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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

Shih-Hwa Chiou,⁎ Jorn-Hon Liu,⁎ Wen-Ming Hsu,⁎ Steve S.-L. Chen,⁎ Sui-Yuan Chang,** Li-Jung Juan,** Jung-Chun Lin,** Yu-Ting Yang,** Wing-Wai Wong,§ Cheng-Yi Liu,§ Young-Sun Lin,† Wu-Tse Liu,‡ and Cheng-Wen Wu#**

Human CMV (HCMV), a member of the β subfamily of herpesviruses, contains a dsDNA genome of 229,354 bp with a potential to encode >200 proteins (1). HCMV infection usually develops asymptomatic lifelong infection in healthy individuals, but can cause severe clinical complications such as HCMV retinitis when reactivated in immunocompromised patients (2–4). Although the detailed mechanisms of HCMV latency and reactivation are not yet well understood, accumulating evidences suggest that the virus can use a panel of viral proteins to escape from cellular immune control and, thus, successfully survive and replicate in host cells (5–8).

HCMV infection 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 infected 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 HEL-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'-CAATCAGTAGCTCATCAGG-3'); hFasL reverse (5'-CTCCTGGAAGCCCGCGACACCAC-3'); hFas forward (5'-CTCCTGGAAGCCCGCGACACCAC-3'); hFas reverse (5'-CTCCTGGAAGCCCGCGACACCAC-3'); hFas forward (5'-CTCCTGGAAGCCCGCGACACCAC-3'). PCR amplification was performed for 35 cycles at 94 °C for 1 min, 52 °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. After virus absorption at 37 °C for 30 min, the cells were washed with PBS twice, fed with fresh culture medium, and grown in a 37 °C incubator supplied with 5% CO2.

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 A450 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 pre-treated 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 protocol was performed as described previously (17). The hybridization signals were read as positive when the in situ hybridization signals were predigested with nuclease-free protease 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'-GAGCTTGAACATCCCGAGCCCATTCACAAAGCAGC-3' (antisense primer, 486 bp) and 5'-GGTGTGATCTCCCGAGCCCATTCACAAAGCAGC-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 with 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 5′/H11032 region from /H11002 to /H11032 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-dependent 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.

**FIGURE 2.** Specific activation of FasL promoter by HCMV IE2. A, HCMV IE2, but not IE1, activates the FasL promoter in transient transfection assays. FasL promoter-luciferase constructs were transfected into HRPE cells along with plasmids encoding IE1 or IE2. After harvesting the cells, lysates were assayed for luciferase activity. All relative luciferase activity was normalized with control Renilla luciferase expression. To ensure the expression of the effectors, Western blottings with mAb (MAB810; Chemicon) specifically recognizing the third exon, which is shared by HCMV IE1 and IE2 proteins, are shown. The expression level of /H9252-actin was used as an internal control. B, IE2, without its C-terminal 80 residues, is unable to activate the FasL promoter. Transfections were done as described in A except that a truncated form of IE2 which loses its C-terminal 80 residues (IE2CD80) was used as indicated. C, 5′ deletion mutants of FasL promoter fail to respond to IE2 activation. Transfections were applied as described in A. The transcriptional activity of wt and two mutants of FasL promoter with serial 5′ deletion (~370 and ~339) were assayed in the presence of IE2. The relative fold of luciferase activity has been normalized with the luciferase expression in the absence of IE2. Data shown here are the mean ± SE of two independent experiments, each with triplicate luciferase results.
Increased expression of FasL in vitreous and retina of HCMV retinitis patients

To examine whether our previous observation is physiologically relevant, we investigated whether sFasL could be released from HCMV-infected retina into a vitreous body. Diagnosis of HCMV retinitis is rapid-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 nercotizing 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. To exclude the potential effects of HIV infection on the release of sFasL, seven AIDS patients without HCMV retinitis were also examined. We found that the sFasL levels in these patients were all below the detection limit (Table I).

To test further, the postmortem enucleation of the eyeball was performed on patients with or without HCMV retinitis. The specificity of HCMV infection in these areas was confirmed by detection of HCMV IE Ags in all layers of retina within cytomelagic cells, a typical sign of HCMV infection, and in many nonmegal cells (Fig. 4E) as compared with that obtained from AIDS patients without HCMV retinitis (Fig. 4B). To examine whether apoptosis occurs in these regions, TUNEL assay was performed. We observed many apoptotic cells in the foci of HCMV-infected retina (Fig. 4F). In contrast, no apoptotic cells were identified in HCMV IE-negative samples (Fig. 4C). Consistent with our observation that HCMV IE2 up-regulates the Fasl expression, we detected higher levels of FasL in HCMV IE-positive specimens (Fig. 4, G and J) as compared with the basal level of FasL in AIDS patients without HCMV retinitis (Fig. 4D). Dual immunohistoestaining for FasL and HCMV IE Ag was also conducted to determine whether increased expression of FasL does correlate with HCMV infection (Fig. 4, H and I). We found that the FasL and HCMV IE Ags were colocalized in infected neuroretina and HRPE cells of HCMV-infected retina (Fig. 4F). A number of HRPE cells with intranuclear and typically cytoplasmic inclusions in the lesions of HCMV-in- fected retina were also observed under the microscope (Fig. 4I). Arguing against the involvement of HIV in the lesions of HCMV-infected retina, immunostaining with HIV Ag-specific Abs was performed on samples collected from AIDS patients with or without HCMV retinitis. We found that no HIV-1 p24 Ag was present in HRPE cells among all specimens examined (data not shown). The rarely detected HIV-1 p24 Ag was found to localize at few neuroretinal cells (Müll cells; 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 nercotizing 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.

This report provides the first evidence that HCMV infection can induce apoptosis of Fas-expressing cells via IE2 induction of FasL expression in virally infected human retina. Nevertheless, whether IE2 activation of FasL promoter is modulated directly through DNA binding of IE2 to a specific site on the FasL promoter or represents a result of indirect effects mediated by other proteins is largely unknown. As shown in Fig. 2C, a truncated version of the FasL promoter (−370) that loses at least a putative NF-κB binding site responded to IE2 activation to a smaller degree. Note that HCMV IE2 induction of NF-κB promoter has been reported previously in many studies (22). Whether NF-κB is involved in IE2-mediated up-regulation of FasL is currently under investigation.

Because we also observed the basal expression of Fas receptor on the surface of HCMV-infected cells (Fig. 1A), one can argue that cis-ligation of Fas by FasL might induce cell death of the
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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<th>Patient</th>
<th>Vitreous sFasL level (pg/ml) Before ganciclovir treatment</th>
<th>Vitreous sFasL level (pg/ml) After ganciclovir treatment</th>
<th>AIDS Without HCMV Retinitis</th>
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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).
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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