Kinetics of US28 Gene Expression during Active Human Cytomegalovirus Infection in Lung-Transplant Recipients

Jasper M. Boomker,1,2 Erik A. M. Verschuuren,2 Marja G. L. Brinker,1,3 Lou F. M. H. de Leij,1,3 T. Hauw The,2 and Martin C. Harmsen1,3

1Department of Pathology and Laboratory Medicine, Medical Biology Section, and 2Department of Clinical Immunology, University Medical Center Groningen, and 3Groningen University Institute for Drug Exploration, University of Groningen, Groningen, The Netherlands

The ubiquitous human cytomegalovirus (HCMV) causes one of the most frequent infections after organ transplantation. In particular, transplant recipients, a group of patients who receive high-dose immunosuppressive medication, are susceptible to active HCMV infection that arises from primary infection or from reactivation [1]. HCMV expresses homologues of host G protein–coupled receptors (GPCRs), to promote viral replication and maintain persistence. In this respect, the GPCRs encoded by HCMV have been given special attention [2]. Of the 4 GPCRs encoded by HCMV, US28 is considered to make the most significant and most diverse contribution to HCMV infection (reviewed in [3]). US28 has been identified as an early gene during in vitro infection, with transcripts appearing shortly after the immediate-early (IE) 1 gene was transcribed [4]. Others have reported the presence of US28-specific transcripts in semipermissive and latently infected cells in vitro [5]. Ligand binding to US28 triggers an intracellular calcium flux and activates p44/p42 mitogen–activated protein kinase activity [6]. Binding of RANTES/CC-motif chemokine ligand (CCL) 5 or monocyte chemoattractant protein 1/CCL2 to US28 induces a migratory response in human vascular smooth-muscle cells [7], which may ultimately lead to accelerated transplant atherosclerosis [8]. Furthermore, via a high-affinity interaction with the membrane-bound chemokine fractalkine/CX3CL1, US28 acts as an anchor by which virus particles or infected cells can attach to the cell membrane [9]. Moreover, US28 constitutively activates proinflammatory transcription factors, such as NF-kB; nuclear factor of activated T cells, or NFAT; and cAMP-responsive element-binding protein, or CREB [10, 11]. Recently, we found that constitutive signaling activity of US28 transactivates the major IE promoter/enhancer of HCMV in vitro [12].

On the basis of these observations, we recognized that blocking US28 function—for instance, by use of the novel synthetic antagonist that was reported by Casarosa et al. [13]—could limit HCMV pathogenicity. In contrast to conventional antiviral targets—such as the DNA polymerase UL54 or the kinase UL97, which are generally expressed during the late stages of viral replication and are located inside the infected cell—US28 is expressed on the cell surface and is easily accessible to antiviral drugs. In view of this, we wished to determine the expression kinetics of US28-specific transcripts in the blood of lung-transplant recipients and compare them with viral load during primary and secondary HCMV infection (reviewed in [3]). US28 has been identified as an early gene during in vitro infection, with transcripts appearing shortly after the immediate-early (IE) 1 gene was transcribed [4]. Others have reported the presence of US28-specific transcripts in semipermissive and latently infected cells in vitro [5]. Ligand binding to US28 triggers an intracellular calcium flux and activates p44/p42 mitogen–activated protein kinase activity [6]. Binding of RANTES/CC-motif chemokine ligand (CCL) 5 or monocyte chemoattractant protein 1/CCL2 to US28 induces a migratory response in human vascular smooth-muscle cells [7], which may ultimately lead to accelerated transplant atherosclerosis [8]. Furthermore, via a high-affinity interaction with the membrane-bound chemokine fractalkine/CX3CL1, US28 acts as an anchor by which virus particles or infected cells can attach to the cell membrane [9]. Moreover, US28 constitutively activates proinflammatory transcription factors, such as NF-kB; nuclear factor of activated T cells, or NFAT; and cAMP-responsive element-binding protein, or CREB [10, 11]. Recently, we found that constitutive signaling activity of US28 transactivates the major IE promoter/enhancer of HCMV in vitro [12].
years) had received organs from HCMV-seropositive donors. Four of the patients were seronegative before transplantation and developed primary HCMV infection afterward. For RT-PCR analysis, 1 mL of EDTA-treated whole blood was mixed thoroughly with 9 mL of nucleic acid sequence–based amplification lysis buffer (Organon Technika) within 4 h of collection and was stored at −80°C until RNA isolation. For determination of pp65 antigenemia by routine methods, 1.8 mL of EDTA-treated whole blood was used. All patients received standard immunosuppressive treatment with rabbit anti–thymocyte globulin (3 mg/kg 2–5 times postoperatively; Thymoglobulin, Mériex), azathioprine (1.5–3 mg/kg/day), cyclosporin A (dose adjusted to whole-blood trough levels of 400 μg/L within 3 weeks, tapering to levels of 150 μg/L), prednisolone (125 mg 3 times on the first day, 0.2 mg/kg/day from day 2 through the third month, and 0.1 mg/kg/day thereafter), Pneumocystis carinii prophylaxis with cotrimoxazole (960 mg on alternate days), and acyclovir (200 mg 4 times a day for 6 months), to prevent herpesvirus infection. Acute rejection was treated with methylprednisolone pulse therapy (500–1000 mg intravenously [iv] daily for 3 days). Recurrent rejection was treated by replacement of cyclosporin A with tacrolimus (Prograf; Fujisawa) and, subsequently, by replacement of azathioprine with methylycyclophololate mofetil (Cellcept; Roche). Patients who were seronegative before transplantation and who developed primary infection received antiviral therapy on confirmed positive antigenemia assay result. Patients who were seropositive before transplantation received antiviral therapy when clinical symptoms became apparent, which was confirmed by a pp65 antigenemia of >10 positive cells/50,000 leukocytes. HCMV-related disease was treated with iv ganciclovir (Cymevene; Roche) or foscarnet (Foscavir) until pp65 antigenemia levels decreased to below the limit of detection. In some cases, hyperimmunoglobulin (Megalotect; Biotest) was given in addition to ganciclovir or foscarnet.

Results of the HCMV antigenemia assay were used as measures of the HCMV loads in infected patients. The assay was performed by specific staining of the pp65 epitope in isolated peripheral-blood leukocytes, in accordance with a protocol described by The et al. [15]. The total number of pp65-positive cells was scored and expressed per 50,000 leukocytes. Routine serological analysis was performed as described by van der Giessen et al. [16]. Detection of IgG and IgM to HCMV was done by semiquantitative ELISA, using alkaline glycine–extracted HCMV antigens from HCMV AD169–infected fibroblasts and, in parallel, extracts from mock-infected fibroblasts. RNA was isolated from an equivalent of 100 μL of whole blood by use of activated silica beads, in accordance with a protocol described by Boom et al. [17]. All DNA was digested by use of RNase-free DNase (Fermentas) at 37°C for 30 min. Synthesis of cDNA was performed on 100 ng of RNA by use of 200 U of Superscript II Reverse Transcriptase II (Invitrogen), in accordance with the manufacturer’s protocol. One microliter of cDNA was used as a template for quantitative RT-PCR, which was performed in a 10-μL reaction volume containing 1 μL of cDNA added to 5 μL of Universal Master Mix (Applied Biosystems), 0.5 μmol/L.

Figure 1. Comparison of immediate-early (IE) 1 and US28 gene transcript levels (shown as the relative quantity of transcripts, compared with that of a housekeeping gene) and viral load (pp65 antigenemia; shown as the no. of positive cells/50,000 leukocytes) in whole-blood samples from lung-transplant recipients with primary or secondary human cytomegalovirus infection. The bottom panel shows the correlation between IE1 and US28 gene transcription levels.
Figure 2. Time course and level of immediate-early (IE) 1 and US28 gene transcripts (shown as the relative quantity of transcripts, compared with that of a housekeeping gene) in whole blood from 4 lung-transplant recipients with primary or secondary human cytomegalovirus (HCMV) infection. The course of HCMV infection was monitored by pp65 antigenemia (shown as the no. of positive cells/50,000 leukocytes). Time points at which pp65 antigenemia was determined are indicated by circles on the X-axes. Immune responses against HCMV antigens were determined by measurement of IgM and IgG levels. Antiviral treatment administered (ganciclovir, megalotect, foscarnet, and methylprednisolone for rejection treatment) is shown in the boxes at the top.

primer pair, and 0.2 μmol/L probe. PCR consisted of uracil–N-glycosylase treatment at 50°C for 2 min, denaturation at 95°C for 10 min, and 40 cycles of denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min. All reactions were done in triplicate, using 3 reaction wells for each template DNA. As a control for viral DNA contamination in the RNA sample, for each sample we included an equivalent amount of non-transcribed RNA. PCR was performed using the ABI 7900HT system (Applied Biosystems), and data were analyzed using SDS software (version 2.2; Applied Biosystems). The IE1 and US28 primer pairs were obtained from the Assays-by-Design service of Applied Biosystems and were designed as follows: IE1 forward, 5′-CATTGAGGAGATCTGCATGAAGGT-3′; IE1 reverse, 5′-ATCCACACTAGGAGAGCAGACT-3′; IE1 reporter, 5′-FAM-CTTTGCCCAGTACATTCT-TAMRA-3′; US28 forward, 5′-TGCTCTGCTGGCGAGTTC-3′; US28 reverse, 5′-GACGCGAAAAGCTCATGCT-3′; and US28 reporter, 5′-FAM-CCCGCGATGTATCCTT-TAMRA-3′. Premixed primers and probes for the detection of human β2 microglobulin were obtained from Applied Biosystems (Assays-on-Demand Gene Expression Products). Relative quantification was determined by normalizing against expression of the human β2 microglobulin housekeeping gene and adjusting for efficiency of amplification. For determination of primer efficiency, HCMV-infected fetal lung fibroblasts were diluted in whole blood at a range of 1–100,000 cells/mL; fibroblasts were infected at an MOI of 1 and were diluted in whole blood 1 day after infection. The lower limit of detection was 100 infected cells/mL in whole blood. Correlations between relative quantities of gene transcripts and viral load were calculated using Pearson's correlation coefficient.

Results. The specificity of amplification was determined
using samples from 5 HCMV-seronegative lung-transplant recipients who had received a lung from an HCMV-seronegative individual and 5 healthy HCMV-seronegative individuals. No false-positive results were detected for these samples (data not shown), indicating that the primers were specific for the detection of IE1 or US28 gene transcripts in whole blood. In AD169-infected fibroblasts, we detected US28-specific transcripts within 3 h after infection (data not shown), which is in line with previous observations [4].

Blood samples from 4 patients with primary infection (n = 48 samples) and 8 patients with HCMV reactivation (n = 85 samples) were analyzed. Active HCMV infection was routinely monitored on the basis of pp65 antigenemia. In figure 1, the normalized levels of IE1 and US28 gene transcripts are depicted relative to viral load (i.e., pp65 antigenemia). During primary infection and reactivation, the levels of IE1 gene transcripts correlated with viral load (Pearson’s r = 0.68 and Pearson’s r = 0.65, respectively; P < 0.001). Compared with that for IE1 gene expression, the correlation between US28 gene expression and viral load was weaker for primary as well as secondary infection (Pearson’s r = 0.91; P < 0.001) and secondary (Pearson’s r = 0.62; P < 0.001) infection (figure 1).

Representative examples of 2 patients with primary infection and 2 patients with secondary infection are shown in figure 2. Generally, acute rejection episodes occurred soon after lung transplantation and were treated with high-dose methylprednisolone (figure 2, arrows). Active HCMV infection, as determined by positive pp65 antigenemia, was initially treated with ganciclovir, but recurring infection was treated with foscarnet or megalotect until pp65 antigenemia levels became negative. As was expected, the resolution of HCMV infection was associated with an increase in HCMV-specific IgG titers or with seroconversion. After primary and secondary infection, increases in viral load (i.e., pp65 antigenemia) coincided with the presence of IE1 gene transcripts in leukocytes from patients. Transcription of the IE1 gene appeared to follow the course and level of infection. In addition, US28 gene transcripts were also readily detected, and,
similar to IE1 gene transcription, US28 gene transcription matched the course and level of infection. Moreover, the levels of transcription of the IE1 and US28 genes were comparable during the majority of the infection.

Discussion. We have shown that the HCMV-encoded chemokine-receptor homologue US28 is expressed in the peripheral-blood leukocytes of lung-transplant recipients during active HCMV infection. Furthermore, we have shown that the US28 gene is coexpressed with the IE1 gene, which implies that it is already expressed at immediate-early or early stages of clinical HCMV infection. To our knowledge, this is the first report to describe expression of the US28 gene in relation to the progression of viral infection during primary and secondary HCMV infection. Our observations in patients correlate with the early expression kinetics of the US28 gene that was found in infected fibroblasts in vitro [4]. In line with previous observations [18], only a weak correlation was found between IE1 gene expression levels and pp65 antigenemia among our patients. US28 gene transcription levels did not correlate with pp65 antigenemia. Nevertheless, IE1 and US28 gene expression was mostly followed by an increase in viral load. A limitation of our study is that measurements were performed in whole-blood samples. Thus, expression levels of HCMV genes reflect an average of a pool of different infected cell types, which may have different transcription characteristics for these HCMV genes.

We conclude that the level of US28 gene expression is highly associated with the level of IE1 gene expression but is unrelated to HCMV disease activity, as measured by pp65 antigenemia. Although a strong correlation between US28 and IE1 gene transcript levels is not formal proof of causality per se, our findings appear to provide in vivo evidence that US28 and IE1 may interact. Pleskoff et al. have reported that US28 induces apoptosis in vitro [19], which could be prevented by coexpression of IE1. In light of their results, it would seem that IE1 expression is mandatory for US28 gene transcription. Conversely, as we mentioned before, US28 signaling may also enhance major IE promoter/enhancer activity. The mutual interaction between US28 and IE1 might be relevant for amplification of gene expression. We have demonstrated that the US28 gene is abundantly expressed during the early stages of clinical infection and that its expression kinetics resembles that of the IE1 gene. The fact that US28 is a cell-surface molecule that is present on HCMV-infected cells during the early stage of infection renders it an attractive and easy accessible target for anti-HCMV therapy.

Acknowledgments

We thank Dr. J. M. Middeldorp and Dr. S. Stevens (Free University of Amsterdam, The Netherlands), for providing some of the blood samples and the reagents.

References