Induction of Both Membrane-Bound and Soluble HLA-G Expression in Active Human Cytomegalovirus Infection

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Background. Alteration of HLA expression or cytokine production plays a crucial role in the pathogenesis of human cytomegalovirus (HCMV) infection. HLA-G has been suggested to be involved in HCMV infection, and modulation of HLA-G expression by interferon (IFN)–γ and interleukin (IL)–10 has been reported. However, the clinical relevance of HLA-G in HCMV infection remains unknown.

Methods. The study included 75 patients with active HCMV infection (age range, 1–4.5 years) and 150 sex- and age-matched healthy control subjects (age range, 1–5 years). HLA-G expression in peripheral monocytes from patients (n = 38) and control subjects (n = 20) was analyzed using flow cytometry. Plasma levels of soluble HLA-G (in 75 patients and 150 control subjects), IL-10 (in 75 patients and 40 control subjects), and IFN-γ (in 75 patients and 40 control subjects) were determined using enzyme-linked immunosorbent assay.

Results. The mean percentage of HLA-G–positive monocytes among patients with active HCMV infection was dramatically increased, compared with that among healthy control subjects (6.33% vs 1.64%; P < .001). Similarly, significant increases were observed in soluble HLA-G level (median, 54.91 vs 21.32 U/mL; P < .001) and IL-10 level (median, 9.24 vs 1.82 ng/mL; P < .001). Although the expression of IFN-γ was higher in patients with active HCMV infection than in healthy control subjects, the difference was not statistically significant (median, 1254.46 vs 887.05 ng/mL; P = .070). Furthermore, no correlation was established between HLA-G expression and levels of IL-10 or IFN-γ.

Conclusions. HLA-G expression in monocytes and plasma soluble HLA-G and IL-10 levels were increased during active HCMV infection.

Human cytomegalovirus (HCMV) is a β-herpesvirus type 5 that causes widespread, persistent human infection [1]. After initial infection, HCMV remains persistent in the host, proposing a delicate balance between the virus and the host’s immune system [2]. HCMV rarely causes complications in immunocompetent individuals, but it can have damaging clinical effects in those with an immature or suppressed immune system, such as fetuses, neonates, and immunocompromised patients [3].

HCMV has evolved a multitude of strategies to subvert host immune surveillance and responses by both the innate and adaptive arms of the immune system [4]. Strategies, such as the down-regulation of classic major histocompatibility complex (MHC) class I and class II antigen expression, inhibit MHC molecule–restricted antigen presentation [5]. In principle, HCMV-infected cells with down-regulated MHC molecules should be vulnerable to natural killer (NK) cell–mediated cytolysis. However, HCMV has developed various mechanisms to impede NK cell recognition by inducing expression of the nonclassic HLA class I antigens, such as the immunotolerant HLA-G [6], which can suppress the function of various immune cells, including T lymphocytes, NK cells, and antigen-presenting cells [7]. Recent studies also highlighted induction...
by HLA-G of suppressor cells, such as regulatory T cells, dendritic cells, and NK cells, providing a long-term immunomodulatory function [8]. Consequently, decreased immune function of the host often leads to an advantage for virus replication and activation.

The nonclassic HLA class I antigen HLA-G differs from the other HLA class molecules I by its lower polymorphism, restricted tissue distribution, expressed as both membrane-bound and soluble isoforms, and biological immunosuppressive properties [9]. To date, 7 isoforms of HLA-G have been generated by alternative splicing of its primary transcript. HLA-G1, -G2, -G3, and -G4, are membrane bound, whereas HLA-G5, -G6, and -G7 are soluble molecules. Numerous findings have revealed that both membrane-bound and soluble isoforms of HLA-G bear similar immunosuppressive properties via their receptors, such as immunoglobulin (Ig)–like transcript (ILT)–1, ILT-2, and KIR2DL4 [10]. Beyond its initial relevance in fetal-maternal immunotolerance, HLA-G has been reported to be involved in tumor immune escape, inflammatory and autoimmune diseases, and allograft acceptance [9]. In viral infection, induction of HLA-G expression by virus-infected cells was proposed as a mechanism that helps viruses subvert host antiviral defenses [11]. The role of HLA-G after infections with cytomegalovirus (CMV) [6], human immunodeficiency virus (HIV) [12], and neurotropic viruses, such as rabies virus, has been discussed [13]. Induction of HLA-G expression in HCMV-infected cells was observed in vitro, but this finding remains controversial [6, 14]. Furthermore, alteration of cytokine production is another strategy for virus evasion of host immune responses [15]. Two of the most important cytokines in HCMV infection, interleukin (IL)–10 and interferon (IFN)–γ, reportedly have the capacity to up-regulate HLA-G messenger RNA, affecting both cell surface and soluble HLA-G protein levels [16–19].

In the current study, we analyzed HLA-G expression in monocytes and plasma levels of soluble HLA-G, IL-10, and IFN-γ in patients with active HCMV infection. Our findings revealed that the percentage of HLA-G–expressing monocytes and plasma soluble HLA-G and IL-10 levels are dramatically increased during active HCMV infection, which might explain how HCMV evades host antiviral immune responses.

**PATIENTS, MATERIALS, AND METHODS**

**Patients and control subjects.** Seventy-five consecutive patients with active HCMV infection (age range, 1–4.5 years) were enrolled in this study. Specimens were collected from the Taizhou Hospital of Zhejiang Province, affiliated with Wenzhou Medical College. Virological proof of active HCMV infection was obtained for all patients by laboratory detection of pp65 antigenemia, HCMV DNA, and HCMV-specific IgM and IgG antibody. Active HCMV infection was defined by any detection of CMV pp65 antigen–positive leukocytes and/or CMV DNA in peripheral blood leukocytes. Patients’ conditions were diagnosed according to clinical symptoms; diagnoses included HCMV hepatitis (n = 34), HCMV pneumonitis (n = 18), and HCMV gastrointestinal disease (n = 22). A control group included 150 unrelated healthy children (age range, 1–5 years) matched for sex and age. Parents provided informed consent for all patients and control subjects, and the study protocol was approved by the Institutional Ethics Committee.

**Serological and pp65 antigenemia test.** The presence of HCMV-specific IgM and IgG antibodies was demonstrated using a microparticle enzyme immunoassay (Abbott Laboratories). HCMV pp65 antigenemia was identified using an indirect immunofluorescence assay kit (Light Diagnostics; Chemicon), according to the manufacturer’s instructions.

**Real-time quantitative polymerase chain reaction (PCR) assay.** The PCR primers and probe used for this assay were from the immediate early (IE) gene. Real-time PCR was conducted using a TaqMan fluorescence PCR kit (Da An Gene). First, 5 μL of the solution containing DNA extracted from the samples was added to the PCR mixture. After denaturing at 93°C for 2 min, 10 cycles of 45 s at 93°C and 60 s at 55°C were performed, followed by 30 cycles of 30 s at 93°C and 45 s at 33°C, conducted in a 7300 real-time PCR system (PE Applied Biosystems). Real-time fluorescence measurements were taken, and the threshold cycle value for each sample was calculated.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Patients with HCMV infection (n = 75)</th>
<th>Control subjects (n = 150)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age range, years</td>
<td>1–4.5</td>
<td>1–5</td>
</tr>
<tr>
<td>Sex: male/female</td>
<td>41/34</td>
<td>87/63</td>
</tr>
<tr>
<td>No. of patients with HCMV-related disease</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepatitis</td>
<td>34</td>
<td>0</td>
</tr>
<tr>
<td>Pneumonitis</td>
<td>19</td>
<td>0</td>
</tr>
<tr>
<td>Gastrointestinal disease</td>
<td>22</td>
<td>0</td>
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</table>
Figure 1. Distribution and comparison of plasma soluble HLA-G (sHLA-G; A), interleukin (IL)-10 (B), and interferon (IFN)-γ (C) levels in patients with active human cytomegalovirus (HCMV) infection and in healthy control subjects. Dashed lines represent median values. P values were determined for all samples with use of the Mann–Whitney U test.

Flow cytometry analysis. Peripheral blood mononuclear cells (PBMCs) were separated from freshly drawn anticoagulated blood with use of Ficoll-Paque density gradient centrifugation. Peripheral monocyte cell surface HLA-G expression was analyzed in 38 patients with active HCMV infection and in 20 healthy control subjects. PBMCs were gated with phycoerythrin-labeled anti-CD14 (BD), and HLA-G expression in monocytes was analyzed. In brief, 1 × 10⁶ mononuclear cells were incubated in 200 μL of phosphate-buffered saline at room temperature for 30 min in the dark with 10 μL (1 mg/mL) of fluorescein isothiocyanate–conjugated anti-human HLA-G–specific monoclonal antibody MEM-G/9 (IgG1; Serotec), and an isotype IgG was added as a negative control (BD). After 2 washing steps with phosphate-buffered saline, HLA-G expression was quantified by flow cytometry (FACSCalibur; BD); 10,000 cells were analyzed for each patient sample. Data on HLA-G expression were acquired and analyzed using CellQuest software, version 3.3 (BD).

Soluble HLA-G enzyme-linked immunosorbent assay (ELISA). Plasma soluble HLA-G levels in 75 patients with active HCMV infection and in 150 healthy control subjects were determined with the soluble HLA-G–specific ELISA kit (Exbio). Each sample was measured in triplicate. The final concentration was determined by optical density, according to the standard curves; optical densities were measured at 450 nm (SpectraMax 250; Molecular Devices). The detection limit was 1 U/mL. With regard to other details, the assay was performed according to the manufacturer’s instructions.

IL-10 and IFN-γ ELISA. Plasma cytokine concentrations in 75 patients with active HCMV infection and in 40 healthy control subjects were measured in triplicate with the commercially available human IL-10 and IFN-γ ELISA Kit (RayBiotech). The detection limits for IL-10 and IFN-γ were 1.0 and 15 pg/mL, respectively. The assay was performed according to the manufacturer’s instructions.

Statistical analysis. Statistical analysis was performed using SPSS software (version 13.0; SPSS). Levels of soluble HLA-G and cytokines were compared between groups with use of the Mann–Whitney U test. The percentages of HLA-G–expressing monocytes were compared by means of 2-sided Student’s t test. Differences were considered to be statistically significant at P < .05.

RESULTS

Characteristics of patients and control subjects. Characteristics of the 75 patients with active HCMV infection and of the 150 healthy control subjects are shown in table 1. Patients had either HCMV pp65 antigenemia and/or PBMCs positive by determining the point at which the fluorescence exceeded a threshold limit. pGEM-IE, which contains the IE gene, was used as a positive control. A standard graph was constructed; the threshold cycle values from the clinical samples were plotted on the standard curve, and the copy number was calculated. Samples were defined as negative when the threshold cycle values exceeded 30 cycles. The urine DNA concentrations were expressed as copies per milliliter. The detection limit of this assay was 100 copies/mL.
Plasma soluble HLA-G, IL-10, and IFN-γ. Plasma soluble HLA-G, IL-10, and IFN-γ concentrations were determined using ELISA. Soluble HLA-G expression was dramatically increased in patients with active HCMV infection (median level, 54.91 U/mL; range, 6.75–282.72 U/mL), compared with healthy control subjects (median level, 21.32 U/mL; range, 1.07–260.35 U/mL; \( P < .001 \)). Similarly, the IL-10 level was markedly elevated in patients with active HCMV infection (median level, 9.24 pg/mL; range, 1.61–41.77 pg/mL), compared with healthy control subjects (median level, 1.82 pg/mL; range, 1.03–3.98 pg/mL; \( P < .001 \)). Although plasma IFN-γ levels were increased in patients with active HCMV infection (median level, 1254.46 pg/mL; range, 296.71–2019.06 pg/mL), compared with healthy control subjects (median level, 887.05 pg/mL; range, 326.27–1550.07 pg/mL; \( P = .070 \)), the increase was not statistically significant (figure 1).

Other studies have indicated that IL-10 and IFN-γ could upregulate the expression of soluble HLA-G [18, 19]; however, in the current study, no correlation was established between soluble HLA-G expression and levels of these 2 cytokines (data not shown). Furthermore, the levels of soluble HLA-G, IL-10, and IFN-γ were also compared among patients with different types of HCMV diseases. There were no statistically significant differences in the plasma concentrations of soluble HLA-G, IL-10, and IFN-γ among patients with HCMV hepatitis (\( n = 34 \)), patients with HCMV pneumonitis (\( n = 19 \)), and patients with HCMV gastrointestinal disease (\( n = 22 \)) (table 2). Therefore, when variables were compared between patients and healthy control subjects, all patients with active HCMV infection (\( n = 75 \)) were considered as a single group.

Monocyte cell surface HLA-G expression. Cell surface HLA-G expression was analyzed in CD14-gated peripheral monocytes (figure 2). There were no statistically significant differences in the percentages of HLA-G–positive monocytes among patients with HCMV-related hepatitis (median per-

### Table 2. Comparison of Soluble HLA-G, Interleukin (IL)–10, and Interferon (IFN)–γ Levels among Patients with Different Human Cytomegalovirus (HCMV)–Related Diseases

<table>
<thead>
<tr>
<th>HCMV-related disease</th>
<th>Soluble HLA-G, U/mL</th>
<th>IL-10, pg/mL</th>
<th>IFN-γ, pg/mL</th>
<th>( P ^{a} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatitis</td>
<td>57.44 (7.93–264.25)</td>
<td>12.24 (1.61–31.60)</td>
<td>911.51 (332.24–1949.96)</td>
<td>NS</td>
</tr>
<tr>
<td>Pneumonitis</td>
<td>54.02 (6.75–229.17)</td>
<td>11.69 (2.49–41.77)</td>
<td>1361.13 (296.71–2019.06)</td>
<td>NS</td>
</tr>
<tr>
<td>Gastrointestinal disease</td>
<td>49.47 (14.59–282.72)</td>
<td>11.97 (3.34–37.96)</td>
<td>1294.63 (335.23–1814.50)</td>
<td>NS</td>
</tr>
</tbody>
</table>

**NOTE.** Data are for 34 patients with hepatitis, 19 with pneumonitis, and 22 with gastrointestinal disease. NS, not significant.

\( ^{a} \) The Mann-Whitney \( U \) test was used for comparisons.
Figure 3. A, Percentages of HLA-G–positive monocytes among patients with different human cytomegalovirus (HCMV)–related diseases. P values were determined using a 2-sided Student’s t test. B, Percentages of HLA-G–positive monocytes among patients with active HCMV infection and among healthy control subjects. Solid lines represent mean values. NS, not significant.

**DISCUSSION**

HCMV has evolved multiple mechanisms to escape host immune recognition and innate or adaptive immune responses. In response to infection, hosts mobilize both innate and adaptive immunity to ensure appropriate control of the infection [2]. Innate immunity plays a crucial role in limiting early viral replication through activation of NK cells and IFN-γ production. Although the initial infection does not eradicate the virus, cellular immune response is the predominant mechanism for controlling HCMV replication and activation, and HCMV-specific CD8+ T cells, CD4+ T cells, and γδ T cells are crucial for restricting viral replication in hosts with persistent infection [20].

HLA-G is considered to have critical immunotolerant effects in various physiopathological conditions. Induction of HLA-G expression by virus-infected cells could be an additional mechanism that helps viruses subvert host defenses [9]. In HIV-infected patients, HLA-G expression was up-regulated in CD8+ T cells and monocytes, and patients who were long-term progressors expressed higher plasma levels of HLA-G than did long-term nonprogressors, indicating a new mechanism for HIV to evade the cytotoxicity of immune cells [12, 21]. In neurotropic viral infections, herpes simplex virus type 1 and rabies virus up-regulate the neuronal expression of HLA-G isoforms in both infected cells and neighboring uninfected cells [22]. Indeed, up-regulation of HLA-G gene expression was observed in human neuronal line NT2-N cells after in vitro infection by a highly pathogenic strain of rabies virus or a strain of herpesvirus-inducing neuron latency. Cell surface expression of HLA-G was restricted to rabies virus–infected neurons [13]. The findings show that these neurotropic viruses differentially regulate HLA-G expression in human neurons and that HLA-G might be involved in their escape from the immune response in the nervous system.

In the context of HCMV infection, another study showed that both cell surface HLA-G and soluble HLA-G could be produced during viral reactivation in macrophages and that bronchoalveolar macrophages from patients with acute HCMV pneumonitis also express HLA-G molecules [6]. Moreover, only soluble HLA-G could be induced in CMV-infected U-373 MG astrocytoma cells through the cooperative action of the 2 IE1 pp72 and IE2 pp86 products. Of interest, cell surface HLA-G expression was observed to be down-regulated in the HLA-G1 transfectant U-373 with HCMV infection [14]. The different isoform expression pattern in U-373 cells with HCMV infection may be explained by the fact that virus-encoded US2 protein targets membrane-bound HLA-G1 but not soluble HLA-G1 for degradation [23]. Our data revealed that HLA-G–positive CD14+ monocytes are markedly increased during active HCMV infection. However, in HIV infection, the induced HLA-G expression is mainly restricted to CD8+ T cells [12]. This differ-
ence in HLA-G expression between diseases could reflect the fact that CD14+ monocytes are the predominant harboring site for latent HCMV infection [24]. Furthermore, plasma soluble HLA-G concentrations were increased significantly in patients with active HCMV infection, compared with healthy control subjects; similar increases have been observed in HIV-infected patients [21].

The HLA-G expression induced in patients with active HCMV infection could inhibit the functions of various immune cells involved in defense against HCMV infection. During HCMV infection, NK cells play a major role in limiting viral replication and reducing viral load, especially during early acute infection [25]. This finding was supported by evidence from a murine model, in which NK cells were reported to be involved in the clearance of murine CMV infection and the adoptive transfer of NK cells provided protection against murine CMV [26, 27]. Virus-specific CD8+ T cells have an important role in protection against CMV disease. An essential role for T cell immunity was first recognized in studies of murine CMV models in which the elimination of lymphocytes coincided with increased levels of reactivation and dissemination of viral infection [28]. The selective depletion of lymphocyte subsets in mice also revealed CD8+ T cells to be the most important component in the immune control of murine CMV infection [26]. Healthy long-term HCMV carriers have high percentages of circulating HCMV-specific CD8+ T cells, many of which recognize specific peptides within either the viral tegument protein pp65 or the IE proteins, such as IE1 and IE72 [29, 30]. Furthermore, there is increasing evidence that HCMV-specific CD4+ T cells are also an important correlate of protection against HCMV disease [31]. Findings of another study indicated that, in healthy HCMV carriers, typically 1%–2% of all circulating CD4+ T cells are specific for HCMV [32, 33]. The immunosuppressive function of both cell surface HLA-G and soluble HLA-G includes inhibition of the cytotoxic activity of cytotoxic T lymphocytes and NK cells [34, 35], induction of the apoptosis of activated CD8+ T cells and CD8+ NK cells [36], and inhibition of allogeneic CD4+ T cell proliferation and interference with naïve CD4+ T cell priming [37, 38]. Consequently, HCMV-induced HLA-G molecules could be a mechanism that helps HCMV subvert host defenses, favoring virus dissemination and exacerbating the severity of HCMV diseases.

Various factors could regulate HLA-G expression, including cytokines and the epigenetic status of the HLA-G gene [39]. Alteration and modification of cytokine expression is commonly observed during HCMV infection [15]. Two of the most important cytokines in virus infection, IL-10 and IFN-γ, have been reported to induce HLA-G expression [18, 19]. Although we observed no statistically significant difference in IFN-γ levels between patients with HCMV infection and healthy control subjects, IL-10 levels were increased dramatically in the HCMV-infected patients. Despite their roles in HCMV infection, no correlation was established between either IL-10 or IFN-γ and the expression of soluble HLA-G, indicating that increased soluble HLA-G production may not be induced by IL-10 and IFN-γ in HCMV infection.

In conclusion, to our knowledge, this is the first study to demonstrate clinical aspects of HLA-G expression in active HCMV infection. Our data revealed that both HLA-G expression in monocytes and plasma soluble HLA-G levels were dramatically increased in patients with active HCMV infection, suggesting that up-regulation of HLA-G expression in HCMV infection could be an additional mechanism by which HCMV subverts host defenses. More studies are warranted, however, to clarify the clinical consequences of increased HLA-G expression during HCMV infection.

References


