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Murine Cytomegalovirus Infection and Apoptosis in Organotypic Retinal Cultures Short title: MCMV Infection in Retinal Culture

Ming Zhang, M.D., Ph.D., Brendan Marshall, Ph.D., and Sally S. Atherton, Ph.D.

Department of Cellular Biology and Anatomy, Medical College of Georgia, Augusta, Georgia 30912

Abstract

Purpose—An organotypic retinal culture model was used to determine the pattern of murine cytomegalovirus (MCMV) infection and if apoptosis was induced in MCMV infected cultured retinas.

Methods—Retinas harvested from C57BL/6 mice were individually cultured at 37°C on 3 µm filter inserts placed in 24 well plates. Some retinas were infected with MCMV (5×10^5 PFU/well). At day 4, 7, and 11 post infection (p.i.), the culture medium and cultured retinas were collected for examination.

Results—Replicating virus was recovered and viral early antigen (EA) and late antigen (LA) positive cells were observed in the MCMV infected retinal cultures. The majority of MCMV infected cells were glia and horizontal cells. Infection resulted in atrophy of the photoreceptor cells and cytomegaly. Apoptosis of uninfected bystander cells, including photoreceptor cells and horizontal cells, was observed. TNF-α was produced by activated microglia during MCMV infection of the retina. Mouse apoptosis microarray studies, caspase activity studies, and RTPCR studies showed that the genes involved in both the death receptor mediated apoptotic pathway as well as the mitochondrial pathway were upregulated.

Conclusions—Many aspects of MCMV infection of retinal cultures parallel those observed during MCMV retinitis in mice. Thus, this *in vitro* system might be used to explore the role of apoptosis of uninfected retinal cells as well as the contribution of cytokines and other modulators to the pathogenesis of cytomegalovirus retinitis.

Keywords

apoptosis; murine cytomegalovirus; retinitis; caspase; organotypic culture

Introduction

Cytomegalovirus (CMV) infection in immunocompetent individuals usually causes a self-limiting, subclinical disease. However, in adult and pediatric patients who are immunosuppressed (IS) as a result of chemotherapy, malignancy, or the acquired immunodeficiency syndrome, CMV infections are a significant cause of morbidity and mortality (1-8). Prior to the introduction of highly active anti-retroviral therapy (HAART), CMV retinitis was the most common sight-threatening opportunistic infection observed in AIDS patients (4-8). CMV retinitis is also observed in newborn infants with congenital cytomegalovirus infection (9-11). Although the ophthalmologic features of CMV retinitis

have been well-characterized, the pathogenesis of ocular CMV infections has not been fully determined.

Although necrosis is a prominent feature of CMV retinitis, apoptotic cells have been observed during microscopic examination of biopsy specimens of eyes from patients with HCMV retinitis (12, 13). In the mouse model of cytomegalovirus retinitis studied in our laboratory, both apoptotic cells and necrotic cells have been observed in the retina during the evolution of MCMV retinitis (14-16). Immunohistochemistry studies (14, 15) and electronic microscopic examinations (16) have shown that the majority of apoptotic cells are not virus infected. However, apoptosis-inducing factor(s) and possible apoptosis pathway(s) involved remains to be identified. In this study, an *in vitro* MCMV infected organotypic retinal culture system which has many features paralleling those observed during MCMV retinitis in mice was used to determine the pattern of MCMV infection, if apoptosis was observed in MCMV infected cultured retinas, and if apoptosis was observed, the likely mechanism for its induction.

Methods

Virus and virus titration

The original stock of MCMV (K181 strain) was a generous gift of Dr. Edward S. Mocarski (Stanford University School of Medicine, Stanford, CA). Stocks of K181 were prepared in mouse embryo fibroblast (MEF) cells (BioWhittaker, Walkersville, MD) grown in tissue culture medium containing 5% fetal calf serum (HyClone, Logan, UT). The titer of MCMV in virus stocks was determined by duplicate plaque assay on monolayers of MEF cells and virus stocks were stored at -70°C. A fresh aliquot of stock virus was thawed and diluted to the appropriate concentration immediately before each experiment.

Mice

Female three-to-four week old C57BL/6 mice (Taconic Inc., Germantown, NY) were used in all experiments. Mice were housed in accordance with National Institutes of Health guidelines. Mice were maintained on a 12 hour light cycle alternating with a 12 hour dark cycle and were given unrestricted access to food and water. The treatment of animals in this study conformed to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and was approved by the Institutional Animal Care and Use Committee of the Medical College of Georgia.

Organotypic retinal culture

Female C57BL/6 mice were killed by an overdose (5.0 ml/kg) of a mixture of 42.9 mg/ml ketamine, 8.57 mg/ml xylazine, and 1.43 mg/ml acepromazine. The eyes were removed and placed in ice-cold minimum essential medium [(MEM), Gibco, Carlsbad, CA] preparation medium. The anterior portion of the eye was removed by an incision along the ora serrata. After removal of the lens and vitreous, the retina was removed from the eyecup by inversion. Retinal explants were mounted with the photoreceptor side down on a 3.0 µm FCF filter (1 retina/filter) in 12mm culture plate insert (Millipore, Billerica, MA) and covered with one drop of Matrigel (Becton Dickinson, Bedford, MA) and kept at room temperature for 10 minutes to allow coagulation of the Matrigel. A 26 gauge needle was used to make a hole in the periphery (to allow MCMV access to peripheral retinal cells). Each culture plate insert and retina was placed into one well of a 24 well plate containing 550µl/well of Dulbecco's MEM-F12 (supplemented with 10% horse serum, 5 mM glutamine and buffered with 20 mM HEPES, pH 7.4) and incubated at 37°C. Culture medium was changed after 1 day and twice weekly thereafter. Some cultured retinas were inoculated with MCMV (5×10^5 PFU/well) for 3 hours. At day 4, 7, and 11 post infection (p.i.), culture

medium was harvested for virus recovery, and some retinal cultures were collected and fixed in 4% paraformaldehyde for 5 min, immersed in 25% sucrose overnight, snap-frozen, and sectioned on a cryostat and ready for further immunohistochemistry study. Some retinal cultures were prepared for caspase activity, apoptosis microarray and RTPCR studies as described below.

Immunohistochemistry

Monoclonal antibody to a MCMV early antigen (EA) (17) and the antibody rat anti-F4/80, which was used to identify activated microglia, were labeled with FITC (Sigma, St. Louis, MO) or biotinylated with Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL) according to the manufacturer's instructions. Mouse monoclonal antibody to a MCMV late antigen (LA) was kindly provided by Dr. John Shanley (University of Connecticut Health Center, Framington, CT). Retinal glial cells including activated Müller cells and astrocytes were stained with mouse anti-glial fibrillary acidic protein (GFAP) (BD Pharmingen, San Diego, CA) or with rabbit anti-GFAP (Chemicon, Temecula, CA). Mouse anti-Go- α (Chemicon) was used to identify both rod and cone bipolar cells. Rabbit anti-calbindin (Chemicon) was used to stain horizontal cells. A rabbit anti- γ -aminobutyric acid (GABA) (Sigma) was used to stain GABAergic amacrine cells. Mouse anti-neurofilament (NF) (Sigma) was used to stain ganglion cells as well as some horizontal cells. FITC-labeled anti-TNF- α and rabbit anti-active caspase 3 was purchased from BD Pharmingen.

Double staining of MCMV EA and retinal cell antigens or TUNEL and triple staining of MCMV EA, GABA and GFAP was performed as described previously (16). For double staining of TUNEL and retinal cell antigens, the sections were first detected with TUNEL staining (15); they were then stained with a retinal cell-specific antibody followed by reaction with Texas Red-Avidin or Texas Red labeled anti-rabbit IgG as described previously (16). For triple staining of MCMV EA, MCMV LA and retinal antigens for which the antibodies had been made in rabbits (GABA, calbindin, GFAP), the sections were stained first with one of the rabbit anti-retinal cell antibodies and the reaction was developed using AMCA-anti-rabbit IgG. The sections were then stained with anti-MCMV LA and then reacted with biotinylated anti-mouse in DAKO ARK kit (Dako Corporation, Carpinteria, CA) (16); the immunolabeling was detected using Texas Red-Avidin. Finally, the slides were reacted with FITC-anti MCMV EA and mounted with Vectorshield mounting medium (Vector Laboratories), and examined microscopically.

Apoptosis Microarray and RT-PCR

RNA was extracted from MCMV infected retinal cultures and control retinal cultures (day 7 p.i.) using TRIzol (Invitrogen-Gibco) according to the manufacturer's instructions. Apoptosis genes were detected with Oligo GEArray® Mouse Apoptosis Microarray (SuperArray Bioscience Corporation, Frederick, MD) which profiles the expression of 112 genes involved in apoptosis according to the manufacturer's instructions. Three retinas were pooled for each group. Gene expression was analyzed by using GEA Suite software (SuperArray Bioscience Corporation). The mean of selected control genes was adjusted to value of 100. The minimal positive value of gene expression was set as more than 5% of the mean of control genes. All the negative genes of which the value was equal or less than 5% of the mean of control genes were counted as 5 for the calculation.

RNA-PCR was performed with Access RT-PCR System (Promega Corporation, Madison, WI) according to the manufacturer's instructions. The primers for TNF- α , caspase 3, caspase 8, BCL2-associated X protein (Bax), apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), and β -actin were as follows:

TNF- α : 5'-TTCTGTCTACTGAACTTCGG-3' (298-317)

5'-GTATGAGATAGCAAATCGG-3' (633-651)

Caspase 3: 5'-GGGCCTGTTGAACTGAAAAA-3' (457-476)

5'-CCGTCCTTTGAATTTCTCCA-3' (669-698)

Caspase 8: 5'-GGCCTCCATCTATGACCTGA-3' (1269-1288)

5'-GCAGAAAGTCTGCCTCATCC-3' (1461-1480)

Bax: 5'-ATCGAGCAGGGAGGATGGCT-3' (221-240)

5'-CTTCCAGATGGTGAGCGAGG-3' (671-691)

ASC: 5'-GATGGACGCCATAGATCTCAC-3' (232-252)

5'-GTCTCTGCACGAACTGCCTG-3' (527-546)

β -actin: 5'-TCCTTCGTTGCCGGTCCACA-3' (44-63)

5'-CGTCTCCGGAGTCCATCACA-3' (534-552)

Caspase 3 activity

The enzymatic activity of caspase 3 was measured by modified enzymatic assay using the fluorogenic peptide substrate carbobenzoxy-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin (DEVD. AFC) (Enzyme Systems Products, Dublin, CA) (18, 19). MCMV infected and uninfected control cultured retinas were treated with 1% Triton X-100. Eighty micrograms of protein from each lysate were added to the enzymatic reactions containing 25 μ m DEVD.AFC. After incubation at 37°C for 60 minutes, fluorescence at excitation 360 nm/emission 530 nm was monitored by a GENios plate-reader (Tecan US, Research Triangle Park, NC). For each measurement, a standard curve was constructed using free AFC. Based on the standard curve, the fluorescence reading from each enzymatic reaction was translated into the nanomolar amount of liberated AFC to quantify caspase activity.

Western Blotting for Caspase 8

Western blotting for caspase 8 was performed as previously described (20). Briefly, proteins were extracted from freshly isolated normal retinas and from MCMV infected and uninfected control cultured retinas using modified radioimmunoprecipitation (RIPA) buffer with an inhibition cocktail (complete Mini Protease Inhibitor Cocktail; Roche, Basel, Switzerland). Eighty μ g of protein from each sample was loaded onto a 12% SDS-polyacrylamide gel (Bio-Rad, Hercules, CA). The separated proteins were transferred onto a nitrocellulose membrane (GE Healthcare, Piscataway, NJ) which was blocked with 5% nonfat dry milk for 1 hour at room temperature. The membrane was then incubated overnight at 4°C with rabbit anti-caspase 8 antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA). Binding of HRP-conjugated secondary antibody (goat anti-rabbit IgG-HRP, 1:2000; BD PharMingen, San Diego, CA) was performed for 1 hour at room temperature. The immune complex was detected by the ECL chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ) and exposure to x-ray film. To verify equal loading among lanes, the membranes were stained with the intrinsic protein actin (mouse monoclonal anti- β -actin antibody; Sigma) after staining for caspase 8.

Results

Organotypic retina cultures

Cultured retinas were observed using an inverted microscope. Even after several exchanges of culture medium, the retinas remained attached to the filters. Hematoxylin and eosin staining of cultured retinas demonstrated that the overall architecture of the cultured retina was maintained throughout the culture period (Figure 1A). The staining patterns of Calbindin (Figure 2A), Go- α (Figure 2B), GABA (Figure 2C), GFAP (Figure 2D) and NF (not shown), which are located in horizontal cells, bipolar cells, amacrine cells, glial, and ganglion cells, respectively, and the morphology of individual cells appeared to be identical to that of freshly isolated normal mouse retina. In addition, no F4/80 positive activated microglia were observed in uninfected cultured retinas (not shown).

MCMV infection of the cultured retina

Following inoculation of MCMV, replicating virus was recovered from cultures (Figure 3) and MCMV EA and MCMV LA positive cells were observed in the retina. As early as day 4 p.i., a few MCMV infected cells were observed in the peripheral retina and by day 11, infected cells were observed not only in peripheral retina but also in the central retina. As shown in Figure 4, the majority of MCMV infected cells in the cultured retina were GFAP positive glia. Some virus infected cells were calbindin positive horizontal cells (Figure 5). Many GABA positive cells were also observed; however, since these cells were also GFAP positive, they were glia and not amacrine cells (Figure 6). A similar pattern of staining of infected cells has been observed in the retinas of MCMV infected immunosuppressed mice (16). F4/80 positive activated microglia, which were usually EA negative, were also observed in the retina (not shown). Photoreceptor atrophy and virus-infected cytomegalic cells were observed only in MCMV infected retinas (compare Figure 1A and Figure 1B).

Apoptosis in MCMV infected cultured retinas

To determine if apoptosis was induced and to identify which retinal cells underwent apoptosis after MCMV infection, cultured retinas were double stained with TUNEL and with antibodies specific for retinal cell antigens or for MCMV antigens. In the absence of MCMV infection, only a few apoptotic cells were noted in the cultured retina (Figure 7). Many TUNEL positive cells were observed in all layers of the MCMV infected retina (Figure 8). Consistent with previous observations *in vivo* (14-16), the majority of apoptotic cells were uninfected bystander retinal cells (not shown). Many apoptotic cells observed in the outer nuclear layer were photoreceptor cells (Figure 8) while some of them were horizontal cells (Figure 8) or bipolar cells (not shown). Since both caspase dependent and independent apoptosis were reported in the retinal apoptosis (21), slides of MCMV infected retina and control retina were detected with active caspase 3. Active caspase 3 positive cells were observed in the MCMV infected retina (Figure 9 D, E, F), but not in the control retina (Figure 9 A, B, C). Although TUNEL positive cells were observed in all layers of the retina (Figure 8), the majority of active caspase 3 positive cells were only noted in the inner nuclear layer and ganglion cell layer (Figure 9 D, E, F).

A mouse apoptosis microarray was used to confirm that apoptosis was induced and to explore the possible inducer(s) of apoptosis in cultured MCMV infected retinas. Of the 112 genes analyzed, 29 genes were upregulated more than 2 fold and 5 genes were downregulated more than 2 fold by MCMV infection. Among the 29 upregulated genes, 19 are directly involved in apoptosis (Table 1). Some genes involved in the death receptor mediated apoptotic pathway, such as caspase 3, caspase 8, TNF- α , and Apaf1 (22), were upregulated. Although Fas and Tnfrsf22, a TRAIL receptor, were upregulated, Fas ligand and TRAIL were not detected in MCMV infected retinas. Some genes involved in the

mitochondrial pathway of apoptosis, for example, Bid, Bax and Trp53 (23), were also upregulated. ASC, an adaptor molecule important for caspase-1 activation (23) was strongly upregulated (17.59 fold) while caspase 1 was increased 7.6 fold in the MCMV infected retinas. Other upregulated apoptosis genes included caspase12 which is usually activated by oxidative stress (24), 3 genes (BCL10, card6 and card14) which mediate activation of NF-kappa B (25, 26), and two BCL2 like genes (Bcl2L11, Bcl2L14) which are initiators of apoptosis (27).

To confirm the microarray results which indicated that genes involved in both the death receptor mediated apoptotic pathway and mitochondrial pathway as well as the ASC gene were upregulated, expression of apoptotic genes including caspase 3, caspase 8, Bax and ASC was analyzed by RT-PCR. The results were similar to microarray results. As shown in Figure 10, more apoptotic gene mRNA was detected in the MCMV infected cultured retinas, compared to mRNA level in the normal retinas and in uninfected control retinal cultures. In addition, protein was extracted from the MCMV infected retinas and control retinas and detected with caspase 3 activity and caspase 8 cleavage. The results showed that caspase 3 activity of MCMV infected retinas was 0.06 nmole/mg protein/hour, while the level of caspase activity in control mice was undetectable. Caspase 8 cleavage was measured with Western blotting. Elevated cleaved caspase 8 was found in the MCMV infected retinas (Figure 11).

Several anti-apoptosis genes were also upregulated (Table 2). Some of these genes function as neuronal apoptosis inhibitor proteins, such as bag4, Cflar and Birc serial proteins (28-32). The gene for IL-10, an anti-inflammatory cytokine, was also upregulated 4.86 fold. In contrast, as shown in Table 3, downregulated genes were those which are usually highly expressed in the normal retina and perform functions such as regulating cell growth (Ark1) (33), epithelial sodium transport (Tsc22d3) (34) and modulating ATP metabolism (Mapk8ip1) (35).

TNF- α in MCMV infected cultured retinas

To determine that the apoptosis inducer TNF- α was produced during MCMV infection of the retina, expression of TNF- α mRNA was analyzed by RT-PCR. As shown in Figure 12, TNF- α mRNA was observed in MCMV infected retinas but not in uninfected control retinas. To determine if TNF- α was produced by the activated microglia which were observed in the MCMV infected retinas, retinas were stained for TNF- α and F4/80. TNF- α positive cells were indeed observed in MCMV infected retinas, and the majority of these cells were activated microglia which were F4/80 positive (Figure 13).

Discussion

Organotypic retinal cultures have been developed and used for investigation and experimental manipulation of the retina under controlled biochemical and treatment conditions (36-38). In contrast to cultures of dissociated retinal cells, organotypic cultures have the advantage of the highest degree of architecture preservation, retaining cell-cell contacts and interactions among different cell populations. Results from the MCMV infected organotypic retinal cultures described herein are similar to observations of the retina from studies using MCMV infected mice (14-16). For example, both in culture and in animals (15, 16), the majority of MCMV infected cells were glia and horizontal cells. MCMV infection in organotypic retinal culture resulted in atrophy of the photoreceptor cells and the appearance of cytomegalic MCMV infected cells.

Apoptosis is a consistent finding in HCMV infection of the retina in human patients (12, 13) and in the studies of MCMV retinitis in the mouse model (14-16, 20). The results from the

current studies of MCMV infected cultured retina confirmed that apoptosis was induced after MCMV retinal infection and the majority of the TUNEL+ apoptotic cells, including photoreceptors and horizontal cells, were uninfected. TNF- α , as well as some genes involved in apoptotic death receptor-mediated pathways, such as caspase 3, caspase 8, and Apaf1, were upregulated during MCMV infection of cultured retina. The results correlate well with our previous observation that TNF- α , which was produced mainly by activated microglia in MCMV infected retina, plays a role in the apoptosis of MCMV infected retina (20). Because the retinal cultures lack inflammatory cells that migrate to the eye during MCMV infection, the results of these studies using cultured retinas provide additional support for our previous suggestion that retinal apoptosis during MCMV infection is not T cell dependent (14, 20). In addition, some genes involved in the mitochondrial pathway, such as Bid, Bax and Trp53, were also upregulated. It has been shown that mitochondrial activation mediated by Bid, a BH3-only pro-death Bcl-2 family protein and the major molecule linking the two pathways, is responsible for the prompt progress of TNF- α induced apoptosis (39-46). Bid, which is activated by caspase 8 following TNF- α stimulation (39, 40), can activate mitochondria via direct interaction with the multidomain pro-death molecule Bax or Bak (40-44) or via the cathepsin B and caspase 2 pathway (45). TNF- α might activate mitochondria in our model since high levels of mRNA for TNF- α , Bid, caspase 8 and Bax were detected.

However, other factors in addition to TNF- α may also contribute to retinal apoptosis. One interesting result is the highly increased expression of ASC in the MCMV infected retina. ASC is a pro-apoptotic protein originally found as a component of a “speck” in apoptotic cells and contains an N-terminal pyrin domain (PD) and a C-terminal caspase-recruitment domain (CARD) (47). Both CARD and PD are protein-protein interaction domains and are structurally related to the death domain (DD) and death effector domain (DED) (48, 49). Recent studies have shown that ASC is key to caspase-1 activation which, in turn, is essential for the processing and release of biologically active IL-1 β (50-55), a proinflammatory cytokine with critical roles in innate and adaptive immunities (56). IL-1 β is also known to be an inducer of cellular NO production through activation of iNOS which could induce retinal apoptosis in some retinal diseases (57-60). In addition to caspase 1 activation, one recent study showed that ASC mediates induction of multiple cytokines, including TNF- α , by *Porphyromonas gingivalis* via a caspase-1-independent pathway (61). The exact role of ASC in apoptosis of MCMV infected retina remains to be determined.

Some anti apoptosis genes were also elevated. These genes probably have two functions. First, many of these genes, for example, bag4, Cflar and Birc serial proteins, function as inhibitors of neuronal apoptosis (27-31), which could counter proapoptotic stimuli and contribute to preventing apoptosis of uninfected retinal neurons. Second, elevated anti apoptosis gene expression in MCMV infected cells induced by virus infection or by specific viral genes could prevent apoptosis in MCMV infected cells. Recently it has been shown that HCMV IE2 induces expression of cellular Cflar (c-FLIP), an antiapoptotic molecule that blocks the direct downstream executor caspase 8 of the FasL/Fas pathway, and prevents HCMV infected cells from apoptosis (62).

In summary, results using the MCMV infected retinal culture system correlate with results from studies of the retina in MCMV infected immunosuppressed mice. In the future, this *in vitro* system could be used to explore the role of apoptosis of uninfected retinal cells as well as the contribution of cytokines and other modulators to the pathogenesis of cytomegalovirus retinitis. The retinal culture system might also be used to test new approaches to prevent/reduce viral replication since it permits manipulation/examination under carefully controlled experimental conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Wentworth BB, Alexander ER. Seroepidemiology of infections due to members of the herpes virus group. *Am J Epidemiol*. 1971; 94:496–507. [PubMed: 4330477]
2. Weller TH, Hanshaw JB. Virologic and clinical observations on cytomegalic inclusion disease. *N Eng J Med*. 1962; 266:1233–1244.
3. Jacobson MA, Mills J. Serious cytomegalovirus disease in the acquired immunodeficiency syndrome (AIDS). *Ann Intern Med*. 1988; 108:585–594. [PubMed: 2831765]
4. Culbertson WW. Infections of the retina in AIDS. *Internat Ophthalmol Clin*. 1989; 29:108–118.
5. Jabs DA. Acquired Immunodeficiency syndrome and the eye. *Arch Ophthalmol*. 1996; 114:863–866. [PubMed: 8660172]
6. Goldberg DE, Smithen LM, Angelilli A, Freeman WR. HIV-associated retinopathy in the HAART era. *Retina*. 2005; 25:633–649. [PubMed: 16077362]
7. Drew WL. Cytomegalovirus infection in patients with AIDS. *Clin Infect Dis*. 1992; 14:608–615. [PubMed: 1313313]
8. Cohen J. New hope against blindness. *Science*. 1995; 268:368–369. [PubMed: 7716538]
9. McCracken GJ, Shinefield HR, Cobb K, Rausen SR, Dische MR, Eichenwald HF. Congenital cytomegalic inclusion disease. A longitudinal study of 20 patients. *Am J Dis Child*. 1969; 117:522–539. [PubMed: 4181125]
10. Stagno S, Reynolds DW, Amos CS, et al. Auditory and visual defects resulting from symptomatic and subclinical congenital cytomegaloviral and toxoplasma infections. *Pediatrics*. 1977; 59:669–678. [PubMed: 193086]
11. Boppana S, Amos C, Britt W, et al. Late onset and reactivation of chorioretinitis in children with congenital cytomegalovirus infection. *Pediatr Infect Dis J*. 1994; 13:1139–1142. [PubMed: 7892084]
12. Chiou SH, Liu JH, Hsu WM, et al. Upregulation of Fas ligand expression by human cytomegalovirus immediate-early gene product 2: a novel mechanism in cytomegalovirus-induced apoptosis in human retina. *J Immunol*. 2001; 167:4098–4103. [PubMed: 11564832]
13. Buggage RR, Chan CC, Matteson DM, Reed GF, Whitcup SM. Apoptosis in cytomegalovirus retinitis associated with AIDS. *Curr Eye Res*. 2001; 21:721–729. [PubMed: 11120560]
14. Bigger JE, Tanigawa M, Zhang M, Atherton SS. Murine cytomegalovirus infection causes apoptosis of uninfected retinal cells. *Invest Ophthalmol Vis Sci*. 2000; 41:2248–2254. [PubMed: 10892869]
15. Zhang M, Atherton SS. Apoptosis in the retina during MCMV retinitis in immunosuppressed BALB/c mice. *J Clin Virol*. 2002; 25:S137–147. [PubMed: 12361764]
16. Zhang M, Xin H, Roon P, Atherton SS. Infection of retinal neurons during murine cytomegalovirus retinitis. *Invest Ophthalmol Vis Sci*. 2005; 46:2047–2055. [PubMed: 15914622]
17. Pande H, Campo K, Shanley JD, et al. Characterization of a 52K protein of murine cytomegalovirus and its immunological cross-reactivity with the DNA-binding protein ICP36 of human cytomegalovirus. *J Gen Virol*. 1991; 72:1421–1427. [PubMed: 1646282]
18. Jiang M, Yi X, Hsu S, Wang CY, Dong Z. Role of p53 in cisplatin-induced tubular cell apoptosis: dependence on p53 transcriptional activity. *Am J Physiol Renal Physiol*. 2004; 287:F1140–7. (Epub 2004 Aug 17). [PubMed: 15315938]
19. Dong Z, Wang J. Hypoxia selection of death-resistant cells: a role for Bcl-XL. *J Biol Chem*. 2004; 279:9215–9221. [PubMed: 14676192]

20. Zhou J, Zhang M, Atherton SS. TNF-alpha-induced apoptosis in murine cytomegalovirus retinitis. *Invest Ophthalmol Vis Sci.* 2007; 48:1691–1700. [PubMed: 17389501]
21. Carmody RJ, Cotter TG. Oxidative stress induces caspase-independent retinal apoptosis in vitro. *Cell Death Differ.* 2000; 7:282–291. [PubMed: 10745273]
22. Chowdhury I, Tharakan B, Bhat GK. Current concepts in apoptosis: The physiological suicide program revisited. *Cell Mol Biol Lett.* 2006; 11:506–525. [PubMed: 16977376]
23. Yu JW, Wu J, Zhang Z, et al. Cryopyrin and pyrin activate caspase-1, but not NF-kappaB, via ASC oligomerization. *Cell Death Differ.* 2006; 13:236–249. [PubMed: 16037825]
24. Wootz H, Hansson I, Korhonen L, Napankangas U, Lindholm D. Caspase-12 cleavage and increased oxidative stress during motoneuron degeneration in transgenic mouse model of ALS. *Biochem Biophys Res Commun.* 2004; 322:281–286. [PubMed: 15313203]
25. Stehlik C, Hayashi H, Pio F, Godzik A, Reed JC. CARD6 is a modulator of NF-kappa B activation by Nod1- and Cardiak-mediated pathways. *J Biol Chem.* 2003; 278:31941–31949. [PubMed: 12775719]
26. Gaide O, Martinon F, Mischeau O, Bonnet D, Thome M, Tschopp J. Carma1, a CARD-containing binding partner of Bcl10, induces Bcl10 phosphorylation and NF-kappaB activation. *FEBS Lett.* 2001; 496:121–127. [PubMed: 11356195]
27. Napankangas U, Lindqvist N, Lindholm D, Hallbook F. Rat retinal ganglion cells upregulate the pro-apoptotic BH3-only protein Bim after optic nerve transection. *Brain Res Mol Brain Res.* 2003; 120:30–37. [PubMed: 14667574]
28. Gotz R, Wiese S, Takayama S, et al. Bag1 is essential for differentiation and survival of hematopoietic and neuronal cells. *Nat Neurosci.* 2005; 8:1169–1178. [PubMed: 16116448]
29. Eichholtz-Wirth H, Fritz E, Wolz L. Overexpression of the ‘silencer of death domain’, SODD/BAG-4, modulates both TNFR1- and CD95-dependent cell death pathways. *Cancer Lett.* 2003; 194:81–89. [PubMed: 12706861]
30. Dutton A, Young LS, Murray PG. The role of cellular FLICE inhibitory protein (c-FLIP) in the pathogenesis and treatment of cancer. *Expert Opin Ther Targets.* 2006; 10:27–35. [PubMed: 16441226]
31. Qiu XB, Markant SL, Yuan J, Goldberg AL. Nrdp1-mediated degradation of the gigantic IAP, BRUCE, is a novel pathway for triggering apoptosis. *EMBO J.* 2004; 23:800–810. [PubMed: 14765125]
32. Lesne S, Gabriel C, Nelson DA, et al. Akt-dependent expression of NAIP-1 protects neurons against amyloid-beta toxicity. *J Biol Chem.* 2005; 280:24941–24947. [PubMed: 15797869]
33. Puig O, Marr MT, Ruhf ML, Tjian R. Control of cell number by Drosophila FOXO: downstream and feedback regulation of the insulin receptor pathway. *Genes Dev.* 2003; 17:2006–2020. [PubMed: 12893776]
34. Fiol DF, Mak SK, Kultz D. Specific TSC22 domain transcripts are hypertonically induced and alternatively spliced to protect mouse kidney cells during osmotic stress. *FEBS J.* 2007; 274:109–124. [PubMed: 17147695]
35. Taru H, Kirino Y, Suzuki T. Differential roles of JIP scaffold proteins in the modulation of amyloid precursor protein metabolism. *J Biol Chem.* 2002; 277:27567–27574. [PubMed: 12023290]
36. Duenker N, Valenciano AI, Franke A, et al. Balance of pro-apoptotic transforming growth factor-beta and anti-apoptotic insulin effects in the control of cell death in the postnatal mouse retina. *Eur J Neurosci.* 2005; 22:28–38. [PubMed: 16029193]
37. Franke AG, Gubbe C, Beier M, Duenker N. Transforming growth factor-beta and bone morphogenetic proteins: cooperative players in chick and murine programmed retinal cell death. *J Comp Neurol.* 2006; 495:263–278. [PubMed: 16440295]
38. Reidel B, Orisme W, Goldmann T, Smith WC, Wolfrum U. Photoreceptor vitality in organotypic cultures of mature vertebrate retinas validated by light-dependent molecular movements. *Vision Res.* 2006; 46:4464–4471. [PubMed: 16979692]
39. Gross A, Yin XM, Wang K, et al. Caspase cleaved BID targets mitochondria and is required for cytochrome c release, while BCL-XL prevents this release but not tumor necrosis factor-R1/Fas death. *J Biol Chem.* 1999; 274:1156–1163. [PubMed: 9873064]

40. Zhao Y, Li S, Childs EE, Kuharsky DK, Yin X-M. Activation of Pro-death Bcl-2 family proteins and mitochondria apoptosis pathway in tumor necrosis factor-alpha-induced liver injury. *J Biol Chem.* 2001; 276:27432–27440. [PubMed: 11369777]
41. Desagher S, Osen-Sand A, Nichols A, et al. Bid-induced conformational change of Bax is responsible for mitochondrial cytochrome *c* release during apoptosis. *J Cell Biol.* 1999; 144:891–901. [PubMed: 10085289]
42. Ding WX, Ni HM, DiFrancesca D, Stolz DB, Yin XM. Bid-dependent generation of oxygen radicals promotes death receptor activation-induced apoptosis in murine hepatocytes. *Hepatology.* 2004; 40:403–413. [PubMed: 15368445]
43. Wei MC, Lindsten T, Mootha VK, et al. tBID, a membrane-targeted death ligand, oligomerizes BAK to release cytochrome *c*. *Genes Dev.* 2000; 14:2060–2071. [PubMed: 10950869]
44. Zhao Y, Ding WX, Qian T, Watkins S, Lemasters JJ, Yin XM. Bid activates multiple mitochondrial apoptotic mechanisms in primary hepatocytes after death receptor engagement. *Gastroenterology.* 2003; 125:854–867. [PubMed: 12949730]
45. Guicciardi ME, Bronk SF, Werneburg NW, Yin XM, Gores GJ. Bid is upstream of lysosome-mediated caspase 2 activation in tumor necrosis factor alpha-induced hepatocyte apoptosis. *Gastroenterology.* 2005; 129:269–284. [PubMed: 16012953]
46. Chen X, Ding WX, Ni HM, et al. Bid-independent mitochondrial activation in tumor necrosis factor alpha-induced apoptosis and liver injury. *Mol Cell Biol.* 2007; 27:541–553. [PubMed: 17101783]
47. Masumoto JS, Taniguchi S, Ayukawa K, et al. ASC, a novel 22-kDa protein, aggregates during apoptosis of human promyelocytic leukemia HL-60 cells. *J Biol.Chem.* 1999; 274:33835–33838. [PubMed: 10567338]
48. Masumoto J, Taniguchi S, Sagara J. Pyrin N-terminal homology domain- and caspase recruitment domain-dependent oligomerization of ASC. *Biochem Biophys Res Commun.* 2001; 280:652–655. [PubMed: 11162571]
49. Richards N, Schaner P, Diaz A, et al. Interaction between pyrin and the apoptotic speck protein (ASC) modulates ASC-induced apoptosis. *J Biol Chem.* 2001; 276:39320–39329. [PubMed: 11498534]
50. Poyet JL, Srinivasula SM, Tnani M, Razmara M, Fernandes-Alnemri T, Alnemri ES. Identification of Ipaf, a human caspase-1-activating protein related to Apaf-1. *J Biol Chem.* 2001; 276:28309–28313. [PubMed: 11390368]
51. Agostini L, Martinon F, Burns K, McDermott MF, Hawkins PN, Tschopp J. NALP3 forms an IL-1 β -processing inflammasome with increased activity in Muckle-Wells autoinflammatory disorder. *Immunity.* 2004; 20:319–325. [PubMed: 15030775]
52. Dowds TA, Masumoto J, Zhu L, Inohara N, Nunez G. Cryopyrin-induced interleukin 1 β secretion in monocytic cells: enhanced activity of disease-associated mutants and requirement for ASC. *J Biol Chem.* 2004; 279:21924–21928. [PubMed: 15020601]
53. Martinon F, Burns K, Tschopp J. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL- β . *Mol Cell.* 2002; 10:417–426. [PubMed: 12191486]
54. Kanneganti TD, Body-Malapel M, Amer A, et al. Critical role for Cryopyrin/Nalp3 in activation of caspase-1 in response to viral infection and double-stranded RNA. *J Biol Chem.* 2006; 281:36560–36568. [PubMed: 17008311]
55. Johnston JB, Barrett JW, Nazarian SH, et al. A poxvirus-encoded pyrin domain protein interacts with ASC-1 to inhibit host inflammatory and apoptotic responses to infection. *Immunity.* 2005; 23:587–598. [PubMed: 16356857]
56. Dinarello CA. Interleukin-1 β , interleukin-18, and the interleukin-1 β converting enzyme. *Ann NY Acad Sci.* 1998; 856:1–11. [PubMed: 9917859]
57. Veluthakal R, Jangati GR, Kowluru A. IL-1 β -induced iNOS expression, NO release and loss in metabolic cell viability are resistant to inhibitors of ceramide synthase and sphingomyelinase in INS 832/13 cells. *JOP.* 2006; 7:593–601. [PubMed: 17095838]
58. Sennlaub F, Courtois Y, Goureau O. Inducible nitric oxide synthase mediates retinal apoptosis in ischemic proliferative retinopathy. *J Neurosci.* 2002; 22:3987–3993. [PubMed: 12019318]

59. Matveeva NY, Kalinichenko SG, Pushchin II, Motavkin PA. The role of nitric oxide in the apoptosis of neurons in the retina of the human fetal eye. *Neurosci Behav Physiol.* 2007; 37:111–118. [PubMed: 17187201]
60. Sanvicens N, Gomez-Vicente V, Masip I, Messeguer A, Cotter TG. Oxidative stress-induced apoptosis in retinal photoreceptor cells is mediated by calpains and caspases and blocked by the oxygen radical scavenger CR-6. *J Biol Chem.* 2004; 279:39268–39278. [PubMed: 15210718]
61. Taxman DJ, Zhang J, Champagne C, et al. Cutting edge:ASC mediates the induction of multiple cytokines by *Porphyromonas gingivalis* via caspase-1-dependent and -independent pathways. *J Immunol.* 2006; 177:4252–4256. [PubMed: 16982856]
62. Chiou SH, Yang YP, Lin JC, et al. The immediate early 2 protein of human cytomegalovirus (HCMV) mediates the apoptotic control in HCMV retinitis through up-regulation of the cellular FLICE-inhibitory protein expression. *J Immunol.* 2006; 177:6199–6206. [PubMed: 17056549]

Figure 1. Photomicrographs of hematoxylin and eosin stained sections of a control (uninfected) cultured retina (A) and an MCMV infected cultured retina (B) at day 11 p.i. The overall architecture of the retina in the control cultures is well preserved (A). Photoreceptor atrophy and loss and cytomegalic virus infected cells are observed in the MCMV infected retina (arrows) (B). The retinas are oriented so that the filter/photoreceptor layer is at the top of each micrograph. Original magnification: 200 $\times$.

Figure 2.

Photomicrographs of double staining for DAPI (A2, B2, C2, D2) and Calbindin (A1), Go- α (B1), GABA (C1), and GFAP (D1), which are located in horizontal cells, bipolar cells, amacrine cells, and glia, respectively in a control (uninfected) cultured retina. The merged images are shown in A3, B3, C3, and D3. Original magnification: 200 $\times$.

Days after MCMV Infection of Cultured Retina

Figure 3.

Titer of MCMV ($\text{Log}_{10} \pm \text{SEM PFU/ml}$) in the MCMV infected cultured retinas at day 4, 7, and 11 days after inoculation of MCMV.

Figure 4. Photomicrographs of staining for MCMV early antigen (EA), late antigen (LA), and GFAP in an MCMV infected cultured retina at day 11 p.i. Many MCMV infected cells in the cultured retina were GFAP positive glia (arrows) (EA –A; LA – B; GFAP – C; merge – D). Original magnification: 200 $\times$.

Figure 5. Photomicrographs of staining for MCMV EA and calbindin in an MCMV infected cultured retina at day 11 p.i. Some MCMV infected cells in the cultured retina were calbindin positive horizontal cells (arrows) (EA –A; calbindin – B; DAPI – C; merge – D). Original magnification: 200 $\times$.

Figure 6. Photomicrographs of MCMV EA (A), GFAP(B) and GABA (C) positive cells (arrows) in an MCMV infected cultured retina at day 11 p.i. As shown in the merged image (D), most of the MCMV-infected, GABA-positive cells were also GFAP positive. Original magnification: 200 $\times$.

Figure 7. Photomicrographs of TUNEL staining in a control retina after 11 days of culture. Isolated areas containing a few apoptotic cells were observed in the cultured retina (circle) (TUNEL –A; DAPI –B). Original magnification: 200 $\times$.

Figure 8. Photomicrographs of staining for TUNEL and Calbindin in an MCMV infected cultured retina at day 11 p.i. Many apoptotic cells observed in the outer nuclear layer were photoreceptor cells; rare calbindin positive horizontal cells were also seen (arrow) (TUNEL –A; calbindin –B; DAPI –C; merge –D). Original magnification: 200 $\times$.

Figure 9. Photomicrographs of uninfected cultured retina (top row) after 11 days of culture and MCMV infected cultured retina (bottom row) at day 11 p.i. DAPI staining (A,D) and active caspase 3 staining (B,E). The merged images of the uninfected and infected retinas are shown in C and F respectively. Original magnification: 200 $\times$.

Figure 10.

mRNA of apoptosis genes in MCMV infected cultured retinas at day 7 p.i. (A). RNA from normal retinas and from non-MCMV infected retinal cultures was used as the controls. More apoptotic gene mRNA was detected in the MCMV infected cultured retinas. Amplification of β -actin was used to verify loading. (B) Ratio of target gene mRNA/ β -actin mRNA.

Figure 11.

(A) Western blot of caspase-8 cleavage in MCMV infected cultured retinas at day 7 p.i. Protein from normal retinas and from non-MCMV infected retinal cultures was used as controls. Cleaved caspase 8 was detected in the MCMV infected cultured retinas. β-actin was used to verify loading. (B) Ratio of cleaved caspase 8/β actin.

Figure 12.

TNF- α mRNA in MCMV infected cultured retinas at day 7 p.i. TNF- α mRNA was detected in MCMV infected retinas but not in control retinas. RNA from MCMV infected eyes of immunosuppressed (IS) and RNA from non IS mice were used as TNF- α positive controls. No TNF- α mRNA was detected in MCMV infected or uninfected RPE cells. A 1kb ladder was used to identify TNF- α RNA and amplification of α -actin was used to verify loading.

Figure 13.

Photomicrographs of staining of TNF- α and F4/80 in an MCMV infected cultured retina at day 11 p.i. Most of the TNF- α positive cells were F4/80 positive activated microglia (arrow) (TNF- α –A, F4/80 – B, DAPI – C, merge – D). Original magnification: 200 $\times$.

Table 1

Increased expression of mRNA for apoptosis related genes during MCMV infection of the cultured retina.

Symbol	Description	Control	MCMV	MCMV/CTR
Casp3	Caspase3	5.49	28.17	5.13
Casp8	Caspase8	36.49	86.48	2.37
Tnf	Tumor Necrosis Factor	5.00	64.17	12.83
TNFRsf22	Tumor necrosis factor receptor superfamily member 22	17.41	119.26	6.85
Fas	Fas (TNF receptor superfamily member)	56.04	127.87	2.28
Apaf1	Apoptotic peptidase activating factor 1	5.00	36.53	7.31
Bax	Bcl2-associated X protein	14.24	43.17	3.03
Bid	BH3 interacting domain death agonist	20.34	43.35	2.13
Trp53	Transformation related protein 53	10.04	38.12	3.80
ASC	apoptosis-associated speck-like protein containing a caspase-recruitment domain	37.03	651.47	17.59
Casp1	Caspase 1	10.54	80.08	7.60
Casp12	Caspase 12	12.41	115.63	9.32
Card6	Caspase recruitment domain family, member 6	5.00	36.72	7.34
Card14	Caspase recruitment domain family, member 14	5.00	17.32	3.46
Bcl10	B-cell leukemia/lymphoma 10	13.28	80.42	6.06
Bcl2l11	Bcl2-like 11 (apoptosis facilitator)	5.00	37.10	7.42
Bcl2l14	Bcl2-like 14 (apoptosis facilitator)	5.00	75.21	15.04
Cideb	Cell death-inducing DNA fragmentation factor, alpha subunit-like effector B	36.26	135.73	3.74
BNIP3	Bcl2/adenovirus E1B interacting protein 1, NIP3	162.67	381.89	2.35

Table 2

Increased expression of mRNA for anti apoptosis genes during MCMV infection of the cultured retina

Symbol	Description	Control	MCMV	MCMV/CTR
Api5	Apoptosis inhibitor 5	44.41	96.93	2.18
Atf5	Activating transcription factor 5	269.59	708.48	2.63
Bag4	BCL2-associated athanogene 4	24.07	118.03	4.90
Birc1a	Baculoviral IAP repeat-containing 1a	5.00	47.90	9.58
Birc1b	Baculoviral IAP repeat-containing 1b	5.00	21.56	4.31
Birc3	Baculoviral IAP repeat-containing 3	5.00	15.57	3.11
Birc4	Baculoviral IAP repeat-containing 4	5.00	13.00	2.60
Birc6	Baculoviral IAP repeat-containing 6	5.00	92.45	18.49
Cflar	CASP8 and FADD-like apoptosis regulator	41.30	255.53	6.19
IL-10	Interleukin 10	23.72	115.29	4.86

Table 3

Genes for which mRNA expression was decreased during MCMV infection of the cultured retina

Symbol	Description	Control	MCMV	MCMV/CTR
Akt1	Thymoma viral proto-oncogene 1	245.09	13.10	18.71
Mapk8ip1	Mitogen activated protein kinase 8 interacting protein 1	1005.06	224.21	4.48
Polb	Polymerase (DNA directed), beta	119.72	30.91	3.87
Prdx2	Peroxiredoxin 2	679.80	183.18	3.71
Rnf7	Ring finger protein 7	573.65	131.10	4.38