Murine Cytomegalovirus Infection Causes Apoptosis of Uninfected Retinal Cells

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PURPOSE. To determine the role of apoptosis in prevention and/or exacerbation of retinal disease in a mouse model of cytomegalovirus retinitis.

METHODS. Immunocompetent or T-cell–depleted BALB/c mice were injected with murine cytomegalovirus (MCMV) by supraciliary injection. On sequential days after infection, mice were killed, and eyes were harvested for cryosectioning or for DNA extraction. Ocular sections were stained with monoclonal antibodies specific for MCMV or for T cells or used in the TdT-dUTP terminal nick-end labeling (TUNEL) assay to detect apoptotic cells.

RESULTS. In immunocompetent BALB/c mice, TUNEL assays revealed that a large area of the retina was apoptotic in relation to the relatively small number of MCMV-infected cells that were observed in the subjacent choroid and/or retinal pigment epithelium. In infected eyes from T-cell–depleted mice, there were more TUNEL-positive cells, and the areas of apoptosis were more extensive than in immunocompetent mice. These observations correlated with the increased extent of MCMV infection that is observed in the eyes of T-cell–depleted mice. However, irrespective of immune status, TUNEL-positive apoptotic cells were present mainly in areas of the retina overlying areas of MCMV-infected choroid and/or retinal pigment epithelium. More intense DNA laddering, indicative of increased apoptosis, was observed in the posterior segments of the eyes of T-cell–depleted mice after supraciliary inoculation with murine cytomegalovirus compared with less intense DNA laddering in the posterior segments of eyes of immunocompetent MCMV-infected mice.

CONCLUSIONS. The ability of the mouse’s immune system to control MCMV infections in some tissues depends on induction of apoptosis in virus-infected cells. However, in the retina, cells undergoing apoptosis were not virus-infected, a finding that suggests that apoptosis of uninfected retinal cells may play a role in the pathogenesis of MCMV retinitis. (Invest Ophthalmol Vis Sci. 2000;41: 2248–2254)

Cytomegalovirus (CMV) infection in immunocompetent individuals usually causes a self-limiting, subclinical disease. However, in immunocompromised patients, such as patients who have received a bone marrow transplant or patients with acquired immune deficiency syndrome (AIDS), CMV infections are a significant cause of morbidity and death. The primary presentation of CMV disease in patients with AIDS is retinitis, which occurs in 29% to 32% of patients with AIDS. Development of CMV retinitis in patients with AIDS has been correlated with the loss of CD4 T cells and CD8 T cells and has been indirectly correlated with the loss of natural killer (NK) cell activity. In a mouse model of CMV retinitis, depletion of either NK cells or CD8 T cells predisposes mice to retinal infection with murine CMV (MCMV).

NK cells and T cells can mediate control of CMV by recognizing infected cells and inducing them to undergo apoptosis and/or by secreting antiviral cytokines such as interferon (IFN)-γ and tumor necrosis factor (TNF)-α. The relative importance of either mechanism for control of CMV depends on the tissue. In nonregenerating cells, such as the neurons of the retina, virus clearance by nonlytic mechanisms would be more beneficial to the host by causing less cell death and tissue destruction than lysis of infected neurons. The purpose of these studies was to determine the role of apoptosis during MCMV infection of the retina. The results of these studies suggest that apoptosis does not facilitate clearance of MCMV from the eye, but rather increases MCMV-induced retinal disease by causing death of uninfected bystander retinal cells.

METHODS

Animals

Female euthymic BALB/c mice, 6 to 8 weeks old, were obtained from Taconic (Germantown, NY). Animals were housed in accordance with National Institutes of Health guidelines, and all study procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Mice were kept on a 12-hour light–dark cycle and given unrestricted access to food and water.
Virus and Virus Titrations

Stocks of MCMV (Smith strain) were prepared from salivary gland homogenates of MCMV-infected BALB/c mice as previously described. The RM461 strain of MCMV was kindly provided by Edward S. Moraski and Cheryl Stoddart (Department of Microbiology and Immunology, Stanford University School of Medicine, CA). RM461 contains a modified Escherichia coli LacZ, β-galactosidase (β-gal) gene at the HindIII L/J site just downstream of immediate-early gene 2. RM461 expresses β-gal as an early viral gene product during the viral replication cycle. Stocks of RM461 were prepared on Swiss Brown mouse embryo fibroblasts. Virus stocks of both strains were titered in duplicate on Swiss Brown mouse embryo fibroblasts. Mock virus stocks were prepared from salivary gland homogenates of uninfected mice. Before injection, the salivary gland homogenates from mock-infected and MCMV-infected mice were diluted identically (1:44, which reduced the titer of gland homogenates from mock-infected and MCMV-infected homogenates of uninfected mice. Before injection, the salivary gland homogenates from mock-infected and MCMV-infected mice were diluted identically (1:44, which reduced the titer of the virus stock from 2 × 10^7 plaque-forming units (pfu)/ml to 4.5 × 10^5 pfu). Therefore, 2 μl of the virus stock solution used for injection contained 9 × 10^7 pfu MCMV.

Immunodepletion

Thymectomies were performed on 6-week-old mice by using a modification of a protocol by Chin. Thymectomized mice were rested for 1 week before T-cell depletion. T-cell depletion was accomplished by intravenous injection of 500 μg anti-CD4 made in ascites from the GK1.5 hybridoma (American Type Culture Collection, Rockville, MD) and 150 μg anti-CD8 made in ascites from the 2.43 hybridoma (American Type Culture Collection). This protocol typically depletes 95% of the CD4+ T cells and 99% of the CD8+ T cells from the mouse as assayed by flow cytometry of splenocytes, and this level of depletion is maintained for at least 4 weeks. In some experiments, euthymic mice were immunosuppressed with methylprednisolone acetate (2 mg per mouse) by intramuscular injection every 3 days and with intravenous injection of antibodies (450 μg anti-CD4 [GK1.5], 100 μg anti-CD8 [2.43], and 10 μg anti-NK cell antisera [anti-asialo GM1; Wako, Richmond, VA] per injection) on days 0, +7, and +14 after MCMV injection.

Supraciliary Injection

Mice were anesthetized by intramuscular injection of a cocktail containing 0.02 ml Rompun and 0.03 ml ketamine per 25 g body mass. The left eyes of mice were injected with 9 × 10^7 pfu MCMV in a volume of 2 μl by the supraciliary route, as previously described. Immunohistochemistry and TUNEL

Animals were deeply anesthetized, killed, and perfused with PBS. Eyes were enucleated and cleaned of all muscle and connective tissue, leaving only the globe with some conjunctival tissue and approximately 1 mm of optic nerve. Eyes were immersed in OCT compound (Tissue-Tek; VWR Scientific, Houston, TX), snap frozen on dry ice, and sectioned on a semiautomatic cryostat (Microm HM505E; Zeiss, Houston, TX). Serial sections were collected on consecutive, coated slides (Superfrost/Plus; Fisher Scientific, Pittsburgh, PA). Monoclonal antibody to an MCMV early gene product, pp56, was precipitated from the supernatant of hybridoma cell line 25G11 (a gift of John Stanley, Department of Medicine, University of Connecticut Health Center, Farmington) by ammonium sulfate, purified by protein G chromatography (Gibco, Grand Island, NY), and biotinylated using the EZ sulfo-nolink system (Sulfo-NHS-LC-Biotin; Pierce, Rockford, IL) according to the manufacturer's instructions. The same method was used for biotinylation of anti-CD4 (from the GK1.5 hybridoma) and anti-CD8 (from the 2.43 hybridoma) antibodies. For TdT-dUTP terminal nick-end labeling (TUNEL), frozen sections were brought to room temperature and fixed in 0.5% glutaraldehyde in phosphate buffered saline (PBS). After washing in PBS, sections were digested in proteinase K (40 μg/ml; Sigma Chemical, St. Louis, MO) for 15 minutes. Slides were treated for endogenous peroxidase by incubation in 0.3% H2O2 for 20 minutes and then incubated with 15 U/ml terminal deoxynucleotidyl transferase (TdT; Gibco) and 10 nM/ml biotin-16-dUTP (Boehringer Mannheim, Indianapolis, IN). Immunohistochemistry and TUNEL assays were conducted using the ABC streptavidin-horseradish peroxidase kit (Vector, Burlingame, CA) and developed using DAB (Sigma). All slides were counterstained with methyl green dye.

DNA Laddering

To detect DNA laddering, eyes were enucleated from PBS-perfused mice. The anterior segment was removed, and the remaining posterior segment was snap frozen. After thawing, the posterior segments from two eyes of each group of mice were pooled for extraction. DNA was extracted from the tissues by using a kit (Apoptosis Lysate Kit; Chemicon, Temecula, CA) according to the manufacturer’s specifications. The extract was ethanol precipitated and resuspended in 10 μl of buffer 3 provided in the apoptosis lysate kit. Because this procedure leaves most of the large DNA unextracted, equivalent volumes (5 μl of the extracted material) from each of the experimental and control groups were loaded in each lane of a 1% agarose gel and electrophoresed for 2 hours at 70 V.

RESULTS

MCMV Infection Causes Apoptosis in Retinal Tissue

Because NK cells and T cells have been shown to modulate MCMV retinitis and because NK cells and T cells can induce apoptosis in virus-infected cells, experiments were performed to test the hypothesis that apoptosis plays a role in MCMV retinitis. To determine whether apoptosis occurs in MCMV-infected eyes, BALB/c mice were injected with 9 × 10^7 pfu MCMV by the supraciliary route. Uninfected and mock-infected mice were used as controls. Three mice from each group were killed and perfused with PBS on days 2, 3, 5, and 7 after infection. Eyes were enucleated, frozen in OCT compound, and sectioned on a cryostat. As described in the Methods section, serial sections were collected so that adjacent sections could be stained on separate slides. A TUNEL assay was conducted on sections from each eye.

Apoptotic cells were not observed in the eyes of uninfected mice (not shown). On postinjection (PI) days 2, 3, and 5, TUNEL-positive cells were only rarely observed in the retinas of mock-infected mice. However, by PI day 7, TUNEL-positive cells were no longer observed in the retinas of mice in this group (not shown). TUNEL-positive cells were never observed in the cornea, iris, choroid, vitreous humor, aqueous humor, or
optic nerve of mock-injected mice. In contrast, in MCMV-infected mice, increasing numbers of apoptotic cells were observed in the choroid and retina on days PI days 3, 5 (not shown), and 7 (Fig. 1A). In MCMV-infected mice, it appeared that neuronal cells of the inner and outer nuclear layers of the retina were undergoing apoptosis and that apoptosis was not occurring in infiltrating inflammatory cells. However, because Griffiths et al.\(^\text{50}\) have shown that activated T cells migrating into ocular tissues after injection of HSV-1 into the anterior chamber undergo apoptosis mediated by Fas–Fas ligand interactions, additional staining was performed to confirm that the cells undergoing apoptosis were not infiltrating T cells.

To determine whether the apoptotic cells were T cells, serial sections adjacent to those used for the TUNEL assays were stained for the T-cell markers CD4 and CD8. In MCMV-injected immunocompetent mice, T cells were first observed at PI day 5 in the anterior segment, ganglion cell layer, and choroid (not shown). At this time, T cells were not observed in either the inner or the outer nuclear layers of the retina, although foci of apoptotic cells were seen in the outer and inner nuclear layers of the retina (not shown). At PI day 7, staining of the adjacent sections of the eye shown in Figure 1A revealed that CD4\(^+\) and CD8\(^+\) T cells were present in all retinal layers but only near the site of injection (not shown). In areas of the retina distant from the site of injection, CD4\(^+\) T cells (Fig. 1C) and CD8\(^+\) T cells (Fig. 1D) were observed predominantly in the choroid, and CD8\(^+\) cells were observed only rarely in the outer nuclear layer (Fig. 1D). Areas of apoptosis did not correlate with the locations of T cells (compare Fig. 1A with Fig. 1C and 1D). T cells were not observed in the eyes of either uninfected- or mock-infected mice at any time (not shown).

Because previous studies have demonstrated that CMV-infected cells are rarely found in the retinas of infected mice earlier than PI day 7, even in immunocompromised mice in which virus replicates and spreads more quickly than in non-immunocompromised mice,\(^\text{15,14}\) and because MCMV-infected cells normally die by necrosis,\(^\text{31}\) it appeared unlikely that the apoptotic cells observed in the present studies were virus-infected. To determine which ocular cells were infected with MCMV and whether these cells were the same as those undergoing apoptosis, serial sections from the eyes of normal immunocompetent MCMV-inoculated mice were stained for virus using a monoclonal antibody to pp56, an early antigen of MCMV. The pp56 antigen was not detected at any time in the ciliary body, iris or choroid of mock-infected mice. In infected mice, pp56-positive virus-infected cells were observed from days 2 through 7 in the ciliary body, iris, and choroid. In the posterior segment, MCMV infection of the choroid was observed mainly in areas near the site of injection. However, in animals in which the choroid distant from the site of injection was infected, the retina overlying the area of infected choroid was apoptotic (Figs. 1A, 1B). The number of MCMV-infected cells in the posterior segment of an infected immunocompetent mouse appeared to be low in relation to the number of retinal cells that were apoptotic (compare Figs. 1A, 1B). In MCMV-infected immunocompetent mice, virus did not spread into the deeper layers of the retina, and among the mice in this group, only one of 12 mice had virus in the outer nuclear layer, and only a single cell in this layer of this mouse (which was harvested on PI day 7) was MCMV-positive (not shown). Although the results shown in Figure 1 are from serial sections from the injected eye of one immunocompetent mouse at PI day 7, similar staining patterns for apoptosis, T cells, and virus-infected cells were observed in the serial sections from the other immunocompetent mice collected at that time.

**T-Cell Depletion Increases Virus Spread and Apoptosis**

T-cell-depleted mice are more susceptible to MCMV retinitis than immunocompetent mice and MCMV-infected cytomegalic cells are frequently observed in the retinas of T-cell-depleted mice on PI day 7.\(^\text{13,14,28}\) If apoptosis is associated with virus infection, then the retinas of T-cell-depleted mice should have more apoptotic cells than retinas of immunocompetent mice after MCMV infection. Alternatively, even though the experi-
ments in immunocompetent mice do not suggest that apoptosis is due to infiltration of T cells, it is possible that infiltrating T cells cause apoptosis and therefore that the retinas of T-cell–depleted mice should have few or no cells undergoing apoptosis. To differentiate between these two possibilities, thymectomized mice were depleted of T cells by injection of monoclonal antibodies to CD4 and CD8. After resting the mice for 28 days after T-cell depletion to allow for the catabolism of the rat monoclonal antibodies, the supraciliary space was injected with $9 \times 10^2$ pfu of MCMV. Three mice were killed on each of PI days 2, 3, 5, and 7; eyes were enucleated, frozen, and serial sections were collected on separate slides for comparative staining.

Immunohistochemistry using monoclonal antibody to MCMV indicated that virus spread through the choroid by PI day 5 (Fig. 2A) and into the retina at PI day 7 (Fig. 2C). Large areas of retinal destruction with cytomegalic cells consistent with retinitis were seen at PI day 7. Although TUNEL-positive cells were observed in most ocular structures, they were especially evident in the choroid and retina on day 5 (Fig. 2B), and by PI day 7, they were interspersed in the areas of virus infection (Fig. 2D). Just as foci of apoptotic cells were found on PI days 5 and 7 in the retina near areas of the choroid containing virus-positive cells in immunocompetent mice (see Figs. 1A, 1B), in T-cell–depleted mice on PI day 5, apoptotic cells in the retina were associated with areas of the choroid that contained virus-positive cells.

Comparison of TUNEL stains of eyes of MCMV-infected immunocompetent mice with those of MCMV-infected T-cell–depleted mice suggested that the eyes of T-cell–depleted mice contained more apoptotic cells than the eyes of immunocompetent mice. To confirm this observation, posterior segments were dissected from the anterior segments of the injected and contralateral eyes of T-cell–depleted and immunocompetent mice 7 days after MCMV injection; low-molecular-weight DNA was extracted and separated electrophoretically. Figure 3 shows that DNA laddering, caused by degradation of DNA into

**FIGURE 2.** MCMV spread and apoptosis in T-cell–depleted mice. Immunohistochemistry for MCMV-infected cells (A, C) and for apoptotic cells (TUNEL, B, D) in the infected eye of a T-cell–depleted mouse 5 (A, B) and 7 (C, D) days after supraciliary injection with $9 \times 10^2$ pfu of MCMV. MCMV-positive cells (A, C) and TUNEL-positive cells (B, D) are indicated by arrows. Magnification, $\times 316$.

**FIGURE 3.** Apoptosis-associated DNA fragmentation is increased in infected T-cell–depleted mice. Agarose gel electrophoresis (1% gel) of low-molecular-weight DNA extracted from the posterior segments of the eyes of uninfected immunocompetent mice (lane 1), infected immunocompetent mice (lanes 2 and 3), and infected T-cell–depleted mice (lanes 4 and 5). Eyes were removed 7 days after injection with $9 \times 10^7$ pfu of MCMV. Lanes 2 and 4 are posterior segments from the injected eyes, lanes 3 and 5 are from the un.injected, contralateral eyes. Markers (123 bp) are to the left of lane 1 and to the right of lane 5. These results are representative of three independent experiments.
nucleosomal fragments, was present in infected eyes of immunocompetent and T-cell–depleted mice. DNA laddering was not detected in the eyes of uninfected mice or in the uninjected eyes of the infected mice. In addition, more nucleosomal length DNA (indicated by increased intensity of 180-bp bands or multiples thereof) was extracted from the posterior segments of MCMV-inoculated eyes of T-cell–depleted mice than from the posterior segments of MCMV-inoculated immunocompetent mice (for example, compare lanes 2 and 4 in Fig. 3). The results shown in Figure 3 are representative of those from three independent experiments, each of which suggested that the amount of apoptosis was increased in the injected eyes of T-cell–depleted mice compared with the injected eyes of normal immunocompetent mice.

**Retinal Apoptosis Is Associated with Virus Infection and Not with Injection Trauma**

To determine whether the presence of virus was sufficient to induce apoptosis, euthymic mice were depleted of T cells by injection of monoclonal antibodies to CD4 and CD8, were depleted of NK cells by injection of anti-asialo GM1 antisera, and were further immunosuppressed by intramuscular injection of methylprednisolone. These mice were then injected in the supraciliary space with $5 \times 10^5$ pfu of the RM461 strain of MCMV. This strain of virus spreads to the uninoculated eye in immunosuppressed mice (Zhang and Atherton, unpublished data). Eighteen days after inoculation of MCMV into the supraciliary space of one eye, mice were killed and perfused with PBS, and the uninjected eyes were removed, frozen, and sectioned. MCMV-positive cells were observed in the choroid but not in the retina of the uninoculated, contralateral eye (Fig. 4A). Staining of adjacent sections for TUNEL revealed TUNEL-positive cells in the retina overlying areas of the choroid containing MCMV-infected cells (Fig. 4B). TUNEL-positive cells were observed only in the areas of the retina where the subjacent choroid was MCMV positive.

**DISCUSSION**

BALB/c mice infected with MCMV by the supraciliary route have been used to study the pathogenesis of MCMV retinitis. In the present studies, a small number of MCMV-infected cells in the choroid and/or retinal pigment epithelium of virus-infected immunocompetent BALB/c mice was associated with a large number of apoptotic cells in the overlying retina. In infected eyes from T-cell–depleted mice, there were more TUNEL-positive cells, and the areas of apoptosis were more extensive than in immunocompetent mice. The observation that more cells were apoptotic in infected eyes of T-cell–depleted mice commensurate with increased replication of MCMV was corroborated by increased laddering of low-molecular-weight DNA extracted from the posterior segments of MCMV-infected, T-cell–depleted mice when compared with low-molecular-weight DNA extracted from posterior segments of MCMV-infected immunocompetent mice. The association of virally infected choroid and retinal pigment epithelial cells observed in injected eyes of both immunocompetent and T-cell–depleted mice was also observed in eyes that had been infected with MCMV by hematogenous spread of the virus. Taken together, the results of these studies suggest that irrespective of immune status of the mouse or the route by which virus infects the eye, MCMV infection of the choroid and/or retinal pigment epithelium causes apoptosis of overlying uninfected retinal cells.

Griffith et al. showed that T cells undergo Fas-mediated apoptosis on entering ocular tissue after anterior chamber inoculation of HSV-1. Activated T cells express Fas, and several ocular tissues, including corneal epithelium and endothelium, iris, ciliary body, and retina, express Fas ligand. However, in immunocompetent mice, the areas of apoptosis in MCMV-infected eyes did not correspond to the locations of T cells, and T cells cannot account for the apoptotic areas in T-cell–depleted mice. Nevertheless, the findings from these experiments do not resolve the question of whether T cells observed in the choroid of immunocompetent mice become apoptotic.
The observation that there were large areas of apoptosis in T-cell-depleted mice argues against a T-cell-secreted factor as the cause of cell death. Additionally, the presence of apoptotic cells in the retinas of MCMV-infected, T-cell-depleted, NK-cell-depleted, methylprednisolone-treated mice argues against T-cell– or NK-cell–secreted products causing apoptosis. Instead, the presence of infected RPE or choroidal subjacent to apoptotic cells in the retina suggests that a viral product or infected cell (or dying cell) product causes apoptosis of the retinal cells. Seigel and Liu have reported that conditioned medium from dying retinal cell cultures contains a substance that can induce apoptosis of cultured retinal cells. Therefore, by extrapolation, virus-infected cells in the choroid and RPE may secrete one or more factors that induce apoptosis of the overlying retinal cells.

In the current studies we used a monoclonal antibody to pp56, an early viral antigen, to identify virus infected cells. Possibly, apoptotic cells of the retina are virus infected, but virus cannot be detected because induction of apoptosis prevents the expression of early virus antigen. This appears to be unlikely for two reasons: First, foci of apoptotic retinal cells were detected on PI days 3, 5, and 7, yet MCMV antigens were not detected in the retinas of immunocompetent mice until day 7. Because MCMV can establish productive infection in retinal cells, it is unlikely that apoptotic cells in the retina were due to virus infection of retinal cells. Second, HCMV has immediate early gene products, IE-1 and IE-2 (homologues for MCMV IE-1 and MCMV IE-3, respectively), which have been shown to block p53-mediated apoptotic pathways. Expression of these gene products in an infected retinal cell may protect infected cells from apoptosis for the 6 to 8 hours needed for the expression of the pp56 early antigen that could be detected by immunohistochemistry.

Retinal degeneration caused by induction of apoptosis of retinal cells has been studied in several animal models. Apoptosis can be induced in the retina of rats by prolonged constant or blinking light, lead poisoning, and ischemia caused by increased ocular pressure. In the present studies, some of the apoptosis observed in the retinas of mock-infected eyes and in the retinas of MCMV-infected eyes at early times after injection may be caused by ischemia due to increased intraocular pressure during and immediately after supraciliary injection. Alternatively, apoptosis could be caused by factors released by cells damaged by the injection trauma. However, at later time points, apoptotic cells of the retina seemed to be located near MCMV-infected areas of the choroid. In vitro studies have shown that human immunodeficiency virus infection induces glial cells to produce TNFα, which induces production of platelet-activating factor in an autocrine fashion; platelet-activating factor, in turn, causes neuronal cells to undergo apoptosis. Additionally, a recent report showed that MCMV infection of neonatal mouse brains results in apoptosis of neuronal cells that are not MCMV infected. Thus, in mice injected with MCMV by the supraciliary route, MCMV infection of choroid and RPE may cause cells in these locations to release a factor or factors that induce apoptosis of retinal cells.

Although the apoptosis-inducing factor(s) and the role, if any, of apoptosis of choroidal T cells in the pathogenesis of this disease remain to be identified, it is likely that apoptosis-induced destruction of retinal cells caused by the virus infection affects the visual axis. Induction of apoptosis in uninfected retinal cells during CMV infection may play an important, but as yet unexplored, role in the pathogenesis of CMV retinitis in human patients.

References