tested positive. (G) RT-PCR assay for MCMV IE1 mRNA in MCMV-infected VEGF-A^{hyper} and WT mice at 6 months p.i. IE1 mRNA was detected in 6 of 13 eyes from VEGF-A^{hyper} mice, while all 15 eyes from MCMV-infected WT mice tested negative.

Figure 2.

MCMV infection exacerbates CNV in VEGF-A^{hyper} but not in WT mice. (A) Representative images of SD-OCT from infected WT (lower left) and VEGF-A^{hyper} mice (lower right) at 6 months p.i.; also age-matched, uninfected WT (upper left) and VEGF-A^{hyper} mice (upper right). CNV lesions were found in MCMV-infected and uninfected VEGF-A^{hyper} mice (indicated by red squares). (B) The numbers (left) and volumes (right) of CNV lesions in MCMV-infected and uninfected VEGF-A^{hyper} mice (**p* < 0.05). (C) Fluorescein angiography images of MCMV-latently-infected and uninfected VEGF-A^{hyper} and WT mice. (D) Photomicrographs of ocular H&E staining of MCMV-infected WT (lower left) and VEGF-A^{hyper} mice (lower right) at 6 months p.i., as well as from age-matched,

uninfected WT (upper left) and VEGF-A^{hyper} mice (upper right). CNV lesions were observed in the subretinal space (upper right) and inside the inner retina (lower right) (scale bars: 100 μ m). (E) Merged photomicrographs of ocular staining for CD31 (red) and DAPI (blue) from infected WT (lower left) and VEGF-A^{hyper} mice (lower right) at 6 months p.i., as well as from age-matched, uninfected WT (upper left) and VEGF-A^{hyper} mice (upper right). CD31-stained neovessels are present in the inner retina (lower right) (scale bars: 100 μ m).

Figure 3. Features of photoreceptor degeneration and CNV lesions in VEGF-A^{hyper} mice. (A, B) Photomicrographs of ocular H&E staining (A) and analysis of ONL thickness (B) among infected and uninfected WT and VEGF-A^{hyper} mice 6 months p.i. A significantly thinner outer nuclear layer (ONL) was observed in MCMV-infected and uninfected VEGF-A^{hyper} mice, compared with infected and uninfected WT mice (scale bars: 400 μm). ** $p < 0.01$; **** $p < 0.0001$. (C–H) Representative electron micrographs of ocular ultrastructure from infected WT (E) and VEGF-A^{hyper} mice (F, G, H) at 6 months p.i.; also age-matched, uninfected WT (C) and VEGF-A^{hyper} mice (D). No remarkable pathological changes were observed in the RPE layer or inner retina of non-CNV areas of both infected (F) and uninfected (D) VEGF-A^{hyper} mice. Some CNV lesions (indicated by asterisks) were observed in the RPE layer, surrounded by pigmented RPE-like cells (G). In addition, the

morphology of nearby photoreceptors was disturbed, with shortening and loss of outer segments (G, indicated by arrowheads). Some CNV lesions (indicated by asterisks) grew into the inner retina (H) and many pigmented RPE-like cells were observed adjacent to new vessels in the inner retina (H, indicated by arrows). No remarkable pathological changes were observed in the RPE layer or inner retina of infected (E) or uninfected (C) WT mice.

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Figure 4.

MCMV-infected VEGF-A^{hyper} mice contain increased ocular VEGF-A compared with uninfected VEGF-A^{hyper} mice at 6 months p.i. (A) Protein levels of VEGF in plasma assayed by ELISA ($n = 6$; $*p < 0.05$; $***p < 0.001$; $****p < 0.0001$). (B) Protein levels of ocular VEGF assayed by ELISA ($n = 6$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$). (C) Merged photomicrographs of ocular staining for F4/80 (red), VEGF (green), and DAPI (blue) of MCMV-infected WT (lower left) and VEGF-A^{hyper} mice (lower middle and right) at 6 months p.i., as well as from age-matched, uninfected WT (upper left) and VEGF-A^{hyper} mice (upper middle and right). A few F4/80-stained cells were observed in CNV lesions, some of which co-stained with VEGF (yellow arrows), while some were VEGF-negative (red arrows). The majority of VEGF-stained cells were not F4/80 macrophages (white arrows) (scale bars: 100 μm). (D) The number of F4/80-stained cells per CNV lesion in MCMV-infected and uninfected VEGF-A^{hyper} mice at 6 months p.i.

Figure 5. Protein levels of several inflammatory/angiogenic factors were increased in eyes of infected VEGF-A^{hyper} mice at 6 months p.i. (A–F) Western blots of RIP3, TGF-β1, TGF-β-R1, TGF-β-R2, and GFAP in eyes of MCMV-infected or uninfected WT and VEGF-A^{hyper} mice at 6 months p.i. Ratio of RIP3 to β-actin(B), TGF-β1 to β-actin(C), TGF-β-R1 to β-actin(D), TGF-β-R2 to β-actin(E), and GFAP to β-actin(F). (G, H) Protein levels of CCL5 (G) and IL-6(H) in eyes of MCMV-infected or uninfected WT and VEGF-A^{hyper} mice at 6 months p.i. Data are shown as mean ± SEM (for western blot, $n = 3$; for ELISA, $n = 4$) and compared by t -test. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.