Haemochromatosis is a genetic disorder of iron overload resulting from loss-of-function mutations in genes coding for the iron-regulatory proteins HFE [HLA-like protein involved in iron (Fe) homeostasis], transferrin receptor 2, ferroportin, hepcidin and HJV (haemojuvelin). Expression of the first four genes coding for these proteins in retina has been established. Here we report on the expression of HJV. Since infection of retina with CMV (cytomegalovirus) causes blindness, we also investigated the expression of HJV and other iron-regulatory proteins in retina during CMV infection. *HJV* (HJV gene) mRNA was expressed in RPE (retinal pigment epithelium)/eyecup and neural retina in mouse. *In situ* hybridization and immunohistochemistry confirmed the presence of *HJV* mRNA in RPE, outer and inner nuclear layers, and ganglion cell layer. Immunocytochemistry with cell lines and primary cell cultures showed *HJV* expression in RPE and Müller cells. In RPE, the expression was restricted to apical membrane. Infection of primary cultures of mouse RPE with CMV increased *HJV* mRNA and protein levels. Under similar conditions, *HFE* (HFE gene) mRNA levels were not altered, but HFE protein was decreased. Hepcidin expression was, however, not altered. These findings were demonstrable in vivo with CMV-infected mouse retina. The CMV-induced up-regulation of *HJV* in RPE was independent of changes in *HFE* because the phenomenon was also seen in *HFE*-null RPE cells. CMV-infected primary RPE cells showed evidence of iron accumulation and oxidative stress, as indicated by increased levels of ferritin and hydroxynonenal. The observed changes in *HJV* expression and iron status during CMV infection in retina may have significance in the pathophysiology of CMV retinitis.

Key words: cytomegalovirus (CMV), haemochromatosis, haemojuvelin (HJV), Müller cell, retina, retinal pigment epithelium.

INTRODUCTION

Hereditary haemochromatosis, an autosomal recessive disorder of iron overload, is primarily caused by mutations within the *HFE* gene, which codes for HFE [HLA-like protein involved in iron (Fe) homeostasis] [1–4]. Iron overload in patients with *HFE* mutations is an age-dependent process, therefore haemochromatosis as a clinical disease manifests only at >50 years of age. There are four other genes involved in iron homeostasis; mutations in these genes also result in haemochromatosis: *HJV* (haemojuvelin) [also known as RGMc (repulsive guidance molecule c) or HFE2], hepcidin [also known as HAMP (hepatic antimicrobial peptide)], TFR2 (transferrin receptor 2) and ferroportin. Irrespective of the genes involved in the pathogenesis of the non-*HFE* haemochromatosis, iron overload is present in various organs, as seen in patients with *HFE* mutations. There is, however, an important difference between the classical haemochromatosis caused by *HFE* mutations and the haemochromatosis caused by mutations in *HJV* and the gene coding for hepcidin. The latter is called ‘juvenile’ haemochromatosis, because pathological iron overload in tissues occurs at a much younger age than is seen in classical haemochromatosis [5,6]. The tissues commonly affected in haemochromatosis include liver, pancreas, heart, kidney and pituitary, and they clinically present in the form of liver cirrhosis, hepatocarcinoma, diabetes, cardiomyopathy, nephropathy and endocrine dysfunction [1–6]. It is generally believed that the central nervous system is not affected in haemochromatosis, despite the fact that existing clinical evidence points to the contrary. A number of reports have described neuropathological changes in this disease [7–9]. Even though the brain is separated from the systemic circulation by the blood/brain barrier, iron accumulation in certain areas of the brain, such as the basal ganglia, has been demonstrated in haemochromatosis patients [10–12]. These findings suggest that the notion of the brain being protected by the blood/brain barrier from systemic iron overload is not accurate. This provides a compelling reason for studying the expression and regulation of haemochromatosis-related genes in the retina, because the blood/retinal barrier may not protect the retina from iron overload in this disease.

There have been previous reports on the expression of some iron-regulatory proteins in the retina [13–17]. These proteins include transferrin, TIR1, ferritin, hephaestin, caeruloplasmin and ferroportin. All these proteins are expressed throughout the retina. This is not surprising, because all cells have a requirement for iron for their function and likely use TIR1 to take up iron in the form of transferrin-Fe<sup>2+</sup>. The expression of ferritin, an intracellular protein involved in the storage of iron in the bound form, in all retinal cell types is also expected. Hephaestin and caeruloplasmin, which are ferroxidases, may play a role in retinal iron metabolism by their ability to oxidize Fe<sup>2+</sup> to Fe<sup>3+</sup>. Among the iron-regulatory proteins whose expression has been reported thus far in the retina, only ferroportin is involved in haemochromatosis. There had been
MATERIALS AND METHODS

Materials

Reagents were obtained from the following sources: RNA extraction reagent (TRizol® from Invitrogen–Gibco, Grand Island, NY, U.S.A.; GeneAmp RT (reverse transcription)–PCR kit from Applied Biosystems, Foster City, CA, U.S.A.; Taq polymerase kit from TaKaRa, Tokyo, Japan; and PowerBlock from Biogenex, San Ramon, CA, U.S.A. DMEM (Dulbecco’s modified Eagle’s medium)/F12 medium (Invitrogen–Gibco), with 10% (v/v) fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin, was used for growing retinal cell lines. Antibodies used were obtained from the following sources: rabbit polyclonal anti-HFE, rabbit polyclonal anti-hepcidin, chicken anti-MCT1 [anti-(monocarboxylate transporter 1)], mouse monoclonal anti-vimentin and rabbit polyclonal anti-hydroxynonenal from Alpha Diagnostic International, San Antonio, TX, U.S.A.; rabbit polyclonal anti-HJV and HJV control/blocking peptide from Professor Paolo Arosio, Dipartimento Materno Infantile e Tecnologie Biomediche, Università di Brescia, Brescia, Italy); goat anti-rabbit IgG coupled to Alexa Fluor 555, goat antichicken IgG coupled to Alexa Fluor 568, goat anti-rabbit IgG coupled to Alexa Fluor 488 and donkey anti-mouse IgG coupled to Alexa Fluor 488 from Molecular Probes, Carlsbad, CA, U.S.A.; Vectashield Hardset Mounting Media with DAPI (4',6-diamidino-2-phenylindole) from Vector Laboratories, Burlingame, CA, U.S.A.; ketamine, xylazine and acepromazine (plaque-forming units) of MCMV in a volume of 0.2 ml. At 14 days after infection, the salivary glands were removed and virus titration was performed by plaque assay. A 50% tissue culture infectious dose (TCID50) was calculated as the reciprocal of the dilution of virus stock giving plaques in 20% of monolayer cultures. The mean TCID50 was calculated from 3 independent determinations. The virus titration was performed according to the protocol supplied by the Jackson Laboratory. All experiments were performed according to the protocols of the Jackson Laboratory. Mice at 3 weeks of age were used to establish primary cultures of RPE cells. The purity of the cultures was verified by immunodetection of RPE65 (RPE-specific protein of 65 kDa), a known marker for RPE cells, and MCMV (mouse CMV) to induce infection in the eyes. Mice were purchased from Harlan–Sprague Dawley (Indianapolis, IN, U.S.A.). All experimental procedures involving these animals adhered to the 1985 revision of the Principles of Laboratory Animal Care (National Institutes of Health publication no. 85-23) and were approved by the Institutional Committee for Animal Use in Research and Education.

Establishment of primary RPE cell cultures from mouse eyes

Primary cultures of RPE cells were prepared as described previously [19]. Age-matched wild-type and Hfe−/− mice were obtained from the same litter originating from the mating of heterozygous mice. Genotyping was performed according to the protocol supplied by the Jackson Laboratory. Mice at 3 weeks of age were used to establish primary cultures of RPE cells. The purity of the cultures was verified by immunodetection of RPE65 (RPE-specific protein of 65 kDa), a known marker for RPE cells.

Primary cultures of Müller cells and retinal ganglion cells

Primary cultures of retinal Müller cells were established from retinas of C57BL/6 mice as we described previously [24]. The purity of the culture was verified by immunodetection of vimentin, glutamine synthetase, cellular retinaldehyde-binding protein and the glutamate transporter EAAT1 (excitatory amino acid transporter 1), all of which are protein markers for Müller cells. Primary cultures of retinal ganglion cells were prepared as described previously [25] from retinas of C57BL/6 mice at 1–2 days of postnatal age. These cells were positive for the neuronal cell marker NF-160 (160 kDa neurofilament protein) and the ganglion cell-specific marker marker NF-160 (160 kDa neurofilament protein) and the ganglion cell-specific marker Thy-1.

Virus and virus titration

The original stock of MCMV (k181 strain) was generously provided by Professor Edward S. Mocarski (Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, CA, U.S.A.) to S.S.A. Virus stocks were prepared from salivary-gland homogenates of BALB/c mice (Taconic, Germantown, MD, U.S.A.) as described previously [26]. Briefly, mice were infected with 2 mg of the immunosuppressant methylprednisolone acetate intramuscularly every 3 days. At 2 days after the first injection of methylprednisolone acetate, mice were infected intraperitoneally with 5 × 106 PFUs (plaque-forming units) of MCMV in a volume of 0.2 ml. At 14 days after infection, the salivary glands were removed aseptically and homogenized (10%, w/v) in DMEM containing 10% fetal bovine serum. Preparations were clarified by centrifugation (2500 g for 20 min), and 0.1 ml aliquots of the supernatants were stored at −80°C. A fresh aliquot of MCMV stock was thawed and used immediately for each experiment.

MCMV infection in primary RPE cells

Primary RPE cells were seeded in 10-cm-diameter dishes and grown in a cell-culture incubator until they reached 60% confluency. A 50 μl portion of the virus stock was added per 5 ml of medium in the dish containing primary RPE cells. After 2 h, fresh primary RPE culture medium containing 5% fetal bovine serum was added. RNA was collected on days 1, 2, 3 and 4 after infection. For dose–response experiments, the virus stock was diluted 1 in 1000 and 1 in 1000 before adding to the primary cell culture.
Ocular inoculation

Mice were anaesthetized by intramuscular injection of a mixture of 42.9 mg/ml ketamine, 8.57 mg/ml xylazine and 1.43 mg/ml acepromazine at a dose of 0.5–0.7 ml/kg body weight. The left eyes of mice were injected with 5 × 10^3 PFUs of MCMV (or PBS in control mice) in a volume of 2 μl by way of the suprachoroidal route, as previously described [26]. Briefly, a superficial transcleral entry wound was made parallel with, and just posterior to, the limbus by introducing the bevel of a sharp 30-gauge needle into the suprachoroidal space. A 2 μl volume of virus (or PBS), followed by 3 μl of air, was injected. The injection was judged successful if ophthalmic observation using the dissecting microscope showed a chorioretinal detachment associated with the appearance of air in the suprachoroidal space immediately after injection. Mice were killed on day 7 after infection, and retinas were processed for preparation of RNA and tissue sections.

Preparation of eye sections

Animals were perfused with PBS under anaesthesia to reduce contamination from red blood cells and then killed. Eyes were enucleated (removed) and embedded in Tissue-Tek® O.C.T. (optimal cutting temperature) Compound (VWR Scientific, Houston, TX, U.S.A.) in individual disposable vinyl specimen moulds and frozen at −30°C for at least 1 h before sectioning. After trimming, serial frozen sections (8 μm thick) were made on a cryostat, mounted on positively charged slides (SuperFrost/Plus; Fisher Scientific, Pittsburgh, PA, U.S.A.) and stored at −80°C before immunostaining.

For preparation of RNA from posterior segments, eyes were cleansed of all muscle and connective tissue after perfusion, leaving only the globe with some conjunctival tissue and approx. 1 mm of the optic nerve. Corneas and lenses were removed under the dissecting microscope, and the posterior cup with retina was collected and used for RNA preparation. All procedures were conducted on ice.

RT–PCR

Neural retina and RPE/eye cup were prepared as described previously [18,19] and used for preparation of total RNA. RT–PCR was carried out under optimal conditions depending on the nature of the specific PCR primer pairs. The following primers were used: mouse HFE: forward primer, 5'-GGC TTC TGG AGA TAT GGT TAT-3'; reverse primer, 5'-GAC TCC ACT GAT GAT TCC GAT A-3'; mouse HJV: forward primer, 5'-GGC TGA GGT GGA CAA TCT TC-3'; reverse primer, 5'-GAA CAA AGA GGG CCG AAA G-3'; mouse hepcidin: forward primer, 5'-GCA CTC AGC ACT CGG ACC C-3'; reverse primer 5'-GGT CAG GA T GTG GCT CTA GGC TA T-3'. The PCR product was subcloned into the pGEM-T Easy vector and sequenced to confirm its molecular identity. The subcloned plasmid was also used for generation of sense and antisense riboprobes for in situ hybridization. Each PCR experiment was repeated at least three times with similar results.

In situ hybridization

Mouse eyes were frozen in Tissue-Tek® O.C.T. Compound and sections were made at 10 μm thickness and fixed in 4% (v/v) paraformaldehyde. Treatment of tissue sections and hybridization with digoxigenin-labelled sense and antisense riboprobes were carried out as described previously [18,19]. The hybridization signals were detected with anti-digoxigenin antibody conjugated to alkaline phosphatase. The colour reaction was developed with Nitro Blue Tetrazolium/5-bromo-4-chloroindol-3-yl phosphate. Cryosections were hybridized with sense riboprobe to determine non-specific binding (negative control). The probes were prepared by in vitro transcription with appropriate RNA polymerases after linearizing the plasmid with suitable restriction enzymes. The specificity of the probe was confirmed by searching the GenBank® database with the nucleotide sequence of the HJV-specific PCR product as the query. The in-situ-hybridization experiment was repeated twice with similar results.

Immunofluorescence analysis

Cryosections of mouse eyes were fixed in 4% paraformaldehyde for 10 min, washed with 0.01 M PBS, pH 7.4, and blocked with 1 × PowerBlock for 60 min. Sections were then incubated overnight at 4°C with one or more of the following primary antibodies: polyclonal anti-HFE (1:1000), polyclonal anti-hepcidin (1:50), polyclonal anti-HJV (1:100) and/or chicken anti-MCT1 (1:1000), mouse monoclonal anti-vimentin (1:100), polyclonal anti-L-ferritin (1:2000) and polyclonal anti-H-ferritin (1:2000). The specificity of HJV antibody was confirmed by using the peptide-neutralized antibody. The HJV antibody was mixed with the antigenic peptide and incubated for 2 h at 37°C and for 24 h at 4°C. The mixture was centrifuged at 3000 g for 15 min at 4°C to pellet the immune complex. The supernatant was incubated with the sections overnight at 4°C to serve as negative controls. Additional negative controls involved omission of the primary antibodies. Sections were rinsed and incubated for 1 h with goat anti-rabbit IgG coupled to Alexa Fluor 555 and/or goat anti-chicken IgG coupled to Alexa Fluor 568, goat anti-rabbit IgG coupled to Alexa Fluor 488 and donkey anti-mouse IgG coupled to Alexa Fluor 488. Coverslips were mounted with Vectashield Hardset Mounting Medium with DAPI (a nuclear stain) and sections were examined with a wide-field epifluorescence microscope (Carl Zeiss, Jena, Germany).

Retinal cell lines and primary retinal cell cultures were grown on 12-mm-diameter cover slides for 24 h in a 24-well cell culture plate at 37°C in a 5% CO2 incubator. Medium was removed and the cells were fixed in ice-cold methanol for 10 min after air-drying. Cells were then washed with 0.01 M PBS, pH 7.4, and blocked with 1 × PowerBlock for 120 min. Cells were incubated overnight at 4°C with polyclonal anti-HFE (1:100), polyclonal anti-hepcidin (1:50) or polyclonal anti-HJV (1:50). After incubation of the cells with primary antibody, the same protocol was followed as used for staining the tissues. Negative control cells were treated likewise, but in the absence of primary antibodies.

Immunohistochemical and immunocytochemical studies were repeated twice, the results from these two experiments being similar.

Western blot

Protein lysates were prepared from primary RPE cells (control and MCMV-infected) and subjected to SDS/10%-(w/v)-PAGE. The proteins were then transferred on to a PVDF membrane and probed with specific antibodies. The positive bands were detected with appropriate secondary antibodies coupled to horseradish peroxidase. The signals were developed using an enhanced-chemiluminescence detection kit (Thermo Fisher Scientific, Waltham, MA, U.S.A.). This experiment was repeated twice with comparable results.
RESULTS
Expression of HJV in retina
To investigate whether HJV is expressed in the retina, RT–PCR was performed with RNA isolated from neural retina (mostly devoid of RPE) and RPE/eye cup of normal mouse. We found evidence for the expression of HJV mRNA in RPE/eye cup as well as in the neural retina (Figure 1A); HPRT1 primers were used as an internal control for RT–PCR. The PCR product was subcloned in pGEM-T Easy vector and sequenced to confirm the molecular identity of the product. RT–PCR was also performed using HIV-specific primers with RNA isolated from primary cultures of mouse RPE, Müller and ganglion cells. RPE and Müller cells showed significantly greater expression of HJV mRNA compared with that of ganglion cells (Figure 1A). We then analysed the expression pattern of HJV mRNA and protein in the retina by in situ hybridization and immunofluorescence. In situ hybridization for the analysis of HJV mRNA revealed the expression of HJV in the inner nuclear layer, outer nuclear layer and RPE cell layer and, to a lesser extent, in the ganglion cell layer (Figure 1B). The signals observed were specific; no positive signals were detected when a sense riboprobe was used in place of the antisense riboprobe. Immunofluorescence analysis of HIV protein showed positive signals almost throughout the retina in a pattern consistent with expression of the protein in RPE, photoreceptor cells, Müller cells and ganglion cells (Figure 1C). Negative controls with omission of the primary antibody or with peptide-neutralized antibody did not yield positive signals, indicating the specificity of the signals obtained with the antibody. The expression pattern of HIV protein corroborates the expression pattern of HJV mRNA. Since RPE and Müller cells are vital for optimal health and function of the neural retina, we focused on the expression of HJV in these two cell types. To confirm the expression of HJV in RPE and Müller cells, we performed immunocytochemistry with primary cultures of mouse RPE and Müller cells and with established human cell lines (ARPE-19, a human RPE cell line, and rMC1, a rat Müller cell line (Figure 1D). These studies confirmed the expression of HJV in RPE and Müller cells.

Further analysis of HJV expression in RPE and Müller cells in retina
To determine whether HJV is expressed in RPE in a polarized manner, double-labelling studies were performed in normal mouse retinal sections with a rabbit polyclonal anti-HIV antibody and a chicken polyclonal anti-MCT1 antibody. MCT1 has been shown to be a marker for the apical membrane of RPE cells [27]. The signals specific for HIV (green) and MCT1 (red) were both co-localized in the apical membrane of RPE (Figure 2B). To confirm the presence of HIV in Müller cells, double-labelling studies of HJV (red) and vimentin (green), a marker for Müller cells, were performed. The two proteins did not co-localize uniformly throughout the Müller cells, but co-localization was evident in the outer plexiform layer and also in parts of the inner plexiform layer (Figure 2C). These data indicate that HIV is expressed in Müller cells in specific regions rather than throughout the cell.

Expression of iron-regulatory proteins in retina during MCMV infection in vitro
It is known that infection of non-retinal cells with CMV is associated with proteosomal degradation of HFE [22,23]. US2 (unique short protein 2) encoded by the CMV genome is responsible for this effect. It is also known that HFE mRNA levels are not affected by CMV infection. Since RPE expresses HFE [18] and also serves as a host cell for CMV infection [26,28], we wanted to determine whether CMV infection of these cells affects HFE as it does in other non-retinal cells. For this, we infected primary mouse RPE cells with MCMV and compared the expression of HFE between uninfected and infected cells. RT–PCR and immunofluorescence were done to monitor HFE mRNA and protein levels. We found no change in HFE mRNA in response to MCMV infection (Figure 3A); however, there was a marked decrease in HFE protein in MCMV-infected cells compared with control cells (Figure 4). These data demonstrate that CMV infection elicits the same effects on HFE expression in RPE as it does in other non-retinal cells. There are, to our knowledge, no reports in the literature on the effects of CMV infection on other iron-regulatory proteins. Since HIV and hepcidin are expressed in RPE (19); the present study), this provided an opportunity to examine the effects of CMV infection on the expression of these two iron-regulatory proteins in these cells. HFE controls the expression of hepaticin in the liver [1–4], therefore, we expected significant changes in hepaticin levels in CMV-infected RPE cells on the basis of the observed decrease in HFE levels. Quite unexpectedly, there were no detectable changes in the levels of hepaticin mRNA or protein in RPE cells infected with MCMV compared with control cells (Figures 3A and 4). Since HIV also regulates hepaticin expression [29,30], we monitored the levels of HJV mRNA and protein in control and MCMV-infected RPE cells. We found robust up-regulation of HJV expression in RPE cells in response to CMV infection (Figures 3A and 4). The increase in HJV mRNA levels was dose-dependent in terms of the virus load and time-dependent in terms of duration of infection (Figures 3A and 3B).

Relevance of HFE to CMV-induced changes in HJV expression
It is known that both HFE and HJV regulate hepaticin expression. MCMV infection in primary RPE cells leads to degradation of HFE protein, but, at the same time, increases the levels of HJV mRNA and protein. To determine whether the increase in HJV expression during CMV infection is in response to decreased HFE protein levels, we infected primary RPE cells from HFE-null mice with MCMV. As expected, there was no HFE protein in either untreated or MCMV-infected HFE-null primary RPE cells (Figure 4). However, there was a significant increase in the levels of HIV protein in uninfected HFE-null RPE cells in comparison with infected wild-type cells (Figure 4). Interestingly, we found that, as observed in wild-type HFE-positive primary RPE cells, HFE-null RPE cells showed an increase in HIV protein in response to MCMV infection (Figure 4). In addition, hepaticin mRNA levels were higher in MCMV-infected HFE-null RPE cells compared with uninfected HFE-null RPE cells (Figure 4). The changes in HIV, HFE and hepaticin protein levels corresponded to changes in respective mRNA levels (Figure 5A). These data show that the MCMV-induced up-regulation of HJV expression is independent of the virus-induced degradation of HFE. To confirm further that the increase in HIV expression during MCMV infection is independent of HFE, we used MG132, a proteosomal degradation inhibitor, which would prevent the CMV-induced degradation of HFE. The concentration of this inhibitor used in our study was 30 μM, which was optimal to block HFE degradation [22]. Irrespective of the presence or absence of MG132, MCMV infection increased HJV expression in primary RPE cells, confirming that the observed change in HIV expression in response to CMV infection is independent of HFE (Figure 5B).
Evidence of iron accumulation in primary RPE cells in response to MCMV infection

It has been speculated that CMV infection in mammalian cells depletes HFE protein as a means to increase cellular iron levels and consequently to promote virus multiplication [22,23]. Here we sought to obtain evidence of iron accumulation in primary RPE cells in response to CMV infection. For this, we compared the levels of various iron-regulatory proteins between control and MCMV-infected primary RPE cells from mouse retina. First, we confirmed the decrease in HFE and increase in HJV protein levels in infected cells (Figure 6). We then monitored the levels...
Figure 2  Localization of HJV in retina

(A) Negative control without primary antibody. (B) The polarized expression of HJV was investigated by comparing its expression pattern with that of MCT1 (an apical membrane transporter in RPE). MCT1 was detected with a secondary antibody conjugated to Alexa Fluor 568 (red) and HJV was detected with a secondary antibody conjugated to Alexa Fluor 488 (green). DAPI was used as a nuclear stain. Merging of the fluorescent signals (yellow) in the RPE cell layer indicates that the expression of HJV is specifically associated with the apical membrane in RPE. The top row shows a representative section at an original 200 × magnification and the bottom row is a representative section at an original 400 × magnification. The inset in the bottom row is a higher magnification of the RPE cell layer. (C) Double-labelling of HJV (red) and vimentin (green), a protein marker for Müller cells in retinal sections shows that the two proteins co-localize in some regions of Müller cells but not throughout the cell. The co-localization is evident particularly near the outer plexiform layer (yellow merged signal). GCL, INL and ONL are defined in the legend to Figure 1.

of TfR1 and ferritin as surrogates of cellular iron status. The steady-state levels of TfR1 and ferritin in cells are reciprocally related in their response to changes in cellular iron levels [31,32]. TfR1 mRNA contains an IRE (iron-responsive element) in its 3′- untranslated region, which binds an IRE-binding protein when cellular levels of iron are low, resulting in enhanced stability of mRNA and increased TfR1 protein levels. By contrast, ferritin mRNA contains an IRE in its 5′-untranslated region, which binds an IRE-binding protein when cellular levels of iron are low, resulting in blockade of translation and hence reduced levels of ferritin. Thus TfR1 protein levels are high and ferritin protein levels are low when cellular levels of iron are low. The reverse is true when cellular levels of iron are high. Iron accumulation leads to decreased stability of TfR1 mRNA and increased translation of ferritin mRNA, leading to a decrease in TfR1 protein levels and an increase in ferritin protein levels. We found clear evidence of decreased TfR1 protein levels and increased ferritin protein levels in MCMV-infected RPE cells compared with uninfected cells (Figure 6). These data indicate that MCMV infection promotes iron accumulation in RPE cells.

Expression of iron-regulatory proteins in retina during MCMV infection in vivo

To determine whether the effects of MCMV infection on iron-regulatory proteins observed in primary cultures of RPE cells hold true in vivo, we injected mouse eyes with PBS (control) or MCMV and monitored for the expression of iron-regulatory proteins. RT-PCR showed that the levels of HJV mRNA were increased in the retina when infected with MCMV (results not shown). Immunohistochemical analysis indicated that HFE protein,
Figure 3  Dependence of the expression of iron-regulatory proteins in mouse primary RPE cells during MCMV infection in vitro on infection time and viral load

(A) RT–PCR analysis showing HFE, hepcidin-gene and HJV mRNA levels in uninfected and MCMV-infected primary RPE cells on days 1–4 after infection. (B) RT–PCR analysis showing HJV mRNA levels in untreated and MCMV-infected primary RPE cells at different viral loads. HPRT1 was used as an internal control for all the reactions.

Figure 5  Effect of MCMV on HJV expression in mouse RPE cells is independent of HFE

(A) RT–PCR analysis showing HJV, HFE and hepcidin-gene mRNA levels in uninfected and MCMV-infected primary RPE cells from wild-type and HFE-null mice. Cells were infected with MCMV and used for RNA isolation on day 4 after infection. (B) RPE cells were infected with MCMV in the absence or presence of the proteasomal inhibitor MG132 (30 μM). RNA was prepared on day 4 after infection. HPRT1 was used as the internal control.

Figure 4  Expression of HFE, HJV and hepcidin in mouse primary cultures of wild-type and HFE-null RPE cells during MCMV infection in vitro

Primary cultures of RPE cells were established from the retinas of wild-type and HFE-null mice. The cells were infected with MCMV and immunocytochemistry was performed on day 4 after infection. Negative controls represent cells with no exposure to primary antibody.

expressed exclusively in RPE, was almost completely depleted in MCMV-injected eyes (Figure 7A). In contrast, the levels of HJV increased markedly throughout the retina in response to MCMV infection (Figure 7B). We also monitored the levels of ferritin to determine whether MCMV infection in vivo promotes iron accumulation. The levels of ferritin (L- as well as H-) were increased markedly throughout the retina in response to MCMV infection (Figures 7C and 7D), suggesting iron accumulation in MCMV-infected retinas. Excessive iron accumulation in cells generates hydroxyl radicals via the Fenton reaction, which then promote lipid peroxidation. 4-Hydroxynonenal is one of the by-products of lipid peroxidation and is widely used as a marker of oxidative stress [33]. Therefore, we monitored the levels of
The only remaining haemochromatosis-causing gene that has not been studied is HJV. With the present study documenting the expression of HJV in the retina, it is clear that all five genes that cause haemochromatosis are expressed in the retina. These findings warrant careful and systematic examination of retinal involvement in haemochromatosis. The expression of hepcidin and HJV in the retina is particularly important, because loss-of-function mutations in these two genes cause juvenile haemochromatosis in which iron overload in tissues occurs at much earlier ages than in the classical haemochromatosis caused by mutations in HFE. The incidence of the juvenile form is very rare in humans, but it is likely that iron accumulation with consequent disruption of retinal structure and function may become apparent very early in life in patients with this form of haemochromatosis. Targeted analysis of retinal function in patients with juvenile haemochromatosis may eliminate the confounding factor of age in establishing the connection between haemochromatosis and retinal dysfunction.

Exciting among our findings are the polarized expression of HFE (basolateral membrane) and HJV (apical membrane) in RPE and the abundant expression of HJV and hepcidin in RPE, Müller cells, ganglion cells and photoreceptor cells ([18,19]; the present study). All cells require iron as an essential nutrient and yet not every cell expresses HFE and HJV. Therefore our findings concerning the expression of HFE and HJV in RPE raise the question: what is the need for the RPE cell to express these regulatory proteins? RPE must have mechanisms to obtain iron from the blood. Iron exists in circulation predominantly in the form of a transferrin–Fe3+ complex, and all cells express TfR1, which facilitates the cellular entry of iron from this complex via receptor-mediated endocytosis. There is evidence for the expression of TfR1 in RPE [15–17]. What makes RPE unique is that this cell is also involved in the handling of iron arising from the continuous phagocytosis of outer rod segments. Thus, RPE must also have mechanisms to export iron to protect against the risk of excessive iron accumulation. Iron export in other cell types is mediated by ferroportin, and the expression of this transporter has been documented in RPE [14]. We have recently demonstrated that hepcidin, which regulates ferroportin, is also expressed in RPE [19]. Since the expression of the hepcidin gene in the liver is known to be under the control of HFE and HJV, we hypothesize that the expression of this iron-regulatory hormone in RPE is also controlled by HFE and HJV. Hepcidin is also expressed in Müller cells, ganglion cells and photoreceptor cells. This raises the question as to the identity of the factors that regulate its expression in these cells. HFE is not expressed in Müller cells, but HJV is. In addition, since HJV is a glycosylphosphatidylinositol-anchored protein, it can be generated from the RPE apical membrane in a soluble form by phosphatidylinositol-specific phospholipase C, and HJV thus released may act on Müller cells. Our findings that the iron-regulatory hormone hepcidin is expressed within the retina and that its expression may be controlled locally by HFE and HJV suggest an obligatory role for these proteins in the maintenance of retinal iron homeostasis.

It is generally assumed that most cells, including those present in the retina, obtain iron primarily through transferring/TfR1-mediated pathway. However, non-transferrin iron delivery is currently being recognized as a significant mode of iron entry in specific cell types. Ferritin, which is composed of L-ferritin and H-ferritin at various stoichiometries, can deliver iron into mammalian cells through receptor-mediated endocytosis. Specific receptors for H-ferritin and L-ferritin have been identified [40,41]. Interestingly, the H-ferritin receptor (known as Tim-2) and the L-ferritin receptor (known as ScarA5) are expressed mostly in developmentally and tissue-specific manner [40,41]. It would be of interest to determine in future studies whether these newly
Figure 7  Effects of MCMV infection in vivo on the expression of HFE and HJV in retina, and consequences of these effects in terms of iron accumulation and oxidative stress

Mice were infected in vivo by intraocular injection of MCMV. Control mice were injected with PBS. Retinal sections were prepared on day 7 after injection and used for immunofluorescence studies.
identified processes of iron entry into cells also operate in any of the cell types within the retina.

In addition to reporting for the first time on the expression of HJV in the retina, we also demonstrate here that the expression of HJV in the retina is markedly influenced by CMV infection. It is known that infection of mammalian cells with CMV leads to down-regulation of HFE protein via proteasomal degradation [22,23]. It is believed that this process is necessary for virus multiplication in the host cell, because HFE depletion will lead to iron accumulation in cells, which is obligatory for virus proliferation. These findings are relevant to RPE and retina, because CMV infection is one of the major causes of retinitis in immunocompromised individuals and leads to serious functional deficits [21]. Since RPE cells express HFE, we asked whether infection of RPE cells with CMV leads to changes in HFE expression. We also wanted to know whether HFE is the only target for CMV infection or whether other key iron-regulatory genes are influenced also by CMV infection. Our present findings that CMV infection decreases HFE levels, but increases HJV expression in RPE, may have significance with regard to the pathophysiology of CMV infection. The current belief is that CMV infection leads to degradation of HFE with the goal of increasing iron levels in the host cell. Supporting evidence for this idea comes from the observations that chelation of cellular iron interferes with virus multiplication [42,43]. The present studies provide additional evidence for iron accumulation in cells as a consequence of CMV infection. The data from the present studies show that CMV infection in RPE cells leads to a decrease in TfR1 levels and an increase in ferritin levels, which are indicative of iron accumulation. These changes are accompanied by evidence of increased lipid peroxidation. Thus, although enhanced iron accumulation may initially favour virus multiplication in the host cell, the process eventually leads to disruption of host cell function through oxidative stress. Changes in cellular iron status as a result of CMV infection seem to play an important role in this process.

Our present data show that, even though CMV infection leads to decreased levels of HFE, there is no significant change in the levels of hepcidin. This is likely to be due to the concomitant increase in the expression of HJV, HFE and HJV have similar effects on the hepcidin gene, both enhancing its expression. The reciprocal changes that occur in the cellular levels of HFE and HJV in response to CMV infection may explain the lack of detectable changes in hepcidin levels. However, additional or alternative mechanisms cannot be ruled out at present. Pro-inflammatory cytokines are known to regulate hepcidin expression [44]. It is likely that infection of RPE cells with MCMV influences expression of such cytokines, which could have an impact on hepcidin expression. Irrespective of the mechanisms involved in the maintenance of hepcidin levels in CMV-infected cells, it is notable that iron levels increase in these cells when there is no change in hepcidin levels. Hecpidin regulates cellular iron levels through its action on ferroportin, an iron exporter. Since hepcidin levels do not change in CMV-infected RPE cells, other mechanisms must participate in the promotion of iron accumulation. The expression of TfR1 is decreased in these cells, which is most likely to be in response to increased iron levels. HFE interacts with TfR2 [45,46]. The interaction between HFE and TfR2 regulates iron entry into cells, but the molecular events involved in the process remain mostly unknown. Since HFE is markedly down-regulated in CMV-infected cells, the potential role of HFE–TfR2 interaction in iron entry into these cells may be relevant to the observed iron accumulation in CMV-infected RPE cells. The present studies show that the levels of L-ferritin and H-ferritin are elevated in RPE cells as a consequence of CMV infection. Even though ferritin is regarded generally as an intracellular protein, it is becoming increasing evident that the L- and H-ferritin are also secreted into the extracellular medium. The secreted L-ferritin and H-ferritin seem to provide alternative routes for iron delivery into cells through specific receptors. Therefore, it is possible that CMV infection influences these non-transferrin modes of iron entry into cells to promote iron accumulation.

It has been demonstrated that one of the CMV genome-encoded viral proteins, namely US2, is directly responsible for promoting proteasomal degradation of HFE. We do not know whether the up-regulation of HJV in CMV-infected cells is a direct effect of any of the viral proteins on HJV gene or whether it is a cellular response to the virus-induced HFE depletion in an attempt to maintain iron homeostasis. What is important, however, is the finding that CMV infection does lead to iron accumulation, despite the up-regulation of HJV. It is also possible that the depletion of HFE in CMV-infected cells has additional roles. HFE, an MHC class-I like glycoprotein, may have cellular functions unrelated to iron homoeostasis. It has been suggested that HFE may serve as a ligand for specific lymphocyte populations and thus modulate immune function in the host to the benefit of the virus [47,48]. Future studies will have to address these different possibilities.

Another interesting finding from the present studies is the difference in the cellular levels of hepcidin in response to CMV infection in wild-type RPE cells compared with HFE-null RPE cells. The expression of HJV is increased in both cell types on CMV infection. This is not the case with hepcidin. In wild-type cells, CMV infection does not alter hepcidin levels, whereas there is a significant increase in hepcidin expression in HFE-null cells in response to CMV infection. The functional significance of this difference remains to be investigated. Since CMV infection leads to depletion of HFE in wild-type RPE cells, there may not be any difference between wild-type and HFE-null RPE cells upon CMV infection in terms of HFE levels. Similarly, the expression of HJV is enhanced in both cell types in response to CMV infection. The expression of hepcidin seems to be the major difference between the wild-type and HFE-null RPE cells. RPE cells express ferroportin, the target for hepcidin [14]. Therefore it is possible that HFE-null RPE cells may accumulate iron at significantly higher levels than wild-type cells on CMV infection. This could affect CMV multiplication as well as eventual oxidative damage to the cells. This may have clinical significance for patients with HFE mutations in terms of complications associated with CMV retinitis.

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