Research Article

Cytomegalovirus (CMV) Infection Causes Degeneration of Cochlear Vasculature and Hearing Loss in a Mouse Model

MATTIA CARRARO,^{1,2} ALI ALMISHAAL,³ ELAINE HILLAS,⁴ MATTHEW FIRPO,⁴ ALBERT PARK,^{4,5} AND ROBERT V. HARRISON^{1,2,6}

¹*Institute of Biomaterials and Biomedical Engineering, University of Toronto, Toronto, ON, Canada*

²*Auditory Science Laboratory, Neuroscience and Mental Health Program, The Hospital for Sick Children, Toronto, ON, Canada*

³*Department of Communication Sciences and Disorders, University of Utah, Salt Lake City, UT, USA*

⁴*Department of Surgery, University of Utah, Salt Lake City, UT, USA*

⁵*Department of Otolaryngology, University of Utah, Salt Lake City, UT, USA*

⁶*Department of Otolaryngology, Head and Neck Surgery, University of Toronto, Toronto, ON, Canada*

Received: 12 October 2016; Accepted: 24 November 2016; Online publication: 19 December 2016

ABSTRACT

Cytomegalovirus (CMV) infection is one of the most common causes of congenital hearing loss in children. We have used a murine model of CMV infection to reveal functional and structural cochlear pathogenesis. The cerebral cortex of Balb/c mice (*Mus musculus*) was inoculated with 2000 pfu (plaque forming units) of murine CMV on postnatal day 3. At 6 weeks of age, cochlear function was monitored using auditory brainstem response (ABR) and distortion product otoacoustic emission (DPOAE) measures. Histological assessment of cochlear vasculature using a corrosion cast technique was made at 8 weeks. Vascular casts of mCMV-damaged cochleas, and those of untreated control animals, were examined using scanning electron microscopy. We find very large variations in the degree of vascular damage in animals given identical viral injections (2000 pfu). The primary lesion caused by CMV infection is to the stria vascularis and to the adjacent spiral limbus capillary network. Capillary beds of the spiral ligament are generally less affected. The initial vascular damage is found in the mid-apical turn and appears to progress to more basal cochlear regions. After viral migration to the inner ear, the stria vascularis is the primary affected structure. We suggest that initial auditory

threshold losses may relate to the poor development or maintenance of the endocochlear potential caused by stria dysfunction. Our increased understanding of the pathogenesis of CMV-related hearing loss is important for defining methods for early detection and treatment.

Keywords: congenital hearing loss, sensorineural deafness, corrosion casting, scanning electron microscopy, spiral limbus, stria vascularis, spiral ligament, endocochlear potential, endolymphatic potential

INTRODUCTION

Congenital cytomegalovirus (CMV) is the one of the most common causes of sensorineural hearing loss in children and an important cause of neurodevelopmental delay (e.g., Demmler 1991; Grosse et al. 2008; Foulon et al. 2008, 2012; Manicklal et al. 2013; Delany et al. 2014). The incidence of CMV infection within various geographic regions is estimated to be about 0.5 %. In the USA, approximately 20,000 infants develop CMV infection annually; of these, 2000 are symptomatic at birth and suffer severe neurologic sequela. Approximately 18,000 are asymptomatic at birth but develop significant handicaps such as hearing impairment (Demmler 1991;

Correspondence to: Robert V. Harrison · Department of Otolaryngology, Head and Neck Surgery · University of Toronto · Toronto, ON, Canada. Telephone: 1 416 813 6535; email: rvh@sickkids.ca

Manicklal et al. 2013). Quality of life and treatment-cost burdens are significant, and the development of new CMV vaccines is a high priority (Delany et al. 2014).

A characteristic of CMV-induced sensorineural hearing loss is that it is progressive (Fowler et al. 1997, 1999; Fowler & Boppana 2006; Royackers et al. 2011; Foulon et al. 2012; Goderis et al. 2014; Park et al. 2014). Many children with unilateral hearing loss diagnosed at school age likely had a missed, asymptomatic infection (Choi et al. 2009; Goderis et al. 2014). Misono et al. (2011) reported congenital CMV infection in about 10 % of cases of children of 4 years of age or older presenting with idiopathic hearing loss. This problem of delayed (missed) diagnosis underlines the importance of understanding the pathogenesis of inner ear CMV infection and of finding early diagnostic biomarkers.

The newborn mouse is analogous to the human fetus in utero (Otis & Brent 1954). At birth, the mouse auditory system is immature and normal hearing thresholds take weeks to develop (e.g., Kasai et al. 1981). We have previously demonstrated that newborn (BALB/c) mice infected with murine CMV (mCMV) results in a labyrinthitis that shares many characteristics of human virus-mediated hearing loss (Wang et al. 2013). Thus, after intracerebral inoculation of newborn mice, 45 % initially showed a 30–50 dB ABR threshold shift at 4 weeks of age that progressed to greater than 80 dB at 6–8 weeks. These findings mirror longitudinal clinical studies that show that 50 % of children with CMV-induced hearing loss have a deterioration of thresholds over time (Fowler et al. 1997, 1999). Further evidence for the validity of our mouse model relates to the onset time of CMV infection. Human data shows an age-dependent effect such that children are more likely to develop hearing loss after maternal CMV infection during the first trimester than later in pregnancy (Pass et al. 2006). By analogy, in our mouse model, all postnatal day 3 (P3) infected mice had elevated ABR thresholds at 4 weeks of age; of those inoculated at P8, only 15 % had ABR threshold shifts. Mice infected at P14 showed no ABR threshold changes (Wang et al. 2013).

While we have extensive documentation on the functional consequences of CMV-related hearing loss, little is known about CMV pathogenesis in the cochlea. Given this background, we present here a detailed assessment of all the capillary networks of the cochlea in the CMV-infected mouse model. Using a modified corrosion casting technique (Carraro et al. 2016; Carraro & Harrison 2016), we have examined spiral ligament vessels, stria vascularis, and the spiral limbus capillary beds associated with the organ of Corti and the spiral ganglion. We describe the degree and extent of capillary degeneration in each capillary

bed and the distribution of damage along the cochlear length.

METHODS

In overview, the cerebral cortex of the Balb/c mouse (*Mus musculus*) was inoculated with mCMV at postnatal day 3 (P3). Cochlear function was monitored at 6 weeks using ABR and DPOAE measures. Histological assessment of cochlear vasculature using a partial corrosion cast technique was made at 8 weeks of age. Inner ear vascular casts were examined using scanning electron microscopy. A total of 25 animals were used in this project. For the corrosion cast study, seven animals were inoculated with mCMV and compared with 10 age-matched controls. For the functional assessments with ABR and DPOAE, four mice were CMV inoculated (identical protocol to the histological study) with four normal controls. All procedures were approved by the Hospital for Sick Children (Toronto) Animal Care Committee (following Canadian Council for Animal Care guidelines) and the University of Utah, Institutional Animal Care and Use Committee (protocol #11-09004). Animals were housed and bred under specific pathogen-free conditions at the Central Animal Facility at the University of Utah.

CMV INFECTION

We use a recombinant mCMV (strain K181 MC.55 [ie2GFP⁺]) that expresses green fluorescent protein (GFP). For preparation of this virus, M2-10B4 murine fibroblast cells (American Type Culture Collection #CRL-1972, Manassas, VA) were grown in complete medium (minimal essential medium, 10 % (v/v) fetal bovine serum (FBS), 2 mM l-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 10 nM HEPES). Once 70 % confluent, the viral inoculum was added and the cells were incubated to reach an 80 to 100 % cytopathic effect. Each was then subjected to three freeze–thaw cycles and the resulting viral supernatant was collected. Virus purification, carried out by Virapur (San Diego, CA), was as follows: cellular debris is removed by centrifugation (1000×g) at 4 °C, and the virus was pelleted (23,000×g; 2 h at 4 °C) in 35 % sucrose solution with Tris-buffered saline (50 mM Tris–HCl, 150 mM NaCl; pH 7.4). The pellet was re-suspended in Tris-buffered saline containing 10 % FBS. Viral stock titers were determined on M2-104B cells as 50 % tissue culture infective doses (TCID₅₀) per milliliter. Virulent, salivary gland-pas-

saged, sucrose gradient-purified virus was used for all intracerebral injections.

At P3, pups were inoculated with 2 μ l of 2000 pfu (plaque forming units) of mCMV by injection into the right cerebral hemisphere (Wang et al. 2013). To achieve anesthesia, pups were placed on ice, then a 30G injection needle was inserted past the calvarium into the mid-parietal cortex. Control mice were not inoculated. Mice were monitored for adverse effects, including mortality, behavioral abnormalities, and developmental delay. Experimental and control animals were housed separately.

ASSESSMENT OF COCHLEAR FUNCTION

At 6 weeks of age, ABR and DPOAE measures of cochlear function were made in experimental ($n = 4$) and control mice ($n = 4$) as we have previously described (Wang et al. 2013). In brief, animals were anesthetized (i.p. injection) with ketamine (100 mg/kg) and xylazine (10 mg/kg). ABR and DPOAE recordings were made in a double-walled sound chamber (IAC, Bronx, NY). Body temperature was maintained at 37 °C. A small incision was made at the tragus to allow direct access to the ear canal. ABR thresholds were obtained to tone pips at 8, 16, and 32 kHz. Responses from stimulation of each ear were measured. Stimuli were presented using a high-frequency electrostatic speaker (EC-1, Tucker-Davis Technology (TDT) Alachua, FL) fitted with a 1.5-cm polyethylene tube positioned into the ear canal. Tone pips were presented at 24–32/s. Stimuli were presented over a 15–90 dB SPL range of intensity in 5 or 10 dB steps. Evoked potentials were measured via needle electrodes in a vertex/mastoid configuration. ABR signals were amplified (TDT RA4), filtered 100 Hz to 3 kHz, and averaged (1024 sweeps; TDT RA16BA controlled by BioSigRP software; Tucker-Davis Technology). Threshold responses were determined by visual inspection of ABR waveforms. If no signal was recorded at 90 dB SPL, the cochlea was considered to be non-responsive. The DPOAEs were measured using a microphone coupled with two speakers (ER-10B+ and 2xEC1 Etymotic Research, Elk Grove, IL). Stimuli of two primary tones f_1 and f_2 ($f_2/f_1 = 1.2$) were presented with $f_2 = f_1 - 10$ dB. Primary tones were stepped from 30 to 80 dB SPL (for f_1) in 10 dB increments and in octave steps at 8, 16, and 32 kHz. The ear canal acoustic emissions were amplified and digitized. Signals at f_1 , f_2 , and $2f_1 - f_2$ were determined by FFT after spectral averaging from 50 waveform traces. The noise floor was also measured. Statistical analysis of ABR and DPOAE data was carried out with GraphPad Prism 7, using Welch's t

test (Gaussian distribution, different SD, two-tailed p value).

CORROSION CASTING

At 8 weeks of age, cochlear vasculature was assessed with a novel, partial corrosion casting method. An identical protocol was applied to mCMV-infected mice ($n = 7$) and untreated control animals ($n = 10$). A detailed description of this technique has recently been published (Carraro et al. 2016); the salient steps are as follows. The mice were anesthetized with i.p. ketamine (100 mg/kg) and xylazine (10 mg/kg). After exposing the heart, the aorta was cannulated for polymer perfusion, and the right atrium was cut to allow blood escape. To achieve complete vascular casting, all perfusion steps were done under strict temperature and pressure control. To flush out blood, we infused 50 ml of heparinized (100 U/ml) PBS at 40 °C and at 80 mmHg pressure. Then, also at 40 °C, Mercor II polymer (Ladd Research Industries, Williston, VT) plus catalyst was infused at 170 mmHg. Twelve hours was allowed for complete polymerization, then the inner ear specimen was immersed in 16 % KOH at 50–55 °C for 30 min. The cochlea was partially corroded with ethylene-diamine tetraacetic acid (EDTA; 10 % at 55 °C) for 25–30 min. Only part of the temporal bone was digested such that a bony scaffold remained to support the cochlear vascular cast.

VASCULAR IMAGE ACQUISITION

For electron microscopy, casts were washed in distilled water, immersed in 100 % ethanol and critical-point dried. Gold-sputtered specimens were viewed under a scanning microscope (Hitachi 3400 s) as illustrated by Figure 1. For analysis, all the images were acquired at the same (250 $\times$) magnification. After image scale calibration, vessel diameters were directly measured with ImageJ software (Schneider et al. 2012). Experimental and control vessel diameters were compared using Welch's t test (Gaussian distribution, different SD, two-tailed p values) implemented with GraphPad Prism 7 software.

RESULTS

Cochlear Dysfunction After CMV Infection

At 6 weeks of age, animals inoculated with mCMV showed clear evidence of elevated ABR thresholds

FIG. 1. Scanning microscopy image of the vascular anatomy of the (normal) mouse cochlea revealed by partial corrosion casting. Most evident are the overlapping stria vascularis and spiral ligament capillary beds of the cochlear wall.

and reduced DPOAE amplitude. Figure 2 (left panels) shows ABR thresholds for CMV-infected mice ($n = 8$ ears, 4 subjects) versus normal age-matched controls ($n = 8$ ears, 4 subjects). In the right panels, DPOAE thresholds for infected and control animals are compared ($n = 8$ ears, 4 subjects for each group). The upper graphs show ABR and

DPOAE thresholds at 8, 16, and 32 kHz; these values are averaged for the lower histograms. In all plots, standard error of the mean is reported. Using Welch's t test (Gaussian distribution, different SDs, two-tailed p value), we find a significant elevation in average ABR thresholds in CMV-infected animals, $t(42) = 12.32$, $p < .0001$, compared with controls. For DPOAE comparisons, there is a significant elevation in averaged thresholds in CMV treated mice, $t(48) = 12.99$, $p < .0001$, compared with untreated controls.

Cochlear Vasculature

In the cochlea, there are three major vascular beds, namely the spiral ligament vessels, stria vascularis, and the spiral limbus vasculature. Vessels of the spiral ligament and the stria are in the cochlear wall. Note in the scanning images of corrosion casts (Figs. 1 and 3) that spiral ligament vessels overlay the stria vascularis, with their respective capillaries running roughly perpendicular to each other. The spiral limbus network is central within the cochlea and feeds organ of Corti and spiral ganglion cell regions. Note that a corrosion cast reflects the dimensions of the vessel lumen and not the complete vessel.

FIG. 2. Upper left panel: ABR thresholds at 8, 16, and 32 kHz, for mCMV-infected mice compared to control animals (eight ears in each group). Upper right panel: DPOAE thresholds determined at 8, 16, and 32 kHz (f_2) for control versus mCMV-infected mice (eight ears in each group). The lower histograms show ABR (left) and DPOAE (right) data averaged across frequency. All plots

show standard error of the mean. There is a significant elevation in average ABR thresholds in CMV-infected animals, $t(42) = 12.32$, $p < .0001$, compared with controls. For DPOAE measures, note a significant elevation in averaged thresholds in CMV treated mice, $t(48) = 12.99$, $p < .0001$, compared with untreated controls.

FIG. 3. Various degrees of cochlear vascular damage caused by mCMV infection (2000 pfu). **a** Normal control; no damage is evident. **b** Mid-apical turn; note the narrow capillary diameters of the stria vascularis (horizontally oriented behind the spiral ligament vessels).

In animal **(c)**, there is extensive degeneration of all the vasculature of the apical turn, including the spiral limbus. For the mouse represented in panel **d**, there is widespread loss of all vascular networks throughout the cochlea.

Stria Vascularis

Our main experimental observation is of significant damage to the stria vascularis in subjects infected with mCMV. Figure 3 provides a graphic overview of the salient findings. In contrast to a normal control mouse (panel a), various degrees of vascular degeneration can be noted 8 weeks after mCMV infection (panels b–d). The range of vascular damage in identically CMV inoculated mice is very broad, but the first region to consistently show pathological change is the mid-apical cochlear turn. In subjects with more extensive lesions, there was apparent progression to the basal turn. Panel b illustrates mild vascular degeneration. There is some loss of the capillary network of the stria (organized horizontally behind the spiral ligament vessels), but most evident is the narrowing of vessels. Spiral ligament vessels (vertically oriented) appear normal. Panel c shows extensive degeneration of the vasculature of the apical turn, including the spiral limbus. In example d, there is extensive loss of the vascular networks throughout the cochlea with just a few feeding vessels of the spiral limbus remaining.

Our subject analyses are made at one time-point (8 weeks) post-infection. While it is not a longitudinal study, we can infer a progression of damage of vasculature from cochlear apex to base. Before there is a total loss of a capillary structure, early evidence of vessel degeneration is revealed by a narrowing of capillary lumen as clearly evident, for example, in panel b of Figure 3. Strial capillary diameters in infected mice versus age-matched untreated controls are plotted in Figure 4. In some CMV-damaged cochleas, there is total loss of apical stria vascularis (e.g., panels c and d of Fig. 3). The changes reported in Figure 4 are only from subjects where capillaries are present to measure and thus under-represents the overall apical strial degeneration in CMV-infected mice. The left panel shows the comparison for the mid-apical turn and on the right for the basal cochlear turn ($n = 500$ samples for each group); error bars report standard error of the mean. Data were compared using Welch's t test (Gaussian distribution, different SDs, two-tailed p values). In the mid-apical turn (left panel), there is a significant reduction in capillary lumen diameter in CMV-infected animals,

FIG. 4. Comparison of stria vascularis capillary diameter between mCMV (2000 pfu)-infected mice ($n = 6$) and control animals ($n = 5$). Measured diameter values for each group = 500; error bars report standard error of the mean. In the mid-apical turn (*left panel*), there is

a significant reduction in capillary lumen diameter in CMV-infected animals, $t(926) = 17.77$, $p < .0001$, compared to controls. In the basal turn, stria vessel diameters in infected mice are not significantly different from control animals, $t(974) = 0.086$, $p = 0.9313$.

$t(926) = 17.77$, $p < .0001$, compared to controls. In contrast, in basal cochlear regions, stria vessel diameters in infected mice are not significantly different from control animals, $t(974) = 0.086$, $p = 0.9313$. We have examined 14 ears of seven mice inoculated with CMV by right-side cerebral injection. We observe differences in degree of vascular between the ears, but find no evidence of laterality, i.e., more damage to the right-side cochlea.

Spiral Limbus Vasculature

The spiral limbus vasculature feeds the modiolar regions of the cochlea including the spiral ganglion and also the organ of Corti. In our vascular casts, examination of the spiral limbus often required micro-dissection of the peripheral spiral ligament and stria vessels as illustrated in the examples of Figure 5. The normal spiral limbus vascular has a very characteristic architecture, as evident in panels b and c, with three “layers” of capillary vessels fanning out to feed the modiolus, spiral ganglion, and organ of Corti (these layers are indicated by the arrows in 5c). In the CMV-infected specimen of panel d, the spiral limbus capillaries have degenerated leaving only the feeding vessels. While our study is not longitudinal, the observed damage patterns are consistent with an apical to basal progression of degeneration.

The spiral limbus vasculature appears to degenerate at about the same time as the stria vascularis. For example, Figure 6 shows a cochlear view from the top in a CMV-infected mouse where the stria is normal in one region (orange arrow) but missing more apically (white arrow). Note that in the region where the stria is damaged, the spiral limbus vasculature is also missing (area indicated by dashed line). We suggest that these degenerative events may be related.

Spiral Ligament Vessels

Spiral ligament capillaries run approximately perpendicular to those of the stria vascularis as can be seen in many of the scanning images. Spiral ligament vessels give rise to only a few arterial ramifications that serve to feed the stria vascularis, and similarly few vessels drain the venous side of stria vascularis. These feeding and drainage vessels for the normal mouse stria vascularis are indicated in Figure 7a by the arrow symbols. This image shows the stria in a basal cochlear region in after removal of the spiral ligament vessels. Both top arrows mark feeding vessels that originate from spiral ligament and the lower arrow shows the drainage vessel, typically located midway between both feeding arterioles. Note the characteristic “fan-out” arrangement of the stria capillary bed. Panel b is from a CMV-infected animal, and again the upper arrows show the stria feeding vessels originating from the spiral ligament. However, the stria vascularis has degenerated with the most damaged region occurring in the area between both feeding branches (highlighted by the colored rectangle). The lower arrow approximates to the expected (but no longer present) position of a stria drainage venule. It is important to note that the spiral ligament vessels are still present in their normal conformation. We generally observe in 8-week post-infection samples that the spiral ligament vasculature is the last to degenerate, often after a complete loss of other vascular networks in the cochlea.

In overview, we show that 8-week post-CMV infection, a wide range of cochlear vessel degeneration, is evident. Mild stria vessel degeneration is evident from capillary narrowing in the mid-apical turn of the cochlea. In other specimens, these areas show complete loss of stria and spiral limbus vasculature. The spiral ligament vessels are the last structures to be affected. Further evidence of an increased vascular fragility in the cochlear apex after CMV infection comes from noting evidence of vessel leakage, almost

FIG. 5. Spiral limbus vasculature in controls (**a–c**) and a CMV-infected mouse (**d**). **a** Low magnification view of normal spiral limbus vasculature where stria vascularis and spiral ligament vessels have been dissected away. **b, c** High magnification views of the spiral limbus vessels in control mice. In **c**, the arrows show the three

characteristic vascular layers of this capillary network. Panel **d** illustrates degeneration of spiral limbus vessels in an mCMV-infected animal. The characteristic vascular pattern of this region is completely lost; the feeding arterioles are abnormal; the draining venules are not present.

exclusively in upper cochlear turns. This leakage is manifest as polymer spheres as illustrated in Figure 8 in a mCMV-infected animal. Because of our temper-

FIG. 6. Apical view of a vascular cast from a CMV-infected mouse. The orange arrow indicates normal stria vascularis. The white arrow marks a more apical region with a degenerated stria, and in this same apical area, the spiral limbus vasculature is also abnormal or missing (dotted outline).

ature and pressure control of polymer infusion, this leakage should not be considered to be an artifact of the methodology; it is not observed in normal control mice. Furthermore, the pattern of arteriole supply to the cochlea suggests that at the apex, fluid pressure would be lower than for other cochlear areas, again suggesting that leakage due to excess perfusion pressure is unlikely. On the issue of artifact, we should note that the vascular casts are very delicate specimens with section diameters in the order of 5 μm . However, we can find no evidence that our observations of degeneration relate to breakage of cast specimens. In scanning images, it is easy to distinguish the sharp fractures of broken casts. Typically, we observe rounded ends to vessel terminals that we interpret as a natural limit to capillary fluid transport. The viscosity of the (un-polymerized) casting perfusate is low, and we suppose that when capillary degeneration prevents its passage, this will approximately reflect the limit of *in vivo* the blood perfusion.

In addition to vessel degeneration after CMV infection, we have occasionally noted evidence of regenera-

FIG. 7. Feeding and drainage vessels of the stria vascularis capillary network. Panel **a** shows the basal stria vascularis in a normal mouse after removal of overlying spiral ligament vessels. The upper arrows mark feeding arterioles from the spiral ligament. The bottom arrow shows the drainage venule, normally located midway

between the feeding arterioles. Panel **b** is from a CMV-infected animal; upper arrows show the feeding vessels. The most stria degeneration has occurred in the region between the feeding branches (orange rectangle). The spiral ligament capillaries have maintained a normal configuration.

tion. In corrosion, cast samples angiogenesis can be indicated by capillary intussusception, often evident from a midline capillary “pinching” to divide the vessel (Macchiarelli et al. 2006; Kus et al. 2014). Figure 9 illustrates such a characteristic signature of capillary vessel regeneration. We have observed such features in virus-infected cochleas suggesting some regenerative potential of the vasculature after CMV infection.

DISCUSSION

We have implemented a strictly standardized corrosion cast methodology, with careful control of polymer perfusion pressure and temperature. This provides complete vessel casting (i.e., full infiltration of capillary beds) with no methodological artifacts to invalidate between-animal comparisons. Capillary filling is not only complete, but the degree of perfusion and the resulting cast

diameters are consistent. Relative differences in measured capillary diameters relate to pathological changes and not variance in perfusion technique (Carraro et al. 2016).

This is the first study to clearly show degeneration of the cochlear vasculature following mCMV infection in an animal model that well approximates to human congenital CMV infection. At 8-week post-inoculation, we show that most damage is to the stria vascularis at the cochlear apex and that more damaged cochleas show further degeneration towards the base. While our study is cross-sectional, the results are consistent with CMV pathogenesis having a temporal progression from apical to basal cochlear areas. When we observe injury to the stria vascularis, we also note spiral limbus vasculature damage in the adjacent region.

Animals with mCMV-related vascular damage have auditory thresholds losses as indicated by ABR and DPOAE measures. We suggest that the most plausible link between vascular degeneration and functional loss is related to a disruption of the endocochlear

FIG. 8. Two views of the apical cochlear turn in an mCMV-infected mouse in which vascular leakage is evidenced by polymer spheres. Note also the loss of stria vascularis adjacent to the leakage site and that the spiral ligament vessels show less damage than the stria in this area.

FIG. 9. Severely damaged spiral ligament vessels in an mCMV-infected animal showing evidence of regenerative angiogenesis in the form of capillary midline pinching or intussusception (high magnification in **b**).

(endolymphatic) potential (EP). Without the accumulation of K ions in scala media, hair cell transduction is attenuated. It is well understood that stria function is essential for Na/K ion pumping and the maintenance of the EP. Numerous studies have explored the link between stria mechanisms and EP generation, for example, those reporting loss of EP in cochlear anoxia or hypoxia (Thalmann et al. 1972; Lawrence et al. 1975; Gafni & Sohmer 1976; Salt et al. 1987; Sohmer et al. 1989; Hibino et al. 2010; Patuzzi 2011) or after a block of ion pumping mechanisms by loop diuretics such as furosemide (Sewell 1984). Also of importance is the maintenance of the integrity of the scala media compartment. The boundaries of scala media have to be impermeable to maintain a high K⁺ ion concentration. A relevant report by Cohen-Salmon et al. (2007) showed the importance of a proper stria vascularis endothelial barrier to maintain the EP. They also noted that only the integrity of stria vascularis and not the spiral ligament vessels was important in this regard. Further to this, we know that the integrity of the intrastrial fluid–blood barrier is important for a proper cochlear function.

The degree of vascular damage revealed in the present study after CMV infection could clearly cause hearing loss. An important question is whether such losses, as measured in our model by ABR and DPOAE recording, or with audiometric tests in human CMV infection, relate solely to EP changes after stria dysfunction, or whether hair cells or cochlear neural elements are directly damaged by viral insult. There is much evidence to suggest that the organ of Corti is not the initial target of the virus (Li et al. 2008; Schachtele et al. 2011; Wang et al. 2013; Bradford et al. 2015; Li et al. 2015). It is important to note that the apical vascular damage in the present study is not reflected in predominantly low frequency ABR or DPOAE threshold changes (Fig. 2). Indeed, there is a “flat” threshold elevation consistent with a cochlear-wide EP reduction, independent of the site of stria lesion

There are many features of our mouse model that reflect clinical observations of congenital CMV-related hearing loss. For example, there is much variability in structural and functional changes in identically inoculated animals; such variability in hearing loss in children with congenital CMV is well reported (e.g., Fowler et al. 2006; Foulon et al. 2008, 2012; Manicklal et al. 2013; Goderis et al. 2014). It is possible that a mild to moderate hearing loss is caused by a change in EP and that a prolonged period of hypoxia/anoxia or inflammation might subsequently result in more severe hearing loss (Billett et al. 1989; Schachtele et al. 2011). In an initial period of stria dysfunction, there is a possibility of recovery, but prolonged loss of EP (when there is severe hypoxia) may result in permanent sensory damage. This notion is based on studies that have reported cochlear dysfunction with partial recovery after short periods of severe hypoxia or anoxia (e.g., Perlman et al. 1959; Schulte and Schmiedt, 1992; Tabuchi et al. 1998) but with permanent sensorineural hearing loss after very long periods of oxygen deprivation (e.g., Billett et al. 1989; Tabuchi et al. 1998; Sawada et al. 2001). If the initial target of a viral infection is the stria vascularis, then with early detection and diagnosis, perhaps, some treatment for recovery is possible. This notion that CMV infection involves an initial phase of stria damage and a loss of EP is consistent with reports that early intervention with anti-viral agents such as gan/valganciclovir can improve hearing outcomes (e.g., Kimberlin et al. 2003, 2015; Bilavsky et al. 2016).

We can state that stria vascularis is an early, if not the initial, site of cochlear damage. However, we are still uncertain about how the virus infiltrates the inner ear. It has recently been reported in a mouse model that mCMV is found in the bone marrow of the inner ear (Bradford et al. 2015). We can confirm that observation; we have detected a significant viral signal in the bone marrow of the cochlear apex at 1–3 days post-inoculation. It has been previously reported that in human CMV infection, one virus reservoir is the

bone marrow (Bego & Jeor 2006). We would suggest that a possible link between bone marrow pathology with EP maintenance of EP involves perivascular macrophage-like melanocytes (PVMs). These are renewed by bone marrow-derived precursor cell migration and are involved in the maintenance of the intrastrial fluid-blood barrier (Shi 2010; Neng et al. 2013). Furthermore, melanocytes appear to be essential for normal stria development and EP generation (Steel & Barkway 1989).

Taken together, these data suggest that an impaired development of stria vascularis blood barrier and the consequent interference with EP generation could lead to dysfunctional cochlear hair cell transduction and an associated sensorineural hearing loss. Importantly, it has been reported that in human CMV infection, one virus reservoir is the bone marrow, and that monocyte differentiation into macrophages is a prerequisite for a successful infection (Söderberg-Nauclér et al., 2001; Bego & Jeor 2006; Manicklal et al. 2013). This supports our hypothesis of an inner ear bone marrow-derived cell involvement in causing stria dysfunction in CMV-related hearing loss.

In summary, after CMV infection in a mouse model, there is a deterioration of auditory function (as reflected in ABR and DPOAE measures), and the stria vascularis is one of the first damaged structures. Strial degeneration is initially evident at the cochlear apex. Our observations are consistent with the hypothesis that early hearing loss relates to the poor maintenance of EP that results from stria dysfunction. It is possible that early detection of viral invasion of the inner ear and the prevention of vascular damage with early intervention might ameliorate hearing loss associated with CMV infection.

ACKNOWLEDGEMENTS

This research was supported by a Canadian Institutes of Health Research (CIHR) grant (RVH) and a Triologic Career Development Award (AP). We thank Jaina Negandhi for her assistance in preparing the final manuscript.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest The authors declare that they have no conflict of interest.

REFERENCE

BEGO MG, JEOR SS (2006) Human cytomegalovirus infection of cells of hematopoietic origin: HCMV-induced immunosuppression, immune evasion, and latency. *Exp Hematol* 34:555–570

- BILAVSKY E, SHAHAR-NISSAN K, PARDO J ET AL (2016) Hearing outcome of infants with congenital cytomegalovirus and hearing impairment. *Arch Dis Child* 101(5):433–438
- BILLETTE TE, THORNE PR, GAVIN JB (1989) The nature and progression of injury in the organ of Corti during ischemia. *Hear Res* 41:189–197
- BRADFORD RD, YOO YG, GOLEMAC M ET AL (2015) Murine CMV-induced hearing loss is associated with inner ear inflammation and loss of spiral ganglion neurons. *PLoS Pathog* 11(4):e1004774. doi:10.1371/journal.ppat.1004774
- CARRARO M, HARRISON RV (2016) Degeneration of stria vascularis in age-related hearing loss; a corrosion cast study in a mouse model. *Acta Otolaryngol* 136(4):385–390
- CARRARO M, PARK AH, HARRISON RV (2016) Partial corrosion casting to assess cochlear vasculature in mouse models of presbycusis and CMV infection. *Hear Res* 332:95–103
- CHOI KY, SCHIMMENTI LA, JUREK AM (2009) Detection of cytomegalovirus DNA in dried blood spots of Minnesota infants who do not pass newborn hearing screening. *Pediatr Infect Dis J* 28(12):1095–1098
- COHEN-SALMON M, REGNAULT B, CAYET N ET AL (2007) Connexin 30 deficiency causes intrastrial fluid-blood barrier disruption within the cochlear stria vascularis. *Proc Natl Acad Sci U S A* 104:6229–6234
- DELANY I, RAPPUOLI R, DE GREGORIO E (2014) Vaccines for the twenty-first century. *EMBO Molecular Medicine* 6(6):708–720
- DEMMLER GJ (1991) Infectious Diseases Society of America and Centers for Disease Control. Summary of a workshop on surveillance for congenital cytomegalovirus disease *Rev Infect Dis* 13:315–329
- FOWLER KB, BOPPANA SB (2006) Congenital cytomegalovirus (CMV) infection and hearing deficit. *J Clin Virol* 35:226–231
- FOWLER KB, DAHLE AJ, BOPPANA SB ET AL (1999) Newborn hearing screening: will children with hearing loss caused by congenital cytomegalovirus infection be missed? *J. Pediatr* 135(1):60–64
- FOWLER KB, MCCOLLISTER FP, DAHLE AJ ET AL (1997) Progressive and fluctuating sensorineural hearing loss in children with asymptomatic congenital cytomegalovirus infection. *J Pediatr* 130:624–630
- FOULON I, NAESSENS A, FARON G ET AL (2012) Hearing thresholds in children with a congenital CMV infection: a prospective study. *Int J Pediatr Otorhinolaryngol* 76:712–717
- FOULON I, NAESSENS A, FOULON W ET AL (2008) A 10-year prospective study of sensorineural hearing loss in children with congenital cytomegalovirus infection. *J Pediatr* 153:84–88
- GAFFNI M, SOHMER H (1976) Intermediate endocochlear potential levels induced by hypoxia. *Acta Otolaryngol* 82:354–358
- GODERIS J, DE LEENHEER E, SMETS K (2014) Hearing loss and congenital CMV infection: a systematic review. *Pediatrics* 134(5):972–982
- GROSSE SD, ROSS DS, DOLLARD SC (2008) Congenital cytomegalovirus (CMV) infection as a cause of permanent bilateral hearing loss: a quantitative assessment. *J Clin Virol* 41:57–62
- HIBINO H, NIN F, TSUZUKI C ET AL (2010) How is the highly positive endocochlear potential formed? The specific architecture of the stria vascularis and the roles of the ion-transport apparatus. *Pflugers Arch* 459:521–533
- KASAI M, YONEDA T, HABU S ET AL (1981) In vivo effect of anti-asialo GM1 antibody in natural killer activity. *Nature* 291(5813):334–335
- KIMBERLIN DW, JESTER PM, SANCHEZ PJ ET AL (2015) Valganciclovir for symptomatic congenital cytomegalovirus disease. *N Engl J Med* 372:933–943
- KIMBERLIN DW, LIN C, SANCHEZ PJ (2003) Effect of ganciclovir therapy on hearing in symptomatic congenital cytomegalovirus disease involving the central nervous system: a randomized, controlled trial. *J Pediatr* 143(1):16–25

- KUS LH, NEGANDHI J, SKLAR MC ET AL (2014) Angiogenesis in costal cartilage graft laryngotracheoplasty: a corrosion casting study in piglets. *Laryngoscope* 124(10):2411–2417
- LAWRENCE M, NUTTALI AL, BURGIO PA (1975) Cochlear potentials and oxygen associated with hypoxia. *Ann Otol Rhinol Laryngol* 84:499–512
- LI L, KOSUGI L, HAN GP ET AL (2008) Induction of cytomegalovirus-infected labyrinthitis in newborn mice by lipopolysaccharide: a model for hearing loss in congenital CMV infection. *Lab Invest* 88:722–730
- LI X, SHI X, WANG C ET AL (2015) Pathological changes of the inner ear cochlea in different time windows of murine cytomegalovirus-induced hearing loss in a mouse model. *Acta Otolaryngol* 135:536–541
- MACCHIARELLI G, JIANG JY, NOTTOLA SA ET AL (2006) Morphological patterns of angiogenesis in ovarian follicle capillary networks. A scanning electron microscopy study of corrosion cast *Microsc Res Tech* 69:459–468
- MANICKLAL S, EMERY VC, LAZZAROTTO T ET AL (2013) The ‘silent’ global burden of congenital cytomegalovirus. *Clin Microbiol Rev* 26:86–102
- MISONO S, SIE KC, WEISS NS ET AL (2011) Congenital cytomegalovirus infection in pediatric hearing loss. *Arch Otolaryngol Head Neck Surg* 137(1):47–53
- NENG L, ZHANG F, KACHELMEIER A ET AL (2013) Endothelial cell, pericyte, and perivascular resident macrophage—type melanocyte interactions regulate cochlear intrastrial fluid-blood barrier permeability. *J Assoc Res Otolaryngol* 14(2):175–185
- OTIS EM, BRENT R (1954) Equivalent ages in mouse and human embryos. *Anat Rec* 120:33–63
- PARK AH, DUVAL M, McVICAR S ET AL (2014) A diagnostic paradigm including cytomegalovirus testing for idiopathic pediatric sensorineural hearing loss. *Laryngoscope* 124:2624–2629
- PASS RF, FOWLER KB, BOPANA SB ET AL (2006) Congenital cytomegalovirus infection following first trimester maternal infection: symptoms at birth and outcome. *J Clin Virol* 35(2):216–220
- PATUZZI R (2011) Ion flow in stria vascularis and the production and regulation of cochlear endolymph and the endolymphatic potential. *Hear Res* 277:4–19
- PERLMAN HB, KIMURA R, FERNANDEZ C (1959) Experiments on temporary obstruction of the internal auditory artery. *Laryngoscope* 69(6):591–613
- ROYACKERS L, CHRISTIAN D, FRANS D ET AL (2011) Hearing status in children with congenital cytomegalovirus: up-to 6 years audiological follow-up. *Int J Pediatr Otorhinolaryngol* 75:376–382
- SALT AN, MELICHAR I, THALMANN R (1987) Mechanisms of endocochlear potential generation by stria vascularis. *Laryngoscope* 97:984–991
- SAWADA S, MORI N, MOUNT RJ ET AL (2001) Differential vulnerability of inner and outer hair cell systems to chronic mild hypoxia and glutamate ototoxicity; insights into auditory neuropathy. *J Otolaryngol* 30:106–114
- SCHACHTELE SJ, MUTNAL MB, SCHLEISS MR ET AL (2011) Cytomegalovirus-induced sensorineural hearing loss with persistent cochlear inflammation in neonatal mice. *J Neurovirol* 17:201–211
- SCHNEIDER CA, RASBAND WS, ELICEIRI KW (2012) NIH Image to ImageJ: 25 years of image analysis. *Nat Meth* 9:671–675
- SCHULTE BA, SCHMIEDT RA (1992) Lateral wall Na, K-ATPase and endocochlear potentials decline with age in quiet-reared gerbils. *Hear Res* 61:35–46
- SEWELL WF (1984) The effects of furosemide on the endocochlear potential and auditory-nerve fiber tuning curves in cats. *Hear Res* 14:305–314
- SHI X (2010) Resident macrophages in the cochlear blood-labyrinth barrier and their renewal via migration of bone-marrow-derived cells. *Cell Tissue Res* 342:21–30
- SÖDERBERG-NAUCLÉR C, STREBLOW DN, FISH KN (2001) Reactivation of latent human cytomegalovirus in CD14(+) monocytes is differentiation dependent. *J Virol* 75:7543–7554
- SOHMER H, FREEMAN S, SCHMUEL M (1989) ABR threshold is a function of blood oxygen level. *Hear Res* 40:87–91
- STEEL KP, BARKWAY C (1989) Another role for melanocytes: their importance for normal stria vascularis development in the mammalian inner ear. *Development* 107:453–463
- TABUCHI K, ITO Z, WADA T ET AL (1998) The effect of mannitol upon cochlear dysfunction induced by transient local anoxia. *Hear Res* 126:28–36
- THALMANN R, MIYOSHI T, THALMANN I (1972) The influence of ischemia upon the energy reserves of inner ear tissues. *Laryngoscope* 82:2249–2272
- WANG Y, PATEL R, REN C ET AL (2013) A comparison of different murine models for cytomegalovirus-induced sensorineural hearing loss. *Laryngoscope* 123:2801–2806