Up-Regulation of Fas Ligand Expression by Human Cytomegalovirus Immediate-Early Gene Product 2: A Novel Mechanism in Cytomegalovirus-Induced Apoptosis in Human Retina


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Up-Regulation of Fas Ligand Expression by Human Cytomegalovirus Immediate-Early Gene Product 2: A Novel Mechanism in Cytomegalovirus-Induced Apoptosis in Human Retina


Human CMV (HCMV) is an important pathogen that causes widespread diseases in immunocompromised individuals. Among the opportunistic HCMV infections, HCMV retinitis is most common in transplant recipients and AIDS patients. It often leads to blindness if left untreated. The question as to how HCMV infection causes retinal pathogenesis remains unresolved. Here, we report that viral immediate-early gene product 2 (IE2), but not IE1, up-regulates the Fas ligand (FasL) expression in HCMV-infected human retinal pigment epithelium cells. Increased secretion of FasL from virally infected cells into cultured medium was observed upon HCMV infection. The capability of such cell-free medium to induce apoptosis of Fas (CD95)-expressing Jurkat cells further implies that Fas-FasL interaction might mediate cell death in the lesion of HCMV retinitis. To support this idea, we observed augmented soluble FasL levels in vitreous from AIDS patients with HCMV retinitis as compared with that from AIDS patients without HCMV infection. In addition, by in situ hybridization and immunohistochemistry, we detected enhanced signals of FasL, the existence of viral IE Ags and apoptotic cells at the same sites in the lesion of HCMV-infected retina. These results strongly suggest that IE2 induction of FasL expression in human retina might be an important event that takes place in the early stage of infection and finally leads to visual loss in individuals affiliated with HCMV retinitis. The Journal of Immunology, 2001, 167: 4098–4103.

Cellular immune reactions and the associated inflammatory responses can be harmful to nearby tissues. Since minor inflammation can result in impaired vision or even blindness, the eye is naturally designed as an immune privileged site where infections usually do not lead to destructive immune reactions (9, 10). The underlying mechanism has been hypothesized to involve Fas ligand (FasL)-mediated programmed cell death (also called apoptosis) of Fas (CD95)-expressing T cells when attracted to the infection sites (9). In this case, activated T cells are eliminated through ligation of Fas by FasL and no serious immune reactions are induced. Thus, the damage to the eye is minimized. However, HCMV infection of human eyes is shown to cause large-scaled cell death and tremendous visual dysfunction (3, 4). Whether HCMV takes the advantage of the FasL-dependent immune evasion to exert its destructive effects remains an important issue. At least two studies argue for this hypothesis. First, in a mouse model of CMV retinitis, Bigger et al. (11) observed apoptosis of uninfected retinal cells in the lesions of retinitis. Second, FasL expression was found up-regulated upon HCMV infection of the human retinal pigment epithelial (HRPE) cells (12). Nevertheless, the correlation of the observed cell death in HCMV-infected human retina to FasL up-regulation is not clear, nor does the role of viral immediate-early (IE) proteins in stimulating the FasL synthesis.

In the present study, we present substantial evidences to show that FasL expression is indeed regulated by HCMV protein IE2 during the early stage of infection. In addition, elevated levels of FasL signals were detected together with HCMV IE Ags and apoptotic cells in the lesions of HCMV retinitis. These findings provide a potential mechanism for the pathogenesis of HCMV retinitis.
Materials and Methods

Cells and HCMV infection

HRPE cell line ARPE-19 and HCMV strain AD169 were obtained from American Type Culture Collection (Manassas, VA). HRPE cells were maintained in DMEM and Ham’s F-12 medium at a 1:1 ratio and supplemented with 10% FCS. HEL-299 cells were maintained in DMEM plus 10% FCS. Jurkat cells were maintained in RPMI 1640 medium plus 10% FCS. All of these cells were grown in a 37°C incubator supplied with 5% CO2. Cell-free virus stock was prepared from the supernatants of AD169-infected HRPE-299 cells as described previously (13). One day before infection, subconfluent HRPE cells were switched to serum-free medium.

HRPE cells were infected with AD169 at a multiplicity of infection (MOI) of 5. After virus absorption at 37°C for 2 h, cells were washed with PBS twice, fed with fresh culture medium, and grown in a 37°C incubator supplied with 5% CO2.

RNA analysis

Total RNA was extracted using the RNeasy kit (Qiagen, Chatsworth, CA). First-strand cDNA synthesis was conducted using oligo(dT) primer and Moloney murine leukemia virus-reverse transcriptase (Promega, Madison, WI). The derived cDNA products (5 μl) were PCR amplified in a 50-μl reaction mixture containing 0.2 mM dNTP, 2 mM MgCl2, 10 pmol of each primer, and 1 U of Taq DNA polymerase. The primer pairs used were: hFasL forward (5'-CAACATGCGTCCATGCTCTC-3') and hFasL reverse (5'-CTG CAGAGAAGCCCGCCAGCAC-3'); hFasL forward (5'-CTCAGAAGCGTGCCCCGGACAC-3') and hFasL reverse (5'-GTTGTTGGAGCAATCTCCCTGGAGTAT-3'); HCMV IE forward (5'-CACAAGGCGCCCTCAGAACAA-3') and HCMV IE reverse (5'-CAGCAAC ATCTCCATCTTCTCTCCTGG-3'); β-actin forward (5'-TCTCTGTTGCTATCCAGGAACACT-3') and β-actin reverse (5'-GAAGATTGGCCTGAGGCGAT-3'). PCR amplification was performed for 35 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. The expected sizes of PCR products for FasL, Fas, and β-actin are 350, 266, and 314 bp, respectively.

Western blot assay

HCMV AD169-infected HRPE cells were harvested at days 1, 2, 3, 5, and 7 postinfection. The cell lysates were prepared as described. Fifteen microliters of sample was boiled at 95°C for 5 min and separated on a 10% SDS-PAGE. The proteins were transferred to Hybond-ECL nitrocellulose paper (Amersham, Arlington Heights, IL) by wet-transfer system. The mAbs used to identify protein products were anti-FasL mAb (F37720/lot 3; BD Transduction Laboratories, Lexington, KY), anti-Fas mAb (F22120; Transduction Laboratories), anti-CMV IE mAb (MAB810; Chemicon International, Temecula, CA), and anti-β-actin mAb (MAB18501; Chemicon International). The reactive protein bands were detected by the ECL detection system (Amersham).

Transfection and dual-luciferase assay

The Lipofectamine 2000 system (Life Technologies, Grand Island, NY) was used for transfection of DNA into HRPE cells. The IE1 and IE2 plasmids had been described previously (14). The Fas promoter constructs (wild-type (wt) and deletion mutants) were kindly provided by Dr. C. Warren (15). Briefly, 106 cells/well were seeded onto 6-well plates 24 h before transfection. DNA solution (250 μl of OPTI-MEM containing 3 μg of pGL2-hFasL reporter construct, 3 μg of pR(IE1), 3 μg of pSIE2 wt (IE2)) or mutant effector plasmids (IE2CD80), and 30 ng of pRL-TK Renilla normalizing luciferase vector) and reagent solution (5 μl of LF200 and 250 μl of OPTI-MEM) were separately preincubated at 25°C for 5 min. The transfections were then followed according to the manufacturer’s instructions. After 48 h of incubation, the cell lysates were prepared using the dual-luciferase reporter assay system (Promega). Both firefly and Renilla luciferase activities were monitored by illuminator (TD2020; Turner Designs, Sunnyvale, CA).

ELISA test and flow cytometry

The concentration of soluble FasL (sFasL) in vitreous samples and in supernatants from infected HRPE cells was determined by ELISA kit (MBL, Nagoya, Japan). The developed reaction was quantified by reading at A490 nm (MRX; Dynatech Laboratories, Chantilly, VA). Each individual sample was analyzed in triplicate. Apoptotic cells were determined by flow cytometry using propidium iodide and FITC-labeled annexin V (Immunotech, Luminy, France). Cells (106 cells/ml) were incubated with relevant Abs in PBS containing 1% BSA. The reaction was conducted at 4°C for 30 min in the dark. After incubation, the cells were washed twice with cold PBS, resuspended to 106 cells/ml in 250 μl of PBS, and analyzed by flow cytometry using a FACSCalibur apparatus (BD Biosciences, San Diego, CA). For blocking assays, the culture supernatants were pretreated with anti-FasL mAb (clone H9P, MBL, Nagoya, Japan) for 60 min at 37°C before the addition of Jurkat cells. Alternatively, Jurkat cells were pretreated with anti-Fas mAb (clone ZB4; MBL) for 60 min at 37°C before the addition of supernatants from AD169-infected HRPE cells.

Immunohistochemistry and in situ hybridization

The research followed tenets of the Declaration of Helsinki. All samples were obtained after informed consent from the patients. Twenty-four eye-balls enucleated from dead AIDS patients were obtained at autopsy and immediately fixed with 4% paraformaldehyde. The 4-μm paraffin sections were deparaffinized in xylene, rehydrated in a series of graded alcohols, and immunostained with Abs against CMV IE Ag (MAB810; Chemicon), FasL (Transduction Laboratories), and HIV-p24 (clone Kal-1; DAKO, Glostrup, Denmark). Immunoreactive signals were detected with a mixture of biotinylated IgG Ab and peroxidase-conjugated streptavidin (LSAB2 system; DAKO). Apoptotic cells in tissue sections were identified by the TUNEL method (In Situ Cell Death Detection kit, POD; Boehringer Mannheim, Mannheim, Germany) as described previously (16). As a negative control, the TdT was omitted from the TdT buffer. Chromogenic development was then applied with 3-amin-9-ethyl-carbazole and slides were counterstained with methyl green. Positive staining was identified under the light microscope as red granules. The RNA in situ hybridization protocols was performed as described previously (17). The slides were deparaffinized slides were predigested with nuclelease-free proteinase K (0.1 mg/ml; Boehringer Mannheim). One hundred microliters of FITC-conjugated antisense and sense probes were added onto each slide. The sequences of FasL probes used were: 5′-GGAGCTTGAACATCCCCGCCACCTACCAAGGCACC-3′ (antisense primer, 461–500 nt) and 5′-GGCTGTTGACCTCCTCCATAC-3′ (sense primer, 486–512 nt). Hybridization products were visualized by alkaline phosphatase conjugated secondary Abs and chromogen nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Sigma, St. Louis, MO). Positive staining was identified as purple granules under the microscope.

Results

Up-regulated expression of FasL in HCMV-infected HRPE cells

Since constitutive expression of FasL is critical for maintaining the immune privilege of the eye, we hypothesized that dysregulated FasL expression by HCMV might be responsible for the pathogenesis of HCMV retinitis. In the eye, HRPE cells are located at the blood-retina barriers (13, 18). Infection of HRPE cells has been shown to result in serious ocular manifestations and suspected to be critical for the development of HCMV retinitis (13). In this study, we first infected a HRPE cell line, ARPE-19, with HCMV AD169 and monitored the expression of FasL. We found that the synthesis of FasL protein in HCMV-infected HRPE cells was enhanced early after infection. The up-regulation of FasL was initiated at day 1 after infection (Fig. 1A). In contrast, the expression level of Fas remained unchanged (Fig. 1A). Consistent to the Western blot data, we observed an increase of FasL mRNA (Fig. 1B). These experiments suggest that HCMV induction of FasL is specific and is determined at the transcriptional level. The early induction of FasL in infected cells further implicates that viral IE genes might play a role in this event. This idea was supported by the evidence that the mRNA level of IE genes was also induced at day 1 after infection (Fig. 1B).

Induction of FasL promoter by HCMV IE2

Since IE2 is a potent transactivator (19, 20), we set up to examine whether HCMV IE2 directly regulates FasL expression. We found that IE2 induced the activity of FasL promoter efficiently in HRPE cells. The activation was specific for IE2 because, first, another HCMV IE protein, IE1, had no effect on the same promoter (Fig. 2A). Note that the inability of IE1 in inducing FasL was not due to a low level of protein being made. In fact, the IE1 protein was more abundant than IE2, based on the Western blot results using a mAb (mAb810) against the common region (exon 3) shared by IE1 and IE2 proteins. Second, removal of IE2 C-terminal 80 residues...
(IE2CD80), which are required for repressing the transactivation activity of the tumor suppressor protein p53 (14), greatly reduced the protein’s activation activity on FasL promoter (Fig. 2B). Third, IE2 no longer activated a truncated version of FasL promoter without its 3'H11032 region from 1147 to 370 nt (Fig. 2C). Thus, we conclude that HCMV IE2 is able to specifically regulate the FasL promoter, providing a molecular mechanism linked to the pathogenesis of HCMV retinitis.

Enhanced sFasL from HCMV-infected HRPE cells caused Fas-independent apoptosis of Jurkat cells in vitro

Identification of the correlation between IE2 and FasL up-regulation in cultured HRPE cells prompted us to examine whether the increased FasL upon HCMV infection induces apoptosis of uninfected cells as observed in HCMV-infected human retina (see below). Consistent with previous data, the levels of sFasL in the cell-free supernatants of infected HRPE cells were increased since day 1 after infection (Fig. 3A). To further investigate whether secreted FasL from HCMV-infected HRPE cells causes Fas-dependent apoptosis in vitro, we cultivated Jurkat cells in medium supplemented with 50% of supernatants from mock or HCMV-infected cell cultures, respectively. After 48 h of incubation, the percentage of apoptotic cells in the cultures was determined by flow cytometry. We found that cells exposed to HCMV-infected supernatants exhibited a higher percentage of apoptosis as compared with those exposed to mock supernatants (Fig. 3B). Furthermore, functional blocking of Fas or FasL with respective mAbs prevented the HCMV-induced apoptosis of uninfected Jurkat cells (Fig. 3B). Interestingly, we found that neither anti-Fas nor anti-FasL could block the apoptosis of ~8% of Jurkat cells when cultured with mock-infected supernatants (Fig. 3B), suggesting that other FasL/Fas-independent cell death pathways were also involved. Our results provide evidences that HCMV-infected HRPE cells are able to release large amounts of FasL, which, in turn, can induce apoptosis of nearby cells, presumably through a Fas-mediated pathway.
To examine whether our previous observation is physiologically relevant, we investigated whether sFasL could be released from HCMV-infected cells into vitreous body. Diagnosis of HCMV retinitis was based on the clinical results of indirect ophthalmoscopy (Fig. 1A) and fluorescein angiography (data not shown). As presented in Table I, before anti-HCMV treatment, the averaged sFasL level of vitreous fluids among the eight AIDS patients with HCMV retinitis was 440 pg/ml, with a SD of 36 pg/ml. In contrast, the sFasL levels of vitreous fluids in these eight patients declined dramatically (Table I). We found that no HIV-1 p24 Ag was present in HRPE cells among all specimens examined (data not shown). Therefore, HIV might not have substantial influence in this instance if there is any.

**Discussion**

HCMV retinitis is the most common ocular opportunistic infection in immunocompromised individuals, including AIDS patients (3, 4). Although the initial symptoms and signs of infection are minimal and often ignored, the destructive and invasive nature of HCMV retinitis can result in blindness if left untreated (3, 4, 21). However, up to date, the pathogenic pathway of the underlying mechanism has not been completely established. It is unclear how HCMV infection causes large-scaled cell death in retina. If transmission of HCMV in retina is through cell-cell contacts, the focal infection would be the major pattern and necrotizing retina could be limited in a small area. However, the progression of HCMV retinitis is rapidly spreading into nearby tissues (21). It is likely that viral products or factors released from infected cells mediate the cell death observed in the lesions of HCMV retinitis.
virally infected cells. To answer this question, we examined whether apoptosis occurs in HCMV-infected HRPE cells. Using the TUNEL assay, we did not observe any apoptotic cells within 3 days after infection (data not shown). Although the underlying mechanisms are not completely understood, several groups have argued against the death of virally infected cells at least in the early stage of infection. It seems that many viruses have evolved unique strategies to avoid its own death while eliminating nearby uninfected cells, e.g., HIV virus. HIV infection also stimulates FasL expression. However, death of HIV-infected cells is inhibited, even Fas receptors are usually abundant on the surfaces of infected cells. This mystery was not resolved until recently by Geleziunas et al. (23). Based on their study, HIV can inhibit the ASK1-dependent signaling pathway, which is downstream of the Fas/FasL pathway. Thus, by impairing functions of downstream regulators, HIV-1 can protect the infected host cells from undergoing apoptosis. As for HCMV, at least two strategies have been reported to overcome apoptosis. First, our laboratory has demonstrated that HCMV IE2 can down-regulate p53 activity, which is critical for the induction of cell apoptosis and is located even more downstream of ASK1 along the cell death signaling pathway (14). Second, a HCMV-encoded cell death suppressor, viral inhibitor of

Table I. Detection of Vitreous sFasL level in AIDS patients with HCMV retinitis

<table>
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<tr>
<th>Patient</th>
<th>Vitreous sFasL level (pg/ml)</th>
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<th>Vitreous sFasL level (pg/ml)</th>
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<tr>
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<tr>
<td>1</td>
<td>844 ± 67</td>
<td>103 ± 19</td>
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<td>7</td>
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<tr>
<td>8</td>
<td>152 ± 14</td>
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*a sFasL concentrations in the eye vitreous from eight AIDS patients afflicted with HCMV retinitis and seven AIDS patients without HCMV retinitis were measured by ELISA (clone 4H9; MBL). Data shown here are the mean ± SD of triplicate determinations. Without ganciclovir treatment, the averaged sFasL level (440 ± 36 pg/ml) of the vitreous in patients with HCMV retinitis was significantly higher than that (below 10 pg/ml) in patients without HCMV retinitis (p < 0.001). The vitreous sFasL levels in six patients determined (patients 1–6) were markedly decreased after 2 mo of i.v. ganciclovir treatment (5 mg/kg every 12 h for 4 wk, and 5 mg/kg/day for 5 days per week in the succeeding 4 wk). b —, Undetermined.
caspase-8-induced apoptosis, has been newly identified (24). It was shown to inhibit Fas/FasL-mediated apoptosis by binding to and subsequently inactivating caspase-8. Therefore, it is possible that HCMV can use different approaches to prevent its own death, even cis-ligation of overexpressed FasL and Fas occurs on the surfaces of HCMV-infected cells.

In summary, our results indicate that Fas/FasL-mediated apoptosis previously thought to be important in the maintenance of immune surveillance within human retina (9, 10, 18) might turn out to be destructive once manipulated by HCMV.

Acknowledgments
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References